Trophic niche and detection of the invasive signal crayfish (*Pacifastacus leniusculus*) in Scotland

Kirsten Jennifer Harper

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School of Natural Sciences

UNIVERSITY *of* STIRLING

Declaration

I hereby confirm that this PhD thesis is an original piece of work conducted independently by the undersigned, except where specified below. No work contained herein has been submitted for any other degree.

- Dr. Jason Newton provided assistance with stable isotope analysis.
- Dr. Zara Gladman provided white-clawed crayfish data set that this author analysed for the stable isotope work.
- Prof. Colin Bean and Dr. Mark Coulson suggested eDNA analysis, but this author, in conjunction with Dr. Michael Leaver and Prof. James F. Turnbull, developed the study and conducted all work.

Signature: _____

Date: **August 31, 2015**

Abstract

Aquatic invasive species are a major threat to native freshwater biodiversity. The North American signal crayfish *Pacifastacus leniusculus* was introduced to Great Britain during the 1970s and is now widely distributed throughout England, Wales and Scotland. First recorded in Scotland in 1995, *P. leniusculus* is now established at more than twenty sites. The only other introduced crayfish species present in Scotland is the white-clawed crayfish *Austropotamobius pallipes*. *A. pallipes* is restricted to only two locations in Scotland, Loch Croispol and Whitemoss Reservoir. *P. leniusculus* negatively impacts macrophytes, invertebrates and fish though ecological and physical processes. Additionally, *P. leniusculus* has displaced *A. pallipes* throughout much of its native range within Great Britain due to competition and disease. Consequently, the two *A. pallipes* populations in Scotland have a high conservation value. This PhD study aimed to improve understanding of *P. leniusculus* invasion success by examining trophic dynamics and to develop methodologies that could improve the detection and control of *P. leniusculus* populations in Scotland.

Stable isotope analysis was used to determine the diet composition, trophic position and whether an ontogenetic dietary shift occurs in the Loch Ken population of *P. leniusculus*. Bayesian mixing models indicated that *P. leniusculus* in Loch Ken do exhibit an ontogenetic dietary shift. Additionally, individuals of all sizes occupied the trophic position of a predator in Loch Ken suggesting that invertebrates and fish constitute an important component of *P. leniusculus* diet.

Stable isotope analysis was used once again to compare the isotopic niche width and diet composition of *P. leniusculus* populations from Loch Ken and *A. pallipes* populations from Loch Croispol and Whitemoss Reservoir. At the species level, *A. pallipes* exhibited a larger

niche width than that of *P. leniusculus*. At the population level, the isotopic signatures of the *A. pallipes* populations were considerably different from each other suggesting an overestimation of *A. pallipes'* niche width at species level. Results showed no dietary overlap between species and Bayesian mixing models suggested *P. leniusculus* and *A. pallipes* were consuming different resources, indicating there would be no direct competition for food resources if they were to co-occur.

A plus-maze study was used to determine if *P. leniusculus* exhibited a preference for one of four food attractants (*Oncorhynchus mykiss*, *P. leniusculus*, beef or vegetation), which could be used to improve trapping efficiency. In the maze system, *P. leniusculus* exhibited no preference for any food attractant presented. This would suggest that either the maze was not a good model or food attractants would not improve trapping efficiency of *P. leniusculus*. Additionally, a comparative investigation into the use of gill nets as a method to control *P. leniusculus* was conducted. Results showed that the net type and the presence of fish entangled in the net influenced the number of *P. leniusculus* caught.

Finally, environmental DNA (eDNA) was used and evaluated for detection of *P. leniusculus*. A robust quantitative Polymerase Chain Reaction (qPCR) assay and DNA extraction protocol were developed. Using the developed qPCR assay, *P. leniusculus* eDNA was detected in controlled aquaria conditions but not in environmental water samples collected from the field. Furthermore, the quantities of *P. leniusculus* eDNA declined in aquaria conditions while individuals were still present suggesting the mechanisms for eDNA release by *P. leniusculus* are complex.

Stable isotope analysis indicates that *P. leniusculus* exhibit an ontogenetic dietary shift, and in each life stage, *P. leniusculus* function as an omnivore but occupy the trophic position of a predator. Niche width analysis revealed that the diet of *P. leniusculus* was less general

than that observed in *A. pallipes* and thus diet of *P. leniusculus* may not be responsible for invasive success. Food attractants will not enhance trapping efficiency but nets may present a potential new method to control *P. leniusculus*. Similarly, eDNA presents a promising new method for rapid detection of *P. leniusculus*.

It will not be possible to eradicate *P. leniusculus* in Scotland but the findings of this PhD may help prevent establishment of new populations. These results should be incorporated into future management strategies for *P. leniusculus* populations in Scotland and may have broader applications in Great Britain and Europe.

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List of Species

All species names mentioned in the text are listed below.

Anguilla anguilla (Linnaeus, 1758)	European eel
Aphanomyces astaci (Schikora, 1906)	Crayfish plague
Astacus astacus (Linnaeus, 1758)	European noble crayfish
Astacus leptodactylus (Eschscholtz, 1823)	Turkish narrow-clawed crayfish
Austropotamobius pallipes (Lereboullet, 1858)	White-clawed crayfish
Batrachochytrium dendrobatidis (gen. et sp. nov.)	Chytrid fungus
Barbatula barbatula (Linnaeus, 1758)	Stone loach
Cambarus robustus (Girard, 1852)	Big river crayfish
Cherax pulcher (sp. n.)	Common name not yet defined
Cherax destructor (Clark, 1936)	Common yabby
Cherax quadricarinatus (von Martens, 1868)	Australian red claw crayfish
Chironomus riparius (Meigen, 1804)	Midge
Cottus gobio (Linnaeus, 1758)	European bullhead
Cryptobranchus alleganiensis (Daudin, 1803)	Eastern hellbender
Cyprinius carpo (Linnaeus, 1758)	Common carp
Esox lucius (Linnaeus, 1758)	Northern pike
Gasterosteus aculeatus (Linnaeus, 1758)	Three-spined stickleback
Oncorhynchus mykiss (Walbaum, 1792)	Rainbow trout
Orconectes immunis (Hagen, 1870)	Papershell crayfish
Orconectes juvenilis (Hagen, 1870)	Kentucky river crayfish
Orconectes limosus (Rafinesque, 1817)	Spiny-cheek crayfish
Orconectes propinquus (Girard, 1852)	Northern clearwater crayfish

Orconectes rusticus (Girard, 1852)	Rusty crayfish
Orconectes sanbornii (Hobbs and Fitzpatrick, 1962)	Sanborn's crayfish
Orconectes virilis (Hagen, 1870)	Virile crayfish
Pacifasticus leniusculus (Dana, 1852)	North American signal crayfish
Paranephrops planifrons (White, 1842)	New Zealand crayfish
Pelobates fuscus (Laurenti, 1768)	Common spadefoot
Perca fluviatilis (Linnaeus, 1758)	Perch
Phoxinus phoxinus (Linnaeus, 1758)	Minnow
Physa gyrina (Say, 1821)	Pond snail
Platichthys flesus (Linnaeus, 1758)	European flounder
Procambarus acutus (Girard, 1852)	White river crayfish
Procambarus clarkii (Girard, 1852)	Red swamp crayfish
Rana catesbeiana (Shaw, 1802)	American bullfrog
Ranina ranina (Linnaeus, 1758)	Spanner crab
Rutilus rutilus (Linnaeus, 1758) x *Abramis brama* (Linneaus, 1758)	Roach/bream hybrid
Salmo salar (Linnaeus, 1758)	Atlantic salmon
Salmo trutta (Linnaeus, 1758)	Brown trout
Triturus cristatus (Laurenti, 1768)	Great crested newt

List of Abbreviations

The most frequently used abbreviations in the text are listed below. Other abbreviated terms found within the text are accompanied by a definition.

$\delta^{13}C$	Isotopic signature of carbon (‰)
$\delta^{15}N$	Isotopic signature of nitrogen (‰)
AIC	Akaike's Information Criterion
ANOVA	Analysis of Variance
bp	Base pair
C	Carbon
CD	Mean distance to centroid
CO1	Cytochrome c oxidase subunit I gene
CR	Carbon range
CR_c	Corrected carbon range
Ct value	Concentration threshold
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
FFG	Functional Feeding Group
GB	Great Britain
GLM	General Linear Model
HCO 2198	Global Reverse Primer
IUCN	International Union for Conservation of Nature
LCO 1490	Global Forward Primer
LOD	Limit of Detection
LOQ	Limit of Quantification
milliQ	Ultrapure water
mtDNA	Mitochondrial DNA

N	Nitrogen
NBN	National Biodiversity Network
NNSS	Non-Native Species Secretariat
NR	Nitrogen range
NTC	No template control
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
qPlCO1F	*P. leniusculus* Forward primer
qPlCO1R	*P. leniusculus* Reverse primer
ROV	Remote Operated Vehicle
SAF	Species Action Framework
SDNND	Standard deviation of the nearest neighbour distance
SE	Standard Error
NERC	Natural Environment Research Council
SEA	Standard Ellipse Area ($‰^2$)
SEA_c	Corrected Standard Ellipse Area ($‰^2$)
SIAR	Stable Isotope Analysis in R
SIBER	Stable Isotope Bayesian Ellipses in R
SNH	Scottish National Heritage
SUERC	Scottish Universities Environmental Research Centre
TA	Convex hull
TEF	Trophic Enrichment Factor
Tp	Trophic position
TpR	Trophic position range

Chapter 1. General Introduction

This PhD study examined the trophic dynamics of an invasive crayfish species to facilitate understanding of invasion success and to investigate potential methodologies that could improve the detection and control of this species in Scotland.

1.1 The definition of 'invasive'

The terminology used within the field of invasion ecology is variable and inconsistent (Colautti and MacIsaac, 2004; Lockwood *et al*., 2007). Consequently, the terminology used throughout this thesis will be defined. Terms like 'alien', 'non-native', 'non-indigenous' and 'exotic' have all been used to define a species moved outwith its usual geographic range (Lochwood *et al*., 2007). However, since these terms have been applied inconsistently within the literature and species not native to an ecosystem do not always result in negative impacts (Colautti and MacIsaac, 2004), Lockwood *et al*. (2007) used the qualifying term 'invasive' to describe a 'non-native' species which negatively impacts an ecosystem or the economy. This PhD thesis uses the term 'invasive' in the same way - to describe a 'non-native' species that causes ecological and/or economical damage.

1.2 Invasive species

1.2.1 A global perspective

The introduction of species outside their native range is recognised as an important component of global environmental change (Lockwood *et al*., 2007; Lodge *et al*., 2000; Sala *et al*., 2000). In recent decades, anthropogenic activity - through either direct or indirect means - has facilitated the introduction and spread of species, resulting in biological invasions occurring at an accelerated rate and on a larger scale (Hulme, 2009, Mack *et al*., 2000). Thus, invasive species now represent the most important threat to global biodiversity

after habitat loss and fragmentation (Lowe *et al*., 2000). Indeed, it has been suggested that invasive species may eventually surpass habitat loss and fragmentation to become the leading threat to global biodiversity in the foreseeable future (Crooks and Soulé, 1999). Through predation, habitat modification, and competition for space and food, invasive species can have negative impacts on native species, communities and ecosystems (Sakai *et al*., 2001). In addition to impacting biodiversity, invasive species can result in high economic cost. For instance, invasive species are estimated to cost in the region of $125 billion annually (approximately £80 billion) in the United States (US) (Pimentel *et al*., 2005), while the cost is estimated to be €12.7 billion annually (approximately £9 billion) in Europe (Kettunen *et al*., 2008).

1.2.2 A British perspective

In 2006, the Great Britain (GB) Non-Native Species Secretariat (NNSS) was formed to co-ordinate the management of invasive species throughout England, Wales and Scotland. In 2008, the NNSS produced a national policy framework, the Invasive Non-Native Species Framework Strategy, which is intended to deal with invasive species at a national level (DEFRA, 2008).

Currently, there are an estimated 2,000 non-native species in GB. Of this 2,000 species, approximately 1,800 are terrestrial and around 80 are found in both marine and freshwater environments (NNSS, 2015). Within the 2,000, 10 – 15 % are considered invasive and detrimental to native species through predation, competition, disease transfer, and reduction of genetic diversity (NNSS, 2015). Furthermore, problems are exacerbated by high economic cost. It is estimated that GB is currently spending an estimated £1.7 billion annually on invasive species.

1.2.3 A Scottish perspective

Scottish National Heritage (SNH) estimate that there are around 988 invasive species present in Scotland, excluding fungi or marine invasive species (SNH, 2001) and the economic cost of invasive species to Scotland alone is estimated to be £264 million per annum (SNH, 2015a). SNH formed the Species Action Framework (SAF) to direct species management within Scotland (SNH, 2007). The invasive species listed in the SAF were deemed to be a threat to Scotland's biodiversity. All six species listed were already established and in need of targeted management. Of the six species listed in the SAF, only one invertebrate was included. The invertebrate listed is known to be one of the worst globally invasive species, the North American signal crayfish *Pacifastacus leniusculus* (Dana, 1852) (Figure 1-1).

Figure 1-1: The North American signal crayfish *Pacifastacus leniusculus*.

1.3 Native distribution of crayfish

Crayfish are the largest mobile freshwater invertebrates (Holdich, 2002). There are over 640 species of crayfish described (Crandall and Buhay, 2008), belonging to three families: Astacidae, Cambaridae and Parastacidae. Astacidae and Cambaridae are found in the Northern hemisphere, while Parastacidae is restricted to the Southern hemisphere (Figure 1.2) (Reynolds and Souty-Grosset, 2012). There are 39 species in the family Astacidae and 180 species in the family Parastacidae, while the largest number of species is found within the family Cambaridae, with over 440 species described (Reynolds and Souty-Grosset, 2012). The greatest species diversity of crayfish is found in North America, with around 382 species occurring there, while about 151 species occur in Australasia (Crandall and Buhay, 2008). In comparison, Europe exhibits the lowest diversity of crayfish with only five known native species (Reynolds and Souty-Grosset, 2012). However, these numbers are constantly changing as new species are described each year (Crandall and Buhay, 2008). For instance, a new species of Parastacidae, *Cherax pulcher* (sp. n.) from Indonesia was recently described in May 2015 (Lukhaup *et al.*, 2015).

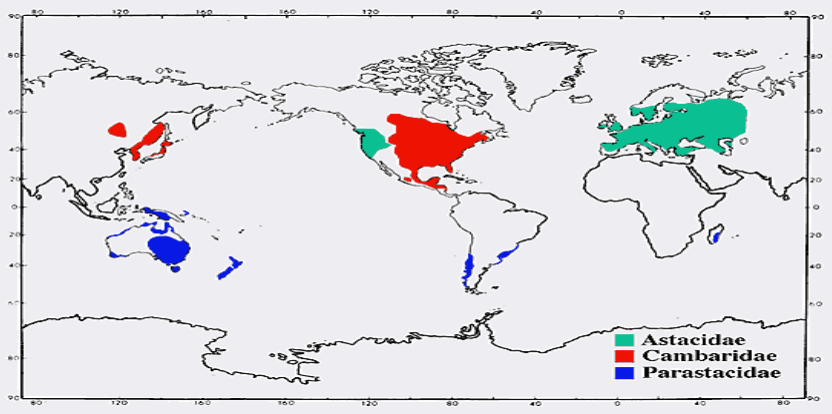


Figure 1-2: Native distribution of crayfish families worldwide. Reproduced from Crandall and Fetzner (2010).

1.4 Invasive crayfish

Invasive crayfish have been introduced around the globe and are today found on nearly every continent (Holdich, 2002), meaning they are amongst the most widely distributed and successful aquatic invasive species (Holdich *et al*., 2014). Crayfish have been largely translocated outside their native range for aquaculture purposes (Gherardi, 2010). More recently, the aquarium trade and recreational fishing activities have also facilitated the spread of invasive crayfish species (Lodge *et al*., 2000). The majority of invasive crayfish species originate from North America and there are now more than twice as many invasive crayfish species compared with native crayfish species in Europe (Holdich *et al*., 2009a).

Invasive crayfish are known to negatively impact freshwater ecosystems as they can change the energy and nutrient flow, physically alter habitats and alter the community composition (Gherardi, 2007; Holdich, 1999; Nyström, 2002; Stenroth and Nyström, 2003; Whitledge and Rabeni, 1997). For example, habitat alteration through the removal of submerged plant material by the invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852) resulted in a 71 % loss of macroinvertebrates and an 83 % loss in amphibia species within a Spanish lake (Rodriguez *et al*., 2005). McCarthy *et al*. (2006) found seven different species of invasive crayfish, each negatively impacting zoo-benthos density in cage experiments conducted across four continents.

Furthermore, negative impacts of invasive crayfish are not restricted to native non-crayfish taxa: they are also detrimental to native crayfish species. An estimated 30 – 50 % of native crayfish species are threatened with population decline or extinction (Taylor, 2002), partly due to anthropogenic activities such as pollution, overharvesting and habitat destruction (Perdikaris *et al*., 2012). However, the greatest threat to native crayfish is from invasive crayfish (Holdich *et al*., 2009a). Direct competition with invasive crayfish has resulted in

the displacement of native crayfish in North America (Taylor, 2002). In Europe, invasive crayfish introduced from North America are vectors for a fungus-like disease known as crayfish plague, caused by *Aphanomyces astaci* (Schikora, 1906). As a result, *A. astaci* has compounded the displacement of native crayfish species further than competition alone (Holdich *et al*., 2009a; Reynolds and Souty-Grosset, 2012). Consequently, all native European crayfish species are listed on the International Union for Conservation of Nature (IUCN) red list, which identifies species faced with high risk of global extinction (IUCN, 2015).

1.4.1 Invasive crayfish in GB

There are currently seven species of invasive crayfish with established populations in the wild within GB; the European noble crayfish *Astacus astacus* (Linnaeus, 1758), Turkish narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823), red swamp crayfish *P. clarkii*, spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817), virile crayfish *Orconectes virilis* (Hagen, 1870), white river crayfish *Procambarus acutus* (Girard, 1852), and the North American signal crayfish *P. leniusculus* (Holdich *et al*., 2014; Stebbing *et al*., 2014). Of the seven invasive crayfish species present, *P. leniusculus* is the most widely distributed within GB (Holdich *et al*., 2009a) (Figure 1-3).

P. leniusculus were introduced to GB waters during the late 1970's and early 1980's for aquaculture purposes (Lowery and Holdich, 1988; Holdich and Reeve, 1991; Holdich *et al*., 1999). Encouraged by the British government, introductions resulted in large breeding populations becoming established in England and Wales (Holdich *et al*., 2014). Additionally, through escapes (Holdich *et al*., 1995; Maitland *et al*., 1996), deliberate introductions from the aquarium and pond trade (Chucholl, 2013; Holdich *et al*., 2009a; Lodge *et al*., 2000), and recreational angling activities (Bean *et al*., 2006; Lodge *et al*., 2000;

Peay *et al*., 2010), *P. leniusculus* has become well established throughout England, Wales (Rodgers and Watson, 2011) and Scotland (Gladman *et al*., 2009) (Figure 1-3).

1.4.2 Invasive crayfish in Scotland

P. leniusculus is relatively recent invader in Scottish waters. Anecdotal records of *P. leniusculus* in Scotland first began in the early 1990's (Freeman *et al*., 2010); however, *P. leniusculus* presence was not officially confirmed until 1995 (Maitland, 1996). Within one decade, *P. leniusculus* established populations in eight river catchments and over 58 km of river (Gladman *et al*., 2009). Thereafter, *P. leniusculus* has spread to more than twenty sites in Scotland, spanning 15 different river catchments (Freeman *et al*., 2010), and now occupies an estimated 174 km of river (SNH, 2015b) (Figure 1-3).

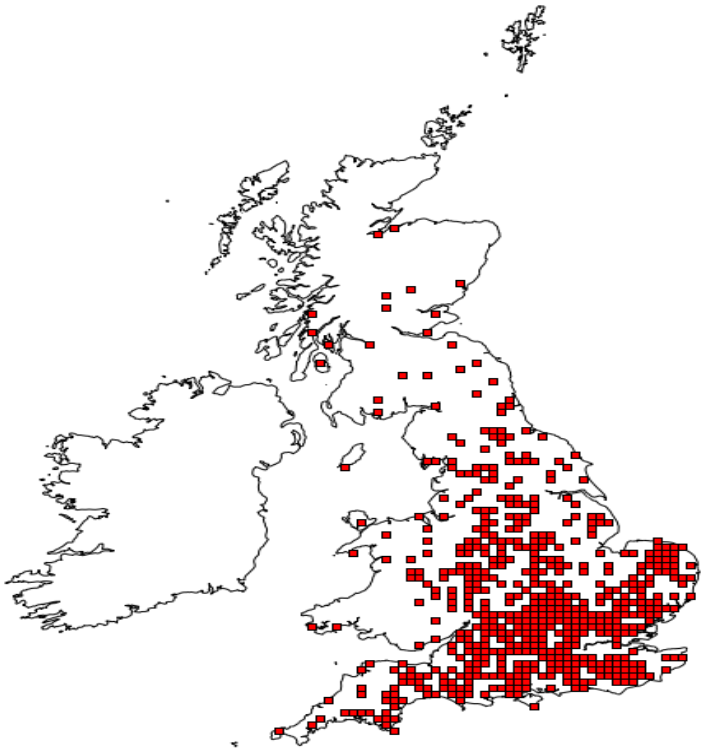


Figure 1-3: Distribution map of *P. leniusculus* in GB (NBN, 2015).

1.5 Environmental Impacts of *P. leniusculus*

1.5.1 Ecological

1.5.1.1 Macrophytes

Aquatic macrophytes play a key role in freshwater ecosystems by influencing water chemistries and providing resources, such as habitat and food, to both invertebrates and vertebrates (Nyström *et al*., 1999, Usio *et al*., 2009).

P. leniusculus has been shown to negatively impact the macrophyte abundance and composition of an ecosystem. In enclosure experiments, Nyström *et al*. (2001) demonstrated *P. leniusculus* had a negative effect on macrophytes through grazing and non-consumptive destruction of stems by cutting with chelae. Nyström *et al*. (1996) used replicated ponds in Southern Sweden and found as *P. leniusculus* densities increased, macrophyte biomass, species richness and coverage decreased. *P. leniusculus* also grazed more voraciously on macrophytes than native crayfish in controlled feeding experiments (Nyström and Strand, 1996). Additionally, *P. leniusculus* exhibited a preference for seedlings over established macrophytes, which could subsequently inhibit recruitment and lead to a decline in macrophyte species (Nyström and Strand, 1996). Usio *et al*. (2009) also found a significant reduction in macrophytes by a variety of *P. leniusculus* sizes using enclosure experiments. However, the authors attributed the decline in macrophytes to non-consumptive destruction rather than feeding.

1.5.1.2 Invertebrates

Larger, less mobile invertebrates are more seriously affected by invasive crayfish than smaller, faster moving species (Ilheu and Bernardo, 1993; Nyström, 1999). For instance, thin-shelled gastropods are negatively impacted by *P. leniusculus* (Nyström *et al*., 1996; Nyström and Perez, 1998; Nyström *et al*., 1999), along with large invertebrate taxa such as

Trichoptera and chironomids (Nyström *et al*., 1996; Usio *et al*., 2009). Additionally, the overall invertebrate biomass and species richness can be negatively impacted by the presence of *P. leniusculus* in freshwater environments (Crawford *et al*., 2006; Moorhouse *et al*., 2014).

P. leniusculus do not only directly affect invertebrate communities by predation. As previously discussed, macrophytes are important habitats for many invertebrate taxa. Therefore, the reduction of macrophyte abundance and species composition by *P. leniusculus* can also indirectly affect invertebrate communities. Usio *et al*. (2009) observed the decline of an Isopod species when *P. leniusculus* were present. However, the authors suggest that rather than being predated, the Isopod simply migrated away from enclosures once the macrophytes had been eliminated. Similarly, Nyström *et al*. (1996) reported a decline in macrophyte-associated invertebrates with reduced macrophyte biomass, caused by increased *P. leniusculus* abundance.

1.5.1.2a Native crayfish

Prior to the introduction of *P. leniusculus* to GB there was only one species of crayfish present: the native white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858). However, *A. pallipes* populations in England and Wales are in serious decline due to *A. pallipes* susceptibility to *A. astaci* which *P. leniusculus* carries (but is itself resistant to unless stressed), and because of competition between the two species (Holdich and Reeve, 1991; Reynolds, 1998). At the current rate of decline, *A. pallipes* is estimated to be extinct within 25 years (Bradley and Peay, 2013). Although native to other parts of GB, *A. pallipes* does not naturally occur in Scotland. However, two separate populations were introduced several decades ago (Maitland *et al*., 2001). Consequently, given the current decline and risk of extinction of *A. pallipes* elsewhere due to *P. leniusculus*, the two Scottish populations

represent potential *A. astaci* free refuge stock for future conservation efforts (Gladman *et al*., 2009).

1.5.1.3 Fish

P. leniusculus can impact fish species by predation, modification of habitat or competition for resources, such as food and shelter (Holdich *et al*., 2014). In laboratory trials, Bubb *et al*. (2009) found *P. leniusculus* to be highly aggressive towards European bullhead *Cottus gobio* (Linnaeus, 1758), causing fin damage and in some cases mortality. The authors also found densities of *C. gobio* to be reduced in rivers where *P. leniusculus* were present. Peay *et al*. (2009) found the densities of brown trout *Salmo trutta* (Linnaeus, 1758) and *C. gobio* to be negatively associated with the presence of *P. leniusculus* in a Northern England stream. Similar results were observed by Guan and Wiles (1997) for *C. gobio* and stone loach *Barbatula barbatula* (Linnaeus, 1758) in the River Great Ouse. Further laboratory experiments by the authors suggested that the reduced densities observed might be the result of the displacement of both species from shelters by *P. leniusculus*, which could increase the risk of predation from other organisms. The displacement of fish species from shelters, and consequently an increased risk of predation, is supported by the findings of Griffiths *et al*. (2004). The authors reported that the presence of *P. leniusculus* resulted in a decrease in the proportion juvenile Atlantic salmon *Salmo salar* (Linnaeus, 1758) sheltering over-winter, noting that *S. salar* without shelter during the day in winter are highly vulnerable to predation. More recently, Edmonds *et al*. (2011) observed *P. leniusculus* to exhibit aggressive behaviour towards emerged *S. salar* fry resulting in moderate fry mortality. Additionally, fish remains have also been reported from the guts of *P. leniusculus* caught in natural conditions (Guan and Wiles, 1998).

1.5.2 Physical

The burrowing and foraging activity of *P. leniusculus* can result in riverbank erosion, producing an increased input of sediment into ecosystems (Holdich *et al*., 2014). Sediment loading can have ecological and economic costs, such as increased risk of flooding and interference with the reproduction of fish and invertebrate taxa (Harvey *et al*., 2011). For example, Findlay (2013) reported that *P. leniusculus* activity resulted in the reduced survival of *S. trutta* eggs due to increased amounts of fine sediment in the water.

1.6 Aims of thesis

The presence of the invasive crayfish *P. leniusculus* is costly, not only economically but ecologically. Therefore, it is critical to prevent the establishment of further *P. leniusculus* populations in Scotland in order to protect native biodiversity.

The aim of this PhD study was to contribute to the understanding of the invasive success of *P. leniusculus* within Scotland through examination of trophic dynamics and to develop methodologies that could improve detection and control of this species.

The thesis aim was attained by:

1. Examining the diet and trophic position of *P. leniusculus* in a Scottish freshwater loch and determining if an ontogenetic dietary shift is exhibited (Chapter 2).
2. Comparing niche width, in order to better understand the invasive potential of *P. leniusculus* and competition with *A. pallipes* (Chapter 3).
3. Determining if trapping efficiency of *P. leniusculus* could be improved with the use of food attractants (Chapter 4).
4. Developing a molecular assay for the detection of *P. leniusculus* DNA in environmental water samples (Chapter 5).

Chapter 2. Using stable isotopes to analyse the trophic ecology of *Pacifastacus leniusculus* in a Scottish freshwater loch: Does an ontogenetic dietary shift occur?

2.1 Introduction

Invasive crayfish species frequently have a detrimental impact on the freshwater ecosystem they invade; this impact is due to changing the energy and nutrient flow, physically altering the habitat, and changing the community composition and diversity (Gherardi, 2007; Holdich, 1999; Nyström, 2002; Stenroth and Nyström, 2003; Whitledge and Rabeni, 1997). As such, crayfish are considered to be keystone species in freshwater environments (Reynolds and Souty-Grosset, 2012).

Since first being discovered in Scotland in 1995 (Maitland, 1996), *P. leniusculus* has become increasingly widespread and is now established at more than twenty sites within Scotland which encompass at least fifteen different river catchments (Freeman *et al*., 2010).

As an opportunistic omnivore, *P. leniusculus* can have a significant effect on the aquatic food web by impacting several trophic levels (Bondar *et al*., 2005; Olsson *et al*., 2008). For example, *P. leniusculus* are known to consume macrophytes (Momot, 1995; Nyström *et al*., 1996; Usio *et al.,* 2009), invertebrates (Nyström *et al*., 1999; Crawford *et al*., 2006), benthic fish (Guan and Wiles, 1997) and amphibians (Axelsson *et al*., 1997). Additionally, as omnivores, crayfish have the potential to occupy different trophic levels in different habitats, for example acting as an herbivore in one system but a predator in another (Stenroth *et al*., 2008). Consequently, the

polytrophic feeding behaviours of *P. leniusculus* can affect freshwater ecosystems in complex ways that are difficult to predict (Bondar *et al*., 2005).

Additionally, an omnivore may not maintain a consistent diet throughout its life (Bondar *et al*., 2005; Vojkovská *et al*., 2014). Dietary shifts at different life stages, known as ontogenesis, may be a consequence of an organism feeding more efficiently on prey of different sizes, different types or in different habitats (Mittelbach *et al*., 1988). It may also result from changes in predation risk, for example being able to feed in less protected habitats as an organism increases with size (Hjelm *et al*., 2000; Mittelbach *et al*., 1988). Consequently, ontogenetic dietary shifts can result in the same organism at different life stages having different ecological impacts on an ecosystem (Bondar *et al*., 2005; Bondar and Richardson, 2009; Mittelbach *et al*., 1988).

Traditionally, gut content analysis and direct field observations were employed to establish trophic dynamics and community structure (Alfaro *et al*., 2006). However, gut content analysis has limitations. For example, overestimation of the quantity of particular food sources is common and identification can be difficult due to size or condition of food sources (Alfaro *et al*., 2006). In addition, gut content analysis does not differentiate between the food items that are consumed and the food sources that are actually assimilated (Alfaro *et al*., 2006; Crehuet *et al*., 2007). Consequently, determining the full range of food sources consumed and their relative proportions to the diet of a consumer can be difficult.

As a result, naturally occurring stable isotopes have become an important tool in the modern ecologists' toolbox to elucidate community structure, trophic relationships and energy flow within an ecosystem (Roth and Hobson, 2000). Stable isotope ratios

provide information regarding food sources assimilated over a long period of time due to isotopes being consistently altered by biological and physical processes as they are transferred through the food web (Carmichael *et al*., 2004). Therefore, the stable isotope ratios of the consumers reflect those of their food sources in a predictable way (Bearhop *et al*., 2004). As a result, stable isotopes provide an estimate of trophic position (Tp) and can help elucidate many of the complex interactions within any given food web, including omnivory (Peterson and Fry, 1987; Post, 2002).

Carbon (C) and nitrogen (N) are the most commonly used elements for stable isotope analysis in food web studies (Whitledge and Rabeni, 1997). Each element has a lighter (^{12}C and ^{14}N) and heavier isotope (^{13}C and ^{15}N). Biochemical processes alter the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios, also known as fractionation or the trophic enrichment factor (TEF), which leads to distinct isotopic compositions in consumer tissue compared with prey (Peterson and Fry, 1987). The $^{13}C/^{12}C$ ratio has been found to change little with trophic position and thus reflects the primary producer consumed by an organism (DeNiro and Epstein 1978), while the $^{15}N/^{14}N$ ratio changes predictably between trophic levels (DeNiro and Epstein 1978; Peterson and Fry, 1987). It is the predictable enrichment of approximately 3 – 4 ‰ in ^{15}N between prey and consumer which allows the trophic position of an organism to be estimated (Fry, 1988; Minagawa and Wada, 1984), and consequently feeding relationships within a food web to be defined (Hill *et al*., 2015).

In addition to defining trophic position, mixing models using stable isotopes have enabled estimates of food source contributions to a consumer diet to be made (Phillips *et al*., 2014). Recent advancements have used Bayesian modelling approaches such as Stable Isotope Analysis in R (SIAR) (Parnell *et al*., 2010) to make inferences

regarding the composition of an organism's diet. SIAR not only enables TEFs to be included for food sources, but also different TEFs to be included for each potential food source as well as dealing with variability within the TEFs (Phillips *et al*., 2012). Additionally, the Bayesian modelling approach enables large numbers of food sources to be included (Phillips *et al*., 2014).

It is also possible to use the position of a consumer within isotopic space to quantify isotopic niche width as a proxy for the consumer's trophic niche width (Karlson *et al*., 2015). Layman *et al*. (2007) developed six metrics intended to measure trophic interactions, infer trophic diversity and quantitatively indicate the niche width occupied by a consumer. Since then, Jackson *et al*. (2011) developed a more robust method of calculating isotopic niche width using Stable Isotope Bayesian Ellipses in R (SIBER). SIBER expands upon the metrics proposed by Layman *et al*. (2007) and in addition uses a plotted ellipse area to represent the niche width of a group of individuals. The larger the ellipse area, the greater the niche width and the more general a diet. Furthermore, the ellipse area is less sensitive to different sample sizes and therefore niche width comparisons can be made between groups (Jackson *et al*., 2011).

Previous stable isotope studies on several crayfish species have differed in their conclusions regarding whether plant or animal material is the main food source. Some authors have found crayfish to feed predominantly on invertebrates (Nyström *et al*., 2006; Whitledge and Rabeni, 1997), whereas others reported detritus to be the main food source (Bondar *et al*., 2005; Evans-White *et al*., 2001; Rudneck and Resh, 2005). Additionally, there are mixed reports within existing literature with regard to the existence of ontogenetic dietary shifts in crayfish. Frequently, an ontogenetic dietary

shift can be detected when conducting gut content analysis but not when examining the stable isotope signature of crayfish tissue. For example, Parkyn *et al*. (2001) observed an ontogenetic dietary shift in the New Zealand crayfish *Paranephrops planifrons* (White, 1942) by examining the gut content of individuals of different sizes but could not detect the same ontogenetic shift when using stable isotope analysis. Guan and Wiles (1998) detected an ontogenetic dietary shift in *P. leniusculus* inspecting gut content, reporting that adult *P. leniusculus* shifted to a more plant and detritus based diet compared with an invertebrate-based diet in juveniles. However, subsequent studies employing stable isotope analysis have produced conflicting results. Bondar *et al*. (2005) were unable to identify an ontogenetic dietary shift in *P. leniusculus*, while Stenroth *et al*. (2008) reported that *P. leniusculus* did exhibit an ontogenetic dietary shift. It is possible that the differences observed between the stable isotope studies relate to whether *P. leniusculus* is acting as a native (Bondar *et al*., 2005) or invasive (Stenroth *et al*., 2008) species in an aquatic system.

The aim of the present study is to describe the trophic position, examine the diet composition and investigate whether or not an ontogenetic dietary shift can be identified in *P. leniusculus* inhabiting a Scottish freshwater loch using stable isotope analysis.

2.2 Methods

2.2.1 Study site

Loch Ken (55.0090° N, -4.0560° W), located near Castle Douglas in Dumfries and Galloway, Scotland (Figure 2-1), is home to the largest known population of the invasive *P. leniusculus* in Scotland (Gladman *et al.* 2010). Within Loch Ken there is an estimated *P. leniusculus* density of between 1.06 – 9.05 crayfish per m^2 (Ribbons and Graham, 2009). *P. leniusculus* has been found to be present from the head of Loch Ken down to Parton House, covering an approximate length of 9,500m (approximately two thirds) of the loch (Ribbons and Graham, 2009). However, as of 2012, *P. leniusculus* have been recorded as far as Crossmichael (Figure 2-1), an advancement of a further 3,000 m since 2009 (Andrew Blunsum, Loch Ken Ranger, Per. Comm. 2012).

Figure 2-1: Location of Loch Ken within Scotland.

Two study sites were selected within Loch Ken (Figure 2-2 for location and Figure 2-3 for photographs); Site 1 (55.0368° N, -4.1120° W) and Site 2 (55.0441° N, -4.1205° W), which was north of Site 1. The sites were located approximately 1,000m apart and both sites were located within the North basin of Loch Ken. The North basin was selected for study, as *P. leniusculus* is known to be particularly dense here. This is due to the population in Loch Ken having originated from the feeder streams within the Water of Ken catchment, which enters into the North basin (Maitland, 1996).

Study sites were also selected away from the Loch's river inflow point as carbon isotope compositions of aquatic flora and fauna could potentially be confounded by terrestrially-derived material, making the trophic interactions difficult to elucidate (Prof. Colin Adams, Per. Comm. 2013).

Figure 2-2: Location of sampling Site 1 and Site 2 within Loch Ken.

a. b.

Figure 2-3: Photographs of Site 1 (a) and Site 2 (b); both photographs are taken facing south.

The study was carried out between July and September 2013. *P. leniusculus*, invertebrate, zooplankton, vegetation and detritus collection took place over a 5 day period during 15th and 19th July 2013, gill netting between 19th and 20th August 2013 and stable isotope analysis between August and September 2013.

2.2.2 Sample collection

2.2.2.1 P. leniusculus

P. leniusculus exhibit ontogenetic shifts in spatial distribution (Harrison *et al*. 2006). Juveniles are restricted to shallow areas with rocky substrate for cover, which minimizes predation by larger crayfish and predatory fish. Adults inhabit deeper depths. To account for this spatial distribution, *P. leniusculus* were collected, under SNH licence, using three separate methods of capture:

1. Crayfish traps
2. Kick sampling
3. Hand searching

2.2.2.1a Crayfish traps

Eighteen Swedish Trappy traps (Figure 2-4), baited with either rainbow trout *Oncorhynchus mykiss* (Wallbaum, 1792) or *S. salar*, were positioned at three points around each site. At each point three equally spaced (1 m) traps were then attached to a buoy and deployed, by hand, at a depth of 0.8 ± 0.1 m. For a total of five days, traps were left overnight and emptied the next day. Traps were then re-baited with fresh fish and redeployed. *P. leniusculus* were counted and sexed on site, placed into a cool box for transport and then frozen until return to the laboratory.

Figure 2-4: Swedish Trappy trap. Length 520 mm, diameter 210 mm and mesh size 20 mm. Reproduced from www.trappy.com.

2.2.2.1b Kick sampling

Kick sampling was primarily used to capture juvenile *P. leniusculus*. Using a standard D-shaped pond net (1 mm mesh), 2 minute kick/sweep samples were performed. This was repeated at multiple locations around each site to ensure all available habitats were sampled. The pond net was placed on the loch bottom and the substrate disturbed (by foot using a kicking motion) just upstream of the net for the allocated 2 minute

period to collect any organisms present. In cases where vegetation was present, the net was swept back and forth through the substrate for the 2 minute period. Contents of the net were then transferred into a white plastic tray with 2 - 3 cm of loch water, and examined for the presence of *P. leniusculus*. *P. leniusculus* were counted, transferred to pots, placed in a cool box for transport and then frozen until return to the laboratory.

2.2.2.1c Hand searching

Hand searching was primarily used to capture juvenile *P. leniusculus*. Each site was actively searched by hand, turning over rocks and vegetation to expose any sheltering *P. leniusculus*, which were then collected with a small hand-net (1 mm mesh). *P. leniusculus* caught in the net were then transferred into a white plastic tray, counted, transferred to pots, placed in a cool box for transport and then frozen until return to the laboratory.

Any individuals caught about to moult, or freshly moulted, were discarded from the study sample, as crayfish are known to avoid foraging for a period before and after ecdysis (Reynolds, 2002).

2.2.2.2 Invertebrates

Aquatic invertebrates were obtained by kick sampling as detailed in section 2.2.2.1b. Contents of the net were then transferred into a white plastic tray with 2 - 3 cm of loch water, and examined. Invertebrates were sorted from organic material, placed in pots and transported in a cool box before being frozen and identified to family upon return to the laboratory.

2.2.2.3 Zooplankton

On the last day at each site, a zooplankton net was pulled through the water horizontally. Organisms were captured in a vial at the bottom of the net and then rinsed with loch water until a 2 litre storage bottle was filled, before being transported back to the laboratory in a cool box for identification.

