A faunistic study of parasites of the barnacle *Semibalanus balanoides* (Linnaeus, 1767) with a focus on the biology of *Maritrema gratiosum* (Nicoll, 1907) (Digenea, Microphallidae)

Thesis submitted for the degree of

Doctor of Philosophy in Aquaculture

By

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UNIVERSITY of STIRLING

Declaration

I hereby declare that the work and results presented in this thesis was conducted by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not been previously submitted for any other degree or qualification.

The literature consulted has been cited and where appropriated, collaborative assistance has been acknowledged.

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Stirling, Scotland, UK

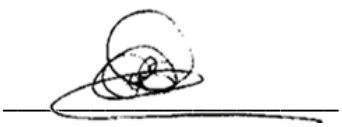
June 2021

This is to certify that this thesis for the degree of Doctor of Philosophy entitled "A faunistic study of parasites of the barnacle *Semibalanus balanoides* (Linnaeus, 1767) with a focus on the biology of *Maritrema gratiosum* (Nicoll, 1907) (Digenea, Microphallidae)" submitted to the University of Stirling (UK), is an original work carried out by Yu-Ching Chuang under our supervision.

Prof. James E. Bron

Stirling, UK

June 2021



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Abstract

This study set out to characterise the fauna and biology of parasites of the barnacle *Semibalanus balanoides.* The dominant parasite species was identified as a microphallid metacercariae *Maritrema* sp. and was determined to be the focus of study. Systematic sampling was conducted to investigate the spatial distribution patterns of *Maritrema* sp. in *S. balanoides* populations across different latitudes, habitat types and scales; and to explore the factors affecting the discovered patterns. An aggregated or over-dispersed distribution was observed at each sampling site, as well as when the data were pooled. The relationship of variance to mean of metacercarial abundance was found to fit Taylor's Power Law. Genuine spatial heterogeneity was detected after correcting for host size, and a decreased trend of abundance towards the sea was revealed. This trend was more apparent at a gully (local scale) than across whole area scale. Possible causes of this phenomenon were discussed. After analysis of several host and environmental factors, a multiple regression model which explained up to 88% of the variance of abundance was developed. The influence of host density and parasite crowding upon infection parameters were also investigated. Afterward, morphology, SEM, and molecular tools were applied to diagnose this dominant digenean in the studied localities and its identity was confirmed to be *Maritrema gratiosum*. To confirm that the specimens collected from three different rocky shore localities along the Scottish coast represented a single species, morphometric and molecular methods were applied. Although subtle differences in the morphology of specimens collected from distant localities were found, with single base pair polymorphism being revealed in their ITS2 rDNA, all specimens were considered to be a single species. Distance between sampling localities, culture conditions, and parasite crowding were found to contribute to morphological variations. Finally, an *in vitro* culture study was performed to obtain ovigerous adults of *M. gratiosum.* The morphology of the mature forms was observed and the effects of different concentrations of serum upon adult longevity, size and egg production was measured. Survival for 10-days was achieved when flukes were cultured in NCTC-109 plus chicken serum and antibiotics. Forty percent chicken serum seemed to provide better results in terms of survival time and in producing flukes with the largest body lengths. Both normal and abnormal eggs were observed among those produced by the adults cultured *in vitro*. The ability of self-fertilization was revealed, and the status of progenesis and its evolutionary and ecological significance have been discussed. CLSM was undertaken to provide detailed study of the development of their internal organs and musculature during the course of their *in vitro* culture. The musculature of *M. gratiosum* was similar to that of other microphallids, however, some additional novel structures were found, most notably a ligament connecting pars prostatica and seminal vesicle and a racket-shaped excretory bladder. Through the thorough study of a single species of digenean, this study has provided baseline distribution information in the chosen localities, provided greater insight into its biology, and including further insights into its role in rocky shore ecosystems. The methodologies described in the present study can provide a tractable model by which the study of the complexity of coastal host-parasite ecology can be continued.

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Chapter 1 Introduction

1.1 Background

The introduction of a parasite to, or elimination of a parasite from an ecosystem can strongly affect the interactions between many species in the community and, therefore, influence biodiversity (Thomas *et al.*, 2005). The roles parasites play, however, have been largely overlooked. The first step in understanding the roles which parasites play in an ecosystem is to recognize their existence. Some basic questions need to be answered first, such as: what are the primary parasites in the system of interest, how prevalent are the parasitic infections, and how does the parasitic infection affect individual hosts, populations, and communities (Sousa 1991). For those that are interested in these questions, intertidal ecosystems provide a good arena for research. Rocky shores represent evolutionary pathways from the sea to life on land, which have resulted in a vast biodiversity of marine life forms (Archer-Thomson & Cremona, 2019). Striking similarities and differences exist among sheltered and exposed rocky shores. For example, animal and plant coverage varies considerably from low to high tide, and some shores are dominated by animals, some are seaweed-covered. Why this difference exists and the spectrum of intermediates between these extremes, and why the variety and types of organism differ significantly according to their vertical position on the shore, are questions that are fundamental to understanding rocky shores (Archer-Thomson & Cremona, 2019). On the other hand, predictable patterns and zonation are observed, and rocky shores can be categorized. There are physical zones and biological zones on shores. Physical zones are based on tidal height, and can be defined as Extreme High Water of Spring Tides (EHWS), Mean High Water of Spring Tides (MHWS), Mean High Water of Neap Tides (MHWN), Mean Low Water of Neap Tides (MLWN), Mean Low Water of Spring Tides (MLWS), and Extreme Low Water of Spring Tides (ELWS). Biological zones can be recognized from horizontal bands of different colours, reflecting the most apparent organisms within the zones. Basic biological zones of British rocky shores comprise lichens, limpets and barnacles, and kelps, from high to low shore, as shown in Figure 1.1. This pattern is further modified according to the exposure of a specific shore. The distribution of organisms on the shore is usually determined by multiple factors that vary with height (Archer-Thomson & Cremona, 2019).

Figure 1.1 Basic rocky shore zones. The orange zone above Mean High Water Spring (MHWS) tides represents a band of *Caloplaca* (Th.Fr., 1871) lichens. Below is the black tar lichen (*Verrucaria maura* Wahlenberg, 1803) zone. The grey/light brown zone between Mean High Water Neap (MHWN) and Mean Low Water Spring (MLWS) tides, is dominated by limpets and barnacles. At the base is the brown zone showing where kelps dominate. After Archer-Thomson and Cremona (2019; p.15).

One way to appreciate the diversity of the seashore's organisms is to divide them into functional groups according to the way they exploit environmental resources (Little *et al.*, 2009). For example, one can consider producers, grazers, suspension feeders, and predators. Among the identifiable functional groups, parasites, although often present in enormous numbers, are usually ignored by biologists working in the intertidal zone. Some authors consider that they act in a similar way to predators, however, they consume prey resources / tissues and under most circumstances do so without directly killing their hosts (Little *et al.*, 2009). Others have pointed out the detrimental effect of parasitism which could be related to species extinction (Zholdasova, 1997). The impact of parasitism has been long overlooked as a parameter affecting host ecology, but has now been recognized as an important factor in the structuring of animal communities and food webs (Fredensborg *et al.,* 2006). According to Sousa (1991), parasites can be quite prevalent in some taxa particularly in intertidal systems, however, the real impacts of parasites on intertidal populations are often unknown and difficult to establish. Some theories based on existing evidence have been proposed. According to Thomas *et al.* (1997), for example, parasitism affects the population ecology of wetland animals in many different ways. There is mutual adaptation between local parasites and host populations. Parasitism can affect mate choice (Vyas, 2013), create patterns of spatial distribution (Mouritsen & Poulin, 2002), increase hosts susceptibility to stress (Beldomenico & Begon, 2015), alter host fecundity (Granovitch *et al*., 2009) and change the normal distribution and migration patterns of hosts (Anderson, 1972; Curtis, 1987; McNeil *et al*., 1995; Mouritsen & Poulin, 2005). The concept of keystone parasites has also been examined by Mouristen and Poulin (2002) using a simulated model. The concept of "keystone species" was discussed by Navarrete and Menge (1996) and was defined as

species at all trophic levels that have disproportionately large effects on their community. For examples, in the *Pisaster ochraceus* (Brandt, 1835) (seastar, the predator) – *Mytilus trossulus* A. Gould, 1850 (mussel, the prey) systems, *P. ochraceus* was identified as keystone predator as its removal has a major influence for the population of *M. trossulus*. The interaction strength between mussel and the other predator, *Nucella emarginata* (whelk), however, was 2-10 times lower than the previous case across sites and wave exposure (Navarrete and Menge, 1996). Under this concept, a keystone parasite was defined as "the parasitic species that affect the local abundance of the key predators or grazers which is the main determinant of the relative abundance and diversity of other organisms in intertidal systems" (Mouritsen & Poulin, 2002). Parasites also play a role in trophic interactions by altering the phenotypes of their host. A process by which parasites alter the phenotype of their intermediate host and thereby increase the risk of predation by the definitive host (defined as favorization by Combes 1991) was discussed by Thomas *et al.* (1997). According to Combes (1991), favorization is an adaptive ethological process modifying the spatiotemporal positions of the parasite and its target in a way that increases the probability of their encounter. It is the product of selection in the parasite gene pool. Thomas *et al.* (1997) discussed the trade-off between the costs or benefits of easier predation, and it is largely decided on the pathogenicity consequence of parasitism. Furthermore, according to Mouritsen and Poulin (2002), for both rocky shores and soft-sediment flats, parasites influence the survival, reproduction, growth, and behaviour of intertidal invertebrates. Parasites can also modify the structure of intertidal communities. They either directly influence the abundance of key host species and their interaction and competition with other species; or indirectly affect the behaviours of their hosts, therefore causing changes in features of the habitat, or leading to either greater success of settlement or local disappearance of certain species. These authors suggested that although parasitism can have many negative effects on individual hosts, at the community level, parasitism can be considered as a factor which can increase intertidal diversity. Consequently, they also stated that parasites should be more widely included in community studies and food web models.

Hudson *et al.* (2006) suggested that a healthy ecosystem is one that is rich in parasites, although the consequence of parasitism for a particular species can by positive or negative. Opposing theories have been proposed. Hudson *et al.* (2006) suggest that parasite-induced competitive exclusion can lead to or accelerate the extinction of a particular species (reduce biodiversity, in the case of generalist parasites), but on the other hand, can also act to increase biodiversity (specialist parasites). Here generalist parasites refer to those can infect divers host species, for examples, most microphallids to their final hosts (Deblock, 2008). On the contrary, specialist parasites refer to those have high host specificity, for examples, gyrodactylid monogeneans (Bakke *et al*., 2002). Thomas *et al.* (2005) also stated that the apparent competition mediated by parasites can influence the structure of an ecological community, but that the net effects on biodiversity can be positive or negative. Contrary to

Hudson *et al.* (2006), these authors suggested that generalist parasites with a preference for the competitively superior host can help to maintain host species diversity, while host-specific parasites which prefer competitively inferior species have detrimental effects for biodiversity. Under the assumption that many parasites are host specific, a community rich with hosts should also be the one rich in parasites (Hudson *et al.*, 2006). To elucidate the "function" of certain parasites in an ecosystem, the first step is to identify their existence and patterns of infection. Marine birds play important roles as final hosts in the life-cycle of many intertidal parasites. A record of adult parasites in individual seabirds does not, however, necessarily mean that the parasites are locally produced at the place where the bird is captured and instead, more reliable information will come from the study of intermediate hosts (Galaktionov & Bustnes, 1999).

Sessile barnacles are ubiquitous taxa in the intertidal zone. Barnacles are recognized as intermediate hosts for a number of digeneans and cestodes (Hadley & Castle, 1940; Arvy & Nifrelli, 1969; Stubbings, 1975; Williams *et al.*, 1981), but the roles barnacles play in parasite life-cycles have been largely overlooked and similarly, their parasite fauna is also rarely studied. According to Galaktionov and Dobrovolskij (2003), the occurrences of certain stages of a parasite species in some geographical regions does not necessarily indicate that their entire life-cycles are completed there. The completion of the life-cycle for a given parasite species requires overlapping of the distribution areas of all hosts involved. The fruitful results of the preliminary survey conducted by Colston (2012) regarding the associated and parasitic fauna of barnacles in Scotland, the abundance and feasibility of easy access to barnacles off the Scottish coast, and the co-location of seabird habitats, make them good targets for research examining rocky shore parasite-host systems. A diverse array of parasitic and associated fauna for barnacles (*Semibalanus balanoides* Linnaeus, 1767 and *Austrominius modestus* Darwin, 1854) from Scottish coast was discovered in Colston's study. More information regarding the influence of these parasitic fauna, especially the most common digenean parasite *Maritrema gratiosum* Nicoll, 1907, on the host health and population dynamic, however, was not explored further. Most importantly, regarding the high prevalence, intensity, and abundance of *M. gratiosum* in their hotspot, *i.e*., Dunbar, the absence of the first intermediate host and the cercarial stage is rather bizarre. How does this prevalent digenean species develop and maintain its populations at a particular locality? Besides barnacles, are there any other host species can maintain its populations? These questions are still not clear. Furthermore, the morphology and biology of *M. gratiosum* collected in UK deserve further study as the publications so far were either fragmented, from other geographical areas, or out of date (Nicoll, 1907; Rankin, 1939; Hadley and Castle, 1940; Deblock and Tran Van Ky, 1966; Deblock and Rausch, 1972; Irwin *et al*., 1990). In Colston's work the author provided no molecular data for *M. gratiosum* either, which can be very important for species diagnosis. This introductory chapter, will first introduce barnacle biology and their taxonomic characters, and describe

what is known concerning the major parasites of barnacles and their life history. Second, given that intertidal molluscs are important primary intermediate hosts of many digeneans, this chapter will also examine records of Digenea in key British intertidal gastropods. Third, this chapter will briefly introduce the final hosts of intertidal digeneans and cestodes, especially shorebirds within the sub-orders Charadrii (plover-like waders) and Lari (gulls and allies), which are vital in their life-cycle, as this is where the adult stages are located and sexual reproduction and egg dispersal commence. While the high mobility of birds makes them the main dispersal agent in the life-cycle of digeneans and cestodes, due to ethical and logistic constraints, however, many bird species have not been confirmed as definitive hosts (Fredensborg *et al.,* 2006). In this chapter concept of dominant parasite and its relation to distribution patterns of parasite communities will be briefly addressed. The method of choice for digenean parasite diagnosis and the application of morphometrics in the context of parasite ecology will also be mentioned. Finally, due to the difficulty of obtaining adult digeneans from the final hosts, the application of *in vitro* culture of helminth parasites will be briefly address in this chapter.

1.2 Barnacles

1.2.1 Taxonomy, structures, functions, and biology of barnacles

Barnacles are a ubiquitous group of substrate-attaching and parasitic crustacean species. One of the first biologists to recognize the importance of barnacles was Charles Darwin, who authored a seminal work, the monograph “Cirripedia” (Darwin, 1851). In the present day, with respect to their relationship to human interests, they are most well known as fouling agents (Maleschlijski *et al.,* 2012), and some are also important as seafood (Dionisio *et al.,* 2013). They are also a target for biologists who are interested in the recruitment and settlement of intertidal taxa (Wethey, 1984; Roberts *et al.*, 1991; Hills & Thomason, 2003; Jenkins *et al.*, 2008; Fukaya *et al.*, 2010; Bracewell *et al.*, 2013).

1.2.1.1 The taxonomy of sessile barnacle

All barnacles belong to the crustacean subclass Cirripedia Burmeister, 1834 (Anderson, 1994). The classification below Cirripedia, however, are still under revision. Under the subclass there are three infraclass, namely the Acrothoracica Gruvel, 1905, the Rhizocephala Müller, 1862, and the Thoracica Darwin, 1854. The barnacle species in the infraclass Thoracica are the main targets of the present study. According to their morphology, thoracican barnacles can be sub-divided into two groups: pedunculate or stalked barnacles, and those without a stalk, *i.e*., sessile or acorn barnacles (Anderson, 1994). The Order Pedunculata Lamarck, 1818 was not accept now and it was sub-divided by Buckeridge and Newman (2006) into several orders, but the discussion of these is outside the scope of this research and, therefore, will not be discussed further. The sessile or acorn barnacles (Order Sessilia

Lamarck, 1818), however, are the main targets of the present study. Forty thoracican species have been recorded from the shallow waters around the British Isles (Southward, 2008). The taxonomic position of sessile barnacles is illustrated in Figure 1.2.

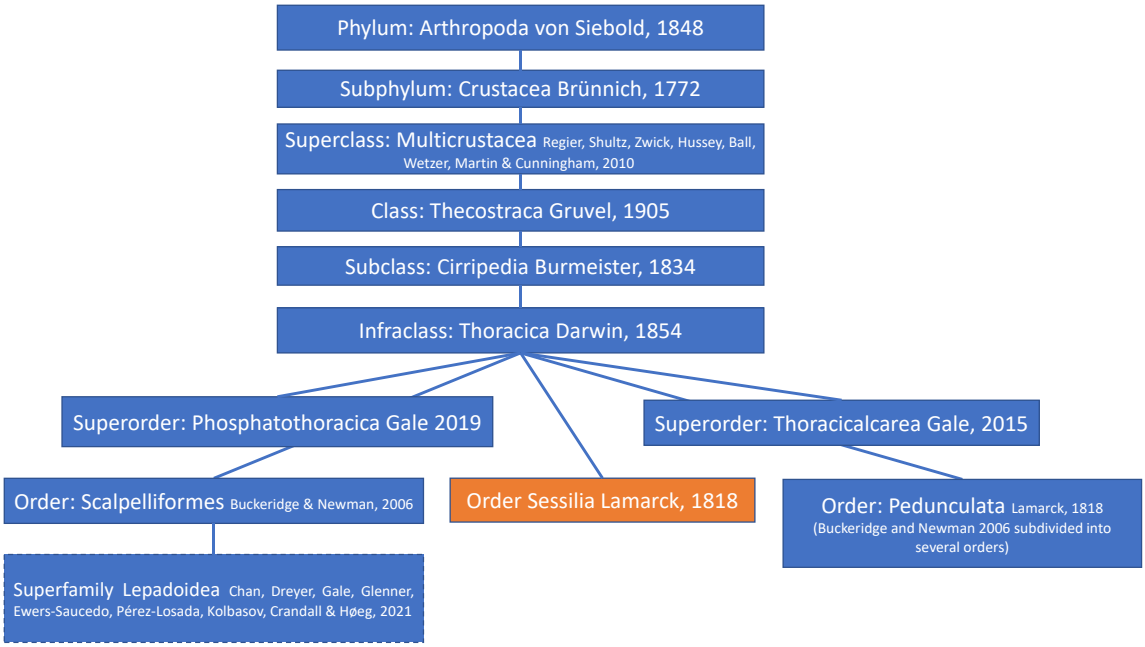


Figure 1.2. Taxonomic organization of barnacles within the infraclass Thoracica Darwin, 1854. Image reproduced from the World Register of Marine Species (WoRMS) on-line database.

It is important to understand the basic anatomy of adult sessile barnacles, due to the need for correct identification of host species. Furthermore, when searching for their parasites, it is important to differentiate normal host tissue and parasites and the potential pathological reaction elicited by the parasites. The anatomical characters can also reflect some parameters related to parasitic infection. For examples, the diameter of the opercular aperture can reflect the size of sessile barnacle, and size is an important parameter for parasite abundance (Carrol *et al*. 1990). Regarding the morphology of digenean parasite, morphological plasticity has been reported to related to host factors. For examples, natural morphological variability of *Phyllodistomum umblae* (Fabricius, 1780) Bakke, 1982 was discovered on specimens from different host and localities (Bakke, 1988); the morphological variabilities of naturally infected *Fasiola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1855 was also found to be related to their hosts species (Ashrafi *et al*, 2015). Furthermore, barnacle cirrus has been identified as the entry point of larval digenean (Irwin *et al*., 1990) and its morphology can reflect environmental conditions such as exposure (Hoch, 2011). The following review provides an overview of the major organ systems and physiology of sessile barnacles.

1.2.1.2 Shell plates structure of sessile barnacles

Adult sessile barnacles typically have a wide conical shape with their main aperture being in the apical position whilst firmly attached, ventrally, to their substrate. The basic structure of a sessile barnacle comprises a series of shell plates and a soft body, termed the prosoma, within as shown in Figure 1.3. The shell plates are sometimes called parietes or compartments (Bassindale, 1964).

The arrangement of the shell plates can be one of the taxonomic identification traits for sessile barnacles. For more information regarding different shell plate arrangements in ancestral and current sessile barnacles and other important shell plate features, please refer to the section provided in the Appendix 1.

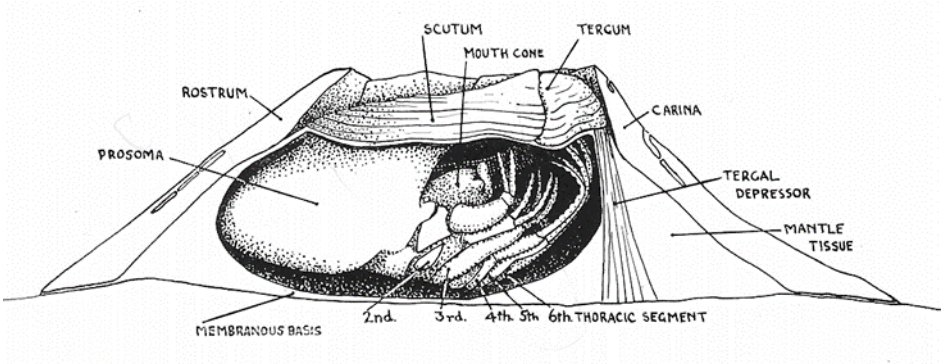


Figure 1.3. A cut away of *Semibalanus balanoides* Linnaeus, 1767 showing the position of the body within its shell. Image reproduced from Bassindale (1964; p. 61).

The systematics of barnacles in Britain and Ireland and the relevant identification keys are comprehensively covered by Southward (2008). *Semibalanus balanoides* and *Austrominius modestus* Darwin, 1854 (syn. *Elminius modestus*) are the commonest species in the intertidal zone of rocky shores in Scotland (Colston, 2012). In brief, *A. modestus* has four wall plates, and the wall plates are bilaterally symmetrical and ridged. The colour of its wall plates is white but often gaining a slaty tinge with age. The operculum is formed by two pairs of scutum and tergum and the mantle opening is along the midline. *Semibalanus balanoides* has six wall plates and the operculum is formed by two pairs of scutum and tergum. The rostrum overlaps the lateral plates and the carina is overlapped by adjacent lateral plates. The shell plates are white when young and the tergum is not beaked. The basis is membranous (Southward, 2008). Schematic drawings of the shell plates of *A. modestus* and *S. balanoides* are shown in Fig. 1.4. Illustrative drawings of adult *S. balanoides* are shown in Fig. 1.5.

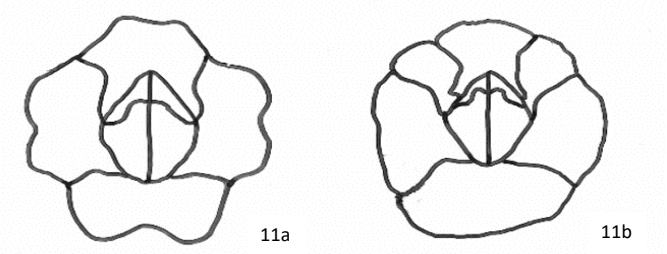


Figure 1.4. Schematic drawings of the shell plates. a. Schematic drawing of the shell plate of *Austrominius modestus*. b. Schematic drawing of the shell plate of *Semibalanus balanoides*.

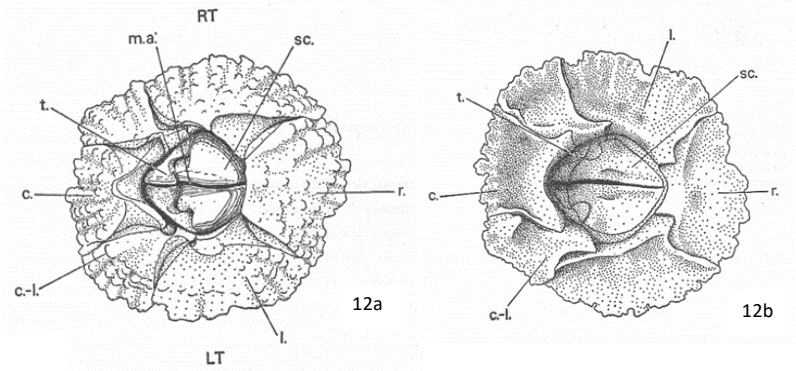


Figure 1.5. Shell plate of adult *Semibalanus balanoides*. a. Adult *S. balanoides* seen from above. b. Internal view of the same shell plates after removal of the soft tissue. c.: carina. c.-l.: carino-lateral plates. l.: lateral plates. LT. left side. m.a.: mantle aperture. r. rostrum. RT.: right side. sc.: scutum. t.: tergum. 11a & 11b redrawn from Southward, 2008; 12a & 12b reproduced from Stubbings, 1975. Plate I.

1.2.1.3 The internal body of sessile barnacles

The structures of the internal body of barnacles may be harder to recognize. As a member of the Crustacea, the sessile barnacles still possess the characters of crustaceans, although modified. The body is a modified version of the fundamental maxillopodan, which comprises five head segments, six thoracic segments and five abdominal segments (Southward, 2008). Barnacles, however, do not have a distinct head and trunk, and in the case of sessile barnacles, the body is upside down with the back at the ventral position. It is convenient to divide the body into an anterior prosoma and a posterior thorax (Stubbings, 1975). The posterior part of the head together with the first thoracic segment forms the prosoma. The oral cone and the first pair of thoracic appendages (cirri) are mounted on the prosoma (Bassindale, 1964). Behind the prosoma, there are five remaining thoracic segments, which each bear a pair of setose cirri (cirri II-VI). The mouthparts comprise a complex structure called the oral cone, carrying the labrum, mandibular palps, mandibles, maxillules and maxillae (Fig. 1.6). The gnathobase of the third cephalic appendage constitutes the mandible and provides important classification characters (Southward, 2008).

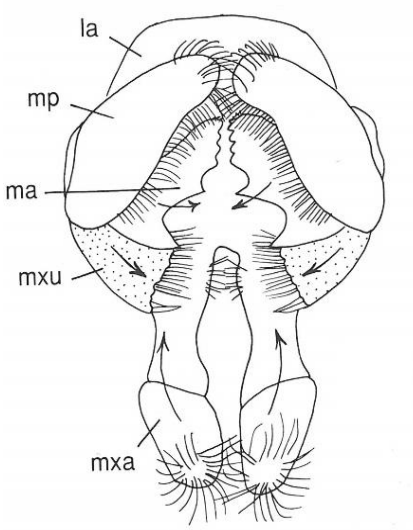


Figure 1.6. Structure of the mouth part of *Perforatus perforatus* Bruguière, 1789. Arrows show direction of next movement. (Reproduced from Anderson, 1994. P:62; the original was from Hunt and Alexander, 1991). la: labrum. ma: mandible. mp: mandibular palp. max: maxilla. mxu: maxillule.

On either side of the oral cone lie the first pair of thoracic limbs (cirri I), modified as maxillipeds, which are shorter and more setose than the remaining cirri. Cirri II and III are modified as maxillipeds as well. The remaining three pairs of cirri serve as fans for food capturing when fully extended. All cirri are biramous and each possesses an exopod and endopod (Southward, 2008). The remaining space between the shell plates and body is termed the mantle cavity, which is lined by a mantle and has a respiratory function. The mantle tissue contains three pairs of large, apicobasal muscles joining the opercular valves to the basis. These muscles consist of rostral scutal depressor muscles, lateral scutal depressor muscles and tergal depressor muscle. Closure of the opercular valves is achieved by contraction of the adductor muscle. Retraction, and the rotational movements of the operculum are the results of contractions of the powerful scutal and tergal depressor muscles in the mantle underlying the shell (Anderson, 1981). The basis of acorn barnacle is enclosed by the secretion of the barnacle and can be membranous or calcareous, depending on species (Anderson, 1994; Southward, 2008). The basic anatomy of barnacle prosoma and mantle muscles are shown in Figs. 1.7-1.8.

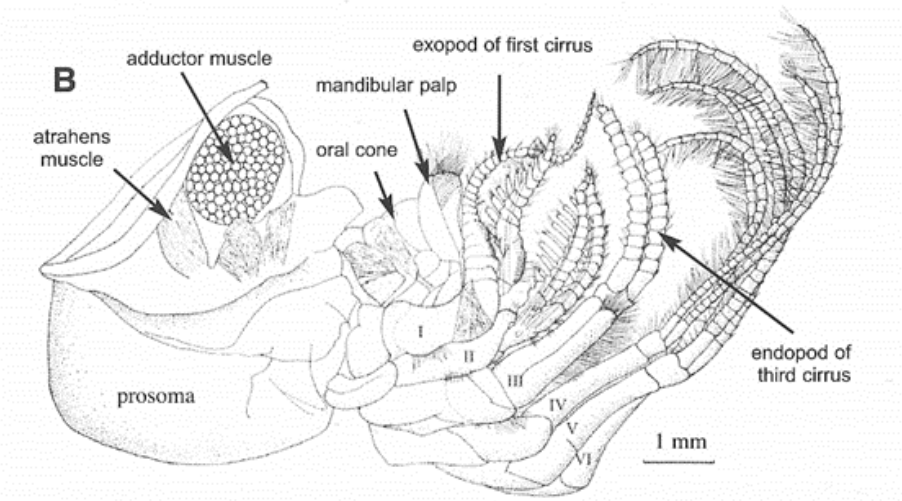


Figure 1.7. General anatomy of the body and limbs removed from the shell of the sessile barnacle *Perforatus perforatus* Bruguière, 1789 in right lateral view. The cirri are labelled in Roman numerals. (Reproduced from Southward 2008; p. 12).

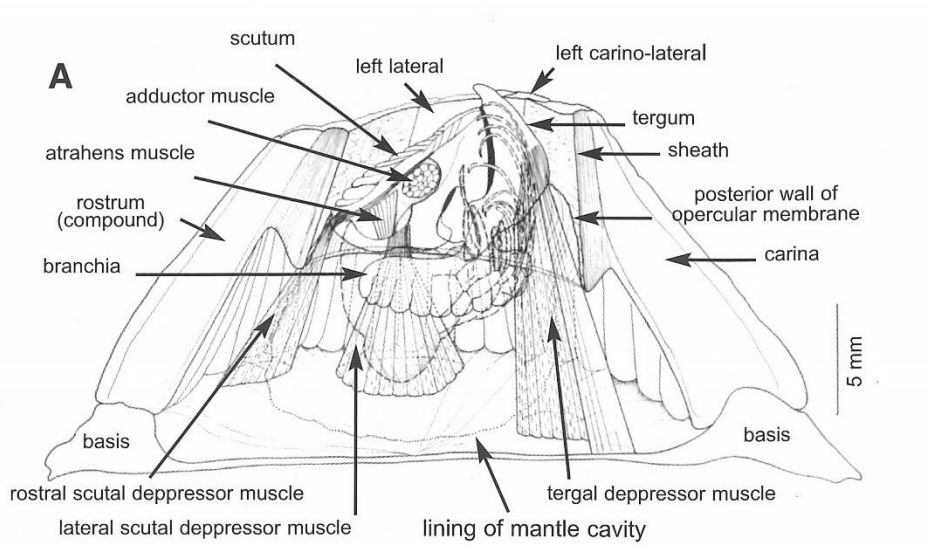


Figure 1.8. General anatomy of *Perforatus perforates* in right lateral view. Body *in situ* the shell showing the muscle. (Reproduced from Southward 2008; p.12).

1.2.1.4 Feeding and digestion

Most thoracicans are captorial planktivores, capturing small zooplankters such as copepods and meroplanktonic larvae such as plutei, veligers, ascidian tadpoles, nauplii and cyprids (Barnes & Reese, 1959; Crisp & Southward, 1961; Young & Gotelli, 1988; Young & Cameron, 1989; Anderson, 1994). In balanomorphs, specialized microfiltration by the maxillipeds is associated with the strong pumping beat characteristic of large active species (Hunt & Alexander, 1991). Small species, such as *S. balanoides*, use the cirral fan to remove small particulate matter from the water, ranging from nauplii to phytoplankton of only a few microns in diameter (Anderson, 1994). During the rhythmic feeding the thorax is elevated and the setose cirri fully extend (mainly by blood pressure) to form a casting net which retains planktonic organism. Species of *Lepas* can also accept pieces of tissue several millimeters in diameter (Bassindale, 1964). In *S. balanoides*, three types of beating have been mentioned. This species performs a respiratory pumping beat and shows prolonged cirral extension to water currents, but in still and slowly moving water, it also exhibits two levels of beating: normal beat and fast beats (Crisp & Southward, 1961; Crisp & Maclean, 1990; Trager *et al.*, 1990; Anderson, 1994), as shown in Fig. 1.9. Pumping beat generates a respiratory current through the mantle cavity, normal beat generates both respiratory current and results in food uptake, and fast beat is used for taking smaller food particles (Anderson, 1994).

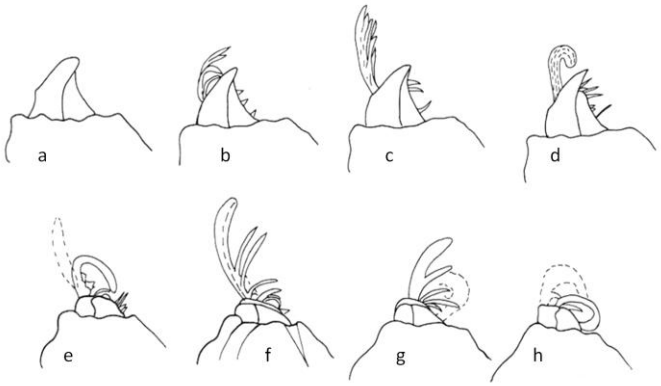


Figure 1. 9. *Semibalanus balanoides* cirral activities: a-d: normal beat cycle; e-h: fast beat cycle. (Reproduced from Anderson, 1994. p: 49. The original was from Crisp and Southward, 1961)

The alimentary canal in barnacles comprises the usual crustacean components, foregut, midgut and a short hindgut (Anderson, 1994). The foregut begins with a short pharynx leading forward from the mouth. The oesophagus of a balanomorph ends in a compact, valvular structure, the ventriculus. The midgut can be divided into two parts, a wide anterior midgut and a narrower posterior midgut. The anterior midgut always carries a pair of midgut glands, which serve as the principal digestive glands (Anderson, 1994, Fig. 1.10). In *Tetraclita squamosa* Bruguière, 1789, these glands discharge secretory products including several carbohydrases and proteases into the foregut ventriculus (Johnston *et al.*,

1993). In balanoids, the anterior midgut is also extended by up to seven tubular midgut caeca, branching into the prosoma (Anderson, 1994). A wide anal chamber after the hindgut leads to the anus. Faecal pellet production is quite rapid in species such as *S. balanoides*, the midgut content being renewed about once every hour during active feeding. Fragments of phytoplankton and zooplankton skeleton are clearly identifiable in faecal pellets (Anderson, 1994). Midgut play the major roles for digestion and adoption (Rainbow and Walker, 1977). Food particles were bound by secretions of salivary glands prior to ingestion. Salivary glands locate at oral cone and the secretions have lubricating and maybe digestive function (Rainbow and Walker, 1977).

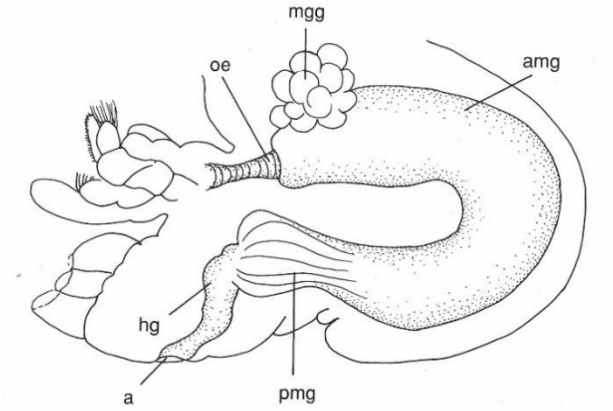


Figure 1.10. Alimentary canal of *Amphibalanus variegatus* Darwin, 1854. (Reproduced from Anderson, 1994. p: 91). a: anus. amg: anterior midgut. hg: hindgut. mgg: midgut glands. oe: oesophagus. pmg: posterior midgut.

1.2.1.5 Respiratory system

Due to the difference of solubility of oxygen in air and water, and intrinsic different physical properties between these two media, aquatic and terrestrial animals have evolved different ways for gas exchange, for example, gills versus lungs. Also, vertebrate and crustaceans use different respiratory pigments if they do possess it, *i.e*., haemoglobin and haemocyanin, respectively. There are needs for changes in respiratory pigment functions when moving between air and water. Decreasing in oxygen affinity and change in haematological parameters (*e.g*., increase in haemoglobin concentration) are two major means utilize by fish when switch from water to air. On the other hand, many bimodal crab species contain haemocyanin with high oxygen affinity but variable tendency for elevation of haemocyanin concentrations (Morris and Bridges, 1994). For sessile barnacles, the entire body surface and limbs and the lining of the mantle cavity can act in respiratory exchange. In addition to this, balanomorphs have paired and vascularized respiratory surfaces termed branchiae. The branchiae, like other surfaces in the mantle cavity, are ventilated by the water flow generated by the pumping beat (Anderson, 1994). There is no evidence of respiratory pigments, however, in the haemolymph (Waite & Walker, 1988). A special adaptation of respiratory exchange exists in intertidal balanoids. When emersed and exposed to air, water is expelled from the mantle cavity and replaced by a bubble of air.

The aperture is then held slightly open as a micropyle to facilitate gas exchange. In the case of desiccation, the micropyle and aperture close, and respiration becomes anaerobic (Grainger & Newell, 1965; Anderson, 1994). The structure of the branchiae and a schematic drawing of the air bubble in balanoids are shown in appendix 2.

1.2.1.6 Reproduction

Most barnacles are hermaphrodite species; however, self-insemination rarely happens and usually one will act as male and the other will act as female during the breeding season (Southward, 2008).

The female gonad is a pair of sac-like, lobulated ovaries, located in the basal mantle tissue in sessile barnacles. Paired oviducts run rostrally and pass to the female opening near the bases of the first pair of cirri (Anderson, 1994). The ovaries consist of much branched structures ending in many finger-like processes where the ova develop (Stubbings, 1975). Sessile barnacles and some pedunculates have a pair of oviduct glands that can produce a sac into which ova are shed at fertilization and in which the embryos develop in the mantle cavity (Southward, 2008). The male gonads, which are follicular testes, are located bilaterally within the body. Numerous small follicles are connected by branched efferent ducts with a pair of sperm ducts in the prosoma. The sperm ducts expand as a pair of seminal vesicles when entering the thorax. The seminal vesicles run carinally through the thorax to the base of the penis and unite with an ejaculatory duct which extends through the penis to a terminal male opening (Anderson, 1994). The hermaphrodite reproductive system is illustrated in Fig. 1.11.

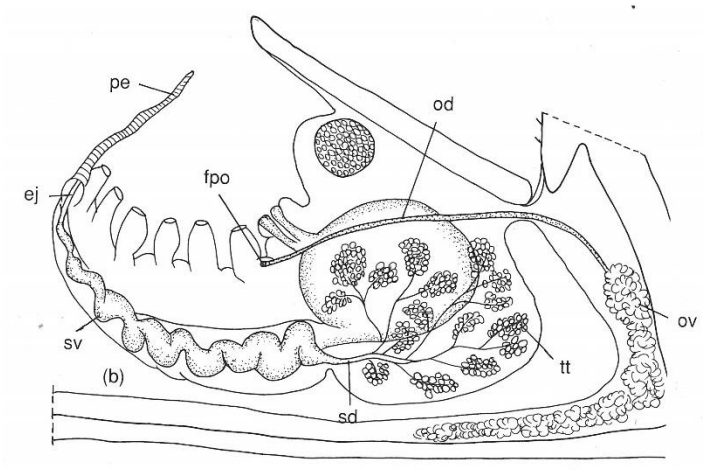


Figure 1.11. Reproductive system of a balanomorph thoracican. Abbreviations: ej: ejaculatory duct. fpo: female pore. od: oviduct. ov: ovary. pe: penis. sd: sperm duct. sc: seminal vesicle, tt: testis. (Reproduced from Anderson, 1994; p.129).

After mating, oocytes are released into paired oviducal sacs and are fertilized in these sacs. Later the sacs separate from the oviducal openings and move to either side of the prosoma as egg lamellae (Anderson, 1994). Each egg is surrounded by two membranes, an outer fertilization membrane and an inner egg membrane (Anderson, 1994). Larval release is species-dependent. In warm water-species, larval release is followed by moulting, and in cold-water species the incubation period is longer and is accompanied by a prolonged period of anecdysis. Growth and moulting are resumed after larval release, often in the summer (Barnes, 1962; Bassindale, 1964; Walker & Yule, 1987; Anderson, 1994). The time and duration of the breeding season varies widely among species. Temperature (Patel, 1959; Patel & Crisp, 1960; Hines, 1978; El-Komi & Kajihara, 1991), photoperiod (Barnes, 1989), salinity (Dineen & Hines, 1992), acidification (Pansch *et al.*, 2018) and food supply (Hines, 1978; El-Komi & Kajihara, 1991) are factors influencing seasonal breeding, which act in different ways in different species. *Semibalanus balanoides* is a synchronized breeder which breeds once annually and releases its larvae in the spring (King *et al.*, 1993).

Larval development typically includes six feeding naupliar stages and a non-feeding cyprid stage, which is specialized for habitat selection and attachment (Southward, 2008). The I to V stage naupliar larvae are similar morphologically, but the size increases after each moulting. The basic structures of a stage IV naupliar larva comprise a pear-shaped body with a dorsal shield, an anterior-ventral nauplius eye, two frontal filaments and frontal-lateral horns, three pairs of appendages furnished with setae for swimming and feeding (antennules, antennae and mandibles), a labrum, a mouth, a caudal process and a caudal spine (Bassindale, 1964; Stubbings, 1975; Anderson, 1994; see Fig. 1.12).

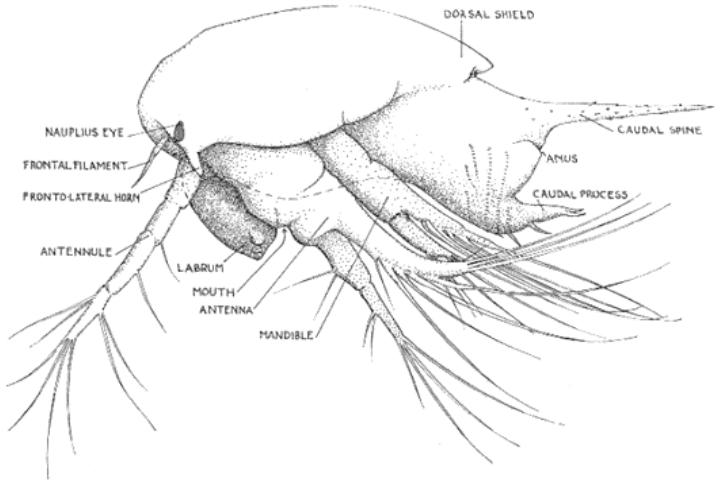


Figure 1.12. Side view of a stage IV nauplius of *Semibalanus balanoides*. The mandible is displaced ventrally to show the caudal process. (Reproduced from Bassindale, 1964; p.65).

The complexity of larvae increases with each moult until the VI stage when two special structures appear: the penultimate segment of the antennule swells to become the future attachment organs, and the rudiments of paired compound eyes of cyprids (Bassindale, 1964; Stubbings, 1975). A

schematic illustration to show the size ratio and general shape of the I-VI naupliar and cypris stages is provided in Fig. 1.13. The VI stage will moult into a morphologically quite different non-feeding cypris stage, which is specialized for settlement on a suitable substrate. Cypris have a bivalved boat-shaped carapace, a pair of anteriorly protruding antennules, and a posterior caudal appendage (Stubbings, 1975). Other organs are covered by the carapace, which are illustrated in Fig. 1.14.

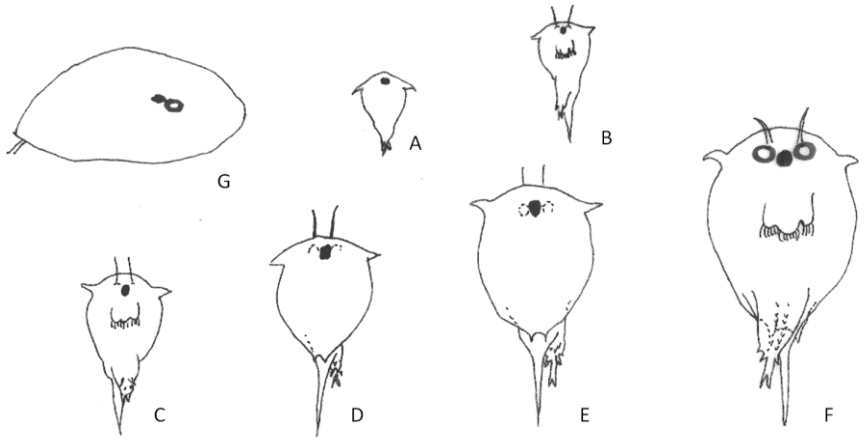


Figure 1.13. Outline drawing of nauplius stage I-VI and cyprids (at the same magnification: X35). A: nauplius I. B: nauplius II. C: nauplius III. D: nauplius IV. E: nauplius V. F: nauplius VI. G: cyprids. Redrawn from Stubbings (1975; Plate XXVI).

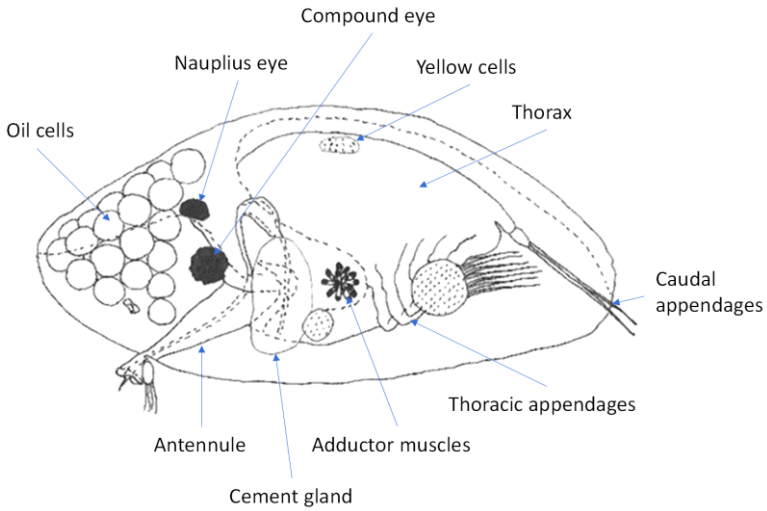


Figure 1.14. The main features of the cyprids larva of *Semibalanus balanoides*, visible in the living organism. Redrawn from Stubbings (1975, Fig. 177).

1.2.1.7 Barnacle settlement

Cypris settlement is a complex behaviour, which involves a sequence of events including attachment by the antennules, exploration through walking on the antennule, fixation by the antennules; or alternatively swimming away from unsuitable substrates (Crisp, 1955; Liang *et al*.,

2019). There are two phases in a cyprid's life: free-swimming and exploratory crawling phases. The factors concerning cyprids settlement involve recognition of an appropriate environment include: physical factors such as light, flow velocity and the degree of disturbance, surface texture, and topography of the substratum (Crisp, 1955; Rittschof *et al.*, 1984; Wethey, 1984; Chabot & Bourget, 1988; Eckman *et al.*, 1990; Mullineaux & Butman, 1991; Dineen Jr. & Hines, 1992; Young, 1991). Regarding the physical signals, a recent study revealed that cyprids of the intertidal barnacles *Amphibalanus amphitrite* Darwin, 1854 can detect conspecific adults by vision and can discriminate between different surface colours of settlement sites. The shell plates of adults, which emit a red fluorescence, may act as a signal to attract cyprid larvae for settlement (Matsumura & Qian, 2014). Biotic factors such as age of cyprids, presence of cyprids or adult conspecifics, settlement factors, density dependent mortality (intra-species competition), and competition with other taxa, predation, *etc*. (Rittschof *et al.*, 1984; Wethey, 1984; Hui & Moyse, 1987; Dineen Jr. & Hines, 1992; Johnson & Strathmann, 1989; Hills & Thomason, 2003; Ellrich *et al.*, 2015) also affect the pattern of cyprid settlement. After attachment, cyprids undergo metamorphosis and dramatic morphological changes into the generally recognizable adult barnacle form.

The settlement of cyprids of *S. balanoides* is a classic example. The cyprids of *S. balanoides* are at first photopositive and congregate at the sea surface, and later they become photonegative and swim down to seek a suitable substrate (Knight-Jones & Crisp, 1953; Barnes, 1955; Stubbings, 1975). Upward movement in the initial swimming phase brings cyprids to surface currents and aggregation with other plankters; currents will carry those cyprids to the shore. When cyprids contact a substrate, the crawling phase is initiated and a cyprid makes active comparisons and choices between various settlement positions. If a suitable position is not found, the cyprid will return to the water column and wait for another chance. This behaviour leads to the gregariousness of barnacle populations (Hui & Moyse, 1987). On settling on a surface, the cyprids become indifferent to light and proceed to explore the surface (Stubbings, 1975). When contacting a suitable surface, the crawling phase initiates and they move over the surface, examining it, until a suitable settlement site is found. The suitable site can be a small depression or fissure in the surface. The tendency of cyprids to settle in grooves or pits on a surface was defined as rugophilic (Crisp & Barnes, 1954; Stubbings, 1975). Also, a wide range of chemical cues are found to attract cyprids to settlement sites (Stubbings, 1975; Gabbott & Larman, 1987; Anderson, 1994; Liang *et al*, 2019). Among these chemical factors, "settlement factors" related to release of compounds from conspecific adult integuments plays a crucial role (Larman *et al.*, 1982; Gabbott & Larman, 1987; Maki *et al.*, 1988; Raimondi, 1989; Johnson & Strathmann, 1989; Tegtmeyer & Rittschof, 1989; Maki *et al.*, 1990; Robert *et al.*, 1991; Maki *et al.*, 1992; Anderson, 1994; Dreanno *et al.*, 2006; Abramova *et al.*, 2019). Crisp & Meadows (1963) have shown that this factor might be arthropodin, which is a water-soluble fraction of the arthropod cuticle and heterogenous in

composition. Dreanno *et al.* (2006) revealed that these proteins (called "barnacle settlement pheromone" or settlement-inducing protein complex (SIPC)") are present in the naupliar, cypris larval stages and in the adult. In adults, SIPC is present in the shell and in all organs that are lined by cuticular tissues. Other chemical cues include the mucus of predators or competitors (e.g., limpets, nudibranchs, whelks, cnidarian, etc.) (Raimondi, 1988; Johnson & Strathmann, 1989; Ellrich *et al.*, 2015). Also, the microbial flora composed by different bacterial species exerts different effects on different barnacle species (Maki *et al.*, 1988, 1990, 1992). The behaviour of larval barnacles is also responsible for their pattern of settlement, these include territoriality and species avoidance behaviour (Hui & Moyse, 1987). The cyprids of some barnacle species, especially the intertidal forms such as *S. balanoides*, *Balanus crenatus* Bruguière, 1789 and *A. modestus*, exhibit territoriality towards conspecifics. The size of the territory is little greater than the sum of the radius of the previously settled spat and the length of the settling cyprid (Crisp 1961). This distance allows neighbouring spat to establish before the onset of direct spatial competition, whilst preventing any further individuals from settling in between. The Balanomorph also exhibit specific avoidance behaviour. Their cyprids will avoid settling on the top of another conspecific (Hui & Moyse, 1987). For *S. balanoides* will avoid settlement in contact with the edge and on top of conspecific adults. No such avoidance was noted when *S. balanoides* cyprids settled near adults of alien species (Knight-Jones & Moyse, 1961). Species avoidance behaviour does not occur in *A. modestus* and *Chthamalus montagui* Southward, 1976 (Hui & Moyse, 1982, 1987).

Although many factors contribute to the final pattern of gregariousness, the combined effects of all these factors result in a preferred habitat for each species, these then resulting in a unique geographical distribution and occupation of a particular zone on the shore. For example, intertidal species such as *A. modestus* occupy bare rock from the sub-littoral zone up to near HWST, and *S. balanoides* are rarely found above HWNT (Connell, 1961a; Hui & Moyse, 1987). The habitat of intertidal species can overlap and interspecies competition can constrain the distribution each species. Three species, *S. balanoides*, *A. modestus* and *C. montagui*, are found with overlapping distributions around the British coastline, with their upper and lower limits and latitudinal preferences differing (Connell, 1961 a & b). Figure 1.15 illustrates the position of adults of these species taken across a specific shore profile. Desiccation, intraspecies competition and predation are the factors considered to shape these patterns (Connell, 1961b; Hui & Moyse, 1987). According to Svensson *et al*. (2006), when *S. balanoides, C. montagui* and *Chthamalus stellatus* Poli, 1791 cohabit the intertidal zone, *S. balanoides* is the stronger competitor within the mid and lower shore, while *C. montagui* is more likely to be found at the higher shore, and *C. stellatus* is more common in the mid to high shore zones.

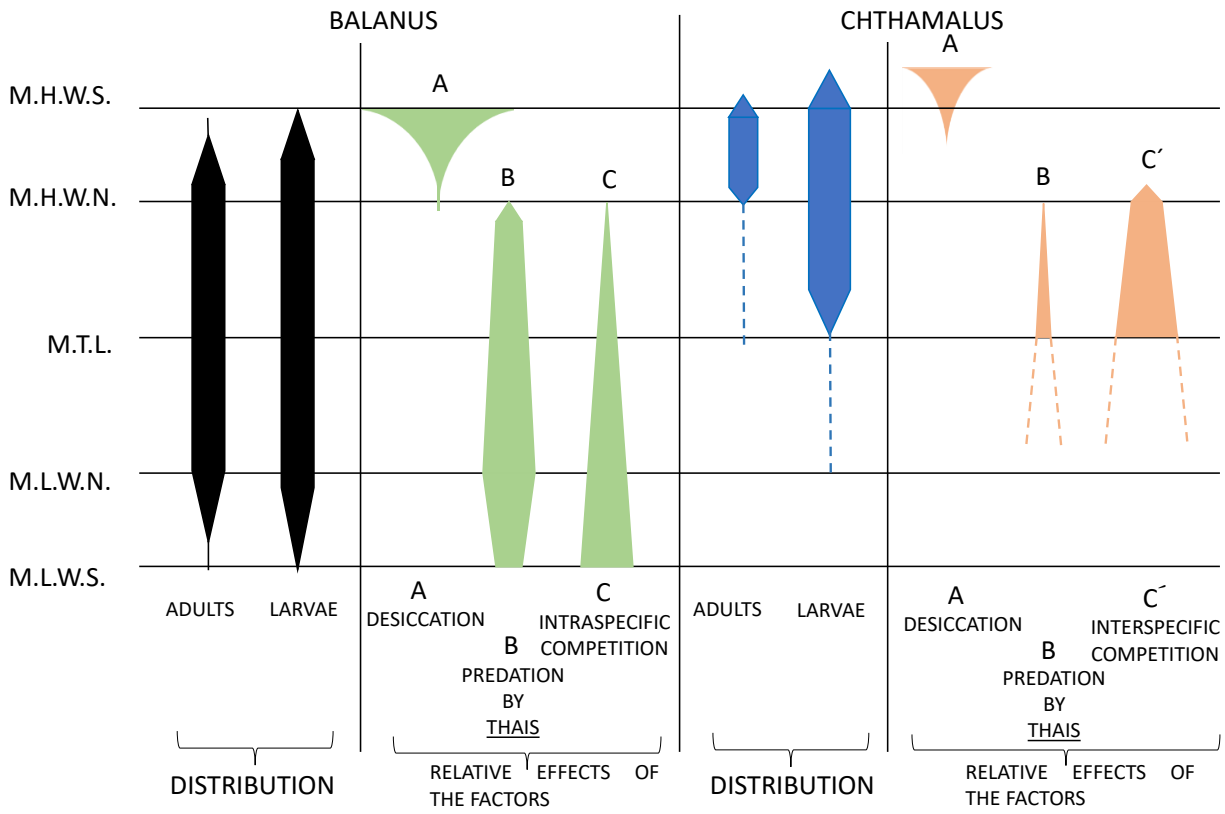


Figure 1.15. Comparison of patterns of distribution of adults and newly settled cyprids of *Semibalanus balanoides* and *Chthamalus montagui* at Millport (Scotland), with diagrammatic representation of factors that result in adult pattern. A. Desiccation, B. Predation, C. Intraspecific competition, C´.Interspecific competition. (Reproduced from Hui & Moyse, 1987, the original was from Connell, 1961b).

More detailed distribution studies have been conducted by recent researchers and provide further insights. In a study of larval recruitment and adult abundance for *S. balanoides*, a strong density-dependent mortality shaped the patterns of adult abundance (Jenkins *et al.*, 2008). The relationship, however, was not simply linear. Under lower recruitment, the recruit-adult relationship is positive whilst in contrast, under high recruitment, the relationship is negative. The authors provide two initial hypotheses concerning the recruit-adult relationship. One is that increases in recruitment lead to increases in the local adult population size, while the other is that populations are recruitment-limited only when post-settlement mortality is entirely density-independent. In this study both assertions were seen to be correct at some point. Although the relationship between adult density and recruit density was not clear, there was a positive correlation between recruit density and instantaneous mortality at the early stage of settlement. Other factors observed to impact the settlement patterns include, but are not limited to: latitudinal gradient (Okuda *et al.*, 2009), seasonality (Fukaya *et al.*, 2010), scale of sampling (Jenkins *et al.*, 2001), and climate change (Svensson *et al.*, 2006).

1.2.2 Predators of barnacles

Barnacles have several recognised predators, and these predator-prey relationships also provide channels of parasite transfer between hosts. The aim of this section is to review the known predators of barnacles and to try to outline potential pathways for multi-host parasite *e.g.,* digenean life-cycles through initial barnacle infection to infection of the final host, *e.g.* seabirds. A summary of the major non-avian predators is listed in appendix 3.

From the literature cited in appendix 3, most predators which impact on the distributions of intertidal barnacles are the gastropod molluscs, notably *Nucella* Röding, 1798 spp. Other predators either exist in low numbers, such as nudibranchs; or prefer larger prey, *e.g*., sea stars. The importance of some predators is hard to estimate, for example crabs and fish, which only come and feed at high tide making their direct observation difficult. The main route of digenean infection, in intertidal systems, is through the feeding activity of wading birds, however, detailed pathways are largely hypothetical. Except for a rare example of ruddy turnstones feeding directly on barnacles (Hadley & Castle, 1940), waders feeding directly on barnacles are not documented. A more probable pathway, however, is through the trophic web in the intertidal zone and the ability of digeneans to use paratenic hosts can maintain or increase transmission (Marcogliese, 2007). The more mobile predators such as birds, crabs, and fish act as secondary carnivores but also eating species that are grazers and suspension feeders. For example, birds will take crabs and crabs will take whelks (Little & Kitching, 1996). Under such circumstances, one can deduce that if the major predators of intertidal barnacles consume a parasitized barnacle, the infective stages inside can be carried to a whelk and enter the trophic web. This might contribute to the successful transmission of digeneans to their definitive host, waders. Further evidence, however, is needed to support this hypothesis.

1.3 Barnacle parasites

Several barnacle parasites have been observed and reported in the literature and the following provides an overview of those previously described. It should be noted in reading this, however, that often the lines that demarcate free-living species from symbionts and mutualists / commensals from parasites are poorly defined. It is also important to note that many species may switch between apparent life modes *e.g.,* facultative parasites.

Regarding associated and/or parasitic fauna, halacarid mites were found on the outer surface of the shell plates of both barnacle species in Colston's work (2012), with at least four species of mite being found. There were also planarians, nematodes and polychaetes found to be associated with the barnacles. Prior to this more recent work, there have been surprisingly few references concerning the

parasitic or associated fauna of barnacles. According to Arvy and Nigrelli (1969), which is the most comprehensive review so far, several groups were described from barnacles. These included: gregarines, ciliates, trematodes, isopods, fungi, lichens and turbellarians. There was also a report of a rhizocephalan parasitizing *Chthamalus* Ranzani, 1817 and *Balanus* Costa, 1778 (see Deichmann & Høeg, 1990).

In terms of obligated parasite, several categories were reported, including protozoans, digeneans, cestodes, and crustaceans (Belopol'skaya, 1953; Arvy & Nigrelli, 1969; Irwin & Irwin, 1980; Williams *et al.,* 1981; Zaben, 1988; Blower & Roughgarden, 1989; Southward, 2008; Colston, 2012; Fong *et al.*, 2019). Colston (2012) reported two types of gregarine chromistans (Alveolata, Apicomplexa) from the intestines of *S. balanoides* and *A. modestus* (0.47%; n= 3 / 633). One type of gregarine was considered to be in the genus *Cephaloidophora* Mawrodiadi, 1908, and the other type is most likely to be in the genus *Pyxinioides* Tregouboff, 1912. Ectocommensal or free-living ciliated protozoans were also observed on the body surface and shell plates of both barnacle species, as were at least two different species of sessile peritrich.

In terms of metazoan parasites, platyhelminths comprising digenean cysts and cestode larvae were found by Colston (2012) within the body cavity of both barnacle species. Metacercarial cysts were commonly found around the intestine of both types of barnacles. Encysted larval cestodes (cysticercoids) were found in the outer intestinal tract of *S. balanoides* only (7.59%; n= 61 / 803). Five different forms of cysticercoids were found and were speculated to belong to the order Cyclophyllidea van Beneden in Braun, 1900 and the families Dilepididae Railliet & Henry, 1909 and / or Hymenolepidae Fuhrmann, 1907. Type 1 cestode had 10 rostellar hooks. Type 2 cestode had at least 13 rostellar hooks. Type 3 cestode had 22 rostellar hooks and was identified as *Acanthocirrus retrirostris* Krabbe, 1869. Type 4 cestode had 9 rostellar hooks. Type 5 cestode had 24 rostellar hooks and 6 spinlets.

The presence of larval cestodes was also reported in earlier reports, *i.e.*, by Williams *et al.* (1981) and Belopol'skaya (1953). Cysticercoids of *Acanthocirrus retrirostris* (Cyclophyllidea, Dilepididae) were found in *S. balanoides* with opercular apertures exceeding 2.25 mm (Williams *et al*., 1981). Belopol'skaya (1953) described another three species of cysticercoids in *S. balanoides*: *Fimbriarioides intermedia* Fuhrmann, 1913; *Anomotaenia clavigera* Krabbe, 1869; and an unidentified species. A more recent study provided a possible explanation for the interesting phenomenon that larval cestode can only be found in host larger than certain size. That is body size of the host as well as their parasites constrain the demographic parameters of the parasite community. In other words, there was optimal size of parasite and host for the range of parasite species that can invade and establish of any host species (De Leo *et al*., 2016). Further evidences though, are needed for this inference.

The following describes in more detail some of the key groups of obligate parasites reported from barnacles.

1.3.1 Gregarine parasites of barnacles

Gregarines are obligate unicellular parasites that infect the intestines, reproductive organs and body cavities of a variety of invertebrates and are reported several times in barnacles (Arvy & Nigrelli, 1969). In all, there are about 1650 described species (Rueckert *et al.*, 2011). Aquatic gregarines belong to the chromistan phylum Myzozoa Cavalier-Smith et Chao, 2004, subphylum Apicomplexa, infraphylum Sporozoa Leuckart, 1879, class Conoidasida, subclass Gregarinasina Dufour, 1828, order Eugregarinorida Léger, 1900 (World Register of Marine Species, WoRMS). These are common parasites of arthropods and annelids and they possess a life-cycle consisting of several different stages, with some species having multiple hosts. Information regarding gregarine life cycle is provided in appendix 4.

Several species of gregarines are reported in barnacles. According to Arvy and Nigrelli (1969), the following genera of gregarines have been reported in barnacles of the genus *Balanus* spp*., Chthamalus* spp*., Pollicipes* sp. Leach, 1817 and *Capitulum* Gray, 1825 (syn. *Mitella*)*.* These gregarines include the genera *Gregarina* Dufour, 1828*, Nematoides* Mingazzini, 1891*, Cephaloidophora* Mavrodiadi, 1908*, Frenzelina* Nowlin et Smith, 1917*, Pyxinioides* Trégouboff, 1912 *and Bifilida* Tuzet et Ormières, 1964. According to Henry (1983), eight species of barnacles were found to be affected and seven gregarine species belonging to the genera *Cephaloidophora, Pyxinoides* and *Gregarina* were involved. Negative effects of gregarines for barnacles are possible, such as a delay in the release of nauplii (Barnes, 1953) and, necrosis and desquamation of gut epithelium (Arvy & Nigrelli, 1969).

1.3.2 Digenean parasites of barnacles

Until now only one digenean species has been reported in barnacles, which is *Maritrema gratiosum* (Microphallidae) (Hadley & Castle, 1940; Ching, 1978; Irwin & Irwin, 1980; Williams *et al.,* 1981; Carrol *et al.*, 1990; Zaben, 1988; Galaktionov & Bustnes, 1999; Sari & Malek, 2000; Colston, 2012). The digenean microphallids belong to a speciose family of digenean flukes. The taxonomy of the family Microphallidae Ward, 1901 has a long history, with the Microphallidae being initially recognized as a subfamily in the family Heterophyidae Odhner, 1914 (Deblock, 2008). Fundamental research concerning the excretory system of the larval form, however, changed this concept (Rothschild, 1937). Flame cells and the related collecting ducts and an excretory bladder consisting the excretory system of digenean, and the flame cell patterns in larval and adult digenean can be applied to separate groups among different taxonomic levels (Dawes, 1968). The patterns, however, is often difficult to discern and more readily seen in live specimens. In Rothschild's report, the basic flame cell formula was identified among cercariae of *Maritrema* Nicoll, 1907; *Microphallus* Ward, 1901;

Levinseniella Stiles et Hassall, 1901 and *Spelotrema* Jägerskiöld, 1900. Rothschild stated that the members of the Microphallinae have xiphidiocercariae which develop in sporocysts, possess a "mesostoma" type of excretory system and encyst in arthropods. In contrast, members of the Heterophyidae and Opisthorchidae have pleurolophocercous cercariae, develop in rediae, possess a "stenostoma" type of excretory system and encyst in fish. The distinction between these two groups, therefore, appeared clear. The author also mentioned that the larval forms of Lechithodendriinae Loss, 1896 and Pleurogenetinae Loss, 1899 are similar to those of *Maritrema*, as is their life-cycle. It is now generally accepted that the Microphallidae and the Heterophyidae + Opisthoorchidae are two distinct groups. The anatomy of the genitalia, specifically the presence of a cirrus sac and eversible cirrus, points to the ancestry of plagiorchids for microphallids (Deblock, 1971).

The genus *Maritrema* was established by Nicoll in 1907, and later in 1909 the same author erected the subfamily Maritreminae. In 1923, however, he suppressed his own subfamily and included *Maritrema* in the subfamily Microphallinae Ward, 1901 (Rankin, 1939). The genus *Spelotrema* Jägerskiöld, 1900 was raised during the study of *Levinseniella* Stiles et Hassall, 1901 by Jägerskiöld in 1901 (Rankin, 1940). Baer (1943) reduced *Spelotrema* as being synonymous with *Microphallus* Ward, 1901 and separated species possessing a cirrus sac into the family Maritrematidae and then species without a cirrus sac into the family Microphallidae. Cable and Kuns (1951) suggested that, from an evolutionary point of view, the reduction of a protrusible cirrus and cirrus sac would be accompanied by the development of corresponding accessory structures to facilitate copulation and therefore, a diverse array of different copulatory organs is seen in the Microphallidae. Apart from the structure of copulatory organs, the morphology of the microphallids is rather homogeneous, so these authors did not agree with Baer's point of view (1943). Instead, they proposed only one family, the Microphallidae which contains: *Maritrema* Nicoll, 1907, *Microphallus* Ward, 1901, *Gynaecotyla* Yamaguti, 1939, *Levinseniella* Stiles et Hassall, 1901, *Carneophallus* Cable et Kuns, 1951 (syn. current *Microphallus*), *Spelophallus* Jägerskiöld, 1908 (syn. *Microphallus*), *Microphalloides* Yoshida 1938, and *Pseudospelotrema* Yamaguti, 1939 (Cable et Kuns, 1951). Deblock *et al.* (1961) made several genera synonymous with *Maritrema*, namely *Mritreminoides, Odhneria, Numeniotrema, Pseudomaritrema, Pseoduspelotrematoides*. Etges (1953) also made *Pseudospelotrema* synonymous with *Maritrema*; and Baer (1943) made *Maritreminoides* a synonym of *Pseudospelotrema* (Ching, 1963). According to Deblock (1971), who provided the most comprehensive diagnostic key for the Family Microphallidae, the originality and diversity of copulatory apparatus is of primary importance to differentiate members in this family, and the second important character is the configuration of vitellaria. Other characters, e.g. sucker, digestive tract, position of the genital glands, extensions of the uterus, excretory formular, arrangement of the prostate gland and *pars prostatica*, metraterm, etc, might have less value in higher taxonomy but still useful in genus level.

The members of the family Microphallidae are parasites of vertebrates, especially seabirds (Charadriiformes, Lariformes, Anseriformes). Marine molluscan and crustaceans are common first and second intermediate hosts of Microphallidae (Etges, 1953). The taxonomy of the family Microphallidae Ward, 1901, its ancestors, and its relationship downstream including the genus *Maritrema* Nicoll, 1907, is shown in Fig. 1.16. According to the information obtained from World Register of Marine Species (WoRMS), there are eight subfamilies within the family Microphallidae, and the genus *Maritrema* is positioned within the subfamily Maritrematinae Deblock et Heard, 1970. There is another version of the classification of Microphallidae proposed by Deblock (2008) was provided in appendix 5. This latter classification system, however, has not been formally accepted.

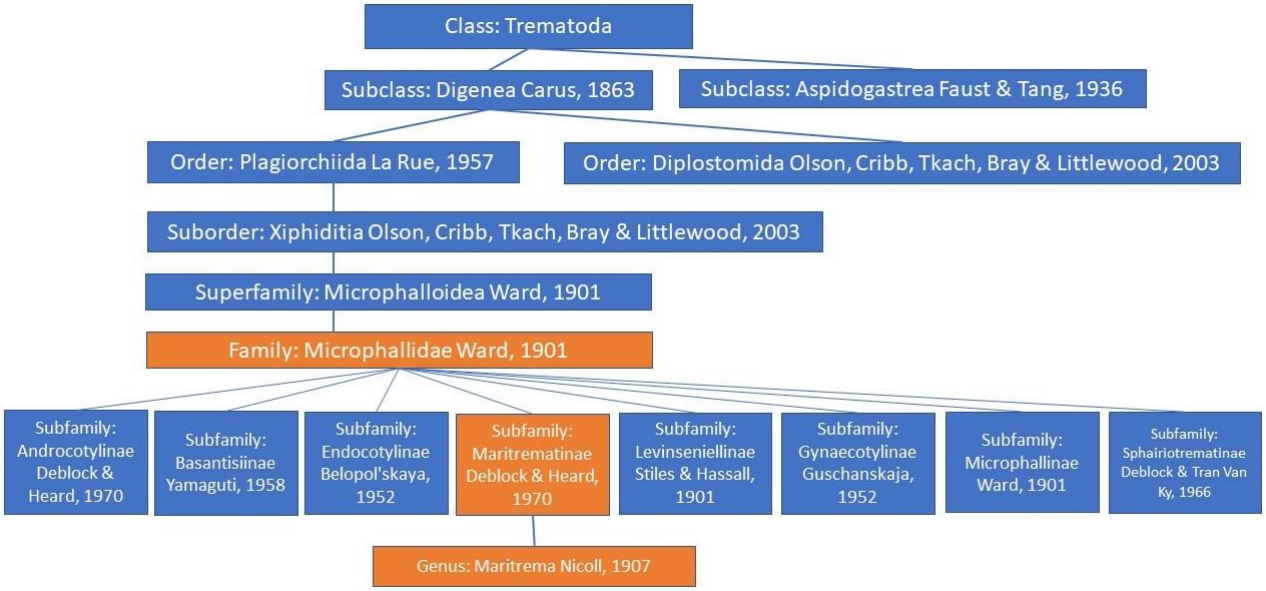


Figure 1.16. Taxonomic position of the genus *Maritrema* Nicoll, 1907 in the Class Trematoda. Extracted from the WoRMS online database and from Olson, 2003.

The genus *Maritrema* Nicoll, 1907 is the type-genus of the subfamily Maritrematinae, and has defining characters of a linguiform body, post-ovarian testes, intercaecal cirrus sac, eversible cirrus, simple genital pore sinistro-lateral to ventral sucker, medium to dextral ovary, post-caecal uterus, and most significantly, a horseshoe-shaped or ring-shaped vitellarium close to the margin of the hind body (Fig. 1.17).

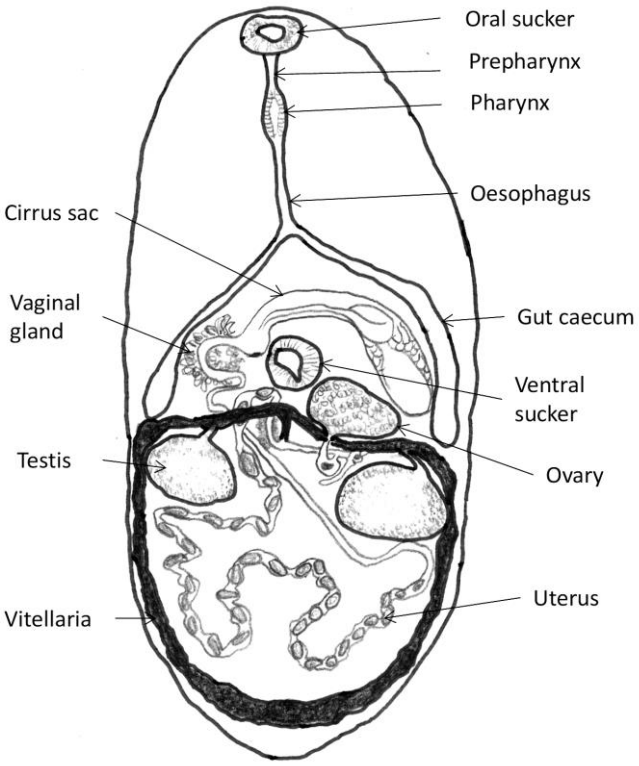


Figure 1.17. Adult *Maritrema gratiosum* Nicoll, 1907, ventral view. Redrawn from Hadley & Castle (1940).

Speciating members belonging to the genus *Maritrema* on morphology can be difficult because of the general similarity of features. Several "groups" within this genus are distinguished, however, based on prominent morphological features, for example the "eroliae complex" and the "acadiae group". Understand these groupings can help researchers not only to diagnose the species correctly, but also to find the affiliations of the target parasite with its related species. This regard will be important to understand the evolution of morphology, biology or even pathogenicity of the target parasite, as these traits can be related to closely related species. Furthermore, correct speciation based on morphological ground will be vital for molecular phylogeny, because current molecular information for the genus *Maritrema* is fragmented and incomplete. A proper molecular phylogeny inference for members in this genus or even in the family Microphallidae will have to base, at least partially, on correct morphological diagnosis.

The eroliae complex as defined by Deblock and Canaris (1992) include species that possess an eversible cirrus with spines. This group included approximately 19 species with a morphology like that of the Japanese species *Maritrema eroliae*, Yamaguti, 1939. The eroliae complex is further classified into three groups by their body size, or into five groups by their relatedness: namely the "*patulus", "eroliae", "urayasensis", "echinocirrata",* and "*macropharynga"* groups (Deblock & Canaris, 1992). These *Maritrema*, however, possesses a thorny cirrus, while the remaining species possess a glabrous

cirrus of various lengths and thicknesses. The “acadiae group”, also first described by Deblock and Canaris (1992), includes a small group of *Maritrema* that possess an elongated fusiform body and small cirrus sac. These included: *Maritrema acadiae* Swales, 1933; *Maritrema longiforme* Kifumed *et al.*, 1972; *Maritrema opisthometra* Leonov, 1958; *Maritrema paracadiae* Ching, 1974; *Maritrema prothometra* Deblock et Heard, 1969; and *Maritrema chiriacae* Deblock, 1975. The acadiae group was also mentioned by Cremonte and Martorelli (1998) who stated that the species they had described, *Maritrema orensensis* Cremonte et Martorelli, 1998 shared similarity with several species that possessed a simple genital atrium and a cirrus without spines, such as *M. acadiae* Swales, 1933; *Maritrema laricola* Ching, 1963 (syn. *M. longiforme*); and *M. paracadiae* Ching, 1974. They did not include *Maritrema gratiosum* Nicoll, 1907 in this group but they stated that their species was most similar to *M. gratiosum* (see Cremonte & Martorelli, 1998). Later, Rauque *et al.* (2013) on describing their new species *Maritrema patagonicum* Rauque, Flores et Brugni, 2013, stated that it also belongs to the “acadiae group”. They defined this group as having an unarmed cirrus and have decapods as their second intermediate host and included the species listed above except *M. gratiosum*.

Some detailed morphology of the genitalia also has been applied for diagnosis, for example, a seminal vesicle of the “*subdolum* type” (without obvious *pars prostatica*), or species with a long cirrus sac whose bottom is introduced between the ovary and right caecum (*gratiosum* and *acadiae*), or the male genital duct of the “*linguilla* type” (with differentiated *pars prostatica*) (Deblock, 1975). Alternatively, different types of the cirrus sac and the accessories are mentioned: the “*subdolum* type” (distal part of the seminal vesicle folds itself and continues without interruption as an ejaculatory duct, often forming a loop, which represent the invaginated cirrus and with no *pars prostatica* visible) versus the “*gratiosum* type” (an ovoid seminal vesicle carries a short narrow canal distally and falls into the ampullary dilation of the *pars prostatica* and another short canal connects to the *pars prostatica* with sinuous ejaculatory duct, and having a thick wall, representing the invaginated cirrus) (Deblock, 1971).

Although members of the genus *Maritrema* are, in general, avian parasites, some species are found in mammals or even in reptiles; these are not listed here but are provided in appendix 6. Furthermore, some *Maritrema* species are characterized by uniquely abbreviated life-cycles. For example, *Maritrema oocysta* Lebour, 1907 encysted in their first intermediate host *Peringia ulvae* Pennant, 1777*, Littorina rudis* (syn. *L. saxatilis*) and *Littorina obtusata* Linnaeus, 1758 (Lebour, 1907); *Maritrema murmanica* Galaktionov, 1989 were found encysted or unencysted in *L. saxatilis* (Galaktionov, 1989); and *Maritrema syntomocyclus* Deblock et Tran Van Ky, 1966 were found encysted in *Hydrobia acuta* Draparnaud, 1805 (Deblock and Tran Van Ky, 1966b). Whilst most are marine, it should be recognized that not all *Maritrema* species are marine species. According to Kinsella and

Deblock (1994) and Kudlai *et al.* (2015), *Maritrema* spp. which have a freshwater life-cycle, are summarized in appendix 7.

1.3.3 Cestode parasites of barnacles

In Colston's research (2012), at least five different cestode larvae with differing morphologies were found attached to the outer intestinal tract of *S. balanoides* found in Scotland. One cysticercoid was diagnosed as *A. retrirostris*. The remaining forms were diagnosed as most likely belonging to the family Dilepididae or Hymenolepidae within the Cyclophyllidea. Knowledge of cestodes in barnacles is scant. Here some general aspects of the life-cycle of cyclophyllideans and some relevant morphological features of the cysticercoid stage found in barnacles are discussed. A life cycle for cestode using crustacean as intermediate host was provided below (Fig. 18).

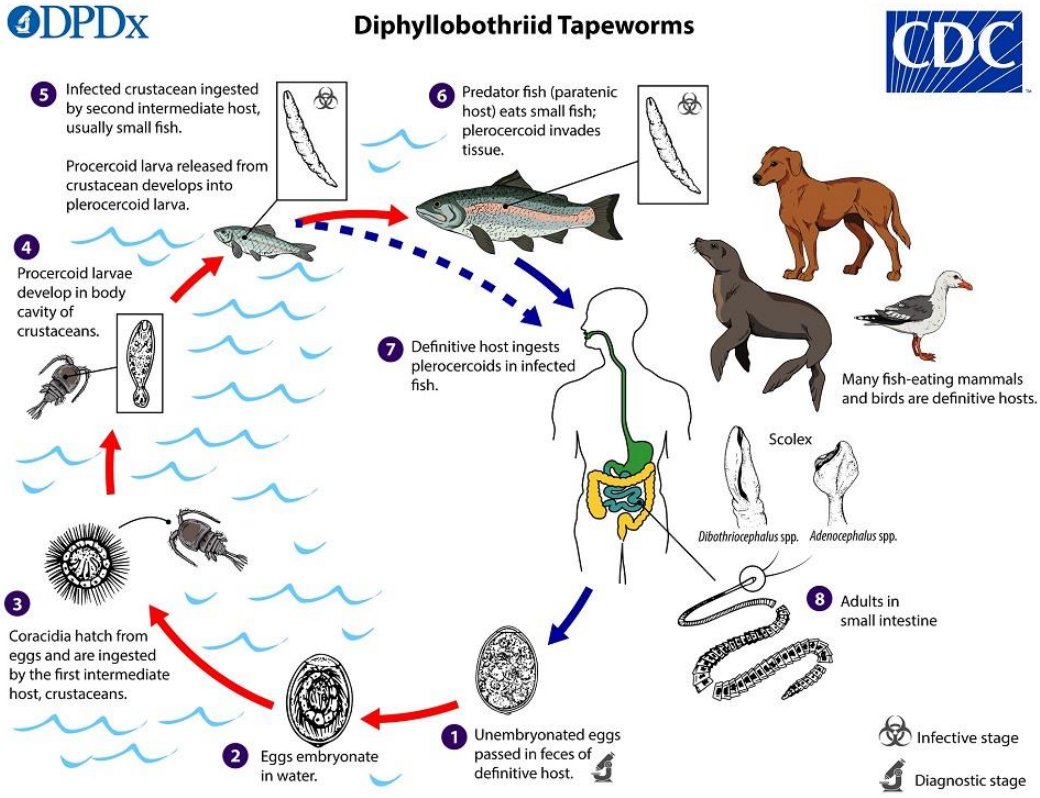


Figure 1. 18. The life cycle of Diphyllobothriid cestode. From webpage of Centers for Disease Control and Prevention of USA. https://www.cdc.gov/parasites/diphyllobothrium/biology.html.

The morphological identification of cestode species is usually based on the features present in adults. Identification based on larval morphology is not easy and often depends on the experimental development of adult stages in the final host. For adult morphology, members of the Cyclophyllidea possess a scolex, which bears four muscular suckers and an apical extension (rostellum) often with hooks. The vitellaria are compact and unpaired (Yamaguti, 1958; Caira & Reyda, 2005). Adult marine cyclophyllideans are generally parasitic in the digestive tract of final hosts and are restricted to birds

of the following orders: Charadriiformes, Gruiformes, Ciconiiformes, Podicipediformes, Pelecaniformes, Falconiformes and Sphenisciformes (Caira & Reyda, 2005). There are three stages in their life-cycle: the first stage being the hexacanth larva within the egg, the second stage being the cysticercoid larva in intermediate hosts, and the third stage comprising adults in the avian final host. For this group, intermediate hosts include: polychaetes, amphipods, copepods, branchiopods, cirripedes, euphausids and gastropods (Caira & Reyda 2005). According to Yamaguti (1958), the key identification traits for the family Dilepidae are: genitalia single or double, genital pores unilateral or alternating regularly or irregularly. Testes are usually more than four. Gravid uterus is more or less saccular or reticular, and sometimes breaks down into egg capsules containing one egg or more, or provided with a paruterine organ. The key identification traits for the family Hymenolepidae are: proglottids are usually longer than wide, inner longitudinal muscle bundles well developed and forming two layers. Usually has single set of reproductive organs. Genital ducts are usually dorsal to excretory systems, testes are 1, 2, 3 or more but not numerous, in dorsal medulla. External and internal seminal vesicles are usually present. Genital pores are usually unilateral. Ovary is in ventral medullar, medium, sub-medium or lateral, lobed or not. Uterus commonly saccular, rarely reticular or breaking down into egg capsules. Vitelline gland is usually post-ovarian. Eggs have three membranes.

Although there has been little research on cestodes in barnacles, some studies have been conducted for other marine cestode species which utilize brine shrimps (*Artemia* spp. Leach, 1819) as intermediate hosts. In the report of Vasileva *et al.* (2009), cysticercoids of two cestodes, namely *Fimbriarioides tadornae* Maksimova, 1976 and *Branchiopodataenia gvozdevi* Maksimova, 1988 (both in the Hymenolepididae), were identified morphologically. The authors combined the research results of Georgiev *et al.* (2005) and proposed identification keys for cestodes from *Artemia* spp. from the western Mediterranean. The cysts of both species are oval to lemon-shaped and with a thick wall consisting of three layers. The important indices measured include: length and width of cyst, scolex, rostellum sheath, rostellum and rostellar hooks and the diameter of suckers. The number and shape of rostellar hooks, the length and shape of blade, distance between handle-tip and guard-tip and distance between blade-tip and guard-tip were also measured. The other important characters include the presence of an outer larval envelope and the modification of the cercomer (Vasileva *et al.,* 2009). Morphology of the rostellar hooks for larval cestode can be important identification characters and a schematic drawing of rostellar hooks is provided in figure 19.

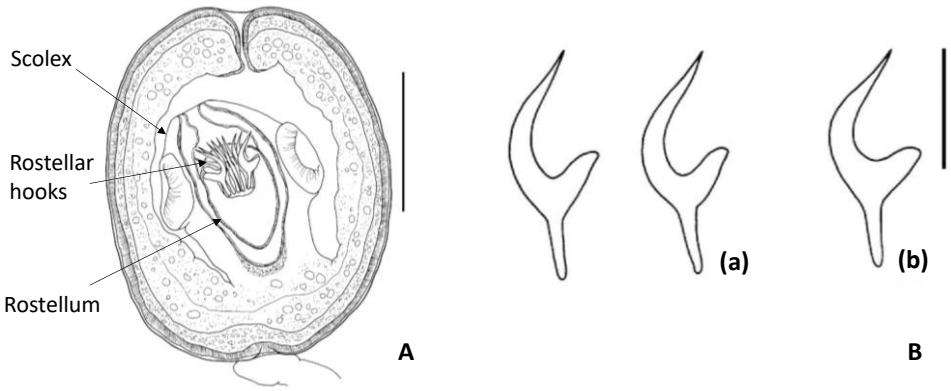


Figure 1. 19. *Branchiopodataenia gvozdevi* (Maksimova, 1988) Bondarenko et Kontrimavichus, 2004 from *Artemia parthenogenetica*, Bras del Port, Spain (a) and *A. franciscana*, River Ebro Delta, Spain (b). A. Cysticercoid. B. Rostellar hooks of cysticercoids. Scale-bars = 100 µm (A), 20 µm (B). (Reproduced from Vasileva *et al*., 2009, p. 145).

In the *Artemia*-cestode systems, cestode infection seems to be closely related to physiological changes in the brine shrimp. According to research into saltpan brine shrimp (*Artemia parthenogenetica* Bowen & Sterling, 1978) (see Sánchez *et al.*, 2006), it has been shown that red brine shrimp have a more important role for the circulation of avian cestodes than transparent shrimp as the red brine shrimp have a significantly higher intensity and abundance of cysticercoids. Interestingly, red brine shrimp were found to have a higher lipid content, and infected brine shrimp were positively phototactic rather than photophobic. These characters all favour the choice of infected shrimp for predators. Also, the water concentration of saltpans makes cestode eggs float naturally, which could lead to higher uptake rate for positively phototactic individuals. Distribution studies have also been conducted in the *Artemia*-cestode system. In the study of Sánchez *et al.* (2013), the relationship between prevalence of cestodes in *Artemia* spp. and the abundance of avian final hosts was investigated. The team found that seasonal changes in bird counts were weak predictors of cestode dynamics. Only particular species of cestode in *Artemia* were associated with the number of their bird hosts. Individual cestode species abundance, however, showed a marked seasonal pattern. There could be multiple reasons for the deviation from the original hypothesis, which was that the density and biomass of final hosts is a potential driver of the variation of cestode distribution within brine shrimp. Firstly, the precision of bird counts could lead to bias, also the density of brine shrimp populations within the sampling saltpan. Moreover, some bird species selectively remove infected shrimp. This behaviour will counteract the effect of bird density on transmission. The third possibility is that the brine shrimp population undergoes seasonal change naturally. Other environmental factors, such as temperature, salinity, and dissolved oxygen, can also affect the distribution pattern of cestodes. On the other hand, multiple infections were more common than expected, which indicated

that the cestode community is not randomly structured. In conclusion, the author suggested that although the prevalence of cestodes in brine shrimp is weakly correlated with bird count, the high prevalence and richness of cestodes in brine shrimp populations can be an indication of high diversity and abundance of birds in an ecosystem. The author also suggested that prevalence of cestodes in brine shrimp is a positive indicator of bird conservation efforts. Although these interesting suggestions and hypotheses might be or not be applicable to other similar parasite-host systems, at least their study revealed that the pattern of cestode distribution within intermediate hosts is an outcome of the interaction of many factors.

1.3.4 Crustacean parasites of barnacles

A single isopod species *Hemioniscus balani* Buchholz, 1866 has been reported to infect barnacles (Crisp, 1968; Blower & Roughgarden, 1988; Blower & Roughgarden, 1989; Arnott, 2001; Southward, 2008; Fong, 2016; Fong *et al.*, 2019). Infection by *H. balani* results in the castration of the adult sessile barnacle. The host species is simultaneously hermaphroditic, while the parasite is a protandrous hermaphrodite. The life-cycle of *H. balani* is illustrated in Fig. 1.20. Infection of more than a single specimen of *H. balani* is rare; a single specimen, however, is sufficient to result in the complete castration of the host barnacle host (Blower and Roughgarde, 1989). This isopod can possess the same biomass as the barnacle body itself in infected barnacle populations. Infection results in a decrease in barnacle reproduction per area and thus has an impact on barnacle populations (Fong *et al.*, 2019).

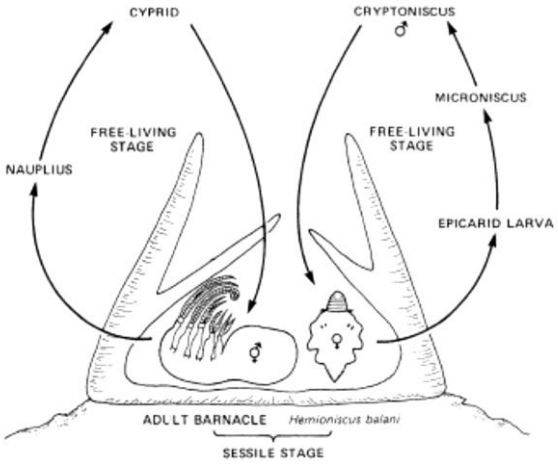


Figure 1.20. Host *Chthamalus fissus* Darwin, 1854 or *Chthamalus dalli* Pilsbry, 1916 and parasite (*Hemioniscus balani*) life-cycles. The sessile stage of *H. balani* occurs within the shell of an adult barnacle which is attached to rock in the intertidal zone. Both hosts and parasites have a free-living oceanic stage (Reproduced from Blower & Roughgarden, 1989).

1.4 Larval digeneans in littorinid molluscs found in Britain

There are numerous studies concerning aspects of larval digenean infection of marine molluscs. Here I will focus only on Digenea infecting molluscs of the family Littorinidae Children, 1834 around British coasts. Littorinids are important first intermediate hosts of *Maritrema* spp., *Microphallus* spp. and many other Digenea. In the UK, there is only one genus in the family Littorinidae, namely *Littorina* Ferussac 1822. Four species and several subspecies, however, exist in this genus. The four species are *Littorina littorea* Linnaeus, 1758*, L. littoralis* (accepted as *Littorina obtusata littoralis* Linnaeus, 1758)*, L. neritoides* (accepted as *Melarhaphe neritoides*, Linnaeus, 1758) and *L. saxatilis* (Graham, 1988). The four species and the related subspecies can be differentiated by several characters, such as their typical distribution along the shore, shell shape, size, height, and thickness, aperture width and pigmentation on head and tentacles, *etc*. For detailed identification keys for these four species, one can consult the work of James (1968). According to James (1968, 1969), 22 species of larval digenean were identified in *Littorina* species collected in Pembrokeshire and Cardigan Bay, Wales, UK. The author provided the identification keys and images for these larval digeneans and these works are probably the most comprehensive study for larval digeneans infecting *Littorina* so far. The 25 species of larval digenean described by James are provided in Table 1.1. In addition to the species listed in the table, Popiel (1976b) also described *Cercaria littorinae saxatilis V* from *L. saxatilis rudis* Maton, 1797 (accepted as *L. saxatilis*) from Wales.

Table 1. 1. Larval digeneans from *Littorina* species in Pembrokeshire and Cardigan Bay, Wales, UK described by James (1968, 1969)

Cercaria brevicauda Pelseneer, 1906	*Cercaria roscovita* Stunkard, 1932
Cercaria emasculans Pelseneer, 1906	*Cercaria ubiquitoides* Stunkard, 1932
Cercaria lebouri Stunkard, 1932	*Crytocotyle linguan* Creplin, 1825(=*Cercaria lophocerca* Lebour, 1911)
Cercaria linearis Lespes, 1857	*Himasthla leptosoma* Creplin, 1829 (cercaria of *Echinostomum secundum* Nicoll, 1896)
Cercaria littorinae obtusatae Lebour, 1911	*Himasthla littorinae* Stunkard, 1966
Cercaria littorinae Rees, 1936 (=*C. buccini*, Lebour, 1911 sensu Rees, 1935)	*Microphallus claviformis* Brandes, 1881
Cercaria littorinae saxatilis I James, 1968	*Microphallus pygmaeus* Levinsen, 1881 (=*Cercaria littorinae rudis* Lebour, 1906
Cercaria littorinae saxatilis II James, 1968	*Microphallus similis* Jagerskiold, 1900(=*Cercaria ubiquita*, Levour, 1907
Cercaria littorinae saxatilis III James, 1968	*Notocotyloides petasatum* Deslongchamps, 1824
Cercaria littorinae saxatilis IV James, 1968	*Parapronocephalum symmetricum* Belopolskaia, 1952
Cercaria of *Spelotrma excellens* Nicoll, 1907 (*sensu* Lebour, 1911)	*Parvatrema homoeotecnum* James, 1964
Cercaria parvicaudata Stunkard et Shaw, 1931	*Podocotyle atomon* Rudolphi, 1809

1.5 Records of some digenean and cestode parasites of Charadriiformes Huxley, 1867 and Laridae Rafinesque, 1815 birds

A large number of digenean and cestode parasites of birds belonging to the Charadriiformes (including waders and gulls) birds have been recorded and these are summarised in Appendix 8. In addition to the host-parasite records, some ecological patterns were also observed. The reasons behind these observed patterns, *i.e*., the presence of one dominant species and 4-5 associate species, and the high abundance or intensity of microphallids in some charadriiform or lariform birds, is rather obscure. One possibility comes from the concept of "generalists" and "specialists". Canaris *et al.* (2003) mentioned that in the United States, a large majority of helminth parasites in charadriids are generalists. Helminths lacking specificity at the adult stage (generalist) might more easily be present in various hosts, while highly specific parasites are more likely to be present in a particular species with high intensity. Another possibility is based on trophic webs. The success of a cestode or digenean life-cycle depends on trophic webs; based on the presence of appropriate intermediate and definitive hosts. The high prevalence and abundance of microphallids in charadriiforms might be related to their foraging habitats, including those of rocky shores, mud flats and salt marshes (Forrester *et al*., 2012) where marine microphallids can complete their life-cycle. Furthermore, the main diet of these waders consists of invertebrates, crustaceans, molluscs and insects (Forrester *et al*., 2012), which might serve as candidate second intermediate hosts for various microphallids. Canaris and Kinsella (2007) pointed out that, in general, digenean species are dominant in marine habitats while cestodes are dominant in freshwater habitats. They also pointed out that the lack of habitat for some direct cycles in nematodes and the lack of intermediate hosts for acanthocephalans, nematodes and digeneans, can explain their low abundance at certain localities. It is plausible that the habitats characteristics of intermediate and final hosts contribute most to the observed infection patterns among shore birds, but the causes are yet to be validated. Close observations based on local distribution, feeding behaviour and diet data of candidate hosts, and sophisticated surveys of the distribution of second intermediate hosts are needed, as these are the first step to reveal host-parasite dynamics, which can provide clues for the discovered patterns.

1.6 Distribution study and morphometrics as a tool to elucidate parasite ecology

In Colston's work (2012), a digenean parasite, *Maritrema arenaria* Hadley et Castle, 1940 (accepted as *M. gratiosum*), had been recognized as the most "common" parasites in barnacle host collected from Scotland. In her work the definition of "common parasite", although the author didn't specify, can be referred to "numerical dominance", and this fact can be inferred from her data for abundance. The author although describing a phenomenon of dominance of a species of parasite in a

particular host-parasite system, didn't try to elucidate the cause of this dominancy. The concept of dominant parasite in a host-parasite system is not as strait forward as it looks superficially. This dominancy can base on biomass or on count data (abundance), and the two aspects can both be quantified (Poulin *et al*., 2008, Lambden & Johnson, 2013). Furthermore, in parasitology, dominance is the contrary of evenness, and both terms can be related to a pattern of "core and satellite species" in parasite communities. These parameters can both be quantified as well (Poulin, 1995). A general pattern of a few numerically dominant species and many low abundance species in parasite communities had been revealed in a research based on metadata analysis of fish host (Poulin *et al*., 2008), The cause of this pattern, however, is complicated and is not easy to discover. By analysis large sets of data, Poulin *et al*. (2008) revealed that ratio between numerical abundance of the most and second or most and third species (indices of dominance) in gastrointestinal helminth communities of fish hosts, is not influenced by species richness, mean numbers of parasite per host and taxonomy identity of the dominant species. The dominance indices, however, is related to species interactivity and point to the influence of differences in recruitment rates. In an earlier research about the evenness of parasite communities in different types of hosts (fish, birds and mammal, which also based on metadata analysis), had shown that patterns of evenness (or dominancy) is not the same between different host types (Poulin, 1995). Several questions therefore raised from these pioneer researches: 1. What is the cause of dominancy of a digenean parasite in a crustacean host (barnacle)? 2. is this pattern one of the intrinsic characters of a particular host-parasite system on a particular habitat type (rocky shore)? 3. is the pattern of dominancy a long-term trend? Attempt in the present study is not to analyse large sets of data but try to identify the distribution patterns of parasite populations in a small geographical scale, *i.e*., within km^2. Field-based observational method carried out in rocky shore ecosystems was chosen in the present study. After the patterns being identified, potential factors which might influence the distribution of the target parasite were analyzed. The confounding factors related to the environment in the chosen locality were minimized by a systematic sampling strategy, which will be further explained in chapter three.

This systematic sampling approach also provide a chance to examine whether the target digenean parasite specimens belong to the same species. In Colston's research (2012) a preliminary diagnosis of the digenean specimens collected from Scotland and the morphology by light microscopy and scanning electronic microscopy (SEM) were provided. In her work, however, didn't explore the possibility of morphological plasticity and the potential causes, and didn't confirm whether these specimens belong to the same species. Morphometric techniques are based on numerical measurements of morphological features and have been applied in parasitology for species differentiation (Hernández-Mena *et al*., 2014; Gracenea and Ga´llego, 2017), detecting different morphotypes and / or cryptic species (Cavaleiro *et al*., 2012) and confirmation of single species (Caffara

et al., 2016). These techniques are mostly based on statistics and can be as simple as descriptive to sophisticated analysis. There are many factors can influence the precision of morphological diagnosis, such as fixation (Bakke. 1988), state of maturations (Valero *et al*., 2005), host types and infection locations within the host (Höglund and Thulin, 1992; Martorelli and Ivanov, 1996; Ashrafi *et al*., 2015,), or environment (Shinn *et al*, 2004; Valero *et al*., 2012; Ashrafi *et al*., 2015,). Morphometric technique although can be applied to species diagnosis, it is not practical in most circumstances as large specimens are usually needed and an existing morphological standard based on full ranges of morphological difference is prerequisite (Becerra and Valdecasas, 2004). Final confirmation of the species of specimens collected in present study is therefore based on molecular method. Molecular diagnosis techniques have their own caveat either, because the information of the member in the genus *Maritrema* is incomplete and fragmented. In the present study there was an attempt to fill this gap by providing more molecular data for *Maritrema* specimens collected from different localities in Scotland and New Zealand and generating more data from different molecular markers and conducting phylogenetic analysis. These will be further explored in chapter four.

1.7 *In vitro* cultivation and its applications in helminthology

One of the initial aims of this research is to elucidate the life cycle stages, including confirming known hosts and searching for potential paratenic hosts for *M, gratiosum* on its hot spot, *i.e*., Dunbar. This goal although didn't achieve at the end, ovigerous adults were still obtained via *in vitro* culture method, and this *in vitro* model was proven to be a good model for parasitic fluke with potential wider applications. Understand the natural life history of the members in Microphallidae is, however, the foundation to explore their biology and potential applications in other scientific fields, *e.g*., medical applications. Research into the life-cycle of the genus *Maritrema* Nicoll, 1907 is fragmented but began early on. The information sometimes came from field observations and often needed to be proven experimentally. Rankin (1939) concluded that in general, the life-cycle of *Maritrema* started with miracidia that penetrated molluscs and developed into a sporocyst, and that xiphidiocercariae produced in the sporocyst then penetrated various species of crustacean after which, following ingestion of the crustacean host, they matured in the intestine of vertebrate final hosts, primarily shore birds. This general rule is still true, however moderate differences exist among individual species.

In vitro culture as a technique to provide research materials are more familiar in the field of virology and bacteriology. The related technologies are well developed, for examples, cell culture and media related business. The applications of *in vitro* culture in parasitology especially for macroparasite, on the contrary, are not so common and are only familiar with a few experts, for example, J.D. Smith, the author of "In Vitro Cultivation of Parasitic Helminths (1990)". According to Smith, *in vitro* culture

techniques for helminth have wider application than immunobiology and chemotherapy, instead, it can be a tool for understand fundamental biological phenomenon. He summarized the contribution of these techniques as follow: 1. Replacement of definitive or intermediate hosts. 2. Identification of unknown larval stages. 3. Immunobiology. 4.Biochemistry. 5. Physiology. 6.Chemotherapy. 7. Cell or tissue differentiation (Smith, 1990). The same author also summarized that *In vitro* culture had been applied to several categories of helminths, *i.e*., Digenea, Cestoda, Filariodiea, and Acanthocephala (Smith, 1990). *In vitro* culture techniques had been applied to the members in Microphallidae for several times in the decade, e.g., for the purpose of completing life cycle (Pung, 2009), obtaining adult stage (Fujino *et al*., 1977; Davies and Smyth, 1979; Zaben, 1988), or simply to develop or improve *in vitro* culture conditions (Fredensborg and Poulin, 2005;. Pung *et al*., 2011. West *et al*., 2014). The influence of culturing conditions for the morphology of the adult stage, however, are not studied for Microphallids. *Maritrema gratiosum* had been successfully cultured to ovigerous adult by Zaben (1988), since his work, however, there was no attempt to culture this species. Furthermore, in Zaben's work *in vitro* culture technique was treated as a research topic itself to compare with *in vivo* cultivation, further applications deserve more researches. For examples, for exploiting developmental and reproductive biology, understanding neuro-musculature systems, and potential application for this ecologically important species to the field of pharmacology and morphometric.

1.8 Aims and Objectives

The principal aim of this study has been to gain a better understanding of the parasitic and associated fauna of UK barnacles and to examine the potential interactions that occur between the target digenean parasites and their intertidal crustacean hosts at designated rocky shore locations. A numerical dominance of the digenean was discovered, the cause of this patterns however, was not clear. The distribution patterns of the digenean populations within chosen host populations were considered the outcome of parasite-host interactions, and the potential causes of the discovered patterns were analyzed by means of a systematic sampling strategy. Morphological plasticity of the digenean parasite and the potential causes were also investigated, and final species diagnosis was performed. The aim of this study has also been to investigate aspects of the life history of the target digenean and to complete its life cycle *in vitro*. The *in vitro* cultivation technique also provides a chance to further explore the development of muscular system of the digenean from juvenile to adult and to explore more for the biology of the adult stage.

To summarize, the key objectives of the research reported in this thesis have been to:

1) In the absence of first intermediate hosts of *M. gratiosum* in its hotspot and absence of a clear link between different life-cycle stages, the parasitic fauna of the molluscan hosts and barnacle hosts (*S. balanoides* and *A. modestus*) collected in Scotland of this target species was characterized,

2) In order to elucidate the cause of numerical dominant of *M. gratiosum* in its hotspot, examination of the spatial distribution patterns of *M. gratiosum* in barnacle populations collected in a small-scale rocky shore area was conduct under a systematic sampling strategy. The factors affecting the discovered patterns were analyzed,

3) To achieve final species identification of *M. gratiosum,* morphological and molecular methods were applied. Furthermore, to confirm whether the specimens of *M. gratiosum* collected from different localities in the present study belong to the same species under a morphological ground, a morphometric study was carried out. The detected morphological plasticity and the potential causes of this plasticity were analyzed,

4) Attempt to complete the life-cycle of *M. gratiosum in vitro* in order to obtain ovigerous adults and to examine organs and musculature development of adult forms by means of laser scanning confocal microscopy.

Chapter 2 Faunistic survey of parasites infecting *Semibalanus balanoides* (L., 1767) and some intertidal gastropods in Scotland

2.1 Introduction

According to Galaktionov and Dobrovolskij (2003), the completion of digenean life-cycles relies on the intersection of three factors: the parasite, the host, and the environment. The spatial distribution of parasite populations can be more complicated than that of free-living species because the spatial structuring of a parasitic population is influenced by the spatial distribution of the intermediate and definitive hosts, and only in the zone of overlap between the distribution areas of all hosts can a parasite complete its life-cycle, as illustrated in Figure 2.1.

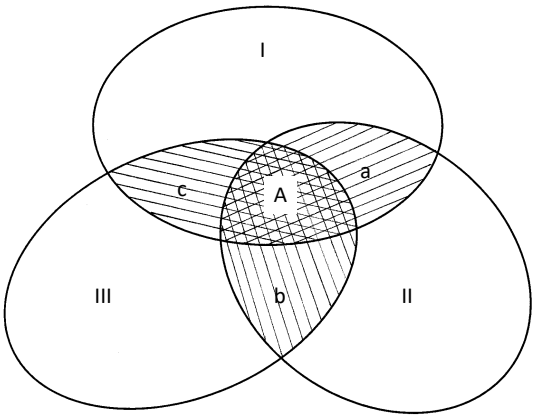


Figure 2.1 Diagram showing the correlation between the distribution area of a parasitic species with a three-host life-cycle and the distribution areas of its hosts. I, II and III, *e.g*., distribution areas of the primary, second intermediate and definitive hosts, respectively. A: the distribution area of the parasitic species; a, b, c, zones of co-occurrence of the host species (redrawn from Galaktionov and Dobrovolskij, 2003; p. 352).

As noted in Chapter 1, knowledge of the parasites of acorn barnacles is surprisingly limited given the ubiquity of these hosts in rocky shore environments worldwide. The most comprehensive review of parasites of barnacles to date was the report of Arvy and Nigrelli (1969). According to their report, gregarines, digeneans, isopods, turbellarians, fungi and lichens were observed to be the major parasites and predators of barnacles. Gregarines were

reported repeatedly in many species of *Balanus*, in *Chthamalus stellatus*, in two species of *Pollicipes* Leach, 1817, and in *Pollicipes polymerus* Sowerby, 1883 (syn. *Mitella polymerus*) (see Table 1 in Arvy & Nigrelli, 1969). According to Stubbings (1975), seven species of gregarine parasites (protists belonging to three genera in the order Eugregarinorida Léger, 1900: *Cephaloidophora* Mavrodiadi, 1908*, Pyxinoides* Trégouboff, 1912 and *Gregarina* Dufour, 1828 have been recorded from several barnacle species. Other protists found on the surface of the barnacle prosoma are probably commensal. Vorticellid colonies and the peritrich ciliate *Epistylis horizontalis* Chatton, 1930 have been recorded in *Semibalanus balanoides* (see Colston, 2012). A single species of isopod, *Hemioniscus balani,* was reported from many species of *Balanus*, from *Chthamalus dalli,* and from *Austrominius modestus* (see Crisp, 1968; Arvy & Nigrelli, 1969; Blower & Roughgarden, 1988, 1989; Arnott, 2001; Southward, 2008; Fong, 2016; Fong *et al.*, 2019). It was also claimed to be the major parasite of *S. balanoides* in western European waters, according to Stubbings (1975). Southward (2008) also mentioned that *S. balanoides* is frequently parasitized by this species and that the prevalence can reach 100%. This isopod, once it has infected a barnacle, will transform from male to female, and feed on the ovarian fluid of the barnacle, resulting in the castration of the host (Blower & Roughgarden, 1988). Knowledge of digenean parasites in *S. balanoides* is comparatively better developed than for other parasitic taxa. The records of generic digeneans in Arvy and Nigrelli's report (1969) are likely to have largely comprised *Maritrema gratiosum* (syn. *Maritrema arenaria*) parasitizing *Balanus balanoides* L., 1767 (syn. *S. balanoides*), except for one report describing parasitisation by *Maritrema linguilla* Jägerskiöld, 1908. Besides this, there are numerous further records of *M. gratiosum* metacercariae in *S. balanoides* (see Hadley & Castle, 1940; Stubbings, 1975; Irwin & Irwin, 1980; Williams *et al.,* 1981; Zaben, 1988; Carrol *et al.*, 1990; Galaktionov & Bustnes, 1999; Colston, 2012).

The life-cycle of *M. gratiosum* is completed in the intertidal zone of rocky shores. Eggs deposited in the intertidal zone are ingested by the first intermediate host, *Littorina* spp. (periwinkles), and hatch inside this host. Within the periwinkle, asexual reproduction occurs and numerous clones of parthenita are produced, cercariae are subsequently released into the intertidal zone, and a short free-living stage begins. These cercariae then penetrate the cirri of the second intermediate host, *S. balanoides* (Irwin *et al.*, 1990), and encyst in its prosoma as metacercariae. The barnacles are eaten by the final hosts, various waders and gulls, within which the metacercariae develop in the small intestine to gravid adults. Adults release eggs, which pass into faeces, and the eggs are thereby resuspended in the water column, and the life-cycle completes (Figure 2.2).

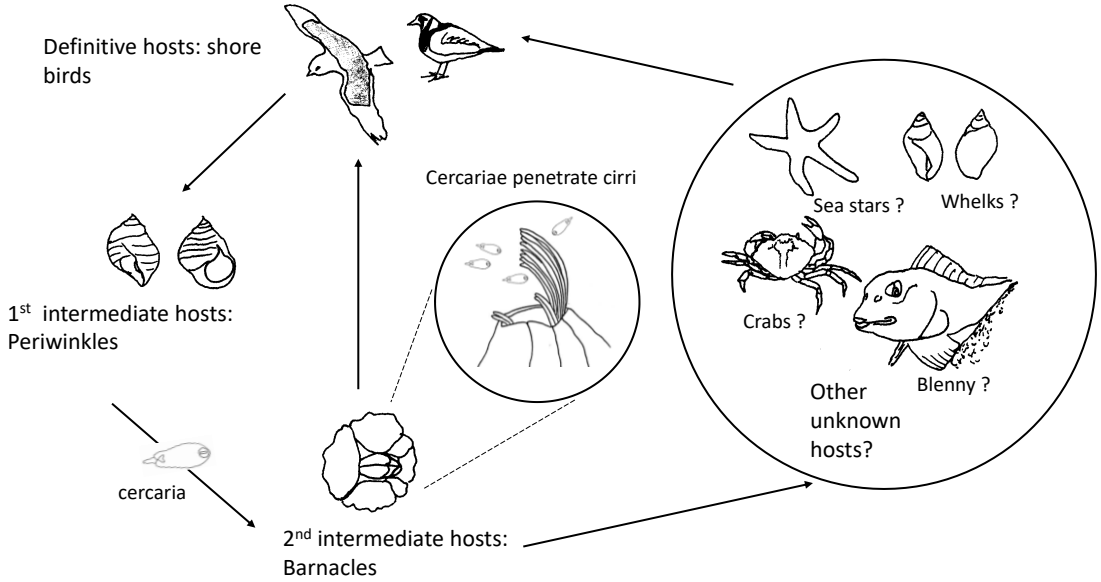


Figure 2.2. Extrapolated life-cycle of *Maritrema gratiosum*. Cercariae are released from molluscan hosts and penetrate a barnacle via its cirri. Potential intermediate / paratenic hosts in the trophic web are added to reveal possible pathways to the definitive hosts.

Several species of larval cestode have also been reported in *S. balanoides*. Four species have been recorded: *Acanthocirrus retrirostris* (Cyclophyllidea) (see Williams *et al.*, 1981), *Fimbriarioides intermedia* Fuhrmann, 1913 (Hymenolepididae), *Anomotaenia clavigera* Krabbe, 1869 (Dilepididae) and an unidentified species (Belopol'skaya, 1953). According to De Murguia and Seed (1987), 12 species of mite (Arachnida) have also been found as epifauna associated with *S. balanoides*, *Chthamalus montagui* Southward, 1976 and *A. modestus* on rocky shores in North Wales. The rhizocephalan parasite *Boschmaella japonica* Diechmann et Høeg, 1990, was found to infect *Chthamalus challengeri* Hoek, 1883 and *Amphibalanus amphitrite amphitrite* Darwin, 1854 and other rhizocephalans, *Boschmaella balani* Bocquet-Védrine, 1967*, Chthamalophilus delagei* Bocquet-Védrine, 1957 and *Bocquetia rosea* Pawlik, 1987 were found to infect other barnacle species as well (Deichmann & Høeg, 1990). Turbellaria belonging to the genus *Stylochus* have been reported as being observed free on the internal wall of the opercular valve or deep within the host. These are predators of oysters, barnacles, pagurid crabs and other invertebrates. Besides animals, some algal species can grow on or penetrate the shells of *S. balanoides* as well (Arvy & Nigrelli, 1969). According to the same authors, two species of lichens (*Didumella balani* [syn. *Pharcidia balani*] (G. Winter) Bausch, 1936) and *Pharcidia marina*) were found on the shell of *B. balanoides* and *C. stellatus* and one species of virulent fungus, *Lagenidium chthamalophilum* Johnson, 1958, was

reported to infect ova of *Chthamalus fragilis* Darwin, 1854. Stubbings (1975) mentioned two species of Chlorophyceae and four species of Cyanophyceae infecting barnacles.

As mentioned in Chapter 1, a more recent survey of barnacle-associated fauna was carried out by Colston (2012). In her work, carried out in Southeast Scotland, several associated and parasitic taxa of barnacles were found. In brief, two types of gregarine, metacercarial cysts of *M. gratiosum*, cysticercoids of *Acanthocirrus retrirostris* and a further four unidentified cyclophyllidean cestodes were found in the soft tissues of two barnacle species, namely *S. balanoides* and *A. modestus*. In addition, several associated marine organisms were found outside the shell plates or on prosoma of barnacle. These included ectocommensal *Zoothamnium* Bory de St. Vincent, 1824 sp., mites, planarian worms, polychaete worms and nematodes. Colston (2012), however, did not confirm the presence of the cercarial stage of *M. gratiosum*, which is *Cercaria littorinae saxatilis V* (Popiel, 1976b), nor did she identify the final host of *M. gratiosum* at her sampling localities. Considering the high prevalence of *M. gratiosum* metacercariae at Dunbar, it is surprising that she did not find the cercarial stages during her study. According to the concept that "the overlap of the distribution areas of all hosts is needed for a parasite to complete its life-cycle" as suggested by Galaktionov and Dobrovolskij (2003), several questions are, therefore, raised:

1. How can *M. gratiosum* complete its life-cycle and become highly prevalent at certain localities?
2. Why is it less successful at other localities?
3. Is the parasitic fauna and the specific infection parameters associated with a host an intrinsic character of a locality?

To answer these questions, a pilot study was conducted and this is presented in this chapter.

To answer the first question, one possibility is the existence of another host species that was overlooked at the study site. Several key marine molluscs, *e.g., Littorina* Férussac, 1822 spp., were found co-habiting with *S. balanoides* and were recognised in the present study. The broad range of digenean cercariae that they have been observed to play host to in the UK (see Chapter 1) demonstrates the potential for them to be first intermediate hosts for the barnacle parasite *M. gratiosum* and possibly others. One periwinkle, *Littorina saxatilis* Olivi, 1792, had been previously shown, for instance, to serve as a first intermediate host of *M. gratiosum* (see Popiel, 1976a). Other species of *Littorina* may also serve as first intermediate hosts, but no evidence for this has been provided thus far and will be explored in this pilot study. *Nucella lapillus* Linnaeus, 1758 is known to be a paratenic host for *M. gratiosum*, in which

metacercarial cysts can be found. This connection was suggested to be the consequence of predation, as *N. lapillus* is a major predator of *M. gratiosum* (see McCarthy, 1999). In the present study, there is an attempt to reveal the "distribution area of *M. gratiosum*" in an area where high prevalence of the metacercariae of a digenean parasite is known. This requires demonstrating the presence of mature digeneans in the definitive hosts and the presence of cercariae in the 1st intermediate host in the study locality. Where it is not possible to achieve this fully, one can nevertheless characterise the area where likely overlapping of the distribution of these three stages occurs and the existence of other paratenic hosts. Hadley and Castle (1940) had found the adult stages of *M. gratiosum* in the intestine of turnstone (*Arenaria intrepres morinella*), however this digenean species has also been reported from other Charadriidae Vigors, 1825 and Laridae Vigors, 1825 hosts such as *Charadrius hiaticula* L., 1758*, Charadrius essentiellemen, Haematopus ostralegus* L., 1758*, Larus ridibundus* L., 1766*, Limosa lapponica L., 1758, Calidris alpina L., 1758* (Nicoll, 1907; Rankin, 1939; Deblock & Tran Van Ky, 1966). Furthermore, several species of microphallids have been reported in certain lariform birds (Threlfall, 1967; Irwin & Prentice, 1976; La Sala *et al.*, 2009; Diaz & Cremonte, 2010). Since a kittiwake (*Rissa tridactyla* L., 1758) breeding site is in one of the localities (Dunbar), kittiwakes are therefore considered as a potential definitive host of the target parasites in the present study.

In answering the second question, the infection parameters of *M. gratiosum* and its potential 1st and 2nd intermediate hosts at different types of locality were compared. The criteria chosen were based on several assumptions, such as differences in exposure, the existence / absence of final hosts, and the extent of anthropological influence. Environmental implications on parasitism have become popular research topics in recent years, and have led to a discipline called "Environmental Parasitology". This discipline deals with topics such as anthropogenic impact on parasitism and using parasite as indicators for environmental health; among these, research studies related to pollution are especially promising (Sures *et al.*, 2017). Anthropological influences, however, are not limited to chemical pollution. In the ongoing climate change scenario, thermal stress is another important topic. For example, Casas-Mulet *et al*. (2021) has shown that exposures to combinations of continuous high temperature (i.e., global warming) and intermittent thermal peaks (i.e., hydropower production) can significantly increase the mortality of brown trout (*Salmo trutta*) infected by the oomycete *Saprolegnia parasitica* (Coker, 1923). Colston (2012) found *M. gratiosum* and several types of larval cestodes in both *S. balanoides* and *A. modestus*. *Austrominius modestus*, as an invasive species from Australia, which probably arrived during World War II

(Southward, 2008), has potential to influence the ecology and parasitism status of the native species *S. balanoides*. Interactions between native and invasive species under the scope of parasite ecology can be complicated. For example, invasive species populations can outcompete local species by introducing new parasites (spillover) or by an absence of natural enemies (enemy release hypothesis). The establishment of invasive species can also be supressed by parasite infection (suppressive spillover) (Chalkowski *et al*., 2018). The present study also provides a chance to examine whether *A. modestus* has outcompeted *S. balanoides* in chosen localities and whether this is related to parasite infection or not. The aforementioned research, however, need to be developed upon a thorough understanding of the "baseline" data of the hosts and their parasites, *i.e*., an understanding before pollution exposure or invasive species are introduced or before rises in temperature begin, otherwise, laboratory simulations are needed to gain meaningful results. One of the aims of the present study is to gather such baseline data for barnacle-parasite systems at chosen localities. Environmental influences at both large and small scale, for example between localities and within a locality, are investigated in the present study. At the large scale, different types of rocky shores were explored. An exposed coast at Machrihanish, a sheltered coast under the Forth rail bridge at South Queensferry, and a moderately exposed coast containing diverse terrain at Dunbar were chosen. Factors that favour the circulation of complex life-cycle parasites such as digeneans are examined here. At the small scale, biotic factors are studied. Gathering in crevices at low tides is the natural behaviour of periwinkles (Cummins *et al.*, 2002). Reduced mobility of parasitized molluscs has been reported in some research (Lambert & Farley, 1968; Williams & Ellis, 1975; Davies & Knowles, 2001), therefore it was hypothesized that periwinkles in open areas may have more pronounced digenean infections than those in rock crevices. Parasite prevalence of barnacles attached to biotic and abiotic substrates, for example limpets on seaweeds versus rocks, were compared. The barnacles attached on organisms might be hypothesised to have a higher prevalence of parasites, as these individuals are attached on movable objects compared to stationary rocks, and might therefore have more opportunities to encounter cercariae or other sources of parasites. Furthermore, molluscs to which barnacles attach, might increase local water current, and increase the chance for food particles/ parasites to enter barnacles.

To answer the third question, the associated / parasitic fauna of barnacle populations at several localities studied in Colston's work were investigated again, to reveal temporal differences. The existence of an aggregated distribution pattern for different parasite populations is frequently observed and is an accepted aspect of parasite ecology (Crofton,

1971a; Shaw & Dobson, 1995; Poulin, 2007). The present study provides an opportunity to validate this principle among digenean-barnacle system. The infection parameters of *M. gratiosum* were further explored to reveal potential causes of the observed differences in infection. From the first field trip, differences between infection parameters for digeneans were detected between the different tidal levels on the shore. Several researchers had also reported the difference of infection between high and low tide in barnacle-digenean systems (Irwin & Irwin, 1980; Mitchell & Dessi, 1984; Carrol *et al*., 1990; Sari & Malek, 2000). Two contrasting hypotheses were proposed for digenean infections. It was hypothesized that the infection pressure is proportional to the distance from the definitive hosts, therefore, sites closer to shore will favour infection. In contrast to this, it was also proposed that residence at low tide levels will favour infection, as immersion time is longer and the infective miracidial or cercarial stage will have greater opportunity to infect molluscan or barnacle hosts. Several sites were chosen based on the relative position from the reference point where the potential final host (kittiwake) live, and infection parameters were compared from high to low shore sampling sites. Furthermore, in order to conduct a simple estimation of the degree of aggregation of metacercarial cysts of each barnacle population for the sites visited in Dunbar, variance-to-mean ratio was calculated for the samples collected from the five different sampling sites at Dunbar. According to Carrol *et al*. (1990), the variance-to-mean ration (D), is defined as the ratio between the variance (s^2) and the mean (m) of the number of metacercarial cysts. Values of D close to 1 indicate a random dispersion, D <1 indicate a uniform dispersion and values D>1 indicate a clumped or aggregated dispersion (Taylor *et al*., 1978; Kamdem *et al.*, 2012).

Finally, Colston (2012) pointed out that the potential influence of *M. gratiosum* infection on barnacle health deserved further study. A brief survey regarding the reproductive success of infected and uninfected barnacles was also conducted in this pilot study.

Aims

This chapter examines the associated and parasitic fauna of *S. balanoides* and other acorn barnacles and some intertidal gastropods from selected rocky shores in Scotland, to fulfil the following aims:

1. To discover the cercarial stages of *M. gratiosum* and its potential paratenic hosts and to determine why this digenean species is abundant at certain localities. To investigate the

macroparasite fauna of molluscan hosts living alongside the barnacles and analyse the differences in infection parameters between sites.

2. To chronologically compare the associated / parasitic fauna of barnacle populations, in order to validate the hypothesis that the parasitic fauna and associated infection parameters for a species is an intrinsic characteristic of an ecosystem.
3. Compare digenean infection parameters in barnacle populations between and within localities, to test the following hypothesis:
    A. The degree of infection by digeneans in primary or second intermediate hosts is correlated to the presence of, and distance from, concentrations of definitive hosts.
    B. Anthropological disturbance and high exposure have negative impacts on the completion of digenean life-cycles, therefore sheltered and / or high biodiversity localities favour infection when compared to exposed and / or anthropologically influenced localities.
    C. Barnacles attached on organisms are more likely to contract digenean infections, and therefore have higher prevalences and / or abundances of digenean infection.
4. To determine if digenean infections have a negative impact on barnacle health, with a particular focus on their reproductive success.

In this chapter, these questions will be addressed using various techniques, *i.e*., dissection of the host species, light microscopy, and scanning electron microscopy (SEM) to study any recovered parasites in detail, and molecular-based techniques as a tool to confirm species identification. Statistical analysis of the infection parameters and reproductive status of the host species from the different locality types will also be performed. The results of this pilot study will be a basis for more detailed studies in the remainder of this thesis.

2.2 Materials and methods

2.2.1 Sample collection and calculation of infection indices

A total of six field trips were carried out between February 2013 to April 2014, with three localities of rocky shore in Scotland being chosen, based on predicted high or low abundance of trematode parasites and exposure of the coast, diversity of terrain, anthropological influence, existence of final hosts, *etc*. The chosen localities were South Queensferry, Machrihanish and Dunbar (see Figure 2.3 for localities on the map, Figure 2.4 for the local terrain at each locality, and Table 2.1. for details regarding the sampling date, sample size, geographical information, and locality characteristics). The chosen localities had to be within easy reach for one-day sampling trips. The coast at Machrihanish represents an exposed coast with few anthropological disturbances. The coast under the Forth bridge of South Queensferry represents a sheltered coast near an estuary and is under high anthropological influence as the bridge pier is directly located at the site and it is situated beside a village. Finally, a moderately exposed coast containing diverse terrain was chosen at Dunbar. This locality is characterized as having both a sheltered bay near a leisure pool and an exposed rocky outcrop (named “Red Rock” in the present study for convenience). The cliff beside the leisure pool is a breeding site for kittiwakes, *Rissa tridactyla* Linnaeus, 1758, in summer and the famous seabird habitat Bass Rock is only 11.1 km away. The coast at Dunbar has a moderate anthropological influence as it is sits beside the town and provides a recreational environment for people.

Table 2. 1. Details of sampling trips that were conducted.

No.	*Date*	*Locality*	*Purpose*	*Sample size*	*Locality Grid Reference*	*Locality characteristics*
1	02/2013	South Queensferry Right side of the ForthBridge	General survey for barnacle parasites	66	55°59'26.0"N 3°22'59.7"W	Sheltered rocky shore, more anthropological influences
2	03/2013	Machrihanish	General survey for barnacle parasites	46	55°25'21.5"N 5°44'06.3"W	Exposed rocky shore, less anthropological influences
3	06/2013	Dunbar Leisure Pool	General survey for barnacle parasites	300	56°00'19.5"N 2°31'04.2"W	Moderated exposed rocky shore, high biodiversity, near potential final hosts
4	07/2013	Dunbar Leisure Pool	General survey for mollusc parasites	117	56°00'19.5"N 2°31'04.2"W	Moderated exposed rocky shore, high biodiversity, near potential final hosts
5	09/2013	Dunbar Red Rock and Leisure Pool	General survey for barnacle and mollusc parasites	200 (barnacles) 129 (molluscs)	56°00'19.9"N 2°31'33.4"W	Moderated exposed rocky shore, high biodiversity, near potential final hosts, elevated rocky platform
6	04/2014	South Queensferry Left side of the Forth Bridge	General survey for barnacles	400	55°59'26.0"N 3°22'59.7"W	Sheltered rocky shore, more anthropological influences

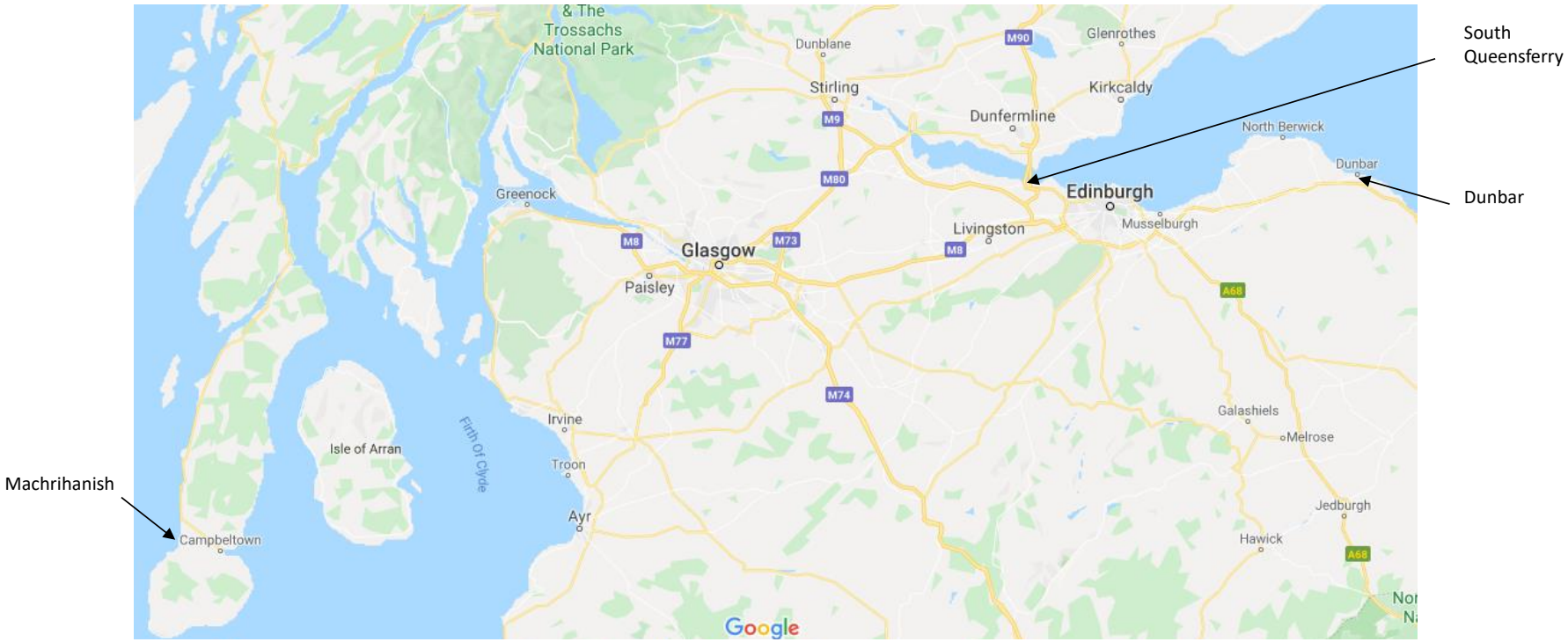


Figure 2.3. The three sampling localities referred to in this chapter (Image source: Google Maps).

Figure 2.4. Local terrain of the three sampling localities. A: South Queensferry. B: Machrihanish. C: Dunbar Red Rock (middle) and Leisure Pool (top right). D: Overview of Dunbar Red Rock. E: Overview of Dunbar Leisure Pool.

2.2.1.1 Sampling trip no. 1 and 2

Two field trips were carried out at South Queensferry and Machrihanish separately in February and March of 2013 (Table 2.1). For the local terrain of these two localities see Figure 2.6. In total 112 barnacles were dissected, 66 from South Queensferry and 46 from Machrihanish. The terminology of locality and site was comparable to that defined by Bush *et al*. (1997). According to Bush *et al*. (1997), a locality is the geographic locale of the external environment where the parasite is found, and a site is a spatial location in a host where a particular sample of parasites is collected. Here I use locality for a city / town where target parasite exists, for example Dunbar, and site refer to places within a locality.

- Field sampling

For trip no. 1 the chosen site was the shoreline at the eastern side of the Forth Bridge, which was covered in small rock fragments interspersed with mud. Small pieces of rock with barnacles on the surface were collected by hand. The rocks with barnacles were chosen randomly. Samples were brought back to the lab in local seawater and maintained in small aquaria with the same local seawater (~35‰, 10°C) and a bubbled air supply. In addition, rocks, seaweeds, limpets, and mussels with barnacles attached were also collected and maintained in separate aquaria. For trip no. 2 the chosen site was an exposed shore with large rock outcrops. The sampling method was similar to that previously described, except that fewer portable rocks could be found on the shore and therefore barnacle-colonised pieces of rock were retrieved using hammer and chisel.

- Dissection of barnacles

The barnacles were observed for the presence of commensal or parasitic organisms outside the shell plates and / or inside the shell. Once the barnacles were detached from their substrate (*e.g.*, a rock), a single barnacle was placed in an embryo glass and submerged in filtered seawater. Species was diagnosed according to the number, shape, pattern and position of shell plates, the nature of the base, the size and shape of the operculum, the colour of the tergo-scutal flap, and the structure of the shell plate. The final diagnosis was based on the key produced by Southward (2008). After the species of barnacle was diagnosed, the surface of each barnacle was screened and any commensal organisms were recorded. The barnacle was turned upside down, the base was removed and the eggs / nauplii were removed by means of tweezers, and if it was gravid (with eggs or unreleased nauplii), a record was made. The inner surface of the shell plate was then observed under a stereo microscope (SZ-PT Olympus) for any possible parasites. After that the prosoma was extracted from the shell

using fine forceps and it was placed on a slide. The prosoma was observed by a compound microscope (Olympus BX51) by adding a drop of seawater and using slight pressure applied to the coverslip. The numbers of metacercarial cysts or larval cestodes were counted using phase contrast under an 4-10 X objective.

- Observation of commensals and parasites for barnacles

Commensal protists on the surface of the prosoma were recorded. Parasites found inside the prosoma were also recorded, photos were taken using a Zeiss MRc camera and AxioVision capture software (Zeiss) and the numbers of larval digeneans or cestodes were counted. Digenean and cestode cysts were extracted from the prosoma by cutting the oral cone and the cirri off and were released by tearing the gut and body wall. The extracted cysts of digeneans or cestodes were preserved in 0.1M phosphate buffered saline (PBS, Sigma), either excysted directly or excysted one day after refrigeration at 4°C. The digenean cysts could be excysted in 0.1 M PBS in a 40°C water bath for 2 ~ 4 hours. The excysted metacercariae can survive in PBS for at least 12 hours. The excysted metacercariae can therefore be stained for morphological diagnosis, fixed for SEM observation, or fixed for molecular diagnosis, depending on the experimental requirements. Some excysted metacercariae were flattened in Berland's fluid, stored in 80% ethanol, stained with Mayer's paracarmine and fixed as a whole mount (see Appendix 9) and observed under compound microscope. Photographed specimens were measured using FIJI ImageJ 1.52p (National Institutes of Health, USA; Schindelin *et al*., 2012). The molecular-based investigations exploring the target digenean will be introduced in detail in Chapter 4. The discovered cestode cysts were similarly excysted in 0.1 M PBS in a 40°C water bath for 2 ~ 4 hours. Whether successfully excysted or not, they were temporally fixed in a drop of seawater under coverslip pressure and observed alive using a compound microscope. An attempt was made to measure the rostellar hooks of cestodes, but no cleaned specimens of the hooks were available, therefore only some hook images were measured. Specimens were photographed using FIJI ImageJ 1.52p.

- Data analysis

Summary statistics were calculated and visualized using Excel (Microsoft Office Professional Plus 2016) or SPSS (IBM SPSS v.23 or v. 25). The significance level employed for all tests was $p<0.05$. These applied to all the following statistics in the present study.

To compare differences between associate organisms or parasitic infection between the two contrasting localities, namely South Queensferry (sheltered and with anthropological influence, trip no.1) and Machrihanish (exposed and with less anthropological influence, trip

no. 2), the prevalence of different categories of associated or parasitic species (protozoan, digenean, cestode, nematode and mite) were compared using a chi-square test or a Fisher's exact test when sample size was small. For samples from South Queensferry (trip no.2), in order to compare the differences between associated organisms or parasitic infection between barnacles on different substrata (organic versus inorganic), the prevalence of different categories of associated or parasitic species (protozoan, digenean, cestode, nematode and mite) were compared using Chi-square test or Fisher's exact test when sample size was small.

2.2.1.2 Sampling trip no. 3

Trip no. 3 (see table 2.1) was carried out at a coastal area near the Dunbar Leisure Pool, which was also near a kittiwake breeding habitat (termed “kittiwake cliff” in the present study). Three sampling sites were explored in total (see Figure 2.5) and the sites were chosen according to the vicinity of kittiwake cliff. The distance between site, however, was not measured. One hundred barnacles were sampled per site and a total of 300 barnacles were dissected. Cyst numbers were counted and infection parameters (prevalence, abundance, and intensity) were recorded. According to Margolis *et al.* (1982) and Bush *et al.* (1997; p. 576-578), prevalence is defined as the number of hosts infected with 1 or more individuals of a particular parasite species divided by the number of hosts examined for that parasite species; abundance is defined as the number of individuals of a particular parasite in a single host regardless of whether or not the host is infected, mean abundance is the total number of individuals of a particular parasite species in a samples of a particular host species divided by the total number of hosts of that species examined; intensity is defined as the number of individuals of a particular parasite species in a single infected host, and mean intensity is the total number of parasites of a particular species found in a sample divided by the number of hosts infected with that parasite.

- Field sampling and laboratory processing for barnacles

Field sampling and laboratory processing procedures were the same as trip no. 1 and 2 except that reproductive status was not recorded.

- Data analysis

Differences between infection parameters for digeneans were detected between different tidal levels on the shore. Two contrasting hypotheses were proposed for digenean infections. It was hypothesized that the infection pressure is proportional to the distance from the definitive hosts, therefore, sites closer to shore will favour infection. In contrast to this, it

was also proposed that residence at low tide levels will favour infection, as immersion time is longer and the infective miracidial or cercarial stage will have greater opportunity to infect molluscan or barnacle hosts. Three sites were chosen based on the relative position from the reference point, the kittiwake cliff. “Cliff side, lower water edge” represents the site near the definitive host at low tide level. “Cliff below birds” represents the site near the definitive hosts and at high tide level. “Open bay, lower water edge” represents sites far from the definitive hosts at low tide level (Figure 2.5). Prevalence was compared between sites using a chi-square test. The abundance data (cyst numbers / animal) were over-dispersed; therefore, the abundance was compared between sites with a negative binomial regression using site as the factor.

Figure 2.5. Sampling sites at Dunbar Leisure Pool (06/2013). (Image source: Google Maps)

2.2.1.3 Sampling trip no. 4

In trip no. 4 (see table 2.1) only mollusc samples were collected. Four sampling sites, from low to high shore, were chosen from the coast near kittiwake cliff (Figure 2.6).

- Field sampling

The distances between these four sites were measured by a marked rope to get a precise distance. Approximately 30 molluscs were picked randomly by hand at each site. Three types of mollusc were collected, namely dog whelks *Nucella lapillus* L. 1758, periwinkles *Littorina* spp. and limpets *Patella* spp. Molluscs were diagnosed to genus level according to the keys provided by Graham (1988). The number of each species per site varied, therefore the numbers of each type was decided according to availability *in situ*.

Figure 2.6. Sampling sites at Dunbar Leisure Pool (07/2013). Distance between Site 1 & 2= 24.3m, site 2 & 3= 11.5m, site 3 & 4= 15.3m. (Image source: Google Maps)

- Morphological observation and species identification for cercariae from different molluscan host

The collected molluscs were sorted by sampling site and placed in small aquaria separated from barnacles from the same site. Seawater and air were supplied, and the aquaria were placed in an incubator at constant temperature (10°C) and a 12:12 hour dark / light cycle. No food was supplied because the whole incubation time was usually not more than two weeks and there remained organisms in the water.

The molluscs were dissected and different stages of parasite were recorded, and morphological diagnoses made, where possible. For dog whelks and periwinkles, the shell was crushed with a mortar and pestle, the soft body was isolated by tweezers, and the different organs (*i.e.*, digestive tract, reproductive, respiratory organs, or mantle tissue) examined by wet mount between two slides. Parasites were observed alive, preserved for scanning electron microscope (SEM) observation, or preserved for molecular diagnosis. For limpets, the soft body was cut from the edge of the foot by tweezers and scissors, and isolated from the shell. Different organs were examined by wet mount methods and parasites were observed alive or preserved for SEM and molecular diagnosis.

Larval stages found in the mollusc hosts included: rediae, sporocysts, cercariae, or metacercariae. These stages can be differentiated by morphology (see Figure 2.7 for examples). The rediae/sporocysts and/or cercariae were diluted in 0.1 M PBS (Sigma) in embryo glasses and observed under a SZ-PT Olympus stereo microscope. Some specimens were placed on a slide with a drop of PBS, and observed alive, under slight coverslip pressure, by a compound microscope. Photographs and measurements were taken as previously described.

