

A comparative assessment of health and immune
response between triploid and diploid
Atlantic salmon (*Salmo salar*)

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Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Lynn Chalmers
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“So long and thanks for all the fish...”

List of abbreviations

α2-M	α2-macroglobulin
°D	degree days
2N	diploid
3N	triploid
4N	tetraploid
ACH50	alternative complement activity
ACH50%	alternative complement activity
ACP	alternative complement pathway
ADJ	adjuvant group
AGD	amoebic gill disease
ANOVA	analysis of variance
aq	aqueous
ARF	Aquaculture Research Facility
ATP	Adenosine triphosphate
BAPNA	Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride
BCP	1-Bromo-3-chloropropane
BHIA	brain heart infusion agar
BKD	bacterial kidney disease
BSA	bovine serum albumin
<i>cat</i>	catalase
cDNA	complementary DNA
CFU	colony forming units
CMS	cardiomyopathy syndrome
<i>crp/sap1a</i>	C-reactive protein (CRP)/serum amyloid P 1a
<i>crp/sap1b</i>	C-reactive protein (CRP)/serum amyloid P 1b
DAB	3, 3-diaminobenzidine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DO	dissolved oxygen

List of abbreviations

d.p.i	days post infection
dsRNA	double-stranded ribonucleic acid
dV	deformed vertebrae
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FTU	feed trial unit
FW	freshwater
G-CFB	gelatine-complement fixation buffer
GFDS	gill filament deformity syndrome
GLM	General linear model
<i>gpx1</i>	glutathione peroxidase 1
<i>gr</i>	glutathione reductase
h	hours
H ₂ O ₂	hydrogen peroxide
h.p.e	hours post-exposure
HRP	horse radish peroxidase
HSMI	Heart and Skeletal Muscle Inflammation
<i>hsp70</i>	heat shock protein 70
HSWB	high salt wash buffer
IFAT	indirect fluorescent antibody technique
IFN	interferon
Ig	immunoglobulins
IgM	immunoglobulin M
<i>il1β</i>	interleukin 1-beta
IP	intraperitoneally
IPN	Infectious Pancreatic Necrosis
IPNV	Infectious Pancreatic Necrosis Virus
ISA	Infectious Salmon Anaemia
ISAV	Infectious Salmon Anaemia Virus
K	condition factor
KOH	potassium hydroxide
LD	light/dark
LJD	lower jaw deformity

List of abbreviations

LL	continuous light
LPS	lipopolysaccharide
LSWB	low salt wash buffer
LT ₅₀	median Lethal Time
MERL	Marine Environmental Research Laboratory
MHC	major histocompatibility complex
min	minutes
MMC	melanomacrophage centres
MS-222	Tricaine mesylate
MT	metric tonne
NaCl	sodium chloride
Na ₂ CO ₃	sodium carbonate
NBFRF	Niall Bromage Freshwater Research Facility
NBT	nitroblue tetrazolium
NRU	normalised relative unit
NTC	no template control
OD	optical density
PA	phagocytic activity
PBS	phosphate buffered saline
PC	phagocytic capacity
PCr	phosphocreatine
PCR	polymerase chain reaction
PD	Pancreas Disease
PI	phagocytic index
P.I.T	passive integrated transponder
PMA	phorbol myristate acetate
PMCV	piscine myocarditis virus
ppm	parts per million
PRV	piscine orthoreovirus
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
R	Region
RBC	red blood cell
RNA	ribonucleic acid

List of abbreviations

ROS	reactive oxygen species
RPS	relative percent survival
rRNA	ribosomal ribonucleic acid
RTFS	Rainbow Trout Fry Syndrome
RT-PCR	real-time polymerase chain reaction
RUs	relative units
s	seconds
S0+	out-of-season Atlantic salmon smolts
S1+	in-season Atlantic salmon smolts
<i>saa5</i>	serum amyloid A-5
SAV	salmon alphavirus
SAV3	salmonid alphavirus subtype 3
SD	standard deviation
SEM	standard error mean
<i>sod1</i>	superoxide dismutase 1
<i>sod2</i>	superoxide dismutase 2
sp	species
SPDV	salmon pancreas disease virus
SRBC	sheep red blood cell
subsp	subspecies
SW	seawater
TGC	thermal growth coefficient
Th	T helper cells
TMB	3'3'5'5'-tertamethylbenzidine dihydrochloride
USD	United States Dollars
V	vertebrae
v/v	volume per volume
VACC	vaccinated group
w/v	weight per volume
WBC	white blood cell

Abstract

Sterile triploid Atlantic salmon represent a solution to the issues of pre-harvest sexual maturation and mature escapees from open aquaculture systems. Although the initial problems of reduced performance and increased deformities in triploids have been thoroughly researched, there is a continued lack of information on their susceptibility and response to disease and routine on-farm treatments compared to diploids. Thus, the main aim of this thesis was to enhance the current understanding of triploid health and immunity through experimental disease challenges and treatments, and aid in determining their robustness and, therefore, suitability for aquaculture.

A commercial furunculosis vaccine equally protected diploids and triploids against challenge with *Aeromonas salmonicida*, and adhesion scores were similar between ploidy (Chapter 2). Interestingly, triploids had lower white blood cell counts but increased cellular activity, e.g. respiratory burst, compared to diploids. Following experimental cohabitation infection with *Neoparamoeba perurans*, causative agent of Amoebic Gill Disease (AGD), ploidy did not affect the manifestation or severity of AGD-associated gill pathology, or the serum innate immune response (Chapter 3). Hydrogen peroxide, used to treat against parasitic diseases, elicited similar primary and secondary stress responses in both ploidy, but led to differences in the expression of stress (*cat*, *gpx1*, *gr*, *hsp70*, *sod1*, *sod2*) and immune (*saa5*, *crp/sap1a*, *crp/sap1b*, *il1 β*) genes (Chapter 4). Finally, vaccination with different vaccine treatments (4 commercial vaccines, 6 different vaccine combinations and a sham-vaccinated control) showed no ploidy differences in adhesion score or antibody response, although vertebral deformities remained higher in triploids (Chapter 5). Increasing severity of vaccine treatments negatively affected weight, length and thermal growth coefficient in both ploidy. Triploids were heavier than diploids at smolt (+ 14 %) and post smolt (+ 32 %).

Overall, this research shows that triploid Atlantic salmon respond as well as diploids to disease and treatment challenges, and supports their application into full-scale commercial aquaculture.

Key words: Atlantic salmon; Triploid; Immunity; Disease Challenge; Vaccination

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Chapter 1

Introduction

1.1. GLOBAL AQUACULTURE

The term ‘aquaculture’ refers to the farming of aquatic organisms such as fish, crustaceans, molluscs and plants with intervention into the rearing process to enhance production *e.g.* regular stocking, feeding and predator protection (Food and Agriculture Organization of the United Nations (FAO), 2002). With the decline in production from capture fisheries over the years, aquaculture has played an increasing role in meeting the global demand for food fish and shellfish, and has quickly become the fastest growing food-producing sector (Figure 1.1) (Ashley, 2007; Subasinghe *et al.*, 2009). Currently, cultured food fish supplies make up almost 50 % of that consumed globally (FAO, 2014; Marine Harvest, 2015), with this expected to increase to more than 60 % (or 132 million metric tonne (MT)) by 2020 (FAO, 2008; Gjedrem *et al.*, 2012). As a result of this rapid industry expansion, several hundreds of aquaculture species are now being successfully cultured (Bostock *et al.*, 2010; FAO, 2015a).

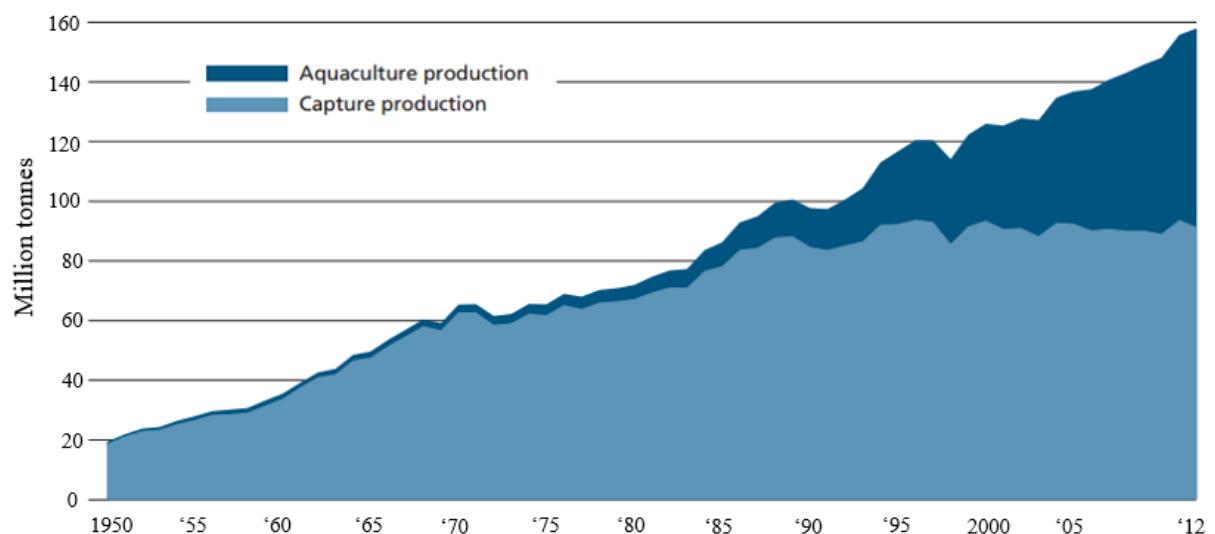


Figure 1.1: Total production from capture fisheries and aquaculture between 1950 and 2012 (FAO, 2014).

1.1.1. Salmonid aquaculture with a focus on Atlantic salmon production

The farming of salmonids is considered one of the most important examples of commercially successful intensive aquaculture (Knapp *et al.*, 2007). Salmonid aquaculture can be traced throughout the 18th and 19th centuries to the early part of the 20th century when Danish and Norwegian salmonid farmers made significant breakthroughs in farming technologies (Knapp *et al.*, 2007; Araneda *et al.*, 2008). As wild capture fisheries declined throughout the latter part of the 20th century, the production of salmonid species grew in importance and further technological advances allowed salmonid aquaculture to become

industrialised (Araneda *et al.*, 2008). Production from salmonid aquaculture currently represents about 4 % of total aquaculture production (Asche and Bjørndal, 2011).