2.2.2.4 Vegetation

At each site, samples of all aquatic vegetation were collected by hand, placed in a clear plastic bag, transported in a cool box and frozen until return to the laboratory.

Samples of terrestrial vegetation (either overhanging the water or growing close to the water's edge) were collected by hand, placed in a clear plastic bag, transported in a cool box and frozen until return to the laboratory.

2.2.2.5 Detritus

Detritus was collected at each site by use of a grab. The grab was deployed by hand, where upon reaching the loch bottom a weight was sent down the line causing the 'jaws' of the grab to close and consequently scoop up a sediment sample. Once brought back to the surface, the contents of the grab were run through a sieve using loch water to remove any rock material. Grabs were repeated at multiple locations for each site. Samples were then transferred to a clear plastic bag, transported in a cool box and frozen until return to the laboratory.

2.2.2.6 Fish

Any small fish present at each site were captured using net sweeps through any vegetation. Fish were then transferred to pots, transported in a cool box and frozen until return to the laboratory.

Gill nets were used to capture larger fish species present in Loch Ken. Gill netting was carried out under Marine Scotland licence. Four nylon Nordic multimesh gill nets (monofilament) were deployed from a boat, two at each site. Nets were deployed and left overnight at depths of 2.5 m and 7 m at Site 1, and depths of 2.5 m and 14 m at Site 2. Upon collection of nets the following day, all fish present were dead. Fish were removed from the net, transferred to clear plastic bags and placed in cool boxes for return to the laboratory.

In addition to fish, large numbers of *P. leniusculus* had also become entangled. Samples of *P. leniusculus* from each depth were also removed from the net, transferred to clear plastic bags and placed in cool boxes for return to the lab. All other *P. leniusculus* remaining entangled in the nets were transferred to clear plastic bags and appropriately disposed of upon return to the laboratory.

After completion of sample collection each day, and especially before leaving Loch Ken to return to the laboratory, equipment was checked for contamination and nets thoroughly washed.

2.2.3 Sample preparation

2.2.3.1 P. leniusculus

Carapace length was measured from the tip of the rostrum to the posterior margin of the carapace using Vernier calipers (± 0.1 mm). After measuring, *P. leniusculus* were

sexed as male or female. If *P. leniusculus* were too small to be able to distinguish sex, it was classed as unknown. Tail muscle was then removed, placed into a pot and frozen at -70°C until further analysis.

The total number of *P. leniusculus* caught was 366 from Site 1 and 452 from Site 2, while the carapace length ranged from 5.5 mm - 69.2 mm and 3.5 mm - 72.6 mm at Site 1 and Site 2 respectively. Additionally, *P. leniusculus* removed from gillnets totalled 55 and 57 from Site 1 and Site 2 respectively. The carapace length ranged from 28.4 mm - 66.8 mm at Site 1, while at Site 2 it ranged from 32.8 mm – 79.7 mm.

Based on carapace length, *P. leniusculus* were separated in to five distinct size classes as follows:

1. 0 mm – 9 mm
2. 10 mm – 20 mm
3. 21 mm – 39 mm
4. 40 mm – 50 mm
5. 51 mm – 70 mm

Due to the large numbers of *P. leniusculus* collected, it was necessary to sample proportional to the total number of *P. leniusculus* individuals collected for each size class. As a result, each size class contributed 20 % to the total number of *P. leniusculus* to be analysed. Within each 20 %, there was a further break down for males and females, proportional to the number that had been caught. As a result, the number of *P. leniusculus* used for stable isotope analysis was 75 for Site 1 and 90 for Site 2.

Individuals caught by gill net were not included in the size class breakdown; they were instead all included for analysis separately as they had been caught at a different depth.

2.2.3.2 Invertebrates

Invertebrate families present at each site were determined to be Chironomidae, Daphniidae, Gammaridae, Lumbriculidae, Corixidae and Ephemeroptera. For each site, invertebrate families were placed in separate pots and frozen at -70°C until further analysis.

2.2.3.3 Zooplankton

Upon arrival to the laboratory, samples were taken in order to identify the organisms present to family level. Cyclopoida, Bosminidae and Daphniidae were determined to be present at each site. Zooplankton samples were pooled for each site and then extracted by vacuum filtration through a membrane filter, before washing the cells off the filter paper into a pot using a small amount of distilled water. Samples were then frozen at -70°C for later analysis.

2.2.3.4 Vegetation and detritus

All vegetation was washed thoroughly with distilled water to remove any sediment and epibionts, and searched a second time for *P. leniusculus* or other invertebrates. Aquatic vegetation was separated into submersed macrophyte and algae, homogenised, placed in separate pots and frozen for later analysis.

For each site, all terrestrial vegetation collected, either overhanging the water or growing close to the water's edge, was homogenised in order to give an approximate representation of food sources present, and placed in pots before being frozen at -70°C for later analysis.

2.2.3.5 Fish

All fish caught were rinsed with distilled water, identified to species level where possible and had the fork length measured to the nearest mm. A 5 mm biopsy punch was used in order to take a dorsal muscle sample, which was then frozen at -70°C for later analysis.

Minnows *Phoxinus phoxinus* (Linnaeus, 1758) were caught using net sweeps while, only three species of fish were caught in the gill nets: Pike *Esox lucius* (Linnaeus, 1758), Perch *Perca fluviatilis* (Linnaeus, 1758) and a Roach/Bream hybrid *Rutilus rutilus* (Linnaeus, 1758) x *Abramis brama* (Linnaeus, 1758). A total of 23 fish were caught at Site 1, while 10 fish were caught at Site 2. Fork length ranged from 92 mm to 427 mm at Site 1, while at Site 2 it ranged from 102 mm – 536 mm.

2.2.4 Stable isotope measurements

All *P. leniusculus*, invertebrate, fish, vegetation, zooplankton and detritus samples were put in a freeze dryer at -60°C for 24 hours. After freeze drying was complete, samples were ground into a fine powder using a mortar and pestle. In order to have enough material for zooplankton and invertebrate analysis, samples were pooled for each site. Dependent on sample type, 0.7 mg or 1.5 mg (for animal and vegetation material respectively) were weighed into tin capsules using a Mettler-Toledo MX5 microbalance accurate to 1 µg. Isotopic analysis was performed at the East Kilbride node of the NERC Life Sciences Mass Spectrometry Facility hosted by the Scottish Universities Environmental Research Centre (SUERC) using a continuous flow isotope ratio mass spectrometer. The system comprises a Thermo Delta XP Plus stable isotope ratio mass spectrometer (IRMS), with an Elementar Pyrocube elemental

analyser (EA) for combusting and purifying the gas species of interest. The analytes are N_2 and CO_2 for nitrogen and carbon stable isotope ratio measurement respectively.

Isotopic ratios are expressed by the standard delta (δ) notation in parts per thousand (‰) using the following equation;

$$\delta X = [(R_{sample}/R_{standard}) - 1] * 1000$$

where X is ^{13}C or ^{15}N and R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio.

Delta values are acquired using N_2 and CO_2 cylinder gases, and are corrected/calibrated using three laboratory standards – gelatin, "alagel" (a mixture of alanine and gelatin), and glycine. The standards have been chosen for a wide range of isotope ratios and are checked against international standards (from the NIST, Washington and the IAEA, Vienna). One of the international standards, USGS 40 which is a glutamic acid, is run daily and forms the basis of calculating N and C elemental abundances. Furthermore, twenty-seven gelatin laboratory standards are run daily ensuring analytical precision of $\delta^{13}C$ and $\delta^{15}N$ expression within 0.1 ‰.

2.2.5 Stable isotope analysis

2.2.5.1 Lipid correction

Before further stable isotope analysis could take place, the $\delta^{13}C$ isotopic signature of organisms were arithmetically corrected for lipid content. Variation in lipid contents can affect the measured carbon isotope ratios (Ehrich *et al*., 2011). Additionally, the estimation of diet contributions by mixing models can be influenced by the lipid content of prey or consumer tissue (Kiljunen *et al*., 2006). However, lipid correcting samples of whole body invertebrates has been advised against (Kiljunen *et al*., 2006).

Even though high quantities of chitin and non-lipid energy stores may result in C:N ratios similar to those observed in high-lipid content tissue such as fish tissue, lipid correction models do not produce equivalent changes in the $\delta^{13}C$ isotopic signature (Kiljunen *et al*., 2006). Therefore, only the $\delta^{13}C$ isotopic signatures obtained for *P. leniusculus* individuals and fish species sampled were arithmetically corrected for lipid content.

Lipid content was arithmetically corrected using the equation recommended by Kiljunen *et al*. (2006):

$$\delta^{13}C' = \delta^{13}C + D * (I + (3.9/1 + 287/L))$$

where $\delta^{13}C'$ is the lipid corrected $\delta^{13}C$ of the organism, D is the isotopic difference between protein and lipid (7.018), I is a constant (0.048) and L is the C:N ratio of the organism's tissue.

2.2.5.2 Isotopic mixing models

Prior to analysis, a hierarchical cluster analysis, based on the Euclidean distance and wards criterion, was performed in R (R Core Team, 2014) on the mean isotopic ratios ($\delta^{15}N$ and $\delta^{13}C$) of each food source obtained from Loch Ken. Cluster analysis produced a dendrogram, which identified groups of organisms with similar isotopic ratios. Cluster analyses were performed on plant and animal material separately. The dendrogram produced for animal material was then used to further group organisms together based on functional feeding groups (FFG) and the organisms' location within $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot space (Figure 2-5). This enabled groups created to be ecologically meaningful, as well as identical at each site. Using cluster analysis, and

further grouping criteria, potential food sources being used in the model were reduced from 16 to 9 (Table 2-1).

The Bayesian mixing model package SIAR (Parnell *et al.*, 2010) was run in R to produce a mixing model, which provided estimates of the proportions of nine different potential food source groups, based on hierarchical cluster analysis results, to *P. leniusculus* diet for each size class.

The isotopic mixing model was run separately for each site within Loch Ken and potential food source groups used were as detailed in Table 2-1. Only data collected for *P. leniusculus* at shallow depths (< 1 m) were used in the isotopic mixing model as only all size classes of *P. leniusculus* and all potential food sources were obtained at shallow depths. Isotopic ratios for each food source group were adjusted using an appropriate trophic enrichment factor (TEF) to account for trophic fractionation. As no crayfish specific TEFs are available, commonly used values collected from literature were used instead. Following Ercoli *et al.* (2014), TEF values of 3.4 ± 0.98 ‰ for $\delta^{15}N$ and 0.39 ± 1.23 ‰ for $\delta^{13}C$ for animal source groups (Post, 2002), and 2.4 ± 0.42 ‰ for $\delta^{15}N$ and 0.40 ± 0.28 ‰ for $\delta^{13}C$ for plant source groups (McCutchan *et al*., 2003) were used.

Table 2-1: Food source groupings for Site 1 and Site 2 based on hierarchical cluster analysis results, known information about FFG and location of organisms in $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot space. Food source groups represent clusters and bear no relationship to trophic position of the organism.

Food source group	Site 1	Site 2
1	Pike, perch, roach/bream hybrid	Pike, perch
2	Minnow	Minnow
3	Gammaridae	Gammaridae
4	Corixidae, Chironomidae, Lumbriculidae	Corixidae, Chironomidae, Lumbriculidae
5	Ephemeroptera	Ephemeroptera
6	Daphniidae, zooplankton	Daphniidae, zooplankton
7	Algae	Algae
8	Submersed macrophyte	Submersed macrophyte
9	Terrestrial vegetation, detritus	Terrestrial vegetation, detritus

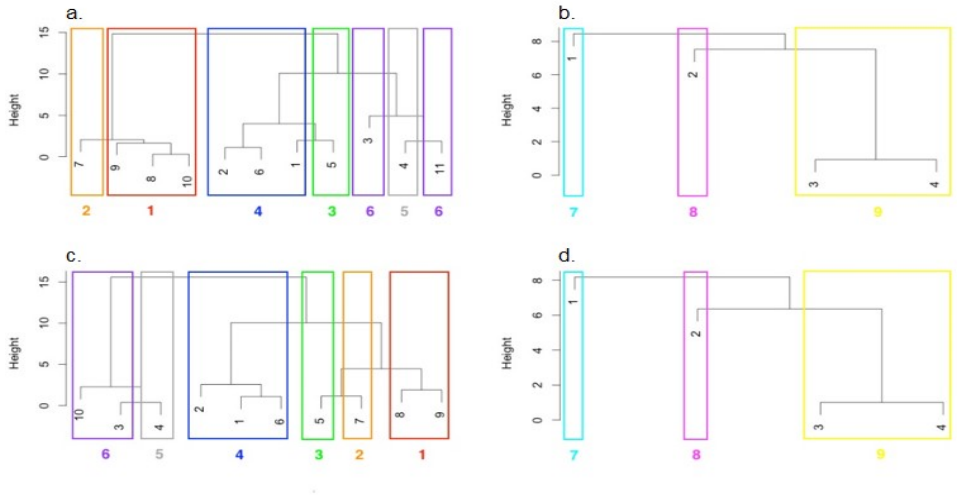


Figure 2-5: Dendrogram of hierarchical cluster analysis of potential food sources collected from Loch Ken at Site 1 (a = animal sources; b = plant sources) and Site 2 (c = animal sources; d = plant sources). Each node represents an individual organism. Animal sources: 1 = Chironomidae, 2 = Corixidae, 3 = Daphniidae, 4 = Ephemeroptera, 5 = Gammaridae, 6 = Lumbriculidae, 7 = minnow, 8 = perch, 9 = pike, 10 = roach/bream hybrid, 11 = zooplankton. Plant sources: 1 = algae, 2 = submersed macrophyte, 3 = terrestrial vegetation, 4 = detritus. Colours represent groupings used in the isotopic mixing model: red = group 1, orange = group 2, green = group 3, blue = group 4, grey = group 5, purple = group 6, aqua = group 7, pink = group 8, yellow = group 9.

2.2.5.3 Trophic position

A trophic baseline is critical to establishing any spatial and/or temporal changes to the trophic dynamics of an ecosystem (Cabana and Rasmussen, 1996), and is also required when estimating the trophic position of an organism.

Traditionally primary producers have been used to establish trophic baselines; however, such organisms can vary widely on a seasonal basis. It is therefore recommended that long-lived primary consumers, such as filter feeding mussels, be used to determine a trophic baseline (Post, 2002). Primary consumers are recommended for use when establishing a trophic baseline as isotopic values are better integrated over time compared with primary producers (Post, 2002).

However, no mussels or snails were collected at either of the sites sampled within Loch Ken. As a result, the mean $\delta^{15}N$ isotopic ratio of the non-predatory invertebrate, Gammaridae, was used as the baseline organism for this study (Jackson and Britton, 2013).

Consequently, the trophic position of *P. leniusculus*, and potential food sources, were calculated as recommended by Anderson and Cabana (2007) using the following equation:

$$Tp_{consumer} = ((\delta^{15}N_{consumer} - \delta^{15}N_{baseline})/3.4) + 2$$

where $Tp_{consumer}$ is the trophic position of an organism, $\delta^{15}N_{consumer}$ is the isotopic ratio of the organism in question, $\delta^{15}N_{baseline}$ is the mean isotopic ratio of a primary consumer, 3.4 is the TEF (Post, 2002) and 2 is the trophic position of the organism used to estimate the baseline.

2.2.5.4 Niche width

Previously, the total area (TA) encompassing the data for a species, plotted by fitting a convex hull around the most extreme data points within a $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot space, was used as a measure of niche width (Layman *et al*., 2007). However, the convex hull method has since been shown to be highly sensitive to different sample sizes (Jackson *et al*., 2011, Syväranta *et al*., 2013). As a result, the standard ellipse area (SEA) was used to compare the mean core isotopic niche width of each size class of *P. leniusculus*. The SEA contains approximately 40 % of the data and therefore reveals the core niche area, which is relatively insensitive to variations in sample size (Jackson *et al*., 2011). As many of the size class sample sizes were small in this study (n = 4 – 42), a sample size corrected version of the standard ellipse area (SEA_c) was employed (Jackson *et al.*, 2011). However, as Jackson *et al*. (2011) recommended a minimum sample size of 10, caution must be taken when drawing inference from results where the sample size used is less than 10.

Niche widths for each size class of *P. leniusculus* were estimated separately for Site 1 and Site 2 by calculating the SEA_c using SIBER. The $\delta^{15}N$ and $\delta^{13}C$ isotope signatures from all individuals collected from all size classes sampled were used.

2.2.5.5 Population metrics

Using the stable isotope signatures from all individuals in all size classes, a $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot was constructed using SIBER, with SEA_c ellipses and convex hull polygons representing the niche width for each size class of *P. leniusculus*. Metrics suggested by Layman *et al*. (2007) were also calculated to quantify trophic structure and resource use. Population metrics calculated for each size class were; $\delta^{15}N$ range (NR) and $\delta^{13}C$ range (CR), the trophic diversity as measured by the mean

distance to the isotopic centroid (CD), trophic evenness as measured by the standard deviation of the nearest neighbour distance (SDNND), as well as the niche width described by the total area encompassed by the convex hull polygon (TA) (Jackson *et al*., 2012). All population metrics were calculated using SIAR.

2.2.6 Statistical analysis

A multiple linear regression model was constructed using site, sex, depth and carapace length to explain variation in $\delta^{15}N$ and $\delta^{13}C$ respectively. Interactions between depth and sex were not examined as crayfish sexed as unknown were not obtained at intermediate and deep depths.

For each response variable, $\delta^{15}N$ or $\delta^{13}C$, a full model was created using all possible variables and subsequent interactions. The 'best' multiple linear regression models were selected for $\delta^{15}N$ and $\delta^{13}C$ by removing non-significant variables from each full model through a series of backward, stepwise deletions with the purpose of minimizing the final model's Akaike's Information Criterion value (AIC; Akaike, 1973). The smaller the AIC, the more variability the model explains. Model validation was systematically applied by checking for normality and homogeneity in model residuals, with no violation of independence (Zuur *et al.*, 2007). All values are ± standard error (SE) unless otherwise described. Significance level was defined as $p < 0.05$.

2.3 Results

2.3.1 Multiple linear regression model selection

The full model for both $\delta^{15}N$ and $\delta^{13}C$ comprised; carapace length, depth, sex, site, carapace length * depth, carapace length * sex, carapace length * site, site * sex, and depth * site. In addition to using the aforementioned AIC value to remove non-significant variables, an F-test statistic was used to compare the model with and without a variable to assess significance. For $\delta^{15}N$, site and carapace length were the only significant variables contributing to the model (Table 2-2), while for $\delta^{13}C$, depth was the only significant variable contributing to the model (Table 2-3). The inclusion of any other variables reduced the models explanatory power. Stepwise deletion of variables from the full model leading to the final multiple linear regression model using the AIC values and F-test statistic can be observed in Table 2-2 ($\delta^{15}N$) and Table 2-3 ($\delta^{13}C$).

Table 2-2: Summary of backwards stepwise deletion of variables for $\delta^{15}N$ multiple linear regression model using AIC values. Bold denotes final model.

Variables	Variables removed	Df	F value	P value	Cumulative model AIC
Carapace length, Depth, Sex, Site, Carapace length * Depth, Carapace Length * Sex, Carapace length * Site, Site * Sex, Depth * Site	Depth	2	-	-	-277.24
	Carapace length * Depth	2	0.0821	0.921	
	Carapace length * Site	2	1.0844	0.299	
	Site * Sex	1	0.7434	0.477	
	Depth * Site	2	1.4703	0.231	
Carapace length, Sex, Site, Carapace length * Sex	Carapace length * Sex	2	2.0778	0.127	-288.11
Carapace length, Sex, Site,	Sex	2	0.2775	0.757	-287.88
Carapace length, Site	**-**	**2**	**8.453**	**< 0.005**	**-291.32**

Table 2-3: Summary of backwards stepwise deletion of variables for $\delta^{13}C$ multiple linear regression model using AIC values. Bold denotes final model.

Variables	Variables removed	Df	F value	P value	Cumulative model AIC
Carapace length, Depth, Sex, Site, Carapace length * Depth, Carapace Length * Sex, Carapace length * Site, Site * Sex, Depth * Site	Sex	2	-	-	-44.91
	Carapace length * Sex	2	0.067	0.935	
	Carapace length * Depth	2	4.835	0.008	
	Site * Sex	1	0.944	0.391	
	Depth * Site	2	0.188	0.829	
Carapace length, Depth, Site, Carapace length * Site	Carapace length * Site	1	1.886	0.171	-48.73
Carapace length, Depth, Site,	Carapace length	1	0.000	0.995	-48.81
	Site	1	0.000	0.984	
Depth	**-**	**2**	**23.64**	**< 0.005**	**-52.81**

2.3.2 Stable isotope analysis

2.3.2.1 Nitrogen

The mean $\delta^{15}N$ of *P. leniusculus* individuals at Site 2 (7.28 ± 0.06 ‰) was found to be significantly lower than the mean $\delta^{15}N$ of individuals at Site 1 (7.52 ± 0.08 ‰) ($t = -2.99$, $p < 0.005$). Carapace length was observed to be weakly, positively related to $\delta^{15}N$ at both sites ($t = 2.484$, $p = 0.01$), with $\delta^{15}N$ increasing with *P. leniusculus* size suggesting a dietary shift (Figure 2-6). The overall model was significant ($F_{2,276} = 8.453$, $p < 0.005$, $R^2 = 0.058$), yet only explained approximately 6 % of the variation within the data.

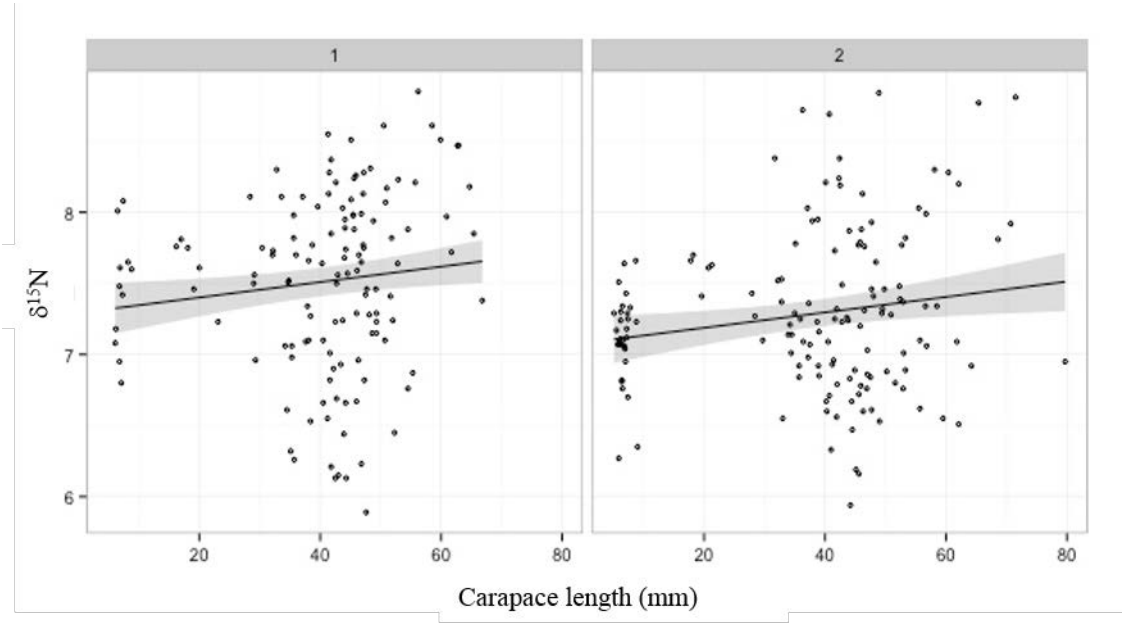


Figure 2-6: The relationship between carapace length (mm) and $\delta^{15}N$ (‰) in *P. leniusculus* for Site 1 (1) and Site 2 (2) collected from Loch Ken, Scotland. Solid black line denotes the fitted linear regression line and the grey area represents the upper and lower bounds of the 95 % CI.

2.3.2.2 Carbon

There was a significant depth effect for $\delta^{13}C$ ($F_{2,276} = 23.64$, $p < 0.005$, $R^2 = 0.146$), with the final model explaining approximately 15 % of the variation within the data. A post hoc test using a Bonferroni adjustment showed that intermediate and deep depths were significantly less enriched in $\delta^{13}C$ ($p = 0.001$ and $p < 0.001$ respectively) than shallow depths. Intermediate and deep depths did not significantly differ from one another ($p = 0.122$) (Figure 2-7). This indicates that *P. leniusculus* are utilising energy sources of different carbon isotopic composition as they move into deeper waters.

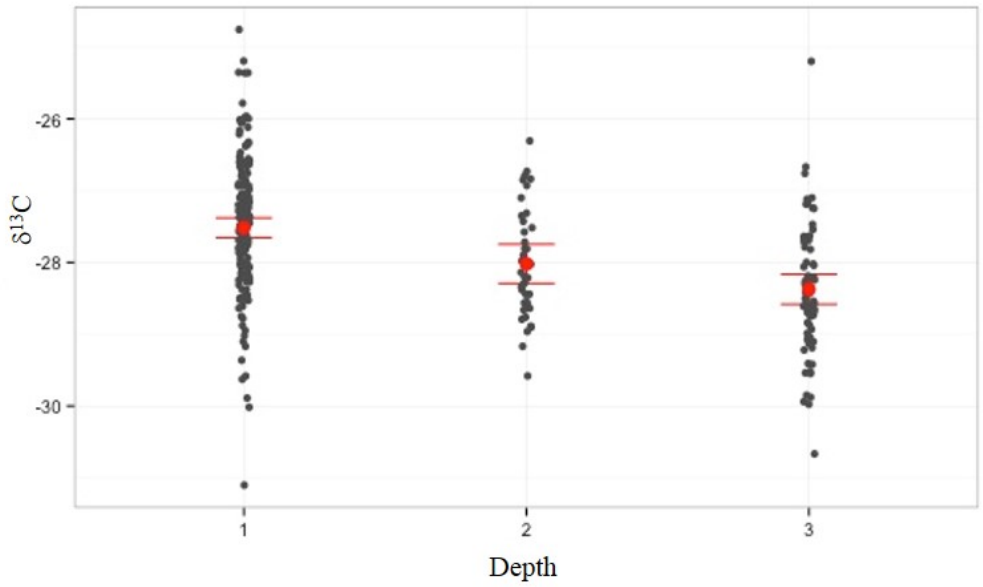


Figure 2-7: Distribution of *P. leniusculus* $\delta^{13}C$ (‰) values at each depth. Each point represents individual measurements. Site 1 and Site 2 are combined. 1 = Shallow (< 1 m), 2 = Intermediate (1 – 7 m), 3 = Deep (> 7 m). Red bars represent mean ± SE.

2.3.3 *P. leniusculus* diet

Only *P. leniusculus* individuals caught at shallow depths (< 1 m) were used when investigating dietary choices as all potential food sources were obtained in water less than 1 m deep.

2.3.3.1 $\delta^{15}N$ and $\delta^{13}C$

Four potential carbon sources were available to *P. leniusculus* individuals in Loch Ken: algae, submersed macrophytes, detritus and terrestrial vegetation (food source groups 7, 8 and 9 respectively). The isotopic ranges of the three food source groups did not overlap and were therefore clearly distinguishable as separate sources of carbon when visualised within a $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot space (Figure 2-8).

The mean $\delta^{13}C$ signatures of size classes 1 - 5 of *P. leniusculus* at Site 1 and Site 2 ranged from -26.61 to -28.07 ‰ and -27.25 to -27.70 ‰ respectively (Table 2-4). Mean $\delta^{13}C$ signatures of *P. leniusculus* were within the range of detritus, terrestrial vegetation and submersed macrophyte (Table 2-4), suggesting that the $\delta^{13}C$ signature of *P. leniusculus* in Loch Ken is a result of consuming, either directly or indirectly, one of these sources.

For all size classes of *P. leniusculus* combined, $\delta^{15}N$ values ranged from 7.39 – 8.01 ‰ at Site 1 and 7.11 – 7.88 ‰ at Site 2 (Figure 2-8). This was less than a 1 ‰ enrichment at both sites, which is not enough to indicate a trophic level change between size classes. In addition to this, the Tp of each *P. leniusculus* size class was relatively similar (Table 2-4). However, there is variation in $\delta^{15}N$ values between size classes, which would suggest diet is not consistent throughout an individual's life.

P. leniusculus were more enriched in $\delta^{15}N$ and $\delta^{13}C$ than all other invertebrates collected from Loch Ken at both Site 1 and Site 2, indicating that they are a trophic step above all other invertebrates but a trophic step below large predatory fish (pike, perch and roach/bream hybrid) (Figure 2-8). This would suggest that *P. leniusculus* are the top invertebrate predator within Loch Ken.

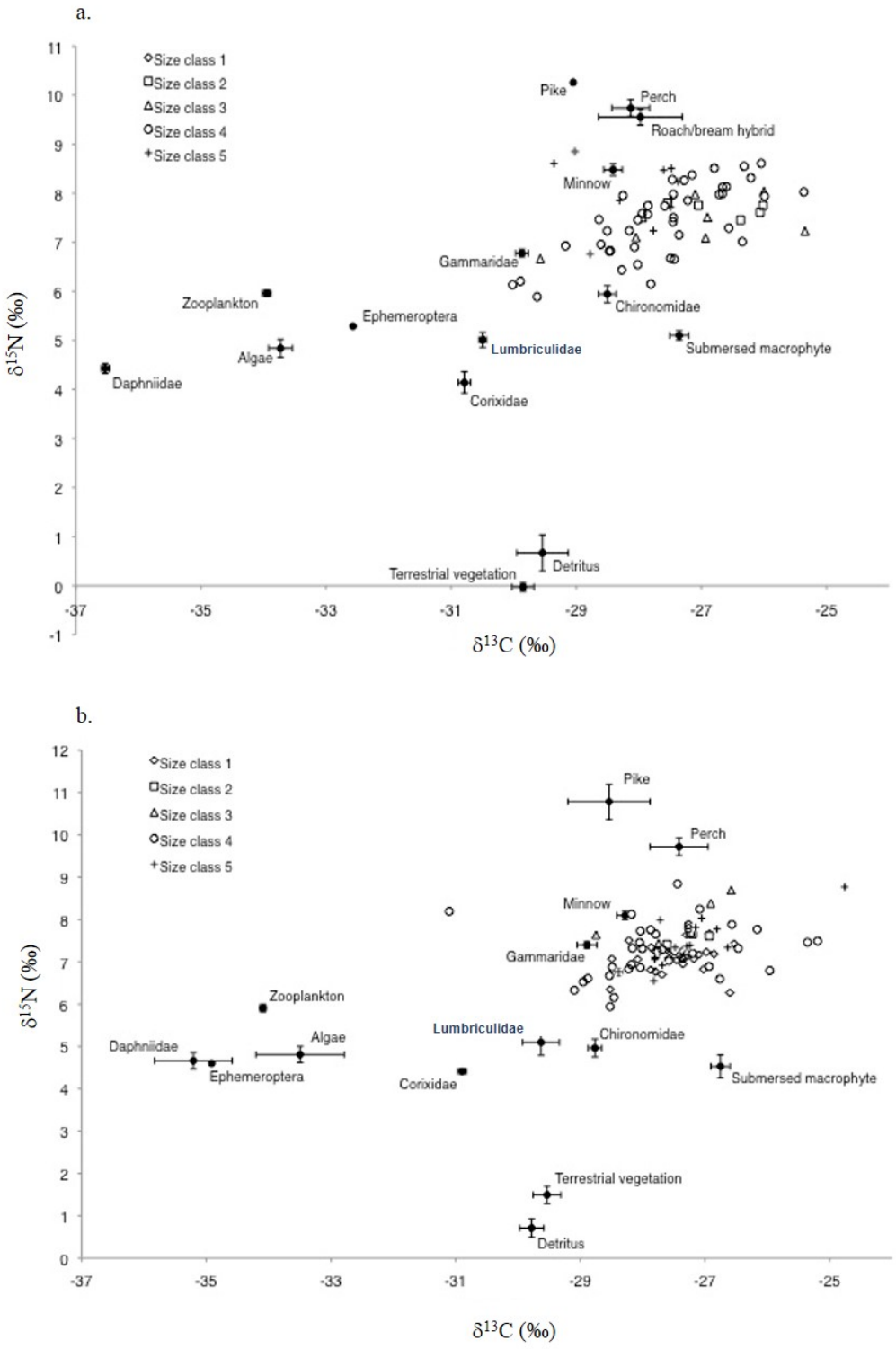


Figure 2-8: $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot for *P. leniusculus* for Site 1 (a) and Site 2 (b). Size classes 1 – 5 are individual data points and potential food sources are mean ± SE.

Table 2-4: Mean $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) values (± SE), as well as estimated mean trophic position (Tp) for *P. leniusculus* and each potential food source group from Site 1 (a) and Site 2 (b) in Loch Ken.

a.

	n	**Mean $\delta^{15}N$ (‰)**	**Mean $\delta^{13}C$ (‰)**	**Mean Tp**
Food source group				
1	23	9.27 (0.64)	-28.14 (1.26)	2.9
2	12	8.48 (0.42)	-28.41 (0.51)	2.5
3	12	6.78 (0.27)	-29.87 (0.35)	2.0
4	27	5.00 (0.99)	-29.88 (1.13)	1.5
5	1	5.29 (0.00)	-32.57 (0.00)	1.6
6	7	5.30 (0.83)	-35.06 (1.39)	1.6
7	6	4.84 (0.45)	-33.73 (0.19)	1.4
8	6	5.10 (0.10)	-27.36 (0.15)	1.5
9	11	0.29 (0.20)	-29.71 (0.20)	0.1
P. leniusculus	76	7.52 (0.08)	-27.50 (0.12)	2.2
Size class 1 (0 mm - 9 mm)	11	7.44 (0.12)	-27.25 (0.25)	2.2
Size class 2 (10 mm - 20 mm)	5	7.68 (0.06)	-26.61 (0.30)	2.3
Size class 3 (21 mm - 39 mm)	8	7.39 (0.17)	-27.23 (0.46)	2.2
Size class 4 (40 mm - 50 mm)	42	7.44 (0.11)	-27.60 (0.16)	2.2
Size class 5 (51 mm - 70 mm)	10	8.01 (0.21)	-28.07 (0.23)	2.4

b.

	n	**Mean $\delta^{15}N$ (‰)**	**Mean $\delta^{13}C$ (‰)**	**Mean Tp**
Food source group				
1	10	10.04 (0.76)	-27.75 (1.26)	2.8
2	12	8.10 (0.34)	-28.28 (0.45)	2.2
3	11	7.40 (0.26)	-28.89 (0.52)	2.0
4	28	4.80 (0.65)	-29.71 (1.04)	1.2
5	1	4.60 (0.00)	-24.91 (0.00)	1.2
6	10	5.16 (0.74	-34.76 (1.27)	1.3
7	3	4.81 (0.19)	-33.49 (0.71)	1.2
8	9	4.53 (0.27)	-26.75 (0.15)	1.2
9	12	1.10 (0.19)	-29.66 (0.14)	0.1
P. leniusculus	89	7.28 (0.06)	-27.52 (0.09)	2.0
Size class 1 (0 mm - 9 mm)	30	7.11 (0.06)	-27.50 (0.09)	1.9
Size class 2 (10 mm - 20 mm)	4	7.60 (0.06)	-27.25 (0.14)	2.1
Size class 3 (21 mm - 39 mm)	5	7.88 (0.28)	-27.46 (0.38)	2.1
Size class 4 (40 mm - 50 mm)	36	7.25 (0.11)	-27.70 (0.19)	2.0
Size class 5 (51 mm - 70 mm)	14	7.44 (0.16)	-27.26 (0.23)	2.0

2.3.4 Isotopic mixing models

2.3.4.1 Food source contribution

Submersed macrophyte (food source group 8) contributed the most to *P. leniusculus* diet for size classes 1, 4 and 5 across both Site 1 and Site 2, relative to other potential food source groups. The largest contributor to diet for size classes 2 and 3 shifted from submersed macrophyte to terrestrial vegetation and detritus (food source group 9) at both sites. The second largest contributor for size classes 1, 4 and 5 was terrestrial vegetation and detritus, while for size classes 2 and 3 the second largest contributor was submersed macrophyte. This finding was echoed across both Site 1 and Site 2 (Figure 2-9; Table 2-5).

At Site 1 and Site 2, Daphniidae and zooplankton (food source group 6) was one of the smallest contributors to *P. leniusculus* diet across all size classes, along with Ephemeroptera (food source group 5) (Figure 2-9; Table 2-5). Although algae (food source group 7) was one of the smaller contributors to *P. leniusculus* diet for size classes 1, 4 and 5, it contributed more to the diets of *P. leniusculus* falling within size classes 2 and 3 (Figure 2-9; Table 2-5)

Size classes 2 and 3 at both sites exhibited an increase in the contribution of animal protein sources to their diet compared to other size classes, except size class 5 at Site 1 which retained an animal protein contribution equal to or higher than that observed for size classes 2 and 3. In particular, Gammaridae (food source group 3) and Corixidae, Chironomidae and Lumbriculidae (food source group 4) contributed more to the diets of individuals within size classes 2 and 3 (Figure 2-9; Table 2-5). Large predatory fish (food group 1) and minnow (food source group 2) accounted for the

increase in animal protein contribution to size class 5's diet at Site 1 and the resulting drop in submersed macrophyte contribution when compared to Site 2 (Table 2-5).

It is possible that sizes classes 2 and 3 consume more terrestrial vegetation/detritus and algae than all other sizes, but it is also possible that they consume more terrestrial vegetation/detritus and algae due to indirect consumption as a result of consuming greater numbers of invertebrates, which feed at lower trophic levels.

As a result of grouping potential food sources together, it is impossible to determine which food source alone within a group is actually contributing to the diet of *P. leniusculus*. Therefore, results indicate that in addition to Gammaridae, an increase in animal protein in the diet of size classes 2 and 3 at both sites is being obtained by consuming larger amounts of either one or more of the following macroinvertebrates; Corixidae, Chironomidae or Lumbriculidae (Figure 2-9).

Consequently, results from the isotopic mixing model would suggest an ontogenetic dietary shift for *P. leniusculus* exists. This fits well with the $\delta^{15}N$ and $\delta^{13}C$ values observed for each size class (Table 2-4), as well as the position of *P. leniusculus* within isotopic bi-plot space (Figure 2-8).

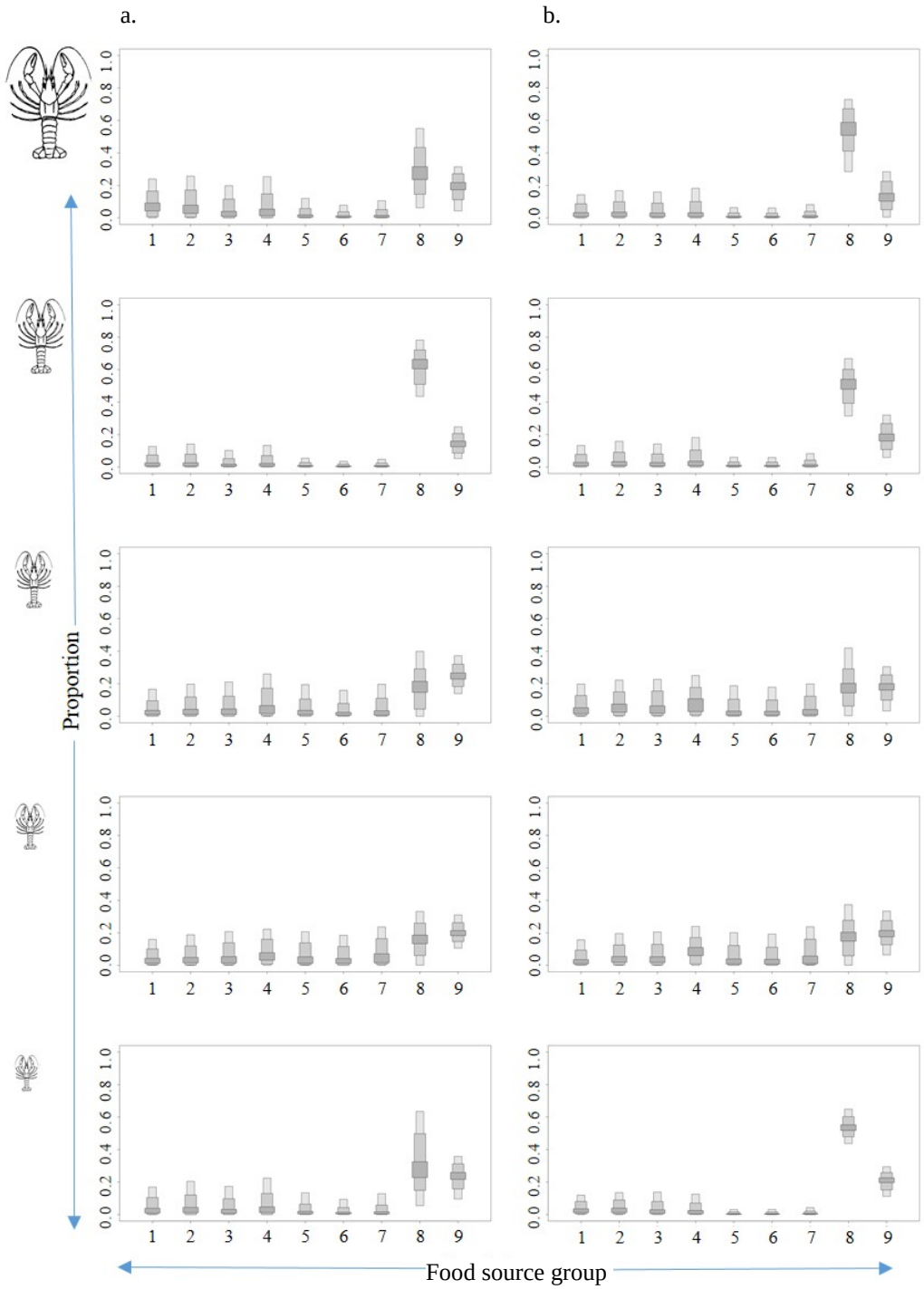


Figure 2-9: Mean contribution (%), expressed as a proportion, of each potential food source group (1 – 7) to the diet of each size class of *P. leniusculus* at Site 1 (a) and Site 2 (b) as estimated by SIAR. Values shown are the 50 %, 75 % and 90 % CI.