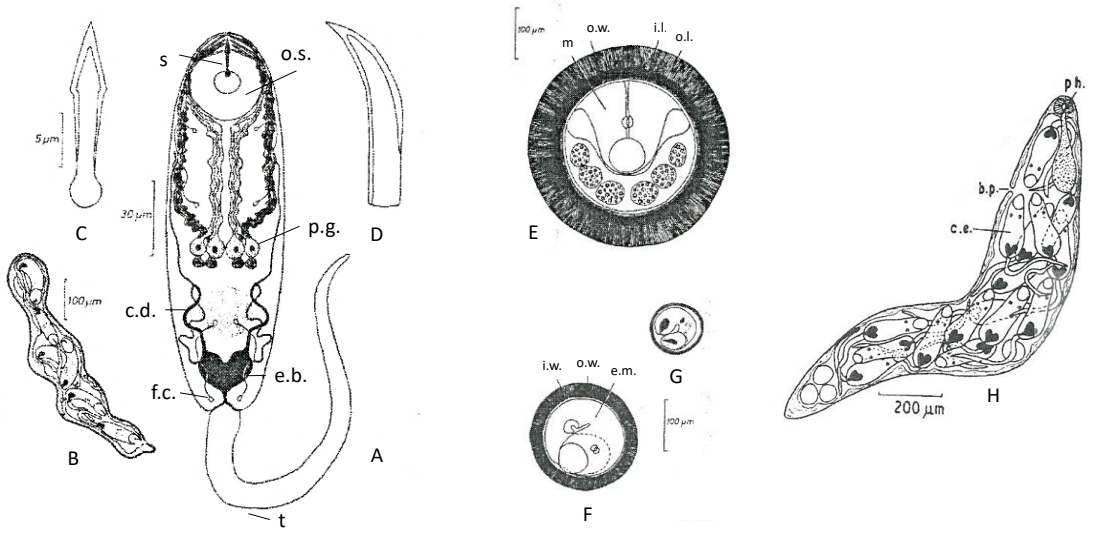


Figure 2.7. Morphology of different stages of larval digenean. A. Cercarial stage of *Cercaria littorinae saxatilis* IV. B. Sporocyst of *C. littorinae saxatilis* IV containing fully formed cercariae. C & D. Stylet, lateral and dorsal view. E. Fully formed encysted metacercaria for *Microphallus similis*. F & G. Later and early stages of the developing metacercarial cyst for *M. similis*. H. Redia for *Cryptocotyle lingua* containing cercariae at various stages in development. b.p.: birth pore, c.d.: collecting duct, c.e. fully formed cercaria, e.b.: excretory bladder, e.m.: early stage metacercaria, f.c.: flame cell, i.l. inner layer of inner cyst wall, i.w.: inner cyst wall, m: metacercaria, o.l. outer layer of inner cyst wall, o.s.: oral sucker, o.w.: outer cyst wall, p.g.: penetration glands, ph: pharynx, s: stylet, t: tail. Reproduced from James (1968, p. 636, fig. 56 and 1969, p. 277, fig. 9-11, p.285, fig. 29-32).

Some of the cercariae, sporocysts and rediae that were found were washed in filtered sea water and fixed in glutaraldehyde for further SEM study. Samples were first fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate at 4°C for 2~3 days before further processing. The samples were then washed in buffer (0.1 M cacodylate rinse + 0.1 M sucrose) and the remaining procedures were carried out in a fume cupboard. Samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 hours. Afterwards they were dehydrated through an ethanol series (30%, 60%, 90%, 100%, 100%) for half an hour each. After dehydration they were packed in BioWraps and were placed in a container for critical point drying (CPD). After CPD, the samples were mounted on stubs and sputter coated with gold using an Edwards S150B sputter coater. The samples were examined using a JEOL JSM6460LV SEM at an accelerating voltage of 10 kv.

Some of the cercariae, sporocysts and rediae that were found were washed in filtered sea water and fixed in 90% ethanol for a subsequent confirmatory molecular diagnosis based

on ITS2 ribosomal rDNA. Methods will be described in more detail in Chapter 4. The species of each specimen were confirmed by direct sequencing of the obtained amplicons.

- Data analysis

The relationship between distances from bird cliffs and digenean infection parameters of potential primary molluscan hosts was analysed. Overall prevalence of parasitic infection and prevalence of each larval stages was compared between sites and mollusc type. The same hypotheses "that infection pressure is proportional to the distance from the definitive hosts, and that low tide will favour infection as immersion time is longer" is applied. Considering that the mobility of molluscs is limited, and that their infection comes from digenean eggs or miracidia, which have limited, or no movement ability compared to cercariae; molluscan infections are typically acquired on the local scale, therefore, the correlation of infection parameters between the distance from the definitive host and the degree of infection should be similar with that of immobile 2nd hosts. The prevalence of digenean infection was compared between sites and analysed. In this preliminary attempt, the infection in molluscs was not diagnosed to species; instead, the infection was categorized by the larval types, such as sporocysts, rediae, metacercariae and mixed infections. The numbers of these larvae are hard to quantify, therefore only prevalence was compared between sites. The prevalence was compared between sites by chi-square tests or by Fisher's exact test when sample size was small.

2.2.1.4 Sampling trip no. 5

Trip no 5 (Table 2.1) was carried out on an elevated rocky platform opposite Dunbar Leisure Pool. This site is termed "Red Rock" for convenience (see Figure 2.4D for local terrain). Three sampling sites were chosen, barnacles were collected from Sites 2 and 3 and molluscs were collected from Sites 1 and 3 (Figure 2.8). One hundred barnacles were dissected per site and in total 200 barnacles were dissected. Forty periwinkles were sampled per site. Fifteen dog whelks were taken at Red Rock but were not microhabitat specific, and 20 dog whelks and 16 periwinkles were taken from a position near kittiwake cliff for the purposes of comparison. In total 96 periwinkles and 33 dog whelks were dissected and examined for digenean infection.

- Field sampling and laboratory processing

Methods for field sampling and laboratory processing for barnacle and mollusc specimens were the same as that described in trip no. 1, 2 and 4.

- Data analysis

The local terrain of Red Rock was a rocky platform with a roughly rectangular shape, therefore the tidal level was not as clear as that for the bay at Dunbar Leisure Pool. It is assumed that the immersion time is roughly the same along the edge of the platform, and decreased moving inland. Specific hot spots for the definitive hosts were not observed at this rocky platform, therefore the influence of definitive hosts is suggested to be negligible. Intrinsic environmental factors, such as topology at small scale, wave action or local fauna, however, can result in patterns of parasite distribution. The analysis was conducted to reveal whether heterogenicity of digenean infection exists in different populations of barnacles found in this rocky platform and the degree of this heterogenicity. The null hypothesis is that the distribution of the digenean parasite is homogeneous. The prevalence between sites was compared by a chi-square test. The abundance between sites were compared by negative binomial regression and/or by a Poisson regression using site as the factor.

The infections in molluscs were also examined and analyzed. Prevalence of digenean infection in molluscs was compared between sites by chi-square test. In addition, digenean infection in the periwinkles in relation to one of their behaviours was examined. A sampling strategy according to the difference of micro-habitat was conducted in order to reveal the differences in parasitic infection of periwinkles from open areas and crevices. Twenty periwinkles were taken from an open area and 20 taken from crevices (Figure 2.9). The levels of infection for different stages of digenean within molluscs, between different micro-habitats (open areas versus crevices), was also compared by chi-square test or by Fisher's exact test when sample size was small.

Figure 2.8. The three sample sites at Dunbar Red Rock in September 2013. (Image source: Google maps)

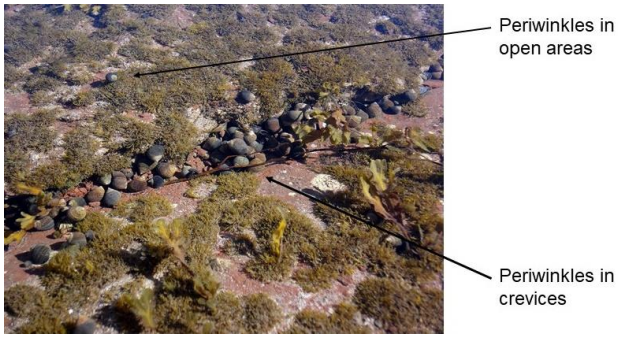


Figure 2.9. Periwinkles occupying different habitats.

Finally, in order to conduct a simple estimation of the degree of aggregation of metacercarial cysts of each barnacle population for the sites visited in Dunbar, variance-to-mean ratio was calculated for the samples collected from the five different sampling sites at the two localities in Dunbar: the three sites near Dunbar Leisure Pool (June 2013) and the two sites at Dunbar Red Rock (Sept. 2013). According to Carrol *et al*. (1990), the variance-to-mean ration (D), is defined as the ratio between the variance (s^2) and the mean (m) of the number of metacercarial cysts. Values of D close to 1 indicate a random dispersion, D <1 indicate a uniform dispersion and values D>1 indicate a clumped or aggregated dispersion (Taylor *et al*., 1978; Kamdem *et al.*, 2012).

2.2.1.5 Sampling trip no. 6: barnacle faunal survey in South Queensferry

Sampling was conducted at a site on the South Queensferry coast to the right-hand side of the Forth Rail Bridge (trip no. 6 in table 2.1). A transect sampling strategy was applied (Figures 2.10). Sampling was conducted across several transverse lines running parallel to the shoreline from low shore to high shore separated by fixed increments (*i.e*., 20 metres), and samples were collected from sampling sites along each transverse line at 40-metre increments (Figures 2.10). As part of this process, first, a site datum was positioned on an obvious object on the shore (a concrete cylinder in this case, Figure 2.11B), and the direction to the furthest sampling site down shore was indicated using a 1m ruler (Figure 2.11C). Sampling started at the furthest sampling site at low tide (Site 15 in Figure 2.10) on the first transverse line. Distance between the first site and the datum was measured by a portable laser measurement system (Leica DISTO classic[5], Leica Geosystems AG, Heerbrugg, Switzerland). A straight line can be measured by the laser measurement with one person holding the apparatus and a second person holding a piece of white board. A tripod was used to fix the laser beam and to maintain it at a fixed height (Figure 2.11E). By means of reflection from a white board, the distance can be calculated. Once the desired distance was reached, the person holding the

white board stopped and put a marker (a yellow fix pot filled with water (Figure 2.11E) on that site and started sampling. Later, a square quadrant (50 x 50 cm, Figure 2.11F) was placed at the position of the marker, and a photo was taken to document all the barnacles in the quadrant.

A decision of whether to accept this site was made when barnacles were present in more than 3 grid-squares, *i.e.*, ensuring sufficient individuals to sample. For quadrants, with barnacles covering less than 3 grid-squares, or where the site was not assessable, the site was discarded and an adjacent surface was chosen instead. Once the site was recorded by photograph, a piece of rock with barnacles attached within the quadrant, where barnacles could be physically sampled, was taken. When the sampling was finished, sampling team then moved to the next sampling site on the same transverse axis. Once all sampling sites on the transverse axis were completed, sampling team then moved 20 metres towards the site datum to the next transverse line. The steps were repeated until the upper limit of the barnacle zone was reached. In total, five transverse lines and 12 sampling sites were created (Figures 2.10). For this particular trip, the focus was on the distribution of barnacles rather than on the parasites within the barnacles. A total of 400 barnacles were collected.

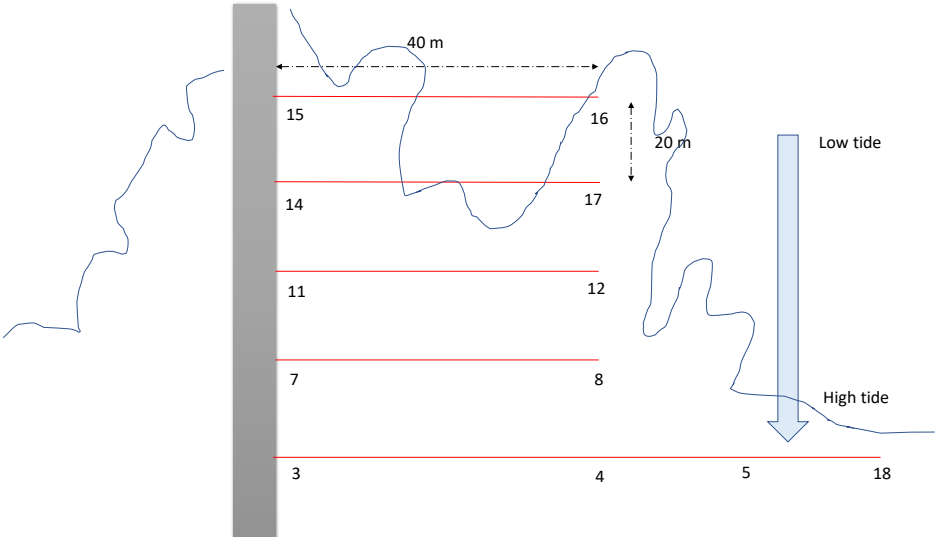


Figure 2.10. Schematic drawing of the transect sampling strategy at South Queensferry. Grey bar represents the Forth Rail Bridge. The distance between each transverse line (red line) is 20 metres. The increment between each point is 40 metres, except between point 4-5-18 which was 20 metres. Barnacle samples were collected from sampling sites along each transverse line at 20 or 40 metre increments.

Figure 2.11. The South Queensferry sample locality. A: Sampling locality overview. B: The concrete cylinder used for site datum. C: Site datum and the reference for the furthermost site. D: Part of the sampling team. E: A laser fixed to a tripod was used to measure the site. Yellow pots were used to mark each sample site. Barnacle covered rocks were collected in buckets. F: The 50 cm × 50 cm quadrant used for gathering information at each sampling site.

2.3 Results

The results of data analysis are summarized in table 2.2 and will be described in detail in the following sections along with the morphology for the parasitic fauna discovered.

Table 2. 2. Summary of the statistical analyses conducted during each trip (Trip no. details are given in Table 2.1.).

Trip no.	*Locality*	*Analysis*	*Target*	*Statistical methods*	*Sample size*	*Results*
1 & 2	South Queensferry Right side of Forth Bridge (SQ) and Machrihanish (MH)	1. Prevalence of different parasitic or commensal taxa of *S. balanoides* from SQ and MH.	Parasitic and commensal taxa in barnacles	1. Bar chart	1. 107	1. Fig. 2.21.
		2. Comparison of prevalence of different parasitic or commensal taxa between *S. balanoides* from SQ and MH.		2. Chi-Square test and bar chart	2. 107	2. Fig. 2.22.
		3. Comparison of prevalence of different parasitic or commensal taxa between *S. balanoides* from organic and inorganic substrate (SQ only).		3. Chi-Square test and bar chart	3. 107	3. Fig. 2.23.
		4. Comparison of reproductive status and metacercarial infection of *S. balanoides* from SQ and MH.	Metacercariae in barnacles	4. Fisher's exact test and bar chart	4. 65	4. Fig. 2.24 and appendix 10
3	Dunbar Leisure Pool	1. Comparison of prevalence of metacercarial infection for *S. balanoides* from three sampling sites near Leisure Pool.	Metacercariae in barnacles	1. Chi-square test and bar chart	1. 300	1. Fig. 2.25
		2. Goodness of fit test of Poisson distribution for abundance and intensity of metacercarial infection for *S. balanoides* from three sampling sites near Leisure Pool.		2. Histogram and goodness of fit test for Poisson distribution	2. 300 for abundance and 141 for intensity	2. Fig. 27

		3. Comparison of mean abundance and mean intensity of metacercarial infection for *S. balanoides* from three sampling sites near Leisure Pool.		3. Negative binomial regression	4. 300	3. Appendix 11
4	Dunbar Leisure Pool	1. Comparison of prevalence of larval digenean infection between mollusc species (pooling data from 4 sites)	Larval digeneans in molluscs	1. Chi-square test and descriptive	1. 116	1. Table 2.3
		2. Comparison of prevalence of larval digenean infection between sampling sites and mollusc species.		2. Chi-square test and descriptive	2. 116	2. Table 2.4
5	Dunbar Red Rock and Leisure Pool	1. Comparison of the prevalence of metacercarial infection for *S. balanoides* from sites 2 and 3 at Red Rock.	Metacercariae in barnacles	1. Fisher's exact test and bar chart	1. 191	1. Fig. 2.28
		2. Goodness of fit test of Poisson distribution for the abundance and intensity of metacercarial infection for *S. balanoides* from site 2 and 3 at Red Rock.		2. Histogram and goodness of fit test for Poisson distribution	2. 191	2. Fig. 2.29
		3. Comparison of the mean abundance and mean intensity of metacercarial infection for *S. balanoides* from site 2 and 3 at Red Rock.		3. Bar chart, descriptive and negative binomial regression	3. 191 for abundance and 189 for intensity	3. Fig. 2.28 and appendix 12
		4. Comparison of the prevalence of larval cestode infection for *S. balanoides* from site 2 and 3 at Red Rock.	Larval cestodes in barnacles	4. Fisher's exact test and bar chart	4. 191	4. Fig. 2.30
		5. Goodness of fit test of Poisson distribution for the abundance and intensity of larval cestode infection for *S. balanoides* from site 2 and 3 at Red Rock.		5. Histogram and goodness of fit test for Poisson distribution	5. 191	5. Fig. 2.31
		6. Comparison of the mean abundance and mean intensity of larval cestode infection for *S. balanoides* from site 2 and 3 at Red Rock.		6. Bar chart, descriptive and Poisson regression	6. 191 for abundance and 6 for intensity	6. Fig. 2.30 and appendix 13
		7. Comparison of the prevalence of the	Larval digeneans in	7. Chi-square test or	7. 129	7. Fig. 2.32

		different stages of larval digeneans found in periwinkles and dog whelks collected from Red Rock and from Leisure Pool.	molluscs	fisher's exact test, and bar chart		
		8. Comparison of the prevalence of the different stages of larval digeneans in periwinkles or dog whelks collected from Red Rock and from Leisure Pool.		8. Chi-square test or fisher's exact test, and bar chart	8. 96 for periwinkles and 33 for dog whelks	8. Fig. 2.33 and 2.34
		9. Comparison of the prevalence of the different stages of larval digeneans in periwinkles collected from Site 1 and 3 at Dunbar Red Rock		9. Fisher's exact test and bar chart	9. 80	9. Fig. 2.35
		10. Comparison of the prevalence of the different stages of larval digeneans in periwinkles within Site 1 or 3 (open rock areas vs. crevices) at Dunbar Red Rock.		10. Fisher's exact test and bar chart	10. 40 for site 1 and 3	10. Figure 2.36 and 2.37
3 & 5	Dunbar Red Rock and Leisure Pool	Comparison of variance-to-mean ratio of abundance of metacercarial infection in *S. balanoides* from the five sampling sites at Dunbar	Metacercariae in barnacles	variance-to-mean ratio (D)	500	Table 2.5
6	South Queensferry Left side of Forth Bridge	Spatial distribution of different barnacle species at each sampling sites.	Barnacles	Pie chart	400	Fig. 2.48

2.3.1 Commensal and parasitic fauna of *Semibalanus balanoides*

The following reports on the commensal and parasitic fauna of *S. balanoides* for the study period Feb. 2013 – Dec. 2018 but with a focus on the findings of trip no. 1 & 2 (Table 2.1.). Mites were regularly found on the shell plates; as were turbellaria, oligochaetes, larval nematodes, and juvenile periwinkles but only occasionally. The mites that were encountered were not speciated in the present study, nor were the ciliates or the algal species that were found on the surface of the prosoma. Digenean metacercariae and cestode cysticercoids were found inside the prosoma but outside the guts of barnacles. Trophozoites of gregarines were found inside the gut of barnacles. Diagnosis of the species of ciliates and gregarines was not made in the current study but photos were taken using a camera attached to a stereo microscope (Figures 2.12-2.13).

With respect to the prevalence of all observed commensal and parasitic taxa, the Protozoa had the highest prevalence. If, however, only the parasitic fauna is considered, then the digeneans were the dominant parasite, followed by cestodes, with gregarines being encountered only occasionally. Nematodes were found only once, and this was most likely an accidental infection. The nematode was found inside the prosoma and the species was not diagnosed (Figure 2.15). The morphology of the larval cestodes that were encountered throughout the study will be discussed later in this thesis. Whole mounted, stained metacercariae were diagnosed to the genus level as *Maritrema* sp. (Figure 2.16), according to their small body size, characteristic ring-shaped vitellaria and the presence of a cirrus sac (Deblock, 2008). While these most likely represent *Maritrema gratiosum* Nicoll, 1907 based on their dimensions and morphology of their internal organs, this will be addressed in detail in Chapter 4 and, therefore, will not be discussed here. The species is provisionally referred to as *Maritrema* sp. until a robust species diagnosis can be achieved.

Comparing the associated and parasitic fauna of barnacles found in Colston's (2012) study and the findings from the present study, the range of parasite taxa found are very similar. Both studies found gregarines, sessile peritrichs, digenean cysts, larval cestode cysts, mites, planarians, nematodes, and polychaetes. The nematode cyst found inside the prosoma is unique in the present study, and the types of larval cestodes that were found in the present study were morphologically different to those reported by Colston. Beside these, no obvious differences were detected. The species of larval cestodes, mites, nematodes and polychaetes were not further investigated in the present study due to constraints of time and resources. The composition of the associated and parasitic fauna of barnacles found in South

Queensferry and Dunbar during 2013-2014 were very similar to those reported by Colston in 2012. Machrihanish was not investigated by Colston, and so is not compared.

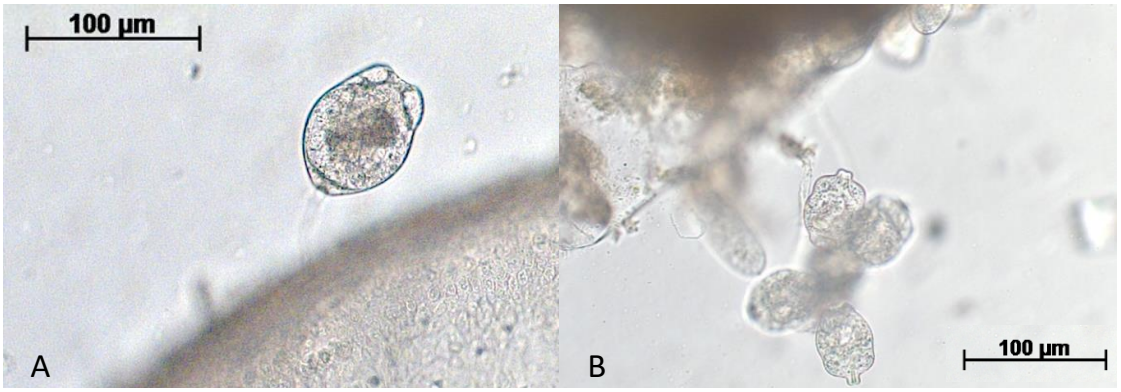


Figure 2.12. Colonies of the stalked peritrich *Epistylis* sp. found attached on the prosoma of barnacles. It had an ellipsoid body and was attached to a branched and non-contractile stalk. A: a single zooid. B: a colony with four zooids.

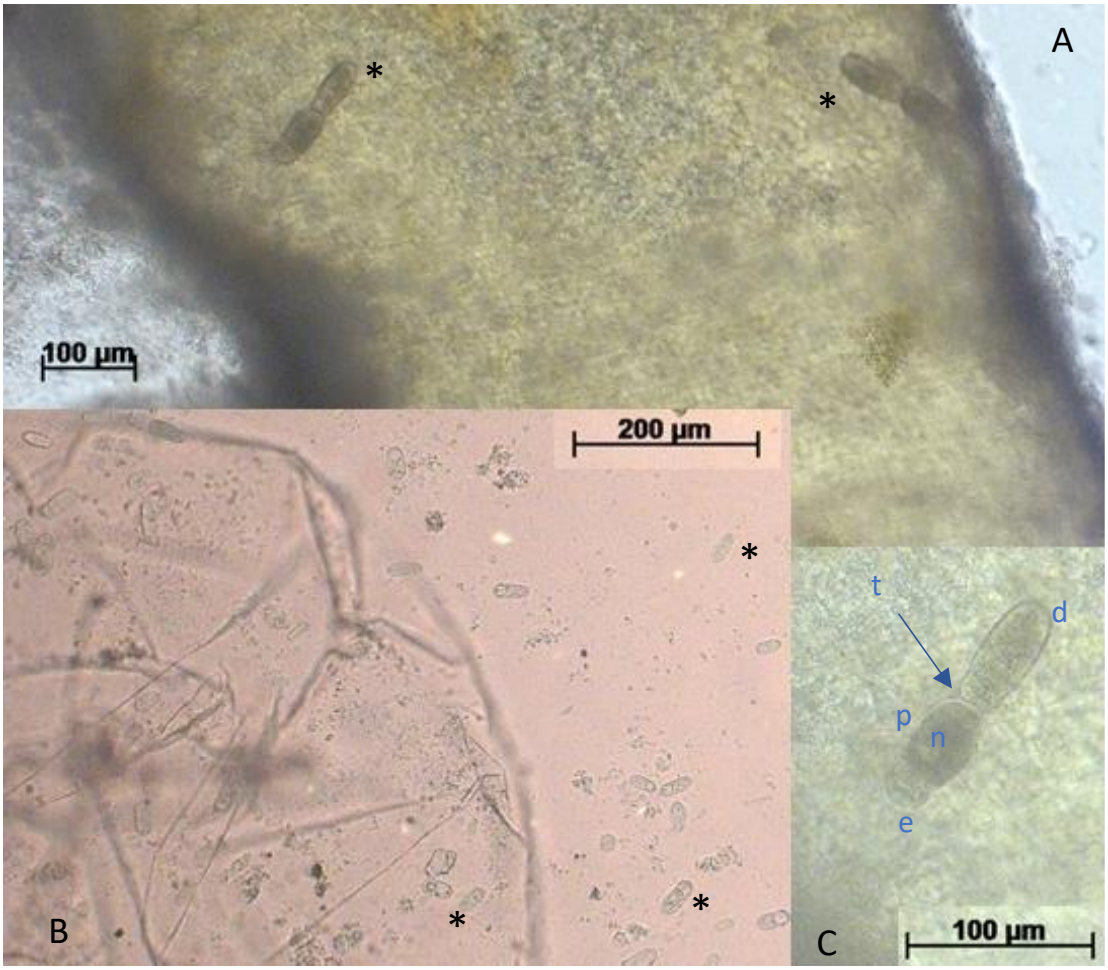


Figure 2.13. Gregarines found in the prosoma of barnacles *Semibalanus balanoides*. A: two pairs of trophozoites in head-to-tail association inside the guts of barnacles. B: numerous elongate ovals, probably oocysts of gregarines, were found outside the prosoma but inside the mantle of barnacles. The gregarines are identified by asterisks (*). C: higher magnification of trophozoites from A. Epimerite (e, 16.4 x 10.2 µm), protomerite (p, 27.7 x 22.5 µm) transverse septum (t), deutomerite (d, 36.2 x 50.2 µm), and a large nucleolus (n) can be seen.

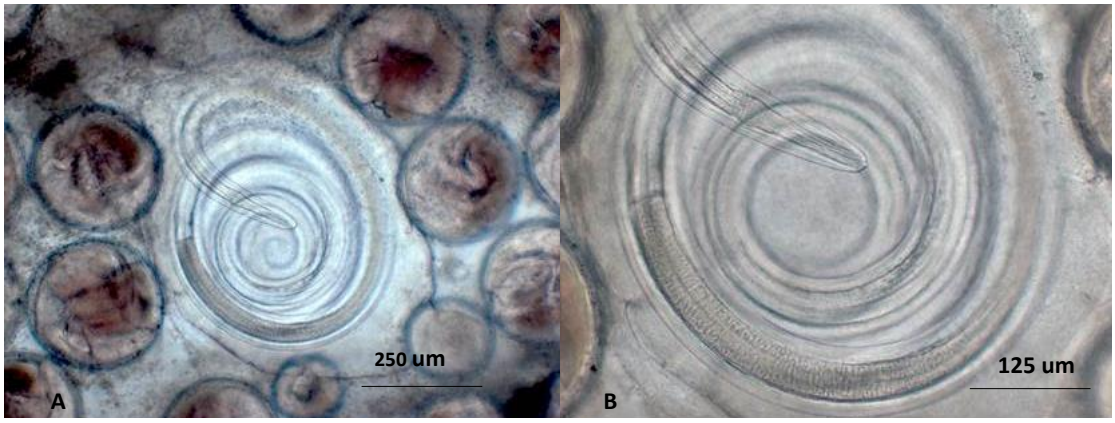


Figure 2.14. A single nematode found inside the prosoma of a barnacle *Semibalanus balanoides*. It was surrounded by a thin membrane, and surrounded by many metacercarial cysts. A: lower magnification. B: head region at higher magnification.

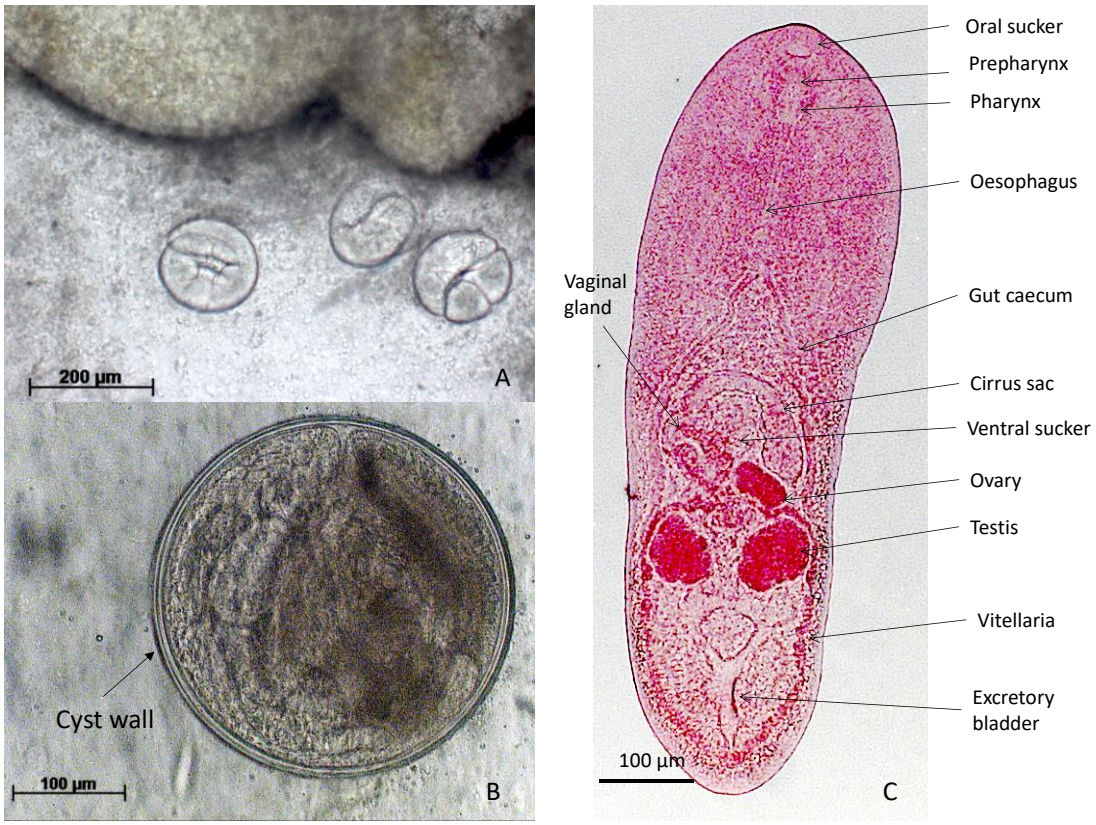


Figure 2.15. Encysted and excysted digenean metacercariae found outside gut wall of *S. balanoides*. A: immature metacercariae. B: a matured metacercaria, noted the 2-layered cyst wall. C: a metacercaria flattened in Berland's fluid and stained with Mayer's paracarmine.

2.3.1.1 Morphology of larval cestodes

Four types of larval cestode were found in the prosoma of barnacles during the study. They were nearly always found as co-infections with digeneans and seldom infected barnacles

alone. They were usually found in larger and more heavily infected barnacles. The first cysticercoid type is the most common, although the prevalence was not calculated. According to the definition by Chervy (2002), three of the four types of larval cestode observed are probably cysticercoid larvae, however, the fourth type of larva, which is more problematic, had the appearance of a plerocercoid larva that was enclosed by a cyst. The first to third type all possessed the characters of cysticercoids: primary lacuna present and scolex retracted.

2.3.1.1.1 Cysticercoid type I

This was the most common type. When in the prosoma of *S. balanoides*, the inner cyst was enclosed by a membranous outer cyst which was full of yellowish fluid. The scolex was retracted in the inner cyst with four unarmed suckers, and a retracted and armed rostellum. The rostellar hooks were arranged in a single row and directed upwards when retracted in the scolex. Corpuscles near the neck region of the scolex and inner cyst wall were evident. When manually removed from the outer cyst, a long cercomer was found (Figures 2.16 - 2.17). Outer cysts measured 373.57 µm L × 276.09 µm W (n=1). Inner cysts measured 220.14 µm L ± 20.55 × 163.93 µm W ± 30.01 (n=3). Scolex measured 135.26 µm W ± 27.89 × 133.15 µm L ± 36.50 (n=2). Sucker measured 59.76 µm L ± 2.87 × 44.25 µm W ± 2.22 (n=2). Rostellum measured 51.67 µm L × 38.26 µm W (n=1). Rostellar hook length 25.48 µm ± 1.47 (n=4). At least 8 rostellar hooks were counted.

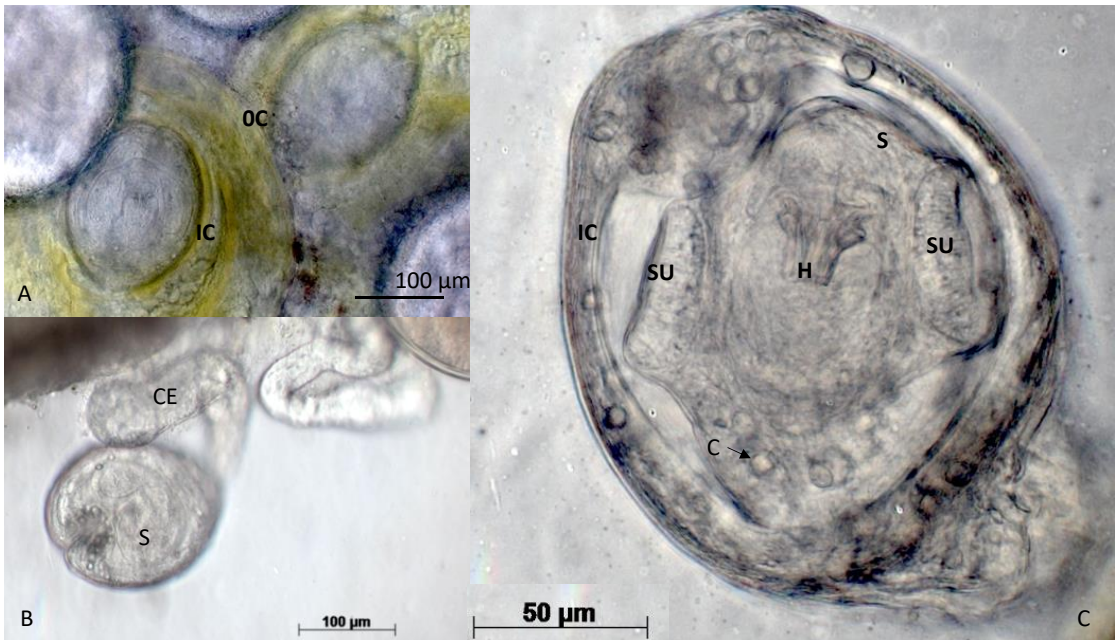


Figure 2.16. Type I cysticercoid. A: mixed infection of cysticercoids and metacercariae *in situ* in the prosoma of *Semibalanus balanoides*. Note the cysticercoids gathered in a group and the space between the outer cyst (OC) and inner cyst (IC) which was filled with a yellow fluid. B: the scolex (S) of a cysticercoid released from its inner cyst. Note a long cercomer (CE). C: higher magnification of a single cysticercoid. Note the scolex (S), rostellar hooks (H), inner cyst (IC) and corpuscles (C) near the bottom of the scolex.

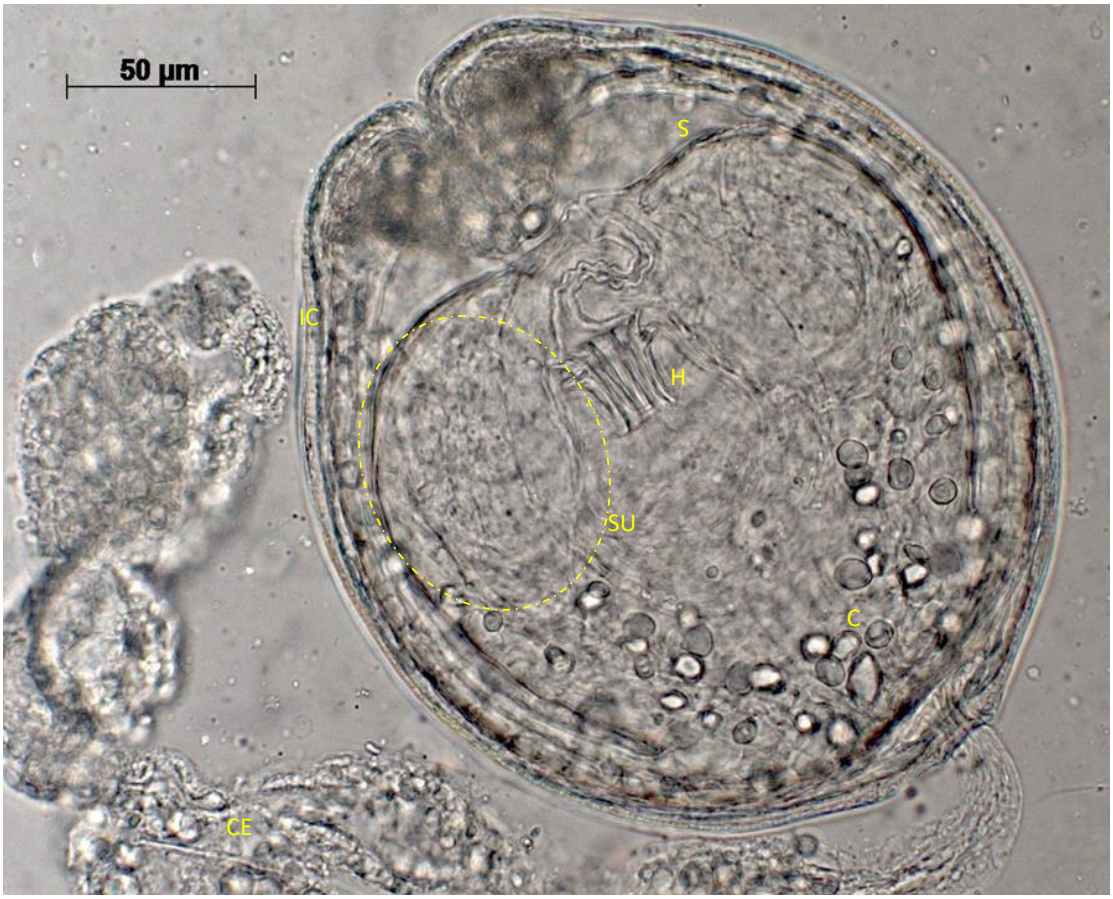


Figure 2.17. Type I cysticercoid. Higher magnification of a single cysticercoid. Note the inner cyst (IC), scolex (S), sucker (SU, indicated by a broken line), rostellar hooks (H), corpuscles (C) and cercomer (CE).

2.3.1.1.2 Cysticercoid type II

Type II was larger than type I and was found in *S. balanoides*. The inner cyst was also enclosed by a membranous outer cyst which was full of fluid, which was frequently yellow in colour. The scolex was retracted in the inner cyst and had four unarmed suckers, and an armed, retracted rostellum. The rostellum was stronger and larger than the first type. The rostellar hooks were directed downward when retracted in the scolex. There seemed to be two rows of rostellar hooks, arranged in a staggered manner. Corpuscles were seen in the neck region of the scolex and inner cyst wall. No cercomer was seen (Figures 2.18). The outer cyst measured 559.67 µm L × 538.44 µm W (n=1); the inner cyst 440.40 µm L ± 31.08 × 441.68 µm W ± 18.58 (n=2). The scolex measured 339.53 µm L × 288.30 µm W. Each sucker measured 113.38 µm L ± 13.44 × 100.23 µm W ± 15.59 (n=2). Rostellum measured 314.18 µm L × 86.39 µm W (n=1). Rostellar hook length 71.82 µm ± 1.20 (n= 6); with at least 10 rostellar hooks present.

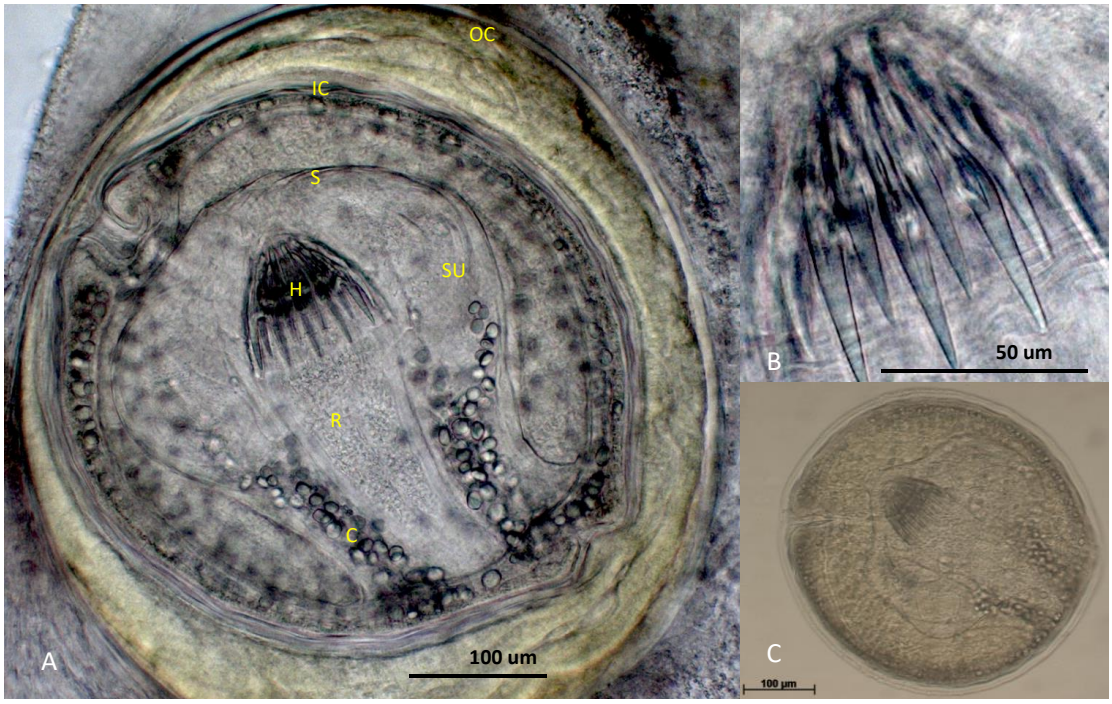


Figure 2.18. Type II cysticercoid in a *Semibalanus balanoides*. A: a single cysticercoid with outer cyst (OC) and inner cyst (IC). Note the scolex (S), sucker (SU), rostellar hooks (H), and corpuscles (C). The space between OC and IC was yellow. B: higher magnification of the rostellar hooks. Note there were two rows of rostellar hooks. C: a cysticercoid without an outer cyst.

2.3.1.1.3 Cysticercoid type III

Type III, found in *S. balanoides,* was quite similar to type I, but the space between the scolex and the inner cyst wall was noticeably larger as were the cyst dimensions (inner and outer cyst) and the scolex. The inner cyst was also enclosed by a membranous, fluid-filled outer cyst. The retracted scolex within the inner cyst had four unarmed suckers and a retracted armed rostellum though the latter was not obvious. The rostellar hooks were directed upward when retracted in the scolex. Only a single row of, at least 8, rostellar hooks was observed. There were very few corpuscles in the neck region of scolex and inner cyst wall (Figures 2.19). Outer cyst measured 753.45 µm L ± 76.90 × 610.73 µm W ± 229.67 (n=2), while the inner cyst measured 450.14 µm L ± 161.84 × 333.87 µm W ± 89.34 (n=3). Scolex measured 217.26 µm L ± 70.55 × 216.86 µm W ± 72.46 (n=3). Rostellum measured 88.73 µm L (n=1). The suckers could not be measured. Rostellar hook length 21.29 µm ± 1.14 (n=4).

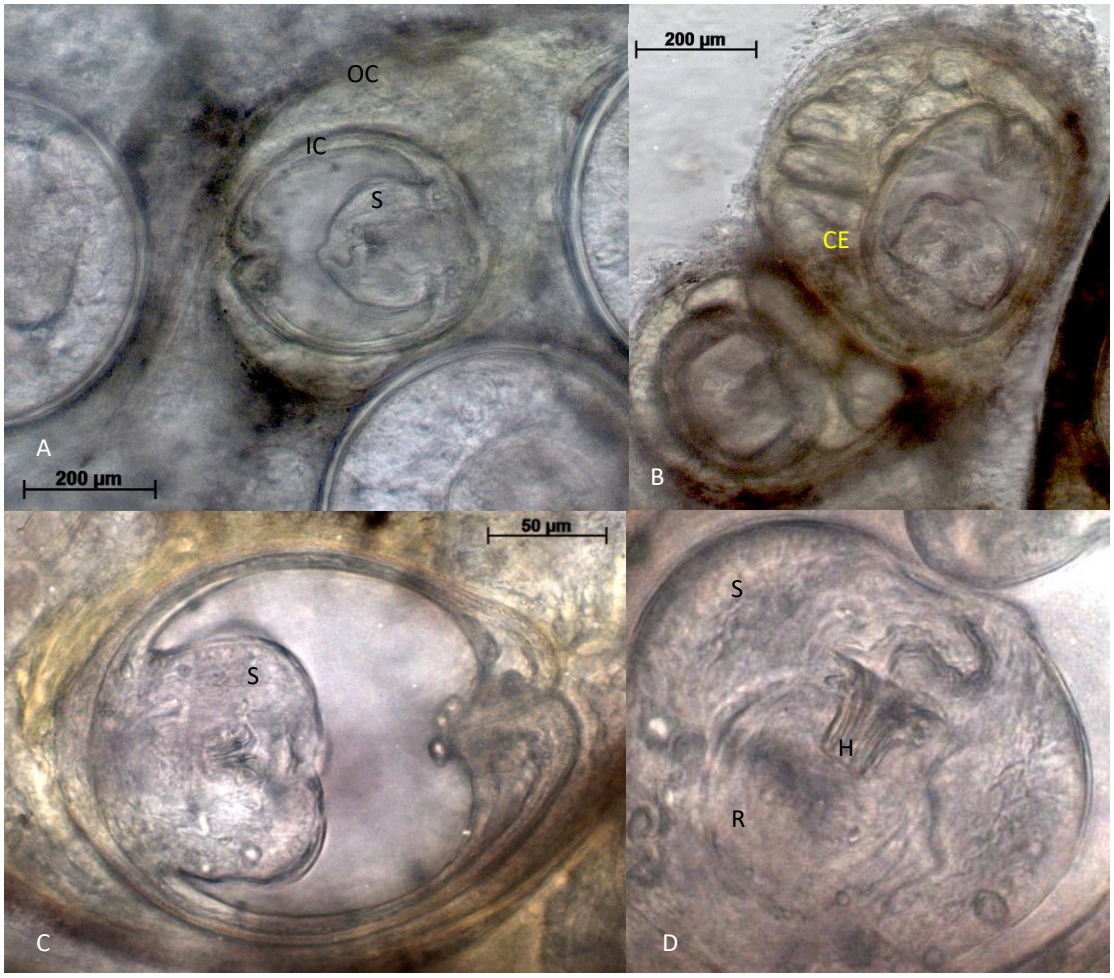


Figure 2.19. Type III cysticercoid. A: mixed infection of a cysticercoid and metacercariae *in situ* in the prosoma of *Semibalanus balanoides*. B: cysticercoids outside the gut of a *S. balanoides*. C & D: individual cysticercoids. CE: cercomer, H: rostellar hooks, IC: inner cyst, OC, outer cyst, R: retracted rostellum, S: scolex.

2.3.1.1.4 Cysticercoid type IV

This type was found only once at Dunbar in a *S. balanoides*. The morphology was closer to that of a plerocercoid, but it may be a developing cercocysticercoid, according to the definition of Chervy (2002). There was only one cyst enclosing the larva, the latter which seemed to be comprised of an everted rostellum, scolex and a cercomer. No primary lacuna or bladder was seen. The rostellum was strong and well-armed. The rostellum hooks were arranged in only one row and directed downward. The scolex had two suckers. There were some corpuscles in the neck region of the scolex (Figure 2.20). The cyst measured 441.25 µm × 436.96 µm (n=1). The scolex measured 370.14 µm L × 266.96 µm W (n=1), neck length 294.76 µm (n=1). Sucker measured 184.08 µm ± 10.60 (diameter only, n=2). Rostellar hook length 135.82 µm ± 4.44 (n=5). At least 12 rostellar hooks were present.

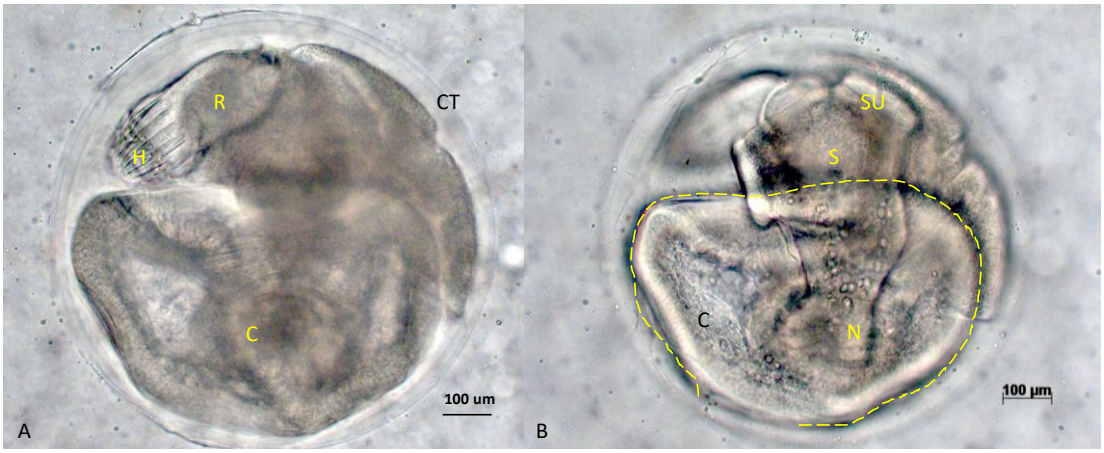


Figure 2.20. Type IV cysticercoid isolated from a *Semibalanus balanoides*. A & B: individual cysticercoid manually removed from the prosoma. Note the cyst (CT), scolex (S), neck of scolex (N), rostellum (R), rostellar hooks (H), sucker (SU), and cercomer (C, indicated by a broken line).

2.3.2 Comparisons of infection parameters between samples from South Queensferry and Machrihanish (trip no. 1 & 2)

A total of 112 barnacles were dissected, 66 were from South Queensferry and 46 from Machrihanish. In total, 107 samples were analysed. The prevalence of the different parasitic or commensal taxa (regardless of sampling sites) was calculated (Figure 2.21). The results showed that the Protozoa (*i.e.*, commensal ciliates and gregarines) were the most prevalent, followed by the digeneans, mites, nematodes and finally cestodes. The prevalence of parasitic or commensal taxa at different sampling sites (South Queensferry vs. Machrihanish) was compared (Figure 2.22). The prevalence of commensal taxa such as protozoans, mites and nematodes were higher in Machrihanish. Protozoan prevalence is significantly higher (Pearson Chi-Square= 25.554, $p<0.001$), and for nematodes and mites it was marginally significant (Pearson Chi-Square= 5.453 & 4.317 $p= 0.02$ &0.038, respectively). While the prevalence of digeneans was higher in South Queensferry than in Machrihanish (Pearson Chi-Square= 21.668, $p<0.001$), the prevalence of cestodes, however, was higher in the Machrihanish samples but not significantly so (Pearson Chi-Square= 1.445, $p= 0.229$). Regarding the taxonomic diversity of the commensal Protozoa in the barnacles from South Queensferry, 30.8% of the barnacles carried ciliates, 1.5% ciliates and gregarines, 0.5% gregarines, 1.5% other unknown Protozoa, while 21% of samples were negative for Protozoa.

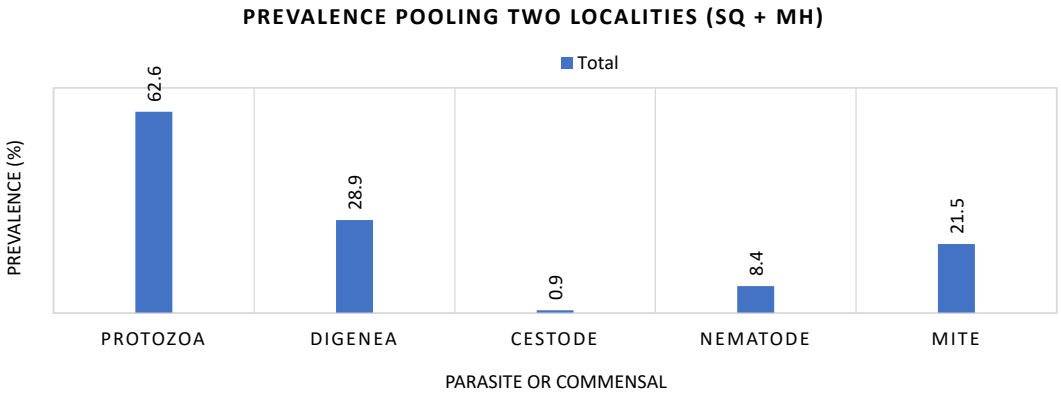


Figure 2.21. Faunistic survey of parasitic / commensals of *Semibalanus balanoides* (South Queensferry (SQ) + Machrihanish (MH), total 112 barnacles). Data revealed the prevalence of various parasitic or commensal taxa found inside the prosoma (protozoans, digeneans and cestodes), on the prosoma (Protozoa) or outside the shell plates (nematodes and mites) of barnacles.

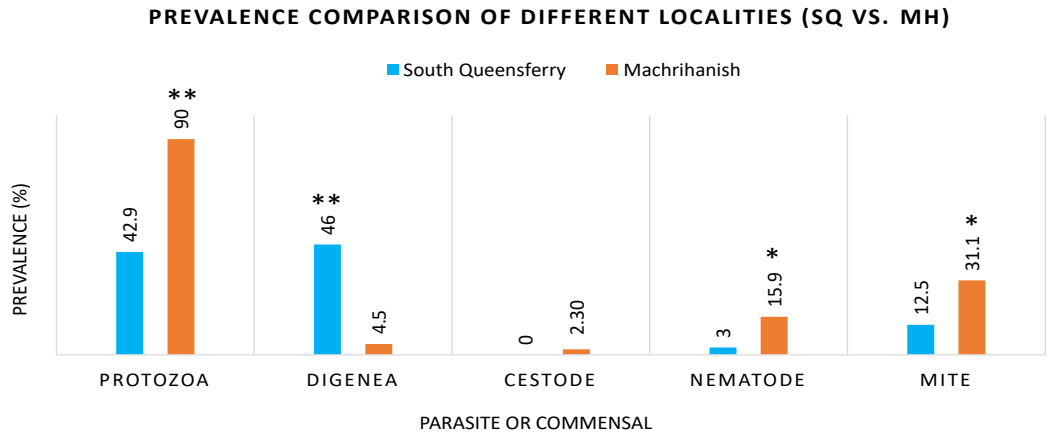


Figure 2.22. Comparison of the prevalence of the different parasitic or commensal taxa from barnacles collected from South Queensferry and from Machrihanish. Asterisks (**) represent p<0.001, and an asterisk (*) represents p<0.05.

The prevalence of the parasitic and commensal taxa of barnacles from different substrates (inorganic = rocks v.s. organic = molluscs or algae) was also compared (Figure 2.23). The prevalence of the parasitic and associated taxa, digeneans, cestodes and mites were found to be higher in the barnacles collected from organic substrates. For Digenea, it was significantly higher (Pearson Chi-Square= 17.238, p<0.001) but for cestodes the difference was not significant (Pearson Chi-Square= 0.491, p= 0.484). For the barnacles collected from the inorganic substratum, the Protozoa and the mites were significantly higher (Pearson Chi-

Square= 22.285, *p*<0.001; and Pearson Chi-Square= 16.509, *p*<0.001 respectively). There was no significant difference in the number of nematodes (Pearson Chi-Square=2.330, *p*= 0.127).

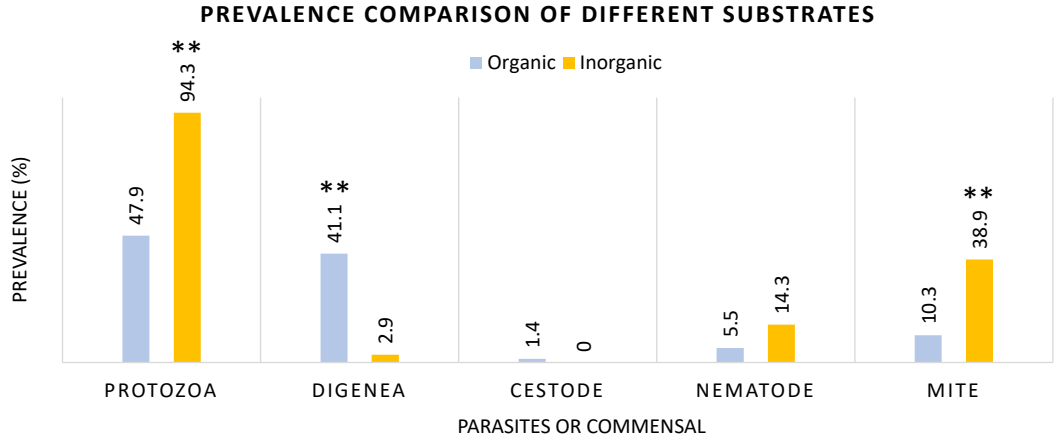


Figure 2.23. Comparison of the prevalence of the different parasitic or commensal taxa from the barnacles collected from different substrates (organic versus inorganic). Asterisks (**) represent p<0.001.

The presence of digenean infection (prevalence) relative to the reproductive status of barnacles (gravid or not) between South Queensferry and Machrihanish was also compared (Figures 2.24A & 2.24B). The prevalence of Digenea in South Queensferry reached almost 50% while that in Machrihanish was 10 times lower. The percentage of gravid barnacles was similar between localities; however, more gravid barnacles were found in the barnacles collected from Machrihanish. An analysis of the pooled data from the two localities using Fisher's Exact Test, *p*= 0.346) or by separate analyses (Fisher's Exact Test, *p*= 0.395 for South Queensferry; Fisher's Exact Test, *p*= 0.493 for Machrihanish, Appendix 10), indicated that digenean infection had no significant association with the reproductive status of their barnacle hosts. As shown in Figure 2.24B, however, the proportion of infected non-gravid individuals is higher than infected gravid individuals at both sites.

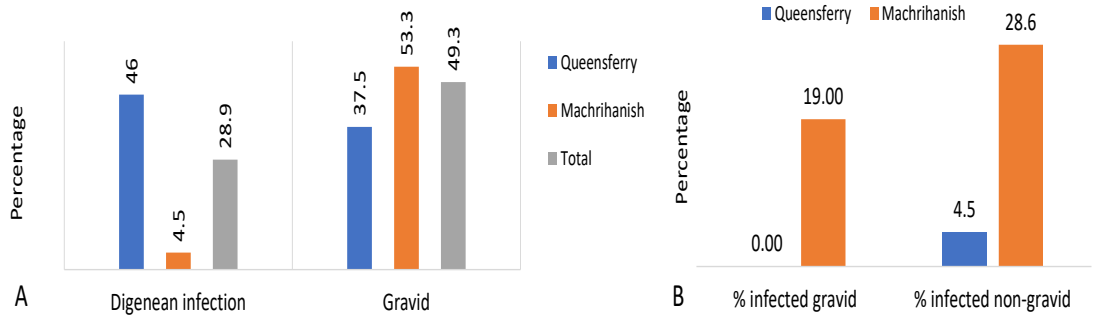


Figure 2.24. The prevalence of digenean infection relative to the reproductive status of *Semibalanus balanoides* collected from South Queensferry and Machrihanish. A: a comparison of the reproductive status and digenean infection in *S. balanoides*. B: a comparison of infected gravid and infected non-gravid *S. balanoides*.

2.3.3 Comparison of infection parameters for digeneans in barnacles among sites from samples collected in Dunbar Leisure Pool (trip no. 3)

In this field trip three sampling sites were explored (Figure 2.5). One hundred barnacles from each site were dissected. All the species were *S. balanoides*, except for one specimen of *A. modestus* which was excluded from the data. Only digenean metacercariae were found at this location. Two types of metacercarial cyst (large and small) were observed. They were assumed to be different developmental stages of the same species, because no substantial morphological difference was found except that the larger cysts had thicker cyst walls. The calculation of infection indices, therefore, included both types.

The prevalence, mean abundance, and mean intensity of metacercariae between the sites were compared and the results are shown in Figures 2.25-2.26. A decreasing trend in prevalence, mean abundance, and mean intensity from kittiwake cliff towards the sea was found. This pattern was similar for both mean abundance and mean intensity. Samples from the "cliff below birds (the kittiwake nesting site)" had mean intensity and abundance that was 6 to 7 times higher than the other two sites lower on the shore. The prevalence of parasites in the barnacles collected at "Cliff below birds" is significantly higher than at the other two sites (Pearson Chi-Square= 93.456, $p<0.001$). The frequency distribution of abundance and intensity show a pattern of overdispersion (Figures 2.27A & 2.27B). The data of abundance does not fit a Poisson distribution (One-Sample Kolmogorov-Smirnov Test, Poisson Parameter= 2.47, $p<0.001$); variance (29.621) is not equal or close to the mean (2.47). The data of intensity does not fit a Poisson distribution either (One-Sample Kolmogorov-Smirnov Test, Poisson Parameter= 5.25, $p<0.001$); variance (48.559) is not equal or close to the mean

(5.25). Under a negative binomial regression model with log link function for abundance data using “Open bay low water edge” as a reference, it was revealed that, “Cliff side lower edge” had a significantly lower abundance (0.62 times incidence ratio, p=0.001), while the sample from the “Cliff below birds” had an abundance that was significantly higher (10.38 times incidence ratio, p<0.001) than the first site (Appendix 11).

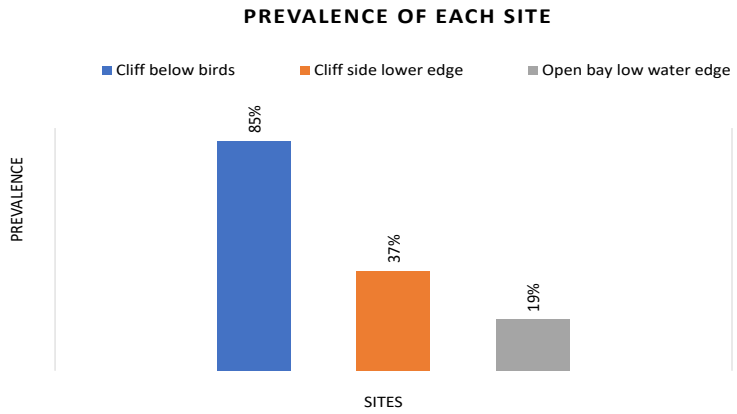


Figure 2.25. A comparison of the prevalence of digenean infection between the three different sampling sites at the coast near the Dunbar Leisure Pool (n = 100 barnacles per site).

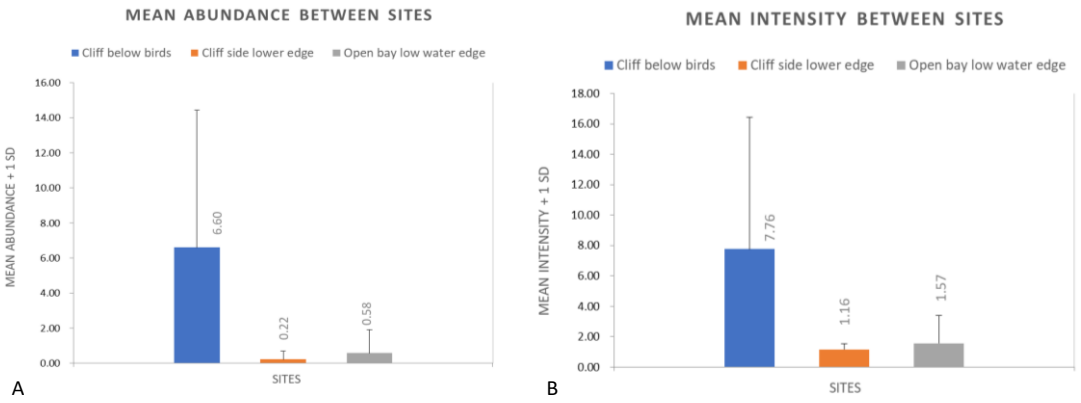


Figure 2.26. Comparison of the mean abundance (A) and intensity (B) of metacercariae in *Semibalanus balanoides* between three different sampling sites at the coast near the Dunbar Leisure Pool (n = 100 barnacles per site for abundance, n = 67, 19, 37 barnacles from left to right for intensity).

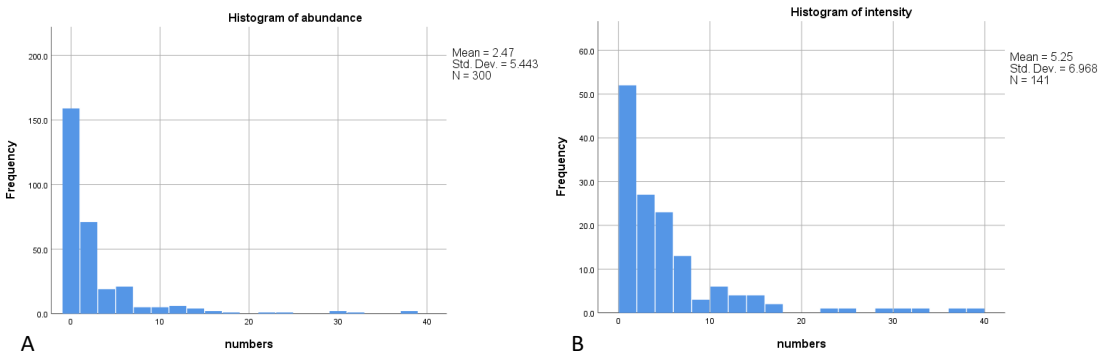


Figure 2.27. Histogram of the abundance (A) and intensity (B) of metacercarial cysts in *Semibalanus balanoides* from three sampling sites at the coast near the Dunbar Leisure Pool using data pooling from three sites and showing a negative binomial (right skewed) distribution.

2.3.4 **Comparison of infection parameters for digeneans in molluscs among sites from samples collected in Dunbar Leisure Pool (trip no. 4)**

During this field trip, only mollusc samples were taken. Four sampling sites were chosen from the shore near kittiwake cliff (Site 1-4, see Figure 2.6). Approximately 30 molluscs from each site were examined. Three types of mollusc were diagnosed to genus level by morphology of shells (using the identification keys of Graham, 1988): namely the dog whelk (*Nucella lapillus*), the periwinkle (*Littorina* spp.) and the limpet (*Patella* spp.). The numbers of each type varied between sites. Overall prevalence of parasitic infection was compared between sites and between mollusc types. Positive parasitic infection was defined as the existence of any stage of the larval digeneans, such as: cercariae, metacercariae, rediae and / or sporocysts, or the combination of them. The results are shown in Tables 2.3 and 2.4. Overall, the prevalence of larval digeneans among all molluscs collected from the shore nearest to kittiwake cliff was the highest, but it did not show a regular decreasing trend towards the lower shore and a chi-square test was not significant among sites (Pearson Chi-Square= 4.451, p= 0.217). If a comparison between the different mollusc types was made, then the limpets had the highest prevalence, followed by dog whelks, and then periwinkles, however, the chi-Square test was only marginally significant (Pearson Chi-Square= 6.500, p= 0.039). The compositions of larval digeneans varied considerably. Only metacercariae were found in the limpets whereas all stages of larval digeneans could be found in the dog whelks and periwinkles (data not shown).

Table 2.3. Comparison of prevalence of parasitic infection[1] between sites and mollusc species.

Site[3] (see Fig.2.6)	Sample size	Overall prevalence (% infection)	Prevalence of dog whelks (% infection)	Prevalence of periwinkles (% infection)	Prevalence of limpets (% infection)
1	30	23.3	50	20	0
2	26	23.1	0	28.6	NA[2]
3	30	13.3	0	18.2	NA
4	30	36.7	NA	18.2	87.5

1 Parasitic infection: including cercaria, rediae, sporocysts and metacercariae

2 NA: no molluscs of this genus present

3 Site: the four sites are arranged from shore to land and are all near kittiwake cliff

Table 2.4. Comparison of the prevalence of parasitic infection between mollusc types for samples collected from site near kittiwake cliff.

Mollusc types	prevalence (% infection)	sample size
Dog whelk	21.7	23
Periwinkle	21.3	80
Limpet	53.8	13

2.3.5 Comparison of infection parameters for digeneans in barnacles among sites from samples collected in Dunbar Red Rock (trip no. 5)

In this field trip three sampling sites were chosen; barnacles were collected from Sites 2 and 3 (see Figure 2.8). One hundred barnacles were dissected per site and a total of 200 barnacles were dissected. Among them, nine were *Austrominius modestus*, and therefore were excluded. Periwinkles were collected from Sites 1 and 3 with 60 individuals taken per site. At Site 1 and 3, twenty periwinkles were collected from open rock areas and another 20 from crevices. Fifteen dog whelks were collected at Red Rock but non-site specific, and 20 dog whelks were collected from the coast at Dunbar Leisure Pool for the purposes of comparison. In total, 96 periwinkles and 33 dog whelks were dissected.

The results for digenean infection in barnacles are shown in Figures 2.28 and 2.29. Almost all the barnacles that were dissected (Sites 2 *vs.* 3) displayed a metacercarial infection. The prevalence was similar between sites and was close to 100 % (Fisher's Exact Test, p= 0.476). Site 2, however, had a mean intensity and mean abundance that was more than three times higher than Site 3 (Figure 2.28). The frequency distribution of abundance and intensity shows a pattern of overdispersion (Figures 2.29A & 29B). The data for abundance do not fit a Poisson distribution (One-Sample Kolmogorov-Smirnov Test, Poisson Parameter= 33.92,

p<0.001; variance (1116.504) is not equal or close to mean (33.92)). The data for intensity do not fit a Poisson distribution either (One-Sample Kolmogorov-Smirnov Test, Poisson Parameter= 34.28, p<0.001; variance (1116.011) is not equal or close to mean (34.28)). Under a negative binomial regression model using "Site 3" as a reference, it was found that, "Site 2" had an abundance that was significantly higher (5.41 times incidence ratio, p<0.001) than Site 3 (Appendix 12).

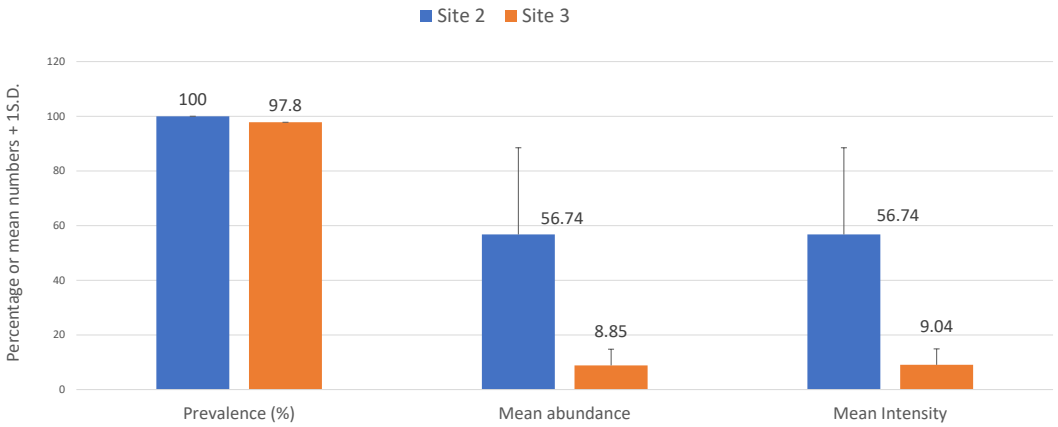


Figure 2.28. Comparison of infection indices (prevalence (%), mean abundance and mean intensity) of metacercariae in *Semibalanus balanoides* from Sites 2 and 3 at Dunbar Red Rock (For abundance:100 barnacles per site. For intensity: 100 barnacles at Site 2 and 89 at Site 3).

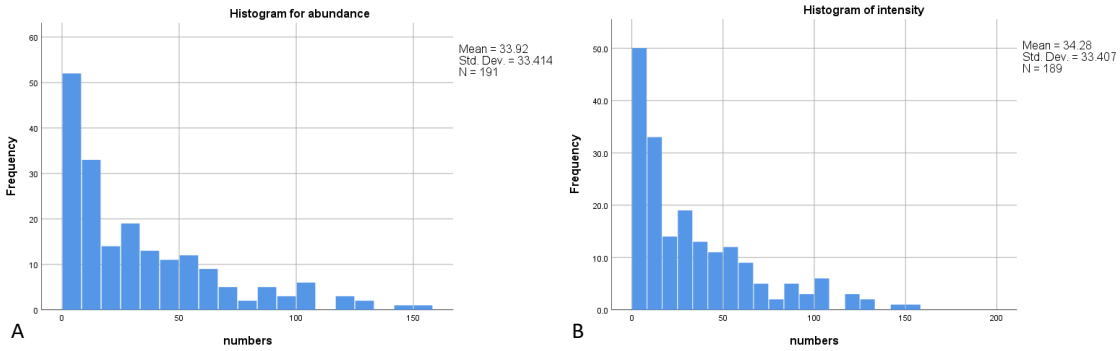


Figure 2.29. Histogram of abundance (A) and intensity (B) of metacercariae in *Semibalanus balanoides* from Sites 2 and 3 at Dunbar Red Rock using data pooled from Sites 2 and 3 and both showing a negative binomial (right skewed) data distribution.

2.3.6 Comparison of infection parameters for cestodes in barnacles among sites from samples collected in Dunbar Red Rock (trip no. 5)

In this field trip the same 191 *S. balanoides* collected from Sites 2 and 3 (see Figure 2.8) were analyzed. The prevalence of larval cestodes in barnacles from Dunbar Red Rock was quite low in general; not more than 4% across the two sampling sites (Fisher's Exact Test, *p*= 0.685). Site 2 also had a higher mean intensity and mean abundance of cestodes when compared to Site 3 (Figure 2.30). The frequency distribution of abundance shows a pattern of zero inflation and for intensity shows a nearly normal distribution (Figure 2.31). The intensity data does fit a Poisson distribution (One-Sample Kolmogorov-Smirnov Test, Poisson Parameter= 5, p=0.895; variance (22.4) is not equal or close to mean (5)) The abundance data does not fit a Poisson distribution either (One-Sample Kolmogorov-Smirnov Test, Poisson Parameter= 0.16, *p*=0.014; variance (1.354) is not equal or close to mean (0.16)) but a goodness-of-fit shows that a Poisson regression provides a better model. Under a Poisson regression model using "Site 3" as a reference revealed that, "Site 2" had an abundance that was significantly higher (4.92 times) than Site 3 (Appendix 13).

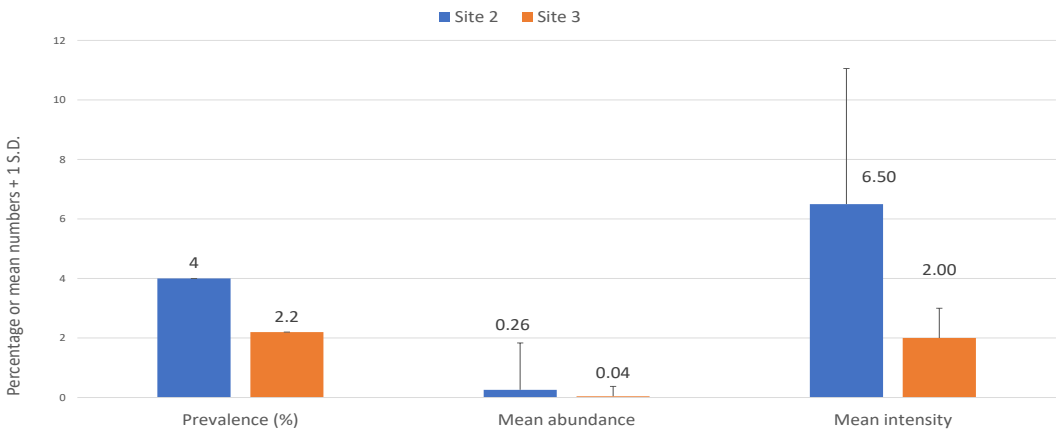
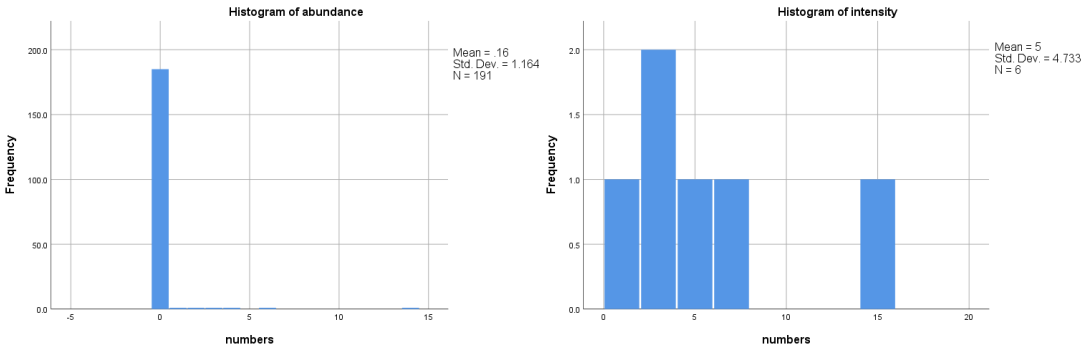


Figure 2.30. Comparison of infection indices (prevalence (%), mean abundance and mean intensity) of larval cestodes in barnacles collected at Sites 2 and 3 at Dunbar Red Rock (For abundance:100 barnacles per site. For intensity: 4 barnacles at Site 2 and 2 barnacles at Site 3).



A B

Figure 2.31. Histogram of abundance (A) and intensity (B) using data pooled from Sites 2 and 3. Abundance shows a zero-inflated data distribution, and intensity shows a slight right-skewed distribution.

2.3.7 Comparison of infection parameters for larval digenean in molluscs among sites from samples collected in Dunbar Red Rock and Leisure Pool (trip no. 5)

Molluscs infected by larval digeneans were further subdivided into different categories: 1) containing any number of metacercarial cysts, 2) more than two metacercarial cysts, 3) containing sporocysts and cercariae, and 3) containing rediae and cercariae. When looking at the composition of larval digeneans in molluscs from Sites 1 and 3 together, it was found that periwinkles had a significantly higher percentage of infection when the data was pooled and then compared to infections in dog whelks (Pearson Chi-Square= 6.802, *p*= 0.009). Metacercarial cysts were quite common in both type of molluscs, but usually appeared in low numbers. Sporocysts / cercariae reached 10% in periwinkles and 6% in dog whelks; while the prevalence of rediae / cercariae were less, ~3% in both types of molluscs (Figure 2.32). Metacercarial cysts were significantly higher in periwinkles (Fisher's Exact Test, p= 0.026). The remaining categories were not significantly differentiated.

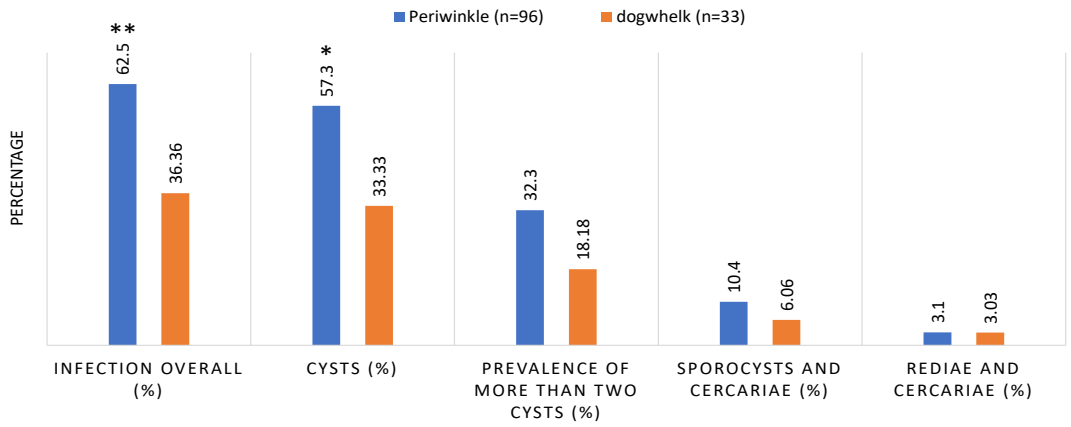


Figure 2.32. A comparison of the prevalence of the different stages of larval digeneans found in periwinkles and dog whelks at Dunbar Red Rock (using data pooled from Site 1, Site 3, non-site specific and Leisure Pool). Asterisks (**) represent p<0.01, and an asterisk (*) represent p<0.05.

When comparing the different categories of larval digenean infection in molluscs collected from Red Rock and Leisure Pool, it was found that the infection in all categories was higher in dog whelks (Figure 2.33) or in periwinkles (Figure 2.34) from Red Rock. Rediae / cercariae and sporocyst / cercariae were only found in molluscs from Red Rock. Differences between Red Rock and Leisure Pool were less dramatic in periwinkles, perhaps due to the higher infection rate at both sites.

For dog whelks, the overall digenean infection was significantly higher in samples from Red Rock than from the Leisure Pool (Fisher's Exact Test, *p*= 0.027). Metacercarial cyst infection was not significant (Fisher's Exact Test, *p*= 0.065), and category “more than two cysts” was marginally significant (Fisher's Exact Test, *p*= 0.049). The remainder of the categories were not significantly different. For periwinkles, the overall digenean infection was found to be higher in the samples from Red Rock than from Leisure Pool but not significantly so (Pearson Chi-Square= 2.880, *p*= 0.09). The remainder of the categories were not significantly different.

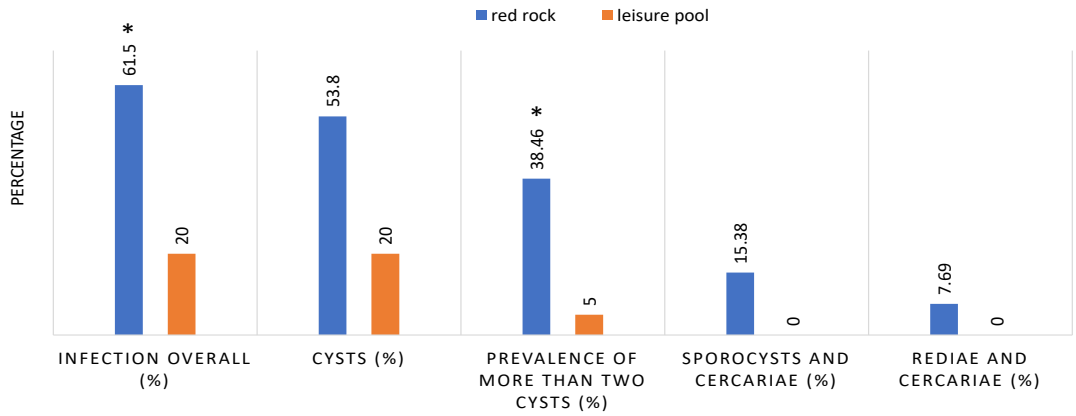


Figure 2.33. A comparison of the prevalence of the different stages of larval digeneans in dog whelks collected from Dunbar Red Rock (n=13) and from the Leisure Pool (n=20). An asterisk (*) represent p<0.05.

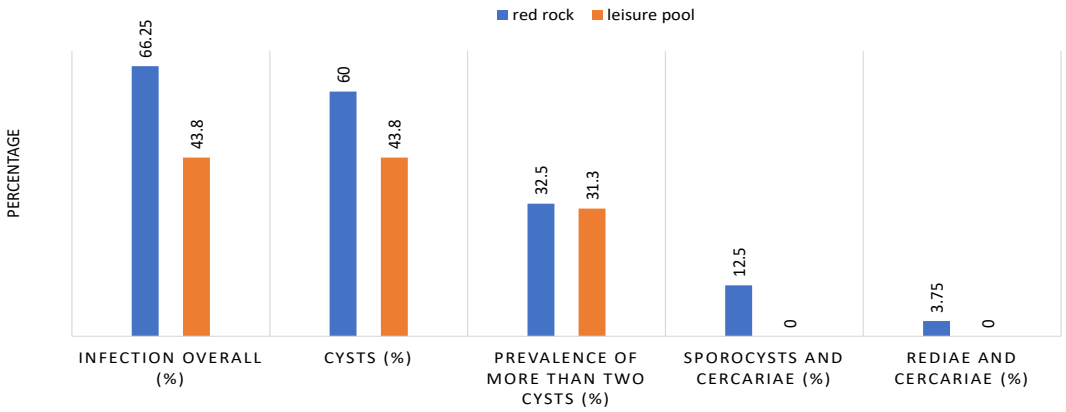


Figure 2.34. A comparison of the prevalence of the different stages of larval digeneans in periwinkles collected from Dunbar Red Rock (n=80) and from the Leisure Pool (n=16).

A more detailed comparison was performed on the periwinkles collected from Red Rock. The percentage of different larval digenean categories were similar between Sites 1 and 3 (Figure 2.35) with all categories showing no significant differences.

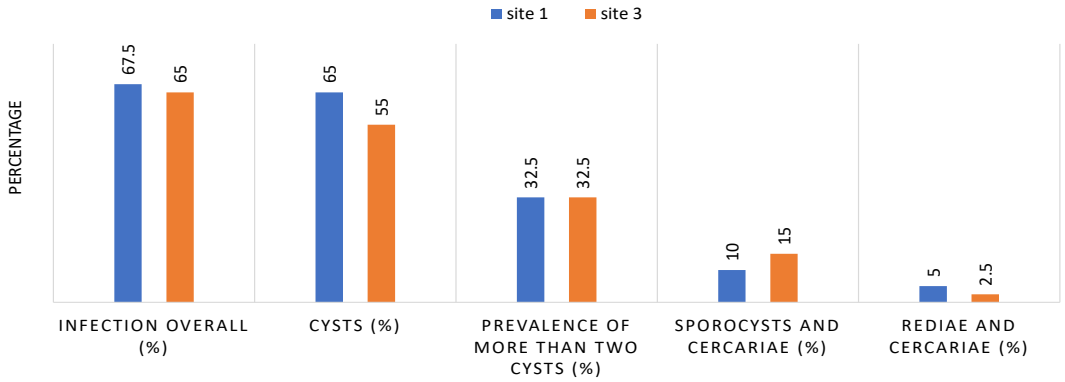


Figure 2.35. A comparison of the prevalence of the different stages of larval digeneans in periwinkles collected at Site 1 (n=40) and Site 3 (n=40) at Dunbar Red Rock.

Different categories of larval digenean infection were also compared between different microhabitats (*i.e.*, open areas versus crevices). Periwinkles collected in crevices were found to have higher larval digenean infections regardless of categories. However, when these were looked at in detail, those from crevices had more metacercarial cysts, while those from open rock areas had more sporocysts / cercariae and rediae / cercariae (Figure 2.36 for Site 1 and Figure 2.37 for Site 3). For both Site 1 and 3, all categories did not differ significantly (Fisher's Exact Test).

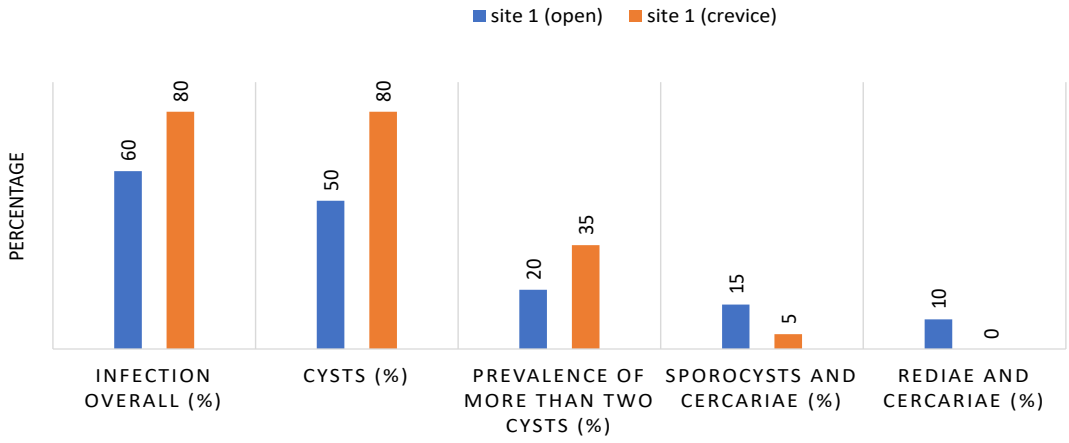


Figure 2.36. A comparison of the prevalence of the different stages of larval digeneans in periwinkles within Site 1 at Dunbar Red Rock. The comparison was made between open rock areas (n=20) and crevices (n=20).

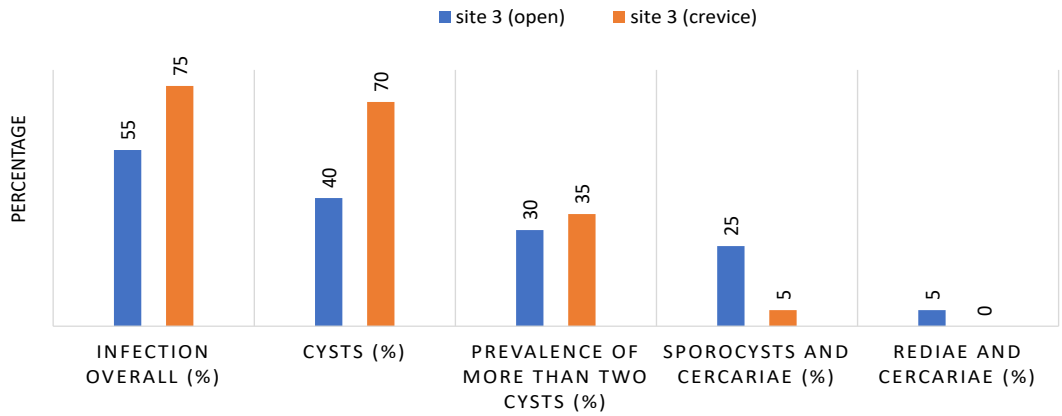


Figure 2.37. A comparison of the prevalence of the different stages of larval digeneans in periwinkles within Site 3 at Dunbar Red Rock. The comparison was made between open rock areas (n=20) and crevices (n=20).

2.3.8 Comparison of variance-to-mean ratio of abundance of metacercarial infection in barnacles from the five sampling sites at Dunbar

In order to conduct a simple estimate of the level of aggregation of metacercarial cysts within barnacle populations, variance-to-mean ratios were calculated for samples from five different sampling sites: including three sites near Dunbar Leisure Pool and two sites at Red Rock. For samples from South Queensferry and Machrihanish, there were no count data for metacercarial cysts so the ratio could not be calculated. The results were shown in Table 2.5. According to the categories defined by Kamdem *et al*. (2012), metacercarial distribution in all sites were clumped (aggregated), except for those from the cliff side lower edge near Dunbar Leisure Pool, which had a distribution closer to random.

Table 2.5. Comparison of variance-to-mean ratio (D) of the five sampling sites at Dunbar.

Sampling sites	Dunbar Leisure Pool			Dunbar Red Rock	
Parameters	Cliff below birds	Cliff side lower edge	Open bay low water edge	Site 2	Site 3
Sample size	100	100	100	100	91
Mean abundance of metacercarial cysts (m)	6.60	0.22	0.58	56.74	8.85
Sample variance (s^2)	61.47	0.23	1.80	996.85	34.77
Variance/mean ratio (D)	9.31	1.05	3.10	17.57	3.93
Distribution	Clumped	Random	Clumped	Clumped	Clumped

2.3.9 Comparison of the infection indices for the number of digenean cysts and larval cestodes in barnacles collected during the current study and from past data

In Colston's (2012) study, both *S. balanoides* and *A. modestus* were studied, but the localities were South Queensferry, Dunbar and Culross, therefore only the former two localities can be compared. The exact locality in South Queensferry (right or left side of the Forth Rail Bridge) was not mentioned in her work, and the locality in Dunbar seems to be the same rocky platform in the present study (Red Rock) justified by the photo in her work. It seems that 73 *A. modestus* and 514 *S. balanoides* were dissected by Colston, but *A. modestus* were all from South Queensferry. In the present study, too few infected *A. modestus* were found, therefore only data from *S. balanoides* were comparable. Readers would note that in her work abundance was called "intensity of infection" and intensity was called "mean intensity of infection". Two tables are provided below for the ease of comparison. From Tables 2.6 and 2.7, the chronological difference between years (2012 vs. 2013) is not obvious. Digenean infections at Dunbar were in general higher than at South Queensferry and the cestode infection, regardless of locality, were low but slightly higher at Dunbar. The prevalence of digenean cysts was higher at both localities in 2013, but the abundance and intensity was lower in 2013 (only data from Dunbar was available). The prevalence and the abundance of larval cestodes was higher at Dunbar for both years but the intensity between localities was quite similar in Colston's work. In the present study, no cestodes were found at South Queensferry. The infection indices for larval cestodes were, however, in general higher in 2012.

Table 2.6. A comparison of infection indices for the digenean cysts collected from *Semibalanus balanoides* in the present study (2013) and by Colston (2012)

Data source	Locality	Sample size	Prevalence	Abundance	Intensity
Present study (2013)	South Queensferry	63	46.0	NA*	NA*
Colston (2012)		337	24.3	0.43	1.77
Present study (2013)	Dunbar (Red Rock)	191	99.0	33.9	34.3
Colston (2012)		177	93.2	45.2	48.5

*No count data available.

Table 2.7. A comparison of the infection indices for the larval cestode cysts collected from *Semibalanus balanoides* from the current study and that of Colston (2012)

Data source	Locality	Sample size	Prevalence	Abundance	Intensity
Present study (2013)	South Queensferry	63	0	0	0
Colston (2012)*		337	0.3	0.02-0	6-1
Present study (2013)	Dunbar (Red Rock)	191	3.1	0.16	5
Colston (2012)*		329**	15.5-0.3	0.95-0.03	6.14-1

*Five types of cestode were analyzed separately.

**Two batches of samples were analyzed. The author did not specify whether they are all from *S. balanoides* or not.

2.3.10 Morphology of cercariae and metacercariae from different molluscan hosts

2.3.10.1 Type I cercaria

This type of cercaria was found in the digestive glands of dog whelks. Numerous rediae containing cercariae were present in the viscera. The distome cercariae had a characteristic tail (L = 521.92 µm; n= 1), its anterior three-quarters looked glandular under a light compound microscope, and a tubular structure was noticed in the posterior quarter. When observed under SEM, the anterior part of the tail was resolved as a fin-like structure with or without ribs, and the posterior tubular structure was still prominent. The body was filled with large glandular cells and was brown in colour when observed alive. An oesophagus bifurcated into two parts in front of the ventral sucker with the two gut caeca extending to the posterior end of the body. Two conspicuous gland ducts terminated on either side of the oral sucker. Under SEM, the body surface was seen to be covered by grid-like protrusions, and between these protrusions were numerous pores, which were also arranged in symmetric manner. The oral sucker and ventral sucker had no such projections and pores. Instead, the lip of the oral sucker had numerous papillae and the lip of the ventral sucker had radial striations (Figure 2.38). Sometimes a few encysted cercariae could be found in the mass of cercariae and rediae. The body (554.67 µm L × 316.59 µm W at widest part; n=1) was wider near the oral sucker (87.68 µm L × 87.84 µm W; n=1), slightly constricted at the ventral sucker (100.78 µm L × 102.97 µm W; n=1) level. The oral and ventral suckers were both unarmed.

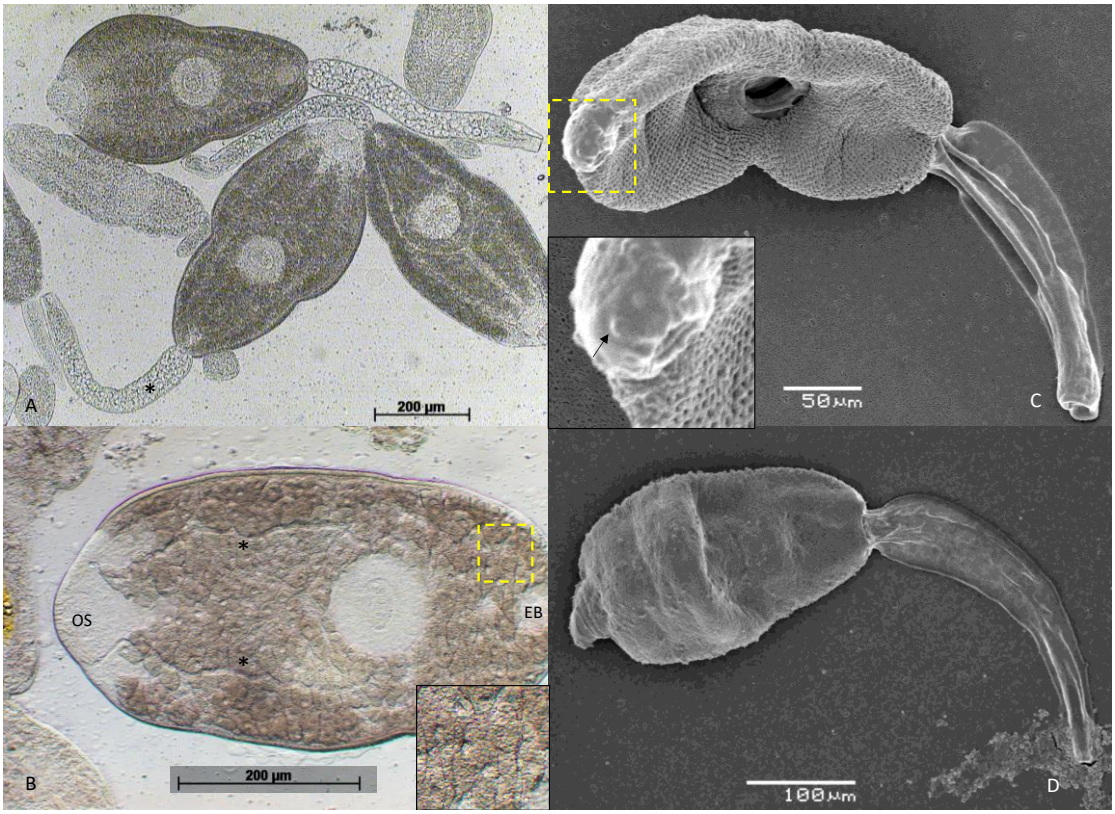


Figure 2.38. The morphology of type I cercariae. A: living cercariae released from the rediae. Note the glandular cells in the tail (*). B: cercarial body which was filled with gland cells (brown in colour). Note the two collecting ducts (*) which terminated on either side of the oral sucker (OS). EB = excretory bladder. Insert: Higher magnification of the brown glandular cells in cercarial body (from the yellow rectangle). C: ventral view under SEM. Note the fin-like part and tubular part of the tail. There were papillae on the oral sucker (arrow) and the body surface was covered by grid-like protrusions (See insert from the yellow rectangle). D: dorsal view under SEM.

2.3.10.2 Type II cercaria

This type of cercaria was found in the digestive glands of periwinkles. Numerous rediae containing cercariae were found in the viscera. The redia was quite elongated and had a small mouth. The cercariae were monostome (body 201.48 µm L × 71.51 µm W; n=1), light brown in colour, and with two eye spots posterior to the oral sucker (17.63 µm L × 21.99 µm W; n=1). The cercariae possess a long tail (413.26 µm; n=1) compared to the body, and when alive the tail beats in a characteristic way, *i.e.*, short but high frequency vibration in pulses. The tail is bordered by a thin membranous fin. There was no sign of a gut, while a kidney-shaped bladder can be seen near the base of the tail. Under SEM, delicate cilia were observed across the body surface, but some cercariae did not have these cilia. For those without cilia, the body surface was spineless, and slightly corrugated when contracted. When stained with neutral red, two

masses of deeply stained gland cells were noticed in the middle of body, and four ducts arose from these cells, which exited in the oral sucker region (Figures 2.39-2.40). A species diagnosis was made using morphology; however, ITS2 rDNA sequence data was generated for confirmatory purposes (Appendix 14).

Figure 2.39. The morphology of type II cercariae. Living cercariae stained with neutral red. Note the two eyespots and the two masses of deep red stained gland cells in the middle of the body. Four ducts (arrows) rise from these glands and terminate near the oral sucker region.

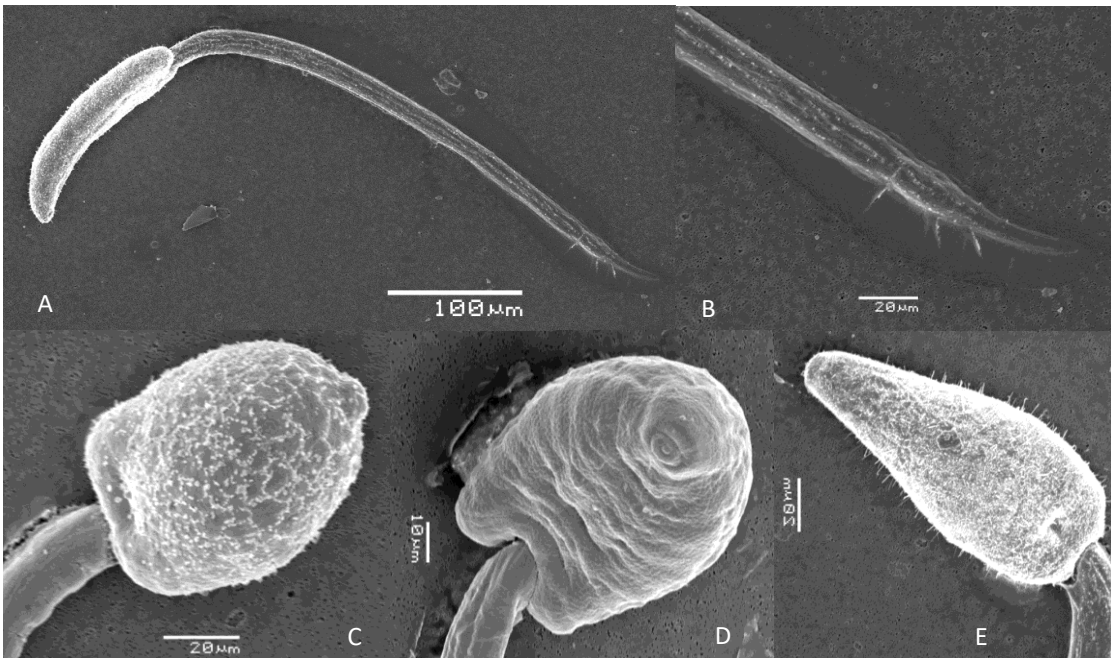


Figure 2.40. The morphology of type II cercariae under SEM. A: whole cercaria, side view. B: tail. Note the fin surrounding the tail. C & E: ventral view of the body. The surface was covered by cilia. D: a cercaria without cilia on the body surface.

2.3.10.3 Type III cercaria

This type of cercaria was found in the digestive glands of periwinkles. Numerous rediae containing cercariae were found in the viscera. The rediae were slightly orange, with a dark

brown gut caecum. Cercariae at all levels of maturation can be found in rediae (2749.36 µm L × 594.69 µm W; n=1). The distome cercaria had a robust body (548.61 µm L × 185.81 µm W; n= 1) and a thin tail (555.54 µm L; n=1). A collar around the oral sucker was noticed. When observed alive, the rediae were yellow to orange and the cercariae were brown in colour. Both oral (65.81 µm L × 63.22 µm W; n=1) and ventral suckers (109.29 µm L × 110.44 µm W; n=1) were strongly muscular. Symmetric, ramified lateral canals with refractive granules were found at both sides of the body, which began at the bladder and extend up to the head collar region. These were the collecting ducts of the excretory system. A prominent pre-pharynx, pharynx, oesophagus, and gut caeca were noticed. Under SEM, the body surface was sometimes spineless and smooth, while sometimes the tegument had prominent grid-like corrugations and pores. This might reflect different levels of maturation or might reflect a certain degree of preparation artefact. The collar under SEM was sometimes surrounded by a ring of symmetrical papillae, while sometimes it was surrounded by a ring of radial ridges each with a hole at the base. These structures might develop into collar spines when mature. In those with relatively smooth surfaces, symmetrically distributed papillae can be found on the lip of the oral sucker and on the ventral side of the forebody and the whole dorsal body wall. The ventral sucker was larger than the oral sucker and strongly muscular, with radial striations on the lip (Figures 2.41-2.42). Species identity was based on morphology; however, ITS2 rDNA sequence data was to generated for confirmation (Appendix 15).

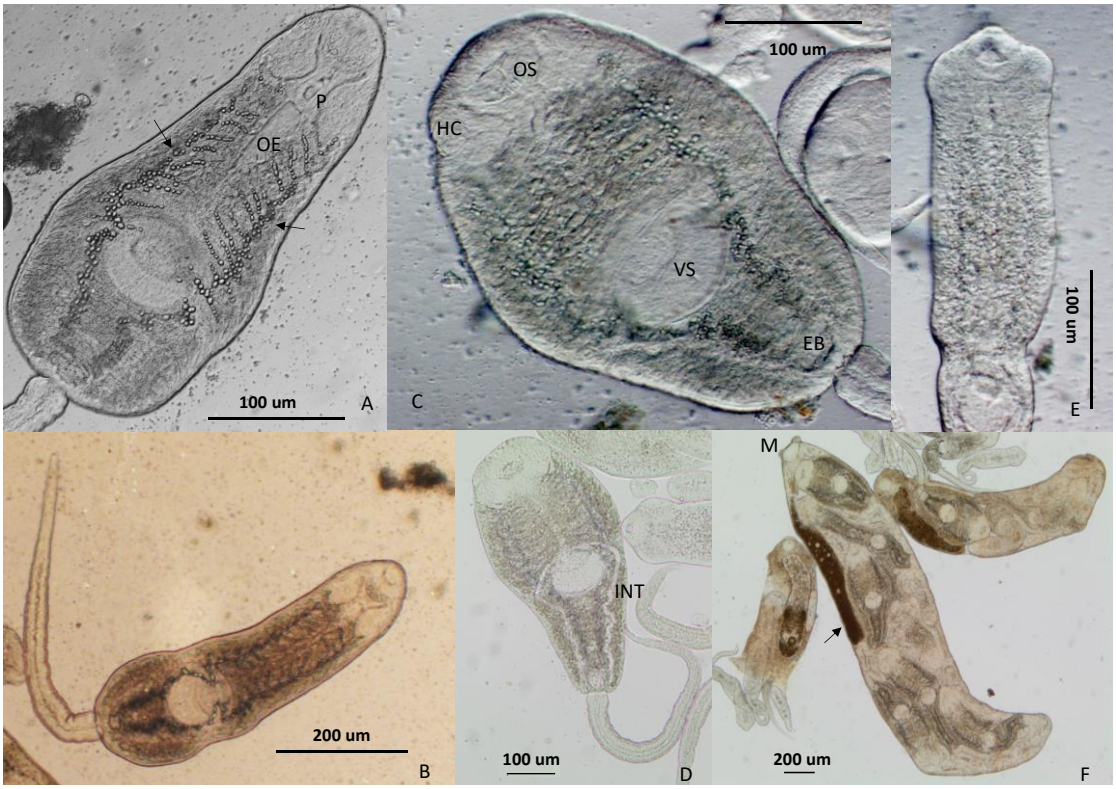


Figure 2.41. The morphology of type III cercariae. A-D: a mature live cercaria. Note the oral sucker (OS), pharynx (P), oesophagus (OE) and intestine caeca (INT). A large ventral sucker (VS) situated in the posterior third of the of body, and two ramified collecting ducts (arrows) with refractive granules extended from the excretory bladder (EB) to the head collar region (HC). E: immature cercariae. Note the body was more elongated and transparent. F: rediae. Note the "sock-shaped" appearance, the light orange colour, the mouth (M) and the dark brown gut (arrow).

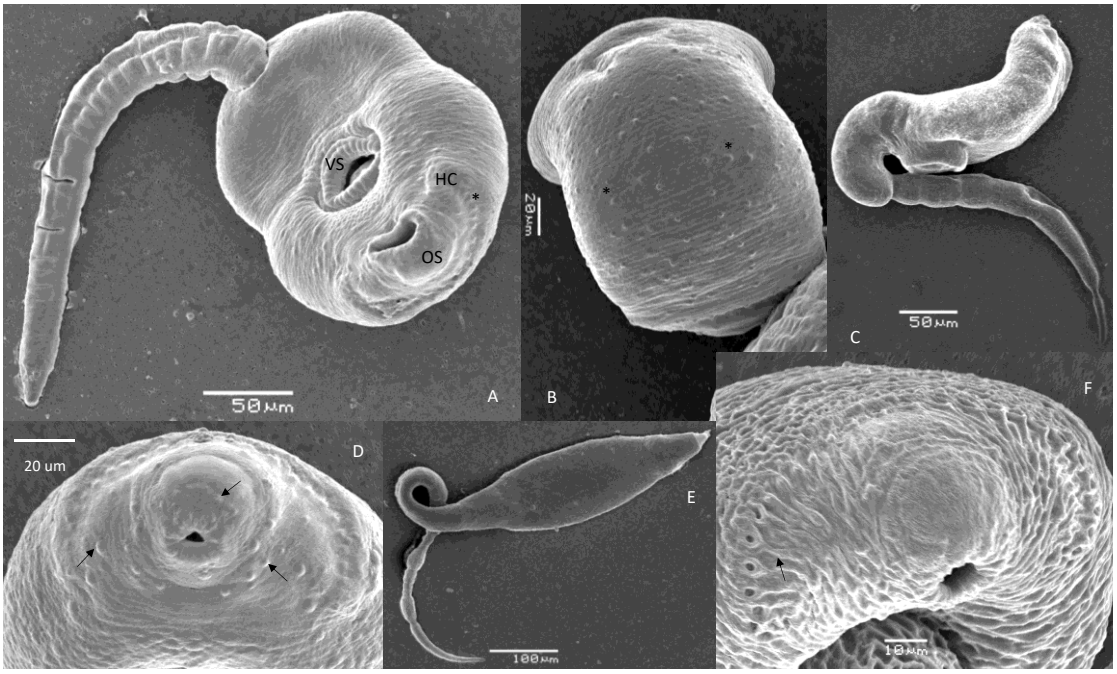


Figure 2.42. The morphology of type III cercariae under SEM. A: ventral view of the whole cercaria. Note the strong oral and ventral sucker (OS & VS), head collar (HC) around the oral sucker, and a row of papillae (*) on the head collar. The ventral sucker was covered with radial striations. B: dorsal side of the body wall, which has many symmetrically distributed papillae (*). C: side view of a cercaria. D: closer view of the oral sucker, head collar and papillae. Note more symmetrical papillae (arrows) scattered on and near the lip of the oral sucker. E: an immature cercaria. Note the surface was quite smooth. F: a cercaria with grid-like corrugation on the surface. Note the spine-like protuberances (arrow) on the head collar each has a pore on the base.

2.3.10.4 Type IV cercaria

This type of cercariae was found twice, in the digestive gland of a periwinkle and a dog whelk respectively. Numerous sporocysts containing cercariae were found in the viscera. They were small, distome, colourless cercariae. The tail was shorter (119.58 µm ± 32.27 S.D.; n=2) than the body (310.37 µm L ± 93.84 S.D. × 135.99 µm W ± 25.70 S.D.; n=2). The oral sucker (54.01 µm L ± 18.07 S.D. × 50.56 µm W ± 20.92 S.D.; n=2) was almost equal to the ventral sucker (52.23 µm L ± 14.33 S.D.× 50.80 µm W ± 10.33 S.D.; n=2). The oral sucker was equipped with a stylet. There were no signs of a gut but a Y–shaped bladder could be seen. There seemed to be penetration glands at both sides of the forebody, but their structures and ducts were not clear. The body was filled with large parenchymal cells. Under the SEM, the body surface seemed to be covered with small scale-like spines. The specimen found in the dog whelk was larger. It was not clear whether a pharynx and gut caeca were present. The excretory bladder was Y-shaped (Figure 2.43).

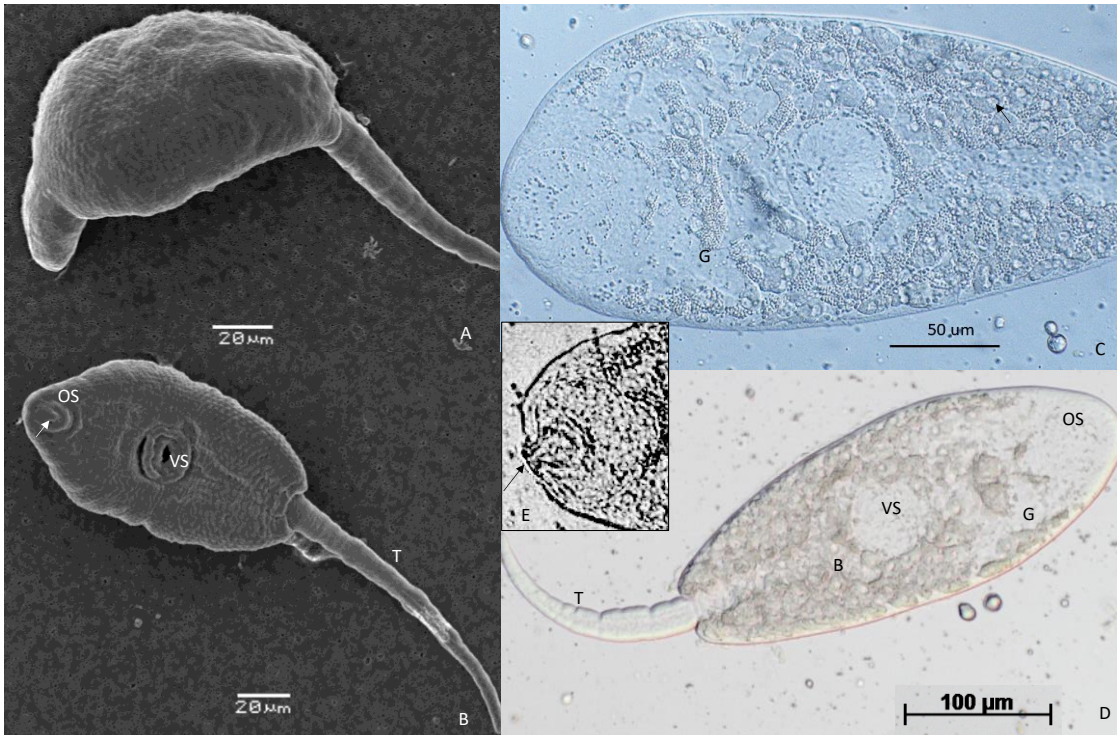


Figure 2.43. The morphology of type IV cercariae. A: Dorsal view of whole cercaria under SEM. Note the surface was covered with minute scale-like spines. B: ventral view of whole cercaria under SEM. Note the oral sucker (OS) with a stylet (*), the ventral sucker (VS) and the thin tail (T). C: forebody of a living cercaria. Note transparent gland cells (G) near the oral sucker and granular parenchymal cells (arrow) in the body. D: a living cercaria ventral view. Note the short tail (T), penetration glands (G), oral sucker (OS), ventral sucker (VS) and Y-shaped bladder (B). E: higher magnification on the oral sucker region. Note the stylet (arrow).

2.3.10.5 Metacercaria

A peculiar type of metacercaria was found only once in periwinkles. These metacercariae were each enclosed in a thin cyst, with sometimes up to 5~10 metacercariae within a single cyst. When stained with neutral red these formed a spherical ball of metacercariae. When in the cyst, the metacercaria were folded, and their bladders were conspicuous. These metacercariae were excysted in 0.1M PBS in a 40°C water bath or released from the cyst using dissection needles. When released, the metacercariae were pyriform with the posterior end being the widest (358.73 µm L × 273.52 µm W at the level of the ventral sucker; n=1). The bladder became Y-shaped and much smaller than that in the cyst. The metacercariae could be maintained in 0.1M PBS at 40^{o}C for up to 48 h during which there were no further changes in their appearance. The ventral sucker (42.69 µm L × 45.48 µm W; n=1) was slightly larger than the oral sucker (45.79 µm L × 53.34 µm W; n=1). A pharynx, oesophagus (pharynx + oesophagus 100.99 µm; n=1), and bifurcated gut with each caecum measuring approx. 139 µm. The ventral sucker was positioned in the posterior third of the body. A male papilla (17.94 µm L × 25.23 µm W) was sinistral to the ventral sucker, a pair of testes (left 31.75 × 77.38 µm, right 52.28 78.22 µm) were noticed at each side, below the ventral sucker near the edge of body, but other male reproductive organs were not clear. A dextral ovary was present (36.89 µm W × 27.07 µm L, n=1) at the same level as the ventral sucker. Some entangled ducts were seen below the ventral sucker, which could relate to the egg-producing apparatus, but they are not clear in the specimens (Figures 2.44-2.45). Specimens were identified based on their morphology; their identification was later confirmed by their ITS2 rDNA sequence (Appendix 16).

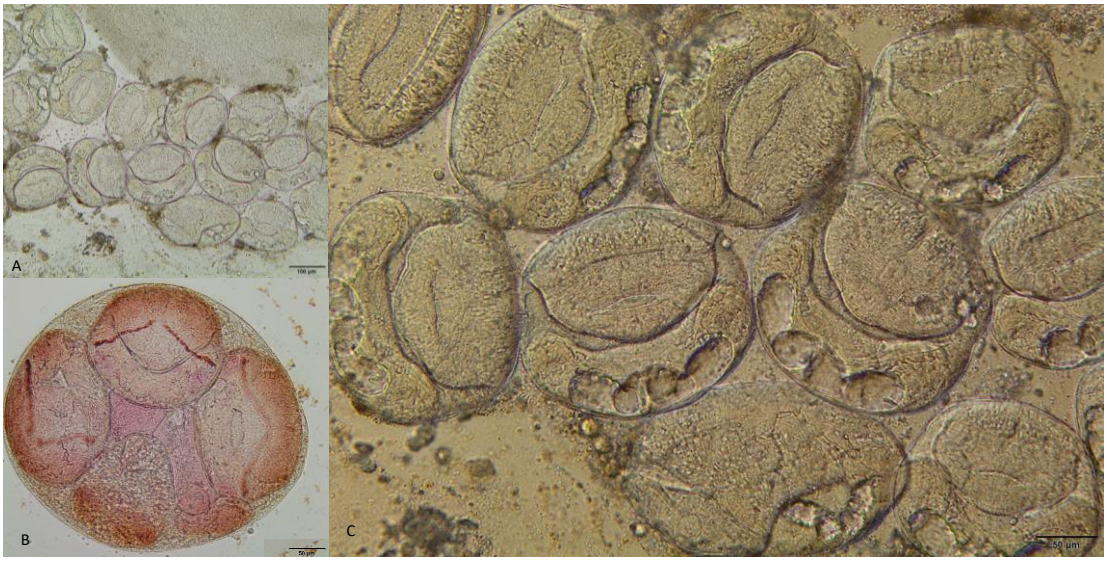


Figure 2.44. The morphology of the metacercariae found in periwinkles. A: many metacercariae enclosed in a thin-walled cyst were released from the digestive gland of a periwinkle. B: some cysts were grouped in a ball in a larger membranous cyst. When stained with neutral red, the gut caeca were clearly visible. C: higher magnification of A. The metacercaria with a conspicuous bladder folded upon itself while in the cyst.

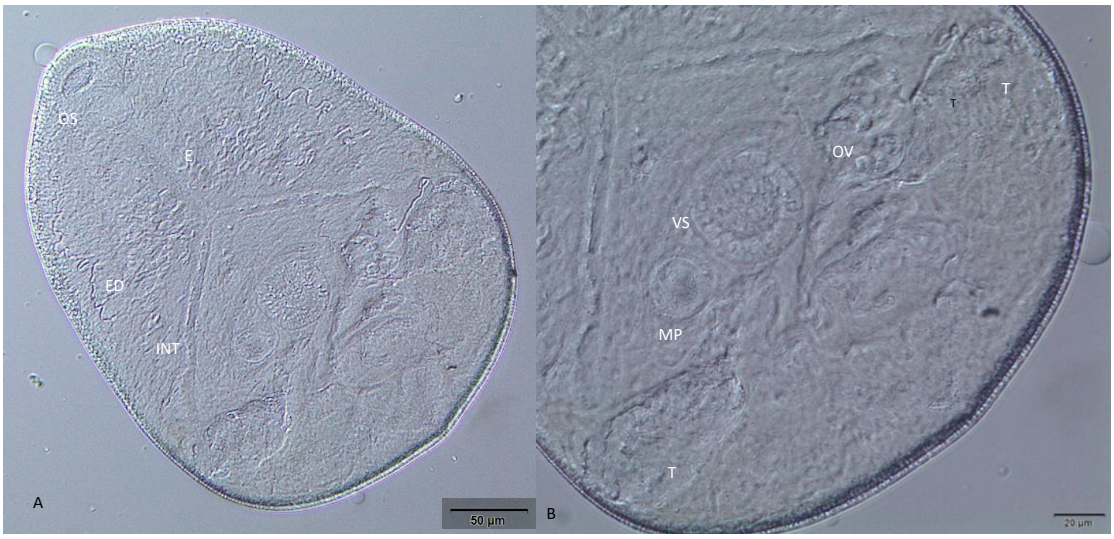


Figure 2.45. The morphology of the metacercaria found in periwinkles. The metacercaria was excysted in 0.1 M PBS at 40°C. A: living worm. Pyriform shaped body. Part of the excretory system can be seen. B: higher magnification of the specimen in A. The reproductive organs were moderately developed. E: oesophagus, ED: excretory ducts, INT: intestine caecum, MP: male papilla, OS: oral sucker, OV: ovary, T: testis, VS: ventral sucker.

2.3.11 Transect study at South Queensferry

In this study (trip no. 6 in Table 1), 400 barnacles were dissected (40 barnacles per site; total 10 sites) and only 12 digenean metacercariae were found. Barnacle species other than *S. balanoides* were also found. In this study, therefore, the focus was on barnacle species distribution. Four species of barnacles were discovered, and the diagnosis was made based on the keys of Southward (2008). Their distribution along the coast was mapped. The first species was *S. balanoides* (Figures 2.46 A & B). It was diagnosed by the following characters: having 6 shell plates, membranous base, rostrum plate overlapped lateral plates and carina plate overlapped by adjacent lateral plates. The shells of young individuals were white while older and larger individuals were brownish or greenish and porous. The tergum was not beaked. The second species was *A. modestus* (Figures 2.46 C & D). It was diagnosed by the following characters: having four shell plates, membranous base, white in colour when young, operculum with two pairs of two plates, and a mantle opening in the middle. The third species was *Balanus crenatus* (Figures 2.47 A, B & D). It was diagnosed by the following characters: having six shell plates, rostrum plate overlapping lateral plates and carina plate overlapped by adjacent lateral plates, tergum not beaked, calcified base, shell plates and basis with pores, and ribbed wall plates. The fourth species was *Verruca stroemia* O.F. Müller, 1776 (Figure 2.47 C). It was diagnosed by the following characters: having four shell plates, a very squat shell, crenulated wall plates, and an asymmetrical mantle opening. When compared by number, then *S. balanoides* was the most abundant species; which was found at every site and in the greatest abundance. The second most abundant species was *A. modestus*, followed by *B. crenatus*. *Verruca stroemia* were found at only one site. When their spatial distribution is considered (Figure 2.48), the abundance of *S. balanoides* decreased toward the sea, while *A. modestus* seemed to increase towards the sea. *Balanus crenatus* only occurred at the lower shore, and *V. stroemia* only occurred at one site up shore.

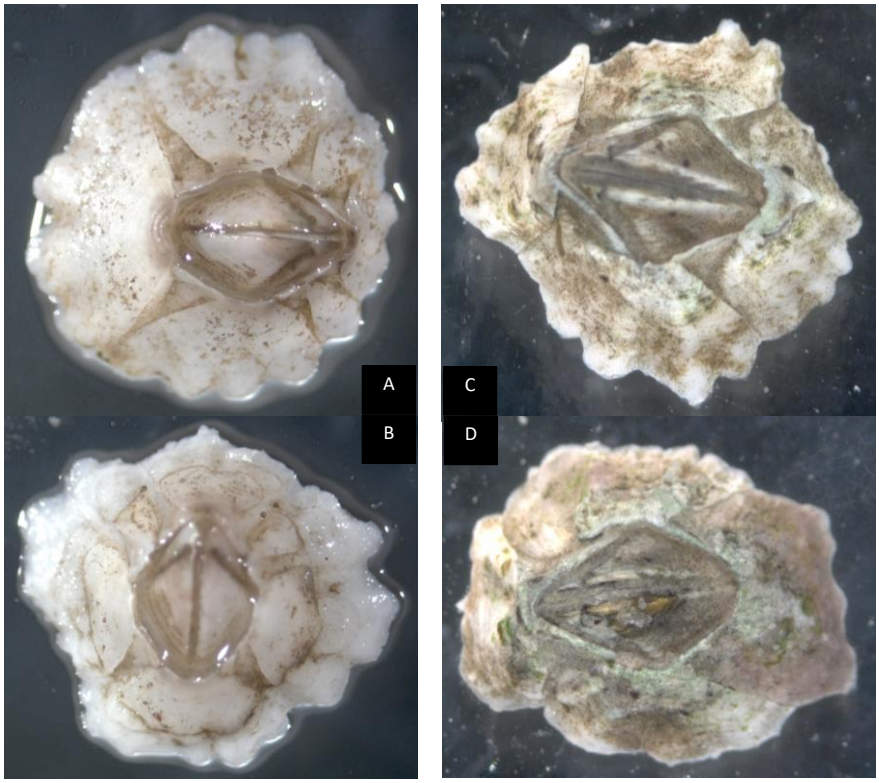


Figure 2.46. Morphology of the different barnacle species found in South Queensferry. A & B: *Semibalanus balanoides.* C & D: *Austrominius modestus*.

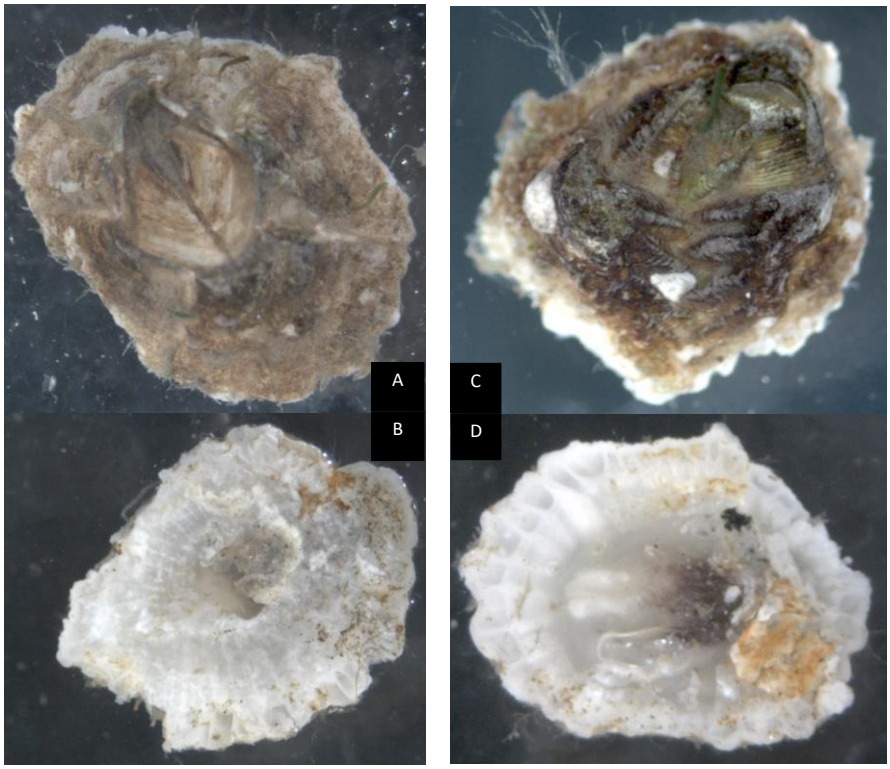


Figure 2.47. Morphology of the different barnacle species found in South Queensferry. A, B & D: *Balanus crenatus*. B & D shows the calcareous base and pores in the wall plates. C: *Verruca stroemia*.

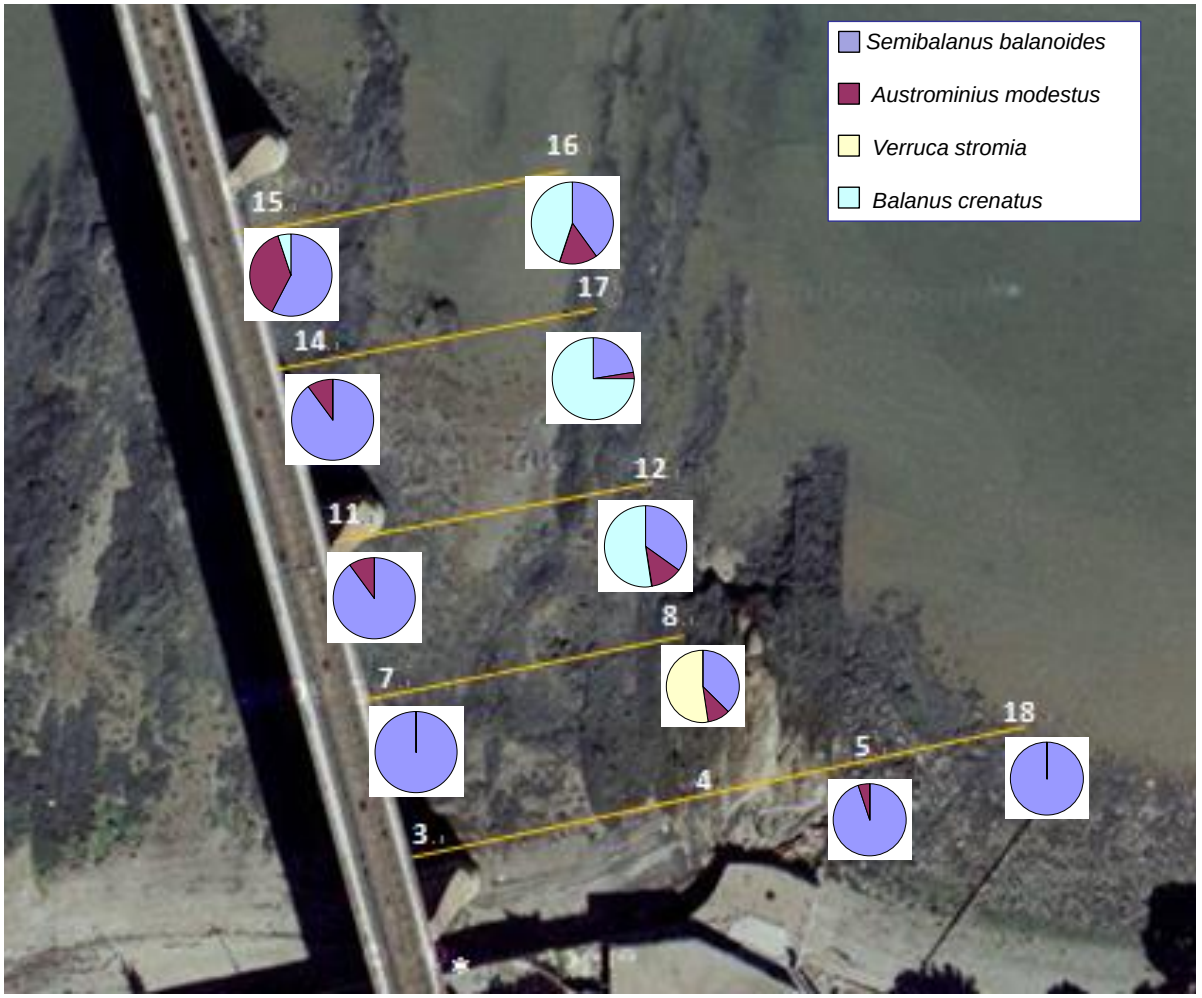


Figure 2.48. Spatial distribution of the different barnacle species at each sampling site in South Queensferry.

2.4 Discussion and conclusions

2.4.1 Comparison for the associated and parasitic fauna and their infection status for barnacles in study localities, and the choice of target parasite

In the present study, the associated fauna of barnacles (largely *S. balanoides*) such as mites, turbellarians, oligochaetes, larval nematodes, amphipods, and juvenile periwinkles were observed. The parasitic fauna encountered included: digeneans (*Maritrema* sp.), cestodes (4 types, species unidentified), gregarines (2 types, species unidentified), and nematodes (1 type, species unidentified). The ciliates were most likely commensal. Algal infections were observed but were not further investigated. The associated and parasitic fauna found on the barnacles at the three investigated localities, namely South Queensferry, Machrihanish and Dunbar, were like those found by Colston (2012), except for the case of the parasitic nematode. The fauna found in the present study also covered the major taxa mentioned by Arvy and Nigrelli (1969) and Stubbings (1975), but the isopod *H. balani* and the rhizocephalans were never found. There are limitations in the chronological comparisons of the infection indices determined for the present study and that of Colston (2012) (see Tables 2.6 & 2.7). Limitations include, for example, the exact locality that was sampled in South Queensferry is unknown and, the barnacle species sampled for the cestode analysis is not clear in Colston's study. It seems that although the data fluctuated between years, a general pattern can still be captured. There is an intrinsic difference between South Queensferry and Dunbar in the profiles of the parasitic fauna for barnacles. According to data in both years, Dunbar is a hotspot for *M. gratiosum*. Furthermore, when there are higher digenean infections in a barnacle host, the infection of rarely found cestode is also seen to increase. There were higher cestode infections in 2012 and higher intensity and abundance but not prevalence for digenean infection in 2012. Should this be the consequence of bias of sampling and counting methods, or reflecting real population changes remains to be determined. It might be that a core and satellite species combination was found. According to Poulin (1996), the core and satellite species concept is important in explaining species interactions and the factors contributing to an even or dominant community is vital for interpreting parasite community structure. Although there are not many species of parasitic helminths that can be found in *S. balanoides* and the appearance of cestodes is not stable, there is a potential that a study at the community level can be conducted. The current comparison between years shows that temporal difference in the parasitic fauna for a particular locality deserves further study, and that the methods of choice should be consistent and balanced in order to get a more objective interpretation.

The major parasites of the dominant barnacle species *S. balanoides* in the study localities are digeneans, in terms of prevalence, abundance and intensity; and this is especially true in Dunbar.

Protozoa were quite common but were most likely commensal ciliates; parasitic gregarines were rare. The biomass of these species was not calculated, but considering the small size of protistan organisms, the digeneans may represent the "dominant taxa" among commensal and parasitic fauna, especially in high prevalence areas. Poulin (2008) pointed out that the reason for one or a few species being dominant in a community but not showing a pronounced dominance in other communities is basic and important in parasite ecology. Furthermore, Sures *et al*. (2017) highlighted that heteroxenous parasites such as digeneans and cestodes are good indicators of a healthy ecosystem, *i.e*., functioning, and resilient. Changes in composition and diversity of heteroxenous communities can provide information about changes in food web structures that lead to disruption or enhancement for transmission. Considering that the digeneans are the numerical dominant species in the chosen system, and considering their ecological importance, digeneans were chosen to be the target taxon for the remainder of this study. Further distributional, diagnostic, and physiological studies were performed on this group. Cestodes can also be an interesting topic for study, and as a satellite taxon in barnacle hosts, their interactions between digeneans in the 2nd and final host will make insightful research topics. The cestodes, however, were not studied further in this study due to their unstable appearance and rarity.

Comparing the infection indices of parasitic/commensal fauna in South Queensferry (SQ) and Machrihanish (MH), the more exposed locality MH had a higher prevalence of Protozoa than the more sheltered locality at SQ. According to the Bathing Water Report by Scottish Environment Protection Agency (https://www2.sepa.org.uk/bathingwaters/), the water quality during 2013-2014 in the area around Dunbar (classified as a guideline area) was better than the area near South Queensferry (the majority classified as mandatory). For Machrihanish, unfortunately no data was available. Ciliate protozoans are common epibionts on crustaceans, *e.g*., on cladocerans, copepods, cirripedes, isopods, amphipods, and decapods (Fernandez-Leborans & Tato-Portoa, 2000). Better water quality might favour the presence of particular ciliate protozoans. Some studies have revealed that polluted and non-polluted habitats have different functional groups of ciliates, and this has been demonstrated in both marine (Xu *et al.*, 2011; Jiang *et al.*, 2013) and fresh water (Shukla & Gupta, 2001) systems. Significant correlations between gill ectocommensal protozoans and water quality were observed on farmed crawfish, *Procambarus clarkii* Girard, 1852 (see Scott & Thune, 1986). The level of ciliate infestation on farmed shrimp in a tropic costal system was reported to be related to stocking density, survival, temperature, and turbidity (López-Téllez *et al*, 2009). To date, it is still not clear the major groups and ecological association of commensal ciliates on *S. balanoides* in natural habitats. Higher prevalence of ciliate protozoans at MH, however, might not be explained by water quality alone and more stringent data are needed to make secure conclusions.

In earlier field trips, regarding the helminths, only cestodes were found at MH, while digeneans were present at both SQ and MH, but their prevalence was 10 times higher at SQ than at MH. The difference in infection rates between the two localities might be related to the presence of 1st and final hosts, the biology of the specific parasitic stage, the local terrain, and water quality related to anthropological influence. Sheltered habitats may favour the transmission of digenean as the infective stage, cercariae, have limited swimming ability and cannot survive long (Combes *et al.*, 1994; Haas, 1994). Many parasite taxa can also serve as indicators of environmental health. Acanthocephalans and cestodes are especially good sentinels for metal pollution as they exhibit high accumulation potential and high resistance to metal pollution (Sures *et al.*, 2017). Likewise, digeneans are promising indicators of ecosystem health due to their complex life-cycles (Marcogliese, 2005; Aguirre-Macedo *et al.,* 2011; Sures *et al*., 2017). They are reliable indicators of free-living species diversity, and their prevalence and intensity in their intermediate and definitive hosts are inversely related to the degree of pollution and disturbance of the local aquatic ecosystem (Sures *et al.,* 2017). For individual parasite-host systems, however, the scenarios vary case by case. As an example (see Cross *et al*., 2001), heavy metal pollution has negative impacts on swimming and longevity in the cercariae of *Cryptocotyle lingua* (Creplin, 1825) Fischoeder, 1903. *Cryptocotyle lingua* from *L. littorea* collected from polluted habitats have a significantly lower horizontal swimming rate. Mortality starts to increase at 20 hours in metal treatment groups, while the control group have an extremely low mortality over 60 hours. The authors suggest the reduction in swimming ability and longevity can result in transmission failure, and therefore can alter parasite populations and communities. Eutrophication, on the other hand, can have a positive effect on parasitism (see Zargar *et al.,* 2017). *Adenoscolex oreini* Fotedar, 1958 (a caryophylleid cestode) infection levels in three fish species were found to be highest in eutrophic lakes, with parasitism and habitat quality having effects on the health traits of host fish in an interactive way. In the present study, it is not possible to link the difference of infection indices of digeneans and cestodes in barnacle populations directly to pollution levels or to the degree of anthropological influence; these might be considered as contributory factors, as the water quality at the two study localities (SQ and MH) was not measured, and other environmental factors such as temperature, exposure or salinity and host abundance can all contribute to the observed differences. Conversely, the observed differences in the parasite profiles might itself provide an indication that the two localities have different ecological characters.

It was also noted that at SQ, the barnacles on organic substrates had a higher prevalence of digeneans and cestodes but lower protozoa than those on rocks. Regarding the digenean infections, similar findings were reported by Irwin and Irwin (1980) and by Mitchell and Dessi (1984). The higher prevalence of metacercarial infection on the shell of *L. littorea* is suggested to be a result of *L. littorea*

co-habitation with *L. saxatilis* and *N. lapillus,* the potential host of the digeneans (Irwin & Irwin, 1980). While Mitchell and Dessi (1984) interpret the consequence of this phenomenon to be an increase in the possibility of transmission to the final host by accidental consumption when definitive hosts prey on these molluscs, they did not provide a possible cause. In the present study, it was suggested that barnacles on mobile objects such as limpets or algae might have a greater chance of encountering cercaria compared to those attached on rocks. One of the possible reasons is the filtering capacity of intertidal bivalves, which are commonly found alongside barnacles. Van Duren *et al.* (2006) and Lassen *et al.* (2006) demonstrated that the physical roughness of mussel beds and their exhalent jets can change local hydrodynamics under experimental conditions. This happens at a small scale, *i.e*., at several centimetres above the mussel bed. Currently, it is hard to predict whether this change in local water flow has a positive or negative impact on digenean transmission.