While ‘salmonids’ comprise many commercially important aquaculture species such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha*), the most dominant farmed species is Atlantic salmon (*Salmo salar*) (Araneda *et al.*, 2008; Benfey, 2015; Marine Harvest, 2015). From 1950 to 1990, the majority of Atlantic salmon was supplied by capture fisheries (Figure 1.2a) but the decline in capture fisheries production from 1990 onwards resulted in the production of Atlantic salmon from aquaculture becoming increasingly important and regularly employed. From 225,642 MT in 1990, farmed Atlantic salmon production exploded to 2.32 million MT in 2014 (Figure 1.2b) (FAO, 2015b; Marine Harvest, 2015). Atlantic salmon production now represents more than 90 % of the farmed salmon market and over 50 % of the total global salmon market (FAO, 2004; Whyte, 2007). Despite this dramatic increase in production and the growing success of salmonid aquaculture and, indeed, the entire aquaculture industry, it is recognised that there are still numerous problems to contend with including nutrient pollution *e.g.* waste from feed and faeces and chemical pollution *e.g.* use of chemical treatments (FAO, 2004), welfare issues (Stevenson, 2007) and disease outbreaks (European Food Safety Authority, 2008; Weber *et al.*, 2013).

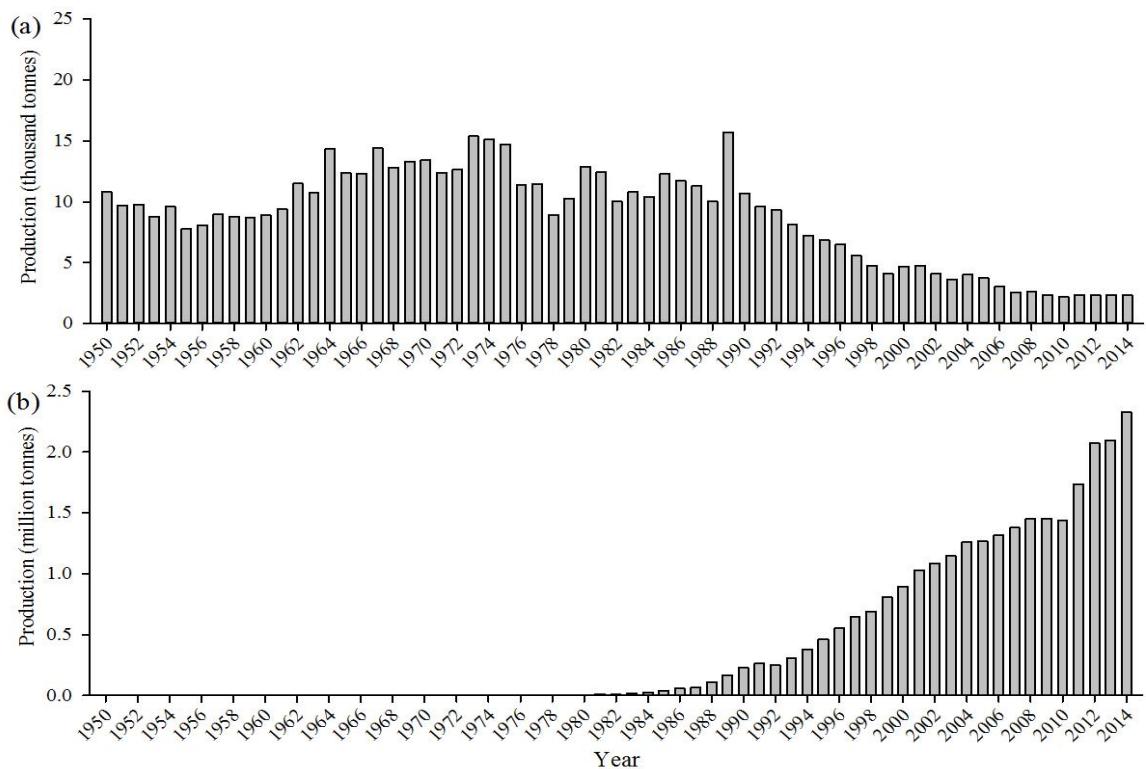


Figure 1.2: Total production of Atlantic salmon (*Salmo salar*) between 1950 and 2014 from (a) Capture fisheries and (b) Aquaculture (FAO, 2015b). Note a 100-fold change in scale between 1.2 (a) and (b).

1.2. ATLANTIC SALMON

1.2.1. Life cycle and life history traits

The term ‘life cycle’ refers to the series of key life events through which an organism passes from the beginning of its life to its death (Collins Dictionaries, 2017a), while the term ‘life history’ is used to describe the sequence and timing of key life events, especially those related to development, reproduction and species preservation (Good and Davidson, 2016). Atlantic salmon are an anadromous species and, as such, have an extremely complex life cycle (Figure 1.3). In late autumn/ early winter, **adult** Atlantic salmon travel from seawater to freshwater to spawn. The female will lay her **eggs** into a spawning nest called a redd, where they will be fertilised by a male. The eggs then undergo a lengthy incubation until the following spring when they will hatch as **alevins**. From the point of hatching, juvenile Atlantic salmon will remain in their natal freshwater for 1 to 6 years, undergoing changes from **alevin** (newly hatched, yolk sac attached) to **fry** (yolk sac exhausted, swim bladder inflated, swimming and feeding freely) and then to **parr** (development of dark vertical stripes, or parr marks) (Aas *et al.*, 2011; Good and Davidson, 2016; U.S. Food and Drug

Administration, 2015). In springtime, parr undergo smoltification, a series of morphological and physiological changes in preparation for the journey to seawater (Good and Davidson, 2016). During this process, silver colouration and darkened fins replace parr marks and **smolts** develop the ability for hypo-osmotic and ion regulation, which will aid in seawater adaptation. From late spring to early summer, smolts then travel from their native rivers to seawater and will remain there for up to 5 years until they reach reproductive maturity (Ayllon *et al.*, 2015). At this point, the parental migration to freshwater will begin again. The Atlantic salmon life cycle has now been extensively studied and, within the aquaculture industry, each life stage can be successfully maintained under controlled, optimised conditions. An Atlantic salmon production cycle will normally last from 24 to 40 months, with 10 to 16 months in freshwater and 14 to 24 months in seawater (Marine Harvest, 2015). However, while Atlantic salmon tend to conform to this general life cycle, many variations in life history traits are often exhibited such as duration in freshwater and seawater and age at smoltification (Good and Davidson, 2016). Of particular concern for the Atlantic salmon aquaculture industry is the plasticity and variation in the timing of sexual maturation, particularly that which occurs prior to harvest.

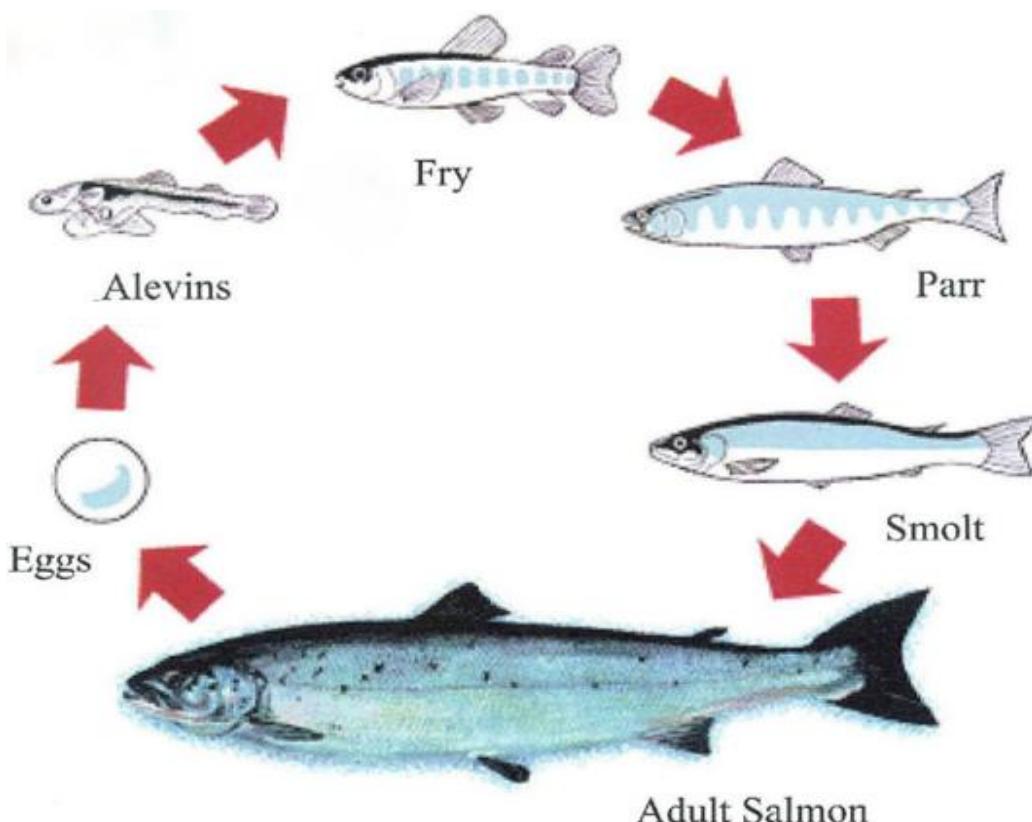


Figure 1.3: Atlantic salmon life cycle (Marine Institute, 2015)

1.2.2. Sexual maturation in Atlantic salmon aquaculture

The term ‘sexual maturation’ refers to the transition of an organism to a developmental state capable of reproduction (Taranger *et al.*, 2010). In Atlantic salmon, this is a complex process in which numerous factors are capable of influencing the time and prevalence of maturation, as well as promoting and/or inhibiting sexual development (Good and Davidson, 2016). In nature, Atlantic salmon can spend 1 to 5 years in seawater until they reach sexual maturity. While most adults mature after 2 to 3 years, some will only spend 1 year in the sea before becoming sexually mature. This type of early maturation is more commonly known as ‘grilsing’ (Good and Davidson, 2016; The Atlantic Salmon Trust, 2016). In addition, sexual maturity can also occur before the fish have left freshwater. This most often occurs in male salmon and these very early maturers are termed “precocious parr” (Saunders *et al.*, 1982; Skilbrei and Heino, 2011).

In aquaculture, sexual maturation is considered a key event compromising the performance of the stock through a range of negative effects (Leclercq *et al.*, 2011). Of all the problems faced by the Atlantic salmon aquaculture, the industry has paid particular attention to sexual maturation prior to harvest and the associated issues (Friars *et al.*, 2001; Oppedal *et al.*, 2003; Fraser *et al.*, 2012a). Sexual maturation in fish causes the transfer of energy from normal somatic growth to gonadal development. This is known to have adverse effects on body growth rates and flesh quality, and may increase incidences of disease and mortality (Ojolick *et al.*, 1995; Benfey, 2001; Leclercq *et al.*, 2011). Sexually mature fish that escape from production sites also pose a threat as their potential interactions with wild fish may impact negatively on genetics and fitness of wild populations (Glover *et al.*, 2013).

1.2.3. Management strategies for controlling pre-harvest sexual maturation

As pre-harvest sexual maturation is known to have a range of negative impacts for aquaculture, the suppression of sexual maturation is a key priority for the industry. Maturation can be controlled through a range of management strategies, which can be applied at different stages in the life cycle.