Table 2-5: Mean contribution (%) of food source groups to the diet of *P. leniusculus* at Site 1 (a) and Site 2 (b) as estimated by SIAR.

a.

	Size class 1			Size class 2			Size class 3			Size class 4			Size class 5		
Food source group	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %
1	0.01	7.1	0.04	0.01	6.8	0.04	0.01	6.6	0.04	0.01	5.0	0.03	0.04	11.0	0.09
2	0.01	8.2	0.04	0.02	8.0	0.05	0.01	8.1	0.04	0.01	5.5	0.03	0.03	11.4	0.08
3	0.01	6.7	0.03	0.01	9.3	0.05	0.01	8.5	0.05	0.01	3.6	0.02	0.01	7.9	0.04
4	0.01	8.9	0.04	0.03	10.5	0.08	0.02	11.4	0.07	0.01	4.9	0.02	0.01	10.2	0.05
5	0.00	4.6	0.02	0.01	9.2	0.05	0.01	7.3	0.04	0.00	2.0	0.01	0.01	4.2	0.02
6	0.00	3.2	0.01	0.01	7.8	0.04	0.01	5.6	0.02	0.02	1.3	0.01	0.00	2.8	0.01
7	0.00	4.2	0.02	0.02	10.8	0.07	0.01	7.6	0.03	0.00	1.8	0.01	0.00	3.7	0.02
8	0.23	33.7	0.32	0.13	17.1	0.19	0.15	19.6	0.21	0.6	61.2	0.66	0.24	30.1	0.32
9	0.22	23.1	0.26	0.18	20.6	0.21	0.23	25.3	0.27	0.13	14.9	0.16	0.17	18.7	0.22

b.

	Size class 1			Size class 2			Size class 3			Size class 4			Size class 5		
Food source group	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %
1	0.01	5.4	0.04	0.01	6.4	0.04	0.02	8.6	0.05	0.01	5.3	0.03	0.01	5.9	0.03
2	0.01	5.9	0.04	0.02	8.4	0.06	0.02	10.0	0.08	0.01	6.3	0.03	0.01	6.8	0.04
3	0.01	5.4	0.03	0.02	8.7	0.05	0.02	10.2	0.06	0.01	5.6	0.03	0.01	6.3	0.03
4	0.01	5.0	0.03	0.06	11.3	0.11	0.03	11.0	0.11	0.01	7.2	0.04	0.01	6.9	0.03
5	0.00	1.1	0.01	0.01	8.2	0.04	0.01	7.0	0.03	0.00	2.2	0.01	0.00	2.2	0.01
6	0.00	1.2	0.01	0.01	7.6	0.04	0.01	6.7	0.03	0.00	2.1	0.01	0.00	2.2	0.01
7	0.00	1.5	0.01	0.01	10.6	0.06	0.01	8.3	0.04	0.00	3.0	0.02	0.00	2.9	0.01
8	0.52	54.2	0.55	0.15	18.7	0.20	0.14	19.7	0.20	0.48	49.3	0.54	0.51	52.1	0.59
9	0.2	20.4	0.23	0.18	20.0	0.21	0.16	17.5	0.20	0.16	19.0	0.20	0.10	14.6	0.15

2.3.5 Niche width

There was a high degree of overlap of trophic niche width, measured by SEA_c, observed for all size classes of *P. leniusculus* at both Site 1 and Site 2 (Figure 2-10). This indicates that each size class does not occupy its own distinct trophic niche and that the same food sources are being utilised by all five size classes. Although the same food sources are being utilised, the variation in SEA_c suggests that different size classes are utilising them differently. At Site 1 size class 3 had the largest SEA_c (1.85 ‰^{2}) and size class 2 had the lowest (0.34 ‰^{2}), while size class 4 had the largest SEA_c (2.23 ‰^{2}) and size class 1 had the lowest (0.50 ‰^{2}) at Site 2. A distinct jump in SEA_c between size class 2 and size class 3 at Site 1 and Site 2 (1.51 ‰^{2} and 1.57 ‰^{2} respectively) was also observed (Table 2-6). This would suggest a change in the diet between these size classes, with subsequent size classes consuming a greater variety of food sources. At both sites, size classes 4 and 5 were found to have the largest NR (Table 2-6). This again suggests that the larger size classes exhibit a greater degree of trophic diversity and consume organisms from a greater number of trophic levels than smaller size classes. The diversity of *P. leniusculus* diet, as measured by CD, was variable and further confirms that the same diet was not consumed by each size class diet at each site (Table 2-6). This finding is consistent with the switch to an increased consumption of animal protein at smaller size classes, before returning to a diet with large contributions from vegetation sources at larger size classes.

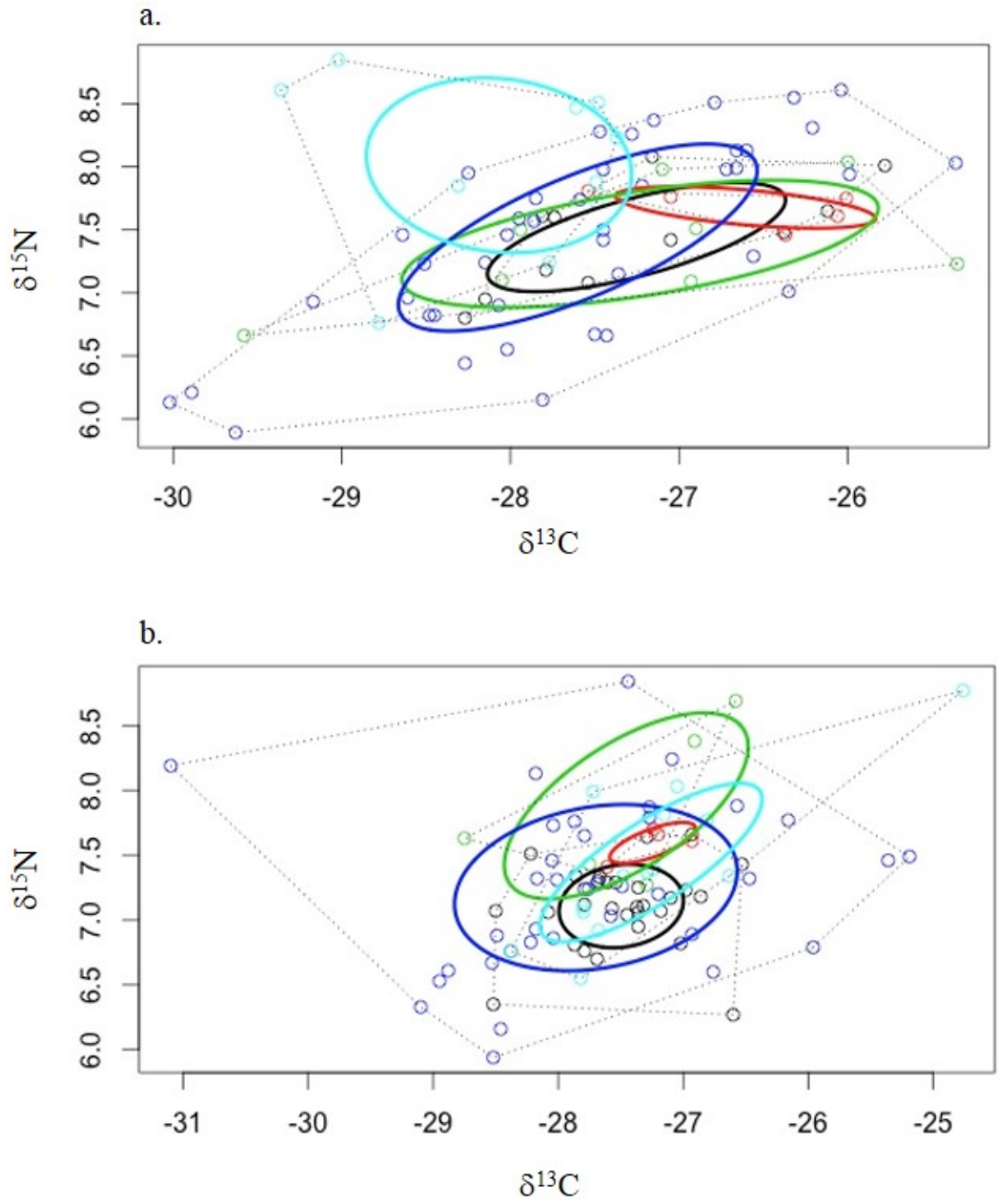


Figure 2-10: $\delta^{13}C$ - $\delta^{15}N$ bi-plot for each size class of *P. leniusculus* at Site 1 (a) and Site 2 (b). Each symbol represents a single individual. Black = size class 1 (0 mm – 9 mm), red = size class 2 (10 mm – 20 mm), green = size class 3 (21 mm - 39 mm), blue = size class 4 (40 mm – 50 mm), aqua = size class 5 (51 mm – 70 mm). Standard ellipses corrected for small sample size (SEA_c) and represent the main niche area of each size class (Jackson *et al.*, 2011). Convex hulls (TA), denoted by the dashed line, represent overall niche diversity and encompass all data points (Layman *et al.*, 2007). Both SEA_c and TA were estimated using SIBER in SIAR. Note the different scale on the $\delta^{13}C$ axis for Site 1 (a) and Site 2 (b).

Table 2-6: Summary of the mean population metrics for each size class of *P. leniusculus* adopted from Layman *et al.* (2007) and the standard ellipse area corrected for sample size to quantify trophic niche width (Jackson *et al.*, 2011). NR = δ^{15}N range; CR = δ^{13}C range; CD = mean distance to centroid; SDNND = standard deviation of the nearest neighbour distance; TA = total area encompassed by convex hull; SEAc = standard ellipse area corrected for sample size (‰^{2}).

a.

	N	NR	CR	CD	SDNND	TA	SEA_c
Size class 1 (0 mm - 9 mm)	11	1.28	1.49	0.80	0.20	1.39	0.88
Size class 2 (10 mm - 20 mm)	5	0.35	1.53	0.57	0.17	0.25	0.34
Size class 3 (21 mm - 39 mm)	8	1.38	4.23	1.09	0.44	2.55	1.85
Size class 4 (40 mm - 50 mm)	42	2.72	4.66	1.09	0.147	6.22	1.73
Size class 5 (51 mm - 70 mm)	10	2.09	1.99	0.86	0.33	2.68	1.70

b.

	N	NR	CR	CD	SDNND	TA	SEA_c
Size class 1 (0 mm - 9 mm)	30	1.39	1.99	0.42	0.18	2.40	0.50
Size class 2 (10 mm - 20 mm)	4	0.29	0.68	0.23	0.20	0.06	0.12
Size class 3 (21 mm - 39 mm)	5	1.42	2.17	0.88	0.25	1.16	1.69
Size class 4 (40 mm - 50 mm)	36	2.90	5.91	1.03	0.44	9.67	2.23
Size class 5 (51 mm - 70 mm)	14	2.22	3.62	0.74	0.57	2.61	1.03

2.4 Discussion

The present study utilised stable isotope analysis to elucidate the trophic ecology of *P. leniusculus* within Loch Ken and determine whether or not an ontogenetic dietary shift was exhibited. Stable isotope values revealed intra-specific isotopic variation between size classes indicating that each size class was utilising each potential food source to a different extent. In this study, the intermediate size classes of *P. leniusculus* (10 mm – 20 mm and 21 mm – 39 mm) consumed more animal protein sources than the smallest (0 mm – 9 mm) and the largest size classes (40 mm – 50 mm and 51 mm – 70 mm) of *P. leniusculus*. Thus supporting the existence of an ontogenetic dietary shift within this population.

2.4.1 Nitrogen

A difference in the $\delta^{15}N$ isotope signature was only detected between sites. *P. leniusculus* at Site 1 had a higher mean $\delta^{15}N$ isotope signature than Site 2 (Table 2-4). Invertebrate food source groups 4, 5, and 6, food source group 2 (minnow) and food source group 8 (macrophyte) also had higher mean $\delta^{15}N$ isotope signatures (Table 2-4). If *P. leniusculus* were feeding on any of these food source groups extensively, that would account for the higher mean $\delta^{15}N$ isotope signature observed (Stenroth *et al*., 2006). Additionally, there may have been food sources that were not sampled in this study which were contributing to the higher $\delta^{15}N$ isotope signature of *P. leniusculus* at Site 1. However, despite the difference in mean $\delta^{15}N$ isotope signature being significant, the actual difference was small (0.24 ‰) and not enough to clearly distinguish Site 1 from Site 2. Therefore, it is likely that the assimilation of $\delta^{15}N$ from potential food sources is similar at both sites regardless of any variation in $\delta^{15}N$ isotope signature (Stenroth *et al*., 2006).

2.4.2 Carbon

The $\delta^{13}C$ isotope signature differed between depths but not between sites for *P. leniusculus*. Individuals obtained at the shallowest depth sampled were the most enriched in carbon (-27.52 ‰) compared with those obtained at intermediate (-28.02 ‰) and deep (-28.37 ‰) depths. There has been previous evidence of spatial variation of $\delta^{13}C$ within single ecosystems (Syväranta *et al*., 2006). For example, Vander Zanden and Rasmussen (1999) reported $\delta^{13}C$ isotopic signatures of primary producers becoming less enriched with increasing depth (littoral-pelagic-profundal), and more recently Ruokonen *et al.* (2012) examined the diet of *P. leniusculus* in a Finnish boreal lake at four depths (0 m – 3 m, 3 m - 6 m, 6 m – 9 m and > 9 m) using stable isotopes. The authors reported distinct and consistent differences in the $\delta^{13}C$ isotope signature for *P. leniusculus* caught at different depths, with individuals caught in the littoral and sub-littoral area (0 m – 3 m and 3 m – 6 m) consuming food sources largely from the littoral area, while individuals caught in the profundal area (> 9 m) largely consumed profundal food sources. This suggests that the $\delta^{13}C$ isotope signature can be used as an indication of foraging habitat, as is common in many marine studies where it is used to determine if dietary sources are from inshore or offshore habitats (Bearhop *et al*., 2004). Although the change in the $\delta^{13}C$ isotope signature in this study was not as clear, nor as distinct as that reported by Ruokonen *et al*. (2012), it is still likely that the variation observed was the result of different basal food sources being utilised at different depths by *P. leniusculus*.

Due to the present study's sampling limitations, further investigation into the effect of depth on the diet of *P. leniusculus* within Loch Ken was not possible. Had sampling permitted collection of potential food sources from multiple depths, greater inference could be drawn regarding how *P. leniusculus* are foraging and exploiting the available food sources within Loch Ken. Future studies should seek to understand how the diet of *P. leniusculus* changes

with depth to further understand resource utilisation and the potential for niche partitioning within a population, which could subsequently reveal mechanisms for habitat expansion by the invasive *P. leniusculus*.

2.4.3 *P. leniusculus* diet

2.4.3.1 Trophic position

All size classes of *P. leniusculus* fed approximately one trophic level above all other benthic invertebrates except for Gammaridae, which would suggest that *P. leniusculus* within Loch Ken are secondary consumers, functioning as omnivores but occupying the trophic position of a predator. As such, *P. leniusculus* are most likely deriving energy from primary or other secondary consumers rather than plant material. Other authors have found other crayfish species to function similarly within their respective ecosystems. For example, Parkyn *et al.* (2001) found *P. planifrons* to function as an omnivore but occupy the trophic position of a predator as *P. planifrons* was more enriched in $\delta^{15}N$ and $\delta^{13}C$ than all other invertebrates but less so than eels; Olsson *et al*. (2008) found *A. astacus* to also occupy the position of a predator; and Taylor and Soucek (2010) found Northern clearwater crayfish *Orconectes propinquus* (Girard, 1852) and rusty crayfish *Orconectes rusticus* (Girard, 1852) to occupy a trophic position between invertebrates and fishes. This study echoes these findings, with *P. leniusculus* in Loch Ken being more enriched in $\delta^{15}N$ and $\delta^{13}C$ than all other invertebrates but less so than the fish species collected.

2.4.3.2 Feeding ecology

Previous stable isotopes studies have revealed conflicting results regarding crayfish diet. Some found crayfish to be utilising detritus as the main food source, while others reported invertebrates to be the main food source in crayfish diet (Crehuet *et al*., 2009; Stenroth *et al*., 2006). In the present study, the mixing model revealed that all size classes of *P. leniusculus* consumed terrestrial detritus and macrophytes, with the model output suggesting

that terrestrial detritus and macrophytes were the most important food sources for size classes 1, 4 and 5 accounting for approximately 50 %, or more, of the total diet. For size classes 2 and 3, detritus and macrophytes were still an important food source despite a reduction in contribution to the diet due to an increase in the consumption of animal protein sources. This was true for individuals caught at both Site 1 and Site 2. Additionally, the $\delta^{13}C$ isotopic signature of *P. leniusculus* falls within that observed for the terrestrial detritus and macrophyte isotopic signatures. This suggests that all size classes of *P. leniusculus* feed extensively on terrestrial detritus and macrophytes within Loch Ken. The heavy reliance on terrestrial vegetation and macrophytes by size classes 1, 4 and 5 of *P. leniusculus* is likely due to the low energetic cost involved in capture and handling as well as potentially being a method of avoiding predation (Parkyn *et al*., 2001; Roth *et al*., 2006). This may be especially true for the smallest *P. leniusculus* (> 10 mm) captured during this study as they were caught in water less than 2 – 3 inches deep by sweeping a net through the vegetation present.

Traditionally, smaller individuals were viewed as largely carnivorous while larger individuals rely mostly on plant material (Hollows *et al*., 2002; Nyström *et al*., 2002; Parkyn *et al*., 2001). The $\delta^{15}N$ isotopic signatures obtained in this study indicate that individuals of all sizes are dependent, to some degree, on animal protein sources (Stenroth *et al*., 2006). The results of the mixing model output in this study further confirmed the utilisation of animal protein sources at all sizes. It also suggested that the larger *P. leniusculus* (size classes 4 and 5) consume similar amounts of, and in some instances more, animal protein in their diet than the smallest individuals sampled (size class 1) (Table 2-5). This is in agreement with the findings by Stenroth *et al*. (2006) and within Loch Ken adult *P. leniusculus* are at least as carnivorous as juvenile *P. leniusculus*.

Few stable isotope studies to date have included fish as a potential food source for crayfish and to the best of present knowledge, only Taylor and Soucek (2010) have included fish in

mixing models when determining source contributions to crayfish diet, finding that fish are an important energy source in the diet of *O. propinquus* and *O. rusticus*. Fish remains have been reported from crayfish stomachs for over 125 years (Guan and Wiles, 1998; Taylor and Soucek, 2010), and several studies have shown crayfish to predate upon small benthic fish (Guan and Wiles, 1997; Guan and Wiles, 1998; Renz and Breithaupt, 2000; Reynolds and O'Keeffe, 2005). *P. leniusculus* in Loch Ken have also been observed to predate upon large predatory fish caught in gill nets (Chapter 4).

The mixing model output in this current study estimated that overall, fish (food source groups 1 and 2 combined) contributed anywhere between 10.5 % and 22.4 % of *P. leniusculus* diet (Table 2-5). In particular, food source group 2 (minnow) contributed more than food source group 1 (pike, perch and roach/bream hybrid) to all size classes of *P. leniusculus* (Table 2-5). The inclusion of fish in the diet of *P. leniusculus* may well be the reason for such high $\delta^{15}N$ signatures compared with other invertebrates sampled.

Since the current study observed *P. leniusculus* to occupy the trophic position of a predator yet have a diet dominated by plant material, it is likely that multiple food sources are being assimilated and incorporated into *P. leniusculus* biomass. This finding is supported by Stenroth *et al*. (2006) who suggested that for an omnivore, such as *P. leniusculus*, the assimilated carbon and nitrogen in tissue might come from separate sources i.e. plant material may contribute to the carbon profile while invertebrates may contribute to the nitrogen profile. In the current study, animal protein sources such as invertebrates and fish, may have contributed to the observed $\delta^{15}N$ isotopic signature whilst macrophytes and terrestrial detritus are likely influencing the $\delta^{13}C$ isotopic signature of *P. leniusculus* within Loch Ken.

2.4.3.3 Ontogeny

Any observed ontogenetic dietary shift in crayfish has previously been explained as a function of juvenile crayfish requiring larger amounts of protein for growth or by the perceived inability of large crayfish to capture fast moving invertebrate prey, as well as not being capable of accessing prey refugia (Alcorlo and Baltanás, 2013; Hollows *et al*., 2002; Parkyn *et al*., 2001).

Although there are no clear isotopic distinctions between size classes, the contribution of each food source group to *P. leniusculus* diet (as estimated by the mixing model) indicates an ontogenetic dietary shift in individuals measuring between 10 mm – 39 mm, with plant material and detritus contributing less to the diet and animal protein increasing. However, the lack of any significant difference between the $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures of each size class suggests that overall the energy sources being utilised are similar. Therefore, any observed ontogenetic dietary shift between size classes may be due to the preferential assimilation of animal protein sources into *P. leniusculus* tissue over plant material (Burress *et al*., 2013; Hollows *et al*., 2002; Whitledge and Rabeni., 1997). Yet, the increase in contribution of terrestrial detritus and algae to the diet of these individuals could in fact be further evidence of an ontogenetic dietary shift in *P. leniusculus* measuring between 10 mm – 39 mm. Since protein-rich diets consisting of invertebrates have been shown to result in increased growth rates for crayfish (Bondar *et al*., 2005), consuming more animal protein over plant material would be beneficial to growing *P. leniusculus*. Consequently, the increase in contribution of terrestrial detritus and algae to *P. leniusculus* diet during size classes 2 and 3 may be a result of indirect consumption while increasing feeding on animal protein sources, such as invertebrates (Burress *et al*., 2013). An increase in the consumption of animal protein sources by juvenile crayfish may be to support the need for larger amounts of protein that are needed for growth (Bondar *et al*., 2005; Momot, 1995; Paglianti and Gherardi, 2004).

Although an ontogenetic dietary shift, whereby *P. leniusculus* switch to increased amounts of animal protein before returning to a more general omnivorous diet dominated by plant material and detritus was observed, all size classes of *P. leniusculus* occupied highly similar trophic positions (Table 2-4). Therefore, it is unlikely that there is any intra-specific niche partitioning between size classes. This is supported by the high degree of niche overlap observed in Figure 2-10.

2.4.4 Niche width

Niche width was measured by the SEA. SEA values can be influenced by sample size, in particular when stable isotope data is not normally distributed as is common in ecological studies (Ercoli *et al*., 2014; Jackson *et al*., 2011; Newsome *et al*., 2012; Syväranta *et al*., 2013). As a result, the corrected SEA, SEA_c, was used in the present study. Jackson *et al*. (2011) recommended a minimum sample size of at least 10, and in the current study, the number of individuals in size classes 2 and 3 at both sites was less than 10. Consequently, the values obtained may be less reliable for these size classes (Ercoli *et al*., 2014).

Even though no clear trend was discernible with regard to the effect on size on the niche width of *P. leniusculus*, size class 2 individuals consistently exhibited the lowest NR, CR, CD and SEA_c values (Table 2-6). Consequently, size class 2 individuals have a less diverse diet and are utilising prey across fewer trophic levels than all other size classes. This would support a dietary shift by this size class of *P. leniusculus* due to increased consumption of animal protein sources and is further corroborated because all invertebrate groupings in this study, apart from Gammaridae, exhibited very similar $\delta^{15}N$ isotopic signatures and near identical trophic positions (Table 2-4).

2.4.5 Limitations of stable isotope studies

One limitation encountered during the present study was the small sample sizes used for each size class, particularly sample sizes that were < 10 individuals. It would have been

preferable to use equal sample sizes for each size class in order to allow a more robust comparison of diets between size classes. This would have ensured that any observed changes in the population metrics calculated were more attributable to changes in the diet of *P. leniusculus* than differences caused by sample size.

Another limitation is that the trophic dynamics of *P. leniusculus* were investigated using individuals from only one location. It would have been advantageous to have another site to compare how *P. leniusculus* diet changes with size. However, in doing so there may confounding variables, including different food sources available at each location. Additionally, a species may function differently in different locations. For example, a species may be a detritivore in one water body but more omnivorous in another (Olsson *et al*., 2008).

Detailed dietary information regarding the consumption of specific food sources can be difficult to obtain using stable isotope analysis alone and therefore may be more effectively obtained by combining stable isotope analysis and with other techniques (Mao *et al*., 2015), such as gut content analysis (Hollows *et al*., 2002; Parkyn *et al*., 2001), fatty acid analysis (Antonio and Richoux, 2014) or RNA-DNA ratios (Olsson *et al*., 2008). By using a combination approach, dietary differences and trophic ecology of *P. leniusculus* could be more effectively interpreted. For example, performing gut content analysis first could inform which food sources should be entered into the isotopic mixing model and thus provide more robust estimates of dietary contributions to *P. leniusculus* diet due to fewer food sources being included. In future, a combined approach may aid the interpretation of *P. leniusculus* diet, and subsequent role within the food web (Cummings *et al*., 2012).

Finally, the actual TEF values for *P. leniusculus* may deviate from the TEF values used in the present study. To the best of current knowledge, no species-specific TEF values exist for *P. leniusculus*. TEFs are critical to be able to draw inference about diet composition

using isotopic mixing models, yet they are often cited as a weak link in stable isotope ecology (Bond and Jones, 2009; Burress *et al*., 2013; Post, 2002). In order to quantify TEF values, a consumer must be fed an isotopically constant diet for the period of time it takes for complete turnover of the tissue to be used for stable isotope analysis (Bond and Jones, 2009); however, it was outwith the capacity of this study to experimentally determine species-specific TEF values for *P. leniusculus*. Using incorrect TEF values may change the estimates of the contribution of each food source to the diet of the target consumer (Bond and Jones, 2009). Therefore, future studies should seek to develop species-specific TEF values for *P. leniusculus* to ensure mixing model dietary proportion estimates for each food source to *P. leniusculus* diet are as representative as possible.

2.4.6 Conclusion

The results of this study show that *P. leniusculus* are opportunistic omnivores who occupy the position of a predator, consuming a broad diet of plant and animal material. Stable isotope results emphasised the importance of animal protein for growing *P. leniusculus*, indicative of an ontogenetic dietary shift, despite overlapping niches and the exploitation of similar dietary resources by all size classes.

This study provides valuable information about the trophic ecology of *P. leniusculus* in a Scottish freshwater loch, as well as providing information on how different sizes of crayfish utilise available resources. However, further research is required to fully understand the implications of population metrics and mixing model outputs with regard to the management of invasive species such as *P. leniusculus*.

Chapter 3. Comparing the isotopic niche width and diet composition of the crayfishes *Pacifastacus leniusculus* and *Austropotamobius pallipes* in Scotland using stable isotopes.

3.1 Introduction

Between one-third and one-half of the world's crayfish species are estimated to be threatened with declining populations or extinction (Taylor, 2002). One of the biggest threats to crayfish diversity is the introduction of invasive crayfish species (Holdich *et al*., 2009a). Invasive crayfish pose a serious threat to the displacement of many native crayfish species through aggressive competition for resources and transmission of disease (Holdich *et al*., 2014).

Scotland has no native crayfish species (Bean *et al*., 2006; Maitland, 1996). However, two introduced species are known to be present – the invasive North American signal crayfish *P. leniusculus* and the white-clawed crayfish *A. pallipes*.

As an invasive species, *P. leniusculus* has become widespread throughout Europe (Holdich *et al*., 2009a). After being brought from Sweden and introduced to England for aquaculture purposes during the 1970's (Holdich and Reeve, 1991), breeding populations became rapidly established in England and Wales (Holdich *et al*., 2014). In addition to introduction for aquaculture farming, escapes (Holdich *et al*., 1995; Maitland, 1996), introductions by the aquarium and pond trade (Chucholl, 2013; Holdich *et al*., 2009a; Lodge *et al*., 2000), and the use of live crayfish as bait by anglers or as food for fish stocks have all resulted in the spread of *P. leniusculus* throughout GB (Bean *et* al., 2006; Lodge *et al*., 2000; Peay *et al*., 2010). It is believed that *P. leniusculus* arrived in Scotland sometime during the 1980's, however, their arrival was not officially reported until 1995 (Maitland, 1996). Since then, *P. leniusculus* has become established at more than twenty different sites (Freeman *et al*., 2010) and in at least 174 km of river length, as well as standing waters (SNH, 2015a). It is likely that many more populations will be discovered as other sightings are awaiting confirmation (Freeman *et al*., 2010).

Conversely, *A. pallipes* is established at only two sites - Loch Croispol, Durness and Whitemoss Reservoir, Renfrewshire (Maitland *et al*., 2001). The point of introduction of *A. pallipes* into Loch Croispol was believed to be from a feeder stream in 1945 (Thomas, 1992), while the Whitemoss Reservoir population is suggested to have originated from stock introduced to Scotland over 150 years ago (Maitland *et al*., 2001).

A negative impact upon native flora and fauna is often a consequence of introduced species; however, *A. pallipes* does not appear to have any known negative impacts in Scotland (Gladman *et al*., 2009). For example, the Loch Croispol population of *A. pallipes* is known to co-exist successfully with both *S. trutta* and *O. mykiss* (Bean *et al*., 2006). However, *P. leniusculus* has significant negative effects cementing its status as invasive. English populations of *P. leniusculus* have been shown to reduce the abundance of *S. trutta* (Peay *et al*., 2009) and as a direct result of the introduction of *P. leniusculus, A. pallipes* has suffered serious population declines (Peay, 2001). Despite *A. pallipes* being an introduced species in Scotland, both known populations are protected by law and represent potential refuge populations for the rest of GB. Current legislation lists *A. pallipes* in Appendix III of the Bern Convention and on Annexes II and V of the Habitats Directive, Annex II requires the designation of Special Areas of Conservation (SAC) and Appendix III and Annex V manage exploitation (Holdich *et al*., 2009b). *A. pallipes* is also protected under Schedule 5 of the Wildlife and Countryside Act (1981), which prohibits the taking of *A. pallipes* from the wild and selling throughout Britain. *A. pallipes* is also a species of importance under the Nature Conservation (Scotland) Act 2004. Additionally, *A. pallipes* is classified as endangered on the IUCN Red List (Grandjean *et al*., 2015). In this study, the Scottish populations of *A. pallipes* are considered to be native due to the protection *A. pallipes* is afforded, combined with the lack of negative impact and the duration of the population's presence within Scotland.

By understanding why an invasive species is so successful, it may be possible to predict future invasions, determine effects on native species and subsequently develop eradication protocols (Bodey *et al*., 2011). Diet plasticity has been cited as a key reason for the success of invasive species.

For example, *P. leniusculus* are thought to be more adaptable and able to switch diet, subsequently utilising food sources from lower trophic levels (Holdich *et al*., 2014). Furthermore, invasive species have been shown to occupy a larger niche width than native species (Shea and Chesson, 2002). The increased niche width is often a result of a more generalist diet. By utilising a more generalist diet, an invasive species can gain competitive advantage over native species (Crowder and Snyder, 2010; Hänfling *et al*., 2011).

Traditionally, niche width was quantified using gut content analysis across a population in combination with measurements of prey biomass and diversity (Bearhop *et al*., 2004). However, gut content analysis is biased towards food sources recently consumed and therefore does not provide information regarding which food sources are actually assimilated, nor does it provide direct information on foraging activity in an organism (Carmichael *et al*., 2004). Additionally, using gut content analysis results in the abundance of food sources in a consumer's diet being frequently over- or under-estimated (Bearhop *et al*., 2004).

Consequently, Bearhop *et* al. (2004) proposed using the variance observed within the stable isotope ratios $\delta^{13}C/\delta^{12}C$ and $\delta^{15}N/\delta^{14}N$ of an organism's tissue as a measure of determining trophic niche width within a population. The $\delta^{15}N$ isotope increases in a predictable step-wise enrichment of 3 – 4 ‰ with each trophic level, due to $\delta^{14}N$ being preferentially excreted by biochemical processes (Minagawa and Wada, 1984; Peterson and Fry, 1987). While $\delta^{13}C$ also increases as the trophic level increases, it does so to a lesser magnitude of 1 - 2 ‰ (Cabana and Rasmussen, 1994). As a result stable isotope analysis has since become the

preferred method of determining niche width, as isotopic values describe the assimilated diet of an individual, providing evidence of long-term diet rather than a ‘snap shot’ of diet as provided by gut content analysis (Bearhop *et al*., 2004).

The present study aims to compare the niche width of the invasive *P. leniusculus* and native *A. pallipes* in Scotland. More specifically, this study focused on determining whether *P. leniusculus* exhibited a larger niche width than *A. pallipes* and how resource use differed between the two species. Using stable isotopes, the Bayesian modelling approach SIAR (Parnell *et al*., 2010) and the Bayesian ellipse method SIBER (Jackson *et al*., 2011) were used to quantify diet composition and niche width. The hypothesis being tested was that there is no difference between the niche width of *P. leniusculus* and *A. pallipes*. If *P. leniusculus* exhibit a larger niche, this may indicate greater diet plasticity, which could potentially explain the overwhelming success of *P. leniusculus* as an invasive species and the resulting displacement of *A. pallipes* throughout its native range.

3.2 Methods

3.2.1 Crayfish

3.2.1.1 P. leniusculus

$\delta^{13}C$ and $\delta^{15}N$ isotope values obtained for *P. leniusculus* during Chapter 2 were used for further analysis and comparison in this part of the study.

To briefly recap, *P. leniusculus* were obtained from two sites in Loch Ken, Dumfries and Galloway, Scotland during July 2013 (Figure 2-1 and 2-2) by a mixture of kick sampling, hand searching and trapping. In addition to collecting *P. leniusculus*, fish, invertebrate, zooplankton, vegetation and detritus samples were collected at the same time. Upon arrival back at the laboratory, the carapace length of *P. leniusculus* individuals were measured from the tip of the rostrum to the posterior margin of the carapace using Vernier calipers (± 0.1 mm). Individuals were then sexed as male, female or unknown (too small for sex to be determined) and the tail muscle was removed and frozen at -70ºC until further analysis.

Full methodology can be found in Chapter 2, sections 2.2.2.1 and 2.2.3.1.

3.2.1.2 A. pallipes

Dr. Zara Gladman obtained $\delta^{13}C$ and $\delta^{15}N$ isotope values for *A. pallipes* during the course of her PhD study 'Non-native crayfish in Scotland' (2012). Stable isotope data that was not used in her thesis and information on sampling methodology was provided with her permission for use during this PhD.

A. pallipes is present in only two sites in Scotland, Loch Croispol and Whitemoss Reservoir. The first, Loch Croispol is located close to Durness in Sutherland in the north-west corner of Scotland. The second site is Whitemoss Reservoir, located in Renfrewshire, west central Scotland (Figure 3-1).

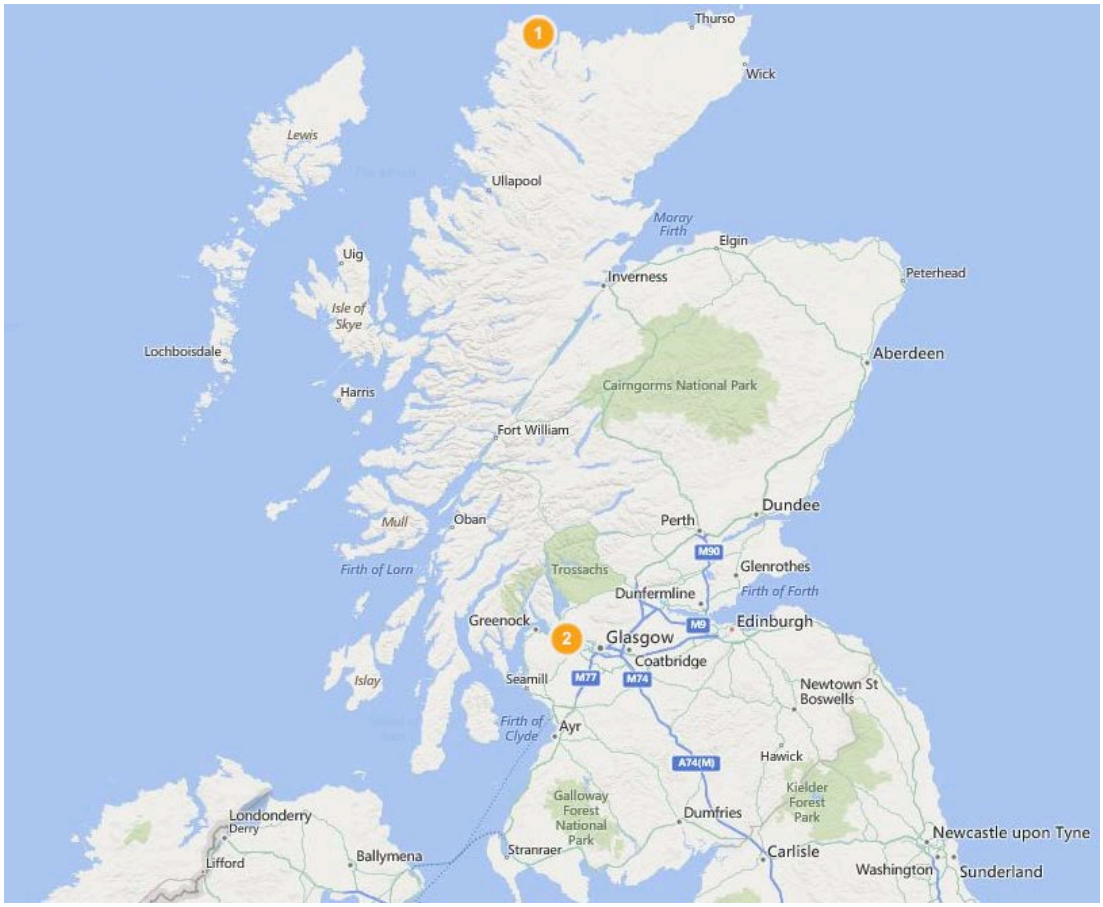


Figure 3-1: Location of Loch Croispol (1) and Whitemoss Reservoir (2) within Scotland.

A. pallipes were collected from Loch Croispol during May 2009 by hand searching in water 1 m deep or less. Potential predator and prey organisms were also collected. Fish species; *S. trutta*, sticklebacks *Gasterosteus aculeatus* (Linnaeus, 1758) and eels *Anguilla Anguilla* (Linnaeus, 1758) were caught using a gill net. Any invertebrates present were collected by kick sampling and later identified to family level. Primary producers in the form of macrophytes and algae were also collected. All samples were frozen at -20ºC until further analysis.

During August 2009, *A. pallipes* were collected from Whitemoss Reservoir by hand searching in water 1 m deep or less. At the same time, invertebrates were obtained by kick sampling and later identified to family level. Algae were also collected as examples of

primary producers. A return visit in 2011 collected *O. mykiss*, *G. aculeatus*, and *P. phoxinus* by seine netting. All samples were frozen at -20ºC until further analysis.