2.4.2 Influence of parasitic infection on the health and reproduction of barnacle host

From the results of the present study, digenean and / or cestode infections do not appear to be associated with the reproductive status of *S. balanoides*, or only have light impact on reproduction, even in high intensity infections. Whether barnacle reproductive status is related to infection or not still needs to be validated. However, in the present study only the prevalence of gravid barnacles was recorded; therefore, the potential impact on the quantity or quality of eggs or larvae that parasitism might provoke, cannot be estimated. The production of egg masses places a great burden on the parent barnacles, as revealed by the reproductive anecdysis of *S. balanoides,* induced as the result of a debilitating condition following the discharge of reproductive material (Barnes, 1962). Therefore, should the "fecundity" be measured, which under the definition of Barnes and Barnes (1968) is defined as the number of eggs produced per unit time per standard increase in body size, a negative impact of metacercariae infection on barnacle fecundity might be found. *Maritrema gratiosum* can infect a range of hosts but the barnacle is only its 2nd intermediate host. *Maritrema gratiosum*, as a precocious species undertakes most of its development in the barnacle rather than in the final host (Hadley & Castle, 1940). Hence, the higher the intensity of infection, the higher the likely energy requirements and therefore the burden to the host is likely to be great. Another barnacle parasite, *H. balanis*, is known to impair barnacle egg production (Arnott, 2001). In cases of major parasite impact, parasitic castration can occur, which is a common outcome when larval digeneans infect snails, and when certain isopods and parasitic barnacles infect crustaceans (Lafferty & Kuris, 2009). The biomass or size of eggs/nauplii was not, however, measured in the present study, therefore the potential negative effects of infection on egg production and development could not be assessed and this might therefore be an important necessary adjustment to the study design warranted in further studies. As a further observation, visible immune responses (*e.g.,* melaninization in response to infection) were rarely

observed in the barnacle parasitised by metacercaria. If it did occur, it was clearly not severe. It is speculated here that a prominent defensive response does not occur in the parasitized barnacles. Although lacking adapting immunity as in vertebrates, crustaceans still possess a diverse array of immune mechanisms to react with pathogens, *i.e*., agglutination, encapsulation, phagocytosis, clottable proteins, and bactericidal activity; and these mechanisms all require the involvement of haemocytes (Vazquez *et al*., 2009). Research on barnacle haemocytes is very rare. According to Waite and Walker (1988), the haemolymph of balanomorph barnacles contains very few haemocytes (the main type is hyaline cells), and their efficiency in eliminating bacteria is not good when compared to decapod crustaceans. This result, however, does not mean that barnacles are poor at coping with bacterial infection. In a recent study, Essock-Burns *et al*. (2017) found that proteinaceous materials with the appearance of coagulated liquid which possessed cytotoxicity to bacteria were observed during the settlement and metamorphosis of the sessile barnacle *Amphibalanus amphitrite*. The authors suggested that these materials are secreted from cuticle layers upon activation by pattern recognition proteins. Based on the above research, some plausible explanations for the lack of an immune response in barnacles infected with digeneans are suggested here. There are three possibilities, one is that barnacle pattern recognition proteins respond less when they encounter digenean surface proteins than bacterial surface proteins. The second possibility is that the cercariae actively secrete materials which can suppress the barnacle's immune responses. The final possibility is that the invasion of cercariae do not cause prominent immune responses in the beginning, and those who can withstand the infection fail to launch an immune response later due to the formation of a metacercarial cyst. Which mechanism is used is still to be determined. According to the observations of the present study, infection of metacercariae does not seem to increase barnacle mortality, therefore the virulence of *Maritrema* sp. to its barnacle host seems low. It is also possible that weak or heavily infected / affected barnacles die at the beginning of infection therefore the survivors are those that can withstand the infection.

2.4.3 Preliminary distribution study for *Maritrema sp*. metacercariae in the study localities

After further investigating the spatial distributions of *Maritrema* sp. at a high prevalence locality (Dunbar), some clear trends were observed. At Dunbar Leisure Pool, potential final hosts were present and infection indices were much higher at the site closest to the highest densities of the final hosts. Negative binomial regression models revealed an abundance of parasites that was more than 10 times higher at the site nearest to kittiwake cliff than at the site furthest from it. For the two hypotheses proposed, *e.g*., "infection pressure is proportional to the distance from concentrations of definitive hosts" versus "residence at low tide levels will favour infection", the first one is supported and the second one is rejected. It seems from the data obtained, that infection pressure is proportional to the

distance from areas with highest levels of definitive hosts, therefore sites closer to the shore will favour infection. Although low tide sites will favour infection, as the immersion time is longer and the infective miracidial or cercarial stage will have more chance to infect mollusc or barnacle hosts, the influence is less than that resulting from the presence of final hosts. Similar distribution patterns were reported in the same parasite-host system at other localities and in similar systems. The volume of *Echinostephilla patellae* (Lebour, 1912) Kollien, 1996 in the tissues of the limpet *Patella vulgata* L., 1758 increased with height on the shore (Copeland *et al.*, 1987). Bartoli and Holmes (1997) found that the prevalence of *Maritrema misensis* A. Palombi, 1940 is not proportional to the population density of its molluscan host *Cerithium mediterraneum* Deshayes, 1843. *Cerithium mediterraneum* is restricted to the infra-littoral zone and was found in their highest density at the lower reaches. The prevalence of *M. misensis*, however, is highest in *C. mediterraneum* individuals occupying upper infralittoral zones. The prevalence and intensity of infection of *Maritrema madrynense* Diaz & Cremonte, 2010 and hemiuroid digenean in two sympatric pulmonate molluscs were both higher at an upper intertidal site (Di Giorgio *et al.*, 2017). A higher prevalence and intensity of metacercarial cysts of *M. gratiosum* was also reported in barnacles collected from the higher shore, in larger barnacles and from barnacles on limpets or on *L. littorea* (see Irwin & Irwin, 1980; Mitchell & Dessi, 1984; Carrol *et al.*, 1990). At Dunbar Red Rock, such a trend was not, however, apparent. A heterogeneity in the digenean distribution, however, was detected between the two selected sites. A negative binomial regression model revealed an abundance that was more than 5 times higher at Site 2 when compared to those at Site 3. The variance to mean ratio were rather high for the two sites (17.57 and 3.87 respectively). The importance of final hosts is unknown here. Although there were no signs of aggregation of avian hosts at Red Rock, potential avian hosts, such as gulls, terns, oyster catchers, and smaller charadriiform waders, were regularly observed. Furthermore, Bass Rock, which is the home of largest gannet population in the world and other seabirds, is just 11 kilometres away. Bartoli and Holmes (1997) commented on the transmission restriction in protected shallow water ecosystems in cases where the first intermediate host occurs in an area separated spatially from the definitive host. The lack of tidal currents and very gentle slopes can be overcome by the behaviour of the cercariae and the host. In the present study, this might not be the case at Dunbar Red Rock, as it is a moderately exposed site. The flat, elevated terrain of Red Rock is still a barrier for the transmission of digeneans. One of the questions to ask is how the prevalence and infection intensity can reach such a high level at Dunbar Red Rock. Furthermore, it was noticed that the site with the highest abundance and intensity of digeneans also had a high abundance and intensity of cestodes. This seems to fit the hypothesis suggested by Poulin (2007), *i.e.,* the existence of nested patterns of parasite communities such that parasite species with a

high prevalence are found in all sorts of infracommunities, while rare parasite species only occur in species-rich infracommunities.

The interpretation of variability in parasite infection data should be undertaken with caution. There are some intrinsic characters which make these types of data different from data acquired from microparasites such as viruses, bacteria, or protozoans. The distribution patterns of macroparasites are almost always aggregated across their host populations (Wilson *et al.*, 2002; Poulin, 2013). This character means that the abundance or intensity data for macroparasite infection is not normally distributed and often is zero inflated, therefore the usual statistical methods under normal distribution assumptions cannot be applied, or can only be applied after data transformation. These data are often best described by a negative binominal distribution (Shaw & Dobson, 1995). Furthermore, for complex macroparasite life-cycles, the dynamics will be different for direct life-cycle, two host life-cycle or three-host life-cycle parasite populations. The presence of vectors, free-living stages or stages that transmit through trophic chains, can add on more variables that affect the dynamics of macroparasite transmission (Dobson, 1988). By means of metadata analysis, Poulin (2013a) revealed that the aggregation levels of helminth parasites on fish hosts are tightly constrained by mean infection levels, which account for over 80% of the explained variability. The author also suggested that heterogeneity in exposure or susceptibility are the main factors generating aggregation, and variation in host body size or parasite taxon or stage have no significant effect on aggregation. In his article, the main subject is fish-parasite systems, therefore the dynamic might be different from the crustacean-parasite systems in the present study. For example, Poulin used host body size as a proxy for susceptibility to infection because of the acquired immunity and larger external surface which develops with age, and aging means longer accumulation time. In the review article of Wilson *et al.* (2002), further factors are considered to be related to the heterogeneities in macroparasite infection, *e.g*., sample size, biased sampling, specificity and sensitivity of indirect measures of parasitism, statistical methods applied, host factors (e.g., age, sex, body conditions, behaviours, genetics etc.), and parasite genetics. For example, the distribution of *Plagiorchis elegans* (Rudolphi, 1802) Braun, 1902 in a natural wood mouse population (*Apodemus sylvaticus* L., 1758) was determined by age-group and sex (Boyce *et al.*, 2014). *Cephalogonimus yamunii* Upadhyay, Jaiswal, Malhotra & Malhotra, 2013 (Digenea) distribution in *Xenentodon cancila* Hamilton, 1822 (garfish) populations is highest in late summer, and size, weight and sex are all influential (Upadhyay *et al.*, 2013). Stephenson *et al.* (2017) found that the transmission of *Gyrodactylus turnbulli* Harris, 1986 (Monogenea) to the next host was related to infection load, but that the relationship was not linear. Resistant and experienced donors transmitted more parasites, while parasites from experienced donors showed lower growth rates in recipients than those from inexperienced donors. The role of parasite genetics has also been identified. In the "*Maritrema*

novaezealandense Martorelli, Fredensborg, Mouritsen & Poulin, 2004 - *Zeacumantus subcarinatus* G. B. Sowerby II, 1855" system, the timing of infection and / or intraspecific competition among parasite clones within molluscs determines the genetic diversity of parasite clones that molluscs harbour. Numbers of infected molluscs and tidal mixing effects resulting in high numbers of the genotype that the 2nd host can carry (Keeney *et al.*, 2007). Very high genotypic diversity of metacercariae was found within the cockle *Austrovenus stutchburyi*. The 2nd intermediate host in this case was considered an accumulator of genetic diversity in the digenean life-cycle (Leung *et al.,* 2009). In the present study, the target host is an invertebrate, in which 'classical' acquired immunity does not exist (Melillo *et al.*, 2018), therefore the assumption that body size/ age is related to susceptibility might not be true, although these authors suggested that immunological memory is also present in invertebrate innate immune responses. If the parasites do not cause mortality or a prominent immune response in barnacle hosts, we can expect that the larval digenean/ cestode will only accumulate through the aging process until the space inside a barnacle is saturated. Furthermore, the target barnacle is a hermaphrodite species, so is the target parasite, therefore the influence of sex does not exist. The method to detect infection is a direct one (by dissection), therefore the impact of indirect diagnosis does not exist either. However, other factors such as host behaviour, body conditions and genetics might contribute to parasite burdens but these are harder to detect. Currently we do not know whether host avoidance behaviour exists by means of special cirral activity, whether the barnacle can detect the existence of cercariae, or whether the barnacle will take them as food or not. Different types or speed of cirral activity might lead to different degrees of infection. Alternatively, parasite infection might increase the metabolic needs of the host barnacle and the consequences might be to increase the beating rate for respiration or feeding. The geographical difference of host or the parasite genome within the same species (*e.g*., different strains or genotypes) might produce heterogeneities as well, as has been demonstrated in a vertebrate-nematode system (*e.g*., the Soay sheep study, conducted by Smith *et al*., 1999), and a mollusc-digenean system (*e.g*., the *Microphallus* Ward, 1901 study of Lively & Dybdahl, 2000). The above-mentioned theories deserve further study. The entry site of cercariae are the cirri (Irwin *et al.*, 1990), therefore it is reasonable to predict that longer cirri have a higher risk of infection. In *S. balanoides*, it was observed that different phenotypes of cirrus length related to the exposure conditions of the environment (Arsenault *et al.*, 2001), therefore the length of cirri can be a proxy of both biotic and abiotic variables influencing infection parameters. This variable will be investigated in Chapter 4 of this thesis.

In terms of understanding the population dynamics of the target digeneans, the two localities might offer different insights. In the simulated population model mentioned by Dobson (1988), the author suggests that at lower intermediate host population densities, both the parasite and the

intermediate host population display a tendency to oscillate; in contrast to this, in the region of high intermediate host population density, transmission proceeds at maximum efficiency, and the dynamics of the intermediate host stage might be ignored, and the model has the property of a simple direct life-cycle macroparasite. In the present study, the different characters of the infection indices of the target parasite in the barnacle populations at Dunbar Red Rock and the Leisure Pool suggested that the dynamics of the target digenean parasite in the two localities might be different. For example, the prevalence at the Red Rock locality is quite uniform and generally high, whereas at the Leisure Pool locality the difference in prevalence at separate sampling sites can be several times higher. The highest mean abundance value at Red Rock (56.74) is 8.6 times higher than the that at Leisure Pool (6.6). The highest variance to mean ratio at Red Rock (17.57) is 1.9 times higher than that at Leisure Pool (9.31). Although the two localities are not far away, both localities have their own distinctive terrain. These two localities provide a chance to examine Dobson's assumption (Dobson, 1988) regarding the different population dynamics occurring in low versus high intermediate host density scenarios. The author's theories were based on R_0, the basic reproduction rate of parasite when first introduced into the host population, and the effects on R_0 of host behavioural or reproductive change induced by parasite were investigated using mathematic model. The author suggested that both parasite and intermediate host populations show oscillatory patterns of abundance. When the density of the intermediate host is low, parasite induced host behaviour change increases the tendency to oscillate. On the contrary, when intermediate host population densities are high, change in the behaviour of host are less important for parasite population density and the major determinant is host-parasite interactions. Although there is not enough data in this study to test his model, *e.g.*, 1st and 2nd host population density, as well as the current study involving a three-host rather than a two-host life-cycle, it would nevertheless be interesting to investigate the role that the second intermediate host plays in the studied system, and to actually calculate the R_0 value of the target parasite. These data might provide some explanation as to why certain parasites thrive in a particular ecosystem but not in others. It can also be applied to predict the minimum host population size, for each stage of the life cycle, that can sustain the existence of the parasite population in a particular ecosystem; or to determine the influence of parasite of host populations under different infection pressure.

2.4.4 Larval cestode in barnacles

Larval cestodes in barnacles have rarely been studied. At the cysticercoid stage, there are not many diagnostic characters that can be resolved. The most important characters are the morphology of the scolex and the rostellar hooks, also the host species is important (Williams *et al.*, 1981; Chervy, 2002). Unfortunately, at the time these samples were isolated, no such knowledge / resource was available, therefore the hooks were not cleared and measured. Cestodes would be an interesting

subject for a further study as well. A non-exhaustive list of cestodes from several sea birds is provided below for the interests of future researchers (Appendix 17).

2.4.5 Diagnosis of cercariae and metacercariae found in molluscs

In the attempt to find the first intermediate host(s) of the digenean *Maritrema* sp., many other larval digeneans were found but no cercariae of *Maritrema* sp. were identified. The cercariae of *M. gratiosum* have been reported a few times (Popiel, 1976b; Zaben, 1988; Irwin *et al*., 1990), but not in this decade. Cercariae for other *Maritrema* have been reported occasionally (Lebour, 1907; Ching, 1963; Deblock & Tran Van Ky. 1966b; Prevot *et al.*, 1976; Benjamin & James, 1987; Martorelli *et al*., Casanova *et al.*, 1998; 2004; Alda *et al*., 2013; Presswell *et al*., 2014; Kudlai *et al*., 2015). The discovered cercariae in the present study included: type I cercariae (probably *Cercaria purpurae* Lebour, 1911 described by Lebour (1912)), type II cercariae (*C. lingua*), type III cercariae (closest to *Himasthla littorinae* Stunkard, 1966), type IV cercariae (probably a plagiorchid cercaria), and a species of metacercaria (belonging to the Microphallinae). Some of their identity was further confirmed by ITS2 rDNA sequencing data. Given the high prevalence and intensity of microphallid metacercariae in the study locality, it was quite surprising that no xiphidiocercariae belonging to the microphallid group were found. The difficulty of finding them might due to their small size, their spatial distribution, or the timing of their release. Molecular techniques, *e.g*., environmental DNA (eDNA), can shed more light on this (Bass *et al*., 2015). Molecular detection is also useful in parasite diagnostics in identifying cercariae to the species level (Hust *et al*., 2004; Al-Kandari *et al*., 2011, 2014) and could have been used to determine the larval *M. gratiosum* stages in molluscs, but this was not carried out as the morphology suggested they were not *Maritrema* sp.

Type I cercariae morphologically resembles *Cercaria purpurae* described by Lebour (1912), which was found in the digestive gland of *Purpura lapillus* (syn. *Nucella lapillus*). The present specimens differed from that described in lacking the undulating crown of spines in the ventral surface. Type II cercariae fits perfectly with the described morphology for *Cercariae lophocerca* Lebour, 1907 escribed by Lebour (1912), which she also found in the digestive gland of periwinkles. The only difference was that the body surface of the samples in the present study was covered by cilia instead of spines. It also fits perfectly with the morphology of *Cryptocotyle lingua*, according to Stunkard (1930). Considering that *C. lophocerca* is the synonym of *C. lingua*, therefore, this type of cercaria is likely to represent *C. lingua*. This finding was further supported by sequence data of ITS2 rDNA region (Appendix 14). Type III cercariae certainly belong to the *Echinostomum* group. It most closely resembled *Echinostomum secundum* Nicoll, 1906 and *Echinostomum leptosomum* described by Lebour (1912). *Echinostomum leptosomum* was isolated from *Paludestrina stagnalis* Baster, 1765 while *E. secundum* was isolated from periwinkles. The collar spines in the samples analysed in the present study were never clear,

therefore, the number and size cannot be measured. According to the key of James (1968), this type most closely resembled *Himasthla littorinae*, in that it developed inside orange rediae rather than inside colourless rediae and its tail was longer than that of *H. littorinae*. The sequence data based on ITS2 rDNA confirmed its identity to the genus level (Appendix 15). Type IV cercariae: according to their small body size, weak tail, the presence of a stylet, and the equal sized suckers could belong to the "*Spelotrema* group" defined by Lebour (1912). The cercariae of *Microphallus* and *Maritrema* are both included in this historic group. However, according to James (1969), all marine microphallid xiphidiocercariae are monostome, while plagiorchid xiphidiocercariae are distome. It also possessed other characters typical of the plagiorchid group, including a very small stylet, numerous penetration glands and spherical granular cystogenous gland cells almost filling the whole body. Unfortunately, at the time the samples were processed, the stylet was not measured and the penetration gland was not stained. Further identification to the species level cannot be achieved. The metacercariae found in a periwinkle: according to the following characters; the small body size, the absence of a male genital pouch, fleshy male papilla, and ventral sucker not affected by a genital atrium, is proposed to belong to the subfamily Microphallinae (Deblock, 2008). Other characters are not clear, therefore further diagnoses could not be made based on morphological ground. Sequence data based on ITS2 rDNA, however, suggests that the identity of the specimens as *Microphallus pygmaeus* (Appendix 16).

2.4.6 The infection profile of larval digenean in molluscs and the search for first intermediate host

The profile of digenean infections in molluscs co-habiting with *S. balanoides* was also investigated. Metacercarial cysts, while almost always present in the molluscs that were examined, were usually present in low numbers, attached to the membrane of viscera and were hard to isolate. Without thorough examination, it was hard to quantify the number of metacercariae, therefore, only a rough estimation can be made. Without excystment, the species were not possible to determine either. Furthermore, metacercariae in molluscs were not related to the life-cycle of *Maritrema* sp., therefore they were not further investigated here. In general, molluscan hosts collected at Dunbar Red Rock had higher digenean infection rates than those collected from the Leisure Pool (Figure 2.34). The data obtained further support the fact that Dunbar Red Rock locality is a "hot spot" of digenean infection.

Concerning the population dynamics of the digeneans, we cannot ignore the role that the mollusc host plays. At Dunbar Red Rock, larval digenean infections regardless of the stages found were higher in periwinkles than in dog whelks. The cercarial, sporocyst and redial infections were also higher in periwinkles. In periwinkles, the prevalence of cercariae and sporocysts can reach 10%. The data obtained might simply reflect the fact that periwinkles are hosts of many digenean species (see Section

1.4 in Chapter 1). If comparing larval digenean infections in periwinkles between Red Rock and the Leisure Pool, then Red Rock had a higher prevalence of digeneans regardless of the stage or cercariae / sporocyst / rediae infection. The prevalence of cercariae / sporocyst infection could reach 12% and cercariae / rediae could reach 7%. From the data obtained during trip no. 4 (Table 2.3), the same hypotheses, that "the infection pressure is proportional to the distance from the definitive hosts", and that "low tide will favour infection as immersion time is longer" are both not completely supported. For the first hypothesis, the infection pressure is not proportional to the distance to the major concentration of definitive hosts. This is because only the highest prevalence of overall digenean infection was observed at the site closest to kittiwake cliff, but it did not show a decreasing trend toward the low shore. On the other hand, the low shore level does not favour infection as no increasing trend can be seen from low shore to high shore. It is possible that the mobility of molluscan hosts dilutes the effect of tidal influence and only a strong influence from the final host can overcome this dilution. Contrary to the data concerning the digeneans in barnacles, different degrees of infection were not observed for the different types of larval digenean recovered from the periwinkle populations collected from Site 1 and 3 from Red Rock (Figure 2.35). This could again be a result from the dilution effect due to the mobility of the periwinkles.

The differences in the infection profile in the periwinkles from the different microhabitats (open areas versus crevices) is worth discussing (Figures 2.36-2.37). Although the overall infection of larval digeneans was higher in periwinkles collected from rock crevices, so was the metacercarial infection; periwinkles from open areas had a higher prevalence of cercaria / sporocyst / rediae. The observed trend, however, is not statistically significant. The prevalence of metacercariae in molluscan hosts is not precise, therefore this is not discussed here. The observed phenomenon of higher sporocyst / cercariae infection seems to be congruent with the finding of several researchers that larval digenean infections can change host behaviours. There are several studies that demonstrate that digenean infections have either a positive or negative effect on the mobility of their molluscan hosts. We should consider the natural movement of the target mollusc host before we discuss any impact of the parasitism on their behaviour. Newell (1958) found that although moving about, *L. littorea* are predominantly found at the middle shore. The mechanism bringing them back to their original position (homing) is found to be their U-shaped moving track, therefore maintaining their zonation position. They tend to aggregate and form clusters, especially at the junction of two surfaces, and their crawling speed was 2~10 cm/min depending on temperature and substrate. Their activity level is influenced by light intensity and temperature; therefore, they are inhibited during winter (Newell, 1958). Seasonal movement of *L. littorea* was detected in populations in parts of North America and Europe, but not in Britain (Williams & Ellis, 1975). On the other hand, *L. saxatilis*, the other important British species,

exhibit different behaviours and habitat preferences. Pardo and Johnson (2006) revealed that although they often utilize surface irregularities for shelter, no homing behaviour was observed for this species. Movement decreased with increased water flow and maximum wave force. The most interesting feature was that gravid females use crevices more often than non-gravid females and males, and that gravid females carrying more embryos appear to remain in the shelters. The same authors also revealed that *L. saxatilis* are abundant at the mid-intertidal zone, and are most active during high tide, and can move a metre a day. Such data are important to understanding the impact of parasitism. Knowledge regarding to their movement patterns, the distance of their movements, cercaria emission time and dispersion ability, can help people to predict the 'transmission area" of a parasitized mollusc in a particular habitat, therefore enhancing the understanding of the population dynamics of both parasites and hosts in this particular habitat.

The concept of "transmission area" is further complicated by the fact that parasitized molluscs might behave differently with uninfected molluscs. Davies and Knowles (2001) found that *L. littorea* can detect *C. lingua* cercariae, cercarial homogenate in conspecific mucus trails and guano at low concentrations and avoid it. Lambert and Farley (1968) found that parasitized *L. littorea* move slower and shorter distances when responding to environmental threats, therefore were spatially separated from non-infected specimens. Williams and Ellis (1975) found that *L. littorea* infected with *C. lingua* or *Renicola roscovita* (Stunkard, 1932) Werding, 1969 move significantly shorter distances than uninfected molluscs. Lower mean rates of locomotion and different migratory activity was observed in the mud snail *Nassarius obsoletus* Say, 1822 infected by several species of larval digenean (Stambaugh & McDermott, 1969). Individual *Ilyanassa obsoleta* Say, 1822 infected with *Gynaecotyla adunca* (Linton, 1905) Yamaguti, 1939 tend to deviate from their normal movement patterns and make repeated excursions to the upper shore where the next host (i.e., amphipod beach-hoppers *Talorchestia longicornis* Say, 1818) dwell (Curtis, 1987, 1990). *Littorina littorea* infected by *C. lingua* consume less ephemeral macroalgae than uninfected specimens. The population loss of *L. littorea* due to castration and mortality caused by *C. lingua* can have a positive effect on algae populations in an indirect way. Wood *et al.* (2007) revealed that this trait-mediated indirect effect is one of the key factors shaping community structure. The schistosome infected snail *Bulinus truncates* Audouin, 1827 and *Biomphalaria glabrata* Say, 1818 move less quickly and less often than uninfected snails and are less likely to avoid predation (Swartz *et al.*, 2015). Similarly, the climbing distances of the marine snail *Cerithidea scalariformis* Say, 1825 are reduced when parasitized by certain species of digenean, which impedes their ability to avoid predatory crabs (Belgrad & Smith, 2014). Bartoli and Holmes (1997) found, through employment of small-scale studies, that only a small part of the host population that occupies the margin of the distribution area can serve for transmission; due to the small or non-overlap

of the distribution area between 1st, 2nd and definitive host. In the case of *M. misenensis*, this situation was overcome by specific behaviours by the cercariae and the host (Bartoli & Holmes, 1997). The cercariae were emitted by the 1st host which dwell in the infralittoral zone. After emergence, these cercariae swim up to the water surface, attach to the surface film and capture an air bubble. Wind and wavelets will bring these cercariae to beached algal debris where they can encounter an amphipod 2nd intermediate host. The amphipods live above the water line but will enter the upper littoral zone occasionally to hydrate themselves, and this is when the infection can be achieved. The above-mentioned studies reveal part of the diversity of parasite manipulation and adaptation behaviour which can increase transmission success. These studies also revealed that adaptation and host manipulation is specific to each host-parasite system, its consequences can, however, extend beyond the involvement of host-parasite systems alone to affect wider aspects of the studied ecosystem.

Although the 1st intermediate host of *Maritrema* sp. was not found at either Red Rock or at Leisure Pool, Red Rock did have a higher prevalence (up to 100%) and mean abundance (up to 56.7 cysts) of *Maritrema* sp. metacercariae than Leisure Pool (prevalence up to 85%, mean abundance up to 6.6 cysts). Based on morphology, the target parasite in the present study is very likely to be *M. gratiosum*. The life-cycle of *M. gratiosum* was not clear until the stages infecting the 1st intermediate host were proven experimentally to be *Cercaria littorinae saxatilis* V (see Popiel, 1976b) by Irwin in 1990. In the report of James (1969) regarding the digenean fauna of *L. saxatilis*, five types of microphallid cercariae were found: namely *M. similis*, and *Cercariae littorinae saxatilis* I, II, III & IV. The author also found that the prevalence of these cercariae varied with *L. saxatilis* varieties, with height above the chart datum, and with size of the host. In the case of *M. similis*, the larvae do not only show host preference (more in *L. saxatilis rudis*), but also show environmental preferences (sheltered shores). The prevalence (described as "incidence" in the original reference) of most shores is usually below 3%, but in the regions where the final host is present, the prevalence can reach 63.6%. The other four types of microphallid cercariae also showed different degrees of host specificity, with some being rare and only found in certain *L. saxatilis* subspecies (James, 1969). Considering the high prevalence and abundance of the target parasite *Maritrema* sp. at Dunbar, its cercariae are not likely to be rare. It is more likely that in the search for molluscan hosts, different regions of the shore need to be explored. According to James (1968), the distribution of different subspecies of *L. saxatilis* in Dale, Pembrokeshire, varied on the shore. The distribution of *L. saxatilis tenebrosa* is usually above MHWS, which is well above the barnacle zone; and they concentrate in highly exposed areas. The distribution of *L. saxatilis neglecia* is between MHWS and MLWS and these also concentrate in highly exposed areas. The other two subspecies, *L. saxatilis rudis* and *L. saxatilis jugosa*, tend to concentrate in more sheltered regions, and can be found between MHWS to MLWN. These results show that further

surveys for mollusc hosts are needed to reveal the local distribution of *L. saxatilis* in Dunbar. Once the 1st intermediate host can be identified locally, the infection profile of *C. saxatilis* V can be revealed and the ecology of the target parasite at chosen localities would be much clearer.

An attempt to isolate and identify digenean eggs in kittiwake faecal samples by collecting fresh faeces on the beach and using a flotation method (data not shown) was unsuccessful. More efficient modern techniques may be applied in the future. For example, combining techniques of environmental DNA (eDNA) and next generation sequencing (NGS). Environmental DNA has been successfully used for the detection of parasite diversity in other host-parasite systems (Tanaka *et al*., 2014; Bass *et al*., 2015; Sengupta *et al*., 2019) and could be applied in the future to look at the distribution of *M. gratiosum* in the field. eDNA techniques have a potential to detect alternative hosts, mechanical vectors, and free-living infective stages in the environment for complex life-cycle parasites (Bass *et al*., 2015). For example, techniques based on cytochrome oxidase markers for the detection of *Schistosoma mansoni* Sambon, 1907 with a detection limit of 10 cercariae per litre of water has been successful (Sengupta *et al*., 2019). Assessing the parasite in the final host may be achieved by looking at bird abundance and species in relation to *M. gratiosum* prevalence in the final or in intermediate hosts. For example, *Fredensborg et al.* (2006) found that the digenean prevalence in the snail host *Zeacumantus subcarinatus* was positively and significantly correlative with bird abundance across bays, but not within a smaller scale (50 x·25 m plots).

2.4.7 The transect study in South Queensferry

The transect study conducted in South Queensferry (trip no. 6) provided some local information regarding the spatial distribution of the different species of barnacles. The distribution of the four observed species, *S. balanoides*, *A. modestus*, *B. crenatus* and *V. stroemia*, fits their original zonation, according to Southward (2008). *Austrominius modestus*, as an invasive species, still did not surpass *S. balanoides* in coverage in the study localities. Seventy-three *A. modestus* were found by Colston in South Queensferry in 2012 and 21.9% of them were infected with digenean metacercariae. In the present study, 41 *A. modestus* were found in the transect study but none of them were infected with digeneans or cestodes. In other localities, e.g., Dunbar in the present study, there were very few *A. modestus*. In could be that as an invasive species adapting warmer water, *A. modestus* were still competing with other local species. Furthermore, it seems that *A. modestus* can be infected by digenean metacercariae to similar degrees as *S. balanoides*, but no cestode can be found in this species. The potential for parasite spillback (i.e., acquiring parasites from native hosts and then transmitting them back to a native host) by *A. modestus* and a lack of an enemy deserves further study.

2.4.8 Final remarks

Only four localities were investigated in this preliminary study and they are all rocky shores, however, the chosen localities already represent diverse habitats covering major factors of interest, *e.g*., the relation to definitive hosts, anthropological influence, different terrains, different exposure to the elements, *etc*. Although concrete conclusions cannot be drawn and more evidence is needed to reveal the relationship between biotic and abiotic environment factors and the abundance of digeneans in barnacle populations, a diverse ecosystem harbouring an ideal parasite-host system has been identified. One thing that is clear is that a high heterogeneity of digenean distribution does exist in barnacle populations at Dunbar. Comparing the variance to mean ratio of all 5 selected sites at Dunbar (Table 2.5), aggregation of the digenean infections was observed at almost every site, except the low abundance site, which was close to random. In the present study, the choice of sampling sites was based on local conditions or existing information. A larger, more systematic study is needed to reveal the spatial patterns of metacercariae distribution in the "hot spot", *i.e*., Dunbar Red Rock, and to resolve the cause of this heterogeneity. More sophisticated sampling of barnacles and molluscs could be conducted in the breeding season of kittiwakes in the future, with the hope of revealing the degree of influence of avian hosts and the generation time of the full life-cycle.

This preliminary faunistic study described in this chapter provides critical background information regarding the associates and parasitic fauna of *S. balanoides* in the chosen localities. In the following chapters, several questions arising will be explored. In particular: 1. What species of *Maritrema* are present in the targeted host populations? Do they belong to a single species? 2. What is the spatial distribution pattern of the target parasite and what is the potential cause of such patterns? 3. Is it possible to obtain gravid adults and complete the life-cycle *in vitro* to provide a model system for further studies? To answer these questions, the following studies were undertaken and are described in the following chapters: 1. A study of the morphological and molecular identification of the target parasite *Maritrema* sp. 2. A more sophisticated spatial distribution study to reveal the spatial patterns of the target species in the population of *S. balanoides*. 3. An *in vitro* culture study of the target parasite to reveal some aspects of the biology of development and an opportunity to complete the life-cycle.

2.5 Conclusions

Compared with past studies, the associated and parasitic fauna of *S. balanoides* in selected localities were similar. The target parasite *Maritrema* sp. was chosen based on its numerical dominance and its ecological importance as an indicator of ecosystem health. The cercarial stage of *Maritrema* sp. was not found and further downstream research including the use of eDNA were

suggested to investigate its existence. An aggregated distribution of metacercariae in barnacle populations at the studied localities was confirmed and a heterogeneity of aggregation between and within localities was noticed. An ideal ecosystem, *i.e.*, Dunbar, with a high *Maritrema* sp. infection for investigating host-parasite interaction, was confirmed. In Dunbar, higher infections of metacercariae in barnacles occupying the upper shore was found. Two contrasting hypothesis, one focus on host effect and one on tidal effect, were proposed. The two localities in Dunbar with different terrains, *i.e*., the Leisure Pool and Red Rock, possess different infection patterns, and therefore may provide different insight for host-parasite interactions. A more systematic survey at Dunbar Red Rock is needed to reveal the potential cause of the observed distribution patterns in the target parasite at different scales. More sophisticated studies are also needed to confirm the species of the target parasite (*Maritrema* sp.) and to reveal any morphological and genetic heterogeneity in different populations. Under the difficult situation of trying to obtain adult *Maritrema* sp. from avian hosts, an *in vitro* culture study is also proposed to obtain ovigerous adults so that the life-cycle can be closed in the laboratory.

Chapter 3 Spatial distribution of metacercariae of *Maritrema gratiosum* (Nicoll, 1907) in *Semibalanus balanoides* (Linnaeus, 1767) populations on the Scottish coast

3.1 Introduction

With respect to the distribution of *Maritrema gratiosum* in the field, little information is available. The most detailed report of the distribution of this species in the UK is the one published by Carrol *et al.* (1990) who conducted their work in north-east Ireland. They found that the distribution of metacercarial cysts in *Semibalanus balanoides* was aggregated, and the phenomenon of over-dispersion was more pronounced at higher abundance. They also noticed higher prevalence in human activity related sites and that larger barnacles had significantly more cysts than smaller ones, however, the trend was only significant at heavier infections. The dispersal was affected by location in the barnacle zone and host size, and these two factors acted independently. They also investigated morphometric parameters reflecting barnacle size and found that both the basal plate length and the opercular aperture length can be good indicators, although the behaviour of these two parameters in terms of location on the shore were not the same. Later in Colston's work (2012) focusing on the associated and parasitic fauna in barnacles, some information regarding infection parameters (*i.e.*, prevalence, abundance, and intensity) for metacercariae and larval cestodes in *S. balanoides* and *Austrominius modestus* collected from several localities in Scotland were provided. Her findings included: pronounced heterogeneity in infection parameters between localities and within a locality, a typical over-dispersion (right-skewed) histogram in intensity and abundance, and a positive relationship between operculum length and abundance. In this work, however, it seems that only one site was made per locality, and according to the author, the choice of sampling site required better judgement. The bias from the sampling procedure will bring some concerns since earlier studies had already demonstrated that the characters of locality and position on shore will affect the infection parameters of digeneans infecting barnacles (Carrol *et al*., 1990). Interestingly, in Colston's work the author did consider the spatial effect of digenean infection in barnacles, but on a very small scale, *i.e.*, barnacle host distribution on a single rock. The possible ecological meaning, however, was not discussed, *e.g*., differences in exposure of the barnacle hosts on a scale of a single rock. The author did suggest, however, that a more systematic way of sampling, *e.g.*, along a transect line from lower to upper shore, could be applied in the future study. The inclusion of spatial distribution elements in parasite distribution studies is that most typically lacking (however, see Yang *et al*., 2013) and so its application in digenean-barnacle systems will be explored in the present study.

An aggregated distribution is ubiquitous in parasitology, but its causes and consequences are rather complicated (Poulin, 2006, 2007). What does an aggregated infection mean to both parasite and host ecology and evolution? If moving focus from parasite to a more general spectrum, *i.e*., free-living species, we can find a similar hyperbolic-shape (hollow curve) on species abundance distribution (SADs) studies. The discrepancy between the proportion of rare and abundant species at the community level has been the research interest of ecologists for a long time, and many theories were proposed purely on theoretical grounds or in trying to integrate empirical findings (McGill *et al*., 2007). Some prospective directions were raised to resolve the issue of failure to reject the numerous models proposed and to patterns that go beyond the hollow curve that are generated in empirical or theoretical works. These include, as examples, environmental gradient analysis, successional (temporal) analysis, deconstruction, or sub-setting samples, comparing high and low diversity systems, measure currencies other than abundance, *etc*. (McGill *et al*., 2007). It is assumed that the same principles work as well in parasitic species as in free-living species, because McGill *et al*. (2007) mentioned a study based on "species labelled SADs" is informative, which is much the same as a distribution study for a known parasite species. Currently the applications of these novel ideals in parasite distribution study are lacking and so these will also be explored in the present study.

Not as many theoretical outputs have been generated in parasitology regarding the characteristic distribution patterns as those related to free-living species, however, parasitologists are still trying to explain the empirical patterns and to determine the ecological meanings behind them. Why do we need to know the cause and degree of aggregation? It is necessary because aggregation in parasite populations can influence effective population size and genetic diversity, sex ratio, and on the probability of local extinction (Poulin, 2006). There are a vast number of research studies that have addressed the topic of distribution in either free-living or parasitic species. In the present study, however, the focus will be on digenean-barnacle systems, especially microphallids. Firstly, relevant distribution studies for digenean-barnacle systems will be introduced. Secondly, the distribution in other microphallid-host systems will be addressed to find out what are the parameters of most interest to explain the discovered patterns. Finally, some methodological issues in the distribution study that can jeopardize the analysis will be discussed.

3.1.1 Distribution studies in digenean-barnacle systems

Regarding the location within the shore's barnacle zone, Carrol *et al.* (1990) found that the greatest number of cysts per host was found in the mid to upper part of the barnacle zone. The density of final hosts was the proposed cause of these observed patterns of distribution, although other causes such as the age of barnacle host, distribution of mollusc hosts, type of cercariae, also contributed to the patterns. In earlier research conducted on the east coast of Northern Ireland (Irwin and Irwin,

1980), different prevalence and intensities of metacercariae of *M. gratiosum* were observed on coasts having different exposures. Both prevalence and intensity (infection indices) were higher on sheltered coasts than exposed coasts, but within relatively sheltered coasts, those displaying higher human activity had the highest infection indices. The authors also found that within a particular coast, infection indices were higher at the upper shore than lower shore. The substrates that the barnacles were attached to also seemed to influence infection indices. The coast with the highest infection indices consisted of greywacke and slate of Silurian origin, while the coast with the lowest infection indices was comprised of flint nodules protruding from chalk surfaces. The authors also found that barnacles attached on *Littorina littorea* had higher infection indices compared to those attached to rocks, although no cercariae of *M. gratiosum* were found in these molluscs. *Littorina littorea* was, however, found cohabitating with *Littorina saxatilis*, which is the potential host of *M. gratiosum* (see Popiel, 1976b). After the study of Irwin and Irwin (1980), a small survey was carried out for the same host-parasite system in north-east England. Once again, the pattern of higher prevalence and intensity on the upper shore areas were found, and barnacles attached to living creatures (*e.g.*, common limpets, *Patella vulgate*) also had higher infection indices (Mitchell & Dessi, 1984). There is temporal/seasonal variability of infection indices in *M. gratiosum*-barnacle systems as well. In the study of *M. gratiosum* in *Balanus perforatus* Bruguière, 1789 collected from south-west Wales and conducted by Sari and Malek (2000), the authors found that the prevalence and abundance were significantly different between collection years and between seasons. They found two peaks in March and September and two troughs in February and July. The same differences previously noted between the upper and lower shore levels by other authors were also noticed. Prevalence was found to be significantly correlated to tidal level but not with temperature. Abundance increased with host size and host size was represented by scutum length. The biannual peaks that were observed were explained by the biannual tidal cycle. The correlation between infection level and tidal level will be included and examined in the present study.

3.1.2 Distribution studies in other microphallid-host systems

The distribution of other microphallid-host systems has also been studied by various authors. The distribution of *Microphallus turgidus* (Leigh, 1958) Deblock, 1971 in its second intermediate host, the grass shrimp (*Palaemonetes* spp.) was investigated by Pung *et al.* (2002). Two different hosts, *P. pugio* Holthuis, 1949 and *P. vulgaris* Say, 1818 were surveyed and the authors found a higher prevalence, mean intensity, mean abundance, and population density (no. cysts cm^{-1} host body length) in the former species. Environmental factors, *e.g.*, salinity, had a positive effect on *M. turgidus* prevalence and density. Host size was found to be positively correlated with parasite density, but only in high abundance hosts for *P. pugio*. In another parasite-host system including *Himasthla elongata* Mehlis,

1831 and *Cercariae parvicaudata* Stunkard and Shaw, 1931 and blue mussels *Mytilus edulis* Linnaeus, 1758, the effect of age and size of the second intermediate host was investigated (Nikolaev *et al.*, 2006). The authors found that the prevalence reached 100% in 3–4-year-old mussels and remained at this level until they were 9 years old. The intensity increased with age and only slightly decreased in the oldest mussels. Among mussels of the same age, higher intensities occurred in larger mussels. Most of the metacercariae at the final stage of the experiment were still alive with little signs of aging. This indicated a long life-span of metacercariae in the species examined.

Temporal variation is also reported for parasite-host systems other than *M. gratiosum* (see Yoon *et al.*, 1997; Granovitch *et al.*, 2000; Prinz *et al.*, 2010; Repullés-Albelda *et al.*, 2013). In the research of Prinz *et al.* (2010) for *Echinostephilla patellae* (Lebour, 1911) Kollien, 1996 and the limpet *Patella vulgate* Linnaeus, 1758, the concept of the transmission window was addressed. The transmission window is the period during which cercariae are abundant and possess infectivity. There is a threshold temperature for cercarial release (14 °C in the case of *E. patellae*). The distinct peak in September was suggested to be the result of the abundance of upstream hosts (birds) coinciding with the higher temperature of seawater at this time. The authors also found a sudden decline in infected limpets following the peak. This phenomenon was explained by the pathological effect of heavy infection on hosts. The negative effects that parasites impose on host health may play a regulatory role for both host and parasite populations.

3.1.3 Distribution on barnacle host level and the effect of host factors

Poulin (2006) highlighted that parasite distribution is ever-changing and an estimation of aggregation at any time point is merely a snapshot of this dynamic. The habitat of the parasite is their host, which is fragmented, and is not like the habitats used by free-living species which are regarded a continuum. The distribution of the hosts themselves, however, is also patchy. Therefore, when considering the distribution of *M. gratiosum*, it is important to consider the distribution of barnacle hosts at the same time. The concept of an “infra-population” was mentioned in the work of Bush *et al.* (1997). It refers to all individuals of a parasite species in an individual host at a particular time. Not all individuals in a host population will harbour infra-populations of the target parasites. In the case of *S. balanoides*, which is only parasitized by one species of digenean, only one type of infra-population can be found. More than two species of infra-population, however, can exist in other host-parasite systems. Another similar concept, one of “hemi-populations”, was suggested by Galaktionov and Dobrovolskij (2003). This is defined as populations formed by animals passing through different phases of their life cycles in different habitats. Local hemi-populations of parasites refer to a group of certain life-stages in an individual host, or free-living phases in a microhabitat. In the present study, the term infra-population will be used for ease of discussion, but the concept of hemi-populations will also be

discussed. Study of the distribution of *M. gratiosum* populations is the study of the distribution and characters of infra-populations. The distribution of *S. balanoides* is generally as follows. The limit of zonation for adult *S. balanoides* is between minimal low water spring tides (MLWS) and maximum high water spring tides (MHWS), but they settle predominantly between minimum low water neap tides (MLWN) and maximum high water neap tides (MHWN). Factors that influence adult patterns include: desiccation, predation, and intraspecific competition (Hui & Moyse, 1987). In the British Isles, the maximum abundance is around the mid-tide level (Southward, 2008). According to Jenkins *et al.* (2008), the population dynamics of *S. balanoides* are not linear. Density dependent mortality was found at the early phase of settlement (i.e., during the summer time), then changed to a density independent pattern of mortality afterwards. As an annually breeding species, new cohorts of *S. balanoides* larvae start their settlement during the 4-6 week period in the spring. The continuous gradient of density dependent mortality throughout time maintains the optimum adult population density. Other factors affecting the distribution of metacercariae of *M. gratiosum* include the distribution of the first intermediate host. According to the data of NBA Atlas (National Biodiversity Network Atlas), the principal mollusc host of *M. gratiosum, i.e., L. saxatilis,* has a UK-wide distribution. Regarding the local distribution, over 100 species of molluscs were recorded in Dunbar (see Berry & Smith, 1987), but six species were commonly encountered. These were: *Patella vulgata, L. littorea, L. saxatilis, L. obtusata, Nucella lapillus* and *Mytilus edulis*. Only the counts for *L. littorea* were given but not those for *L. saxatilis*, which is the species of focus in this study. Counts were higher in the mid-shore (i.e., 3397.7 molluscs m^{-2}) than the low shore (i.e., 858.9 molluscs m^{-2}) level, but the shell length was greater within the low shore zone than at the mid shore level.

Host factors are incorporated in the systematic study in the present study to better analyse their importance on digenean distributions. Some of the host factors considered in the present study such as host size and cirrus length, maximum basal plate length and width and maximum height and length of the opercular aperture were used as indicators for the size of *S. balanoides* off north-east Ireland (Carrol *et al.*, 1990). It was found that only the basal length and operculum length had significant effects on the cyst numbers of *M. gratiosum*. Basal length increased towards low-tide level while operculum length fluctuated. Another morphological indicator that might reflect the environment of barnacle settlement is the morphology of the cirrus. Morphological plasticity of the cirrus was found in *Balanus glandula* Darwin, 1854 off the British Columbian coast. A precise relationship was found between cirrus length and average wave exposure: the stronger the wave exposure, the shorter the cirrus length (Arsenault *et al.*, 2001). In the case *of S. balanoides* in the harbour in New York, variation in the morphology of the cirri was detected between sites, however, this was not directly related to wave exposure and had no relationship to barnacle density (i.e., crowding). Despite this, a general

pattern of shorter cirrus length with stronger wave exposure was still detected (Hoch, 2011). In the present study, maximum basal plate length and maximum operculum length were applied as indicators for host size, and length and width of the sixth cirrus was applied as an indicator for local exposure.

3.1.4 Technical issues in the distribution study

Historically, parasite distribution in different digenean-host systems has been analysed by use of a range of different statistics methods, such as goodness-of-fit and variance to mean ratios for detecting aggregation of *M. gratiosum* (see Carrol *et al.*, 1990); chi-square test to compare prevalence of *M. turgidus* at different salinities, two-way ANOVA to test for the interaction between species and sex with respect to intensity, abundance and parasite density, interaction between species and salinity with respect to parasite density, and interaction between host species and body length with respect to parasite density (Pung *et al.*, 2002); regression and correlation for host size effect versus intensity of *H. elongata* and *C. parvicaudata*, one-way ANOVA for intensity between different host age groups (Nikolaev *et al.*, 2006); and one-way ANOVA for the number of developmental stages in rediae of *E. patellae* in different months (Prinz *et al.*, 2010). A general linear model (GLM) was applied to examine the effect of fish host length and conditional factors (K) on the abundance of *Zeuxapta seriolae* (Meserve, 1938) Price, 1962, and factorial ANOVA for examining seasonal and yearly effects on size-corrected abundance (Repullés-Albelda *et al.*, 2013). Due to the over-dispersion character of parasite distribution data, the assumption of normality cannot be fulfilled and data transformation is often required for proper application of these statistical tools. Also sample size, sampling procedure, sensitivity for parasitism measurement, host factors (size, age, sex, health, etc.), will affect the precision of estimation of distribution patterns (Wilson *et al*., 2002; Poulin, 2013).

There are many ways to capture the infection profile in a parasite-host system. The most intuitive way is to calculate the average number of parasites per host. This value can be referred to as abundance or as intensity and depends on whether uninfected hosts are included or not. Poulin (2006) mentioned that due to the high variance of this value, it is a less meaningful value for describing infection level. The variance, or the degree of aggregation, however, is more important, as the degree of aggregation can reflect heterogeneity in host exposure and susceptibility. There are many ways to measure parasite aggregation, e.g., variance-to-mean ratio, negative binomial parameter *k*, patchiness index of Lloyd (1967), and index of discrepancy (D) (Wilson *et al*., 2002; Poulin, 2006). Among these parameters, Lloyd's patchiness index (or mean crowding), although used less frequently, can quantify average aggregation experience by an individual parasite. This index incorporates an important element in the sampling procedure, that is "average density per quadrat" and "variance in density among quadrat" and has the advantage of easy calculation (Wade *et al*., 2018). When used properly, *i.e.*, incorporating the information regarding spatial relationships (spatially explicit), then this index can

help reveal parasite "hot spots" in habitats. More sophisticated spatially explicit models have been applied in medically important parasites such as *Schistosoma japonicum*; spatial autocorrelation (Moran's I) was applied for assessing whether the pattern of infected snails, humans and livestock populations were clustered or not, and directional distribution (standard deviational ellipse) for assessing the directional trend each year, and compactness and orientation of the dispersion of the infected snails, humans and livestock (Yang *et al*, 2013). In a study of aggregation of malarial vectors, spatially implicit methods, namely variance to mean ratios and Morisita's index for dispersion, were applied for accessing patchiness. Spatially explicit statistics, such as correlograms (Moran's I) and variograms, were applied to summarize spatial pattern of the full extent of the study area, and local indicators of spatial association (LISA) and spatial analysis by distance indices (SADIE) were applied to capture local patterns and clusters within individual sampling units (*i.e.*, hot, and cold spots in the sample space) (Kamdem *et al.*, 2012).

3.1.5 Aims

The information described above, crossing space and time, are fragmented, though have already provided a general profile of the distribution for *M. gratiosum* in its barnacle host. As commented on in Chapter 2, Dunbar is an ideal locality with distinct terrain that is suitable for a larger distribution study. In the present study, a more systematic survey combining environmental and host factors will be conducted for the distribution of *M. gratiosum* in selective localities, including Dunbar, to gain a more precise infection profile *in situ*, and to identify the potential causes of the discovered patterns. In addition, and most importantly, I will include spatial information and use random tables in the sampling procedure to reduce sampling bias, and at the same time to reveal local hotspots and in relating high spots of infection to environmental and host factors. The aim of the present study is to provide baseline distribution data in this digenean-barnacle system and to develop a predictable model in the chosen habitat, which in the future can be applied to longer-term population dynamic studies. This can also provide baseline data for further investigations looking at the diversity of genes and the morphology of both the parasite and host populations. Furthermore, the distribution data regarding the digenean population can be related to the population dynamics of their avian final hosts, which could therefore be useful in conservation programmes.

3.2 Materials and methods

The locality for the systematic study described in this chapter is Dunbar in Scotland and the choice of this locality is based on the well-established barnacle-parasite system, closeness of intermediate hosts to final hosts, the high prevalence and abundance of *M. gratiosum* found during the preliminary

study, and two contrasting areas of terrain it contains (see Chapter 2). Dunbar is characterized by a maritime climate and has more sunshine than anywhere else in Scotland. The annual hours of sunshine exceed 1500 hours, and Dunbar has an extreme range of temperatures extending from 31 °C to -12 °C. Its geological features include: volcanic deposits and dykes, cliffs, a raised shore platform and raised beaches, features associated with a glacial-isostatic uplift. Human activities nearby have been extensive, as the area is a popular seaside holiday destination and has been a golfing resort since the 19th century. The famous bird habitat Bass Rock is only 11 km away. The distribution of *Maritrema* sp. at Dunbar and the relevant ecological significance, however, have not been thoroughly studied.

In order to obtain a more accurate profile of *M. gratiosum* distribution, it is necessary to consider two types of distribution, namely host population distribution and the infra-population of parasites, both contributing to the overall distribution of the targeted parasite in the field. Assuming that most of the barnacle population at Dunbar was represented by *S. balanoides* (which is the case if only sampled in their zonation), the effect of the distribution of this single species must be considered. Based upon the survey of barnacle species over several sampling trips, it was clear that most barnacles at Dunbar were *S. balanoides* and that their zonal distribution was quite typical for this species. If the distribution of the barnacle population is homogenous, then the effect of host distribution can be ignored as a background value. However, the distribution of *S. balanoides* is substantially patchy. Their distribution is affected by predators, local exposure, algal competition, local terrain, larval recruitment, and post settlement mortality, *etc*. To understand the cause of the patchy distribution of the barnacle hosts is not the purpose of the present study because what is of interest is the distribution pattern of the digeneans parasitizing them. The potential factors that affect barnacle distribution such as host size, distance from the land and host density, however, are incorporated in the following analysis.

Systematic sampling strategies were applied in the present study, the relationship between host size and metacercarial numbers was investigated first, and both biological and environmental factors were included later for building a model for predicting abundance and in examining the underlying causes of the patterns observed. Here, a “systematic survey” means comparing infection parameters at different latitude gradients, comparing the degree of aggregation in the different habitat types, across different scales within the sampling area, and in different subsets of samples. Systematic sampling ensures the results can be quantified and analysed in a logical manner and relevant bias can be reduced. Furthermore, the count data for metacercariae from two additional localities at higher latitudes in Scotland, *i.e*., Rosehearty and Stonehaven, were included to elucidate the effect of a latitude gradient for degrees of aggregation. Sampling trips to Rosehearty and Stonehaven took place in January 2018, in which 60 barnacles were dissected at both localities using the same methods mentioned in Chapter 2. These samples that were collected were for the purpose of morphometric

and an *in vitro* culture study, and the same systematic sampling strategies were not applied; the samples, however, are still included in the present study.

In the present study, the description of infection parameters and terms used for geographic information complies with the terminology provided by Bush *et al*. (1997).

3.2.1 Sampling strategies in systematic study

The sampling sites in the pilot study (see Chapter 2), as a scoping exercise, were chosen according to more arbitrary criteria. In present study, a more systematic distribution study was conducted at Dunbar. The distribution of metacercariae in *S. balanoides* across a 120 m × 160 m rocky platform at Dunbar Red Rock was investigated at both the "whole area" (*i.e.*, looking at overall site data) and the "local scale" (*i.e.*, looking at data within a small area within the larger area) levels to determine indicators of spatial distribution. This design, by looking at different scales, can detect potential scale effects and hopefully determine an optimal sampling distance. Furthermore, this design can provide distribution information along a subset of samples from a gully (local scale, from low to high tide), to reveal tidal effect. The platform at Dunbar Red Rock, although elevated from the sea and roughly horizontal, has many crevices which allow sea water to penetrate the larger structure. The distance between low water and high-water during spring tide is approximately 5 m and allows the platform to be totally submerged at high tide. The barnacles, therefore, are not only distributed at the edge and along the slope but are also have a patchy distribution towards the top of the shore. The sample area that was explored was divided into a grid. To incorporate the distance factor, a proper "distance lag" needed to be decided. Distance lag is the term applied in spatial statistical analysis, which refers to the interval between neighbouring sampling or analysis units (Dungan *et al.*, 2002). In a malarial vector study, a spatial resolution of 5 km was decided upon based on the maximum flying distance of adult mosquitoes (Kamdem *et al.*, 2012). In the present study, the distance between sampling sites on the map represented the distance lag, the chosen value was based on the movement distance of mollusc hosts within a day. A proper distance lag can be very difficult to find (Kamdem *et al.,* 2012). The strategy applied in the present study was to use a smaller distance lag first, which was the distance approximating to the maximum movement distance of the first intermediate molluscan host. According to Janson's (1983) research, the average daily movement distance of *L. saxatilis* in the intertidal zone is within the range of 1-4 metres. The creeping speed of *L. saxatilis* is around 0.5 mm per second, so they can theoretically move much further than 4 metres within a day. It may be, however, that some environmental or behavioural factors (such as home-ranging) constrain their actual dispersal rate. In the present study, 5-10 metres were employed as the distance lag in the smaller scale study (local scale), followed by a 40-metre distance lag in the larger scale study (whole area). Although the main purpose of setting difference distance lags is to find an optimal sampling

distance in systematic sampling, this difference will also lead to an increase in "resolution" from the whole area scale to the local scale.

For the "local scale" study that was conducted in late May 2014, samples of 40 barnacles were collected from 12 nodes at the intercepts of adjacent 5 × 10 m grid squares (see Figure 3.2). For the "whole area" study that was conducted in mid-July 2014, samples of 40 barnacles were collected at 16 nodes at the intercepts of adjacent 40 × 40m grids (see Figure 3.3). A small rock sample with attached barnacles was taken at each sampling point using a hammer and chisel, with 40 barnacles being isolated from each sample, and the number of parasite cysts within each barnacle being determined. The prevalence, intensity, and abundance of parasites at each site were analysed. Several environmental and / or host factors were also recorded and analysed. These factors included: operculum and basal length of the barnacle, the cirrus length and the width of the barnacle, mollusc coverage and mollusc species, algae and barnacle coverage, and parasite cyst density among barnacles on the rock sample. The locations chosen for the whole area and local scale studies are shown in Figure 3.1. The gridded sampling points for the whole area study and local scale study are shown in Figures 3.2 & 3.3.

Figure 3. 1 Sampling area at Dunbar (Lat. 56° 0' 24.9"N, Long. 2° 31' 31.1" W). The blue rectangle represents the area of the "whole area" study, while the yellow rectangle represents the "local scale" study.

Figure 3.2. The grid overlay for sampling sites in the "whole area" study. The distance lag for the whole area study is 40 x 40 m. Sampling sites are at the nodes of the grid (numbers in white are sampling sites). Numbers in yellow are four sampling sites chosen arbitrarily on the edges of the rock platform.

Figure 3.3. The gridded sampling sites of the "local scale" study. The distance lag is 5 x 10 m. Sampling sites are on the nodes of the grid.

Other problems needed to be solved before starting. The first issue was that the scale of the present study was much smaller than that of other distribution studies, which often extend to a hundred kilometres or more. The study area in the present study was not more than 200 m^2. In such a small area, GIS (geographic information system) systems cannot be applied, therefore other methods needed to be applied to precisely locate the sampling sites. Also, a specific problem for barnacle sampling was caused by their attachment to the substrate. When barnacles attach to a fixed rock bed, it can be difficult to detach a piece of rock for further processing. If successfully detached, the size of the rock fragment was variable. This would lead to a variable size of sampling unit. In the study of Kamdem *et al.* (2012) investigating mosquito distributions, the sampling unit was the sleeping room within a sample house. Each sleeping room had the same size. In the present study, the confounding effect of different sizes of sampling unit had to be minimised.

As a GIS system could not be used for the present study, a portable laser distance measuring system (Leica DISTO classic 5) was employed to establish sampling sites. The sampling grid was positioned relative to a selected site datum and was expanded by moving along a pre-determined axis reflecting shore topology. The site datum was located at an obvious and permanent object, *e.g.*, prominent rock. A straight-line distance was then measured using the laser measurement tool with one person holding the apparatus and the other person holding a piece of white board. A tripod was used to mount the laser measurement tool and maintain it at a fixed height (see inset in Figure 3.4). By means of reflection from the white board, the distance could be calculated by laser measurement. Once the desired distance was attained, the person holding the white board placed a marker at the sampling point (i.e., a fluorescent yellow fix pot filled with water) on that site and started sampling. When the sampling was finished, the team established the next sampling site along the same axis. Once sampling sites along the first axis were completed, the team turned the measuring device 90^0 to establish the first point along the next parallel sampling axis. For the local scale study, a square quadrant (50 cm^2) was centred at the sample point and a photo was taken to include all barnacles under the quadrant. A decision of whether to accept this site was then made: when barnacles covered more than 3 grids of the quadrant, the site was accepted; when barnacles covered less than 3 grids of the quadrant or the site was not accessible, then this site was discarded and an adjacent point was chosen instead. Once the photo was taken, a piece of rock with barnacles was taken ideally within the range of the quadrant. For the whole area study, a circular quadrant (1-m radius) was placed encircling the marker and a photo was taken to include all organisms in the quadrant. The photo of the circular quadrant was also used for capturing environmental information at the chosen site. Later a square quadrant measuring 50 cm^2 was placed at the position of the marker, and another photo was taken to document all barnacles in the quadrant. A decision whether to accept or discard the site was made

before taking a photo using the same criteria mentioned above. After all the photos were taken, a piece of rock with barnacles was taken within the range of the square quadrant whenever possible. Schematic and real-life images of sampling strategies are shown as Figures 3.4-3.6.

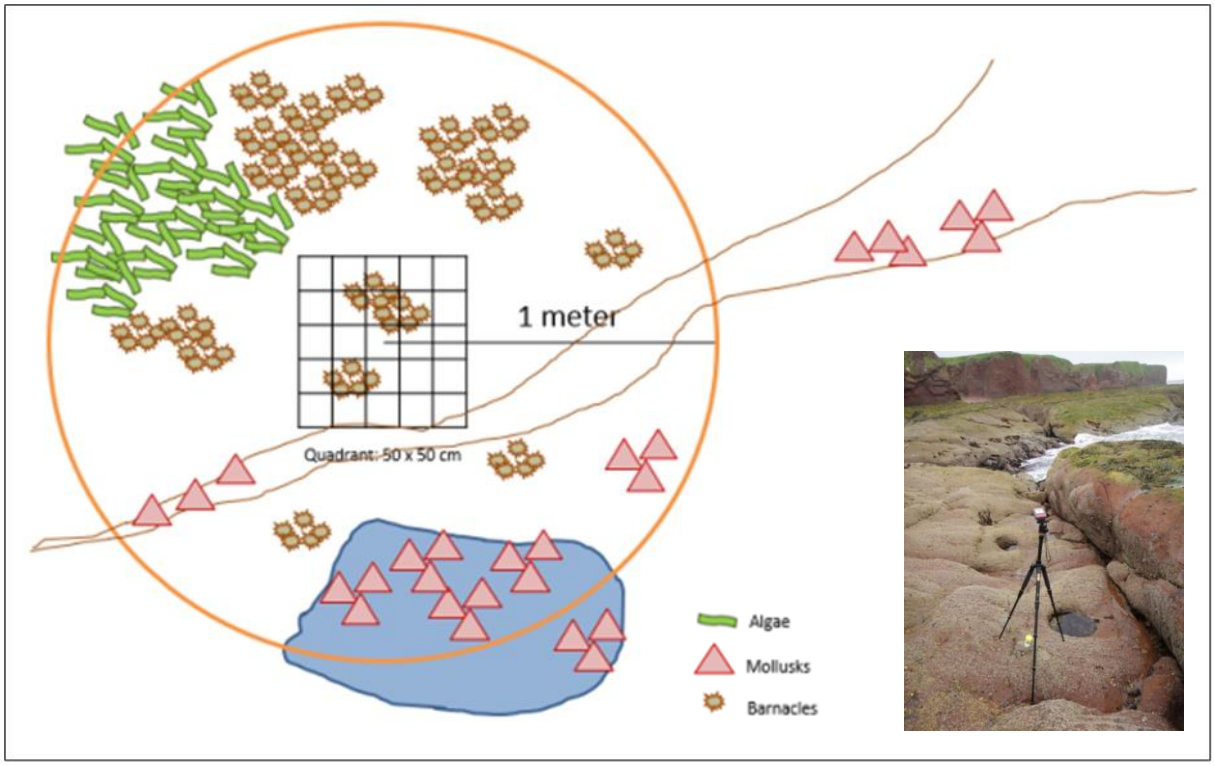


Figure 3.4. Schematic overview for samples and environmental data collection. A square quadrant (50 cm^2) is placed in the middle of a sampling site and a circular quadrant (1 m radius) is placed outside. Several environmental factors recorded in the circle are estimated and analysed. Inset: laser measurement tool fixed on a tripod at the field.

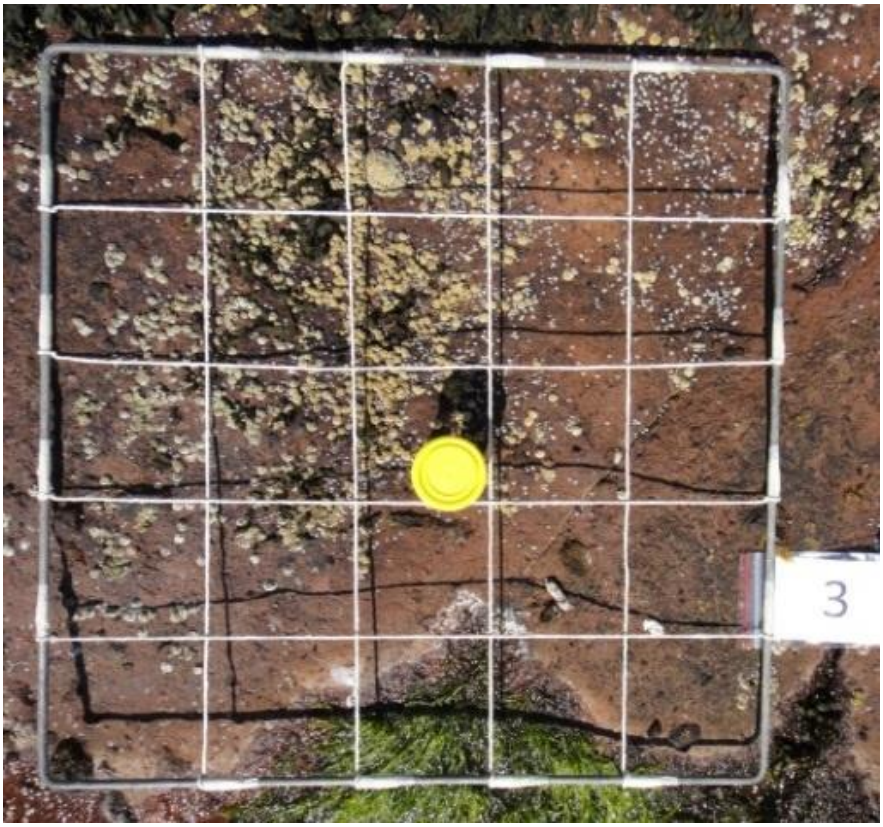


Figure 3.5. The square quadrant (50 cm^2) *in situ* at the sample site. The density of the barnacles or total barnacle count can be analysed using this quadrant.

Figure 3.6. A photo showing the circular quadrant (1 m radius) *in situ* at the sample site. The barnacle coverage, topography of this site, density/ species of mollusc, and coverage of algae can be analysed using this quadrant.

The rocks brought back to the lab in the present study, which functioned as “sampling units”, varied in size and shape, and were covered in barnacles at different densities. To reduce sampling error, and to achieve a better “random” sampling of 40 barnacles at each sampling site, a method combining image analysis and random tables was applied. The photo of a single rock with barnacles on the surface was taken and analysed using FIJI ImageJ 1.52p (National Institutes of Health, USA; Schindelin *et al*., 2012) (see Figure 3.7). The number of barnacles was counted and the surface area of the rock was calculated by ImageJ. Barnacles were numbered one by one from left to right on the photo. Afterwards, 40 numbers among the total count were chosen using a random number table. The 40 selected numbers designated barnacles to be dissected for infection indices to be calculated. If the chosen barnacle could not be taken from the rock, the one beside it was taken.

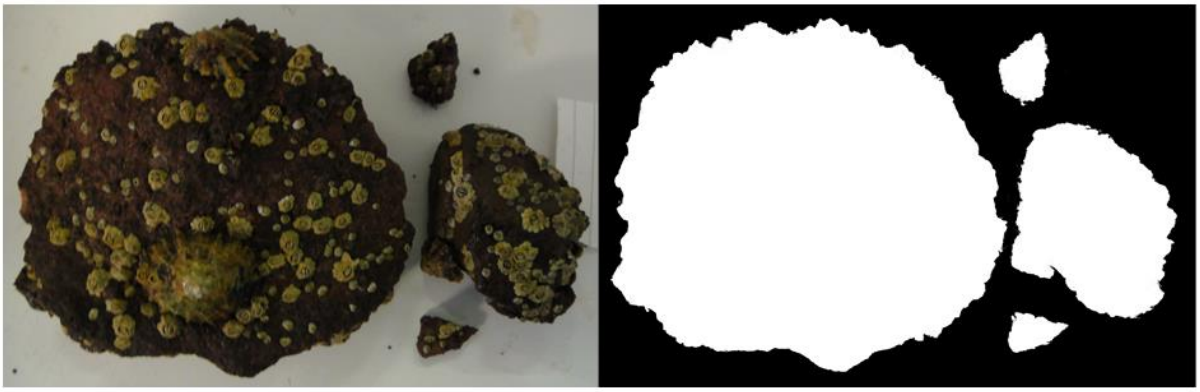


Figure 3.7. Example for estimating mean barnacle density on the sampled rock. The numbers of barnacles were counted on the original photo (left). The area of the sampled rock (sampling unit) was estimated by transforming the image using the “binary” function in Image J (right).

3.2.2 The effect of repeat sampling

The effect of repeated sampling of barnacles from the same rock twice was studied to understand the potential bias from repeat sampling. In the whole area study, two sets of forty barnacles were detached from the sampled rock at sites 11, 12 and 14 in the lab (first and second sampling) using random number tables and the metacercariae were counted. The data from the three sites was pooled and the number of metacercariae per barnacle of the first and second samplings were compared using a paired sample T test. Correlation analysis was applied to investigate the relatedness between the two samplings.

3.2.3 The feasibility of using formaldehyde-fixed barnacles

On some occasions, the samples could not be processed immediately for practical reasons, such as a lack of rearing facilities or a lack of time. As a potential solution to this, the feasibility of using formaldehyde-fixed barnacles was studied. Two sets of barnacle samples (n = 60 each set) were

collected from the rocks sampled at sites 4, 7 and 20 using random tables. The number of cysts in the barnacles was determined and then analysed. The number of metacercariae per barnacle in the formaldehyde-fixed barnacles and fresh barnacles was then compared by paired sample T-tests and histograms. The purpose of this test is to investigate the technical issues of sampling. In the result sections, however, all metacerariae except those included in this trial are from barnacles that were not fixed (i.e., fresh).

3.2.4 Analysis of metacercarial counts versus host size

3.2.4.1 Count data of the metacercariae in the local scale study

In the local scale study, systematic sampling was carried out along a gully (see Figure 3.3). Descriptive statistics covering the mean number of metacercariae per barnacle at each site was analysed by means of a box and whisker plot, histogram, and mean plot. Count data were found to be non-normally distributed and so a distribution fit was conducted. Several methods were compared for data transformation, including: Log, LN, X^0.25 and X^0.5. A one-way ANOVA was carried out to investigate differences between sites using the transformed count data. *Post hoc* analysis was applied to reveal which site were significantly different from others.

The count data for the number of metacercarial cysts were plotted on the map to reveal patterns of spatial distribution. Three types of count data were generated for the evaluation of their distributional pattern. The first method was the most intuitive, showing the mean number of cysts per barnacle (i.e., mean abundance). The second method employed was to generate Lloyd's mean crowding (Wade *et al*., 2018) and the obtained values were then plotted on the map of the sample site. According to Wade *et al*. (2018), this index m* can be calculated as follows, where m*= Lloyd's mean crowding, m= mean abundance in 40 barnacles, and V_m= variance in 40 barnacles, to give the following equation: $m^* = m + ([V_m/m]-1)$. Under Lloyd's original definition (for free-living species), m* measured on average how many other individuals an individual will encounter in a quadrat, and the original assumption is a random spatial distribution among individuals (Wade *et al*., 2018). Here, in a parasitology study, the rock substitutes the quadrat. The third method revealed an estimated number of total metacercariae at each site within a quadrant. Total metacercarial numbers in a quadrant (50 cm^2) was calculated as follows. Total metacercarial numbers were obtained by multiplying the mean number of metacercariae per barnacle with the total number of barnacles per quadrant. The total barnacle number per quadrant was obtained by multiplying the mean barnacle density per cell with the numbers of cells covered by barnacles within a quadrant. Mean barnacle density per cell was an estimated value; it was obtained by calculating the average barnacle number of 5 cells where possible.

A ranking system was designed to represent the distance of each site to the land for use in later analysis in order to detect the influence of distance from land for abundance. Each sampling site was given a rank by measuring the distance of the site to site 21, which was closest to the shore, and which was designated as the reference site. The "rank" of each site was proportional to the real distance between site 21 measured on the map (see Figure 3.8).

Figure 3.8. Ranking system in local scale study. Each sampling point was given a rank by measuring the distance from site 21. Site 21 was designated as the reference site.

3.2.4.2 Relationship of host factors versus metacercarial count in the local scale study

To better understand the biotic and abiotic factors, more variables such as host factors and environmental factors were considered in the whole area study. This cannot be achieved directly because the relationship of the host factor itself such as size must be analysed first. Barnacle size was estimated by two indices: maximum operculum length (MOperculumL) and maximum basal length (MBasalL). As shown in Figure 3.9, maximum basal length is the maximum length from the edge of the rostral plate to the carinal plate. Maximum operculum length is the maximum length of the operculum aperture. After photos of a barnacle were taken under a stereomicroscope (Olympus SZ-PT), the length was measured using image analysis software (ZEISS AxioVision). Descriptive statistics and a mean plot by site were examined first to reveal any structuring within the data. Thereafter, an ANOVA and *post hoc* analysis were performed to detect differences between the maximum operculum length at the different sites. Scatter plots were used to reveal the relationship between the log host size and log abundance. A Pearson correlation was performed to investigate the relationship between MOperculumL and MBasalL and also to describe the relationship between these two parameters and

the number of metacercariae per barnacle. A GLM was performed thereafter to investigate the relationship between the number of cysts per barnacle (abundance, x^2.5 transformed), barnacle size (by MOperculumL), and site. Finally, a simple linear regression was performed to examine the relationship between MOperculumL and abundance. A negative binomial regression of the raw data of abundance using the maximum operculum length as a predictor was performed to investigate model fit.

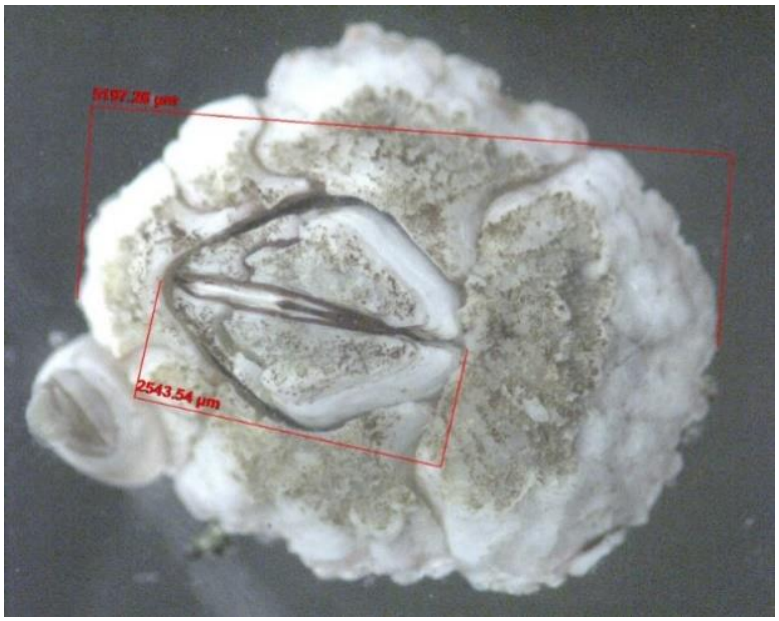


Figure 3.9. Measurement of the maximum basal length and maximum operculum length. Maximum basal length is the maximum length from the edge of rostral plate to the carinal plate. Maximum operculum length is the maximum length of the operculum aperture.

3.2.4.3 Count data for the metacercarial cysts in the whole area study

Descriptive statistics, box and whisker plots, histograms and mean plots were used for the whole area study. A one-way ANOVA and *post-hoc* analysis were applied to look for differences of the transformed count data between the sites. In the whole area study, the number of metacercarial cysts were plotted on the map to reveal the patterns of spatial distribution, using the same three methods described for the local scale study. A fourth method was applied in whole area study. Weighted Lloyd's patchiness index (P_w), was generated to reveal the impact of host density on abundance. According to Wade *et al*. (2018), the original Lloyd's patchiness index (*P*) was generated by the following equation. P= m*/m. Then the index "barnacle density on the sampled rock (d)" was calculated. To obtain this value, the total number of barnacles on each rock that was sampled were counted, and the area from which the rock was sampled was calculated by Image J (see Figure 3.7). Value "d" is obtained by dividing the total number of barnacles on each rock, by rock area. Finally, the patchiness index

multiplied by the barnacle density on the sampled rock which is then plotted on the map. This value will become a weighted version of Lloyd's patchiness index (P_w): $P_w = P$ x d. Using this method, the effect of host density and the potential strength of the host-parasite interaction (*e.g.*, recruitment rate, and degree of intra-species competition), will be incorporated

3.2.4.4 Relationship of host factors versus metacercarial count in the whole area study

Host indices were investigated first, as in the local scale study, using descriptive statistics, mean plots by site and by one-way ANOVAs and *post hoc* analyses. Scatter plots were applied to reveal the relationship between log host size and log abundance. A Pearson correlation was performed to investigate the relationship between host size and abundance (x^0.25 transformed). A negative binomial regression of the raw data of abundance using the maximum operculum length as a predictor was applied to examine the fit of the model.

A ranking system was designed to represent the distance of each site to the land for use in a general linear model (GLM) in order to detect the influence of distance from land for abundance. Each sampling site was given a rank by measuring the distance of the site to site 18, which was closest to the shore and which was designated as the reference site. The "rank" of each site was proportional to the real distance between site 18 measured on the map. To obtain simpler values, the starting point (site 18) was given a value of 2 but not 1 (see Figure 3.10). To assess the relationship between host size, distance to the shore and the number of metacercariae per barnacle, a GLM was applied using operculum length as co-variables. Between subjects, the effect of operculum length was examined by using abundance (X^0.25 transformed) as the dependent variable. The source of variance included site rank, operculum length and site rank*operculum length. A profile plot estimating the marginal mean was obtained using maximum operculum length as a co-variant.

Figure 3.10. Ranking system in the whole area study. Each sampling point was given a rank by measuring the distance from site 18. Site 18 was designated as the reference site

3.2.5 Relationship between host density and abundance

Host density is a potential factor contributing to the heterogeneity of abundance (Thieltges & Reise, 2007) and their relationship will be investigated. Host density was calculated as the mean number of barnacles per cm^2 on the sampled rock. The value was plotted against the site rank and the mean abundance per site to reveal any patterns. Data was only available from the whole area study.

3.2.6 Relationship of host and environmental factors and parasite abundance

Multiple regression was applied to investigate further factors which might affect the mean number of metacercariae per barnacle. More host and / or environmental factors were added to the model, such as operculum length, cirrus length and width, mollusc coverage and species, algae and barnacle coverage, and cyst density on the sample rock. The relationship between the cirrus length and the width was studied first. For each barnacle that was dissected, the sixth pair of cirri was cut at the base, mounted in sea water under a coverslip, and photos were taken under a compound microscope (Olympus BX51). The length of the outer contour of the cirrus was measured using ImageJ. Both rami were measured and the mean of these was used as the final value for analysis. The width of

the cirrus refers to the width of the base of the first segment (Figure 3.11). The definition of all the factors can be seen in Table 3.1. The concept of the grading system for some factors (*i.e.*, barnacle, mollusc, and seaweed coverage) can be referenced to Mettam (1994). To sum up the methods of obtaining these parameters: an estimated rank for mollusc coverage (by percentage) and species number (count by genus only), algae and barnacle coverage (by percentage) was decided by visual estimation by area. Cyst density on the sample rock was calculated by the method mentioned above. After obtaining all the data for these parameters, a multiple regression was performed. Enter, forward, backward, and stepwise methods of regression were applied to investigate the importance of each parameter.

Figure 3.11. An image of the 6th pair of cirri of the barnacle *Semibalanus balanoides*. The length of the outer contour of the two rami were measured and averaged

Table 3. 1 Definition of the parameters for the multiple regression analyses

Parameter	Definitions	Parameter	Definitions
Cyst number	count data for mean cyst number per barnacle, 5 barnacles / site	**Mollusc coverage**	estimated mollusc coverage area (%) in the circular quadrant: <10% and 10% (appendix 2 in Mettam, 1994)
Operculum length	measurement of 5 barnacles / site	**Mollusc species**	number of mollusc species present (*Littorina* sp. and *Patella* sp.) in the circular quadrant: 0, 1 & 2
Cirrus length	measurement of 5 barnacles / site	**Algal coverage**	estimated algae coverage area (%) in the circular quadrant: 0-90 % (appendix 2 in Mettam, 1994)

Cirrus width	measurement of 5 barnacles / site	**Barnacle coverage**	estimated barnacle coverage area (%) in the circular quadrant: 10-90 % (appendix 2 in Mettam, 1994)
Cirrus W/L ratio	cirrus width/ cirrus width of 5 barnacles / site	**Cyst density of the sampled rock**	mean cyst number per barnacle (from 40 barnacles) x total number of barnacles on the rock / rock area

3.2.7 Comparison of infection indices and degree of aggregation of digenean infection in barnacle populations between environmental gradient and habitat types

Infection indices, namely prevalence, abundance, and intensity, will be compared between different latitudes, between locality types, and between sampling scales. First, the relationship of prevalence and abundance was investigated by a scatter plot according to the locality at different latitudes (See Figure 3.12). Two rocky shore localities at higher latitudes, i.e., Rosehearty and Stonehaven, were included for comparison. These two localities were visited for the purpose of a morphometric and an *in vitro* culture study, but infection parameters were also recorded. Data used for Dunbar Leisure Pool were collected from a previous sampling trip (trip no. 3, see Table 2.1 in Chapter 2).

To reveal differences between infection parameters and the degree of aggregation, data are compared in different combinations according to the questions of interest, *i.e*., between different latitudes, between locality types, and between sampling scales. The parameters evaluated included: prevalence, abundance, intensity, variance-to-mean ratio (D), negative binomial (k), Lloyd's mean crowding (m*), and weighted Lloyd's patchiness index (P). Negative binomial k is calculated as the following formula according to Wade *et al*., (2018): $k= m^2/(V_m-m)$.

Prevalence was compared by a chi-square test. Intensity and abundance were compared by ANOVA using X^0.25 transformed data with *post hoc* analysis. Finally, to further investigate the agreement of Taylor's Power Law in distribution pattern for this digenean-barnacle system, a scatter plot was produced by plotting log variance and log mean of abundance. Afterwards, a curve estimate was performed to reveal which model had a better fit to the data.

Figure 3.12. The four localities for Investigation of the relationship between prevalence and abundance, namely Rosehearty (57°41'52.5"N 2°6'51.5"W), Stonehaven (56°58'16.1"N 2°12'34.5"W), Dunbar Red Rock (56°00'19.9"N 2°31'33.4"W) and Dunbar Leisure Pool (56°00'19.5"N 2°31'04.2"W).

Table 3. 2 Strategies for comparison of infection and aggregation parameters

	Parameters		
Comparison	Prevalence, abundance, intensity	Variance-to mean ratio D	Negative binomial k
Between different latitudes	Data from Rosehearty, Stonehaven, and Dunbar Red Rock		
Between locality types	Data from Dunbar Red Rock (Rocky platform) and Leisure Pool (Sheltered Bay)		
Between sampling scales	Data from whole area scale and local scale in Dunbar Red Rock P.S. Lloyd's mean crowding (m*) are calculated by sites for local scale and whole area study. Lloyd's patchiness index (P) is only calculated in whole area study.		

3.2.8 Statistical tools

All analyses were performed using Excel (Microsoft Office Professional Plus 2016) and SPSS (IBM SPSS Statistics 23 or 25).

3.3 Results

3.3.1 The effect of replicate sampling

Two replicate samples (first and second replicate) each of forty barnacles were collected from the rocks sampled at sites 11, 12 and 14 using random number tables with the number of metacercariae per barnacle being recorded. The data from the three sites were pooled and compared. The mean, median and sum of the cyst number from the first and second replicates were not the same, however, the variance and SE were similar (see Appendix 18 for descriptive statistics). Also, the histogram of the first and second samplings had approximately a similar shape (Figure 3.13). As the data for the first and second replicate was found to be not normally distributed, a Wilcoxon Rank Sum test was applied, which found no significant difference between the two (p = 0.072). A non-parametric correlation was also applied. Using Kendall's tau method, the correlation coefficient was 0.435 (p<0.001). This analysis revealed that although replicate sampling would not yield very similar results, the difference was not significant, therefore indicating that no experimental bias was introduced.

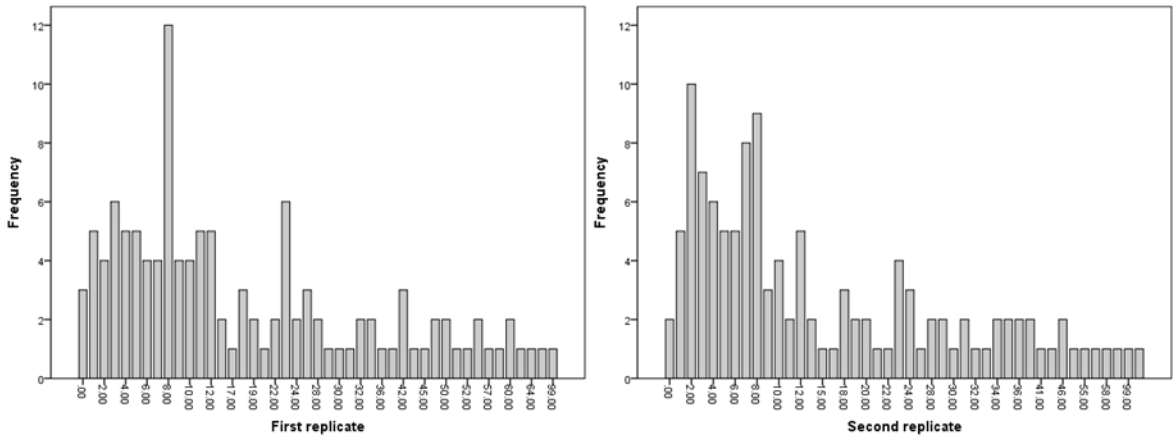


Figure 3.13. Frequency histogram comparison of the distribution of cysts per barnacle in the first (left) and second (right) replicate.

3.3.2 The feasibility of using formaldehyde-fixed barnacles

A comparison between formaldehyde-fixed barnacles and fresh barnacles was carried out. A total of 120 samples were collected from the rocks at sites 4, 7 and 20. The cyst number was counted and analysed as previously described. The data from the fresh barnacles was identified as cyst number 1 while those from the formaldehyde-fixed barnacles were identified as cyst number 2. The descriptive statistics for cyst number 1 and 2 (pooled for the 3 sites) is shown in Appendix 19. The data histogram for cyst numbers 1 and 2 looked similar and both were skewed to the right (Figure 3.14). By looking at the histogram for each site separately, the histograms for the fresh and formaldehyde fixed samples were not very similar (Figure 3.15). A Wilcoxon Rank Sum test of the raw data and transformed data found no significant difference between the two (p = 0.542). The results indicated that formaldehyde-

fixed samples can be used for analysis, however, they might not be the best samples as the dissection of the barnacle and the counting of the cysts was more difficult than processing fresh samples.

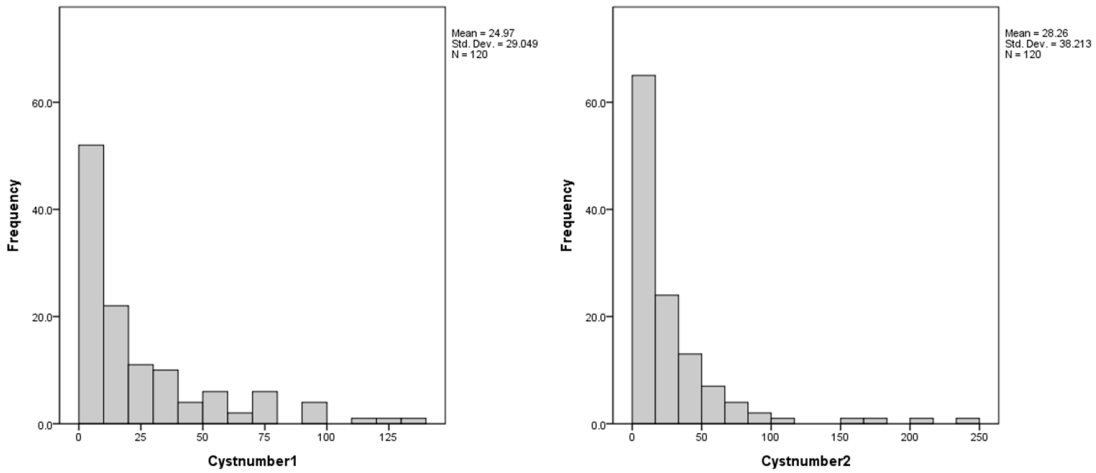


Figure 3.14. Histograms for fresh samples (left) and formaldehyde-fixed samples (right) using pooled data from three sites.

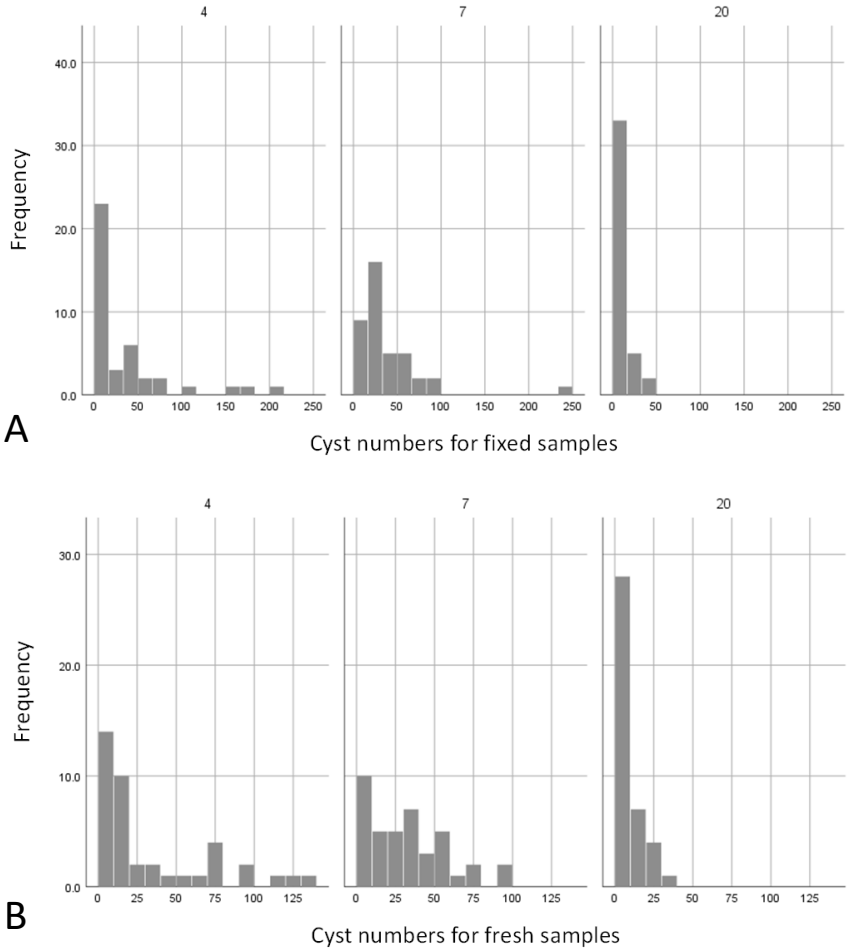


Figure 3.15. Frequency histograms for cyst numbers for fixed samples (A) and fresh samples (B) collected at sites 4, 7 and 20.

3.3.3 Local scale study

3.3.3.1 Infection indices and metacercarial count

Infection indices (prevalence, abundance, and intensity) in the local scale study can be referred to in Appendix 20 and descriptive statistics of the number of metacercariae per barnacle (referred to as abundance in the following analysis) can be referred to in Appendix 21. In the local scale study, 480 samples were included, with a mean parasite abundance of 21.01 ± 23.70 (mean ± 1 SD). The prevalence of each site was in general high, ranging from 80%-100%. Of the 480 barnacles, 461 were infected, and the mean parasite intensity was 21.86 ± 23.83 metacercariae per barnacle (mean ± 1 SD). Mean abundance at each site ranged from 8.93 ± 8.03 to 36.23 ± 33.17 metacercariae per barnacle (mean ± 1 SD), minimum to maximum range 0-133 metacercariae per barnacle. A mean plot of the abundance per site is shown in Figure 3.16. A scatter plot of the total metacercariae per site presented in Figure 3.17 shows an approximately similar but not identical patterns in mean cyst number per site. The equation for the total number of metacercariae per 50 cm^2 = mean abundance per barnacle × total barnacle numbers per quadrant. The equation for the total number of barnacles per quadrant = mean barnacle numbers per cell × numbers of cells covered by barnacles within a quadrant. Mean barnacle numbers per cell is the average number of barnacles from 5 cells where possible. For site 12, however, only 3 cells were available.

The distribution of cyst numbers was over-dispersed, and the data were not normally distributed, regardless of whether the data is analysed by site or as pooled data (Figures 3.18 & 3.19). A distribution fit test rejected the null hypothesis that the distribution of cyst number per barnacle had a binomial, Poisson, or exponential distribution (p<0.001 respectively). Several methods for data transformation were compared, including: Log, LN, X^0.25 and X^0.5. A value of 0.5 was add to the raw data to prevent problematic calculations associated with zero values. It was found that the best approach to data transformation was X^0.25, which transformed the data to give a normal distribution. A one-way ANOVA was performed to investigate differences in abundance between sites (using X^0.25 transformed data, one-way ANOVA: F= 7.932, p<0.001). *Post hoc* analysis (Tamhane test) showed that some sites were significantly different from others. Site 23 was different from sites 12, 18, 19 and 20; sites 4, 7 and 15 were different from sites 18, 19 and 20, and site 12 was different from site 15.

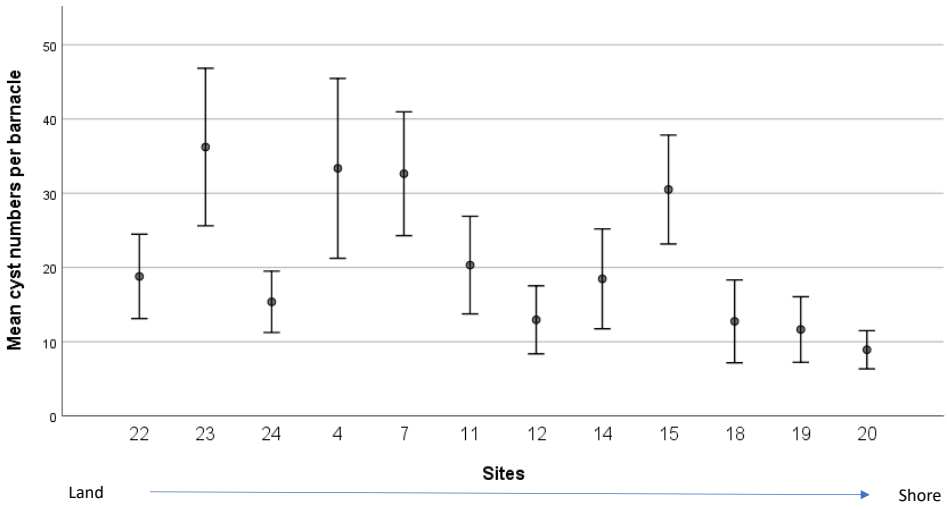


Figure 3.16. Local scale study. Mean plot of the mean cyst numbers per site. Sites are arranged from land to shore.

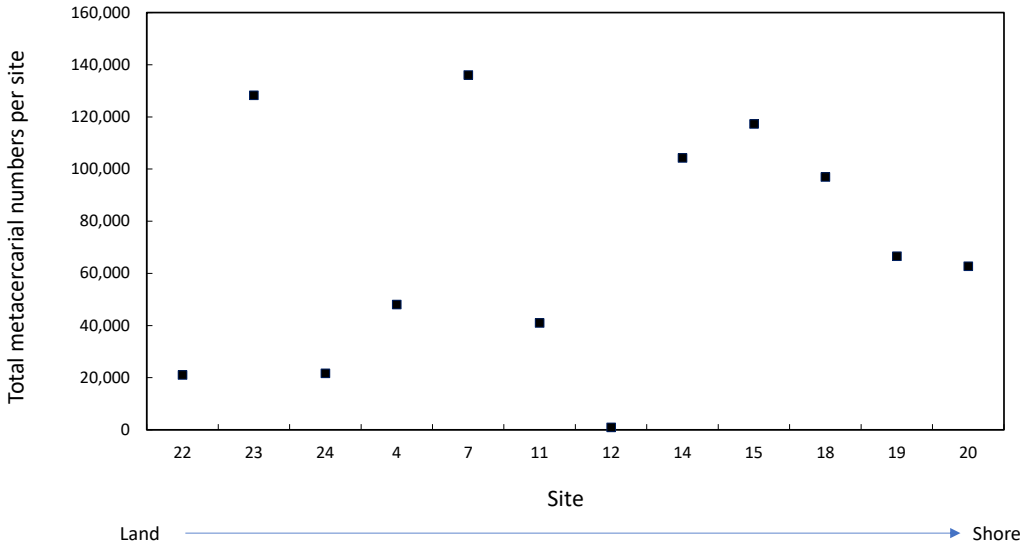


Figure 3.17. Local scale study. Scatter plot of the total number of metacercariae per 50 cm^2 per site. The numbers were obtained by multiplying mean cyst numbers with the estimated total number of barnacles within the quadrant. Sites are arranged from land to shore.

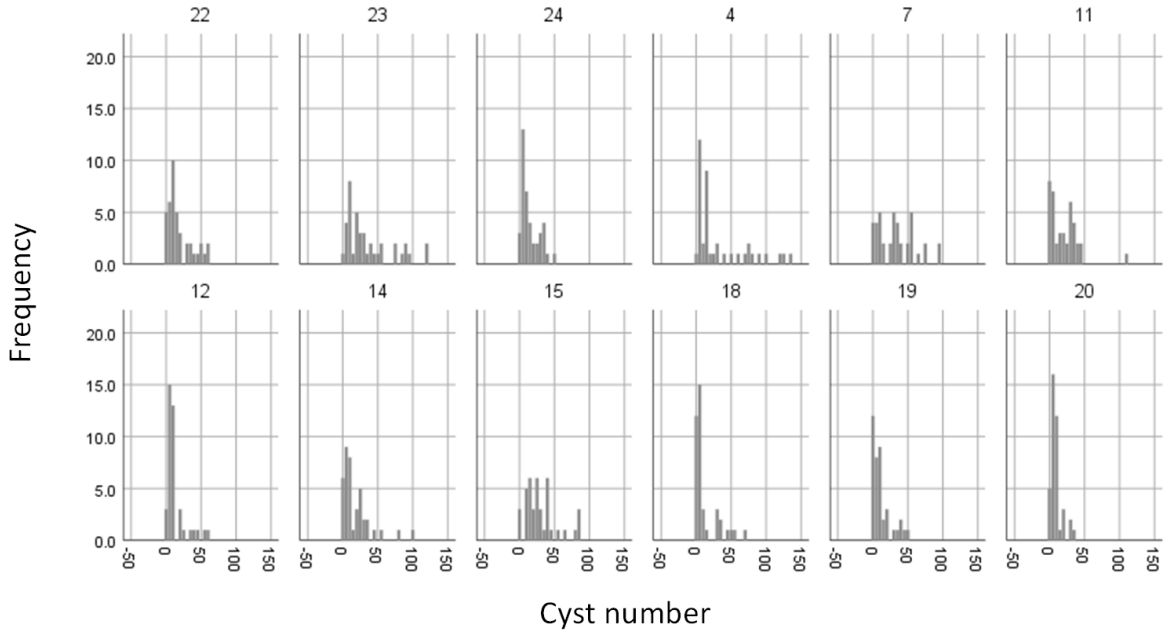


Figure 3.18. Frequency histograms of the cyst number per barnacle for the 40 barnacles sampled at each site in the local scale study. The order is from land (site 22) to shore (site 20).

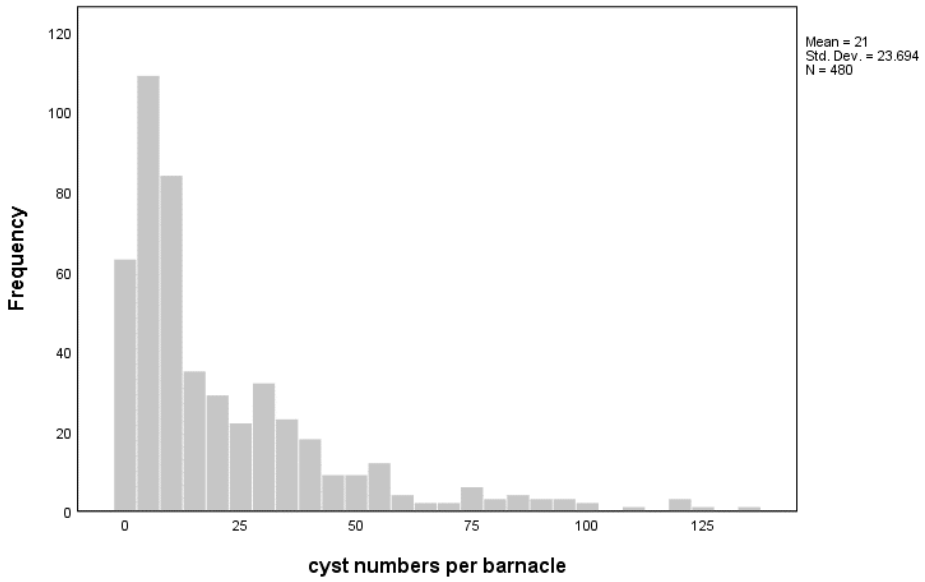


Figure 3.19. Histogram of cyst number frequency for the 480 barnacles sampled in the local scale study.

The count data of metacercarial cysts provided a chance to investigate the patterns of spatial distribution. Three methods were applied. The first method, “abundance”, is shown in Figure 3.20A. As the sample sites were arranged in a “grid pattern”, the abundance along the longitudinal and transverse axis can be compared. Although abundance fluctuated, a slight but significant decreasing trend can be observed from the land to the shore (simple linear regression, R^2= 0.061, p<0.001, used x^0.25 transformed abundance as dependent and site rank as independent variable). No trend, neither

decreasing or increasing, was observed when focusing on individual transverse axes. The second method was to plot the estimated total number of metacercariae in the quadrant. The second method is to plot Lloyd's mean crowding (see Section 3.2.4.1) on the map to show which site has a higher intra-species competition (Figure 3.20B). A slight decreasing trend was also observed from the land to the shore but not significant (simple linear regression, R^2= 0.25, *p*=0.098, used Lloyds mean crowding as the dependent and site rank as the independent variable). In Figure 3.20C, the total number of metacercariae per 50 cm^2 at each site are shown. By plotting the total number of metacercariae in the defined area (a 50 cm^2 quadrant) in the selected site, the quantity of metacercariae available to the final host in the defined area would be better demonstrated. The total number of metacercariae per site revealed a more variable profile than using mean cyst number per barnacle per site. In the plot of the total number of metacercariae per site, the decreasing trend towards the shoreline along the longitudinal axis disappeared. By this method, no decreasing trend was observed (simple linear regression, R^2= 0.023, *p*=0.639, used total metacercariae per 50 cm^2 as the dependent and site rank as the independent variable). These three methods each provides a different biological perspective.

Figure 3.20. Spatial distribution of metacercariae in the local scale study. The numbers are shown in different colours that reflect the numeric scale provided in the legend. A. Mean cyst number per barnacle at each site in the local scale study. Forty barnacles were counted for each site. B. Lloyds mean crowding. This parameter was calculated as m + ([V_m/m]-1), where m= mean cyst number per barnacle per site, V_m= variance at each site. C. Estimated total metacercarial numbers at each site pre 50 cm^2 (unit: 1000 metacercariae). Total metacercarial numbers were obtained by multiplying the mean number of metacercariae per barnacle with the total number of barnacles per quadrant. The total barnacle number per quadrant was obtained by multiplying the mean barnacle density per cell with the numbers of cells covered by barnacles within a quadrant. Mean barnacle density per cell was an estimated value; it was obtained by calculating the average barnacle number of 5 cells where possible.

3.3.3.2 Analysis of barnacle size versus metacercarial numbers

The relationship between the barnacle size indices MOperculumL (maximum operculum length) and MBasalL (maximum basal length) and metacercarial numbers was analysed for 474 barnacles. The descriptive statistics for barnacle size are shown in Appendix 22. From the skewness, kurtosis, and histogram (Figure 3.21), the data were roughly normal distributed. ANOVA and *post hoc* tests (Tamhane test) were performed for MOperculumL and MBasalL to investigate differences between sites. For MBasalL, significant differences between the sample sites were detected (ANOVA, F= 5.181, p<0.001); site 23 was different from 24; site 24 was different from sites 4, 15, 18, 19 and 20; site 4 was different from 12, site 12 was different from sites 15 and 19. For MOperculumL, significant differences between the sample sites were also detected (ANOVA, F= 7.551, p<0.001); site 22 was different from sites 23 and 19; site 23 was different from 24; site 24 was different from sites 4, 7, 14, 15, 18, 19 and 20; site 11 was different from 19; site 12 was different from sites 15, 19 and 20. The mean plot of MOperculumL and MBasalL (Figures 3.22 & 3.23), although this fluctuated, it revealed a slight increasing trend toward the shore for both indices.

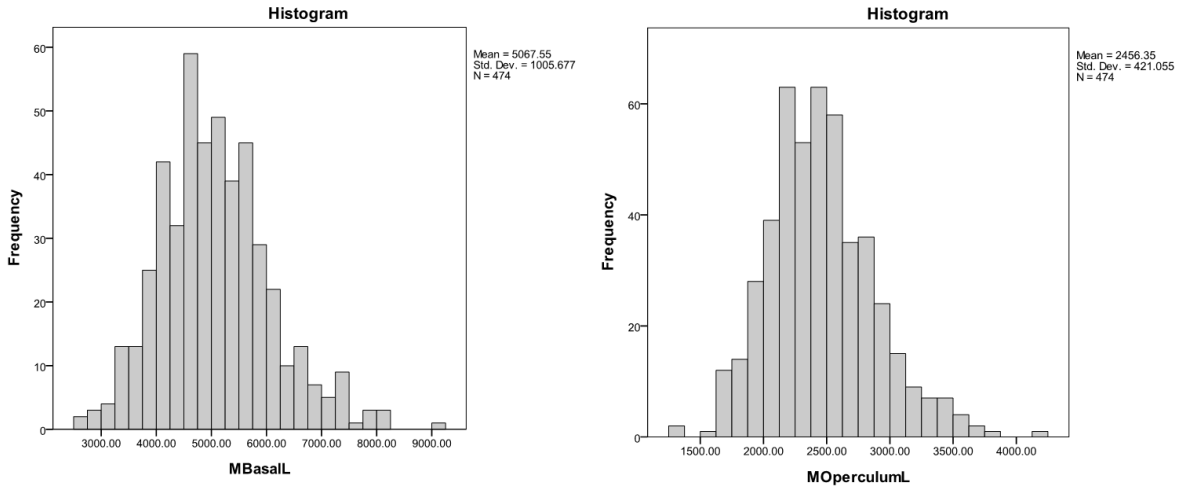


Figure 3.21. Frequency histogram of the maximum basal length (left) and maximum operculum length (right) for the barnacles sampled in the local scale study.

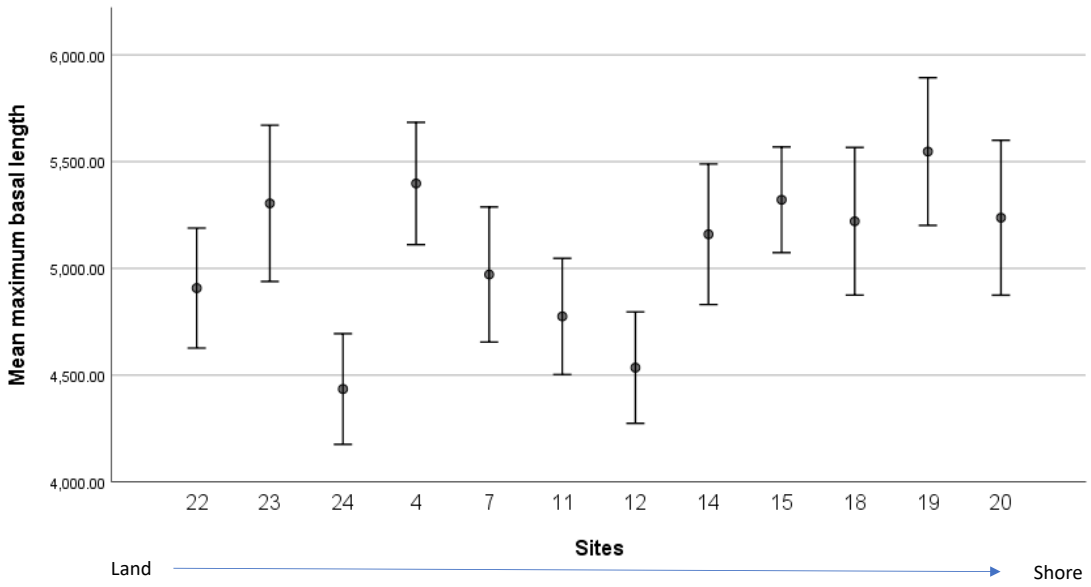


Figure 3.22. The mean plot of maximum basal length (µm) by site in the local scale study. The error bars indicating 95% confidence interval. Sites are arranged from land to shore. Sample N=40 per site.

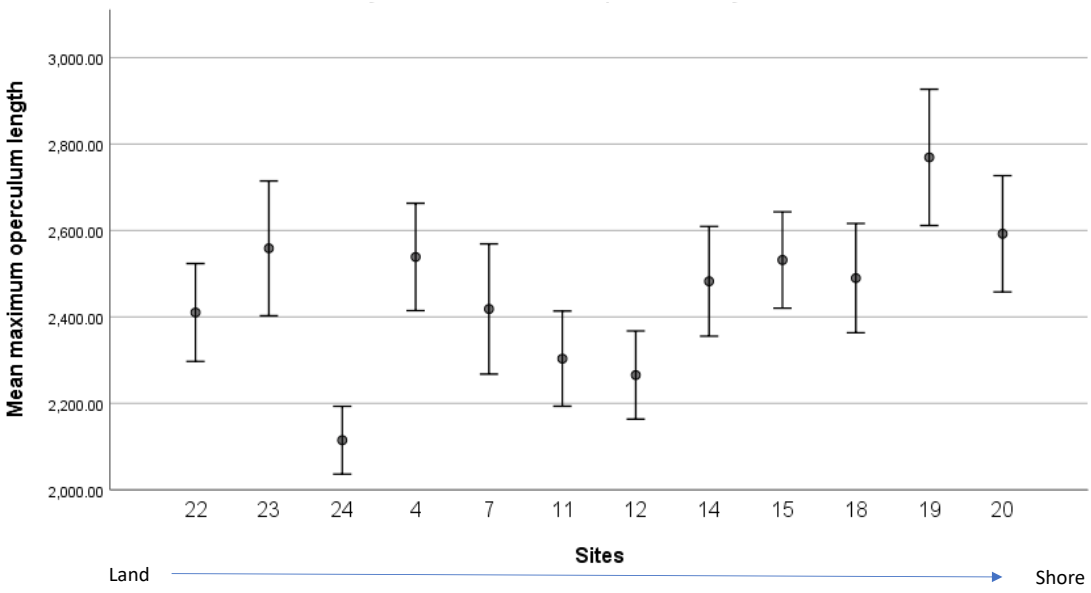


Figure 3.23. The mean plot of maximum operculum length (µm) by site in the local scale study. The error bars indicating 95% confidence interval. Sites are arranged from land to shore. Sample N=40 per site.

After investigating the structure of the data for MBasalL and MOperculumL, the relationship between these two parameters and their relationship between abundance were studied. A highly significant correlation between MBasalL and MOperculumL was found (Pearson Correlation = 0.817, p<0.001) and an obvious positive linear correlation was also seen in the scatter plot (Figure 3.24). As MOperculumL had a higher correlation with cyst number, and lower SEs and SDs, and because these two size indices were highly correlated, MOperculumL was chosen over MBasalL for the host size index for the following analysis.

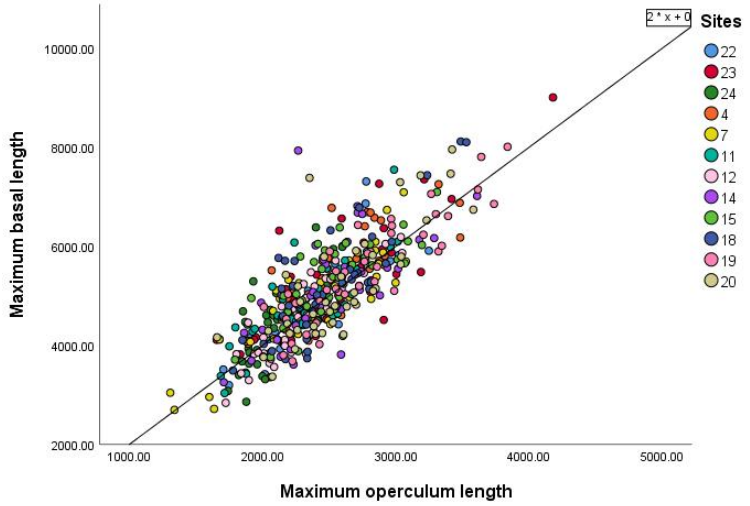


Figure 3.24. Scatter plot for maximum basal length vs. maximum operculum length of local scale study. A linear least squares regression line was added to show the correlation.

Some degree of correlation between transformed host indices and abundance was found: for MBasalL (log10) vs. abundance (log10) (Pearson correlation = 0.419, p<0.001), and for MOperculumL (log10) vs. abundance (log10) (Pearson correlation 0.439, p<0.001). Scatter plots were produced to reveal the relationship of host indices and abundance (LOG-LOG scatter plot, Figure 3.25). A positive relationship was found between log host size (both maximum basal length and operculum length) and log abundance. When the relationships by site were examined (Figure 3.26), positive relationships between log host size (operculum length) and log abundance were consistent, however, different slopes between sites were seen indicating heterogenous relationships between these two parameters among sites.

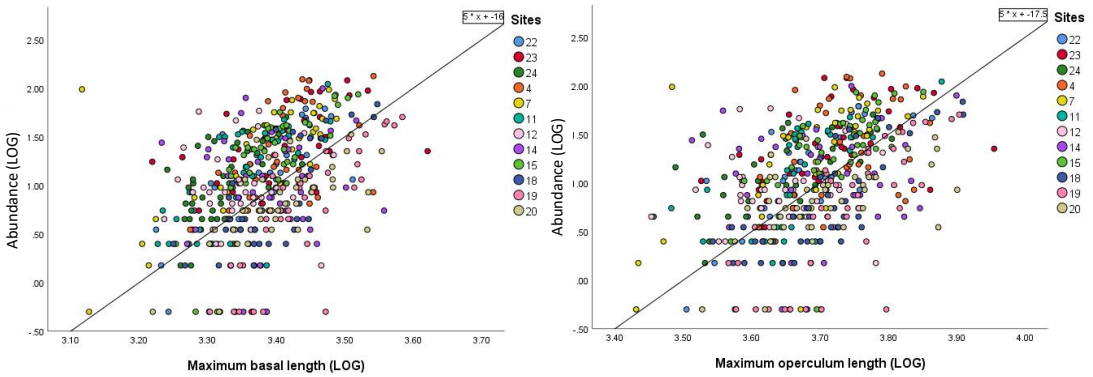


Figure 3.25. Scatter plot for log10 maximum basal length vs. abundance (left) and log10 maximum operculum length vs. log 10 abundance (right) within the local scale study. The numbers referring to site which are mapped in Figure 3.3. A linear least squares regression line was added to show the correlations.

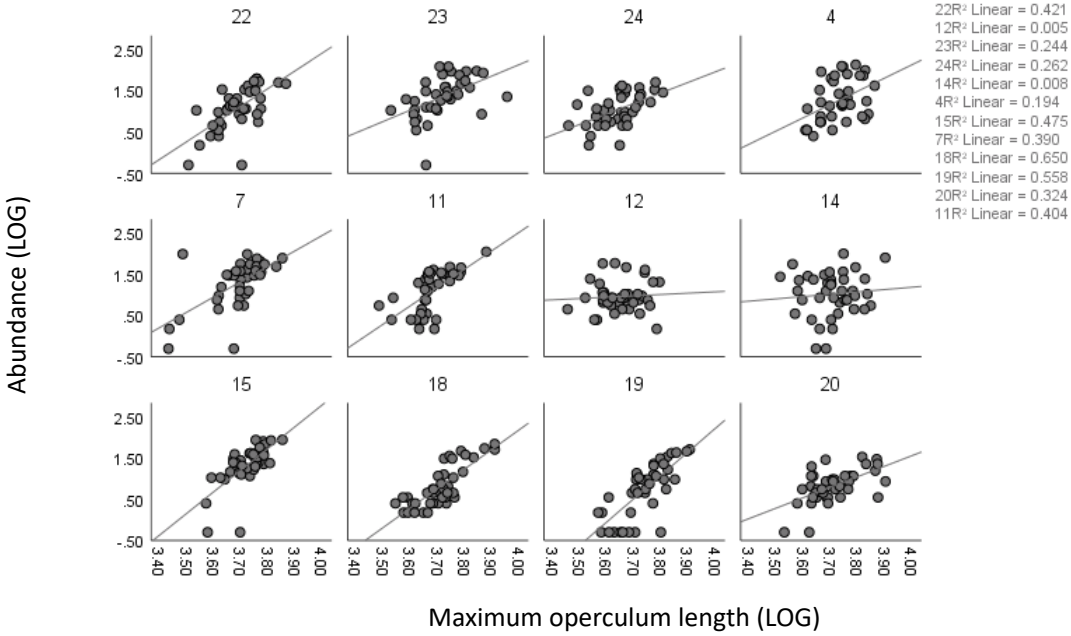


Figure 3.26. Scatter plots of the log 10 maximum operculum length versus log 10 abundance by site within the local scale study. The numbers on each plot refer to the grid reference presented in Figure 3.3. Linear least squares regression lines were added to indicate the relationship.

To investigate the relationship between host size and abundance further, a simple linear regression for the operculum length and the transformed abundance was performed first. The correlation coefficient was moderated for maximum operculum length versus transformed abundance (Pearson correlation= 0.482, p<0.001). The R square was 0.23, which means 23% of the variance of the transformed abundance can be accounted for by operculum length (Table 3.3). The results of ANOVA (F= 142.61, p<0.001) and the correlation both showed that the correlation value was statistically significant. The coefficients table (Table 3.4) indicates

that the standard regression coefficient was 0.482, and that a one µm increase in the length of the operculum can lead to an increase in abundance ((X+0.5)^0.25 transformed) of 0.001 unit. From the residue analysis, the residual mean was zero, and the histogram and normal P-P plot showed a normal distribution, indicating that there was no violation of the assumptions required for the regression (data not shown). This was supported by a scatter plot of the regression standardised predicted values vs. regression standardised residuals, which was found to be homogeneous (data not shown). For the local scale study, the simple linear regression function for abundance and maximum operculum length can be expressed as:

Abundance ((X+0.5)^0.25 transformed) = 0.346 + 0.001 x maximum operculum length

After re-organisation, the equation can be given as:

Abundance (cyst no per barnacle) = (0.346 + 0.001 x maximum operculum length)^4 - 0.5

Table 3.3. Simple linear regression model summary [b] for maximum operculum length and abundance of local scale study

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	0.482[a]	0.232	0.230	0.493	1.533

a. Predictors: (Constant), MOperculumL

b. Dependent Variable: cyst number (X^0.25 transformed)

Table 3.4. Coefficients [a] for maximum operculum length and abundance

Model	Unstandardised Coefficients		Standardised Coefficients	T	Sig.	95.0% Confidence Interval for B		Collinearity Statistics	
	B	Std. Error	Beta			Lower Bound	Upper Bound	Tolerance	VIF
(Constant)	0.346	0.134		2.583	0.010*	0.083	0.610		
MOperculumL	0.001	0.000	0.482	11.942	<0.001****	0.001	0.001	1.000	1.000

a. Dependent Variable: cyst number (X^0.25 transformed)
Level of significance: <0.05*, <0.01**, <0.005***, <0.001****

Furthermore, due to the over-dispersion nature of the abundance data, a negative binominal regression of the maximum operculum length and abundance was performed to investigate the fit of this model and to obtain an optimal k value. By using raw data of the maximum operculum length and site rank as the predictor for abundance, a model with a dispersion coefficient of 0.803 was produced with a reasonable goodness of fit (deviance = 1.144, df = 469). Dispersion coefficient is equal to the negative binomial parameter k, which is referred to as the aggregation parameter. The smaller the k, the more aggregated the population. When k approaches infinity, the Poisson distribution results (Young & Young, 1990). When k = 1, it has a property of geometric distribution. In most biological situations, the value of k is less than 5 (Crofton, 1971b).

To understand more about the impact of the host indices and site upon abundance, a general linear model (GLM) was conducted using maximum operculum length as co-variable. The host index was examined for between-subject effects. The dependent variable was abundance (x^0.25 transformed), and the source of variance that was included was site, MOperculumL and site * MOperculumL. The resulting model explained 49.4% (MOperculumM) of the variability in abundance (R square = 0.494, see Table 3.5). MOperculumL was found to have a significant interaction with site. This means that the host indices and sites are not independent. The estimated marginal mean for size corrected for abundance was produced (Appendix 23). The profile plot for estimating marginal means of abundance using MOperculumL as a co-variant revealed a significant decreasing trend (simple linear regression, $R^2 = 0.558$, $p = 0.005$, used estimated marginal mean as dependent and site rank as independent variable) in abundance towards the sea (Figure 3.27).

Table 3.5. Tests of between-subjects effects of cyst number (x^0.25 transformed) and maximum operculum length within the local scale study.

Dependent Variable: cyst number (x^0.25 transformed)					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	73.696[a]	23	3.204	19.068	<0.001****
Intercept	0.004	1	0.004	0.023	0.879
Sites	8.247	11	0.750	4.462	<0.001****
MOperculumL	38.706	1	38.706	230.337	<0.001****
Sites * MOperculumL	7.894	11	0.718	4.271	<0.001****
Error	75.619	450	0.168		

Total	1906.349	474
Corrected Total	149.315	473

a. R Squared = 0.494 (Adjusted R Squared = 0.468)

Level of significance: <0.05*, <0.01**, <0.005***, <0.001****

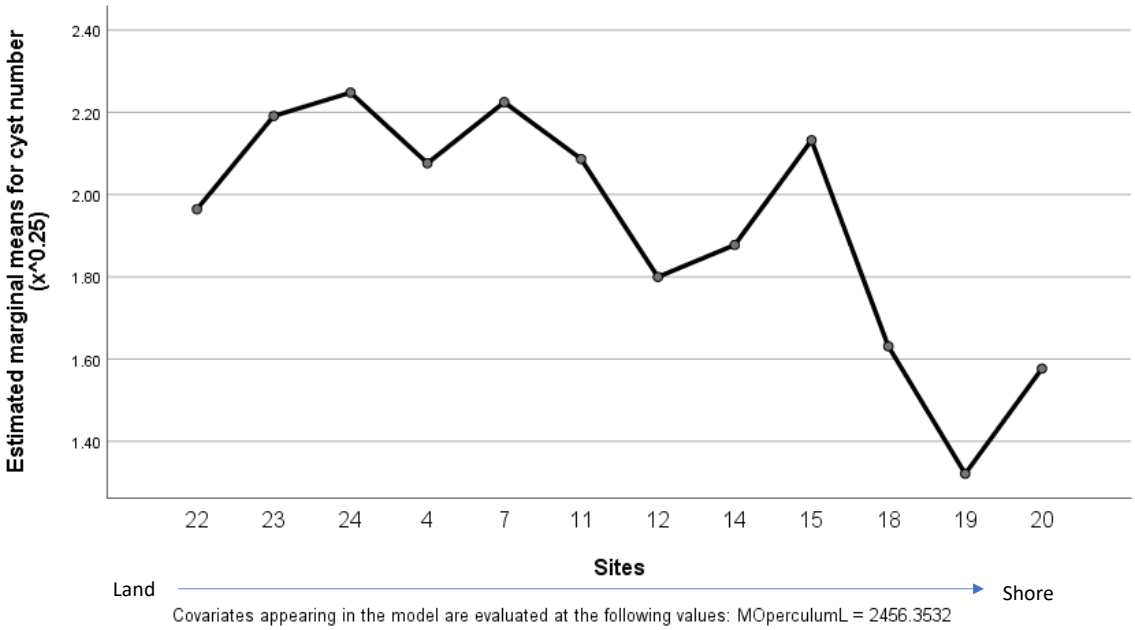


Figure 3.27. Profile plot for estimated marginal mean of abundance (x^0.25) of local scale study. Maximum operculum was the co-variant. Sites are arranged from land to shore.

3.3.4 Whole area study

3.3.4.1 Metacercarial cyst distribution on site and infection indices

The infection indices at each site in the whole area study are shown in Appendix 24 and descriptive statistics for abundance in Appendix 25. In the whole area study, a total of 640 samples were included; mean abundance was 30.45 ± 38.23 S.D. Among the 640 barnacles, 623 were infected, and the mean intensity was 31.28 ± 38.41 S.D. Mean abundance at each site ranged from 5.88 ± 5.99 S.D. to 125.05 ± 63.39 S.D., minimum to maximum number of cysts was 0-287. The prevalence at each site was in general high, ranging from 82.5%-100%, and mean intensity ranged from 7.12 ± 5.88 S.D. - 125.05 ± 63.39 S.D. As expected, the raw data were not normally distributed, and this was revealed by the shape of histogram of the abundance, no matter by site (Figure 3.28) or by pooled data (Figure 3.29). A different shaped histogram between sites though was noticed, and this indicated the heterogenous distribution

among sites. From the results of the local scale study, an (X + 0.5)^2.5 transformation was found to be the best way to transform abundance.

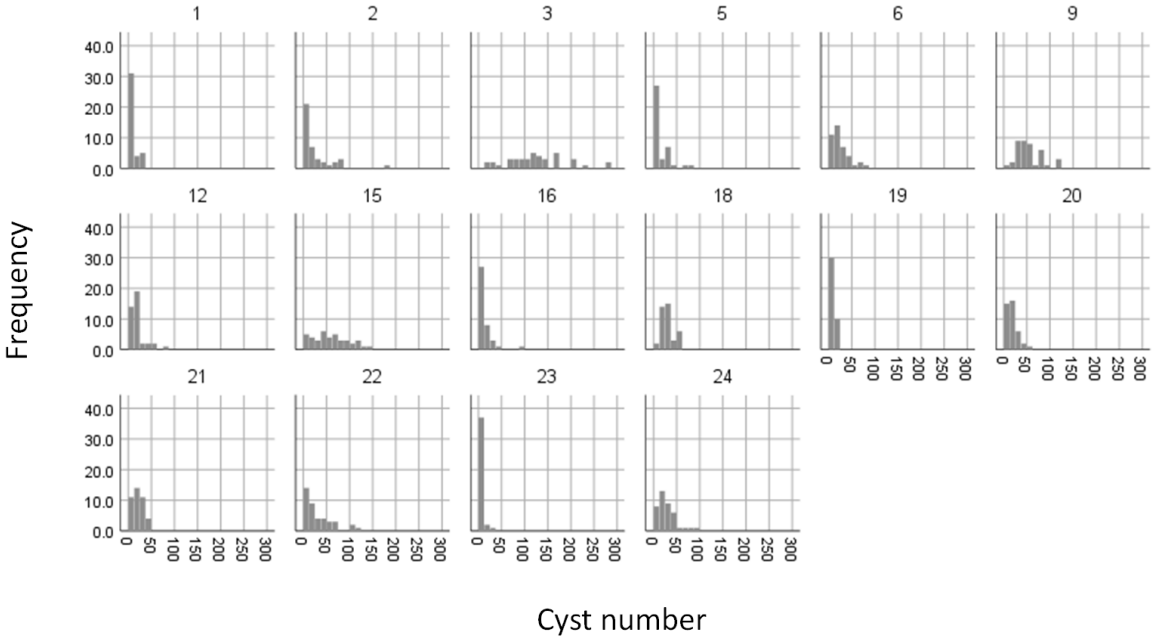


Figure 3.28. Frequency histograms for abundance by sampling site within the whole area study.

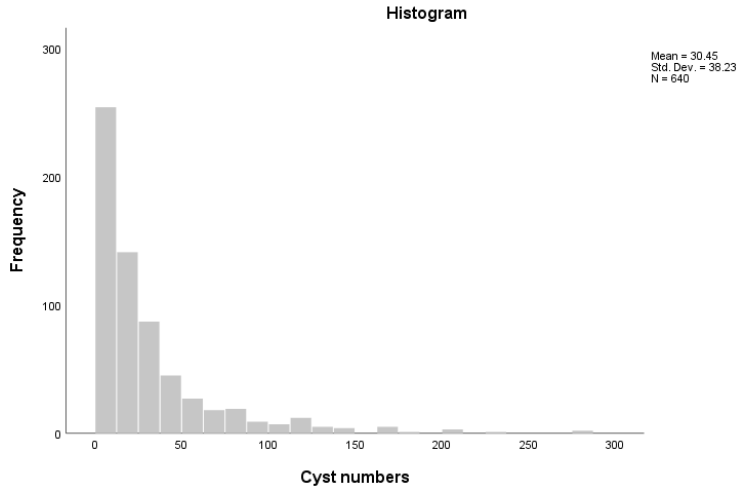


Figure 3.29. Frequency histogram for abundance using pooled samples within the whole area study.

The count data of metacercarial cysts was plotted on the map to reveal the pattern of spatial distribution, as for the local scale study. The same three methods used in the local scale study were applied again, namely “mean cyst number per barnacle”, “Lloyd’s mean crowding”, and “total metacercarial numbers per 50 cm^2” (see Figures 3.30A-C). The fourth index, weighted Lloyd’s patchiness index, was applied to reveal the “patchiness” under the influence of host density (see Figure 3.30D). Unlike what was found in the local scale study, *e.g.*, a slight decreasing trend of metacercarial numbers towards the sea, no obvious patterns

could be immediately observed by these three methods in the whole area study (i.e., the simple linear regression for the four parameters were non-significant, data not shown). What could be confirmed was the heterogeneous nature of metacercarial distribution in real space, as revealed by all three methods. Also, the patterns of distribution were not the same when comparing the four calculation methods. The highest number at a certain site by the first method would not necessarily be the highest by the second method, and *vice versa*.

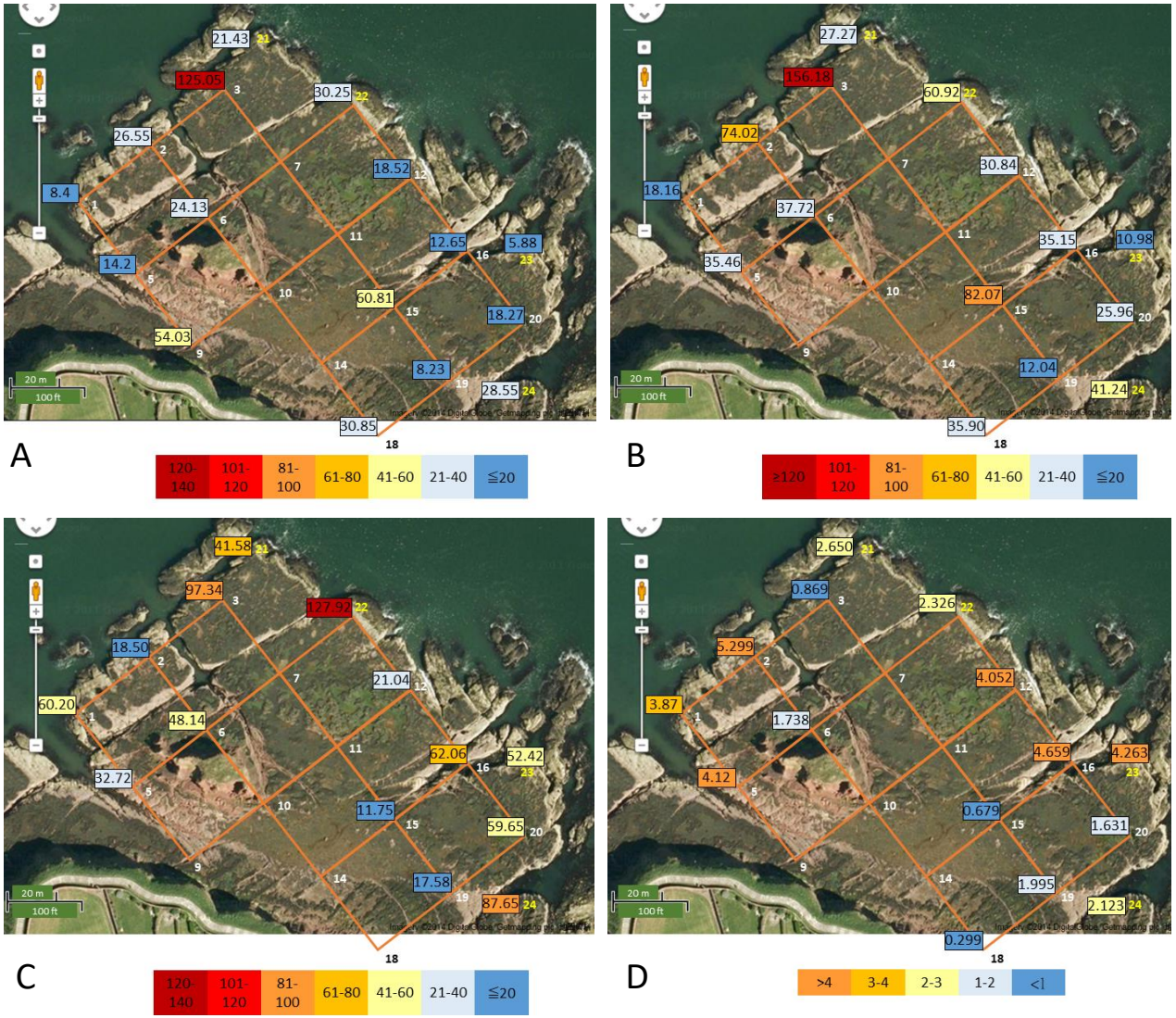


Figure 3.30. Spatial distribution of metacercariae in the whole area study. The numbers are shown in different colours that reflect the numeric scale provided in the legend. A. Mean cyst number per barnacle (m) at each site. Forty barnacles were counted for each site. B. Lloyds mean crowding (m*). This parameter was calculated as m + ([V_m/m]-1), where V_m= variance of the 40 barnacles at each site. C. Estimated total metacercarial numbers at each site per 50 cm^2 (unit: 1000 metacercariae). This value was obtained by multiplying the mean number of metacercariae per barnacle with the total number of barnacles per quadrant. The total barnacle number per quadrant was obtained by multiplying the mean barnacle density per cell with the numbers of cells covered by barnacles within a quadrant. Mean barnacle density per cell was estimated; it was obtained by calculating the average barnacle number of 5 cells where possible. D. Weighted Lloyd's patchiness index (P_w). The index was calculated as P_w= P x d. Lloyd's patchiness index (P) was generated by the following equation. P= m*/m. Barnacle density on the sampled rock (d) is obtained by dividing the total number of barnacles on each rock, by rock area.

3.3.4.2 Data exploration of metacercarial numbers and operculum length for the whole area study

Differences in abundance between the sites was detected by a one-way ANOVA using transformed data ((x+0.5)^0.25, one-way ANOVA: F = 44.68, p<0.001). A *post hoc* analysis (Tamhane test) showed that all sites were significantly different from others (data not shown). The mean plot of abundance at each site was plotted using raw data (Figure 3.31), and this revealed a highly fluctuated profile of mean abundance among sites.

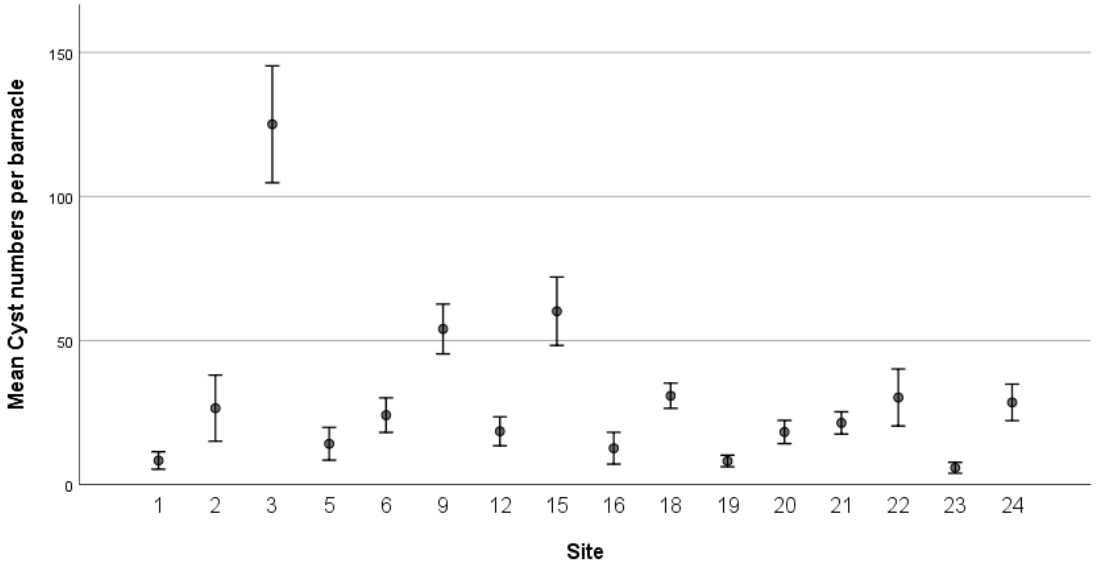


Figure 3.31. Mean plot for abundance by site within the whole area study. Error bars indicating 95% confidence interval.

Similar analyses to those conducted for the local scale study were performed for the host index operculum length. The descriptive statistics for the maximum operculum length are shown in Appendix 26. From the results of the local scale study, it was already established that the raw data of the operculum length was normally distributed, this being supported by skewness and kurtosis values (see Appendix 26). Histograms of the raw data showed a normal distribution (Figure 3.32). The mean operculum length was quite variable by sites as shown in the mean plot (Figure 3.33). By the mean plots, it was noted that the profile of maximum operculum length among sites was not the same as the abundance. Significant differences between sites were demonstrated using a one-way ANOVA (one-way ANOVA: F = 46.264, p<0.001). *Post hoc* analysis (Tamhane test) showed that each site was significantly different from at least one other site (data not shown).

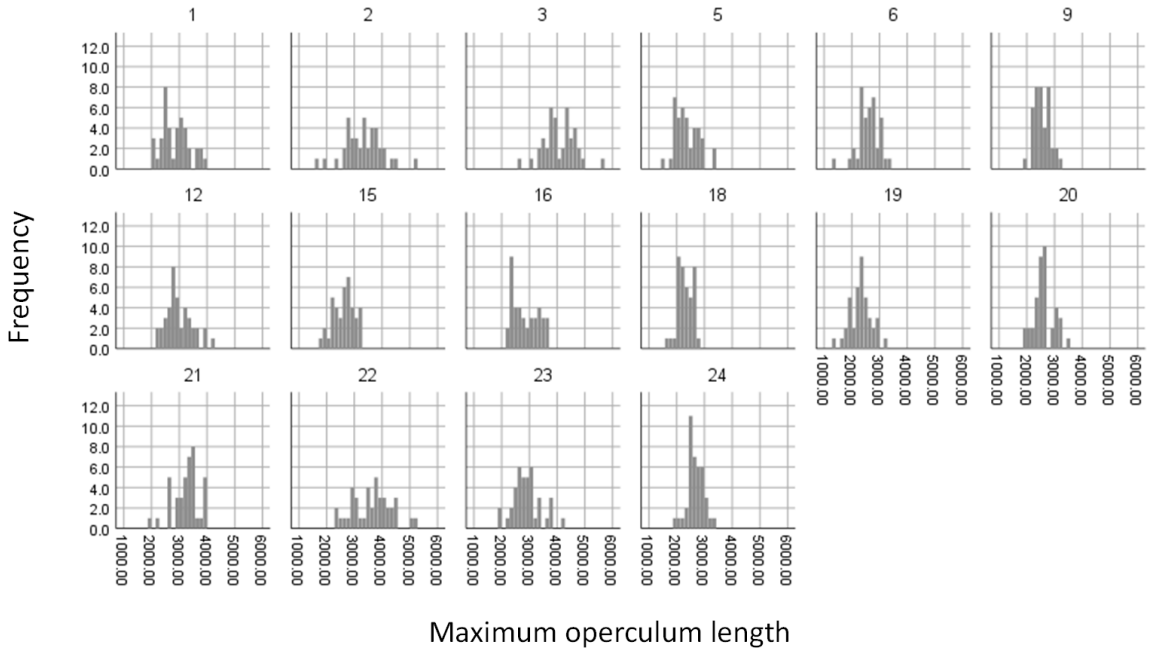


Figure 3.32. Frequency histogram of the maximum operculum length by site within the whole area study.