The term ‘selective breeding’ is used to describe the process by which animals and/or plants are bred to selectively develop particular genetic or phenotypic characteristics (Collins Dictionaries, 2017b). Modern breeding programmes were initiated for plants around 1900 with breeding programmes for farmed animals following in 1915 (Gjedrem *et al.*, 2012). Since the first report of selective breeding in fish (Embody and Hayford, 1925), numerous studies have been undertaken to select for characteristics such as improved

growth and disease resistance (Gjedrem *et al.*, 2012). While it is now recognised that selective breeding programmes are adopted in the aquaculture industry to improve genetic performance (Gjøen and Bentsen, 1997), studies and surveys have concluded that only around 10 % of total aquaculture production is based on genetically improved stocks (Gjedrem *et al.*, 2012). However, since the implementation of the first Atlantic salmon breeding programme in 1975, there is evidence to suggest that 97 % of world production from Atlantic salmon aquaculture is based on improved stocks (Gjerde, 2006; Gjedrem and Baranski, 2009). Initially, the breeding programmes focussed on selecting only for improved growth but interests have now expanded and current traits selected for include age at maturity and disease resistance as well as numerous characteristics associated with flesh quality. Due to the negative impacts associated with sexual maturation, in selecting for age at maturity breeding programmes must aim to reduce fecundity and stop maturation. It is recognised that salmonid breeding programmes have successfully selected for high age and size at maturity (Gjerde, 1984; Gjedrem, 2000; Taranger *et al.*, 2010). However, the strong phenotypic link between growth rate and early maturation continues to be a difficult problem to fully overcome (Taranger *et al.*, 2010). Improved feeds and husbandry practises to facilitate increased growth are considered to have the potential to negate the results of selective breeding (Taranger *et al.*, 2010). In addition, the selection of late maturing age may have a negative impact on production timings, not only for commercial aquaculture but also in terms of broodstock for breeding programmes (Ayllon *et al.*, 2015). Considering this, it is essential that conflicts of interest between growth and maturation be avoided in order for aquaculture to fully benefit from selective breeding programmes.

Photoperiod manipulation is another method which can be employed in aquaculture to delay or inhibit sexual maturation. The term “photoperiod manipulation” refers to the act of artificially altering the daily duration of light and darkness that an organism is exposed to. It has been extensively demonstrated that this method can be an effective tool in the delay or advance of maturity in farmed fish and has successfully altered sexual maturation in a large number of commercial fish species including rainbow trout, Chinook salmon, Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*) and Nile tilapia (*Oreochromis niloticus*) (Hansen *et al.*, 2001; Imsland and Jonassen, 2003; Biswas *et al.*, 2005; Unwin *et al.*, 2005; Taylor *et al.*, 2008; Taranger *et al.*, 2010). The method has also been shown to successfully alter sexual maturation in the Atlantic salmon (Hansen *et al.*, 1992; Oppedal *et al.*, 1997; Taranger *et al.*, 1998; Endal *et al.*, 2000). When used in Atlantic salmon aquaculture, continuous light (LL) treatment is generally applied from mid-winter

in seawater onwards with the aim to postpone maturation in fish until the following year and that fish will remain immature for the duration of the production cycle (Endal *et al.*, 2000). This technique is readily being standardised and applied in aquaculture, having proved an effective way to reduce the occurrence of early maturation in commercial scale salmon cages (Porter *et al.*, 1999; Hansen *et al.*, 2000). However, despite the clear benefits of photoperiod manipulation (Taranger *et al.*, 2010), there are a number of potential problems that need to be considered. These can include potentially high running costs, fish welfare issues and the overall difficulty of achieving full photoperiod control in sea cages as well as the knowledge that maturation is affected by a range of factors (*e.g.* age, feeding, photoperiod history) and not photoperiod alone (Migaud *et al.*, 2007; Leclercq, 2010; Taranger *et al.*, 2010). As such, these factors must be assessed for photoperiod manipulation to be fully suitable for the control of maturation in commercial farming settings

Of all the methods currently considered and utilised for the management of sexual maturation, artificial sterilisation through triploidisation has been shown to be the only method to completely and permanently suppress maturation.

1.3. ARTIFICIALLY STERILE, TRIPLOID SALMON

The term '*ploidy*' refers to the number of chromosome sets present within the nucleus of a biological cell (Beaumont *et al.*, 2010; Yildiz, 2013). The most common chromosome arrangement found in animals is two sets (2N) and is referred to as '*diploid*'. However, not all organisms have this number of chromosome sets – those possessing more are known as '*polyploids*' and specific terms such as triploid (3N) and tetraploid (4N) are used to describe organisms with 3 and 4 chromosome sets, respectively (Campbell and Reece, 2005).

1.3.1. History & production of triploid fish

In nature, triploidy in fish is caused by disturbances in egg ripening such as extreme increases or decreases in temperature which result in second polar body expulsion being blocked during meiotic division (Flajšhans *et al.*, 1993; Maxime, 2008). The unpaired third chromosome set then causes irregular meiotic division and is responsible for their sterility (Tiwary *et al.*, 2004; Campbell and Reece, 2005). This functional sterility represents a number of economic and ecological advantages to the aquaculture industry (Galbreath and Thorgaard, 1995; Maxime, 2008; Weber *et al.*, 2013). Economic advantages include the potential for faster growth and shorter production cycles, reduction in quantity of downgrades at harvest as flesh quality is not reduced and less need for potentially costly

photoperiod regimes (Taranger *et al.*, 2010; Taylor *et al.*, 2013, 2014, 2015). Ecological advantages of triploidy include preventing the potential interactions and introgression of escapees with wild populations (Cotter *et al.*, 2000; Taranger *et al.*, 2010; Taylor *et al.*, 2012, 2013). Importantly, only females appear to be fully sterile as males have been found to develop gonads, although the sperm is aneuploid and, as a result, unable to produce viable offspring (Benfey *et al.*, 1986). As such, since the first attempts at artificial induction of triploidy in the 1940's and 1950's (Makino and Ozima, 1943; Swarup, 1959a), and further research involving aquaculture species during the 1980's, triploidy can now be readily induced through chemical, temperature (hot or cold) or hydrostatic pressure shocks at a specific time-point post-fertilisation, although this is dependent on the species (Figure 1.4) (Chourrout, 1980, 1984; Thorgaard, 1986; Piferrer *et al.*, 2009). This method of egg shocking is the quickest, most cost-efficient way to produce large batches of triploid fish. The induction of triploidy through the application of a hydrostatic or temperature 'shock' to newly-fertilised eggs has now been improved and perfected for a wide range of commercially important aquaculture species including Atlantic salmon, rainbow trout, brown trout, turbot and grass carp (*Ctenopharyngodon idella*) (Tiwary *et al.*, 2004; Maxime, 2008; Piferrer *et al.*, 2009; Dunham, 2011; Preston *et al.*, 2013).

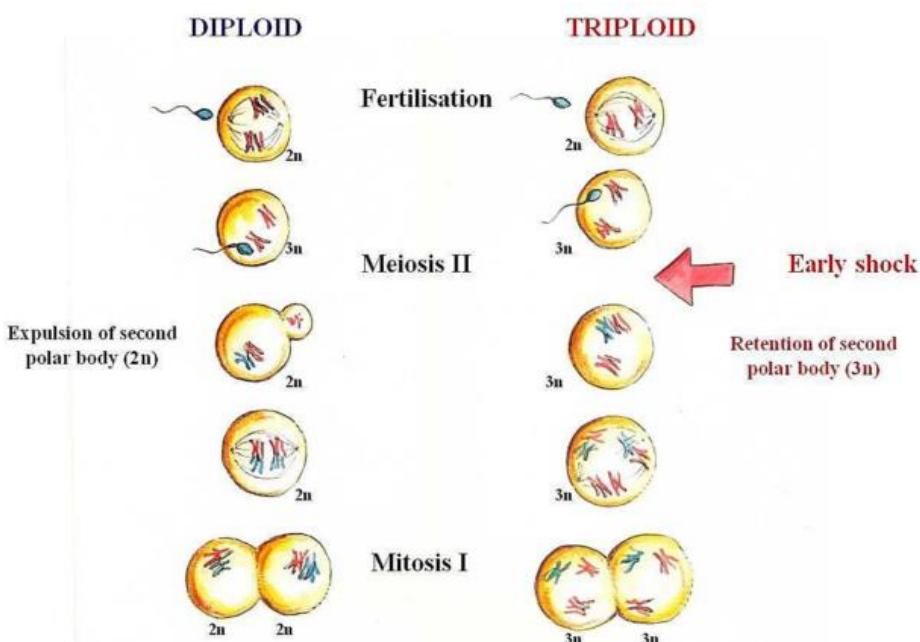


Figure 1.4: Ploidy manipulation in fish. Eggs released at metaphase of meiosis II. Fertilisation resumes meiosis. A physical or chemical shock at meiosis can suppress cell division while allowing chromosomal division, producing triploids by retention of the second polar body (Adapted from Dr. Tom Hansen, IMR, Norway).

1.3.2. Triploids in aquaculture and resultant research

Despite the many advantages that triploids present for aquaculture, to be considered for commercial production they must be able to perform at least as well as diploids in all aspects of their biology (O’Flynn *et al.*, 1997; Cotter *et al.*, 2002; Oppedal *et al.*, 2003). To address this concern, extensive research has been ongoing to enhance the current knowledge and understanding of triploid Atlantic salmon biology, physiology, production and welfare.

In terms of overall appearance, diploid and triploid Atlantic salmon are generally morphologically identical (Benfey, 2001; Taylor *et al.*, 2007). However, triploids have been shown to differ from diploids in three major ways: cell size and number, prevalence of morphological deformities and environmental tolerance (Ojolick *et al.*, 1995; Benfey, 2001; Taylor *et al.*, 2007; Atkins and Benfey, 2008; Maxime, 2008). Triploids have three sets of chromosomes rather than the diploid two and, as such, the nuclei of cells must be larger to accommodate this extra DNA (Dunham, 2011; Fraser *et al.*, 2012b). To compensate for these larger cells, triploids have been found to possess fewer cells compared to diploids. As a result, they are able to maintain the cell’s cytoplasm to nucleus ratio as well as relative organ size (Swarup, 1959b; Cogswell *et al.*, 2002; Tiwary *et al.*, 2004; Dunham, 2011). In one of the earliest studies examining triploid fish, Swarup (1959b) consistently found that the average nuclei and cell size of triploid three-spine stickleback (*Gasterosteus aculeatus*) were noticeably larger than that of diploids. The study also revealed that, while organ size had the same dimensions in both diploids and triploids, the cells were, again, larger in size but also fewer in number. A study by Benfey and Sutterlin (1984) found further evidence to support the findings of Swarup (1959b). Their study examined the haematology of diploid and triploid Atlantic salmon and found that triploids had a larger mean erythrocyte volume but had lower erythrocyte counts than diploids. Further studies have continued to consistently find reduced cell number and increased cell size in triploid Atlantic salmon as well as highlighting the tendency for a more elongated appearance in erythrocytes (Sadler *et al.*, 2000a; Benfey, 2001; Cogswell *et al.*, 2002; Taylor *et al.*, 2007). However, Fraser *et al.* (2012b) commented that the increased size and reduced number of cells found in triploids has not yet been confirmed in all cell types. The authors suggest that the increase in size and reduction in number may not apply to cells in which the nucleus occupies a small portion of the total cell volume and as such, this is an area that requires further study.