Upon return to the laboratory, the carapace length of all *A. pallipes* individuals were measured from the tip of the rostrum to the posterior margin of the carapace using Vernier calipers (± 0.1 mm) and all individuals were sexed as either male or female.

3.2.2 Sample preparation

3.2.2.1 P. leniusculus

In total, 818 individuals were removed from Loch Ken across two sites. Of that total, only 165 of those individuals were selected for stable isotope analysis as described in section 2.2.3.1. Samples were prepared for stable isotope analysis as detailed in section 2.2.4.

3.2.2.2 A. pallipes

Ten *A. pallipes* individuals were selected for stable isotope analysis from Loch Croispol, while 33 individuals from Whitemoss Reservoir were selected. Invertebrates identified to be present at Loch Croispol were Corixidae, Trichoptera, Gammaridae, Sphaeriidae, Ephemeroptera, Sialidae and Chironomidae. Invertebrates identified at Whitemoss Reservoir were Corixidae, Trichoptera, Gammaridae, Sphaeriidae, Ephemeroptera, Sialidae and Zygoptera.

After samples were defrosted, all samples (crayfish, fish, invertebrates, macrophytes and algae) were prepared for analysis. The tail muscle was dissected from each *A. pallipes* individual, a piece of abdominal muscle was cut from each fish and bivalves were removed from their shells. Samples were dried in an oven at 60ºC for 48 h. Once dried, samples were ground into a fine powder using a mortar and pestle.

3.2.3 Stable isotope analysis

3.2.3.1 Measurments

Aliquots of 0.7 mg or 1.5 mg (for animal and vegetation material respectively) were weighed into tin capsules using a Mettler-Toledo MX5 microbalance accurate to 1 µg. Both *P. leniusculus* and *A. pallipes* isotopic analysis was performed at the East Kilbride node of the NERC Life Sciences Mass Spectrometry Facility hosted by the SUERC using a continuous flow isotope ratio mass spectrometer, as detailed in section 2.2.4.

3.2.3.2 Lipid correction

The $\delta^{13}C$ isotopic signatures for *P. leniusculus* and *A. pallipes* were arithmetically corrected for lipid content, as variation in lipid contents can affect the measured carbon isotope ratios (Ehrich *et al*., 2011). Additionally, the estimation of diet contributions by mixing models can be influenced by the lipid content of prey or consumer tissue (Kiljunen *et al*., 2006). Kiljunen *et al*. (2006) also advise against lipid correcting samples of whole body invertebrates. Therefore, only the $\delta^{13}C$ isotopic signatures obtained for both species of crayfish were corrected for lipid content as whole body invertebrates were used for stable isotope analysis.

Lipid content was arithmetically corrected using the equation recommended by Kiljunen *et al*. (2006):

$$\delta^{13}C' = \delta^{13}C + D * (I + (3.9/1 + 287/L))$$

where $\delta^{13}C'$ is the lipid corrected $\delta^{13}C$ of *P. leniusculus* or *A. pallipes*, D is the isotopic difference between protein and lipid (7.018), I is a constant (0.048) and L is the C:N ratio of *P. leniusculus* or *A. pallipes* tissue.

3.2.3.3 $\delta^{13}C$ and $\delta^{15}N$ corrections between sites

As $\delta^{13}C$ and $\delta^{15}N$ of basal resources can differ amongst water bodies, a baseline correction was made for both $\delta^{13}C$ and $\delta^{15}N$ in order to allow comparisons between sites. The correction for $\delta^{15}N$ was in the form of estimating the trophic position (Tp) of crayfish, using the following equation recommended by Anderson and Cabana (2007):

$$Tp = ((\delta^{15}N_{crayfish} - \delta^{15}N_{baseline})/3.4) + 2$$

where Tp is the trophic position of *P. leniusculus* or *A. pallipes*, $\delta^{15}N_{crayfish}$ is the isotopic ratio of *P. leniusculus* or *A. pallipes*, $\delta^{15}N_{baseline}$ is the mean isotopic ratio of a primary consumer, 3.4 is the trophic level increment (Post, 2002) and 2 is the trophic position of the organism used to estimate the baseline. Post (2002) recommended using long lived primary consumers as baseline organisms, such as mussels and snails, as these show reduced spatial and temporal variations in isotopic values compared to primary producers. However, although mussels were collected from Whitemoss Reservoir and Loch Croispol, none were found to be present at either site in Loch Ken. Therefore, the mean $\delta^{15}N$ isotopic ratio of the non-predatory invertebrate, Gammaridae, was chosen as the baseline organism as it was present at all sites sampled (Jackson and Britton, 2013).

Following Olsson *et al.* (2009), differences in carbon basal resources were corrected for using the following equation:

$$\delta^{13}C_c = (\delta^{13}C_{crayfish} - \delta^{13}C_{meaninv})/C_{Rinv}$$

where $\delta^{13}C_c$ is the baseline corrected $\delta^{13}C$ of *P. leniusculus* or *A. pallipes*, $\delta^{13}C_{crayfish}$ is the $\delta^{13}C$ of *P. leniusculus* or *A. pallipes*, $\delta^{13}C_{meaninv}$ is the mean $\delta^{13}C$ calculated from invertebrates collected at each site and C_{Rinv} is the $\delta^{13}C$ range ($\delta^{13}C_{max} - \delta^{13}C_{min}$) for the same invertebrate chosen for the baseline when calculating the trophic position, in this instance Gammaridae.

3.2.3.4 Isotopic mixing model

The Bayesian mixing model package SIAR was run in R (R Development Core Team, 2014) to produce a mixing model, which provided estimates of the proportions of five different potential food source groups, in the diet of *P. leniusculus* and *A. pallipes*. Although many other potential food sources were available at each site, only food sources available across all sites sampled were considered for input into the mixing model. The five common potential food sources available at all four sites were; algae, macrophyte, Corixidae, Gammaridae and Ephemeroptera.

The mixing model was run separately for each site sampled. Isotopic ratios for each potential food source were adjusted for using an appropriate TEF to account for trophic fractionation. As no crayfish specific TEFs are available, commonly used values collected from literature were used instead. Following Ercoli *et al*. (2014), TEF values of 3.4 ± 0.98 ‰ for $\delta^{15}N$ and 0.39 ± 1.23 ‰ for $\delta^{13}C$ for animal source groups (Post, 2002), and 2.4 ± 0.42 ‰ for $\delta^{15}N$ and 0.40 ± 0.28 ‰ for $\delta^{13}C$ for plant source groups (McCutchan *et al*., 2003) were used.

3.2.3.5 Niche width

As previously described in section 2.2.5.4 niche width was traditionally estimated by calculating the convex hull area (TA) which encompassed all data points within a $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot space (Layman *et al.*, 2007). However, the TA has subsequently shown to be highly sensitive to sample size and recent work by Jackson *et al.* (2011) recommended adopting the standard ellipse area (SEA) as a measure of niche width instead. The SEA contains approximately 40 % of the data, therefore revealing the core isotopic niche of an organism as well as being relatively insensitive to different sample sizes.

Using a sample size corrected version of the standard ellipse area (SEA_c), the niche width of *P. leniusculus* and *A. pallipes* was estimated for each population sampled and for each species within the corrected $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot space using SIBER in SIAR. The

Tp and $\delta^{13}C_c$ isotope signatures from all individuals collected from all sites sampled were used.

3.2.3.6 Population metrics

Five population metrics from Layman *et al*. (2007) were adapted in order to elucidate trophic structure. These metrics were: Tp range (TpR) and $\delta^{13}C_c$ range (CR_c) defined as the distance between the two individuals with the highest and lowest corresponding values within the population; the trophic diversity as measured by the mean distance to the isotopic centroid (CD); trophic evenness (spread of individuals within isotopic space) as measured by the standard deviation of the nearest neighbour distance (SDNND); as well as the niche width described by the total area encompassed by the convex hull polygon (TA) (Jackson *et al*., 2012). All population metrics were calculated using SIAR.

3.2.4 Statistical Analysis

In order to compare *P. leniusculus* and *A. pallipes* appropriately, any *P. leniusculus* that were obtained at depths greater than 1 m or fell outwith the size range of *A. pallipes* (15.2 mm – 47.6 mm) across both sites, were excluded from analysis. Therefore, in total 48 *P. leniusculus* from Loch Ken Site 1, 36 *P. leniusculus* from Loch Ken Site 2, 33 *A. pallipes* from Whitemoss Reservoir and 10 *A. pallipes* from Loch Croispol were included in statistical analysis.

Differences among species, site and sex were explored separately for $\delta^{13}C$ and $\delta^{15}N$, with carapace length (mm) as a covariate, using a univariate GLM. Subsequent post hoc tests were performed using a Bonferroni adjustment to identify significant interactions. Raw data was used in analyses as transformations failed to improve the few non-normal data, and visual inspection of residuals indicated normality. Additionally, GLMs are considered to be robust to deviations from normality (Field, 2005).

All statistical analyses were conducted using SPSS (V 22.0) and values reported are mean ± SE unless otherwise stated. Significance level was defined as $p < 0.05$.

An isotopic bi-plot for $\delta^{13}C$ and $\delta^{15}N$ was constructed with standard ellipses and convex hull polygons for each crayfish species using SIBER in SIAR. In addition, metrics from Layman *et al.* (2007) were adapted in order to elucidate trophic structure using SIAR (Jackson *et al.*, 2012).

3.3 Results

3.3.1 Trophic position

The estimated Tp was found to be significantly different between species ($F_{1,122}$ = 826.003, $p < 0.001$, $n^2 = 0.871$), with *A. pallipes* having a higher Tp (3.6 ± 0.03) than *P. leniusculus* (2.1 ± 0.03) ($p < 0.001$). The estimated Tp of male and female crayfish were not found to be significantly different ($F_{1,122}$ = 1.095, p = 0.297, n^2 = 0.009). However, there was a significant interaction between species and sex ($F_{1,122}$ = 6.072, p <0.05, n^2 = 0.047), with both male and female *A. pallipes* having a higher Tp (3.6 ± 0.05 and 3.5 ± 0.05 respectively) than male and female *P. leniusculus* (2.1 ± 0.04 and 2.1 ± 0.03 respectively) which followed the overall trend observed between species. There were no significant differences detected for $\delta^{13}C_c$ for either species ($F_{1,122}$ = 1.422, p = 0.235, n^2 = 0.012) or sex ($F_{1,122}$ = 0.199, p = 0.656, n^2 = 0.002). Consequently, there was no significant interaction of species and sex ($F_{1,122}$ = 0.156, p = 0.694, n^2 = 0.001).

There was a significant difference between sites ($F_{3,122}$ = 377.259, $p < 0.001$, n^2 = .903), with Bonferroni post-hoc tests revealing that the estimated Tp for all sites were significantly different from one another (all p values < 0.001; mean Tp ± SE found in Table 3-1). A significant difference was also found for $\delta^{13}C_c$ between sites ($F_{3,122}$ = 35.323, $p < 0.001$, n^2 = .465). Bonferroni post-hoc tests revealed that Loch Ken Site 1, Loch Ken Site 2 and Whitemoss Reservoir were all significantly different from one another (all p values < 0.001; mean $\delta^{13}C_c$ ± SE found in Table 3-1), however Loch Ken Site 2 and Loch Croispol were not significantly different (p = 0.481).

An estimated Tp of around 3 suggests that *A. pallipes* diet consists of mainly invertebrates (Figure 3-3; Table 3-1), while an estimated Tp of around 2 for *P. leniusculus* suggests individuals are feeding at lower trophic levels (Figure 3-2; Table 3-1).

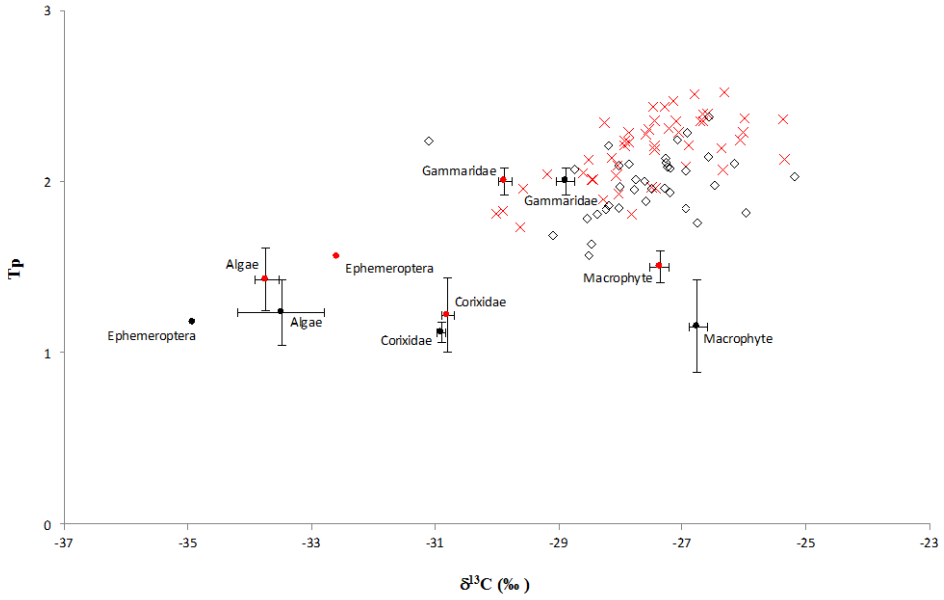


Figure 3-2: $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot for each population of *P. leniusculus*. *P. leniusculus* populations are individual data points and potential food sources are mean ± SE. Red = Loch Ken Site 1; Black = Loch Ken Site 2; × = *P. leniusculus*, Loch Ken Site 1; ◇ = *P. leniusculus*, Loch Ken Site 2; ● = potential food sources.

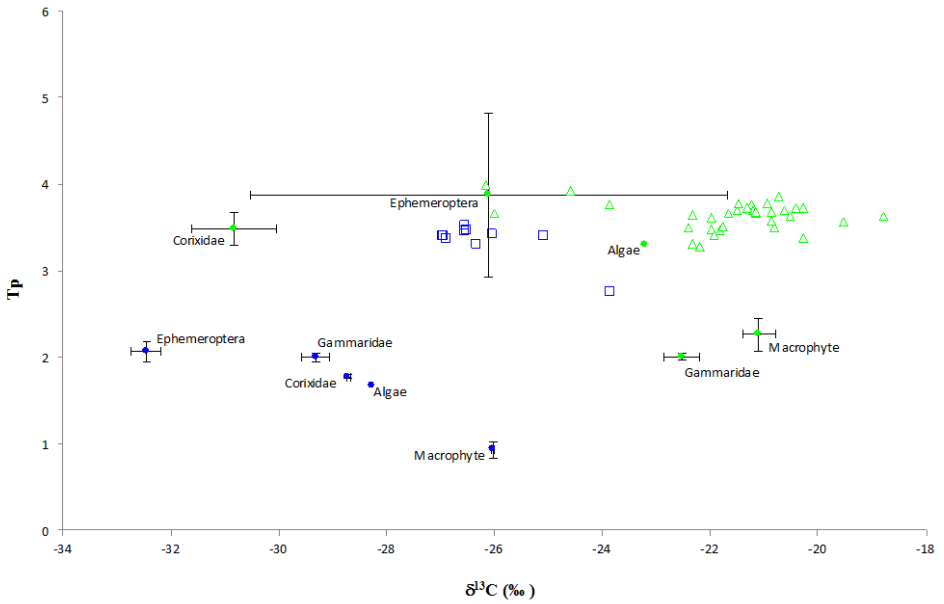


Figure 3-3: $\delta^{13}C$ - $\delta^{15}N$ isotopic bi-plot for each population of *A. pallipes*. *A. pallipes* populations are individual data points and potential food sources are mean ± SE. Green = Whitemoss Reservoir; Blue = Loch Croispol; △ = *A. pallipes*, Whitemoss Reservoir; □ = *A. pallipes*, Loch Croispol; ● = potential food sources.

Table 3-1: Mean values (± SE) of trophic position (Tp) and baseline corrected carbon ($\delta^{13}C_c$) (‰) for *P. leniusculus* and *A. pallipes* at all sites sampled.

Species	*n*	Mean Tp	Mean $\delta^{13}C_c$ (‰)
P. leniusculus	84	2.1 (± 0.02)	2.51 (± 0.10)
Loch Ken Site 1	48	2.2 (± 0.03)	2.86 (± 0.14)
Loch Ken Site 2	36	2.0 (± 0.03)	2.05 (± 0.12)
A. pallipes	43	3.5 (± 0.02)	3.28 (± 0.22)
Whitemoss Reservoir	33	3.6 (± 0.03)	3.87 (± 0.19)
Loch Croispol	10	3.3 (± 0.07)	2.62 (± 0.21)

3.3.2 Isotopic mixing models

3.3.2.1 The diet of P. leniusculus and A. pallipes

As previously described, four models (one for each site sampled) were run to estimate the contribution of each potential food source to the diet of *P. leniusculus* and *A. pallipes*. In order to then compare dietary contributions at the species level, the overall mean contributions (%) of each food source to *P. leniusculus* (Site 1 and 2 at Loch Ken combined) and *A. pallipes* (Whitemoss Reservoir and Loch Croispol combined) were calculated.

At the species level, outputs from SIAR suggested a difference in the use of putative food sources. Both species predominantly consumed primary producers. Macrophytes constituted the largest proportion of *P. leniusculus* diet (78 %), while algae and macrophytes constituting the largest proportion to the diet of *A. pallipes* (34 % and 27 % respectively) (Figure 3-4). However, although plant material (comprising both algae and macrophytes) contributes the largest proportion to *A. pallipes* diet (61 %), invertebrate food sources make a significant contribution too. Gammaridae contributes 18 % to the diet of *A. pallipes*, followed by Corixidae at 13 %. Ephemeroptera contributes the least (8 %).

This is in contrast to the invertebrate contribution to *P. leniusculus* diet where Corixidae contributes the most at 9 %, followed by Gammaridae and Ephemeroptera at 5 % and 3 % respectively. Overall invertebrates contributed an estimated 39 % to the diet of *A. pallipes*,

yet only 17 % to *P. leniusculus*. This would suggest at the species level that *A. pallipes* are more predatory.

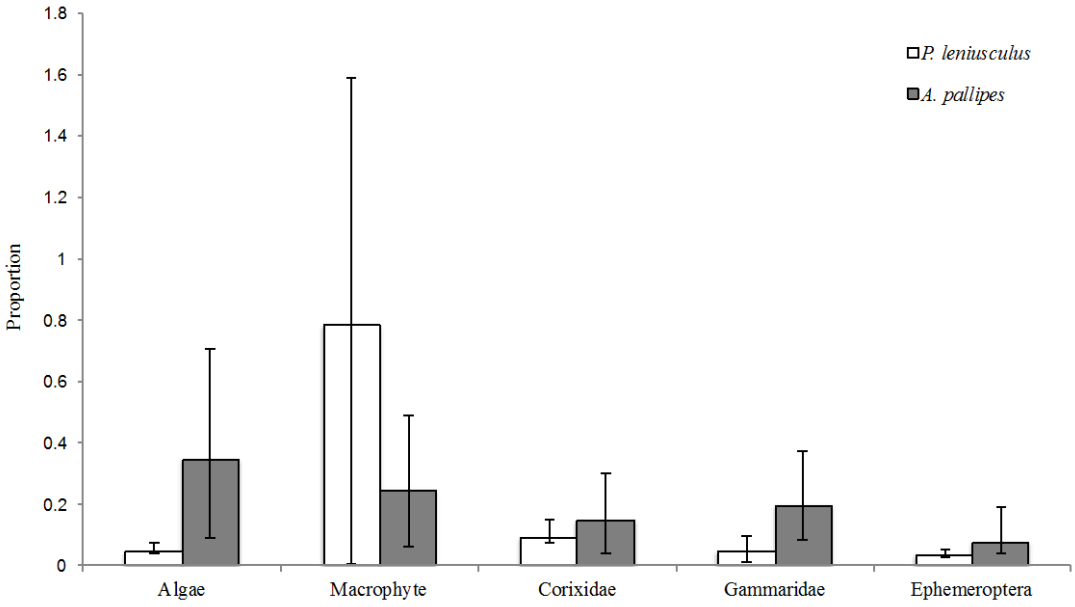


Figure 3-4: Mean overall contribution (%), expressed as a proportion, of each potential food source group to the diet of *P. leniusculus* and *A. pallipes* as estimated by SIAR. For each species, values are mean ± SE from all sites sampled containing that species.

The estimated dietary contributions of each food source did not follow the same trend at the population level as observed at the species level. For *P. leniusculus*, macrophytes still contributed the largest proportion to *P. leniusculus* diet (Figure 3-5; Table 3-2); however, for *A. pallipes* differences were observed. At Whitemoss Reservoir, plant material still contributed the most to *A. pallipes* diet, but at Loch Croispol the largest contributor to *A. pallipes* diet was Gammaridae, followed by Corixidae (Figure 3-5; Table 3-2). At Whitemoss Reservoir the only other significant contribution to diet was from the invertebrate, Gammaridae (10 %) (Figure 3-5; Table 3-2).

Plant material (algae and macrophytes) at Whitemoss Reservoir contributed 83 % to *A. pallipes* diet, invertebrates (Corixidae, Gammaridae and Ephemeroptera) only contributed an overall total of 17 % (Table 3-2). The converse was observed for *A. pallipes* at Loch Croispol with plant material contributing an overall 39 % and invertebrates contributing an

overall 59 %, more than half the diet in this instance (Table 3-2). For both sites containing *P. leniusculus,* plant material contributed an overall 89 % at Site 1 and 78 % at Site 2 (Table 3-2). In both instances, plant material contributed to more than half the diet for *P. leniusculus*. Therefore, when investigating individual populations, *P. leniusculus* and *A. pallipes* at Whitemoss Reservoir consume more plant material while *A. pallipes* at Loch Croispol were more predatory.

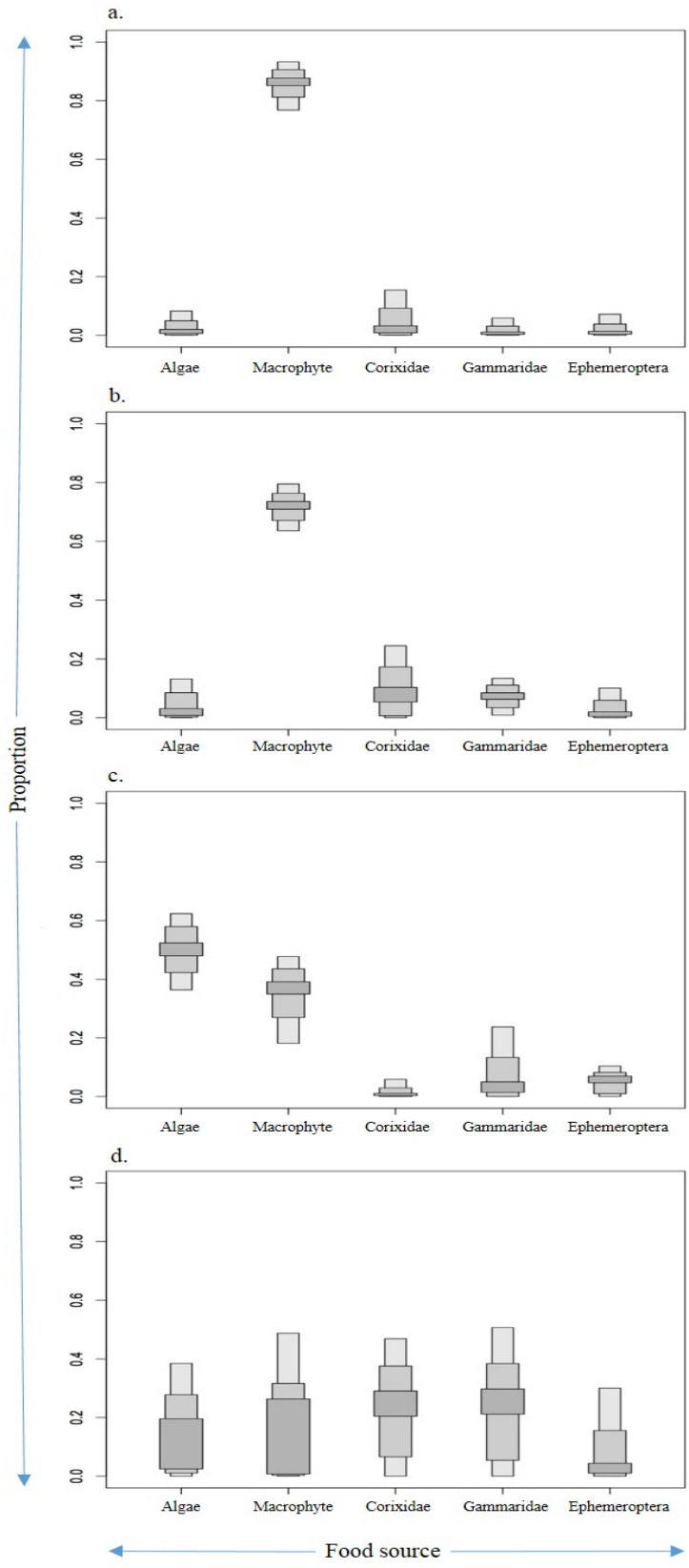


Figure 3-5: Mean contribution (%), expressed as a proportion, of each potential food source to the diet *P. leniusculus* at Loch Ken Site 1 (a) and Loch Ken Site 2 (b), and *A. pallipes* at Whitemoss Reservoir (c) and Loch Croispol (d) as estimated by SIAR. Values shown are the 50 %, 75 %, and 90 % CI.

Table 3-2: Mean contribution (%) of each food source to the diet of *P. leniusculus* and *A. pallipes* as estimated by SIAR.

	P. leniusculus **Loch Ken Site 1**			***P. leniusculus*** **Loch Ken Site 2**			***A. pallipes*** **Whitemoss Reservoir**			***A. pallipes*** **Loch Croispol**		
Food source group	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %	Lower 95 %	Mean % contribution	Upper 95 %
Algae	0.01	3.8	0.02	0.01	5.7	0.03	0.48	49.7	0.52	0.03	18.4	0.20
Macrophyte	0.84	84.7	0.87	0.71	71.8	0.74	0.35	33.4	0.4	0.01	21.1	0.25
Corixidae	0.01	7.0	0.04	0.03	10.9	0.08	0.01	2.1	0.01	0.21	24.6	0.30
Gammaridae	0.00	1.8	0.01	0.06	7.4	0.08	0.01	10.3	0.05	0.21	25.4	0.31
Ephemeroptera	0.00	2.5	0.01	0.01	4.2	0.02	0.05	5.3	0.07	0.01	10.2	0.04

3.3.3 Niche Width

The total niche width, as measured by SEA_c, for *P. leniusculus* at the species level (all individuals from both sites combined) was 0.53 ‰^{2}, which was less than that obtained for *A. pallipes* (0.77 ‰^{2}) (Figure 3-6). There was no overlap between species, which suggests that *P. leniusculus* and *A. pallipes* occupy distinct trophic niches and are not only unlikely to be utilising the same resources, but that *A. pallipes* are also consuming resources at higher trophic levels. In contrast, when examining niche width at the population level there was a greater variation observed within and between species. *P. leniusculus* individuals from Site 1 within Loch Ken exhibited a larger niche width (0.48 ‰^{2}) compared with Site 2 (0.40 ‰^{2}) (Figure 3-7). However, there was a degree of overlap suggesting individuals occupy a similar niche and are utilising some of the same resources. When examining niche width for both populations of *A. pallipes* sampled, there was a distinct difference between the SEA_c values obtained for the population at Whitemoss Reservoir compared with Loch Croispol. The niche width obtained for individuals from Whitemoss Reservoir was 0.55 ‰^{2}, which is more than double that observed for individuals from Loch Croispol (0.27 ‰^{2}) (Figure 3-7). Neither was there any overlap observed between *A. pallipes* populations (Figure 3-7). This would imply that each population of *A. pallipes* occupies a distinct trophic niche and utilises different resources from one another. Additionally, the population of *A. pallipes* at Loch Croispol had the lowest SEA_c value overall yet the Whitemoss Reservoir population had the highest overall SEA_c value with both *P. leniusculus* populations falling in between.

At the species level, *A. pallipes* was found to have the largest TpR (1.23), while *P. leniusculus* had the smallest (0.95). The larger TpR suggests that *A. pallipes* exhibit a greater degree of trophic diversity and consume organisms from a greater number of trophic levels than *P. leniusculus*. *A. pallipes* and *P. leniusculus* had similar CR_c (Table 3-3). The CD was greater for *A. pallipes* than *P. leniusculus* (Table 3-3), suggesting that *A. pallipes* consume a greater variety of resources in their diet. The higher SDNND value obtained for *A. pallipes*

indicates greater trophic variation within individuals sampled compared to *P. leniusculus* (Table 3-3).

Investigating the trophic dynamics of individual populations of crayfish produced contrary evidence compared with investigating crayfish trophic dynamics at species level. The TpR for all populations were similar, however both populations of *P. leniusculus* exhibited the largest Tp range (Table 3-3). This would indicate that at the population level, *P. leniusculus* exhibits a marginally greater degree of trophic diversity than *A. pallipes*. The CR_c was largest for the *A. pallipes* population at Whitemoss Reservoir (5.04), followed by *P. leniusculus* at Loch Ken Site 1 (4.21), Loch Ken Site 2 (3.94) and finally *A. pallipes* at Loch Croispol (2.10). The larger the CR_c value, the greater the number of basal resources being utilised by a population. The diversity of crayfish diet at population level (CD) indicated that *P. leniusculus* from Loch Ken Site 1 had the greatest degree of trophic diversity, while *A. pallipes* from Loch Croispol had the smallest (Table 3-3).

Table 3-3: Summary of the mean population metrics adopted from Layman *et al.* (2007) for each species and population of crayfish sampled and the standard ellipse area corrected for sample size (SEA_c) to quantify trophic niche width (Jackson *et al.*, 2011). TpR = Tp range; CR = $\delta^{13}C_c$ range; CD = mean distance to centroid; SDNND = standard deviation of the nearest neighbour distance; TA = total area encompassed by convex hull; SEA_c = standard ellipse area corrected for sample size ($‰^2$).

	n	**TpR**	**CR_c**	**CD**	**SDNND**	**TA**	**SEA_c**
P. leniusculus	84	0.95	5.13	0.78	0.12	2.82	0.53
Loch Ken Site 1	48	0.78	4.21	0.80	0.08	1.82	0.48
Loch Ken Site 2	36	0.81	3.94	0.54	0.25	1.86	0.40
A. pallipes	43	1.23	5.04	0.93	0.16	3.28	0.77
Whitemoss Reservoir	33	0.71	5.04	0.77	0.15	2.16	0.55
Loch Croispol	10	0.75	2.10	0.49	0.34	0.45	0.27

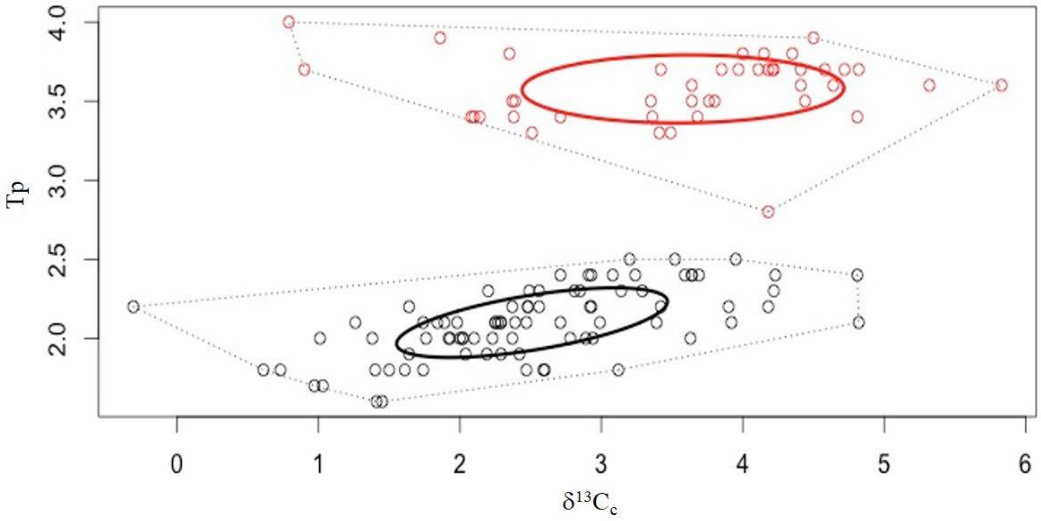


Figure 3-6: Tp - $\delta^{13}C_c$ bi-plot for each species of crayfish. Each symbol represents a single individual. Black: *P. leniusculus*, Loch Ken Site 1 and Site 2; Red: *A. pallipes*, Whitemoss Reservoir and Loch Croispol. Standard ellipses corrected for small sample size (SEA_c) and represent the main niche area of each size class (Jackson *et al.*, 2011). Convex hulls (TA), denoted by the dashed line, represent overall niche diversity and encompass all data points (Layman *et al.*, 2007). Both SEA_c and TA were estimated using SIBER in SIAR.

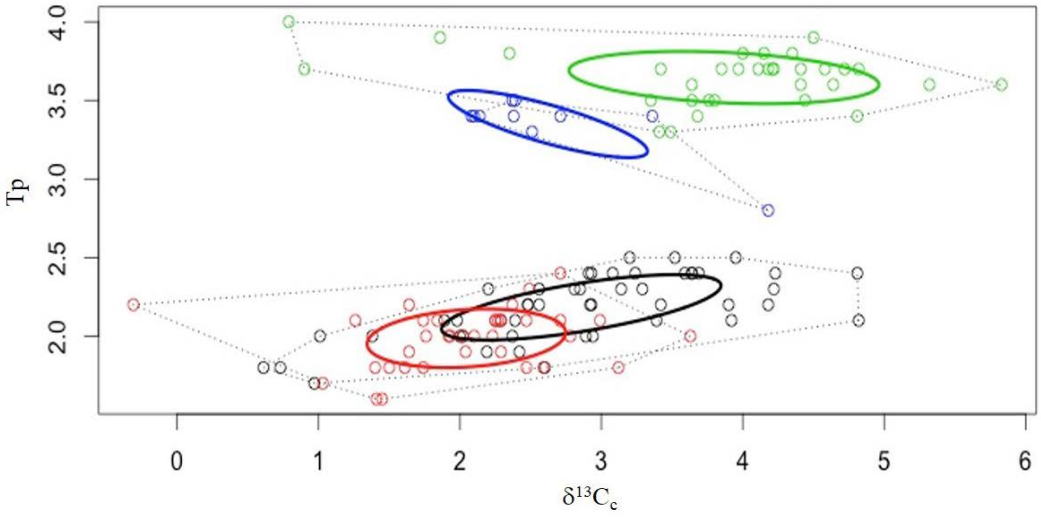


Figure 3-7: Tp - $\delta^{13}C_c$ bi-plot for each population of crayfish. Each symbol represents a single individual. Black = *P. leniusculus*, Loch Ken Site 1; Red = *P. leniusculus*, Loch Ken Site 2; Green = *A. pallipes*, Whitemoss Reservoir; Blue = *A. pallipes*, Loch Croispol. Standard ellipses corrected for small sample size (SEA_c) and represent the main niche area of each size class (Jackson *et al.*, 2011). Convex hulls (TA), denoted by the dashed line, represent overall niche diversity and encompass all data points (Layman *et al.*, 2007). Both SEA_c and TA were estimated using SIBER in SIAR.

3.4 Discussion

"Invasive species often show high plasticity of niche width in terms of habitat use, feeding ecology or behaviour" (Bodey et al., 2011).

It has been suggested that invasive species often have a broader diet and consequently a larger niche width than native species (Shea and Chesson, 2002).

3.4.1 Niche width

Previous studies have compared the trophic dynamics and niche width of a native and invasive crayfish species using stable isotopes. Both Ercoli *et al*. (2014) and Olsson *et al*. (2009) compared the niche width of the native *A. astacus* and invasive *P. leniusculus* within Finnish boreal lakes and Swedish streams respectively. Unlike those studies, the present study did not find the niche width of the invasive *P. leniusculus* to be greater than that of the native *A. pallipes*. At the species level these authors found the invasive crayfish species to have a niche width 2 – 3 times larger than that of the native crayfish species, however, in this study *A. pallipes* had a larger niche width (as measured by SEA_c) than *P. leniusculus*. Additionally, *A. pallipes* had a greater TpR, further supporting the finding of a wider niche (Ercoli *et al*., 2014; Olsson *et al*., 2009). There was no overlap of niche width between *A. pallipes* and *P. leniusculus* (Figure 3-6) which would suggest that each species is utilising different resources and indicates that the two species would be unlikely to directly interact for food resources if found co-occurring presently (Jackson *et al.*, 2012). Ideally, in order to draw the best comparison of niche width between *A. pallipes* and *P. leniusculus*, samples would be taken of each population from the same site. However, there are no known sites in Scotland where the two species co-exist due to the apparently inevitable displacement of *A. pallipes* by *P. leniusculus*.

At the population level *P. leniusculus* from both sites and *A. pallipes* from Whitemoss Reservoir exhibited similar niche widths according to the estimated SEA_c values (Table 3-

3), which suggests all individuals are utilising resources similarly despite differences in environment or species (Olsson *et al*., 2009). *A. pallipes* from Loch Croispol was the exception. This population had a niche width approximately half of that exhibited by the other populations sampled suggesting *A. pallipes* populations are utilising resources differently. Furthermore, there is no overlap of niche width between the *A. pallipes* populations while the niche widths of *P. leniusculus* from each site have a high degree of overlap (Figure 3-7). This indicates dietary differences between each *A. pallipes* population but suggests that *P. leniusculus* are utilising similar resources at each site. For *P. leniusculus*, this would be expected since it is a single population from two sites at one location. In order for real inference to be drawn and wider application of niche comparison between *A. pallipes* and *P. leniusculus*, more than one population of *P. leniusculus* would need to be tested. Guan and Wiles (1998) explain that diet can vary within a single species in different habitats as the biomass and availability of prey resources will differ. Therefore, including multiple populations would give a more accurate representation of the true niche width of *P. leniusculus* in comparison to *A. pallipes*.

The wider niche exhibited by *A. pallipes* at the species level may be due to the wider spread of Tp and $\delta^{13}C_c$ values observed for individual populations of *A. pallipes*, which could potentially be causing an overestimation of niche width (Figure 3-7). In contrast, it can be seen that the Tp and $\delta^{13}C_c$ values observed for *P. leniusculus* at each site are similar. Niche width has also shown to be influenced by many factors including the surrounding environment, competition, population density and resource density and diversity (Bearhop *et al*., 2004). Such factors may affect search and handling time, change the amount of energy gained and cause alternate foraging strategies to be applied in response to differing local conditions (Bearhop *et al*., 2004; Svänback and Bolnick, 2005). Consequently, environmental conditions can affect the community structure and potentially impact the diet of the studied species (Ercoli *et al*., 2014). In the current study abiotic and biotic variables

such as water chemistry, substratum type and temperature were not assessed at each sampling location. Therefore, it is unclear if differences in niche width between *P. leniusculus* and *A. pallipes* are due to actual differences in trophic ecology or if population metrics are reflecting environmental differences (Ercoli *et al*., 2014). For example, Johnston *et al*. (2011) reported that the same crayfish species could exhibit different diets and trophic positions dependent on location and environmental conditions. Consequently, inferences should be made with caution about niche width at the species and population level between *P. leniusculus* and *A. pallipes*. Future studies comparing the niche width between *P. leniusculus* and *A. pallipes* should seek to ascertain that populations are obtained from environments with similar environmental characteristics, as well as ensuring all populations are well established. By selecting an established population, it can be assumed that it has stabilised within the environment (Ercoli *et al*., 2014) and thus diet is not changing nor is the overall community in a state of fluctuation, which could potentially lead to inaccurate measurements of diet composition and niche width.

Ultimately, the isotopic variation among consumers is driven by the variation amongst prey resources available (Jackson *et al*., 2012; Newsome *et al*., 2012). If metrics are calculated for consumers with no regard for the variation exhibited by prey resources, it may lead to incorrect interpretations of dietary variation, specialisation and food-web structure (Newsome *et al*., 2012). In this study, there was variation amongst the $\delta^{15}N$ and the $\delta^{13}C$ isotope signatures of the five putative prey resources from each location used to estimate the population metrics in the current study. Newsome *et al*. (2012) suggested accounting for the isotopic variation of prey available to the studied consumer. The authors calculated the population metrics for the available prey resources and determined what percentage of the area the consumer occupied. In future, when comparing *A. pallipes* and *P. leniusculus* it may be useful to account for the isotopic variation between prey resources obtained from different locations.