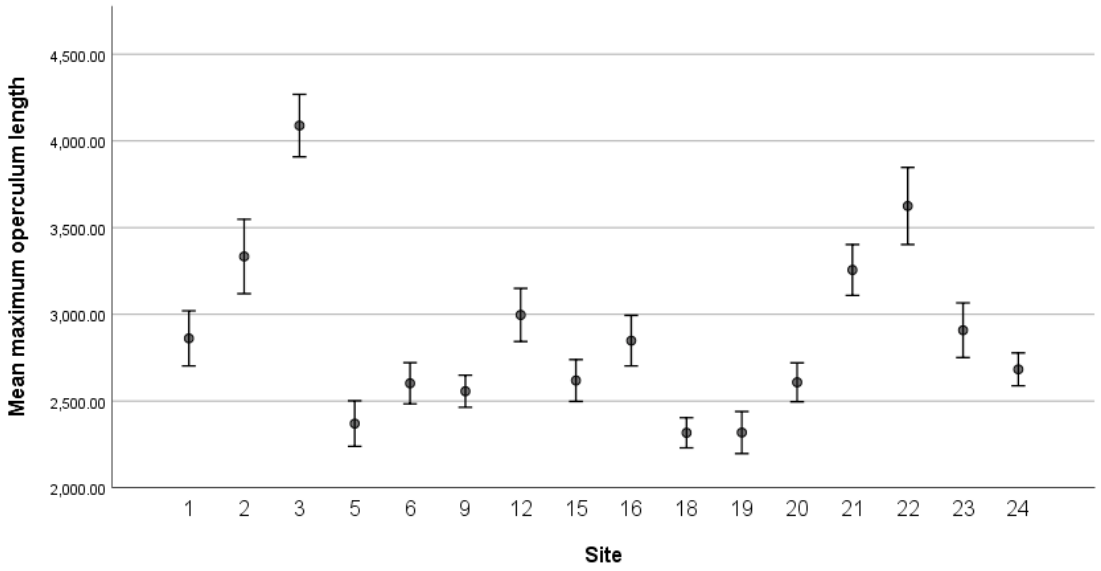


Figure 3.33. Mean plot of the maximum operculum length by site within the whole area study. Error bars indicating 95% confidence interval.

The relationship between host size (maximum operculum length, log) and cyst number (log) was studied by scatter plots. Using pooled data from each site, a positive relationship between the two variables was found (Figure 3.34). A simple linear regression of the maximum operculum length (LOG) can explain 23% of the variance for abundance (LOG) (R^2 = 0.23, F = 190.11, p<0.001). A moderate correlation between the maximum operculum length and cyst

number was revealed using X^0.25 transformed data (n = 640, Pearson correlation = 0.516, p<0.001). When examined by site, the relationship between the log host size and log cyst number (Figure 3.35), positive relationships were also consistent, however, the slope of the line of best fit at each site was different. This difference indicated a heterogeneous relationship between host size and abundance among sites.

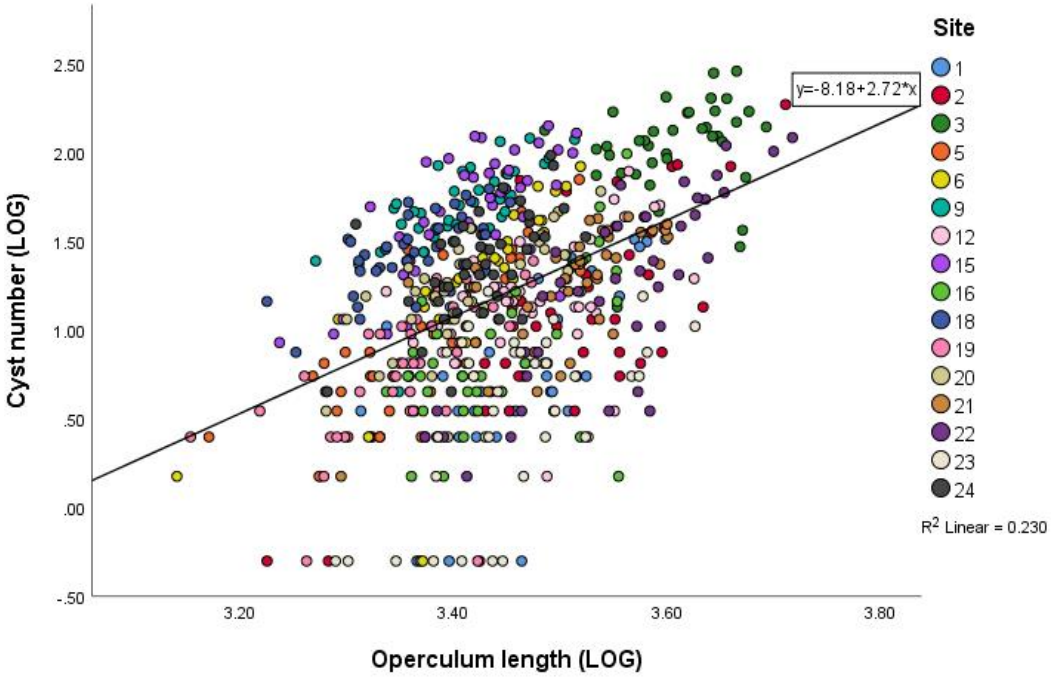


Figure 3.34. Scatter plot of the maximum operculum length (log) vs. cyst numbers (log) for the whole area study. A line of best fit was added to show the correlation.

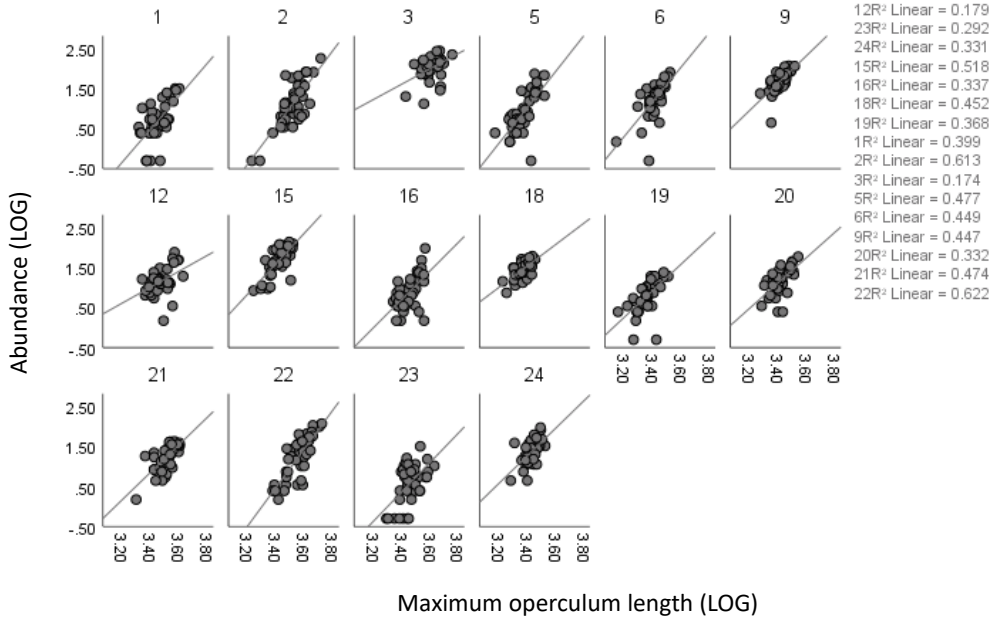


Figure 3.35. Scatter plots of the maximum operculum length versus mean abundance (x^0.25) by site within the whole area study. Lines of best fit show the correlations.

A similar analysis by a negative binominal regression as what was conducted in the local scale study was performed for the abundance data for the whole area study to investigate the possible fit of this model and to find an optimal k value as well. By using raw data of the maximum operculum length and site rank as the predictor for abundance, a model with a dispersion coefficient of 0.741 was produced with a reasonable goodness of fit (deviance = 1.226, df = 635).

3.3.4.3 Operculum length as a co-variable

A general linear model (GLM) was applied to investigate the relationship between the site rank, host size and abundance, using operculum length as a co-variable. Between-subjects effect for operculum length was examined, the dependent variable was cyst numbers (X^0.25 transformed), and source of variance included: site rank, operculum length and site rank * operculum length. By testing for between subject effects (Table 3.6), significant effects for site rank, maximum operculum length and the interaction between operculum and site rank were detected. The model explained 73.8% of the variability of the transformed abundance (R square = 0.738). The estimated marginal mean for size corrected abundance at each site rank was calculated (see Appendix 27). The profile plot for estimated marginal mean using maximum operculum length as a co-variant revealed no decreasing trend in cyst numbers with larger site rank values (*i.e.*, closer to the sea, a simple linear regression was non-significant, data not shown) (Fig. 3.36).

Table 3.6. Tests of Between-Subjects Effects for abundance (x^0.25 transformed) in the whole area study using operculum length as a co-variable

Dependent Variable: Abundance (X^0.25 transformed)					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	184.573[a]	31	5.954	55.241	<0.001****
Intercept	0.845	1	0.845	7.843	0.005**
Site rank	4.257	15	0.284	2.633	0.001***
Operculum length	42.245	1	42.245	391.952	<0.001****
Site rank* Operculum length	3.974	15	0.265	2.458	0.002***
Error	65.531	608	0.108		
Total	3065.746	640			
Corrected Total	250.104	639			

a. R Squared = 0.738 (Adjusted R Squared = 0.725)
Level of significance: <0.05*, <0.01**, <0.005***, <0.001****

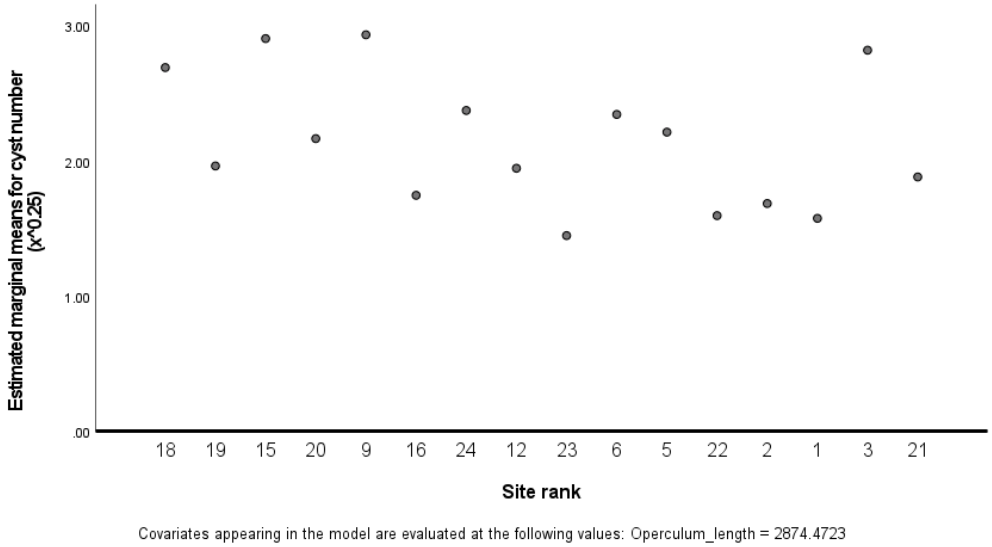


Figure 3.36. Estimated marginal means of cyst numbers (X^0.25 transformed) vs. site rank in the whole area study.

3.3.4.4 The effect of host density

To further examine the effect of host density, the mean number of barnacles per cm^2 on the sampled rock at each site was calculated and plotted against the mean parasite abundance per site or site rank. The results revealed that, host density was negatively correlated with parasite abundance (Figure 3.37), and positively correlated with the distance from land to the shore (Figure 3.38).

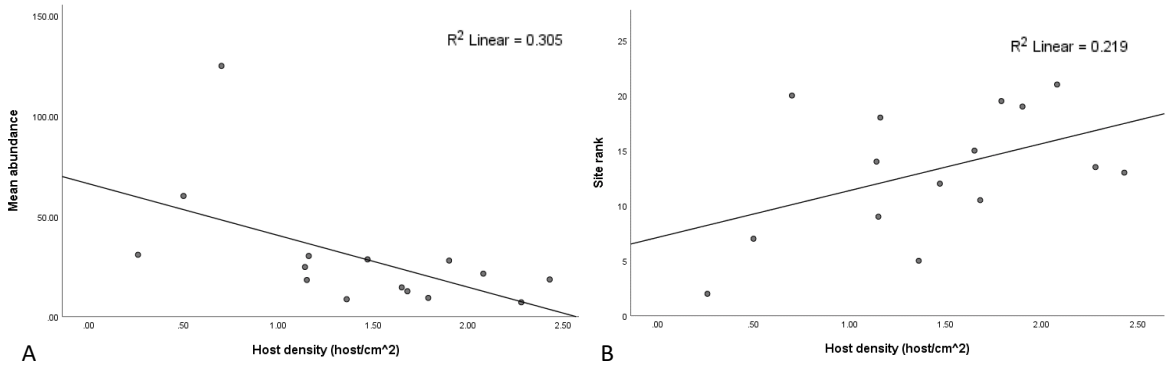


Figure 3.37. A. Scatter plot for host density (no. barnacle/cm^2 on the sampled rock) versus mean abundance at each site for whole area study. B. Scatter plot for host density (no. barnacle per cm^2 on the sampled rock) versus site rank in the whole area study.

3.3.4.5 Multiple regressions for host and environmental factors and cyst numbers

A multiple regression was applied, and more factors were considered. More host or environmental factors were added to the model, such as operculum length, cirrus length and width, mollusc coverage and species, algae and barnacle coverage, and cyst density on the sampled rock. Data were not available for all these factors from all sample sites, as a result, only sites 1, 3, 9, 12, 15, 18 were included in the analysis. The relationship between cirrus length and width was studied first. A scatter plot was produced for 29 samples, which revealed a non-linear pattern between cirrus length and width (Figure 3.38). A Pearson correlation showed a moderate value, 0.387 (p=0.038), indicating a moderate correlation between these two parameters. Cirrus length and width, although correlated, are all retained in the following multiple regression analysis.

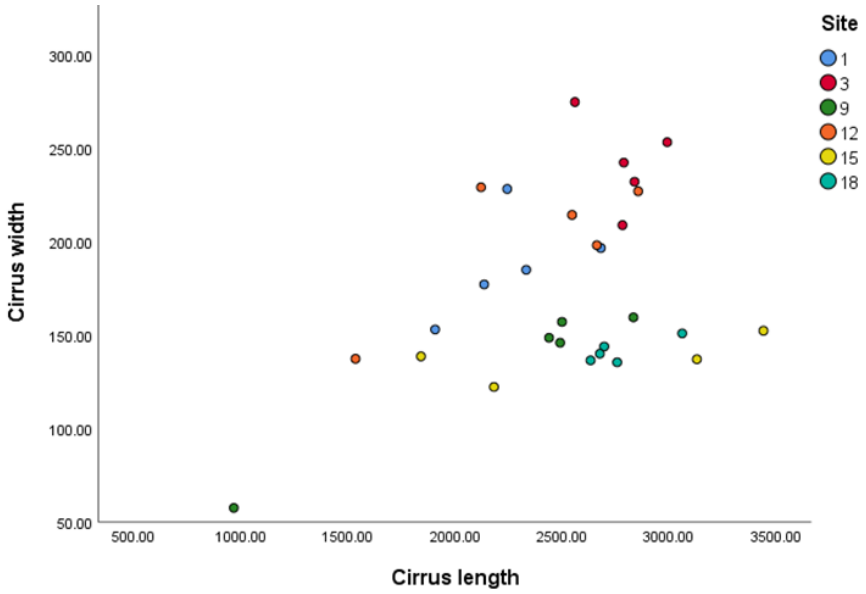


Figure 3.38. Scatter plot of cirrus length and width (n=29) in the whole area study.

The definition of all the factors and the methods for obtaining these parameters is shown in Table 3.1 and in Figures 3.4 - 3.6 and 3.11. Table 3.7 shows the descriptive statistics for all the variables that were included. After obtaining data for these parameters, multiple regressions were applied to abundance (X^0.25 transformed). Correlations of each variable were examined first (see Appendix 28). Although the sample size is small, some interesting correlations were revealed. Abundance correlated with operculum length, which was not a surprise; it was, however, also highly negatively correlated with barnacle coverage, and moderately positive correlated with cyst density on the sampled rock. Operculum length was highly correlated with cirrus width but not with cirrus length, and moderately correlated with

cirrus width / length ratio. This might suggest that in this case, cirrus width when compared to cirrus length, is more appropriate to reflect barnacle size. Operculum length was found to be negatively correlated with mollusc coverage and species. The possible explanation is that the mollusc in this case is a space competitor for barnacles (*e.g.*, limpet), or that the presence of molluscs simply reflects the fact that more algae (also a space competitor for barnacles) was covering the sampling site. Cirrus length was negatively correlated with cirrus width / length ratio, and barnacle coverage. Cirrus width, on the contrary, positively correlated with cirrus width / length ratio, but negatively correlated with mollusc coverage and species. Cirrus width / length ratio was highly negatively correlated with mollusc coverage and species. Mollusc species was completely correlated with mollusc species, which will be a problem of collinearity, therefore, in the following analysis, one or both of the variables were excluded.

Table 3.7. Descriptive statistics for parameters included in the multiple regressions for abundance in the whole area study

	Mean	Std. deviation	N
Cyst numbers	54.54	41.88	24
Operculum length	3311.19	701.99	24
Cirrus length	2559.61	445.99	24
Cirrus width	184.04	45.68	24
Cirrus W/L ratio	0.07	0.02	24
Mollusc coverage	1.38	0.50	24
Mollusc species	1.38	0.50	24
Algae coverage	40.83	19.32	24
Barnacle coverage	27.71	25.62	24
Cyst density on sampled rock	37.29	29.24	24

Enter, stepwise, forward, and backward methods of regression were tested, and the results are summarized (see Appendix 29). All the models tested produced a good value of Durbin-Watson test (1.5-2.5, representing model fit), and the ANOVA was highly significant (p<0.001). For all the models that were tested, a reasonable frequency histogram of regression standardized residual and a fairly linear P-P plot for regression standardized residuals were produced. All the scatter plots for regression standardized predicted values versus regression standardized residuals did not show a pattern. These residual analyses suggest that the model analysed were all statistically acceptable (data not shown). After considering the relationship between the independent variables, the included and excluded

variables, and the R square and F values of each model, the full variable enter model was selected as the best model. The multiple regression function of the enter model can be expressed as:

Cyst number (x+0.5)^0.25 = 0.001 operculum length + 0.001 cirrus length -0.016 cirrus width

Or expressed as:

Cyst number = (0.001 operculum length + 0.001 cirrus length -0.016 cirrus width)^4 -0.5

3.3.5 Comparison of infection indices and degree of aggregation of digenean infection in barnacle populations between environmental gradient and habitat types

3.3.5.1 Relationship between prevalence and abundance

The relationship of prevalence and abundance was investigated by combining data from the whole area and the local scale studies for Dunbar Red Rock. The results show a positive but non-linear relationship between log abundance and prevalence (Figure 3.39), the positive relationship complied with the abundance-occupancy relationship described by (Morand & Guégan, 2000).

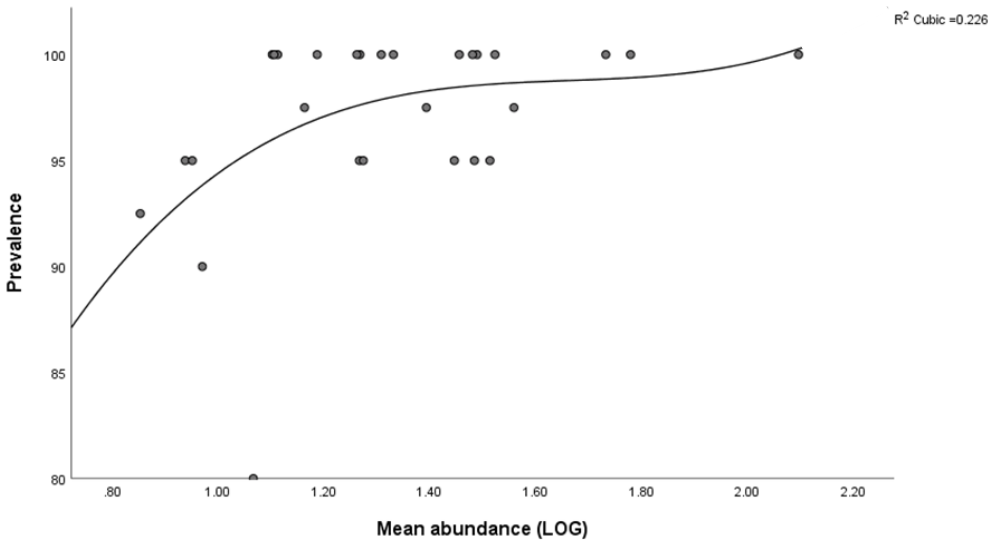


Figure 3.39. Scatter plot of the log mean abundance versus prevalence combining data from the whole area study and local scale study.

3.3.5.2 Comparison between latitude, locality type, and scale

The results are summarised in Table 3.8. The prevalence of the three localities at the different latitudes are similar and all reaching 100%. The abundance and intensity between

localities, however, are significantly different when compared by ANOVA using x^0.25 transformed data (for abundance, F = 18.467, df = 759, p<0.001; for intensity, F = 21.431, df = 739, p<0.001). A *post hoc* analysis (Tamhane) found that each locality was different from the others. The abundance and intensity both increased towards lower latitudes. Variance-to-mean ratio also increased towards lower latitudes, negative binomial k, in a contrast, decreased towards lower latitudes.

The two types of habitats at Dunbar, i.e., a rocky platform and a sheltered rocky bay, show quite distinct patterns. The prevalence on the rocky platform was almost twice higher than that in the sheltered bay. The differences in abundance and intensity were even more dramatic (i.e., an independent sample T test using x^0.25 transformed data, for abundance, t = 30.087, df = 888.259, p<0.001, two-tailed; for intensity, t = 19.025, df = 360.938, p<0.001, two-tailed), and both parameters were higher on the rocky platform. For the aggregation indices, variance-to-mean ratio was also higher on the rocky platform; however, the negative binomial k was lower in the sheltered bay, indicating a higher degree of overdispersion in this type of habitat.

When comparing the two sampling scales at Dunbar Red Rock, the prevalence between the whole area and the local scale study was not significantly different (Pearson chi-square = 1.495, p = 0.221, two-sided). The abundance and intensity between the whole area and the local scale studies were shown to be significantly different (i.e., by independent 2-sampled T test using x^2.5 transformed data, for abundance, t = 4.686, df = 1083.03, p<0.001, two-tailed; for intensity, t = 4.557, df = 1050.31, p<0.001, two-tailed). The abundance and intensity in the whole area study was higher than that in the local scale study. The variance-to-mean ratio was also higher in the whole area study; the negative binomial k, in a contrast to this though was higher at the local scale.

Finally, several comparisons were made between mean abundance, Lloyd's mean crowding, variance-to-mean ratio, Lloyd's patchiness index and host density among sites in the local scale and the whole area scale (Appendix 30).

Table 3.8. Comparison between infection and aggregation indices by latitude, habitat types and sampling scale.

Comparison	Locality	Sample size	Prevalence	Abundance			Intensity		Variance-to mean ratio D	Negative binomial *k*	Lloyd's mean crowding
				Mean	S.D.	Variance	Mean	S.D.			
Between different latitudes	Rosehearty (higher latitude)	60	98.33	7.72	4.48	20.037	7.85	4.40	2.595	4.84	6.43
	Stonehaven (medium latitude)	60	96.67	17.58	15.68	245.874	18.19	15.60	13.986	1.35	14.34
	Dunbar Red Rock (lower latitude)	640	97.34	30.45	38.23	1461.500	31.28	38.41	47.997	0.65	47.64
Between locality types	Dunbar Red Rock (rocky platform)	640	97.34	30.45	38.23	1461.500	31.28	38.41	47.997	0.65	47.64
	Dunbar Leisure Pool (sheltered Rocky Bay)	300	47.00	2.47	5.44	29.621	5.25	6.97	11.992	0.22	11.22
Between sampling scales	Dunbar Red Rock (whole area)	640	97.34	30.45	38.23	1461.500	31.28	38.41	47.997	0.65	47.64
	Dunbar Red Rock (local scale)	480	96.04	21.01	23.70	561.411	21.86	23.78	26.721	0.82	26.54

3.3.5.3 Fitness of Taylor's Power Law

It would be interesting to examine the fitness of Taylor's Power Law (Taylor 1978) for the target parasite-host system. According to Taylor's equation: Log (s^2) = Log a + b Log X, where s^2 = variance and X = mean number of parasites per organism examined (= mean abundance). In the present study, if plotting Log (mean abundance) and Log (variance) of each site and trying to get a regression line, would generate plots like those shown in Figures 3.40 and 3.41. A positive, although not completely linear relationship, can be seen in the scatter plot (Figure 3.40), and the cubic function provided the highest curve fit for the data set (R^2 = 0.894, p<0.001 for the local scale and 0.811, p<0.001 for the whole area study). The slope of the linear regression line is 1.69 for data from the local scale study and 1.51 for the whole area study, which means that the intensified effect of abundance upon variance is slightly stronger at the local scale. The variance-to-mean ratio was summarized at the local and whole area scale in Appendix 24, which revealed that each sample site had a variance-to-mean ratio >1, indicating an aggregated distribution regardless of scale. Furthermore, variance-to mean ratio can be applied to examine the degree of aggregation. Therefore, the whole area scale study has a higher value of variance-to-mean ratio (48.00) when compared to the local scale study (26.73), indicating a higher degree of aggregation.

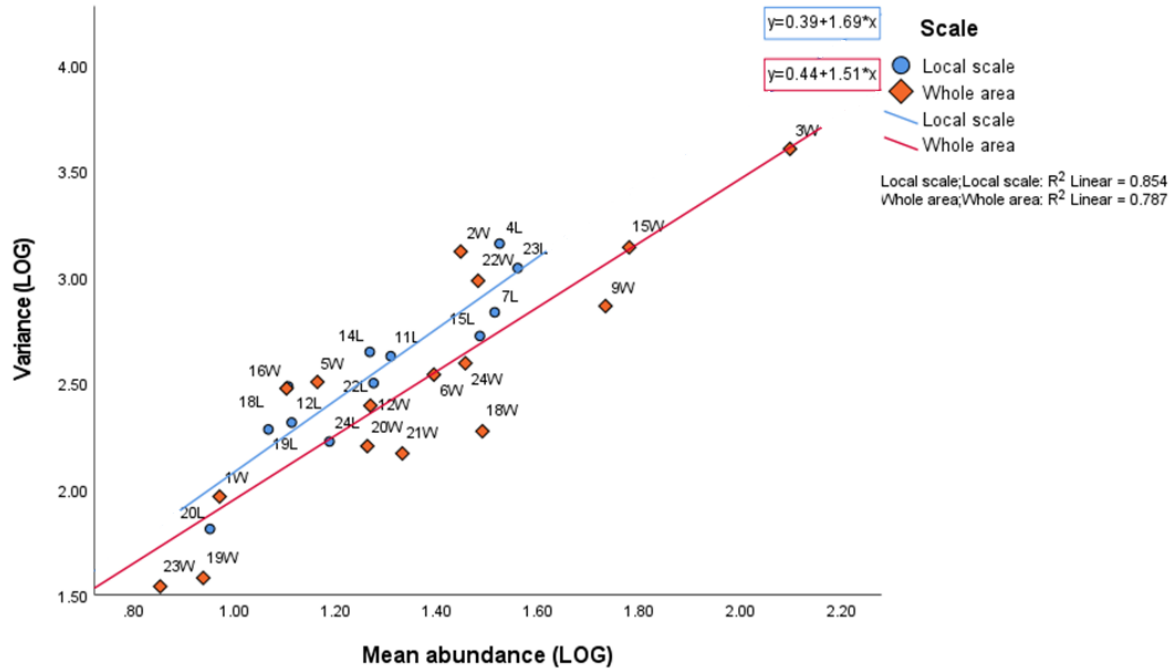


Figure 3.40. Scatter plot for Log 10 mean abundance to Log 10 variance in the local scale and whole area study with respective linear regression lines.

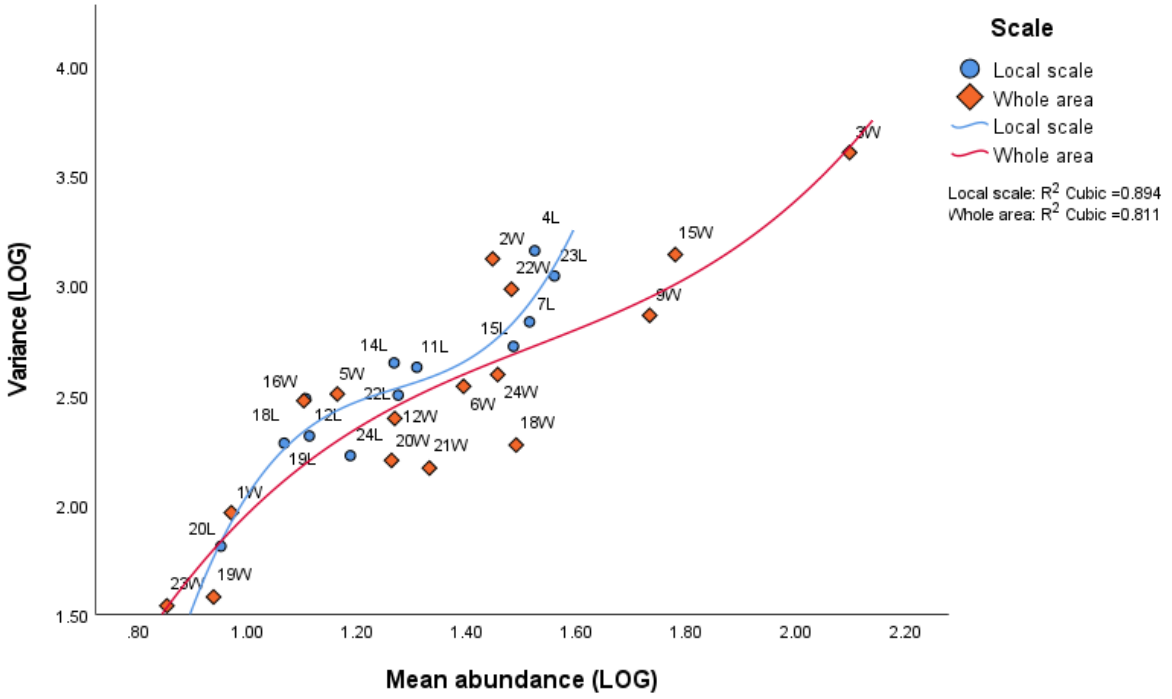


Figure 3.41. Scatter plot for Log 10 mean abundance to Log 10 variance at local scale and whole area study with respective cubic regression lines.

3.4 Discussion

In this chapter, the infection patterns, level of aggregation, and the potential factors (host and other environmental factors) contributing to *M. gratiosum* aggregation in the barnacle host populations, have been examined in a systematic way, allowing insights to be gained into several aspects of parasite ecology. The comparisons of infection patterns were made between latitude, locality type and scale. Three aspects, the infection parameters and patterns of aggregation, the observed spatial trend, and the source of heterogenous distribution, are discussed separately. Studies in parasitology which apply systematic sampling strategies are rare (but see Fredensborg *et al*., 2006; Thieltages, 2007; Thieltages & Reise, 2007; Hartson *et al*., 2011; Fong, 2015). The present study is believed to be the first distribution study applying a systematic sampling strategy for *M. gratiosum* populations in its barnacle host *Semibalanus balanoides*.

3.4.1 Infection parameters and the patterns of aggregation

3.4.1.1 Relationship between abundance and prevalence

A positive but non-linear relationship between log mean abundance and prevalence was identified at the study locality (Dunbar Red Rock, see Figure 3.39). This positive relationship was described in several empirical works (Morand & Guégan, 2000; Calvete *et al.,* 2004; Thieltges *et al.,* 2009; Pérez-del-Olmo *et al.*, 2011), covering different geographical scales and taxa of helminths and

hosts, and can be referred to the abundance-occupancy relationships which was well described in the free-living organism (Gaston *et al*., 2000; Morand & Guégan, 2000; Pérez-del-Olmo *et al.*, 2011). Abundance-occupancy relationships includes two main phenomena. The first one is commonly recognized intra-specifically, that an increase in abundance for a species is often accompanied by an increase in the number of sites in which it is resident. The second one is recognized inter-specifically, that widespread species tend to be abundant, while rare species have a narrower distribution. These phenomena have impacts on several areas, such as conservation, invasion, and biodiversity studies (Gaston *et al*., 2000). Do parasite distributions follow the same rule? The answer may not always be positive. For example, Fong (2015) found that and increase in density and aggregation of the host barnacle *Chthamalus fissus*, did not result in an increase in the prevalence of its isopod parasite *Hemioniscus balani*. This kind of finding highlights the uniqueness of parasite ecology. In the following discussion, several infection patterns discovered in the present study will be addressed from the perspective of parasite ecology.

3.4.1.2 Impact of latitude on parasitic infection

In the present study, an increase in abundance and intensity and their variance towards lower latitudes was observed, despite prevalence being very similar in the three studied localities (see Table 3.8). In parallel to this, the variance to mean ratio also increased towards lower latitudes, in congruence with a decrease in negative binomial k values. These trends maybe represent genuine biological patterns, *i.e*., *M. gratiosum* or its hosts adapt better in habitats at 56 degrees north latitude than at 57 degrees. The major determinant is believed to be climate, i.e., total time covered by ice and sea temperature. The observed trends can also be biased by other factors. For example, although the three localities are all rocky shore habitats, the observed trend did not account for host size or other environmental factors, as these data were not available in Rosehearty and Stonehaven. The bias from sample size is perhaps not negligible either, as the sample size in Dunbar Red Rock was 10 times higher than at the other two localities, and this fact may be accounted for the observed higher variance in Dunbar Red Rock. When comparing the species richness, comparatively few empirical or theoretical studies focus on the abundance of organisms along latitude gradients (Johnson & Hass, 2021). Latitude gradients in biodiversity are a well-known pattern in free living taxa, where diversity increases towards lower latitudes (Willig *et al*., 2003). Species richness of parasite communities, however, do not always follow the same rule (Poulin & Leung, 2011; van der Mescht *et al*., 2018; Fecchio *et al*., 2019; Preisser, 2019; Cuevas *et al*., 2020; Johnson & Hass, 2021). For example, Poulin and Leung (2011) revealed that the taxonomic composition of helminth communities varied by host type and latitude, the variation is, however, complicated and without consistency. They found that the proportion of digeneans in helminth communities in bird and mammals increased towards higher latitudes. Johnson and Hass

(2021) also found a reversed trend for latitude-diversity-gradients in helminth parasites of lentic-breeding amphibians. There are many factors that need to be considered when analysing parasite abundance and latitude gradients, for example, host size, host density, host types, habitat types, land-cover diversity, etc., as these factors are not isolated from latitude and are often correlated with each other (Johnson & Hass, 2021). While in the present study attempt was made to include more factors, however, data was not available and therefore the results were not analyzed further. Furthermore, the above-mentioned studies are often global- or continent-scaled meta-analyses, while the present study represents a small scale, local study focusing on a single host species. The finding in the present study, however, might be unique for this host-parasite system; and a larger scale study covering wider latitude ranges, higher sample numbers, and more environmental factors, should be able to clarify the observed trend.

3.4.1.3 Impact of locality characters for parasitic infection

In the present study, the high abundance of the data from Dunbar in the preliminary survey (see Chapter 2) further supports the observation that Dunbar is a "hotspot" for the target parasite, the distribution was spatially heterogeneous and the infection parameters of the two localities (Red Rock and Leisure Pool) differed substantially. When examining the infection parameters closely (i.e., abundance and its variance, see Table 3.8), it was noticed that the increase of mean abundance and variance between the two habitat types is not proportional, and this was reflected in the higher variance-to-mean ratio at Red Rock. The negative binomial k, however, is lower at the Leisure Pool, which shows a different trend compared to the variance-to-mean ratio. Due to the vicinity of the two localities, environmental variables such as temperature and salinity cannot differ substantially. Therefore, other environmental or host factors might contribute to the observed differences, *e.g.*, local terrain, exposure, or host fauna. The Leisure Pool is a breeding site of kittiwakes (a potential final host) and can provide a new source of *M. gratiosum* annually. It is speculated that at the Leisure Pool the observed infection patterns reflected an early establishment of the *M. gratiosum* life-cycle, while at Red Rock the observed patterns were the results of long-term accumulations. The terrain of a rocky platform at Red Rock may provide an optimal environment for long-term survival for both host and parasite species, while a cliff terrain at the Leisure Pool may result in a higher turnover rate for both host and parasite species. A general lower abundance and intensity while higher aggregation parameters (lower k in this case) at the Leisure Pool may support this theory. More specifically, lower k value might reflect a pulsed release resulting in clumped infections of barnacle hosts at the early stage.

The influence of habitat characters to parasite diversity is a subject for conservation studies, as habitat fragmentation and habitat types are often correlative to parasite prevalence, abundance, and

species richness (Laurance *et al.*, 2013; Francová & Ondračková, 2014; Morand & Bordes, 2015; Kiene *et al*., 2021), as which were observed in the present study. The characters of habitat can be determinants for parasite ecology. For example, the fragility of heteroxenous parasites to habitat fragmentation were demonstrated (Kiene *et al*., 2020). Furthermore, habitat types were proven to be correlative to genetic structures in different populations of *S. japonicum* (Rudge *et al*., 2009). In the present study, habitat type seems to be a determinant for parasite dispersal patterns. The casual relationships of parasite diversity regarding habitat types can be complex and are often multi-factorial and interactive between host and parasite. For example, in the case of *S. japonicum* (Rudge *et al*., 2009), it has been proven that the difference of major reservoir hosts in marshland and hilly regions contributed to the observed genetic patterns. While in a rodent-helminth parasite study, host habitat specialization was applied to explain parasite species richness, and this parasite diversity was suggested to drive host traits, such as immune investment and sexual size dimorphism (Morand & Bordes, 2015). Base on the same principals, it is possible that different genetic structures can be identified in both *M. gratiosum* and *S. balanoides* populations from the two habitat types studied in the present study, but this still need to be validated. In a study by Thieltges *et al.* (2009) that compiled large datasets representing 85 host-parasite pairs of crustacean and bivalves; prevalence, intensity, and abundance were found to possess repeatability in host-parasite species pairs; especially, the repeatability of intensity and abundance of infection in crustacean hosts was strong. These infection parameters were considered by the authors to be a character of a given host-parasite species pair. In the present study, such predictability was detected between samples from Dunbar and South Queensferry in 2013 and 2013 (see Chapter 2, Section 2.3.9.). Are these infection parameters representing intrinsic characters for Red Rock and Leisure Pool? It is likely, but the best way to prove it is to perform a long-term, follow-up study. Studies on the effect of habitat characters on parasite abundance are rare, such as that of Francová and Ondračková (2014). Furthermore, a study for a single parasite species within a small scale, as in the present study is lacking, and might reveal more subtle parasitological phenomena and warrant further investigation in the future.

3.4.1.4 Impact of scale for parasitic infection

The difference of abundance and intensity between local and whole area scale, although significant, was not large. It is reasonable to expect seeing higher variance and variance-to-mean ratio at the whole area scale, as the sample size is larger, and the sampling extent is wider at this scale. The negative binomial k, however, is lower in the whole area scale, indicating a higher degree of overdispersion at this scale. This can be explained by the inclusion of more extreme values in the whole area study. But the observed patterns can also reflect true differences in the infection patterns, for

example, the size of the barnacle hosts were smaller at the local scale (see Appendix 22 and 26), therefore this might contribute partially to the observed differences.

Observation scales can influence ecological inference, for examples, inference for the population mean and variance and other spatial statistic parameters are dependent on the size and shape of sampling units, distance lag between sampling sites and extent of sampling. In spatial statistical analysis, the scale is defined as "extent", which refers to the total length, area or volume that is observed, analysed or that exists (Dungan *et al.*, 2002). Thieltges and Reise (2007) found conspicuous spatial heterogeneity in the metacercarial distribution over a distance of 50 km in a muddy flat habitat. Moreover, these authors mentioned that the effect of host density on metacercariae distribution seems to act on a very small scale (50 cm), and they point out the effect of small-scale aggregation of the host (cockles) is important. At a larger scale, this effect seems to be overridden by the stronger effect of the density of the upstream host. In contrast to this, Fredensborg *et al*. (2006) found that on a large scale (across 12 bays), the relationship between digenean prevalence in molluscan hosts and the abundance of avian hosts was positive, while on a small scale (i.e., at different tidal heights), this relationship disappeared. Studies conducted at different scales are actually looking at different phenomena. For example, Calvete *et al.* (2004) revealed that under the local scale, the parasite community of the investigated avian host showed low spatial repeatability and predictability, and they suggested that the lack of spatial repeatability is common. While Pérez-del-Olmo *et al.* (2011) found that the prevalence, mean abundance, mean intensity, and variance-to-mean relationship of a marine fish-parasite system, although they varied substantially among sites, they were predictable in terms of taxonomic categories, host specificity, regional distribution patterns, and the mode of transmission. To capture the desired biological phenomenon, the extent of the study area must be large enough to reveal spatial patterns, but not too large to dilute the local patterns (Dungan *et al.*, 2002). Also, the sampling should be balanced, that ideally covers the whole range of sizes, age, sexes, reproductive states, and populations of the host (Wilson *et al.*, 2002). For example, these authors demonstrated that the age-related decrease in sample sizes should be corrected for to allow proper inferences to be made from the data. In the present study, the focus was on the locality of Dunbar and the sampling area covered by "Red Rock", with the chosen area being shown to be appropriate in terms of the fact that this scale was able to reveal heterogeneity and patterns of distribution of the target parasite. The chosen distance lag was justified in terms of the movement ability of the first intermediate host and the cercarial stage of the target parasite but not the host, because the host is a sessile species. The sampling procedure is balanced, by fixing numbers of host per site, covering different age groups by randomized collection, and accounting for host population density. Therefore, the results should

nominally be capable of revealing the source of heterogeneity of metacercarial abundance in the barnacle population from the rocky shore ecosystem.

Further factor relevance to scale effects may come from the dynamics of the host population of the studied system. In the report of Jenkins *et al*. (2000), the variation in settlement (unmetamorphosed cyprids) and recruitment (metamorphosed individuals) of *S. balanoides* populations was detected at different spatial scales, namely localities (100s of kilometres), shore (1000s of metres) and site (10s of metres). They found that the settlement period varied in length and time among locations. The patterns of settlement and recruitment showed substantial variations among locations. Furthermore, recruitment showed significant variation between the lower shore scale and site scale. In a similar study conducted by Jenkins *et al.* (2001), they found that locations contributed most variability to the population parameters of *S. balanoides*. However, within a locality, it is the smaller "site" scale rather than the shore scale, which contributed most to the greater variability. The smallest scale "replicate" within the site (<0.5m), however, accounted for little variability. This study shows the importance of scale when studying the population dynamics of acorn barnacles. Therefore, the distribution of the digenean population among barnacle populations can covary with host population and can be reflected on infection parameters at different scale, as what was found in the present study.

3.4.1.5 Patterns of aggregations and the fitness of Taylor's Power Law

By examining the pattern of count data of metacercarial cysts per barnacle (abundance), an aggregated distribution of metacercarial cysts in barnacle populations was confirmed, regardless of latitude, habitat types, and scale of the study. This was proven by the right-skewed shape of the histogram of the raw data, the variance-to-mean ratio, the negative binomial k, and the Lloyd's mean crowding. Phenomenon of aggregation was observed in one research using the same parasite-host system as the present study and can be directly comparable with. In the research of Carrol *et al.* (1990), the dispersion of *M. arenaria* (syn. *M. gratiosum*) in barnacle populations was found to be aggregated by variance-to-mean ratio when the data from 17 localities along the coastline of the Ards Peninsula and Belfast Lough in Northern Ireland was analysed. They found that "localities" and "variation within samples" contributed to most of the variance of the dispersion patterns observed. Here the influence of "scale" was identified again. "Between localities" represented a source of variance from the larger scale and "within samples" represented the source of variance from the small scale. They also found that the dispersion pattern did not fit either a Poisson or a negative binomial distribution.

In the present study it was noticed that the relationship of variance to the mean was cubic rather than linear when compared to that described by Carrol *et al.* (1990). From the results of the present

study, it seems that the slope of the log variance to log mean for abundance is maintained until the mean approaches a value equal to log 1.0 (local scale) and 1.2 (whole area), it then plateaus briefly and then starts to increase again at a mean value around log 1.25 (local scale) and 1.45 (whole area), and then finally plateaus again. This means that for a certain narrow range, the distribution is closer to random than aggregated. Furthermore, this pattern, although not obvious, can be identified when focusing on the local scale and the whole area scale separately (see Figure 3.47) or when examining pooled data (data not shown). This difference of variance-to-mean relationship between Carrol *et al.*'s (1990) study and the present study, might originate from the scale between the two studies. In the study of Carrol *et al*. (1990), the distance between localities must be substantially larger than in the present study, because the scale in the present study ranged from 160 x 120 m to 50 x 15 m only. The biological meaning of this cubic curve is still obscure, and might result from bias in the sampling method or from a too small sample size. However, the most likely explanation of the cubic curve in the present study is that at the finer scale in the studied system, it is the local interaction between host and parasite that is being characterised, and this pattern is not linear. It was speculated that the probability of contracting this digenean infection is not equal at this scale. At certain ranges of abundance (mean ≈ 12.8-30.5 for the local scale and, 12.7-30.9 for the whole area study), aggregation is not obvious, but below or beyond this range, aggregation intensified. As prevalence is often positively correlated with abundance (Shaw & Dobson, 1995; Behnke *et al.,* 2008; McVinish & Lester, 2020), it is possible that for a certain range, the infection strength is higher and contributes to a more even distribution of parasites in host populations. Below or beyond that range, infection strength decreases and this decrease leads to a higher level of overdispersion, therefore parasites tend to concentrate in a smaller number of individuals. This range of abundance might reflect age, size, parasite resistance by the host, a particular parasite behaviour, extrinsic factors, or a combination of these, which will ultimately create an ideal environment for transmission. The "infection strength" described here might be similar to the "achievement factor" defined by Crofton (1971b), which is a composite parameter combining reproductive rate of the parasite and their potential for infection.

The aggregation of parasites among hosts is recognized as one of the biological "laws" (Poulin, 2007; Gourbiére *et al.,* 2015). Although there are several parameters that can be applied to measure aggregation (Wilson *et al*., 2002; McVinsh & Lester, 2020), variance-to-mean ratio is applied widely. Examples include those of Pennycuick (1971) for three species of parasites in three-spined sticklebacks, Carrol *et al.* (1990) for *M. gratiosum* in barnacles, that of Repullés-Albelda *et al*. (2013) for *Z. seriolae* in greater amberjack, and that of Kamdem *et al*. (2012) for anopheline mosquitoes. It is also applied in the numerous theoretical works (Crofton, 1971a,b; Anderson & May, 1978; Wilson *et al*., 2002; Poulin, 2007, 2013a; Gourbiére *et al*., 2015; McVinish & Lester, 2020). The variance-to-mean ratio was derived

from Taylor's model for the density-dependent spatial behaviour in animals (Taylor *et al*., 1978). The hypothesis of this model is that spatial variance is proportional to a fractional power of mean population density; this has been validated in 174 sets of data from diverse free-living taxa (Taylor *et al*., 1978). The authors concluded that spatial behaviour is density-dependent in most species and true randomness is biologically rare. The spacing between an individual and its neighbours is governed by the behaviour of the organism, which reflects fundamental biological processes. Although this model was built using data from free-living animals, the patterns of variance versus the mean observed in the present study conformed to what had been observed for other taxa by Taylor *et al*. (1978). McVinish and Lester (2020) suggested that this parameter is a measurement of departure from a Poisson dispersion, and the assumption of a host-parasite model that fits the Poisson dispersion is: 1) that encounters between the host-parasite interaction follow a Poisson process, 2) that no parasite reproduces within the host, and, 3) that the parasite dies at a density-dependent rate. Any departure from the Poisson dispersion suggests the violation of one or more of these assumptions. Under this condition, it was suggested that the encounter and /or death rate in the studied digenean-barnacle system does not follow the Poisson process, because no metacercariae reproduce in the barnacles. Since most parasite distribution data follows a negative binomial distribution (Shaw *et al*., 1998), its exponent (negative binomial k) is also useful to characterise the degree of overdispersion of the parasite population (Wilson *et al*., 2002; Poulin, 2006). This parameter was less intuitive than variance-to mean ration, therefore had been applied less in empirical works (Pennycuick, 1971; Shaw *et al*., 1998; Brooker *et al.*, 2006). The usefulness of this parameter in modelling, however, makes it popular in theoretical works (see Crofton, 1971; Anderson & May, 1978; May & Anderson, 1978; Wilson *et al.*, 1996; Alexander *et al*., 2000; Pullan *et al*., 2012; Collyer & Anderson, 2021). It was interesting to note that the variance-to-mean ratio and negative binomial k in the present study mostly shown a reversed relationship (table 3.8), while variance-to-mean ratio and Lloyd's mean crowding shown a similar but not the same pattern (see Appendix 30) in the present study. Both variance-to-mean ratio and negative binominal k can be a good indicator for parasite aggregation (also see the comparison by Poulin, 2006, Figure 6.4). The discrepancy between variance-to-mean ratio and Lloyd's mean crowding was considered to be the result of mathematical calculation, furthermore, they have different biological meaning either.

The level of parasite aggregation can have significant ecological relevance. Poulin (2013) stated that the difference in exposure to parasite and susceptibility for host are the main factors determining aggregation in parasitism. From the perspective of the parasite, aggregation can promote competition and mate encounter; furthermore, the crowding effect is reflected on parasite morphology, fecundity, sex ratio, effective population size, and genetic diversity (Poulin, 2006). Relationship between

morphology and crowding will be explored further in chapter four. From the perspective of the host, the degree of aggregation is also related to the dynamics of the host population shaped by parasite-induced mortality (Stanko *et al*., 2006), and this had been quantified in theoretical work. In the theoretical work of Crofton (1971b), this was proven in a simulation model that, when the negative binomial k is between 1-3, parasitism can exert a highly significant effect and a continual control on the host population; whereas when k <1, the parasitism can be regarded as relatively harmless. Gourbiére *et al.* (2015), pointed out that although a negative binomial distribution is important for interpreting aggregations in data, the goodness of fit to this distribution does not provide information about the cause of aggregation. This will be discussed further in the following sections.

3.4.2 The spatial trend of distribution

3.4.2.1 The observed trend

In the present study, a decreasing trend in abundance towards the sea was observed at both the local scale and in the whole area study. This finding agreed with the findings of the previous distribution studies for *M. gratiosum,* however, the uniqueness of the habitat in Dunbar Red Rock has been addressed here.

In the local scale but not the whole area scale study, a slight but significant trend (simple linear regression, $R^2 = 0.558$, $p = 0.005$, used host size corrected values) of a decreasing abundance towards the sea indicates that local factors influence the accumulation of metacercariae within barnacles, and these factors are relative to the distance between the sample site and the shore. A similar but not the same trend was observed by Carrol *et al.* (1990) in their study conducted on the coast of County Down, N. Ireland. They found that basal length increased towards low tide but the operculum length fluctuated, and that the height on the shore can explain more than 50% of the variability seen in abundance, with the central part of the barnacle zone bearing the highest abundance. The results in their study may reveal different phenomena when compared to the present study. First, the sample sites (*i.e.*, 8 sites at 0.5 m increments for a total length of ~4 m, from the high limit of the barnacle zone to low tide) cover the highest limit of barnacle distribution, whereas in the present study, the highest limit of the barnacle zone may not be reached and the total length of the study area (i.e., ~50m) is much longer. Second, the local terrain was not mentioned in their study. In the present study, however, this trend was observed both within a gully (i.e., at the local scale) and across a rocky platform (i.e., at the whole area scale), which means that it is the tidal level but not the "slope" between the upper and lower shores contributing to the decreasing trend (*e.g.*, the trend is more obvious across different tidal

levels). Barnacle size can be considered as a co-variable that co-varies with the tidal level, therefore contributing to part of the variability in both studies. This was proven after correcting for the effect of host size, after which this trend was still apparent (*i.e.*, in the present study). In the study of Irwin and Irwin (1980), only two levels, *i.e.*, the upper shore and lower shore, were examined, and barnacles on the lower shore had a lower prevalence but a similar abundance to those on the upper shore. The same trend was found in England, but in this case the difference was more extreme, such that no infection was established in barnacles on the lower shore (Mitchell, 1984).

3.4.2.2 Possible explanations for the observed trend

The reasons for this decreasing trend toward the sea in the digenean-barnacle system can be complicated. Carrol *et al.* (1990) suggested that the higher numbers of the first intermediate host (*L. saxatilis*) along the upper shore and the faster growth rate and lower survival rates of barnacles along the lower shore, both contributed to this trend. Irwin and Irwin (1980) found that the existence of the principal final host, gulls in this case, affected the prevalence and abundance at different localities. They also suggested that the upper shore was exposed for longer periods in terms of visiting avian hosts, and had more abundant *L. saxatilis*; these two factors were both suggested to contribute to the higher prevalence found in the upper shore. The real causal relationship only can be proven experimentally. For example, in the study of bivalve-metacercarial systems (Thieltges, 2007), the effect of tidal level and the density of the upstream host was investigated experimentally. The author found that the infection level at the mid-tide or the low tide was species-dependent, and the upstream host density was proven by field experiments to be the major determinant of observed infection levels. The author therefore suggested that the observed difference in the infection levels between tidal levels reflected a difference in the population density of upstream hosts. Whether the same principle applied to the present study is not known, because the habitat in Thieltges's (2007) work was a mud flat. Based on the above studies, however, most researchers suggested that the influence of the upstream or downstream host is the major determinant for the observed patterns.

In the present study, the local distribution of *L. saxatilis* or avian hosts at Red Rock was unknown, but the effect of barnacle population dynamics on digenean distribution was addressed. It was found that host density increased toward the shore (Figure 3.38) and was negatively correlated with parasite abundance (Figure 3.37), and adding a host density factor can intensify the Lloyd's patchiness index (see appendix 30). These findings suggested that host density should be included in the analysis of parasite distribution in the digenean-barnacle system. In the study of Jenkins *et al*. (2008), significantly higher recruitment of *S. balanoides* on the low shore compared to the high shore was detected at some sites. Calcagno *et al*. (1998) found that the mortality of the recruited barnacle decreased with age.

Johnson *et al*. (1998) found that the probability of encounter with a barnacle above medium size always increased with distance from a crevice, and the odds ratio gradient for encountering a barnacle above medium size is significantly greater in high shore crevices than in low shore crevices. The authors assumed that mortality caused by whelk predation is the most important factor contributing these observed patterns. These findings suggested that complex factors affect the turnover rate for barnacle populations across different tidal levels. As new *S. balanoides* recruits will not have parasites and older settled barnacles have more time to accumulate metacercariae, a higher prevalence / abundance could also be a contributing factor to a different turnover rate of barnacle populations relative to their position on the shore.

On the other hand, the distribution of parthenita in mollusc hosts can provide extra information explaining the observed patterns of parasite distribution. For example, the prevalence of *M. similis* in *L. saxatilis* in the same coastal area was found to be highest along the lower intertidal zone, and was found to decrease towards the upper intertidal zone. This pattern was suggested to be related to the distribution of the final host, the eider duck *Somateria mollissima* Linnaeus, 1758 (see Galaktionov & Dobrovolskij, 2003). The significance of *M. similis* parthenita distribution in mollusc hosts relative to the distribution of metacercariae in the second intermediate host is not clear. Currently the distribution pattern of parthenita of *M. gratiosum* in *Littorina* spp. is still unknown. It would be interesting to know more details of this pattern for the sampling locality. Furthermore, the distribution and behaviour of cercariae are likely to be a critical factor in determining the distribution of metacercariae, *e.g.*, their swimming behaviour (Haas, 1992; Rea & Irwin, 1992) or in their patterns of releasing cercaria (Thieltges *et al.,* 2008; Koprivnikar & Poulin, 2009; Prinz *et al.,* 2010). Wilson *et al.* (2002) mentioned that parasite aggregation does not act in isolation, and it is the result of the dynamic between parasite and host factors. Particularly, parasite virulence can have a regulatory effect on host populations, therefore, affecting host density. This can be related to the k value of negative binomial distributions; for example, when k increases and the parasites become more evenly distributed in the host population, then parasite-induced host mortality also increases, and host density declines. In the present study, it is supposed that the same principle also applied, but the degree of influence was unknown because the virulence of *M. gratiosum* toward *S. balanoides* is also unknown. If the virulence *M. gratiosum* toward *S. balanoides* is very low, an extreme aggregation can be expected because metacercariae can continually accumulate without killing the host. This dynamic between parasite and host might be partially reflected on the cubic curve of variance-to-mean ratio, for example, the interval showing a steadier slope might reflect an equilibrium between host and parasite populations.

3.4.2.3 Host patchiness and digenean distribution

In the present study, four methods were applied for producing digenean distribution maps at Dunbar Red Rock, and each has a unique biological meaning. The first method, plotting mean abundance in 40 barnacles at each site, is one of the most intuitive types of presentation. The second method, plotting Lloyd's mean crowding value of these 40 barnacles at each site, reveals the degree of intra-specific competition at each site. Whether these 40 barnacles were representative for the chosen site, however, is not known. Therefore, a third method was applied. By plotting the total number of metacercariae in the sample area (*i.e.*, the 50 cm^2 quadrant) per site, this more closely reflected the numbers of parasites available to infect final hosts. This value may be closer to the infection risk for final hosts at a particular site in real life. The trend of decrease disappears when the data are presented as the total metacercariae per site, which suggested that host populations play a role in dispersion of parasite populations. The fourth method which can only be applied to the whole area study, is to plot weighted Lloyd's Patchiness Index per site. Lloyd's Patchiness Index in its original form is used to present how individuals are dispersed in the quadrat. When the value is greater than 1, then the dispersion is aggregated. When the value is equal to 1, then the dispersion is random, and when the value is smaller than 1, then the dispersion is over-dispersed (Wade, 2019). In the present study the influence of the local host population was included by adding a "weight" (i.e., host density) to this index.

Comparing mean abundance and Lloyd's mean crowding, we find that the two parameters show a similar trend (see Appendix 30), while Lloyd's mean crowding was always larger than mean abundance. This finding was congruent with the finding of Reiczigel *et al*. (2005) on simulated data. On the other hand, Lloyd's Patchiness index showed no similar trend with the variance-to-mean ratio (see Appendix 30) at both the local and whole area scale. Furthermore, we can find that all sites had a variance-to-mean ratio and Lloyd's patchiness index greater than 1, indicating an aggregated distribution. Adding a "weight" to Lloyd's patchiness index intensifies the original index, and by doing so, the variation increased and some sites had a value less than 1 (Appendix 30). A decision of how to apply a "weight" is a difficult one. It is not clear whether host density will have a positive or negative influence on the studied system, although a negative relationship between host density and abundance was found in the present study (Figure 3.37).

In parasitology, the importance of host density on infection level has seldom been mentioned (but see Thieltges & Reise, 2007; Buck *et al*., 2017). A high host density can have both positive and negative impact on parasitic infection at the same time. In the work of Thieltges and Reise (2007) looking at a bivalve-metacercariae system, this factor was studied by a field experiment, and it was found that cockles from high host density plots had a lower infection intensity than those from low host density plots. Buck *et al*. (2017) found that an increase in molluscan host density in a patch can

result in an increase in the density of digenean infected molluscs, but a decrease in the overall prevalence in the mollusc population. The discovered patterns suggested that there is a balance between an elevation in transmission rate and a dilution effect that comes from host patchiness. The characters of aggregated distribution might provide a further explanation. In a flea-mammalian host study (Stanko *et al*., 2006), the authors found that flea abundance or prevalence or both, decreased with an increase in host abundance in major data sets (i.e., 34/57 sets). Furthermore, there was only a decreasing trend (i.e., in 8 data sets) or no identified trend in flea mean crowding versus host abundance in the 57 sets of data, and the relationship was asymptotic in half of the data sets with a decreasing trend. On the other hand, in most data sets a positive relationship was found between flea mean crowding and flea abundance. The revealed patterns were explained by the authors as relating to a differential reproduction rate between host and parasites. The authors also mentioned the importance of parasite-induced host mortality or host-induced parasite mortality on the observed patterns, but these appear to be inferences rather than evidence proven conclusions. In the present study, the weighted parameter, host density on the sampled rock, was decided to have an additive impact on mean crowding, and so was plus rather than divided by Lloyd's patchiness index. This decision assumed that *S. balanoides* can tolerate *M. gratiosum* to a very high degree, therefore including more hosts will result in an increase with more extreme values of abundance and variance, which will therefore result in an increase in mean crowding and patchiness index.

Although Lloyd's mean crowding has been cited more than 250 times since its appearance (Wade, 2019), its use has been ignored by parasitologists in general (Reiczigel *et al*., 2005). This index was originally designed for spatial ecology, and several researchers have pointed out some interesting properties of this index in its application in parasitology (Wilson *et al*., 2002; Reiczigel *et al*., 2005; Poulin, 2006). Reiczigel *et al*. (2005) mentioned that mean intensity can roughly predict mean crowding, but an increase in mean intensity will not necessarily lead to an increase, and sometimes even a decrease in mean crowding. Thus Reiczigel *et al*. (2005) suggested that these two parameters should be analyzed separately. More importantly, Lloyd's mean crowding index includes variance-to-mean ratio and negative binomial k at the same time, and therefore this can have a wider biological application and can be extended to community-level studies. A more recent survey applied an infra-community crowding index, which was based on the concept of Lloyd's mean crowding, as a measure of degree of interaction in ruminant parasite communities (Ferrari *et al*, 2016). In this research, by applying a quantifiable tool based on a degree of interactivity, the study was able to reveal that the degree of interaction is dynamic and sensitive to host factors.

Host patchiness can explain parasite spatial distribution patterns. Shaw *et al*. (1998) highlighted that the aggregation level of the host should be considered when analysing parasite aggregation.

Brooker *et al*. (2006) revealed that parasitic infection patterns in human populations at both the small and large scales differed between urban (i.e., high population density) and rural (i.e., low population density) areas. Hartson *et al*. (2011) investigated the effect of land use and wetland types and spatial distribution on amphibian parasite abundance. They found that parasite infection parameters and diversity were strongly influenced by variables at the watershed and regional scales. The authors suggested the observed trend is relative to the abundance of the intermediate host in certain habitat types and to the vicinity of the Mississippi Flyway highlighting the importance of habitat alteration in parasite ecology. Other examples relative to habitat fragmentations are discussed in Section 3.1.4.3, while the effects of host abundance and parasite distribution and crowding have been discussed by Stanko *et al*. (2006). Besides the apparent effect on parasite distribution, host patchiness can influence parasite genetic diversity and evolution. Poulin (2006) suggested that a decrease in the relative number of hosts occupied can increase the risk of extinction in local parasite populations, and that an elevation in the degree of aggregation and the resulting host mortality can increase the risk of loss of major parasite populations. Aggregated distribution also means that only a small proportion of the parasite population can enter final hosts and complete their life-cycle. Furthermore, parasite morphology often covaries with crowding (Reiczigel *et al*., 2005), the decrease in body size due to crowding can influence parasite fecundity, and only a small proportion of the parasites can be responsible for reproduction output (Poulin, 2006). These notions bring up the unique influence on parasite genetic diversity regarding the mode of transmission relative to parasite aggregation and host patchiness. Cornell *et al*. (2003) revealed that a characteristic aggregated transmission (i.e., bursts of infective larvae released by a host denoted by the authors), can explain the spread of rare recessive gene in sheep-nematode systems. Keeney *et al*. (2007) found that a lower number of digenean genotypes found in molluscan 1st hosts compared to those found in crab 2nd intermediate host, were markedly different. Interestingly, they also found that most crabs were infected by small numbers of cercariae from multiple sources, occasionally there were crabs that harboured large numbers of cercariae from a single mollusc. These findings indicate that a combination of spatial distribution and parasite genetic research can be an interesting and serves as highly promising topics for further future research. The spatial distribution patterns discovered in the present study provide an immediate foundation for further future work.

3.4.3 The source of heterogenous distribution and the multiple regression model

In the present field-based study, the potential factors contributing to the observed abundance were examined by multiple regressions incorporating different host and environmental factors. In the multiple regression, using the full variables enter method, a regression function was developed, provided a good prediction as it explained 89.8% of variance in the studied parasite-host system. The

variables that were included were: operculum length, cirrus length and cirrus width. Host size and exposure were also identified as important factors for metacercarial abundance in the studied system. This result partially echoes the suggestion mentioned above that the influence of the tide can be important at this scale. Host size was included as a fundamental contributor to abundance. Location on the shore (presented as site rank), although showing a mild influence on abundance, was not significant in the whole area study, therefore, this was not included. The potential effect of exposure was incorporated in terms of cirrus length and width of the barnacle. Higher exposure was considered to have a negative effect for abundance because stronger waves can flush the cercariae away. The competition effect of neighbouring non-host taxa was estimated by the percentage covering of algae and the mollusc host. This competition effect reflects on barnacle density / coverage and, therefore, has an indirect negative effect for digenean abundance. However, mollusc hosts, which could be the source of cercariae, may also be contributors to higher abundance. Finally, as mentioned above, metacercarial density on the sampled rock, which could reflect the degree of infection success, was incorporated in the model as well.

Although observational or theoretical studies can reveal patterns of aggregation, they do not answer a fundamental question of why parasites aggregate. Poulin (2013) suggested that most of the observed aggregation (variance of abundance) can be explained by the abundance itself. This statement, however, seems to be a chicken-and-egg conundrum. In empirical works, researchers have investigated the heterogeneity of parasite distributions at different levels, for example, at the community level (Mouritsen & Poulin, 2002; Calvete *et al.,* 2004; Thieltges *et al.,* 2009; Pérez-del-Olmo *et al.,* 2011; Kanarek & Zaleśny, 2013), at the infracommunity level (Calvete *et al.,* 2004; Behnke *et al.,* 2008), and / or the individual species level (Irwin & Irwin, 1980; Carrol *et al.,* 1990; Sari & Malek, 2000; Pung *et al.,* 2002; Thieltges, 2007; Thieltges & Reise, 2007; Prinz *et al.,* 2010; Repullés-Albelda *et al.*, 2013; Parietti *et al.,* 2015; Pérez-Chi *et al.,* 2015; and, the present study). According to the scale of the study and the system examined, different variables contributing to the observed patterns have been identified and therefore should be chosen accordingly. For example, the position on the shore and host size was suggested as important factors for infection parameters in the barnacle-digenean system (Irwin & Irwin, 1980; Carrol *et al.,* 1990). In mobile crustacean systems, seasons, host size, water temperature, dissolved oxygen, salinity were found to be related to different infection parameters (Pong *et al.,* 2002; Parietti *et al.,* 2015; Pérez-Chi *et al.,* 2015). In bivalve systems, the tidal level, the density of the upstream host, host size and small-scale physical properties were considered in the observed metacercarial infection patterns (Thieltges, 2007; Thieltges & Reise, 2007). For mammalian hosts, the site, age, and year were identified as important parameters affecting the community structure of parasites in bank voles (*Myodes glareolus* Schreber, 1780). In avian hosts, the host

population density, host body condition, habitat types, seasons and age were identified as important factors for parasite community structures (Calvete *et al.,* 2004; Kanarek & Zaleśny, 2013). The decision to include the parameters listed in the above-mentioned studies seems to be based on prerequisite knowledge of the chosen host-parasite systems. Theoretical works emphasize more on testing / simulating the suggested models, and are therefore, are also based on prerequisite knowledge of biological processes. For example, Crofton (1971b) developed a model of host-parasite relationships by incorporating several important parameters for a host-parasite system, *i.e.*, the negative binomial k, the lethal level, the transmission rate of the parasite, and host density. Gourbiére *et al.* (2015) developed a mathematical framework for the distribution of parasites in hosts by breaking down the infection process in to two parts: the exposure of the host to the parasite and the infection success of the parasites. Under this model, they found that aggregation decreased linearly with the number of encounters, but this depends non-linearly on parasite success. They also found that random variation can generate an aggregated distribution, encounter and success are not equal filters, and that host heterogeneity can produce asymmetric effects on encounter and success.

When larger data were available in multivariate analysis, e.g., big data analysis, it seems to be better to include all available data without prior assumption. Due to the constraint of time and resource, the process of data collection is always assumption-based. This will have an impact on data analysis afterward. Although there has been some criticism regarding the use of stepwise regression as a technique due to the biasing effect of using a large number of explanatory variables (Smith, 2018), this methodology appears justified in the present study. In the present study, enter, forward, backward, and stepwise methods were applied and compared, and the best method was chosen based on the percentage of the variance explained and the analysis of residuals. Furthermore, the inclusion / exclusion of variables was not entirely dependent on the default settings, but was also adjusted by the existing knowledge of the parasite-host system and by the correlation coefficient between the variables. Therefore, the results of the multiple regression can be considered relatively robust. Host size and exposure were identified as the major determinants in the multiple regression model and will be discussed further in the following sections.

3.4.3.1 Host size and age

In the present study, larger sized barnacles were found to have a higher abundance. Operculum length was applied as a proxy for barnacle size. There was, however, only moderate correlation between barnacle size and abundance, and in the GLM model including size and sites as the variables, with only 39.3% (operculum length) of the variance in abundance explained. In the report of Carrol *et al.* (1990), abundance was found to be positively related to host size, but abundance related less closely

to host size at sites of low prevalence than sites with high prevalence. This could be explained by Taylor's model which suggested that in low abundance areas, the distribution is closer to random (Taylor *et al.*, 1978). Larger *S. balanoides* were found to have a higher prevalence and abundance of *M. gratiosum* in England (Mitchell & Dessi, 1984), and the same was seen for the *B. perforatus* - *M. gratiosum* system in Wales (Sari & Malek, 2000). The positive relationship between host size and parasite abundance has been demonstrated in other crustacean-digenean systems as well. The density of *M. turgidus* metacercariae increased with the length of heavily infected species *Palaemonetes pugio* Holthuis, 1949 but not the more lightly infected *P. vulgaris.* It was assumed that shrimp size increased with shrimp age, and that the positive relationship between host size and metacercarial density indicated that parasite density does not affect host survival (Pung *et al.,* 2002). A positive relationship between host size and parasite abundance could also be found in digenean species using molluscs as a second intermediate host. Prevalence and intensity of metacercariae of both *H. elongata* and *C. parvicaudata* increased with the size of their mussel hosts. For *C. parvicaudata*, however, numbers of young and old metacercariae were not affected by size, only mature metacercariae increased significantly in larger mussels. Notably, increasing the probability of infection was suggested to be associated with the higher pumping rate of larger mussels (Nikolaev *et al.*, 2006). In a contrast to this, in a Monogenea-teleost system, an opposite trend was observed. A significant negative association was found between the total length of fish hosts and the abundance of *Z. seriolae*. This was probably associated with the immune capabilities of younger fish and parasite-induced host mortality, where heavily infected fish were removed at early ages (Repullés-Albelda *et al.*, 2013). The negative correlation between host length and parasite abundance was also observed in the *Microcotyle sebastis* Goto, 1894 - rockfish system (Yoon *et al.*, 1997). The negative correlation between abundance and host age seems to be consistent in monogenean-fish systems. In terrestrial animals, age is also an important factor for helminth infection parameters, however, whether young animals or adult animals will have higher abundance or more diverse parasites is host-species and parasite-species dependent (Calvete *et al.,* 2004; Behnke *et al.*, 2008; Kanarek & Zaleśny, 2013).

Many reasons could contribute to the positive relationship between host size and parasite abundance in a barnacle-digenean system. The simplest situation is the accumulation of parasites with age, in the case that pathogenicity is low, and the space limit has not been reached. There is also the possibility of parasite induced host gigantism, as has been demonstrated in mollusc-parthenita systems (Galaktionov & Dobrovolskij, 2003), and this cannot be ruled out, and would need to be proven by use of laboratory experiments utilizing cultured barnacles and mollusc hosts. It is also possible that parasite-induced mortality occurred in younger / smaller barnacles, although this mortality cannot be observed on a single sampling. More tolerant individuals might grow larger and accumulate parasites.

This would also need to be proven experimentally and could be achieved by removal methods in the field utilized by other researchers (Jenkin *et al.*, 2008; Bracewell *et al.,* 2013). A theory has been proposed with regard to the density of metacercarial infra-populations and host age. According to Galaktionov and Dobrovolskij (2003), if the host growth is not accompanied by drastic changes in its biology, and the host does not pass to another biotope, and also, if the heavy infection does not cause host death, the number of metacercariae it harbours will increase with age. Their theory seems to fit the barnacle-digenean system well. Once the cypris larva has settled on a substrate and transformed to its adult morphology, the barnacle will just enlarge in size and will not move, the primary structural change will occur in its reproduction organs only (Zardus, 2012). Moreover, due to their sessile and gregarious nature, there are limited possibilities for growth, this being constrained by space competition from conspecifics. In respect of the latter, an exponential decline of growth rate with density was found by Jenkins *et al.* (2008). This might also explain why there is only a moderate correlation between barnacle size and parasite abundance. Infection with metacercariae does not seem to have an effect on a barnacle's health nor does a strong immune response occur. Another factor related to host size / age in terms of metacercarial accumulation could be the mode of cercarial penetration. It is already known that the entry of cercariae to the crustacean second intermediate host in microphallids is by active penetration (Irwin *et al.*, 1990). Two mechanisms of penetration of cercariae were suggested by Bartoli (1981). One is limited penetration, with the number of metacercariae increasing up to a limit and stabilising with host age, as all possible spaces are occupied. The second pattern is unlimited penetration, the number of metacercariae grows consistently throughout the host's life, in which penetrated cercariae leave the host and free space for new larvae. These two mechanisms could be switched as alternatives, according to the local ecosystem. In sheltered locations, where infection pressure is high, limited penetration dominates; while in exposed ecosystems where infection pressure is low, unlimited penetration is triggered. According to Bartoli's theory, in the present study, limited penetration could be present in a high prevalence locality such as Dunbar Red Rock, while unlimited penetration could be present in a lower prevalence locality such as Dunbar Leisure Pool. The probability of eliminating a metacercaria is unknown. No obvious signs of an immune reaction were detected in infected barnacles, and only occasionally were dead cysts surrounded by a layer of melanized tissue observed. Also, no obvious adverse effects on health and reproduction of barnacles could be found. The barnacles with heavy infections seem to be morphologically and functionally normal. As synchronised breeders, almost 100% gravid barnacles can be found in the breeding season despite the coexisting high prevalence and intensity of infection (personal observation).

3.4.3.2 Exposure and other extrinsic factors

Subtle environmental conditions such as tide level, exposure and food supply can have effects on post-settlement interactions as well. For example, Thieltges and Reise (2007) suggested that small-scale physical properties such as residual water at low tide in small pools can have an impact on the exposure time and cercarial release rate, thus contributing to parasite distribution heterogeneity. Barnacle behaviours might contribute to different infection levels. It is known that the beating rate of barnacles is affected by current types, size, age and temperature, and different types of beating that exist (Crisp & Southward, 1961; Anderson, 1981; Anderson & Southward, 1987). In extension feeding, the cirrus fan will stay still and be exposed for a long time. Also, in normal and fast beating, barnacles can create water currents which will carry the cercariae into proximity. Furthermore, the length cirrus is positively related to wave exposure (Arsenault *et al.*, 2001). As the entry point of cercariae of *M. gratiosum* is via the cirrus, then more actively beating barnacles or those that possess longer cirri might be more exposed to cercarial infection. A similar concept was suggested by Thieltges and Reise (2007) in a cockle-metacercariae system, where the higher filtration rate of larger bivalves resulted in a higher exposure to cercariae since they contract infection via the filtration current. Barnacles, as sessile suspension feeders, might be in some way like bivalve. Furthermore, from the parasite perspective, a rarely considered factor was brought up by Wilson *et al.* (2002), which is the possible feedback of recruitment produced by parasite themselves. If this mechanism does exist, then the infection event cannot be considered independent, and the resulting abundance will depart from a negative binomial distribution. This non-independence of infection was already observed in *S. mansoni* cercarial infection. Here, it was found that *S. mansoni* cercariae tend to penetrate its host in a group, and this tendency was due to a highly specific intraspecific chemical signal (Haas, 1992). More sophisticated research studies are needed to investigate the dynamic between host and parasite populations if the uncertainty mentioned above is to be confirmed.

Other factors, although excluded from the multiple regression model at the end, deserve a discussion. The dynamics of the host population alone can play a role on parasite aggregation. In the study of density-dependent mortality and growth of *S. balanoides* (see Jenkins *et al.*, 2008), it was found that patterns of adult abundance were dynamic and were maintained through the action of density-dependent mortality. The abundance of barnacles was suggested to be determined by two forces: first a positive cyprid-adult relationship under low recruitment conditions and second, a negative relationship under high recruitment conditions. The dynamics of host barnacle populations will certainly affect distribution patterns of parasite infra-populations contained. According to density-dependent mortality patterns, however, a single population of barnacles in a fixed area undergoes turnover through a period (turnover time might depend on the local environment) and so does the

parasite population resident inside. It is suggested that if other biotic or abiotic factors are fixed, simply the fluctuation of barnacle populations at certain sites will provide a limit to prevalence, abundance and intensity of the parasites contained. In addition, the heterogeneity of survival rates of older barnacles in the space can influence the heterogeneity of parasite abundance in that space.

Although mollusc species / coverage was not included in the final regression function, the importance of mollusc hosts to metacercarial distribution in the second intermediate host should not be overlooked. Behavioural factors will affect the distribution of molluscs. In the study of Davies and Knowles (2001) for *L. littorea – C. lingua* system, *L. littorea* was found to be able to detect and avoid the presence of cercariae and guano. The authors suggested that because aggregation is the natural behaviour of *L. littorea* and parasitized snails move slower and for shorter distances, the infected snails tend to scatter outside a flock of snails in a tidal pool. It can therefore be speculated that barnacles in open area and crevices are subject to different infection risks. Furthermore, the grazing area of *Littorina* spp. is markedly affected by the settlement of barnacles and macroalgae. In the present study, it was found in multiple regressions models that the area covered by molluscs and observed molluscs species was negatively correlated with barnacle coverage, cyst density on the sampled rock, and operculum length, cirrus width and cirrus width / length ratio. One interpretation could be that areas with more or larger barnacles will have less space for molluscs, while in more exposed areas where barnacles have shorter and wider cirri, fewer molluscs will be present as most of them will search for shelter. In the study of Janson (1983), examining the behaviour of different phenotypes of *L. saxatilis,* shelter seeking behaviour was observed in both phenotypes studied. Snails in exposed areas ("E morph") were especially prone to aggregate in cracks and crevices. Also, home-ranging behaviour was found in the "E morph" but not in snails in sheltered areas ("S morph"). These factors relating to molluscs could contribute to difference in the aggregation of parasite infra-populations. A further complication of the situation relates to the behaviour of cercariae. The cercariae of *Maritrema* belong to the xiphidiocercaria type and have poor swimming abilities. According to a study of *M. novaezealandense* (see Studer & Poulin, 2013), the survival time of cercariae of this species is around 6-8 hours. This means that they cannot last longer than one tidal cycle. Cercariae die faster under higher temperature, salinity, and ultraviolet radiation. These authors suggested that the main transmission windows for this species would occur during low tide in warmer months in tidal pools. Whether a similar scenario could occur for *M. gratiosum* is still unknown. Currently, however, too little information is available concerning the cercariae of *M. gratiosum*, such as their longevity in intertidal areas, time of release, phototaxic and geotaxic behaviours, and their presence in the mollusc host and the species of potential mollusc host in the study localities. Further researches are needed to investigate these aspects. Furthermore, what is also unknown is the species and frequency of potential

final hosts of *M. gratiosum* in the study localities. It is likely that the model could explain more of the observed variability if the final host factors and more precise environmental factors could be included.

3.4.4 Final remark and conclusions

The present study is the first study to apply a systematic sampling strategy for the spatial distribution of *M. gratiosum* metacercariae in its crustacean host *S. balanoides* off the Scottish rocky shore. A negative trend of infection was found along a latitudinal gradient. Heterogeneity of distribution was detected across a rocky platform and a moderately sheltered bay, *i.e.*, Dunbar Red Rock and the Leisure Pool, and at different scales. The infection patterns of the two studied habitat types, i.e., the rocky platform and sheltered bay, may provide an example of long-term accumulation and early development of the digenean-population respectively. In the two studied scales, *i.e.*, a local scale and a whole area scale, a significant decreasing trend of abundance was identified along a gully (i.e., the local scale) but not in the whole area after correction of host size effect. These results indicates that the effect of distance to the land upon abundance is more apparent in the smaller scale, although it was not possible to confirm if the effect of the tide or the density of the upstream host contributed more. Four parameters, mean abundance, Lloyd's mean crowding, total metacercariae, and weighted Lloyd's patchiness index, were applied for visual presentation of the spatial trends in real space; and their biological meanings were discussed separately. Aggregations of *M gratiosum* in *S. balanoides* population was confirmed by means of variance-to-mean ratio, negative binomial k, and Lloyd's mean crowding. The relationship between the variance and the mean of the abundance complied with Taylor's Power Law, but a subtle discrepancy was noticed, and this was suggested to be related to host-parasite interactions. The multiple regression models applied to the whole area study explained a high percentage of the variance of abundance, with host size and exposure (cirrus length) being the major determinants. The uniqueness of the present study are the methodologies applied and the scale studied. Multi-factorial analysis was suggested in a field-based parasite distribution study, as these factors were often correlative. A strategic sampling plan and prudent choice of the analysed factors helped to eliminate potential confounding factors. A robust understanding of why each factor is included, however, is prerequisite. Each of the biotic factors examined can be explored further in the future, and more abiotic factors could be included. The distribution data obtained can provide a reliable baseline data for further researches in the study system; more specifically, temporal changes in distribution and spatial genetic diversity analysis relative to distribution should be promising research topics.

Chapter 4 Diagnosis of *Maritrema gratiosum* Nicoll, 1907 (Digenea: Microphallidae) from *Semibalanus balanoides* L. in Scottish coastal waters

4.1. Introduction

4.1.1. General features of *Maritrema gratiosum* Nicoll, 1907

Maritrema gratiosum Nicoll, 1907 belongs to the family Microphaliidae Ward, 1901 and is the type species of the genus *Maritrema* Nicoll, 1907. The original description of Nicoll (1907) included a detailed description and measurements of the body and major organs but did not describe the cirrus and related ducts inside the cirrus sac, neither did it include a figure. At the same time, this species was also described as *Cercaria balani* by Lebour (1907) for the metacercariae from the "common barnacle" *Balanus balanoides* (now accepted as *Semibalanus balanoides* Linnaeus, 1767) from Northumberland (Lebour, 1907). The description, however, was simple and did not mention the cirrus or the related ducts. It was not until 1940 that a comprehensive description of the morphology of the adult and larval stages, along with the life-cycle, was provided by Hadley and Castle (1940) under the name *Maritrema arenaria*. According to these authors, the final host of *M. arenaria* in the USA was a marine wading bird, the ruddy turnstone, *Arenaria interpres* Linnaeus, 1758, and the second intermediate host was *S. balanoides*. Large numbers of adults were found in the bird intestines and when the guts of the birds were examined, they contained partially digested barnacles and partially and freshly excysted metacercariae, from which the author built the connection between the adult and metacercarial stages. In 1966, Deblock and Tran Van Ky reconsidered the systematics of several microphallid species and stated that *M. arenaria* Hadley et Castle, 1940 should be the synonym of *M. gratiosum* Nicoll, 1907. They also pointed out that *Maritrema* spp. should share the feature of an eversible cirrus. The "papilla" described by some authors (such as in the case of Hadley & Castle, 1940) reflects the condition of cirrus eversion during fixation. The lack of a precise description regarding the male copulatory organs has a profound influence on the systematic of this genus, because the origin and diversification of the male copulatory organ (i.e., cirrus sac and its accessories) provide key taxonomic criteria for the classification of members in the Microphallidae (see Deblock, 1971). Later the cercariae and metacercariae were reported by Popiel (1976a, b) from *Littorina saxatilis rudis* (syn. *Littorina saxatilis* Olivi, 1792) and *S. balanoides*, respectively. However, it was not until the 1990s that the connection between the cercarial stages and metacercarial stages was established. In 1990, Irwin *et al.* (1990) identified the cercarial stage of *M. gratiosum*, which was previously described by Popiel (1976b) as *Cercaria littorinae saxatilis* V. The cercarial stage was confirmed through experimental infection. These cercariae possess a short tail, a clear penetration gland, and a characteristic spear-

shaped stylet. By light microscope observation, Irwin *et al.* (1990) found that the diagnostic spear-shaped stylet was visible in the cercariae and young metacercarial stages inside barnacles. The authors also pointed out that the dispersal of *C. littorinae saxatilis* V might depend on currents and waves due to its short tail.

To this point, the life history of *M. gratiosum* was confirmed and can be summarised as follows. The final hosts of *M. gratiosum* are charadriiform birds; the second intermediate hosts are cirripede crustaceans, and the first intermediate hosts are molluscs belonging to the Littorinidae. Barnacles are the sole second intermediate host for *M. gratiosum* but numerous species of final hosts are reported. This phenomenon fits the general lack of specificity of final hosts for microphallids. Appendix 31 summarises the hosts of *M. gratiosum* reported in the literature. From the literature cited above, there is a knowledge gap in that there is a lack of contemporary morphological studies applying modern technologies for *M. gratiosum*; especially given that there is some disagreement regarding the morphology of the cirrus which needs to be resolved. Furthermore, a lack of molecular studies of the different developmental stages for this species hinders a robust species diagnosis and an appreciation of its genetic diversity and evolution. Finally, due to an aggregated and heterogeneous distribution of *M. gratiosum* in the study space (see Chapter 3), the morphological plasticity of the species will be investigated here by morphometric analysis, and the relevance of crowding effects and other factors on the morphological diagnosis will be analysed.

In the following sections, some aspects concerning the diagnosis of digeneans in barnacles and relevant methodologies will be introduced.

4.1.2. Electron microscopy as a tool for studying digenean morphology

Electron microscopy is a powerful tool for revealing fine surface structures, ultrastructure at a cellular level, and shedding light on biological processes and taxonomy (Jamjoom & Shalaby, 2006). The surface structures of the adult and metacercarial stages of Digenea are conserved, such as the presence of spines, papillae, and corrugations on the tegument. Dependent on the species, the tegumental structures vary. For example, the tegument of the adult eye fluke *Philophthalmus megalurus* Cort, 1914 has no spines but possesses cobblestone like protuberances (Edwards *et al.,* 1977). The newly excysted *Clonorchis sinensis* Looss, 1907 has a spined tegument but transforms to a spine-less, knobbed, or lobulated surface when matured as an adult (Fujino *et al.*, 1979). The reasons for the loss of spines on the adult is unknown, but the author found that these spines were absorbed rather than abraded during migration. Newly excysted *Fasciola hepatica* Linnaeus, 1758 are also equipped with a spined tegument (Bennett, 1975a), and the spines increase in numbers and transform from single pointed to multi-pointed throughout development (Bennett, 1975b). The authors pointed

out that the metamorphosis of these spines during maturation are important to the survival of *F. hepatica.* For example, they are important in locomotion in the host, for anchoring the body while freeing the oral sucker for feeding, and for creating a hyperplastic state to the epithelium of the bile duct which consequentially facilitates feeding. More specifically, the author suggested that the increase in the numbers and digit of the spines during maturation are likely to increase nutritious cellular debris, and the nutrients can be absorbed directly by the parasite's tegument.

Sensory organs (*i.e*., various types of papillae) are distributed across the body of digeneans, and the topology of these papillae, the shapes, arrangement, and distribution of the tegmental spines have taxonomic importance (Jamjoom & Shalaby, 2006). Chaetotaxy, the pattern of papilla distribution, is sometimes applied to species differentiation. In the study of Komalamisra *et al.* (2005), chaetotaxy was used to separate five species of *Paragonimus.* Ultrastructural features and chaetotaxy have also been investigated in the cercariae of a species of *Allopodocotyle* and their diagnostic value discussed (Bogéa & Caira, 2001). Kostadinova (1999) investigated the chaetotaxy of *Echinostoma miyagawai* Ishii, 1932 and compared with other *Echinostoma* species to re-evaluate the applicability of chaetotaxy in the species of the '*revolutum*' group. To understand what is the normal morphology of a digenean species under SEM can have medical importance. Senft *et al*. (1978) revealed that tegumental development of *Schistosoma mansoni* in permissive and non-permissive hosts were different. Basch and Basch (1982), however found that there were no differences in the surface morphology of adult *S. mansoni* cultured *in vivo* and *in vitro*. Soliman and Ibrahim (2005) found that treatment can also lead to ultrastructural differences in the tegument of *Schistosoma haematobium* Bilharz, 1852.

In general, the surfaces of microphallids are armed with spines that are visible via light microscopy. Only fragmented information is available relating to the surface ultrastructure of species belonging to the Microphallidae. The teguments of *Maritrema* spp. are in general covered with spines and the spine number and size decreases toward their posterior end (Zaben, 1988; Diaz & Cremonte, 2010; Pina *et al.*, 2011a; Alda *et al.,* 2013). There is, however, no evidence that these spines will increase or decrease in number or change in shape during maturation (*i.e*., from newly excysted young adults to ovigerous adults). The first scanning electron microscopy (SEM) observation of *M. gratiosum* was conducted by Zaben (1988). According to the author, the tegument of this species was covered by alternate rows of backwardly directed digitiform spines. These are more spines on the ventral than the dorsal side, and their teeth numbers decrease from 8-13 to 2-6 posteriorly. The spines also extend into the oral cavity and the floor of the ventral sucker. A single type of sensory papilla was found and characterised as a wart-like boss with an apical pit through which a cilium protrudes (Zaben, 1988). The author found 9 ciliated papillae in the floor of the cavity of the ventral sucker. Genital pores can be seen beside the ventral sucker, and Laurer's canal and excretory pore were evident on the dorsal

surface; the former near the middle of the body but closer to the left and the latter near the posterior side. Zaben (1988) found that there were no consistent patterns to the sensory papillae except for areas around the ventral and oral sucker. Furthermore, the author found that there were signs of degradation of the surface ultrastructure in adults cultured *in vitro* from 9th day, but Zaben was not able to follow their development beyond this because of financial constraints to project funding. His findings suggested that a more systematic survey and a long-term follow up study (both *in vitro* and *in vivo*) of changes to the tegument in *M. gratiosum* are needed. In addition to a study of their distribution of sensilla (i.e., chaetotaxy), a detailed study of the shape and size of spines, and possibly, the morphology of the cirrus could be conducted. For examples, SEM observations of the surface of *Microphallus prima* (Jägerskiöld, 1908) Stunkard, 1951 metacercariae (Pina *et al.*, 2011b) revealed tegumental spines with a squamous arrangement and having 17 teeth. The size of the spines reduces and the number of teeth decreases posteriorly. The edge of the posterior ventral region is spineless but equipped with microvilli. Uniciliated sensory papillae are distributed on the oral sucker and are scattered across the body, with more on the ventral surface. *Maritrema portucalense* Pina, Russell-Pinto et Rodrigues, 2011 is equipped with tegumental spines bearing 20 teeth in the anterior region. Six uniciliated papillae lie on the lip of the ventral sucker (Pina *et al.*, 2011a). The presence of "hand-shaped" spines with 7-9 fingers distributed across the body of *Maritrema orensensis* was revealed by SEM, and similarly there were 9-10 fingered spines in *Maritrema bonaerensis* Etchegoin et Martorelli, 1997. The use of SEM also highlighted the morphology of the everted cirrus of this latter species (Alda *et al.*, 2013). SEM also revealed the tubular, filiform, unspined, everted cirrus of *Maritrema corai* Hernández-Orts, Pinacho-Pinacho, García-Varela et Kostadinova, 2015 (Hernández-Orts *et al.*, 2016).

Furthermore, SEM observations combined with morphometrics provide the means to differentiate all stages of digenean species. For examples, the metacercariae of *Microphallus pygmaeus* (Levinsen, 1881) Baer, 1944 and *Microphallus piriformes* Galaktionov, 1983, which are morphologically similar. Plate-like, 7-9-digit spines are found on the fore body of *M. piriformes*, while hand-shaped spines with 4-6 digits are found on *M. pygmaeus*. Also, the distribution of papillae around the oral sucker of these two species is different (Saville *et al.*, 1997). Detailed observations of the cercariae of *M. arenaria* (syn. *M. gratiosum*) was performed by Irwin *et al.* (1990). A characteristic 10 µm long, spear-shaped stylet was visible in the cercarial stage and remained throughout the process of penetration and early encystment. Following penetration of the barnacles (*S. balanoides*), the cercaria lose their tails and the depleted penetration glands. SEM images also revealed the similarity of surface structures of cercariae before and after entering barnacles. They were all covered by numerous, closely-packed, short spines. Sensory papillae were observed at the anterior region on snail-released cercariae or cercariae inside barnacles.

An SEM study will be conducted on newly excysted, young adult *M. gratiosum* in the present study, to compare these with the surface structures revealed by Zaben (1988), and to evaluate the use of these structures by comparing them in adults derived from *in vitro* cultures and *in vivo* infections (natural and experimental). These comparisons are likely to reveal any developmental changes and whether they are affected by the experimental host or the culture conditions.

4.1.3. Application of molecular techniques to digenean diagnosis

Traditionally the taxonomic position and description of parasites has relied on methods other than molecular techniques, such as morphology, interbreeding studies, and geographic ranges (McManus & Bowles, 1996). These methods are subject to several difficulties: a lack of robust taxonomic characters, uncertainty of those characters, morphological similarity between closely related species, phenotypic plasticity, the lack of distinctive morphological characters in certain life-cycle stages, the small size of adult stages, and a lack of conserved and hard structures, *etc*. Molecular techniques have, therefore, in more recent times, been used in conjunction with traditional methods to strengthen parasite taxonomy. With continuing advances of molecular technologies, the use of a molecular approach for the recognition of parasites has become common (Nolan & Cribb, 2005). Protein-based approaches, such as protein sequencing, protein electrophoresis (isoenzyme and allozyme analysis) and serotyping were popular previously. These latter techniques have, however, been largely superseded by DNA-based approaches, which often provide the most direct, rapid, and cost-effective means for the characterisation of species. Several DNA-based methodologies have been developed for different purposes, each possessing some advantages and disadvantages. DNA-DNA hybridisation can be applied in higher level comparisons but it is quite expensive and difficult to set up (Sarich *et al.*, 1989; Springer & Krajewski, 1989; Bledsoe & Sheldon, 1990). DNA hybridisation by dot-blot systems can be applied for parasite identification under field conditions. Ribosomal RNA sequencing was previously more common but has now been replaced by polymerase chain reaction (PCR)-based approaches for direct ribosomal DNA amplification. In comparison with other molecular methods, PCR is direct, requires only trace amounts of samples, is comparatively non-laborious, and is relatively cheap. Modified methods based on PCR techniques, such as PCR-linked restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), nested-PCR, random amplified polymorphic DNA (RAPD), loop-mediated isothermal amplification (LAMP), and real-time PCR, have now been developed for specialist purposes. They are applied for different purposes and present different powers and constraints. Primary sequencing comparison is a desirable method because the sequences of regions of interest can be revealed directly, different taxa can be compared, and the evolutionary distance can be calculated. Some knowledge of the target sequence, however, is required in advance to enable the development of primers. In addition, this method is only feasible for

small numbers of samples, and the cost may also be considerable. Methods utilising arbitrary primers, such as RAPD, can be applied to detect DNA polymorphism without prior information on the sequences, but it is less informative; an anonymous pattern of fragments being compared, rather than the exact base composition. RAPD and the similar AFLP analyses, can both search sections of the entire genome and can be used for description of strains in epidemiological studies. LAMP, when compared to PCR, is faster and even more sensitive, and can be achieved without extracting DNA from the samples. The advantage of LAMP makes it applicable in eDNA samples and field-based studies (Williams *et al*., 2017; Davis *et al.*, 2020). qRT-PCR, is a simple, fast, closed, and automated approach, that can be applied not only to identify a parasite, but also to quantify the amount of infection. The power of this method makes it applicable to parasite detection in various samples and to quantitation of the expression and regulation of a particular gene, to allelic discrimination, and to therapy efficacy assessment. The disadvantage of this approach, however, is the high cost. Microsatellite techniques, which are based on short segments of DNA sequences composed of tandem repeats, can be applied to detect intraspecies variation in a parasite population (McManus & Bowles, 1996; Tavares *et al.*, 2011). 18S rDNA-based metagenomics, which builds on next generation sequencing (NGS), has, for example, been used to successfully estimate helminth biodiversity in wild rats, and is reliable and useful compared to standard microscopy and individual sequencing (Tanaka *et al.*, 2014).

As mentioned above, primary sequencing via PCR is the most direct method for parasite species diagnosis, however, its utility depends on the purpose of the research, as the choice of target genes need to be considered carefully. Regions unconstrained by functions, for example introns and non-coding regions, usually evolve more rapidly than coding regions. Choosing a region with too little variation renders the distinction between groups difficult but on the contrary, highly divergent sequences are not robust because of increased homoplasy and the number of acceptable alignments becomes too large. A region displaying similarities between 70~100% is suggested (Nolan & Cribb, 2005). Commonly used genes / regions include the ribosomal DNA gene and the mitochondrial genome (McManus & Bowles, 1996). Ribosomal RNA genes and their spacers are collectively called ribosomal DNA (rDNA). In the eukaryotic genome, this multigene family concentrates in the nucleolar organizer region and is composed of hundreds of tandem repeat copies of the transcription unit, the non-transcribed spacer and two internal transcribed spacers (Figure 3.1). These repeats are nearly identical because the entire rDNA evolves in a concerted way, through gene conversion and unequal crossing-over. Thus, by rapid homogenisation and fixation of new variants, sequences from individuals are generally found to be representative of the larger interbreeding group from which they derive (Nolan & Cribb, 2005). rDNA includes regions which are highly conserved (the small subunit gene, 18S rDNA and 5.8S rDNA) and those which are highly variable (the internal transcribed spacers, ITS 1 and 2),

making it particularly useful across the range of taxonomic study (McManus & Bowles, 1996). In the rDNA family, the 18S rDNA evolves slowest and is suitable for inferring deep phylogenetic relationship while the 28S rDNA (large subunit) evolves at a faster rate, and the ITS rDNA evolves the fastest. Analysis of the ITS rDNA region has been applied to explore species boundaries in digenean families and has become the default region of choice for species diagnosis. Among the ITS regions, ITS 1 shows the highest variability and give greater resolution than ITS2 in some cases. The high variability of the 5' end of ITS1 allows species identification, and the more conservative 3' end is useful in systematic study (Schulenburg *et al.*, 1999; Nolan & Cribb, 2005). Due to its sequence being highly conserved and the extensive knowledge database available, 18S rDNA, has been applied to the large-scale phylogenetic study of the phylum Platyhelminthes (Littlewood & Olson, 2001), and applied to the phylogeny of the Digenea at the class level in combination with morphology (Cribb *et al.*, 2001). 18S rDNA and 28S rDNA together has also been used to study the phylogeny of 163 digenean and 7 aspidogastrean species (Olson *et al.*, 2003). The mitochondrial genome, on the other hand, has several characteristics that differ from rDNA. It is a small, circular, maternally inherited genome. It is quite compact, with no introns and minimal non-coding regions, and evolves independently of the nuclear genome. In vertebrates, mitochondrial genes evolve ~5-10 times faster than nuclear genes, but it is not necessarily the case in other animals (McManus & Bowles, 1996). A short DNA sequence of 600 bps in the mitochondrial gene for cytochrome c oxidase subunit 1 (CO1) has been accepted as a standardized species-level barcode for animals (Kress & Erickson, 2008). Primers targeting CO1 regions have been developed for the identification of a wide range of digeneans (23 families) and cestodes (6 orders) (van Steenkiste *et al.*, 2015). Mitochondrial markers including 12S rDNA are also applied to the phylogenetic study of cestodes (von Nickisch-Rosenegk *et al.,* 1999). Using large amounts of sequencing to characterise 18S rDNA, 28S rDNA and fragments of the mitochondrial genome, a comprehensive phylogenetic study of the Cestoda was achieved enabling some long-standing conflicts to be resolved (Waeschenbach *et al.*, 2012).

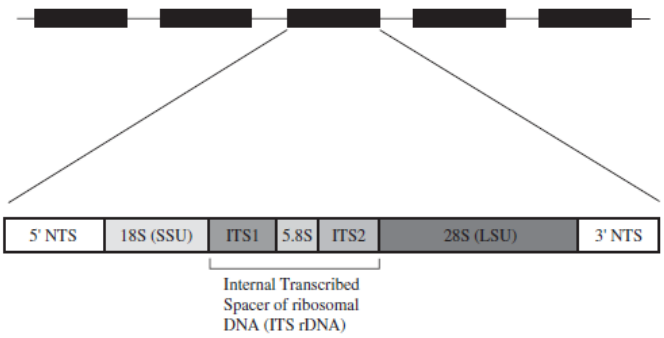


Figure 4.1. Diagrammatic representation of ribosomal DNA within the nucleolar organizer region of the eukaryotic genome (the black boxes represent tandem repeats), the transcription unit, the non-transcribed spacers and the two internal transcribed spacers. Taken from Nolan and Cribb (2005).

Molecular techniques have been applied as powerful tools to complement traditional morphological taxonomy and are especially useful to link the morphologically distinct life-cycle stages of Digenea. Molecular approaches have been applied to smaller scale studies concerning a few species, such as in the discrimination of similar cercarial stages between co-occurring microphallids (Hust *et al.*, 2004; Al-Kandari *et al.*, 2011, 2014), and to establish links between life-cycle stages of other families (Bartoli *et al.*, 2000; Locke *et al.*, 2011; Heneberg *et al.*, 2015). On the other hand, larger scale studies have focused on the phylogeny at higher taxa, also providing valuable information or even re-shaping digenean phylogeny based on morphology. In the study of Olson *et al.* (2003), by applying the combined data of the small and large subunit ribosomal RNA genes (ss rDNA and ls rDNA), two major lineages were identified, namely the Diplostomida and the Pagiorchiida, and a new order the Xiphidiata including 4 superfamilies: Gorgoderoidea, Allocredioidea, Plagiorchoidea and Microphalloidea was developed. In the work of Tkach *et al.* (2003) concerning the phylogeny of the Microphalloidea, three sub-clades were detected, namely: Lecithodendriidae, Microphallidae and the final sub-clade including Pleurogenidae + Prosthogonimidae. In an intriguing piece of research relating to a group of microphallids possessing a 2-host life-cycle (the *pygmaeus* group), two clades were detected within the Microphallidae: the *Microphallus* clade and the *Maritrema* clade. *Maritrema gratiosum* (syn. *M. arenaria*) sits well in the *Maritrema* clade and is closest to *Maritrema prosthosmetra* Deblock & Heard, 1969*, Maritrema oocysta* and *Maritrema subdolum* Jägerskiöld, 1909 depending on the gene marker applied (Galaktionov *et al.*, 2012). Gene markers belonging to the ribosomal rRNA families such as ss rDNA, ls rDNA, ITS regions, and several candidates of the mitochondrial gene are commonly used markers, whether used individually or combined, as in the abovementioned studies. In the present study, ribosomal DNA approaches are applied to confirm the identity of the target parasite and to compare the phylogenetic relationship of the target parasite with other members of the family Microphallidae. A link between genetic relatedness and their respective morphologies for the analysed microphallids will also be discussed.

4.1.4. Morphometric approaches and their application in digenean diagnosis

Morphometric approaches provide another way to compare morphologically similar platyhelminth species, or to compare individuals of the same species sampled from different geographical locations. They differ from the morphological methods discussed above, which are based on observations of form, colouration, and anatomy. Morphometric methods depend on measurements and counts, generally require larger data sets, and employ statistical methods to differentiate individuals / populations. Applications of morphometric analysis to the study of digeneans encompass a wide range of study approaches. They range from merely comparing the range

of meristic values between target species / strains, to sophisticated analyses applying statistical methods such as multivariate analysis. As an example, such methods have been applied to the identification of the metacercarial stages of a new *Diplostomum* species from Portugal (Cavaleiro *et al.*, 2012). Using principal component analysis (PCA) and cluster analysis, differences were revealed between the new species and previously described species. Two morphotypes were found within this new species, these being suggested to be nominally "identical" by molecular methods. Applying morphometric analysis can also confirm the existence of a single species. Caffara *et al.* (2016) used PCA to prove their target species *Euclinostomum heterostomum* (Rudolphi, 1809) Travassos, 1928 from Israel to be a single species, morphometrically like that of another described species *E. heterostomum,* but not the same. Hernández-Mena *et al.* (2014) used PCA to distinguish a new strigeid species, *Parastrigea plataleae* Hernández-Mena, García-Prieto et García-Varela, 2014*,* from another three described species of *Parastrigea* recorded in Mexico, and a clear separation was revealed (see Figure 4.2 below).

Morphometric analysis is also useful in medically important digenean species. In the study of Valero *et al.* (2012), two phenotypic patterns, the valley pattern and the altiplanic pattern, were distinguished among adult *F. hepatica* from Peru and Bolivia. The size and shape of adults were analysed by geometrical morphometric measurement of a single variable, the "centroid size", to capture the allometric extension of conspecifics. The morphometric variation among samples was analysed by size-free canonical discriminant analysis. This study revealed no apparent relationship between adult shape and altitude or geographical distribution. The authors argued that allometry-free shape seems to be more stable than size in fasciolid species. Later, in the study of Ashrafi *et al.* (2015), the relationship between metric traits and geographical and altitudinal distribution of two different liver flukes, *F. hepatica* and *Fasciola gigantica* Cobbold, 1855, were studied. Morphometric variation

was accessed again by the two methods mentioned above; and it was found that both altitude and host type influenced fasciolid distribution, and host type influenced fasciolid shapes.

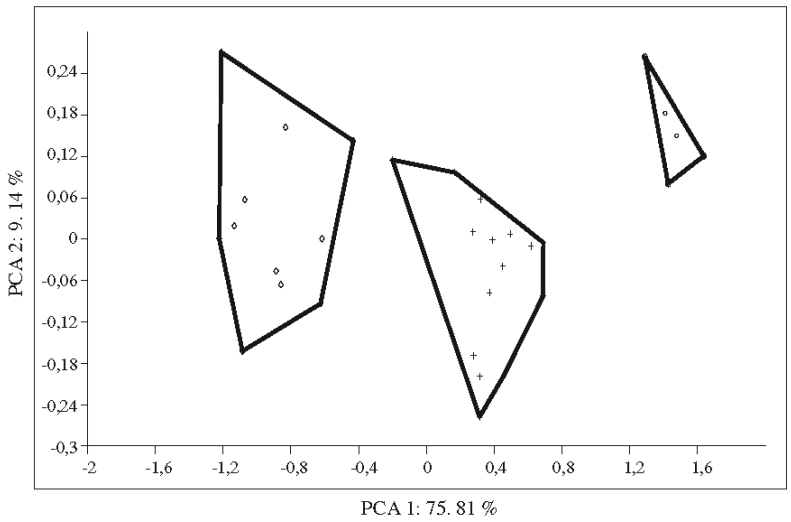


Figure 4.2. Principal component analysis using 16 morphometric variables from 15 specimens of *Parastrigea plataleae* (+), five specimens of *Parastrigea cincta* (o), and 11 specimens of *Parastrigea diovadena* (◊) (reproduced from Hernández-Mena *et al.* (2014), Figure 3).

Morphometric analysis is also applied extensively for identification of monogenean species, as the hard parts of the haptors are important for taxonomy. Soo and Lim (2015) analysed morphometric data of two new *Ligophorus* species by PCA and concluded that the two new species can be differentiated based on separated or combined data from anchors, ventral bars, and copulatory organs. Shinn *et al.* (2010) developed a standard operating procedure to achieve rapid identification of the notifiable monogenean pathogen *Gyrodactylus salaris* Malmberg, 1957, by visual, morphometric, and molecular methods. Linear discriminant analysis-based classifiers were used for morphometric analysis. Hahn *et al.* (2011) applied PCA and canonical variate analysis (CVA) to differentiate similar *Gyrodactylus* species, *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 and *G. salaris,* and discussed the possibility of using an automated diagnostic approach.

In some studies, metric measurements have been employed as a means to compare the dimensions of organs or body parts between the target species and the described species (either conspecifics or different species), and to compare larval stages for which few characters can be applied for species discrimination. Examples include discrimination of *M. bonaerensis* and *M. orensensis* (see Alda *et al.*, 2013); *Maritrema brevisacciferum* Shimazu et Pearson, 1991 (see Kudlai *et al.*, 2015); *Lepidapedon* species (Lepidapedidae) from deep-sea fish (Dallarés *et al.*, 2013); *Aphanurus mugilus*

Tang, 1981 from mullet fish (Atopkin *et al.*, 2017); and *Skrjabinolecithum pyriforme* Besprozvannykh, Atopkin, Ermolenko et Beloded, 2016 from mullet (Besprozvannykh *et al.*, 2017). Multivariate analysis of variance (MANOVA) is applied on rare occasions to compare morphometric data to reveal differences between variable data sets, and at the same time to access univariate effects by pairwise comparison of the individual variables between data sets (Gállego *et al.*, 2014). Following the analytical methodology applied by Tkach (1998) in the study of *Maritrema neomi*, the coefficient of variance (CV) was used to estimate the stability of each variable in the metric data.

Finally, as crowding and host geographical origins can influence digenean morphology (Stillson & Platt, 2007; Hildebrand *et al*., 2015; Falcón-Ordaz *et al*., 2019), and different degrees of crowding had been detected in the studied areas in the present study (i.e., between localities and among localities, see Chapter 3, Table 3.8, Figures 3.20 & 3.30). In this chapter, therefore, a MANOVA study will be conducted for specimens from different scales at Dunbar Red Rock (the local scale versus the whole area scale), and a principal component analysis will be conducted for specimens from the same locality but different scale, and for localities of different latitude (Dunbar, Stonehaven, and Rosehearty). An assumption, that morphological plasticity is positively correlative to geographical distance, will be examined. Beside natural variability, the method of fixation used is also an important factor affecting morphological diagnosis (Bakke, 1988; Falcón-Ordaz *et al.*, 2019). Depending on the subsequent methods that are applied, common fixation methods include: heat fixation, cold fixation, and anaesthetization (physical, chemical and press fixation) (details can be found in Bakke, 1988, Table 1), and various chemicals and procedures can be applied. Two fixation methods (Berland's fluid for stained specimens and coverslip pressure for live specimens) will be applied in this study, and the influence of the fixation methods will be discussed later.

4.1.5. Aims of the present study

The present study aims to perform a morphological study of the metacercariae and adult *M. gratiosum* found in *S. balanoides* collected from the Scottish coast. By applying modern technologies, such as light microscopy and electron microscopy, a redescription of this type species in the genus *Maritrema* will be conducted, with special focus given to the description of cirrus morphology and, where possible, their arrangement of sensilla and possible application of chaetotaxy. Furthermore, molecular tools will be applied in order to link morphological and genetic evidence in order to achieve a robust species diagnosis, and to contribute to the body of evidence regarding the phylogeny and relatedness with other microphallid species in existing databases. The connection between phylogeny and morphology, therefore, will be discussed. Finally, due to an aggregated and heterogeneous distribution of *M. gratiosum* throughout the study area, morphometric studies will be conducted to

investigate morphological plasticity relative to crowding effects, and to establish the relationship of morphological plasticity with geographical distance.

4.2. Materials and methods

From a preliminary study, the metacercariae found in the prosoma of *S. balanoides* were diagnosed morphologically as *Maritrema* sp. (see Chapter 2). The target parasite, therefore, is provisionally referred to as *Maritrema* sp. until a final diagnosis can be made.

4.2.1. Collection and excystment of metacercariae of *Maritrema* sp. from *Semibalanus balanoides*

Samples of barnacles were collected from three different localities throughout 2014-2018 (Figures 4.3 & 4.4). The first batch of samples was collected in 2014 for a larger scale distribution study at a rocky platform of a coastal area at Dunbar (called "Red Rock", see Figure 3.1 and location details in Chapter 3). Samples from multiple sampling points covering different scales at Red Rock were taken (see Figures 3.2 & 3.3, detailed sampling methods see Chapter 3). Consecutive samplings were carried out in the winters of 2017 and 2018. One batch of samples collected from Dunbar Leisure Pool in 2017 for the purposes of species diagnosis (the present study and *in vitro* culture, see Chapter 5). The second and third batches of samples that were collected from localities at higher latitudes, namely Stonehaven and Rosehearty, in 2018 were to expand the geographical range (see Figure 3.12 and location details in Chapter 3). The geographical information and infection indices are summarised in Table 4.1. All the hosts used in the present study were confirmed to be a single species, *S. balanoides*. The methods of collecting barnacles from the rocks, barnacle dissection, extraction and excystment of digeneans were the same as described in Chapter 2.

Figure 4.3. Sampling localities for the morphological and morphometric study. A. The three localities at different latitudes. B. The two sampling localities in Dunbar. Source: Google Maps

Figure 4.4. Details of the sample sites for the morphometric study at Dunbar Red Rock. The map to the left shows the entire study site whole extent (i.e., the blue box denotes that for the whole area scale study, while the yellow box for the local scale study) which was then sub-divided into smaller sampling units as shown by the orange and yellow units to the right. Source: Google Maps.

Table 4.1. Geographical information and infection indices at the four sample localities

Locality/year of sampling/no. of barnacles dissected	**Numbers of newly excysted young adult / cultured adult for morphometric analysis**	**Geographical information**	**Prevalence (%)**	**Mean intensity**	**SD**	**Mean abundance**	**SD**
Dunbar-Red Rock (2014, n = 640)	133 for local scale, 119 for whole area scale (young adult)	56°00'19.9"N 2°31'33.4"W	97.34	31.28	38.41	30.45	38.24
Dunbar-Leisure Pool (2017, n = 59)	22 for cultured adult	56°00'19.5"N 2°31'04.2"W	100	7.08	8.70	7.08	8.70
Stonehaven (2018, n = 60)	20 for cultured adult	56°58'17.1"N 2°11'59.1"W	96.67	18.19	15.60	17.58	15.68
Rosehearty (2018, n = 60)	20 for cultured adult	57°41'49.0"N 2°06'24.9"W	98.33	7.85	4.40	7.72	4.48

4.2.2. Morphological diagnosis of the metacercarial and adult stages of *Maritrema* sp.

4.2.2.1. Morphological observations via light microscopy

The excysted metacercariae were observed live under a stereomicroscope to assess their body movement and gross morphology. Live metacercariae were relaxed and fixed in Berland's fluid by immersing them in the solution for about 30 seconds. Berland's fluid is composed of 1 volume of 40% formalin + 19 volumes of 99% glacial acetic acid. After the specimens were relaxed, the metacercariae were preserved in 80% ethanol. They were then stained with Mayer's paracarmine (Cable & Hunninen, 1942) and then processed through graded alcohols and then mounted in Canada balsam for long-term preservation (see Appendix 9). Stained samples were observed under a compound light microscope (Olympus BX51) with and without phase contrast and photographs were taken using Olympus cellSens software and their morphology subsequently described. The morphology was further confirmed for the adult stages. The culture of the adult parasites is detailed in the methods section of Chapter 5. Live and stained samples of 2~5-day old experimental adults from the three different localities were observed, described, and compared. Diagnosis to the species level based on their morphology was accomplished using the diagnostic key for the genus *Maritrema* (see Deblock, 2008) and the original descriptions of Hadley and Castle (1940) and Deblock and Tan Van Ky (1966a).

4.2.2.2. Structural observations via scanning electron microscopy (SEM)

Samples of excysted metacercariae were stored in 2.5 % glutaraldehyde at 4℃. Then, the samples were fixed in 2.5 % glutaraldehyde buffered with 0.1 M sodium cacodylate at 4℃ for 2~3 days before further processing. The samples were washed in buffer (0.1 M cacodylate rinse + 0.1 M sucrose) and the remaining procedures were carried out in a fume cupboard. The samples were then post-fixed in 1% osmium tetroxide in the same buffer for 2 hours. Samples were then dehydrated through an ethanol series (30%, 60%, 90%, 100%, 100%) for half an hour each. At this stage, two methods were applied to the metacercarial samples, either they were prepared for critical point drying (CPD) or were processed using hexamethyldisilazane (HMDS) (Nation, 1983).

4.2.2.2.1. Critical point drying (CPD) method

After the above steps, samples were packed in BioWraps and placed in containers for CPD (Leica EM CPD030). After CPD, the samples were mounted on 13 mm diameter aluminium stubs and sputter coated with gold using an Edwards S150B sputter coater. The samples were examined using a JEOL JSM6460LV SEM at an accelerating voltage of 10kv.

4.2.2.2.2. Hexamethyldisilazane (HMDS) method

This method is particularly useful for fragile specimens; the samples were collected on membrane filters (Cyclopore track etched polyester membrane). After the above steps, the samples were

transferred to 50:50 100% ethanol / HMDS for two changes, 30 minutes each. Thereafter, the samples were put on a membrane filter instead of in an Eppendorf tube. The samples together with the membrane were air dried at room temperature in a fume cupboard. After drying, the samples were mounted on an aluminium stub and then sputter coated and then viewed as detailed above.

Surface structures and measurement of the tegumental spines was performed on the SEM and photomicrographs taken using FIJI ImageJ 1.52p software (National Institutes of Health, USA; Schindelin *et al*., 2012).

4.2.3. Molecular diagnosis of *Maritrema* sp.

The small subunit and internal transcribed spacer regions of the ribosomal RNA gene were selected as the gene markers for molecular diagnosis and were amplified by polymerase chain reaction (PCR). Two primer sets were applied for both genes.

For the ribosomal RNA gene markers, the targets were a partial sequence of the 18S rRNA gene (referred to as 18S rDNA in the present study), and a region covering partial sequences of the 5.8 S, the complete sequence of the second internal transcribed spacer, (called ITS2 rDNA in the present study) and the partial sequence of the 28S rRNA gene. The primers were sourced from the published literature and two primer sets, namely TC and 3S, were employed (see Table 4.2). DNA of individual barnacles and metacercariae were extracted using the HotShot method (Truett *et al.*, 2000; see Appendix 32).

Polymerase chain reactions (PCR) were performed on samples collected from two different localities. For metacercariae collected from Dunbar, Scotland (2016), most of the work was completed in the lab of Professor S.C. Chen in the Veterinary School of the National Pingtung University of Science and Technology (NPUST) in Taiwan. DNA from 16 metacercariae from two barnacles were examined. A 25 µL reaction was carried out for primer set TC comprising 0.125 µl of Blend Taq–Plus– (Toyobo, Osaka, Japan; 2.5 U μ^{-1}), buffer for Blend Taq 2.5 µL (10 × concentration), 2 µL dNTPs (2 mM each), forward primer 0.625 µL, reverse primer 0.625 µL, DDW 16.125 µL and DNA 3 µL. The PCR conditions for primer set TC were: initial denaturing at 95°C 120 seconds, follow by denaturing at 95°C for 30 sec., annealing at 48°C for 40 sec., extension at 72°C for 80 sec., 35 cycles, and final extension at 72°C for 600 sec. followed by 4°C for 300 sec. A 25 µL reaction was carried out for primer set 3S comprising 0.125 µL of Blend Taq–Plus– (Toyobo, Osaka, Japan; 2.5 U μ^{-1}), buffer for Blend Taq 2.5 µL (10 × concentration), 2 µL dNTPs (2 mM each), forward primer 0.625 µL, reverse primer 0.625 µL, DDW 15.125 µL and DNA 4 µL. The PCR conditions for primer set 3S were: initial denaturing at 95°C for 120 seconds, followed by denaturing at 95°C for 30 sec., annealing at 54°C for 40 sec., extension at 72°C for 80 sec., 35 cycles, and final extension at 72°C for 600 sec., followed by 4°C for 300 sec. All PCR

reactions were performed in a TaKaRa PCR Thermal Cycler Dice Gradient (Takara Bio Inc., Japan). PCR amplicons were electrophoresed (1-2 uL product; 1.2-1.5% agarose gel; 0.5 X TAE buffer) and visualised under UV light using a Gel DOC 2000 (Bio Rad). Following confirmation of the presence of a tight single band, the remainder of the PCR amplicons, were sent directly for Sanger sequencing (Genomics, New Taipei City Taiwan), using, each original PCR primer as the sequencing primer.

For the metacercariae collected from Stonehaven and Rosehearty, Scotland in 2018, most of the work was completed at the Institute of Aquaculture in University of Stirling, Scotland; only the partial 5.8S + full ITS2 + partial 28S rDNA fragment was targeted and analysed. Eight individual metacercariae from eight different barnacles per site were used for this study. A 15 µL reaction was carried out for primer set 3S containing MyTaq master mix 6.25 µL (Bioline), forward primer 0.5 µL (1 nmol μL^{-1}), reverse primer 0.5 µL (1 nmol μL^{-1}), ultra-pure water 6.75 µL and DNA 1 µL. The PCR conditions were: initial denaturing at 95°C for 70 seconds, follow by denaturing at 95°C for 15 sec., annealing at 57°C for 20 sec., extension at 72°C for 40 sec., 36 cycles, and final extension at 72°C for 120 sec., followed by 25°C for 1 sec. All PCR reactions were performed in a Biometra thermocycler (Biometra GmbH). Following gel electrophoresis (as above), PCR amplicons were visualised using a GeneGenius Gel Imaging System (Syngene) and photographed using Genesnap (Syngene). Confirmed single banded PCR products were purified by a magnetic bead clean-up method (AxyPrep Mag PCR Clean-up kit, modified method, see Appendix 33) and PCR products sent for bidirectional Sanger sequencing (Eurofins Genomics, GmbH), using each original PCR primer in turn as the sequencing primer. Several microphallid species were also sequenced and used for the phylogenetic analysis. Samples of metacercariae of *Maritrema poulini* Presswell, Blasco-Costa & Aneta Kostadinova, 2014 and adults of *Maritrema deblocki* Presswell, Blasco-Costa & A. Kostadinova, 2014 were kindly provided by Dr. Bronwen Presswell from the University of Otago, New Zealand. DNA from these specimens were prepared in the same way as detailed above; one specimen for each sample were processed. The agarose gel quality control of the two *M. deblocki* samples, however, revealed multiple bands (PCR amplicons). For these samples it was necessary to separate and purify the amplicons by band excision from the agarose gel (1.9%), using the MinElute Gel Extraction Kit, Qiagen, UK, according to the manufacturer's instructions) followed by cloning (Promega, pGEM®-T and pGEM®-T Easy Vector Systems) into DH5α competent cells (Thermo Fisher, UK), following the manufacturers' instructions. Standard blue / white selection from Xgal / IPTG / ampicillin LB agar plates was used initially to identify clones likely to contain recombinant products (white colonies) while the presence of amplicons of the expected sizes was confirmed by PCR for templates, using the original PCR primers. Representative recombinants with different-sized inserts were selected and grown in standard LB / ampicillin broth overnight and plasmids purified using a commercial kit (GenElute Plasmid Miniprep kit, Sigma-Aldrich).

The purified plasmids were sent for bidirectional Sanger sequencing (Eurofins Genomics, GmbH), using T7 and SP6 promoter oligos that flank the cloning site as the sequencing primers.

Table 4.2. Details of the primers used to target the ribosomal RNA gene

Primer set	Primer name	Sequences	Target gene	Literature
TC	TC_F	ATGGCTCATTAAATCAGCTAT	18S rDNA	Routtu *et al.*, 2014
	TC_R	TGCTTTGAGCACTCAAATTTG		
3S	3S_F	GGTACCGGTGGATCACGTGGCTAGTG	5.8S + full ITS2 + partial 28S rDNA	Galaktionov *et al.*, 2012
	ITS2_R	CCTGGTTAGTTTCTTTTCCTCCGC		

4.2.3.1. Data analysis

The bi-directional, paired sequences were first aligned using SeqMan Pro (Lasergene) to form a contig comprising the full amplicon region. Primer sequences were identified and trimmed and any ambiguous positions were confirmed manually by visual inspection and comparison of the 'forward' and 'reverse' chromatogram traces. Sequences with excessive noise were discarded. Contigs from different individuals / groups were then aligned/compared using MegAlign (Lasergene). The resulting consensus for each species was then used to search for homologous sequences using the Basic Local Alignment Search Tool (BLAST) of National Center of Biotechnology Information (NCBI) to identify the most similar sequences.

For phylogenetic inference, analysis of the higher taxa was performed using 18S rDNA sequences. Three species of Opisthorchiata, 2 species of Troglotremata, 1 species of Allocreadoidea, 1 species of Plagiorchoidea, and 8 species of Microphalloidea available from the NCBI nucleotide database were used. In addition, *Fasciola gigantica* (Fasciolidae, Echinostomatoidea) was chosen as an outgroup. The selection of taxa and outgroup can be referred to the study of Olson *et al.* (2003). These sequences along with the consensus sequences derived from the Dunbar, Stonehaven and Rosehearty samples were aligned using MUSCEL (Molecular Evolutionary Genetics Analysis, MEGA X). Phylogenetic trees were constructed by the neighbour joining (NJ), maximum likelihood (ML) and maximum parsimonious (MP) methods with bootstrapping by MEGA X (Kumar *et al.*, 2018). Family level analysis aimed at revealing relationships between selected species was performed using the partial 5.8S + full ITS2 + partial 28S rDNA fragment. Sequences of 8 species of *Microphallus* and 6 species of *Maritrema* from GenBank together with sequences generated from 2 *Maritrema* species from New Zealand, shared by Dr. Presswell, were aligned using MUSCEL. NJ, ML and MP trees were constructed and compared. Details of the sequences used for the phylogenetic analyses are listed in Tables 4.3 and 4.4.

Table 4.3. Sequences used for the 18S rRNA gene phylogeny

Accession no.	Species	Family	Superfamily/Suborder	Stage	Host	Locality	Source
JF823988	*Clonorchis sinensis* strain VNM	Opisthorchiidae	Opisthorchiata	adult	*Homo sapiens*	Viet Nam	Unpublished
HM004211	*Opisthorchis viverrini* strain SK	Opisthorchiidae	Opisthorchiata	adult	*Mesocricetus auratus*	Thailand	Unpublished
HQ874608	*Centrocestus formosanus*	Heterophyidae	Opisthorchiata	adult	*Mesocricetus auratus*	Thailand	Unpublished
FJ357163	*Synthesium tursionis*	Brachycladiidae	Allocreadioidea	NA	NA	NA	Unpublished
AJ287572	*Rubenstrema exasperatum*	Omphalometridae	Plagiorchioidea	NA	*Crocidura leucodon*	NA	Cribb *et al.*, 2001
AJ287551	*Orchipedum tracheicola*	Orchipedidae	Troglotremata	NA	*Cygnus olor*	NA	Cribb *et al.*, 2001
HM004210	*Paragonimus pseudoheterotremus*	Troglotrematidae	Troglotremata	metacercaria	*Larnaudia larnaudii*	Thailand	Unpublished
AY222149	*Prosthogonimus ovatus*	Prosthogonimidae	Microphalloidea	adult	*Pica pica*	Ukraine	Cribb *et al.*, 2001
AY222150	*Schistogonimus rarus*	Prosthogonimidae	Microphalloidea	adult	*Anas querquedula*	Ukraine	Olson *et al.*, 2003
AY222151	*Pleurogenoides medians*	Pleurogenidae	Microphalloidea	adult	*Rana lessonae*	Ukraine	Olson *et al.*, 2003
AY222152	*Pleurogenes claviger*	Pleurogenidae	Microphalloidea	adult	*Rana temporaria*	Ukraine	Olson *et al.*, 2003
AJ287534	*Maritrema oocysta*	Microphallidae	Microphalloidea	NA	barnacle	NA	Cribb *et al.*, 2001
AJ287541	*Microphallus primas*	Microphallidae	Microphalloidea	NA	*Carcinus maenas*	NA	Cribb *et al.*, 2001
AJ287531	*Microphallus fusiformis*	Microphallidae	Microphalloidea	NA	*NA*	NA	Littlewood & Olson, 2001
EU825773	*Microphallus turgidus*	Microphallidae	Microphalloidea	NA	*Palaemonetes pugio*	NA	Unpublished
MF077354	*Fasciola gigantica* strain NB	Fasciolidae	Echinostomatoidea	NA	Cattle	Vietnam	Unpublished

Table 4.4. Sequences used for the partial 5.8S + full ITS2 + partial 28S rRNA gene fragment phylogeny

Accession no.	Species	Genus	Family	Stage	Host	Locality	Sources
HM584196	*Microphallus triangulatus* isolate Mtri	*Microphallus*	Microphallidae	sporocyst containing metacercariae	*Littorina saxatilis*	Russia	Galaktionov *et al.*, 2012
HM584181	*Microphallus piriformes* isolate Mpir2	*Microphallus*	Microphallidae	sporocyst containing metacercariae	*Littorina saxatilis*	Iceland	Galaktionov *et al.*, 2012
KT355829	*Microphallus minutus* isolate V13	*Microphallus*	Microphallidae	Cercaria	*Posticobia brazieri*	Australia	Kudlai *et al.*, 2015
HM584183	*Microphallus calidris* isolate Mcal2	*Microphallus*	Microphallidae	sporocyst containing metacercariae	*Littorina sitkana*	Russia	Galaktionov *et al.*, 2012
HM584198	*Microphallus pseudopygmaeus* isolate Mpse	*Microphallus*	Microphallidae	sporocyst containing metacercariae	*Onoba aculeus*	Russia	Galaktionov *et al.*, 2012
HM584190	*Microphallus pygmaeus* isolate Mpyg	*Microphallus*	Microphallidae	sporocyst containing metacercariae	*Littorina saxatilis*	Iceland	Galaktionov *et al.*, 2012
HM584180	*Microphallus similis* isolate Msimilis	*Microphallus*	Microphallidae	Metacercaria	*Carcinus maenas*	Ireland	Galaktionov *et al.*, 2012
HM584174	*Microphallus abortivus* isolate Mab	*Microphallus*	Microphallidae	sporocyst containing encysted metacercariae	*Hydrobia ulvae*	Ireland	Galaktionov *et al.*, 2012
KT355825	*Maritrema brevisacciferum* isolate V11	*Maritrema*	Microphallidae	Cercaria	*Posticobia brazieri*	Australia	Kudlai *et al.*, 2015
HM584170	*Maritrema oocysta* isolate Moo	*Maritrema*	Microphallidae	sporocyst containing encysted metacercariae	*Hydrobia ulvae*	Ireland	Galaktionov *et al.*, 2012
HQ650132	*Maritrema eroliae*	*Maritrema*	Microphallidae	NA	*Clypeomorus bifasciatus*	Kuwait	Al-Kandari *et al.*, 2011
KJ540203	*Maritrema novaezealandense* isolate AB2014-Maritr	*Maritrema*	Microphallidae	Cercaria	*Zeacumantus subcarinatus*	New Zealand	Born-Torrijos *et al.*, 2014
HM584172	*Maritrema subdolum* isolate Msub	*Maritrema*	Microphallidae	sporocyst containing cercariae	*Hydrobia ulvae*	Russia	Galaktionov *et al.*, 2012

Accession no.	Species	Genus	Family	Stage	Host	Locality	Sources
HM584171	*Maritrema arenaria* isolate Maa	*Maritrema*	Microphallidae	Metacercaria	*Semibalanus balanoides*	Ireland	Galaktionov *et al.*, 2012
Present study	*Maritrema poulini*	*Maritrema*	Microphallidae	Metacercaria	*Austridotea annectens*	New Zealand	Presswell (Unpublished)
Present study	*Maritrema deblocki*	*Maritrema*	Microphallidae	Adult	*Anas platyrhynchos*	New Zealand	Presswell (Unpublished)
MG902996	*Prosthogonimus cuneatus* isolate *Anas platyrhynchos*	*Prosthogonimus*	Prosthogonimidae	NA	*Anas platyrhynchos*	Vietnam	unpublished

4.2.4. Morphometric study

For selected batches of samples, morphometric studies were carried out to detect potential morphological heterogeneity within and between localities.

First, the morphometric variation in the metacercariae was studied in a single locality, Dunbar Red Rock (see Figure 4.4). Samples from multiple sampling points covering different scales at Red Rock were taken (see Figure 4.4, detailed sampling methods see Chapter 3). The metacercariae were extracted and excysted by the methods described in Chapter 2. In the present study, the metacercarial specimens were those collected in 2014 during the distribution study (see Chapter 3 and detailed methodology of the sampling). In brief, two scales of study were conducted: on the "local scale" and the "whole area". On the local scale, 8 barnacles were selected for each point, and 5 metacercariae per barnacle, where possible, were measured. Five sampling points were selected at the local scale study, *i.e*., a total of 200 metacercariae were measured. For the whole area scale study, similar techniques were applied, but with only 5 barnacles being dissected per point and 5 metacercariae per barnacle were measured. Six points were selected and a total of 150 metacercariae were measured. The variables measured were chosen according to the literature and followed those detailed in Hadley and Castle (1940), Otachi *et al.* (2015) and Atopkin *et al.* (2017). A total of 15 measured variables were used: length and width of the body, the length and width of the oral sucker, length of the prepharynx, length of the pharynx, length of the oesophagus, length and width of the ventral sucker, average length, and width of the two testes, length and width of the cirrus sac, and length and width of the ovary (Figure 4.5). Body width is taken as the length through the middle of the ventral sucker. The mean width of the testes and ovary were the widest distance measured transversely, and the length for both organs was the longest distance measured perpendicular to the transverse axis. The width of the cirrus sac was the longest distance measured from left to right, and the length was measured perpendicular to the width from anterior to posterior.

Descriptive statistics were analysed first to reveal the general structure of the data. The measurements for the metacercariae collected from Dunbar Red Rock were compared with the original description from Hadley and Castle (1940) and to those provided by Deblock and Tran Van Ky (1966a) to confirm the identity of the species. A multivariate analysis of variance (MANOVA) was carried out to compare the two scales at Dunbar Red Rock. The purpose of the MANOVA was to detect the potential impact of crowding effect and sampling scale for within locality morphological difference. The degree of crowding of *M. gratiosum* infrapopulation will be presented as Lloyd's mean crowding (detail calculation see Chapter 3). The coefficient of variance (CV) was used to estimate the stability of each variable in the metric data; a CV value larger than 0.15 will be considered unstable. Principal

component analysis (PCAs) were conducted to investigate morphological heterogeneity within the specimens collected from Dunbar Red Rock.

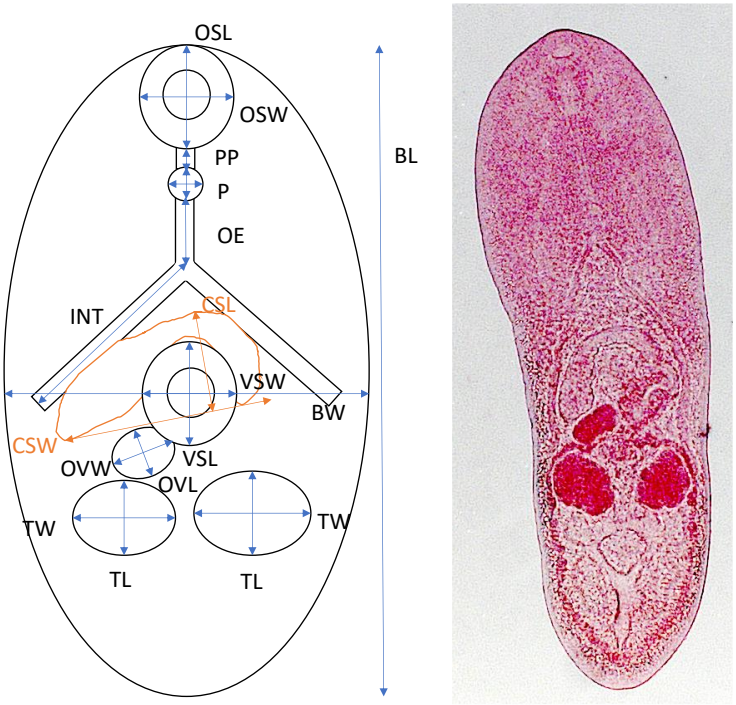


Figure 4.5. Left: Schematic overview of the morphometric variables included in the principal component analysis. BL: body length. BW: body width. CSL: cirrus sac length. CSW: cirrus sac width. INT: intestine caecum length (included in later analysis). OE: oesophagus length. OVL: ovary length. OVW: overay width. P: pharynx length (pharynx width was included inlater analysis). PP: prepharynx length. TL: testis length. TW: testis width. VSL: ventral sucker length. VSW: ventral sucker width. Right: A stained specimen is included for comparison.

Thereafter, geographically distanced samples collected from Stonehaven and Rosehearty in 2018 were compared with samples from Dunbar Leisure Pool collected in 2017. In this analysis, the adults raised in culture were studied; the number of samples available, however, were smaller than those used in the previous analysis. The analysed variables were the same as those given above but measurements regarding the width of the pharynx, the length of the caecum, and the sucker ratio were included, to give a total of 18 variables for analysis. The sucker ratio is defined as the oral sucker length / ventral sucker length. Egg morphology was also studied; egg counts, mean egg length and width (i.e., values derived from an average of 100 eggs with 10 mature eggs collected from 10 individuals) were included. Descriptive statistics were explored first and then the structure of the data was examined. Morphometric measurements for samples from Stonehaven and Rosehearty were compared with the original description from Hadley and Castle (1940) and Deblock and Tran Van Ky (1966a).

Although the sample size was smaller (i.e., 20 to 22 samples per site; a total of 62 samples used in the analysis), PCAs were still carried out for adult samples from the three distanced localities to detect differences among localities. An independent two sample T-test was applied to compare the egg counts from Stonehaven (n = 14) and Rosehearty (n = 12 individuals). Scatter plots with a linear correlation line were used to reveal the relationship between egg morphology and certain body measurements. The influence of crowding on parasite morphology was also evaluated by including the values of Lloyd's mean crowding (see Chapter 3 for calculation details). All analyses were conducted using Excel (Microsoft Office Professional Plus 2016) and SPSS (IBM SPSS statistics 23 & 25).

4.3. Results

4.3.1. Dissection and excystment of the metacercariae of *Maritrema* sp.

Different sizes of metacercarial cysts were noticed in the prosoma of *S. balanoides* and were presumed to represent different developmental stages (Figures 4.6-4.7). These metacercariae possessed thin-wall cysts in general. The metacercariae inside the cyst tended to fold on themselves and only a few organs such that only the sucker and / or the caeca were visible. While observing the prosoma of barnacles, larger cysts had relatively thicker walls with two layers, and smaller cysts had thinner walls and only one layer. Some freely moving cercariae without tails were noticed inside the prosoma of barnacles and were assumed to be newly penetrated cercariae (Figure 4.6). When focusing on these cercariae, traces of collecting ducts of the excretory system, the excretory bladder, and the stylet were observed, but not the penetration glands (Figure 4.7).

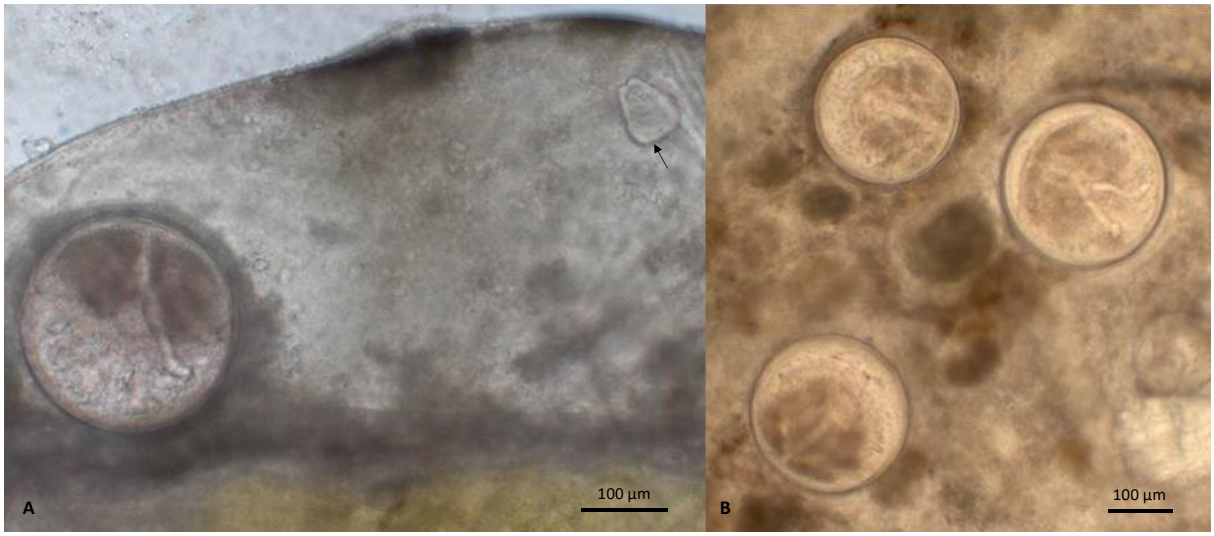


Figure 4.6. Metacercariae of *Maritrema sp.* inside the prosoma of a barnacle (*Semibalanus balanoides*). A. A mature cyst on the left and a cercaria without a tail (arrow) on the right in the prosoma of a barnacle. B. Three mature cysts in the prosoma of a barnacle.