In addition to altered cell size and number, triploidy is regularly associated with skeletal and other anatomical deformities (Maxime, 2008; Leclercq *et al.*, 2011; Hernández-Urcera *et al.*, 2012). While it is recognised that deformities also occur in diploid Atlantic

salmon, there appears to be increased prevalence in their triploid counterparts (Benfey, 2001; Witten *et al.*, 2009; Fjelldal *et al.*, 2012). This is an important consideration for the aquaculture industry in terms of commercial triploid production and welfare (Piferrer *et al.*, 2009; Fraser *et al.*, 2012b). In an early study assessing shortened opercula and lower jaw deformities (LJD) in triploid Atlantic salmon, Sutterlin *et al.* (1987) found that, while ploidy did not affect the prevalence of shortened opercula, it had a significant impact on LJD with triploids showing higher levels (26 - 51 %) than diploids (0 - 4 %). The authors suggest that the increased deformities may be linked to a dietary deficiency or environmental factors. Over the years, studies continued to find increased prevalence of deformities, including LJD, vertebral deformities and cataract formation, in triploid Atlantic salmon compared to diploids (O'Flynn *et al.*, 1997; Sadler *et al.*, 2001; Fjelldal and Hansen, 2010; Leclercq *et al.*, 2011; Taylor *et al.*, 2013). However, as with Sutterlin *et al.* (1987), all authors suggest that the prevalence of deformities in triploids may be linked to other influencing factors, particularly environmental factors and nutritional deficiencies. Considering this, more recent research advances have demonstrated that deformity prevalence in triploid Atlantic salmon can be decreased with improved temperatures and the supplementation of dietary components. Taylor *et al.* (2015) showed that the supplementation of a high histidine level (17.4 g kg^{-1}) in seawater diets reduced the progression of cataract formation in triploids compared to those fed a low histidine diet (12.6 g kg^{-1}). In the same year, Fraser *et al.* (2015a) incubated diploid and triploid egg batches at 6, 8 and 10 °C until first feeding to assess the effect of temperature on deformity in triploids. The results showed that, although triploids continued to have more deformed vertebrae and jaws than diploids, decreasing temperature caused a significant reduction in triploid deformities, with the 6 °C group showing the lowest number and 10 °C showing the highest. As a result, subsequent studies reared both diploid and triploid Atlantic salmon to first feeding at lower temperatures (5 – 8 °C). Fjelldal *et al.* (2016) assessed the short and long-term effects of low (7.1 g kg^{-1}), medium (9.4 g kg^{-1}), and high (16.3 g kg^{-1}) phosphorus diets on triploids when fed from first feeding to seawater transfer. The authors found that as the dietary phosphorus increased, the prevalence of deformities decreased. While triploids in the low and medium phosphorus diet groups continued to show more deformities than the diploids, the high phosphorus diet resulted in comparable deformity levels between ploidy. Smedley *et al.* (2016) also found similar proportions of severely affected individuals (> 10 deformed vertebrae) in both the triploids fed a boosted nutrient diet (increased protein and phosphorus) and diploids fed a standard diet, while triploids fed a standard diet showed a significantly higher proportion of

severely affected individuals. However, it should be noted that, as with Fjelldal *et al.* (2016), the general trend was for triploids to show increased deformities compared to diploids. As such, continued work to optimise rearing conditions and nutrition for triploids is required to reduce the gap between ploidy.

Further to cell size, cell number and deformity prevalence, triploidy can also be associated with reduced tolerance to environmental changes (Ojolick *et al.*, 1995; Atkins and Benfey, 2008; Hansen *et al.*, 2015). Alterations in environmental conditions are commonplace in salmon aquaculture, particularly in seawater, and the tolerance of triploids to these changes represents an important point in terms of their application in the industry. In an early study, Myers and Hershberger (1991) found that a temperature spike (average 18.4 °C) in the latter half of their study caused increased mortality and disease susceptibility in triploid rainbow trout. The authors suggest that, while survival and growth were similar between ploidy, triploids have a lower thermal tolerance than diploids and that suboptimal rearing conditions may have a negative effect on their growth response. A subsequent study by Ojolick *et al.* (1995) assessing the effects of chronic high temperature (average 21 °C) on triploid rainbow trout would support this suggestion. Significantly higher mortality was observed in the triploids (68.5 %) compared to the diploids (39 %) at 21 °C, with diploids significantly heavier (+ 23.9 %) and longer (+ 4.8 %) than the triploids by the end of the 23 day study. However, the authors suggest that the cause of these effects may not have been suboptimal temperature alone. It is recognised that increasing water temperature can limit the availability of oxygen as well as increase the metabolic rate of fish (Johnston and Dunn, 1987; Wetzel, 2001). As such, it is suggested that triploids, due to the high temperature experienced and lower blood oxygen content, have not been able to cope with the chronic demand for oxygen caused by increased metabolism. However, the authors concluded that further study would be required to elucidate these relationships (Ojolick *et al.*, 1995). Subsequently, Hyndman *et al.* (2003a and b) assessed the effects of exhaustive exercise at 9 °C and 19 °C in diploid and triploid brook trout (*Salvelinus fontinalis*). At 9 °C, the authors observed similar anaerobic capacities between diploids and triploids. However, ploidy differences were observed when fish were exercised at 19 °C: As in previous studies, they found significantly increased mortality in triploids (90 %) compared to diploids (0 %). While diploids and triploids were shown to respond similarly in terms of plasma glucose and lactate, ploidy had a significant effect on the utilisation and recovery of muscle metabolites. Triploids utilised less phosphocreatine (PCr) and more glycogen than diploids and also had difficulty restoring these metabolites to pre-exercise levels. These findings

would suggest that triploids have difficulty utilising anaerobic pathways. In continued support of the previous findings, Atkins and Benfey (2008) assessed the effects of acclimating Atlantic salmon to 12, 15 and 18 °C. They found that routine metabolic rate was higher in triploids than in diploids at low temperatures (12 and 15 °C) while the opposite was observed at high temperature (18 °C), so highlighting the lower thermal optimum for triploids. However, they also highlighted that increased cell size in triploids results in reduced cell surface area to volume ratios and suggest that the lower metabolic rates of animals with larger genomes and cell size is caused by this ratio reduction. However, further studies would be required to assess this effect.

Hansen *et al.* (2015) subsequently assessed the effects of high temperature (19 °C) combined with normoxia (100 % O₂ saturation) or hypoxia (70 % O₂ saturation) on production performance parameters, such as feed intake, growth and mortality, in Atlantic salmon. They found that triploids had lower feed intake than diploids at both levels of oxygen saturation, with the lowest feed intake observed in the triploid hypoxic group. The same was observed for growth rate, as the triploid hypoxic group show significantly lower growth rate than the other three groups. While mortality was generally low in all groups (< 4 %), it was again the triploid hypoxic group which was the worst affected, with significantly higher mortality (3.35 %) than all other groups (0.11, 0.23 and 0.97 %). Behavioural changes were also observed between diploids and triploids, with diploids behaving normally and triploids exhibiting ram ventilation and swimming against the current. It is clear that the combination of high temperature and low oxygen availability severely affected triploid salmon. The authors of the study concluded that, consistent with previous studies, triploids have lower anaerobic metabolic scope than diploids at high temperature and that reductions in feed intake and behavioural changes in response to hypoxia may be indicative of changes to reduce O₂ demand and negate the consequences of negative changes in temperature and/or O₂ availability. They also suggest that the increased sensitivity of triploids to environmental changes may affect the geographical distribution of triploid salmon farming. Most recently, and in further support of Hansen *et al.* (2015), Sambraus *et al.* (2017) also assessed the impact of temperature and O₂ availability on triploid Atlantic salmon. They found that, while mortality between groups was similar, the highest mortalities were observed in the triploid group with high temperature (18 °C) and low O₂ (60 % saturation). In addition, triploids were found to have higher feed intake at < 9 °C but lower feed intake at ≥ 15 °C compared to diploids. In terms of the triploid groups, feed intake at both high and low temperatures was lower with 60 % O₂ saturation than 100

%. The authors conclude that triploids have a lower temperature optimum for feeding and growth and may be seriously affected by suboptimal conditions. They suggest that to optimise performance and welfare for commercial triploid aquaculture, production sites should be carefully selected based on environmental profiles (*e.g.* seasonal temperature range, oxygen saturation). Overall, it can clearly be seen that triploids appear to be more sensitive to environmental changes than diploids but it is recommended that further studies be undertaken to elucidate the utilisation of anaerobic pathways in triploids. Additionally, work is required to determine any relationships between environmental extremes and disease susceptibility.

In terms of performance, growth and survival are key aspects for the consideration of triploid Atlantic salmon in full commercial production (Tiwary *et al.*, 2004). It would be expected that triploids show improved growth and survival over diploid counterparts as the lack of sexual development avoids the growth depression and increases in mortality often associated with the pre-harvest sexual maturation of diploids (Tiwary *et al.*, 2004; Piferrer *et al.*, 2006, 2009; Maxime, 2008). However, a number of studies have reported reduced performance in triploids compared to diploids with impaired growth in freshwater (Johnstone, 1992; Galbreath *et al.*, 1994; Taylor *et al.*, 2011) and seawater (Galbreath and Thorgaard, 1995; McCarthy *et al.*, 1996) previously described for triploid Atlantic salmon. Despite this, a recent study by Taylor *et al.* (2014) investigating the effects of rearing triploid Atlantic salmon communally with diploids or in isolation could provide an explanation for these differences. The study found that, while triploid weight was slightly lower than diploids when raised in isolation (- 6.7 %), communal rearing severely impacted the triploids and their resultant weight was 26.4 % lower than their diploid counterparts. Additionally, it was found that triploids reared communally weighed significantly less (29.6 %; approximately 1 kg) than their isolated counterparts. As such, communal rearing of diploids and triploids in these previous studies along with the more dominant nature of diploids could explain the suboptimal growth performance reported for triploids (Galbreath and Thorgaard, 1995; Taylor *et al.*, 2014). In addition, studies have also found reduced survival during the early freshwater life stages (egg to first feed) (Johnstone, 1992; McGeachy *et al.*, 1995; Withler *et al.*, 1998; Cotter *et al.*, 2002; Fjelldal and Hansen, 2010; Taylor *et al.*, 2013). However, a range of factors have been suggested which may attribute to this decreased freshwater survival including egg/gamete quality, poor fertilisation, the triploidisation procedure and associated handling as well as suboptimal conditions.