Finally, as mentioned in Chapter 2, sample size among populations or species is an important factor when interpreting population metrics (Ercoli *et al*., 2014; Jackson *et al*., 2011; Newsome *et al*., 2012; Syvaranta *et al*., 2013). If equal sample sizes are unattainable in future studies, a bootstrap modeling approach could be applied (Jackson *et al*., 2012; Newsome *et al*., 2012). Bootstrapping randomly selects *X* number of individuals within the larger sample size, where *X* is equal to the total number of individuals within the smallest sample size. Population metrics are then calculated many times in order to attain a conservative estimate of the mean and variance for a subset of the individuals within the larger sample size (Newsome *et al*., 2012). Bootstrapping would then allow comparisons to be made between samples of varying sizes with greater confidence.

3.4.2 Diet composition

The use of mixing models to convert $\delta^{15}N$ and the $\delta^{13}C$ isotope signatures into estimates of dietary contribution provides data that can be effectively compared with more traditional types of data regarding diet, such as gut content analysis (Newsome *et al*., 2012).

Mixing models estimated the dietary composition of *A. pallipes* and *P. leniusculus* at the population and species level. At the species level, *P. leniusculus* appeared to use more plant material than *A. pallipes*, while *A. pallipes* used more of each invertebrate source (Corixidae, Gammaridae and Ephemeroptera) (Figure 3-4). Therefore, although both species are clearly omnivores, the mixing model suggests that *A. pallipes* are more predatory than *P. leniusculus*. However, the population at Loch Croispol influenced the dietary estimates at the species level for *A. pallipes*. When diet composition is viewed at the population level, it can be seen that the diet of *P. leniusculus* is similar at both sites with macrophytes contributing the largest amount to diet (Figure 3-5; Table 3-2). In contrast, the two populations of *A. pallipes* are very different (Figure 3-5). *A. pallipes* at Loch Croispol were more predatory with Gammaridae contributing most to diet, while *A. pallipes* at Whitemoss

Reservoir were more herbivorous with algae contributing the most to diet. A more carnivorous diet in *A. pallipes* has previously been reported. For example, Reynolds and O'Keeffe (2005) report *A. pallipes* to be more carnivorous than most other crayfish species. The authors found *A. pallipes* (carapace length of 40+ mm) to have a diet comprised of 40 % animal material, as measured by gut content analysis, compared with only 20 % in *P. leniusculus* of a similar size (Mason, 1975). Correspondingly, sub-adult *A. pallipes* were reported to have a diet consisting of 80 - 85 % animal material (Reynolds and O'Keeffe, 2005) compared with 65 % in *P. leniusculus* (Mason, 1975).

It is unclear as to why there are differences at the population level for *A. pallipes*. It is possible that isotopic variability between locations may be confounding any observed differences (Cummings *et al*., 2012) or that the differences in diet observed are reflecting differences in the availability of each food source at each location (Ercoli *et al*., 2014, Reynolds and O'Keeffe, 2005). Additionally, in the present study only five common food sources present at each location were included in the mixing model. As a result, it is likely that other important prey resources have been missed, which may have influenced the mixing model outputs causing biased results (Newsome *et al*., 2012).

Invasive crayfish species typically have a stronger impact through predation than native crayfish species on native prey species (Haddaway *et al*., 2014; Peay *et al*., 2009). Invasive crayfish species alter ecosystems through modification of habitat, consumption of macrophytes, increased cycling of nutrients through detrital consumption and predation of invertebrates, amphibian larvae and fish fry (Gherardi, 2007). *P. leniusculus* is known to negatively affect the biomass and diversity of macroinvertebrates (Crawford *et al*., 2006; Guan and Wiles, 1998; Nyström *et al.*, 1999; Nyström, 2002; Stenroth and Nyström, 2003). Since *P. leniusculus* has been established in Loch Ken for over 10 years (Ribbens and Graham, 2009), it is possible that it has reduced the diversity and availability of many invertebrate species present. For example, *P. leniusculus* is known to preferentially feed on

molluscs (Nyström and Pérez, 1998), yet none were collected during the course of this study. It was expected that molluscs would be present in Loch Ken and be a potential food source for *P. leniusculus*. As no molluscs were collected, this could suggest that *P. leniusculus* had previously depleted any mollusc population. A reduction in the biomass and diversity of invertebrates in Loch Ken would result in *P. leniusculus* utilising macrophytes more heavily, and consequently produce a decrease in niche width (Olsson *et al*., 2009). However, since no survey information on the invertebrate community composition prior to the introduction of *P. leniusculus* is available, it is not possible to confirm how the invertebrate community has been affected or whether *P. leniusculus* diet has shifted away from invertebrates towards plant material. If this is the case and the study is repeated in the future, an even narrower niche width and reduced diversity in diet may be observed as *P. leniusculus* continues to deplete resources within Loch Ken.

3.4.3 Invasion success

It is often expected that invasive species exhibit a wider niche width than native species (Shea and Chesson, 2002). A wider niche width indicates greater diet diversity and thus an ability to occupy more variable habitats. As a result of the wider niches exhibited by invasive species, they are more likely to impact a greater number of species (Goodell *et al*., 2000).

In the current study, a combination of stable isotope derived population metrics and mixing models revealed that the invasive *P. leniusculus* does not occupy a wider niche width than the native *A. pallipes* at the species level. This suggests that direct competition for resources is not a key factor in *P. leniusculus* displacing *A. pallipes*. Butler and Stein (1985) reached a similar conclusion for other crayfish species. The authors postulated that displacement of Sanborn's crayfish *Orconectes sanbornii* (Hobbs and Fitzpatrick, 1962) by *O. rusticus* was the result of a faster growth rate and higher fecundity of the invasive *O. rusticus*, as well as juvenile *O. sanbornii's* greater susceptibility to predation, rather than competition for food.

Therefore, if direct competition for resources is not driving the displacement of *A. pallipes*, why is *P. leniusculus* so successful?

Despite direct competition for resources appearing not be a factor in the displacement of *A. pallipes* by *P. leniusculus*, *P. leniusculus* may cause indirect negative effects on *A. pallipes* by limiting the invertebrate community through consumption of macrophytes. Changes in the macrophyte biomass or diversity can impact the whole ecosystem, including reducing the invertebrate biomass and diversity (Guan and Wiles, 1998; Nyström *et al*., 1996; Nyström and Perez, 1998; Usio *et al*., 2009). Therefore, if macrophytes were reduced through *P. leniusculus* consumption, there would be less invertebrate resources available for *A. pallipes* resulting in indirect competition between species.

There are additional factors contributing to *P. leniusculus* success as an invasive species. For example, when compared with *A. pallipes*, *P. leniusculus* grows faster and has a higher fecundity rate (Lodge and Hill, 1994), reaches higher densities (Guan and Wiles, 1996), is more aggressive (Holdich *et al*., 1995), exhibits a greater tolerance for a wide range of environmental conditions (McMahon, 2002) and can populate habitats not suitable for *A. pallipes* (Sibley *et al*., 2011). Moreover, it is believed the main reason for *A. pallipes* displacement by *P. leniusculus* is due to *P. leniusculus* being a vector for the crayfish plague *A. astaci* (Holdich *et al*., 2014). *P. leniusculus* is largely unaffected by *A. astaci* but leads to mass mortalities in *A. pallipes* (Dunn *et al*., 2009). However, Holdich *et al*. (1995) has also suggested that where *P. leniusculus* and *A. pallipes* have been found in mixed populations, believed to be free of *A. astaci*, the eventual displacement of *A. pallipes* may be the result of inter-specific mating resulting in sterile eggs. Although both *P. leniusculus* and *A. pallipes* may suffer recruitment loss, high fecundity rates would enable *P. leniusculus* to recover. Dunn *et al.* (2009) has since refuted reproductive interference as a mechanism of displacement and instead suggested that *A. pallipes* are competitively excluded from refuges by *P. leniusculus*, which leads to predation.

3.4.4 Conclusion

In conclusion, the overwhelming success of *P. leniusculus* as an invasive species and the mechanisms surrounding the displacement of *A. pallipes* by *P. leniusculus* is likely to be the result of a complex interaction between the multiple factors discussed above.

Chapter 4. Controlling invasive crayfish: Methods to improve the trapping efficiency of *Pacifastacus leniusculus*

4.1 Introduction

Invasive species are considered a major threat to native biodiversity (IUCN, 2000). Invasive crayfish species are globally distributed and some of the most detrimental to freshwater ecosystems (Holdich *et al*., 2014; Stebbing *et al*., 2014). The impact of invasive crayfish can affect several levels of ecological organisation and result in the loss of native species, including native crayfish (Gherardi *et al*., 2001; Gherardi *et al.*, 2011a). In GB, there are currently seven known species of invasive crayfish with established populations in the wild (Holdich *et al*., 2014; Stebbing *et al*., 2014). Of these, the North American signal crayfish *P. leniusculus* is the most widely distributed in GB and is well established in waters throughout England, Wales (Rodgers and Watson, 2011) and Scotland (Gladman *et al*., 2009).

The control or eradication of invasive crayfish species is not only difficult but also expensive (Gherardi *et at*., 2011). *P. leniusculus* is estimated to cost GB in the region of £2 million annually in management, research and habitat restoration activities (Williams *et al*., 2010). At one point in time, Scotland alone was spending £250,000 every five months on a continuing eradication program (Gherardi *et al.*, 2011a).

Freeman *et al*. (2010) concluded that it is unlikely there will be a single solution to contain and eradicate *P. leniusculus* in Scotland. Since populations have spread to a wide range of habitats, including both lentic and lotic systems, the control and eradication of *P. leniusculus* will require each habitat to be treated using different techniques.

Consequently, eradication of *P. leniusculus* in Scotland seems unfeasible and future efforts should be directed towards controlling and containing current populations (Freeman *et al*., 2010). As such, any technique that would enable *P. leniusculus* to be maintained at low

enough densities to mitigate their negative effects on the native biota in Scottish waters would be useful (Gherardi *et al.*, 2011a).

The IUCN released guidelines in 2000 for the prevention of biodiversity loss caused by an invasive species. They recommend that any control method must not negatively affect native flora and fauna, be efficient, be non-polluting and be acceptable socially, ethically and culturally (IUCN, 2000). Holdich *et al.* (1999) released similar guidelines solely aimed at selecting control methods for managing invasive crayfish. The authors suggest that control methods should be evaluated for effectiveness, environmental safety, public safety, cost, labour requirements and be easily accepted by the public. At present, there are no methodologies available for the control of invasive crayfish that fully meet the criteria suggested by either the IUCN (2000) or Holdich *et al.* (1999) (Bean *et al.*, 2006; Stebbing *et al.*, 2014). However, many methods show potential and have been investigated including traps, biocides and biological controls (Stebbing *et al.*, 2003). Current methods of management for invasive crayfish species can be categorised into six broad categories: mechanical, physical, biological, biocidal, autocidal and legislative (Freeman *et al.*, 2010; Gherardi *et al.*, 2011a; Stebbing *et al.*, 2014).

Although it has been suggested that mechanical control such as trapping has limited success in controlling *P. leniusculus* (Holdich and Sibley, 2009), a long-term trapping program carried out in the River Clyde in Scotland over an eight-year period considerably reduced the number of *P. leniusculus* trapped, from 10,625 individuals in 2001 – 2002 to 5,335 individuals caught in 2006 – 2007 (Freeman *et al.*, 2010). This was a reduction of almost 50 % in the trappable *P. leniusculus* population present in the River Clyde. Additionally, a short-term trapping project in Loch Ken in Scotland removed over 650,000 *P. leniusculus* individuals in just 56 days, which ultimately resulted in a reduction of approximately 60 % in the population of males (Ribbons and Graham, 2009).

Although trapping may not provide a complete solution, given the significant reduction in populations of *P. leniusculus* observed, anything that can be done to improve trapping efficiency should be investigated. For example, Gherardi *et al*. (2011a) suggested that trapping efficiency might be improved by using more attractive baits. Therefore, the aim of this part of the PhD was to improve trapping efficiency of *P. leniusculus* through the identification of a preferred food source. Initially, a decision chamber was used as the tool to investigate the potential for selecting more effective baits.

4.2 Methods

4.2.1 Laboratory Investigation

4.2.1.1 Crayfish

Adult *P. leniusculus* were collected, under SNH licence, from Daer Water in the upper catchment of the River Clyde at Elvanfoot using Swedish Trappy traps baited with *S. trutta* and *O. mykiss*. *P. leniusculus* were transported in cool boxes with ice packs from capture location to the laboratory at the University of Stirling under said SNH licence. All crayfish were blotted dry and weighed (± 0.1 g) and carapace length (CL) was measured from the tip of the rostrum to the posterior margin of the carapace using Vernier calipers (± 0.1 mm).

Fourteen adult *P. leniusculus* (CL = 52.8 ± 0.88 mm), seven male and seven female with all appendages intact were selected for use during experiments. The *P. leniusculus* were held individually in secure aerated holding tanks (Ferplast Geo Medium tank, L 23.2 cm x W 15.3 cm x H 16.6 cm), which contained large plastic piping to provide some shelter. Holding *P. leniusculus* individually ensured that appendages remained intact for the duration of experiments by avoiding antagonistic interactions between individuals. Doing so also prevented familiarity with other crayfish being established, which could potentially have influenced arm selection within the maze. No berried females or recently moulted male or female adults were used in experiments in order to avoid any potential underlying olfactory cues, which may have influenced arm selection within the maze environment. Additionally, crayfish were marked with a spot of Tipp-Ex (white correctional fluid) on the dorsal view of the carapace to provide a visual aid under dimly lit experimental conditions. After completion of experiments, all individuals were euthanised by freezing.

4.2.1.2 Experimental setup

P. leniusculus were maintained, and experiments were conducted in the temperature control room of the Institute of Aquaculture at the University of Stirling, Scotland. Air and water temperatures were maintained at a constant 12°C for the duration of the experiments.

A 'flow through' plus-maze was constructed out of clear acrylic plastic. Although the plus-maze was comprised of two 'long' arms (L 28.7 cm x W 12.5 cm x H 13.5 cm) and two 'short' arms (L 20.2 cm x W 12.5 cm x H 13.5 cm), the space available to crayfish was equal in each (L 15.0 cm x W 12.5 cm x H 13.5 cm) (Figure 4-1). Arms were large enough that crayfish could turn around freely. The plus-maze was lined with white gravel substrate (particle size 6 mm – 8 mm) to aid crayfish mobility within the maze, as well as to define the maze and crayfish under dimly lit experimental conditions.

The plus-maze was filled with 9 cm of water and fed by four reservoir tanks (L 48 cm x 34.5 cm x H 28 cm). Each tank supplied one arm of the plus-maze with water (gravity fed) through plastic tubing (L 1 m x Dia. 1.2 cm).

Prior to experiments, dye trials using commercial food colouring were run to visualise water flow and ensure that the odour plume within the plus-maze would be separate and equal when travelling through each arm. It took several attempts to obtain a water flow rate that would enable *P. leniusculus* to remain within the plus-maze without it overflowing or for the gravity fed flow rate to be impeded. Eventually, a water flow rate of 1:02 minutes ± 0.03 s for dyes to reach the centre of the maze was achieved. Water exited the maze though an outflow pipe located in the centre of the maze.

Water temperature within the plus-maze, as well as the water supplied from the reservoirs, was maintained at the same temperature to ensure that the vertical position of the odour plume was sustained throughout the entire length of the arm.

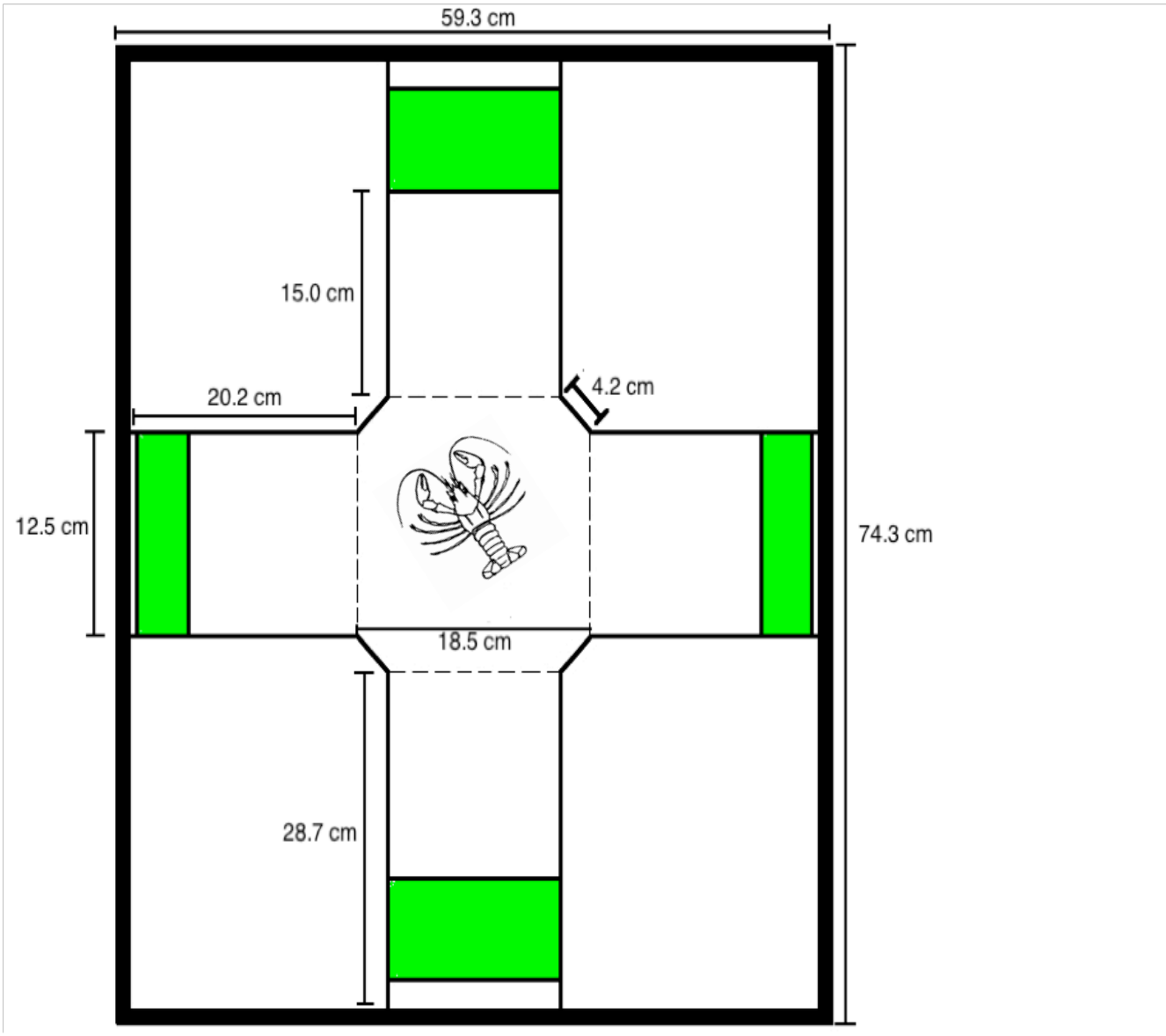


Figure 4-1: Schematic drawing of the 'flow through' plus-maze. Crayfish represents where *P. leniusculus* were placed at the start of every trial, dashed lines indicate sliding doors and the 'threshold' over which *P. leniusculus* had to cross in order to be scored as an 'arm choice' and green areas indicate area where attractants were placed, inaccessible to *P. leniusculus*.

4.2.1.3 Attractant preparation

Four food sources (*O. mykiss*, *P. leniusculus*, beef and aquatic vegetation) were tested, first in a 'fresh' condition (i.e. recently dead) and secondly in a decomposed state. Both beef and *O. mykiss* were obtained from a local supermarket, *P. leniusculus* were taken from those initially caught for experiments and the aquatic vegetation was obtained from a local freshwater pond. Each food source was homogenized and 5 g of food material placed in an

empty filter tea bag. Filter tea bags were chosen as they prevented breakup of food and/or any solid material from entering the plus-maze, while still permitting the food odour to permeate the maze. Half of each food source was frozen immediately after homogenization and the remaining half placed inside an incubator under a constant temperature of 25°C for 5 days in order for decomposition to occur and then subsequently frozen. This process allowed standardisation of food sources for the total period of experimentation.

4.2.1.4 Procedure

P. leniusculus were contained in the centre of the plus-maze for a 3 minute acclimation period by use of plastic sliding 'doors' (Figure 4-1), which blocked the entrance to each arm of the plus-maze. Each door was constructed out of two acetate sheets, with each pair containing several 6 mm diameter flow holes. The holes allowed the attractant odour to diffuse into the central holding area where *P. leniusculus* could detect the odour prior to the start of trial. Doors were held in place using a plastic tube, which had been sliced vertically, top to bottom, on each side of the arm entrance to create a plastic guide. Thin plastic fishing line was attached to the top of each door, allowing them to be pulled upwards smoothly and quickly in the plastic guides after the acclimation period had ended. Doors rose uniformly and equally when vertically pulled, and *P. leniusculus* exhibited no signs of alarm.

Each trial ran for 23 minutes and comprised of a 3 minute contained acclimation period and a 20 minute maze exploration period. A trial length of 20 minutes was selected as water flow rate was observed to be impeded after longer lengths of time during preliminary dye trials.

Upon completion of each trial, water from the plus-maze was discarded and the maze and substrate cleaned with distilled water to prevent odour carry-over from influencing subsequent 'arm choice' by *P. leniusculus*. Maze and reservoirs were refilled to total volume.

All trials were conducted under an artificial reversed photoperiod, with 12 h of darkness during daytime periods and 12 h of light during night periods. *P. leniusculus* were given a period of seven days to acclimate to the reversed photoperiod before trials commenced. Experiments were then carried out during the "nocturnal" hours of the reversed photoperiod.

Experiments were recorded using a DigiLife 00V-H71Z HD 1080p video camera. Since the camera was not an infra-red unit, a red light bulb was used to illuminate the maze and *P. leniusculus* activity during "nocturnal" periods, enabling video capture. The video camera was suspended directly above the maze ensuring a whole maze view. The plus-maze was screened off with black plastic to minimise disruption to *P. leniusculus* during trials.

Video recordings were analysed using Observational Data Recorder (ODRec) software (v1.00 beta 2, Samuel Péan, France, 2013). The position of *P. leniusculus* within the maze was noted every 30 s for the duration of the trial and individuals were considered in or out of an arm once the rostrum of *P. leniusculus* had passed the sliding door 'threshold' separating the arm and the centre of the maze (Figure 4-1).

4.2.2 Experiments

4.2.2.1 Experiment 1

In order to determine if fresh or decomposed attractants were preferred by *P. leniusculus* and in an effort to ensure no memory bias influenced 'arm choice', a total of five treatment scenarios were tested:

1. Control: all fourteen crayfish were presented with only water and no attractant.
2. Fresh non-memory: ten crayfish were presented with each of the four fresh food attractants a total of three times, each one week apart.
3. Fresh memory: four crayfish were presented with each of the four fresh food attractants a total of three times on three consecutive days.

4. Decomposed non-memory: ten crayfish were presented with each of the four decomposed food attractants a total of three times, each one week apart.
5. Decomposed memory: four crayfish were presented with each of the four decomposed food attractants a total of three times on three consecutive days.

The same four *P. leniusculus* individuals were used in both the fresh and decomposed food attractant memory trials, and the same ten *P. leniusculus* individuals were used for both the fresh and decomposed food attractant non-memory trials.

Each food attractant was randomly allocated to an arm of the plus-maze during each of the treatments. Food attractants were anchored in the green shaded area of each arm (Figure 4-1) behind a plastic mesh barrier, enabling *P. leniusculus* to detect the odour but not physically access food.

4.2.2.2 Experiment 2

In order to determine if four different food attractants were overwhelming and inhibiting 'arm choice', only one food attractant (*O. mykiss*) was placed in the green shaded area of an arm (Figure 4-1). The remaining three arms were empty. *O. mykiss* was chosen as *P. leniusculus* are known to be attracted to it in field trapping conditions (personal observation). The plus-maze was set up as above and the trial lasted 23 minutes and comprised of a 3-minute acclimation period and a 20 minute maze exploration period. Six of the twelve *P. leniusculus* individuals, three female and three male, were randomly selected and the food attractant randomly allocated to an arm. This experiment was carried out only once on each of the six *P. leniusculus* individuals. The plus-maze was cleaned after use as described in section 4.2.1.4.

4.2.2.3 Experiment 3

To establish if being able to physically access the food attractant influenced 'arm choice', the plus-maze was once again set up as before. This time *O. mykiss* was placed inside an

arm, accessible to *P. leniusculus*. Four of the twelve *P. leniusculus* individuals were randomly selected, two female and two male, and the food attractant was randomly allocated to an arm. This trial was carried out only once on each of the four *P. leniusculus* individuals and the plus-maze cleaned after use as previously described in section 4.2.1.4.

4.2.3 Field investigation

During late August 2013, four nylon Nordic multimesh gill nets were deployed at two sites in Loch Ken in Dumfries and Galloway, Scotland (Figure 2-2) to obtain fish specimens for stable isotope analysis (section 2.2.2.6). Gill nets were deployed for 24 hr and upon retrieval the following day there was an unexpected finding. Along with several species of fish, gill nets were teeming with *P. leniusculus* (Figure 4-2).

Subsequently, it was decided that further investigation as to why such large numbers of *P. leniusculus* were observed was warranted and whether gill nets could potentially be used as an alternative method to trapping for detection and control of *P. leniusculus*.

Figure 4-2: Examples of *P. leniusculus* entangled in a nylon Nordic multimesh gill net (monofilament).

4.2.3.1 Experimental set-up

Less than two weeks later during the first week of September 2013, a SeaBotix LBV 150SE Remote Operated Vehicle (ROV) was taken to Loch Ken to observe *P. leniusculus* underwater response to a cotton multimesh gill net over a 24 hr period. The ROV was connected by cable to a shore-based screen allowing live observation of crayfish behaviour in response to the gill net, as well as recording for later analysis.

The gill net used had two panels, one with a mesh size of 1 cm and the other a mesh size of 2 cm. Each panel was split so that one-half of the panel was baited with several pieces of *O. mykiss* (Figure 4-3). Pieces of *O. mykiss* were attached to the net by cable ties.

The gill net was deployed from a boat at Site 2 in Loch Ken (Figure 2-2) at approximately 2 m depth. On either end of the gill net a set of three Swedish Trappy traps baited with *O. mykiss*, were deployed. Traps were deployed to ensure that crayfish were attracted to the *O. mykiss*. Each trap was placed approximately 10 m from either end of the gill net, and within each set of three traps, individual traps were set approximately 1 m apart.

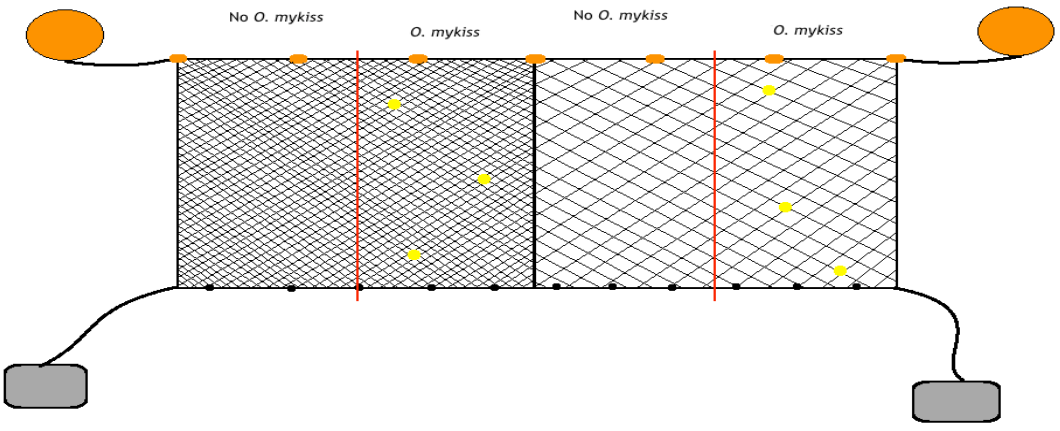


Figure 4-3: Schematic diagram of cotton multimesh gill net (multifilament) as would be viewed underwater. Top of gill net: large orange balls represent buoys and small orange ovals represent floats. Bottom of gill net: Large grey blocks represent cement anchors and small black dots represent lead weights. Red lines indicate how panels were split. Yellow circles represent pieces of *O. mykiss*. L-R: 1 cm mesh panel, 2 cm mesh panel.

The gill net and traps were deployed at 12:00 hr. There were four ROV observation periods over the 24 h period that followed. Observations took place from: 12:00 – 17:00 hr, 21:00 – 22:30 hr, 05:00 – 08:00 hr and 10:30 – 12:30 hr.

During observations, it became increasingly difficult to locate the deployed traps with the ROV due to water conditions. As a result, a single Swedish Trappy trap was baited with *O. mykiss* and deployed in < 1 m of water for observation.

After the 24 hr observation period ended, the gill net and all traps were recovered. Any trapped *P. leniusculus* were removed before being euthanised by freezing upon return to the University of Stirling.

4.2.4 Statistical analysis

For experiment 1, a mixed Analysis of Variance (ANOVA), which included within-subject and between-subject terms, was used in order to examine the mean time spent within the plus-maze. The within-subject terms were: treatment (fresh or decomposed), trial number (1, 2 or 3), and attractant type (*O. mykiss*, *P. leniusculus*, beef or vegetation). The between-subject terms were: memory or non-memory and sex (female or male). Subsequent post hoc tests were performed using a Sidak adjustment to identify any significant interactions. Residuals were visually inspected for normality. Raw data was used as any transformations failed to improve normality.

For experiment 2, experiment 3 and the control, a one-way repeated measures ANOVA, which included within-subject and between-subject terms, was performed to examine differences in the mean time spent within the plus-maze. The within-subject term was arm selection and the between-subject term was sex (female or male). Residuals were visually inspected for normality. Raw data was used as any transformations failed to improve normality.

All statistical analyses were conducted using SPSS (V 22.0). Values reported are mean ± SE unless otherwise stated. Significance level was defined as $p < 0.05$.

4.3 Results

4.3.1. Laboratory investigation

4.3.1.1 Experiment 1

4.3.1.1a Control

There was no significant difference in the mean time *P. leniusculus* spent in any of the four arms within the plus-maze ($F_{3, 36} = 1.55$, $p = 0.22$, $n^2 = 0.11$), nor was there any difference in the mean time spent in any of the four arms between sexes ($F_{1, 12} = 0.33$, $p = 0.58$, $n^2 = 0.03$). Consequently there was no significant interaction between arm selection and sex ($F_{3, 36} = 1.47$, $p = 0.24$, $n^2 = 0.12$). This indicates that that there was no underlying preference for any arm within the plus-maze before the addition of attractants (Figure 4-4).

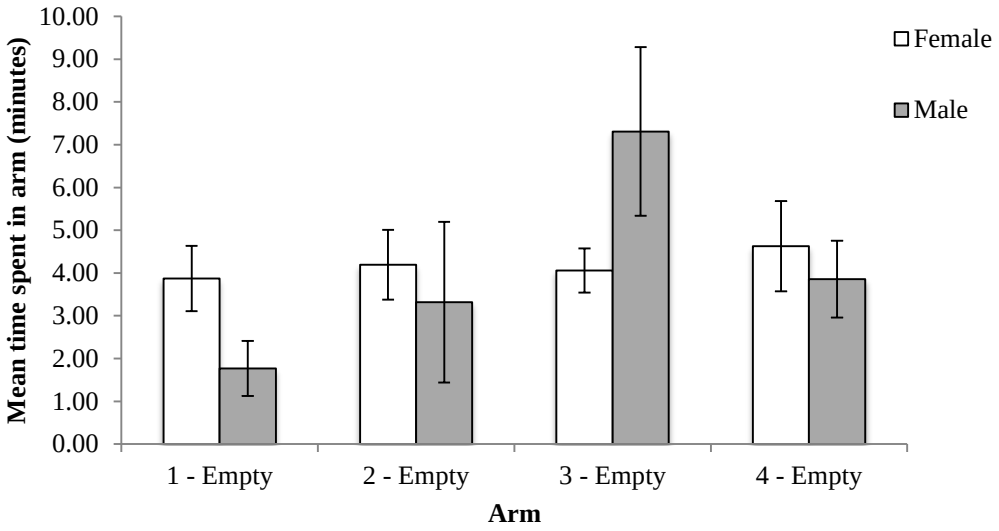


Figure 4-4: Mean time in minutes (± SE) female and male *P. leniusculus* spent in each empty arm of the plus-maze.

4.3.1.1b Attractants

Results revealed no significant difference between memory or non-memory treatments ($F_{1, 12} = 0.032$, $p = 0.862$, $n^2 = 0.00$) or for sex ($F_{1, 12} = 1.009$, $p = 0.335$, $n^2 = 0.08$). The mean time spent in an arm was not significantly influenced by the treatment type, fresh or decomposed ($F_{1,12} = 3.84$, $p = 0.07$, $n^2 = 0.24$), or by the type of attractant used – *O. mykiss*, beef, *P. leniusculus* or vegetation ($F_{3, 36} = 2.04$, $p = 0.13$, $n^2 = 0.15$). However, the overall mean time spent in an arm was found to be significantly different between trials ($F_{1.35, 16.23}$

= 10.124, $p < 0.005$, $n^2 = 0.46$). No interactions were found to significantly influence the mean time spent in any arm.

Post hoc tests using a Sidak adjustment revealed that the overall mean amount of time spent in any given arm was significantly different between trial 1 (4.28 ± 0.11) and trial 2 (4.87 ± 0.15) ($p < 0.01$), as well as between trial 2 and trial 3 (4.38 ± 0.10) ($p < 0.05$). This would suggest that *P. leniusculus* were less active and increased the amount of time spent in one or more arms during trial 2 compared to trials 1 and 3. The overall mean time spent in any given arm between trial 1 and trial 3 was not significantly different ($p = 0.60$). Although no significant difference in the mean time spent in each arm between attractants was detected, less time was spent in the arm containing *O. mykiss* as the trials progressed, while increasing amounts of time were spent in arms containing beef and *P. leniusculus*. Additionally, only in trial 1 did *P. leniusculus* spend more time in one attractant above all others available (Figure 4-5).

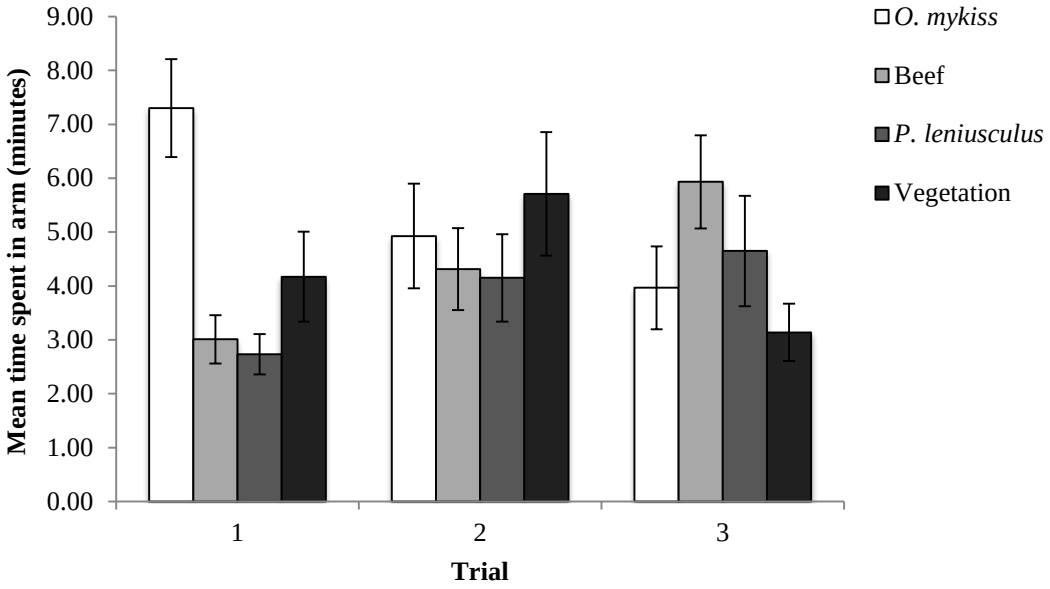


Figure 4-5: Mean time in minutes (± SE) *P. leniusculus* spent in an arm of the plus-maze, containing either *O. mykiss*, beef, *P. leniusculus* or vegetation as an attractant, across three individual trials. Memory and non-memory trials, as well as sexes are combined.

4.3.1.2 Experiment 2

There was no significant difference in the mean time spent in the arm containing the *O. mykiss* attractant compared with the three other empty arms ($F_{3, 12} = 1.46$, $p = 0.28$, $n^2 = 0.27$). There was also no significant difference observed between female and male *P. leniusculus* with regard to the mean time spent in the arm containing *O. mykiss* attractant compared with the empty arms ($F_{1, 4} = 1.10$, $p = 0.35$, $n^2 = 0.22$) (Figure 4-6). Consequently, there was no significant interaction between arm selection and sex ($F_{3, 12} = 2.58$, $p = 0.10$, $n^2 = 0.39$). It can be seen in Figure 4-6 that although no preference was detected, male crayfish appeared to spend a large proportion of time in one particular empty arm, arm 4.

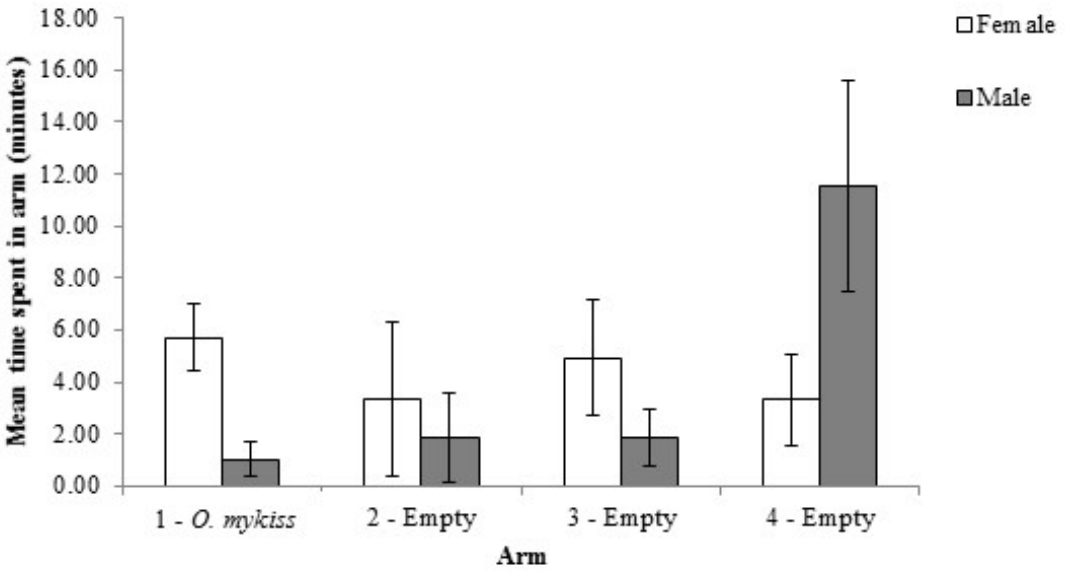


Figure 4-6: Mean time in minutes (± SE) female and male *P. leniusculus* spent in each arm of the plus-maze, one containing an attractant (*O. mykiss*) and three with no attractant.

4.3.1.3 Experiment 3

There was no significant difference detected between the mean time spent in each arm of the plus-maze, despite one arm containing *O. mykiss* that was accessible to *P. leniusculus* for the entire duration of the trial ($F_{3, 6} = 1.99$, $p = 0.22$, $n^2 = 0.50$). There was also no significant difference in the amount of time spent in any given arm between sexes ($F_{1, 2} = 1.22$, $p = 0.38$,

n^2= 0.38). As a result, there was no significant interaction detected between arm selection and sex ($F_{3, 6}$ = 0.32, p = 0.81, n^2 = 0.14).

Although no significant difference was detected for the mean time spent in the arm containing trout compared to the empty arms, it can be seen from Figure 4-7 that both female and male *P. leniusculus* spent a large proportion of their time in the arm containing *O. mykiss*.