Figure 4.7. Different developmental stages of the metacercarial cysts released from the prosoma of a barnacle (*Semibalanus balanoides*). A. Arrow: thicker matured cysts. Arrowhead: younger cyst with thinner wall. Asterisk: freely moving cercaria without tail. B. Higher magnification of the cercaria inside the barnacle. Arrow: collecting ducts. Arrow head: excretory bladder. Asterisk: the stylet.

After incubation in PBS at 40℃, most of the metacercariae readily excysted within 2 hours. After excystment, actively moving young adults and empty cysts were observed under a stereomicroscope (Figure 4.8). The bodies of newly excysted metacercariae were seen actively contracting.

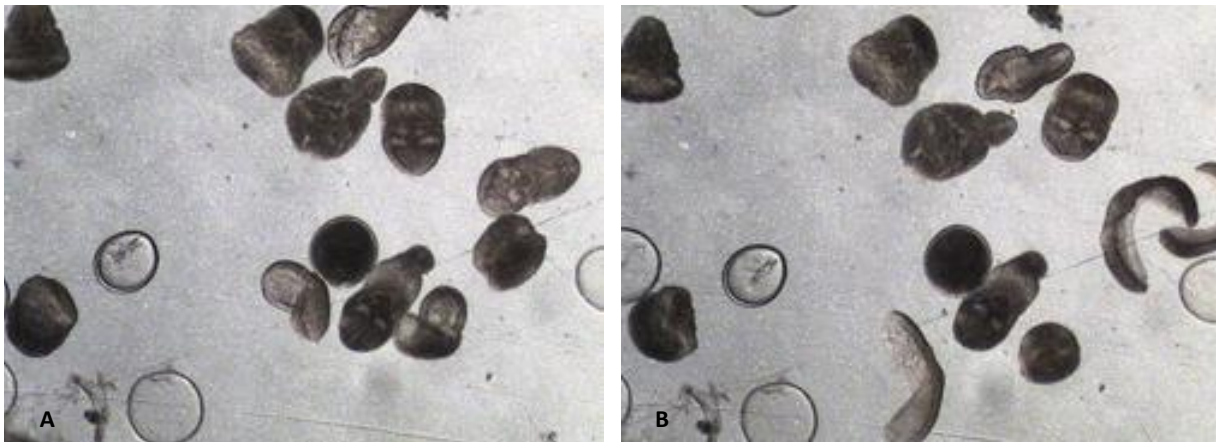


Figure 4.8. A & B. Newly excysted young adults of *Maritrema* sp. The flukes were actively moving. Numerous empty cysts can be observed.

4.3.2. Morphological description and diagnosis of *Maritrema* sp.

4.3.2.1. Descriptions of newly-excysted young adults

This description is based on the newly encysted metacercariae collected from Dunbar Red Rock in 2014 (Figure 4.9). The newly excysted young adults that were first relaxed using Berland's fluid and then stained with Mayer's paracarmine appeared to possess an elongated tongue-like shape, with a

slightly tapered anterior end and a rounded posterior end. The oral sucker was sub-terminal and the ventral sucker situated at the mid body level; the two suckers were almost equal in size. The oral sucker leads into a short prepharynx, which sometimes could not be seen but was sometimes quite elongated. Below the prepharynx was an oval and muscular pharynx and a slender oesophagus which extends to about one-third of the body length. The oesophagus bifurcates into a pair of gut caeca which extend close to the posterior edge of the testes. The bifurcated caeca circumvent the ventral sucker, the cirrus sac, the vagina, and the ovary. A curved, thin-walled cirrus sac lies transversely anterior the ventral sucker, which is wider at the dextral side where the seminal vesicle is located, and it is narrow in the middle and sometimes enlarges again before finally constricting at the genital pore. The posterior edge of the cirrus sac extends beyond the ventral sucker to the level of the ovary. The vagina is curved in a C-shape and joined to the genital pore on the left. The genital atrium is simple and small; the genital pore is unarmed, sinistral and not always discernible. A mass of glandular tissue, which stains light pink with Mayer's paracarmine, surrounding the vagina appeared to be consistent with the vaginal gland identified in Hadley and Castle's (1940) original description. An elongated, oval to triangular-shaped ovary was located dextrally between the cirrus sac and vitellaria, close to and sometimes overlapped by the posterior margin of the cirrus sac, its transverse axis somehow oblique to the mid-body line. The vitellaria follicles form a complete ring which encircle the edge of the hindbody, but always behind the ovary. There are a pair of testes located in the posterior third of the body. The testes appear transversely oval to sub-lobed, their long axis occasionally oblique to the mid-line of the body line. The two testes are not necessarily equal in size. The uterus is already visible in the young adults but there are no signs of eggs being present. The excretory bladder is y-shaped and seems to fuse at the upper end, posterior to the testes. The egg-producing apparatus between the ovary and the testes is a pink mass in stained specimens but is more readily seen in living specimens. The male copulatory organs are also better seen in living specimens. The measurements of the body and internal structures are provided in Table 4.5. Measurements from the original description presented in Hadley and Castle (1940) are included in the table for the purposes of direct comparison.

Table 4.5. Descriptive statistics of the body measurements for the newly excysted young adults from Dunbar Red Rock (n = 120) and the original description from Hadley and Castle (1940) (based on a single live specimen, n = 1). Values in purple represent ranges that do not overlap with the original description.

	Present study			Hadley & Castle (1940)
Variables	Mean	SD	Range	Value
Body length	636.80	71.72	455.50-883.60	576
Body width	209.52	27.42	130.28-269.78	360
Body width/length ratio	0.33	0.05	0.24-0.44	0.625
Oral sucker length	42.09	4.90	28.10-53.21	
Oral sucker width	40.44	4.18	28.93-54.28	55
Prepharynx length	27.81	15.68	0.00#-56.12	19
Pharynx length	22.49	2.99	15.46-33.14	28

Oesophagus length	120.36	22.44	77.85-239.32	150
Ventral sucker length	46.67	5.47	30.21-58.81	
Ventral sucker width	44.94	6.12	26.99-58.86	55
Testes length mean	56.74	7.87	28.79-74.18	80 (left) 74 (right)
Testes width mean	70.81	9.17	32.41-88.83	110 (left and right)
Cirrus sac length	58.85	7.26	38.55-79.90	
Cirrus sac width	137.25	22.35	88.22-312.73	
Ovary width	48.19	6.69	30.95-68.89	70
Ovary length	33.60	5.22	18.58-54.09	50
Sucker ratio	0.91	0.10	0.63-1.20	1

#Value in zero represents organ which is not measurable.

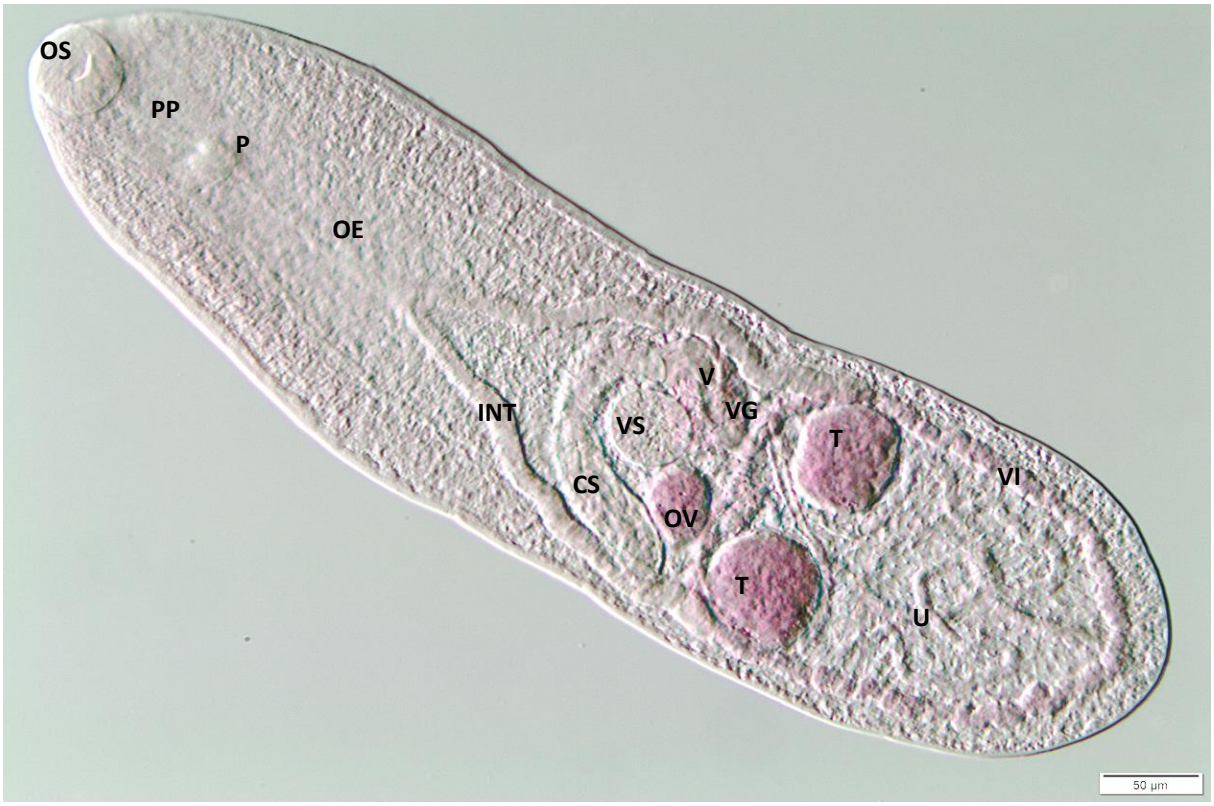


Figure 4.9, Newly excysted young adult relaxed in Berland's fluid, fixed in 80% alcohol and stained with Mayer's paracarmine, ventral view. CS: cirrus sac. INT: intestine caecum. OE: oesophagus. OS: oral sucker. OV: ovary. P: pharynx. PP: prepharynx. T: testes. U: uterus. VS: ventral sucker. V: vagina. VG: vaginal gland. VI: vitellaria.

4.3.2.2. Descriptions of cultured adult of *Maritrema* sp.

These adults were from excysted metacercariae cultured in NCTC 109 medium with antibiotics and 20 or 40% chicken serum at 40 °C for various time. The method used for culturing and the results of the *in vitro* culture will be addressed in Chapter 5.

4.3.2.2.1. Samples from Dunbar

The experimental adults were cultured from metacercariae collected at Dunbar Leisure Pool from *S. balanoides* in 2017. The samples were relaxed in Berland's fluid, fixed in 80% alcohol, and

subsequently stained with Mayer's paracarmine as mentioned above. In general, the morphology of the cultured adults (n = 22) was the same as the newly excysted young adults (n = 120), but the copulatory organs were clearer in detail, the development of the uterus was more advanced with numerous eggs being present, the testes and ovary stained less intensely, the vaginal gland was obscured and stained less intensely (Figure 4.10). The eggs in the distal uterus were brown in colour (Figure 4.10).

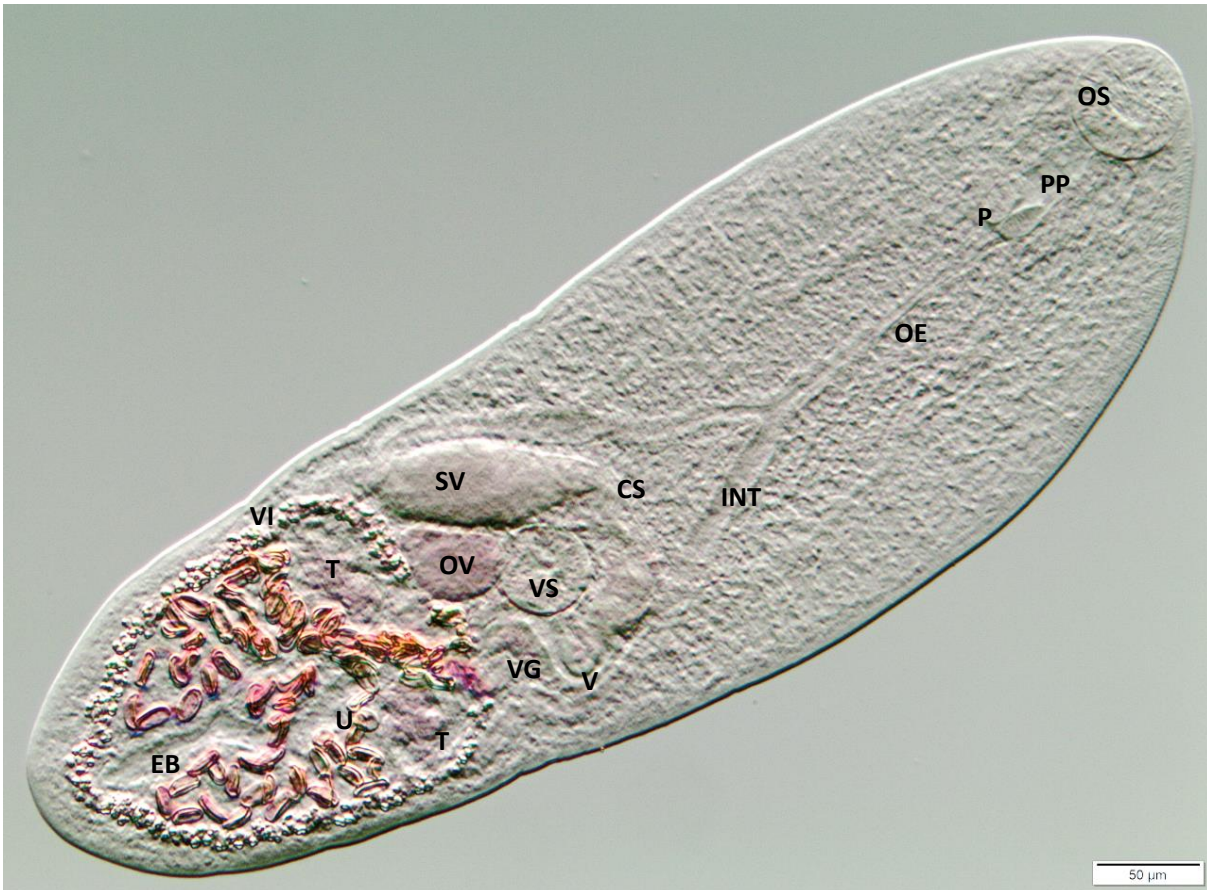


Figure 4.10. A cultured adult (120 hours old, 20% chicken serum) relaxed in Berland's fluid, fixed in 80% alcohol, and stained with Mayer's paracarmine, ventral view. The adult resulted from a cyst collected from *Semibalanus balanoides* collected at Dunbar Leisure Pool in December, 2017. CS: cirrus sac. EB: excretory bladder. INT: intestine caecum. OE: oesophagus. OS: oral sucker. OV: ovary. P: pharynx. PP: prepharynx. SV: seminal vesicle. T: testes. U: uterus. VS: ventral sucker. V: vagina. VG: vaginal gland. VI: vitellaria.

The measurements of the body and the internal organs are shown in Table 4.6, and measurements of the original description from Hadley and Castle (1940) and from Deblock and Tran Van Ky (1966a) are included to permit a direct comparison. The general morphology of the cultured adults was like the fixed specimens, but more details relating to the copulatory organs could be seen, and traces of the collecting ducts of the excretory system could be seen.

The cirrus sac enclosed a small to medium oval seminal vesicle, which was surrounded by prostate gland cells. The seminal vesicle narrowed in the distal end to a tubular region; this tube connected to a bulbous *pars prostatica*. The *pars prostatica* led to an eversible, smooth, and tubular cirrus which contained the ejaculatory duct (Figure 4.11). The cirrus could be occasionally seen protruding from the genital pore. The *pars prostatica* was active, moving back and forth in the living specimen; the moving *pars prostatica* led to convolution of the tubular region between the seminal vesicle and the cirrus. The testes contained some vacuoles; however, this may have been an artefact because of the culture conditions. A fine *vas efferens* could be seen to the anterior side of each testis but could not be tracked further.

The posterior end of the ovary led to a short oviduct, and then immediately posterior to this was an oval fertilization chamber, followed by a *receptaculum seminis* of about the same size, and finally a thin, curved Laurer's canal (Figure 4.11). The proximal end of the uterus joined the *receptaculum seminis* and made an ascending loop across the transverse vitellaria which then extended down to the posterior half of the body and became tortuous in the area encircled by the vitellaria. The distal part of the uterus ascended obliquely between the two testes towards the left to join the vagina.

Table 4.6. Descriptive statistics for the body measurements of five-day old experimental adults (n = 22) collected from *Semibalanus balanoides* from Dunbar Leisure Pool (for egg length and width n = 15 adults, mean for 10 eggs from each adult); and the original description from Hadley and Castle (1940) (preserved adults*, n = 10; living adult**, n = 1); from Deblock and Tran Van Ky (1966a) (sample size unknown). Values in purple represent measurement ranges / values that do not overlap.

	Present study			**Hadley and Castle (1940)**		**Deblock and Tran Van Ky (1966a)**
Variables	Mean	SD	Range	Range*	Value**	Range
Body length	694.28	90.18	560.74 - 901.00	303-566	858	520-570
Body width	228.85	23.07	200.98 - 272.40	192-266	380	240-280
Body width/length ratio	0.34	0.06	0.23 - 0.48	0.47-0.63	0.44	0.41-0.49
Oral sucker length	58.75	4.79	52.03 - 72.62			
Oral sucker width	54.98	4.27	44.70 - 61.33	38-54	55	40-48
Prepharynx length	42.62	20.62	0.00# - 80.52	10-14	34	30-64
Pharynx length	32.56	3.7	27.27 - 41.68	20-30	34	20-30
Pharynx width	24.81	3.05	17.84 - 28.54			20
Oesophagus length	152.46	34.72	92.07 - 209.77	64-100	153	80-110
Caecum length	262.81	24.69	218.99 - 312.36	142-208	220	160-200
Ventral sucker length	50.04	4.92	42.85 - 61.71			
Ventral sucker width	49.31	5.8	42.21 - 60.85	38-50	55	40-50

Testis length mean	44.99	8.23	28.67 - 59.37	36-64 (left) 40-60 (right)	87	50-80
Testis width mean	67.11	15.01	50.09 - 96.90	76-88 (left) 70-100 (right)	112	75-100
Ovary length	37.69	7.13	25.97 - 50.85	30-62	50	40-50
Ovary width	58.51	8.4	36.59 - 72.70	54-82	70	70-80
Cirrus sac length	69.78	8.37	56.22 - 86.38			40-50
Cirrus sac width	156.83	13.38	133.93 - 182.24			140-210
Egg numbers	75.62	55.55	0.00 - 189.00			
Egg length	16.3	2.75	11.98 - 20.22	16-20	20	20
Egg width	8.56	1.04	7.15 - 11.13	6-7	10	10-12
Sucker ratio	1.18	0.09	1.01-1.37	1-1.08	1	1-1.6

#Value in zero represents organ which is not measurable.

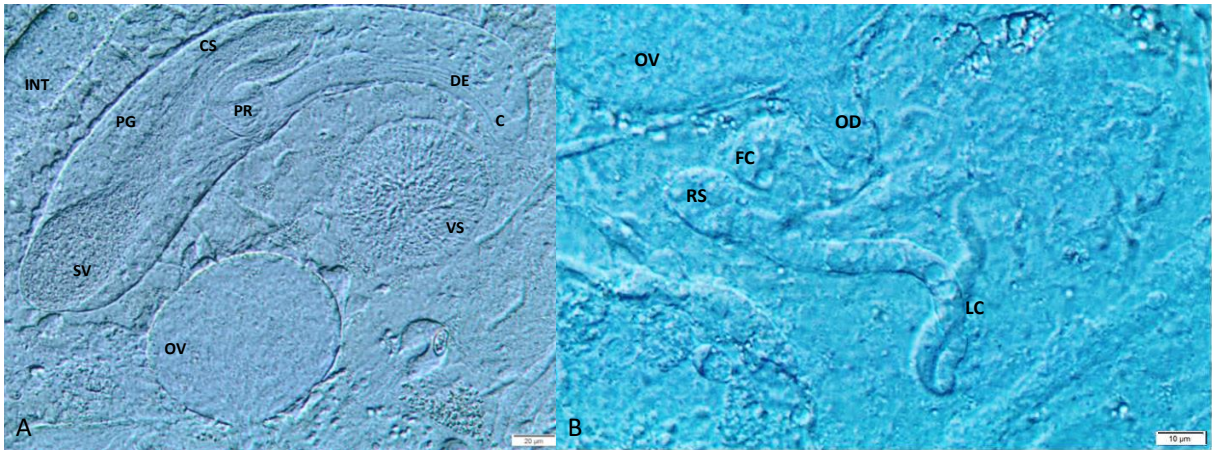


Figure 4.11. A. Male copulatory organs of an adult cultured in NCTC-109 with antibiotics and 20% chicken serum for 24 hours, ventral view. The adult result from cyst collected from *Semibalanus balanoides* collected at Dunbar Leisure Pool in December, 2017. B. Female copulatory organs of adult cultured in NCTC 109 with antibiotics and 40% chicken serum for 72 hours, ventral view. C: cirrus. CS: cirrus sac. DE: *ductus ejaculatorius*. FC: fertilization chamber. INT: intestine caecum. LC: Laurer's canal. RS: *receptaculum seminis*. OD: oviduct. OV: ovary. PG: prostate glands. PR: *pars prostatica*. SV: seminal vesicle. VS: ventral sucker.

The eggs were elongated oval to ovate; these tended to collapse under fixation and only a few eggs contained a large cell, presumably an ovum, were seen. Traces of vitelline globules / cells could be seen in some eggs (Figure 4.12). The eggs in the distal uterus were often brown in colour. In living specimens, eggs were frequently observed full of cells or globules and some of these formed larger clumps or masses concentrated at the two poles (Figure 4.12). Finger-like structures were distributed in the tegument of the forebody in some specimens and these were assumed to be glandular structures and were refractive under light microscopy (Figure 4.13).

Figure 4.12. A. Eggs within an adult cultured in NCTC 109 with antibiotics and 40% CS for 72 hours. The eggs were full of cells or globules with some of these aggregating together. The adult derives from a cyst from *Semibalanus balanoides* collected at Dunbar Leisure Pool in December, 2017. B. Eggs with an ovum in five-day old experimental adults (cultured in NCTC-109 with 20% CS) collected from *Semibalanus balanoides* from Dunbar.

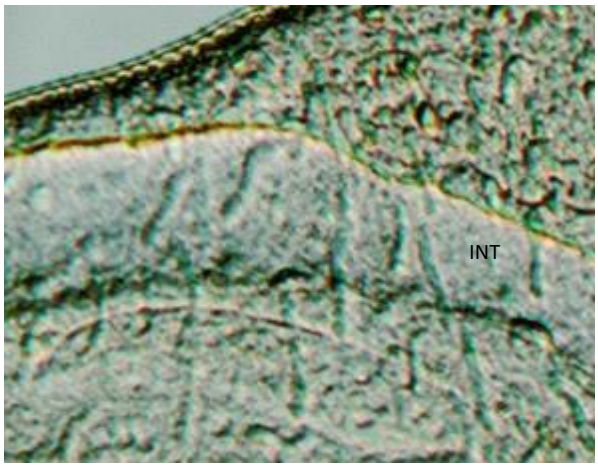


Figure 4.13. Refractive finger-like structures in the tegument observed by phase contrast microscopy in adults cultured in NCTC109 with antibiotics and 20% CS for 24 HRS. These structures were assumed to be glandular structures. INT: intestine caecum.

4.3.2.2.2. Samples collected from Stonehaven and Rosehearty

Metacercarial samples collected from Stonehaven and Rosehearty were also cultured to ovigerous adults; these were cultured in NCTC-109 supplemented with 20% chicken serum. The morphology of these adults was like the adults that were obtained by culture from Dunbar. The same morphometric variables were measured and are summarised in Table 4.7. Measurements of the original description from Hadley and Castle (1940) are also included to permit their direct comparison.

Table 4.7. Descriptive statistics for the body indices of the four-day old adults obtained from experimental culture of material collected from Stonehaven and Rosehearty (n = 20 at both localities, 20% chicken serum.) alongside the measurement values from Hadley and Castle (1940) (preserved adults*, n = 10; live adult**, n = 1) and Deblock and Tran Van Ky (1966a). Values in purple represent measurement ranges / values that do not overlap.

Source	**Stonehaven**			**Rosehearty**			**Hadley and Castle (1940)**		**Deblock and Tran Van Ky (1966a)**
Variables	Mean	SD	Range	Mean	SD	Range	Range*	Value**	Range
Body length	666.08	55.19	559.84 – 752.34	693.83	59.15	603.00 – 808.30	303-566	858	520-570
Body width	213.11	14.33	187.43 – 243.24	214.71	15.74	192.43 – 248.78	192-266	380	240-280
Body width/ length ratio	0.32	0.03	0.28 – 0.36	0.31	0.02	0.27 – 0.36	0.47-0.63		0.41-0.49
Oral sucker length	55.22	5.65	41.70 – 63.99	55.86	4.47	45.94 – 63.95			
Oral sucker width	54.92	2.34	50.59 – 59.76	54.96	3.48	47.04 – 62.79	38-54	55	40-48
Prepharynx length	31.61	12.59	9.33 – 53.22	29.07	14.44	10.36 – 57.72	10-14	34	30-64
Pharynx length	30.12	3.94	23.32 – 41.86	29.1	3.51	24.66 – 37.47	20-30	34	20-30
Pharynx width	26.57	1.44	23.93 – 28.57	26.96	2.09	22.13 – 29.90			20
Oesophagus length	149.01	19.64	123.31 – 191.36	154.94	23.18	114.22 – 192.17	64-100	153	80-110
Caecum length	285.29	29.24	227.71 – 330.81	291.85	27.52	235.94 – 344.27	142-208	239	160-200
Ventral sucker length	49.22	2.34	45.20 – 53.29	49.38	3.53	42.02 – 55.52			
Ventral sucker width	47.12	3.35	40.46 – 54.27	45.8	4.23	39.43 – 53.14	38-50	55	40-50
Testes length average	38.78	6.22	30.34 – 54.57	42.08	4.82	36.32 – 54.10	36-64 (left) 40-60 (right)	87	50-80
Testes width average	56.63	6.56	45.26 – 69.70	57.79	5	50.51 – 69.82	76-88 (left) 70-100 (right)	112	75-100

Ovary length	34.04	6.03	26.72 – 51.19	33.23	3.52	28.74 – 43.83	30-62	50	40-50
Ovary width	53.69	6.63	31.49 – 62.17	52.63	7.01	39.56 – 62.02	54-82	70	70-80
Cirrus sac length	70.51	6.09	57.79 – 78.09	72.13	6.28	61.16 – 87.48			40-50
Cirrus sac width	154.03	12.08	130.49 – 180.87	155.65	8.68	142.55 – 181.48			140-210
Egg numbers	58.29 (n = 14)	29.124	0 – 98	69.75 (n = 12)	57.763	0 – 157			
Egg length	16.04 (n = 10)	1.22	14.31 – 18.55	15.89 (n = 10)	1.25	13.77-17.63	16-20	20	20
Egg width	8.81	0.3	8.36 – 9.37	8.49	0.47	7.92 - 9.17	6-7	10	10-12
Sucker ratio	1.12	0.13	0.88-1.37	1.13	0.1	1.01-1.47	1-1.08	1	1-1.6

4.3.2.3. Study of egg morphology versus body width and length

The relationship of egg length to width was investigated using a linear regression on the data taken from the experimental adults sampled from Stonehaven and Rosehearty. A positive correlation was observed between the mean egg length and the mean egg width (10 individuals, Figure 4.14A), also in the body length and egg number (Figure 4.14B). The relationships between the body width and egg number (Figure 4.14C), the body length and the mean egg length (Figure 4.15A), and the body width and the mean egg length (Figure 4.15B), however, were not clear. A negative correlation was observed between the body length and the mean egg width (Figure 4.16A) and a positive correlation between the body width and the mean egg width (Figure 4.16B). Apart from the relationship between the body length and the egg number in the samples collected from Rosehearty (Figure 4.14B), none of the remaining variables regarding egg morphology and body length / width were significantly correlated. The trend between body length / width and egg morphology is similar between the two localities. Independent sample T-tests of the differences in egg count and egg lengths in the samples collected from Stonehaven and Rosehearty were compared and were not significantly different (p>0.05) despite the mean egg count from Rosehearty being higher (Table 4.8). An independent sample T-test of the egg widths though was significantly different ($t_{177.69}$= 2.3, p = 0.023) with the eggs from Stonehaven being wider (Table 4.8). The samples collected from Dunbar were not included in this analysis due to multiple culture conditions that were applied.

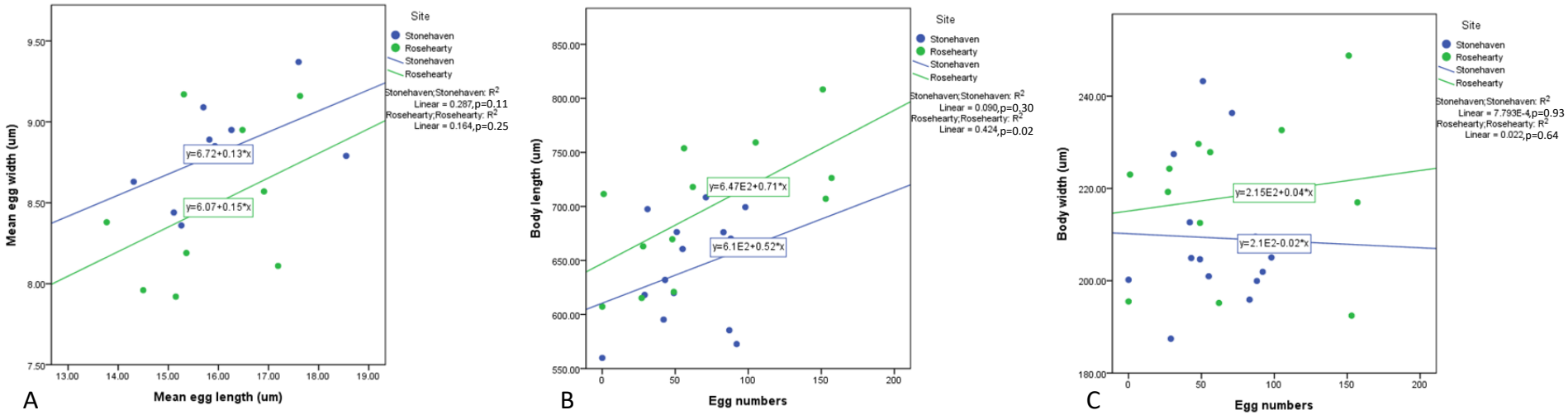


Figure 4.14. A. Scatter plot of the mean egg length vs. the mean egg width. B. Scatter plot of the egg number vs. the body length. C. Scatter plot of the egg numbers vs. the body width. Samples were collected from Stonehaven and Rosehearty. A linear regression line is applied to each data set.

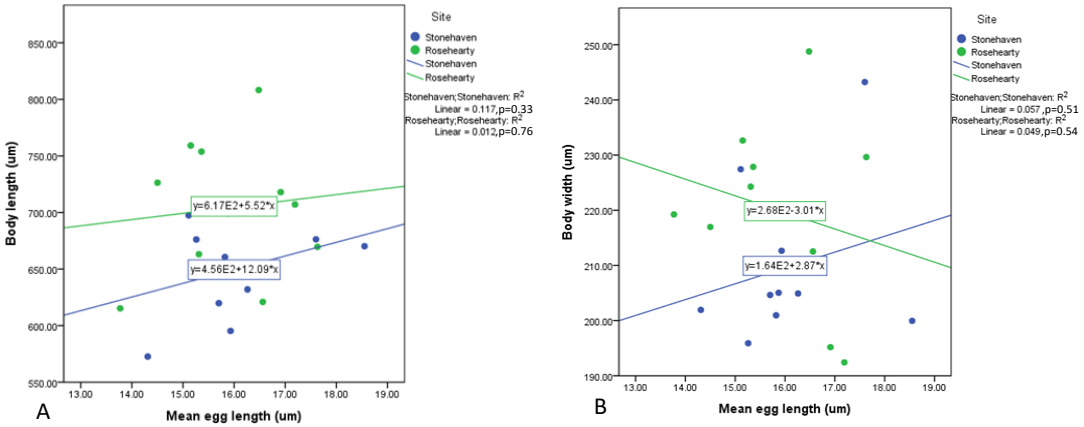


Figure 4.15. A. Scatter plot of the mean egg length vs. the body length. B. Scatter plot of the mean egg length vs. the body width. Samples were collected from Rosehearty and Stonehaven. A linear regression line is applied to each data set.

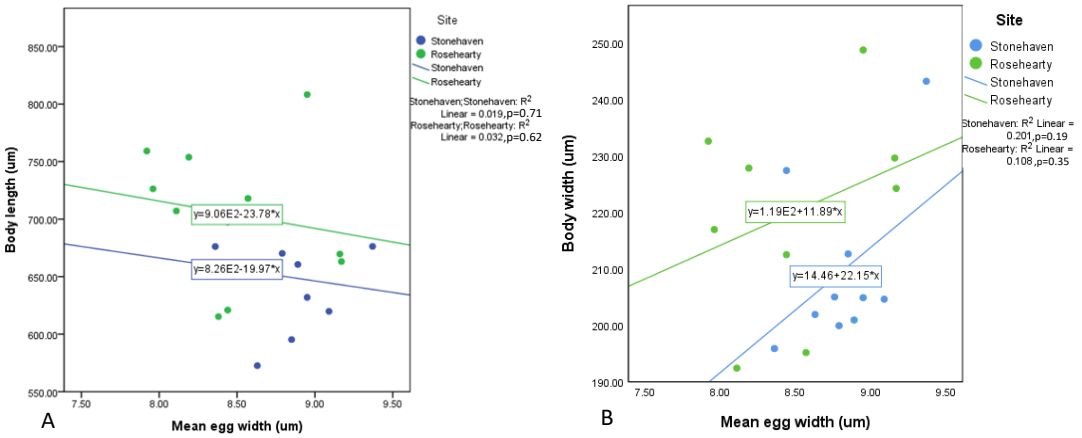


Figure 4.16. A. Scatter plot of the mean egg width vs. the body length. B. Scatter plot of the mean egg width vs. the body width. Samples were collected from Rosehearty and Stonehaven. A linear regression line is applied to each data set.

Table 4.8. Group statistics applied to the egg measurements on samples collected from Stonehaven and Rosehearty.

	Site	N	Mean	SD*	SE*
Egg numbers	Stonehaven	14	58.50	28.88	7.72
	Rosehearty	12	69.75	57.76	16.68
Egg width	Stonehaven	100	8.81	0.82	0.08
	Rosehearty	100	8.48	1.17	0.12
Egg length	Stonehaven	100	16.04	2.04	0.20
	Rosehearty	100	15.89	2.42	0.24

*SD = standard deviation, SE = standard error.

4.3.3. Morphological observation by scanning electron microscope

Under SEM, the metacercariae were often curled or folded in on themselves to the ventral side. There were no appreciable differences between the images acquired by CPD or HMDS methods of preparation therefore the observations from both methods are combined below. The body was oval and a bowl-shaped posture toward the ventral side was sometimes observed. At the anterior end, there was a weakly developed oral sucker and the mouth was observed. A ventral sucker was seen in the middle of the body. (Figure 4.17).

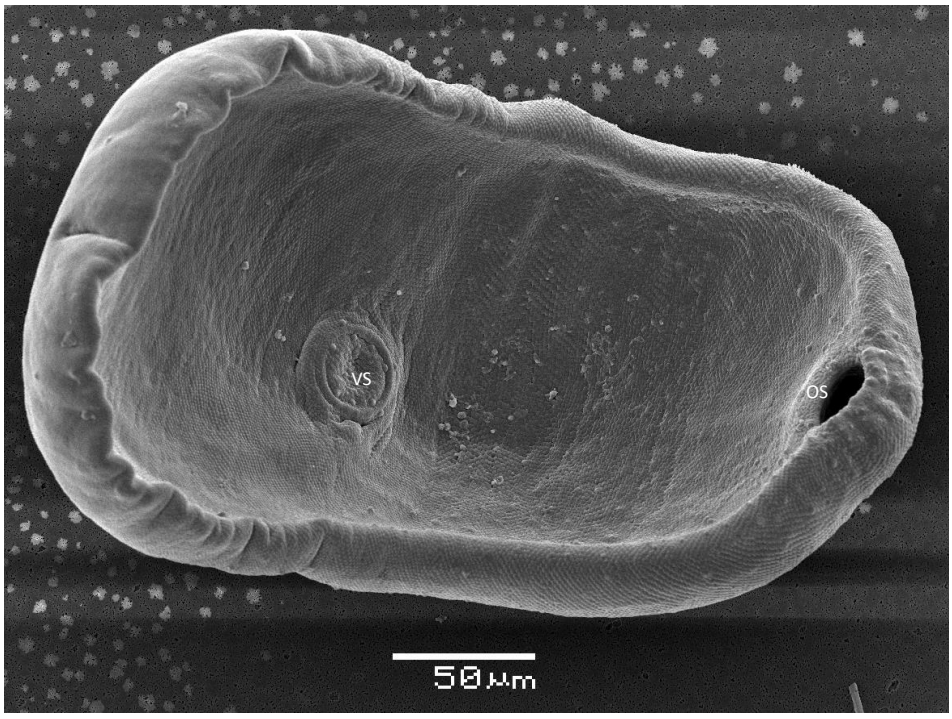


Figure 4.17. Newly excysted young adult under SEM. Whole worm ventral view showing oral and ventral suckers. OS: oral sucker. VS: ventral sucker.

The body was entirely covered with staggered, scale-like spines which were distributed from the anterior to the posterior end on both dorsal and ventral surfaces. The spines were larger and denser anterior to the acetabulum. Below the ventral sucker, the spines diminished and become smaller but did not entirely disappear. The oral sucker was sub-terminal to the anterior end and was surrounded by numerous papillae. The anterior lip of the oral sucker lacked spines whereas the posterior lip had spines with some spines also visible inside the buccal cavity. Whorl-like patterns and ridges were observed in the spine-less region of the anterior lip. The papillae around the oral sucker were symmetrical and contained a single cilium. There were two papillae on the posterior lip, four at the transverse mid-line of the oral sucker, four marking the inner row of the anterior lip, and approximately eight on the outer row of the anterior lip. Observed from the dorsal side, there were also symmetrical papillae on the upper lip of the oral sucker (Figure 4.18).

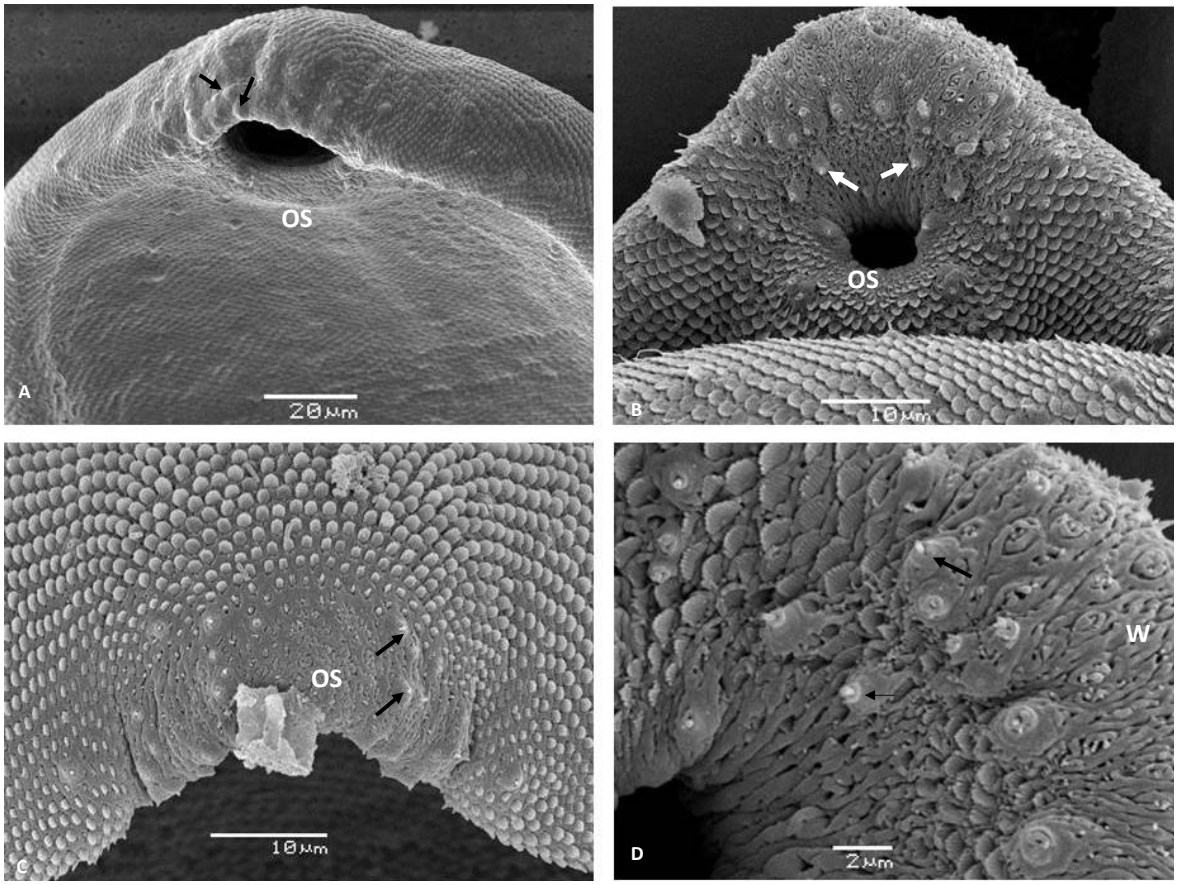


Figure 4.18. Oral sucker (OS) and related papillae on a newly excysted young adult collected from Dunbar in 2013. A. Oral sucker ventral view. Note numerous papillae without cilium (arrows) around the oral sucker. B. Oral sucker ventral view. Note numerous uniciliated papillae (arrows) around the oral sucker. C. Oral sucker dorsal view. Note numerous uniciliated papillae (arrows). D. Higher magnification of image B. Note uniciliated papillae (arrows) and the whorl-like patterns (W) of spineless tegument.

Papillae at the same position on different specimens were found sometimes seen with cilia and sometimes without. The ventral sucker was circular and covered by small spines. Six uniciliated papillae were normally observed on the inner surface of the sucker ring (but in Figure 4.19, seven papillae were found). There were 3-4 uniciliated papillae on the outer surface of the sucker ring (Figure 4.19).

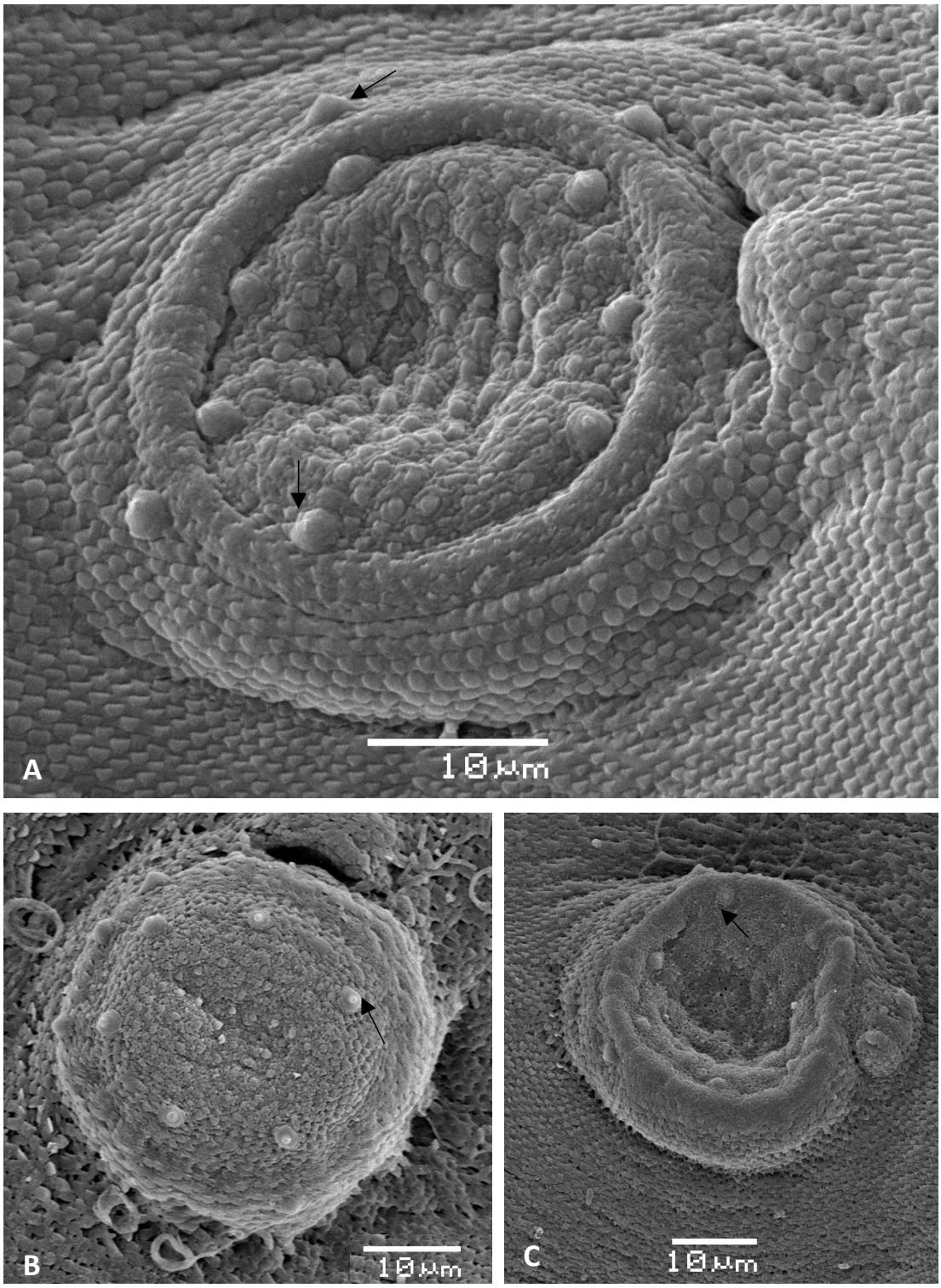


Figure 4.19. Ventral sucker and related papillae on a newly excysted young adult collected from Dunbar in 2013. A. Ventral sucker. There were seven uniciliated papillae (arrow) marking the inner ring of the ventral sucker and three marking an outer ring. B. A protruding ventral sucker. Six uniciliated papillae (arrow) describe an inner ring and 3-4 mark an outer ring. C. Ventral sucker. Six uniciliated papillae (arrow) describing an inner ring on the ventral sucker.

The spines of the anterior ventral surface were larger and wider at their base, measuring and average 1.42 L (±0.14 SD, range 1.27-1.73, n = 10) x 1.66 W (±0.11 SD, range 1.51-1.84, n = 10) µm, and had 10-11 teeth. The spines of the posterior ventral surface were smaller and wider at their base, measuring an average 0.82 L (±0.06 SD, range 0.70-0.90, n = 10) x 0.89 W (± 0.05 SD, range 0.80-1.00, n = 10) µm; the teeth were not clear in the images obtained (Figure 4.20). The spines of the anterior dorsal surface were also larger and wider at their base, measuring an average 1.72 L (± 0.09 SD, range 1.55-1.81, n = 10) x 2.21 W (± 0.06 SD, range 2.15-2.23, n = 10) µm, and had 11-13 teeth (Figure 4.21). The spines of the posterior dorsal surface were smaller and narrower at their base, measuring an average 1.22 L (±0.05 SD, range 1.13-1.31, n = 10) x 0.63 W (±0.05 SD, range 0.56-0.73, n = 10) and had 5-6 teeth (Figure 4.22).

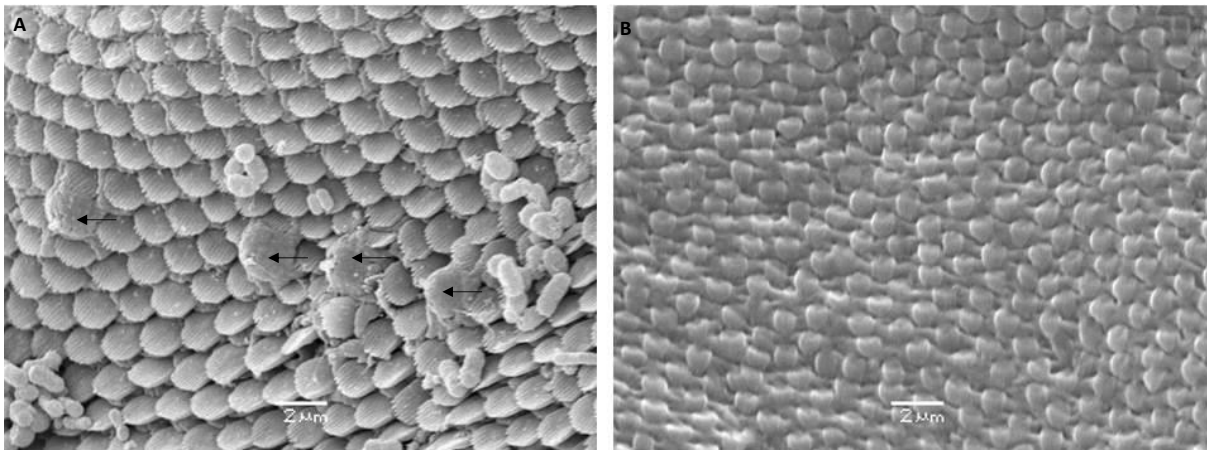


Figure 4.20. Spines on the ventral surface of a newly excysted young adult collected from Dunbar in 2013. A. Spines on the anterior ventral surface. Note the four uniciliated papillae (arrows). B. Spines on the posterior ventral surface.

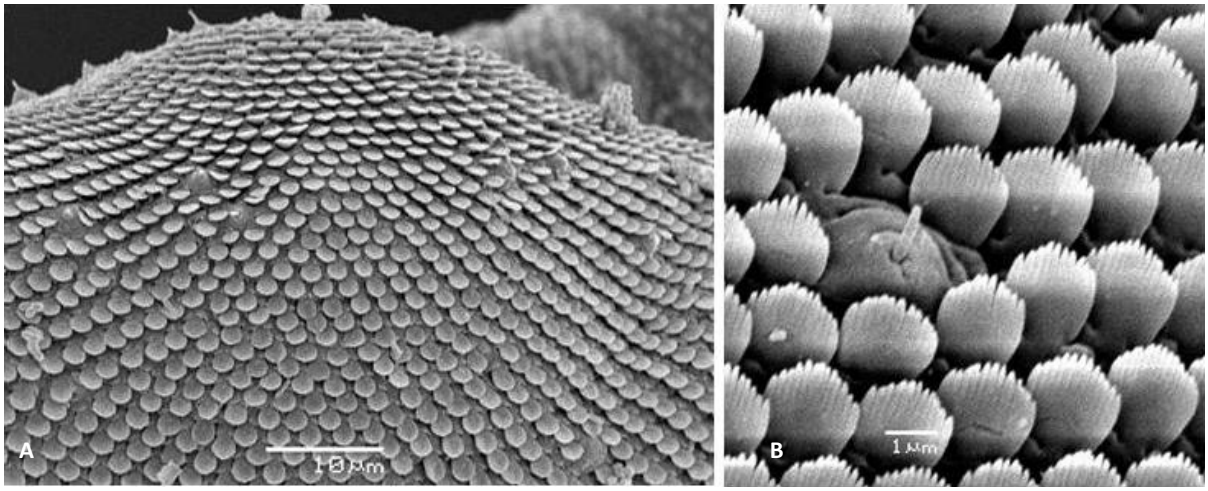


Figure 4.21. A & B. Spines on the anterior dorsal surface of a newly excysted young adult collected from Dunbar in 2013. Note the uniciliated papilla (arrow) in B.

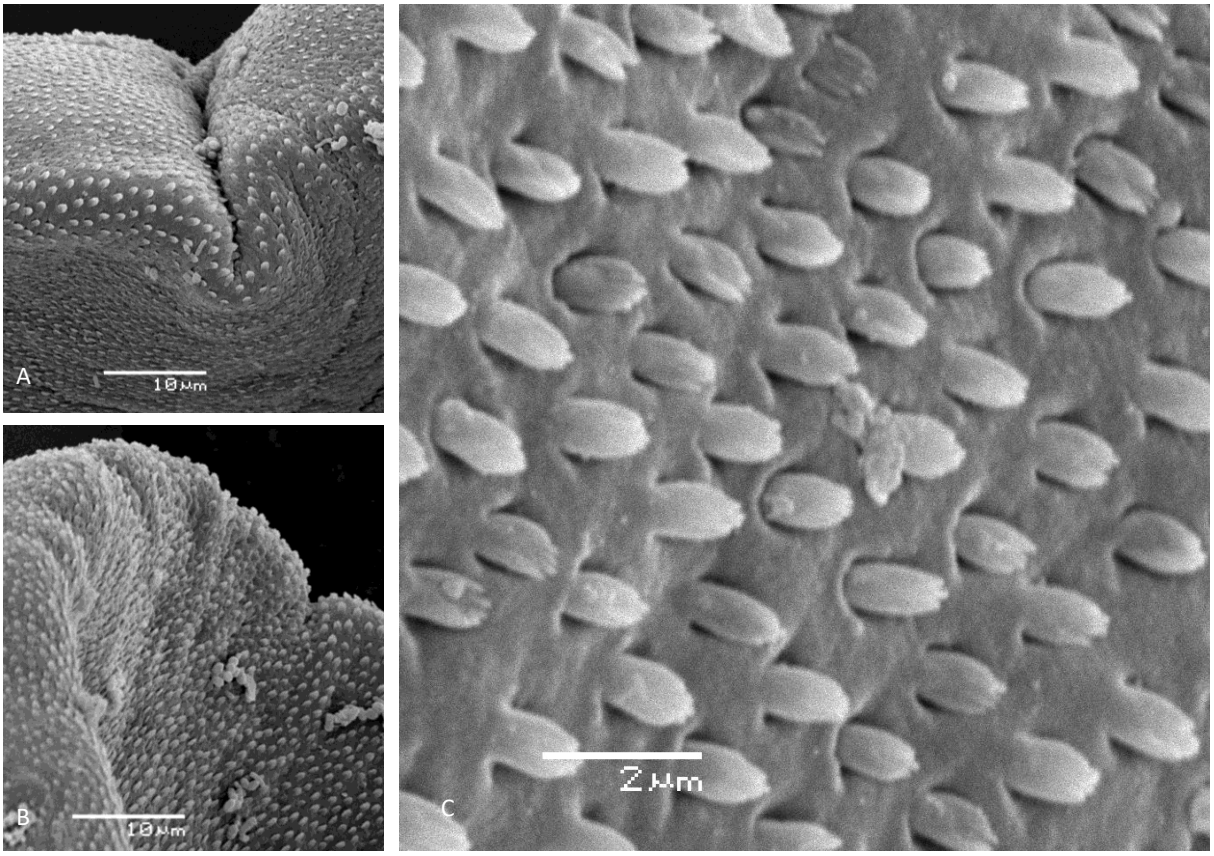


Figure 4.22. Spines on the posterior dorsal surface of a newly excysted young adult collected from Dunbar in 2013. A. Dorsal view. Note the cleft at the middle of posterior end. B. Spines on the posterior dorsal surface. Ventral side at the right. C. Higher magnification of the spines on the posterior dorsal surface.

There were two types of papillae observed. The first type was a uniciliated papilla, which consists of a protuberance with a central pit from the centre of which a cilium protrudes. This type of papillae was found distributed across the body but were numerous around the oral and ventral suckers. A pair of uniciliated papillae were also observed on the posterior ventral surface. The second type of papillae that were seen were dome-shaped papillae. This type of papillae was found, notably, in a group on the ventral surface near the oral sucker. Dome-shaped papillae, however, were seen sparsely distributed on both sides of the body (Figure 4.23). An excretory pore was found dorsal surface at the posterior end of the body. The tegument around excretory pore was spineless but had microvilli (Figure 4.24).

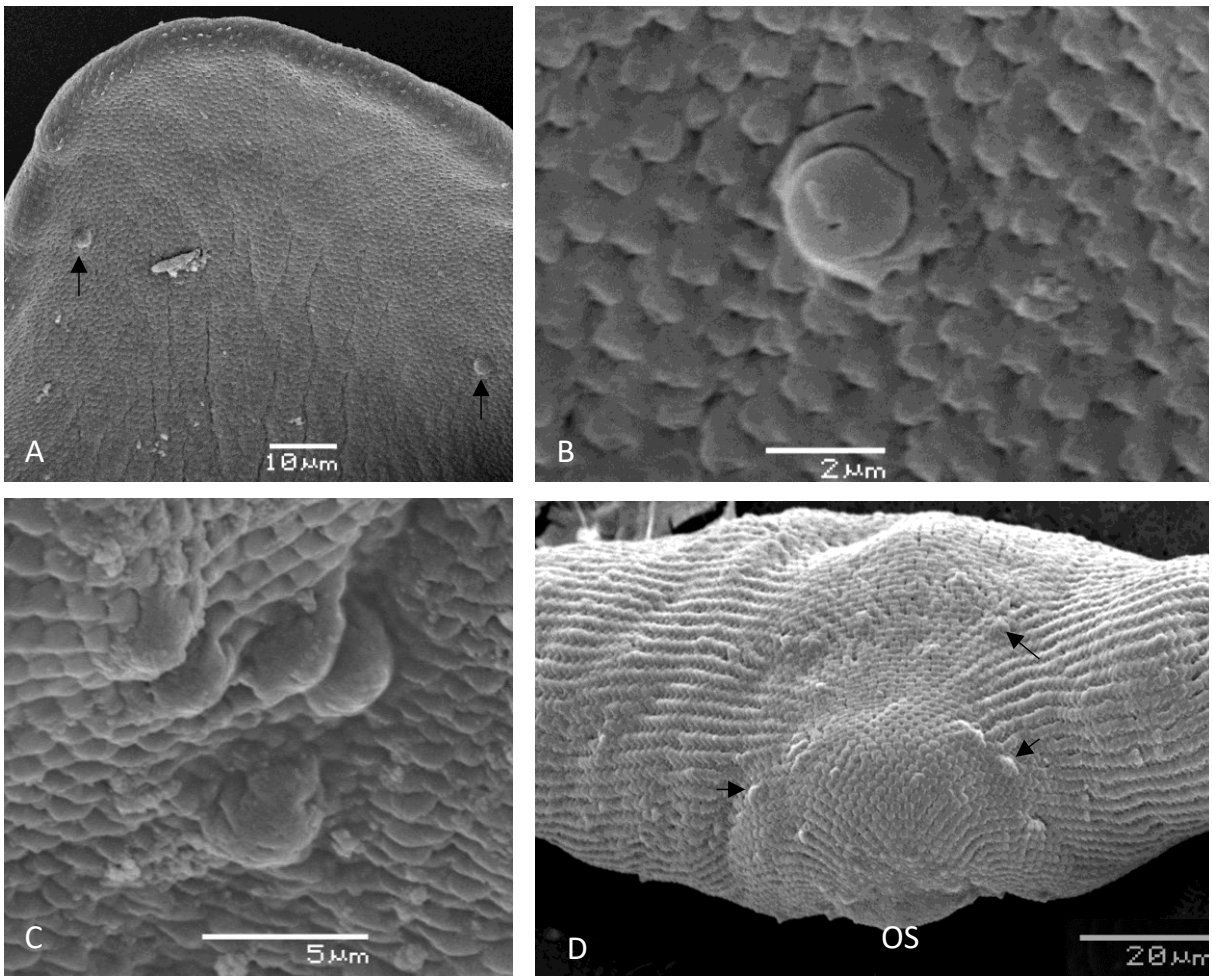


Figure 4.23. Papillae on the body of a newly excysted young adult collected from Dunbar in 2013. A. A pair of papillae (arrows) on the ventral surface in the posterior part of the body. B. Higher magnification of image A revealing the unciliated papilla. C. A group of dome-shaped papillae on the ventral surface at the anterior and near the lateral edge of the body. D. Papillae (arrows) scattered across the anterior dorsal surface. Dorsal view. OS: oral sucker.

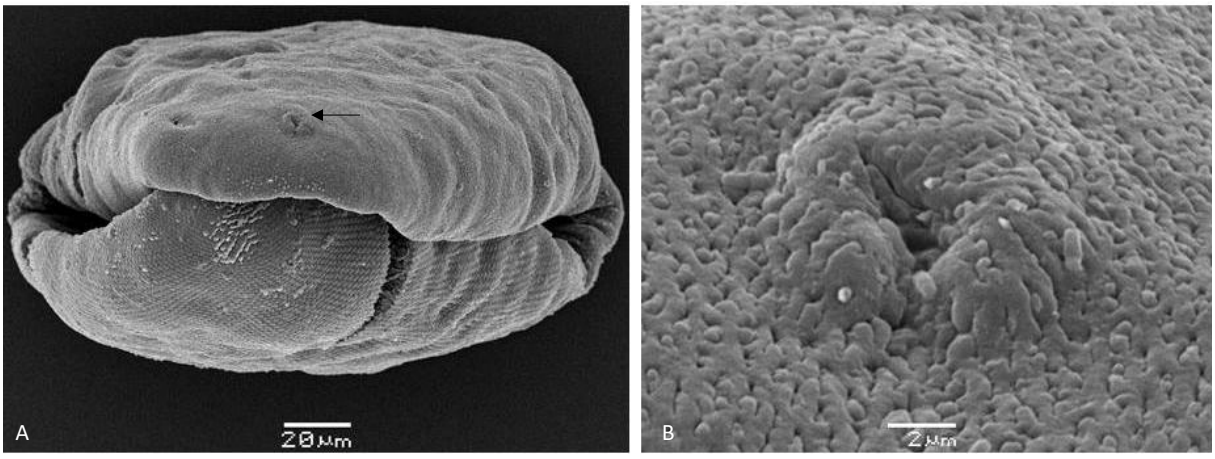


Figure 4.24. An excretory pore on newly excysted young adult collected from Dunbar (2013). A. A folded specimen showing an excretory pore (arrow) on the dorsal surface. B. Higher magnification of the excretory pore. Note that the tegument is spineless in this region and possesses microvilli.

4.3.4. Molecular diagnosis of the metacercariae of *Maritrema* sp.

4.3.4.1. Ribosomal DNA

4.3.4.1.1. 18S rDNA

Trimmed sequences from 16 amplicons (each 739 bases) were obtained from the metacercariae collected from *S. balanoides* at Dunbar in 2016 and were found to be identical in base composition. Comparing the consensus sequence (see Appendix 34) with those on GenBank, the most similar sequence was *Maritrema oocysta* (AJ287534; 96% identity, 100% coverage), including 12 variable sites and 5 gaps. The top 30 hits (identity from 92%-96%), however, cover species across many digenean families. This suggests that the marker of choice is rather conservative. Also, the primer set TC (see Table 4.2) was found to amplify the 18S rDNA from the host (a 705 bp fragment, see Appendix 35, 100% identical to *Semibalanus balanoides* isolate MT01231 (KJ193741; 100% coverage)), and thus is unsuitable for robust molecular diagnosis. A phylogenetic inference at the superfamily level was conducted by comparing sequences from GenBank (Figure 4.26, sources of sequences see Table 4.3). *Fasciola gigantica* (Fasciolidae) was chosen as an out group. In Figure 4.25A, the evolutionary history was inferred using the Neighbour-Joining method (NJ) (Saitou *et al*., 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985), with a total of 763 positions in the final dataset. In Figure 4.25B, the evolutionary history was inferred using the Maximum Likelihood method (ML) based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985), with a total of 719 positions in the final dataset. In Figure 4.25C, the evolutionary history was inferred using the Maximum Parsimony method (MP). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985), with a total of 719 positions in the final dataset. All three analyses involved 17 nucleotide sequences.

In the three algorithms used (NJ, ML & MP), the target parasite (*Maritrema* sp.) from Dunbar always grouped with *M. oocysta*, while *Microphallus turgidus* always grouped with *Microphallus primas*. The other *Microphallus* species, i.e., *Microphallus fusiformis* grouped with the two Pleurogenidae and two Prosogonimidae species with low nodal support in the NJ and ML trees. In the MP tree, partition between *M. fusiformis*, the *Maritrema* clade, the *Microphallus* clade and the Pleurohenidae + Prosogonimidae clade collapsed, with these four clades forming a single large group.

In the three algorithms applied, although the topology obtained was not the same, the Microphallidae + Pleurogenidae + Prosogonimidae is monophyletic, and form a sister group with the Troglotrematidae + Orchipedidae + Omphalometridae + Brachyladiidae. These two sister groups were well separated from the more basal Opisthorchiidae + Heterophyidae clade. In the MP tree, the

Microphallidae + Pleurogenidae + Prosogonimidae was the most derived group but not in the NL and ML trees. *Maritrema* always appeared more basal than *Microphallus* in all three topologies that were obtained.

4.3.4.1.2. The partial 5.8S + full ITS2 + partial 28S rDNA fragment

Amplicons from the 8 metacercariae collected from Dunbar in 2016, 1 metacercariae from Dunbar collected in 2017, and 8 metacercariae collected from both Stonehaven in 2018 and from Rosehearty in 2018 were successfully sequenced by primer set 3S (see Table 4.2). They were all identical in length (524 bases, see Appendix 36) and near identical in their base composition, except that two of the samples from Stonehaven were detected to have a base substitution at position 293 (A / G polymorphism - A instead of G consensus). Comparing the consensus sequence to others on GeneBank, the sequence was identical to *Maritrema arenaria* (HM584171) (100% identity and coverage), and the remaining hits either displayed large gaps or lower identity. Furthermore, this primer set was used to successfully amplify other *Maritrema / Microphallus /* echinostomid / heterophyid species. One amplicon from *M. poulini* (kindly shared by Dr. Bronwen Presswell from the University of Otago, New Zealand, sequences see Appendix 37) showed 97% identity to *Maritrema brevisacciferum* isolate V11 (KT355825), with 11 variable sites and 5 gaps. One amplicon from a *Microphallus* sp. metacercaria ex *Littorina* sp. isolated during this study showed 100% identity to *Microphallus pygmaeus* (HM584190) (see Appendix 16). As the *M. pygmaeus* sequence is already available on GenBank, the sequences obtained here were not used for a phylogenetic study.

Specimens of adult *M. deblocki* (shared by Dr. Presswell) were detected with multiple bands from the preliminary PCR amplification, therefore, gel extraction and cloning were conducted to investigate each band more closely. The electrophoresis gel including the two *M. deblocki* specimens prior to cloning are shown in Figure 4.25. Of the two, a sequence corresponding to *M. deblocki* and one to a fish were obtained from the first specimen. From the second specimen, eight clones were obtained - 4 products matched *Microphallus* spp., 3 products matched the target parasite, and 1 product matched *Littorina* sp. The target sequence from the first specimen comprised 470 bases (named *Maritrema deblocki* Md-3), and was 95% identical to *Maritrema brevisacciferum* (KT355825) (coverage 100%), with 15 gaps and 9 variable sites when compared to KTW55825. The fish sequence comprised 548 bases and was most similar to *Coregonus fontanae* (JQ731757) (93% identical, coverage 98%). When the 8 cloned sequences from the second specimen were aligned, 3 distinct contigs were identified. The consensus sequence for Contig1 (442 bases) gave a perfect match (100% identity, 100% coverage) to *Microphallus pygmaeus* (HM584190), the consensus for Contig 2 (470 bases) were most similar to *Maritrema brevisacciferum* (KT355825) (94% identical, coverage 100%), and Contig 3 (497 bases) was most similar to *Littorina littorea* (AF494750) (99% identity, coverage 86%). When aligned (see

Appendix 38), the 3 clones of *M. deblocki* and the sequence extracted from the gel, resulted in 10 polymorphic sites being identified, and the consensus (see Appendix 39) was 93% identical to *Maritrema brevisacciferum* (KT355825, 100% coverage).

Figure 4.25. Electrophoresis of the *Martitrema deblocki* samples. Clonal sequence analysis of the Md-1 specimen produced 3 distinct contigs, while two contigs were identified for Md-3.

A phylogenetic inference at genus level was conducted by comparing sequences retrieved from GeneBank (Figure 4.27, sources of sequences see Table 4.4). *Prothogonimus cuneatus* (Prosthogonimidae) was chosen as an outgroup. In Figure 4.27A, the evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). In Figure 4.27B, the evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). In Figure 4.27C, the evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). All three analyses involved 23 nucleotide sequences, and a total of 540 positions were included in the final dataset.

It was clear from the three algorithms (NJ, ML and MP trees) that *Maritrema* spp. always formed a monophyletic group, and *Microphallus* spp. always formed a monophyletic group. The 4 sequences of *M. deblocki* always clustered together, and the 3 sequences of *Maritrema* sp. always clustered together with the *M. arenaria* isolate Maa from GeneBank (HM584171). The topology of the MP and ML trees were similar: *M. eroliae* clustered with *M. novaezealandense* and with *M. oocysta,* which formed the most basal group. This group was closer to the group formed by *M. brevisacciferum* + *M.*

poulini and the four *M. deblocki* samples. The two groups mentioned above together with *M. subdolum* formed a monophyletic group and was the sister group of *M. arenaria* + the three *Maritrema* sp. provided through this study. In the NJ tree, *M. eroliae* + *M. novaezealandense* was still the most basal species, followed by *M. oocysta*, and next by *M. poulini* + *M. brevisacciferum* + the 4 *M. deblocki* samples. These 3 clades formed a monophyletic group. *Maritrema subdolum* is the sister species of the above-mentioned groups. These three clades + *M. subdolum* were the sister group of *M. arenaria* + the three *Maritrema* sp. generated in this study (see Figure 4.27).

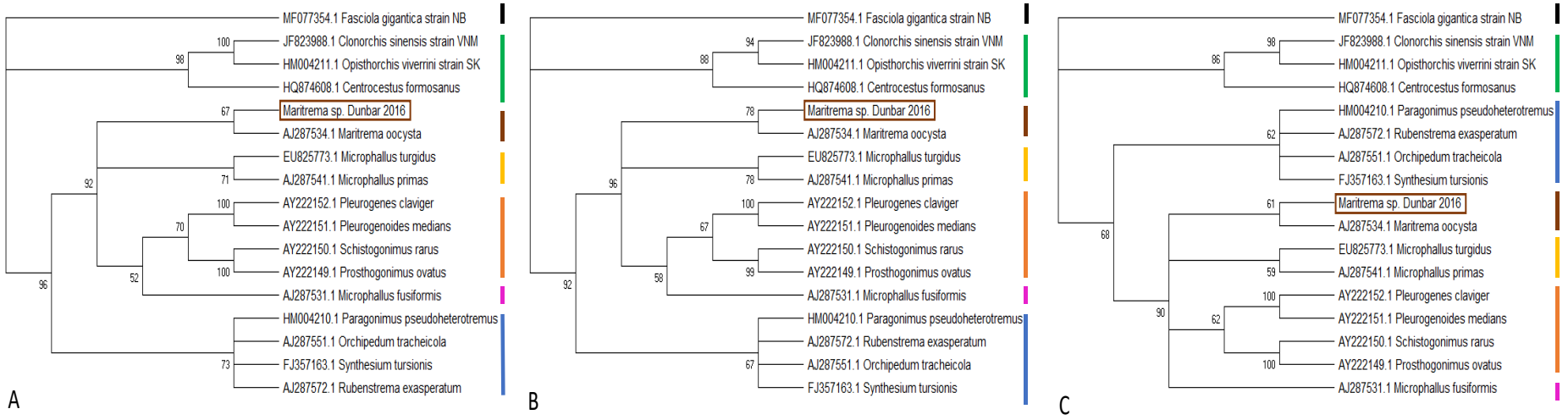


Figure 4.26. Molecular phylogenetic analysis using 18S rDNA for *Maritrema* sp. collected from Dunbar. Taxa are grouped by a unique colour code to facilitate a comparison between them. The target sequence from the present study is indicated by a brown rectangle on each tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). A. Neighbour-Joining method. B. Maximum Likelihood method. C. Maximum Parsimony method.

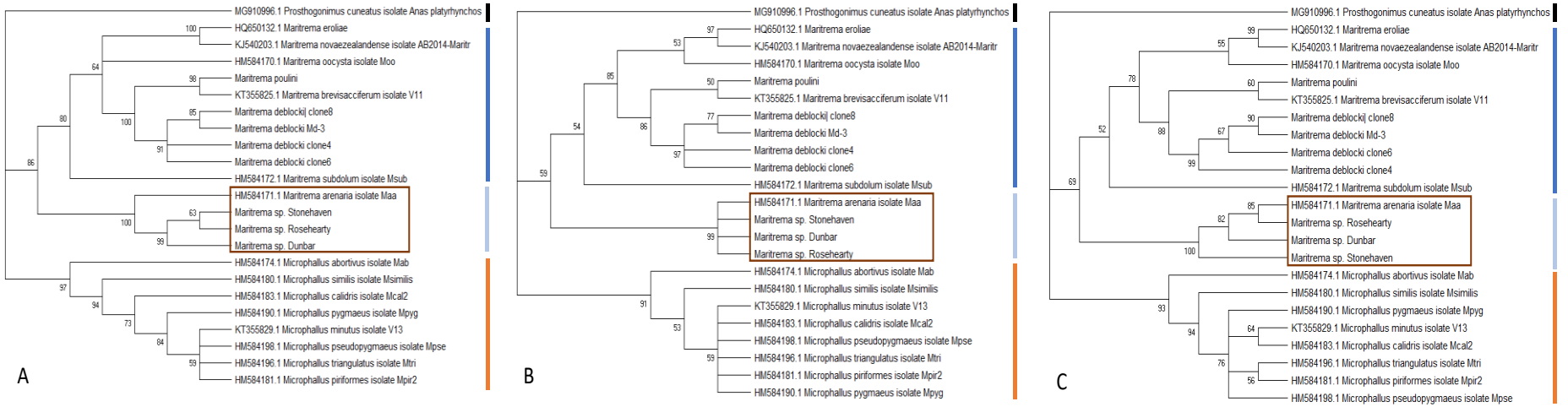


Figure 4.27. Molecular phylogenetic analysis using partial 5.8S + full ITS2 + partial 28S rDNA fragment for *Maritrema* sp. collected from Dunbar. Taxa are grouped by a unique colour code to facilitate a comparison between them. The target sequence from the present study is indicated by a brown rectangle on each tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). A. Neighbour-Joining method. B. Maximum Likelihood method. C. Maximum Parsimony method.

4.3.5. Morphometric study

4.3.5.1. MANOVA for whole area and local scale study

The MANOVA test for the local scale and whole area study of metacercariae from Dunbar revealed a significant multivariate main effect; with Wilks' Lambda = 0.618, F = 9.781, p <0.0001, partial eta squared= 0.382, observed power = 1. The Box's test for covariance matrices was highly significant (F = 3.113, p<0.0001), leading to rejection of the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups. A significant univariate main effect was found for oral sucker L, oral sucker W, pharynx L, ventral sucker L, testis L, testis W, cirrus sac L and ovary W (Table 4.9). When comparing these variables at the two study scales, samples from the local scale study were always larger than those from whole area study. A comparison was made for the coefficient of variance (CV) of each variable for the two studied scales (Table 4.10). A value over 0.15 was considered large CV. The CVs in the whole area scale were often larger than those in the local scale, with more CV values larger than 0.15 being found in the whole area scale. Lloyd's mean crowding for the local scale study was 11.22, while for the whole area scale was 47.64, showing that metacercariae in the whole area scale on average experience more intense crowding (intra-species competition) than those in the local scale.

Table 4.9. Tests of between-subject effects of metacercariae from the Dunbar-local scale study and from the whole area study. Significant values are shown in a bold font.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[p]
Scale	Body length	3466.74	1	3466.74	0.65	0.419	0.00	0.65	0.13
	Body width	751.64	1	751.64	1.00	0.319	0.00	1.00	0.17
	Oral sucker L	985.70	1	985.70	44.41	**<0.001**	0.15	44.41	1.00
	Oral sucker W	260.24	1	260.24	14.24	**<0.001**	0.05	14.24	0.96
	Prepharynx L	535.95	1	535.95	2.28	0.133	0.01	2.28	0.32
	Pharynx L	722.19	1	722.19	64.79	**<0.001**	0.21	64.79	1.00
	Oesophagus L	939.70	1	939.70	1.54	0.216	0.01	1.54	0.24
	Ventral sucker L	245.66	1	245.66	9.33	**0.002**	0.04	9.33	0.86
	Ventral sucker W	44.31	1	44.31	1.43	0.233	0.01	1.43	0.22
	Testis L average	253.02	1	253.02	4.97	**0.027**	0.02	4.97	0.60

Testis W average	599.94	1	599.94	8.04	**0.005**	0.03	8.04	0.81
Cirrus sac L	328.46	1	328.46	4.58	**0.033**	0.02	4.58	0.57
Cirrus sac W	19.98	1	19.98	0.06	0.806	0.00	0.06	0.06
Ovary W	474.36	1	474.36	10.99	**0.001**	0.04	10.99	0.91
Ovary L	23.75	1	23.75	0.94	0.334	0.00	0.94	0.16

Table 4.10. Comparison of the coefficient of variance (CV) of the metacercariae collected for the local scale and the whole area studies. Values over 0.15 are given in a bold, purple font.

Variables	CV (local scale)	CV (whole area)
Body length	0.11	0.11
Body width	0.13	0.13
Oral sucker length	0.10	0.12
Oral sucker width	0.10	0.10
Prepharynx length	**0.49**	**0.56**
Pharynx length	0.14	0.13
Oesophagus length	**0.21**	**0.19**
Ventral sucker length	0.10	0.12
Ventral sucker width	0.11	0.14
Testis width average	0.11	0.13
Testis length average	0.11	0.14
Cirrus sac width	0.10	**0.16**
Cirrus sac length	0.15	0.12
Ovary width	0.13	0.14
Ovary length	0.14	**0.16**

4.3.5.2. Principal component analysis of metacercariae

The following analysis was to test the hypothesis that there may be minor morphological differences between metacercariae collected from different localities. Although the differences are not large enough to separate them as different species, the degree of morphological differences may be correlated to distance between sampling localities.

4.3.5.2.1. Local scale study

For the local scale study, 133 metacercariae were analysed in total and 15 variables were included. Firstly the mean, SD and the range of these variables were examined (Table 4.11). Normality tests were carried out for these variables and most of them were normally distributed and therefore no transformation was performed. Based on the eigenvalues suggested that 2-3 informative components could be extracted.

The correlation matrix was provided for reference (Table 4.12). The results of a Kaiser-Meyer-Olkin measure of sampling adequacy was 0.854 (with 1 being best) and Bartlett's Test of sphericity was highly significant (p<0.0001) indicating there are underlying factors contributing to the variances and that there are correlations among the variables. The 15 variables that were included had a CV of around 0.10-0.15, except for the prepharynx and oesophagus which had higher CV values; the CV for the oesophagus was notably high (see Table 4.10). This is reasonable because the length of these two organs is largely affected by body contraction. Also, body length was highly correlated with oesophagus length in the correlation matrix (see Table 4.12). Three components were extracted according to eigenvalue (>1). When examining the compositions of the three components extracted (principal components one, two and three; PC1-3, see Table 4.14), the values were mostly positive. The first three components together explain 62.47 % of the total variance, with PC1 accounting for 41.87 %, PC2 accounting for 12.80 %, and PC3 accounting for 7.75 % of the variance (Table 4.13). When looking at the pattern matrix (Table 4.14), it can be seen that PC1 comprised body W, testis W, ventral sucker W, ventral sucker L, cirrus sac W, ovary W, testis L, and oral sucker L; PC2 comprised body length, oesophagus L, prepharynx L, cirrus sac L, and ovary L; while PC3 comprised pharynx L only. Oral sucker W and L was found to be cross correlated (showing up in two components with high values). There were no clusters relating closely to the five sampling sites. When coding the sampling sites using different colours according to their distance from land, no structures were evident from the PCA plots either (see Figures 3.35-3.36).

Table 4.11. Descriptive statistics of metacercarial morphometric variables from local scale study (n = 133)

Variables	Mean	SD	Range
Body length	644.21	73.73	449.22-875.60
Body width	212.97	27.50	136.97-295.37
Oral sucker L	46.04	4.53	33.99-64.12
Oral sucker W	42.47	4.36	31.07-54.87
Prepharynx L	30.72	15.04	0.00-63.21
Pharynx L	25.87	3.63	17.28-37.25
Oesophagus L	124.22	26.59	69.33-241.41
Ventral sucker L	48.64	4.80	35.24-65.69
Ventral sucker W	44.10	5.01	31.19-56.49
Testis W average	73.89	8.13	51.41-99.66
Testis L average	58.74	6.41	41.06-73.48
Cirrus sac W	137.81	13.31	94.52-174.98
Cirrus sac L	61.14	9.43	38.31-99.68
Ovary W	50.93	6.46	37.04-66.57
Ovary L	34.21	4.87	22.77-61.79

Table 4.12. Correlation matrix of the body morphometrics made on the metacercariae collected from Dunbar – the local scale study (n = 133). Values >0.7 that are correlated are highlighted in a bold font while values <0.1 suggest they are independent of one another.

		Body length	Body width	Oral sucker L	Oral sucker W	Prepharynx L	Pharynx L	Oesophagus L	Ventral sucker L	Ventral sucker W	Testis W	Testis L	Cirrus sac W	Cirrus sac L	Ovary W	Ovary L
Correlation	Body length	1.00														
	Body width	0.08	1.00													
	Oral sucker L	0.33	0.52	1.00												
	Oral sucker W	0.43	0.40	**0.78**	1.00											
	Prepharynx L	0.42	0.02	0.12	0.27	1.00										
	Pharynx L	0.25	0.07	0.33	0.31	0.01	1.00									
	Oesophagus L	0.66	-0.01	0.20	0.36	0.16	0.17	1.00								
	Ventral sucker L	0.42	0.58	0.57	0.47	0.15	0.20	0.29	1.00							
	Ventral sucker W	0.34	0.65	0.51	0.41	0.12	0.14	0.22	**0.77**	1.00						
	Testis W	0.23	**0.76**	0.53	0.41	0.08	0.09	0.16	0.55	0.62	1.00					
	Testis L	0.56	0.45	0.54	0.50	0.24	0.16	0.39	0.60	0.61	0.63	1.00				
	Cirrus sac W	0.36	0.62	0.53	0.50	0.17	0.17	0.28	0.67	0.62	0.61	0.59	1.00			
	Cirrus sac L	0.45	0.07	0.25	0.28	0.26	0.18	0.23	0.23	0.28	0.09	0.31	0.30	1.00		
	Ovary W	0.33	0.50	0.41	0.39	0.15	0.17	0.21	0.53	0.48	0.55	0.55	0.47	0.28	1.00	
	Ovary L	0.37	0.19	0.28	0.31	0.17	0.00	0.30	0.29	0.33	0.29	0.46	0.31	0.19	0.19	1.00

Table 4.13. Total variance explained among samples analysed from the local scale study.

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings[a]
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total
1	6.28	41.87	41.87	6.28	41.87	41.87	5.74
2	1.92	12.80	54.67	1.92	12.80	54.67	3.53
3	1.16	7.75	62.42	1.16	7.75	62.42	1.81

Extraction Method: Principal Component Analysis.

a. When components are correlated, sums of squared loadings cannot be added to obtain a total variance.

Table 4.14. Pattern matrix[a] for the local scale study showing the relative contribution of each morphometric measurement to the overall separation of specimens. Values >±0.6 are highlighted in purple suggesting a strong contribution, while parameters <±0.1 suggest they make a negligible contribution to the separation of specimens.

	Component		
	1	2	3
Body width	0.96*	-0.32	-0.05
Testis W	0.91	-0.11	-0.09
Ventral sucker W	0.82	0.07	-0.04
Ventral sucker L	0.76	0.12	0.08
Cirrus sac W	0.75	0.12	0.05
Ovary W	0.63	0.10	0.06
Testis L	0.63	0.40	-0.04
Oral sucker L	0.62	0.02	0.47**
Body length	0.05	0.85	0.12
Oesophagus L	-0.04	0.73	0.09
Prepharynx L	-0.06	0.62	-0.11
Cirrus sac L	0.02	0.53	0.21
Ovary L	0.28	0.50	-0.29
Pharynx L	-0.02	0.02	0.86
Oral sucker W	0.43**	0.25	0.47**

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.[a]

a. Rotation converged in 6 iterations. *Value in purple shows the major contributors for each component. **Value in orange shows the variable that is cross correlated.

When plotting the three factor scores of each component on the X and Y axes and annotating by site, no particular grouping can be detected, neither for factor score 1 vs. 2 (Figure 4.28) nor factor 2

vs. 3 (Figure 4.29). For each plot, samples from all five sampling points were distributed evenly and overlapped.

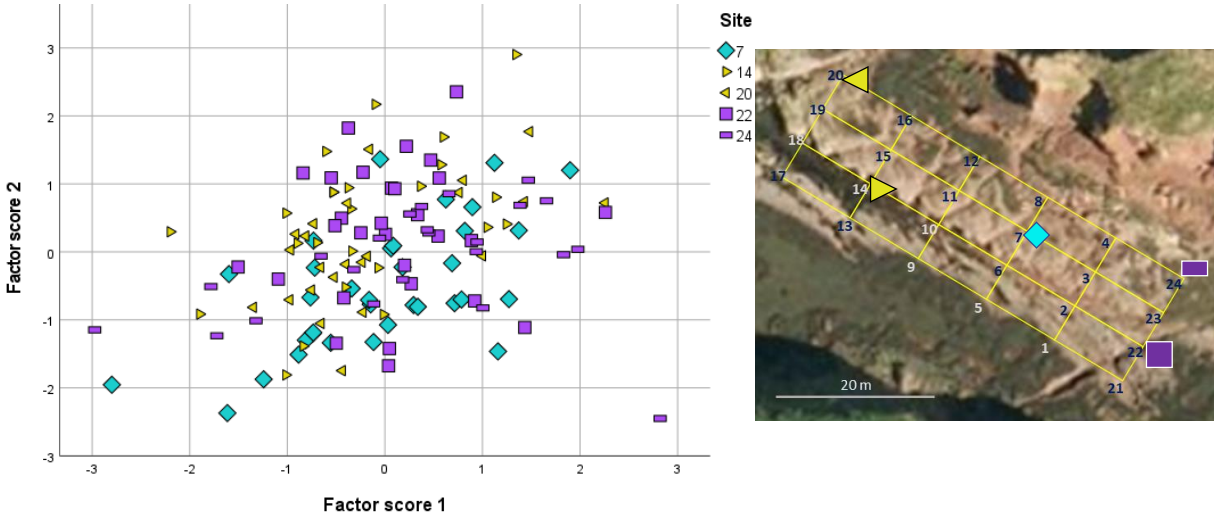


Figure 4.28. Principal component analysis for metacercaria from Dunbar for the local scale study where the factor scores 1 are plotted against the scores for factor 2. A map of the sampling sites is provided on the right. The sampling sites were coded using different colours according to their distance from land.

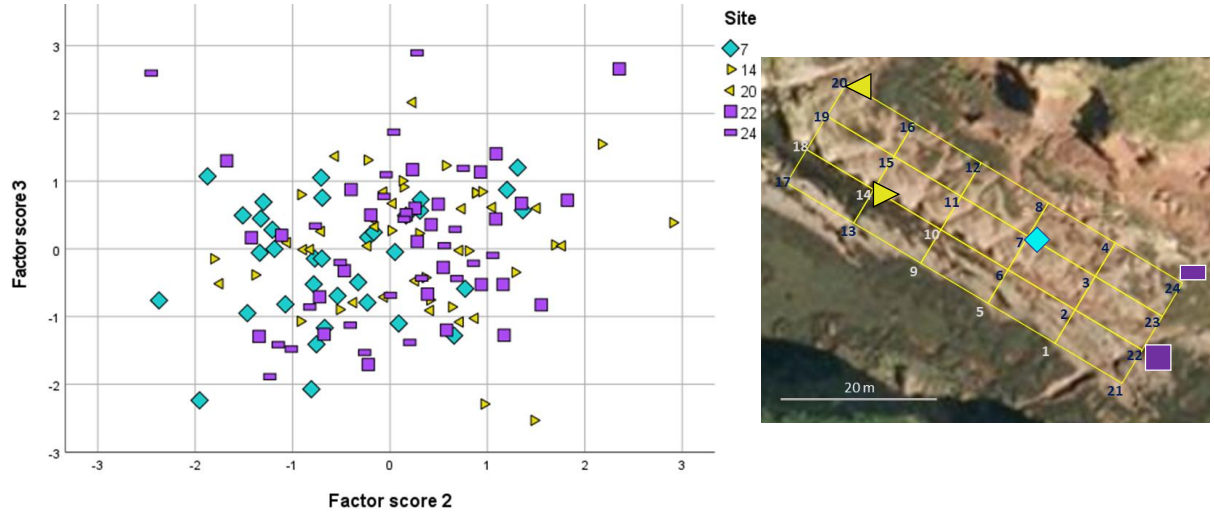


Figure 4.29. Principal component analysis for metacercaria from Dunbar for the local scale study where the factor scores for PC2 are plotted against those for factor 3. A map of the sampling sites is provided on the right. The sampling sites were coded using different colours according to their distance from land.

4.3.5.2.2. Whole area study

For the whole area study, a total of 120 metacercariae were analysed and the same 15 variables were included. In the first analysis an outlier was identified, therefore, this outlier was excluded in the

following analysis and 119 samples were included. Up to 4 components could be extracted based on the eigenvalues.

The mean and standard deviation (SD) were calculated for each variable (Table 4.15). The correlation matrix was provided for reference (Table 4.16). The result of Kaiser-Meyer-Olkin measure of sampling adequacy was 0.86 (1 is best) and Bartlett's Test of sphericity was highly significant (p<0.0001) indicating there are underlying factors contributing for the variance and there are correlations among the variables. All variables had CV values around 0.10-0.15. By checking individual CV values, the variables chosen in this data set were equally stable; but the prepharynx length, the oesophagus length and probably the cirrus sac width and ovary width were highly variable (see Table 4.10).

Four components were extracted which together explained 68.83% of the variance; the first component explained 44.8%, the second accounted for 9.58 % of the variance, the third explained 7.55%, and the fourth for 6.9% of the variance (Table 4.17). The correlation matrix (see Table 4.16) revealed the correlation between the body length and the oesophagus length, the ventral sucker length, and the testis width. A high correlation was found between the body width and the ventral sucker, testes, and ovary. The organs near the ventral sucker, such as the testis and the ovary were correlated with the ventral sucker, and the testis and ovary were also correlated with each other. When looking at the pattern matrix (Table 4.18), quite a clean separation of the four components was found: the first one consisted of the body W, ventral sucker W, testis W, cirrus sac W, ovary L, testis L, ventral sucker L, ovary W and the cirrus sac L; the second comprised the variables oesophagus L and body L; the third one was influenced by the prepharynx L only, while the separation of specimens by the fourth were influenced by the pharynx L, oral sucker L and the oral sucker W. PC1, PC2 and PC3 were mostly positive, but several negative values appeared in PC4 (see Table 4.18). PC1 again was dominated by body width and by morphometrics taken on organs around the ventral sucker. PC2 was dominated by the oesophagus length and the body length. In this analysis, the separation of samples through PC3 was influenced by the prepharynx alone (see Table 4.18). PC4 was dominated by the pharynx length, the oral sucker length, and its width, but these were all negative.

In the PCA plots, no obvious grouping could be detected when using two factors score or three factors together, neither were the five sampling sites marked by different colour according to their distance from land (see Figures 4.30 & 4.31).

Table 4.15. Descriptive statistics of the metacercarial morphometric variables made on specimens collected from barnacles from the whole area study conducted at Dunbar Red Rock (n = 119).

	Mean	Std. Deviation	Analysis N
Body length	634.72	68.31	119
Body width	209.45	27.52	119
Oral sucker L	42.01	4.84	119
Oral sucker W	40.33	4.00	119
Prepharynx L	27.61	15.60	119
Pharynx L	22.43	2.93	119
Oesophagus L	119.36	19.67	119
Ventral sucker L	46.59	5.42	119
Ventral sucker W	44.83	6.04	119
Testis L average	56.70	7.89	119
Testis W average	70.82	9.21	119
Cirrus sac L	58.85	7.29	119
Cirrus sacW	137.17	22.42	119
Ovary W	48.10	6.64	119
Ovary L	33.52	5.17	119

Table 4.16. Correlation matrix of the morphometric variables measured on the metacercariae collected for the Dunbar for the whole area study (n = 119). Values >0.7 that are correlated together are highlighted in a bold font while values <0.1 suggest they are independent of one another.

		Body length	Body width	Oral sucker L	Oral sucker W	Prepharynx L	Pharynx L	Oesophagus L	Ventral sucker L	Ventral sucker W	Testis L average	Testis W average	Cirrus sac L	Cirrus sac W	Ovary W	Ovary L
Correlation	Body length	1														
	Body width	0.29	1													
	Oral sucker L	0.47	0.43	1												
	Oral sucker W	0.52	0.37	0.64	1											
	Prepharynx L	0.33	-0.04	-0.01	0.03	1										
	Pharynx L	0.37	0.14	0.32	0.32	0.14	1									
	Oesophagus L	0.65	0.32	0.33	0.37	0.01	0.13	1								
	Ventral sucker L	0.60	0.65	0.55	0.47	0.13	0.44	0.38	1							
	Ventral sucker W	0.43	0.8	0.36	0.37	0.13	0.27	0.35	**0.72**	1						
	Testis L average	0.55	0.54	0.42	0.53	0.14	0.38	0.36	0.58	0.61	1					
	Testis W average	0.38	**0.72**	0.36	0.46	0.02	0.21	0.38	0.55	0.69	**0.71**	1				
	Cirrus sac L	0.38	0.51	0.31	0.38	0.06	0.20	0.26	0.48	0.46	0.39	0.40	1			
	Cirrus sac W	0.25	0.49	0.26	0.20	0.09	0.12	0.11	0.39	0.47	0.39	0.36	0.31	1		
	Ovary W	0.38	0.56	0.46	0.42	0.04	0.34	0.15	0.52	0.53	0.56	0.48	0.29	0.39	1	
	Ovary L	0.46	0.57	0.43	0.44	0.12	0.25	0.34	0.65	0.53	0.61	0.50	0.4	0.41	0.45	1

Table 4.17. Total variance explained for the specimens analysed for the whole area study.

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings[a]
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total
1	6.72	44.80	44.80	6.72	44.80	44.80	5.83
2	1.44	9.58	54.38	1.44	9.58	54.38	2.85
3	1.13	7.55	61.93	1.13	7.55	61.93	1.22
4	1.03	6.90	68.83	1.03	6.90	68.83	3.81

Extraction Method: Principal Component Analysis.

a. When components are correlated, sums of squared loadings cannot be added to obtain a total variance.

Table 4.18. Pattern matrix[a] for the whole area study showing the relative contribution of each morphometric measurement to the overall separation of specimens. A value of >±0.5 is highlighted in purple suggesting a strong contribution, while parameters <±0.1 suggest they make a negligible contribution to the separation of specimens.

	Component			
	1	2	3	4
Body width	0.94*	0.01	-0.19	0.09
Ventral sucker W	0.87	0.06	0.08	0.04
Testis W average	0.76	0.16	-0.11	0.01
Cirrus sac W	0.76	-0.24	0.12	0.06
Ovary L	0.59	0.15	0.07	-0.17
Testis L average	0.57	0.14	0.08	-0.30
Ventral sucker L	0.57	0.17	0.09	-0.32
Ovary W	0.53	-0.23	-0.08	-0.48
Cirrus sac L	0.52	0.23	0.02	0.01
Oesophagus L	0.09	0.90	-0.01	0.08
Body length	0.11	0.65	0.35	-0.31
Prepharynx L	0.04	0.09	0.92	0.02
Pharynx L	-0.07	-0.13	0.21	-0.82
Oral sucker L	0.11	0.21	-0.24	-0.66
Oral sucker W	0.09	0.33	-0.19	-0.61

Extraction Method: Principal Component Analysis.

Rotation Method: Oblimin with Kaiser Normalization.

a. Rotation converged in 15 iterations. *Value in purple shows the major contributors for each component.

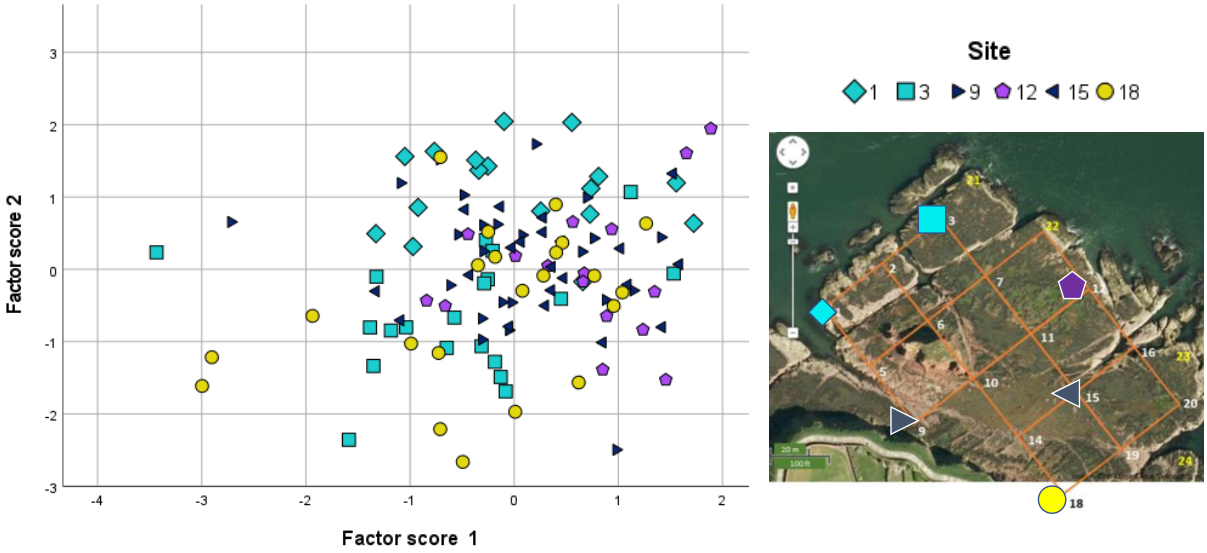


Figure 4.30. Principal component analysis of the metacercaria collected from Dunbar for the whole area study - factor score 1 vs. 2. A map of the sampling sites is provided on the right. The sampling sites were coded using different colours according to their distance from land.

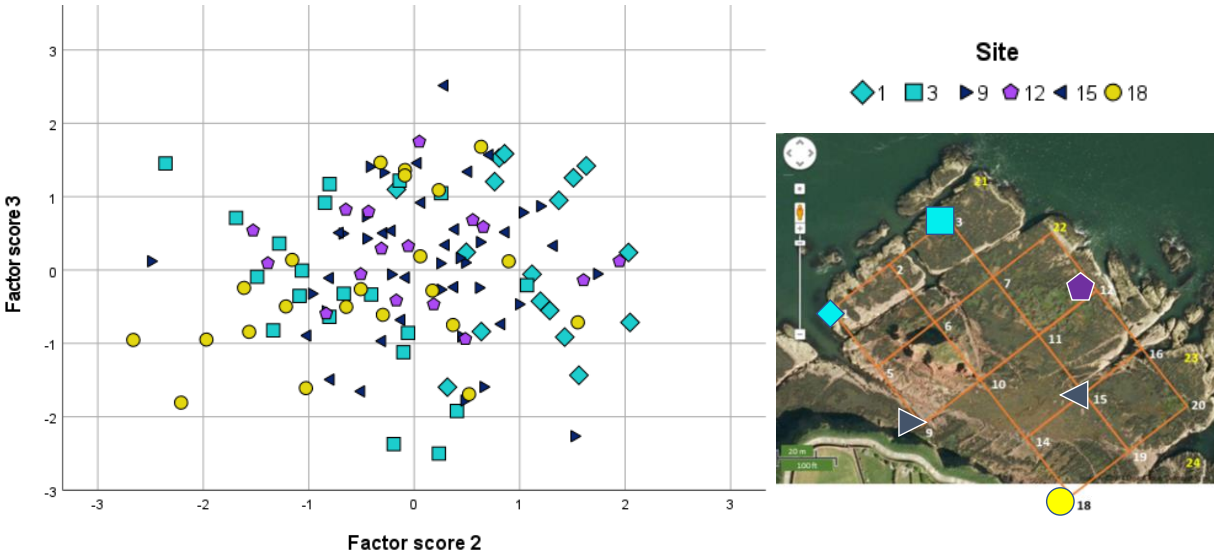


Figure 4.31. Principal component analysis of the metacercaria collected from Dunbar for the whole area study - factor score 2 vs. 3. A map of the sampling sites is provided on the right. The sampling sites were coded using different colours according to their distance from land.

4.3.5.2.3. Cultured adults from Dunbar, Stonehaven and Rosehearty

In this study, the samples size was smaller (total n = 62: n = 20 for Stonehaven, 20 for Rosehearty and 22 for Dunbar), and 18 variables were included. Sucker ratio, caecum length and pharynx width were new variables that were included. The correlation matrix was provided for reference (Table 4.19).

Four components were extracted with eigenvalues >1, explaining 67.47% of the total variance and accounting for 31.87%, 16.58%, 10.28% and 8.74% of the total variance for the first four principal components (Table 4.20). The results of a Kaiser-Meyer-Olkin measure of sampling adequacy showed a value of 0.653 and Bartlett's Test of sphericity was highly significant (p<0.0001) indicating there are underlying factors contributing for the variance and there are correlations among the variables. Only a small number of variables were highly correlated as revealed by the correlation matrix (see Table 4.19). Again, the body length was found to be highly correlated with the oesophagus length.

In the pattern matrix (Table 4.21), the first component comprised the ventral sucker W, body W, testis W, testis L, ovary W, and the ovary L; the second comprised the cirrus sac L, cirrus sac W, caecum L, oral sucker W, and the pharynx W; the third comprised the oesophagus L, body L, and the ventral sucker L; and the fourth comprised the sucker ratio, oral sucker L, pharynx L, and the prepharynx L. PC1 was dominated by the ventral sucker width and the body width and included the round organs near the ventral sucker. In PC1, the pharynx width had a high negative value. PC2, which comprised the cirrus sac, the caecum, the oral sucker, and the pharynx, did not reflect shape. PC3 was related to the oesophagus, the body length, and the ventral sucker length. PC4 included the remaining variables (see Table 4.21). These patterns seemed quite random except for the first component.

Lloyd's mean crowding value for Dunbar was 47.64, for Stonehaven this was 14.31, and for Rosehearty the value was 6.43, showing that the degree of crowding decreased toward higher latitude, indicating that metacercariae experience less intense crowding (intra-species competition) toward higher latitudes. The observed difference on crowding seems to have some degree of relevance for the morphological variation on cultured adults from the three localities, as the compactness of the data were in a reversed order to the crowding index (see Figures 4.32 & 4.35). However, culture condition is a confounding factor and cannot be eliminated at the current time.

Table 4.19. Correlation matrix of the morphometric variables measured on the cultured adults (n = 62). Values >0.7 that are correlated are highlighted in a bold font while values <0.1 suggest they are independent of one another.

		Body L	Body W	Oral sucker L	Oral sucker W	Prepharynx L	Pharynx L	Pharynx W	Oesophagus L	Caecum L	Ventral sucker L	Ventral sucker W	Sucker ratio	Testis L	Testis W	Cirrus sac W	Cirrus sac L	Ovary L	Ovary W
Correlation	Body L	1.00																	
	Body W	0.06	1.00																
	Oral sucker L	0.53	0.46	1.00															
	Oral sucker W	0.47	0.29	0.28	1.00														
	Prepharynx L	0.34	0.26	0.43	0.33	1.00													
	Pharynx L	0.45	0.32	0.56	0.30	0.33	1.00												
	Pharynx W	0.13	-0.37	-0.12	0.29	-0.18	0.03	1.00											
	Oesophagus L	**0.82**	-0.05	0.45	0.33	0.02	0.38	0.05	1.00										
	Caecum L	0.53	0.02	0.21	0.34	0.06	0.05	0.37	0.38	1.00									
	Ventral sucker L	0.46	0.23	0.38	0.07	0.11	0.28	-0.31	0.39	0.21	1.00								
	Ventral sucker W	0.19	**0.71**	0.26	0.27	0.25	0.23	-0.29	0.02	0.08	0.39	1.00							
	Sucker ratio	0.17	0.27	0.67	0.21	0.32	0.32	0.13	0.14	0.03	-0.43	-0.06	1.00						
	Testis_ L	0.44	0.44	0.48	0.15	0.31	0.21	-0.35	0.26	0.12	0.42	0.49	0.14	1.00					
	Testis W	0.28	0.63	0.42	0.23	0.42	0.29	-0.42	0.15	0.01	0.43	0.59	0.07	**0.71**	1.00				
	Cirrus sac W	0.47	0.52	0.36	0.49	0.39	0.27	0.05	0.15	0.38	0.26	0.50	0.14	0.37	0.47	1.00			
	Cirrus sac L	0.34	-0.03	0.18	0.50	0.28	0.15	0.47	0.13	0.37	-0.09	-0.06	0.24	-0.03	-0.07	0.51	1.00		
	Ovary L	0.26	0.32	0.36	0.16	0.27	0.26	-0.26	0.24	0.10	0.29	0.33	0.11	0.46	0.48	0.18	-0.10	1.00	
	Ovary W	0.25	0.40	0.29	0.29	0.26	0.32	-0.31	0.22	-0.16	0.34	0.32	0.02	0.32	0.45	0.23	-0.15	0.39	1.00

Table 4.20. Total variance explained between the cultured adults (n = 62)

	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings[a]
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total
1	5.74	31.87	31.87	5.74	31.87	31.87	4.78
2	2.98	16.58	48.45	2.98	16.58	48.45	2.84
3	1.85	10.28	58.73	1.85	10.28	58.73	3.49
4	1.57	8.74	67.47	1.57	8.74	67.47	2.71

Extraction Method: Principal Component Analysis.
a. When components are correlated, sums of squared loadings cannot be added to obtain a total variance.

Table 4.21. Pattern matrix[a] for the cultured adults study showing the relative contribution of each morphometric measurement to the overall separation of specimens. A value of >±0.41 is highlighted in purple suggesting a strong contribution, while parameters <±0.1 suggest they make a negligible contribution to the separation of specimens.

	Component			
	1	2	3	4
Ventral sucker W	0.88*	0.14	0.09	0.18
Body W	0.85	0.05	0.24	-0.19
Testis W	0.83	-0.06	-0.09	-0.04
Testis L	0.61	-0.08	-0.29	-0.07
Ovary W	0.47	-0.22	-0.23	-0.13
Ovary L	0.41	-0.19	-0.31	-0.15
Cirrus sac L	-0.06	0.82	0.05	-0.16
Cirrus sac W	0.63	0.65	0.03	0.01
Caecum L	-0.05	0.64	-0.41	0.20
Oral sucker W	0.25	0.64	-0.12	-0.15
Pharynx W	-0.52	0.63	-0.01	-0.05
Oesophagus L	-0.22	0.07	-0.92	-0.13
Body L	0.02	0.36	-0.80	-0.12
Ventral sucker L	0.41**	-0.08	-0.64	0.41
Sucker ratio	-0.12	0.06	0.10	-0.96
Oral sucker L	0.22	0.00	-0.40	-0.65
Pharynx L	0.12	0.01	-0.38	-0.49
Prepharynx L	0.40	0.18	0.05	-0.42

Extraction Method: Principal Component Analysis.
Rotation Method: Oblimin with Kaiser Normalization.[a]
a. Rotation converged in 22 iterations. *Value in purple shows the major contributors for each component. **Value in orange shows the variable that is cross correlated.

In the principal component analysis, it is interesting to know whether this difference between localities reflected the morphological difference brought about by culture conditions or true differences between localities. Specimens were marked according to locality, and specimens from Dunbar were marked according to the different culturing conditions used (Figures 4.32-4.34). Dunbar 1 represents specimens that were incubated for 48 hours in 20% chicken serum, Dunbar 2 specimens incubated for 120 hours in 40% chicken serum, Dunbar 3 for 48 hours incubated in 40% chicken serum, and Dunbar 4 for 120 hours incubated in 20% chicken serum. Although samples from the three sites still overlap, the samples from Dunbar were spread more widely than those from Stonehaven and Rosehearty. When focusing on specimens from Dunbar, culturing conditions clearly contributed to morphological variability. It seems that culturing time (i.e., specimens of a younger age) is a factor separating these specimens (i.e., Dunbar 1 & 3) from the others. The trend can be seen when plotting factor score 1 & 2 (Figure 4.32) but not 2 & 3 (Figure 4.33). When looking at the loading plots of component 1 vs. 2 (Figure 4.34), it seems that variables in component 1 are dominated by variables near the middle part of the body (i.e., ventral sucker W, body W, testis W, testis L) important for separating these younger specimens.

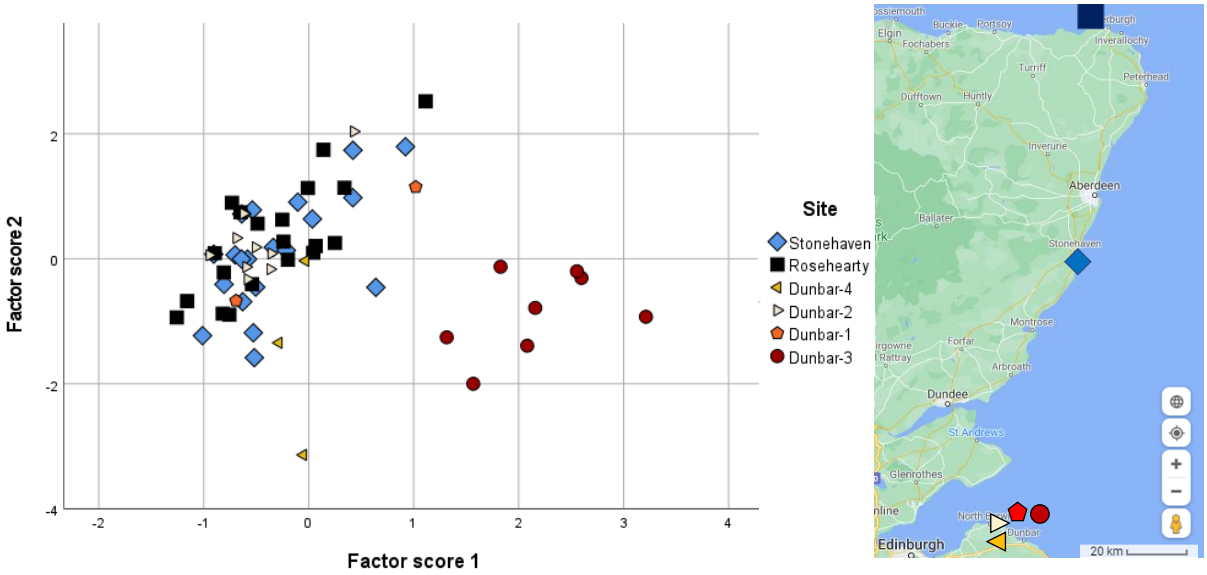


Figure 4.32. Principal component analysis for cultured adults and exploring the different culturing methods used. The plot shows the scores for factor 1 plotted against those of factor 2. A map of the sampling localities is provided on the right. The sampling sites were coded using different colours according to their distance from land and culture conditions (see text).

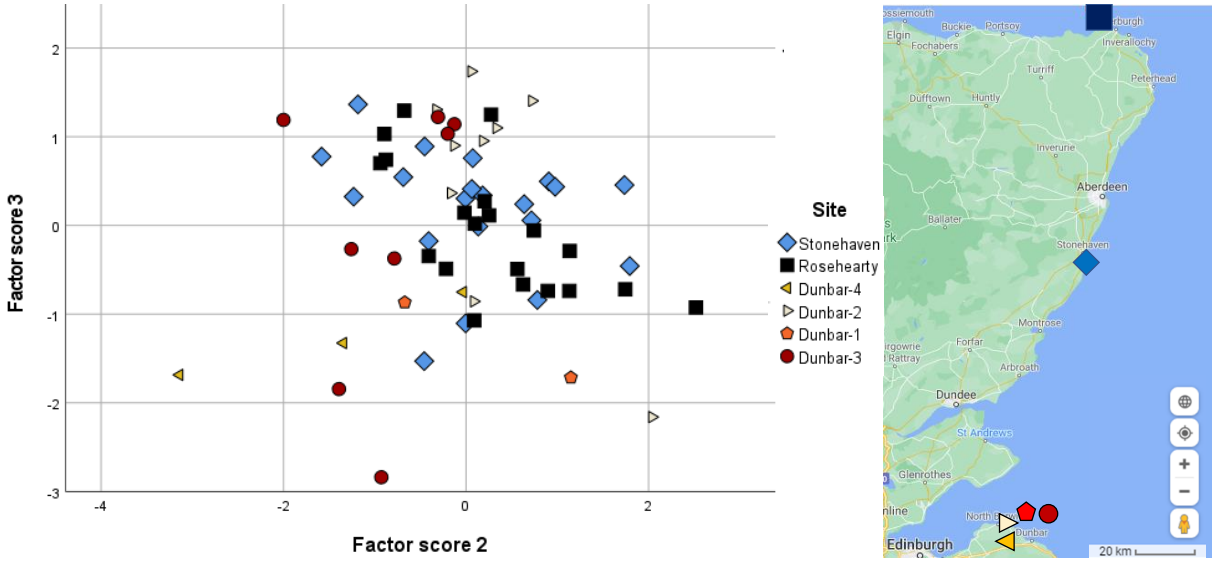


Figure 4.33. Principal component analysis of the adults raised in culture. The plots show the scores for factor 2 plotted against factor 3. A map of the sampling localities is provided on the right. The sampling sites were coded using different colours according to their distance from land and culture conditions (see text).

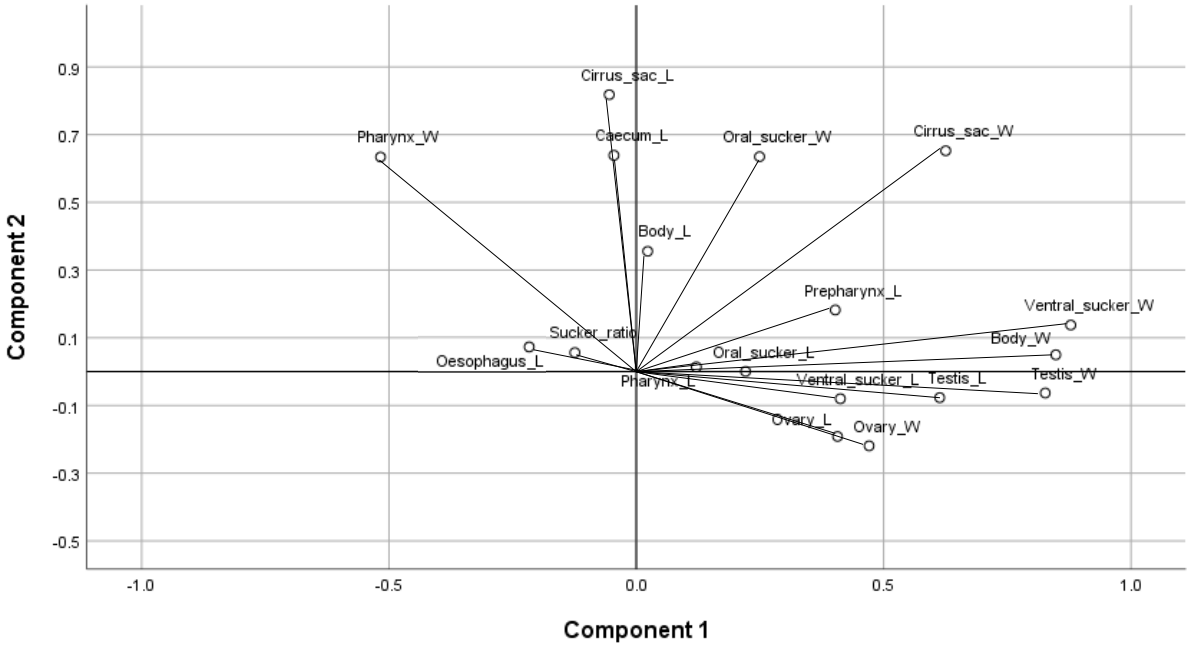


Figure 4.34. Loading plot in rotated space for adults produced in culture media - component 1 vs. 2

To further confirm the importance of culture condition, another set of PCAs were conducted by removing samples Dunbar 1 & 3. In the second set of PCAs (Figures 4.35-4.36), most of the samples from the three localities overlapped, except those from Dunbar 4 (i.e., 120 hours in 20% chicken serum). From the loading plot (see Figures 4.37 & 4.38), it was the second component which was dominated by ovary W, pharynx L and oesophagus L, that pulled the specimens in group Dunbar 4 apart. It is speculated here that age is the major contributor for the observed morphological differences, because the samples that overlapped all had a similar age (i.e., 96 hours *vs.* 120 hours).

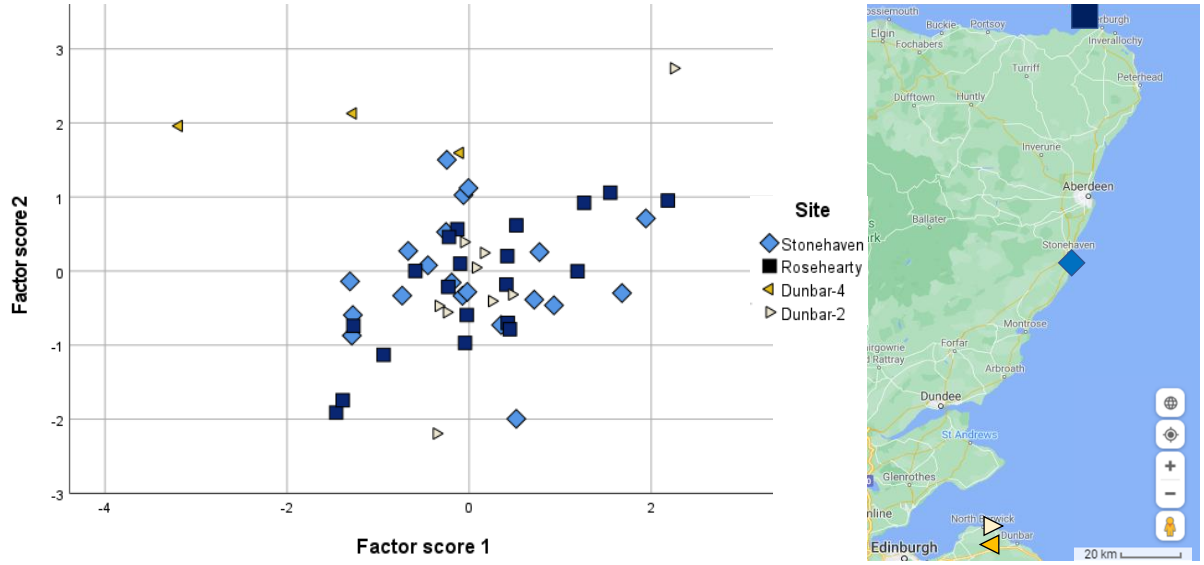


Figure 4.35. Principal component analysis of adults raised in culture (excluding those from Dunbar 1 & 3). The plot is of the scores for factor 1 against factor 2. A map of the sampling localities is provided on the right. The sampling sites were coded using different colours according to their distance from land and culture conditions (see text).

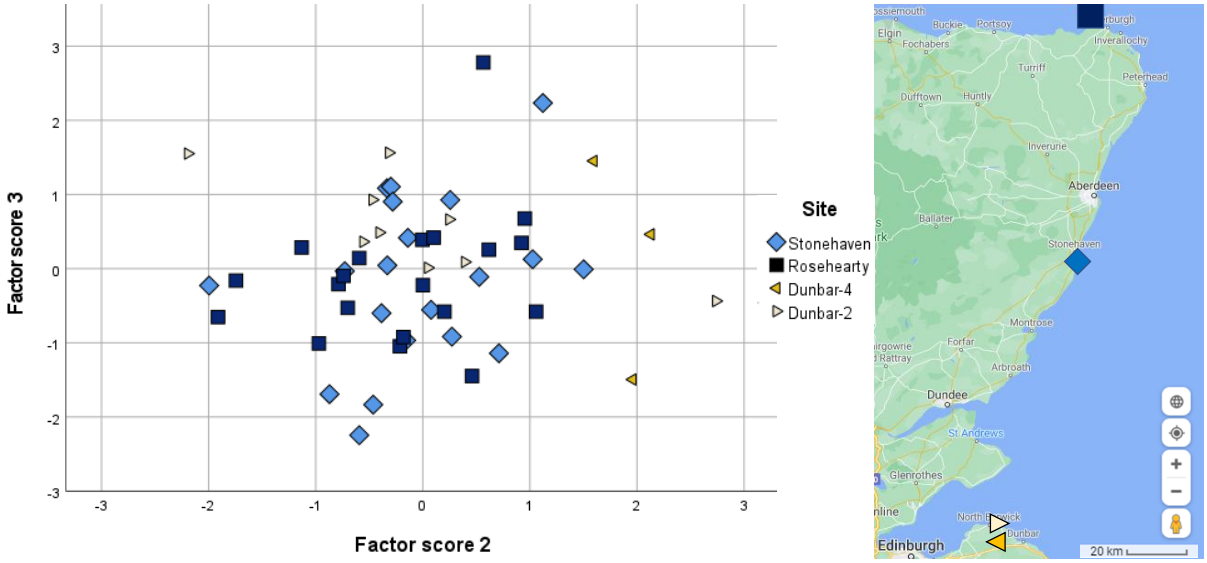


Figure 4.36. Principal component analysis for cultured adults (excluding those from Dunbar 1 & 3) - factor score 2 vs. 3. A map of the sampling localities is provided on the right. The sampling sites were coded using different colours according to their distance from land and culture conditions (see text).

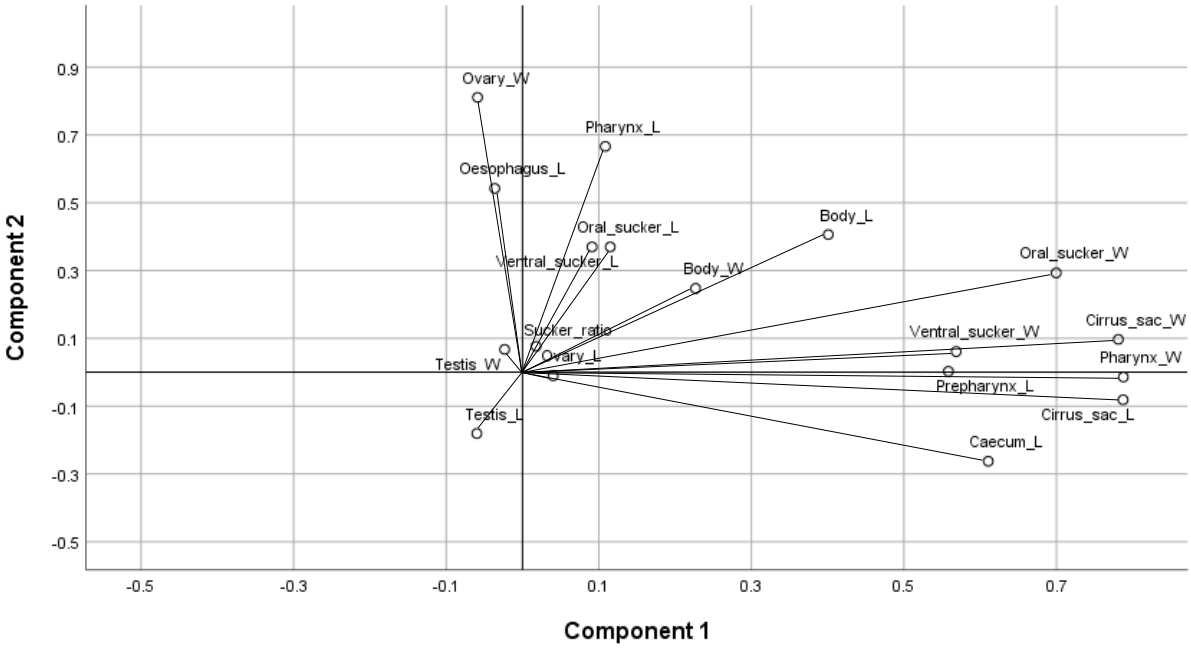


Figure 4.37. Loading plots for cultured adults investigating the culturing methods that were used. This analysis does not include samples from Dunbar 1 & 3. The plot is of component 1 vs. component 2.

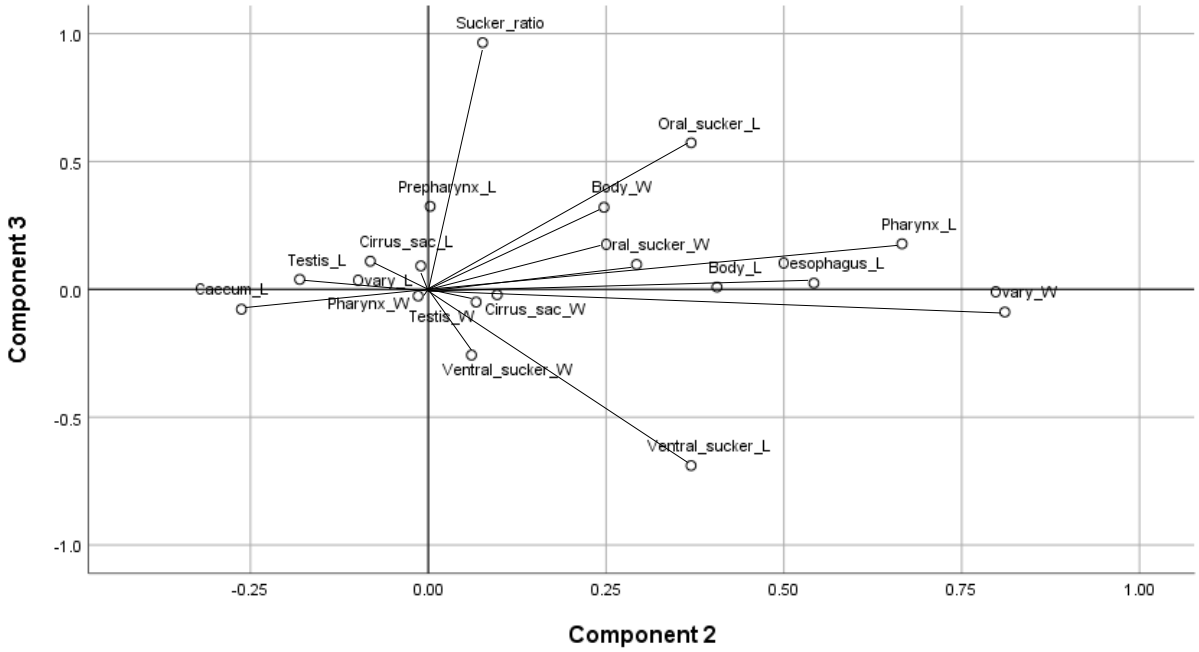


Figure 4.38. Loading plots for cultured adults investigating the (breaking down culturing methods that were used. This analysis and does not include samples from excluding Dunbar 1 & 3. The plot is of component 2 vs. component 3.

4.4. Discussion

4.4.1. Diagnosis of *Maritrema* sp. collected from Scottish coastal waters by morphological and molecular techniques

4.4.1.1. Morphological observation by light microscopy

4.4.1.1.1. Newly excysted young adults from Dunbar

The metacercariae and adults obtained in the present study possessed the typical morphology of *Maritrema*, especially in terms of the presence of a cirrus sac, and a complete ring of postacetabular vitellaria. The morphology of the newly excysted young adults (sometimes called metacercaria in other literature) under the light microscopy is, in general, congruent to the original description of Hadley and Castle (1940) and Deblock and Tran Van Ky (1966a) (see Table 4.6). In the present study, however, the body shape is more elongated and some measurements differ from some of the measurements made by these authors. For metacercariae only data from Hadley and Castle (1940) is comparable; the range of body width, body width / length ratio, oral sucker width, testis width, ovary width in the present study is slightly smaller; and the mean body width / length ratio is only half of theirs. Nearly all the measurements are proportionally smaller in the present study except body length and prepharynx length. The variabilities in body shape may be partially explained by different fixation methods, furthermore, the prepharynx length is subject to variability caused by contraction. It is also not clear whether their sample was representative, because they used a living (unfixed) adult and only one specimen was measured.

The effect of preparation and fixation on digenean morphology had been investigated in a systematic way (Bakke, 1988). The author suggested that the variabilities of whole mount specimens derived from various sources: phenotypic variabilities, age, muscular contraction / relaxation, handling methods, and genetics. Bakke (1988) also pointed out that flattening has a pronounced effect on both body and organ measurements. On the other hand, the author also stated that flattening (or stretching) was often necessary for descriptions for taxonomic work. Numerous chemical and physical methods can be applied to fixing / flattening, and hot fixation (e.g., hot 10% formaldehyde) is recommended by the author. If unable to perform hot fixation, cold (ca. 20°C) fixation is also an option but only a few methods gave acceptable results, such as Berland's fluid, which was used in the present study. The glacial acetic acid in Berland's fluid functions as a swelling agent due to its capacity to swell proteinaceous gels and tissues. The samples treated by Berland's fluid will, therefore, stretch and reach a maximum size. This effect was obvious in the samples of the present study. The young adults treated in such a way were usually more elongated than samples prepared by other methods (e.g., cold 10% formalin fixation) or than live specimens. Among *Maritrema* species, only one species description, *Maritrema portucalense*, mentioned the use of Berland's fluid (Pina *et al*., 2011a). Interestingly, *M.*

portucalense is also described as having an elongated body shape. The fact that both *M. portucalense* and *M. gratiosum* in the present study flattened with Berland's fluid have the elongated body shape might indicate that this shape is indeed the result of applying Berland's fluid. It is, therefore, important to compare morphologies using the same fixation methods. In the present study, all the specimens used for morphological diagnosis and morphometric studies were fixed flat using Berland's fluid. Although this method gave the samples an elongated appearance, it is still an acceptable method, as recommended by Bakke (1988), which should reveal fine internal structures and reduce variability in the morphology.

4.4.1.1.2. Cultured adults from Dunbar

When comparing the morphology of five-day-old experimental adults from Dunbar in the present study, more pronounced variabilities were found between the measurements made in this study and the measurements from the two original descriptions (*i.e.*, Hadley & Castle, 1940; Deblock & Tran Van Ky, 1966a; see Table 4.7). The width and length of the testes, ovary and eggs are all smaller than that of the living specimen from Hadley and Castle (1940) and from the fixed samples of Deblock and Tran Van Ky (1966a). The length of the cirrus sac from the present study is larger than that from Deblock and Tran Van Ky (1966a). Relatively few differences, however, were found between the measurements made on the cultured adults in the present study and that of the preserved specimens from Hadley and Castle (1940), except for the body length, body width/length ratio and the caecum length. The fixation methods employed in the two original descriptions were not clearly described. The observed differences in the measurement of the ovary, testes and eggs could be because in the present study experimental adults were measured while in the other two studies natural adults were measured. The method by which the cirrus sac length and width were measured in the study of Deblock and Tran Van Ky (1966a) are unknown. These could all contribute to the variability that was observed.

4.4.1.1.3. Cultured adults from distanced localities

When comparing the measurements of specimens collected from Stonehaven and Rosehearty to the original description, more pronounced variations were detected (see Table 3.9). The mean body length was smaller than the living specimen from Hadley and Castle (1940) and larger than fixed specimens of Deblock and Tran Van Ky (1966a). The body width and the body width / length ratio were in general were smaller than those in these two original descriptions. The pharynx width, oesophagus length and caecum length were larger than the two original descriptions. The testes, ovary and egg length again were smaller than the two original descriptions, and cirrus sac length was larger than that reported by Deblock and Tran Van Ky (1966a). The larger variability found in samples from Stonehaven and Rosehearty compared to the samples collected from Dunbar with reference to the original descriptions, indicates potential differences between these localities, and these are supported by the

morphometric analyses, which will be discussed later. It was noticed that the live specimen under coverslip pressure had a more elongated body shape compared to preserved adults in Hadley and Castle's (1940) report, and the measurement of Deblock and Tran Van Ky (1966a) were closer to the preserved adults of Hadley and Castle's (1940). This again illustrates the potential effects of fixation and flattening. In the present study fixation methods affected the body length, particularly the measurements of contractable organs like the oesophagus and the intestinal caecum. It is also interesting to note that in the present study the vaginal gland in adults stained less intensely with Mayer's paracarmine (see Figure 4.10). The presence of a clear vaginal gland in the metacercarial stage but not in the adult stage of *M. gratiosum* was mentioned by Deblock and Tran Van Ky (1966a). Carmine is known as a purely nuclear stain (Gower, 1939); therefore, it is suggested that the vaginal gland cells may have less nuclear materials after maturation and might indicate that the composition of the cells had changed. The observed reduction in staining in the present study may be related to the functional change of the vaginal gland, or may be related to the culture conditions that were used but this remains to be established. There is no information about the function of the vaginal gland, but it is speculated that this might relate to copulation. The lack of certain nutrients in the artificial rearing medium might also result in poorer development of the parenchymal organs such as the testes, ovary, or glandular tissues such as the vaginal gland. This factor could lead to smaller / abnormal appearance of these organs. The vaginal gland is not usually described in other *Maritrema* species (see Appendix 40 for species possessing this structure) and it is not therefore considered a constant structure in *Maritrema* species.

4.4.1.1.4. Reproductive and excretory organs

Some detail of the reproductive organs is only visible in living specimens, such as the movements and morphology of the *pars prostatica* (see Figure 4.11A), and ootypic junction (this is called the ootypic crossroad in the description of Deblock, 1971; see Figure 4.11B). The structure of the ootypic junction observed in the present study agrees with that described in Hadley and Castle's (1940) report. What is not described in the previous studies is the movement of the *pars prostatica,* which is in a back-and-forth manner. It was also noticed that this structure can move without the contraction force of the cirrus sac. This can be seen in flattened samples when alive. The "male papilla" observed by Hadley and Castle (1940) is probably the *pars prostatica* as it sometimes moves to the place quite near the genital pore and when fixed, it looked like a papilla. This situation was supported by the finding in the present study that *pars prostatica* can move by itself. The issue has already been discussed by Deblock and Tran Van Ky (1966a). The cirrus was, in general, in an invaginated state in the present study, only under rare occasions could the evaginated cirrus be observed outside the genital pore. According to the finding in the present study, the male copulatory organ of *M. gratiosum* is composed

of a series of elongated structures which are enclosed in a cirrus. It seems most likely that there is an evaginable, smooth, and tubular cirrus rather than a male papilla. An ampullar-shaped *pars prostatic* which can move by itself serves the connection between the seminal vesicle and the cirrus.

4.4.1.1.5. Eggs of *Maritrema*

In the case of live or fixed samples, the presence of an operculum on the eggs was not confirmed (see Figure 4.12). Most of the specie descriptions for *Maritrema* do not include egg morphology, however, Zaben (1988) described normal eggs of *M. gratiosum* as having operculi. On the other hand, some species of *Maritrema* were reported to have an operculum (see Appendix 41). Cribb *et al.* (2001) classified the Microphallidae as having egg operculi and eggs that are unembryonated when laid. It seems that the eggs observed in the present study were indeed unembryonated in the uterus, although the discharge of eggs was never observed. Given that cultured adults often produce abnormal eggs (Smyth & Halton, 1983), it is possible that operculi of the samples in the present study were not properly developed. The egg number found in this study was positively related to body length but not to body width (see Figure 4.14). The possible explanation is that longer worms represent larger or more mature worms and allow more eggs to accumulate in the uterus. This might be related to the degree of crowding. As the degree of crowding was higher in Stonehaven than in Rosehearty (see Section 4.3.2.2.2.), a larger (longer) body size can be expected for specimens from Rosehearty, which is true in the present study (see Table 4.7). Poulin (2006) pointed out that crowding can have an effect on the fecundity of a parasite, the finding in the present study is that individuals from Rosehearty (where there is less crowding) had a higher egg number than those from Stonehaven (where there was greater crowding) (see Table 4.8), seems to fit this statement. This phenomenon deserves further study. No obvious relationship was found between mean egg length and body width or to body length. Mean egg width, however, is slightly negatively correlated with body length, and mean egg width positively correlated with body width. This may have something to do with the crowding effect within the parent fluke but the reason is not clear.

4.4.1.1.6. Conclusions on morphological observation by light microscopy

To conclude, the present study provides the most comprehensive re-description of the morphology of *M. gratiosum* in recent decades extending current knowledge. No obvious morphological differences were noticed when comparing the present description with the previous two descriptions, confirming that the specimens collected in the present study represent the same species described by Hadley and Castle (1940) and Deblock and Tran Van Ky (1966a). On morphological grounds, although there are some measurements of the present study out with the ranges of the two original descriptions, these differences can be explained, and the differences are not large enough to consider the measured specimens to belong to a new / different species. Furthermore, no obvious

structural differences, such as the shape of the *pars prostatica* and cirrus, and topology of the internal organs, were observed. An evaginable, smooth and tubular cirrus rather than a male papilla was confirmed, and the cirrus is connected to the seminal vesicle by an ampullar-shaped *pars prostatic*. Therefore, morphological data indicate the metacercariae collected from the three sites off Scottish coast, namely Dunbar, Stonehaven and Rosehearty in the present study were *M. gratiosum*. Subtle differences in morphometric data were revealed via statistical methods and will be discussed later.

4.4.1.2. SEM observation *of Maritrema* sp. collected in the present study

Several fixatives have been used for the SEM study of digenean or monogenean samples. In the report of Naem *et al.* (2012) for adult *Fascioloides*, samples fixed by AFA solution (alcohol, formaldehyde, acetic acid, distilled water, and glycerine) were used. In the report of Lorsuwannarat *et al.* (2014) for newly excysted and juvenile *Fasciola*, the samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer solution (PBS) pH 7.4 overnight prior to the following washing and dehydration steps. Galaktionov *et al.* (2006) used 3% glutaraldehyde made up in filtered sea water to fix parthenogenetic metacercariae from bivalve hosts. Irwin *et al.* (1990) used 2% paraformaldehyde and 2.5 % glutaraldehyde in 0.1M sodium cacodylate buffer containing 3% sucrose to fix cercariae of *M. arenaria*. Whittington *et al.* (2001) used boiling seawater to kill adult *Benedenia* and fixed them unflattened in 10% buffered neutral formalin. The above-mentioned methods all appear to provide reasonable results for SEM study. Inappropriate preparation procedures can, however, lead to less optimal results for SEM observation. Examples of this include the use of hypertonic solutions which can cause shrinkage, while hypotonic solutions can cause artificial inflation. Other commonly encountered situations, such as the presence of bacteria, debris, and fractures of the teguments, can affect the results in SEM observations (González *et al*., 2012). Besides the regular procedure of CPD to prepare SEM specimens, some researchers use HMDS instead (Irwin *et al.*, 1990; Whittington *et al.*, 2001; Galaktionove *et al.*, 2006). There were no appreciable differences observed between the results applying CPD and HMDS in the present study, which demonstrated the applicability of both methods. The HMDS method, however, is more convenient for small samples as it avoids the need for transfer to the critical point dryer, which sometimes leads to the loss of samples. Bray *et al.* (1993), compared HMDS, Peldri II and CPD methods for animal and plant tissues and found these three methods produced identical results for animal tissues but not for plant tissues. They also suggested that HMDS was a time-saving and inexpensive method compared to CPD.

There are few SEM studies for metacercariae or adults of *Maritrema* spp. The results obtained in the present study were in general agreement with the first SEM study of *M. gratiosum* (Zaben, 1988), such as a piriform and fully-spined body, digitiform spines which teeth numbers decreased posteriorly, and more spines on the ventral than the dorsal side. Subtle differences, however, were noticed. The

protrusible lips of the genital pore, protrusible rim of the excretory pore and exit of the Lauren's canal on the dorsal side observed by Zaben were not found in the present study. The range of teeth numbers of the spines also differs; 8-13 anteriorly and 2-6 posteriorly observed by Zaben, and 10-11 anteriorly and 5-6 posteriorly in the present study. There were nine papillae observed on the floor of the cavity of the ventral sucker by Zaben, but only six to seven were found in the present study. Furthermore, there was only one type of papillae (ciliated) observed by Zaben, rather than two types observed in the present study. No measurement of the spine of *M. gratiosum* was mentioned by Zaben (1988), but judged by the images provided, the spines in his specimens had a similar width to those in the present study. For *M. portucalense* (see Pina *et al.,* 2011a), a fully spinated body surface was found, reflecting the spine morphology observed in *M. gratiosum* in the present study. The spines of the anterior body of *M. portucalense* had more teeth (up to 20), and the oral sucker was surrounded by uniciliated papillae. The ventral sucker was spinated, and had 6 uniciliated papillae on the lip as seen for *M. gratiosum* in the present study. For *M. bonaerensis* and *M. orensensis* (see Alda *et al.*, 2013), a measurement of the spines was given. Both latter species were totally covered by spines. For *M. orensensis* metacercariae, "hand-shaped" spines measuring ~0.7 µm in length with 7-9 "fingers" were mentioned, without stating their position. For *M. bonaerensis* metacercariae, the same kind of spine was also found, measuring ~0.7 µm in length with 9-10 fingers. The morphology of spines of *M. bonaerensis* and *M. orensensis* were quite like those of *M. gratiosum* in the present study, however, the spines of *M. portucalense* were wider and shorter and had more teeth, according to the figure provided. Similar uniciliated papillae were observed for *M. portucalense* and they were also distributed more densely around the oral sucker. For both studies, no dome-shaped papillae were observed, unlike the situation in the present study (see Figure 4.23C). A few reports of other microphallids have revealed similar surface structures. For *Microphallus primas* (Jägerskiöld, 1908) Stunkard, 1951, the tegumental spines of the forebody had more teeth (mean = 17) compared to the spine on the hindbody, while the smaller spines of the hindbody had less teeth (i.e., 1-4). Microvilli were also observed at the posterior end as seen for *M. gratiosum* in the present study (see Figure 4.24B) but they covered a larger area in *M. primas*. Symmetrical uniciliated papillae were also noticed on the lip of the oral sucker but were lower in number (*i.e*., only 4) and throughout the body surface especially on the ventral side (Pina *et al.*, 2011b). For *Gynaecotyla squatarolae* (Yamaguti, 1934) Yamaguti, 1939 (see Lim *et al.*, 2008), "scale-like" tegumental spines covering the whole body were observed. The anterior side of the oral sucker had no spines and the posterior side was covered by spines with 6-9 "digits". The tegumental spines just posterior to the oral sucker were wider and had more digits (i.e., 16-17), but decreased to 13-14 in front of the ventral sucker and further decreased to 3-5 on the hindbody. The ventral sucker was also armed with spines and papillae. It is noteworthy that two types

of papillae were mentioned: type I (ciliated, knob-like) and type II (aciliated round swelling) (see Lim *et al*., 2008). From the images provided it is not clear whether these two types of papillae were the same as those observed in the present study (uniciliated and dome shaped papillae; see Figures 4.23B & 4.23C). Similar surface structures were found in *Gynaecotyla longiintestinata* Leonov, 1958 (syn. *Gynaecotyla adunca*) as well but without mentioning the aciliated papillae (Pina *et al.*, 2007). To ascertain the functions of fixation / movement / irritation of tegumental spines, a histological study is needed in the future, to reveal histological / pathological changes to the host tissue and the orientation of the flukes *in situ*.