Despite these negative findings, more data exists to support equal or better performance in triploids. In the early study by Swarup (1959b), growth was not significantly different between diploid and triploid three-spine sticklebacks. Concurringly, Johnson *et al.* (1986) measured the body weight, fork length and condition factor of coho salmon in freshwater and seawater, and found no significant differences between diploids and triploids. Johnson *et al.* (1986) also found that ploidy did not have a significant effect on seawater survival. Subsequent studies in triploid Atlantic salmon continued to report comparable or improved growth (McGeachy *et al.*, 1995; O’Flynn *et al.*, 1997; Cotter *et al.*, 2002; Oppedal *et al.*, 2003; Leclercq *et al.*, 2011; Taylor *et al.*, 2011; Fraser *et al.*, 2012a) and survival post-first feeding and in seawater (Johnstone, 1992; McGeachy *et al.*, 1995; Withler *et al.*, 1998; Leclercq *et al.*, 2011; Taylor *et al.*, 2011, 2013) compared to diploids. A very recent study by Fjelldal *et al.* (2016) investigated the short- and long-terms effects of feeding diploid and triploid Atlantic salmon during freshwater with diets containing low (7.1 g kg^{-1}), medium (9.4 g kg^{-1}) and high (16.3 g kg^{-1}) levels phosphorus. The study found that the use of low phosphorus in the diet of triploids reduced survival and weight compared to all other groups. In both the medium and high phosphorus diet groups, triploids were found to have significantly higher weight and length than their diploid counterparts throughout the study. However, at harvest (trial termination), the weight of all triploid groups was significantly lower than their respective diploids. It could be suggested that this may have been a result of the groups being fed a standard commercial diet during their time in seawater and that they require consistently enhanced diets throughout the lifecycle. To support this suggestion, Smedley *et al.* (2016) investigated the effects of supplementing the feed of triploids with increased protein and phosphorus throughout seawater. They found that triploids fed the supplemented diet were significantly heavier at harvest than both triploids and diploids fed a standard commercial diet. These findings continue to support equal or better performance in triploids compared to diploids but further research is required to fully elucidate the optimal conditions and nutritional timeline for triploid Atlantic salmon.

However, despite the recent research advances and continued work to fully elucidate the biological and physiological differences between diploid and triploid Atlantic salmon, little work has been undertaken on triploid health and immune response.

1.4. FISH IMMUNE SYSTEM

The term ‘immune system’ refers to the system of biological structures and processes within all living organisms which is able to detect and protect against a range of disease agents,

and distinguish them from healthy body tissue (Rauta *et al.*, 2012). The immune system is generally divided into two, depending on the specificity and speed of the response (Dixon and Stet, 2001; Parkin and Cohen, 2001; Roberts, 2012). These are termed the innate (or natural) and the adaptive (or specific) immune systems (Fearon and Locksley, 1996; Campbell and Reece, 2005; Roberts, 2012). However, it is widely recognised that the two are not separate entities and complex interactions occur between them (Parkin and Cohen, 2001; Watts *et al.*, 2001; Rauta *et al.*, 2012). Innate immunity is evolutionarily older and is considered to be found in some form in all multicellular organisms (Fearon and Locksley, 1996). Present from the time of birth, it provides rapid, non-specific defence against a wide range of organisms, regardless of their identity. Adaptive immunity is considered to have occurred later in evolution than innate immunity and is only present in vertebrates (Fearon and Locksley, 1996; Tort *et al.*, 2003). It develops only after exposure to a foreign stimulus *e.g.* microbes, pathogens, abnormal body cells or toxins, and involves an adaptive change in the lymphoid system resulting in specific immune memory, making it essential in protection against recurring infections (Rauta *et al.*, 2012; Roberts, 2012).

Due to their basal position in vertebrate phylogeny, fish are considered to form an important link between higher vertebrates and invertebrates as well as occupying the apparent evolutionary crossroads between the innate immune system and the development of the adaptive immune system (Levraud and Boudinot, 2009; Herath, 2010). However, while physiologically similar, a number of important differences exist between the immune systems of fish and higher vertebrates. For example, fish are in permanent contact with the surrounding aquatic environment and as such, may be challenged by pathogens from very early life stages (Ellis, 2001; Rombout *et al.*, 2005; Uribe *et al.*, 2011). As such, while fish possess both innate and adaptive immunity, it is recognised that their innate immunity is stronger and they are highly dependent on it for protection and survival (Whyte, 2007; Uribe *et al.*, 2011; Rauta *et al.*, 2012). An overview of the fish immune system is presented in Figure 1.5.

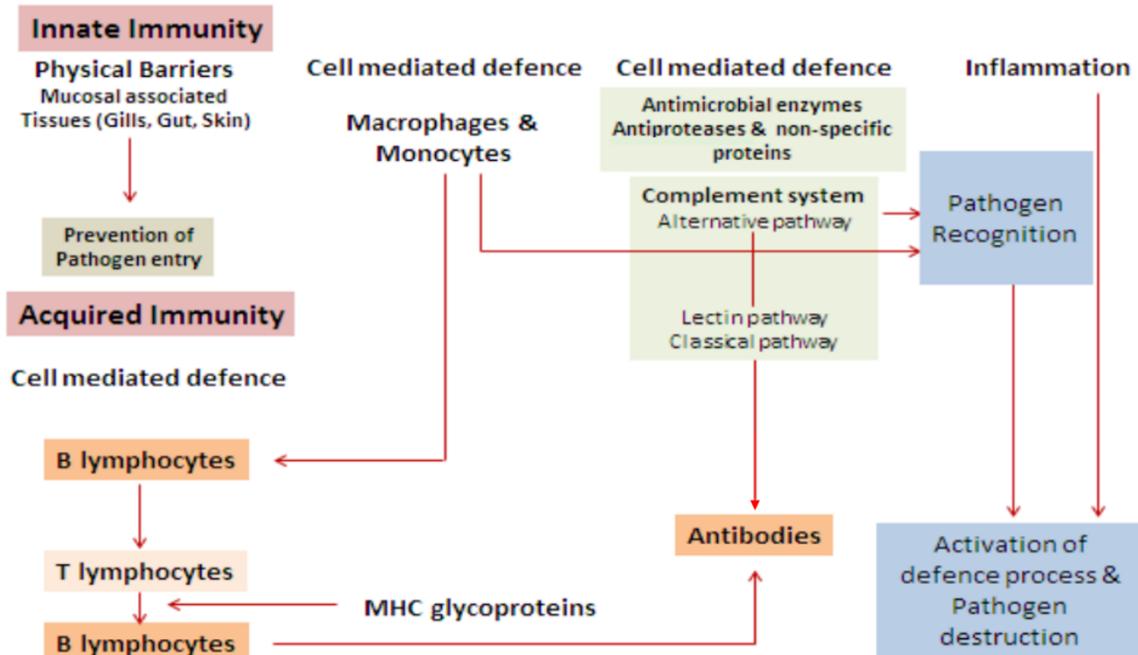


Figure 1.5: Overview of the fish immune system (Metochis, 2014)

1.4.1. Lymphoid organs in fish

In higher vertebrate groups such as mammals, there are numerous lymphoid organs including the thymus, bone marrow, lymph nodes and the spleen (Lymphoma Association, 2012). In fish, while many of the same organs are present, they lack bone marrow and lymph nodes. As a result, the roles played by these organs are taken over by the anterior kidney, spleen, thymus and mucosa-associated lymphoid tissues (Press and Evensen, 1999; Alvarez-Pellitero, 2008; Uribe *et al.*, 2011).

The anterior or head kidney is morphologically similar to mammalian bone marrow and is a known lymph node analogue and, as such, assumes haemopoietic functions and serves as a secondary lymphoid organ (Dalmo *et al.*, 1997; Press and Evensen, 1999; Uribe *et al.*, 2011). The head kidney also plays a critical role in phagocytosis, the trapping and clearance of particles from the bloodstream and antibody (immunoglobulin M, IgM) production (Press and Evensen, 1999; Herath, 2010; Uribe *et al.*, 2011; Metochis, 2014). Furthermore, melanomacrophage centres (MMCs) in the head kidney are known to trap and retain antigens thus, aiding in the development of immunological memory (Press and Evensen, 1999; Agius and Roberts, 2003).

The spleen plays a secondary role compared to the head kidney in terms of both innate and adaptive immune responses (Kiron, 2012). It is made up of a reticular cell network supporting blood-filled sinusoids that contains diverse cell populations including

blood cells, endothelial cells, reticular cells, macrophages and MMCs (Press and Evensen, 1999; Kiron, 2012). As well as haematopoietic functions, the spleen is also known to be involved in macromolecule clearance, antigen degradation and antibody production (Dalmo *et al.*, 1997; Kiron, 2012).

The thymus is a paired organ located near the opercular cavity in fish and is essential in the development of a fully functional immune system (Romano *et al.*, 1999; Rauta *et al.*, 2012). Two parts can usually be distinguished, the outer layer or cortex and the inner layer or medulla, although the division of the two is highly species-specific and often not clear (Press and Evensen, 1999; Uribe *et al.*, 2011). It is essential in the production of T lymphocytes, which are involved in phagocyte stimulation and antibody production by B cells.

1.4.2. Innate immune system

1.4.2.1. Physical barriers

Physical barriers, provided by the skin, gills and mucosal membranes, offer fish essential first line defence against invading pathogens (Ellis, 2001; Subramanian *et al.*, 2007; Huang *et al.*, 2011; Esteban, 2012). The defensive role of skin mucus has been well studied in fish (Magnadóttir, 2006). This unique barrier, consisting of glycoproteins, proteoglycans and proteins, is continually produced and sloughed off, effectively trapping pathogens and preventing attachment (Ellis, 2001; Esteban, 2012; Secombes and Ellis, 2012). Also essential in the prevention of tissue colonisation and invasion is the diverse range of antimicrobial factors found in mucus, such as lectins, complement proteins and lysozyme (Magnadóttir, 2006; Kiron, 2012). If pathogens successfully breach the physical barriers, they are traditionally considered to encounter the cellular components and humoral parameters of the innate immune system (Bayne and Gerwick, 2001; Ellis, 2001; Nürnberger *et al.*, 2004; Whyte, 2007; Secombes and Ellis, 2012).

1.4.2.2. Cellular components

In fish, the key cellular components involved in the innate immune system are neutrophils and monocytes/macrophages as well as non-specific cytotoxic cells and, to a lesser extent, epithelial and dendritic cells (Dalmo *et al.*, 1997; Magnadóttir, 2006). Neutrophils and monocytes/macrophages are highly phagocytic, able to engulf a wide range of particles including carbon, bacteria and yeast. Phagocytosis is one of the most important defence mechanisms possessed by fish as it is the least influenced by temperature (Uribe *et al.*,

2011). Respiratory burst, important in bactericidal activity, is also induced during phagocytic processes due to the synthesis of oxygen and nitrogen free radicals (Dalmo *et al.*, 1997; Roberts, 2012).