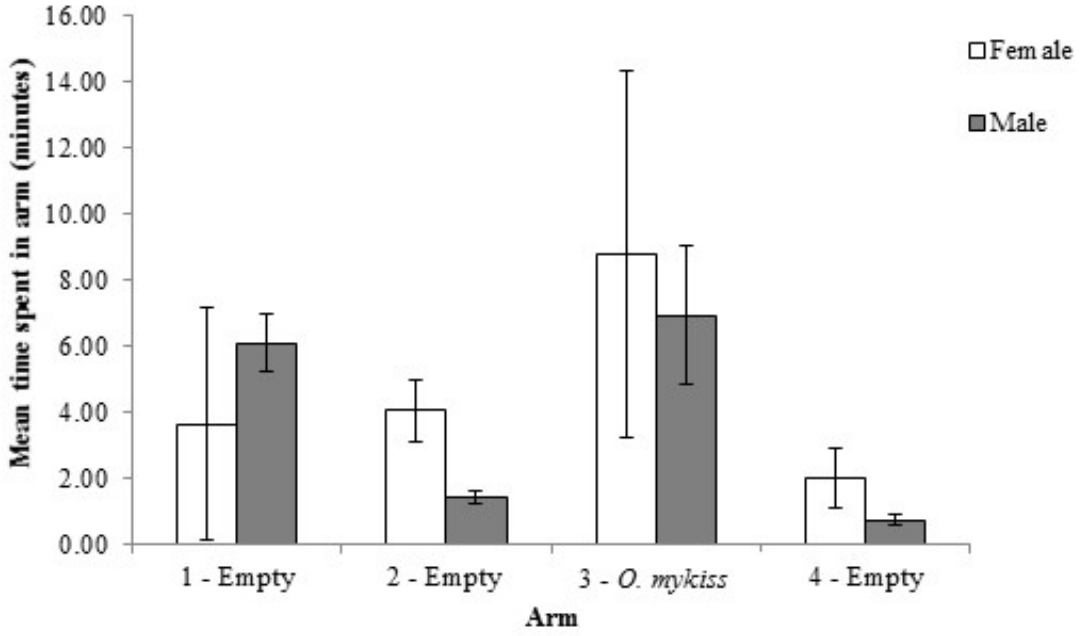


Figure 4-7: Mean time in minutes (± SE) female and male *P. leniusculus* spent in each arm of the plus-maze, one containing an attractant (*O. mykiss*) accessible within the arm and three with no attractant.

In addition to the results and statistical tests reported, several other approaches were used to explore the data but no significant associations were detected.

4.3.2 Field investigation

4.3.2.1 Accidental by-catch

Quantitative information on the number of *P. leniusculus* found entangled within the nets is not available. The first set of four nylon Nordic multimesh gill nets (monofilament) were not placed under planned experimental conditions. In addition, the very large numbers of

animals captured in the nets would have taken several days to remove and quantify. Since technical assistance with boat and net handling was only available for one day to set the nets and one day to retrieve them, numbers are estimated. These were in the thousands rather than the hundreds (Figure 4-2). It is also important to note the condition of fish specimens caught when nets were retrieved after the 24 hr period ended. All fish caught appeared to have had flesh damaged by *P. leniusculus* to varying degrees, and in some cases nothing but skeletal remains were present (Figure 4-8).

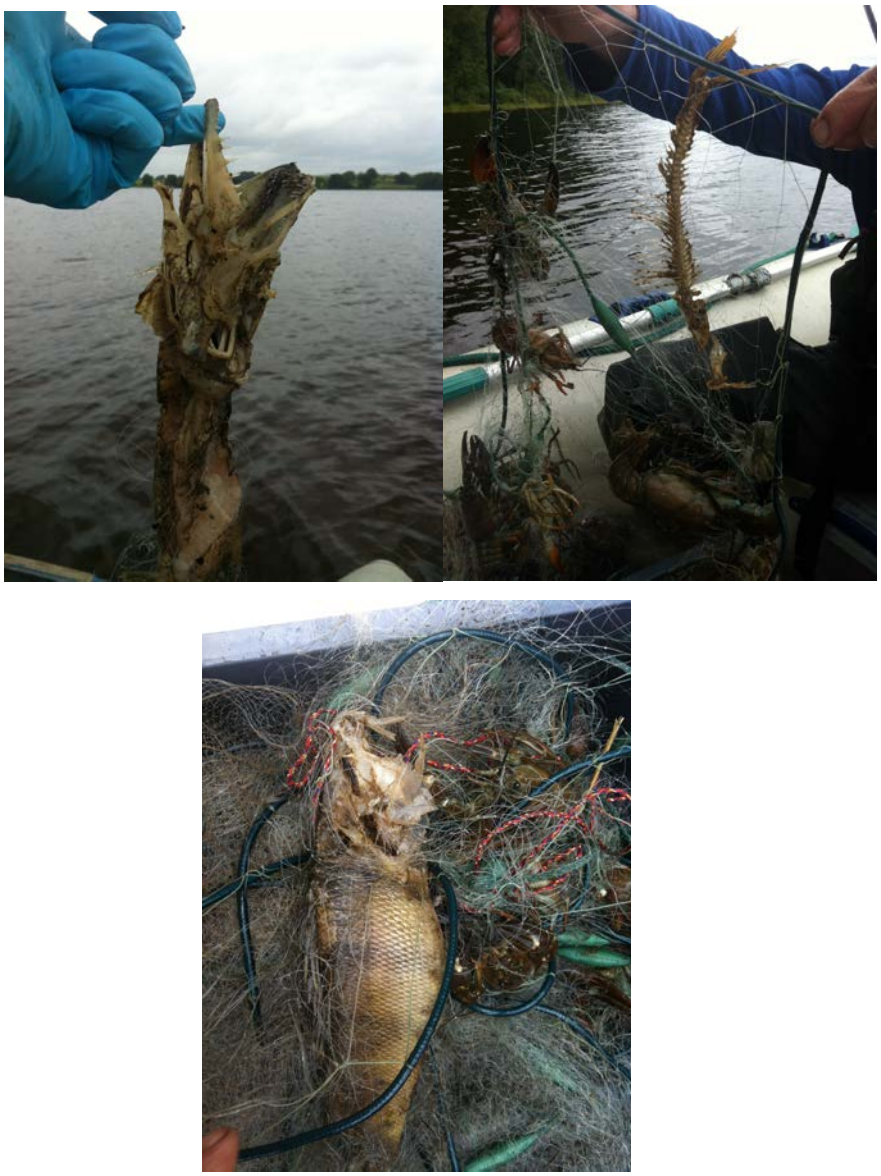


Figure 4-8: Examples of flesh damage by *P. leniusculus* to fish specimens present when nylon Nordic multimesh gill nets (monofilament) were retrieved after a 24 hr period.

4.3.2.2 Baited gill net

Before observations of the baited cotton multimesh gill net (multifilament) began, one set of the Swedish Trappy traps was located and checked in order to confirm that: a) *P. leniusculus* were present in the area of Loch Ken where observations were taking place, and b) *P. leniusculus* were attracted by the *O. mykiss* used as bait on the gill nets. *P. leniusculus* were found to be present in traps as well as wandering outside of traps. A single Swedish Trappy trap was deployed in shallow water for ease of observation when two sets became difficult to locate in unfavourable water conditions. *P. leniusculus* were found within the trap and the area surrounding the trap. Interestingly, individuals outside of the trap were observed actively trying to obtain the *O. mykiss* bait through the wall of the trap.

After several attempts, conditions prevented checks of the full length of the gill net during observation periods. High turbidity and flow impeded visibility and the ability to keep the ROV within range of the gill net. The ROV was positioned on the Loch bottom at the end of the gill net, alongside the 2 cm mesh panel, to stabilise it and prevent disturbance to sediment and also to any *P. leniusculus* present. When the ROV was positioned on the Loch floor, only a small portion of the 2 cm mesh panel was visible.

The *O. mykiss* bait was not observed on video and so *P. leniusculus* were not observed consuming the *O. mykiss* bait that had been secured to the mesh panel. However, where *P. leniusculus* were observed to be moving around on the net, their location corresponded to the side of the 2 cm mesh panel where *O. mykiss* bait had been secured. In total, seven *P. leniusculus* individuals were observed on the portion of the gill net being monitored. On one occasion, two *P. leniusculus* individuals were entangled before freeing themselves. All other individuals walked freely over the 2 cm mesh panel (Figure 4-9). No fish were seen near or within the gill net during these observation periods. Furthermore, no *P. leniusculus*

were found to be present on or entangled within the gill net when it was retrieved at the end of the 24 hr period. Nor were any fish found to be present.

Figure 4-9: Video stills showing *P. leniusculus* on and around the baited cotton multimesh gill net (multifilament).

4.4 Discussion

4.4.1 Laboratory investigation

The intended aim of the present study was to enhance *P. leniusculus* trapping efficiency through the use of food attractants. Originally, plans were made to determine the preferred fresh and decomposed food attractant to *P. leniusculus* using a plus-maze. Preference would then have been determined in the plus-maze between the previously demonstrated preferred fresh and decomposed food attractants. Following this, the amino acid and biogenic amine composition of the overall preferred fresh or decomposed food attractant would be obtained. The resulting amines would then be tested in a field study to determine if trapping efficiency of *P. leniusculus* could be improved through the use of a targeted food attractant. However, during the initial stage of the study intended to determine a preferred fresh and decomposed food attractants, no obvious preference by *P. leniusculus* was exhibited. As a result, this discussion will explore the lack of preference observed during this study.

P. leniusculus showed no apparent preference for any of the four food attractants when presented in either a fresh or decomposed state. There was no difference in food attractant preference between sexes and previous experience did not influence arm choice. Also, there was no preference exhibited by male or female *P. leniusculus* when presented with three empty arms of the plus-maze and one containing fresh *O. mykiss*. Finally, having fresh *O. mykiss* available within one arm of the plus-maze did not result in *P. leniusculus* exhibiting a preference for the *O. mykiss* arm over the remaining empty arms.

The lack of any apparent preference to any of the four food attractants exhibited by *P. leniusculus* in this study, whether behind a mesh barrier or accessible within an arm, was unexpected. Many previous studies using various crayfish species in maze environments have reported preferences when individuals were presented with different food stimuli (Correia *et al*., 2007, Kominoski *et al*., 2007; Loya-Javellana *et al*., 1993).

There may be several reasons to explain the results of this study. For instance this is the first study, to the best of current knowledge, to use a four arm plus-maze to investigate preferences, either food or pheromones, in any crayfish species. Previous studies used two arm Y- or T-maze shapes to investigate preference (e.g. Adams *et al*., 2005; Correia *et al*., 2007; Kominoski *et al*., 2007; McMahon *et al*., 2005; Tierney and Atema, 1988). Consequently, it may be harder to detect any preference when presenting more than two choices. It is also possible that the range and/or quantities of attractants were not enough to illicit a response, or were even foods which *P. leniusculus* were attracted to (Kenning *et al*., 2015). Additionally, the plus-maze may be spatially restrictive, thus not representing a comparable ecological environment and hence influencing the behaviour of *P. leniusculus* (Kenning *et al*., 2015).

4.4.2 Attractant limitations

Meakin *et al*. (2008) reported that in food choice experiments the common yabby *Cherax destructor* (Clark, 1936) preferred live zooplankton over inert food sources, indicating moving prey stimulate and/or influence crayfish feeding preferences. In the present study, all food attractants presented to crayfish were inert. Therefore, it is possible if *P. leniusculus* were presented with live food sources that a preference may have become apparent. Live food sources could potentially create stronger olfactory cues, which could persist in the flow through plus-maze for a longer period of time. Future studies should therefore seek to compare live and inert food sources within the plus-maze environment. However, if *P. leniusculus* were to prefer live food sources, there would be challenges associated with how to incorporate live bait into traditional trapping methods.

Crayfish rely on sensory capacities and learning mechanisms when selecting prey (Correia *et al*., 2007; Arzuffi *et al*., 2000). Hazlett (1994a) reported that *O. virilis, O. rusticus* and big river crayfish *Cambarus robustus* (Girard, 1852) did not respond to animal protein

odours if they had not had previous experience with that food. It was concluded that if no previous association between smell and taste had been formed, smell alone did not signal food availability to an individual. It was also reported that repeated exposure to a food item was needed to form an association. *P. leniusculus* in the present study were not exposed to the four food attractants prior to the start of the study and it is unknown to what extent they may have experienced them in natural conditions prior to capture. Therefore, it is possible that *P. leniusculus* may not have recognised the four food attractants used in this study as food, subsequently influencing behaviour within the plus-maze environment.

It is also known that detecting one type of stimulus can cause an animal to inhibit responses to any other stimuli present; this is the most common reaction when detecting multiple stimuli (Hazlett, 1999). For example, when the crayfish *O. virilis* was presented with food and alarm odours simultaneously, the food response was greatly reduced (Hazlett, 1999). In the current study there were two food attractants used which could potentially result in altered behaviour of *P. leniusculus* within the plus-maze. For instance, *O. mykiss* are known to predate *P. leniusculus* (Nyström *et al.*, 2001) and the food consumption behaviour of crayfish has been shown to be affected by the presence of predatory fish (Blake and Hart, 1993; Gherardi *et al.*, 2011b; Nyström and Åbjörnsson, 2000; Stein and Magnuson, 1976). It is possible that potential preferences were obscured during this study due to *P. leniusculus* altering behaviour in response to detecting *O. mykiss*. In addition to *O. mykiss*, crushed conspecifics were used as a potential attractant during this study. Sensitivity to crushed conspecifics has been reported in crustaceans (Pijanowska, 1997), including some crayfish species (Adams and Moore, 2003; Hazlett, 1990; Hazlett, 1994b). The alarm odour received from crushed conspecifics has been suggested to serve as a warning, signaling danger from predators (Pijanowska, 1997). Furthermore, it has been reported that freeze thawing of crushed individuals does not eliminate the signal released (Hazlett, 1994b). Therefore, it is possible that the behaviour of *P. leniusculus* within the plus-maze was influenced by the

detection of an alarm odour received from crushed conspecifics. As a result, the response to the alarm odour may be masking any potential preference for the food attractants presented in this study.

4.4.3 Plus-maze limitations

Correia *et al*. (2007) used a Y-maze to examine prey detection in the crayfish *P. clarkii*. The prey Midge *Chironomus riparius* (Meigen, 1804) was either placed behind a mesh barrier to examine chemical and visual cues or behind a transparent solid barrier to examine visual cues alone. The authors observed *P. clarkii* to detect a stimuli and attack but after being unable to physically obtain the *C. riparius* no detection or attack behaviours were exhibited during the remainder of the trial. The authors surmised that *P. clarkii* learned *C. riparius* was unavailable for consumption and therefore ignored the prey and continued to search the Y-maze. Furthermore, Sacristan *et al*. (2014) reported that the Australian red claw crayfish *Cherax quadricarinatus* (von Martens, 1868) wandered around an aquarium without regard to the position of the food. The authors noted that it was only when *C. quadricarinatus* was close to or in contact with the food, that it accepted or rejected the food presented. Based on these findings, it is possible that *P. leniusculus* were exhibiting similar learned behaviour, which resulted in no apparent preference during the first two food attractant trials conducted in the present study. However, when the food attractant *O. mykiss* was available within the maze there was still no clear preference exhibited by *P. leniusculus*. It is unclear why no preference for the arm containing *O. mykiss* was observed. Even though the *O. mykiss* was available within the arm of the plus-maze, the food attractant was still contained within a filter tea bag. Therefore, it is possible that the filter tea bag acted as a barrier resulting in *P. leniusculus* exhibiting the learned behaviour described above. Future studies should investigate how barriers between *P. leniusculus* and food influences foraging behaviour and perceived preference in *P. leniusculus* within a maze environment. Additionally, it is possible that the composition of the filter tea bag prohibited the attractant odour from

permeating the maze. A larger mesh size or different material could be explored in future studies.

Finally, it is possible that some parameters of the plus-maze were not suitable for detecting preference in *P. leniusculus*. Odour plumes are known to be affected by flow dynamics, which can influence the detection by crustaceans such as *P. leniusculus* (Hazlett *et al*., 2006; Weissburg, 2011). It is possible that the flow rate in this study was too slow and that the odour plume did not permeate the maze fully and/or reach *P. leniusculus* during the acclimation period, despite dye trials revealing water flow reaching the center uniformly between arms. Consequently, the acclimation period of 3 minutes and the trial period of 20 minutes may not have been long enough for *P. leniusculus* to exhibit a clear preference. Additionally, water was gravity fed into the plus-maze from reservoir tanks, which would have resulted in a slowing of flow as time continued. The addition of a water pump feeding the plus-maze at a constant water flow rate would be advisable in future studies. Furthermore, the camera in the present study was positioned at a height that enabled observation of *P. leniusculus* at all times within the plus-maze. The camera's wide angle view did not allow detailed observation of crayfish movement. Only position of *P. leniusculus* and amount of time spent in various arms of the plus-maze could be observed. Previous studies investigating preference in crayfish utilised finer motor movements to define preference, for example antennule movement (Correia, 2003), specific feeding behaviours (Montemayor *et al*., 2002) or a suite of behavioural responses, including body orientation to a stimulus and number of times walking legs are touched to mouth (Kreider and Watts, 1998; Tierny and Atema, 1988). Future studies seeking to define preference using a plus-maze would benefit from additional camera angles and/or visual observations to capture more detailed behavioural responses, which could better define preference in *P. leniusculus*.

4.4.4 Field investigation

After the unexpected by-catch of large numbers of *P. leniusculus* in gill nets while obtaining fish specimens for Chapter 2, plans were made to investigate if nets may provide a more efficient alternative to traditional trapping methods.

Only a few documented instances of interactions between crayfish and gill nets exist. For example, the Minnesota Department of Natural Resources (DNR) describe crayfish being caught in gill nets during fisheries lake surveys as far back as 1985, when 300 *O. propinquus* or *O. rusticus* were found entangled in gillnets in Basswood lake (DNR, 2015). Additionally, on a forum found on the Association for the Sciences of Limnology and Oceanography (ASLO) website, a photograph taken at Lost Creek Reservoir in Utah was shared of a gill net containing *O. mykiss* and several crayfish of an unidentified species. The poster explains that when gill netting for fish, crayfish frequently eat the fish and become entangled in the net (ASLO, 2015). However, to the best of current knowledge there is only one study investigating the relationship between gill nets and crayfish by-catch. Moonga and Musuka (2014) discuss the effect of the invasive *P. clarkii* as gill net by-catch in the Kafue River, Zambia. The authors analysed questionnaire responses by local fishers about *P. clarkii* by-catch. There was some seasonal aspect to the by-catch biomass with larger numbers of *P. clarkii* caught during warmer months. In addition, gill nets closest to shore, and gill nets closest to the bottom of the River had higher numbers of *P. clarkii* present. Furthermore, around 60 % of fishermen reported disfiguration of their catch by *P. clarkii*. Crayfish are known to consume fish (Guan and Wiles, 1998; Taylor and Soucek, 2010), but determining if crayfish consume fish by means of scavenging or predation has been debated (Niemiller and Reeves, 2014). The report by Moonga and Musuka (2014) and the findings in this study (Figure 4-8) provide further support for crayfish predating live, large predatory fish.

However, these recent findings do not indicate why crayfish, such as *P. clarkii* and *P. leniusculus,* become entangled in such large numbers. The present study attempted to repeat the *P. leniusculus* by-catch observed initially by using a gill net baited with *O. mykiss* and observing the process with an ROV. The second netting attempt did not yield any *P. leniusculus,* although several individuals were observed moving around on the net by the ROV. The stark difference in by-catch numbers presented a puzzle.

Moonga and Musuka (2014) report that *P. clarkii* were entangled in gill nets and similarly, this study found *P. leniusculus* entangled in gill nets. This did not occur with the gill net used in the second attempt - in fact *P. leniusculus* were observed to move freely over the net (Figure 4-9). This may have been due to the different types of gill nets used. The gill nets used in the first instance were monofilament nylon gill nets, while the second gill net was made of cotton. The nylon gill nets entangle organisms to a greater degree, which may be one possible reason for the difference observed. Furthermore, Moonga and Musuka (2014) found a mesh size of 5 cm or greater to catch the most *P. clarkii*. The cotton gill net only used two mesh sizes, 1 cm or 2 cm. This suggests that the mesh size of the gill net used the second time was too small for *P. leniusculus* to become entangled.

Alternatively, the presence of fish caught in the monofilament nylon gill net were responsible for attracting *P. leniusculus* to the net. Many animals release chemicals when injured, and crayfish are known to be highly sensitive to these chemical cues (Dickey and McCarthy, 2007). For example, chemical signals released from injured pond snails *Physa gyrina* (Say, 1821) resulted in Kentucky river crayfish *Orconectes juvenilis* (Hagen, 1870) becoming more active (Dickey and McCarthy, 2002). Therefore, *P. leniusculus* may have detected chemical signals released by fish caught in the gill net and consequently become entangled due to the construction of the net when searching for the source. In contrast, no fish were caught in the cotton gill net. Furthermore, the addition of *O. mykiss* bait to the cotton gill net did not appear to attract *P. leniusculus* in the same manner as it did when

applied the nylon gill net. This may be because the *O. mykiss* bait was dead and subsequently did not release the same chemical signals. However, it should also be noted that *P. leniusculus* did appear attracted to the same *O. mykiss* bait in the Swedish Trappy trap and were observed trying to remove it from outside of the trap.

4.4.5 Conclusion

It is unclear as to why *P. leniusculus* were found in such large numbers on the nylon gill net and attracted to the Swedish Trappy trap using *O. mykiss* as bait, yet no apparent attraction was exhibited when *O. mykiss* was attached on the cotton gill net and no preference observed when presented in the plus-maze environment.

The findings of the current study suggest that attraction to food by *P. leniusculus* is subject to multiple chemical signals. Future studies should seek to explain the mechanisms behind the attraction of *P. leniusculus* to the gill nets with fish, which could subsequently be utilised to increase the efficiency of traditional trapping methods.

Chapter 5. Can environmental DNA (eDNA) be used for the early detection of *Pacifastacus leniusculus* in Scotland?

5.1 Introduction

Gherardi *et al*. (2011a) state that species which "have been introduced outside their native range (alien or non-indigenous species) have the potential to cause irreparable ecological and economic damages." In fact, invasive species are recognised as a significant threat to freshwater biodiversity (Dextrase and Mandrak, 2006; Lodge *et al*., 2000; Sala *et al*., 2000), with freshwater ecosystems containing around 10 % of all described species despite covering < 1 % of the earth's surface (Strayer and Dudgeon, 2010).

P. leniusculus is endemic to North America but was introduced to GB in the 1970's (Holdich *et al*., 2014), and anecdotal records of introductions to Scotland begin during the 1990's (Freeman *et al*., 2010). As a non-indigenous species, *P. leniusculus* is not only difficult to detect but can colonise new aquatic environments rapidly (Gherardi *et al.*, 2011a). This can make finding, controlling or eradicating *P. leniusculus* populations problematic as well as costly (Gherardi *et al.*, 2011a).

As a result, early detection of invasive species is key to enabling the best chance of eradication and prevention of spread (Takahara *et al*., 2013). After the initial introduction, invasive species occur at low densities, which can make detection difficult using traditional sampling methods (Herder *et al*., 2014). Recently, environmental DNA (eDNA) has emerged as a new tool to detect and monitor invasive species (Jerde *et al*., 2011; Scriver *et al*., 2015).

eDNA is defined as DNA extracted directly from environmental samples such as soil, sediment or water, without any visual signs of the biological source material from

where it came (Thomsen and Willserslev, 2015). Organisms release DNA into the environment through faeces, skin, hair, mucus, urine, gametes, insect exuviae or decomposing individuals (Bohmann *et al*., 2014; Herder *et* al., 2014; Pedersen *et al*., 2015; Thomsen and Willserslev, 2015). eDNA persistence in aquatic environments is variable. Dejean *et al*. (2011) showed that after the removal of an organism, eDNA persisted for up to four weeks in natural pond conditions. However, most of the eDNA present was shown to degrade within two weeks of the organism being removed. In controlled aquaria conditions, eDNA persistence was determined to range from 7 to 14 days (Piaggio *et al*., 2014; Thomsen *et al*., 2012a). As such, eDNA is indicative of the contemporary presence of a target species within any given aquatic environment.

The detection of a target species within aquatic environments using eDNA is non-invasive and rapid (Eichmiller *et al*., 2014; Goldberg *et al*., 2015; Sigsgaard *et al*., 2015). eDNA can also increase the accuracy of detection compared to traditional sampling methods, as well as decreasing sampling costs (Dejean *et al*., 2012; Jerde *et* al., 2011; Sigsgaard *et al*., 2015; Takahara *et al*., 2013). Furthermore, eDNA allows detection of a target species at any age and of either sex (Herder *et al*., 2014). This could prove especially useful for invasive species such as *P. leniusculus*, as traditional sampling methods using traps tend to size select larger individuals as well as being biased towards males (Freeman *et* al., 2010; Gherardi *et al*., 2011a; Lawrence *et al*., 2006).

Ficetola *et al*. (2008) were the first to successfully detect the presence of an invasive species, the American bullfrog *Rana catesbeiana* (Shaw, 1802), using eDNA. Subsequent studies have used eDNA to successfully detect other invasive species including fish (Takahara *et al*., 2013), reptiles (Piaggio *et al*., 2014), gastropods (Goldberg *et al*., 2013) and the invasive crayfish *P. clarkii* (Tréguier *et al*., 2014). As

such, eDNA has the potential to increase management efficiency of invasive species in freshwater ecosystems.

The present study examines whether eDNA has the potential to be used as a reliable method for detection of *P. leniusculus* in Scotland. The aims of the current study were to:

1. Develop a species-specific quantitative PCR (qPCR) assay for the detection of *P. leniusculus*.
2. Investigate the persistence of *P. leniusculus* eDNA under controlled laboratory conditions.
3. Determine the presence or absence of *P. leniusculus* using the developed qPCR assay on water samples obtained under natural field conditions.

The results and implications of using eDNA as a method to detect and potentially monitor *P. leniusculus* in Scotland are discussed.

5.2 Methods

5.2.1 Crayfish

P. leniusculus were collected in July 2014 under SNH licence from Loch Ken (55.0090° N, 4.0560° W), located near Castle Douglas in Dumfries and Galloway, Scotland and transported to the University of Stirling under said license. *P. leniusculus* were kept in secure aerated plastic holding tanks containing large plastic piping to provide shelter and fed on a diet of fish flakes (Goldfish Flake Food, Aquarian). In August 2014, all remaining individuals (n = 30) were euthanised by freezing. Following freezing, 2 legs were removed from each individual crayfish and placed into a Bijou sample container containing 7 ml of absolute ethanol. Prepared samples were then stored at room temperature until DNA extraction could be completed.

5.2.2 DNA extraction

Total DNA was extracted by removing a leg from ethanol storage, blotting dry and cutting a 100 - 200 mg portion. This was added to a 2 ml microtube containing 1 ml DNA Lysis Buffer (50 mM Tris/HCl pH 8, 120 mM NaCl, 20 mM EDTA, 0.5 % SDS). Immediately thereafter, 1 µl of 10 ng μl^{-1} RNAase A was added and the sample was incubated at room temperature for 30 minutes. Following this, 1 µl of 50 ng μl^{-1} Proteinase K was added and the tubes mixed gently by end-over-end rotation at 55^{o}C overnight. Protein and other contaminants were then precipitated by the addition of 1 ml of 4 M Ammonium acetate, mixed by inverting the tube several times, followed by centrifugation at 14,000 rpm for 5 minutes. DNA was precipitated by taking 0.6 ml of the supernatant to a fresh 2 ml microtube, adding 1.2 ml of 95 % ethanol and inverting the tube several times. Following incubation at -20°C for 20 minutes, DNA was collected by centrifugation at 14,000 rpm for 15 minutes. After removal of supernatant, the DNA pellet was washed by adding 0.5 ml of 75 % Ethanol, vortexed and then

centrifuged for 5 minutes at 14,000 rpm. All traces of supernatant were removed with a pipette, and pellets were allowed to dry in the open tube at room temperature for 10 minutes. DNA was then re-dissolved in 50 µl of milliQ water. Upon completion of extraction, the concentration (ng μl^{-1}) and quality (A_{260}/A_{280} ratio) of DNA for each sample was measured using a Nanodrop ND-1000 Spectrophotometer. Samples were then stored at -20°C.

5.2.3 Primers

5.2.3.1 Primer design

Two primer pairs were employed to amplify two mitochondrial DNA (mtDNA) sequences of differing lengths. The first was a 710 base pair (bp) region of the cytochrome c oxidase subunit I gene (CO1), employed to verify the sequences of CO1 genes in the target species. For this, the published 'global' primer pair used was HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'), taken from Folmer *et al*., (1994).

The second primer pair, designed to amplify a much shorter internal region of the CO1 gene for eDNA assay, was designed by consideration of an alignment of crayfish CO1 sequences generated from in-house CO1 sequencing and sequences obtained from publically available resources. CO1 sequences for *P. leniusculus*, for native *A. pallipes* and for several other species recorded in GB were downloaded from NCBI GenBank (Table 5-1).

These sequences were then aligned using the MAFFT (v7.182) multiple sequence alignment program and moved to a word document.

Table 5-1: List of eight crayfish species with established populations in the wild in GB (Holdich *et al.*, 2014) and two crayfish species with potential to be found in the wild present in the GB aquarium trade (Pöckl *et al.*, 2006), and their corresponding CO1 gene sequence accession numbers in GenBank. '*' denotes species present within the aquarium trade.

Crayfish species	GenBank accession number
Pacifastacus leniusculus	JF437997.1
Austropotamobius pallipes	AY667115.1
Astacus leptodactylus	JQ421504.1
Astacus astacus	GU727619.1
Procambarus clarkii	KJ645845.1
Orconectes virilis	FJ608578.1
Orconectes limosus	JF911577.1
Procambarus acutus	KF773892.1
*Cherax quadricarinatus**	HG942364.1
*Procambarus fallax**	JF438007.1

By viewing the CO1 sequences of several species aligned simultaneously, it was possible to identify regions by eye, where differences existed. These differences could then be used to distinguish *P. leniusculus* from related taxa. These regions were used as input to PrimerBLAST (NCBI) with specificity searching for *P. leniusculus*. Of the primer pairs returned, only one matched suitable criteria, namely matching T_ms >60^{o}C, with minimal identity between species, and which produced amplicons < 100 bp with internal regions of divergent sequence between species. The selected primer pair, which amplified an 87 bp region of the CO1 gene, was as follows: qPlCO1F (5'-ATAGTTGAAAGAGGAGTGGGTACT-3') and qPlCO1R (5'-TAAATCAACAGAAGCCCCTGCA-3') (Figure 5-1). Amplification primers were ordered from Eurofins.

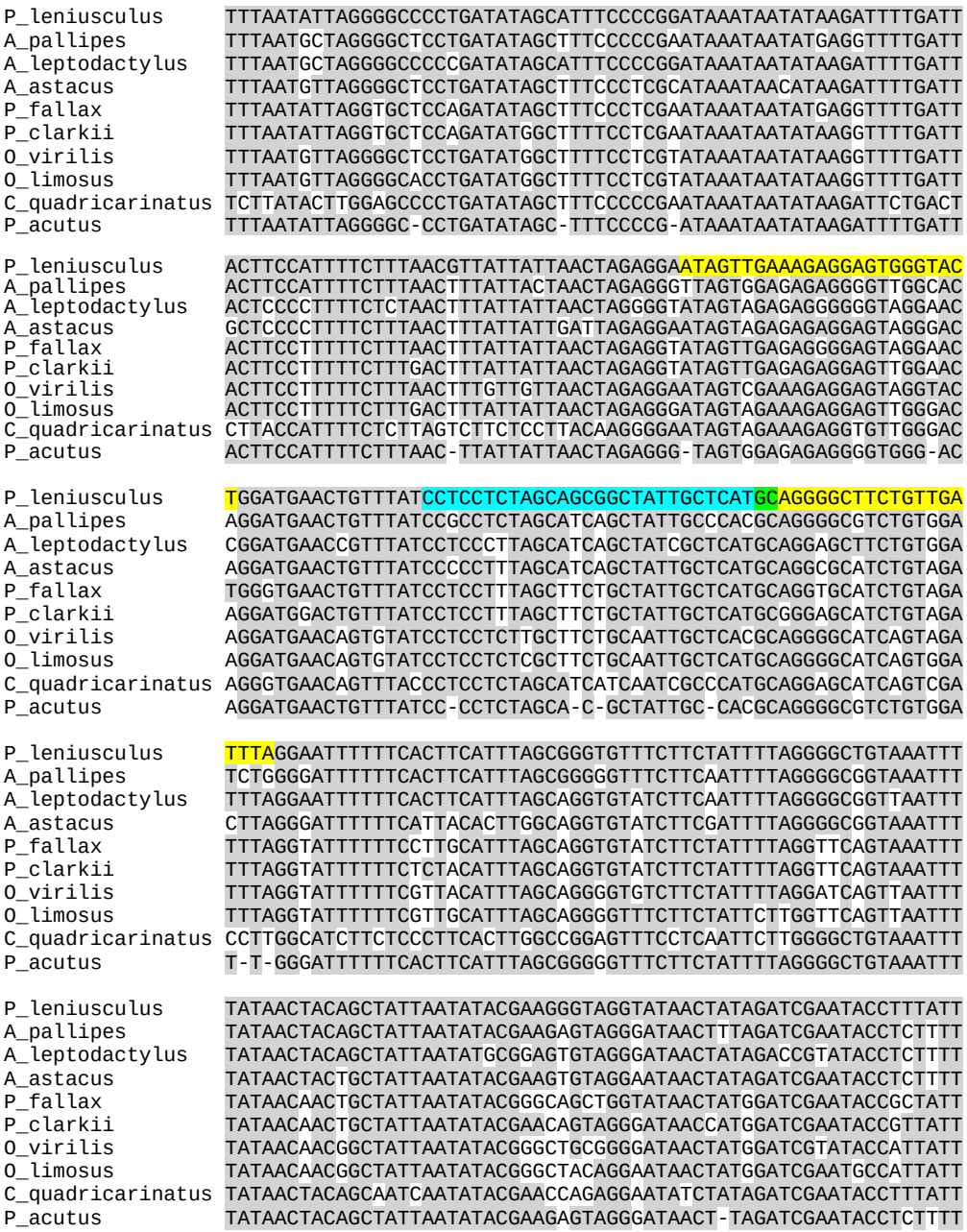


Figure 5-1: Alignment of target region of the CO1 sequences for *P. leniusculus* and other species of crayfish known to be currently present within GB, either in the wild or within the aquarium trade. Greater consideration when designing primers was given to *A. pallipes*, the only other species of crayfish known to be present in Scotland. Grayed residues represent those that are identical to *P. leniusculus* in any other of the aligned species. CO1 primer annealing positions are highlighted in yellow, TaqMan probe annealing position is highlighted in aqua and any primer/probe overlap is highlighted in green.

5.2.3.2 In vitro testing of primers

Following *in silico* testing, qPlCO1F/qPlCO1R and HCO 2198/LCO 1490 were tested *in vitro* using the DNA extracted from the Loch Ken population of *P. leniusculus*, as detailed in section 5.2.2. Using a Thermocycler Biometra (Tgradient 96), Polymerase Chain Reaction (PCR) was used to amplify mtDNA markers. The reaction mixture for each individual sample consisted of 10 µl 2x of MyTaq HS Mix (Bioline), 0.4 µM of each primer and 7.4 µl milliQ water. The reaction mixture was premixed and added to 1µl of stock DNA (80 ng μl^{-1}) to give a total reaction volume of 20 µl. Optimal PCR thermal cycle conditions for qPlCO1F/qPlCO1R were determined to be 95°C for 1 minute, followed by 35 cycles, each containing a denaturation step of 95°C for 15 seconds, primer annealing step of 60°C for 15 seconds and an extension step of 72°C for 20 seconds. A final extension step of 72°C for 1 minute was then completed. The PCR thermal cycle for HCO 2198/LCO 1490 was identical to qPlCO1F/qPlCO1R, except that an annealing temperature of 55°C was applied.

Following PCR, gel electrophoresis was used to analyse and quantify the PCR products. All samples were run on a 2 % agarose gel in 1X TAE buffer and visualised using a Syngene UV Transilluminator.

5.2.3.3 Sequencing PCR products

Full CO1 gene PCR products that were successfully amplified were prepared for commercial sequencing using a NucleoTraPCR kit (Macherey Nagel), following manufacturer's guidelines. Once PCR product purification was complete, the resulting supernatant concentration and quality was measured using a Nanodrop ND-1000 Spectrophotometer. DNA sequencing was outsourced (Lightrun, PCR products, GATC Biotech). Lightrun specified purified PCR products must contain between 20 – 80 ng

μl^{-1} of template DNA. If samples successfully met this condition, they were suitable for sequencing and 5 µl of purified PCR product was added to 2.5 µl of primer 1 (10 µM; HCO 2198) or 2.5 µl primer 2 (10 µM; LCO 1490) to give a total reaction volume of 10 µl.

5.2.4 Developing a TaqMan probe

Using the CO1 gene sequence consensus (Figure 5-1) created to design a more specific primer pair for *P. leniusculus*, the aligned CO1 gene sequences were constricted to the region bound by the two amplification primers, qPlCO1F and qPlCO1R. Within this area, a region of the *P. leniusculus* CO1 gene with minimal similarity to related crayfish taxa, especially *A. pallipes*, was identified. This region also had a melting temperature of at least 8ºC higher than the amplification primers. The resulting region became the TaqMan probe sequence (Figure 5-1). This probe was ordered from Eurofins: FAM-5'-CCTCCTCTAGCAGCGGCTATTGCTCATGC-3'-BHQ1.

The TaqMan probe is a fluorogenic duel-labeled probe consisting of a fluorophore attached to the 5'-end and a quencher attached to the 3'-end. In this case, the fluorophore was 6-carboxyfluorescein, also known as FAM, and the quencher a Black Hole Quencher (BHQ) dye. BHQ serves to quench the fluorescence emitted by FAM. The TaqMan probe anneals within the region amplified by a specific primer pair and during qPCR is degraded. As degradation occurs, the fluorophore is released from the probe, in the process moving away from the quencher. Consequently, the quencher's effect is lessened while the fluorophores fluorescence signal increases. As a result, the fluorescence detected during qPCR is directly proportional to the amount of FAM and the amount of target DNA present in the sample.

As with the amplification primers, the specificity of the TaqMan probe was tested *in silico* using Primer-BLAST by pairing the probe sequence with either the forward or reverse primer sequence. *In silico* testing showed that this TaqMan probe had five mismatches to the native *A. pallipes* and at least 4 mismatches to all other possible non-native species found in GB (Figure 5.1).

5.2.5 Testing primer and probe specificity

In order to be certain that the primer pair and TaqMan probe are amplifying a region of the CO1 gene specific to *P. leniusculus*, they were also tested on DNA from *A. pallipes*, the only other species of crayfish known to be present in Scotland. *A. pallipes* tissue samples were provided by Moneycarragh Fishfarm, Co Down, Ireland. DNA was extracted from *A. pallipes* leg tissue as detailed in section 5.2.2.

5.2.5.1 Standard PCR

The resulting DNA was tested alongside *P. leniusculus* DNA under the optimal PCR conditions detailed in section 5.2.3.2, using the designed primer pair, qPlCO1F/qPlCO1R. The reaction mix was as described in section 5.2.3.2. PCR products were then run on a 2 % agarose gel (section 5.2.3.2) to confirm primer pair specificity to *P. leniusculus*.

5.2.5.2 Quantitative PCR

During the course of this study, quantitative PCR (qPCR) was performed twice in all instances. First using SYBR Green and no probe and secondly using the sequence specific designed TaqMan probe. The primer pair, qPlCO1F/qPlCO1R, was used in all qPCR assays unless otherwise stated. The reaction mixtures and thermal cycle conditions for each qPCR assay were the same each time and are listed in Table 5-2. A Mastercycler ep *realplex* (Ependorf) was used to perform all qPCR assays.

SYBR Green and TaqMan probe qPCR assays were performed on *P. leniusculus* and *A. pallipes* DNA. The reaction mixture and thermal cycle conditions are as described in Table 5-2. The resulting qPCR products were run on a 2 % agarose gel to visualise primer pair and TaqMan probe specificity.

Table 5-2: qPCR assay reaction mixture compositions (final volume of 10 µl) and thermal cycle conditions for both SYBR Green and the designed TaqMan probe.

	SYBR Green	**TaqMan Probe**
Reaction mixture	5 µl Luminaris colour Hi-green qPCR Master Mix (Thermo Scientific); 0.4 µM of each primer; 1.2 µl milliQ water; 3 µl DNA template	5 µl SensiFAST Probe No-ROX (Bioline); 0.4 µM of each primer; 0.1 µl TaqMan probe (0.1µM); 1.1 µl milliQ water; 3 µl DNA template
Thermal cycle conditions	50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles each containing a denaturation step of 95°C for 15 seconds, annealing step of 60°C for 15 seconds and an extension step of 72°C for 20 seconds. This was followed by a melt curve step.	95°C for 5 minutes, followed by 40 cycles each containing a denaturation step of 95°C for 15 seconds, annealing step of 60°C for 15 seconds and an extension step of 72°C for 20 seconds.