From the few studies of microphallids above, one can tell that the surface structures of microphallids are quite similar. The differences exist in details such as the length and width of spines, density of spines on the body wall, teeth number of spines, and supination of oral and ventral sucker, and the topology and types of papillae. Therefore, applying chaetotaxy to confirm the identity of *M. gratiosum* is possible, due to predictable patterns that have been identified, especially for the papillae around suckers. A more systematic SEM study that covers the whole body and both sides of the studied and referenced specimens, and consistent sample preparation procedures, however, are required in future studies. More specimens from wider geographical extent may be needed to reveal spatial heterogeneity in surface characters.

Note that "morphologically similar papillae" do not mean "identical" papillae. Whether these papillae are the same or not can only be confirmed by TEM study. Two types of papillae around the oral sucker of the eye fluke *Philopthalmus megalurus* Cort, 1914, ciliated and aciliated, were found under SEM (Edwards *et al.*, 1977). Under TEM, however, three types of papillae were discernible. Similarly, three types of papillae were found in newly excysted *F. hepatica by* TEM: spiral of tegument with a pit, ciliated type, and domed type (Bennett, 1975a). Four types of papillae of *Gorgoderina vitelliloba* Olsson, 1876 were revealed by TEM: button, rosette, ciliated and domed types (Hoole & Mitchell, 1981). The authors also found that these papillae types were not always present in both metacercarial and adult stages, while the morphology of some of the papillae changed in the adult stage. Although subtle differences of ultrastructure of these papillae were revealed by TEM, they nevertheless comprise a basic form. This basic form was revealed in the sister group of the Digenea, in the aspidogastrian *Cotylogaster occidentalis* Nickerson, 1902 in which five types of papillae were found (Ip & Desser, 1984). The five types of papillae are basically variants of a basic bulb cell anchored by a rootlet. The rootlet, which is divided into two parts, the apical cap, and a column of striated fibres, is variable in shape (Ip & Desser, 1984). The bulb cell, ciliated or not, has dense rings at the top and a long extension at the bottom, which will eventually connect to nerve networks (Edwards *et al.*, 1977). The basic anatomy of these papillae reveals their function as sensory organs. Various functions of

digenean papillae are suggested by different researchers. For example, the ciliated papilla is proposed to be a tangoreceptor while the domed papilla is a pressure receptor for *F. hepatica* (see Bennett, 1975a); the ciliated papilla around the oral sucker were described as tango-, rheo-, and chemoreceptors for *P. megalurus* (see Edwards *et al.*, 1977); the button papilla near the oral sucker was considered to be a mechano-receptor or stretch receptor for *G. vitelliloba* while the dome-shaped papilla on the ventral sucker functioned as a tangoreceptor (Hoole & Mitchell, 1981). The real function of these papillae can only be proven by electrophysiological experiments (Hoole & Mitchell, 1981). It is interested to note that six papillae on the ventral sucker were observed in distantly related families; in gorgoderids (see Hoole & Mitchell, 1981), in *F. hepatica* (plagiorchiid, see Bennett, 1975a, 1975b), and in microphallids (see Pina *et al.*, 2011a). This may reflect functional similarity of the ventral sucker and might suggest that this pattern is quite conservative. In the present study, the observation data were not sufficiently robust to confirm whether the "dome-shaped" papillae really exist or not, because papillae in the same position near the oral sucker were ciliated or aciliated in different specimens. There is a possibility that the cilia were destroyed during processing therefore giving an aciliated appearance, or it might reflect differences in maturity. In absence of further data, they will be considered as being present. The functions of these uniciliated papillae or done-shaped papillae may only can be demonstrated experimentally. Their functions can be speculated, however, to be tango-, chemo-, rheo-, or pressure- receptors, due to conservation of papilla functions between digenean species. A ventral concavity was observed by confocal microscopy study in *M. bonaerensis* and *M. orensensis* and was observed under SEM in specimens in the present study (see Figure 4.27). This will be discussed later in Chapter 5.

4.4.1.3. Molecular diagnosis of *Maritrema* sp. collected in the present study

Besides traditional morphology, molecular techniques were also applied in the present study to confirm the identity of the target parasites and to provide phylogenetic inferences within the genus and between families. For the purposes of inferring phylogeny, small and large subunit rDNA are often chosen, due to the characters of constant and variable sites in a single fragment allowing homologous alignment but still retaining phylogenetic signals (Littlewood & Olson, 2001). The database of small subunit (SSU) rDNA is well developed and is therefore often the first choice for exploratory phylogenetic analysis of a wide range of organisms. It has, however, been demonstrated that parsimony-informative sites are not evenly distributed throughout this gene (*i.e.*, 16S), and that regions with high rescaled consistency indices are scattered through the more conserved regions, therefore Littlewood and Olson (2001) suggested sequencing of the entire gene. It is well known that 18S and 28S regions have evolved most slowly (*i.e.*, are highly conserved) within the ribosomal DNA sequence, hence they are more suitable for higher taxonomy (Nolan & Cribb, 2005). According to the

same authors, for the purposes of species distinction for digeneans, ITS rDNA is the "default" region, and ITS1 gives greater resolution than ITS2 due to the presence of variable repeat units. Intraspecies variation is usually low in the ITS1 regions and geographical variation is generally limited. It is suggested by these authors to sequence the entire ITS region to get better distinction between species. Although the ITS region was suggested as the default region for species discrimination, in a phylogenetic study of the Microphalloidea, the authors employed LSU instead of SSU or ITS regions (Tkach *et al.*, 2003). Additionally, in the phylogenetic study of the *pygmaeus*-group of microphallids (Galationove *et al.*, 2012), the authors compared the results of 28S, ITS1 and ITS2 regions. The results suggested that ITS2 was the most unstable and probably represents the least parsimony-informative region of the ones available. More sequence data certainly gives better resolution of phylogenetic inferences. Using concatenated sequences of SSU and LSU rDNA, the relationships of 170 digenean taxa were resolved (Olson *et al.*, 2003). Furthermore, "combined evidence" analysis (combining molecular and morphological data for analysis) was applied to reveal the interrelationships more accurately among digeneans (Cribb *et al*., 2001), although the topologies of the trees were more strongly influenced by the molecular characters than the morphological ones. The authors suggest that this greater influence results from the greater number of informative positions in molecular data relative to the number of characters in molecular data.

In the present study, by comparing the sequences derived from the more conservative marker 18S rDNA, the membership of the target parasite within the Microphallidae was confirmed. The reason explaining why only 16 metacercariae from Dunbar were collected from only two barnacle hosts was a logistic one, but may not have an obvious impact for the analysis. Leung *et al*. (2009) demonstrated that the genetic diversity of metacercariae in sessile second intermediate hosts was very high, and suggested that the second intermediate host to be an accumulator of genetic diversity for the studied digenean *Gymnophallus* sp. Therefore, it is likely that the 16 metacercariae from the two hosts can still be representative of the variation within the population, although using metacercariae from multiple sources would be preferential. Metacercarial samples from Dunbar always grouped with other microphallids and formed a well-supported monophyletic group. The topology revealed in the present study (see Figure 4.25) was congruent with other larger scale studies which analysed more taxa and utilised longer sequences (Olson *et al.*, 2003; Tkach *et al.*, 2003). The phylogenetic trees obtained in the present study can only be compared with the lower half of Figure 6 in the report of Olson *et al*. (2003). The topology obtained by three algorithms (NJ, MP, ML) in the present study was congruent to the topology of Olson *et al.* (2003) in that the Echinostomatoidea (*i.e*., the outgroup used in the present study) is the most basal, followed by the Opisthorchiodea, then the Microphalloidea. This topology supports previous findings that microphallids are more derived Digenea (by molecular evidence of

Olson *et al.*, 2003; and by life-cycle and morphological evidence of Cribb *et al.*, 2003). Also, in the present study, the Pleurogenidae and Prosthogonimidae were found to be even more derived than the Microphallidae within the Microphalloidea clade; this conformed to the results of Tkach *et al.* (2003). The sequence of *Microphallus fusiformis* Reimer, 1963*,* however, is problematic. It was not included in the Microphallidae clade in the NL and ML trees. In the MP tree, four lineages, namely *Maritrema, Microphallus,* Pleurogenidae + Prosthogonimidae, and *M. fusiformis,* were apparent. Also *M. fusiformis* was always the most derived in the three algorithms. In the trees generated by partial 5.8S + full ITS2 + partial 28S rDNA fragment, however, *M. piriformes* always grouped with other *Microphallus* species (see Figure 4.27). To resolve this anomaly, more information is needed, such as checking the identity of the samples generating *M. fusiformis* data again*.*

By comparing sequences of partial 5.8S + full ITS2 + partial 28S rDNA fragment, the consensus sequences of the target parasite obtained from the three study localities (Dunbar, Stonehaven and Rosehearty) were 100% identical to the only ITS2 rDNA sequence of *M. gratiosum* available on GenBank (HM584171 *Maritrema arenaria* isolate Maa). In the phylogenetic trees, all the *Maritrema* species formed a monophyletic group with different degrees of bootstrap support (see Figure 4.27). This evidence demonstrated that the target parasite belongs to the genus *Maritrema* and is most like *Maritrema arenaria* on GenBank. If the morphological diagnosis of the sequence (accession no. HM584171) is correct and because *M. arenaria* is the synonym of *M. gratiosum*, it is considered here, on the grounds of molecular evidence, that the metacercariae collected off the Scottish coast were *M. gratiosum*.

For the phylogeny of the lower taxa at the genus level (*i.e*., trees built from partial 5.8S + full ITS2 + partial 28S rDNA fragment), it is possible to compare present results with the study of Galaktionov *et al.* (2012), in which 28S, ITS1 and ITS2 rDNA were applied to infer phylogeny. There were only three species of *Maritrema* included in their study. By adding more species in the present study, further molecular evidence of the relatedness within the genus *Maritrema* was revealed. In the present study, *M. gratiosum* from Scotland is not closest to *M. oocysta* (see Figure 3C of the ITS2 tree presented in Galationov *et al.*, 2012 and Figure 3.34 in the present study) but to *M. subdolum* (see Figure 3B of the ITS1 tree in Galationov *et al.*, 2012 and Figure 3.34 in the present study). The *M. gratiosum* group is separated from all other *Maritrema* species included in the present study. The phylogenetic inference by 28S rDNA by Galationov *et al.* (2012), Kudlai *et al.* (2015) and Hernández-Orts *et al.* (2016) showed similar results. *Maritrema gratiosum* is closest to *Maritrema prosthometra* Deblock & Heard, 1969 followed by *M. oocysta*. *Maritrema subdolum* is more basal and far away from *M. gratiosum*. In the study using 28S rDNA and 28S + ITS1 by Presswell *et al.* (2014); the 28S inference shows a similar pattern to the above. On the other hand, in the analysis combining sequence data from 28S and ITS1

regions, *M. gratiosum* was closest to the group formed by *M. poulini* + *P. deblocki*, but not so close to *M. oocysta* and quite distant from *M. subdolum*. This highlights the observation that considerable variation of *Maritrema* spp. topology appeared when using different combinations of genes and taxa.

The presence of a polymorphic site in the 18S rDNA and different copies of ITS2 rDNA identified in the current study deserves some discussion. The single polymorphic site in 18S rDNA found in samples from Stonehaven may reflect genetic polymorphism within this locality. As 18S rDNA is highly conservative, and usually suitable for inferring deep phylogenetic relationships (Nolan & Cribb, 2005), this single polymorphic site could possibly be a hint of higher genetic heterogeneity. More evidence, however, would be needed to support such a conclusion. In the present study, contamination was detected in the *M. deblocki* samples. Two samples each obtained from individual flukes (confirmed visually under stereomicroscope) were used for DNA extraction. In the first specimen two bands, one for fish sequence and one for parasite sequence, were detected by direct PCR. In the second specimen, three distinct products, two species of microphallid and one *Littorina* sp. sequence, were detected by cloning. Also, different clones of ITS2 copies were detected for two microphallids (potentially *M. deblocki* and *M. pygmaeus*). It was not possible to trace the sources of contamination for the *M. pygmaeus,* therefore it is beyond the scope of discussion here. For *M. deblocki*, however, it was confirmed visually that there was only one mature adult per tube. Why do different copies of ITS2 with low levels of variation (i.e., 1-4 bps different among 470 bps) exist? There are four possible explanations. The first is that there was trace amount of *M. deblocki* tissue from other co-habiting flukes contaminating the sample employed. The second is that PCR errors occurred and were detected in the sequence. The third is that sequencing errors occurred. This latter seems unlikely because the sequence obtained by cloning was quite clean and the signal was good. There was also no such degree of sequencing errors detected for other products sequenced by the same company. The fourth potential explanation, possibly the most likely in this instance, is that the sequence differences detected reflect a low level of variability within the ITS2 clusters of this species, that became evident from the amplicon cloning / sequencing strategy employed. This variability would not be apparent from direct sequencing of PCR amplicon due the dilution effect within multiple copies. Ribosomal RNA is encoded by a series of tandemly repeated rDNA sequences. Though these repeats are generally considered to be identical in base composition, a result of concerted evolution (Krebs *et al.*, 2011), mutation / variability among these repeat units does occur, particularly within non transcribed regions. The confirmation of genetic variability within the ITS2 region of *M. pygmaeus* would require a much more extensive survey of cloned amplicons.

4.4.1.4. Interpretation of the phylogeny of *Maritrema* in the context of additional morphological and life-cycle data

From the molecular analysis in the present study, the species with spined cirri, e.g., *M. eroliae* and *M. novaezealandense* (the *eroliae* group), clustered together and were most basal in the genus (see Figure 4.27). *Maritrema oocysta*, the species with an abbreviated life-cycle but an unspined cirrus, was closely related to the *eroliae* group. On the other hand, *M. poulini* and *M. brevisacciferum*, which are both freshwater species, clustered together. The remaining three species, *M. deblocki, M. subdolum* and *M. gratiosum* are more difficult to assign on morphological grounds. Although they all have unspined cirri, the *pars prostatica* is not obvious in the first two species compared to the ampulla-shaped one in *M. gratiosum*. Also, the vitellaria is incomplete in *M. deblocki* and *M. pulini* but complete in *M. subdolum* and *M. gratiosum*. The same pattern, that species with spined cirri grouped together, was also found in the analysis of Presswell *et al.* (2014), Kudlai *et al.* (2015), and Hernández-Orts *et al.* (2016). For the information currently available, it is hard to prove the principle of microphallid systematics proposed by Deblock (1971) on molecular grounds. Fragmented information, however, showed a trend towards agreement of the systematics based on morphology and life-cycle. Deblock (1971) mentioned in his diagnostic key that the conformation of the copulatory organs is the most important character that separates genera; the more primitive genus (*Maritrema*) possesses a cirrus sac and an evaginable cirrus, however, the more derived genus (*Microphallus*) has lost the cirrus sac and developed a male papilla to replace the function of the cirrus. From the present study and evidence from other studies, it can be argued that armed or unarmed cirrus is a good taxonomic character for species differentiation and probably a fundamental difference within the genus *Maritrema.* This theory is supported by molecular evidence from the present study. According to Deblock (1971), the shape and position of the vitellaria is of secondary importance. Other characters, such as configuration of the ootypic junction and extension of the uterus, have less importance. The species included in the molecular analysis in the present study only represent a small fraction of existing *Maritrema* species (comprising up to 62 species according to World Register of Marine Species (WoRMS)), so obviously the phylogenetic comparisons are not rigorous. The relationship would be clearer if more species and more markers could be included. The results of the present study, however, have already demonstrated that, with the limited information available, molecular phylogeny can reflect biological and life-cycle characters of *Maritrema* species.

4.4.1.5. Conclusion for species diagnosis

Taking all the evidence provided by combining morphological (both light microscopy and SEM), life-cycle and molecular methods, metacercariae collected off the Scottish coast in the present study can be confirmed as belonging to *Maritrema gratiosum* Nicoll, 1907.

4.4.2. Morphometric study of metacercariae and adults of *Maritrema gratiosum* collected from Scottish coastal sites

4.4.2.1. The effect of sampling scale and crowding

In the present study, a MANOVA was applied before the PCA to detect differences between the two data sets from the same locality but at the different scales (*i.e*., local scale and whole area scale, see Table 4.9). The similar methodology can be found in the study of Gállego *et al.* (2014), in which MANOVA was applied to detect morphometric differences between two sets of data, and PCA was applied subsequently to access the contribution of each variable in distinguishing species. In the present study, a difference was detected as a significant multivariate main effect, the metacercariae from the local scale study being significantly larger than those from the whole area study for many morphometric measurements. The differences found here were not considered to be differences at the species level as the metric measurements for samples from these two scales overlapped. No difference in any morphological parameter to separate specimens from the two scales to different species could be identified either.

The possible cause of this intraspecies level difference can only be speculated upon, but because in the MANOVA the variables which were different between the two data sets did not include body length and width but covered suckers, testes, ovary, and cirrus sac; and the difference was not related to overall size. Crowding is a possible explanation for the difference of these organs, as there is evidence in other digenean systems that crowding can affect the development of reproductive organs (Tandon, 1973; Swarnakumari & Madhavi, 1992; Stillson & Platt, 2007). Stillson and Platt (2007) suggested that competition for nutrients, inhibitory compounds secreted by the competitive flukes, and inflammatory reactions from the host, can be responsible for the reduction in overall size and reproductive organs. Host identity is another factor to create morphological variability in digeneans (Hildebrand *et al*., 2015), however, this factor may not be relevant to the present study. In the present study, the differences between the two scales although significant, however, were not large. This finding partially supports the assumption that distance between sampling sites is proportional to the morphological variability. One of the reasons that there was higher intraspecific competition in the whole area scale than the local scale can be technical. As there are more samples in the whole area scale, and these samples were from a wider range, it could be that more extreme values are included. Just by adding more extreme values can result in a higher value of the crowding index obtained. Other possible causes for the difference between scales include unique local factors such as final host identity and the size / age of the barnacle hosts.

The size of the digenean is not considered an important diagnostic parameter, as many factors, such as host size and parasite age, can influence body size in digeneans (Bakke, 1988). Sometimes

phenotypic plasticity in digeneans can be so high as to be misleading for taxonomists to consider the specimens include different species or even genera (Stillson & Platt, 2007; Hildebrand *et al*., 2015; Falcón-Ordaz *et al*., 2019). For examples, phenotypic plasticity and morphological similarity are noted in diplostomid metacercariae and many factors were suggested to contribute to it, such as species, size and age of host, and age, population size and density of metacercariae. The existence of "morpho-types" does not necessarily represent distinct species, and this can be established by molecular methods (Otachi *et al.*, 2015). Stillson and Platt (2007) proved experimentally that morphological measurements of *Echinostoma caproni* Richard, 1964 in Institute of Cancer Research (ICR) mice was inversely correlative to the inoculum size. Hosts and geography-related morphological variability were noticed in *P. umblae* but was not quantified (Bakke, 1988). Hosts and geography-related variability were also noted in *L. cruzi, F. hepatica* and *F. gigantica* and were quantified by ANOVA (Martorelli & Ivano, 1996) and other morphometric tools (Valero *et al.*, 2012; Ashrafi *et al.*, 2015). Some researchers have pointed out the importance of "scale" in biological studies. Wilson *et al.* (2002) mentioned the effect of sample size biases on field-based studies of host-parasite interaction. They suggested that samples should be collected from appropriate demographic groups and sampling units, such as years, population densities and locations, etc. Grabowski and Porto (2017) studied the appropriate sample size for evolutionary theory inferred by morphological data and discussed the effect of sampling effort and inaccuracy in evolvability and integration statistics. The results in the present chapter already demonstrate the importance of sampling scale and that local populations can have unique characteristics.

4.4.2.2. Principal component analysis

Three PCA analyses were conducted on three datasets in the current study: for specimens from the local scale of Dunbar, the whole area of Dunbar, and specimens from three distanced localities to investigate morphological heterogeneity within and among localities. The results shown that morphology of metacercariae collected within the same locality were quite homogeneous. A comparison of the cultured adults from the different localities in general support the hypothesis that minor morphological differences exist between localities, but the differences are not large enough to separate them as different species. A cofounding factor of culture condition was also detected. By visual inspection, the degree of morphological differences was correlated to distance between sampling localities, however, the difference was not large (see Figures 4.28, 4.30, 4.32, 4.35).

For the results of the PCA of the specimens collected for the local scale study, no obvious grouping can be detected. This indicated that metacercarial specimens for the local scale study are morphometrically similar and no partition could be achieved at this scale of sampling. It is interesting to find the first component which explains the most variance, and commonly recognised as reflecting

"size effects" (Flores & Baccalá, 1998; Gállego *et al.*, 2014), was dominated by variables related to body width but not body length. The variables in the first component, which accounted for 41.87% of the variance, were largely measurements associated with body width and round organs near the ventral sucker, i.e., testis width, ventral sucker width and length, cirrus sac width. In contrast, the second component, which accounted for 12.8% of the variance and is often considered to reflect shape, was dominated by body length. It was largely comprised of vertical measurements of organs. Pharynx length itself which accounted for 7.75% of the variance, accounted for the third component, indicating that this measurement is not affected by body length and width (see Table 4.14). These findings revealed that the morphology of the reproductive organs and ventral sucker contribute most for the intra-species morphological variation in the local scale study. For the whole area study, similar patterns were found but these were not the same as for the local scale study. Four instead of three components, however, were extracted (see Table 4.18). The first component (44.8% of variance) was similarly dominated by body width and round organs near the ventral sucker, the second component (9.58% of the variance) was dominated by the body length and the length of the oesophagus, the third component (7.55% of the variance) was dominated by the pre-pharynx, while the fourth component (6.9% of the variance) was dominated by the pharynx and the oral sucker. From the two sets of analyses above, dimension reduction was achieved from 15 variables to 3-4 components by means of PCA. These revealed that the metacercariae from Dunbar Red Rock were morphologically homogeneous and can be considered a single species morphologically. The most distinguishing features were body length and round organs near the ventral sucker.

When using adult samples from the three distant localities (*i.e.*, Dunbar, Stonehaven and Rosehearty), the results of the PCA told a slightly different story. In this analysis, three extra variables were included – the sucker ratio, the caecum length, and the pharynx width. Also, the sample size was smaller (*i.e.*, 62 vs 133 in the local scale study and 120 in the whole area study) because there were not too many cultured adults available to be included. Some degree of separation was detected for specimens from the three localities (see Figures 4.32 & 4.35). From the loading plots (Figures 4.34 & 4.37), it is interesting to note that the variables which separated specimens from Dunbar from those from Stonehaven and Rosehearty were the organs near the ventral sucker, indicating that the degree of morphological differences were still within the intra-species level. Furthermore, the morphological difference between specimens from Stonehaven and Rosehearty, however, were quite homogeneous. This finding seems to indicate that besides the factor of distance, culture conditions such as culture time and nutrient supply were also important for morphological variation. These two factors should be investigated separately in the future, which was combined unintentionally in the present study. Furthermore, it is possible that larger samples size and more stringent experimental design, i.e.,

applying the same sampling strategies, can elucidate the importance of distance better. It is generally accepted that a larger sample size is ideal for PCA and the ratio between sample numbers vs. variables should be high. Previous research has indicated that when the sample size increases, subject to variable ratio was less important, and vice versa (Osborne & Costello, 2004). However, even under this principal, clear differentiation of digenean or monogenean species using small sample sizes has been achieved in several cases (Rubtsova *et al.*, 2007; Hernández-Mena *et al.*, 2014; Soo & Lim, 2015). Finally, although not quantified, the degree of separations between groups are wider than that observed in specimens from the local scale or whole area study. This finding supported the hypothesis that the degree of morphological differences may be correlated to distance between sampling sites.

PCA is a tool to simplify complexity in high dimensional data, while retaining the patterns and trends at the same time. It allows easier data exploration and can serve to identify key variables and discover outliers. Furthermore, it is an unsupervised multivariate analysis tool, which means that prior knowledge of the data set is not required (Lever *et al*., 2017). In parasitology, the separation of species by means of PCA are most successful in studies based on hard parts, for examples, for monogeneans (Shinn *et al*., 2010; Hahn *et al*., 2011, Soo & Lim, 2015). For digeneans, morphometrical partition is more difficult due to contraction of soft body parts and the lack of hard parts, especially for larval stages which lack diagnostic characters (Cavaleiro *et al*., 2012; Caffara *et al*., 2016). In a contrast to the Monogenea where some measurements of the hard parts (based on no overlap of the measurements) can be achieved (Hahn *et al*., 2011); morphometric measurements often overlapped between different populations or species in digeneans. A prior exploration by a PCA using full variables and a subsequent PCA by the diagnostic variables were applied by Caffara *et al*. (2016) for differentiation *Euclinostomum* metacercariae; however, the efficiency of species partition was not very good. Maybe in the case of digeneans, a combination of more variables in PCA is a better way to separate groups or species. In the present study, characters which contribute most to the intra-species variability were identified. It is worthwhile to include different species in PCAs in the future, to reveal characters which can separate species better. Furthermore, it is suggested that variables with higher CV value (*e.g*., oesophagus length and pre-pharynx length) to be removed, as these variables can create noise rather than taxonomically relevant signals. PCA is a powerful tool, but it has its own limitations as well, for examples, it assumes linear structure of the underlying structures, and its goal is to maximize variance rather than to find clusters (Lever *et al*., 2017). Furthermore, it cannot include conformation information which is often important in digenean taxonomy, e.g., the ampule-shaped *pars prostatica* in *M. gratiosum*.

Geographical and host induced morphological differences within species have been found in other microphallids (Martorelli & Ivanov, 1996). In the present study, all samples were from the same

host species, *S. balanoides*, but the possibility of the existence of unique local populations relating to topological / microenvironmental features cannot be ruled out. Subtle genetic differences in parasites and phenotypic plasticity can lead to unique characters within local populations (Poulin, 2006). These phenotypic differences are often not large enough for species discrimination but could possibly be detected by more powerful / extensive analytical methods. It is important to bear in mind that although PCA methods have been applied to species differentiation they should not be used as the sole method of species diagnosis. As argued by Soo and Lim (2015), metrical analyses are only complementary and supplementary in clustering of species. It is generally not practical for taxonomy as it requires large sample sizes. In the research on landmark superimposition, Becerra and Valdecasas (2004) stated that morphometric identification by landmark configurations, which is based on statistics, gives continuous values from almost identical to any separate distance. Landmark analysis can quantify the difference between two or more specimens / species candidates and can provide continuous distance variables that describe the range from near identity to substantial difference; it is therefore employed to ascribe a degree of similarity rather than a diagnosis. A database for a range of intraspecies variation is required if "virtual morphometric types" equivalent to taxonomic types can be established. Therefore, PCA and relevant techniques are suitable as auxiliary tools and better when applied in conjunction with other tools (e.g., molecular tools) for species diagnosis.

4.4.2.3. Conclusions for the morphometric study

For the metric measurements of *M. gratiosum* metacercariae from Dunbar, a MANOVA revealed a multivariate main effect between the local scale and the whole area scale studies. Specimens from the local scale study were in many aspects larger than the samples from the whole area study. Crowding was considered one of the factors among others contributing to the observed differences. The inclusion of crowding index in morphological analysis is unique in digenean studies. By means of PCAs, no significant morphological heterogeneity was detected for the metacercariae collected from Dunbar, either at the local scale or in the whole area study. Cultured adults *M. gratiosum* from the three localities off the Scottish coast, namely Dunbar, Stonehaven and Rosehearty, were morphologically very similar, and PCAs failed to separate specimens from the three localities into different groups. Culture condition, however, was found to be a confounding factor in the PCAs and this factor should be analysed separately in the future. Contrary to the conventional finding, PC1 was found to reflect shape and PC2 to reflect size in the present study. Measurements of reproductive organs and ventral sucker were identified to contribute most to the intra-species variation.

4.5. Conclusion

In this chapter, metacercariae collected from *S. balanoides* at three localities off the Scottish coast, namely Dunbar, Stonehaven and Rosehearty, were diagnosed to the species level as *Maritrema gratiosum* by a combination of techniques, i.e., light microscopy, SEM, and molecular methods. A comprehensive redescription of the morphology of live and fixed metacercariae were performed. The morphology conformed to the general characters recorded for the genus *Maritrema* and cultured adults were largely congruent with the two existing descriptions of *M. gratiosum* Nicoll, 1907. The presence of an eversible, tubular, and smooth cirrus rather than a male papilla was confirmed. The subtle morphological differences detected between the current results and original descriptions could be explained by fixation techniques, culture conditions and local environmental factors. The morphology and topology of surface spines, papillae and suckers were observed by SEM. The results of SEM showed that the fine surface structures of *M. gratiosum* off the Scottish coast were in agreement with the first descriptions of Zaben (1988) for specimens collected from Wales and they were similar to those observed in other microphallids. This is the first SEM study for *M. gratiosum* from Scotland. Sequencing results for partial sequences of the 18S rDNA showed that the metacercariae collected belonged to the genus *Maritrema*. The consensus sequences of a region covering partial 5.8S, complete ITS2 and partial 28S rDNA of the target parasites were 100% identical to the only sequence of *M. gratiosum* in GenBank (*Maritrema arenaria*, HM584171). The molecular phylogeny of the Microphallidae and other closely related or distantly related families was also investigated, as was the molecular phylogeny within the genus *Maritrema*. Results were interpreted through comparison to previous studies and by comparisons of morphology or life-cycle data. This is the first molecular study of *M. gratiosum* from the UK.

Subtle morphological differences were detected by means of morphometric analysis-based methods, but these variations were intra-species level differences. Multivariate main effect between the local and the whole area scale was detected by MANOVA and the factor of crowding was deduced to be an important factor contributing to the observed differences. By means of PCAs, morphological partition cannot be achieved for metacercariae from the local and the whole area scale. The cultured adult specimens from the three distanced localities in the PCA plots overlapped, indicating the presence of a single species. The adults cultured from Dunbar, however, showed greater variability / spread than the adults cultured from Stonehaven and Rosehearty. All the metacercariae and cultured adults obtained in the present study nevertheless fell within the range of a single species. The influence of distance for morphological variation was confirmed and the confounding effect of culture conditions were discussed. The subtle morphometric differences detected within the same localities but at different scales and among the three geographically distanced localities could be explained by

aggregation-related crowding effects, though potential environmental, host, and genetic factors could not be excluded. This is believed to be the first study to include a crowding index in a morphological analysis of digeneans.

Chapter 5 *In vitro* culture and confocal microscopy study of *Maritrema gratiosum* Nicoll, 1907: from metacercaria to ovigerous adult

5.1. Introductions

5.1.1. *In vitro* culture for digenean

In vitro culture is an important technique for gaining an insight into the biology of parasites. It can be applied to immunology, taxonomy, physiology, developmental and pharmacology-based studies, or simply be employed to provide parasite materials for routine use (Stephenson, 1947; Davies and Smyth, 1978; Davies, 1980; Smyth & Halton, 1983; Zaben, 1988; Ivanchenko *et al.*, 1999; Keiser, 2010; Presswell, 2014; McCusker *et al*., 2016). *In vitro* culture techniques for Digenea, however, are less advanced compared to those employed for Protozoa or bacteria, partly due to their complex life-cycles and due to the general neglect of this area of research (Keiser, 2010). Most efforts have been devoted to medically important species such as *Schistosoma* spp. and *Fasciola* spp. for which diverse techniques have been developed (Ractliffe *et al.*, 1969; Davies & Smith, 1978; Basch, 1981 a & b; Yoshino & Laursen, 1995; Augot *et al.*, 1997; Ivanchenko *et al.*, 1999; Keiser, 2010; McCusker *et al*., 2016).

Due to the complexity imposed by multiple life stages and the diversity of hosts, the major challenge for digenean culture is the completion of the life-cycle in the laboratory. Different life stages of Digenea usually have rather different requirements for cultivation. This reflects the fact that hosts for different life stages of Digenea are quite diverse and can cover different phyla (*e.g.*, Mollusca and Chordata). The nutritional requirements, culture temperature, oxygen level, osmolality, *etc*., thus differ accordingly. The situation is further complicated in that even within the same genus, growth requirements between species can differ considerably. Currently, individual life stages or several life stages of one species can be cultured and maintained in the lab for different lengths of time using axenic or synxenic culture.

Excystment is the first step for *in vitro* culture of metacercariae. Metacercarial excystment is a well-established subject for research, and comprehensive reviews for methodologies across families have been conducted several times (Lackie, 1975; Smyth & Halton, 1983; Fried, 1994). Many species require special chemical and physiological conditions for excystment, but some need only simple triggers such as optimal temperature. For microphallids, some species readily excyst in warm saline but some require enzymatic treatments (Fried, 1994). In the study of *in vitro* excystment of *Maritrema arenaria (*syn. *M. gratiosum*) (see Irwin, 1983), the addition of Hanks Balanced Salt Solution (HBSS)

with 0.22% sodium bicarbonate, 0.3% sodium taurocholate and 0.5% trypsin at 41°C, triggered the excystment of metacercariae after 15 minutes. The author also found evidence suggesting that excystment of this species was achieved by the release of enzymatic materials (acid phosphatase in this case) by metacercariae, while the excystment fluid functioned as a "trigger" for larval activity. A later study by Zaben (1988) tested several excystment conditions including Irwin's (1983) solution on the same species and found that 40% sea water (38 ± 1 °C) can achieve the same percentage (86-87%) of excystment as Irwin's solution. This finding indicates that the excystment of *M. gratiosum* does not require digestive fluid. The same situation does not apply to other microphallids. The excystment of *Microphallus abortivus* Deblock, 1974 was achieved in 15 minutes by applying a freshly made solution consisting of a mixture of Solution A: 5 mL bicarbonate saline containing 0.8% sodium taurocholate and 0.3% trypsin, and Solution B: 5 mL 0.02 M HCL containing 0.8% L-cysteine, the metacercariae being incubated 40-42°C (Saville & Irwin, 1991). Excystment of *Microphallus similis* was achieved by incubation in 0.5% pepsin in HBSS (pH 2) for 30 minutes at 38°C, followed by 0.5% trypsin in HBSS (pH 7) for 10~20 minutes at 38°C (Davies & Smyth, 1979). Extrinsic factors such as temperature, gas phase, pH, oxidation-reduction agents, osmotic pressure, enzyme, and bile, are important for digenean excystment. Intrinsic factors such as substances secreted by the metacercaria or its muscular activity are also involved in excystment (Fried, 1994).

The *in vitro* culture *of S. mansoni* is important for studying schistosomiasis and is also an important model for *in vitro* culture of digeneans in general. Each life stage of this species, from miracidia, cercariae, schistosomules to adult, can be cultivated under laboratory conditions, and different life stages have been maintained worldwide either *in vitro* or *in vivo* (Keiser, 2010). *Schistosoma mansoni* has been successfully cultivated *in vitro* from cercariae to pairing adults, which consequentially produced infertile eggs (Basch, 1981 a & b), however, up to now, the completion of the life-cycle *in vitro* has not yet been fully achieved (Milligan & Jolly 2011) and lab animals are still important for maintaining adults. A specific cell line, "Bge cell line", which is derived from the first intermediate host *Biomphalaria glabrata*, for the purposes of a synxenic culture of *S. mansoni* sporocysts, was developed in 1974 (Hansen, 1975). Long-term proliferative culture of *S. mansoni* sporocysts capable of generating cercariae *in vitro* has been developed using mixtures of medium F, DME / F12 and Bge medium co-cultured with Bge cells. The cercariae that were produced possess the ability to parasitise hosts (Ivanchenko *et al*, 1999). The mechanical transform method developed by Basch seems to be the easiest and most reliable way to gain schistosomula from cercariae *in vitro*. Following this study, a variety of media were tested for schistosomula, young adults and adults; such as Bash, MEM, DMEM, medium 199, Hanks' or EARLE'S saline, medium 169, NCTC 135, Leibovitzs' L-15 and RPMI 1640 (Basch 1981 a & b; Keiser, 2010). Serum and red blood cells are needed for the

development of adults, and antibiotics should be added and the whole procedure should be performed under sterile conditions (Basch, 1981a).

For *Fasciola hepatica*, early work demonstrated the possibility of maintaining adults from naturally infected ruminant hosts *in vitro* with a continuation of egg-laying based on Earle's balanced salt solution and HBSS (Ractliffe *et al.*, 1969). *In vitro* cultivation of metacercariae to partially developed flukes has been achieved with NCTC 135 medium plus 50% heat inactivated chicken serum and sheep red blood cells. The authors, however, encountered difficulty in obtaining mature adults and the animals appeared to be in a state of "suspended animation" with somatic development more advanced than germinal development (Davies & Smyth, 1978). More recent work, however, has shown that mother rediae of *F. hepatica* can be cultured *in vitro* and can produce daughter rediae and cercariae in media based on L-15 Leibovitz medium (Augot *et al.*, 1997). Newly excysted juveniles can be maintained in medium PRMI 1640 supplemented with antibiotics and serum or red blood cells or RPMI 1640 alone (Ibarra & Jenkins, 1984; Keiser *et al.*, 2006). Juvenile or adult *Fasciola* can be collected from abattoirs or from lab animals and maintained *in vitro* with RPMI 1640 supplemented with rabbit serum and rabbit red blood cells and antibiotics (Anderson & Fairweather, 1994). Up until now, the difficulties encountered by Davies and Smyth (1978) in culturing metacercariae through to fully mature adults remains unresolved but some improvement has been achieved by extending the length of maintenance and adult-like forms can be obtained (McCusker *et al.,* 2016).

Echinostoma caproni, an avian parasite, has been widely used as an experimental model due to the ease of cultivation in the laboratory. The life-cycle can be maintained between snails (*B. glabrata*) and laboratory mice (Fried & Huffman, 1996). The metacercariae can be excysted and maintained *in vitro* for a few days in a medium such as NCTC 135 or RPMI 1640, supplemented with antibiotics and other nutritive products, although the flukes were found to deteriorate quickly (Fried, 2000; Keiser, 2010). The larval stage can also be cultivated *in vitro*. Loker *et al.* (1999) cultured rediae of *Echinostoma caproni* using Bge and / or 199 media supplemented with antibiotics and foetal calf serum co-culture with Bge cells, which yielded rediae and / or motile cercariae. Some cercariae even encysted in the medium but the viability of these metacercariae was not mentioned.

Compared to the species mentioned above, progenetic digenean species are an easy target for *in vitro* culture. The rise in temperature alone is sufficient to stimulate spermatogenesis, oogenesis and vitellogenesis (Smyth & Halton, 1983). For *M. similis*, excysted metacercariae developed to ovigerous adults after several days in Hanks' saline or in NCTC 135 supplemented with foetal calf serum (Davies & Smyth, 1978). In recent work, through optimisation of culture conditions, *Microphallus turgidus* cultivated *in vitro* were able to produce fertile eggs which were capable of infecting snail hosts *Spurwinkia salsa* Pilsbry, 1905. The excysted metacercariae were incubated in HBSS overnight and

then cultivated in RPMI-1640 plus 20% horse serum (Pung *et al.*, 2011). The other microphallid species *Gynaecotyla adunca* can also be cultured from metacercariae through to ovigerous adults *in vitro* in HBSS, Dulbecco's Modified Eagle medium / F-12 (DME/F-12) and RPMI 1640, but the eggs that were produced were infertile (West *et al.*, 2014). *Maritrema novaezealandense* can be cultured from metacercariae to ovigerous adults *in vitro* in NCTC-109 supplemented with chicken serum and antibiotics although both normal and abnormal eggs were found (Fredensborg & Poulin, 2005). Zaben (1988) was the first to successfully culture *M. gratiosum in vitro,* and since then no attempt has been made to culture this species. Zaben focused on the survival of metacercariae to adults and on egg production rather than the developmental change for this species under culture conditions. In exploring the culture media required, Zaben tested several non-nutrient and nutrient-rich media (see Appendix 43) and found the best medium to be medium 199 supplemented with 10% foetal bovine serum. A 24-day survival time was achieved with oviposition, with both normal and abnormal eggs being observed. In the present study, natural adults of *M. gratiosum* were not available due to ethical reasons surrounding the sampling of birds and legislation covering their protection, but adults were still required for morphological diagnosis (see Chapter 3) and for the developmental biological study (this chapter). An *in vitro* culture study will be conducted in the present study, in order to optimise culture conditions, to obtain ovigerous adults with normal eggs, and to provide materials for studies of their musculature and developmental morphology.

Appendices 42 and 43 summarise several examples of *in vitro* culture for non-progenetic and progenetic Digenea, respectively. It is not the purpose here to give a comprehensive review of the *in vitro* culture of Digenea, but rather to capture some important progress and give some examples of *in vitro* culture techniques based on different life stages.

5.1.2. Study of the muscular systems in Digenea by laser scanning confocal microscopy

As mentioned above, the development of *M. gratiosum* from excystment through to the adult stage has not been thoroughly studied using modern techniques; furthermore, no study has been performed on the musculature of *M. gratiosum* during this development. Understanding the structure of muscular systems deepens our understanding of the biology and ontogeny of the Digenea. Basic patterns of the muscular system of parasitic flatworms are similar. Muscular systems can be divided into three principal parts in adult worms: somatic muscle, the muscle of adhesive organs and the muscle of the alimentary and reproductive systems. The muscle is in general of a smooth type (Mair *et al.*, 1998a). To observe muscular systems, a fluorescent phalloidin stain, which binds to muscle actin, is applied in conjunction with fluorescent light microscopy, particularly laser scanning confocal microscopy (LSCM) to provide a powerful method to reveal the structures of flatworm muscular systems. Phalloidin belongs to a family of toxic peptides obtained from the mushroom *Amanita*

phalloides (Vaill. ex Fr.) Link (1833). It binds to actin filaments (F-actin or polymerized actin) rather than monomeric actin and can be chemically modified by fluorescent dyes without the loss of actin binding ability and is therefore used widely for F-actin visualization in cell culture (Huang *et al.*, 1992). In digenean studies, this technique is often applied in combination with immunofluorescent techniques to reveal the neuromusculature or is used alone for the study of muscular systems. It has been applied to adult stages (Mair *et al.*, 1998b, 2000), to metacercarial stages (Stewart *et al.*, 2003a), and to follow changes to morphology throughout parasite development (Stewart *et al.*, 2003b; Šebelová *et al.*, 2004; Bulantová *et al.*, 2011; Petrov & Podvyaznaya, 2016; Borges *et al.*, 2017). Very few studies have been conducted concerning the muscular system of microphallids. According to the analysis of body musculature by confocal microscopy, Krupenko and Dobrovolski (2018) stated that the whole ventral surface of microphallid parasites (e.g., the metacercariae of *M. piriformes* and *M. pygmaeus*, and the adult of *Levinseniella branchysoma* (Creplin, 1837) Stiles et Hassall, 1902 acts as an attachment organ (ventral concavity). Generally, a three-layer arrangement of the somatic muscle (i.e., circular, longitudinal, and diagonal) was identified, however, extra sets of muscle on the hind body support the shape and function of the ventral concavity. Thicker additional internal muscles were found near the ventral sucker. The authors also suggested that dorso-ventral muscles function to generate negative pressure while attaching. These muscles couple with surface spines to strengthen the attachment of these small digeneans, which possess relatively weak ventral suckers.

DAPI (4,6-diamidino-2-phenylindole), is a fluorescent dye which can be observed by confocal microscope and is believed to associate with the minor groove of double-stranded DNA (Chazotte, 2011). It is therefore used for staining nucleic acids in nuclei of cells, thereby highlighting the nuclei themselves and the cell distribution in tissue. In parasitology, DAPI is sometimes applied to the diagnosis of Protozoa, such as for *in vitro* malaria parasite stage classification and viability quantification (Moon *et al.*, 2013) and for *in vitro* viability assays for *Cryptosporidium* oocysts (Vande Burgt *et al.*, 2018). There were very few applications of DAPI to help elucidate tissue structures of digenean parasites (Greiman *et al*., 2016; Pila *et al*, 2016; Fischer *et al*., 2017); in the present study DAPI is used to highlight tissues / organs and as an aid for characterising the muscular system.

Aim

The aim of the present study was to culture metacercariae of *M. gratiosum* under *in vitro* conditions, to optimise culture conditions, to obtain ovigerous adults with normal eggs and to provide materials for studies of their musculature and developmental morphology. The growth in the chosen culture medium was analysed by statistical methods to assess aspects of survival, body size and egg production. Finally, by means of confocal microscopy, the present study investigates the muscular systems of *M. gratiosum* during development from metacercariae to ovigerous adults, and relevant biological functions were deduced.

5.2. Materials and methods

5.2.1. Barnacle samples collection and maintenance

Three sampling trips were carried out, the first and the third took place at Dunbar Red Rock (56° 00' 19.9"N; 2° 31' 33.4"W) on the 09 / 2017 and on the 11 / 2018. Three sampling sites, site 1, 2 and 3 were chosen for these two trips, according to the prevalence data determined from previous trips (Figure 5.1). These samples were for a smaller scale pilot study. The second trip was undertaken on the 12 / 2017. Barnacle samples were collected from Dunbar Leisure Pool (56° 00' 19.5"N; 2° 31' 04.2"W, Figure 5.1). A vertical sampling strategy was conducted: a vertical line at approximately the mid-tide level of the rock wall under a kittiwake breeding cliff was chosen (no kittiwakes breed over winter), rock samples with *S. balanoides* attached were taken from the bottom, middle and top positions separated by about 1 metre (Figure 5.2). After checking the prevalence, the samples from the top position with a higher prevalence and abundance were used for the larger scale *in vitro* culture experiments. For both trips, small pieces of rock with barnacles attached were taken and maintained in small aquaria with seawater in the lab. The aquaria were placed in an incubator, an air supply was provided and maintained at 14 °C. Sea water was changed once per week (35 ppt). The maintenance time was limited to two months.

Figure 5.1. The sample sites at Dunbar for the *in vitro* culture experiments. A. Two different localities at Dunbar. B. Three sample sites at Red Rock.

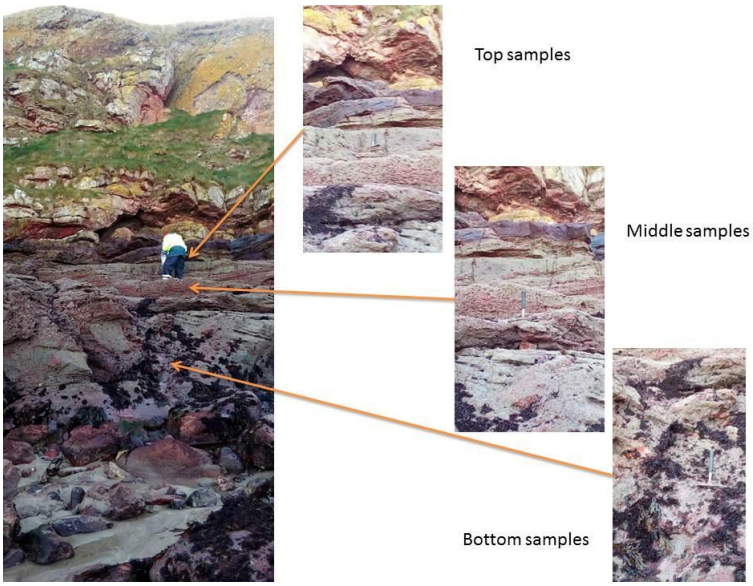


Figure 5.2. Vertical sampling strategy undertaken at Dunbar Leisure Pool under the "kittiwake breeding cliff". Three sites, top, middle, and bottom were chosen.

5.2.2. Parasite material collection

Barnacles were detached from the rock using a scalpel and tweezers. The whole barnacle was placed in an embryo glass with seawater, and the prosoma was extracted from the shell plates using tweezers. The prosoma was examined under pressure of a coverslip and the cyst numbers were recorded. Afterwards, the coverslip was removed and the oral cone was cut by scalpel. The prosoma with the cysts inside was roughly torn, to partially release the cysts, and then the cysts together with the prosoma were placed in a 1.5 mL Eppendorf tube with 500 µL 0.01 M phosphate buffered saline (PBS, Sigma). The tube containing the barnacle and their parasites were incubated at 4°C overnight.

5.2.3. Preparation of excystment fluid and culture media

PBS was prepared by dissolving a tablet (Phosphate Buffered Saline Tablet, P4417 Sigma-Aldrich) in 200 mL distilled water to reach a concentration of 0.01M. Commercially available cell culture media, such as Eagle's Minimum Essential Medium complete medium (EMEM, Gibco) and NCTC 109 (ThermoFisher Scientific, no glutamine, formula see Appendix I4) were tested. Different concentrations of chicken serum (CS, Sigma) and 20% foetal bovine serum (FBS, Gibco) were used as the food source in the experiments using NCTC 109. In experiments using EMEM, EMEM complete medium (contained L-glutamine 2mM) plus 10% FBS plus non-essential amino acid (1x) were used. Different concentrations of antibiotics (penicillin and streptomycin, Sigma) were added to prevent bacterial contamination. To prepare 200 mL NCTC 109 supplemented with 20% chicken serum, 40 mL chicken serum was added to 160 mL NCTC 109. Chicken serum was inactivated at 56°C for 30 minutes before use. NCTC 109 supplemented with 20% foetal bovine serum was prepared using the same method, except that heat inactivation of the serum was not needed. A

commercial penicillin / streptomycin solution was used as a bacterial disinfectant (solution with 10,000-unit mL^{-1} penicillin and 10 mg mL^{-1} streptomycin, sterile-filtered, Sigma). An aliquot of 20 mL of the antibiotic solution was added to 200 mL culture medium to reach a concentration of 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. A total medium volume of 500 µL per tube was used for subsequent culture experiments. Preparation of the culture media and the antibiotic solutions were conducted under laminar flow to prevent bacterial contamination.

5.2.4. Calculations of excystment percentage

The samples used for measuring excystment rate were from site 3 at Dunbar Red Rock. The excystment percentage of metacercariae incubated in 0.01M PBS at 40°C were calculated. Eight barnacles each with between 10-30 cysts were chosen, with the cysts from a single barnacle being placed in one tube. At 15, 30, 60, and 120 minutes, the excystment percentage of the metacercariae from two barnacles were counted.

5.2.5. Tests undertaken with different culture media and excystment fluids

Different combinations of excystment fluid and culture medium were tested in small scale experiments. After the conditions were optimised, a standard excystment fluid and culture medium were used in larger scale experiments to test the effects of different concentrations of chicken serum.

5.2.5.1. Small scale preliminary experiments for culture conditions

Initially, the need for aseptic operations, methods for killing and flattening excysted metacercariae and the feasibility of using different culture media were assessed. The cysts kept in PBS at 4°C overnight were excysted in a 40 °C water bath for several hours, and were then transferred to the desired culture media (500~1000 µL per tube) in an Eppendorf tube. The living flukes in culture medium were then incubated in a 40°C incubator. During culture, several parameters concerning the development of the adults *in vitro* were observed; such as the percentage survival and the survival duration, morphology at different ages, and egg production and the duration over eggs were produced. The percentage survival was calculated by counting live flukes in each tube at each time point, and then by dividing this by the number of live flukes in each tube at time 0. The percentage survival was defined as: the fluke survival per tube at the chosen time point / the fluke survival at time 0 (0 hour, the time the metacercarial cysts were put in the culture medium). At each time point, the mean of the percentage survival of the available tubes was calculated. Some tubes were taken for morphology and for egg counts at certain time points and, therefore, were unavailable at the next time point. This results in the cohort for calculating survival rate gradually decreasing. If a tube was unavailable at a time point, the data from that tube will be excluded but not counted as 0 survival. The viability of the metacercariae was defined as: moving within 30 seconds or following contact stimulation and displaying a transparent body wall. The variation of the pH of the culture medium was also checked at each time point by observing the colour of the culture medium, which contained a phenol red indicator (low pH yellow, pH 7.4

red, high pH purple). The percentage survival was recorded for as long as the worms were alive, but this was not beyond 12 days.

Different methods for fixing and flattening the live metacercariae were also tested at this stage for the purpose of obtaining optimal fluke specimens for permanent preservation and morphological / confocal microscopy observation. Some flukes were observed live by phase contrast light microscopy (Olympus BX51) under coverslip pressure (22 x 22 mm, 0.13 - 0.17 mm thick). Some flukes were fixed directly in 80% ethanol or were fixed first in 10% formalin and then stored in 80% ethanol. Some flukes were heat flattened in culture medium by putting the tube in a near boiling water bath for 1 minute, and then stored in 80% ethanol. Some flukes were flattened in Berland's fluid (1 part formalin plus 19 parts glacial acetic acid) for ~10 seconds and then stored in 80% ethanol. The photos of live flukes were taken using Olympus cellSens standard software. The fixed flukes were stained with Mayer's paracarmine (for the method, see Chapter 4 and Appendix 9) with photos being taken using the same system.

In small scale pilot studies, the following combinations were tested (Table 5.3). The metacercariae were not equally divided in each tube at this stage; instead, the cohort from each individual barnacle were put in the same tube. The medium was not changed during each experiment.

Table 5.1. Treatment groups for the small scale *in vitro* culture experiment

Excystment fluid and condition	**Culture medium**	**Replicate**	**Serum**	**Antibiotics**
PBS 2 hours	PBS	Site 1 & 2	None	None
PBS 2 hours	PBS +EMEM 1:1		None	None
PBS 2 hours	EMEM		None	None
PBS 4 hours	NCTC 109		None	None
NCTC 109 4 hours	NCTC 109		None	None
PBS 4 hours	NCTC 109		None	Penicillin (1 U mL^{-1}) and streptomycin (1 µg mL^{-1})
PBS 4 hours	NCTC 109		20% foetal bovine serum	Penicillin (1 U mL^{-1}) and streptomycin (1 µg mL^{-1})
PBS 4 hours	NCTC 109	Replicate 1 & 2	20% foetal bovine serum	Penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1})
PBS 4 hours	NCTC 109		20% chicken serum	Penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1})

*The physical conditions for all treatment groups were the same: 40°C

5.2.5.2. Larger scale major *in vitro* culture experiment

A more systematic experiment with an appropriate number of samples was conducted to test the effect of chicken serum on egg production and body growth in the chosen culture medium. NCTC109 was chosen as the standard cultured medium, based on the results obtained from the pilot study. Four parameters, namely percentage survival, body length, body width and egg number, were chosen as indicators of the *in vitro* performance of *M. gratiosum*. Morphological differences during the *in vitro* culture were captured by light microscopy (Olympus BX51).

Two treatment groups were assigned: treatment group 1 with NCTC109 supplemented with 20% chicken serum, penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}), and, treatment group 2 with NCTC109 supplemented with 40% chicken serum, penicillin (200 U mL^{-1}) and streptomycin (200 µg mL^{-1}). Chicken serum was inactivated at 56°C for 30 minutes before use. Metacercariae were extracted from barnacles and excysted in PBS for 4 hours as described above. After excystment, five metacercariae were allocated to each tube containing 500 µL culture medium.

The 20% chicken serum treatment group started with 26 tubes (130 metacercariae), the 40% chicken serum group started with 24 tubes (120 metacercariae); the surviving metacercariae were counted every day for 10 days. At 4, 24, 48, 72, 120, 168 and 216 hours, two tubes of metacercariae were taken and observed. The survival of the flukes in the two tubes were counted first, followed by the measurement of their body length and width from photos taken by Olympus cellSens software under a 10X objective. Egg numbers were counted under 20 / 40X objectives. Morphological differences between the major organs and tissues, especially the copulatory organs, were recorded by light microscopy with or without phase contrast. All images were obtained from live flukes under coverslip pressure to prevent fluke movement. Pairs of tubes were only used for single timepoint observations. The media were changed every two days and all operations were carried out under non-sterile conditions as the initial source material was non-sterile. A schematic overview of the major *in vitro* culture experiment is provided below in Figure 5.3.

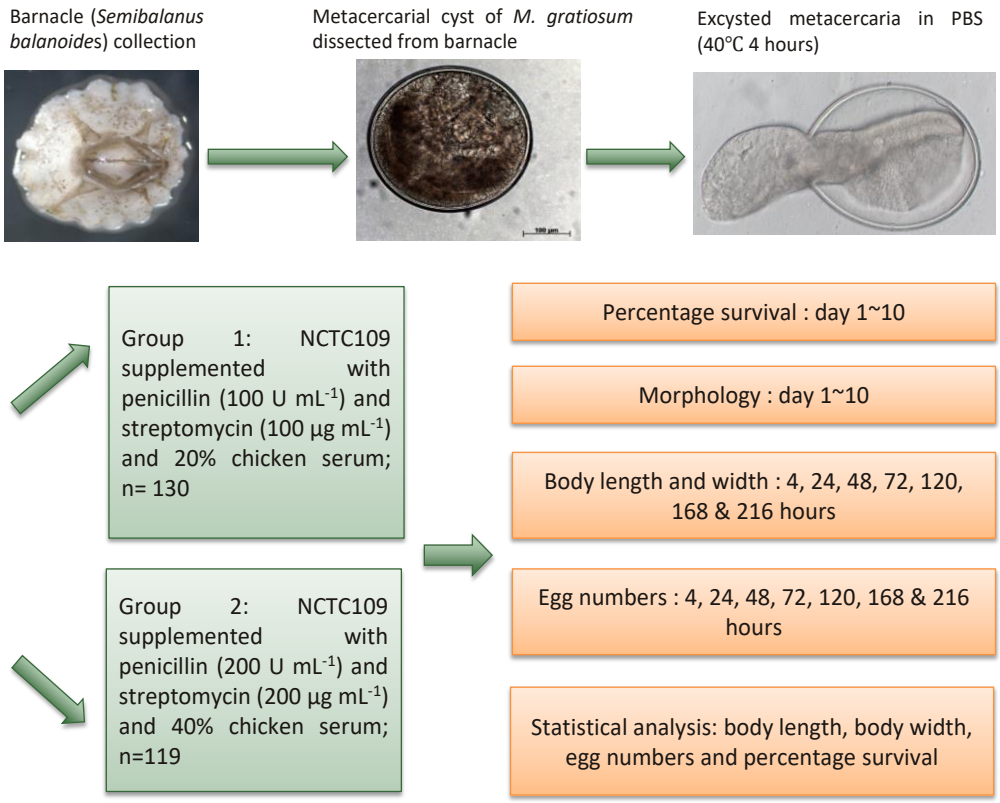


Figure 5.3. Schematic overview of the experimental design used for the *in vitro* culture experiments. All flukes were measured from photos taken from live specimens under coverslip pressure.

5.2.5.3. Statistical analysis for *in vitro* culture

Statistical analysis was only performed for the major *in vitro* culture experiment as the sample sizes for the small-scale experiments were small and variable. The body length and width were compared by independent sample T tests with a Bonferroni correction to avoid random significance due to repetitive comparison (significant at $p<0.007$). The egg number were compared using a Mann-Whitney U test with Bonferroni correction to avoid random significance due to repetitive comparison (significant at $p<0.008$). Survival was presented in two ways. The first method comprises a bar chart showing the percentage of survival compared to time 0. The second method is by means of survival analysis. The survival curve for each group was calculated using the Kaplan-Meier method and curves compared by a Log Rank test (Mantel-Cox). All statistical work was conducted in IBM SPSS statistics version 25 or 27.

5.2.6. Observations of spermatogenesis

Samples were from site 3 at Dunbar Red Rock. Individual cysts (30 replicates) were placed in individual Eppendorf tubes, each containing 200 µL 0.01M PBS. These metacercariae were excysted in a 40°C water bath. Individual flukes were checked for the presence of spermatozoa at 15, 30, 60 and 120 minutes, with 6 tubes being sampled and examined at each time point.

5.2.7. Confirmed the possibility of self-fertilisation

The possibility of self-fertilisation was confirmed by culturing individual metacercaria in individual Eppendorf tubes containing 200 µL of NCTC109 supplemented with 20% chicken serum, penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}). Each metacercaria was excysted individually and transferred to a tube containing culture medium, and cultured at 40 °C under normal gas phase for 2 days. After 4 days, all flukes were checked for the presence of eggs (30 metacercariae were examined).

5.2.8. Extra adult specimens for permanent preservation

The first set of flukes were cultured for 5 days in NCTC-109 supplemented with 40% chicken serum and penicillin (200 U mL^{-1}) and streptomycin (200 µg mL^{-1}), then fixed in a hot water bath and stored in 80% ethanol, and then stained with Mayer's paracarmine. The second set of flukes were cultured for 5 days in NCTC-109 supplemented with 20% chicken serum and penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}), then fixed in Berland's fluid and stored in 80% ethanol, and then stained with Mayer's paracarmine (for the staining methods see Chapter 3). Another two sets of metacercariae were cultured, fixed, stained, and mounted as permanent specimens for species confirmation. The morphological measurements made on the permanent preparations are described and discussed in Chapter 4.

5.2.9. Laser scanning confocal microscopy (LSCM) observation

Metacercarial samples for confocal microscope observations were obtained from *S. balanoides* from Dunbar Leisure Pool and Red Rock (2017 & 2018). Adult samples were from two major groups; group 1 cultured in NCTC-109 supplemented with 20% chicken serum and antibiotics, group 2 cultured in NCTC-109 supplemented with 40% chicken serum and 2X antibiotics. In the 20% chicken serum group, two tubes were taken at 72 and 120 hours, 5 flukes per tube. In the 40% chicken serum group, two tubes were taken at 48, 72 and 120 hours. The live metacercariae were transferred from the culture medium to PBS first, flattened and fixed under coverslip pressure in 5% neutral buffered formalin (NBF) for 5 minutes in a humid chamber. After 5 minutes, the coverslip was lifted and the flattened specimens were flushed back to PBS. The worms in PBS were then transferred to a phalloidin stain (CytoPainter phalloidin-iFluor 594 Reagent, ABCAM) and stained for 3 to 4 days in the dark at 4°C. To make the 2X phalloidin conjugate working stock solution, 2µL of 1000X phalloidin conjugate stock was added to 1 mL of PBS, mixed and centrifuged (1000 rpm for 1 mins). To make a 1 mL phalloidin stain working stock solution, 30 µL of the 2x phalloidin conjugate, 0.2 µL of DAPI (10 mM solution in water, ABCAM), and 9 µL of 10% Triton X100 (Sigma) were added to 900 µL of 5% NBF and kept at 4°C. After 4 days of staining in the dark, worms were mounted individually by placing them in the centre of a slide, then air drying them for about 10 minutes. The stained samples were then mounted in a fluorescent protected mount medium, VECTASHIELD (Vector Laboratories, Inc.), and sealed by nail varnish. The sealed samples were then observed by confocal microscopy (Leica TCS 2 AOBS LSCM) and photographs

were taken using the embedded software. Selected samples observed after several months of the first staining were re-stained with 8x phalloidin as described above, were re-mounted and were scanned again. Following this, in 2018, the re-stain method was determined to be less than ideal, and a new staining protocol was applied using freshly excysted or cultured samples. For this, 4% paraformaldehyde or 10% NBF was used as the fixative, and parasite samples were fixed directly for 18 hours at 4°C or fixed after flattening under a coverslip. 10X phalloidin was used to replace the 8X or 2X phalloidin. Before staining, flukes were washed in PBS with 0.1% Triton-X100 for 30 minutes; and the flukes were stained for ~3 days. After staining for 3 days, samples were washed in PBS for 30 minutes, and mounted in VECTASHIELD. Heat killing the flukes using hot PBS was tried but did not result in any notable improvement of the results and was not considered to be a good method for the flattening of specimens.

5.3. Results

5.3.1. Small scale preliminary experiments

5.3.1.1. Observation of excystment percentage

The excystment percentage at the four time points is shown in Table 5.4. In total, 149 metacercariae were used. At 15 minutes in 0.01 M PBS at 40°C, the excystment rate was quite low, but after 2 hours nearly all the metacercariae had excysted. The same percentage of excystment (nearly 100%) can be achieved in NCTC-109 at 40°C after 2 hours. The excystment percentage in NCTC 109 was like PBS, reaching nearly 100% after 2 hours, but the excystment percentage in the NCTC 109 medium was not formally quantified. In the following experiments, PBS therefore became the standard excystment fluid unless otherwise stated.

Table 5.2. Excystment percentage of metacercariae in 0.01 M PBS at 40 °C.

Time	15 minutes	30 minutes	60 minutes	120 minutes
Excystment rate	10.5%	66%	81%	97.5%

5.3.1.1.1 Eagle's Minimum Essential Medium (EMEM)

For experiments using EMEM complete medium plus 10% foetal bovine serum plus L-glutamine 2 mm plus NEAA (1X), the sample size was small and varied for each treatment group, therefore, a statistical analysis was not performed. Excysted metacercariae survived until the second day in all treatment groups containing EMEM, but the culture medium showed signs of contamination from the first day due to no antibiotics being added. All parasites were dead by the third day, but in the treatment groups that contained EMEM and PBS (1:1), eggs were noticed. An enlarged excretory bladder and numerous vacuoles in the testes were sometimes noticed when the flukes were observed under light microscopy. The uterus was less developed in this nutrient medium, as shown in Figures 5.4. All other organs seemed to be normal.

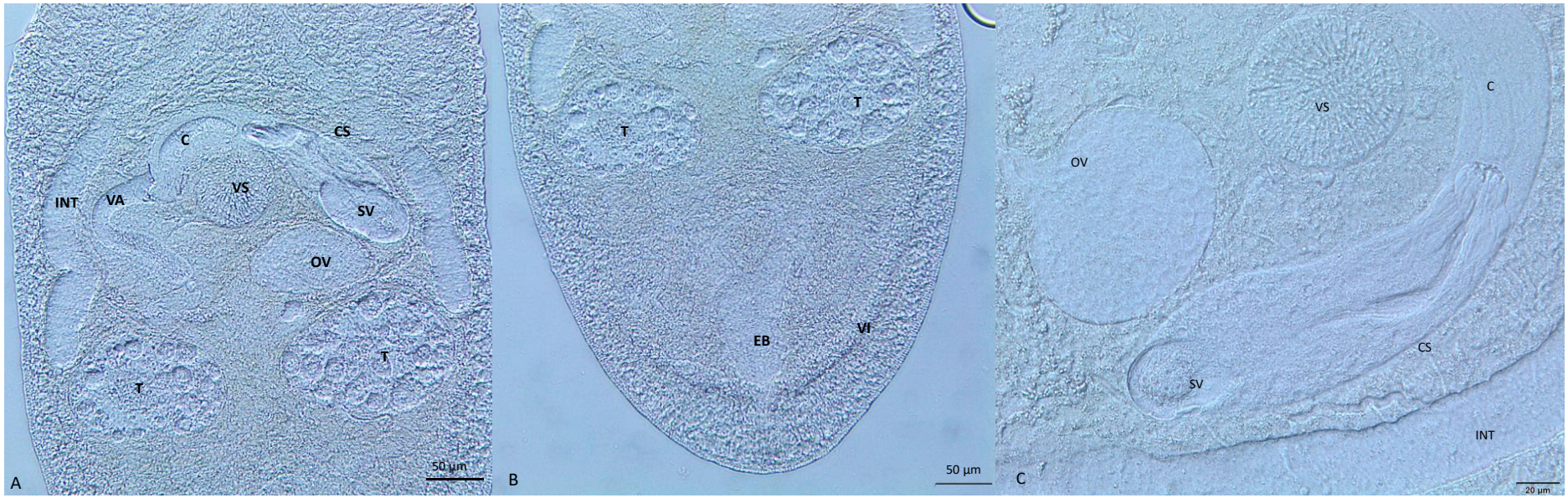


Figure 5.4. Excysted metacercaria cultured in EMEM medium for 40 hours. A. Region of the cirrus sac and testis. Note the bloated caecum and testes containing many vacuoles. B. Region of the uterus; the uterus was not developed and the excretory bladder was enlarged. C. Region of the cirrus sac and ovary, ventral view, anterior side to the right. C: cirrus. CS: cirrus sac. EB: excretory bladder. INT: intestine caecum. OV: ovary. SV: seminal vesicle. T: testis. VA: vagina. VI: vitellaria. VS: ventral sucker.

5.3.1.1.2 PBS

In this experiment, the percentage survival of young adults in 0.01M PBS was calculated. Some tubes were taken for morphological observation and for each time point the sample size (*i.e.*, n = number of tubes) was thus not the same. Therefore, the survival is expressed as percentage survival. The percentage survival rate was calculated by counting live flukes in each tube at each time point, and then by dividing the number by the number of live flukes in each tube at time 0. The same principle was applied for the following experiments. For both sites, the percentage survival decreased rapidly after 24 hours, and by the third day the percentage survival was below 5%. The survival patterns from both sites were similar (Figures 5.5). In this experiment, all parasites from a single barnacle were pooled together in one tube, therefore each tube contained different numbers of parasites. Different fixation methods were tested, *e.g.*, fixed in 80% ethanol, fixed in 10% formalin or no fixing, and the results were observed. The flukes fixed in 80% ethanol were totally opaque and not suitable for microscopy observation. The flukes fixed in formalin had an opaque body wall obscuring internal morphology, which was similarly not good for microscopy observation. Observation of live worms under coverslip pressure provided the best results and revealed details not observable on fixed or stained samples, notably the copulatory organs. Afterwards, images of the developing flukes were taken from live specimens only. The morphology of young adults at 24-48 hours was mostly normal, most organs especially copulatory organs can be seen clearly. The uterus showed minor development but no eggs were observed. No bloated intestine or excretory bladder were observed but the testes still contained vacuoles (Figures 5.6).

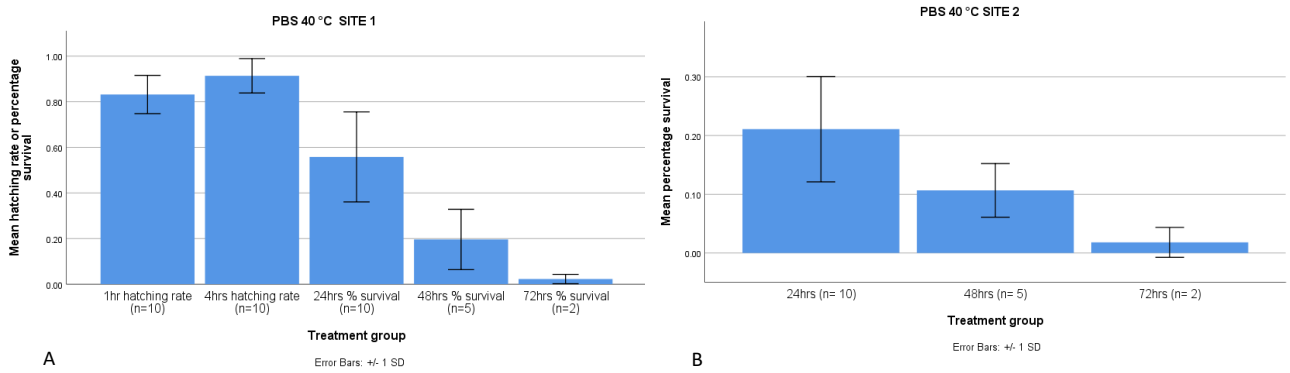


Figure 5.5. Mean hatching rate or percentage survival of the young adults cultured in PBS. A. Site 1. B. Site 2. N = tube numbers. Each tube contains all the metacercariae collected from an individual barnacle (n = 10 in total).

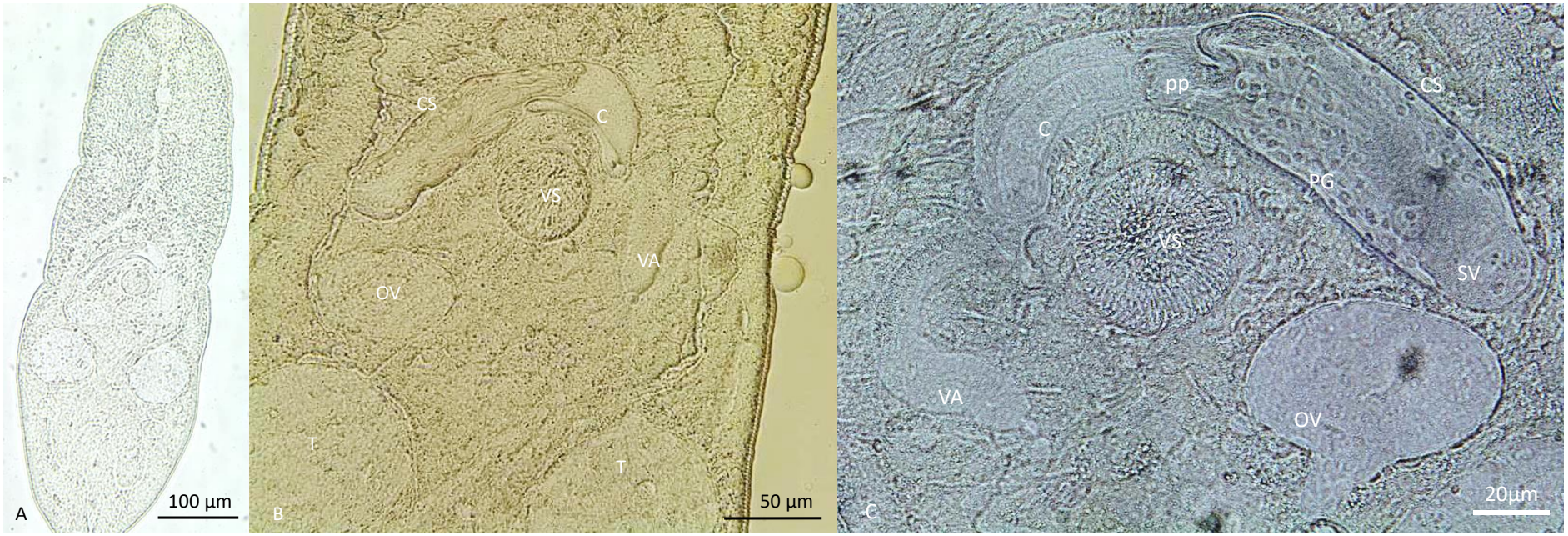


Figure 5.6. Excysted metacercaria cultured in 0.01 M PBS for 24 hours. A. Ventral view of the whole worm. B. Higher magnification showing the region of cirrus sac, ovary, and testes. C. A young adult cultured in 0.01 M PBS for 48 hours. Dorsal view showing the region of the cirrus sac and the ovary. C: cirrus. CS: cirrus sac. OV: ovary. PG: prostate glands. pp: *pars prostatica*.SV: seminal vesicle. T: testis. VA: vagina. VS: ventral sucker.

5.3.1.1.3 NCTC 109

5.3.1.1.3.1 NCTC 109 without antibiotics and serum

In this experiment, the metacercariae in five tubes were excysted in 0.01M PBS and 5 tubes were excysted in NCTC 109. In this experiment, no antibiotics were added; some tubes containing NCTC 109 turned orange denoting a drop in pH (these contained phenol red as a pH indicator) at 24 hours indicative of contamination and acidification of the medium. All flukes were transferred to new tubes containing fresh NCTC 109 but at 48 hours all the tubes turned orange again. The percentage survival dropped rapidly at 24 hours and at 72 hours almost all flukes were dead (Figure 5.7A). It was concluded that the operation under non-sterilised conditions was not capable of supporting the growth of excysted metacercariae, therefore antibiotics were added to all cultures following this experiment.

The internal organs looked like those cultured in PBS or EMEM, but the contours were clearer and no abnormalities, such as a bloated gut or bladder or vacuoles in the testis, were observed. Eggs started to appear at 24 hours and more flukes containing eggs were observed at 48 hours

5.3.1.1.3.2 NCTC 109 with penicillin (1 U mL^{-1}) and streptomycin (1 µg mL^{-1})

In this experiment, a low concentration of antibiotics was applied. At 24 hours, all tubes were in good condition and the flukes were seen actively moving and most were alive. At 48 hours, most tubes turned orange to yellow but the percentage survival was still high. All flukes were transferred to new tubes with fresh medium, however, contamination persisted after 72 hours, despite fresh media being provided. The percentage survival dropped drastically after 72 hours but their survival times extended to 144 hours (Figure 5.7B). The morphology of the flukes was like that of those cultured in NCTC 109 alone. Eggs started to appear at 24 hours and increased thereafter.

5.3.1.1.3.3 NCTC 109 supplemented with 20% foetal bovine serum and penicillin (1 U mL^{-1}) and streptomycin (1 µg mL^{-1})

In this experiment the percentage survival was like that obtained for those cultured in NCTC 109 with a low concentration of antibiotics; however, the percentage survival at 72 hours was higher (50 %). After 72 hours, the percentage survival dropped rapidly but survival times extended up to 144 hours (Figure 5.7C). Contamination started from 24 hours and persisted thereafter, despite provision of fresh media. Morphology of the flukes was like that of those cultured in NCTC 109 alone. Eggs started to appear at 24 hours and increased afterwards. Morphology was like that of those cultured in NCTC 109 alone.

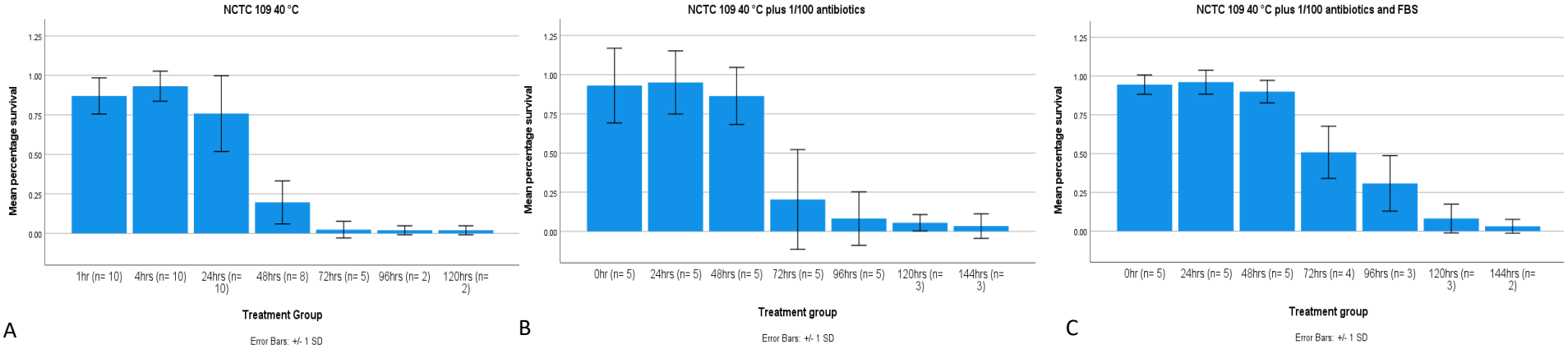


Figure 5.7. Mean percentage survival of young adults cultured in: A. NCTC 109; B. NCTC 109 with 1/100 antibiotics; C. NCTC 109 with 1/100 antibiotics and 20% FBS. N = tube numbers. Ten tubes or five tubes were used at the beginning, and each tube contained all the metacercariae collected from an individual barnacle. The concentration of antibiotics is compared to the routine concentration used in the major *in vitro* culture experiment.

5.3.1.1.3.4 NCTC 109 supplemented with 20% foetal bovine serum and penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1})

In this part of experiment, two replicates were performed. In the first replicate, small sample sizes (*i.e.*, 37 metacercariae from two barnacles divided among four tubes) were employed. In the second replicate, 104 metacercariae were employed, these were collected from two barnacles and were divided among 10 tubes. For both replicates, the percentage survival, body length, body width and egg numbers were monitored. For the first replicate, the percentage survival varied between 100~94% until the end of experiment (186 hours, Figure 5.8A). The media were changed on the third day. For the second replicate, the percentage survival varied between 100-81% until 192 hours and then dropped rapidly at 240 hours (30%, Figure 5.8B). The media were changed on the fourth and tenth day. For both replicates, no signs of contamination were seen (based on the colour of the culture medium). The concentration of antibiotics used here was then used routinely for all subsequent experiments.

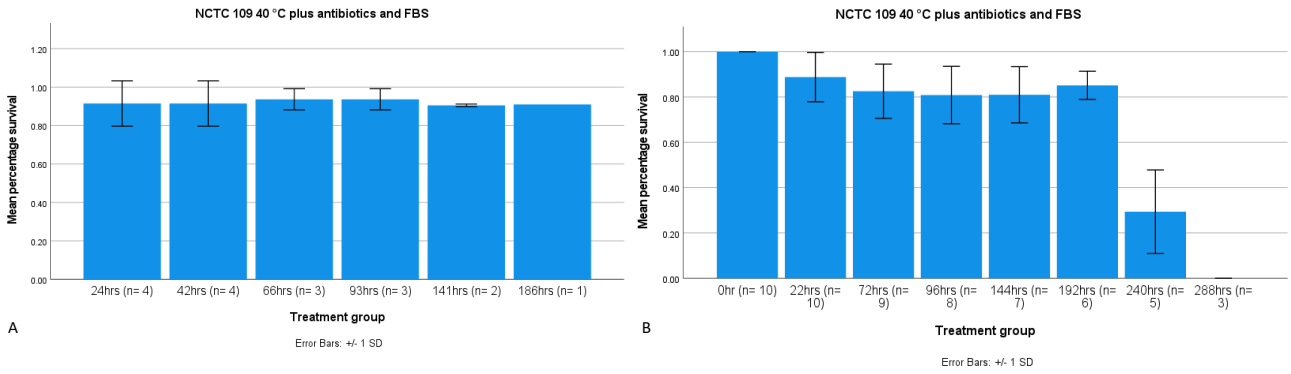


Figure 5.8. Mean percentage survival of the young adults cultured in NCTC 109 with antibiotics and 20% FBS (A. replicate 1, B. replicate 2). N = tube numbers. Four or 10 tubes were used at the beginning, and each tube contained 8-13 metacercariae.

Two parameters were used for measuring the performance under *in vitro* conditions: body length and body width. All the numbers were obtained from photos taken by light microscopy under coverslip pressure when the flukes stopped moving, therefore body size was a bit larger than directly fixed samples. For the body length and width in the first replicate, four time points were monitored. The body length varied between 689.71-1416.49 µm, mean 1005.31 µm ± 35.55 SE; body width varied between 265.55~374.06 µm, mean 327.57 µm ± 5.91 SE. No trend of increase or decrease was observed. For the body length and width in the second replicate, six time points were monitored. The body length varied between 489.34~1324.47 µm, mean 1056.85 µm ± 37.20 SE; body width varied between 244.61~443.08 µm, mean 321.49 ± 7.63 µm. The body length seemed to increase and peak at 196 hours and decrease afterward. No trend of increase or

decrease of body width was observed. Figures 5.9-5.10 show the mean body length and width at each time point for the two replicates.

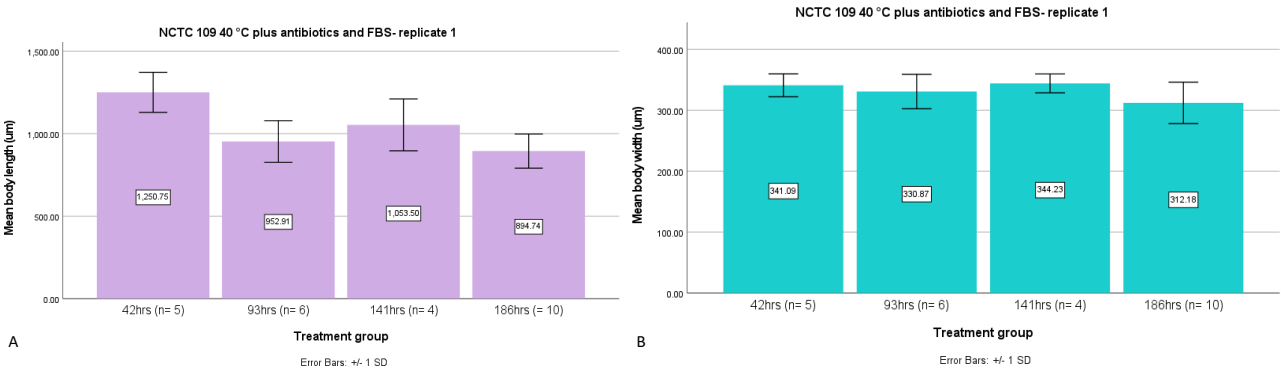


Figure 5.9. Mean body length (A) and mean body width (B) of the young adults cultured in NCTC 109 with antibiotics and 20% FBS at each time point in replicate 1 (n = fluke numbers at each time point).

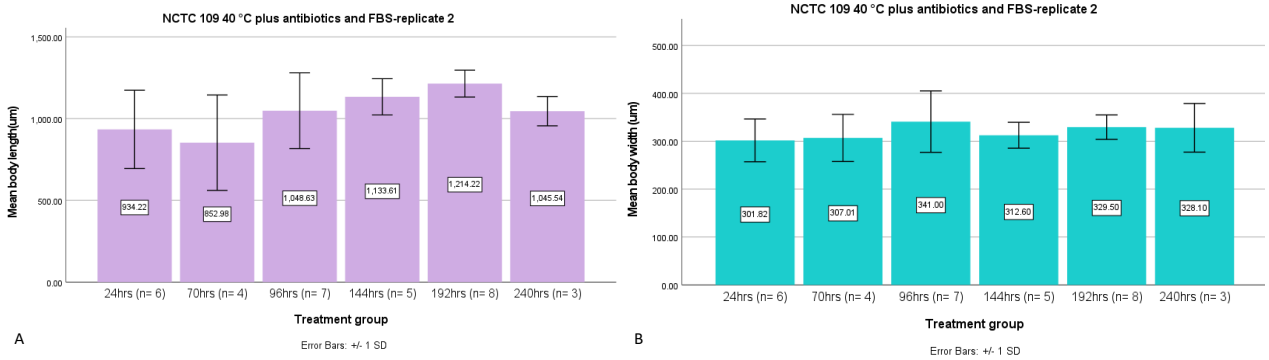


Figure 5.10. Mean body length (A) and mean body width (B) of the young adults cultured in NCTC 109 with antibiotics and 20% FBS at each time point in replicate 2 (n = fluke numbers per time point).

Egg number is another parameter used to assess the performance of newly excysted young adults under *in vitro* conditions. For the first replicate at 8 days, the egg number ranged from 0-282, mean 77.52 ± 16.64 SE. The number peaked at the 4th day and then gradually decreased (Figure 5.11A). For the second replicate at 10 days, the egg number ranged from 0-189, mean 33.39 ± 9.86 SE. The number peaked at the 3rd day and then gradually decreased but at 144 hours there were no eggs at all (Figure 5.11B).

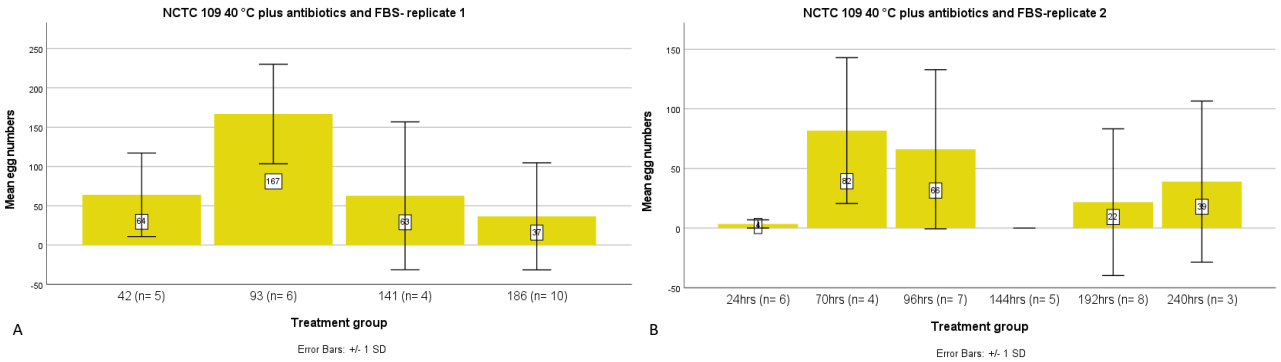


Figure 5.11. Mean egg numbers produced by the young adults cultured in NCTC 109 with antibiotics and 20% FBS (A. replicate 1, B. replicate 2) at each time point (n = fluke numbers per time point).

Morphological observations

In this experiment, more data concerning the morphology of developing, young adults were obtained. All the major organs were clearly visible at 24 hours but there were no eggs in the uterus, as shown in Figure 5.12. Female and male reproductive organs were properly developed at 24 hours (Figure 5.13). At 120 hours, hypertrophied prostate glands and a uterus with numerous eggs was noticed in one fluke (Figure 5.14A). A protruding cirrus which extended to the vaginal chamber was also noticed in this fluke indicating the possibility of self-fertilisation (Figure 5.14B). The development of eggs was also observed (Figure 5.14C). The eggs looked elliptical and there were cells concentrated at one or both poles, which should be vitelline cells or globules. A more homogenous area in the middle is identified as the undivided ovum. No egg operculum could be confirmed.

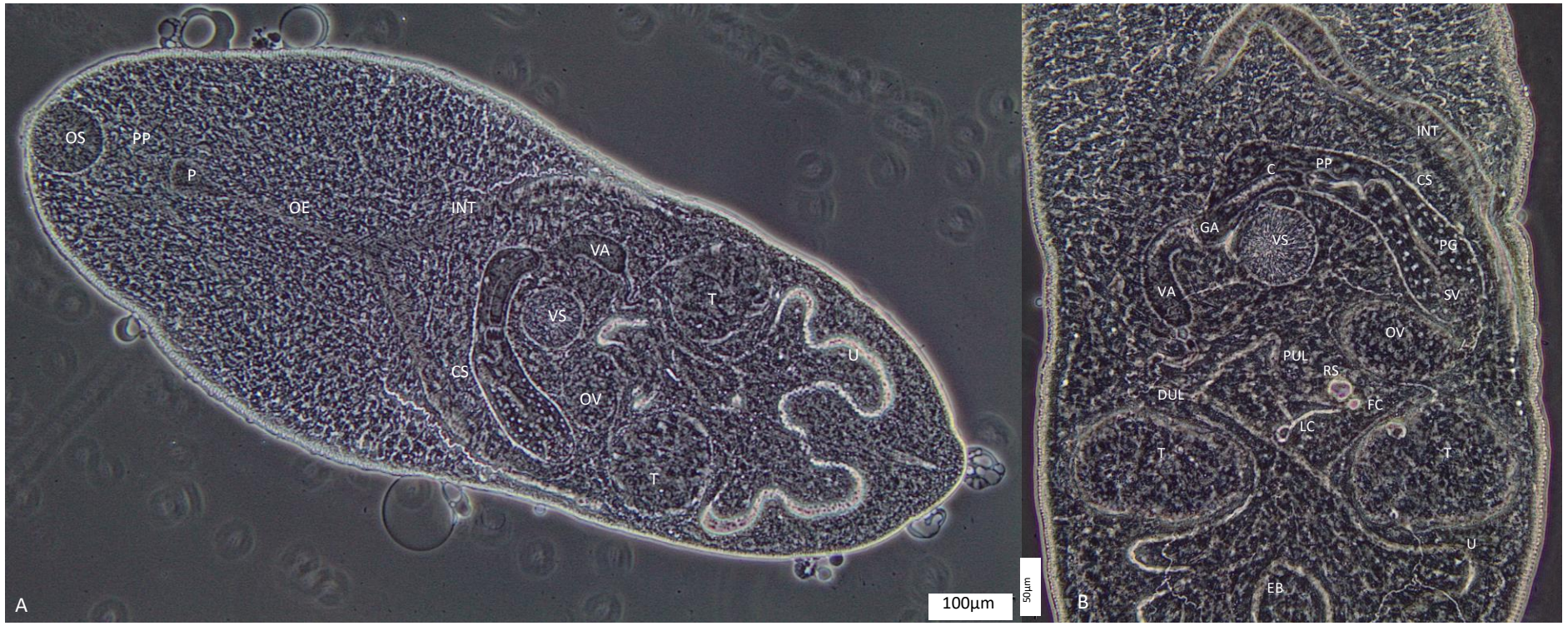


Figure 5.12. A young adult cultured in NCTC 109 supplemented with antibiotics and 20% FBS for 24 hours. A. Ventral view under phase contrast. B. Dorsal view under phase contrast at higher magnification. CS: cirrus sac. DUL: distal uterus loop. EB: excretory bladder. FC: fertilization chamber. GA: genital atrium. INT: intestine caecum. LC: Laurer's canal. OE: oesophagus. OS: oral sucker. OV: ovary. P: pharynx. PG: prostate glands. PP: prepharynx. PUL: proximal uterus loop. RS: *receptaculum seminis*. SV: seminal vesical. T: testis. U: uterus. VA: vagina. VS: ventral sucker.

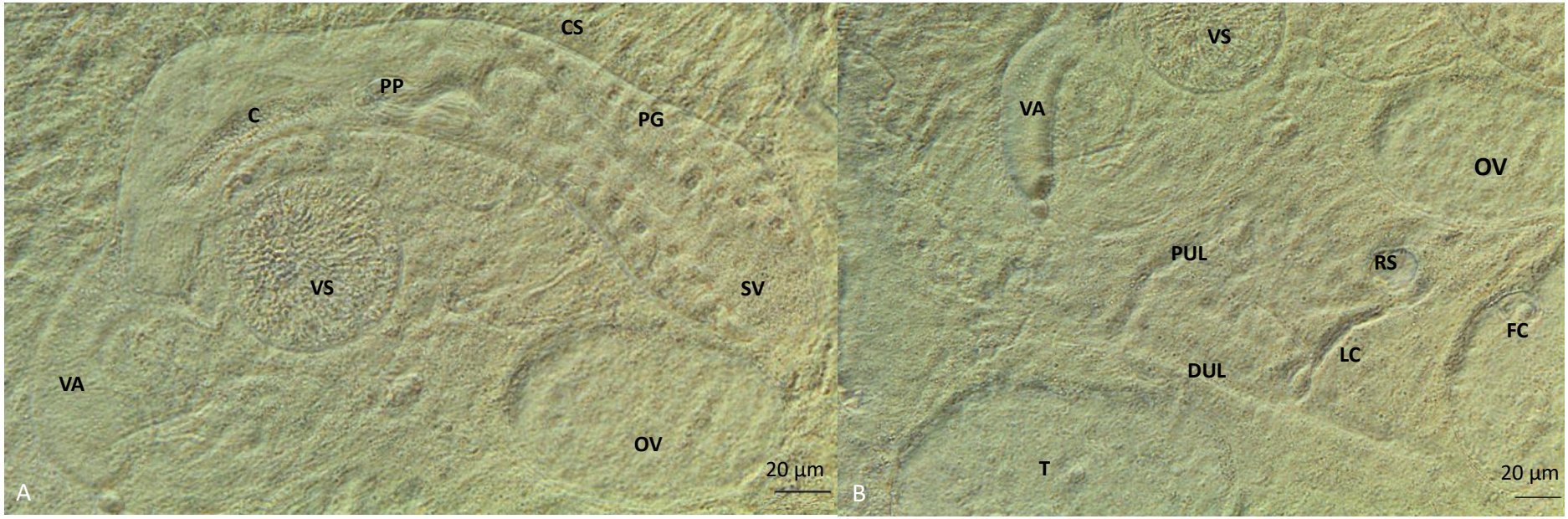


Figure 5.13. Young adult cultured in NCTC 109 with antibiotics and 20% FBS for 24 hours, higher magnification under phase contrast. A. Region of cirrus sac, dorsal view. B. Region of egg producing apparatus, dorsal view. C: cirrus. CS: cirrus sac. DUL: distal uterus loop. FC: fertilization chamber. LC: Laurer's canal. OV: ovary. PG: prostate glands. PP: pars prostatica. PUL: proximal uterus loop. RS: *receptaculum seminis*. SV: seminal vesical. T: testis. VA: vagina. VS: ventral sucker.

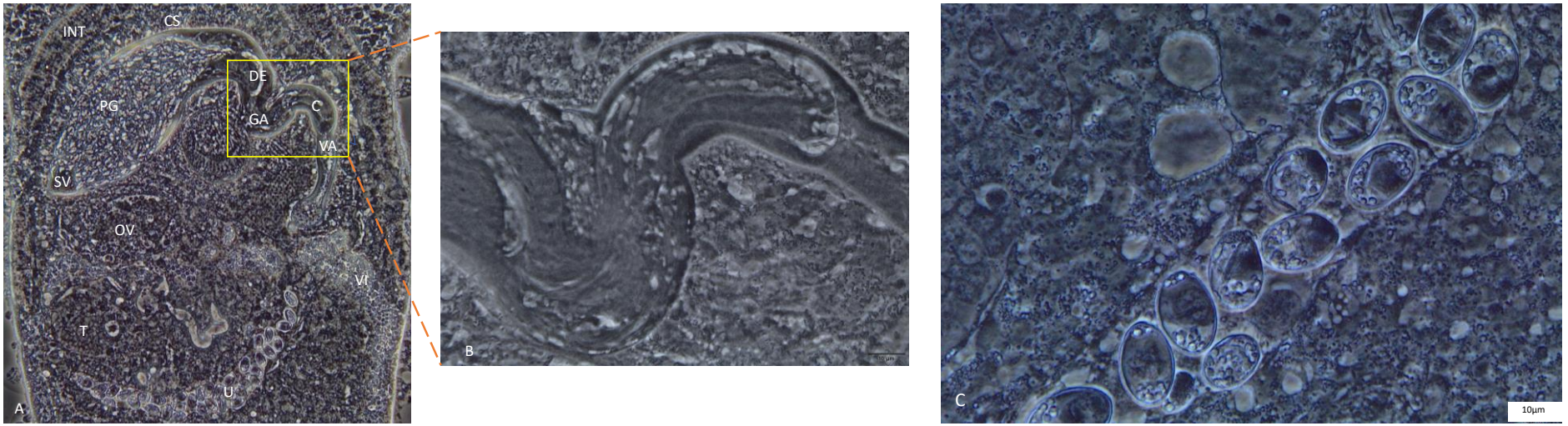


Figure 5.14. Young adult cultured in NCTC 109 with antibiotics and 20% FBS for 120 hours, ventral view at higher magnification under phase contrast. A. Region of the reproductive organs. Note this fluke had hypertrophied prostate glands. B. Region of the vagina showing potential fertilisation. C. Eggs Note the cells at one or both end of the eggs which are vitelline cells or globules, and the ovum in the middle. C: cirrus. CS: cirrus sac. DE: *ductus ejaculatorius* (inside cirrus). GA: genital atrium. INT: intestine caecum. OV: ovary. PG: prostate glands. SV: seminal vesical. T: testis. U: uterus. VA: vagina. VI: vitellaria.

5.3.1.1.3.5 NCTC 109 supplement with 20% chicken serum and penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1})

The methodology of calculating survival rate, growth indices and morphology worked well in the above experiments, therefore the same method was applied again but using chicken serum (CS) as the food source. The survival rate ranged from 97.30-63.11% until 192 hours, and by 236 hours all the worms were dead (Figure 5.15A). The body length ranged from 589.24-1507.79 µm, mean 1035.33 µm ± 60.89 SE. The body width ranged from 205.95-460.31 µm, mean 339.38 µm ± 16.29 SE. Body length seemed to increase first and then decrease, and body width showed no trend of increasing or decreasing (Figures 5.15B & 5.15D). Egg numbers ranged from 0-144, mean 26 ± 10.85 SE. Eggs started to appear at 24 hours and then increased, but given that no eggs were observed, no data were available at 72 hours, and so no conclusions can be drawn (Figure 5.15C).

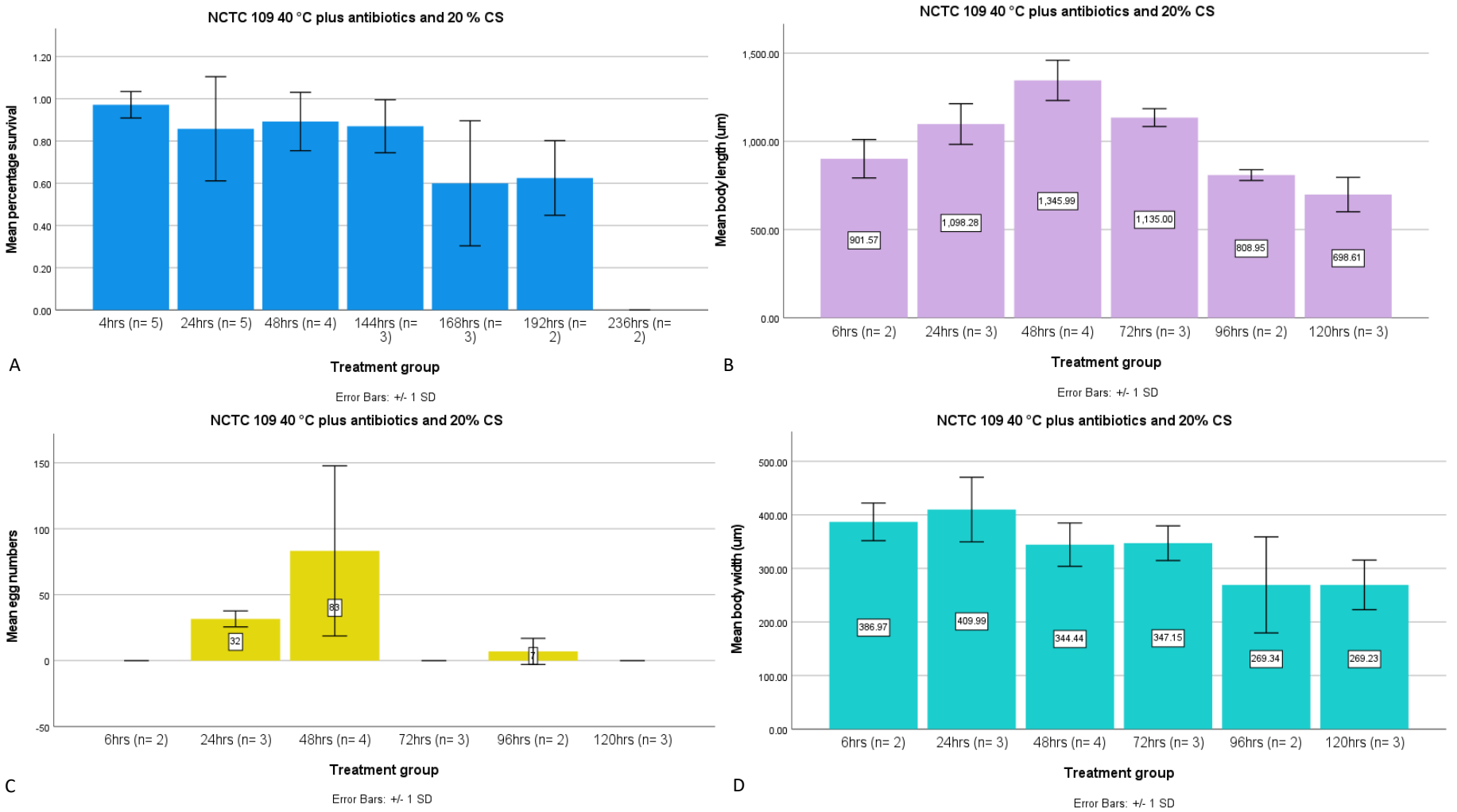


Figure 5.15. A. Mean percentage survival of the young adults cultured in NCTC 109 with antibiotics and 20% CS. N = tube numbers. Five tubes were used at the beginning, and each tube contained 7-8 metacercariae. B. Mean body length of the young adults cultured in NCTC 109 with antibiotics and 20% CS at each time point (n = fluke numbers per time point). C. Mean egg number produced by the young adults cultured in NCTC 109 with antibiotics and 20% CS at each time point. D. Mean body width of the young adults cultured in NCTC 109 with antibiotics and 20% CS at each time point. N = fluke numbers per time point.

Morphological observations

No special features were found when compared to the previous experiments; however, some details are added. All organs were clearly visible at 6 hours and the excretory systems were also visible. The movement of the cirrus was controlled by an obvious *pars prostatica* and the movement of this structure was visible in living specimens under a coverslip, even after the fluke had stopped moving (Figure 5.16). A few vacuoles were noticed in the testes (Figure 5.17). Eggs started to appear at 24 hours as before and those in the distal uterus were light-brown in colour (Figure 5.17A). Eggs at 24 hours were small, elliptical, and thin shelled (Figure 5.18A), and contained very few cells / globules at one end. The eggs seen at 48 hours appeared to contain more cells / globules at each end but no dividing embryo was noticed (Figure 5.18B). At 48 hours, more eggs were seen accumulating in the proximal uterus and materials of cellular origin (assumed to be the ovum) continued to flow from the ovary to the oviduct suggesting that egg formation was in progress (Figure 5.19A). Some finger-like structures were found in the cuticle of the young adults (Figure 5.19B).

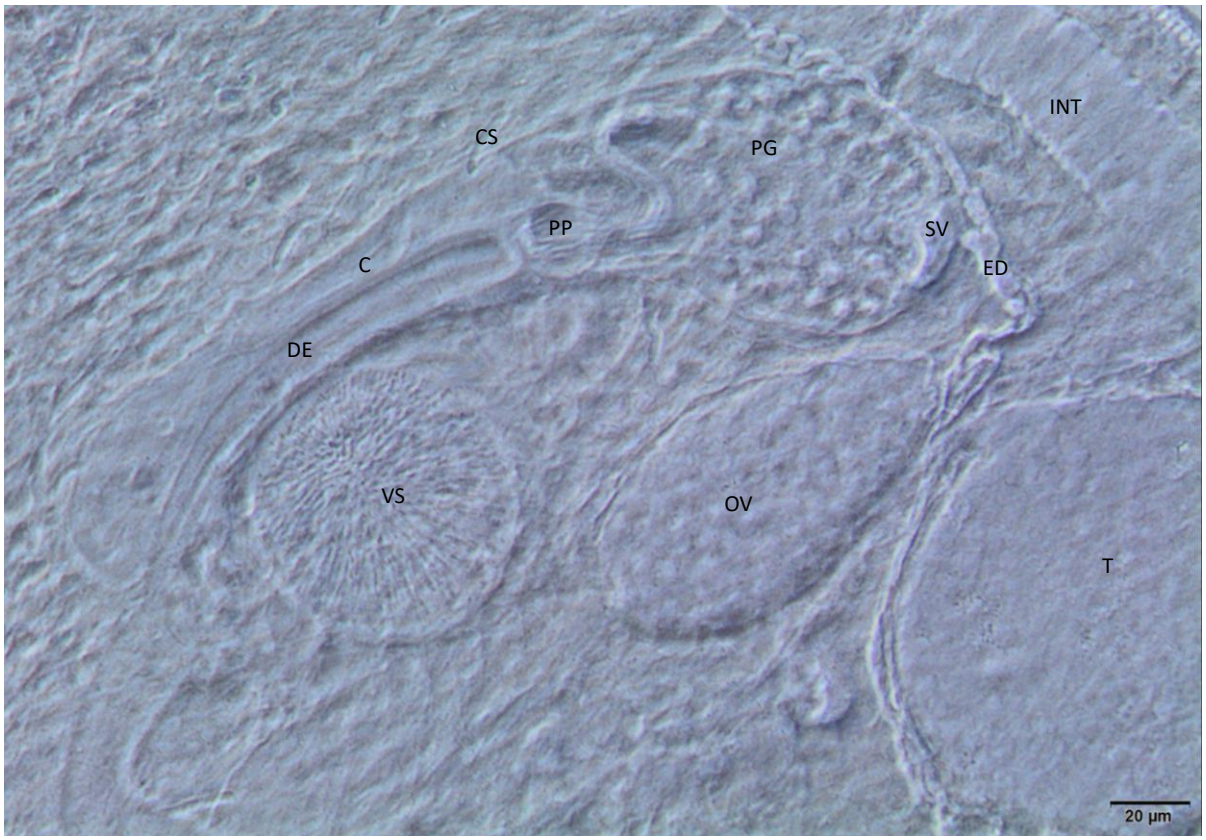


Figure 5.16. Young adult cultured in NCTC 109 with antibiotics and 20% CS for 6 hours under phase contrast, focusing on the male copulatory organs. C: cirrus. CS: cirrus sac. DE: *ductus ejaculatorius* (inside cirrus). ED: excretory duct. INT: intestine caecum. OV: ovary. PG: prostate glands. PP: *pars prostatica*. SV: seminal vesical. T: testis. VS: ventral sucker.

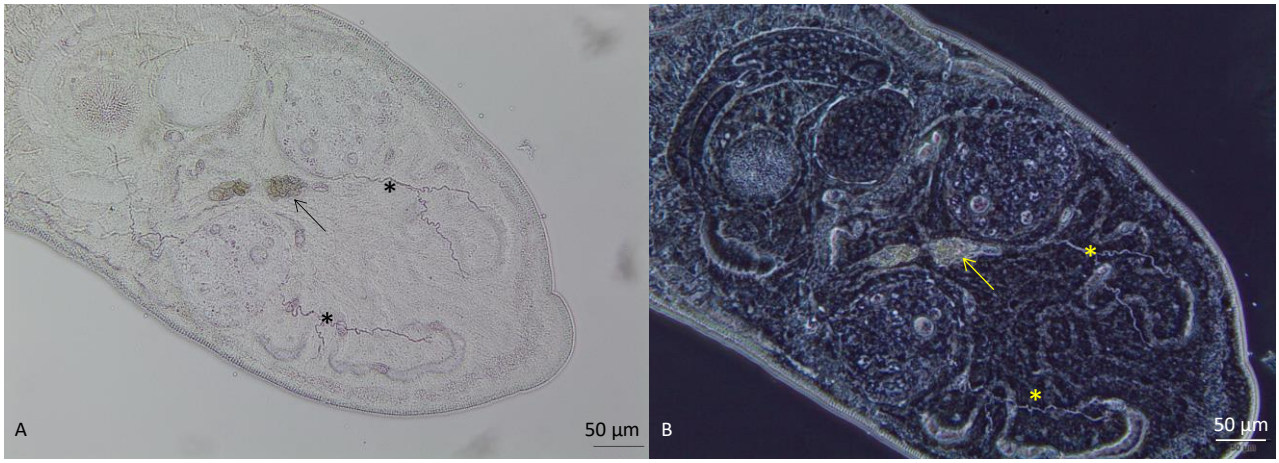


Figure 5.17. Young adult cultured in NCTC 109 with antibiotics and 20% CS for 24 hours, dorsal view. A. Image under normal light. B. Image under dark-field. Note the distal uterus is full of light-brown eggs (arrow). Collecting ducts of the excretory system are visible in the hind body (asterisk).

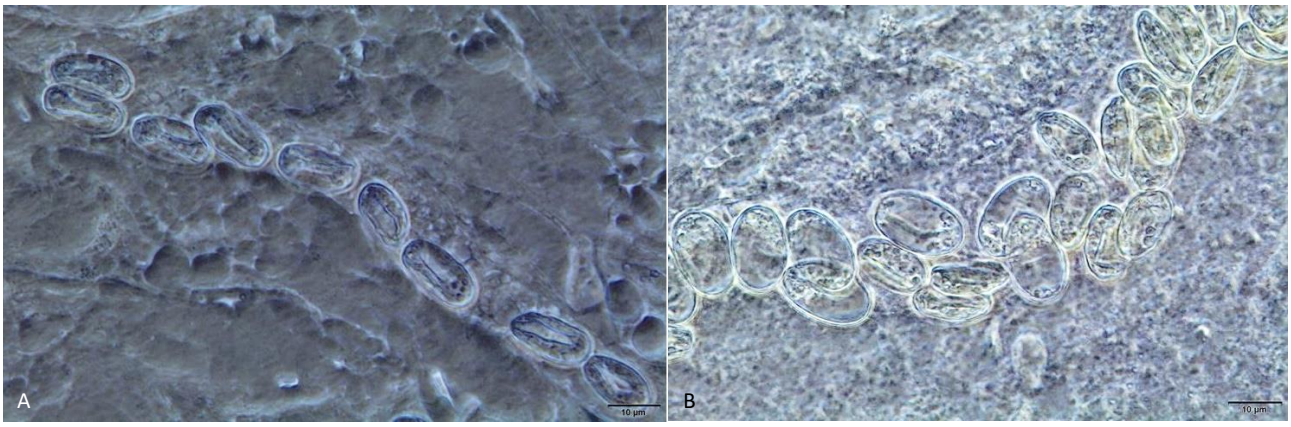


Figure 5.18. Eggs of a young adult cultured in NCTC 109 with antibiotics and 20% CS. A. 24 hours. B. 48 hours.

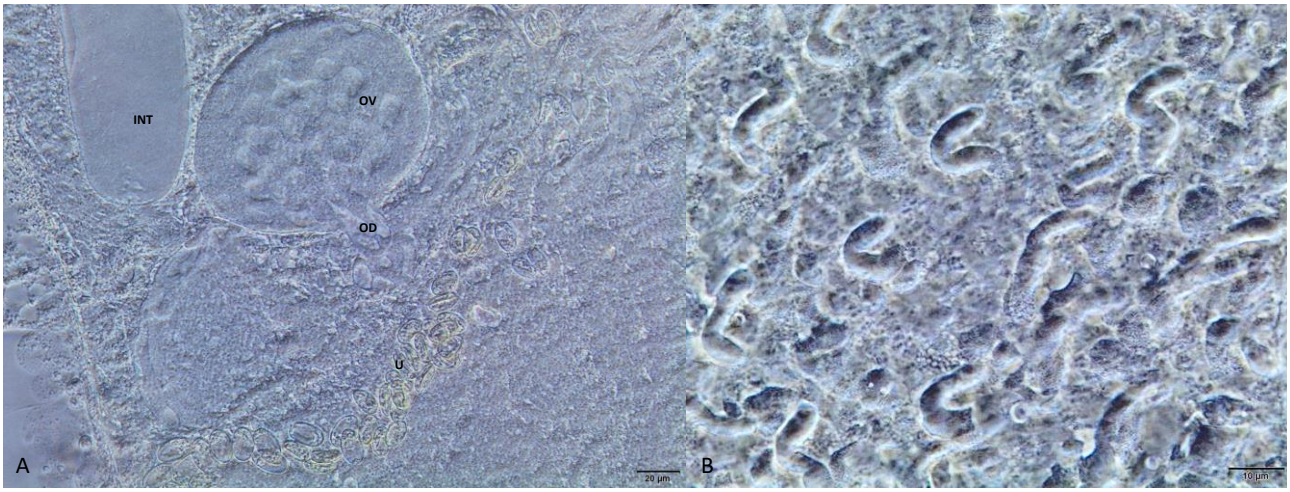


Figure 5.19. Young adult cultured in NCTC 109 with antibiotics and 20% CS for 48 hours. A. Image focuses on the ovary region. Note the flow from ovary to oviduct. B. Finger-like structures in the cuticle. INT: intestine caecum. OD: oviduct. OV: ovary. U: uterus.

5.3.2. Observation of spermatogenesis

In order to confirm spermatogenesis in the early stages of adult development (*e.g.*, inside the metacercarial cyst), each cyst was placed in a single tube to avoid receiving sperm from another individual. Spermatozoa were observed as early as 15 minutes in the excysted flukes. At 15 minutes, spermatozoa were observed in the fertilization chamber and the *receptaculum seminis* but not in the seminal vesicle, also an ovum was seen in the fertilization chamber. The same situation was observed at 30 minutes but with more spermatozoa being seen in the fertilization chamber (Figure 5.20). At 60 minutes, spermatozoa were observed in the seminal vesicles and *ductus ejaculatorius*, but the condition of the flukes was poor and the number of spermatozoa seen were low (Figure 5.21). At 120 minutes, spermatozoa were observed again in the fertilization chamber, but not the seminal vesicle and *ductus ejaculatorius*. The condition of flukes was still poor and the number of spermatozoa low.

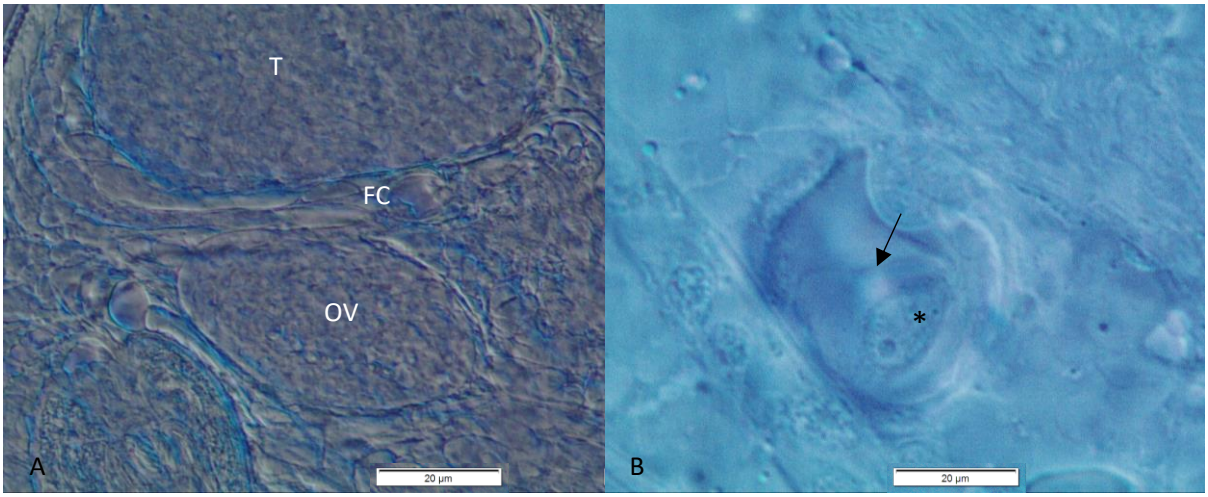


Figure 5.20. Fertilization process. A. A fluke cultured for 30 minutes in 0.01 M PBS. Note the refractive fertilization chamber between the testis and ovary. B. A fluke cultured for 15 minutes in 0.01 M PBS. Note a spermatozoan (arrow) and an ovum (asterisk) in the fertilization chamber. FC: fertilization chamber. OV: ovary. T: testis.

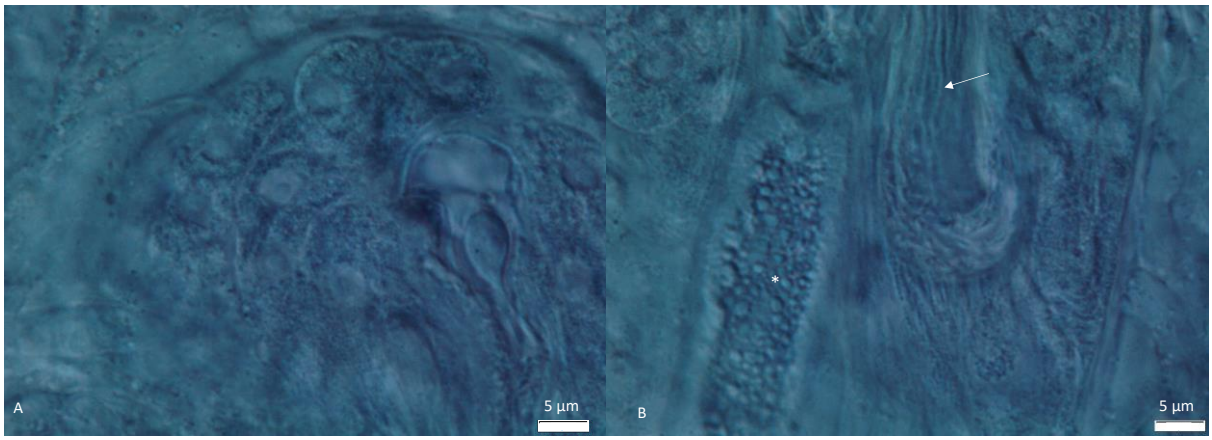


Figure 5.21. A fluke cultured for 60 minutes in 0.01 M PBS, focusing on the seminal vesicle. A. Spermatozoa in the seminal vesicle. B. Spermatozoa in the duct (arrow) between the seminal vesicle and the *ductus ejaculatoris* (asterisk).

5.3.3. Observation of self-fertilisation

In the 30 individually cultured metacercariae, 26 contained eggs by the 4th day and only 1 fluke was dead with no eggs. A high percentage of self-fertilisation (86%) was confirmed. The eggs produced by self-fertilisation looked the same as those produced by cross fertilization. Both "normal" and "abnormal" eggs were seen but no signs of operculi. According to Zaben (1988), normal eggs are eggs with an operculum and contain an ovum surrounded by vitelline globules.

5.3.4. Major *in vitro* culture experiment

In the small-scale pilot studies, the conditions for *in vitro* culture, the need for sterile operation and the intervals and methods required for observation were developed. Here, a systematic, larger-scale experiment was conducted to investigate the parameters of interest. Chicken serum was the choice of food source and two concentrations of serum (20% versus 40%) were compared. Quantifiable parameters such as the percentage survival and growth indices were analysed; and non-quantifiable morphological characteristics were also recorded and described.

5.3.4.1. Survival analysis

For both treatment groups, the number of survivors gradually decreased but the cumulative survival was still ≥ 50 % by day 10 (Figure 5.22). The mean overall survival time reached 7.95 days (Table 5.6). When comparing the two groups with a Log Rank test, they were not significantly different (p= 0.066, Table 5.7), however, in the bar chart, the 40% serum treatment group appeared to have a higher percentage survival at multiple time points especially during the later stages of the experiment (Figure 5.22) and the mean survival time was also higher for the 40% group (Table 5.6, mean= 8.43 days). Due to the experimental design, there was high percentage of censored data, as noticed in Table 5.5. Survival curves were drawn by the Kaplan-Meier method (Stalpers & Kaplan, 2018) shown in Figure 5.23. The curves describing the survival of flukes in both treatment groups did not show any dramatic drops throughout the course of the experiment.

Table 5.3. Comparison of survival for different treatment groups

			Censored**	
serum	Total N	N of Events*	N	Percent
20 %	130	32	98	75.4%
40 %	119	21	98	82.4%
Overall	249	53	196	78.7%

*An event means a death.

**Censored means specimens that leave the test instead of death.

Table 5.4. Comparison of mean survival time of flukes in the two treatment groups.

Serum	Mean for Survival Time (days)			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
20 %	7.509	0.376	6.773	8.246
40 %	8.431	0.316	7.812	9.050
Overall	7.948	0.250	7.458	8.437

Table 5.5. Log rank test on the survival analysis data for the two treatment groups

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	3.376	1	0.066

Test of equality of survival distributions for the different levels of serum.

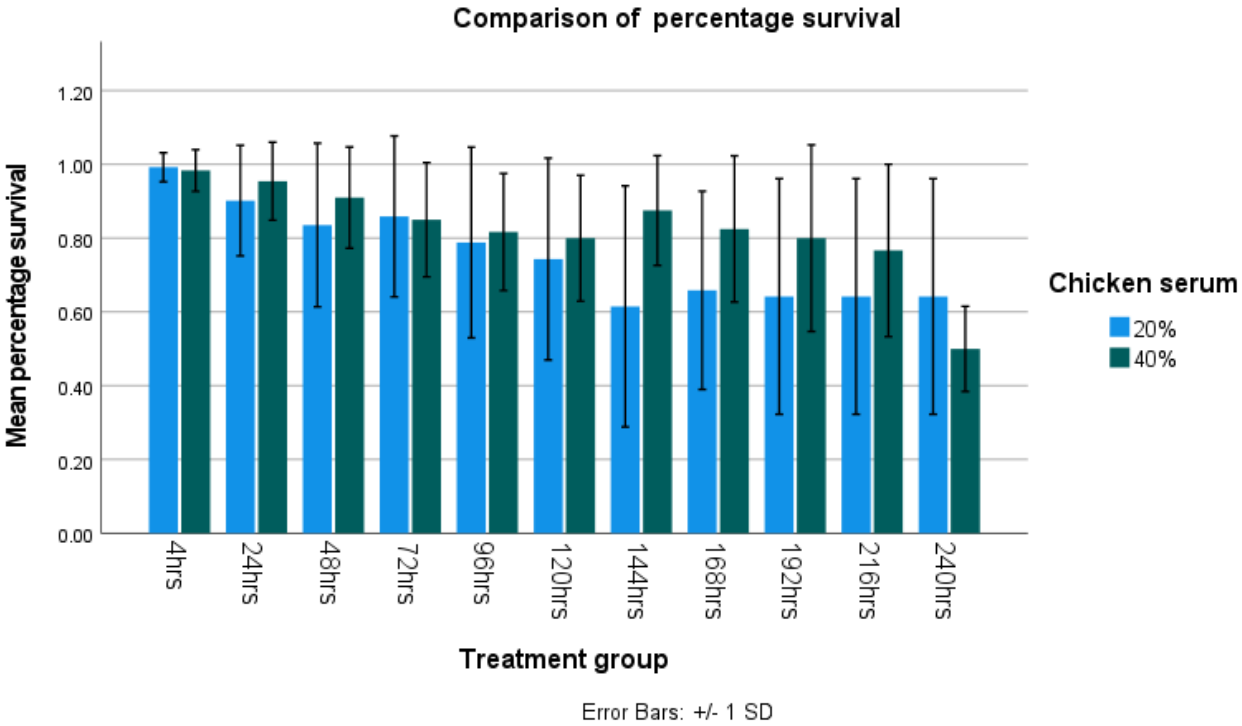


Figure 5.22. A comparison of the mean percentage survival in the young adults cultured in NCTC 109 with antibiotics and chicken serum, 20% and 40% respectively.

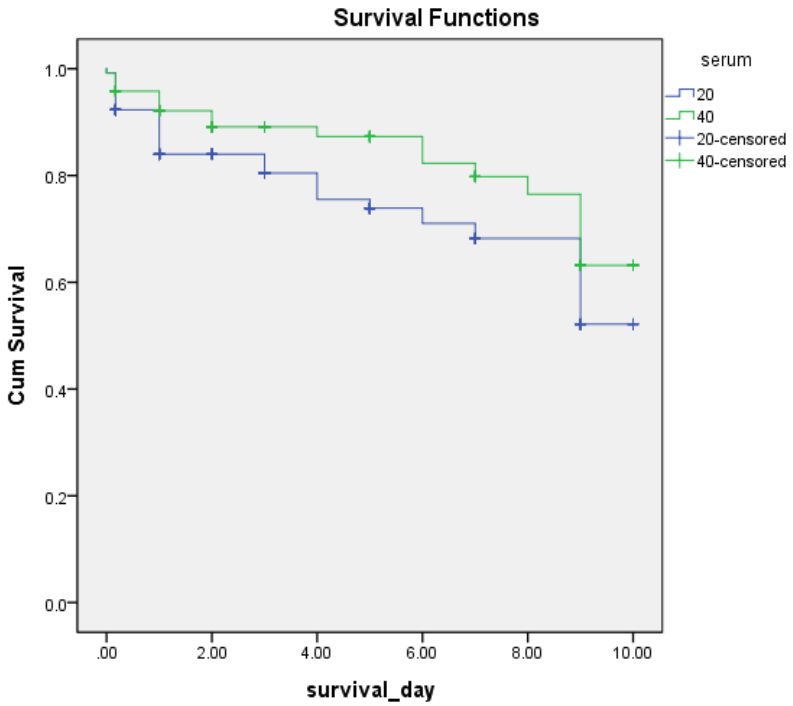


Figure 5.23. Survival curves for the two treatment groups according to the Kaplan-Meier method.

5.3.4.2. Growth indices

The body length of the 20% CS group ranged from 473.76 - 1324.55 µm, mean 853.71 µm ± 23.44 SE; for the 40% CS group it ranged from 566.68 - 1632.14 µm, mean 997.58 µm ± 32.72 SE. The body width of the 20% CS group ranged from 183.46 - 412.97 µm, mean 307.45 µm ± 7.07 SE; for the 40% CS group it ranged from 175.11 - 427.82 µm, mean 315.65 µm ± 7.63 SE. Results of a normality test on the body lengths of the 20% and 40% CS groups, either pooled or separately at each time point, were non-significant. The results of a test for normality on the body width of the 20% CS group were significant as a pooled sample, but were not significant at each time point. A normality test conducted on the body widths of the 40% CS group was not significant, either pooled or separated by time points. Among the 7 time points, body length and width showed no systematic trend of increase or decrease in each group, although body lengths in the 40 % CS group were significantly larger than those in the 20% CS group at 48 and 72 hours (independent sample T test, $p<0.001$, Figure 5.24). Body width was not significantly different for both groups at any time point (Figure 5.25).

Egg numbers for the 20% CS group ranged from 0 - 269, mean 57.22 ± 9.18 SE; for the 40% CS group it ranged from 0 - 428, mean 89.09 ± 15.90 SE. A normality test performed on the egg number was significant, either as a pooled sample or as separate data, and could not be corrected after transformation, therefore a Mann-Whitney U test was conducted. For both groups, eggs could be observed from the second day, peaking at the fifth day in the 20% group and at the second day in the 40% group, and then gradually decreased towards the end of the experiment. Comparing the two groups, egg numbers were not significantly different at any time point (Figure 5.26).

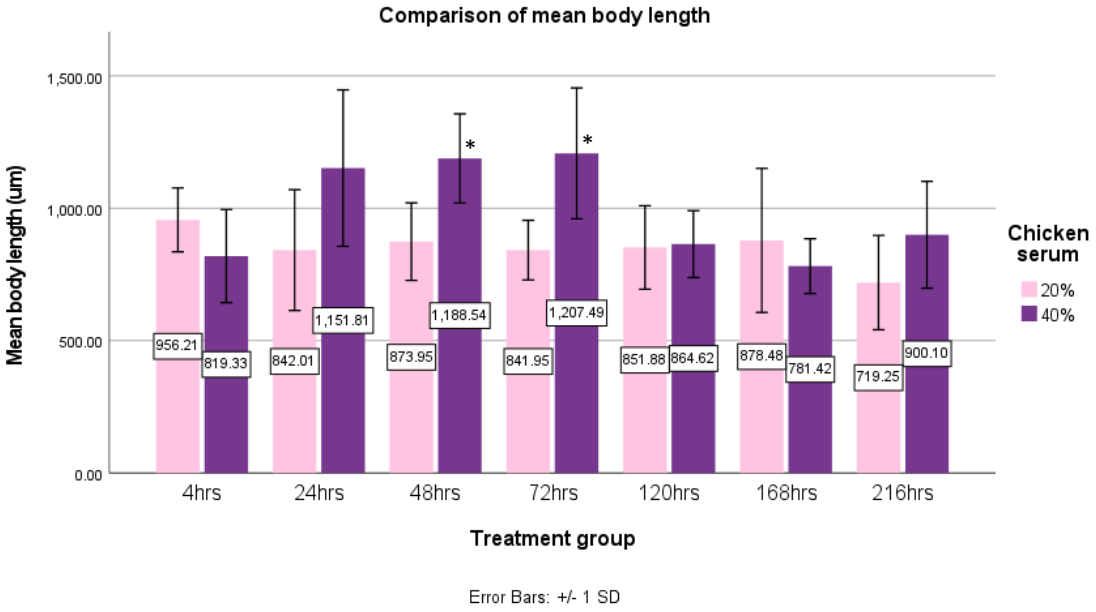


Figure 5.24. A comparison of the mean body length at seven time points made on the young adults cultured in NCTC 109 supplemented with antibiotics and CS, 20% and 40% respectively. * Statistically different ($p<0.001$).

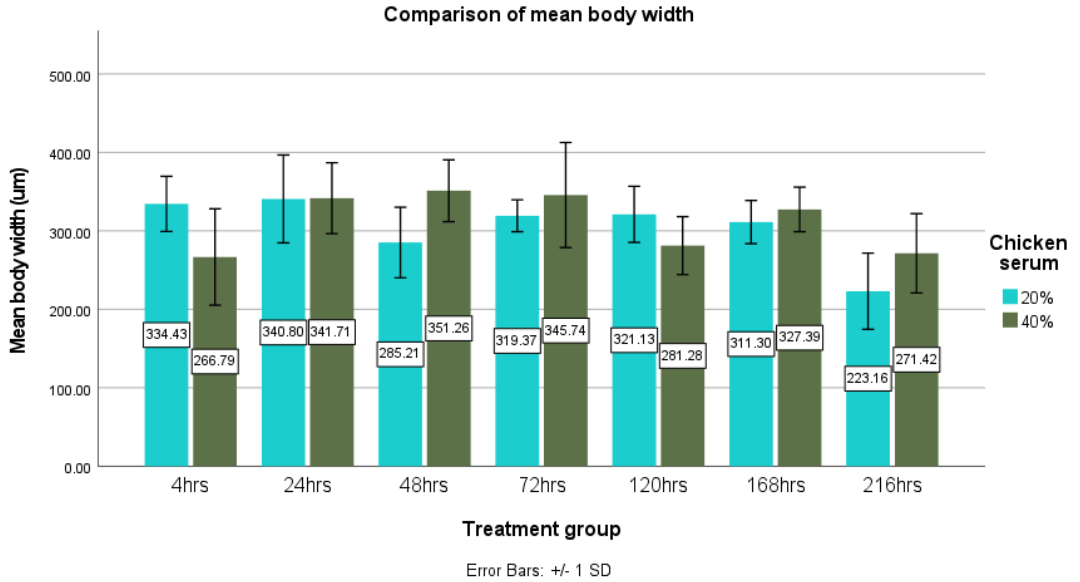


Figure 5.25. A comparison of the mean body width at seven time points made on the young adults cultured in NCTC 109 supplemented with antibiotics and CS, 20% and 40% respectively.

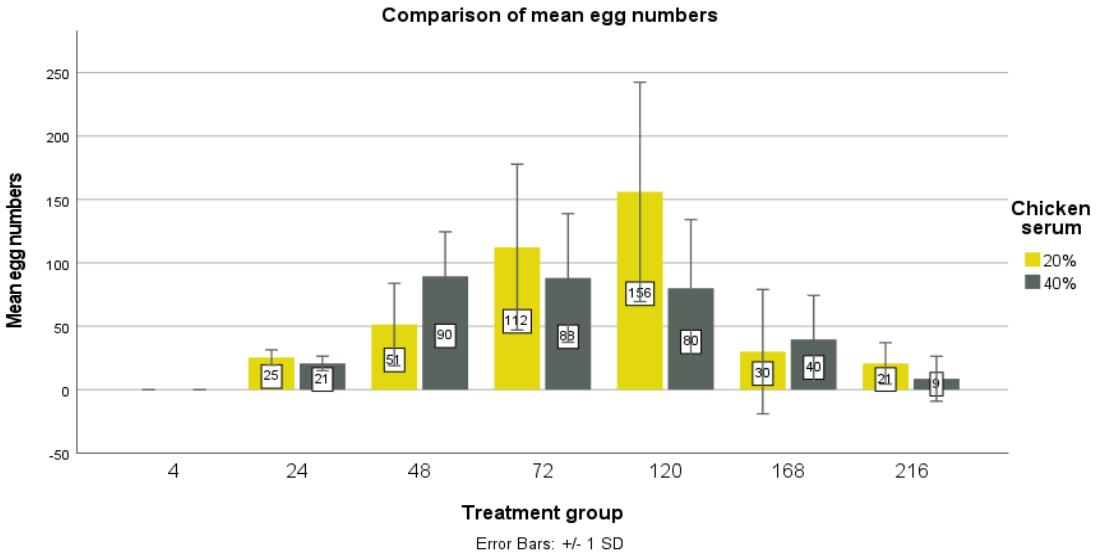


Figure 5.26. A comparison of the mean egg number at seven time points produced by the young adults cultured in NCTC 109 supplemented with antibiotics and CS, 20% and 40% respectively.

5.3.4.3. Developmental morphology

Morphology of the metacercariae in both treatment groups was recorded by light microscopy photography both with and without phase contrast. At pre-determined intervals, the morphology of the whole living fluke and its copulatory organs were recorded, where possible. Egg development was also investigated.

5.3.4.3.1 NCTC109 supplement with 20% chicken serum and antibiotics

At 4 hours, all body organs within the young adults were clearly visible, but no eggs were observable in the uterus (Figure 5.27A). At 24 hours, the internal organs looked like those at 4 hours (Figures 5.27B-5.28), but a few eggs started to appear from the proximal uterus, and the process of egg formation was observed in the fertilization chamber region (Figure 5.29). These eggs were small, oval and possessed a thin shell.

At 48 hours, numerous spermatozoa were visible in the cirrus, in the *ductus ejaculatorius* and in the vagina (Figure 5.30). More eggs subsequently appeared, and each contained numerous cells at one or both ends, these possibly represent vitelline cells. A transparent non-dividing ovum was noticed in some eggs occupying about 2/3 of the egg (Figure 5.31B). At 72 hours, more eggs were seen in the uterus, as were developing eggs and a spermatozoon in the uterus loop (Figure 5.32A). The eggs in the uterus were occupied by cells (Figure 5.32B) and these were assumed to be vitelline cells or globules and the dividing embryo. At 120 hours, the whole uterus was full of eggs (Figure 5.33A), and different egg states were observable. Abnormal eggs without shells were numerous in the proximal uterus and they looked brown and granular, while apparently normal eggs which displayed a shell, vitelline cells / globules and an ovum were more abundant in the distal uterus. The development of the eggs at 168 hours was similar (Figure 5.33B).

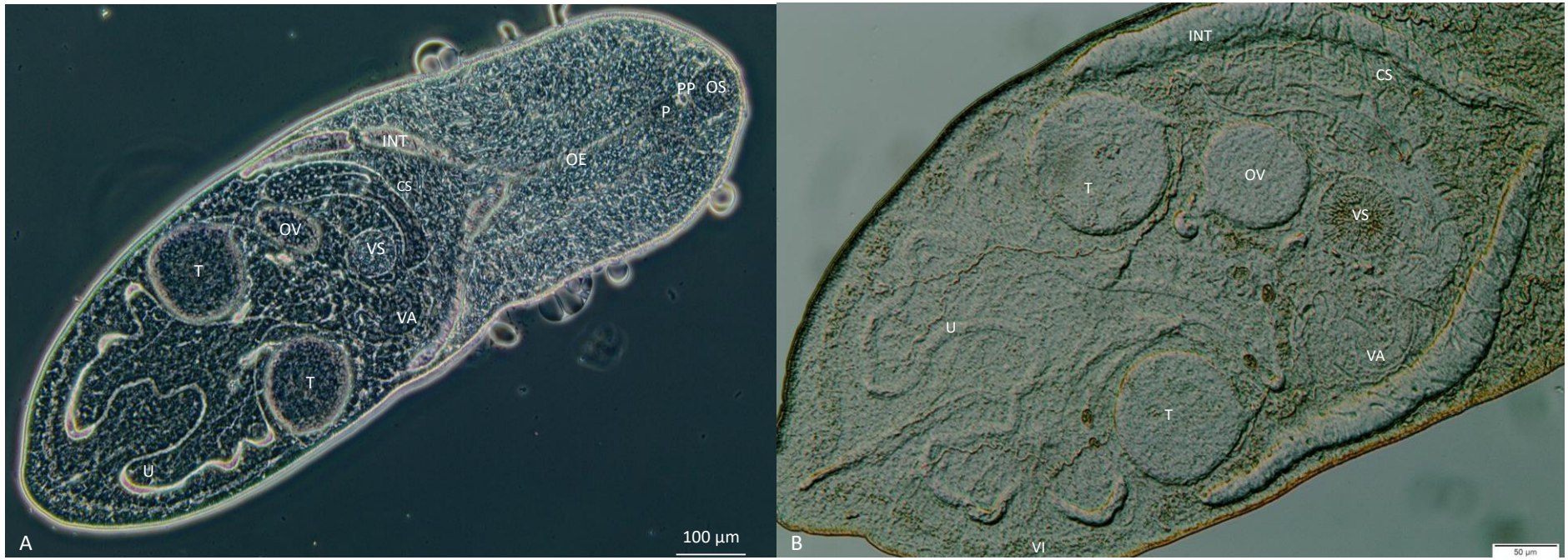


Figure 5.27. A. A young adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 4 hours. Ventral view under dark field. B. A young adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 24 hours. Ventral view under phase contrast. CS: cirrus sac. INT: intestine caecum. OE: oesophagus. OS: oral sucker. OV: ovary. P: pharynx. PP: pre-pharynx. T: testis. VA: vagina. VI: vitellaria. VS. ventral sucker.