1.4.2.3. Humoral parameters

Humoral parameters refer to the aspect of the immune system mediated by macromolecules found in extracellular fluids (Roberts, 2012; Jacobs *et al.*, 2016). These parameters are commonly classified according to their function. Growth inhibitors can either deprive pathogens of essential nutrients or interfere with metabolism. For example, transferrin is a glycoprotein which is known to have high iron binding activity. As iron is essential for pathogen maintenance and infection establishment, the reduction in iron availability for pathogens caused by transferrin is a key protective effect.

Specific enzymes are produced by many pathogens in order to gain entry into, and obtain nutrients from the host. As such, enzyme inhibitors provide an important defence mechanism against these pathogens by neutralising the enzymes they produce. The most extensively studied of these is the anti-protease, α 2-macroglobulin (α 2-M), whose inhibitive properties involve the physical encapsulation of a protease (Magnadóttir, 2006).

The complement system is an enzyme cascade made up of over 30 protein components. It can be activated in three ways: the classical, alternative and lectin pathways. The alternative pathway, which is independent of antibodies, is activated directly by pathogenic material. It has a variety of roles including cell lysis, pathogen clearance through mediation of the inflammatory response and promotion of phagocytosis. The alternative complement pathway in fish is considered the most important for the function of the innate immune response (Whyte, 2007; Uribe *et al.*, 2011; Roberts, 2012).

Lysozyme is a key bacteriolytic enzyme in the innate immune response of fish. (Uribe *et al.*, 2011). Lysozyme is known to have different modes of action against Gram-positive and Gram-negative bacterium (Saurabh and Sahoo, 2008). Against Gram-positive bacteria, lysozyme hydrolyses the bonds in the cell wall peptidoglycans (N-acetylmuramic acid and N-acetylglucosamine) which causes lysis and prevents invasion (Saurabh and Sahoo, 2008). While Gram-negative bacteria are not directly affected by lysozyme, the activation of the complement system by lysozyme disrupts the outer cell wall of the bacteria, exposing the inner peptidoglycan layer which lysozyme can then attack (Magnadóttir, 2006; Saurabh and Sahoo, 2008; Uribe *et al.*, 2011). In addition to antibacterial functions,

lysozyme promotes phagocytosis by directly activating leucocytes and macrophages (Magnadóttir, 2006; Saurabh and Sahoo, 2008; Uribe *et al.*, 2011).

1.4.3. Adaptive immune response

As the innate immune response takes action against a pathogen or foreign body, cells such as macrophages will secrete chemokines (a protein group) in order to activate the adaptive immune response, recruiting cells to the infected areas (Reyes-Cerpa *et al.*, 2012; Wang and Secombes, 2013). This is a key example of interactions that occur between the innate and adaptive immune systems.

Lymphocytes are the key cell group involved in adaptive immunity. Lymphocytes are divided into two main types, T-lymphocytes (or T-cells) and B-lymphocytes (or B-cells). T-cells are responsible for co-ordinating the cell-mediated response, while B-cells direct the humoral response (Shoemaker *et al.*, 2001; Mutoloki and Jørgensen, 2014). Following pathogen invasion, T-cells become activated when antigens have been processed into smaller fragments and are presented on the surface of antigen-presenting cells by major histocompatibility complex (MHC) molecules (Campbell and Reece, 2005; Secombes and Wang, 2012). Then, depending on the class of MHC molecule involved, T-cells will differentiate into either cytotoxic T cells or T helper (Th) cells. Cytotoxic T cells, associated with antigen presentation by class I MHC molecules, are able to directly kill infected or abnormal cells by releasing molecules to induce apoptosis (Laing and Hansen, 2011; Mutoloki and Jørgensen, 2014). The Th cells, associated with antigen presentation by class II MHC molecules, act indirectly by secreting cytokines to activate and co-ordinate the response of other immune cells such as B-cells and macrophages (Laing and Hansen, 2011; Mutoloki and Jørgensen, 2014). The release of cytokines by activated Th cells stimulates B-cell activity. Active B-cells then recognise intact antigens, resulting in cell proliferation and differentiation and, ultimately, the expression and secretion of antibodies by B-cells (Campbell and Reece, 2005; Reyes-Cerpa *et al.*, 2012). Antibodies are a class of proteins called immunoglobulins (Ig) and three classes of Ig isotypes have been identified in fish (IgM, IgD and IgT). The most abundant of isotype is IgM which is used as a marker for protection against several extracellular bacterial diseases as well as viral diseases. In addition, B-cells in certain fish species have also been found to have phagocytic properties (Li *et al.*, 2006).

In aquaculture practices, it is common for fish to be stocked in high numbers and this can result in disease occurrence. The intensive stocking of fish may compromise

physical barriers, through the development of abrasions which can allow the passage of pathogens to the tissues (Kiron, 2012). Systemic defences then take over, providing immediate, non-specific protection. Nonetheless, pathogens may be able to evade these innate responses, at which time the adaptive immune response is activated. The immune system adapts its response during an infection to improve its recognition of the pathogen, which is then retained as immunological memory following pathogen elimination. This allows the adaptive immune response to mount a faster and stronger attack against the pathogen if a repeat encounter occurs.

1.4.4. External factors affecting the immune response

It is widely recognised that a range of factors can affect the immune responses of teleost fish. Many of these factors are external or extrinsic, meaning they do not form part of the essential nature of the organism and operate from outside the organism (Roberts, 2012; Collins Dictionaries, 2017c). The environment is recognised as one of the most important extrinsic factors in modulating the innate and adaptive immune responses in fish, due to the poikilothermic nature of fish and the close relationship they have with their surroundings (Le Morvan *et al.*, 1998; Magnadóttir, 2010). While a wide range of environmental factors such as oxygen level, salinity and pH are known to affect fish immunity, photoperiod and temperature are considered two of the key regulators of immune response.

Photoperiod, the duration of daily light exposure, is one of the key controllers of all living organisms (Bowden, 2008; Collins Dictionaries, 2017d). As animals possess complex systems for monitoring and perceiving light, photoperiod acts as a cue for both circadian (daily) and seasonal rhythms (Bowden, 2008). Changes in these rhythms and in photoperiod causes animals to alter their physiology accordingly, including their immune response (Bowden, 2008). Studies have been undertaken to assess the effect of photoperiod on the immune system. In a study assessing the effect of a constant light/dark (LD) 12:12 photoperiod on the innate immune system of seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax* L.), Esteban *et al.* (2006) found that complement activity in both species was significantly higher during the day than at night. Lysozyme in seabream was found to increase from 08:00 onwards, with the highest activity recorded between 20:00 and 02:00 hours, before decreasing (Esteban *et al.*, 2006). In their 142 day experiment, Burgos *et al.* (2004) also found an increase in lysozyme activity when utilising artificial photoperiods (LD 12:12, 24:0, 14:10) in rainbow trout. The lysozyme activity in the LD 14:10 group increased to day 30 before decreasing while the LD 24:0 group lysozyme

increased to day 7. They suggest that different photoperiods (“illumination protocols”) induce different alterations to the innate immune system over time. Conversely, Bowden *et al.* (2004) found no significant differences in lysozyme activity in Atlantic halibut (*Hippoglossus hippoglossus* L.) during long day (LD 16:8) and short day (LD 8:16) photoperiods at ambient temperature (10 – 14 °C). However, they did note that lysozyme activity was significantly higher in summer than in winter. In addition, photoperiod changes had no effect on tilapia (*Oreochromis niloticus*): No significant difference in lysozyme concentration was observed between a group subjected to decreasing light and temperature or a control group experiencing constant light (LD 12:12) with decreasing temperature (Atwood *et al.*, 2003). From these studies, it can be suggested that different species experience light in different ways. As suggested by Bowden (2008), the effect of photoperiod may also be related to the normal geographical location of a particular species, and photoperiod could be less of a driver for seasonal physiological changes in tropical fish than in temperate species.

As poikilotherms, temperature is a fundamental extrinsic factor in the entire physiology of teleost fish (Le Morvan *et al.*, 1998). It is considered essential in the development and modulation of both the innate and adaptive immune systems in fish and, as such, the effects of temperature on immune responses have been well studied over the years (Le Morvan *et al.*, 1998; Watts *et al.*, 2001; Roberts, 2012). It is now considered that temperature effects on the immune response can vary, depending on factors such as species, life stage, type and species of pathogen and thermal history as well as overall general fish health (Harrah, 2000). It is widely considered that low and high temperatures, within the range for a particular species, can have inhibitory or facilitatory effects on the innate and/or adaptive immune response (Snieszko, 1974; Bly and Clem, 1992; Harrah, 2000; Magnadóttir, 2010). When assessing the effect of low (1 °C), normal (7 °C) and high (14 °C) temperatures of the immune responses of Atlantic cod, Magnadóttir *et al.* (1999) found that innate parameters such as haemolytic activity and anti-protease activity increased at low temperatures while adaptive parameters like IgM concentrations and natural antibody production were increased at high temperatures. Supporting this is a study by Alcorn *et al.* (2002) comparing the immune response of sockeye salmon (*Oncorhynchus nerka*) at 8 and 12 °C. They found increased complement and phagocytic activity at 8 °C and greater antibody response at 12 °C. Different temperatures were also found to affect the immune response of Atlantic halibut: Langston *et al.* (2002) found that increasing temperatures (8, 12, 15 and 18 °C) caused an increase in lysozyme activity but a decrease in packed cell

volumes and circulating leucocytes (%) and suggest that the combination of these parameters indicated the fish were subjected to suboptimal conditions and may reduce the ability to resist disease. A related study of Atlantic halibut by Hoare *et al.* (2002) subsequently showed that increasing temperature resulted in reduced disease resistance to challenge with *Vibrio anguillarum* with 59 % and 29 % mortality in halibut held at 18 and 12 °C, respectively. However, they also showed that antibody production was higher at 18 °C than at 12 °C. Numerous other studies have found this pattern with antibody response (Cecchini and Saroglia, 2002; Nikoskelainen *et al.*, 2004; Martins *et al.*, 2011). Cecchini and Saroglia (2002) also highlighted a difference in the kinetics of antibody response with sea bass showing faster antibody response at 24 and 30 °C (5 days) than at 12 and 18 °C (average 31 days). In a study of channel catfish (*Ictalurus punctatus*) (Martins *et al.*, 2011), while it found the same pattern of higher antibody response at higher temperatures as Hoare *et al.* (2002), it found the opposite pattern of susceptibility with the heaviest infections with *Ichthyophthirius multifiliis* occurring at lower temperatures thus highlighting a key species difference in the immune response to temperature alterations. While there is a general consideration that innate parameters are more active at low temperatures while adaptive parameters are more active at higher temperatures (Bly and Clem, 1992; Magnadóttir, 2006, 2010), the studies discussed suggest great variation in the modulation of the immune response by temperature with innate and adaptive immune parameters being both positively and negatively affected by increases or decreases in temperature. There is clear evidence for a highly complex, species-specific relationship between temperature and immune function.