5.2.6 Limit of Detection and Limit of Quantification

The Limit of Detection (LOD) is defined as minimum amount of DNA of a target species that can be detected in a sample. Conversely, the Limit of Quantification (LOQ) is lowest level of target DNA that is a successful measure of accuracy. Once species specificity of the qPlCO1F/qPlCO1R primer pair had been established (i.e. only *P. leniusculus* mtDNA markers are amplified when used), qPCR was used to determine the LOD and LOQ. LOD and LOQ were calculated using a dilution series of a known amount of *P. leniusculus* DNA. The following dilution series was repeated first using SYBR Green, and then using the TaqMan probe in order to determine if the LOD and

LOQ were different. A known amount of extracted *P. leniusculus* DNA was added to a buffer solution comprising of TE Buffer pH 7.5 and European flounder *Platichthys flesus* (Linnaeus, 1758) DNA (5 ng μl^{-1}). *P. flesus* DNA was added to better reflect the fact that eDNA samples are likely to contain other DNAs, and to protect very low concentrations of diluted crayfish DNA from degradation and adsorption to assay surfaces. The *P. leniusculus* DNA dilution series ranged from 80 ng μl^{-1} to 8 x 10^{-8} ng μl^{-1} through a series of nine 10x serial dilutions. The starting DNA concentration of 80 ng μl^{-1} was added to 90 µl of buffer solution and vortexed thoroughly. Following vortexing, 10 µl of solution was transferred to a fresh eppendorf containing 90 µl of buffer solution and the resulting mixture vortexed. This process was repeated for each dilution step. Reaction mixtures and thermal cycle conditions were as described in Table 5-2. For each assay, there were three replicates per concentration while three negative controls (100 µl TE Buffer) and three no template controls (NTC), whereby the 3 µl DNA template was replaced with 3 µl milliQ water in the reaction mixture, were added to each qPCR 96 well plate.

5.2.7 Water Sampling and DNA Extraction

5.2.7.1 General procedures and precautions

All eDNA work was completed in rooms where crayfish DNA had not been previously handled. DNA extractions were performed in a room dedicated to DNA/RNA extraction, and separated from PCR activities. Working space was wiped with alcohol wipes before and after use. Reaction mixtures for qPCR assays were prepared in a separate room, which was free of any DNA material. Alcohol wipes were also used in this room to wipe bench space thoroughly before and after use. PCR reaction set-up was also prepared in a dedicated space, which had two stations - the first for negative samples and the second for positive. Each station also had separate pipettes and pipette

tips, as well as being cleaned with DNAZap (Invitrogen by Life Technologies) before and after use. Each room had separate equipment including, but not limited to; lab coat, gloves, pipettes, pipette tips, eppendorfs, eppendorf racks and any solutions e.g. ethanol. The qPCR 96 well plates and cover films were kept in the DNA free reaction set-up room.

5.2.7.2 DNA Extraction from Water Samples

All water samples were collected by carefully taking 15ml in a 50 ml sterile polypropylene universal from aquarium tanks, tap water, and environmental sampling locations taking care to avoid cross-contamination. Negative control samples consisted of 15ml of milliQ water. Positive controls consisted of 15ml milliQ water with *P. leniusculus* DNA. The positive control was prepared using the LOD from least sensitive qPCR assay, in this instance, SYBR Green. As a result, a solution was prepared with a final DNA concentration of 8×10^{-3} ng μl^{-1} by adding the required volume of stock DNA (80 ng μl^{-1}) to milliQ water. For some experiments, positive and negative controls also contained *A. pallipes* DNA and *P. flesus* DNA, both at a concentration of 2 ng μl^{-1}. The addition of *A. pallipes* and *P. flesus* DNA investigated if carrier non-target DNA, as might be present in eDNA samples, influenced the qPCR results.

Each 15 ml of water sample or control had 33 ml absolute ethanol and 1.5 ml 3M sodium acetate added. For environmental samples, prior to collection of water from the chosen site at Loch Ken, 50 ml universals were pre-prepared in the laboratory with 33 ml absolute ethanol and 1.5 ml 3M sodium acetate. Loch water (15 ml) was added on site by using individual 15 ml sterile universals to take water from close to the bottom of the water column. Water samples were taken from the edges of Loch Ken in < 0.6

m of water. All water samples were then stored at -20°C until DNA extraction could be performed.

Aquarium samples consisted of water from tanks in which *P. leniusculus* were maintained at varying densities for 7 days without a water change. This provided an opportunity to initially test the primers and probe on eDNA extracted from controlled water samples. Densities in tanks were as follows; 3 crayfish, 2 crayfish or 1 crayfish in approximately 5 L of water.

Samples were grouped into batches based on water source and were processed along with a positive and negative control. Four batches (Airthrey Loch; Glasgow tap water; aquarium tap water; crayfish tank water – 1 crayfish density), each consisting of three replicate water samples, were extracted. Potential for contamination of positive and negative controls was investigated by performing control extractions separately.

DNA was extracted from water samples using a Qiagen DNEasy Blood and Tissue kit: ethanol/sodium acetate treated water samples were centrifuged at 5467 g for 35 minutes at 6°C (modified from Ficetola *et al*., 2008), before removing supernatant in one fluid motion taking care not to disturb the pellet. Excess ethanol was blotted away with sterile tissue and 200 µl of ATL Buffer added before vortexing to re-suspend the pellet. This solution was then transferred to a 1.5 ml eppendorf and 20 µl of Proteinase K added and vortex mixed. Samples were incubated at 56°C for 45 minutes, vortexing occasionally. After incubation, samples were vortexed for a further 15 seconds and 200 µl of Buffer AL added and vortexed to mix. This was followed by the addition of 200 µl of absolute ethanol and vortexing again. This mixture was transferred to a DNeasy column placed in a collection tube and centrifuged at 8000 rpm for 1 minute. The flow through was discarded and the DNeasy column washed again with 500 µl of Buffer

AW1 with centrifugation at 8000 rpm for 1 minute. This was repeated with 500µl of Buffer AW2, but with centrifugation at 14000 rpm for 3 minutes. The DNeasy column was then placed into a fresh, clean and sterile eppendorf and 50 µl of Buffer AE added directly onto DNeasy membrane. This was incubated at room temperature for 1 minute, before centrifugation at 8000 rpm for 1 minute to collect the purified, concentrated eDNA. Upon completion of extraction, samples were stored at -20°C until qPCR could be performed.

SYBR Green and TaqMan qPCR reaction mixtures and thermal cycle conditions were as detailed in Table 5-2. Each qPCR assay had three replicates per water sample per batch, plus three replicates of the positive control and of the negative control. Three no template controls (NTCs) were also added to each qPCR well plate. For selected samples, after the assay was complete, qPCR products were visualized on a 2 % agarose gel. For this, care was taken to ensure that qPCR plates remained sealed until they had been removed to a laboratory at the other end of the building from the water sample processing rooms.

Initial results indicated greater sensitivity when using a sequence specific TaqMan probe qPCR assay vs a SYBR Green qPCR assay. It was therefore decided that only a TaqMan probe qPCR assay would be used during further experiments.

5.2.7.3 Modified methodology

Based on qPCR results from section 5.2.7.2 the existing methodology was modified. It was apparent that when co-processing a positive control alongside a negative control, the negative control value was adversely affected compared to values obtained when negative and positive controls were extracted separately. Consequently, it would be unclear as to whether the environmental water samples themselves were truly positive.

Therefore, after initial trials, when extracting environmental water samples, only a negative control was included. Positive control extracts were still added to the qPCR 96 well plate in order to ensure the qPCR assay was functioning.

Prior to any laboratory or field sampling, sufficient 50 ml universals were prepared simultaneously with 33 ml absolute ethanol and 1.5 ml 3M sodium acetate for the study. Negative controls were prepared with milliQ water from a room where no crayfish DNA had been handled. All universals were prepared in a room with equipment where no crayfish DNA had previously been handled. Pre-prepared universals were stored at -20°C in a room and freezer unit where no crayfish tissue had been previously been stored until required.

5.2.8 In situ testing of the primers and probe

5.2.8.1 Aquarium eDNA trial

Twelve *P. leniusculus* individuals were obtained from Loch Ken under SNH licence during November 2014 and transported to the University of Stirling under said SNH licence. Nine large Ferplast Geo Medium tanks (L 30 x W 20 x H 20.3 cm) were set-up in a temperature-controlled room of 14°C under a 12:12 hr photoperiod. Tanks were filled to a volume of 5.5 L using tap water from another location, based on previous qPCR results to minimise contamination risk and/or false positive results, and an air stone added (Fig. 4-2). *P. leniusculus* were sexed (1 female; 11 male), weighed (± 0.1 g) and carapace length was measured from the tip of the rostrum to the posterior margin of the carapace using Vernier calipers (± 0.1 mm).

Of the nine tanks, three served as a control (0 crayfish), three as low density (1 crayfish) and three as 'high' density (3 crayfish). There were therefore three replicates of each

density. *P. leniusculus* were randomly allocated to each density category and each density was randomly allocated to one of the nine tanks (Figure 5-2).

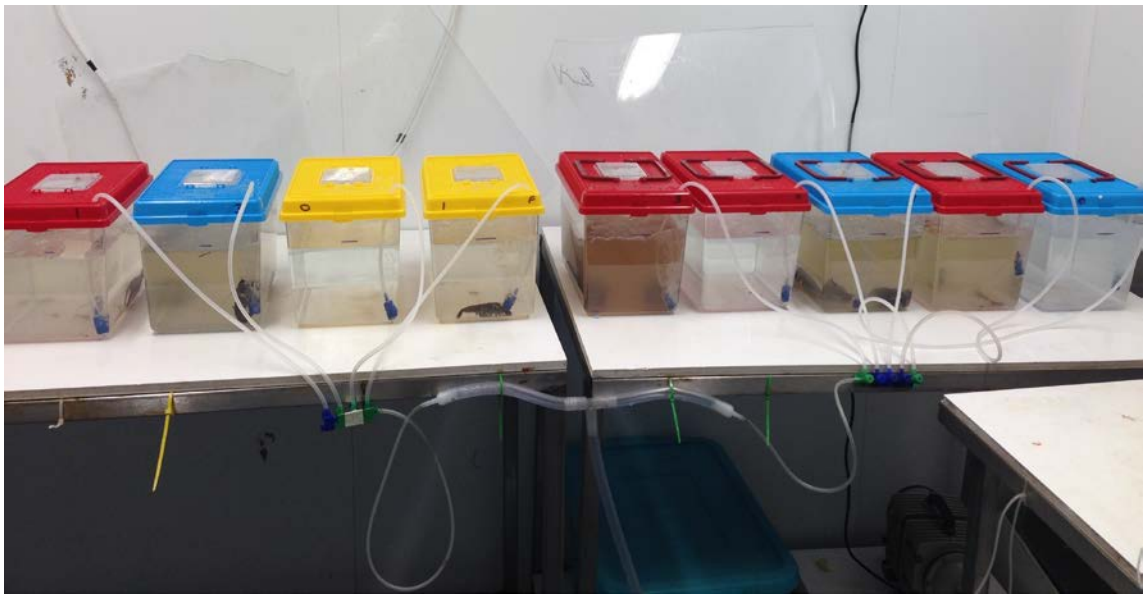


Figure 5-2: Aquaria eDNA trial tank set-up and order of *P. leniusculus* densities. L-R; Tank 1 = 3 crayfish, Tank 2 = 1 crayfish, Tank 3 = 0 crayfish, Tank 4 = 1 crayfish, Tank 5 = 3 crayfish, Tank 6 = 0 crayfish, Tank 7 = 3 crayfish, Tank 8 = 1 crayfish, Tank 9 = 0 crayfish.

The laboratory eDNA trial lasted a total of 14 days. *P. leniusculus* were kept in the tank a total of seven days, and were not fed during this time. On the seventh day, all *P. leniusculus* were removed from the tank and euthanised by freezing.

The laboratory eDNA trial began the first day *P. leniusculus* were introduced to the tanks, however the first samples were not taken until the first time point of 1 day post *P. leniusculus* introduction. There was a second and third sampling time point at 3 days and 7 days respectively. At each time point, three 15 ml water samples were taken from each tank and added to the pre-prepared ethanol/sodium acetate solution, as described in section 5.2.7.2. After the water samples were taken on the seventh day, *P. leniusculus* were removed and the nine tanks maintained for a following seven days. During this time, three 15 ml water samples were taken from each tank and added to the pre-

prepared ethanol/sodium acetate solution at three time points post *P. leniusculus* removal – 1 day, 3 days and 7 days. Clean gloves and sterile universals were used for each tank during each time point in order to minimise possible cross contamination between tanks. Water was taken using individual 15 ml sterile universals from the centre of the water column for each sample within the tank, This was to avoid disturbing the debris on the bottom. All samples were then stored at -20°C until DNA extraction could be completed.

DNA was extracted as per section 5.2.7.2 and a TaqMan probe qPCR assay was performed with the reaction mixture and thermal cycle as detailed in Table 5-2. There were three replicates per water sample and three NTC's were added, along with three replicates of a previously extracted positive control, to the qPCR 96 well plate.

5.2.8.2 Field eDNA trial

Three sites were selected to be included in the field eDNA trial. The first site, Airthrey Loch (56.1472° N, -3.2158° W) was free of *P. leniusculus*. The second and third sites were selected where *P. leniusculus* had well established populations. The sites chosen on this basis were Daer Water in the upper reaches of the River Clyde at Elvanfoot (55.433967° N, -3.648207° W) and once again, Loch Ken (55.0090° N, 4.0560° W) (Figure 5-3). All sites were sampled during late November 2014.

In order to standardise environmental water sampling, a Van Dorn sampler was used at all three sites (Figure 5-4). As *P. leniusculus* is a benthic species, the Van Dorn sampler allowed samples to be taken from the water column as close to the bottom as possible but without disturbing subsurface sediments. Sediment disturbance could lead to the release of "ancient" DNA fragments and lead to false positive results (Tréguier *et al.*, 2014).

Figure 5-3: Location of site 1 (Airthrey Loch), site 2 (Daer Water) and site 3 (Loch Ken) within Scotland.

Figure 5-4: Van Dorn sampler (L 47 cm x Dia. 10 cm) used to sample water column. A heavy brass weight was sent down the rope after positioning equipment causing the top and bottom lids to spring shut. Water samples were collected in individual sterile 15 ml universals from the rubber outflow pipe located at the base of the Van Dorn sampler. L-R; closed Van Dorn sampler and open Van Dorn sampler.

Three 15 ml water samples were taken from each site and added to the pre-prepared universals containing the ethanol/sodium acetate solution. Clean gloves and sterile universals were used at each site to collect the samples and the Van Dorn sampler was cleaned with a 70 % alcohol solution before and after use to minimise the risk of possible cross contamination between sites. Water samples were frozen at -20°C upon return to the laboratory until DNA extraction could take place.

DNA extraction was completed as detailed in section 5.2.7.2 and a TaqMan probe qPCR assay was performed with the reaction mixture and thermal cycle conditions found in Table 5-2. Three replicates were run per water sample. Three NTCs were added along with three replicates of a previously extracted positive control to the qPCR 96 well plate.

5.2.9 Statistical analysis

A Welch two sample t-test was used to test for differences between the mean concentration threshold (Ct) values obtained for positive and negative controls with and without carrier DNA. Differences among density and replicates were then explored separately for the 7 day post *P. leniusculus* introduction and 7 day post *P. leniusculus* removal periods, using a univariate general linear model (GLM). Subsequent post hoc tests were performed using a Bonferroni adjustment to identify significant interactions. Residuals were visually inspected for normality.

All statistical analyses were conducted using SPSS (V 22.0). Values reported are mean ± SE unless otherwise stated. Significance level was defined as $p < 0.05$.

5.3 Results

5.3.1 Primer specificity

Both sets of primer pairs were tested *in vitro* against DNA extracted from *P. leniusculus* tissue alongside DNA extracted from the only other crayfish species known to be present in Scotland, *A. pallipes*.

The global primer pair HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') successfully ampliied, a 710 bp region of the CO1 gene of both *A. pallipes* and *P. leniusculus* DNA following PCR (Figure 5-5). The resulting PCR products from *P. leniusculus* DNA were prepared as described in section 5.2.3.3 and sent for commercial sequencing.

The primer pair qPlCO1F (5'-ATAGTTGAAAGAGGAGTGGGTACT-3')/qPlCO1R (5'-TAAATCAACAGAAGCCCCTGCA-3') designed during the course of this study, as described in section 5.2.3.1, successfully amplified a 87 bp region of the CO1 gene for only *P. leniusculus* DNA (Figure 5-6). As the designed primer pair did not amplify *A. pallipes* DNA, it can be concluded that qPlCO1F/qPlCO1R is specific to only *P. leniusculus*.

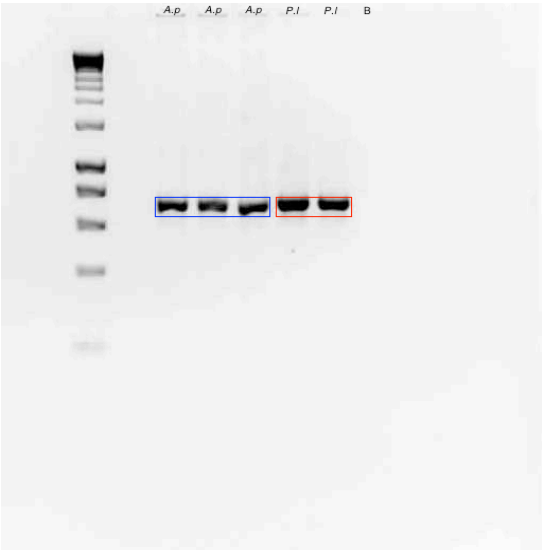


Figure 5-5: Amplification of a 710 bp region of the CO1 gene of both *A. pallipes* and *P. leniusculus* using the global primer pair HCO 2198 and LCO 1490 on a 2 % agarose gel. Bands on the farthest left represent a 1000 bp molecular weight DNA ladder (Hyperladder I, Bioline). A.p = *A. pallipes*; P.l = *P. leniusculus*; B = blank (milliQ water).

Figure 5-6: Amplification of a 87 bp region of the CO1 gene of only *P. leniusculus* using primer pair designed during this study, qPlCO1F and qPlCO1R, under a temperature gradient on a 2 % agarose gel. Bands on the farthest right represent a 100 bp molecular weight DNA ladder (Hyperladder, Bioline). A.p = *A. pallipes*; P.l = *P. leniusculus*; B = blank (milliQ water). L-R: annealing temperature of 60 °C, 58 °C, 56 °C, 54 °C.

5.3.2 CO1 sequence

PCR products were commercially sequenced by Lightrun (GATC Biotech). Upon receipt of results, sequences were reviewed in SeqMan NGen (DNAStar). Sequences were assembled, any base pairs corrected and the sequence ends trimmed. CO1 genes were sequenced from three separate individuals in order to confirm primer/probe specificity (Figure 5-7).

P. leniusculus I:

AACTTTATATTTTATTTTTGGTACTTGAGCTGGTATAGTGGGAACTTCTCTAAGAATAATTATTCGGGTTGAATTAGGT
CAACCTGGAAGATTAATTGGAGATGATCAAATTTATAATGTTGTAGTCACGGCACATGCTTTTGTTATAATTTTTTTTA
TAGTTATGCCAATTATAATTGGAGGATTTGGTAATTGATTAATTCCTTTAATATTAGGGGCCCCTGATATAGCATTTCC
TCGTATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACTTTATTATTAACTAGAGGAATAGTTGAAAGAGGA
GTGGGTACTGGATGAACTGTTTATCCTCCTCTAGCAGCGGCTATTGCTCATGCAGGGGCTTCTGTTGATTTAGGAATTT
TTTCACTTCATTTAGCGGGTGTTTCTTCTATTTTAGGGGCTGTAAATTTTATAACTACAGCTATTAATATACGAAGGGT
AGGTATAACTATAGATCGAATACCTTTATTTGTATGATCTGTATTTATTACAGCAGTCCTTTTATTATTATCTCTACCT
GTTTTAGCAGGGGCTATTACTATATTATTAACAGATCGTAATTTAAATACCTCTTTTTTTGATCCAGCTGGAGGGGGTG
ACCCAATTCTTTATCAACATTTATTTT

P. leniusculus II:

AACTTTATATTTTATTTTTGGTACTTGAGCTGGTATAGTGGGAACTTCTCTAAGAATAATTATTCGGGTTGAGTTAGGT
CAACCTGGAAGATTAATTGGAGACGACCAAATTTATAATGTTGTAGTCACGGCACATGCTTTTGTTATAATTTTTTTTA
TAGTTATGCCAATTATAATTGGAGGATTTGGTAATTGATTAATTCCTTTAATATTAGGGGCCCCTGATATAGCATTTCC
CCGGATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACGTTATTATTAACTAGAGGAATAGTCGAAAGAGGA
GTGGGTACTGGGTGAACTGTTTATCCTCCTTTAGCAGCGGCTATTGCTCATGCAGGGGCTTCTGTTGACTTAGGAATTT
TTTCACTTCATTTAGCGGGTGTTTCTTCTATTTTAGGGGCTGTAAATTTTATAACTACAGCTATTAATATACGAAGGGT
AGGTATAACTATAGATCGAATACCTTTATTTGTATGATCTGTATTTATTACAGCAGTCCTTTTATTATTATCTCTACCT
GTCTTAGCAGGGGCTATTACTATATTATTAACAGATCGTAATTTAAATACCTCTTTTTTTGATCCAGCAGGAGGGGGGG
ACCCAATTCTTTATCAGCATTTATTTT

P. leniusculus III:

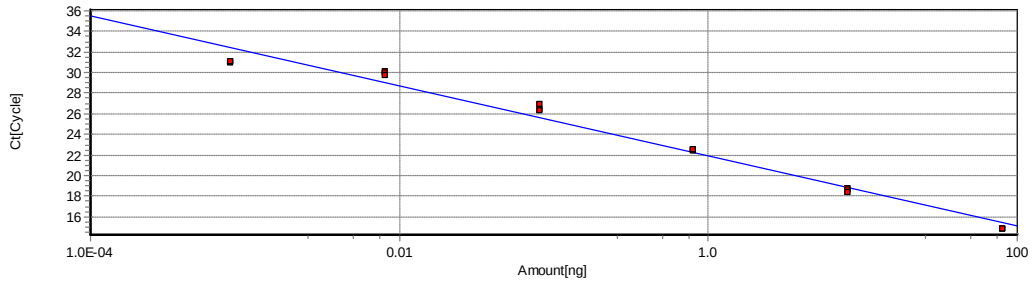
AACTTTATATTTTATTTTTGGTACTTGAGCTGGTATAGTGGGAACTTCTCTAAGAATAATTATTCGGGTTGAGTTAGGT
CAACCTGGAAGATTAATCGGAGACGATCAAATTTATAATGTTGTAGTCACGGCACATGCTTTTGTTATAATTTTTTTTA
TAGTTATGCCAATTATAATTGGAGGGTTTGGTAATTGATTAATTCCTTTAATATTAGGGGCTCCTGATATAGCATTCCC
CCGGATAAATAATATAAGATTTTGATTACTTCCATTTTCTTTAACGTTATTATTAACTAGAGGAATAGTTGAAAGAGGA
GTGGGTACTGGATGAACTGTTTATCCTCCTCTAGCAGCGGCTATTGCTCATGCAGGGGCTTCTGTTGATTTAGGAATTT
TTTCACTTCATTTAGCGGGAGTTTCTTCTATTTTAGGGGCTGTAAATTTTATAACTACAGCTATTAATATACGAAGGGT
AGGTATAACTATAGATCGAATACCTTTATTTGTATGATCTGTATTTATTACAGCAGTCCTTTTATTACTATCTTTACCT
GTCTTAGCAGGGGCTATTACTATATTATTAACAGATCGTAATTTAAATACCTCTTTTTTTGATCCAGCTGGAGGTGGGG
ACCCAATTCTTTATCAACATTTATTTT

Figure 5-7: Alignment of the sequenced region of the CO1 gene for three *P. leniusculus* individuals from Loch Ken. Grayed residues represent those that are non-identical to the designed primer pair or probe in any of the aligned species. CO1 primer annealing position is highlighted in yellow, TaqMan probe annealing position is highlighted in aqua and any primer/probe overlap is highlighted in green.

5.3.3 Limit of detection

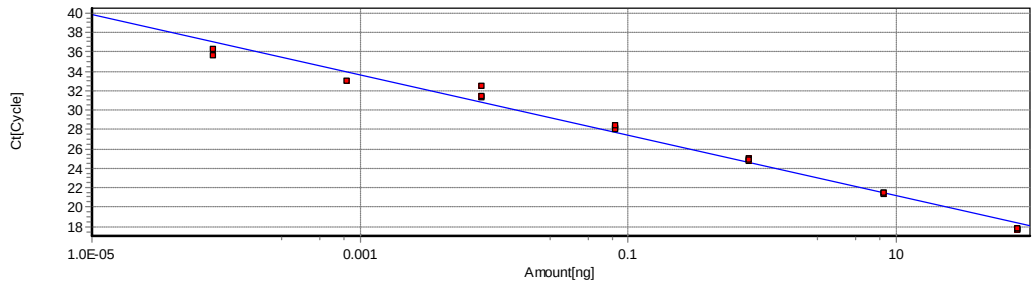
5.3.3.1 SYBR Green vs. TaqMan probe

For SYBR Green the LOD was determined to be 8 x 10^{-3} ng μl^{-1} (Figure 5-8) while for the TaqMan probe LOD was determined to be 8 x 10^{-5} ng μl^{-1} (Figure 5-9). This is an increase in the sensitivity of the TaqMan probe compared to SYBR Green when detecting *P. leniusculus* DNA.



Slope: -3.398
Y-Intercept: 21.9
Efficiency: 0.97
R^2: 0.979

Figure 5-8: Limit of detection of *P. leniusculus* DNA using SYBR Green calculated from a dilution series of known amounts of *P. leniusculus* DNA ranging from 80 ng μl^{-1} to 8 x 10^{-8} ng μl^{-1}. There were three replicates per concentration.



Slope: -3.108
Y-Intercept: 24.32
Efficiency: 1.10
R^2: 0.986

Figure 5-9: Limit of detection of *P. leniusculus* DNA using TaqMan probe calculated from a dilution series of known amounts of *P. leniusculus* DNA ranging from 80 ng μl^{-1} to 8 x 10^{-8} ng μl^{-1}. There were three replicates per concentration.

5.3.4 Initial eDNA extraction trials

5.3.4.1 DNA extraction from water samples

Initial eDNA extractions were carried out on water samples that were obtained from four sources: Airthrey loch, a water body free of *P. leniusculus*; tap water obtained from a house tap in Glasgow; water obtained from a tap located within the temperature control aquarium where crayfish have been held over the last several years; and tank water that had held 1 crayfish and remained untouched for seven days following the removal of crayfish used for initial DNA extraction. Positive and negative controls were also extracted alongside each water sample batch. A qPCR assay was performed on all four water sample batches using both SYBR Green and TaqMan probe. Results are shown in Table 5-3.

Table 5-3: Comparison of mean Ct values obtained for each water sample using both a SYBR Green and a TaqMan probe qPCR assay. The '-' denotes no Ct value obtained.

	SYBR Green	TaqMan probe
Water sample	**Mean Ct value**	**Mean Ct value**
Airthrey Loch	30.81	33.99
Airthrey Loch	31.05	34.00
Negative control	31.20	34.12
Positive control	8.98	11.03
Glasgow tap water	30.17	34.22
Glasgow tap water	31.10	34.96
Glasgow tap water	30.26	22.57
Negative control	23.40	25.36
Positive control	4.72	6.84
Aquarium tap water	26.69	27.79
Aquarium tap water	28.24	29.41
Aquarium tap water	29.29	30.16
Negative control	28.30	29.31
Positive control	-	5.22
1 crayfish density tank water	31.61	31.88
1 crayfish density tank water	29.99	30.75
1 crayfish density tank water	31.63	32.39
Negative control	30.78	23.04
Positive control	5.24	4.21

It was clear from the Ct values obtained for the negative controls, from both the SYBR Green and TaqMan probe qPCR assays, that contamination had occurred at some point

during the process. This was likely during DNA extraction. As a result, the Ct values obtained for each of the four water sample batches were unreliable.

In cases where SYBR Green identified a potential positive result, further analysis indicated non-specific amplification. Therefore, all following results were obtained from only TaqMan probe qPCR assays.

5.3.4.2 Positive and negative controls

In order to confirm that co-processing a positive control along with the negative control was contaminating results, new positive and negative controls were created. A dilution series of *P. leniusculus* DNA was created in order to obtain positive controls at three different concentrations, 0.8 pg^{-1} μl^{-1}, 0.08 pg^{-1} μl^{-1} and 0.008 pg^{-1} μl^{-1}. Four positive controls were produced for each concentration: two containing only *P. leniusculus* DNA and two containing *P. flesus* DNA and *A. pallipes* DNA at a concentration of 2 ng^{-1} μl^{-1} in addition to the *P. leniusculus* DNA. Four negative controls were prepared in a similar fashion, with two containing only milliQ water and two containing *P. flesus* DNA and *A. pallipes* DNA at a concentration of 2 ng^{-1} μl^{-1} in addition to the milliQ water. This enabled investigation into whether or not carrier DNA had any effect on the results obtained during the qPCR assay.

Results indicated that carrier DNA did not influence qPCR results for any of the three concentrations for the positive controls, 0.8 pg^{-1} μl^{-1} (Welch two sample t-test = 0.542, p = 0.589, df = 1.08), 0.08 pg^{-1} μl^{-1} (Welch two sample t-test = 36.587, p = 0.082, df = 1.15), 0.008 pg^{-1} μl^{-1} (Welch two sample t-test = 0.232, p = 0.708, df = 1.111), or for the negative controls (Welch two sample t-test = 0.673, p = 0.550, df = 1.12). Additionally, these results further confirmed the specificity of the TaqMan probe, as negative controls containing *A. pallipes* DNA were not amplified (Table 5-4).

The results obtained in Tables 5-3 and 5-4 informed the decision to modify methodology, as described in section 5.2.7.3, to no longer include a positive control during the DNA extraction stage but to include one only during the qPCR assay for reference.

Table 5-4: Comparison of mean Ct values obtained for positive and negative controls with or without *P. flesus* and *A. pallipes* carrier DNA, at a concentration 2 ng^{-1} μl^{-1}, using a TaqMan probe qPCR assay.

	Without carrier DNA	With carrier DNA
Water sample	**Mean Ct value**	**Mean Ct value**
0.8 pg^{-1} μl^{-1}	13.78	15.15
0.8 pg^{-1} μl^{-1}	13.44	13.39
0.08 pg^{-1} μl^{-1}	17.73	16.47
0.08 pg^{-1} μl^{-1}	17.37	16.37
0.008 pg^{-1} μl^{-1}	19.32	19.44
0.008 pg^{-1} μl^{-1}	20.21	19.65
Negative control (milliQ)	33.34	35.50
Negative control (milliQ)	32.68	32.80

5.3.5 Aquarium eDNA trial

For a result to be positive, the Ct value must be three cycles higher than the negative control value. Even with 0 crayfish in a tank, there was a slight signal compared to the negative control samples. However, this signal was not related to the *P. leniusculus* signal observed in the 1 and 3 crayfish density tanks. Tanks with 0 crayfish density had a consistent qPCR result throughout the entire 14 day period, which included the 7 days' post *P. leniusculus* introduction and 7 days' post *P. leniusculus* removal. Therefore, the value obtained for tanks with 0 crayfish density was taken to be the negative value upon which a positive result for tanks with either 1 or 3 crayfish density was based.

P. leniusculus eDNA was detected in both 1 crayfish and 3 crayfish density tanks for the full 7 day period post *P. leniusculus* introduction. Results from the 3 crayfish density tanks yielded a much stronger positive value than those obtained from the 1 crayfish density tanks (Figure 5-10). During the entire post *P. leniusculus* removal period, eDNA could still be detected in the 3 crayfish density tanks. However, *P. leniusculus* eDNA was only detected in a single 1 crayfish density tank during the 1 day and 3 day post *P. leniusculus* removal sampling periods. *P. leniusculus* eDNA was no longer detectable in 1 crayfish density tanks by the final 7 day post *P. leniusculus* removal sampling period (Figure 5-10).

For the 7 day post *P. leniusculus* introduction period, density was found to be significant ($F_{2,4} = 52.043$, $p < 0.005$, $n^2 = .963$) while replicates were not ($F_{2,4} = 1.422$, $p = 0.342$, $n^2 = .416$). Overall, higher mean Ct values were observed in tanks with 3 crayfish density (23.86) than 1 crayfish density (30.16) and 0 crayfish (36.05), indicating a stronger positive signal for tanks with more *P. leniusculus* present. There was no significant interaction between density and replicates. Bonferroni post hoc tests revealed that all three densities were significantly different from one another (all p values < 0.005; 0 crayfish 36.05 ± 0.77, 1 crayfish 30.16 ± 0.77, 3 crayfish 23.86 ± 0.77). For the 7 day post *P. leniusculus* removal period, there was once again a significant difference between densities ($F_{2,4} = 28.054$, $p < 0.005$, $n^2 = .933$) but no significant difference between replicates ($F_{2,4} = 2.352$, $p = 0.211$, $n^2 = .540$). However, there was a significant interaction between crayfish density and replicates ($F_{4,18} = 4.258$, $p < 0.005$, $n^2 = .486$). The Bonferroni post hoc test revealed that all densities were significantly different from one another (all p-values <0.05; 0 crayfish 37.26 ± 0.43, 1 crayfish 35.42 ± 0.43, 3 crayfish 28.34 ± 0.43). For tanks containing 3 crayfish, the mean Ct value for replicates 1 (31.23 ± 0.75) and 3 (26.32 ± 0.75) were significantly

different ($p < 0.001$) from one another during the final sampling time point (7 days post *P. leniusculus* removal).

Preliminary regression analysis revealed a weak R^2 value (Table 5-5) over time for each water sample analysed, indicating that time at which a sample is taken is not significant in predicting the mean Ct value.

Table 5-5: R^2 value obtained from a linear regression over time for each water sample analysed.

Sample	R^2
Negative control	0.12
0 crayfish density	0.27
1 crayfish density	0.65
3 crayfish density	0.49

It is important to note that the amount of *P. leniusculus* eDNA detected continually decreased after 1 day post *P. leniusculus* introduction through to 7 days' post *P. leniusculus* introduction. This finding was unusual as it was expected that the amount of *P. leniusculus* eDNA present during the 7 day introduction period would increase as time passed, or at the very least level off. This trend continued after *P. leniusculus* were removed, however here it was expected that the amount of *P. leniusculus* eDNA detected would decrease with time after tanks were emptied.

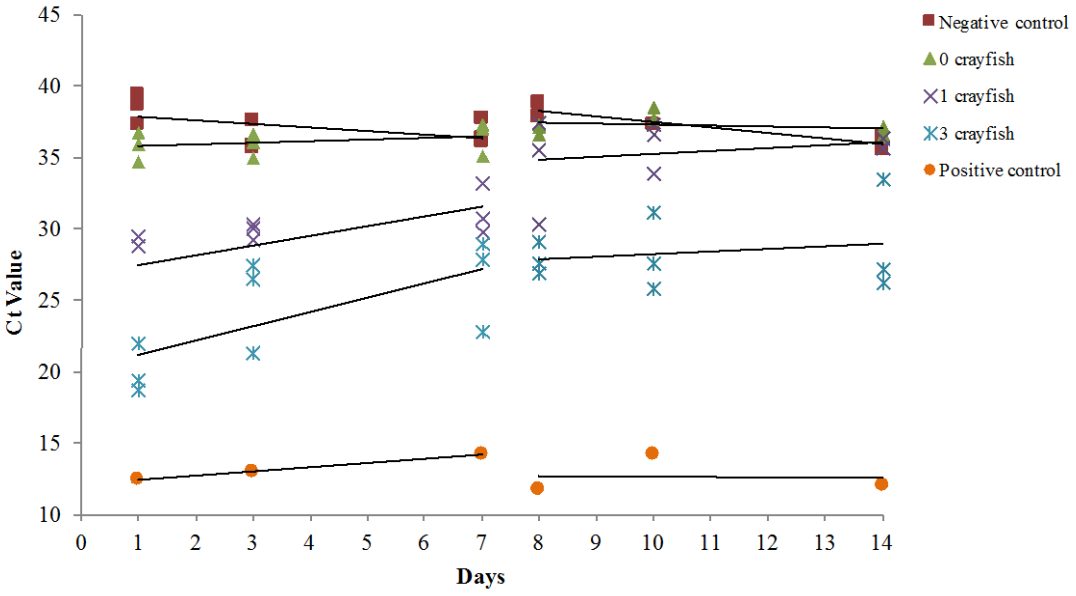


Figure 5-10: Mean Ct value obtained for each tank (0, 1 or 3 crayfish density) sampled, as well as each negative and positive control processed at each of the three time points within the 7 day post *P. leniusculus* introduction period and the 7 day post *P. leniusculus* removal period. Solid black lines represent linear regression.

5.3.6 Field eDNA trial

As previously described, water samples were obtained from one site where crayfish are known to be absent (Airthrey Loch) and two sites where crayfish are known to be present in high densities (Daer Water, River Clyde and Loch Ken). For example, the *P. leniusculus* population within Loch Ken is estimated to be between 1.06 and 9.05 crayfish m^{-2} (Ribbens and Graham, 2009), which is approximately 2 and 5 times less dense than densities used in the aquarium trials conducted in this study. At each site, three 15 ml water samples were collected for the field eDNA trial.

No *P. leniusculus* eDNA was detected at any of the three sites sampled during this study. Negative controls were all negative, indicating that no contamination occurred during the DNA extraction process. One NTC did not register a value during qPCR, however the other two NTCs were negative which confirms no contamination during

the qPCR preparation process. The positive controls registered consistent positive Ct values and served to confirm that the qPCR assay was working as expected.

5.4 Discussion

This study developed a robust and sensitive TaqMan probe assay, which can detect the equivalent of a single *P. leniusculus* cell in 15 ml of water. However, this assay was only successful in detecting *P. leniusculus* eDNA in controlled aquaria conditions. There are many potential reasons why the assay developed in this study was not also successful in detecting *P. leniusculus* in water samples taken in natural conditions. As eDNA fragments found in the environment are commonly less than 150 bp in length (Deagle *et al.,* 2006), extraction, purification and target availability can be problematic. Therefore, primer/probe design, storage of samples, method of extraction and choice of analysis must all be optimal for the target species in question. There is also a need to ensure that appropriate precautions and protocols are taken, similar to those used in studies focusing on ancient DNA, to prevent contamination (Herder *et al*., 2014).

5.4.1 Analysis of samples

5.4.1.1 Primers and probe

Before environmental DNA analysis can begin, the reliability, robustness and specificity of the primers and probe must be tested (Dejean *et al*., 2011; Wilcox *et al*., 2013). This is usually accomplished through a combination of *in silico, in vitro* and *in situ* testing (Bohmann *et al*., 2014; Wilcox *et al*., 2013). Primers and probe must first be tested *in silico* using dedicated software, such as PrimerBLAST (NCBI) as was used in this study, to ensure primer specificity. Once primers have been selected, they must be tested *in vitro* on DNA extracted from the tissue of the target species and PCR conditions should be optimised. Herder *et al.* (2014) recommends that primers should be tested on individuals from several different populations in order to account for any geographic variation, as well as from any closely related species found within the same environment. Primers used in this study were only tested on *P. leniusculus* obtained

from one population - Loch Ken. PCR products obtained from the designed primer pair (qPlCO1F/qPlCO1R) were not sequenced, however PCR products obtained from the global primer pair (HCO 2198/LCO 1490) were. From the sequenced PCR products of three randomly selected individuals (Figure 5-7), it can be seen that even within a single population there is some variation. One individual had a mismatch of a single base pair within both the forward primer (qPlCO1F) and the probe. The number of base pair mismatches in the primers rather than the probe appears to be the most influential factor in determining specificity (Wilcox *et al*., 2013). However, location of the base pair mismatch is also key in determining specificity. When base pair mismatches are located on the 3′ end of the primer or probe, they reduce specificity compared to when present on the 5′ end (Wilcox *et al*., 2013). The base pair mismatches in this *P. leniusculus* individual were located close to the 5′ end on both the primer and the probe, six and seven bp respectively. Thus, it is unlikely that this would impact the specificity of the primers and probe used in this study. However, the observed bp mismatches mean there is no guarantee that the primers and probe would work on 100 % of the population. Consequently, before conducting further eDNA studies it would be advisable to test the primers and probe on other available populations of *P. leniusculus*.