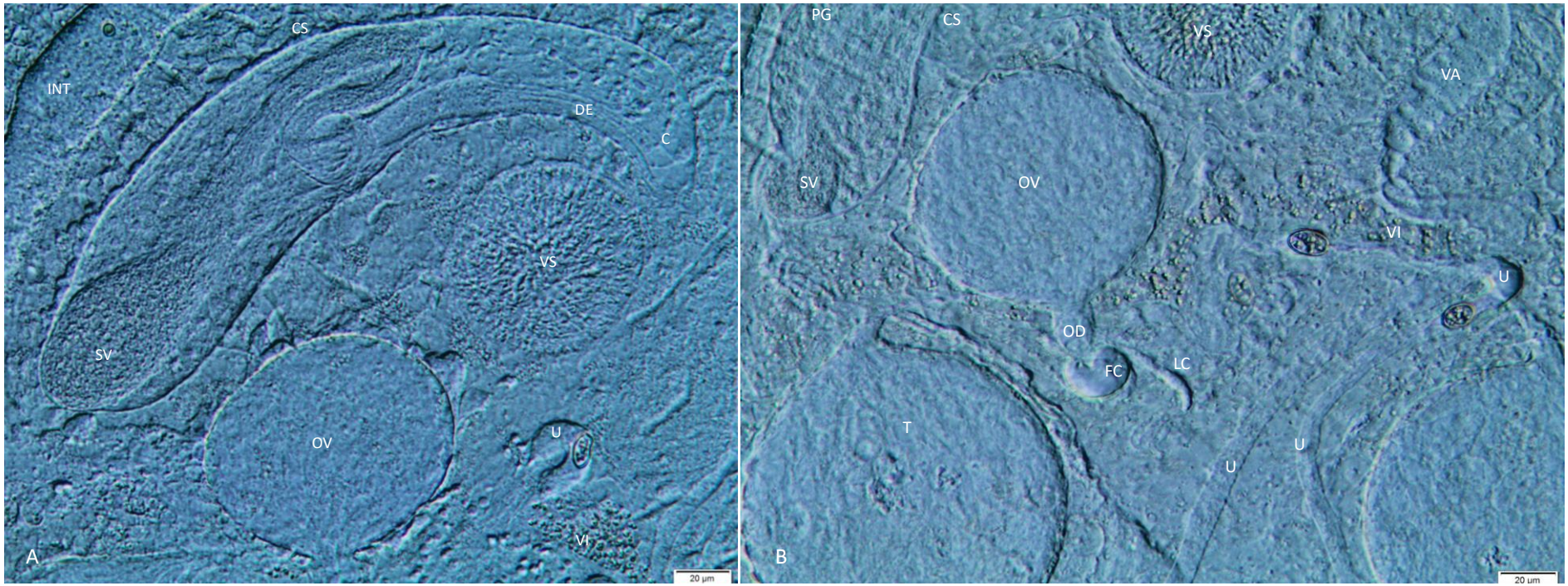


Figure 5.28. A young adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 24 hours. A. At a higher magnification showing the male copulatory organs. B. At a higher magnification and showing the ovary region. C: cirrus. CS: cirrus sac. DE: *ductus ejaculatoris*. FC: fertilization chamber. LC: Laurer's canal. INT: intestine caecum. OD: oviduct. OV: ovary. PG: prostate glands. SV: seminal vesicle. T: testis. U: uterus. VA: vagina. VI: vitellaria. VS. ventral sucker.

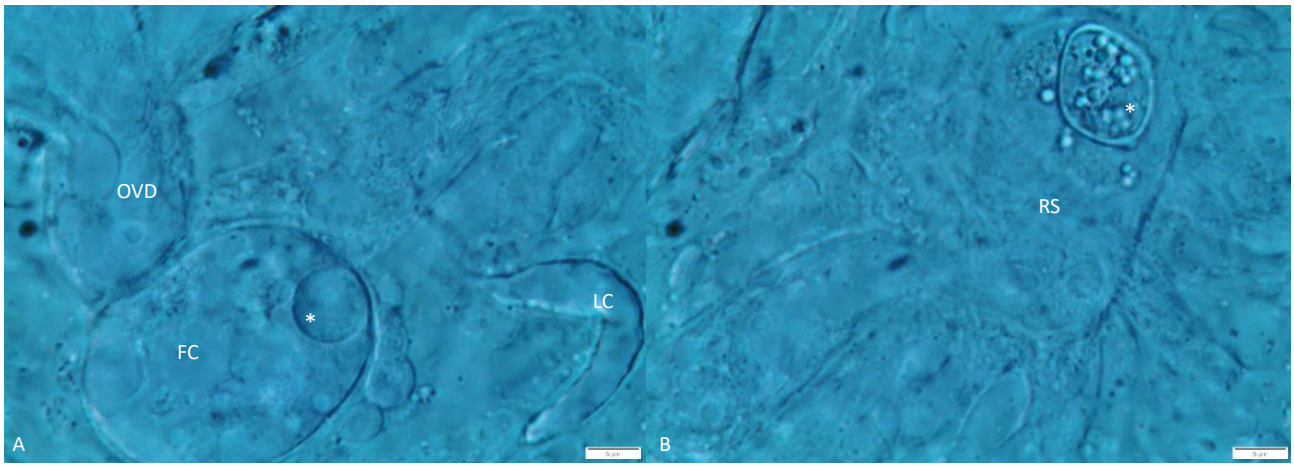


Figure 5.29. A young adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 24 hours, at a higher magnification and showing the oviduct region. A. Fertilization chamber and a developing egg (asterisk) inside with the Laurer's canal on the right. B. *Receptaculum seminis* and a developing egg inside; note the eggshell is visible. FC: fertilization chamber. LC: Laurer's' canal. OVD: oviduct. RS: *Receptaculum seminis*.

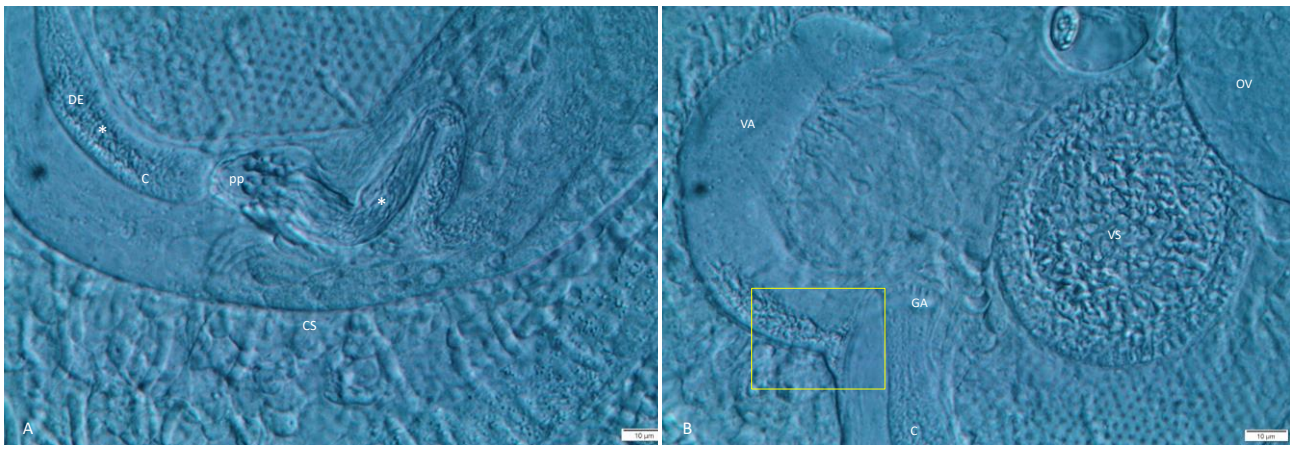


Figure 5.30. A young adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 48 hours. A. At a higher magnification and showing the cirrus sac region. Note numerous spermatozoa (asterisks) in the *ductus ejaculatori*s. Also note the ampulla-shaped *pars prostatica* between the *ductus ejaculatoris* and the seminal vesicle. B. At a higher magnification and showing the genital pore region. Note numerous spermatozoa at the end of the vagina (close to the genital atrium denoted by the yellow square). C: cirrus. CS: cirrus sac. DE: *ductus ejaculatoris*. GA: genital atrium. OV: ovary. pp: *pars prostatica*. VA: vagina. VS: ventral sucker.

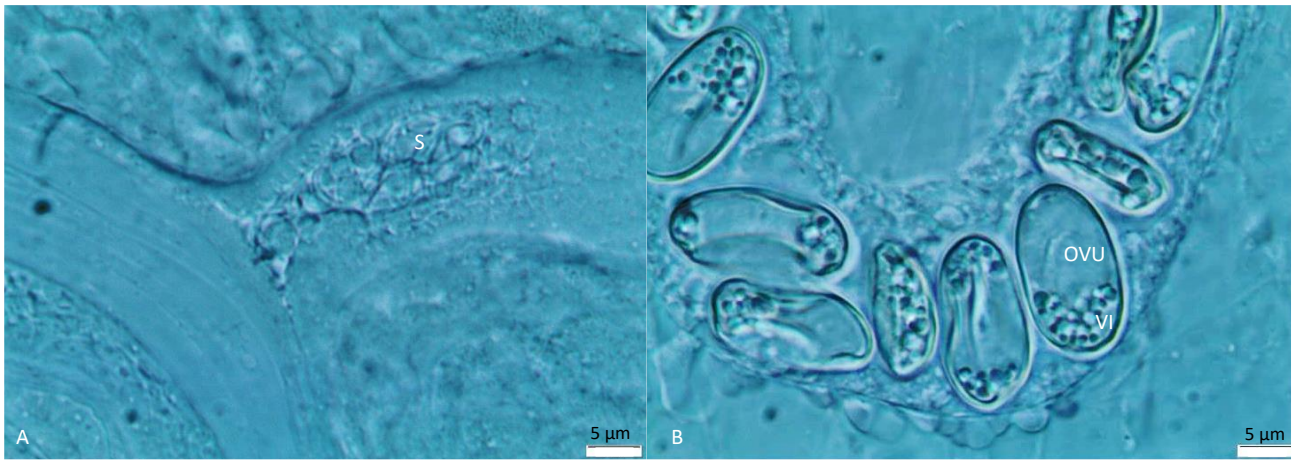


Figure 5.31. A young adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 48 hours. A. Closer look at the highlighted yellow square region presented in Figure 5.30. Note the spermatozoa (S) that have accumulated in the vagina. B. Eggs in the uterus, note the cells at one or both ends of the egg, which are likely vitelline globules (VI) and an ovum in the middle (OVU).

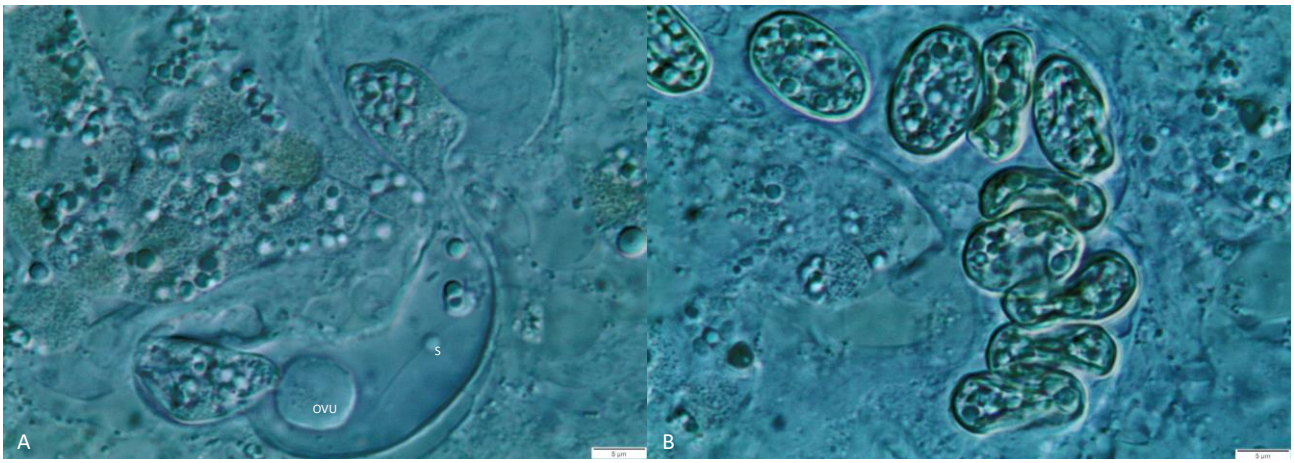


Figure 5.32. Adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 72 hours. A. Higher magnification of the uterus loop. Note an ovum (OVU) and a spermatozoan (S) inside. B. Higher magnification of the uterus loop. Note that the eggs are full of vitelline cells / globules.

At 216 hours, the living flukes appeared to shrink a little and to become less active, and there were wrinkles in their teguments. All copulatory organs were still visible and there were still some intact eggs in the uterus, but the greater part of the uterus contained abnormal eggs and cell debris (Figure 5.34). The presence of an operculum on the egg could not be confirmed throughout the observations and no naturally expelled eggs were observed in the medium. Although there were some eggs attached on tissue debris in the medium, they were considered to have “leaked out” from injured worms

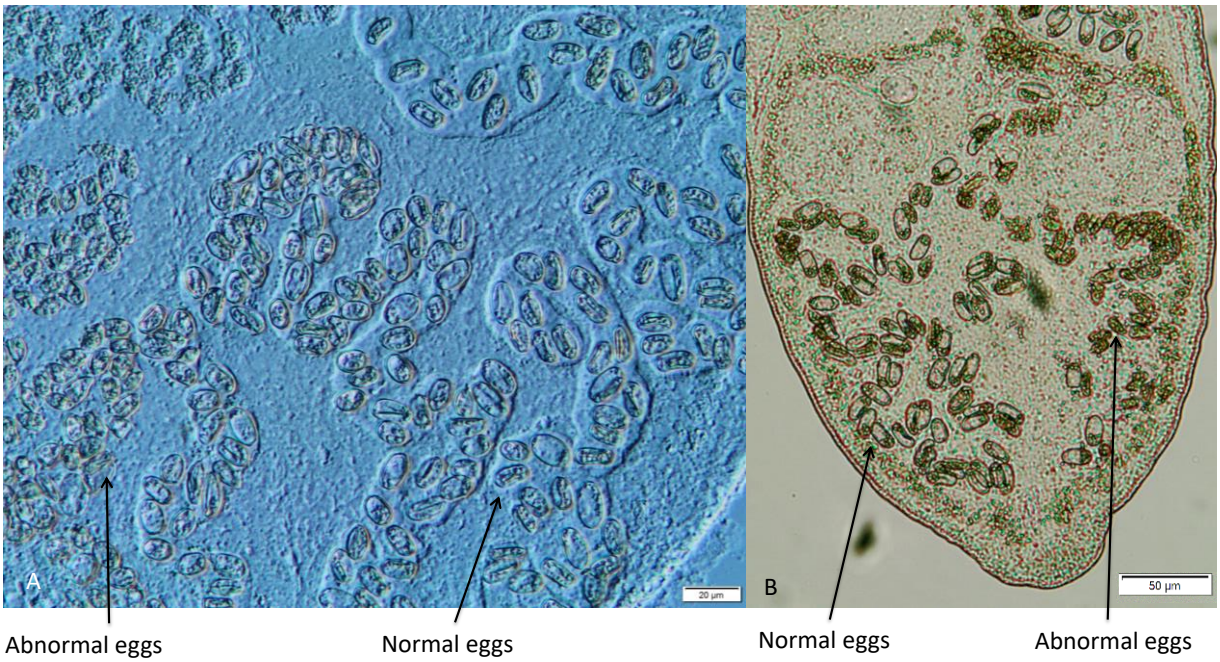


Figure 5.33. A. An adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 120 hours. Higher magnification of the uterus loop. B. An adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 168 hours. Lower magnification of the uterus loop. Note both normal eggs and abnormal eggs can be observed. The abnormal eggs looked granular without shells or had deformed shells and were seen accumulated in the distal part of the uterus.

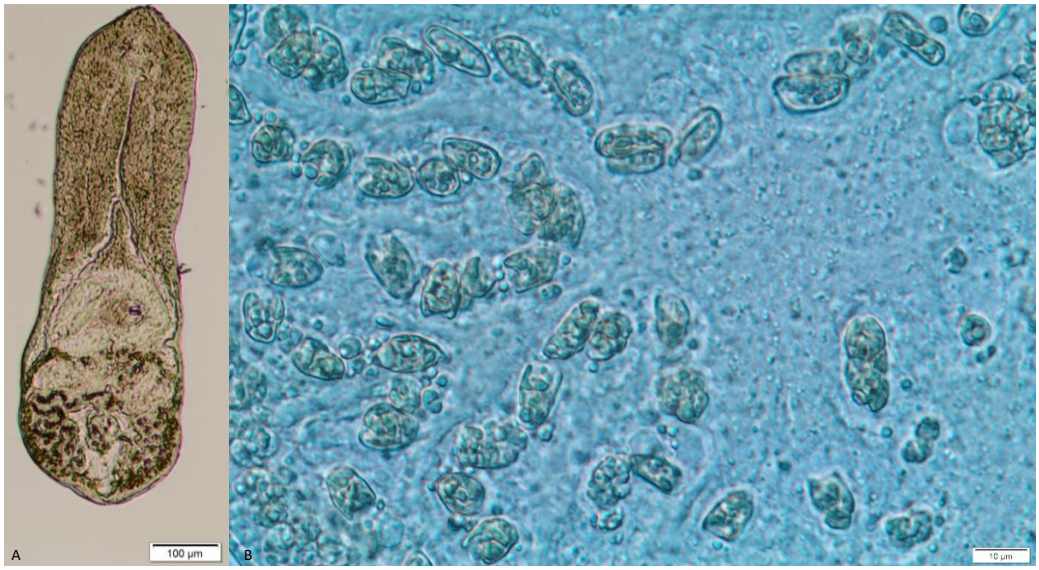


Figure 5.34. An adult cultured in NCTC 109 supplemented with 20% CS and antibiotics for 216 hours. A. Whole worm ventral view. B. Abnormal eggs in the uterus.

5.3.4.3.2 NCTC109 supplemented with 40% chicken serum and antibiotics

In this treatment group, the development of the excysted metacercariae essentially followed the same pattern of that in the 20% chicken serum group with no differences noted. At 4 hours, all the organs had developed except for there being no eggs in the uterus (Figures 5.35A & 5.35B). At 24 hours, eggs started to appear and at 48 hours they increased in number (Figure 5.35C). At the beginning, the eggs looked small, oval and had thin shells (Figure 5.36B). The region of the fertilization chamber and Laurer's canal was difficult to observe and needed to be observed carefully (Figure 5.36A).

Eggs with cells / globules at both ends predominated at 48 hours, which were supposed to comprise vitelline cells / globules and an ovum. Eggs with a presumable dividing embryo accompanying the other cells / globules, however, were also noticed (Figure 5.36B). At 72 hours, a cirrus protruding out of the genital pore was noticed in one fluke (Figure 5.37A). A clear image of the FC and LC region was captured (Figure 5.37B). Moving spermatozoa were observed at the genital pore (Figure 5.38), the *ductus ejaculatorius* and the seminal vesicle (Figures 5.39A & 5.39B). Eggs full of cells were observed in the uterus loop at 72 hours (Figure 5.39C).

At 120 hours, all the copulatory organs were still clearly visible (Figure 5.40A). Spermatozoa were still seen in the vagina, which suggested that fertilization was still carrying on (Figure 5.40B), with eggs still seen in the uterus with vitelline cells at one or both ends and a non-dividing ovum in the middle (Figure 5.40C).

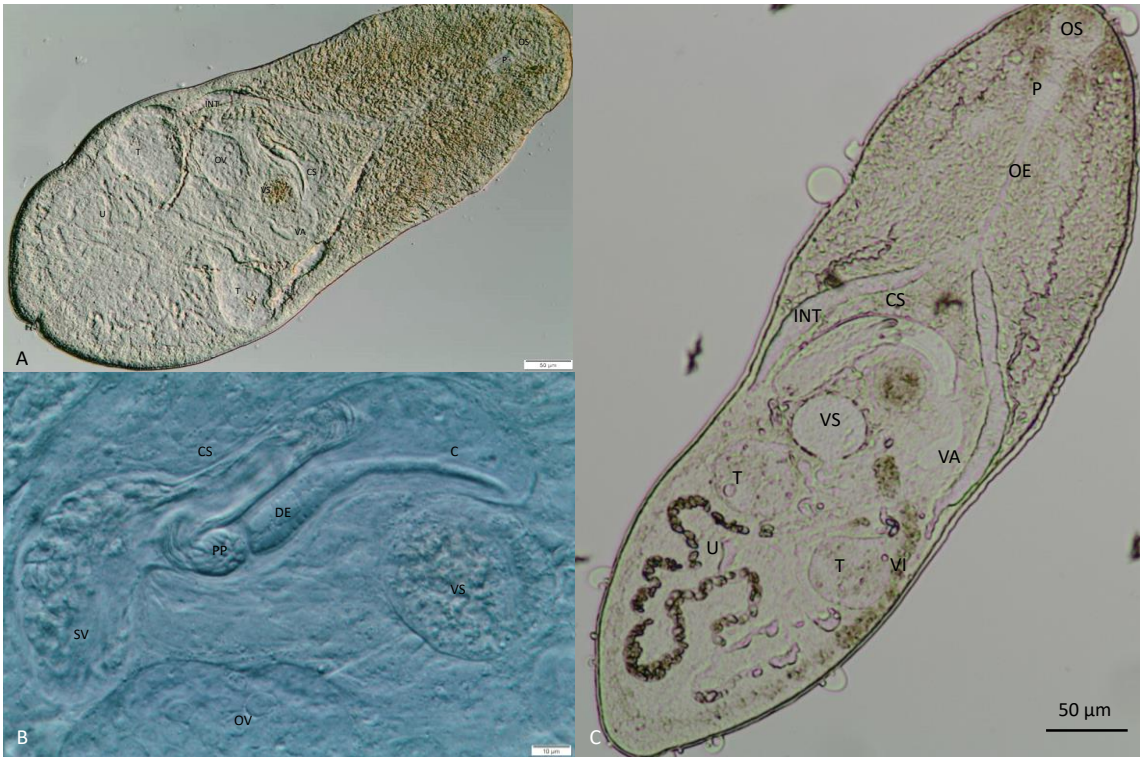


Figure 5.35. A. A young adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 4 hours. B. A young adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 4 hours, viewed at a higher magnification and

showing the cirrus sac region. Dorsal view. C. A young adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 48 hours. Ventral view. Note that the distal uterus is full of eggs. C: cirrus. CS: cirrus sac. DE: *ductus ejaculatoris*. INT: intestine caecum. OE: oesophagus. OV: ovary. P: pharynx. PP: *pars prostatica*. SV: seminal vesicle. T: testis. U: uterus. VA: vagina. VS: ventral sucker.

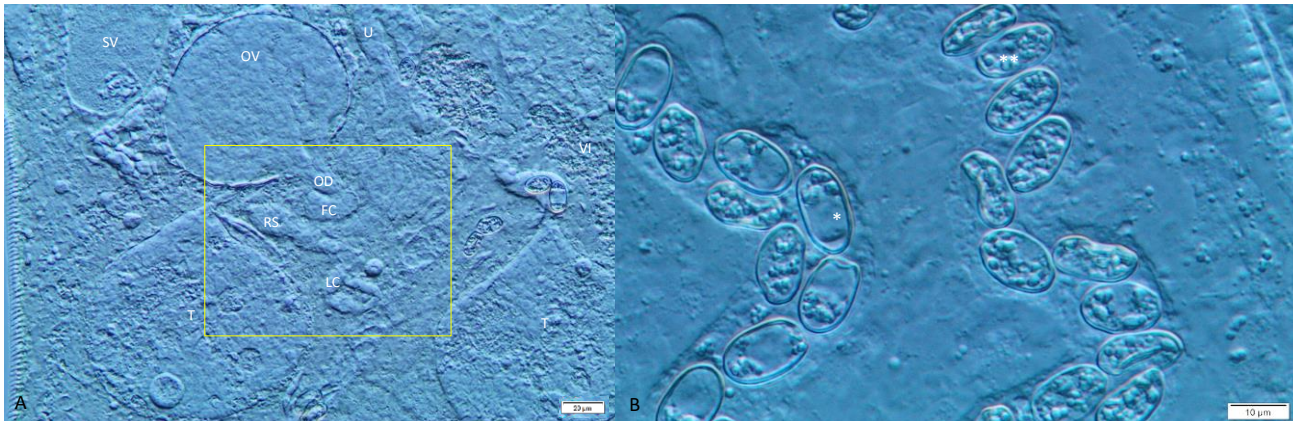


Figure 5.36. A. A young adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 48 hours, showing the female reproductive organs. The yellow rectangle marks the ootypic junction. B. A young adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 48 hours, showing the uterus loop. Note the eggs in the uterus loop, some with vitelline cells / globules at one or both end and an ovum in the middle (*), and some with a dividing embryo accompanied by vitelline cells / globules (**). FC: fertilization chamber. LC: Laurer's canal. OD: oviduct. OV: ovary. RS: *receptaculum seminis*. SV: seminal vesicle. T: testis. U: uterus. VI: vitellaria.

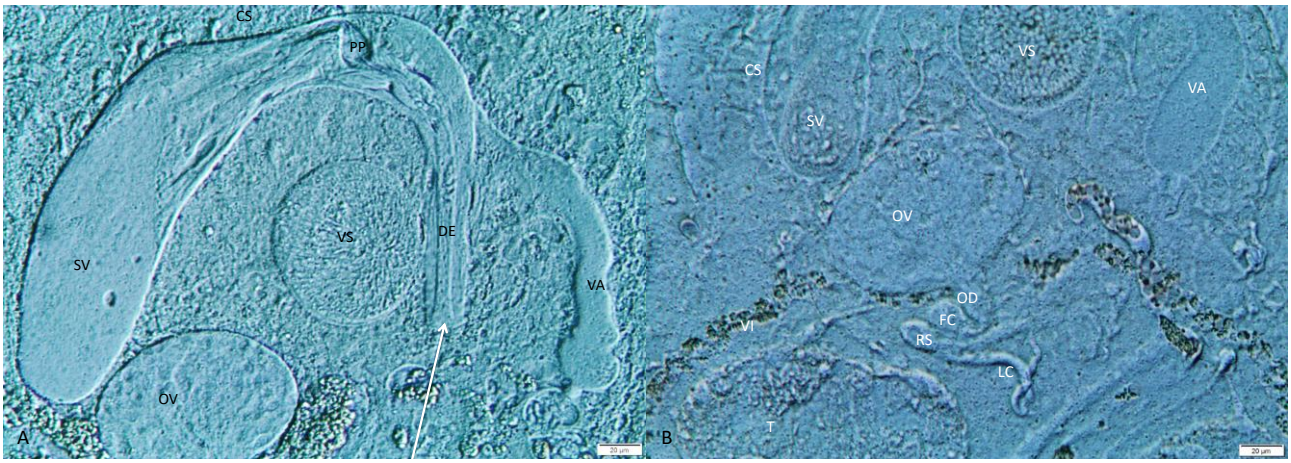


Figure 5. 37. A. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 72 hours, showing the male copulatory organs. Note the cirrus protruding from the genital pore. B. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 72 hours. A higher magnification figure of the ovary region. CS: cirrus sac. DE: *ductus ejaculatoris*. FC: fertilization chamber. GP: genital pore. LC: Laurer's canal. OD: oviduct. OV: ovary. PP: *pars prostatica*. RS: *receptaculum seminis*. SV: seminal vesicle. T: testis. VA: vagina. VS: ventral sucker.

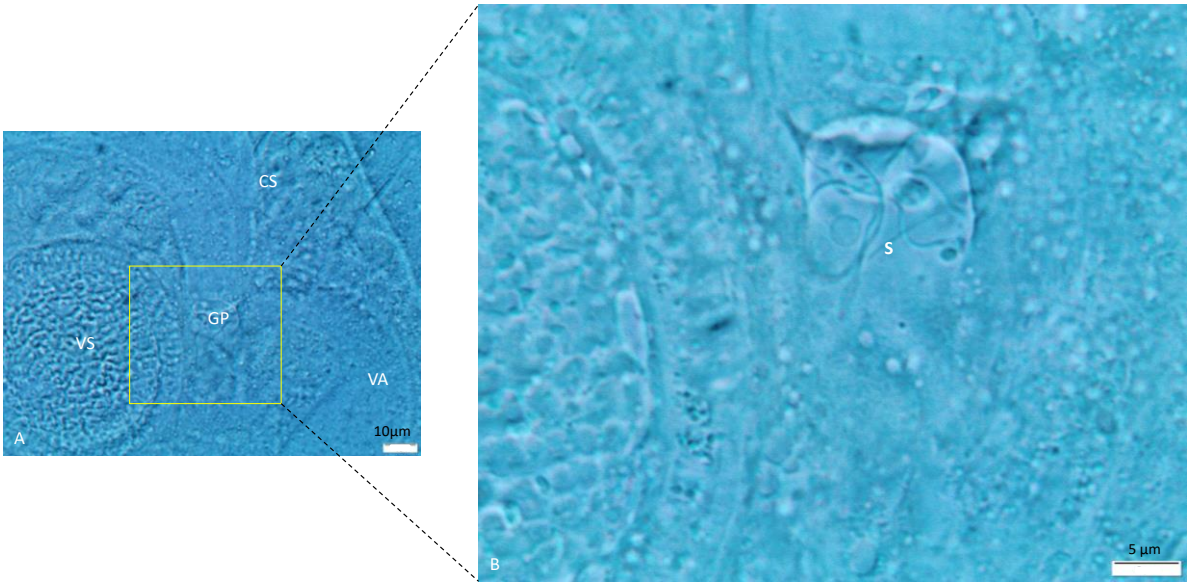


Figure 5.38.An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 72 hours, showing the genital pore. A. Ventral sucker and genital pore. B. Higher magnification of the genital pore region. Note the spermatozoa (S) near the genital pore. CS: cirrus sac. GP: genital pore. S: spermatozoa. VA: vagina. VS: ventral sucker.

At 168 hours, many abnormal eggs started to appear, however, the other body organs looked like those observed at 120 hours (Figures 5.41). At 216 hours, the flukes looked like those in the 20% CS group. They were less active, slightly shrunken and with wrinkles on the tegument, however, all the internal organs were still clearly visible (Figure 5.42A). Atrophy of the testis and ovary were noticed on some occasions (Figure 5.42B). Moving spermatozoa were still visible in the seminal vesicle (Figure 5.42, inset). The cirrus and *pars prostatic*a were seen still moving at 216 hours. Again, no operculum was confirmed, and no naturally expelled eggs were found throughout the observation period.

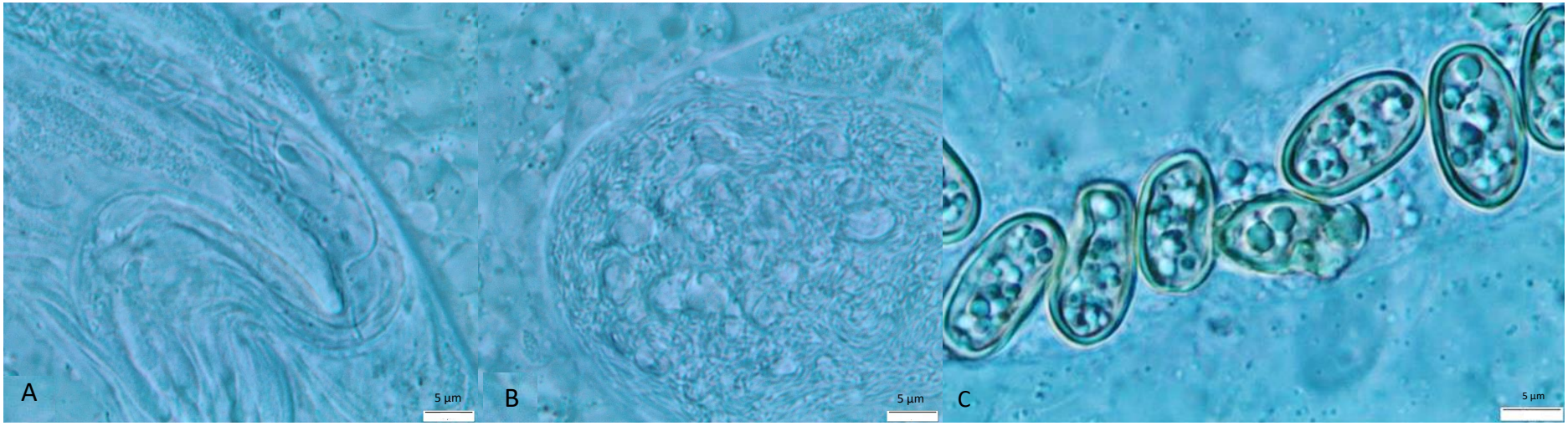


Figure 5.39. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 72 hours, showing the seminal vesicle and *pars prostatica*. A. Spermatozoa in the connecting duct between the seminal vesicle and *pars prostatica*. B. Spermatozoa in the seminal vesicle. C. Eggs produced by an adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 72 hours. These eggs were full of cells / globules and some cells seem to group into larger clumps – these may represent the dividing embryo plus vitelline globules.

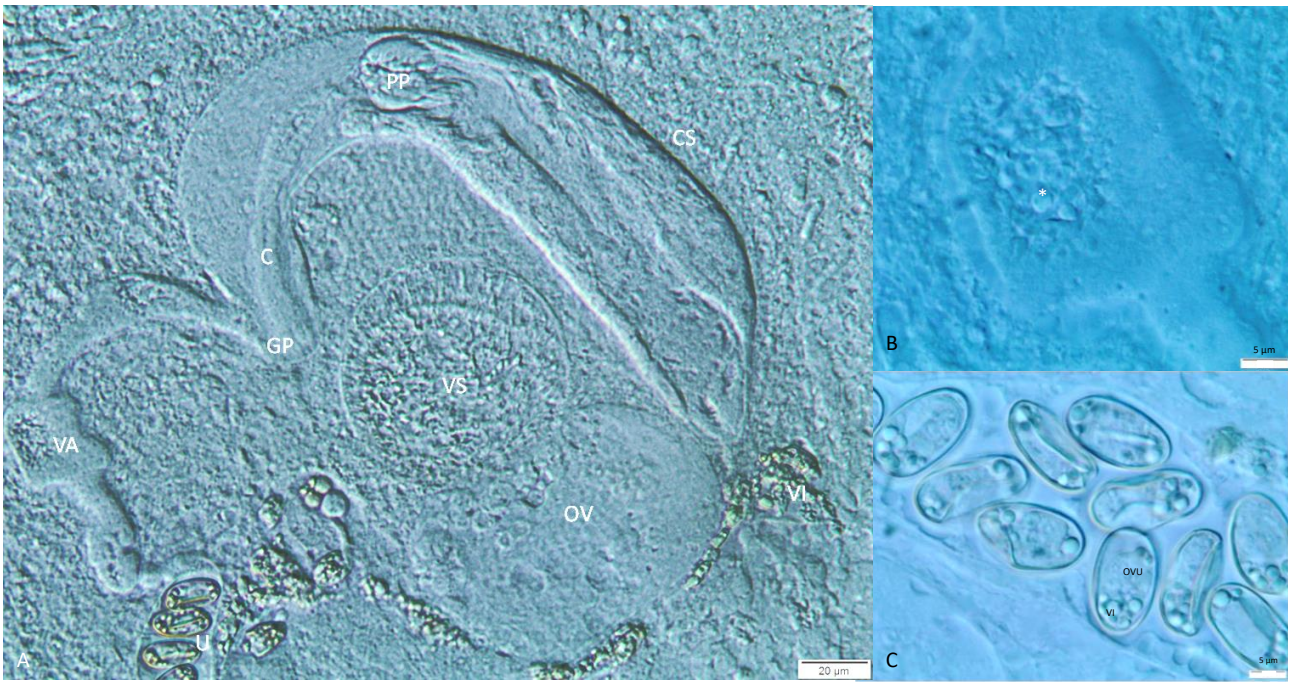


Figure 5.40. A. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 120 hours. Dorsal view and showing the male copulatory organs B. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 120 hours, showing at higher magnification of the junction between the vagina and the uterus. Note the spermatozoa (asterisk) in the vagina. C. Eggs of an adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 120 hours. The eggs comprise vitelline cells / globules at one or both ends and an un-divided ovum in the middle. C: cirrus. CS: cirrus sac. GP: genital pore. OV: ovary. OVU: ovum. PP: *pars prostatica*. VA: vagina. VI: vitellaria. VS: ventral sucker.

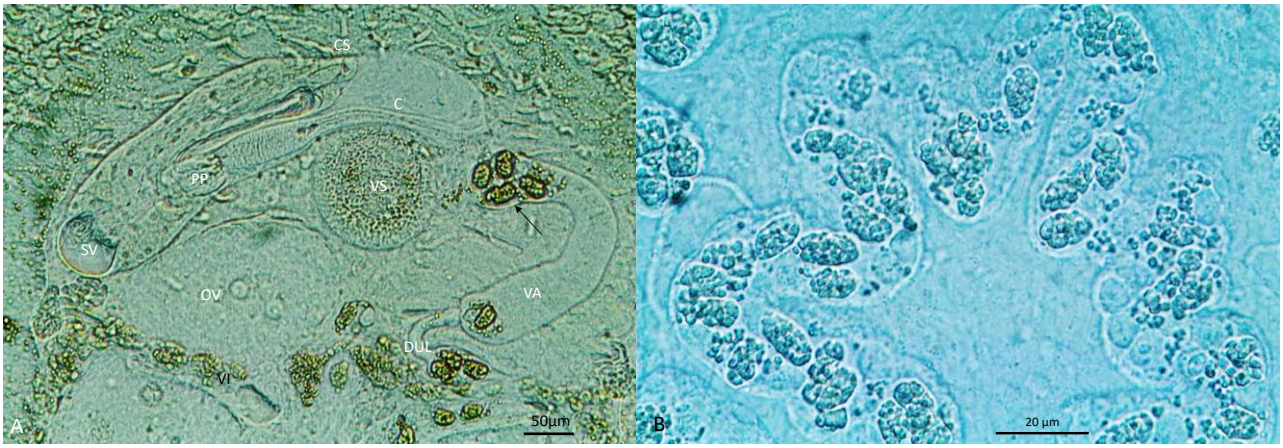


Figure 5.41. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 168 hours. A. Ventral view showing the male copulatory organs. Note there were some eggs in the genital atrium (arrow). B. The uterus. Note that the uterus loop contains many abnormal eggs. C: cirrus. CS: cirrus sac. DUL: distal uterus loop. OV: ovary. PP: *pars prostatica*. SV: seminal vesicle. VA: vagina. VS: ventral sucker.

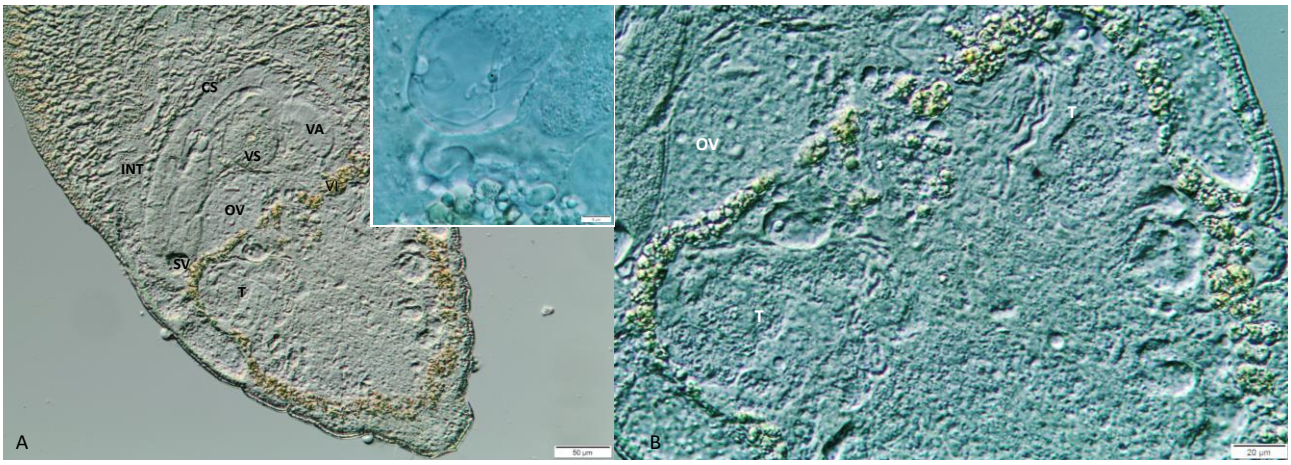


Figure 5.42. An adult cultured in NCTC 109 supplemented with 40% CS and antibiotics for 216 hours. A. The fluke had no eggs. B. Focus on the ovary and testes. Note the ovary (OV) and testes (T) were atrophied, and that the testes contained vacuoles. Inset. Seminal vesicle. Moving spermatozoa were visible in the seminal vesicle but were lower in number. CS: cirrus sac. INT: intestine caecum. OV: ovary. SV: seminal vesicle. T: testis. VA: vagina. VS: ventral sucker.

5.3.5. Structural observation by confocal laser scanning microscopy (CLSM)

Different ages of cultured adults were used for CLSM and these samples were all obtained from Dunbar (2017). There was no difference in the development of the musculature and organs among the different aged parasites (0 to 120 hours), therefore the results will be discussed together. Most images presented here were stained with 2 X phalloidin, however, several images were from re-stained samples using 8-10 X phalloidin (Figures 5.51-5.56). Phalloidin binds specifically to f-actin, which reveals the muscle system; while DAPI binds to DNA and RNA, staining nuclei and helping to define the cellular structure of tissues and organs. By choosing specific channels of laser emission, images for phalloidin or DAPI stain can be presented separately or in combination. The somatic musculature revealed by the phalloidin stain was of a typical digenean type, comprising circular, longitudinal and diagonal muscles. The circular and longitudinal muscle formed lattice patterns, and the diagonal muscles ran obliquely in two opposite directions (Figures 5.43 & 5.44). The longitudinal and circular muscles bands were much denser than the diagonal muscle. Circular muscle bands were thinner than the diagonal and longitudinal muscle (Figure 5.44, inset). Numerous ventro-dorsal muscles were distributed in the forebody but less in the hind body possibly due to body organs occupying the space or the need for rigidity in attachment at the anterior end (Figure 5.43A). At lower magnifications, the oral sucker, pharynx, cirrus sac, vagina, ventral sucker, and flame cells stained strongly (Figure 5.43A). DAPI stain revealed the position and shape of parenchymal organs such as the seminal vesicle, ovary, testes, vaginal glands, and the lining of the intestinal caecum (Figure 5.43 B). The oral sucker comprised compact circular, meridial, and radial muscles and were connected to a muscular pharynx through a short pre-pharynx. The pharynx was oval and comprised thicker radial muscles (Figure 5.44A). The composition of the somatic muscles in the hind body was the same as for the forebody except that there were less ventro-dorsal muscles (Figure 5.44B).

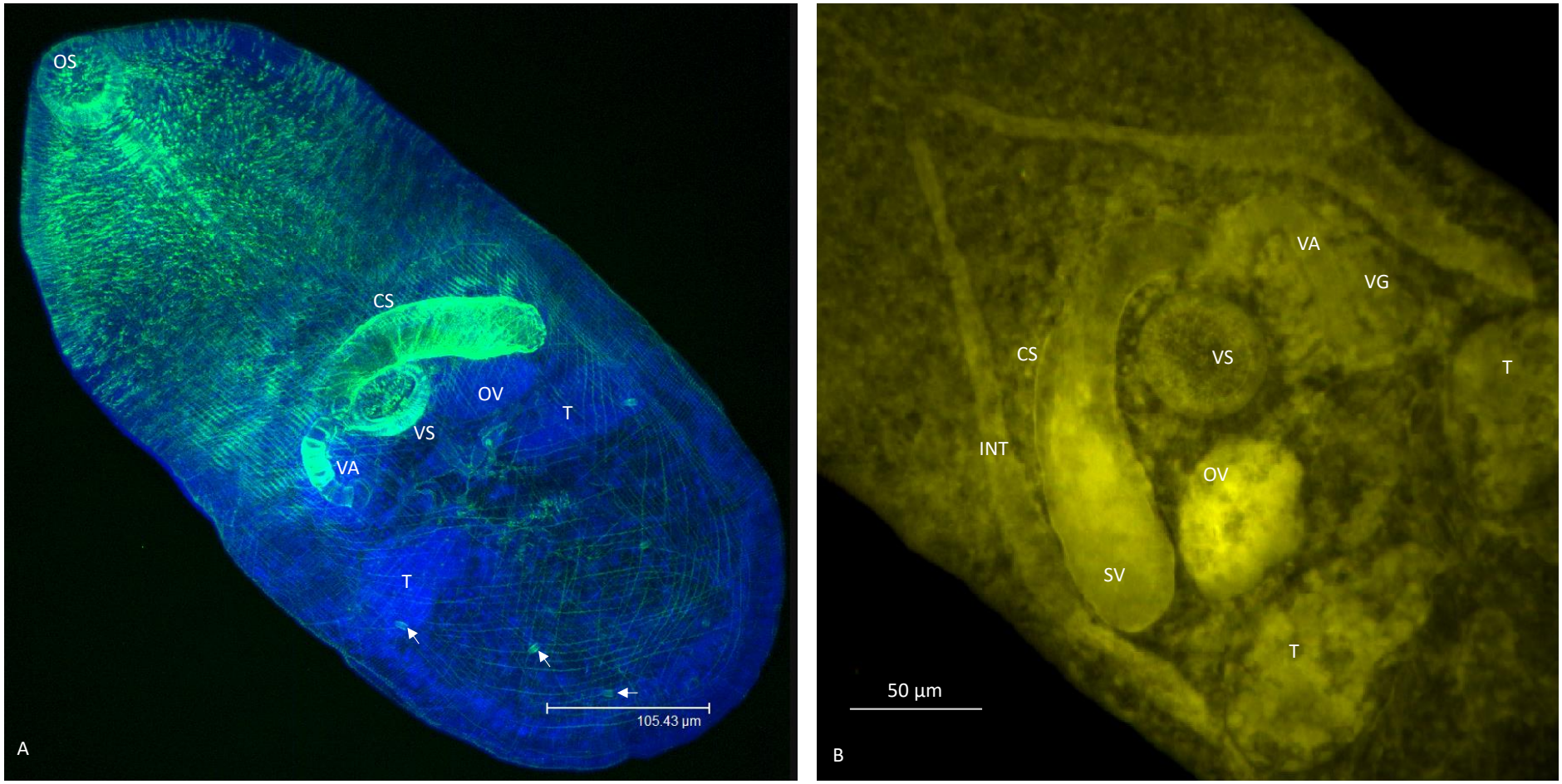


Figure 5.43. *Maritrema gratiosum*-whole worm morphology under LSCM. A. *M. gratiosum* cultured in NCTC-109 supplemented with 40% chicken serum and antibiotics for 120 hours. Whole worm dorsal view. Shows DAPI (blue) and phalloidin (green) stain. Note three pairs of flame cells (arrows) in the hind body. B. *M. gratiosum* cultured in NCTC-109 supplemented with 20% chicken serum and antibiotics for 48 hours. Ventral view focusing on the region containing the reproductive organs stained with DAPI. CS: cirrus sac. INT: intestine caecum. OS: oral sucker. OV: ovary. SV: seminal vesicle. T: testis. VA: vagina. VG: vaginal gland. VS: ventral sucker.

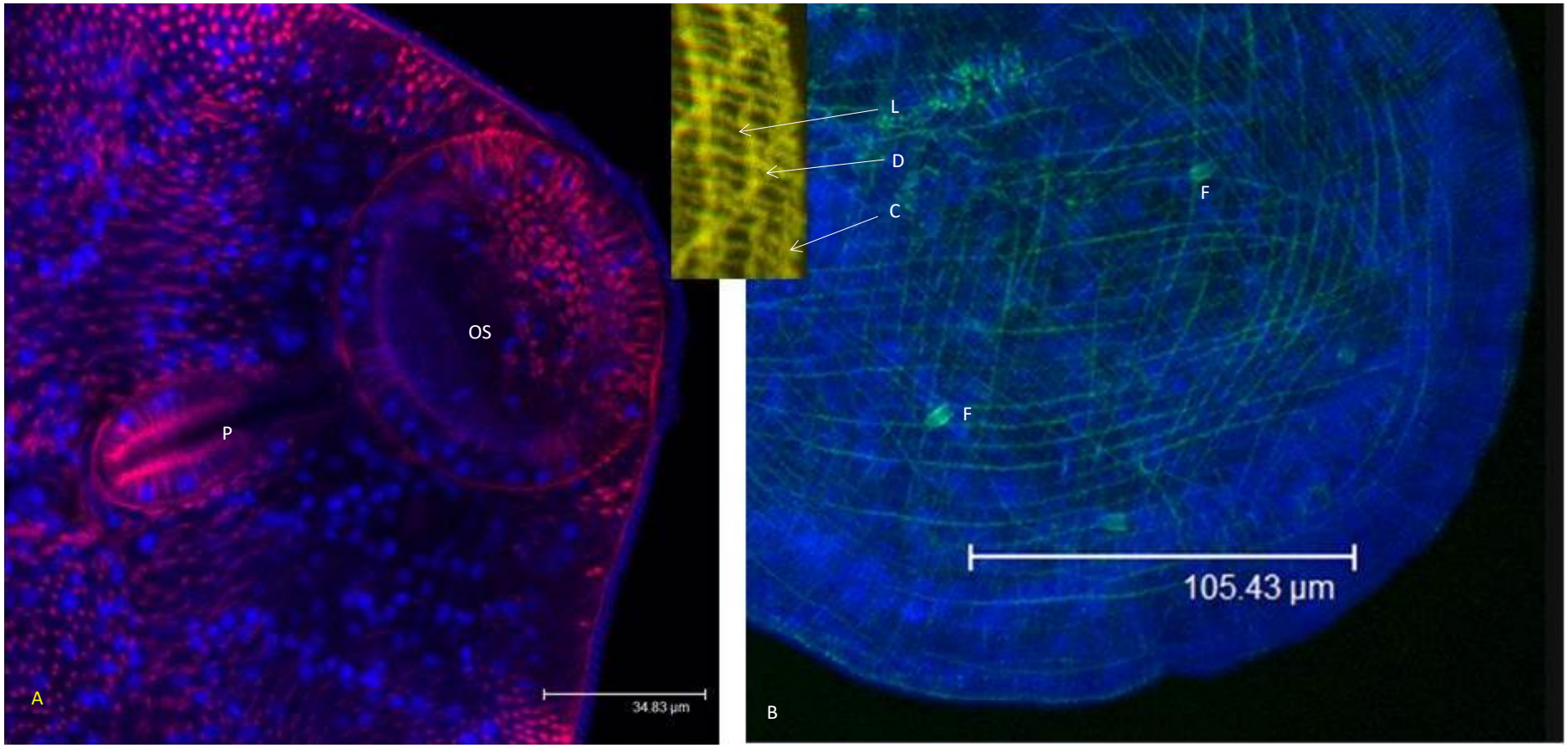


Figure 5.44. Anterior and posterior parts of a specimen of *Maritrema gratiosum*. A. Anterior region of a specimen of *M. gratiosum* cultured in NCTC-109 supplemented with 20% foetal bovine serum and antibiotics for 72 hours. The image shows features stained with DAPI (blue) and with phalloidin (red) stain. The red dots are cross sections of the ventro-dorsal muscles. B. Posterior region. Higher magnification of the hind body from Figure 5.43A, showing features stained with DAPI (blue) and with phalloidin (green) stain. The inset in the middle shows the somatic muscles from the side of the body at a higher magnification (phalloidin stain only). C: circular muscles. D: diagonal muscles. F: flame cell. L: longitudinal muscles. OS: oral sucker. P: pharynx.

In rare preparations, the structures inside the cirrus sac can be seen (Figure 5.45). The cirrus sac, seminal vesicle, *pars prostatica*, cirrus and the ducts connecting these structures were all muscular. The cirrus sac comprised thin layers of diagonal muscle which ran obliquely in two opposite directions (Figure 5.46). The vagina comprised thin layers of circular and longitudinal muscle (Figures 5.45-5.47) and a strong sphincter was noted between the junctions of the vagina and the uterus (Figures 5.45-5.47). The seminal vesicle and the duct connected to the *pars prostatica* also comprised loose circular muscles (Figure 5.47). The connecting duct between the seminal vesicle and *pars prostatica* were folded into an S-shaped loop. A well-developed *pars prostatica* was present, with a "ligament" holding the *pars prostatica* and seminal vesicle together (Figure 5.45). This ligament has never been described before in this species. The *pars prostatica* was ampulla-shaped, and in the DAPI channel it seemed to be divided into several chambers (Figure 5.45B). Much of the cirrus is comprised of compact longitudinal and circular muscles with longitudinal muscles dominating, however, in the most distal part it was narrower and circular muscles were more obvious. The cirrus exited through the male pore at the anterior wall of the small genital atrium. The sinuous and muscular vagina connected to the genital atrium posteriorly. The ovary and vaginal gland stained strongly with DAPI, and cells of the prostate gland were visible throughout the cirrus sac in the DAPI channel (Figure 5.45B). The ventral sucker comprised compact radial muscles in the bulk of the tissue and meridial muscles and circular muscles at the surface, although the circular muscles were not obvious (Figures 5.45-5.47). Several extra thick supportive radial muscle bundles were noticed scattered within the ventral sucker (Figure 5.47D).

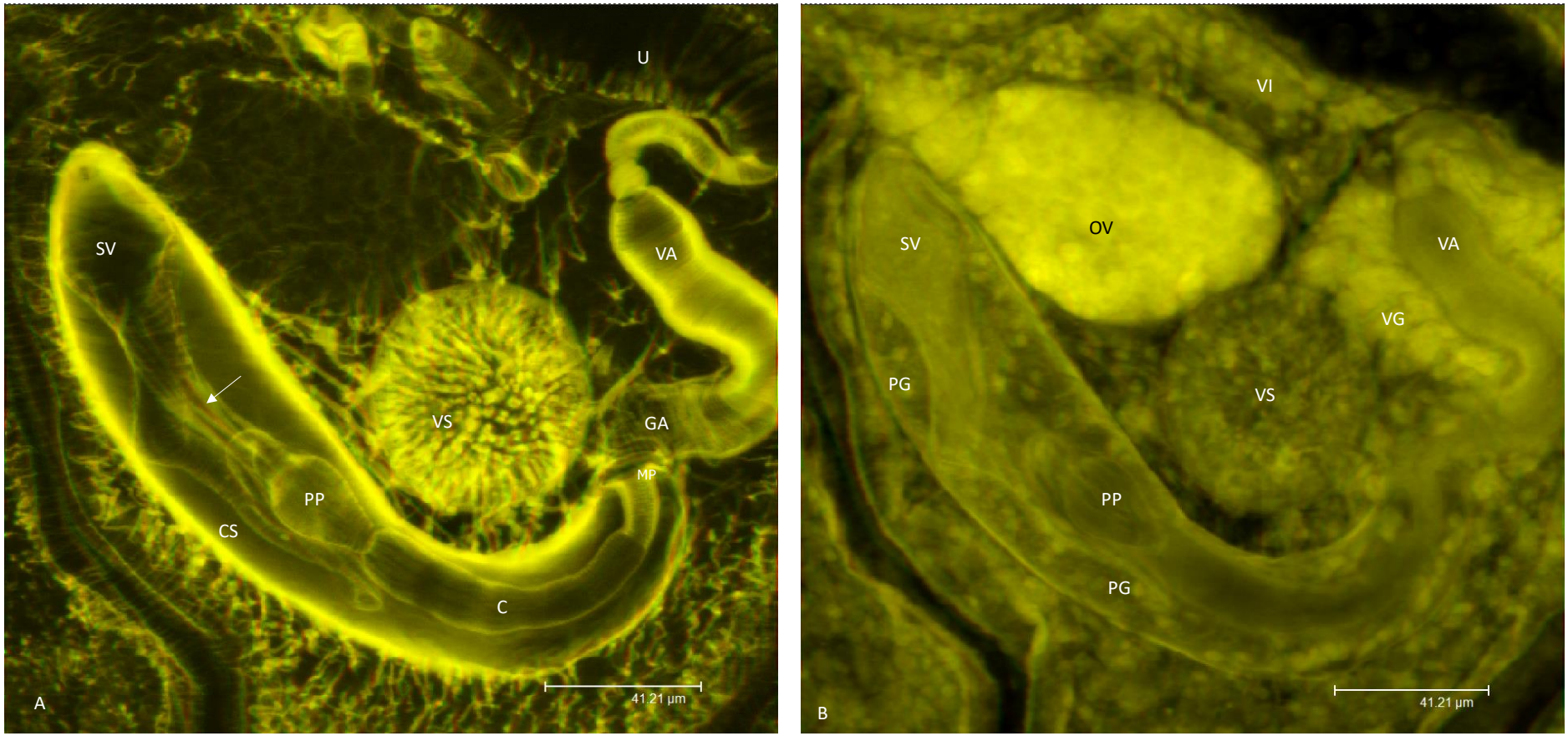


Figure 5.45. Male reproductive system of *Maritrema gratiosum*. Anaglyph 3D image. A. A specimen of *M. gratiosum* cultured in NCTC-109 supplemented with 40% chicken serum and antibiotics for 120 hours. Ventral view of the parasite stained only with phalloidin. Note the ligament connecting the *pars prostatica* and seminal vesicle (arrow). B. *Maritrema gratiosum* cultured in NCTC-109 supplemented with 40% chicken serum and antibiotics for 120 hours. Ventral view of the specimens stained with DAPI only. C: cirrus. CS: cirrus sac. GA: genital atrium. MP, male pore at genital atrium. OV: ovary. PG: prostate glands. PP: *pars prostatica*. SV: seminal vesicle. U: uterus. VA: vagina. VH: vaginal gland. VS: ventral sucker.

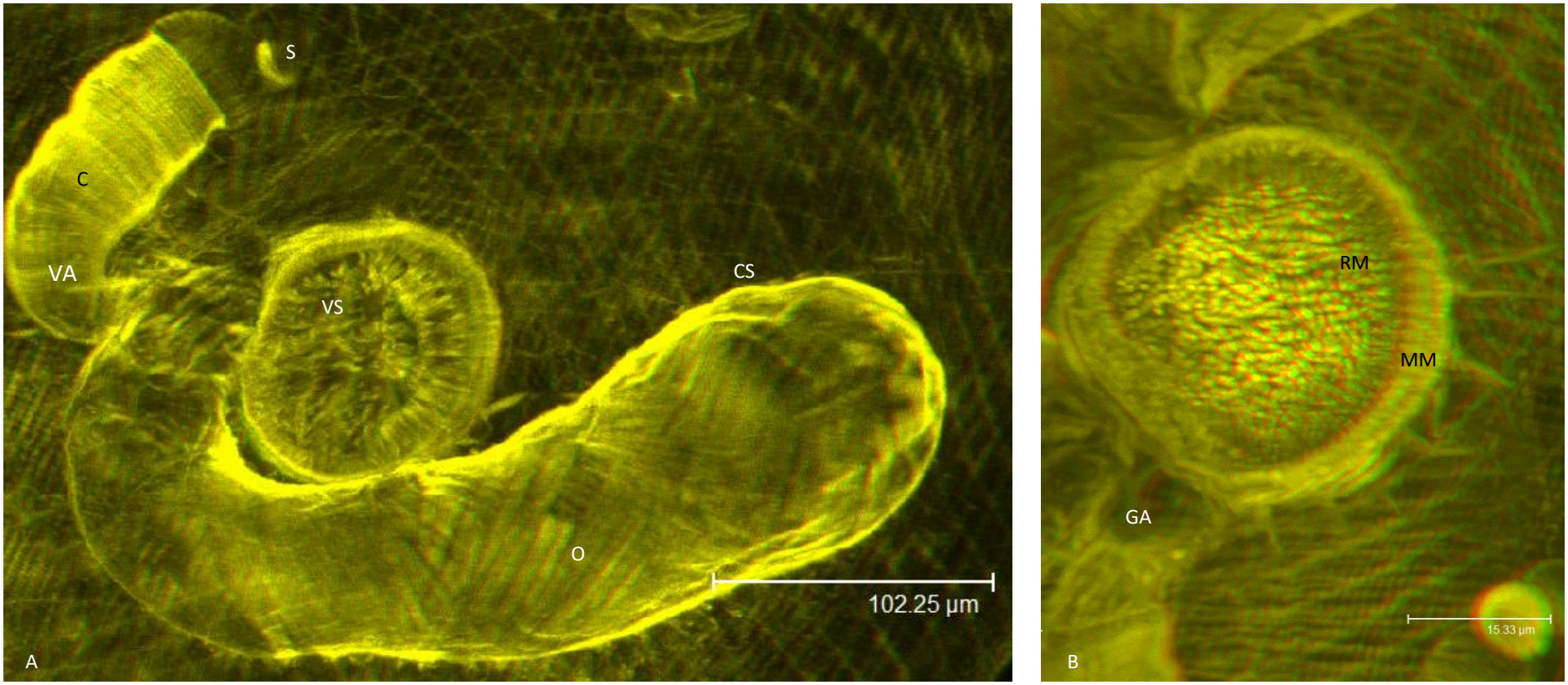


Figure 5.46.. Muscles of the male reproductive system and the ventral sucker of *Maritrema gratiosum*. Anaglyph 3D image. A. *Maritrema gratiosum* cultured in NCTC-109 supplemented with 20 % chicken serum and antibiotics for 72 hours and stained with phalloidin. Dorsal view. Note the circular muscles (C) of the vagina and the oblique muscles (O) of the cirrus sac, and the sphincter between the vagina and the uterus (S). B. Reconstructed image stack for *M. gratiosum* cultured in NCTC-109 supplemented with 40 % chicken serum and antibiotics for 120 hours and stained with phalloidin. Ventral view anterior to the left. Note the radial muscles (RM) in the bulk and meridial muscles (MM) on the side. GA: genital atrium. VA: vagina. VS: ventral sucker.

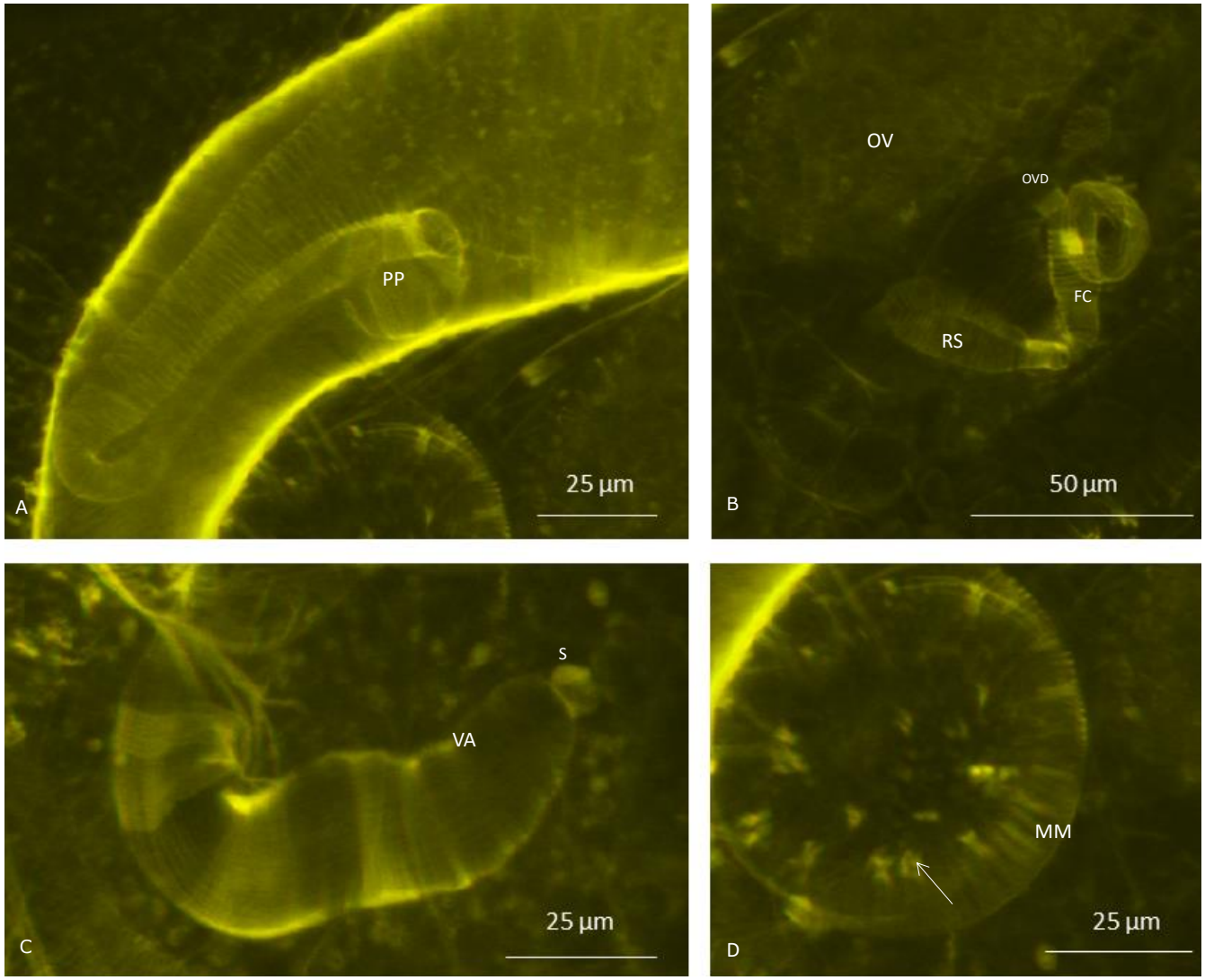


Figure 5.47. Male and female reproductive system and the ventral sucker of a specimen of *Maritrema gratiosum* cultured in NCTC-109 supplemented with 20 % chicken serum and antibiotics for 72 hours and stained with phalloidin only. Anaglyph 3D image. A. *Pars prostatica* (PP) and duct connecting to the seminal vesicle. Note the circular muscles. B. Ovary (OV), oviduct (OVD), fertilisation chamber (FC) and *receptaculum seminis* (RS, not enlarged). C. Sphincter (S) between the vagina (VA) and the uterus. Note the thin layer of circular and longitudinal muscles D. Ventral sucker, meridial muscles (MM) and thicker support muscles (arrow).

The ovary connected to a very short oviduct caudally, leads to a sphincter-like narrow channel, and to a chamber which likely represents the fertilisation chamber described by Hardly and Castle (1940). This chamber was not clearly dilated in the samples that were examined. After the fertilisation chamber, the duct moved posteriorly, narrowed, and then dilated again, and went downwards into a second loop forming another chamber, which fits the description of the *receptaculum seminis* as provided by Hardly and Castle (1940). These authors also used "ootype" to describe this chamber along with the following duct connected to the proximal uterus. Another sphincter was also seen after the *receptaculum seminis*. After the *receptaculum seminis,* a ventrally and upward directed tube was observed; this is identified as Laurer's canal (Figure 5.48A). The intestinal caecum comprised loose but thick longitudinal muscles and more compact circular muscles which then formed a blind end at the level of the testis (Figure 5.48B).

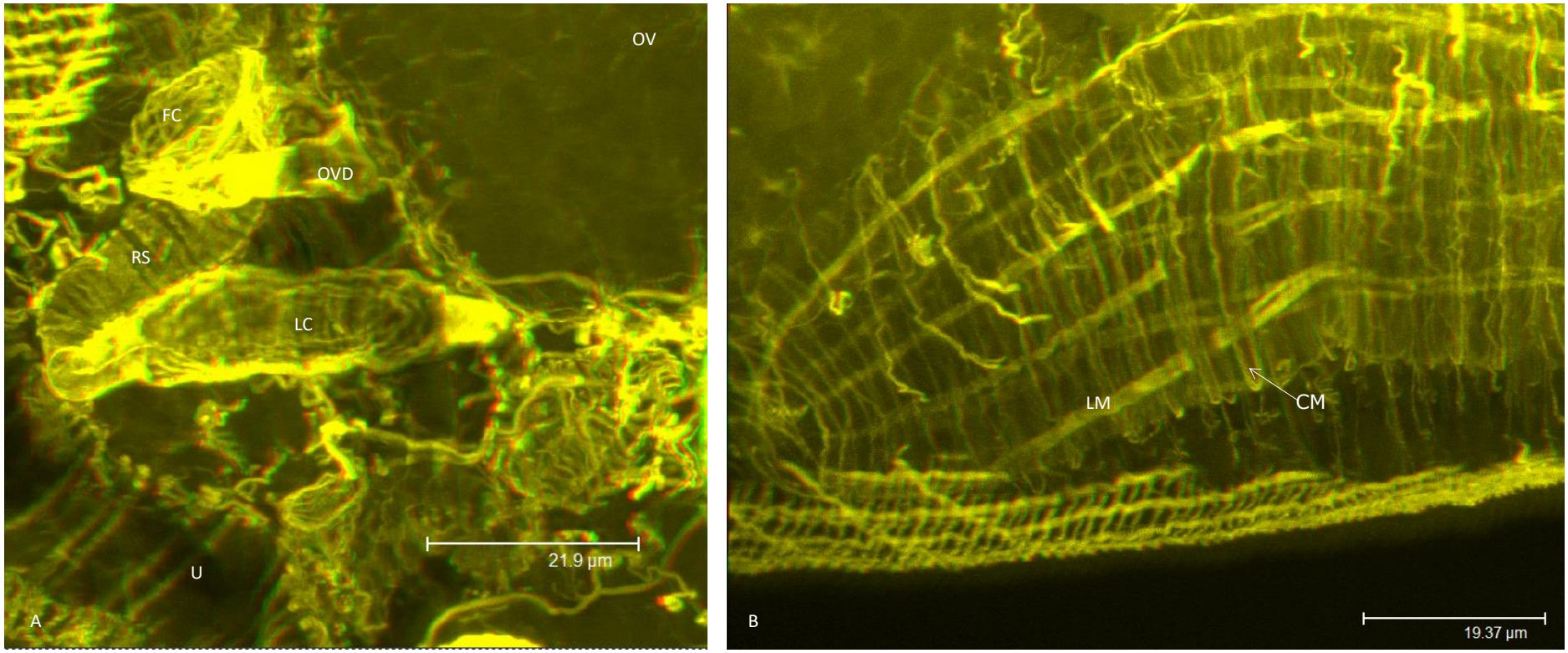


Figure 5.48. A specimen of *Maritrema gratiosum* cultured in NCTC-109 supplemented with 40 % chicken serum and antibiotics for 120 hours. Female system and the intestine caecum. Anaglyph 3D image. A. Ootypic junction. Note the ovary (OV), oviduct (OVD), the fertilisation chamber (FC, not enlarged), the *receptaculum seminis* (RS, not enlarged), Laurer's canal (LC) and the uterus (U). Shows phalloidin stain only. B. Intestinal caecum (Anterior on the right) stained with phalloidin. Note loose circular muscles (CM) and thicker longitudinal muscles (LM), and the body wall at the bottom of the image.

At higher magnification, the flame cells and the ducts connected to them could be seen (Figure 5.49). Finger-like structures projecting from the interior towards the tegument were observed only in specimens stained and viewed for DAPI (Figure 5.50A). A small excretory pore surrounded by radial and circular muscles was also seen at the posterior end of the body (Figure 5.50B).

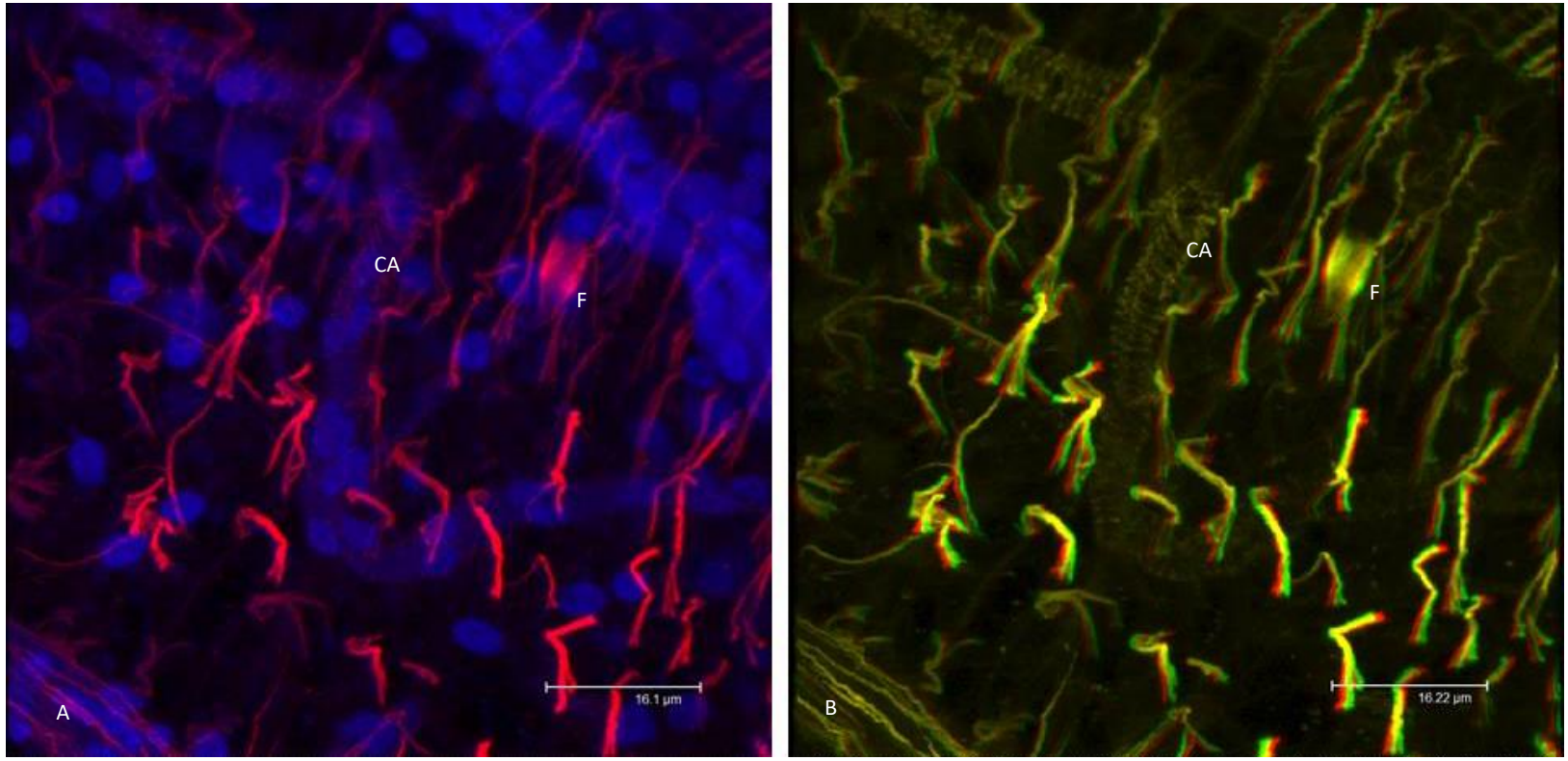


Figure 5.49. Newly excysted specimen of *Maritrema gratiosum* showing a flame cell (F) and the capillary (CA) connected to it. A. Specimen stained with phalloidin (red) and DAPI stain (blue). B. Shows phalloidin stain. Anaglyph 3D image.

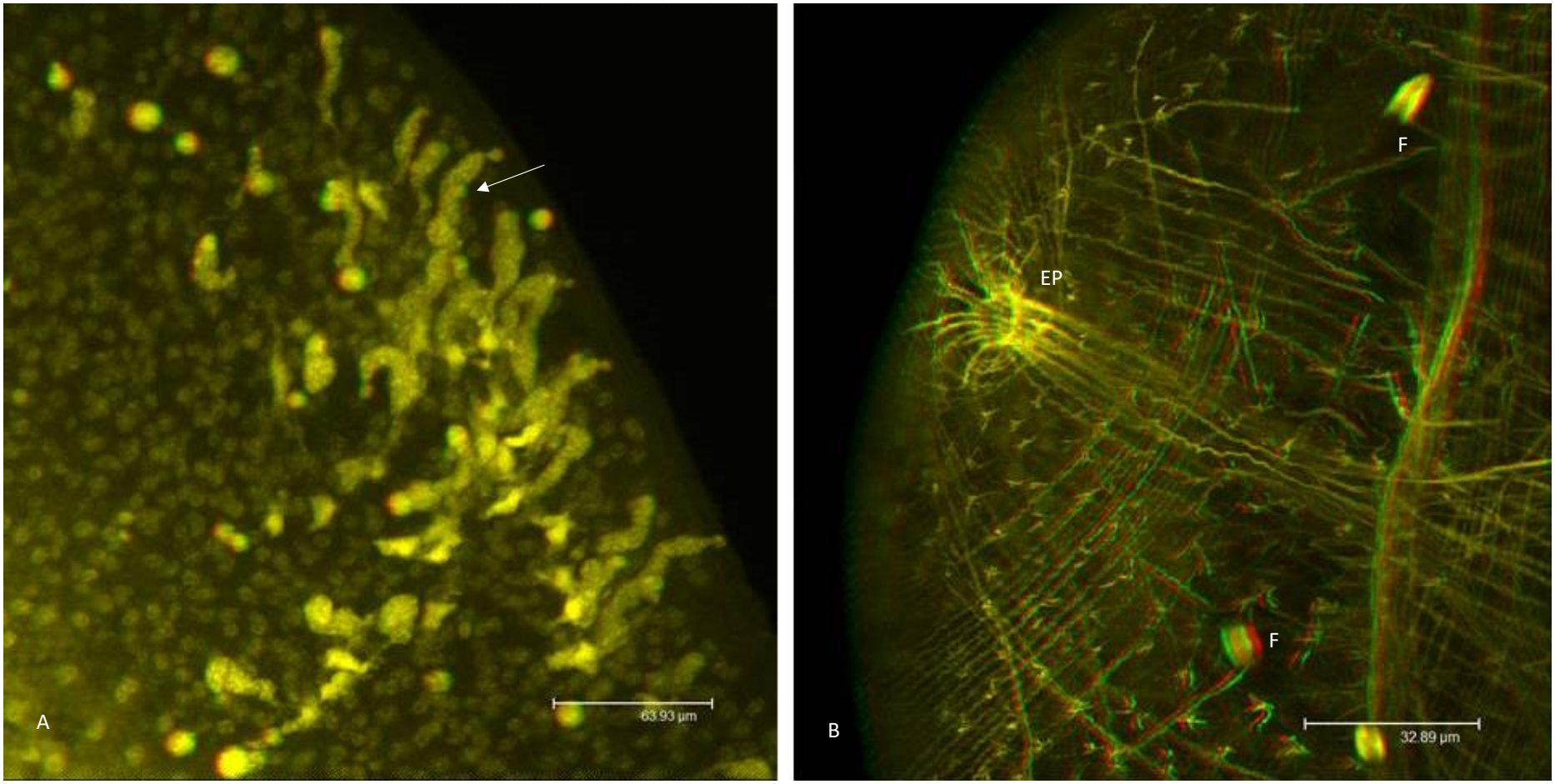


Figure 5.50. Tegumental structures and the excretory pore of *Maritrema gratiosum*. Anaglyph 3D image. A. *Maritrema gratiosum* cultured in NCTC-109 supplemented with 20 % chicken serum and antibiotics for 48 hours and then stained with DAPI stain only. Note the "finger-like" structures (arrow) near the surface of the worm. B. *M. gratiosum* cultured in NCTC-109 supplemented with 40 % chicken serum and antibiotics for 120 hours and stained with phalloidin. Note the excretory pore (EP) which is formed by radial and circular muscles; and three flame cells (F) close to it.

Later in the study, in 2018, and after applying an improved protocol for phalloidin staining, more anatomical structures were elucidated. Symmetrical extra-supportive muscles (at least 4 sets) were observed in the oral sucker in both newly excysted and 4-day-old flukes. These muscles were ventro-dorsally distributed (Figure 5.51). Surface circular and meridial muscles of the ventral sucker were clearly visible and a genital pore on the side was observed. A set of extra-supportive muscles were observed underneath the ventral sucker, but these were arranged as an irregular web (Figure 5.52).

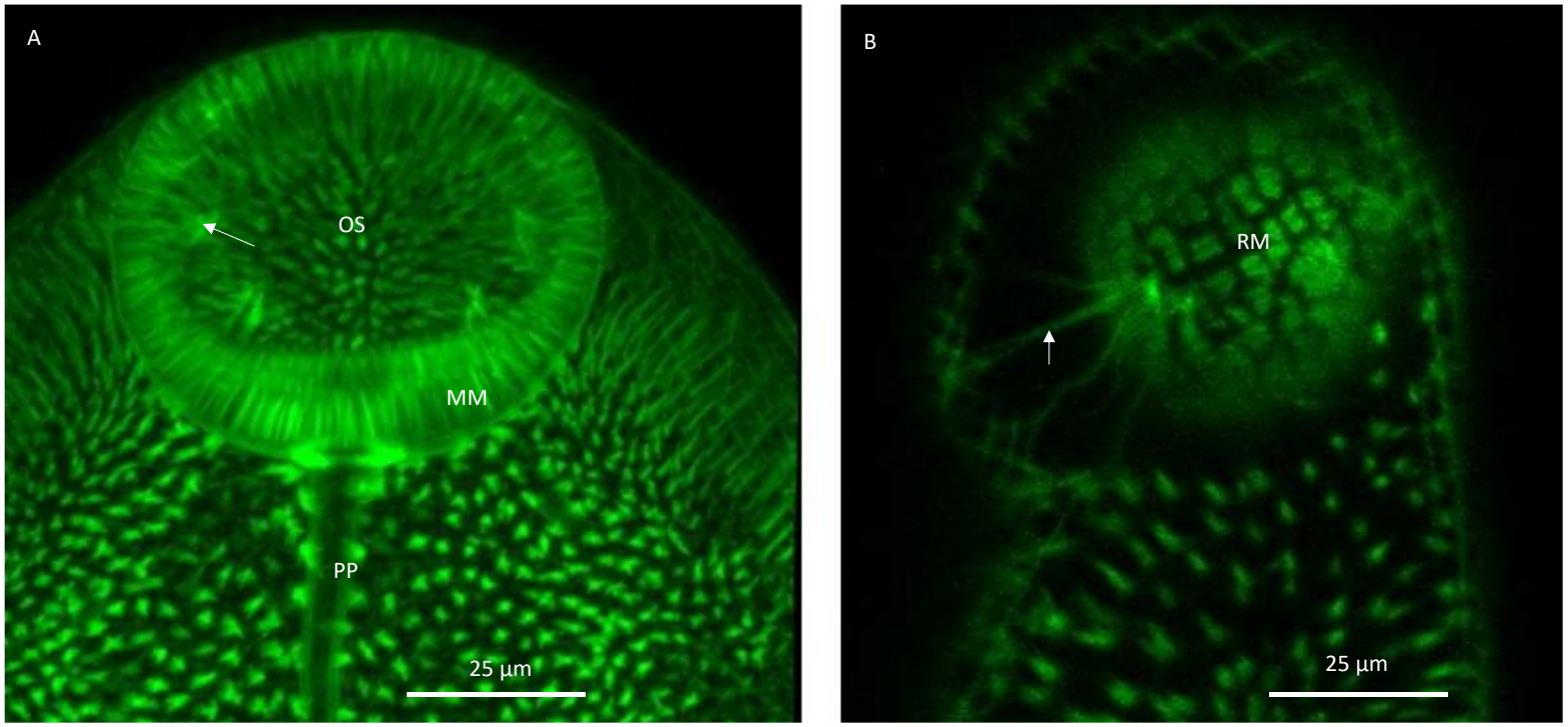


Figure 5.51. Newly excysted specimen of *Maritrema gratiosum* cultured in NCTC-109 supplemented with 20 % chicken serum and antibiotics for 96 hours. A. Specimen stained with x8 phalloidin. Extra supportive muscles (arrow) in oral sucker (OS) region can be seen at dorsal view. MM: meridial muscles. B. Specimen stained with x10 phalloidin. Extra supportive muscles (arrow) in oral sucker region can be seen at oblique section. RM: radial muscles.

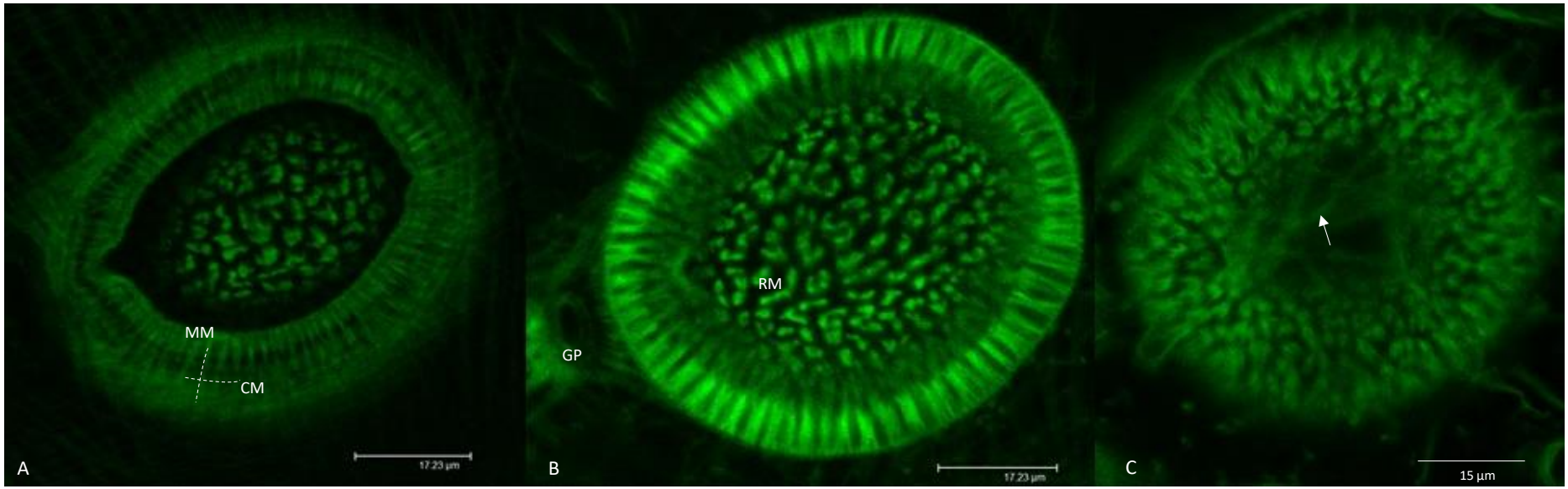


Figure 5.52. Different optical sectioning depths of the ventral sucker of a newly excysted specimen of *Maritrema gratiosum* that was subsequently stained with x8 phalloidin. A. The exterior-most circular (CM) and meridial muscles (MM). B. The radial muscles (RM) inside and the genital pore (GP) on the side. C. Extra supportive muscles (arrow) at the base of ventral sucker.

A normal configuration of the somatic muscles in the hind body were observed using samples which were not flattened (Figure 5.53). By observing the flattened and newly excysted samples, the configurations of the uterus loop, ootypic junction, and excretory bladder were elucidated. In contrast to the generally observed Y-shaped or V-shaped excretory bladder in the Microphallidae, a racket-shaped bladder with two branches at both sides was observed. The exact numbers of flame cells could not be determined; therefore, the flame cell formula cannot be confirmed. The arrangement of the uterus loop agreed with the description by Hardly and Castle (1940) (Figure 5.54).

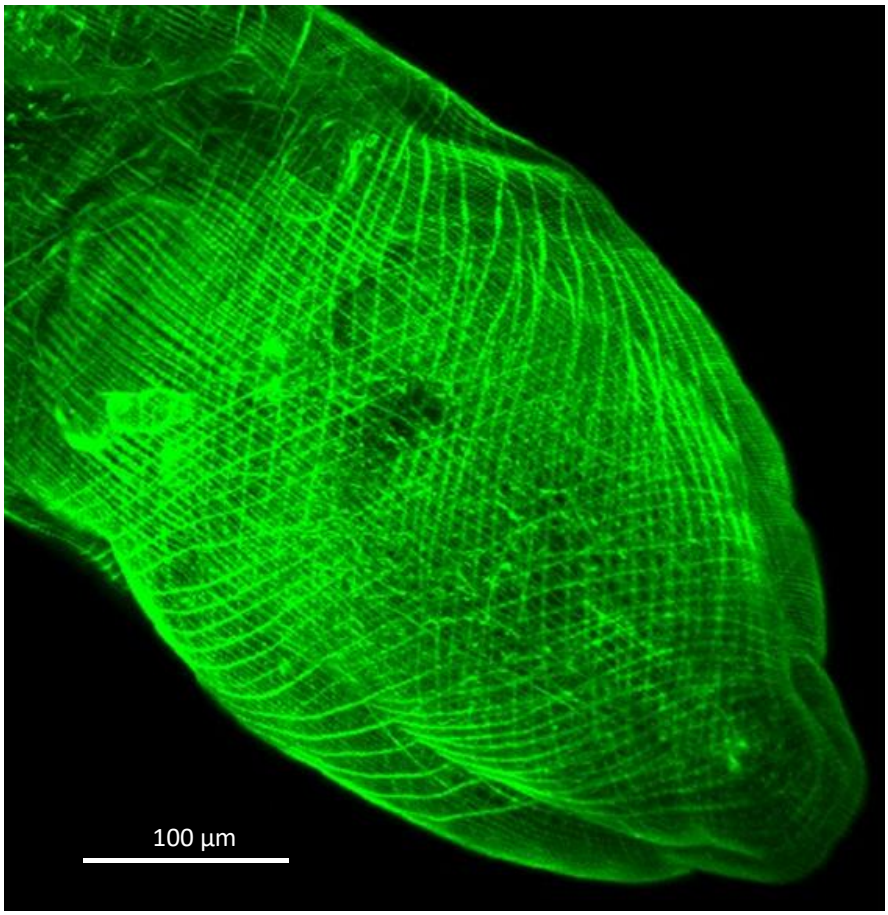


Figure 5.53. Hind body somatic muscles of *Maritrema gratiosum* cultured in NCTC-109 supplemented with 20% CS and ABS for 96 hour and stained with x10 phalloidin. Dorsal view. The sample was not flattened therefore the normal configuration of the fluke and two layers of the body wall could be observed at different focal depths.

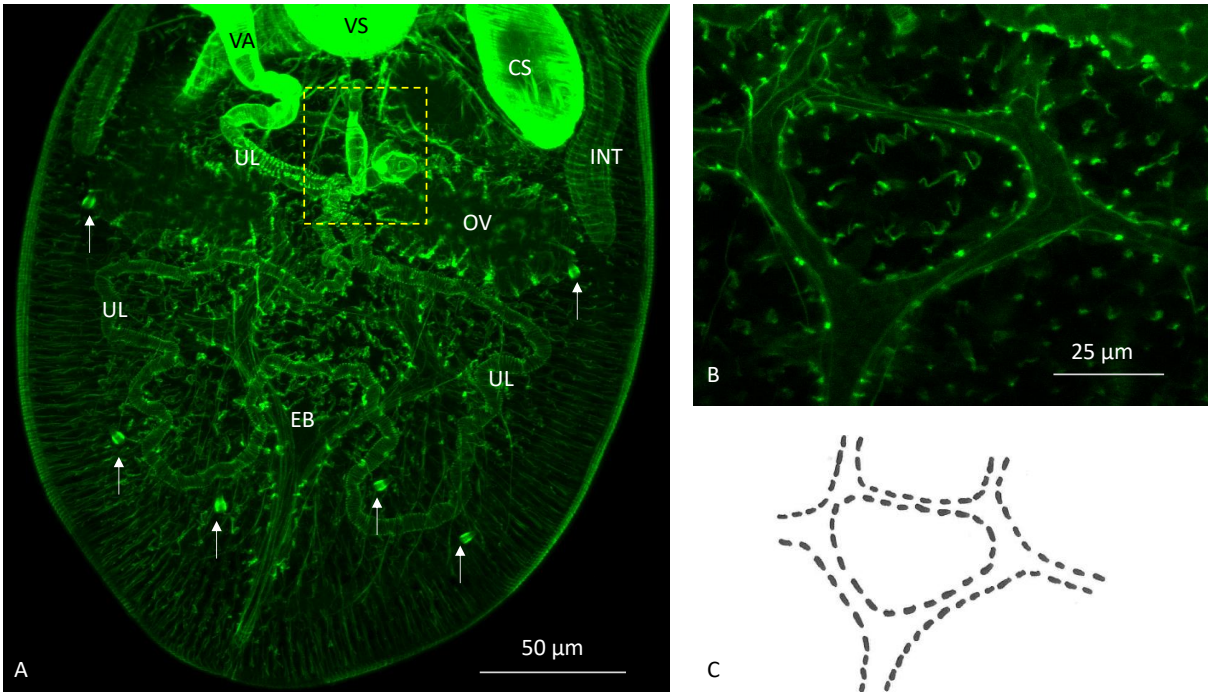
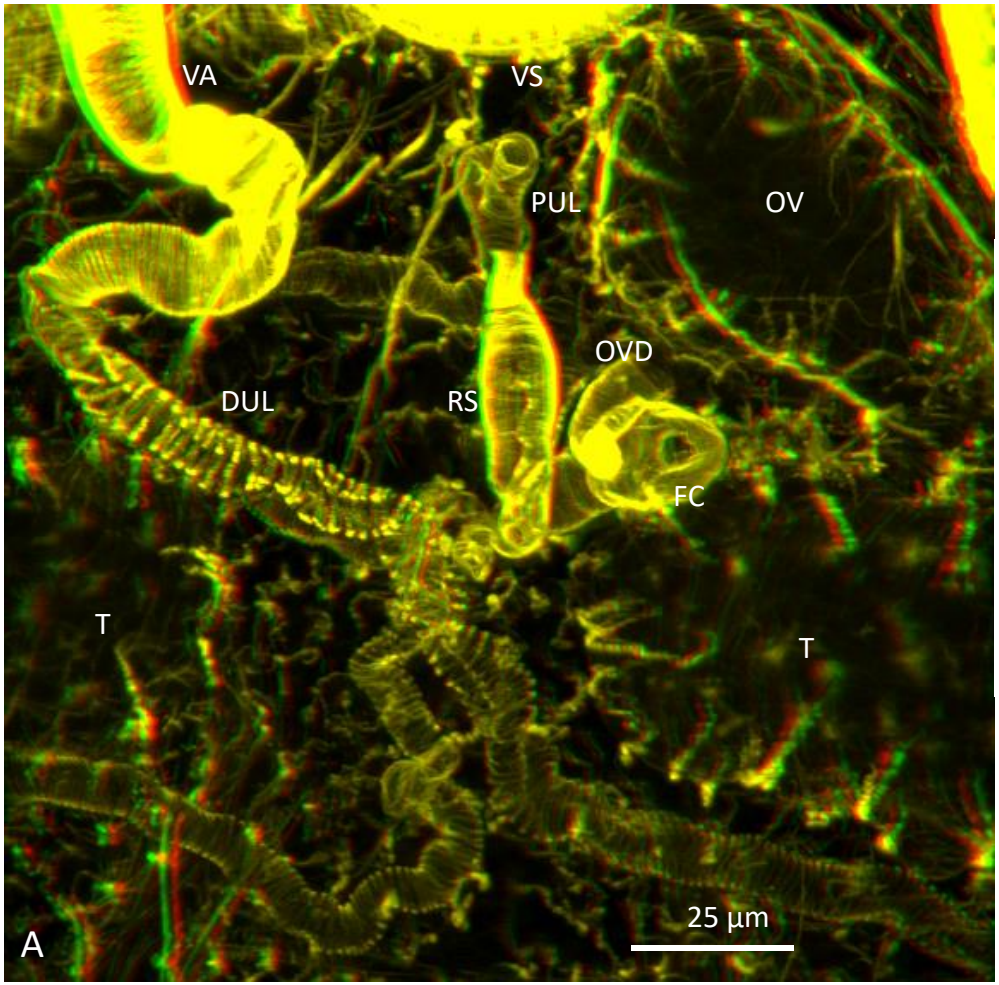
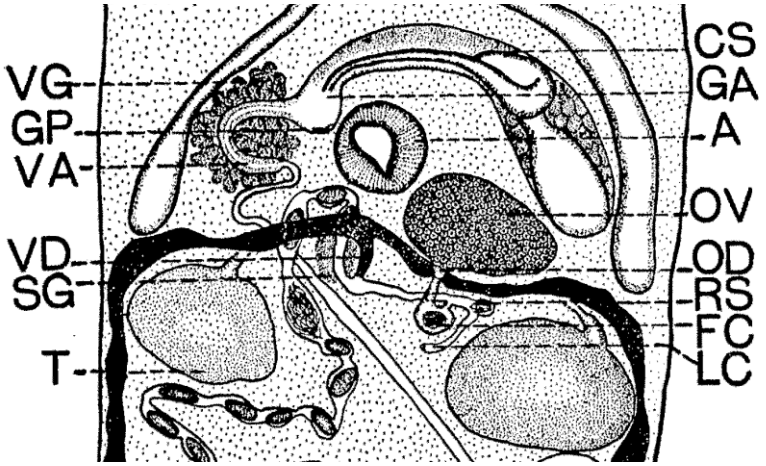


Figure 5.54. Newly excysted specimen of *Maritrema gratiosum* stained with x8 phalloidin. A. Internal organs of the hind body of a newly excysted *M. gratiosum* stained with x 8 phalloidin. Dorsal view. The cirrus sac (CS), excretory bladder (EB), intestine caecum (INT), ootypic junction (yellow square), ovary (OV), vagina (VA), ventral sucker (VS), uterus loops (UL) and six flame cells (arrows) were clearly visible. B. Excretory bladder. Note the four branches on the side. C. Schematic drawing of the racket-shaped excretory bladder with four branches on the side.

The ootypic junction (Figures 5.55-5.56) can be described as follows: after the ovary was a short oviduct, which joined a ventrally directed fertilising chamber. After the fertilising chamber was a transversely directed tube which emitted two tubes: on the ventral side the tube turns upwards and connects to the vitelline duct, before joining the vitellaria; on the dorsal side, the tube connects to the *receptaculum seminis*, which was seen as an upward directed tube-like chamber. The beginning of the *receptaculum seminis* also connected to a caudally directed thinner Laurer's canal, which was not readily seen. After the *receptaculum seminis*, the tube narrowed and then connected to the proximal uterus which then loops and bypasses the vitellaria and then turns downwards to join the main part of the uterus. In the observed specimens, the primal uterus loop almost overlaps the distal uterus loop, which was obliquely directed upwards to the left-hand side to join the vagina. The configuration of the ootypic junction was not the same as that provided in Plate 1 of Hadley and Castle (1940) but the constitutive elements were the same. The major difference is that the fertilisation chamber is to the right-hand side of the *receptaculum seminis*, instead of the left-hand side.





B

Figure 5.55. A. Ootypic junction of a newly excysted specimen of *Maritrema gratiosum* stained with x8 phalloidin. Dorsal view. Anaglyph 3D image. DUL: distal uterus loop, FC: fertilization chamber, OV: ovary, OVD: oviduct, PUL: proximal uterus loop, RS: *receptaculum seminis*, VA: vagina, VS: ventral sucker. B. The original drawing reproduced from Hadley and Castle (1940, figure 1 on plate I) was added to facilitate comparison. A: acetabulum, CS: cirrus sac, FC: fertilization chamber, GA: genital atrium, GP: genital pore, LC: Laurer's canal, OD: oviduct, OV: ovary, RS: *receptaculum seminis*, SG: shell gland, VA: vagina, VD: vitelline duct, VG: vaginal gland.

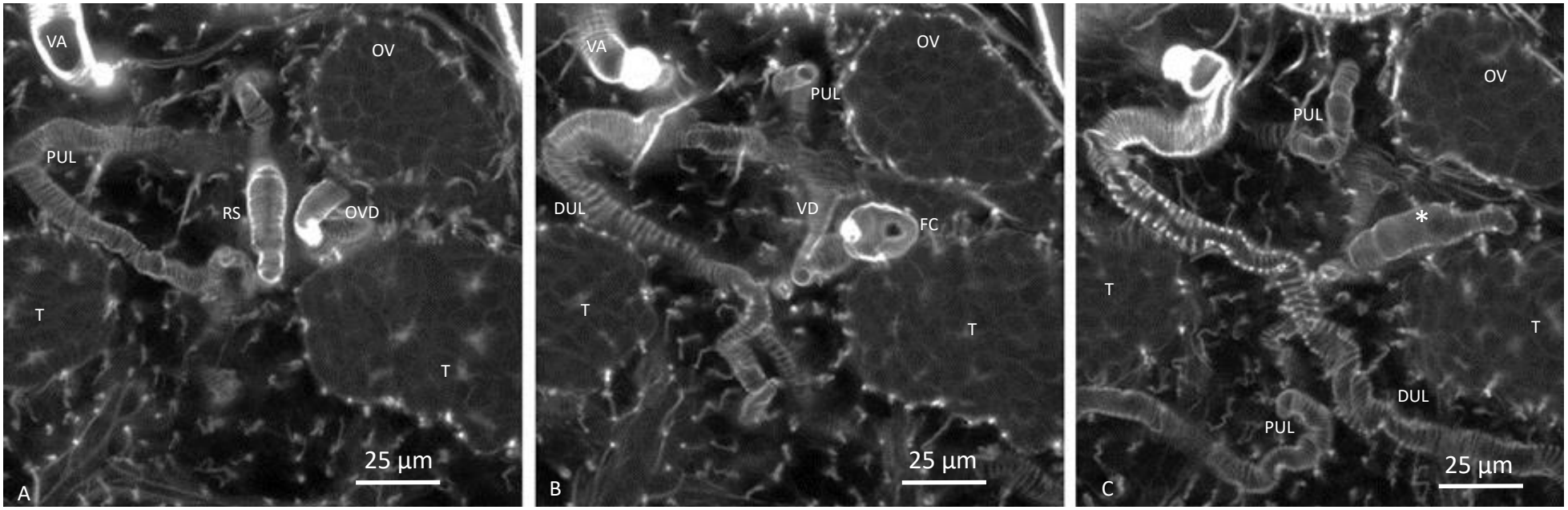


Figure 5.56. Ootypic junction of newly excysted *Maritrema gratiosum* stained with x8 phalloidin moving from the dorsal side (left) to the ventral side (right). A. Ovary (OV), oviduct (OVD), *receptaculum seminis* (RS), proximal uterus loop (PUL) testis (T) and vagina (VA) can be seen. B. Distal uterus loop (DUL), fertilisation chamber (FC), ovary (OV), proximal uterus loop (PUL), testis (T), vagina (VA) and vitelline duct (VD) can be seen. C. The duct (asterisk) connecting the fertilization chamber (FC, can only be seen in B) and *receptaculum seminis* (RS, can only be seen in A) can be seen. DUL: distal uterus loop. OV: ovary. PUL: proximal uterus loop. T: testis.

5.4. Discussion and conclusion

The development of long-term *in vitro* culture techniques for digeneans can reduce the need to use vertebrate animals for maintaining complex life-cycles and facilitate anthelmintic efficacy tests (Pung *et al.*, 2009). *In vitro* culture can provide stable sources of target life stages, which are important for *in vitro* drug screening and *in vivo* pharmacological studies. Recently, *in vitro* culture of *S. mansoni* has been applied to the efficacy test of single chemicals, such as piplartine, upon schistosomula (de Moraes *et al.*, 2012), to the development of medium-throughput phenotypic screening (Abdulla *et al.*, 2009) and, has been incorporated as part of a standardized and systematic approach to high-throughput and whole-organism drug screening (Tavares *et al.*, 2016). *In vitro* culture techniques have been applied to drug screening of liver flukes as well, such as for plumbagin targeting *F. hepatica* (Lorsuwannarat *et al.*, 2014), and for several plant extracts upon *F. gigantica* (Vera-Montenegro *et al.*, 2008). Flukes produced *in vitro* do not necessarily have the same morphology or biology as those produced *in vivo*, and mortality or phenotypic changes are often the major parameters to be evaluated. Techniques for assessing viability and mortality are, therefore, very important in anthelminthic study. The production of eggs and the viability of eggs obtained *in vitro* is another issue of interest when investigating the basic biology. In the present study, building upon earlier research, the effect of serum on *in vitro* survival and egg production of newly excysted metacercariae of *M. gratiosum* collected from *S. balanoides* was investigated. The light microscope-based study also provides more details on parasite morphology, while CLSM provided greater insight into the development of the musculature. The requirements for *in vitro* culture differ greatly between progenetic and non-progenetic species, and even vary between species within the same genus, the choice of culture media reflected those employed in the attempted culture of other microphallids (Fujino *et al.*, 1977; Davies & Smyth, 1979; Zaben, 1988; Fredensborg & Poulin, 2004; Pung *et al.*, 2009; Pung, *et al.*, 2011; West *et al.*, 2014). By including some of these media in the current work, the results were comparable to these former works. In previous studies regarding the *in vitro* culture of *M. gratiosum* and other microphallids (see Appendix 44), the goal of "optimizing culture conditions to obtain ovigerous adults containing normal eggs" was not fully achieved because the survival time was shorter and abnormal eggs were still present. In the present study, however, better-quality images during development were presented, early spermatogenesis was observed, self-fertilization was validated, and better statistical analysis were performed for survival (survival analysis), and performance indices, *i.e.*, body width, length, egg numbers, were included. More importantly, in the present study the *in vitro* culture techniques provide parasitic materials for musculature research by CLSM, and some novel and valuable information were obtained. Some important aspects, *e.g.*, excystment percentage, optimization of culture media, egg development, comparing anatomy, and functional biology for other microphallids, are discussed in the following sections.

5.4.1. Excystment percentage

The excystment of *M. gratiosum* in the present study was successfully achieved by incubating metacercariae in 0.01M PBS at 40°C, but it took 2 hours to achieve ~100% excystment. The temperature was chosen according to the body temperature of avian final hosts. A similar excystment proportion was found by using NCTC-109. Compared to the excystment experiment of *M. arenaria* (syn. *M. gratiosum*) conducted by Irwin (1983), the excystment speed was much slower in PBS used in the present study than in HBSS containing trypsin and bile salt applied by Irwin (1983). In HBSS excystment fluid, excystment can be achieved in 15 minutes with a success rate of 80%. The author, however, did not state whether subsequent excystment of the remaining 20% was possible or not. According to Zaben's results (1988), excystment speed was much slower in 40% (v/v) sea water compared to that seen in the present study, where by 12 hours 86.7% excystment had been achieved. Zaben also mentioned that although Irwin's (1982) solution was highly efficient for excystment, *i.e.,* in 2 hours 87.5% excystment rate, the excysted metacercariae were inactive and showed high mortality. In *M. novaezealandense*, most of the metacercariae excysted within 4 hours in both 0.85% NaCl solution or in NCTC-109 with 20% or 40% serum. The treatment group of 0.85% NaCl, however, had a lower excystment rate when compared to the other two groups (Fredensborg & Poulin, 2005). In *Microphallus japonicus* Osborn, 1919, metacercariae in 0.85% NaCl excysted more rapidly than those in Krebs-Ringer's solution, and the speed increased with temperature (33-41 °C). More than 80% of the metacercariae can be excysted within 1 hour at 37 °C (Fujino *et al.*, 1977).

In the present study, the ease of excystment of *M. gratiosum* suggested that excystment is an intrinsic process and can be triggered merely by elevating temperature. Excystment is also easy for *Microphallus opacus* (Ward, 1894) Ward, 1901, in which metacercariae can be excysted in 0.6% saline, amphibian Ringer's solution, distilled water or conditioned tap water at room temperature (Caveny & Etges, 1971). Irwin (1983) studied structural changes in the of metacercarial cysts of *M. arenaria* (syn. *M. gratiosum)* during excystment. By TEM, he found that the cyst wall of this species is comprised of four layers of heterogeneous materials, and the structures of these four layers changed after excystment. He found that in the metacercariae forced out of the cyst, the same structural changes of the inner two layers of cyst wall did not occur. He also noticed more acid phosphatase activity in the glycocalyx of the tegument of the metacercariae before excystment but less acid phosphatase after excystment. The author suggested that excystment of *M. gratiosum* was accomplished by initial activity of the metacercaria and followed by softening of the cyst wall. Enzymes secreted by the metacercariae might contribute to the softening of the cyst wall while chemicals in the excystment fluid also facilitate excystment by acting on the outer cyst wall. Compared to the relative ease of excystment in *M. gratiosum*, excystment of *M. abortivus* was achieved by a complex excystment fluid. A combination of two freshly prepared fluids was needed: solution A comprised of bicarbonate saline

solution containing sodium taurocholate and trypsin, and solution B comprised 0.02 M HCL containing L-cysteine. The two solutions were combined before use (Irwin *et al.*, 1984). In this recipe, a 95% excystment rate was achieved within 15 minutes at 40°C (Saville & Irwin, 1991). The relative ease of excystment means the flukes can explore any homoeothermic animals they encounter. This might be one of the factors contributing to low host specificity of some microphallid species such as *M. gratiosum*.

5.4.2. The choice of culture medium and optimization of culture conditions

The choice of NCTC-109 was based on the success of Fredensborg and Poulin (2005), who cultured another *Maritrema* species (*M. novaezealandense*) *in vitro* to obtain ovigerous adults. The media applied by Zaben (1988), *e.g.* medium 199 or NCTC 135, was not tested here due to lack of knowledge of the existence of this reference at the time the experiment was conducted, however, the effect of nutrient supplements such as foetal bovine serum and chicken serum were similarly tested. NCTC-109 was originally developed by the National Cancer Institute and formulated for the culture of mouse Strain L sub-lines, NCTC clone 929 (L929 cells) in a serum-free environment. This medium is suitable for generating and maintaining hybridoma cells (Catalogue of NCTC-109, Thermal Fisher Science). NCTC 135 was a medium modified from NCTC 109 and, also developed for L929 cells, but with L-cysteine hydrochloride-H_2O removed, which reduced the toxicity to some cells (Catalogue of NCTC-135, Thermal Fisher Science). NCTC-109 has been applied to the *in vitro* culture of other microphallids (see Appendix 44) and, also proved to be successful for *M. gratiosum* in the present study. In the preliminary experiments, flukes cultured in EMEM supplemented with FBS, L-glutamine and NEAA showed abnormalities in the excretory bladder, testes, and intestinal caecum. All flukes were dead at 3 days, probably due to bacterial contamination, but eggs were observed. Flukes cultured in PBS survived until the 3rd day as well, but abnormalities were noticed in the testes and no eggs were found. Thereafter, NCTC-109 was tested either alone or supplemented with different concentrations of antibiotics and different types of serum. It was found that 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin were needed to prevent contamination, although strict aseptic operation was not needed. In the present study, with this concentration of antibiotics, flukes cultured in NCTC-109 supplemented with 20% FBS maintained >80% survival until 186 hours, and those cultured in NCTC-109 supplemented with 20% CS maintained >60% survival until 192 hours. These data were comparable with Zaben's (1988) results. In his treatment group of "NCTC 130 plus 10% FBS in 38 ± 1 °C", the cumulative mortality at 7-8 days was 40-51% and 100% mortality was reached at the 13th day. In the present study, due to the constraint of time and resources, the experiment was ended on the 10th day. It is possible, however, that the survival time could reach or exceed 13 days.

In both the FBS and CS treatment groups, body length first increased then decreased, body width was consistent, and egg counts peaked at 93 or 70 hours for the FBS group and at 48 hours for the CS group. Although FBS seems to work as well as CS, chicken serum was the final choice of food source given that the final hosts of *M. gratiosum* are avian. In early studies of the physiology of *F. hepatica*, simple saline solutions with no food source were tested and it was proved that flukes cultured in solutions containing food lived longer. This proved the importance of an energy supply for digeneans cultured *in vitro*. In this case, however, the food sources were monosaccharides such as glucose and fructose (Stephenson, 1947). In *M. similis*, several nutrients other than serum were tested such as embryo extract, yeast extract, egg macerate, RBC, and BHK monolayers (Davies & Smyth, 1979). It was found that these supplements were not necessarily beneficial, and some were even inhibitory (*e.g.* mouse and chicken embryo extract, yolk medium + foetal calf serum). While supplements for tissue culture such as chicken plasma, house serum, and beef embryo extract had negative effects on *F. hepatica* (George & Rohrbacher, 1957). Survival was sometimes higher in simple media, for example, *G. adunca* survived longer in HBSS than in RPMI-1640 (West *et al.*, 2014). Some microphallids, such as *M. opacus*, do not require stringent culture conditions. Metacercariae of *M. opacus* can be cultured in conditioned tap water, 0.6% saline or amphibian Ringer's at room temperature for 5 days or at 10°C for 2 weeks to ovigerous adults (Caveny & Etges, 1971). For the choice of culture medium, it seems that no general patterns can be followed for *in vitro* culture of Digenea, but factors such as osmotic pressure, nutrients, aerobic condition, contamination, temperature, pH, hazardous materials, among others, all contribute to the success of culture. Although considerations for the culture of non-progenetic and progenetic species differ markedly, the methodology or findings in non-progenetic species still provide insightful information. Successful culture conditions often need to be obtained in a systematic trial-and-error manner during the early stages. Established conditions for closely related species, however, often provide valuable and useful information.

To compare survival between experiments was not an easy task as no standard methods were applied between research studies. In the literature cited, Zaben (1988) used cumulative mortality, but the exact method of calculation was not provided. Two researchers provided survival number at each time point (George & Rohrbacher Jr., 1957; Fujino *et al.*, 1977), one presented survival time of 50% survival by interpolation (Stephenson, 1947), two presented a survival percentage at the time culture terminated (Pung *et al.*, 2009, 2011), one showed the days of highest survival (Fredensborg & Poulin, 2005), and one showed the longest survival time (30 days in *M. similis*, Davies & Smyth, 1979).

In the present study, the survival was analysed by bar chart and survival analysis. Bar charts gave an easy and general profile for the survival percentage at each time point compared to Time 0. A general trend can be seen and rough comparisons between groups can be achieved. In a contrast to

this, survival analysis is a statistical method, which will show cumulative survival probability in a defined study interval as a curve and makes statistical comparison between different curves (treatment groups) possible. Survival analysis was originally designed for dealing with incomplete observations and has become a method for dealing with data with different survival times (times-to-event) (Rich *et al.*, 2010). Survival analysis is often applied to medical research but can be applied to other fields as well. In the parasitology fields, it has been applied to the efficacy of diagnostic assays for *Plasmodium falciparum* Welch, 1897 (see Phuong *et al.*, 2015), to access the effect of different medicines on the survival of human malarial patients (Allen *et al.*, 2009), to evaluate the effect of *Theileria parva* (Theiler, 1904) Bettencourt, Franca & Borges, 1907 and *Haemonchus* sp. co-infection on the survival of cattle (Thumbi *et al.*, 2014), and to the *in vitro* efficacy testing of drugs against *S. mansoni* (see Almeida *et al.*, 2015). Due to the conceptual similarity, this method was chosen in the present study. For example, some flukes die at certain time point (events) while other flukes "leave the test" due to the need for body measurement, egg counts or fluorescent staining (censored cases). In most *in vitro* culture experiments in the present study, the survival rate decreased slowly, and the cumulative probability of survival was still ≥ 50 % on day 10. This revealed a reasonable high survival probability under the current protocol. Although the slope of the survival curve is not steep, whether the survival probability will drop dramatically after 10 days is not known unless the interval of the experiment can be extended. There are some problems in the present experimental design with respect to the application of survival analysis. The first is the high number of censored data. Ideally it would be better to separate the survival experiment and growth assessment into two sets of experiments. In such a way, the high percentage of censored cases can be avoided. Due to the limitations of time and resources, this was not achieved. High numbers of censored subjects in clinical medical research is a warning sign concerning the target treatment regime (*e.g.*, high numbers of patients leave the trial). Increasing the percentage of censored cases over time also makes the survival curve less accurate when approaching the end (Rich *et al.*, 2010). The second problem is that in the present study there was sub-structuring in the target populations. Although the unit for calculating interval survival is "per fluke", the flukes were cultured five in a tube. There might be subtle differences in each tube as each tube might form a unique microhabitat. This problem is unavoidable using the present experimental design which was tailored according to the need to obtain gravid flukes. In the survival study of *F. hepatica*, only one fluke was placed in a bottle containing 50 mL of culture fluid and it represents the unit at each treatment group (George & Rohrbacher, 1957). Similarly, in the drug efficacy study of *S. mansoni*, one fluke was placed in a well in a 24-well culture plate and it was the unit for survival analysis (Almeida *et al.*, 2015). Although in the present study, the design of each subunit could potentially contribute to bias, in clinical medical studies, data collected from each clinic

also has its own local characters. This confounding effect could be diluted by larger sample sizes. In the present study, the survival probability from these subunits can only be calculated as pooled data.

Regarding survival time, the maximum life span of *M. gratiosum* in the final host in nature is not known, although in Zaben's (1988) work a maximum survival time of 24 days was achieved in medium 199 plus 10 % FBS. It is possible that the adults cultured in the present study could live longer if the culture condition could be further optimized. The final hosts of *M. gratiosum* are largely migratory waders (Deblock, 2008) who only stay in the intertidal zone (where transmission can be completed) for a short time. It is speculated that the adult flukes release their eggs in these birds during their stay throughout the summer to complete their life cycle, thereafter, the flukes die. Another run of transmission starts when these birds arrive again next year. It is also plausible that these flukes live in the intestine of their final hosts for a longer period and can release eggs when conditions are optimal. More evidence is needed to clarify what happens. It is possible for microphallids to achieve long lifespans under *in vitro* culture. For example, *M. similis* can survive for up to 30 days in culture media containing serum medium (Davies & Smyth, 1979) and *M. opacus* can survive for 4 weeks at room temperature *in vitro* and for 5 weeks at 10°C but only for 1 day at 37°C (Caveny & Etges, 1971). Low temperature (to 18 °C) seemed to have little effect on the survival of *F. hepatica* while heat stress (>40 °C) drastically decreased survival (Stephenson, 1947).

In terms of survival, the two groups in the present study, supplemented with 20% and 40% CS respectively, were not statistically different. The 40% CS group, however, could be potentially superior to the 20% CS group, as revealed by the bar chart of survival (Figure 5.41) and the mean survival time by survival analysis (Table 5.6). Also, the largest (specifically longer) flukes, were found in the 40% chicken serum group at two time points. It is not, however, known if this subtle difference resulted from different concentrations of chicken serum, or different concentration of antibiotics as the concentration of antibiotics in the 40% chicken group was also twice than that in 20% CS group, which was a clear flaw in the experimental design. Interestingly, in Zaben's (1988) work, better results, in terms of longevity and egg production, seemed to come from the FBS treatment group but not the CS group cultured in medium 199. The positive effect of chicken serum for egg production had been validated in *M. novaezealandense*, but no significant difference could be detected between the 20% and 40% serum groups with respect to egg numbers, egg volume and fluke size (Fredensborg & Poulin, 2005). The effect of antibiotics on *in vitro* culture of Digenea is rarely studied although antibiotics have been routinely added. In an early study of the physiology of *F. hepatica*, borax was added to simple saline as a bactericide. Other bactericides, bacteriostatics and anthelminthics have been explored, with various effects upon survival. This early work demonstrated the importance of controlling bacterial contamination in the *in vitro* culture of *F. hepatica*. Later, a "bacteria-free" protocol was developed for

F. hepatica and 0.25 mg mL^{-1} aureomycin, 100 U mL^{-1} penicillin or 2.5 mg mL^{-1} streptomycin were applied with beneficial effects for survival (George & Rohrbacher Jr., 1957). There was one report about the effect of the antimycotic agent amphotericin B on larval stages of *S. mansoni*. It was found that amphotericin B reduced the infectivity of miracidia but not their development, growth, viability, and behaviour of the miracidia and primary sporocysts (Moné *et al.*, 2010). In a study of factors affecting the maintenance of *F. hepatica in vitro*, the effect of antibiotics was studied as one of the factors. An effect on egg production but not survival was observed when more than 100 U mL^{-1} penicillin was added. On the other hand, 130 µg mL^{-1} of tetracycline, streptomycin and kanamycin had no effect on egg production. The authors suspected that penicillin inhibited eggshell formation by binding to the lipoprotein secretion of the Mehlis' gland (Ractliffe *et al.*, 1969). In the present study, the effect of antibiotics on the survival and egg production of *M. gratiosum* was not established, but the amount of antibiotic that was applied is like that used in other studies on microphallids, hence the concentration used should be safe for survival under the current protocol. The effect of antibiotics would be an interesting subject to be studied in the future and should be explored by changing one variable at a time.

Another factor important for the assessment of survival is the method for assessing viability. The simplest way to judge whether a fluke is "alive" is by observing movement. In the early work of Stephenson (1947), vitality was first assessed through tissue disintegration, but this method was discarded by the author and movement became the criterion chosen. Dead flukes in the current study typically showed the appearance of an opaque tegument. For movement observation, it was noticed that sometimes after a static period in the culture medium, the fluke stops moving but contraction recovered after gentle touching the fluke or shaking the media. In the *in vitro* culture study of *M. turgidus*, the death of flukes was defined as a lack of movement for 30 seconds. In the opinion of Stephenson (1947), only spontaneous movement indicated a good health status of the fluke, and those with movement which needed to be triggered by vigorous touching were dying. This might also be true for *M. gratiosum,* however, such flukes are still considered viable using the current definition. The viability of flukes is often the major indicator for drug screening. Although microscopic observation of cellular granularity and changes in shape and movement of parasites is the gold standard for drug screening, more sophisticated and precise methods have been developed. The viability can be monitored by video, impedance, enzymatic activity, by colorimety and fluorimetry methods (Tavares *et al.*, 2016). Examples of fluorimetric methods include the quantification of lactate excreted by the parasites, and propidium iodide / fluorescein diacetate staining (Tavares *et al.*, 2016). The motility test is criticized in terms of objectivity and an impedance-based method called xCELLigence worm real-time motility assay (xWORM) that was developed for *S. mansoni*. This system monitored the change in conductivity in the culture medium by an electrode which reflected the movement of the flukes

(Rinaldi *et al.*, 2015). The survival of flukes in the culture medium does not mean that they are normal. Some abnormality had been noticed via the light microscope when culturing in PBS or EMEM, but at the ultrastructural level we do not know whether abnormality occurred or not. Abnormal development has been proven by TEM in *M. similis* under culture conditions (Davies, 1980). Most abnormalities that were seen occurred in secretory cells such as tegumental cells, forebody glands cells, vitellaria and gastrodermis. The premature release of granular material had been noticed in forebody gland cells and large masses of finely granular material accumulated in the secretory ducts. In the vitellaria of cultured adults, ragged vitelline droplets and sometimes large irregular masses of electron dense material were noticed in vitelline cells. These materials were sometimes observed to be associated with the ovum in the uterus, which were only covered by a thin layer of material but not eggshells. These findings support the premature tanning theory suggested by Davis and Smyth (1979). The abnormal tegument and gastrodermis suggest that the normal absorption function cannot be maintained at these surfaces, and therefore that flukes maintained under culture conditions are likely to be suffering malnutrition, cannot produce normal eggs and might have a shorter lifespan.

In the present study, the body length of the fluke seemed to increase at the beginning then decrease afterward, while body width was quite consistent. Fredensborg and Poulin (2004) pointed out that *M. novaezealandense* cultured in media supplemented with serum attained a larger size than those in NCTC-109 alone. They also found that flukes cultured for 5 days started to "contract" and became smaller. The same trend was noticed in the present study. Furthermore, the body size of the adults cultured *in vitro* in the present study is like that obtained from the wild, if comparing the measurements made under a coverslip (Hadley & Castle, 1940). Comparing the data of the fifth day, adult flukes in the present study, had body lengths ranging from 677.46 - 1118.87µm, mean 856.97; and body width ranging from 232.56 - 381.22 µm, mean 305.19. The adult worms collected in the U.S. from the wild by Hadley and Castle had a body size of 0.858 mm length x 0.380 mm width. This similarity of the size suggests that in the current protocol, *M. gratiosum* can grow to a body size like those in the wild, albeit from a different locality / environment.

5.4.3. Egg development

Egg production is very important for the survival and transmission of digeneans, and it is the most important biological process at the adult stage for precocious species such as *M. gratiosum*. In the *in vitro* culture studies, egg production is often considered a better indicator than survival (Fugino *et al.*, 1977; Davies & Smyth, 1979; Kook *et al.*, 1997; Pung *et al.*, 2009, 2011), as the purpose of *in vitro* culture is to sustain the normal biology of healthy flukes. In the present study, egg production started from 24 hours *in vitro*, peaked at the third to fourth day for the two treatment groups respectively and then decreased afterwards. These results were comparable to Zaben's (1988) work, in which, egg

production was considerably reduced after a short time (the 3rd to 5th day) and ceased completely after the 7th to 12th day. What was different from the present study was that in Zaben's work, all the eggs were shed through the genital pore, which did not happen in the present study. Also, the egg numbers obtained by Zaben were less than in the present study. In the present study, the maximum mean egg number (*i.e.*, mean 156 eggs from 9 flukes) was achieved in NCTC 109 plus 20% FBS group at 120 hours, while in Zaben's work the maximum (average of 54.94 eggs from 16 flukes) was achieved in medium 199 plus 10% FBS on the 3rd day. More importantly, he mentioned that the eggs cultured *in vitro* were much lower than *in vivo* (mice), in which 382.2 eggs were recorded on the 6th day with very few abnormal eggs. Given that in the present study, the same culture media was used as that used for the *in vitro* culture of *M. novaezealandense* (see Fredensborg & Poulin, 2004), their results are also comparable. Mean numbers of eggs in the uterus of *M. novaezealandense* during the 5-day development *in vitro* were like those of the present study. Mean egg numbers peaked at 48 hours and decreased afterwards. No differences in egg numbers and egg volumes were found between the two treatment groups of NCTC-109 plus 20% or 40 % chicken serum, but the group maintained in NCTC-109 alone had significantly less egg numbers than the other two groups at 24 and 48 hours. Their results indicated the beneficial effect of serum for egg production. In the present study, similar patterns of egg production were observed. Among the two groups of NCTC-109 supplemented with 20 and 40 % chicken serum, egg numbers were not significantly different at any time point either, this suggested that the concentration of chicken serum at this level did not affect mean egg numbers per fluke. The peak of production, however, was delayed until the 5th day in the 40% group compared to the 2nd day in the 20% group. The prolonged production of eggs (not necessarily more eggs) was observed in the 40% chicken serum group. In the *in vitro* development of *M. japonicas*, eggs were also noticed at 24 hours, and the numbers increased from the 1st day to the 5th day in various media without decreasing. In medium NCTC-109 plus serum, the mean egg number peaked at the 5th day (mean = 45.2) but better egg production was achieved in EMEM plus serum (mean = 94.4 at 5th day) (Fujino *et al.*, 1977). The beneficial effect of animal sera was also shown in *M. turgidus* as well. Flukes produced significantly more eggs in media supplemented with 20% of either calf or horse serum in RPMI-1640 than those with chicken serum or no serum; and higher concentrations of horse serum (up to 40%) worked even better than those with lower concentrations or no serum (Pung *et al.*, 2009). It is too early to conclude that serum provides the essential nutrients for egg production. Where data for *in vivo* production is available, the number and onset of egg production were all better *in vivo* than *in vitro* (*e.g.*, *M. japonicus* in Fujino *et al.*, 1977; *M. similis* in Davies & Smyth, 1979; *M. gratiosum* in Zaben, 1988). Hence serum may be considered as a factor to improve the general health of microphallids and the increase in egg numbers or rather the number of normal eggs reflect a better or more normal physiology under the chosen protocols.

Normal and abnormal eggs were both observed in the present study. A similar observation was made in Zabens' work, in which up to 50% of the eggs observed were normal eggs. Unfortunately, in his work a clear photo of the eggs was not provided, only whole worms containing eggs. The "normal egg" identified and described by him to be "shelled eggs contain an ovum surrounded by vitelline globules", and an operculum also existed. The size and shape of the eggs observed by him was like those of the present study, except that in the present study no operculum was observed. In the present study, it seems that the media NCTC 109 supplemented with 20 or 40 % chicken serum and antibiotics can support the formation of eggs containing an ovum and vitelline cells / globules and can also support embryogenesis in the uterus. In the *in vitro* culture of *M. novaezealandense*, a "normal" egg was defined as having an operculum, ovum, and vitelline cells. Both normal and abnormal eggs were observed in this species (Fredensborg & Poulin, 2004). In the *in vitro* development of *M. japonicus*, abnormal eggs with incomplete or no shells were noticed (Fujino *et al.*, 1977). In the present study, the eggs were found to have a certain degree of similarity to the *in utero* eggs of *M. similis* cultured *in vivo* or *in vitro* (Stunkard, 1957; Davies & Smyth, 1979). According to Stunkard (1957), in a young adult *M. similis* obtained from a mouse after 24 hours post infection, eggs were found in the uterus. The eggs were operculated and contained an ovum. The eggs in the proximal uterus had a thin eggshell and were more transparent, while those further down the uterus were thicker, harder, bright yellow eggs. Similar distributions and colourations of eggs were noticed in the present study as well. What is different from the present study is that the *in utero* eggs obtained from *M. similis in vivo* can develop to a stage containing a miracidium. The miracidia of *M. similis* only hatched when ingested by a mollusc host. According to Davies and Smyth (1979), the "normal eggs" *in utero* of *M. similis* cultured *in vitro* were operculated, oval, thin-shelled, and with an ovum occupying 2 / 3 of the egg, and with yolk granules at one pole of the egg. Eggs containing vitelline globules at one or both ends and an ovum in the middle were also noted in the present study. The presence of an operculum, however, cannot be confirmed. Currently, it is not known whether an operculum exists in the wild type of *M. gratiosum* or not because in existing descriptions of *M. gratiosum* the presence of an operculum on the eggs were not mentioned (Nicoll, 1907; Rankin, 1939; Hadley & Castle, 1940; Deblock & Tran Van Ky, 1966a). If the operculum does exist in the wild type of *M. gratiosum*, the results would have to reflect this and state that no "normal" eggs were obtained in the present study. A SEM or TEM study targeting the eggs of *M. gratiosum* might be able to elucidate the presence of operculum or not in the future. It is interesting to find that the "abnormal" eggs found in *M. similis* developed *in vitro* also had some similarity with those in *M. gratiosum*. According to Davies and Smyth (1979), three types of abnormal eggs were found. The first type consisted of tanned masses of vitelline cells with or without an ovum but without a shell; this type was also found in the present study. In *M. gratiosum* this type of egg always appeared at a later stage of culture (~5 days) and were seen accumulating in the proximal

uterus. Some flukes only had this type of abnormal egg. This agrees with the suggestion of Davies and Smyth (1979) that normal eggs only form at the early stage (1~2 days) of culture. These authors suggested that the formation of abnormal eggs results from the premature tanning of the vitelline droplets and is probably caused by uncontrolled tanning due to excess oxygen. The second abnormal type of eggs are shelled eggs containing vitelline cells but no ovum, and these are probably present in *M. gratiosum* as well. It is not sure if the "dividing" embryos observed in the present study were embryos or embryos plus vitelline globules or vitelline globules only, as the morphology of these cells all looked like, *i.e.*, "a ball of cells". It is not clear, therefore, if the egg morphologies seen in the present study were the same as the second type of abnormal eggs described by Davies and Smyth (1979). The authors did, however, observe the original ovum to form "a ball of cells" in the fluke developed in a mouse but not *in vitro*. It seems likely that some eggs in the present study had achieved a dividing stage as the numbers of cells within the eggs seemed to increase with time. This could probably be supported by the morphological similarity of embryonated eggs deposited by *M. turgidus* in culture medium. These eggs contained clumps of cells, and they were able to develop and subsequently release miracidia which were capable of infecting snails (Pung *et al.*, 2009). In *M. opacus* cultured *in vitro* for 5 days at room temperature or 10 days at 10°C, eggs with 32 cell embryos were observed (Caveny & Etges, 1971).

The accumulation of abnormal eggs in the proximal uterus in flukes at the later stages of *in vitro* culture suggested that the culture conditions applied in the present study could not support the persistent development of normal eggs. The formation of normal eggs requires the early functioning of the vitellaria. The presence of eggshell precursors in the vitellaria was detected in *M. gratiosum* cultured in mice using both catechol and Fast Red 12 hours post infection (Zaben, 1988). The presence of eggshell precursors was detected at day one in *M. similis* using Fast Red Salt B staining, which highlights the presence of alkaline phosphatase (Davies & Smyth, 1979). It would be worthwhile, therefore, to test for the early existence and composition of eggshell precursors in *M. gratiosum* cultured under *in vitro* conditions in the future. No oviposition was observed in the present study. Oviposition under culture condition was, however, achieved in *M. similis* (see Davies & Smyth, 1979) and *M. turgidus* (Pung *et al.*, 2009). The factors lacking, in terms of triggering oviposition in *M. gratiosum*, remain to be established.

Little is known about the intra-uterine development of digenean eggs; however, some valuable information has been obtained recently. According to the work of Świderski *et al.* (2013) concerning the ultrastructure of the intra-uterine eggs of *Maritrema feliui* Gracenea, Montoliu & Deblock, 1993, the eggs of this species belong to the oligolecithal type, and the embryo starts to develop *in utero*. From transmission electron microscopy (TEM) images, the intra-uterine eggs reached a stage of outer

embryonic envelope formation. The outer envelope was formed by the fusion of macromeres at one pole of the egg. While the outer envelope was forming, the remaining blastomeres (*i.e.*, the mesomeres and micromeres) underwent multiplication and degeneration. The authors concluded that the development of the intra-uterine eggs of *M. feliui* is an example of an early stage of ovoviviparity. A similar intra-uterine developmental process was observed in *M. gratiosum* in the current study, but without the evidence of TEM, it is hard to confirm these details. Therefore, TEM could be applied in a future study for investigation of intrauterine egg development for *M. gratiosum*. Not all members of the Microphalloidea have oligolecithal eggs, and not all the species in the same genus reach the same degree of maturation when their eggs are laid. For example, *Brandesia turgida* Brandes, 1888 in the Pleurogenidae has polylecithal eggs and contains fully formed miracidia *in utero*; but *Prosotocus confusus* Looss, 1984 (Pleurogenidae), which also has polylecithal eggs, only reaches early embryo development (Conn *et al.*, 2018). The authors suggested that patterns of embryogenesis and egg formation are similar among digeneans, but that the timing of larvigenesis varies substantially.

5.4.4. The issue of progenesis

In the present study, newly excysted metacercariae of *M. gratiosum* already possess some adult characters, such as well-developed reproductive organs, even though no eggs were observed. Spermatozoa were found in the fertilization chambers as early as 15 minutes after excystment even when flukes were incubated individually. This finding suggested that spermatogenesis of *M. gratiosum* begins early and may start in the second intermediate host. Reproduction by self-fertilisation was confirmed by culturing the flukes individually, showing that the percentage of self-fertilisation was high (86%). Also, no differences in morphology were observed for eggs produced by self-fertilisation, however, whether these self-fertilized eggs are viable, was not confirmed. During the *in vitro* culture of *M. gratiosum*, the internal organs apart from the uterus do not develop further, and the size of the flukes does not change very much. Early stages of egg formation (24 hours) suggested the existence of fully functional or nearly mature ovaries and vitellaria at the time the metacercariae excysted. This precocious sexual development is sometimes called “progenesis” and is one of the characters of the members of the Microphallidae (see Fredensborg & Poulin, 2005). Although under the definition of Lefebvre and Poulin (2005a), true “progenesis” in digeneans is defined in terms of larval stages attaining a degree of maturation to produce viable eggs; progenesis is now widely used in the parasitology literature for conditions of precocious sexual development. Neoteny, in contrast to progenesis, describes a state in which reproduction occurs at a normal age while somatic development is retarded (Lefebvre & Poulin, 2005a). Different degrees of progenesis have been reported in the Microphallidae. For example, *M. opacus* can lay eggs when the metacercariae are still in their metacercarial cysts and still in the crayfish second intermediate host (Caveny & Etges, 1971). Oviposition in metacercarial cysts was observed in *M. japonicus* under *in vitro* conditions (Fujino *et al.*,

1977). *Gynaecotyla adunca* can produce eggs in their amphipod intermediate hosts, *Gynaecotyla longiintestinata* Leonov, 1958 and *Microphallus minus* Ochi, 1928 can produce eggs in their decapod intermediate hosts (Lefebvre & Poulin, 2005a). Other species like *M. similis* (Stunkard, 1957), *M. turgidus* (Pung *et al.*, 2009), *M. novaezealandense* (see Fredensboug & Poulin, 2005), and *M. gratiosum* in the present study, show the appearance of eggs very early on, within 24 hours after excystment. These are considered "facultative progenesis" species according to the definition of Lefebvre and Poulin (2005a) as the definitive hosts still exist. In a contrast to this, in obligate progenetic species, such as *Sogandaritrema progeneticus* Sogandares-Bernal 1962(see Lotz & Corkum, 1983), no definitive host can be found.

Considering the evolutionary benefit of progenesis, a key question is "what is the likely strategic gain?" This could possibly be explained by "reproduction insurance" theory as proposed by Wang and Thomas (2002), which means mechanisms allow the parasite to reproduce even if transmission fails. The most obvious benefit of progenesis would be the abbreviation of the life-cycle, thus reducing the transmission events and making the life-cycle easier to complete (Lefebvre & Poulin, 2005a). In progenesis, all metacercariae could be potential breeders (Lefebvre & Poulin, 2005a). The authors stated that progenesis is secondarily derived from an ancestral three-host pattern and has evolved several times independently among different lineages. The reproduction in obligatory progenetic species, however, relies on the eggs deposited in the cyst having to be inseminated by means of self-fertilisation. This will lead to an extreme inbreeding situation and drastically decrease genetic diversity. The major drawback of progenesis is, therefore, the decreased genetic diversity, which might lead to reduced adaptiveness (Lefebvre & Poulin, 2005a). In a study of the facultative progenetic species *Coitocaecum parvum* Crowcroft, 1945, which can reproduce both in amphipods and in their final fish hosts, more than half of the flukes start to produce eggs in the amphipod hosts. These egg-producing flukes in amphipods attain a larger size than their counterparts in their fish hosts, and the abundance of progenetic flukes increases with amphipod size (Lefebvre & Poulin, 2005c). The possible explanation is: when the flukes sense the possibility of locating a final host is low (*e.g.*, staying longer in older / larger intermediate hosts), some of the flukes start to produce eggs in the intermediate hosts to ensure the possibility of reproduction (Lefebvre & Poulin, 2005a). Despite the superficial benefits of progenesis, the abbreviation of the life-cycle via facultative or obligate progenesis is not widespread. The reason for these evolutionary constraints is not known, but the most plausible explanation would be the decrease of genetic heterogeneity and the quantity of eggs produced. Abbreviation of the life-cycle is favoured when the possibility of transmission to final host is low (Lefebvre & Poulin, 2005b). Another question exists regarding what are the prerequisites for progenesis? A creative idea was suggested by the same authors. The thickness of the cyst wall might be important. For 51 progenetic species that they examined, 10% were found to have a thin cyst wall and 43% to have no cyst. The

reduction or absence of cyst could facilitate the acquisition of host resources, the ease of release of progenetic eggs, or even cross fertilization within the intermediate host (Lefebvre & Poulin, 2005a). Prolonged development in an isopod crustacean intermediate host (*Ligia oceanica* Linnaeus, 1767) has been noted in *M. linguilla* and a delay of encystment was found. The first to third cyst layers formed at 29-38 days post-infection, while the formation of the 4th - 5th layers of the cyst wall and cyst development was not completed until 60 days (Banjamin & James, 1987). Similarly, fully formed cysts containing metacercariae of *M. opacus* were not observed until 46 days of development in crayfish (Caveny & Etges, 1971). In the present study, early spermatogenesis in newly excysted metacercariae was found and eggs started to appear within 24 hours under *in vitro* conditions. The ability to self-fertilise was proven by culturing the flukes individually. Considering the relative ease of excystment, and the ability of self-fertilisation, coupled with high specificity for the second intermediate hosts but not the final hosts, it could be suggested that *M. gratiosum* is on the way to advanced progenesis. This might be related to the short summer in its endemic area (Scotland) and short transmission periods in summer relative to the migratory patterns of the final hosts. Under global range, it is possible that in optimal conditions self-fertilization will not be evolved, therefore, it is assumed that ability of self-fertilization would be different between high and low latitude. Ease of excystment, however, should be an intrinsic character for *M. gratiosum* and will not differ between localities.