1.4.5. Diseases/Pathogens affecting Atlantic salmon and salmonids

Within the Atlantic salmon aquaculture industry, losses can occur due to a range of factors including predation and oxygen depletion but of all the loss-causing factors, disease is considered the most economically significant and production limiting (Meyer, 1991; Subasinghe, 2005). Fish in aquaculture are considered to experience higher infection pressure than their wild counterparts as they are known to experience disruptions to their normal rearing environment such as high stocking densities, extreme temperature fluctuations, insufficient dissolved oxygen (DO) and excessive handling (Meyer, 1991; Bondad-Reantaso *et al.*, 2005; Nicholson, 2006; Kiron, 2012). These can be very stressful conditions and can lead to fish becoming immunocompromised, ultimately allowing pathogens to cause infection (Mock and Peters, 1990; Tort, 2011; Turnbull, 2012). It is now recognised that many disease-causing agents for aquaculture are ubiquitous in the

environment and only become infectious when these unfavourable conditions occur (Cole, 1982; Bortman, 2003). This highlights well the knowledge that the occurrence of disease is a complex series of interactions between the host, pathogen and environment (Carrias *et al.*, 2012; Turnbull, 2012). Infectious diseases causing significant effects for the salmon aquaculture industry are regularly bacterial, viral and parasitic (Rodger, 2016).

1.4.5.1. Bacterial diseases

Bacteria are defined as “microscopic, unicellular organisms lacking a distinct nucleus” (Merriam Webster, 2017). As ubiquitous organisms, bacteria are found in virtually every environment and are known to be highly abundant in aquatic environments (Cole, 1982; Bortman, 2003). In the natural environment, many aquatic bacterial species are saprophytic and play a vital role in synthetic pathways and degradative processes (Roberts, 2012). However, these usually harmless bacteria may become pathogenic if the fish are exposed to poor water quality, low DO, or if they become physiologically stressed (Meyer, 1991). The naturally harmless bacterial species may then become opportunistic pathogens, infecting the fish rendered susceptible by the unfavourable conditions (Roberts, 2012; Purdue University, 2015). Bacterial pathogens can also be obligate and able to live in the environment but ultimately require a host for survival and transmission (Roberts, 2012). Many obligate pathogens can be found as part of the host’s natural flora and are triggered to become pathogenic by the negative conditions previously mentioned (Roberts, 2012). As a result, the interactions between pathogenic bacteria and the host’s immune response have been well studied over the years and it is now recognised that numerous components of both the innate and adaptive immune system protect against bacterial pathogens (Ellis, 1999, 2001; Uribe *et al.*, 2011). This protection begins with skin mucus, which physically traps and removes bacterial pathogens and also contains antimicrobial peptides capable of breaking down bacterial cell walls (Ellis, 2001; Rajanbabu and Chen, 2011; Uribe *et al.*, 2011; Esteban, 2012). If a pathogen gains entry into the fish, an inflammatory response will produce a signal to initiate the onset of phagocytosis (Ellis, 2001). The main cells involved with this response are neutrophils and macrophages which engulf bacterial cells and kill them mainly through the production of reactive oxygen species (ROS) during a process called respiratory burst (Secombes and Fletcher, 1992; Ellis, 1999; Uribe *et al.*, 2011). Complement and lysozyme are also recognised to be involved in protection against bacterial pathogens. Lipopolysaccharides (LPS) in the cell wall of Gram-negative bacteria are known to directly activate the antibody-independent, alternative complement pathway (ACP) and

this results in the lysis of bacterial cell membranes and the recruitment of phagocytes (Ellis, 1999, 2001). Lysozyme is a well-recognised bacteriolytic enzyme that can be found widely throughout the body of fish including in mucus, serum and tissues (Ellis, 1999; Saurabh and Sahoo, 2008; Uribe *et al.*, 2011). This enzyme hydrolyses the N-acetylmuramic acid and N-acetylglucosamine components of the peptidoglycan layer of bacterial cell walls resulting in cell lysis (Ellis, 1999). Furthermore, antibodies are recognised to play a key role in bacterial defence. Antibodies can inhibit the attachment of bacteria to the surface epithelium of the host through the production of anti-adhesins. The production of anti-toxins and anti-invasins by antibodies also provide protection by neutralising toxins produced by bacteria and blocking the invasion of bacteria into non-phagocytic cells, respectively (Ellis, 1999). Finally, antibodies bound to the surface of a bacterium can activate the classical complement pathway allowing for a much greater amount of complement to be activated as well as direct complement to the site of infection (Ellis, 1999). This further highlights the complexity of the immune system of fish and the relationships between innate and adaptive immune response.

Over the years, a range of pathogenic bacterial species have represented great concerns for the Atlantic salmon aquaculture industry, particularly Gram-negative species including *Aeromonas salmonicida* and *Vibrio* species, although successful vaccination has helped to combat many of these diseases (Adams, 2016; Rodger, 2016). *Vibrio anguillarum* is recognised as one of the earliest isolated fish pathogens (Hofer, 1904; Roberts, 2012; Rodger, 2016). The causative agent of Vibriosis, it is known to result in very high levels of mortality in aquaculture as well as experimentally, with reports of 100 % mortality (Austin *et al.*, 2005; Hickey and Lee, 2017). This disease can occur at most times of the year in aquaculture although is most severe in summer and can present in several ways. In young fish, the only signs may be anorexia, darkening and sudden mortality. In terms of older fish, the disease can progress through acute and chronic phases. In acute infection, fish can show dark swollen skin lesions which can become deep ulcers, as well as swelling and liquefaction of the spleen and kidney. Lesions on chronically infected fish become much more granulomatous and can often spread to the mouth and eyes. Immunisation has been widely shown to improve resistance against the disease caused by this pathogen with effective vaccines now readily utilised in aquaculture (Tatner and Horne, 1984; Frans *et al.*, 2011; Roberts, 2012). Previously described in the genus *Vibrio*, *Moritella viscosa* is the causative agent of “winter sore” or “winter ulcer” disease. This disease is associated with prolonged mortalities, which can reach 90 - 100 % in experimental settings, as well as

dowgrades of up to 40 % due to the formation of ulcers on the musculature (Maira *et al.*, 2007; Roberts, 2012). Due to the low temperatures at which this disease occurs along with associated anorexia, it can often be difficult to treat. However, effective vaccines have again been developed to combat this pathogen. An emerging pathogen of concern is *Flavobacterium psychrophilum*, the aetiological agent of Rainbow Trout Fry Syndrome (RTFS) (syn., bacterial cold-water disease, peduncle disease) (Lorenzen *et al.*, 1997). Rainbow Trout Fry Syndrome has been responsible for substantial economic losses in the rainbow trout industry globally, including the UK (Rodger, 2016). Recently the bacterium has also been isolated from Atlantic salmon fry in Scotland following disease outbreaks, causing concern for the industry (Starliper, 2011; Apablaza *et al.*, 2013). The presence of this disease is associated with several external and internal signs including, but not limited to, exophthalmia, abdominal swelling, darkening and anaemia; splenomegaly and swollen kidney (Ekman and Norrgren, 2003; Barnes and Brown, 2011; Boyacioglu and Akar, 2012). This pathogen is known to occur at low temperatures and represents significant economic impact for salmonid aquaculture due to elevated levels of mortality in susceptible fry and fingerlings (Leeds *et al.*, 2010; Boyacioglu and Akar, 2012). As this pathogen generally causes outbreaks in very young fish, often before feeding, it can be difficult to treat as medicated feeds cannot be utilised and antibiotic bath treatments may have little effect (Roberts, 2012). While feeds supplemented with antibiotics can be used post-first feeding, *F. psychrophilum* is considered resistant to many of the antibiotics commonly used and so, the development of effective mitigation & prevention strategies is essential in the control of this pathogen (Roberts, 2012; Hoare *et al.*, 2016; Ngo *et al.*, 2017).

Of all the pathogenic bacterial species, *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of furunculosis, is considered one of the most economically significant for Atlantic salmon aquaculture (McCarthy, 1975; Toranzo *et al.*, 2005; Rodger, 2016). Furunculosis was first described in farmed brown trout in Germany by Emmerich and Weibel (1894). Since the first isolation, *A. salmonicida* is considered to have spread to all areas with wild or farmed salmonids (*e.g.* North America, England, Scotland and Norway), excluding Australia, and has been found in fresh, brackish and sea waters (Novotny, 1978; Roberts, 2012; Midtlyng, 2014). Furunculosis can present a variety of clinical signs depending on whether infection is acute or chronic. In terms of acute infections these signs can include darkening, anorexia, lethargy, skin haemorrhages and hyperaemia as well as swollen spleen and kidney (Bullock *et al.*, 1983; Roberts, 2012; Midtlyng, 2014). More subacute or chronic furunculosis can cause the characteristic furuncles on the flank as well as

tissue and organ necrosis and mortality (McCarthy and Roberts, 1980; Roberts, 2012; Midtlyng, 2014). The severity of this pathogen over the years has made effective disease prevention essential. It is recognised that Duff (1942) undertook the first *A. salmonicida* vaccination trial with cutthroat trout (*Salmo clarkii*), supplementing feed with chloroform-killed cultures of *A. salmonicida* and finding increased antibody response and improved survival in experimental groups (75 %) compared to control groups (25 %). Following this success, numerous studies were undertaken to assess antigen preparation for, antibodies produced and protection elicited by experimental furunculosis vaccines (Krantz *et al.*, 1964; Spence *et al.*, 1965; Michel, 1979; McCarthy *et al.*, 1983) until in the early 1980's licences for the first commercial furunculosis vaccines were obtained (Midtlyng, 1997). Studies then continued to assess vaccine preparation and delivery methods as well as protection efficacy and now, a range of effective commercial vaccines against *A. salmonicida* are now widely used in salmon aquaculture (Lillehaug *et al.*, 1990, 1992; Erdal and Reitan, 1992; Midtlyng *et al.*, 1996a; Adams *et al.*, 1997; Markestad and Grave, 1997).