Although testing of primers and probe on non-target DNA is already standard in eDNA studies (Thomsen *et al*., 2012a), Wilcox *et al.* (2013) recommend an additional step of testing mixed samples of both target and non-target DNA. This study tested the primers and probe on a mixture of *P. leniusculus, A. pallipes* and *P. flesus* DNA and found no amplification of non-target species further indicating specificity.

Once primers and probe have successfully amplified target DNA *in silico* and *in vitro*, *in situ* testing on environmental samples can begin. This study was successful in detecting *P. leniusculus* in controlled aquaria conditions but not in water samples taken

in the natural environment. This would suggest that the primers and probe used in this study are valid and specific to *P. leniusculus* and so the negative results produced by environmental samples taken in natural conditions are the result of other factors, for instance the time of sampling and the quantity of eDNA shed by the target species (Deiner and Altermatt, 2015).

5.4.1.2 PCR vs qPCR

PCR and qPCR require the development of species specific primers and probes and both have been employed in species detection using eDNA (Thomsen and Willersev, 2015). PCR is limited to only presence/absence detection of a target species (Klymus *et al*., 2015), whereas qPCR allows estimation of the amount of target DNA present (Spear *et al*., 2015). In addition, qPCR is more sensitive and more specific when using a TaqMan probe compared to conventional PCR (Pilliod *et al*., 2014). This study used a qPCR assay to analyse eDNA samples.

5.4.1.3 SYBR Green vs TaqMan probe qPCR

There are two types of qPCR available, SYBR Green and TaqMan probe. SYBR Green uses non-specific fluorochromes, which bind to double stranded DNA and therefore targets all DNA with a sample regardless of whether the target species DNA is present or not. TaqMan probe on the other hand utilises a probe that binds specifically to the DNA strand, only releasing fluorescence upon amplification. A signal is thus only emitted when DNA of the target species is present (Herder *et al*., 2014). SYBR Green is very similar to conventional PCR in that only two primers are required. However, this also increases the risk of cross-amplification (Herder *et al*., 2014). This was demonstrated in this study whereby false positive results using SYBR Green during LOD studies were obtained and upon further examination of the melt curves it was found that there was increasing amounts of non-specific amplification (e.g. primer

dimer) as DNA concentration decreased (Figure 5-11). In fact, the observed LOD of 8 x 10^{-3} ng μl^{-1} for SYBR Green was potentially even lower than initially reported. This called into question the reliability of SYBR Green for use in this study and as a result a TaqMan probe assay was developed which added specificity as well as increased the sensitivity (LOD = 8 x 10^{-5} ng μl^{-1}) and reliability of the results obtained.

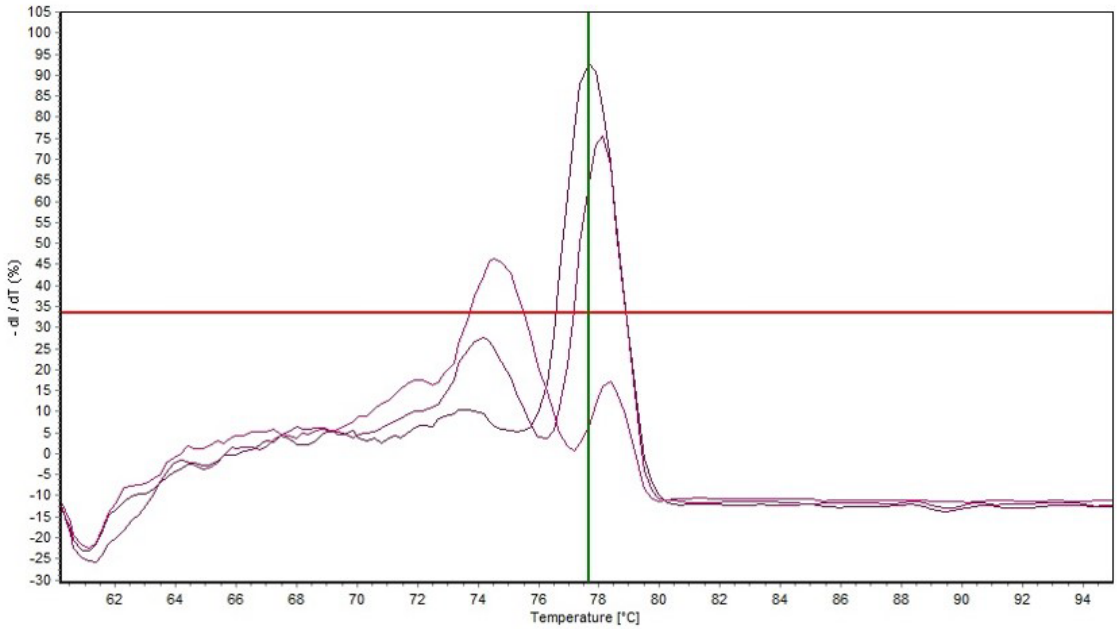


Figure 5-11: Example of melt curve obtained for SYBR Green LOD assay. Highest peaks along green line are *P. leniusculus* DNA amplification. Lower peaks to the left of the green line are amplifications of non-target DNA.

5.4.1.4 qPCR limitations

Despite this study employing qPCR, there are still many issues associated with the reliable detection of species using this method. These include the Ct values being used to define a positive, the number of positive replicates within a plate being used to distinguish a true positive from background noise, the treatment of negative replicates and the optimum standardised number of replicates to be used within a study - currently anywhere between three and twelve (Sigsgaard *et al*., 2015; Thomsen and Willersev, 2015).

Negative replicates are common within eDNA studies due to the low concentration of target DNA and the complex mix of non-target DNA and environmental particles, such as sediment, which can inhibit qPCR efficiency (Thomsen and Willersev, 2015; McKee *et al*., 2015). It is therefore important to consider the amount of template used to avoid inhibition, and yet still retain detectable amounts of DNA (Sigsgaard *et al*., 2015). For example, Takahara *et al.* (2015) found that reducing the template volume from 5 μl to 2 μl, increased detection probability for the common carp *Cyprinius carpo* (Linnaeus, 1758) when using qPCR. The authors surmised that this could be due to increased inhibition encountered when using larger template volumes. Furthermore, Biggs *et al.* (2015) demonstrated a detection rate of only one in twelve replicates for some samples, which indicates very low concentrations of target DNA. This study had only three replicates per sample and therefore any results, negative or positive, may not be reliable (Herder *et al*., 2014). Future studies should include more replicates in analysis as well as identifying the optimum volume of template required.

It has also been suggested that some of the standard detection thresholds defined in literature may need relaxing for eDNA studies (Thomsen and Willersev, 2015). Additionally, Thomsen *et al.* (2012a) suggest that if high Ct values are used to define detection, that sequencing of the resultant product is crucial for confirmation of detection of target species. This was not done for either the aquaria or field trials in this study. Doing so may have improved the reliability of the positive results obtained in the aquaria trials and given a better indication of eDNA persistence for *P. leniusculus*.

Finally, qPCR reproducibility within and between labs has been observed to be poor and may lead to faulty or inconsistent inferences (Nathan *et al*., 2014). Future research should focus on standardising assay procedures.

5.4.2 Contamination

Contamination of eDNA samples can happen either in the field or the laboratory, and procedures must be carefully considered to avoid false positives.

5.4.2.1 Field protocols

Generally when undertaking fieldwork with invasive crayfish, it is crucial to disinfect equipment between sites. For instance, transmission of pathogens, crayfish plague *A. astaci* and chytrid fungus *Batrachochytrium dendrobatidis* (gen. et sp. nov.) may be a risk when sampling *P. leniusculus* populations (Tréguier *et al*., 2014). For eDNA studies, precautions must be even more stringent. Spear *et al.* (2015) identified gear used in eDNA collection, such as wetsuits, to be a potential source of contamination by successfully amplifying Eastern hellbender *Cryptobranchus alleganiensis* (Daudin, 1803) DNA from water samples squeezed from gear after sampling. Therefore, given the potential for transmission of target eDNA by equipment, decontamination between sites is crucial. In the study described here, sampling equipment, boots and Van Dorn sampler, were decontaminated with a 70 % alcohol solution between sites. New sterile gloves and sterile collection vessels were used at each site as recommended by Herder *et al*. (2014).

5.4.2.2 Laboratory protocols

PCR generates billions of copies of DNA, which can be readily spread throughout laboratories if not careful (Turner *et al*., 2015). Many studies advocate the use of separate laboratories for pre- and post-PCR procedures to minimise contamination risk (Dejean *et al*., 2011; Herder *et al*., 2014; Rees *et al*., 2014; Sigsgaard *et al*., 2015; Thomsen and Willersev, 2015). In this study, there was strict separation of pre- and post-amplification procedures. DNA extraction was performed in separate rooms from

the qPCR mix preparation, including preparing the qPCR mix in a DNA free room and adding the DNA template in a third room as suggested by Herder *et al*. (2014). The qPCR assay was then performed in another separate, dedicated room. In addition to controlling where procedures occur, other controls are required for monitoring contamination, such as DNA extraction blanks and qPCR blanks (Sigsgaard *et al*., 2015). Controlling for false positives is a major challenge in eDNA studies and is critical to assess and monitor contamination (Bohmann *et al*., 2014). In this study, negative controls consisting of only 15 ml milliQ water, were extracted at the same time as any water samples (aquaria or field) to monitor contamination during DNA extraction. In fact, this study found that only negative controls should be extracted alongside water samples. When positive results were observed in negative controls they were always associated with batches in which positive controls (milliQ water spiked with a known amount of target DNA) were extracted alongside other samples. Consequently, positive controls should only be added to the qPCR plates to ensure the assay is functioning. Not only should DNA extraction blanks be added to control for contamination, but qPCR blanks should be included in each assay too (Herder *et al*., 2014; Sigsgaard *et al*., 2015). In this study, qPCR blanks (also known as "no template controls" or "NTCs") consisting of milliQ water added at the same volume as the template DNA, were included at the time of preparing the qPCR mix. Ideally field negatives should also be included in eDNA studies whereby water samples are taken into the field to ensure contamination does not occur during transport (Bohmann *et al*., 2014) or samples are taken from water bodies where the target species is not present (Herder *et al*., 2014). This study employed the latter in both the aquaria and field trials conducted. All controls, apart from the positive control, should return negative results to ensure no contamination has occurred. If any negative controls return positive, or the positive control returns negative, all results should be discarded and the analysis

rerun (Herder *et al*., 2015). As the positive and negative controls in this study did not return unusual results, it can be concluded that the laboratory protocols employed were robust and no contamination occurred.

5.4.3 Aquaria trials

The persistence of detectable concentrations of eDNA is important in understanding how eDNA analysis can effectively be utilised for a target species, whether that is simply determining presence/absence or estimating abundance/biomass. This study demonstrated that the persistence of eDNA in *P. leniusculus* is influenced by time and the number of individuals present. When *P. leniusculus* were present, eDNA was continually detected over the course of the 7 day period for all 3 crayfish density tanks. For the 1 crayfish density tanks, eDNA was detected for the entire 7 day period except for one tank on the seventh day. However, despite *P. leniusculus* presence within the tanks, eDNA was observed to decrease as time passed i.e. the concentration of eDNA was lower in all instances on the seventh day compared to the first. This observation was unusual as it was expected that the longer *P. leniusculus* were in the tanks, the greater the concentration of eDNA. Few studies have explored eDNA persistence while the target species is present. Thomsen *et al.* (2012a) conducted a similar experiment with varying densities (0, 1, 2, or 4) of common spadefoot toad *Pelobates fuscus* (Laurenti, 1768) and great crested newt *Triturus cristatus* (Laurenti, 1768) larvae, whereby measurements were taken at 2, 9, 23, 44, 64 days post introduction, before individuals were removed on the 64th day after metamorphosis. Measurements were then taken at 2, 9, 15, and 48 days post removal of individuals. While individuals were present, a significant effect of density and time on eDNA concentration was observed where a greater number of individuals resulted in a greater concentration of eDNA, similar to this study. However in contrast to this study, Thomsen *et al.* (2012a) also

observed increasing concentrations of eDNA over the period of time when individuals were present as well as reporting that *P. fuscus* had consistently higher concentrations of eDNA than *T. cristatus*. The authors suggest this may be due *P. fuscus* larvae being substantially larger and more active than *T. cristacus*. This may explain the decreasing concentrations of eDNA throughout this study. *P. leniusculus* were very inactive during the 7 day period, which may have resulted in eDNA not being released to any great extent. *P. leniusculus* were also not fed and so normal feeding behaviours were not being carried out, which may also have resulted in decreased concentrations of eDNA. High levels of individual variation of eDNA production in other species such as amphibians (Pilliod *et al*., 2014) and fish (Klymus *et al*., 2015) have been reported and it has been suggested that differences in behaviour and physiology may be the cause (Strickler *et al*., 2015). It is therefore crucial to investigate the mechanisms behind eDNA production in *P. leniusculus* before eDNA analysis can be successfully utilised.

After *P. leniusculus* removal, there was a rapid and continuous decrease in eDNA detection. By 7 days' post *P. leniusculus* removal, eDNA could only be detected in two of the 3 crayfish density tanks. Although seven days was not long enough to observe complete degradation, these results are in agreement with other studies that observed rapid degradation of eDNA within 1-2 weeks after removal (Dejean *et al*., 2011; Piaggio *et al*., 2014, Thomsen *et al*., 2012a). However, as no other published studies exist examining the persistence of crustacean eDNA in controlled aquaria experiments, this study cannot be directly compared. Nevertheless, it appears likely that eDNA would be undetectable after 14 days. The low persistence of eDNA suggests that eDNA measurement would be most useful as a tool to assess contemporary presence or absence of *P. leniusculus*.

5.4.4 Field trials

5.4.4.1 Sample collection

"Even if all the genetic procedures are robust, eDNA analysis is not exploitable without a reliable sampling strategy." (Herder et al., 2014).

The reliability of results and ultimately success of eDNA analysis can be dictated to a large extent by sampling method. Although variations on methods of sample collection exist, there are two that are favoured within existing literature. Studies either use pumping and filtration, or collection of water using submersed containers followed by filtration or ethanol precipitation (Rees *et al*., 2014). This study used ethanol precipitation as the chosen method for eDNA sample collection. In both the aquaria and field trial, three 15 ml water samples (total of 45 ml) were taken from each tank/field location for eDNA analysis. The reported success rate for detection of eDNA has been high (> 80 %) using only three 15 ml water samples (Dejean *et al*., 2012; Ficetola *et al*., 2008; Foote *et al*., 2012; Thomsen *et al*., 2012a). However, since *P. leniusculus* were only successfully detected in controlled laboratory conditions but not in natural conditions, it is plausible that the sampling strategy was not appropriate for the environment or for the target species.

It is known for amphibians that detection of eDNA is influenced by an organism's behaviour, habitat, size and volume of secretions (Pilliod *et al*., 2013). Additionally, season can impact many of these factors and consequently influence eDNA detection (Goldberg *et al*., 2011). For example, the epidermal cells of fish and amphibians produce mucus known to be a source of large amounts of DNA, which is consequently deposited into the environment (Tréguier *et al*., 2014). Aquatic arthropods, on the other hand, possess no mucus producing structures and instead form hard exoskeletons made of chitin (Herder *et al*., 2014, Tréguier *et al*., 2014). Furthermore, in some cases the

chitin is combined with calcium carbonate to form an even stronger compound (Tréguier *et al*., 2014). Tréguier *et al*. (2014) suggest that this hard exoskeleton may prevent the release of extracellular DNA into the environment and therefore reduce arthropod detection when using molecular techniques.

To date, much of the available literature on using eDNA to detect organisms focuses on fish and amphibians with studies focusing on aquatic arthropods rare. In fact, only one published study investigating the use of eDNA for detection of crayfish currently exists. Tréguier *et al.* (2014) reported *P. clarkii* were only confirmed in 59 % of ponds where trapping had confirmed *P. clarkii* presence. It was found that eDNA performed better in shallow ponds with high densities of *P. clarkii,* whilst in deeper ponds with low densities (< 2 *P. clarkii* per trap), there was less than a 50 % chance of detecting *P. clarkii* using eDNA. Additionally, eDNA was more successful at detecting *P. clarkii* in ponds that were inhabited by smaller individuals. As Tréguier *et al.* (2014) explain, *P. clarkii* have a rapid growth rate and therefore moulting is frequent. Consequently, frequent moulting is a likely mechanism, which increases the concentration of eDNA within an environment due to the physical act of shedding the exoskeleton and the resulting exuviae left behind. Although ethanol precipitation was also the chosen method of eDNA analysis for Tréguier *et al.* (2014), they collected a total of 800 ml from each of the 158 ponds sampled compared to a total of 45 ml in this study. The 800 ml was a result of 40 ml water samples being collected from twenty evenly spaced locations within the pond and pooled before six 15 ml subsamples were taken for analysis. This would likely have increased the probability of detecting *P. clarkii* considerably.

Findings by Tréguier *et al.* (2014) coupled with existing literature might give insight as to why eDNA did not detect *P. leniusculus* during field trials in this study. The first

location sampled was a fast flowing river (Daer water, the upper reaches of the River Clyde) and was approximately 1.5 m deep and 5 m wide at the point of sampling, while the other location was a 14 km long freshwater Loch (Loch Ken) known to be as deep as 19 m in places (Andrew Blunsum, Loch Ken ranger, Per. Comm. 2012). There is recent evidence to suggest that water body size should be accounted for when sampling (Rees *et al*., 2014b). This was not taken into account during this study, with only three 15 ml point samples taken from both locations despite very different conditions. Thomsen *et al.* (2012a) found differences in detection probabilities between lentic and lotic systems, suggesting that three point samples of 15 ml may not be suitable for running waters such as Daer water. Moreover, there is increasing dilution of eDNA fragments in running water with increasing distance from the source (Pilliod *et al*., 2014). It is therefore likely that increasing the number of samples taken would increase the probability of detection. Furthermore, the main criticism of the ethanol precipitation method is that the volume of water sampled is relatively low and unless a species is found at high densities, and is consequently releasing high quantities of eDNA into the environment, detection by eDNA is unlikely. Also, if an organism does not have a high level of mobility, the area in which eDNA is present is limited (Herder *et al*., 2014). Therefore, if water samples are not taken in close proximity to an organism, the probability of detection by eDNA is low. Based on this reasoning, more samples should have been taken at each site during this study to increase the probability of detecting *P. leniusculus* eDNA. However, if moulting is indeed a mechanism for DNA release in aquatic arthropods such as *P. leniusculus*, as suggested by Tréguier *et al.* (2014), then time of year would also need to be taken into account in any future eDNA studies. The moulting season for *P. leniusculus* occurs between July and September inclusive (Reeve, 2004) and since environmental samples were collected in November during this study, it is unlikely that *P. leniusculus* were releasing any large

quantities of eDNA. This could have potentially limited detection. Moreover, *P. leniusculus* activity varies with season. During winter months *P. leniusculus* spends the majority of time in torpor (a period of inactivity), often in refuges (Peay, 2000). Consequently *P. leniusculus* were not very mobile during the sampling period in this study and therefore DNA is likely to be restricted to certain locations, which may also have further decreased the probability of detection. Spear *et al.* (2015) suggest that where temporal differences may influence detection probabilities, researchers should conduct pilot studies over several months to determine the optimal sampling period. This may be especially useful in species, like *P. leniusculus,* which have a lower baseline rate of eDNA production. Consequently, future research should focus on the mechanisms of DNA release by aquatic arthropods as well as determining how temporal differences affect the eDNA detection rates in *P. leniusculus*.

5.4.4.2 Ethanol precipitation vs filtration

One possible way to overcome many of the problems encountered in this study is to switch to a filtration method when sampling large water bodies or running waters, such as Loch Ken and Daer water. Theoretically by capturing more water, the probability of detection increases (Herder *et al*., 2014). Yet depending on the pore size of the filter used, not all of the eDNA might be retained, which further decreases the detection probability. Whereas when using ethanol precipitation, all eDNA is retained in a sample (Herder *et al*., 2014). Piaggio *et al.* (2014) compared vacuum filtration and ethanol precipitation and found ethanol precipitation to be the optimal method for eDNA analysis. During filtration, they experienced clogging of the filter paper and suggested that this may have affected their ability to detect eDNA. One way to avoid this would be to filter sequentially with filters of decreasing pore size (Turner *et al*., 2014). However, this could potentially result in loss of eDNA, as well as increasing

the risk of contamination and thus provide no real benefit over ethanol precipitation (Santas *et al*., 2013). Additionally, filtration is more time consuming than collecting water for ethanol precipitation. Santas *et al.* (2013) found that filtering a 1 L water sample through a 0.45 µm filter took between 30 minutes and 1 hr, while a 2 L sample took anywhere from 1 – 8 hrs. They also found that detecting *C. alleganiensis* eDNA in 1 L of water was only possible in lotic systems, which would suggest filtration is not the best method for small, still water bodies. If this is the case for amphibians, then it is possibly even more true for aquatic arthropods such as *P. leniusculus*.

One more key difference between filtration and ethanol precipitation is the degree of DNA degradation between water collection and analysis. Rees *et al.* (2014) suggest when using ethanol precipitation, samples are added immediately to ethanol in the field in order to stabilise the DNA before being stored at -20ºC to prevent further degradation until analysis can be completed. This is often not feasible with filtration, as it would increase the already expensive procedure (Biggs *et al*., 2015). Water samples are either collected by filtration, stored on ice and sent to the laboratory for analysis within 24 hrs (Jerde *et al*., 2013) or are frozen until filtration can be performed at a later date (Thomsen *et al*., 2012b). This may have implications on the quality of DNA detected and could ultimately produce false negatives due to DNA degradation.

The samples in this study were added immediately to ethanol in the field and frozen at -20ºC within hours, until later analysis. Therefore, it is highly unlikely that the sampling strategy negatively affected the detection of *P. leniusculus* as a result of any potential eDNA degradation. Consequently, improvements to the sampling strategy for this study would include collecting many water samples from different locations within each site and pooling them for homogenisation, before 15 ml subsamples were taken and added to ethanol for preservation (Biggs *et al*., 2014; Dejean *et al*., 2011; Herder

et al., 2014, Piaggio *et al*., 2014; Tréguier *et al*., 2014). This would account for DNA not being uniformly distributed in water and would have increased the chance of *P. leniusculus* being detected by eDNA. Further development of sampling strategies, including comparing filtration and ethanol precipitation, is necessary to find the optimal method for *P. leniusculus* detection using eDNA

5.4.4.3 Inhibition

Within the literature, inhibition of eDNA extraction and assays is a much discussed topic. Humic acids and other plant degradation products are frequently co-extracted with eDNA in environmental water samples, due to the accidental inclusion of sediment particles (Thomsen and Willerslev, 2015). These compounds can inhibit qPCR reactions, which may in turn affect eDNA quantification (McKee *et al*., 2015). In fact, inhibition can also lead to false negatives, which has consequences for underestimating the occurrence of the target species (McKee *et al*., 2015, Thomsen and Willersev, 2015). Inhibition is a problem for both ethanol precipitation and filtration sampling strategies, although there may be increased risk when using filtration due to the concentration of inhibitors (Piaggio *et al*., 2014).

However, it has been suggested that sediments are an important location for eDNA preservation (Tréguier *et al*., 2014, Turner *et al*., 2015). Furthermore, Turner *et al.* (2015) found carp DNA to be 8 to 1800 times more concentrated in sediments than water and reported eDNA persistence in sediments to be up to 152 days, approximately 5 times longer than records of water column persistence. As a result, there are two divergent trains of thought. Firstly, some studies may want to avoid sediment contamination whereby the results could lead to false positives, due to migratory species, or complicate results of invasive species eradication monitoring or translocation programs. Secondly, other studies may actively include sediment

particles as it may aid detection for species where DNA within the water column may be sparse, e.g. benthic aquatic arthropods.

This study sampled from the centre of the water column using a Van Dorn sampler to avoid disturbing subsurface sediment and releasing ancient DNA following Tréguier *et al.* (2014). However, although the authors advocate avoiding subsurface sediments, they actively disturbed the uppermost sediment to re-suspend any *P. clarkii* DNA fragments before taking water samples. It is possible that this increased the probability of detection in their study and may further explain the lack of detection in this study. As *P. leniusculus* is a benthic organism, future work should modify the sampling strategy to collect water samples closer to the bottom of the water column, after disturbing the top layer of sediment, to investigate the effect of sediment disturbance on *P. leniusculus* detection.

5.4.5 Conclusion

The detection of *P. leniusculus* by eDNA in this present study was only possible in controlled aquaria conditions, however it does highlight future potential for using eDNA to detect and monitor *P. leniusculus*. With increasing resolution of many issues highlighted in this study, such as optimising the current sampling strategy, standardising the qPCR assay and taking into account the influence of season and environment, eDNA measurement represents a promising technique to efficiently and inexpensively monitor the invasion of *P. leniusculus*.

Chapter 6. General discussion

This PhD study aimed to facilitate understanding of *P. leniusculus* invasion success and develop methodologies that could improve the detection and control of *P. leniusculus* populations in Scotland. The success of *P. leniusculus* as a non-native invasive species was explored by: first, examination of the trophic dynamics of *P. leniusculus* in a Scottish freshwater Loch to determine trophic position, diet composition and ontogenesis (Chapter 2); and second, comparison of the niche width occupied by the introduced *P. leniusculus* to the niche width occupied by the only other crayfish species present in Scotland, *A. pallipes*, which is considered by this study to be native (Chapter 3). Methodologies for the detection and control of *P. leniusculus* were then investigated by identifying whether trapping efficiency could be improved with the use of food attractants (Chapter 4). A molecular assay was also developed and evaluated as a tool for rapid detection of *P. leniusculus* DNA in environmental water samples (Chapter 5). In the following chapter, the key findings of each investigation are summarised and their significance to the identified research themes discussed. Additionally, recommendations for future studies are made.

6.1 Trophic dynamics

Stable isotopes not only help researchers to determine the effects of an invader, but can also be used to formulate responses to invasions (Bodey *et al*., 2011). Stable isotopes can be used to identify behavioural patterns by informing researchers about food types being consumed and from where food is foraged (Bodey *et al*., 2011). Additionally, stable isotopes used to calculate the niche width can also be used to predict potential spread and range of an invasive species (Bodey *et al*., 2011). As a result, species management plans can be implemented more effectively by focusing resources based on information gained through stable isotope analysis.

The present study detected an ontogenetic dietary shift when using Stable Isotope Analysis in R (SIAR) to investigate the dietary composition of five size classes of *P. leniusculus* (Chapter 2). However, the shift observed was not linear, as *P. leniusculus* in this study did not shift directly from a juvenile diet of invertebrates to an adult diet comprising mostly of plant material, which is often how the ontogenetic dietary shift is described in existing crayfish literature (Mason, 1975). In the current study, the shift to an increasingly protein based diet occurred in size class 2 (10 – 20 mm) and size class 3 (21 – 39 mm), while size class 1 (0 – 9 mm) exhibited a diet similar to adult *P. leniusculus* in size classes 4 (40 – 50 mm) and 5 (51 – 70 mm), this comprised largely of plant and detrital material. Additionally, it is important to note that the ontogenetic dietary shift could not be distinguished by viewing the $\delta^{15}N$ and $\delta^{13}C$ isotopic signatures alone. The dietary shift only became apparent when viewing the diet contributions of each food source as estimated by the mixing model. There is debate within existing literature as to whether crayfish do in fact exhibit an ontogenetic dietary shift, with different methods reaching different conclusions. For example, gut content analysis could detect an ontogenetic dietary shift in *P. planiforms* whereas stable isotope analysis could not (Parkyn *et al*., 2001). The results from the present study do not provide a definitive answer as to whether *P. leniusculus* exhibit an ontogenetic dietary shift, but the results in combination with existing literature do suggest that stable isotope analysis alone may not be the most suitable method for detecting ontogenetic dietary shifts in crayfish. Future studies should perhaps focus on examining the use of combined methods, such as stable isotope analysis and fatty acid analysis, for determination of crayfish trophic dynamics. For instance, the fatty acid profile of an organism may indicate the type of prey consumed. Lipids, which are transferred from prey to predator without change, release fatty acids during digestion (Parrish *et al*., 2000). The fatty acids released are stored in fat stores such as the adipose tissue in

mammals or the hepatopancreas in decapod crustaceans (Iverson *et al*., 2004). As such, fatty acids provide finer resolution and are more discriminatory than stable isotopes (Iverson *et al*., 2004). Therefore, when used in conjunction with other methodologies, fatty acids may assist in elucidating trophic relationships and ontogenetic dietary shifts in crayfish.

Chapter 2 also found all size classes of *P. leniusculus* occupied the trophic position of a predator within Loch Ken and although some size classes of *P. leniusculus* fed predominately on plant and detrital material (size classes 1, 4 and 5), this indicates preferential assimilation of protein sources by all sizes of *P. leniusculus*. This finding suggests that all sizes of *P. leniusculus* may potentially impact invertebrate and fish assemblages within Loch Ken. Therefore, any future control strategies should target all size classes of *P. leniusculus* to mitigate potential negative effects on community structure. An intensive diversity study of water bodies with and without *P. leniusculus* populations would also be required to confirm reduced invertebrate and fish diversity where *P. leniusculus* are present.

One main limitation of the stable isotope study employed by this PhD, and one that could influence the determination of an ontogenetic dietary shift by analysing the diet composition estimated by an isotopic mixing model, is the lack of species-specific trophic enrichment factor (TEF) values for any crayfish species, including *P. leniusculus*. The successful application of stable isotopes to identify trophic relationships relies on the use of accurate TEFs (Caut *et al*., 2009), and accurate TEFs are critical to the use of mixing models (Parnell *et al*., 2010, Phi1lips and Gregg, 2001). However, TEFs are frequently cited as a weak link in stable isotope studies (Bond and Jones, 2009; Burress *et al*., 2013; Post, 2002). Therefore, future studies should conduct controlled feeding studies on *P. leniusculus* to calculate species-specific TEF values.

Additionally, using the stable isotope values measured during the study and the calculated TEF, the use of Bayesian mixing models to estimate the composition of *P. leniusculus* diet could then be validated.

Chapter 3 compared the niche width and diet composition of the non-native invasive *P. leniusculus* to the native *A. pallipes*. It was expected that *P. leniusculus* would exhibit a wider niche width than *A. pallipes*, as has been observed in other studies comparing the niche width between non-native invasive and native crayfish species (Ercoli *et al*., 2014; Olsson *et al*., 2009). A wider niche width is indicative of a generalist diet and may explain the success of *P. leniusculus* as a non-native invasive species. The results obtained in Chapter 3 indicate that at species level, *A. pallipes* exhibited a larger niche width than *P. leniusculus*. This could imply that dietary breadth and plasticity of *P. leniusculus* are unlikely to be key reasons for the overwhelming success of *P. leniusculus* as an invasive species.

P. leniusculus has been established within Loch Ken for over a decade (Ribbons and Graham, 2009) and it is well documented that invertebrate biomass and diversity are negatively impacted by the presence of this species (Crawford *et al*., 2006; Guan and Wiles, 1998; Nyström *et al*., 1999; Nyström, 2002; Stenroth and Nyström, 2003). It is possible that when *P. leniusculus* were first established in Loch Ken, a wider variety of food sources were available and subsequently consumed. As the population increased, currently estimated to be between 1.06 – 9.05 adult *P. leniusculus* m^{-2} (Ribbons and Graham, 2009), fewer potential food sources would be available. This could subsequently produce the narrower niche width observed by the present study. Therefore, the reduced dietary breadth of *P. leniusculus* in Loch Ken may have been established for several years and the *P. leniusculus* population within Loch Ken is a victim of its own success. Thus, despite the narrower estimated niche width for *P.*

leniusculus observed, *P. leniusculus* would have to exhibit greater diet plasticity to maintain a viable population and achieve population growth when available food sources are reduced. It is greater diet plasticity that could enable success of *P. leniusculus* as an invasive species. Consequently, it is important to determine the niche width of more recent introductions of *P. leniusculus* populations in Scotland to investigate if the amount of time passed since establishment impacts the estimated niche width and diet composition of an invasive species. This would not only allow greater confidence to be placed in the inferences drawn when comparing the niche width and diet composition of *P. leniusculus* and *A. pallipes* populations in Scotland but would further understanding regarding the dietary breadth and plasticity of an invasive species.

Results from Chapter 3 also suggest that there would be a lack of direct competition for resources between *P. leniusculus* and *A. pallipes* if they were to co-occur. If *P. leniusculus* were to be introduced to either Loch Croispol or Whitemoss Reservoir, for example through use as live bait for angling (Bean *et al*., 2006; Lodge *et al*., 2000; Peay *et al*., 2010), during trout hatchery restocking (Bean *et al*., 2006) or through deliberate introduction from the pond and aquarium trade (Chucholl, 2013; Holdich *et al*., 2009a; Lodge *et al*., 2000), then the results obtained in this study indicate that competition for resources may not be the driving mechanism behind the displacement of *A. pallipes* at either site. However, Chapter 3 considered only five common putative food sources in the mixing model used. As a result, it is possible that *A. pallipes* are feeding on other food sources present at Loch Croispol and Whitemoss Reservoir, which were not included in the mixing model. For instance, molluscs are a known food source for both *A. pallipes* (Matthews and Reynolds, 1992; Reynolds and O'Keefe, 2005) and *P. leniusculus* (Nyström and Perez, 1998; Stenroth and Nyström, 2003). However,

molluscs were only present at sites containing *A. pallipes* and so they were not included in the mixing model analysis. Therefore, direct competition for resources between both species may in fact occur, but was not reflected in the results of the present study. Consequently, future studies employing mesocosm experiments with both *A. pallipes* and *P. leniusculus* present together under identical environmental conditions and consuming identical food sources are key to understanding true diet composition and niche width between the two species. This research could then be used to construct and implement species-specific management plans for both *P. leniusculus* and *A. pallipes*.

6.2 Detection and control of *P. leniusculus*

It is generally accepted that eradication of established *P. leniusculus* populations from GB waters, including Scotland, is no longer an achievable goal. Therefore, the development of methodologies to control existing populations is critical in efforts to reduce numbers to levels where mitigation of known negative impacts could occur. However, preventing establishment of populations would be better than control. This requires development of methodologies that enable rapid detection of *P. leniusculus* while eradication is still feasible.

Chapter 4 aimed to improve the trapping efficiency of *P. leniusculus* through the use of a preferred food attractant. However, *P. leniusculus* did not express preference for any of the four food attractants (*O. mykiss*, *P. leniusculus*, beef and vegetation) presented under laboratory conditions. This lack of preference meant that the investigation of food attractants for use in improving trapping efficiency did not continue past plus-maze trials in the laboratory. It is possible that the lack of preference exhibited is evidence of diet plasticity in *P. leniusculus*, although this was not observed in the aforementioned stable isotope study. This lack of preference may be characteristic of a generalist, whereby being able to utilise a wide variety of food sources efficiently

could have enabled *P. leniusculus* to become a successful invasive species. Nonetheless, from this study it is concluded that trapping efficiency of *P. leniusculus* cannot be improved through the use of food attractants.

The large numbers of *P. leniusculus* caught in the monofilament nylon gill nets compared with no *P. leniusculus* caught in the baited cotton gill nets (Chapter 4) reveal potential for the use of nets in controlling *P. leniusculus*. Jansen *et al*. (2009) also report the capture of crayfish (*O. virilis*, *O. rusticus* and the papershell crayfish *Orconectes immunis* (Hagen, 1870)) in gill nets deployed by the Fisheries Assessment Unit (FAU) in Lake of the Woods, Ontario, Canada. Multifilament gill nets were set as part of routine fisheries assessment by the FAU and crayfish were caught incidentally, attracted by fish that had been gilled or become entangled in the nets (Tom Mosindy, FAU, Pers. Comm. 2015). The incidental by-catch of crayfish in that study is echoed by the findings in this PhD. Jansen *et al*. (2009) describe the crayfish as susceptible to entanglement in the nylon gill nets, a finding supported by this study. Based on the results of this study and other studies (Jansen *et al*., 2009; Moonga and Musuka, 2014), tangle nets, which are similar in construction to nylon gill nets and used in the capture of marine Crustacea, could potentially be used to control *P. leniusculus*. Baited tangle nets are placed across the sediment and marine Crustacea such as the spanner crab *Ranina ranina* (Linnaeus, 1758) become entangled as they move across the net (Hill and Wassenberg, 1999). However, tangle nets may only work in lentic water bodies such as Loch Ken. Therefore, future research should also focus on identifying whether fluids released from injured fish, or chemical distress signals released as fish become entangled, are attracting *P. leniusculus* to the nets. Any signals or compounds identified could potentially be used to increase trapping efficiency similar to the use of crayfish pheromones, as described by Stebbing *et al*. (2003).

Early detection presents the best opportunity for eradication and preventing spread of *P. leniusculus* populations before establishment. Chapter 5 reports the development of a protocol to extract DNA from water samples and a robust and sensitive TaqMan probe assay for detection of *P. leniusculus* DNA from water samples. However, *P. leniusculus* were only detected in water samples obtained under controlled laboratory conditions whilst the molecular assay failed to detect *P. leniusculus* eDNA in water samples obtained under natural conditions.

Despite negative results from field trials, this author believes that eDNA represents a potential method for detecting the contemporary presence/absence of *P. leniusculus* in Scotland and on a wider geographical scale, if limitations identified in this study are addressed by future research. For instance, water collection and DNA extraction methods require refinement and standardisation. The results of this study revealed the need to develop strict laboratory protocol to separate pre- and post-amplification procedures to prevent contamination. Separate laboratories are required for each step of the process: DNA extraction, preparing the qPCR mix, adding the DNA template to the qPCR mix, and finally performing the qPCR. The results from Chapter 5 also revealed the importance of appropriate negative controls such as qPCR blanks (known in this study as NTCs) and DNA extraction blanks. Furthermore, it was discovered that extraction of positive controls at the same time as water samples or negative controls could cause contamination. As a result, positive controls should be extracted separately and then included during the qPCR assay to ensure the assay is functioning as expected. Further investigation regarding the volume of water to be sampled is required, as is the effect of season on detection. Water samples were obtained in November, a time period when *P. leniusculus* are known to be relatively inactive, which may have influenced the results obtained. Additionally, research should focus on detection efficiency of *P.*

leniusculus eDNA in water bodies of different sizes, and in lentic versus lotic systems. Finally, a focal area of research identified by the present study and critical to the implementation of eDNA as a method of detection for *P. leniusculus* is the mechanism of DNA release in crustaceans. It is unknown whether DNA is constantly released through daily activities such as feeding or if DNA release is restricted to certain events such as breeding or during growth through shedding of the exoskeleton. Additionally, aquarium trials in Chapter 5 revealed eDNA persistence to decline over time, despite *P. leniusculus* being present. In this study, *P. leniusculus* were observed to be highly inactive and were not fed for the duration of the experiment. Therefore, further research into eDNA persistence is required under controlled conditions that mimic natural environment, for instance artificial ponds, where *P. leniusculus* can exhibit more normal behaviours.

6.3 Conclusion

The stable isotope studies described in this PhD thesis contribute to the understanding of the trophic role of *P. leniusculus* at a variety of sizes, and as a successful invasive species when compared to *A. pallipes*. However, future studies might consider using multiple established populations of *P. leniusculus* across Scotland to help determine if the ontogenetic dietary shift, trophic position and diet composition observed in the Loch Ken population is representative of the species, or whether there is geographic variation in the trophic role of an invasive species. Additionally, niche width comparisons using stable isotope analysis presents a useful tool to predict the effect of *P. leniusculus* on *A. pallipes* in Scotland. Nevertheless, more accurate predictions would have been possible if more populations were compared and/or *P. leniusculus* and *A. pallipes* were maintained in mesocosms under identical conditions.

Improvement of existing methods and discovery of new methods is crucial for the control and detection of *P. leniusculus,* not only in Scotland but also within Europe. Results of this PhD study suggest that trapping efficiency of *P. leniusculus* cannot be improved through the use of food attractants alone but that gill or tangle nets may represent an additional control method yet to be investigated. Additionally, to the best of this author's knowledge, this PhD describes the first use of eDNA as a method of detection for *P. leniusculus*. As a result, the present work provides valuable baseline protocols and information together with suggestions for future researchers to develop the methodology further.

Overall, it is felt this doctoral study has contributed to the fields of astacology and invasion ecology by bringing new insights to the trophic dynamics and providing novel methods for the control and detection of one of the worst globally invasive species, *P. leniusculus*.

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