5.4.5. Confocal microscopy observation of musculature and internal organs

5.4.5.1. Body wall musculature

The body wall musculature of *M. gratiosum* in the present study comprised outer circular, middle longitudinal and inner diagonal muscle fibres (Figure 5.44), which is congruent with the basic pattern found in platyhelminths. This organisation is highly conserved as it has been observed in closely and distantly related digenean species, such as *M. pyriformis*, *M. pygmaeus, Levinseniella brachysoma* (Creplin, 1837) Stiles & Hassall, 1902 (see Krupenko & Dobrovolskij, 2018), *E. caproni* (see Šebelová *et al.*, 2004), *S. mansoni* (see Mair *et al.*, 2000), *F. hepatica* (see Mair *et al.*, 1998b), *Apatemon cobitidis proterorhini* Vojtek, 1964 (see Stewart *et al.*, 2003a) and even in the free-living tubellarian *Macrostomum hystricinum marinum* Rieger, 1977 (see Rieger *et al.*, 1994). The functions of somatic muscle have been the subject of comprehensive discussion. The contraction of the longitudinal and circular muscles shortens and stretches the fluke, and the diagonal muscles are responsible for side-to-side movement and body torsion (Stewart *et al.*, 2003a). The dorso-ventral muscles maintain the characteristic flattened body shape of flatworms, and this is supported by the fact that in *M. hystricinum marinum*, the body region without ventro-dorsal muscles is barrel-shaped (Mair *et al.*, 1998). The somatic muscles, together with suckers and tegumental spines, provide an attachment function which is fundamental to digenean survival. For microphallids, the surface spines were

suggested to facilitate locomotion and attachment functions (Galaktionov *et al.*, 1996; Krupenko & Dobrovolskij, 2018). The force driving these spines to act on the host mucosa is derived from body wall musculature and through the force of ventro-dorsal muscles coupled with the action of ventral concavity. The term "ventral concavity" was mentioned in the study of Krupenko and Gonchar (2017) for notocotylid species which have no ventral sucker. The attachment of notocotylids is by means of a compact muscular wall comprising the whole ventral surface (called the "bottom" of the ventral concavity) which act like a sucker, and well-developed ventro-dorsal muscles which generate negative pressure when contracted (Krupenko & Gonchar, 2017). This ventral concavity is observed in many digenean species (some by SEM) and mainly found in microphallids (Saville *et al.*, 1997; Pina *et al.*, 2007; Pina *et al.*, 2011b; Krupenko & Gonchar, 2017; Krupenko & Dobrovolskij, 2018). Ventral concavity was also noticed in *M. gratiosum* in SEM images in the present study (see Figure 4.17 in Chapter 4). It is speculated that a similar method of attachment is related to this character. The forebody and hindbody of microphallids have different functions. The locomotion / attachment function of the forebody is supported by the existence of denser and larger tegmental spines, body wall musculature and dorso-ventral muscles, and by more active movement of the forebody observed in living worms (Krupenko & Dobrovolskij, 2018). These types of structural differences are more pronounced in strigeids, which have a clear separation of forebody and hindbody. The forebody of strigeids is cup-shaped with, typically, three layers of body wall muscles coupled with various attachment organs. The hindbody, where all the reproductive organs are located, has a body wall with outer circular, intermediate longitudinal and inner circular muscles (Stewart *et al.*, 2003a). In *Cryptocotyle lingua*, the larger spines and solid muscle fibres were found only in the forebody, which shows ventral concavity; while in *Cryptocotyle concava* (Creplin, 1825) Lühe, 1899, the spines become smaller posteriorly but solid musculature covers the whole ventral surface as the whole ventral surface acts as a ventral concavity (Krupenko & Dobrovolskij, 2018).

The musculature of suckers, which is also conserved amongst different species, comprises surface circular and meridial (longitudinal) muscles and inner radial muscles. Contractions of the meridial muscles open the sucker, while contractions of the radial muscles close the sucker into a cup-shape, and contractions of the circular muscles create a suction force (Mair *et al.*, 1998b). Extra supportive muscles were found near the ventral sucker of various species at cercarial or metacercarial stages (Krupenko & Dobrovolski, 2015). In the present study, web-like protractor muscles were also found at the base of ventral sucker (Figure 5.52) and symmetrical ventro-dorsal muscles providing extra support were found in the oral sucker (Figure 5.51).

5.4.5.2. Reproductive organs

As with other species in the Microphallidae, which show different degrees of progenetic development, *M. gratiosum* is also progenetic. The reproductive organs are well developed in newly excysted young adults and thereafter, no further development of either reproductive organs or somatic musculature was noted over the 5-day observation period of the current study. In non-progenetic species, such as *E. caproni*, however, intensive morphogenesis of the reproductive organs can be observed in pre-ovigerous adults, but the somatic muscles show very few changes. It is worth noting that neurotransmitter expression in a complex network of nerve fibres and cell bodies in the female reproductive tract has been reported in post-ovigerous adults of *E. caproni* (see Šebelová *et al*., 2004). It is known that egg formation and passage is achieved through a highly ordered series of rhythmic contractions of the ootype and related ducts (Šebelová *et al.*, 2004). The musculature and sphincters of the egg-producing apparatus, coupled with the timing of neurotransmitter expression in both *E. caproni* (see Šebelová *et al.*, 2004) and *F. hepatica* (see Mair *et al.*, 1998b), indicates that egg formation is achieved with co-ordination of neuromuscular systems. In the present study, neither the "ootype" nor the Mehlis gland were observed. In the original description, however, Hadley and Castle (1940) mentioned the presence of the ootype and described it as the "*receptaculum seminis*" together with the duct connecting to the proximal uterus. These authors also mentioned the "shell gland", referring to the faintly stained gland surrounding the ootype before it connects to the proximal uterus. The shell gland may be the structure homologous to the commonly known Mehlis gland. In the present study, ova were observed to reside and rotate in the fertilization chamber for a period in living specimens, however the exact timing and position of shell formation remains unknown, and the shell gland was not observed even under the DAPI channel. Several sphincters were noted at the "ootypic junction" in the present study (Figure 5.48), indicating the existence of a sequence of events for egg formation. Clearly from the above, more evidence is needed to better elucidate the biology of egg formation in *M. gratiosum*. The difference in configuration of the fertilization chamber and *receptaculum seminis* in the present study (Figure 5.55) compared to the original description of Hadley and Castle (1940) possibly reflects the different techniques employed (confocal versus light microscopy, respectively) and compression differences of the specimens. Modern techniques should give better resolution of the fine structures like these. It is possible that in the future, the fertilization process can be observed by CLSM using live specimens, coupled with the identification of neurotransmitters in the reproductive organs, to reveal sequential changes in the reproductive organs during the process of egg formation.

5.4.5.3. Glandular structures in the tegument

Apparent glandular structures (Figure 5.50A) were observed in the tegument of cultured adults of *M. gratiosum* by confocal microscopy. This is not the first time that tegumental glands have been

described in microphallids. Similar structures have been found several times in *Maritrema* species and are usually described from light microscopy studies. For example, they have been observed in *M. eroliae* (see Smith, 1983); *M. laricola* (see Ching, 1963); *M. linguilla* (see Benjamin & James, 1987); *Maritrema murmanica* Galaktionov, 1989 (see Galaktionov, 1989); *Maritrema neomi* Tkach, 1998 (see Tkach, 1998); *Maritrema patulus* Coil, 1955 (see Coil, 1955); *M. gratiosum* and *M. subdolum* (see Galaktionov *et al.*, 1996). They have also been found in other microphallids such as *M. similis* (see Davies, 1979)*, M. claviformis*; *L. brachysoma* (see Galaktionov *et al.,* 1996); *Gynaecotyla hickmani* Smith, 1983; *Gynaecotyla macrocotylata* Smith, 1983; *Microphallus paragracrapsi* Smith, 1983 (see Smith, 1983) and *Probolocoryphe glandulosa* Coil, 1955 (see Coil, 1955) as well. These structures were usually located in the forebody. Under light microscopy, these structures were described as eosinophilic (stained with Harris' haematoxylin, see Coil, 1955) and appeared refractive in samples in the present study (see Figure 4.13 in Chapter 4). Several types of glands associated with the tegument of cercarial or metacercarial stages have been described by TEM. Galaktionov (1996) described spherical granules in cells (termed tegumental cells by the author) under the tegumental syncytium and muscle layer in newly penetrated *M. gratiosum* (< 18 hours and prior to cyst formation). The cytoplasm of these cells was connected to the tegumental syncytium. At the mid-point of development, when a thin fibrous cyst formed, different types of cells containing electron-lucid vesicles and connected to the syncytium were found. At late stage, however, tegumental cells containing small discoid granules with no connection to the tegument appeared. When the metacercariae were fully formed (with two layers of cyst), the same cells, with small discoid granules, were found with connections to the surface syncytium. Forebody gland cells have also been reported, which seem to be different from tegumental gland cells, having thicker ducts that penetrate the tegumental syncytium (Galaktionov *et al*., 1996). Different types of electron-dense granules (larger) were noticed in these ducts. The term "forebody glands" was first coined by Davies (1979) in a TEM study for *M. similis*. The author described these glands as having a nucleated cell body beneath the tegument and containing electron dense granules, mitochondria, ribosomes, and granular endoplasmic reticulum. The ducts were lined with microtubules and opened to the exterior of the fluke at the bottom of small pits in the tegument. These cells with ducts were also found in the tegument lining the oesophagus. Through cytochemistry, Davies (1979) found these cells to contain complex materials, including diastase-resistant neutral mucosubstances, protein, RNA, and cholinesterase. The author concluded that the secretions of these gland cells of *M. similis* were enzymatic and that the cholinesterase in flukes was much more resistant to inhibitors than vertebrate cholinesterase. Davies (1979) suggested that the presence of the cholinesterase was to compensate for the irritating effect of the spines and to reduce the movement of host intestinal villi to prevent expulsion of the fluke. Other studies indicate that the secretions of forebody glands are heterogeneous. Galaktionov (1996)

suggested that the “dense discoid granules” in tegumental cells appeared at the third stage of development of microphallid metacercariae and were related to glycocalyx production and maintenance. It may therefore function to resist the host immune system. Benjamin and James (1987) described “mucopolysaccharide gland cells” and “small proteinaceous rod-shaped body cystogenous gland cells” in the sub-tegument at the first phase of development of *Maritrema linguilla*. Different types of gland cells appeared over seven developmental phases. The cystogenous gland cells disappeared at the sixth phase and proteinaceous sub-tegumental gland cells reappeared in the forebody at the final phase (7^{th}) of development. These authors suggested that the secretions of these gland cells contribute to cyst formation. Although the development of *M. linguilla* observed by Benjamin and James (1986) was considered abnormal by Galaktionov (1996) since the cercariae used by the former were from sporocysts rather than naturally emitted cercariae; their study still provided some information about the diversity of tegumental glands in microphallids. The “finger-like” structures (see Figure 5.50) observed in the tegument under CLSM in the present study, seem to be homologous to the forebody glands previously mentioned. The observed ducts / glands were sinuous with considerable length and were of homogenous content and moderate intensity of fluorescence emission in the DAPI channel. The cell body, on the contrary, was not observed in the present study. It may fuse with the secretions and therefore not be discernible. It is not clear why this structure is observed in the DAPI channel. According to previous findings by TEM, the forebody gland is a unicellular gland. Although DAPI stains largely DNA / RNA, several other compounds autofluorescence at the same wavelengths as DAPI *e.g.*, chitin and other materials. A possible explanation is the absorptive function of these glands, which can absorb nucleotide from the culture medium (for the ingredients of medium NCTC 109, see Appendix 14), and therefore, can be seen from DAPI channel. So far there has been little systematic use of DAPI for study of structures in microphallids and the present study is the first to describe internal structures of microphallids using DAPI to help delineate tissues / structures. Certainly, more evidence is required to help ascertain the function of the described glands / ducts.

5.4.6. Final remarks and conclusions

The present study provides a comprehensive description of developmental morphology for *M. gratiosum,* the type species for the genus *Maritrema,* from excystment to ovigerous adults. It is the second *in vitro* culture study of *M. gratiosum* after 30 years; the first was that conducted by Zaben (1988). The goal of optimizing culture conditions to obtain ovigerous adults with normal eggs was not fully achieved, however, the present study provided valuable information in other aspects for this common digenean species. Metacercariae extracted from *S. balanoides* can be successfully excysted in PBS at a temperature close to that of the final host (40°C). It can be cultured in NCTC-109 supplemented with chicken serum and penicillin and streptomycin for up to 10 days. By survival

analysis, treatment groups containing 20% and 40% chicken serum proved to be no different in terms of cumulative survival probability. Also, no difference was noticed for egg production between the two groups. Nevertheless, applying 20% chicken serum seems to delay the peak of egg production and result in higher mean egg numbers. Applying 40% chicken serum, however, results in a longer body length and higher mean survival time. Eggs started to appear within 24 hours and egg production peaked at 2-4 days, this conforming to the pattern of other *Maritrema* species cultured *in vitro*. The impact of antibiotics has been discussed and the application and potential weakness of survival analysis were also analysed. Evidence of early spermatogenesis was confirmed and both normal and abnormal eggs could be found. The existence of an operculum cannot be confirmed. Some eggs seemed to reach the stage of early embryogenesis *in utero* while oviposition was not seen. SEM or TEM, however, can be applied to confirm the presence / absence of operculum and intra-uterine development of eggs under different culture conditions. The culture conditions could be improved in the future to increase the longevity of flukes and facilitate normal development of the eggs and oviposition. More chemical and physical factors can be included, such as the use of different food sources, temperatures, gas phase, *etc*. Novel techniques such as xWORM (Rinaldi *et al.*, 2015) can be applied in the future to measure the health / vitality of the cultured adults during culture. Environmental DNA techniques (Bass *et al*., 2015) can be applied to detect the presence of eggs in faecal or sentimental samples, to validate oviposition of *M. gratiosum*. Precocious sexual development of this species was confirmed and the ability to self-fertilise was proven. The status of progenesis of *M. gratiosum* and its evolutionary and ecological significance has been discussed.

Finally, by means of CLSM, the structure and disposition of the musculature of cultured adults of *M. gratiosum* at different ages was described. This is the first CLSM study for *M. gratiosum*. There was no difference detected in terms of musculature development during observation, and this finding conform to the precocious nature of this species. The musculature of the specimens observed in the current study generally agree with the muscular system of other microphallids, and the functional biology of *M. gratiosum* musculature comparing to other digenean species were discussed. In the present study, structures that had not been observed before, a ligament connecting the *pars prostatica* and seminal vesicle, and a racket-shaped excretory bladder with two branches on each side were found. In addition, the configuration of the ootypic junction, which differed from the original description, was described. These findings suggest the capability of modern techniques for contributing to taxonomy based on morphology. Structures resembling forebody glands were revealed by DAPI staining / autofluorescence and the possible biological characters of these structures were discussed.

While impacts of *M. gratiosum* on wading birds are currently unknown, increasing stress on sea bird populations, exerted by changes in marine and coastal environments, may be expected to increase from the additional impacts of parasites, as deteriorated health can exacerbate the hazards of helminth infection on their hosts (Galaktionov, 1996; Sagerup *et al*., 2009). This study has increased the knowledge of the biology of microphallids, and provided new insights and tools to assist future studies of host-parasite interactions.

Chapter 6 Discussion and conclusion

In the pilot study conducted at three different localities in Scotland (*i.e.*, South Queensferry, Machrihanish and Dunbar), a total of 1012 *Semibalanus balanoides* were dissected, and a diverse array of associated and parasitic fauna were found (see Chapter 2). The observed fauna is congruent with the range of taxa reported previously, but parasitic isopods and rhizocephalans were never found. A vital question was raised when comparing the fauna discovered in the present study to that found in an earlier study by Colston (2012), "How can *M. gratiosum* be so successful in the absence of evident cercarial stages?". The numerical dominance of the target parasite *M. gratiosum* at chosen study localities was confirmed, and the need to discover the complete life-cycle stages in their natural environment emerged. Although one of the important goals in the present study was to find the cercarial stage of *M. gratiosum*, the cercarial stage of *Maritrema* sp. was never found throughout the course of the whole study (2012-2018). This finding of "absence" seems to suggest that a special strategy for transmission is utilized by the cercariae of *M. gratiosum*. Novel techniques, *e.g.*, detection of environmental DNA and applying high throughput sequencing, were suggested to investigate their presence. In the pilot study, three research questions were raised: 1. How can *M. gratiosum* complete its life-cycle and become highly prevalent at certain localities? 2. Why is *M. gratiosum* less successful at other localities? 3. Is "the parasitic fauna and the specific infection parameters associated with a host" an intrinsic character of a locality? These questions were answered to some degree in the following research studies, i.e., a spatial distribution study of the metacercariae of *M. gratiosum* (Chapter 3), a study focus on the diagnosis and morphometrics of *M. gratiosum* (Chapter 4), and an *in vitro* culture and developmental morphology study for this precocious species (Chapter 5). The results of these studies revealed that parasites should not be overlooked when studying intertidal ecology, and demonstrated that parasites can develop intricate and fascinating strategies to survive and coexist with their hosts.

In the present study, an aggregated spatial distribution of *M. gratiosum* in its host population was repeatedly observed, i.e., in different environmental gradients, types of habitats, and scale of studies. This aggregated pattern was observed in the pilot study where the choice of sampling site was more arbitrary, and then again later in the more systematic surveys that were conducted afterwards. Although the phenomenon of aggregation in parasite populations has been suggested as a biological "law" (see Poulin, 2007), the cause and consequence of aggregation are more important as they are relevant to the impact of parasitism on host populations, and how parasites interact with their hosts. In the present study two opposing hypotheses were proposed in the pilot study, i.e., "infection pressure is proportional to the distance between the definitive hosts" versus "residence at low tide

levels will favour infection". The results of the study found that the first hypothesis was supported while the second hypothesis was rejected. To investigate the cause of aggregation further, and to incorporate the element of spatial distribution, a more systematic distribution study was conducted. In this study (Chapter 3), some elements inspired by methods applied in free-living species (McGill *et al*., 2007), i.e., environmental gradient analysis, deconstruction (sub-setting) sample, were incorporated. In this field-based study, some phenomena and trends were observed. A model with high predictability was built, however the reason of why this degree of aggregation was observed and the impact on the hosts, remain as difficult questions to answer. Nevertheless, the uniqueness of this study was in the systematic sampling strategies used, the scale of sampling, and the application of Lloyd's crowding indices in the parasite distribution study. The present study is the first distribution study applying a systematic sampling strategy for *M. gratiosum* populations in its barnacle host *S. balanoides*. Furthermore, the distribution study provided a good model for further study and produced a set of baseline data for further application.

In the distribution study (Chapter 3), heterogeneity in the distribution of *M. gratiosum* metacercariae was characterised by the variance to mean ratio, negative binomial k, Lloyd's mean crowding, and by Lloyd's patchiness index. Contrary to the suggestion of Carrol *et al.* (1990), that variation in the abundance between sites at small scales (within 100 m) was minimal and was attributed to the sampling procedure; true heterogeneity was detected at a small scale in the present study. Density-dependent patterns fitting Taylor's power law were observed (a model for density-dependent spatial behaviour of animals; Taylor *et al.*, 1978), however, the heterogeneity of host populations seemed to have a dilution effect on the heterogeneity of distribution of the parasite population. Two scales (i.e., a local scale and a whole area scale) for the distribution study were conducted at Dunbar Red Rock. Significant differences in the abundance and intensity were found between the two scales but not in prevalence. Abundance was positively correlated with prevalence, which fits the abundance–occupancy relationships observed in free-living species (Morand & Guégan 2000). Abundance was found to have a moderate positive correlation with barnacle size, and it showed a negative trend from the land to the shore. This trend of higher intensity and abundance of metacerariae on the higher shore had also been noticed in several earlier studies (Irwin & Irwin, 1980; Mitchell & Dessi, 1984; Carrol *et al.*, 1990). The possible explanations for this trend include: higher numbers of the first intermediate hosts on the upper shore, the existence and distribution of principal final hosts, tidal effects upon the xiphidiocercariae, faster growth rate and lower survival rates of the barnacle on the lower shore, and a higher turnover rate in the barnacle populations in the lower shore. In the present study, larger barnacles (according to their operculum length) were found to have a higher abundance of metacercariae. The reasons for this phenomenon could be: parasite-induced host gigantism, parasite-induced mortality occurring in younger / smaller barnacles, or accumulation effects

with host age. Host density and dynamics are interesting topics for future studies, and this is because the population dynamics of sessile barnacles have already been a popular research area (see Section 1.2.1.7 in Chapter 1). The present study is the first to apply Lloyd's mean crowding and patchiness index to the digenean-barnacle system; these indices, along with other aggregation indices (*e.g.*, variance-to-mean ratio, negative binomial k), deserve further investigation, especially when barnacle population data are included. Potential factors influencing the abundance were explored in the present study via multiple regression, and a model was built to make a prediction of the abundance. Although the results found that exposure and host size were the major determinants for metacercarial abundance, each factor can be further explored individually, and that more factors can be included in the future. This prediction was based on existing parameters which were regarded as relevant to the abundance of *M. gratiosum* metacercariae. The ecology of the rocky shore is complex, and the environment suffers from a degree of instability (see Little *et al.*, 2009). An attempt to build a "good model" for parasite populations would thus be very challenging. It is important to detect key biotic and abiotic factors influencing parasite distribution. This could possibly be achieved by investigating each candidate factor by means of experiments under field conditions or in the laboratory. Like other environmental factors, the parasite itself is a dynamic factor for their host, which can influence host population structure, therefore structuring the surrounding ecosystem. Seasonal fluctuations in recruitment have been recorded in intertidal ecosystems (Mouritsen & Poulin, 2002) and hence temporal (seasonal) changes in distribution would be another worthwhile topic to study in the future. In an era of big-data analysis for ecology (LaDeau *et al*., 2017; Runting *et al*., 2020), the inclusion of diverse arrays of environmental or host variables, whether considered irrelevant or not, may be a powerful way to reveal the "real" causes and consequences of aggregation.

Whether the detected infection parameters and patterns of distribution are true characters of the studied locality remain to be determined. In the present study, latitude appears to have had a negative impact on infection parameters, while the infection parameters in the sheltered bay possessed different characters to those on the rocky platform. The effect of scale was expressed in the distribution data and in the morphology of the metacercariae, and this was shown in the variance of abundance and in the range and variance of each morphological variable. True differences, however, may indeed exist in different types of terrain (*i.e.*, the gully vs. rocky platform). These observations make the answer of "whether infection parameters are true characters of a locality" more obscure, as too many confounding factors exist, e.g., sample size and sampling methods. This is the challenge and beauty of ecological research. Chronological observations appear to be a good approach, as the comparisons are always made between the same host-parasite system in the extent and habitat types. One important requirement is that the methodology applied in chronological studies must be consistent. The present study has provided an efficient and stringent model, along with a set of base-

line data, for direct application in future investigations of this parasite-host system. From the preliminary results of the chronological comparisons made in the present study throughout 2012 to 2013, the infection parameters appear to be the true characters of a locality, because these parameters are the results of the interaction of all the animals in the studied system, and animal fauna in an ecosystem are important characters of it. It is also important to determine the role that parasites play in an ecosystem; as sometimes a particular parasite species can be important as a driving force in shaping community structure, and so may be thought of as a "key-stone parasite" (Thomas *et al.*, 1997; Mouritsen & Poulin, 2002). Whether *M. gratiosum* represents a key-stone parasite in this system remains to be determined, because their influence on their host and on other co-habiting species remains to be established. The influence of *M. gratiosum* on its barnacle host can be investigated by artificially maintaining clean barnacles, if sustainable source of cercarial stages can be found. This can be achieved indirectly by observing the reproductive output of infected and uninfected barnacles. An attempt was made to clean up an area of barnacles on the shore, and then to follow the dynamics of the parasite in the new barnacle recruits and throughout their subsequent development. Although this preliminary trial was not completed (data not shown) due to time constraints, it is believed to be a promising route in investigating the interaction between *M. gratiosum* and *S. balanoides*.

In the pilot study, there were no robust species diagnosis for the target digenean and its identity was confirmed as *Maritrema* sp. based on morphological evidence. A variety of techniques, therefore, were subsequently applied to the diagnosis of the target parasite. These included: morphological description by light microscopy, SEM observations, and molecular characterisation. Based on morphological grounds, the metacercariae collected in Scotland are diagnosed to be *M. gratiosum,* because no obvious structural differences to the accepted descriptions for this species can be found, and the dimension of the body size and organs fits the original descriptions, *i.e.*, those of Hadley and Castle (1940) and of Deblocks and Tran Van Key (1966a). The present study represents the most comprehensive morphological re-description of the species in sixty years. The current study demonstrated the existence of an eversible cirrus connected to an ampullar-shaped *pars prostatica* which can move by itself; and the controversy surrounding the existence of a male papilla was resolved. The study also provided details regarding morphological changes in *M. gratiosum*, e.g., the loss of stain in the vaginal gland in older adults – this may deserve further study. Using 18S rDNA and ITS2 rDNA sequences derived from the metacercariae collected from *S. balanoides* from Scotland, the species were diagnosed as *M. gratiosum.* Identity was based on the consensus of 18S rDNA sequences which sat well within the *Maritrema* clade and the consensus of the ITS2 rDNA which were 100% identical to the only existing ITS2 rDNA sequence of *M. gratiosum* available on GenBank collected from *S. balanoides* from Ireland (see Table 4.4). Furthermore, features obtained by SEM, such as body shape, surface spines and papillae, and sucker papillae and morphology, pointed to their similarity with other

microphallids. These features, however, did not necessarily possess diagnostic value because some are conserved across distantly related species. The functions of the spines and papillae are still obscure and need to be investigated by other methods, *e.g*., TEM, histology, or electrophysiology. This study represents the second SEM observation of the metacercariae of *M. gratiosum*. The first SEM observation was undertaken by Zaben (1988) and the results of the present study largely agree with those of Zaben. A chaetotaxy study in the present study was not possible due to the quality and quantity of specimens available, but its diagnostic application in separating genera and species within the Microphallidae can be further explored in the future. Morphometric techniques were applied to confirm a single species among the specimens collected from the different Scottish localities. A MANOVA analysis for the newly excysted metacerariae revealed a multivariate main effect between samples from the local scale and from the whole area study, both collected from Dunbar. Afterwards, newly excysted metacercariae and cultured adults were analysed separately by means of PCAs. No morphometric heterogeneity could be detected in the metacercarial samples covering the whole area of Dunbar Red Rock. When using adult samples from three distant localities, *i.e.*, Dunbar, Stonehaven and Rosehearty, slight morphological heterogeneity among the specimens was observed. This might reflect differences in habitats among the three localities and different culture condition in specimens from Dunbar, however, specimens from these three localities are morphometrically quite similar and are still considered to be a single species, since cluster analysis showed samples from the three localities to overlap. These results on the whole supported the assumption that "morphological plasticity is positively correlative to geographical distance", although a statistical comparison could be conducted in the future to further validate this assumption. Geographical and host-induced morphological differences within a species have been reported in other microphallids (Martorelli & Ivanov, 1996). In the present study, most of the samples were collected from the same host (i.e., *S. balanoides*), but the possibility of the existence of unique local populations cannot be ruled out. This notion is especially interesting when Lloyd's mean crowding was added for analysis. The effect of crowding seemed to express on the numbers of egg (i.e., the reproductive output) and variation of morphological measurement (PCA), that reproductive organ-related measurements appear to be the main variables which differentiate the two groups (local scale vs. whole area) in a MANOVA. Furthermore, the compactness of specimens in the PCA plots appear to be negatively correlated to crowding (i.e., specimens from distanced localities). As research on the application of crowding index versus digenean morphology has not been conducted, it is not possible to compare the present results with the findings from other studies. The present finding, however, warrants further studies using larger numbers of specimens coupled with stronger statistical analysis. PCA, as a dimension reduction tool, had successfully reduced the dimension from 18 variables to 3-4 components, and identified the variables of most diagnostic utility. Taxonomic non-relevant variables can be removed, and data from

more members within the Microphallidae can be included in the future, to better understand the variables critical to separating genera and species of the Microphallidae. The existence of phenotypic plasticity might reflect local populations with unique genetic characters, and these can be further investigated in the future in conjunction with the distribution data obtained in the present study.

Due to the ethical constraints in obtaining adult stages from avian final hosts, a series of *in vitro* culture experiments were conducted (Chapter 5). The present study is the second *in vitro* culture study for *M. gratiosum*, second only after that of Zaben (1988). A precocious sexual development in this species was confirmed and the ability of flukes to self-fertilise was proven experimentally. The biological importance of this character was also discussed. More chemical and physical factors should be tested in the future to improve culture conditions, and to reduce abnormalities in egg production and to stimulate oviposition. Obtaining the ovigerous adults is the first step towards closing the life-cycle of *M. gratiosum in vitro*. Once viable eggs can be obtained, further experiments, such as the infection of molluscan hosts, can be conducted. In the *in vitro* culture study, the original goal of "optimisation of the culture condition" was not completely achieved, nevertheless it served to provide parasitic materials for the CLSM study which produced several novel findings. This is the first confocal microscopy observation of *M. gratiosum*. Details regarding the configurations of the "oviduct crossroads" were revealed, which is very hard to observe by light microscopy. A new structure, not previously described, was the ligament connecting the *pars prostatica* and the seminal vesicle. New morphological characters of the excretory bladders were also detected. Contrary to the generally accepted "Y-shaped bladder", the bladder under confocal microscopy was shaped like a racket, with two apical lateral branches. The reason for this configuration is unknown, and although it does not seem to affect the phylogenetic position of this species, it deserves further study to elucidate the functional relevance of this configuration. This final research reveals the power of modern techniques and their contribution to parasite morphology and physiology-based studies. Further application of CLSM on *M. gratiosum* and on other digenean species can be wide, *e.g.*, combining neuro-transmitters to reveal sequential neuro-musculature changes at the ootypic junction during the maturation of the metacercaria (see Stewart *et al*., 2003a, b), or to reveal sequential muscular contractions of the ootypic junction during the egg development process, or to elucidate in detail how the egg producing apparatus works.

The present study represents a mixture of field and lab research. It has utilised traditional and novel methods to investigate the life-cycle stages of a particular parasite and associated animals, such as the host and other co-existing parasites. In contrast to controlled laboratory environments, field work provided different kinds of challenges, but the results were fruitful. The greater the time spent in the field, the more the complexity and unpredictability of the environment may be understood,

highlighting the considerable unknowns that remain after the present study. From the point of view of conservation, parasitological research concerning aquatic animals are also important. The parasite infection status is one of the health indicators for the chosen target species. The density and distribution of specific parasites can also be an indicator of biodiversity and can therefore be used to evaluate recovery rates after natural disasters or from human interference. In the report of Kuris *et al.* (2008) for estuarine ecosystems, the authors pointed out that parasites may account for a larger portion of the whole wetland ecosystem (in terms of biomass) then people think. After the investigation of three estuarine ecosystems, the team suggested that the biomass density (kg ha^{-1}) of parasites was superior to that of the highest predators, and digeneans accounted for the highest proportion. The biomass density of digeneans was comparable to that of birds, fish, burrowing shrimp, or oligochaetes (*i.e.*, approx. 10 kg ha^{-1} each). The estimated biomass of free-swimming transmission stages of digeneans was even higher than the biomass of birds. Furthermore, the results revealed that in estuarine systems, parasitic castrators account for a large proportion of the total biomass of parasites, indicating an important ecological role for such parasites. For example, in this study the authors revealed that in castrated *Cerithideopsis californica* Haldeman, 1840 (Haldeman, 1840) snails, digenean parasites comprised between 37-130% of the original soft tissue biomass compared to the uninfected snails. The authors also revealed that across several invertebrate taxa, uncastrated populations only had slightly higher biomass densities than their castrated counterparts. Considering the cost of resisting and maintaining these parasites, the effect on host reproduction, and the net effect on trophic webs and energy flow, parasites in estuaries therefore clearly have a significant impact on the population of their hosts. The high biomass, plus other evidence, proves that parasites play more important and complex roles than previously understood.

Studies of the life-cycle and the relative biology and ecology of a parasite species, provide a great opportunity to explore their role in the ecosystem, and probably as importantly, the roles that humans play. As suggested by Hoeve and Scott (1988) concerning wetland ecosystems, an isolated ecosystem without human disturbance may not always be found. The effects of all sorts of human disturbances upon life-forms in an aquatic environment are complex and dynamic. Environmental factors such as severe climate change make the effects of human disturbance even more unpredictable. There is evidence that increased temperatures can facilitate the transmission of certain parasites, causing the decline of host populations (Mouritsen *et al*., 2018). Furthermore, there is evidence showing that the body temperature of barnacle individuals in a population follow a mosaic pattern, and a rise in temperature can lead to coma in some individuals already suffering from high temperature (Wang *et al*., 2020). The influence of this temperature pattern on the parasitism of barnacle populations and the ecological impact deserves further investigation. Under the ongoing circumstances of global warming, there are stronger needs to elucidate how parasitism influences the structures of aquatic ecosystems.

Invasive ecology is relevant to climate change and is influential on the stability of an ecosystem (Chalkowski *et al*., 2018), and should be incorporated in the future when studying the effect of climate change. Previous research has suggested that parasites can be a good indicator for the wider biodiversity of aquatic ecosystems (Sousa, 1991; Thomas, 1997; Marcogliese, 2005; Hudson *et al.*, 2006; Hechinger *et al.*, 2007). Their ubiquitous presence and complex net effects on ecosystems warrant their inclusion in biodiversity and ecosystem functioning (BD-EF) research (Frainer *et al.*, 2018). There are two further reasons for studying digeneans in aquatic systems. First, is the convenience of accessing intermediate hosts (*i.e.*, snails) and their digeneans. Second, is the fact that the life-cycle of digeneans has an intimate relationship to the invertebrate and vertebrate animals nearby (Hechinger *et al.,* 2007). For example, digeneans can alter community structures by means of controlling the population of dominant grazers in the intertidal zone (Wood *et al.*, 2007). In a study searching for indicators of biodiversity in a wetland ecosystem, it was found that the diversity and abundance of cercariae in snails was positively correlated to the diversity and abundance of birds, and positively related to the diversity and richness of large benthic species, but had no relationship to fish populations. The lack of relationship to fish populations may, however, be due to sampling methods and further research is needed (Hechinger *et al.,* 2007). In other research studying the impact of a hurricane on organisms in coastal lagoons, digenean numbers in snails was utilised as an indicator of the recovery of the lagoon ecosystem. The author mentioned that severe natural disasters like hurricanes have destructive and long-term impacts on ecosystems and that digenean parasites of snails could therefore provide useful indicators of recovery and degradation of costal lagoon ecosystems (Aguirre-Macedo *et al.,* 2011). The above-mentioned principles might also be applied to rocky shore ecosystems. Considering the high prevalence and sometimes also the high intensity of digenean infections in intertidal systems (Sousa, 1991; Mouritsen & Poulin, 2002), the study of digeneans would be a good starting point to appreciate the biodiversity of rocky shore animals. If asking the question about what role parasites play in intra- and interspecific interactions, the answer is often unknown, because people simply do not know about the existence of these parasites, let alone their interactions (Sousa, 1991). The first step in solving some of these intricate puzzles is to screen for the existence of a broad range of intertidal parasites, and furthermore, to investigate the patterns and inter-relationships associated with their existence, a process which has been started for a single species in the present study.

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Appendices

Appendix 1 Different shell plate arrangements in ancestral and current sessile barnacles and other important shell plate features

The ancestral sessile barnacle is believed to have had shell plates comprising eight compartments. At one end was the rostrum and at the other end was the carina. On each side, a lateral plate was flanked by a rostro-lateral and a carino-lateral plate. These plates can be fused or reduced from six to four for different species (Figs. 1) and equipped with different arrangements of ribs and pores (Bassindale, 1964; Southward, 2008; Figs. 2). There are delicate structures belonging to each shell plate called the radius (overlapping flap) and ala (underlying flap), which serve the function of butting between plates (Bassindale, 1964; Figs. 2). The number and shape of the radius and ala can also be keys to identification.

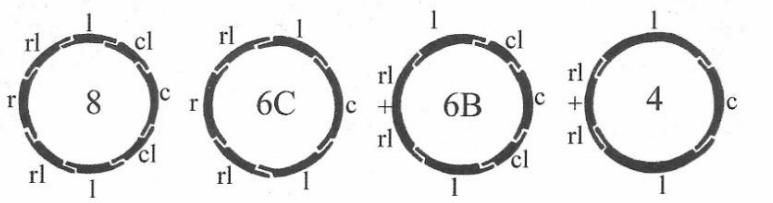


Figure 1. The arrangement of shell plates in four types of sessile barnacle. The "8" is an eight plated ancestral form; 6C: is a six-plated form seen in chthalamoids which is formed by the loss of the carino-lateral plates; 6B: is a six-plated form seen in balanoids – the rostrum is lost and the two rostro-laterals are fused; 4: is a four-plated shell form seen in Elminiinae, and it is presumed that it is derived from the balanoid arrangement and by loss of the carino-laterals. Abbreviations: c: carina. l: lateral plate. r: rostrum. cl: carino-lateral plate. rl: rostral-lateral plate. Reproduced from Southward (2008; p. 8).

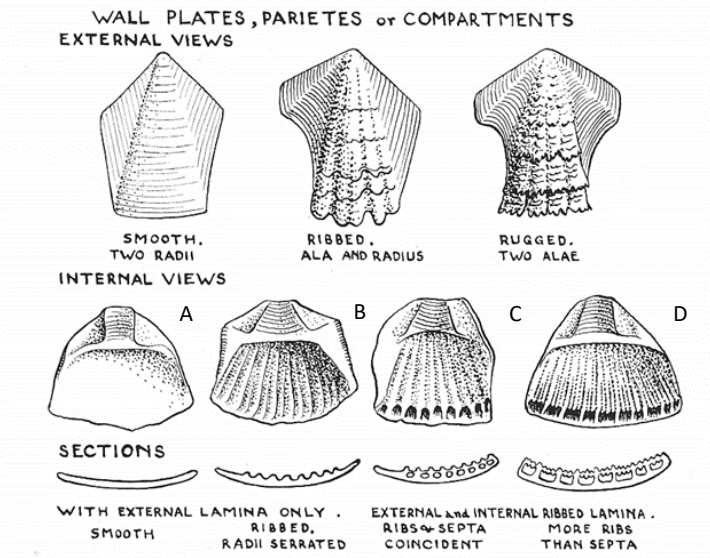


Figure 2. Structure of shell plates of sessile barnacles. A. No ribs or pores; B. Ribs present and also having serrated lateral margins to the radii; C. Pores and coinciding ribs; D. Pores and multiple ribs. Reproduced from Bassindale (1964; p. 56) and Southward (2008; p. 9). The original was from Bassindale (1964; p. 56).

The movable parts which cover the dorsal aperture of sessile barnacles comprise the operculum. The operculum is comprised of two symmetrical plates formed by a scutum (rostrally) and a tergum (carinolly) (Bassindale, 1964; Southward, 2008; Figs. 3-4). Between these two opercular plates, there are fleshy flaps which can seal the opening tightly. These are called tergo-scutal flaps and can be extended to varying degrees and have different colours in different species (Southward, 2008). These features are important identification traits.

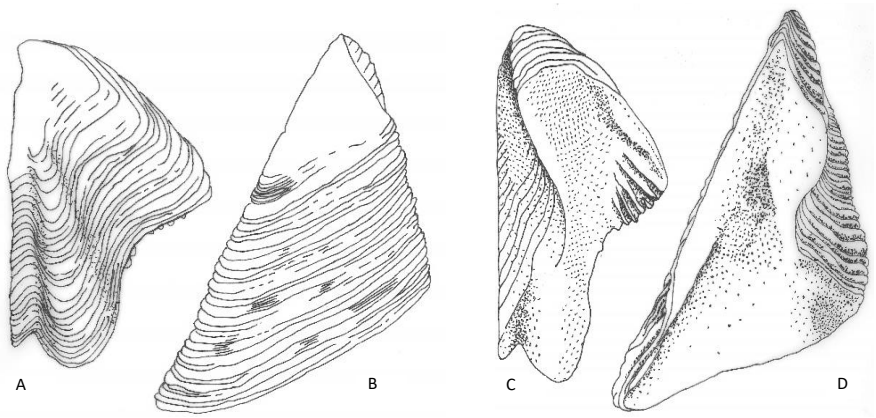


Figure 3. Structures of the scutum and tergum from *Semibalanus balanoides*. A. External surface of the left scutum of a full-grown individual of *S. balanoides*. B. External surface of the left tergum of the same specimen. C. Internal surface of the right scutum of the same specimen. D. Internal surface of the right tergum of the same specimen. Redrawn from Stubbings (1975; Plate VII).

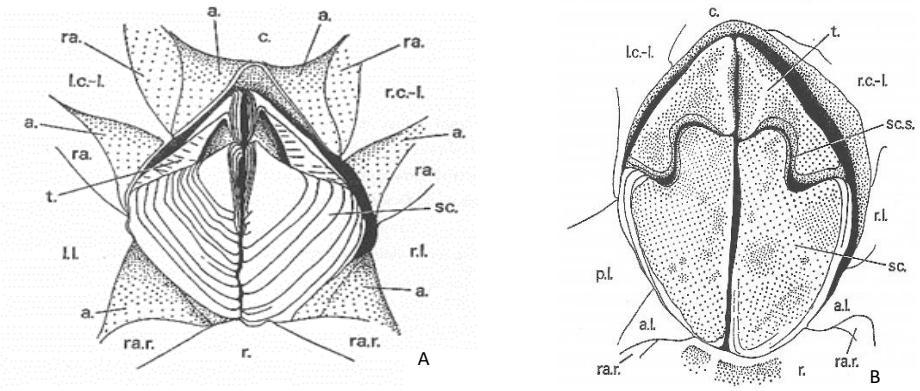


Figure 4. Dorsal view of the scutum and tergum of *Semibalanus balanoides*. A. The opercular region as seen from above in a young uneroded specimen of *S. balanoides*. The scuto-tergal suture has straight margins. B. The opercular region in an old eroded specimen of *S. balanoides*, the scuto-tergal suture describes an S-shaped line (Reproduced from Stubbings, 1975. Plate VI). Abbreviations: a. ala. a.l. ala of lateral compartment. c. carina. l.c.-l. left cranio-lateral. l.l. left lateral. p.l. paries of lateral. r. rostrum. r.a. radius. ra.r. radius of rostrum. r.c.-l. right carino-lateral. r.l. right lateral. r.l. right lateral. s.c. scutum. sc.s. scuto-tergal suture. t. tergum.

Appendix 2 The structure of the branchiae and a schematic drawing of the air bubble in balanoids

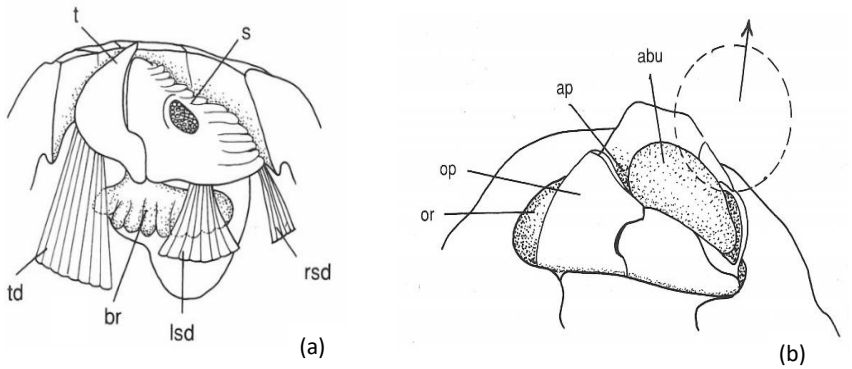


Figure 1. The structures of branchiae and schematic drawing of air bubble in balanoids. (a). Branchiae of *Perforatus perforates*. br: branchia. lsd: lateral scutal depressor. rsd: rostral scutal depressor. t: tergum. td: tergal depressor. (b). Air bubble in the aperture of *Chamaesipho brunnea* Moore, 1944. abu: air bubble. ap: aperture. op: operculum. or: orifice. (Reproduced from Anderson, 1994; p.95-96)

Appendix 3 The major non-avian predators for barnacles listed in the literature

Categories	Common name	Scientific name	Reference
Gastropod molluscs	Dog whelk	*Nucella lapillus* Linnaeus, 1758	Moore (1938); Connell (1961a)
	Emarginate dogwinkle	*Nucella emarginata* Deshayes, 1839,	Connell (1970)
	Hay's rocksnail	*Stramonita canaliculata* Gray, 1839	Connell (1970)
	Frilled dogwinkle	*Nucella lamellosa* Gmelin, 1791	Connell (1970)
	White rock shell	*Dicathais orbita* Gmelin, 1791	Luckens (1975)
	Oyster borer	*Haustrum scobina* Quoy et Gaimard, 1833	Luckens (1975)
Nudibranchs		*Onchidoris fusca* Müller, 1776	Barnes and Powell (1954)
Decapod crustaceans	Velvet crab	*Necora puber* Linnaeus, 1767	Connell (1961a)
	European green crab	*Carcinus maenas* Linnaeus, 1758,	Menge (1983)
	Jonah crab	*Cancer borealis* Stimpson, 1859,	Menge (1983)
	Atlantic rock crab	*Cancer irroratus* Say, 1817	Menge (1983)
Turbellarians		*Stylochus (Stylochus) zanzibaricus* Laidlaw, 1903	Skerman (1960)
		Imogine lateotentare Lee, Beal et Johnston, 2005	Lee, (2006)

		Imogine tripartitus Hyman, 1953	Hurley (1975)
		Pleioplana inquieta Heath et McGregor, 1912	Hurley (1975)
Geophilomorph centipedes		*Strigamia maritima* Leach, 1817	Blower (1957)
Echinoderms	Ochre sea star	*Pisaster ochraceus* Brandt, 1835	Paine (1966)
	Forbes sea star,	*Asterias forbesi* Desor, 1848	Paine (1966)
	Common sea star	*Asterias rubens* Linnaeus,	Paine (1966)
	Giant sea star	*Pisaster giganteus* Stimpson, 1857	Hurley (1975)
		Astrometis sertulifera Xantus, 1860	Hurley (1975)
Pisces	Common blenny	*Lipophrys pholis* Linnaeus, 1758	Qasim (1957)
	Ballan wrasse	*Labrus bergylta* Ascanius, 1767	Connell (1961a)
	Saithe or pollock	*Pollachius virens* Linnaeus, 1758	Nedreaas (1987)
		Oxyjulis californica Günther, 1861	Hurley (1975)

Appendix 4 The life-cycle of a typical gregarine

The life-cycle of a typical gregarine is illustrated in Fig. 1. Like other members in the infraphylum Sporozoa, they produce spores. Once the spores are ingested by an arthropod or other host, a sporozoite invades the intestinal epithelium, and goes through several stages of differentiation. At first, the single cell within the intestinal epithelium grows, intracellularly. Then the posterior region grows and eventually becomes divided into a protomerite and deutomerite. The part within the intestinal epithelium is called the epimerite, which is a structure for attachment and probably nutrition. The whole three-segmented structure is called a cephalont. At the cephalont stage, the protomerite and deutomerite develop microvilli on their outer surface. At some stage the cephalont becomes separated from its host, the epimerite is lost, and sexual reproduction begins. Two cephalonts form a permanent end-to-end association which is called syzygy and often move around in this joined condition. At this stage, gametogony begins and gamatocysts (sporocysts) form and are shed in the faeces. Under appropriate conditions, the sporocysts develop spore ducts and release spores (Smith, 1994).

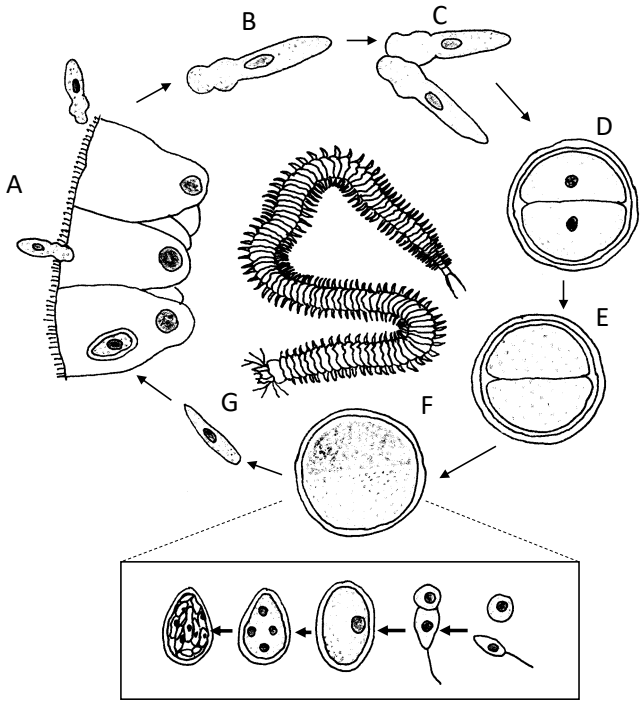


Figure 1. Schematic drawing of the life cycle of the gregarine *Lecudina* in a polychaete. Extracellular trophozoite attached to the host epithelium via an epimerit (A); free trophozoite (B); syzygy (C); early gametocyst (D); formation of gametes (E); cyst filled with separated mature gametes of both sexes (F); the inset shows development in the cyst from morphologically distinguishable female and male gametes that upon fusion grow into an oocyst, to a mature oocyst containing infectious sporozoites; free sporozoite initiating new infection (G). Redrawn from Votýpka *et al.* (1990).

Appendix 5 Systematic of the family Microphallidae proposed by Deblock (2008)

In Deblock's diagnostic key (2008) (Fig. 1), four supersubfamilies are retained under the family Microphallidae, namely the Microphallidi Ward, 1901; the Maritrematidi Nicoll, 1907; the Promicrophallidi Fischthal et Kuntz, 1981; and, the Gynaecotylidi Guschanskaya, 1952. The supersubfamily Maritrematidi is further divided into two subfamilies: the Pseudolevinseniellinae Tsai, 1955 and the Maritrematinae Nicoll, 1907. Three tribes were proposed to belong to the subfamily Maritrematinae: Maritrematini, Nicoll, 1907; Odhneriini Deblock, 1971; and finally, an artificial group of "Vitello-racemina" was created to take those species not considered immediately attributable to a tribe.

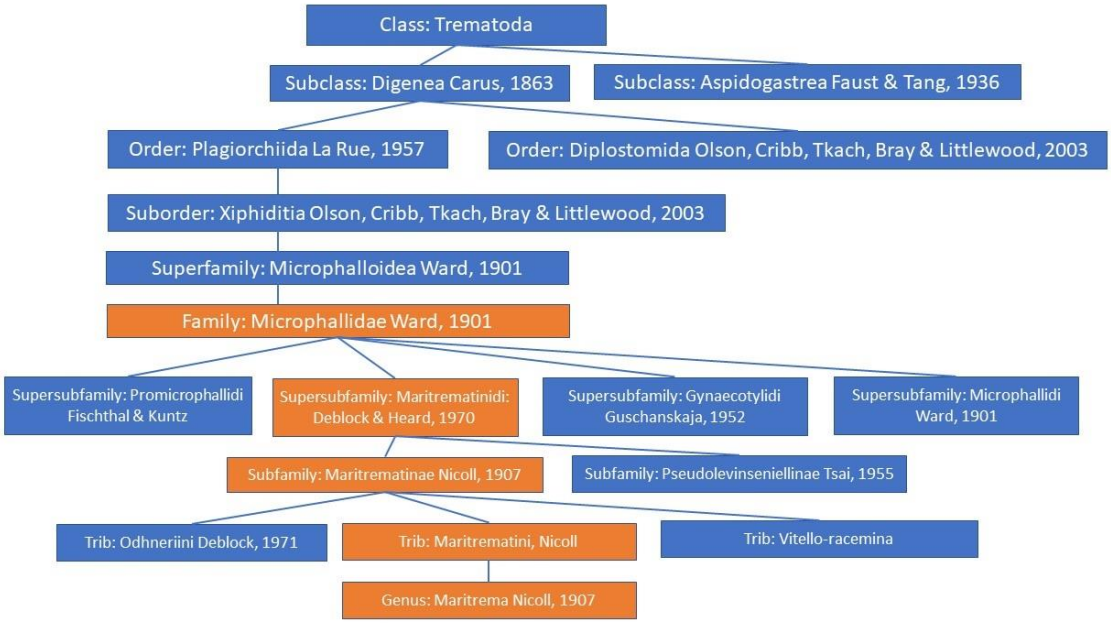


Figure 1.21. Taxonomic position of the genus *Maritrema* Nicoll, 1907 in the family Microphallidae Ward, 1901 as proposed by Deblock, 2008.

According to Deblock (2008), the full taxonomic position of the genus *Maritrema* Nicoll, 1907 is given in the following Table.

Class	**Trematoda**
Subclass	Digenea Carus, 1863
Order	Plagiorchiida La Rue, 1957
Suborder	Xiphidata Olson, Cribb, Tkach, Bray et Littlewood, 2003
Superfamily	Microphalloidea Ward, 1901
Family	Microphallidae Ward, 1901
Supersubfamily	Microphallinae Ward, 1901
Subfamily	Maritrematidi Deblock et Heard, 1970
Tribe	Maritrematini Nicoll, 1907
Genus	*Maritrema* Nicoll, 1907

Appendix 6 *Maritrema* species with mammalian or reptile host

Species	Host	Host common name	Reference
***Maritrema afanasjewi* Belopolskaia, 1952**	*Alopex lagopus* L.	Arctic fox	Deblock and Rausch (1972)
***Maritrema apodemicum* Lewis, 1966**	*Apodemus sylvaticus sylvaticus* L.	Long tail field mouse	Lewis (1966)
***Maritrema carpathica* Matskási, 1984**	*Neomys fodiens* Pennant, 1771	Eurasian water shrew	Matskási (1984)
***Maritrema feliui* Gracenea, Montoliu et Deblock, 1993**	*Crocidura russula* Hermann, 1780	Greater white-toothed shrew	Gracenea *et al.* (1993)
***Maritrema neomi* Tkach, 1998**	*Neomys anomalus* Cabrera, 1907	Mediterranean water shrew	Tkach (1998)
***Maritrema heardi* Kinsella et Deblock, 1994**	*Oryzomys palustris* Harlan, 1837	Marsh rice rat	Kinsella and Deblock (1994)
***Maritrema oocysta* Lebour, 1907**	*Hydromys chrysogaster* Geoffroy, 1804	Water rat	Deblock and Pearson (1968)
***Maritrema pulcherrima* Travassos, 1929**	*Didelphis aurita* Wied-Neuwied, 1826	Big-eared opossum	Deblock (1972)
***Maritrema pyrenaica* Deblock et Combes, 1965**	*Galemys pyrenaicus* Geoffroy, 1811	Pyrenean desman	Casanova *et al.* (1998)
	Neomys fodiens Pennant, 1771	Eurasian water shrew	Casanova *et al.* (1998)
***Maritrema sethsmithi* Canaris, 1971**	*Cryptoblepharus boutonii africanus* Sternfeld, 1918	East-African Snake-eyed Skink	Canaris (1971)

Appendix 7 *Maritrema* species with freshwater life-cycles

Species
Maritrema brevisacciferum Shimazu et Pearson, 1991
Maritrema erpobdellicola Timon-David, 1962
Maritrema feliui Gracenea, Montoliu et Deblock, 1993
Maritrema galloprovinviale Timo-David
Maritrema huillini Flores, Brugni et Pozzi, 2012
Maritrema heardi Kinsella et Deblock, 1994
Maritrema inusitata Leonov et Tsimbalyuk, 1963
Maritrema mapensis Chen, 1957
Maritrema neomi Tkach, 1998
Maritrema parainusitata Kulkina et Beljakova, 1983
Maritrema obstipum Van Cleave et Mueller, 1932
Maritrema patagonica Rauque, Flores et Brugni, 2013
Maritrema poulini Presswell, Blasco-Costa et Kostadinova, 2014
Maritrema pyrenaica Deblock et Combes, 1965.

Appendix 8 Records of some digenean and cestode parasites of Charadriiformes and Laridae birds

Multiple/single host species	Digenean and cestode species	Microphallid species	Ecological data	Locality	Reference
Multiple (focus on Charadriiformes)	Twenty-two digenean and 23 cestode	Three species (2 *Spelotrema* and 1 *Levinseniella*)	NA	British Isles	Williams, 1961
Multiple (10 species of Charadriiformes)	Fifty species: eight families of digeneans consisting of 13 species, and 5 families of cestodes consisting of 25 species	Five species (2 *Microphallus*, 1 *Levinseniella*, l *Maritrema* and 1 *Sphairiotrema*)	92% of the Charadriiformes were infected by one or more species, 74% by digeneans, 70% by cestodes, 28% by nematodes, and 29% by acanthocephalans.	Galway Bay, Ireland	Cabot (1969)
Single: common snipe (*Capella gallinago* Linnaeus, 1758)	Fifty-two digeneans and 46 cestodes	Three species (1 *Levinseniella* and 2 *Maritrema*)	NA	World wide	Threlfall (1970).
Single: long-billed curlew (*Numenius americanus* Bechstein, 1812)	Sixty-one digeneans	Five species (1 *Levinseniella*, 1 *Maritrema*, 2 *Spelotrema*)	NA	World wide	Dronen Jr. and Badley (1979)
Single: dunlin (*Calidris alpina* Linnaeus, 1758)	Six digeneans and 8 cestodes	Two species (1 *Ascorhytis*, 1 *Levinseniella*)	Some temporal and spatial distribution patterns described	California, USA	Ching (1990)

Multiple/single host species	Digenean and cestode species	Microphallid species	Ecological data	Locality	Reference
Single: western willet (*Tringa semipalmata inornata* Brewster, 1887)	Seven digeneans and 2 cestodes	Four species (1 *Ascorhytis*, 1 *Levinseniella*, 1 *Maritrema*, 1 *Odhneria*)	Microphallids occurred in high prevalence and intensity.	California, USA	Ching (1990)
Single: greater yellowlegs (*Tringa melanoleuca* Gmelin, 1789)	Fourteen species: 10 digeneans and 4 cestodes	Zero species	The distribution of helminths was clumped and over dispersed, and the community consisted of 2 core, 3 secondary, and 4 satellite species	Texas and New Mexico, USA	Secord and Canaris (1993),
Multiple: Red-capped plover (*Charadrius ruficapillus* Temminck, 1822), Masked lapwing (*Vanellus miles* Boddaert, 1783), Ruddy turnstone (*Arenaria interpres* Linnaeus, 1758),	Twenty-two species: *C. ruficapillus*: 8 digeneans and 4 cestodes *V. miles*: 2 digeneans and 3 cestodes *A. interpres*: 6 digeneans and 7 cestodes *C. ferruginea*: 1 digenean and 4 cestodes	*C. ruficapillus*: 6 species *V. miles*: 0 species *A. interpres*: 5 species *C. ferruginea*: 0 species	Twenty-five were considered generalists. One to 3 species were dominant in each host. Similarities between the resident *C. ruficapillus* and migrant *A. interpres* were low (32.7%), and *A. interpres* had significantly higher mean numbers of species and helminths. Two microphallids and one cestode were found to be dominant species in the community of *C. ruficapillus* and *A. interpres*. One cestode was dominant in the community of *C. ferruginea* and 1 cestode and 1 nematode were dominant in *V. miles*.	King Island, Tasmania	Canaris and Kinsella, 1998

Multiple/single host species	**Digenean and cestode species**	**Microphallid species**	**Ecological data**	**Locality**	**Reference**
Curlew sandpiper (*Calidris ferruginea* Pontoppidan, 1763).					
Multiple: Ruddy turnstone (*A. interpres*), Snowy plover (*Charadrius alexandrines* Linnaeus, 1758), Semipalmated plover (*Charadrius semipalmatus* Bonaparte, 1825), White-rumped sandpiper (*Calidris fuscicollis* Vieillot, 1819), Black-billed plover (*Pluvialis squatarola* Linnaeus, 1758).	Ten species: *A. interpres*: 4 cestodes and 2 digeneans, *C. alexandrines*: 2 cestodes and 1 digeneans, *C. semipalmatus*: 2 cestodes and 2 digeneans, *C. fuscicollis*: 2 cestodes, *P. squatarola*: 1 cestode and 1 digenean	*A. interpres*: 2 species, *C. alexandrines*: 1 species, *C. semipalmatus*: 2 species, *C. fuscicollis*: 0 species, *P. squatarola*: 1 species	Cestodes were the dominant (8 species), followed by digenean (3 species). Helminth species were clumped and not evenly distributed among hosts. Most of them were generalists.	Belize	Canaris and Kinsella, 2001

Multiple/single host species	**Digenean and cestode species**	**Microphallid species**	**Ecological data**	**Locality**	**Reference**
Multiple: White-fronted plover (*Charadrius marginatus* Vieillot, 1818), Ruddy turnstone (*A. interpres*).	Eighteen species: *C. marginatus*: 6 cestodes, 4 digeneans, *A. interpres*: 6 cestodes, 4 digeneans,	*C. marginatus*: 4 species, *A. interpres*: 3 species,	Similarity indices between the resident (*C. marginatus*) and migratory (*A. interpres*) host were low, but they shared two microphallid species. All digeneans of *C. marginatus* were microphallids, Microphallids accounted for 3/4 of the digeneans in *A. interpres*. A large majority are generalists and aggregated dispersion observed. By compiled data from 17 studies, the authors concluded that digeneans were usually abundant in marine habitat and cestodes in the freshwater habitat.	Namibia	Canaris *et al*,, 2003
Single: spotted sandpiper (*Actitis macularius* Linnaeus, 1766)	Ten species: 7 digeneans and 3 cestodes.	Four species (1 *Gynaecotyla*, 1 *Levinseniella*, 1 *Maritrema*, 1 *Microphallus*	the cestode *Anomotaneia hypoleuci* was the most prevalent species, and the microphallids *Microphallus papillorobustus* (Rankin, 1940) Baer, 1944 and *M. subdolum* were the most abundant species. Most of the helminths were generalists.	Canada	Didyk *et al*., 2007b

Multiple/single host species	**Digenean and cestode species**	**Microphallid species**	**Ecological data**	**Locality**	**Reference**
Multiple: Black turnstone (*Arenaria melanocephala* Vigors, 1829), Ruddy turnstone (*A. interpres*), Dunlin (*C. alpine*).	Thirty-three species: *A. melanocephala*: 12 cestodes, 5 digeneans, *A. interpres*: 13 cestodes, 9 digeneans, *C. alpine*: 12 cestodes, 6 digeneans.	*A. melanocephala*: 0 sepcies, *A. interpres*: 2 species, *C. alpine*: 1 species.	Cestodes were the most prevalent and abundant. Congeneric hosts had more similar parasite communities and black turnstone had significantly higher species richness and abundance than the other two hosts. Difference between congeneric black turnstone and ruddy turn stone are likely to related to different diet and habitats. The infection patterns of these three hosts were similar, with one dominant species, 4-5 associates, and more less-predictable species. Furthermore, high diversity, low evenness, low species richness and continued recruitments of small numbers of rare helminths was consistent over time	Alaska	Canaris & Kinsella, 2007
Single: Ruddy turnstone (*A. interpres*)	Seventy-six species: 46 digeneans and 30 cestodes	Microphallids accounted for 50% of the recorded digenean species	Helminths communities consist of large numbers of 2-3 dominant species and small numbers of rare species. Dominant species tend to differ from region to region. The exaggerated proportion of microphallids may due to intense feeding during migration.	World wide	Didyk *et al*., 2007a
Single: Herring gull (*Larus argentatus* Pontoppidan, 1763)	Twenty-one species: 10 digeneans and 11 cestodes	Two species (2 *Spelotrema*)	Adult birds were generally more heavily infected than the chicks. Only several species dominant and others were	North Wales	Threlfall, 1967

Multiple/single host species	Digenean and cestode species	Microphallid species	Ecological data	Locality	Reference
			rare. Several species had a seasonal pattern of appearance.		
Single: Herring gull (*L. argentatus* Pontoppidan, 1763)	Nine species: 8 digeneans and 1 cestode	Three species: (2 *Spelotrema*, 1 *Maritrema*)	One species dominant and others were at low abundance.	Northern Ireland	Irwin & Prentice, 1976
Single: Olrog's gull (*Larus atlanticus* Olrog, 1958)	Five digenean species	Four species: 2 *Maritrema*, 1 *Odhneria*, 1 *Levinseniella*	*Maritrema bonaerensis* were found with a high prevalence in chicks.	Argentina	La Sala *et al*., 2009
Single: Kelp gull (*Larus dominicanus* Lichtenstein, 1823)	Twelve species: 10 digeneans and 2 cestodes	Three species: 2 *Maritrema* and 1 *Odhneria*	Relationship of the availability of different diet and the abundance of particular helminth species was noticed. Higher number and abundance of the helminth species reflect high prey diversity.	Argentina	Diaz and Cremonte, 2011

Appendix 9 Mayer's Paracarmine stain

1. Protocol:

Collect live worms and observe directly under dissection microscope or observe by wet mount under optic microscope. Fix by 80% ethanol for at least 1 hour. Stain in Mayer's paracarmine for about 10~20 minutes. Transfer to 80% ethanol for 5 minutes. Transfer to 50% acid alcohol for about 30 seconds. Transfer to serious of ethanol for 10 minutes each (80%, 90%, then 100% for twice). Clear the sample with clove oil for at least 30 minutes. Fix the sample in Canada balsam permanently. All steps can be operated in embryo glass.

2. Reagents:

To make 100 mL of Mayer's Paracarmine: add 1 g carminic acid, 0.5 g aluminium chloride and 4 gm calcium chloride to 100 ml 70% ethanol, dissolve well. To make 160 mL of 50% acid alcohol, add 2 mL of hydrochloric acid (concentration about 11.5 M) to 60 mL of distilled water and 100 mL of 80% ethanol.

(Source: Andy Shinn, Institute of Aquaculture, University of Stirling)

Appendix 10 Comparison of reproductive status of infected and non-infected *Semibalanus balanoides* from Machrihanish and South Queensferry

Locality				Gravid -	Gravid +	Total	Fisher's Exact Test	Sig.
Machrihanish	Digenean infection	-	Count	20	22	42	0.493	NS
			Expected Count	19.1	22.9	42.0		
		+	Count	0	2	2		
			Expected Count	0.9	1.1	2.0		
	Total		Count	20	24	44		
			Expected Count	20.0	24.0	44.0		
South Queensferry	Digenean infection	-	Count	7	4	11	0.395	NS
			Expected Count	5.8	5.2	11.0		
		+	Count	4	6	10		
			Expected Count	5.2	4.8	10.0		
	Total		Count	11	10	21		
			Expected Count	11.0	10.0	21.0		
Total	Digenean infection	-	Count	27	26	53	0.346	NS
			Expected Count	25.3	27.7	53.0		
		+	Count	4	8	12		
			Expected Count	5.7	6.3	12.0		
	Total		Count	31	34	65		
			Expected Count	31.0	34.0	65.0		

Appendix 11 Negative binomial regression (with log link function) for the abundance of metacerariae in *Semibalanus balanoides* collected from the vicinity of the Dunbar Leisure Pool (trip no.3)

			95% Wald Confidence Interval		Hypothesis Test				95% Wald Confidence Interval for Exp(B)	
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square	df	Sig.	Exp(B)	Lower	Upper
(Intercept)	-0.54	0.17	-0.87	-0.22	10.89	1.00	0.001	0.58	0.42	0.80
Cliff below birds	2.43	0.20	2.05	2.82	152.58	1.00	<0.001	11.38	7.74	16.74
Cliff side lower edge	-0.97	0.29	-1.53	-0.41	11.36	1.00	<0.001	0.38	0.22	0.67
Open bay low water edge	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									
(Negative binomial)	1[b]									

Dependent Variable: Cyst numbers

Model: (Intercept), Site

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Appendix 12 Negative binomial regression (with log link function) for the abundance of metacerariae in *Semibalanus balanoides* collected from Dunbar Red Rock (trip no.5)

			95% Wald Confidence Interval		Hypothesis Test				95% Wald Confidence Interval for Exp(B)	
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square	df	Sig.	Exp(B)	Lower	Upper
(Intercept)	2.180	.0352	2.111	2.249	3825.622	1	<0.001	8.846	8.256	9.479
site=2	1.858	.0377	1.785	1.932	2435.010	1	<0.001	6.414	5.958	6.905
site=3	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Dependent Variable: Cyst numbers

Model: (Intercept), site

a. Set to zero because this site is the baseline comparator

b. Fixed at the displayed value.

Appendix 13 Negative binomial regression (with log link function) for the abundance of larval cestodes in *Semibalanus balanoides* collected from Dunbar Red Rock (trip no.5)

			95% Wald Confidence Interval		Hypothesis Test				95% Wald Confidence Interval for Exp(B)	
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square	df	Sig.	Exp(B)	Lower	Upper
(Intercept)	-3.125	0.500	-4.105	-2.145	39.052	1	<0.001	0.044	0.016	0.117
[Site=2]	1.777	0.537	0.725	2.830	10.953	1	0.001	5.915	2.064	16.948
[Site=3]	0a	.	.	.	.	.	.	1	.	.
(Scale)	1b									

Dependent Variable: Cyst numbers

Model: (Intercept), Site

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Appendix 14 ITS2 rDNA for *Cryptocotyle lingua* (457 bps)

TTAGTGATATGCTTAAGTTCAGCGGGTATTCACGTCTGATCCGAGGTCAGGAAAGTTAAGCACCGAG
GTGCAAAACAAAATTGCATCGGATGCATTGCCACAACTGAAGCTTCAACCACAGACAAAGAAACGAC
AACGGAGCGCGCAAATTTACTACCACTGAGCCACGACTCCGCCGCCACTCCCTCATCCAGGCAGTCA
GCCAAGATATGGGTGCGTCCGGCACATTGGGGAAAAGCCATAGATCCGGCACCCCGCACAAATGCG
CAGGGAAATCATGCCAGCTGGCAAGACCCAAGCCACGACTTTTTGGGCGTCGTGATAGTTTATAAGC
CGACCCTCGGACAGGCGTGGCCACAGGAAAACCCATGGCCGCAATATGCGTTCAAGATGTCGATGT
TCAAAGCAGTATGCAGTTCACATTAATTCACACAGTTGGCTGCGCTCTTCATCGACA

Top five hits on BLAST (23/10/2020)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Cryptocotyle sp. n. YVT-2019 isolate 17.1 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S large subunit ribosomal RNA gene, partial sequence	767	767	100%	0.0	96.94%	MH025622.1
Cryptocotyle sp. n. YVT-2019 isolate 17.2 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S large subunit ribosomal RNA gene, partial sequence	761	761	100%	0.0	96.72%	MH025623.1
Trematoda sp. RP-2017 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	761	761	91%	0.0	99.76%	KY620038.1
Apophallus donicus isolate MK15 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	712	712	100%	0.0	94.76%	MF447672.1
Apophallus donicus isolate AP5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	712	712	100%	0.0	94.76%	MF438058.1

Appendix 15 ITS2 rDNA for *Echinostoma* sp. (696 bps)

TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTAAACTGCATACTGCTTTGAACATCGACAT
CTTGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAATT
ATCACGACGCCCAAAAAGTCGTGGCTTGGGTCTTGCCAGCTGGCGTGATTTCCTCTATGAGCAATCA
TGTGAGGTGCCAGATCTATGGCGTTTCCCCAATGTATCCGGACGCATCCTCGTCTTGGCTGAAGGCC
ATGGTGCGGTGTGGTAACGGAATCGTGGTTTAATATGGCTATGCCTCGTAATCAGCGTGTTTGGCGC
TATCTAGTCAGCATGCATATGATTTTTGAAGGGAGTCCATACAAGGCACGTTTCGTTATTTGCGCTTC
ATCGTTGGTTGAATGCTGGCTTGGCAATGCATCCGATTCATATTTGAACGCAACCAAAATGGTTAGA
GGTCTCTGTGGGGTACGGTTTCGGAGTGCAGATGTACTTGCTTTGGCCCGTTTACAGCATGTTTGGC
GCTATCTTAGTCGGCATGCACATGTCTTCGGGTGTAGTAATGAGTTCTTCACTCCGGGATTCGTTCTC
TGTAGTTGACTAAAGACTAAGTTTTGGAAATGCATGTTCTCCTGACCTCGGATCAGACGTGATTACCC
GCTGAACTTAAGCATATCACTAA

Top five hits on BLAST (23/10/2020)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Echinostoma trivolvis isolate Etriv3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	564	742	73%	4e-156	95.24%	GQ463126.1
Echinostoma trivolvis isolate Etriv2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	564	742	73%	4e-156	95.24%	GQ463125.1
Echinostoma trivolvis isolate Etriv1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	564	742	73%	4e-156	95.24%	GQ463124.1
Echinostoma trivolvis strain 11B internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	564	742	73%	4e-156	95.24%	AF067851.1
Echinostoma paraensei strain Sumidouro internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	560	736	72%	5e-155	94.96%	AF336232.1

Appendix 16 ITS2 rDNA for *Microphallus* sp. (442 bps)

TTAGTGATATGCTTAAGTTCAGCGGGTAATCACGTCTGATCCGAGGTCAGGAAATAAAATGGAAAGC
GCACCGATTGATCAATCACTGTACTGGTGCAACTAAACCAAAGCCACGAACAACCTACGAGTAATGA
CAGAGCGCGCTTCTATAATTAACCATTAAGCCACGACTCCGCCACCCAAATGCAGGTGTCCGGACAC
ATTAGGGAAAAGCCATAGATCTGGCACCTCACACTCCACAACCATATGGACGGAGGAAATCACGCCA
GCTGGCAAGACCCAAGCCACGACTTTATGGGCGTCGTGATAGTTTATAAGCCGACCCTCGGACAGGC
GTGGCCACAGGCAAACCCATGGCCGCAATATGCGTTCAAGATGTCGATGTTCAAAGCAGTATGCAGT
TCACATTAATTCACGTAGCTGGCTACGCTCTTCATCGACA

Top five hits on BLAST (23/10/2020)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Microphallus pygmaeus isolate Mpyg 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	817	817	100%	0.0	100.00%	HM584190.1
Microphallus sp. nov. 1 IBC-2010 isolate Mkur8C 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	795	795	100%	0.0	99.10%	HM584188.1
Microphallus sp. nov. 1 IBC-2010 isolate Mkur8B 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	795	795	100%	0.0	99.10%	HM584187.1
Microphallus sp. nov. 1 IBC-2010 isolate Mchul2 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	791	791	100%	0.0	98.87%	HM584185.1
Microphallus pseudopygmaeus isolate Mpse 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	778	778	100%	0.0	98.42%	HM584198.1

Appendix 17 Records of cestodes from some seabirds, with a focus on charadriiform species

Cestodes	Hosts	Localities	Reference
Order Pseudophyllidea Carus, 1863 **Family** Diphyllobothriidae Lühe, 1910, Tetrabothriidae Braun, 1900 **Order** Cyclophyllidea Braun, 1900 **Family** Davaineidae Fuhrmann, 1907 Progynotaenidae Fuhrmann, 1936 Hymendolepididae Railliet et Henry, 1909 Dilepididae Railliet et Henry, 1909	Several species of sea birds	Coast of west Wales	Williams (1961)
Family Davaineidae Fuhrmann, 1907, Progynotaeniidae Fihrmann, 1936, Hymenolepididae Fuhrmann, 1907, Dilepididae Fuhrmann, 1907, Choanotaeniidae	Charadriiform birds	Galway Bay, UK	Cabot, 1969
Genus *Amoebotaenia* Cohn, 1899 *Anomotaenia* Cohn, 1900 *Australiolepis* *Choanotaenia* Railliet, 1896 *Diorchis* Clerc, 1903 *Echinocotyle* Blanchard, 1891 *Haploparaxis* *Hymenolepis* Weinland, 1858 *Microsomacanthus* Lopez-Neyra, 1942 *Paricterotaenia* Fuhrmann, 1932 *Profimbriaria* Wolffhügel, 1936 *Shipleyua* *Taenia* Linnaeus, 1758 *Thaparea* Johri, 1953	*Capella gallinago* (Linnaeus) (common snipe)	World wide	William, 1970
Genus *Acanthocirrus* Fuhrmann, 1907 *Anomotaenia* Cohn, 1900 *Aploparaksis* Clerc, 1903 *Austrobilharzia* Johnston, 1917 *Dictymetra* Clark, 1952 *Echinocotyle* Blanchard, 1891 *Kowalewskiella* Baczynska, 1914 *Nadejdolepis* Spasskii et Spasskaya, 1954 *Ophryocotyle* Friis, 1870 *Shipleya* *Trichocephaloidis* Sinitzin, 1896	*Arenaria melanocephala* (black turnstone), *Arenaria interpres* (ruddy turnstone) *Calidris alpine* (dunlin)	Bristol Bay, Alaska	Canaris & Kinsella, 2007

Cestodes	Hosts	Localities	Reference
Wardium Mayhew, 1925			
Genus *Aploparaksis* Clerc, 1903 *Nadejdolepis* Spasskii et Spasskaya, 1954 *Protergynotaenia*	*Charadrius ruficapillus* (red-capped plover)	King Island, Tasmania	Canaris & Kinsella, 1998
Genus *Chitinorecta* Meggitt, 1927 *Davainea* Blanchard, 1891 *Gyrocoelia* Fuhrmann, 1899 *Megalacanthus* Moghe 1926 *Helicoductus* Deblock et Canaris, 2001 *Wardium* Mayhew, 1925	*Charadrius marginatus* (white-fronted plovers)	Coast of Namibia	Canaris *et al.* 2003
Genus *Anomotaenia* Cohn, 1900 *Tetrabothrius* Rudolphi, 1819	*Larus dominicanus* (kelp gull)	Northern Patagonian coast	Diaz *et al.*, 2011
Genus *Anomotaenia* Cohn, 1900 *Aploparaksis* Clerc, 1903 *Dictymetra* Clark, 1952 *Echinocotyle* Blanchard, 1891 *Hymenolepis* Weinland, 1858 *Nadejdolepis* Spasskii et Spasskaya, 1954 *Ophryocotyle* Friis, 1870 *Wardium* Mayhew, 1925	*Arenaria interpres* (ruddy turnstone)	Worldwide	Didyk *et al.*, 2007a
Genus *Anomotaenia* Cohn, 1900 *Kowalewskiella* Baczynska, 1914 *Nadejdolepis* Spasskii et Spasskaya, 1954	*Actitis macularius* (spotted sandpiper)	New Brunswick, Canada	Didyk *et al.*, 2007b
Genus *Aploparaksis* Clerc, 1903 *Dicranotaenia* Railliet, 1892 *Echinocotyle* Blanchard, 1891 *Nadejdolepis* Spasskii et Spasskaya, 1954 *Retinometra* Spasski, 1955 *Trichocephaloides* Braun, 1896	*Calidris alpine* (Dunlin)	Bodega Bay and Bolinas Lagoon, California	Ching, 1990

Appendix 18 Descriptive statistics for replicate sampling

		Abundance of first replicate	Abundance of second replicate
N	Valid	120	120
	Missing	0	0
Mean		20.01	17.25
Std. Error of Mean		1.77	1.73
Median		11	9.5
Std. Deviation		19.45	18.98
Variance		378.5	360.37
Range		99	110
Minimum		0	0
Maximum		99	110
Sum		2401	2070

Appendix 19 Descriptive statistics for fresh and formaldehyde fixed samples

		cyst number 1(fresh samples)		cyst number 2(fixed samples)	
Statistic			Std. Error		Std. Error
Mean		24.97	2.652	28.26	3.488
95% Confidence Interval for Mean	Lower Bound	19.72		21.35	
	Upper Bound	30.22		35.17	
5% Trimmed Mean		21.55		22.25	
Median		12		15	
Variance		843.831		1460.244	
Std. Deviation		29.049		38.213	
Minimum		0		1	
Maximum		133		236	
Range		133		235	
Skewness		1.744	0.221	3.245	0.221
Kurtosis		2.672	0.438	12.566	0.438

Appendix 20 Infection indices (prevalence, mean abundance, and mean intensity) for local scale study. Sites are arranged from land to shore

Site	**22**	**23**	**24**	**4**	**7**	**11**	**12**	**14**	**15**	**18**	**19**	**20**
Prevalence	95	97.5	100	100	95	100	100	95	95	100	80	95
Mean abundance	18.80	36.23	15.38	33.35	32.63	20.33	12.95	18.48	30.50	12.75	11.65	8.93
SD	17.75	33.17	12.92	37.87	26.07	20.55	14.33	21.02	22.93	17.43	13.80	8.03
Mean Intensity	19.79	37.15	15.38	33.35	34.34	20.33	12.95	19.45	32.11	12.75	14.56	9.39
SD	17.66	33.07	12.92	37.87	25.61	20.55	14.33	21.13	22.39	17.43	13.99	7.97

Appendix 21 Descriptive statistics of abundance of local scale study. Sites are arranged from land to shore

statistics	Site											
	22	23	24	4	7	11	12	14	15	18	19	20
Mean abundance	18.80	36.23	15.38	33.35	32.63	20.33	12.95	18.48	30.50	12.75	11.65	8.93
95%CI	13.12	25.62	11.24	21.24	24.29	13.75	8.37	11.75	23.17	7.18	7.24	6.36
	24.48	46.83	19.51	45.46	40.96	26.90	17.53	25.20	37.83	18.32	16.06	11.49
Variance	314.99	1100.33	166.99	1434.23	679.83	422.12	205.28	442.00	525.70	303.68	190.39	64.53
SD	17.78	33.17	12.92	37.87	26.07	20.55	14.33	21.02	22.93	17.43	13.80	8.00
Minimum	0	0	1	2	0	1	1	0	0	1	0	0
Maximum	61	120	50	133	97	110	58	99	86	68	50	33

Appendix 22 Descriptive statistics of barnacle size indices in the local scale study

			Statistic	Std. Error				Statistic	Std. Error
MBasalL N=474	Mean (μm)		5067.55	46.192	**MOperculumL N= 474**	Mean (μm)		2456.35	19.340
	95% Confidence Interval for Mean	Lower Bound	4976.78			95% Confidence Interval for Mean	Lower Bound	2418.35	
		Upper Bound	5158.31				Upper Bound	2494.36	
	Median		4992.91			Median		2428.41	
	Variance		1011385.4			Variance		177287.66	
	Std. Deviation		1005.68			Std. Deviation		421.06	
	Minimum		2697.83			Minimum		1307.45	
	Maximum		9006.79			Maximum		4179.19	
	Range		6308.96			Range		2871.74	
	Skewness		0.53	0.11		Skewness		0.55	0.11
	Kurtosis		0.58	0.22		Kurtosis		0.70	0.22

Appendix 23 Estimated marginal mean for abundance (x^0.25 transformed) by sites in the local scale study

Dependent Variable: cyst number (x^0.25 transformed)

			95% Confidence Interval	
Sites	Mean[a]	Std. Error	Lower Bound	Upper Bound
22	1.964	0.065	1.836	2.093
23	2.191	0.067	2.059	2.323
24	2.248	0.112	2.028	2.468
4	2.076	0.067	1.944	2.208
7	2.225	0.066	2.095	2.354
11	2.087	0.073	1.943	2.231
12	1.800	0.076	1.651	1.949
14	1.878	0.065	1.750	2.005
15	2.132	0.067	2.000	2.264
18	1.631	0.065	1.503	1.759
19	1.321	0.077	1.170	1.473
20	1.577	0.068	1.443	1.711

a. Covariates appearing in the model are evaluated at the following values: MOperculumL = 2456.3532.

Appendix 24 Infection indices of the whole area study

Site	**1**	**2**	**3**	**5**	**6**	**9**	**12**	**15**	**16**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Prevalence	90	95	100	97.5	97.5	100	100	100	100	100	95	100	100	100	82.5	100
Abundance	8.40	26.55	125.05	14.20	24.13	54.03	18.53	60.18	12.65	30.85	8.23	18.28	21.43	30.25	5.88	28.55
S.D.	9.51	35.87	63.39	17.78	18.76	26.98	15.71	37.12	17.24	13.66	6.29	12.60	12.11	30.95	5.99	19.77
Intensity	9.33	27.95	125.05	14.56	24.74	54.03	18.53	60.18	12.65	30.85	8.66	18.28	21.43	30.25	7.12	28.55
S.D.	9.58	36.28	63.39	17.86	18.59	26.98	15.71	37.12	17.24	13.66	6.16	12.60	12.11	30.95	5.88	19.77

Appendix 25 Descriptive statistics of abundance of whole area study

	Sampling sites															
Statistic	1	2	3	5	6	9	12	15	16	18	19	20	21	22	23	24
Mean abundance	8.40	26.55	125.05	14.20	24.13	54.03	18.53	60.18	12.65	30.85	8.23	18.28	21.43	30.25	5.88	28.55
95%CI	5.36	15.08	104.78	8.51	18.13	45.40	13.50	48.31	7.14	26.48	6.21	14.24	17.55	20.35	3.96	22.23
	11.44	38.02	145.32	19.89	30.12	62.65	23.55	72.04	18.16	35.22	10.24	22.31	25.30	40.15	7.79	34.87
Median	5.00	11.50	119.50	6.00	19.00	47.00	14.00	57.00	6.00	29.00	6.00	15.50	21.00	19.50	5.00	23.00
Variance	90.40	1286.87	4017.84	316.06	351.96	727.77	246.77	1377.53	297.26	186.64	39.62	158.82	146.61	957.94	35.86	390.82
S.D.	9.508	35.873	63.386	17.778	18.761	26.977	15.709	37.115	17.241	13.662	6.294	12.602	12.108	30.951	5.988	19.769
Minimum	0	0	13	0	0	4	1	8	1	7	0	2	1	1	0	4
Maximum	32	186	287	76	83	120	78	141	98	62	24	60	43	121	32	96

Appendix 26 Descriptive statistics for maximum operculum length of whole area study

	sampling sites															
Statistic	1	2	3	5	6	9	12	15	16	18	19	20	21	22	23	24
Mean	2861.41	3333.61	4088.36	2370.15	2602.46	2556.59	2996.47	2618.50	2848.28	2317.09	2318.54	2607.95	3255.93	3624.82	2908.43	2682.96
Median	2813.25	3333.39	4007.43	2304.88	2581.76	2521.66	2898.53	2648.67	2754.06	2282.32	2296.48	2565.37	3298.48	3667.19	2827.69	2670.41
Variance	246907.26	449216.07	317563.12	169106.82	137502.61	83722.72	229281.26	142437.23	207633.00	73720.75	143432.76	122955.31	210541.64	484289.19	243530.87	87823.72
S.D.	496.90	670.24	563.53	411.23	370.81	289.35	478.83	377.41	455.67	271.52	378.73	350.65	458.85	695.91	493.49	296.35
Min.	2055.81	1679.01	2664.60	1481.05	1381.97	1864.97	2171.56	1724.96	2172.56	1678.48	1423.33	1907.62	1969.71	2361.02	1946.69	1912.42
Max.	3970.26	5144.12	5664.50	3302.88	3304.23	3263.04	4222.67	3275.43	3651.41	2837.27	3209.00	3451.55	3988.99	5220.87	4230.89	3339.73
Skewness	0.44	0.06	0.08	0.40	-0.74	0.21	0.58	-0.26	0.26	-0.13	0.12	0.36	-0.64	0.11	0.57	-0.20
Kurtosis	-0.51	0.95	0.82	-0.17	1.82	0.08	-0.07	-0.43	-1.35	-0.51	0.19	-0.07	0.57	-0.42	0.47	0.47

Appendix 27 Estimated marginal means for abundance (x^0.25 transformed) of whole area study

Dependent Variable: Abundance (X^0.25 transformed)				
Site rank	Mean[a]	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
2.0	2.689	0.120	2.454	2.925
5.0	1.962	0.093	1.779	2.082
7.0	2.904	0.063	2.780	3.027
9.0	2.164	0.066	2.035	2.293
10.0	2.932	0.078	2.779	3.084
10.5	1.745	0.052	1.643	1.847
12.0	2.372	0.062	2.250	2.494
13.0	1.945	0.054	1.840	2.050
13.5	1.447	0.052	1.345	1.549
14.0	2.343	0.065	2.216	2.470
15.0	2.212	0.083	2.049	2.375
18.0	1.595	0.077	1.444	1.746
19.0	1.684	0.063	1.560	1.808
19.5	1.575	0.052	1.473	1.677
20.0	2.818	0.125	2.573	3.062
21.0	1.881	0.068	1.747	2.014

a. Covariates appearing in the model are evaluated at the following values: Operculum length = 2874.4723.

Appendix 28 Correlations between the variables for multiple regression of abundance of whole area study

		Cyst number(x^0.25)	Operculum L	Cirrus L	Cirrus W	Cirrus W/L ratio	Mollusc coverage	Mollusc species	Algae coverage	Barnacle coverage	Density of sampled rock
Pearson Correlation*	Cyst number(x^0.25)	1.000									
	Operculum L	**0.587**	1.000								
	Cirrus L	**0.591**	0.279	1.000							
	Cirrus W	0.222	**0.828**	0.161	1.000						
	Cirrus W/L ratio	-0.194	**0.518**	**-0.500**	**0.761**	1.000					
	Mollusc coverage	0.236	**-0.568**	0.275	**-0.766**	**-0.845**	1.000				
	Mollusc species	0.236	**-0.568**	0.275	**-0.766**	**-0.845**	**1.000**	1.000			
	Algae coverage	-0.139	-0.242	-0.014	-0.248	-0.168	0.284	0.284	1.000		
	Barnacle coverage	**-0.726**	-0.142	-0.483	0.181	0.488	**-0.633**	**-0.633**	-0.273	1.000	
	Density of sampled rock	0.470	**0.858**	0.167	**0.727**	**0.511**	**-0.530**	**-0.530**	0.085	-0.277	1.000
Sig. (1-tailed)	Cyst number(x^0.25)										
	Operculum L	0.001									
	Cirrus L	0.001	0.093								
	Cirrus width	0.148	0.000	0.226							
	Cirrus W/L ratio	0.182	0.005	0.006	0.000						
	Mollusc coverage	0.134	0.002	0.097	0.000	0.000					
	Mollusc species	0.134	0.002	0.097	0.000	0.000	0.000				
	Algae coverage	0.258	0.127	0.474	0.121	0.217	0.089	0.089			
	Barnacle coverage	0.000	0.254	0.008	0.199	0.008	0.000	0.000	0.099		
	Density of sampled rock	0.010	0.000	0.218	0.000	0.005	0.004	0.004	0.346	0.095	

*Value >0.5 was marked in bold

Appendix 29 Model summary for multiple regression of abundance of whole area study

Model	R square	Durbin-Watson	F (ANOVA)	Sig. (ANOVA)	Included variables (purple variables are non-significant)	Excluded variables
Enter	0.898	2.028	16.460	<0.001	Density of sampled rock, algae coverage, cirrus length, barnacle coverage, cirrus width, operculum length, mollusc species, cirrus w/l ratio	Mollusc coverage
Enter	0.803	1.994	12.205	<0.001	Barnacle coverage, operculum length, algae coverage, cirrus length, mollusc coverage, cirrus w/l ratio, mollusc species	Cirrus width, density of sampled rock
Enter	0.782	1.926	16.549	<0.001	Barnacle coverage, operculum length, algae coverage, mollusc coverage, cirrus w/l ratio	Cirrus width, cirrus length, density of sampled rock
Stepwise	0.843	1.836	35.775	<0.001	Barnacle coverage, operculum length, density of sampled rock	Cirrus length, cirrus width, cirrus w/l ration, mollusc coverage, mollusc species, algae coverage.
Backward	0.883	1.755	21.374	<0.001	Density of sampled rock, algae coverage, cirrus length, barnacle coverage, cirrus width, operculum length, mollusc species, cirrus w/l ratio	Mollusc coverage, density of sampled rock, mollusc species
Forward	0.843	1.836	35.775	<0.001	Barnacle coverage, operculum length, density of sampled rock	Cirrus length, cirrus width, cirrus w/l ratio, mollusc coverage, mollusc species, algae coverage

Appendix 30 Comparison between mean abundance, Lloyd's mean crowding, variance-to-mean ratio, Lloyd's patchiness index and host density

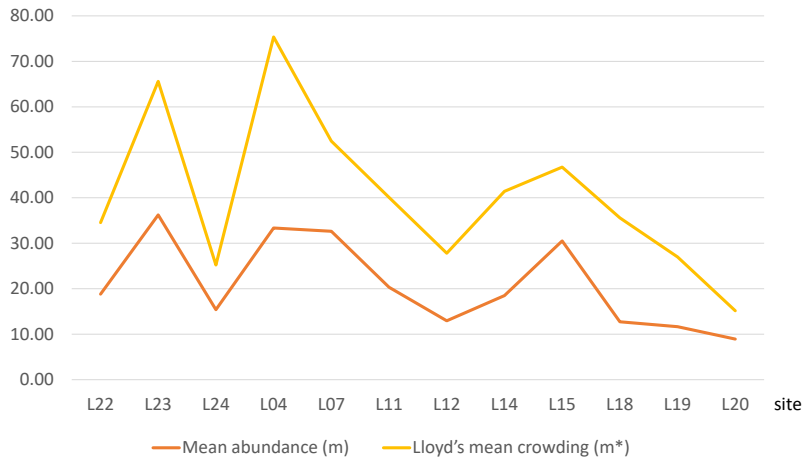


Figure 1. Comparison between mean abundance and Lloyd's mean crowding for Local scale study

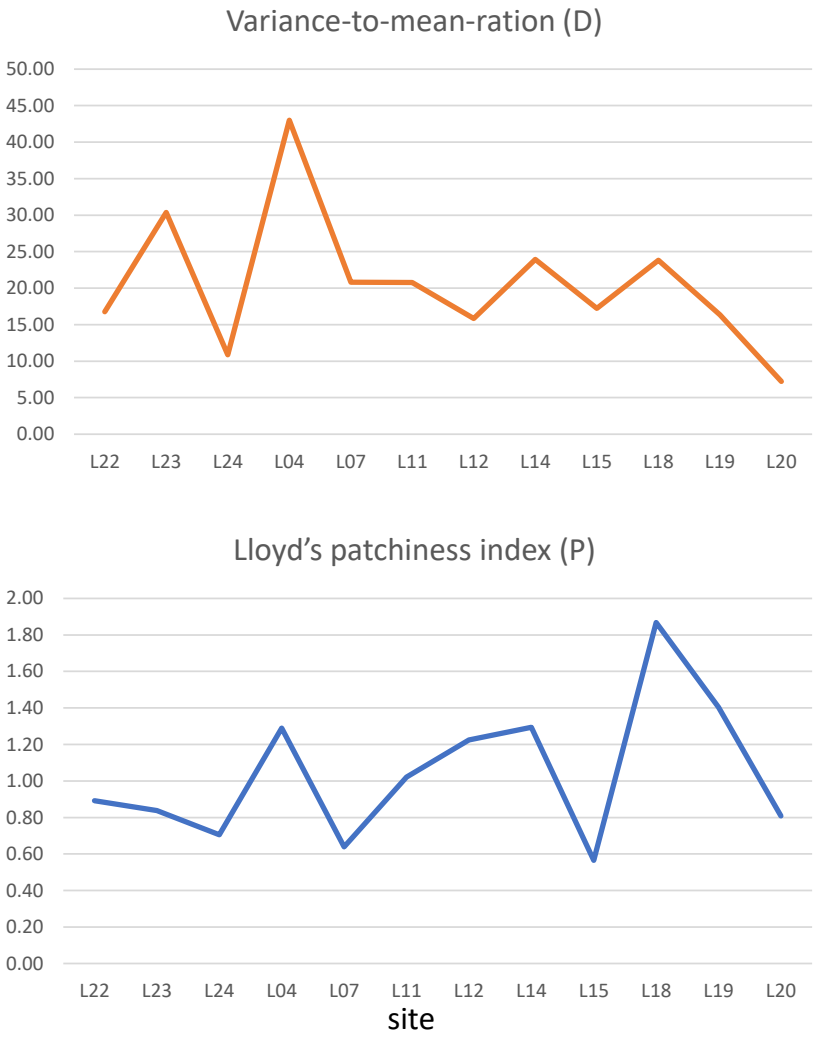


Figure 2. Comparison between variance-to-mean ration and Lloyd's patchiness index for Local scale study

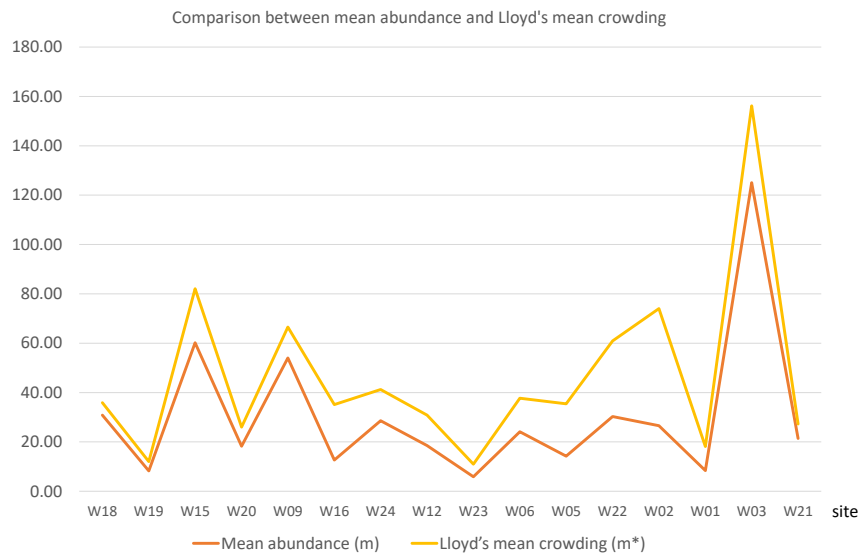


Figure 3. Comparison between mean abundance and Lloyd's mean crowding for Whole area study

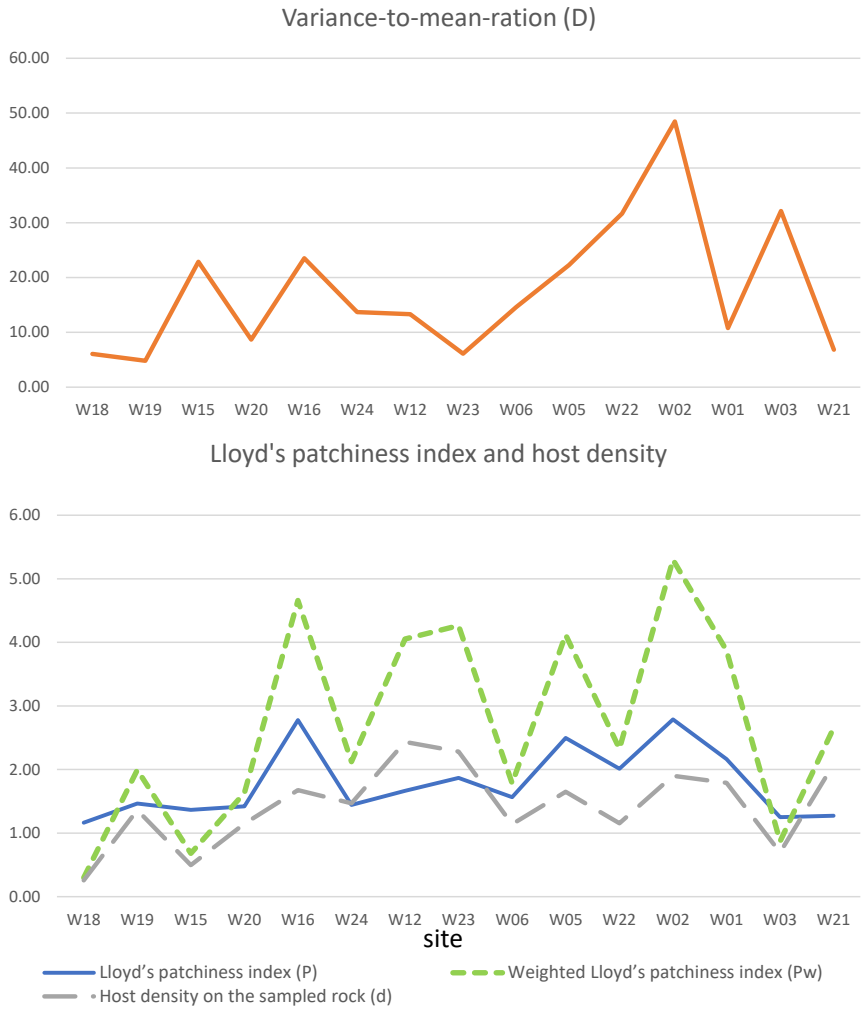


Figure 4. Comparison between variance-to-mean ration and Lloyd's patchiness index for Whole area study

Appendix 31 Host list for *Maritrema gratiosum* Nicoll, 1907

Stage	Host name	Host common name	Host Locality	Source
Final host	*Arenaria intrepres* L., 1758	Ruddy turnstone	USA	Hadley & Castle, 1940
Final host	*Haematopus bachmanni* Audubon, 1838	Black oystercatcher	Alaska	Deblock & Rausch, 1972
Final host	*Charadrius* sp.	NA	NA	Deblock & Tran Van Ky, 1966a
Final host	*Charadrius hiaticula* L., 1758	Common ringed plover	NA	Deblock & Tran Van Ky, 1966a
Final host	*Calidris alpine* L., 1758	Dunlin	Scotland	Deblock & Tran Van Ky, 1966a
Final host	*Limosa lapponica* L., 1758	Bar-tailed godwit	England	Deblock & Tran Van Ky, 1966a
Final host	*Calidris alpine* L., 1758	Dunlin	England	Rankin, 1939
Final host	*Charadris hiaticula* L., 1758	Common ringed plover	England	Rankin, 1939
Final host	*Larus ridibundus* L., 1766	Black-headed gull	England	Rankin, 1939
Final host	*Haematopus ostralegus* L., 1758	Eurasian oystercatcher	England	Rankin, 1939
Final host	*Larus ridibundus* L., 1766	Black-headed gull	Scotland	Nicoll, 1907
Final host	*Calidris alpine* L., 1758	Dunlin	Scotland	Nicoll, 1907
Final host	*Charadris hiaticula* L., 1758	Common ringed plover	Scotland	Nicoll, 1907
	Haeatopus ostralegus L., 1758	Eurasian oystercatcher	Scotland	Nicoll, 1907
Final host	*Bucephala islandica* Gmelin, 1789	Goldeneye		Ching, 1978
Final host	*Haematopus ostralagus* L., 1758	Eurasian oystercatcher		Dawes, 1968
Final host	*Larus ridibundus* L., 1766	Black-headed gull		Dawes, 1968
Final host	*Larus argentatus* Pontoppidan, 1763	Herring gull		Irwin & Prentice, 1976
2nd host	*Semibalanus balanoids* L., 1767	Acorn barnacle	USA, North Ireland, England, North Ireland, Wales? Southern coast of Barent sea, Scotland	Hadley & Castle, 1940; Irwin & Irwin, 1980; Williams *et al.,* 1981; Carrol *et al.*, 1990; Zaben 1988; Galationov & Bustnes, 1999; Colston, 2012
2nd host	*Austrominius modestus* Darwin, 1854	NA	Wales?	Zaben, 1988
2nd host	*Chthamalus montagui* Southward, 1976	Montagu's stellate barnacle	Wales?	Zaben, 1988

Stage	Host name	Host common name	Host Locality	Source
2nd host	*Balanus perforates* Bruguière, 1789	NA	Wales	Sari & Malek, 2000
2nd host	*Balanus glandula* Darwin, 1854	Acorn barnacle		Ching, 1978
1st host	*Littorina saxatilis* Olivi, 1792 (syn. *Littorina saxatilis tenebrosa*)	Rough periwinkle	Wales?	Zaben, 1988
1st host	*Littorina saxatilis* Olivi, 1792 (*Littorina saxatilis rudis*)	Rough periwinkle	Wales?	Zaben, 1988
1st host	*Littorina saxatilis* Olivi, 1792	Rough periwinkle	Southern coast of Barent sea, North Ireland	Galationov & Bustnes, 1999; Irwin *et al.*, 1990;
Paratenic host	*Nucella lapillus* Linnaeus, 1758	Dog whelk	North Ireland	McCarthy *et al.,* 1999

Appendix 32 HotSHOT genomic DNA preparation for digenean parasite

1. Protocol:

Put the target parasitic material (an individual metacercaria in present study) in a 0.2 ml Eppendorf tube containing 100 µl of 90% ethanol as fixing/storage fluid. Before performing the extraction, carefully remove most of the liquid and evaporate the last bit of liquid to prevent losing the parasite. Add 10 µl alkaline lysis reagent and heat sample to 95 ℃ for 30 minutes in a thermal cycler. Cool the sample to 4℃ and add 10 µl neutralization buffer. The DNA can be used immediately.

2. The buffers:

To make the alkaline lysis buffer, add 14.88 mg EDTA (final concentration 0.2 mM) and 200 mg NaOH (final concentration 25 mM) to 200 ml ultrapure water. To make the neutralization buffer, add 1.3 g Tris-HCL to 200 mL ultrapure water (final concentration 40 mM). pH of Alkaline Lysis Reagent will be 12. pH of Neutralization Buffer will be 5.

(Source: Truett *et al*, 2000)

Appendix 33 Modified AxyPrep Mag PCR Clean-up protocol

Add 5 µl of ultrapure water to 15µl of PCR product to a total volume of 20 µl (in 1.5 mL Eppendrof tube). Add the same volume of magnetic beads, mix gently by pipetting. Sit the tube for 5 minutes. Separate beads and DNA from unbound contaminants in a magnetic stand for 5 minutes. Remove the supernatants. Wash the beads with 75% ethanol (190 µl) twice for 30 seconds. Dry the tube in a dry bath incubator if necessary. Elute the DNA by ~16 µl ultrapure water by gently pipetting the pallet. Sit the tube for 3 minutes and transfer the tube back to the magnetic stand. Sit for another 3 minutes. Transfer the supernatants (purified PCR products) to a new 1.5 mL Eppendrof tube.

Appendix 34 18S rDNA for *Maritrema gratiosum* (739 bps)

GGTTCCTTAGATCGTACAAACTACATGGATAACTGTAGTAATTCTAGAGCTAATACATGCCACTATGCCCT
GACCCGCAAGGGGATGGGTGGATTTATTAGAACAGAACCAACCGGTTGTGGCTTCGGTCGCTTCCGTT
GCAATCTGTGATGACTCTGGATAACTTTACTGATCGCAGTCGGCCTTGTGTCGGCGACGGATCTTTCAA
ATGTCTGCCCTATCAATTTACGATGGTAGGTGACCTGCCTACCATGGTGATGACGGGTAACGGGGAATC
AGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACTTCCAAGGAAGGCAGCAGGCGCGC
AAATTACCCAATCCCGGCACGGGGAGGTAGTGACGAAAAATACGGATGCGGGACTCAATAGAGGCTC
CGTAATTCGAATGAGTACAATTTAAATCCTTTAACGAGGACCAACTGGAGGGCAAGTCTGGTGCCAGC
AGCCGCGGTAACTCCAGCTCCAGAAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATC
TGGGTCGCATGGCTACGTGCCGTAGCTTATATTCACGTCCTGGTTACGGCCGGGTCGGGTTTGTGAGTC
GGTTTCGTGGCTGTGTAGCCTTTCAGTCGTGTCTGTTTCGACAGGTGTCGGCGGTTCATCCGTTGGCAT
GCTTCCAGATGCCTTTAACCGGGTGTCGGGGGCGGACGGCTATTTTACTTTGAA

Top five hits on BLAST (7/8/2017)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Maritrema oocysta 18S rRNA gene	1212	1212	100%	0.0	96%	AJ287534.1
Digenea sp. MKP-2016 isolate cercaria small subunit ribosomal RNA gene, partial sequence	1182	1182	100%	0.0	96%	KY417091.1
unidentified digenean 18S, ITS1 and 5.8S rRNA genes (type A1)	1166	1166	100%	0.0	95%	AJ001831.1
Collyriclum faba 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1164	1164	100%	0.0	95%	JQ231122.1
Microphallus primas 18S rRNA gene	1157	1157	100%	0.0	95%	AJ287541.1

Appendix 35 18S rDNA for *Semibalanus balanoides* (703 bps)

TTCAAGGTAACGTCGCCGGTCGCCAACAGGCCCCTCCGAAGaACGGCCCGTCGACGGATCCGGCACG
ATTGAATGCGGCCAGCCGATATTTGGGAGCCCAGCAGTGACGCGCGAGCGCCGCCGCTAGGCATACC
GGACCGGACACGCACTGATATCCAACTACGAGCTTTTTAACCGCAACAGCTTTAATATACGCTACTGGA
GCTGGAATTACCGCGGCTGCTGGCACCAGGCTTGCCCTCCAGTCGATCCTCGTTAAAGGATTCACGTT
GTACTCATTCCGTTTGAGAGATCTCGGTTAACGAGACCTCTGTAAGGTATTTATTGTCACTACCTCCCTG
AACTGGGAGTGGGTAAGTTACGCGCCTGCTGCCTTCCTTAGATGTGGTAGCCATCTCTCAGGCTCCCTC
TCCGGAACCGAACCCAGATTCCCCGTTACCCGTCAGAGCCACGGTCAGCCACTAGCAAACCGTCGAGA
GCTGATAAGGCAGATATTTGAAAGGCGCGTCGCCGGTACAAGACCGTGCGATCCACACAATGTTATTGT
GATTCATCGGAGAACGACCGCCACGAGGGCAGCCATCGGTTTTCAGCCAATAAAAGCGCCCCGGCCA
ACCCGAGAGCTGACACTGGGGCTCGGTTGCATGTATTAGCTCTAGAATTACCACAGTTATCCATGTATCA
CTGTCTCGGCCAGTAAATA

Top five hits on BLAST (17/8/2018)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Semibalanus balanoides isolate MT01231 18S ribosomal RNA gene, partial sequence	1299	1299	100%	0.0	100%	KJ193741.1
Semibalanus balanoides strain C37 18S ribosomal RNA gene, partial sequence	1299	1299	100%	0.0	100%	EU370426.1
Semibalanus balanoides 18S ribosomal RNA gene, partial sequence	1299	1299	100%	0.0	100%	AY520626.1
Argulus foliaceus strain C38 18S ribosomal RNA gene, partial sequence	1291	1291	100%	0.0	99%	EU370428.1
Semibalanus balanoides 18S ribosomal RNA gene, partial sequence	1282	1282	100%	0.0	99%	DQ777622.1

Appendix 36 ITS2 rDNA for *Maritrema gratiosum* (524 bps)

TGTCGATGAAGAGCGCAGCCAACTGTGTGaATTAATGTGAACTGCATACTGCTTTGAACATCGACATCT
TGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCA
CGACGCCCATAAAGTCGTGGCTTGGGTCTTACCAGCTGGCGTGATTTCCCCAGACACACACAGAGACG
CACGCACGTGTGATTGAATTGTTGCATTCTATTGTGTTGTGTTGTGTTGTGTTCGGGGTGCCGGATCTAT
GGTTTTTCCCTAATGTGTCCGGACACCTGCATTCGCGGTGGCGGAGTCGTGGCTTAATGGTGATGAGAT
TTGATGAAAGCGCGCTCCGATTAATCGTGTTTGTGGGTTGTTCGTGTCCCGACGGATGGATGAATTGGT
CTTGCTGCGCTATTGGCTGTTTGTGCGCGCTTGATCCAATTGTCTGCCTGTCCATTGTTCCTGACCTCGG
ATCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAA

Top five hits on BLAST (6/8/2017)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Maritrema arenaria isolate Maa 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	963	963	100%	0.0	99%	HM584171.1
Maritrema brevisacciferum isolate V11 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	351	506	88%	2e-94	100%	KT355825.1
Maritrema brevisacciferum isolate V7 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	351	506	88%	2e-94	100%	KT355824.1
Maritrema novaezealandense isolate AB2014-Maritr 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	351	495	86%	2e-94	100%	KJ540203.1
Maritrema sp. MMIB 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	351	487	86%	2e-94	100%	KC222024.1

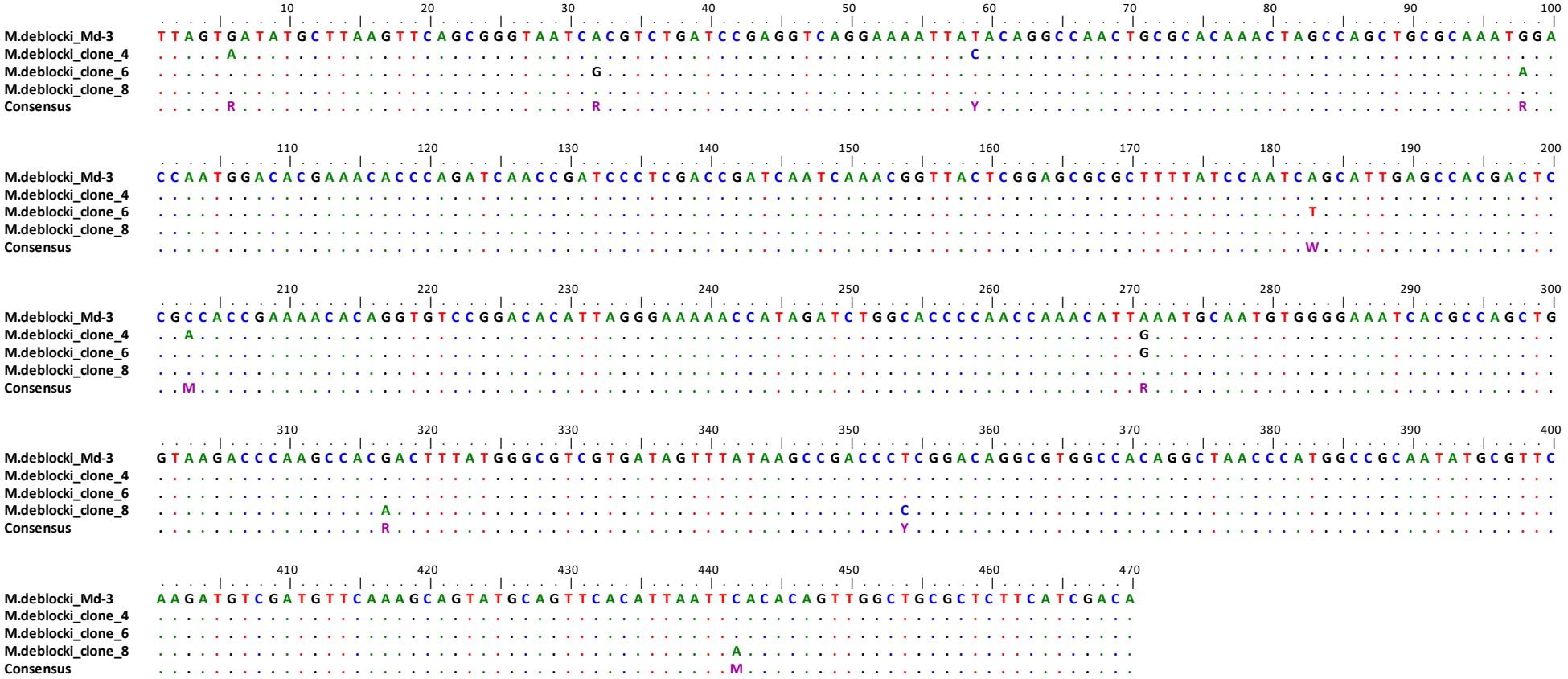
Appendix 37 ITS2 rDNA for *Maritrema poulini* (490 bps)

TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACATCGACATCT
TGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGCCTGTCCGAGGGTCGGCTTATAAACTATCA
CGACGCCCATAAAGTCGTGGCTTGGGTCTTACCAGCTGGCGTGATTTCCCCACATTGCACTGATTTGGT
AAATTAGTCATTGAATGTTTGTTCGGGGTGCCAGATCTATGGTTTTTCCTTAATGTGTCCGGACACCTGT
ATTTGCGGTGTCGGAGTCGTGGCTCAATGCTGATTGGATAAAAGCGCGCTCCGAGTAACCGTTCGATT
GGTCGGTCGAGGGATCGATTGATGTGGGTGTTTCGTGTCCATTGGTCCATTTGCGCAGCCGGCTAATTT
GTGCGCGGTGGGCCTGTATAATTTTCCTGACCTCGGATCAGACGTGATTACCCGCTGAACTTAAGCATAT
CACTAA

Top five hits on BLAST (23/10/2020)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Maritrema brevisacciferum isolate V11 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	811	811	100%	0.0	96.73%	KT355825.1
Maritrema brevisacciferum isolate V7 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	811	811	100%	0.0	96.73%	KT355824.1
Microphallidae sp. 3LF-1471 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	676	676	96%	0.0	92.80%	KM594159.1
Maritrema novaezealandense isolate AB2014-Maritr 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	579	579	100%	7e-161	88.41%	KJ540203.1
Maritrema sp. 2 CC-2013 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	579	579	100%	7e-161	88.41%	KC222022.1

Appendix 38 Alignment of the four sequences of *Maritrema deblocki* generated in the present study



Appendix 39 ITS2 rDNA for *Maritrema deblocki* (470 bps)

TTAGTRATATGCTTAAGTTCAGCGGGTAATCRCGTCTGATCCGAGGTCAGGAAAATTAYACAGGCCAAC
TGCGCACAAACTAGCCAGCTGCGCAAATRGACCAATGGACACGAAACACCCAGATCAACCGATCCCTC
GACCGATCAATCAAACGGTTACTCGGAGCGCGCTTTTATCCAATCWGCATTGAGCCACGACTCCGMCA
CCGAAAACACAGGTGTCCGGACACATTAGGGAAAAACCATAGATCTGGCACCCCAACCAAACATTRAA
TGCAATGTGGGGAAATCACGCCAGCTGGTAAGACCCAAGCCACRACTTTATGGGCGTCGTGATAGTTT
ATAAGCCGACCCYCGGACAGGCGTGGCCACAGGCTAACCCATGGCCGCAATATGCGTTCAAGATGTCG
ATGTTCAAAGCAGTATGCAGTTCACATTAATTMACACAGTTGGCTGCGCTCTTCATCGACA

Top five hits on BLAST (01/11/2020)

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Maritrema brevisacciferum isolate V11 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	712	712	100%	0.0	92.99%	KT355825.1
Maritrema brevisacciferum isolate V7 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	712	712	100%	0.0	92.99%	KT355824.1
Maritrema oocysta isolate Moo 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	630	630	100%	2e-176	90.66%	HM584170.1
Microphallidae sp. 3LF-1471 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	612	612	95%	7e-171	90.41%	KM594159.1
Maritrema sp. DCHF 3847 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	593	593	100%	2e-165	88.70%	MH257762.1

Appendix 40 *Maritrema* species for which a vaginal gland is described

Species	Stage	Source
***Maritrema afanasjewi* Belopolskaia, 1952**	Adult	Deblock & Rausch, 1972
***Maritrema feliui* Gracenea, Montoliu et Deblock, 1993**	Adult	Gracenea *et al.*, 1993
***Maritrema gratiosum* Nicoll, 1907**	Clear in metacercaria	Hadley & Castle, 1940; Deblock & Rausch, 1972; Deblock & Tran Van Ky, 1966a
***Maritrema heardi* Kinsella et Deblock, 1994**	Adult	Kinsella & Deblock, 1994
***Maritrema linguilla* Jägerskiöld, 1909**	Adult	Deblock & Capron, 1960
***Maritrema pacificum* Ching, 1974**	Only in metacercaria	Ching, 1974
***Maritrema paracadiae* Ching, 1974**	Adult	Ching, 1974
***Maritrema prosthometra* Deblock et Heard, 1969**	Adult	Deblock & Heard, 1969

Appendix 41 *Maritrema* species for which an operculum is described

Species	Source
***Maritrema acadiae* Swales, 1933**	Deblock & Rausch, 1972
***Maritrema apodemicum* Lewis, 1966**	Lewis, 1966
***Maritrema bonaerense* Etchegoin et Martorelli, 1997**	Alda *et al.*, 2013
***Maritrema borneoense* Fischthal et Kuntz, 1973**	Fischthal & Kuntz, 1973
***Maritrema carpathica* Matskási, 1984**	Matskási, 1984
***Maritrema deblocki* Presswell, Blasco-Costa et Kostadinova, 2014**	Presswell *et al.*, 2014
***Maritrema formicae* Diaz, Gilardoni et Cremonte, 2012**	Diaz *et al.*, 2012
***Maritrema gratiosum* Nicoll, 1907**	Zaben, 1988
***Maritrema madrynenses* Deblock et Rausch, 1968**	Diaz & Cremonte, 2010
***Maritrema magdalenae* Werding, 1973**	Werding, 1973
***Maritrema misenesis* Palombi, 1940**	Prevot *et al.*, 1976
***Maritrema neomi* Tkach, 1998**	Tkach, 1998
***Maritrema novaezealandense* Martorelli, Fredensborg, Mouritsen et Poulin, 2004**	Martorelli *et al.*, 2004
***Maritrema orensensis* Cremonte et Martorelli, 1998**	Cremonte & Martorelli, 1998
***Maritrema obstipum* Van Cleave et Mueller, 1932**	Etges, 1953
***Maritrema patagonicum* Rauque, Flores et Brugni, 2013**	Rauque *et al.*, 2013
***Maritrema poulini* Presswell, Blasco-Costa et Kostadinova, 2014**	Presswell *et al.*, 2014
***Maritrema subdolum* Jägerskiöld, 1909**	Deblock & Capron, 1960

Appendix 42 *In vitro* culture methods for non-progenetic digenean species

Species	parasitic stage	culture medium	culture condition	results	reference
Schistosoma mansoni	Cercariae to adults	1. Washing medium: Basal Medium Eagle medium (BME) with 15mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) and penicillin 300 µg mL^{-1} streptomycin 300 µg mL^{-1}. 2. Culture medium: Medium 169 sterilized through 0.22-µm membrane filter 3. Human serum and red blood cells	1. Cercariae were washed several times with washing medium and mechanical transformed to schistosomules by repeated aspiration through double-Luer-ended needle. Human serum (5%) was added at late stage of washing. 2. Schistosomules were cultured in culture medium using Petri dishes and incubated at 36 °C humidified, flowing 5% CO2 incubators. After 24-48 hours a drop of red blood cells (RBC) was added in culture medium. Medium and blood cells were changed twice weekly. 3. All procedures were operated under strict sterility.	1. Numerous schistosomules were obtained. 2. Pairing of young adults was first seen at 7th week. 3.Development was slower and flukes were smaller than in mammalian hosts.	Basch, 1981a
S. mansoni	Paired adults to ovigerous adults	Medium 169 with a drop of washed, sedimented human RBC.	The following factors were tested to optimise the culture condition: serum conc., flowing systems and gas phase, amino acids, steroids, reducing agents and antioxidants, adsorbing agents, and adult worm extracts.	1. Best results were obtained with two or three pairs of worms in metal capped Leighton tubes at 37-38 °C in flowing 5 % CO2 with unaltered medium 169 containing 8-10 % human serum and type O RBC. 2. About 10% females produced and shed vitelline conglomerates or/and eggs. 3. The eggs were half size of the normal eggs and lacked germinal disks. 4. The paired male and female failed to achieve complete growth and development	Basch, 1981b

Species	parasitic stage	culture medium	culture condition	results	reference
				and remained in a state of prolonged sub-maturity.	
S. mansoni	Sporocysts	1. Bge cells medium. 2. Sporocyst medium, a mixture of medium F, Dulbecco's Modified Eagle medium/ F-12 (DME/F12), and Bge medium (1:1:2) with the addition of 5% Serum, 5% foetal bovine serum, 0.001% 2-mercaptoethanol, 0.05% chemically defined lipid concentrate, and 20 mg mL^{-1} gentamicin.	1. Bge cells were cultured in Bge cell medium and seeded at least 1 day before addition of sporocysts. 2. Cocultures were maintained at 26°C in sporocyst medium. Both synxenic (mixed) and membrane-separated culture systems were successful. 3. Axenic sporocyst cultures were held in hermetically closed plastic containers in a nitrogen atmosphere maintained by blowing N_2 gas into the chambers. Cultures were maintained in sporocyst medium, either fresh or conditioned 16–20 hours on exponentially growing Bge cells or on synxenic cocultures.	1. Sporocysts became completely covered with Bge cells within 7 days and contained daughter sporocysts after 14 days. Second-generation (daughter) sporocysts appeared 2–5 weeks after initiation of cocultures and were encapsulated by Bge cells. 2. Proliferating cultures in the absence of Bge cells could be achieved if sporocysts were maintained at high density in Bge-conditioned medium and held under reduced oxygen. 3. *In vitro* cultures yielded mostly sporocysts, but cercariae appeared sporadically. 4. Sporocyst development was influenced by both Bge cell numbers and sporocyst numbers in the culture vessels.	Ivanchenko *et al.*, 1999.

Species	parasitic stage	culture medium	culture condition	results	reference
S. masoni	Sporocyst	1.Bge medium supplemented with heat-inactivated 10% foetal bovine serum (FBS) and antibiotics (complete or C-Bge medium). 2. Chernin's balanced salt solution (CBSS) contained penicillin G (100 U mL^{-1}), streptomycin sulphate (0.05 mg mL^{-1}), glucose (1 mg mL^{-1}), and trehalose (1 mg mL^{-1}).	1. Miracidia were washed twice with cold CBSS, followed by re-suspension in complete Bge medium. Larvae were then transferred in C-Bge medium to either 24-well tissue culture plates or 30-mL tissue culture flasks containing previously established Bge cell cultures. 2. *In vitro* miracidial transformations were accomplished by placing aseptically isolated, freshly hatched miracidia into a 24-well plate containing CBSS. 3. Transformed sporocysts were placed into coculture with Bge cells. 4. Comparison of synxenic and axenic cultures: Isolated miracidia were introduced into a 24-well plate containing Bge medium only (axenic cultures), monolayers of Bge cells (synxenic cultures), or Bge cell cultures containing sterile plastic inserts (insert cultures). 5. All plates or flasks were maintained at 26 °C under normal atmospheric conditions. 6. After 14-21 days in culture, medium was changed by replacing 1/2 of the medium in each well. Medium was subsequently changed at 4-5-day intervals.	1. Bge cell medium, supplemented with heat-inactivated FBS and antibiotics, is suitable for both Bge cell line and the extended maintenance and development of *S. mansoni* mother sporocyst (MS). FBS was an essential. 2. MS growth in C-Bge medium was enhanced in the presence of Bge cells or cell supernatants. By day 10, synxenically cultured sporocysts and those cultured in the presence of Bge cell-conditioned medium attained a significantly larger size than larvae maintained in C-Bge medium alone. 3. Production of emergent daughter sporocyst (DS) was observed only in synxenic culture.	Yoshino and Laursen, 1995.
Fasciola hepatica	Adults	1. Washing solution: Earle's balanced salt solution 2. Maintenance media: Earle's balanced salt solution or Hank's balanced slat solution (HBSS).	1. A factorial approach was carried out to test the effect of different medium components such as bile, serum, defibrinated blood, autoclaved liver extract and different combination of antibiotics.	1. Parasites from different host individuals often differed significantly. 2. Some components had positive effects, such as serum, blood and medium 199; some had negative effect, such as penicillin, cholesterol, Eagle's minimal medium amino	Ractliffe *et al.*, 1969

Species	parasitic stage	culture medium	culture condition	results	reference
		3. Basal medium used for factorial experiments: Earle's balanced salt solution plus 30% calf serum plus 0.5% raw liver concentrate plus antibiotics mixture II.	2. Egg count was the outcome variable for evaluation. 3. An automatic apparatus was used to automatically change and collect medium and eggs.	acid, vitamins, 5% CO_2/95% N2; and some had no effect. 3. The effect of some components was altered by the presence of other components.	
F. hepatica	Adults	1. Washing solution: HBSS 2. Routine culture medium (Medium RC): NCTC 135 plus 20% inactivated FBS and 2 drops of defibrinated horse or sheep blood with 100 µg mL^{-1} streptomycin and 100 U mL^{-1} penicillin.	1. Different culture systems are tested, such as sera, diphasic media, supplements (blood cells, embryo extract, liver extract, yeast extract, bile, sodium taurocholate, egg yolk medium), monolayer cell culture, temperature, agitation, gas phase, pH and culture vessels. 2. Newly excysted juveniles were washed in HBSS and culture medium. 3. Flukes were recovered from the abdomens and livers of mice at different time post-infection. 4. Flukes were injected intraperitoneally into mice at different time after culture in medium RC.	1. The best medium was NCTC 135 plus 50% inactivated chicken serum and sheep RBC. 2. The degree of somatic and caecal development was similar to those growth *in vivo*, however the genital rudiment of cultured fluke either failed to develop or similar to those growth 3 days *in vivo*. 3. Fluke growth in cell free culture were generally smaller and less developed than in cell containing medium. 4. Flukes recovered from mice showed somatic and caecal growth but not genital rudiment. 5. Cultured flukes injected into mice resulted in one or more egg-producing adults after 40 days.	Davies and Smyth, 1978

Species	parasitic stage	culture medium	culture condition	results	reference
F. hepatica	Rediae	1. 0.9% NaCl 2. 25% Listerine 3. L-15 Leibovitz medium, the saline fraction was constituted according to the work by Pullin (1971) on the mineral concentration of *Lymnaea truncatula* hemolymph. 4. 10% of brain-heart infusion, 20% of 0.9% NaCl solution, penicillin G (100 IU mL^{-1}), and streptomycin sulphate (100 µg mL^{-1})	1. The larvae were washed in two baths of 0.9% NaCl and were dipped in a third bath of 25% Listerine for 3 minutes before being transferred in Corning flasks measuring 25 cm2 in size. 2. Each flask was overlaid with 1 mm agar (6 mL of agar per recipient) and 6 mL of culture medium. The latter was L-15 Leibovitz medium, but the saline fraction was constituted according to the work by Pullin (1971). 3. To all flasks, 10% of brain-heart infusion, 20% of 0.9% NaCl solution, penicillin G (100 IU mL^{-1}), and streptomycin sulfate (100 µg mL^{-1}) were added. 4. The recipients were maintained in a horizontal position under darkness and constant conditions at 22 °C.	1. A total of 35 daughter rediae exited from the 21 mother rediae of the first and second generations, which were 14±32 days old at the onset of culture. 2. A total of 49 cercariae exited from the 17 mother rediae of the first and second generations, which were 29±50 days old at the onset of culture.	Augot *et al.*, 1997
F. hepatica	Juveniles	1. (1) *Fasciola* saline (2) RPMI 1640 (3) NCTC 135 (4) PBS were tested. 2. These were supplemented with different concentrations of foetal bovine serum or chicken serum. 3. 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, and 0.23 µg mL^{-1} amphotericin were added.	1. Juveniles were placed, 10 per well, in 250 µl relevant media, and incubated in humidified, 37 °C incubator with 5% CO_2 atmosphere.	1. The best culture condition was: RPMI 1640 supplemented with 50% chicken serum. 2. 65% of the juveniles survived after 29 weeks. 3. Significant growth was observed with apparent increased gut complexity and development of reproductive tissue.	McCusker *et al.*, 2016.

Species	parasitic stage	culture medium	culture condition	results	reference
		4. Fatty acids, palmitic acid and BSA were tested		4. Ultrastructural change was observed in the tegument and neoblast-like cells were found to support growth.	
Echinostomo caproni	Rediae	1. Bge or 199 medium with gentamycin (43 pg mL^{-1}). 0, 5 or 10 % heat-inactivated FBS. 2. Bge cells.	1. Snails (*Biomphalaria glabrata*) were crushed in a pool of serum-free medium 199 diluted to half normal strength. Rediae were transferred to fresh medium and rinsed to remove snail debris. 2. From 1 to 22 rediae were placed in individual flat- or round-bottomed wells in 96-well culture plates. Most wells were seeded with Bge cells the day before rediae were to be added. 3. At the time rediae were added, the medium in each well was changed, either to Bge or 199 medium. Depending on the well, the medium contained 0, 5 or 10 % heat- inactivated FBS. 4. Cultures were incubated at 24-27 °C under normal atmospheric conditions and medium in 0.75 mL volumes was changed on a weekly basis. No additional Bge cells were added once the cultures were initiated.	1. Cultures lacking Bge cells can only maintain rediae until 20 days. 2. Rediae in Bge medium initially developed a bloated gut and opaque body wall, but seemed to recover and when the cultures were terminated, many contained developing progeny. Rediae in cultures containing medium 199 did not become bloated and released more progeny, particularly progeny rediae. No obvious effect of FBS was observed. 3. Cultures with rediae derived from snails > 20 days post infection likely contained mixtures of mother and daughter rediae which released progeny rediae and cercariae, or both. 4. Cercariae were observed to be ingested by rediae. Cercariae also encysted in culture, and the resultant cysts were covered by Bge cells. 5. Longest observed interval (days) during which progeny were produced within a single culture was more than 44 days.	Loker *et al.*, 1999

Appendix 43 *In vitro* culture methods for metacercariae of progenetic digenean species

Species	parasitic stage	culture medium	culture condition	Results	Reference
Maritrema gratiosum	Metacercariae	1. Excystment media: (1) 5% pepsin in mammalian saline for 10 mins followed by 0.8% trypsin in mammalian saline for 15 mins. (2) Irwin's solution (1983) for 2 hours. (3) Sea water for 12 hrs (4) 60% (v/v) sea water in distilled water for 12 hrs. (5) 50% (v/v) sea water in distilled water for 12 hrs. (6) 40% (v/v) sea water in distilled water for 12 hrs. 2. Culture media: (1) diluted sea water media (2) NCTC 135 (3) medium 199 with Hank's salts and 0.35 ML^{-1} sodium bicarbonate and L-glutamine. Supplemented with 10% or 30% foetal bovine serum or chicken serum. 3. Both excystment of culture media were treated with 10000 U mL^{-1} penicillin, 10mg mL^{-1} streptomycin and 25 µg mL^{-1} amphotericin B in 0.9% sodium chloride.	Excysted and cultured in covered Petri dishes each with 25-40 worms at 38 ± 1 °C.	1. Irwin's medium was the most effective with 87.5% excystment in 2 hrs but the metacercariae were found to be inactive with rapid mortality. Excystment in 40% sea water was much slower but was as effective in Irwin's medium and the obtained metacercariae remain active and viable. 2. Very few eggs were produced in non-nutrient media but most were normal. 3. In nutrient media egg production began at 20 hrs post excystment. Egg production peaked from 3-5 days post excystment and reduced and ceased at 7-11 days with all eggs being shed through the genital pore. 4. The slowest mortality, longest survival and greatest and most prolonged egg production and best normal egg production occurred in metacercariae excysted in 40% sea water and cultured in medium 199 plus 10 % foetal bovine serum. The worms survived up to 24 days post excystment with highest mean egg numbers of 31.56 (8.84 SE) and 59.44 % normal eggs.	Zaben, 1988
Microphalloides japonicus	Metacercariae	Kreb-Ringer's solution, Eagle's MEM, EMEM supplemented with 20% calf serum, NCTC 109, NCTC 109 supplemented with 20% calf serum. Penicillin (200 U mL^{-1}) and streptomycin (100 µg mL^{-1}) added except EMEM (with kanamycin).	Cultured in tightly sealed tubes at 37 °C in pH 7.2-7.4	1. 0.85% NaCl: died within 24 hours 2. Kreb-Ringer's solution and HBSS: survived until the 5th day with low egg counts 3. EMEM and NCTC-109: survived until the 5^{th} day with higher egg counts	Fujino *et al.*, 1977

Species	parasitic stage	culture medium	culture condition	Results	Reference
				4. EMEM and NCTC-109 with serum: survived until the 5th day with even higher egg counts. Highest egg counts found in EMEM 5. Early spermatogenesis was found in all media 6. Eggs were found in the cyst in flukes cultured in NCTC-109 with serum and Kreb-Ringer's solution	
Microphallus similis	Metacercariae	1. Basic medium (BM) comprised of NCTC 135 plus 20% FCS. 2. Different combinations of basic medium, defined media (HBSS and NCTC 135), sera (FCS), diphasic media, supplements (mouse, chicken, or bovine embryo extract, yeast extract, dithiothreitol, bovine amniotic fluid, egg yolk medium, mouse intestinal mucosal extract, coagulate egg macerate) and cells (rabbit or chicken RBC) were tested.	Different temperature (38-41 °C), gas phase (air or anaerobic), pH (7.4) and culture vessel (Leighton tubes) were tested.	1. Three categories were calculated in each combination of media and conditions: flukes with eggs, flukes with some normal eggs, flukes with some abnormal eggs. 2. The maximum number of normal egg and abnormal eggs/fluke were also recorded. 3. Basic medium produced the highest percent of fluke with eggs (100%). 4. In treatment groups HBSS alone, NCTC 135 alone, BM plus 10% mouse or chicken embryo extract, BM plus 10% mouse intestinal mucosal extract, yolk medium plus 20% FBS, and BM with anaerobic gas, all flukes were dead by day 3. 5. Flukes developed *in vitro* were delayed with respect to those *in vivo* for about one day.	Davies and Smyth, 1979
Gymnophalloides seoi	Metacercariae	RPMI 1640, EMEM, NCTC 109 with 20% FBS and penicillin (200 U mL^{-1}) and streptomycin (1000 µg mL^{-1})	37 °C/5% CO_2, 41 °C/8% CO_2, 41°C/5% CO_2	1. No different with the three media when no serum added. 2. Sexual maturation not achieved in 37 °C/5% CO_2	Kook *et al.*, 1997

Species	parasitic stage	culture medium	culture condition	Results	Reference
				3. More eggs were observed in the group 41 °C/8% CO_2 and 41°C/5% CO_2. Eggs start to appear on 2nd day.	
Maritrema novaezealandense	Metacercariae	NCTC 109, Penicillin (200 units mL^{-1}) and streptomycin (100 ug mL^{-1})	Incubated at 40 ℃, examined at 24, 48, 72, 96, and 120 hours	Adults started to produce eggs from day 1-day 5, maximum at day 2	Fredensborg and Poulin, 2005.
		NCTC 109 supplemented with 20% chicken serum (inactivated at 56 °C for 30 mins), penicillin (200 units mL^{-1}) and streptomycin (100 ug mL^{-1})			
		NCTC-109 supplemented with 40% chicken serum (inactivated at 56 °C for 30 minutes), penicillin (200 units mL^{-1}) and streptomycin (100 ug mL^{-1})			
Microphallus turgidus	Metacercariae	1. Washing solution: HBSS containing penicillin (50 units mL^{-1}) and streptomycin (50 mg mL^{-1}), and 0.005% heat inactivated horse serum. 2. Culture medium: RPMI-1640 (2 mL/well) with HEPES buffer and L-glutamine supplemented with penicillin and streptomycin and either heat-inactivated horse, calf, or chicken serum, all in 20% concentration.	1. Excysted worms were washed 3 times in HBSS and incubated overnight at 37 °C. The next day, worms were transferred to a 24-well tissue culture plate (5 flukes/well) with culture medium of different serum. Worms were incubated at 37 or 42 °C in a humidified incubator with a gas phase of air and monitored for survival every other day. 2. Optimal concentration of horse serum were tested. 3. The effect of culture temperature was examined.	1. *M. turgidus* metacercariae developed into ovigerous adults and began to deposit eggs into the culture after 24 hours in culture medium. 2. The greatest percent survival was observed in worms grown in medium containing calf or horse serum. 3. The number of eggs deposited by worms grown in either 20% calf or horse serum was greater than that of worms in control medium lacking serum or chicken serum. 4. The numbers of eggs deposited by worms cultured in both 20 and 40% serum were greater than that of control cultures.	Pung *et al.*, 2009.

Species	parasitic stage	culture medium	culture condition	Results	Reference
				5. The increase in temperature to 42 °C dramatically decreased worm survival time, but had no effect on total egg production. 6. Most of the eggs produced by *M. turgidus in vitro* (80%) were normal in shape. After incubation for 10 days at 30 °C in brackish water, approximately 30% of the eggs appeared to contain miracidia and the remainder contained embryos in various stages of development. Worms cultured at both 37 and 42 °C produced eggs infective to the hydrobiid snail *Spurwinkia salsa*. The snails were able to produce cercariae and infected *Palaemonetes vulgaris* shrimp.	
M. turgidus	Metacercariae	1. HBSS; with calcium, magnesium, penicillin 50 units mL^{-1}, and streptomycin 50 mg mL^{-1}) containing 0.005% heat-inactivated horse serum. 2. Complete medium (RPMI-1640 with HEPES buffer, L-glutamine, antibiotics, and 20% heat-inactivated horse serum)	1. Excysted worms were washed 3 times in HBSS and incubated overnight. 2. Worms were transferred to 24-well tissue-culture plate wells (5 worms/well) containing 2 mL of complete medium. Worms were then cultured at 41 °C in a humidified incubator with a gas phase of air for 5 days or 10 days. 3. In the gas-phase experiment, egg deposition *in vitro* and infectivity of eggs to snails were tested. Air, 5% CO_2, and anaerobic conditions were tested. 4. The effect of proximity to other worms on *in vitro* egg production by *M. turgidus* and on the infectivity of the eggs to snails was examined.	1. When cultured in a gas phase of air and in anaerobic conditions, nearly all of the worms were dead by day 6 of cultivation, whereas more than 70% of worms in 5% CO_2 were alive and active and deposited the greatest number of eggs. 2. The percentage of snails infected after exposure to eggs deposited by worms cultured in air was significantly higher than that of snails fed eggs produced by worms cultured in 5% CO_2. 3. The highest percentages of fertilized worms were observed at an incubation temperature of 37 °C, a worm density of 50 metacercariae/tube, and when metacercariae were incubated in a conical-bottom tube rather than a flat bottom vessel.	Pung *et al.*, 2011.

Species	parasitic stage	culture medium	culture condition	Results	Reference
			5. The effect of incubation temperature, worm density, and pH on the *in vitro* fertilization of *M. turgidus* were tested.		
Gynaecotyla adunca	Metacercariae	1. HBSS containing penicillin 50 unit mL^{-1} and streptomycin 50 µg mL^{-1}. 2. HBSS or RPMI-1640 or DME/F-12 with HEPES buffer, L-glutamine, and antibiotics.	1. Excysted metacercariae were incubated 24 hours at 42 °C in 15 mL conical bottom polypropylene centrifuge tubes (50 worms per tube) containing 10 mL HBSS to permit fertilization. 2. Worms were then transferred to 48-well polystyrene tissue culture plates (5 worms/well) containing 1 mL/well of HBSS or 1 mL/well of RPMI-1640 or DME/F-12. 3. Medium supplement with horse, new born calf or chicken serum were tested. 3. Worms were cultured at 42 °C in a humidified incubator with a gas phase of air.	1. Worms deposited the most eggs when cultured in DME/F-12 and lived longer in HBSS and DME/F-12 than in RPMI 1640. 2. Worms deposited more eggs in DME/F-12 supplemented with either chicken or horse serum than in DME/F-12 alone and lived longer in DME/F- 12 supplemented with any of the 3 sera tested than in DME/F- 12 alone. 3. Worms deposited more eggs in medium supplemented with 5, 10, or 20% horse serum than in serum-free DME/F-12. 4. When cultured in air at 42 °C in horse serum-supplemented DME/F- 12 medium, excysted metacercariae readily develop into mature adults that survive for up to 2 weeks and deposit embryonated eggs but none of the snail expose to these eggs produce cercariae.	West *et al.*, 2014.