1.4.5.2. Viral diseases

The term “virus” can be used to define ultramicroscopic infectious agents that are only capable of multiplying within the cells of a host and consist of a nucleic acid molecule (DNA or RNA) within a protein coat (Roberts, 2012; Oxford Dictionaries, 2017). The presence of viruses in fish pathology has been described since the mid-1900's but the causative agents were unable to be confirmed (Adedeji *et al.*, 2012). The development of the technique using fish cell lines for the *in vitro* isolation of viruses facilitated viral pathogen identification and is now the standard method for viral detection (Adedeji *et al.*, 2012). The method with which a virus infects a host cell is recognised as a complex process starting first with successful attachment to the cell's surface (Ellis, 2001; Roberts, 2012). The virus then enters the cell membrane, either by endocytosis, fusion or translocation, and engages with the host cell's biochemistry (Ellis, 2001; Roberts, 2012). This interaction facilitates the replication, transcription and translocation (synthesis) of nucleic acid and protein products (Ellis, 2001; Roberts, 2012). These products are then assembled to form new viruses before finally being released into the host (Campbell and Reece, 2005; Roberts, 2012). It is recognised that several immune response parameters can act to combat viruses including one of the first lines of defence, skin mucus, which can actively trap and slough off pathogenic viruses (Esteban, 2012; Collet, 2014). In addition, the production of double-stranded RNA (dsRNA) by viruses during replication is known to initiate the production of interferon (IFN), a group

of cytokines (Ellis, 2001; Adedeji *et al.*, 2012; Collet, 2014). Interferon then induces the synthesis of proteins such as protein kinase 1 and MX proteins, which can block viral replication, and 2',5'-oligoadenylate synthetase, which degrades viral RNA (Robertsen *et al.*, 1997, 2003; Robertsen, 2006; Adedeji *et al.*, 2012). Over recent years, it has become clear that the IFN network present in fish to regulate host defences against viral infection is highly complex (Zou and Secombes, 2011). Furthermore, immune cells are also responsible for protection against viruses including macrophages (Falk *et al.*, 1995; Desvignes *et al.*, 2002), neutrophils (Rønneseth *et al.*, 2006; Montero *et al.*, 2009) and natural killer cells (Ellis, 2001). However, it is recognised that, as with mammalian viruses, fish viruses have developed ways to evade their host's responses such as the suppression of IFN and the inhibition of signalling pathways (Collet *et al.*, 2007; Collet, 2014). These methods are particularly effective when their fish host is already immunocompromised due to unfavourable conditions (Adedeji *et al.*, 2012).

Numerous viral diseases are now recognised to have a devastating economic effect on Atlantic salmon aquaculture. Infectious Salmon Anaemia (ISA), caused by the orthomyxovirus Infectious Salmon Anaemia Virus (ISAV), has been recognised as one of the most serious viral diseases in for salmonid aquaculture since the 1980's (Roberts, 2012; Rodger, 2016). This disease causes external signs such as lethargy, pale gills and exophthalmia with internal signs comprising bloody ascetic fluid in the abdomen and splenomegaly (Roberts, 2012; Rodger, 2016). Following initial infection, mortalities are slow to start and it often takes months before any mortalities occur but spread can cause a rapid increase which may result in up to 90 % mortality (Roberts, 2012; Rodger, 2016). Diagnosis is generally based on the appearance of clinical signs and detection through indirect fluorescent antibody technique (IFAT) and real-time polymerase chain reaction (RT-PCR) (Rodger, 2016). While there are no current treatments for ISA and control is reliant on culling, fallowing and good biosecurity practices on production sites, commercial vaccines to combat ISA have been produced by the vaccine companies Pharmaq (Zoetis) and Novartis Animal Health (Elanco), with work ongoing to further develop location-specific ISA vaccines (Falk, 2014).

Infectious Pancreatic Necrosis (IPN) is a serious disease caused by the birnavirus Infectious Pancreatic Necrosis Virus (IPNV). This viral disease has been problematic for salmonid aquaculture since the early 20th century with reports of losses equating to between 11 and 32 million USD per annum (Roberts and Pearson, 2005; Ruane *et al.*, 2007; Crane and Hyatt, 2011; Munro and Midtlyng, 2011). Known to most seriously affect fry and

fingerlings, the first signs are often sudden and increasingly frequent mortalities along with anorexia and abnormal swimming (Roberts, 2012; Rodger, 2016). External signs include darkening, swollen abdomen and exophthalmia with internal signs comprising pale organs and swollen intestine (Rodger, 2010; Roberts, 2012). Mortalities can vary depending on fish age and can range from 10 – 20 % in older fingerlings, parr and smolts to 70 – 90 % in fry and fingerlings (Roberts and Pearson, 2005; Ruane *et al.*, 2007; Crane and Hyatt, 2011). Survivors of the disease can become carriers, therefore maintaining the virus within the population by either horizontal transmission through shedding to other fish or by vertical transmission through ovarian and seminal fluid, and eggs (Roberts and Pearson, 2005; Ruane *et al.*, 2007; Crane and Hyatt, 2011). Due to this carrier state, it is essential that the spread of this disease be controlled, particularly through good biosecurity, avoiding the use of eggs from IPN positive broodstock, effective egg disinfection procedures and preventing transfer of IPN carrier fish to naïve sites (Ruane *et al.*, 2007; Crane and Hyatt, 2011; Rodger, 2016). More so than control, prevention is even more essential and commercial vaccines have now been developed against this pathogen (Leong and Fryer, 1993; Ruane *et al.*, 2007). While vaccines have been effective in combatting this disease, it has been reported that efficacy can be variable and losses are not completely prevented (Sommerset *et al.*, 2005; Moen *et al.*, 2015). In recent years, genetic methods have become more regularly employed in the prevention of IPN outbreaks. Two research groups from Scotland and Norway independently discovered a major quantitative trait loci (QTL) for IPN resistance, which was found to be responsible for almost all of the genetic variation in IPN resistance in Atlantic salmon (Houston *et al.*, 2008; Moen *et al.*, 2009). Following this discovery, the breeding company AquaGen tested individuals for the high IPN resistance QTL allele, implemented them into their breeding programmes and, in 2009, started marketing “IPN-QTL eggs” (AquaGen, 2010; Moen and Odegård, 2014). From the initial implementation of these egg in 2009/2010, Atlantic salmon production in Norway has reported an 87 % reduction in IPN outbreaks, from 223 sites with outbreaks in 2009 to 30 sites with outbreaks in 2015 (The Norwegian Veterinary Institute, 2016).

Pancreas disease (PD), caused by salmon alphavirus (SAV), is another disease of serious concern for aquaculture (Weston *et al.*, 1999). The gross clinical signs of PD appear as inappetence and abnormal swimming before mortalities occur (Jansen *et al.*, 2017). Post-mortem examination often reveals yellow mucoid gut contents and signs of circulatory disturbance and histological analysis shows complete loss of pancreatic tissue and cardiac myocytic necrosis (Roberts, 2012; Jansen *et al.*, 2017). Pancreas disease is known to

seriously affect salmonids at any time between seawater transfer and harvest, with mortalities (5 – 60 %) generally occurring during the first year at sea (Roberts, 2012; Rodger, 2016; Jansen *et al.*, 2017). Fish that survive an outbreak of PD can continue to show reduced appetite and growth which ultimately results in culls during production or downgrading at harvest (Roberts, 2012; Rodger, 2016). Utilising historical data and economic modelling, it has been estimated that PD can cause between €1.8 million and 35 million worth of losses per annum for the salmonid aquaculture industry, particularly through mortalities, management costs, downgrades and treatment costs (Aunsmo *et al.*, 2010; Rodger, 2016). While the fallowing of sites is known to reduce the severity of PD outbreaks, it is not successful in preventing them (Roberts, 2012). Considering the severity of this pathogen and that prevention is key in its control, vaccine development has been undertaken and commercial vaccines against PD, such as ALPHA JECT micro® 1 PD (Pharmaq) are now available (Roberts, 2012; Skjold *et al.*, 2016; PHARMAQ, 2017). Further viral diseases causing negative economic impacts for the Atlantic salmon aquaculture are cardiomyopathy syndrome (CMS) and heart and skeletal muscle inflammation (HSMI), caused by piscine myocarditis virus (PMCV) and piscine orthoreovirus (PRV), respectively (Palacios *et al.*, 2010; Haugland *et al.*, 2011; Markussen *et al.*, 2013). These pathogens cause the most significant problems through chronic mortalities late in the production cycle and/or near to harvest (5 – 12 months post-seawater transfer) and in 2003 it was estimated that CMS alone, through significantly increased mortality, caused a direct loss of €4.5 to 8.8 million on fish farms (Biering and Garseth, 2012; Roberts, 2012; Bruno, 2013). There are currently no effective prevention methods for these pathogens, although work is ongoing for vaccine development, and the industry is reliant on mitigation strategies as well as early diagnosis and surveillance for the control of these two viral pathogens (Biering and Garseth, 2012; Bruno, 2013).

1.4.5.3. Parasitic diseases

The term “parasite” is used to describe small organisms which live on or in another (the host) for all or part of their life cycle, generally at the expense of the host (Roberts, 2012; Collins Dictionaries, 2017e). In fish, many thousands of parasite species, in different classes and phyla and with varying life cycles, have already been described but it is recognised that very few generally cause disease (Bakke and Harris, 1998; Scholz, 1999; Roberts, 2012). However, those which do are known to have devastating effects for the aquaculture industry (Scholz, 1999; Roberts, 2012; Shinn *et al.*, 2015). As with bacterial and viral diseases, the

high stocking densities associated with aquaculture can prove favourable for certain parasite species ultimately causing them to become pathogenic (Roberts, 2012). Both innate and adaptive immune responses are mounted by fish against parasitic infections (Alvarez-Pellitero, 2008). As with bacteria and viruses, the first response a parasite must contend with is the mucus, which regularly sloughs off to prevent stable colonisation (Alvarez-Pellitero, 2008; Esteban, 2012). It has also been shown that contact with mucus can have a limiting effect on parasite loads as well as a direct killing effect (Buchmann and Bresciani, 1998; Harris *et al.*, 1998). Complement has also been shown to have a protective effect in response to parasites. Levels of complement were found to increase in turbot, sea bass and carp (*Cyprinus carpio*) when infected with *Enteromyxum scophthalmi* (Sitjà-Bobadilla *et al.*, 2006), *Sphaerospora dicentrarchi* (Muñoz *et al.*, 2000) and *Sanguinicola inermis*, respectively (Roberts *et al.*, 2005). In addition, *Gyrodactylus derjavini* and *G. salaris* infecting rainbow trout and Atlantic salmon have been killed *in vitro* by the alternative complement pathway (Buchmann, 1998; Harris *et al.*, 1998), with the same pathway also shown to immobilise and lyse *I. multifiliis* theronts (Buchmann *et al.*, 2001). Furthermore, anti-proteases, particularly α 2M, can also be employed by the host's immune system to combat infectious parasites (Sitjà-Bobadilla *et al.*, 2008; Alvarez-Pellitero, 2008). In charr, α 2M neutralises the virulence-essential metalloprotease produced by *Cryptobia salmonsitica* and causes resistance to the parasite (Alvarez-Pellitero, 2008; Woo and Ardelli, 2014). Increased levels of total anti-proteases and α 2M were also found in turbot and sharpsnout seabream (*Diplodus puntazzo*) when infected with *E. scophthalmi* and *E. leei*, respectively (Sitjà-Bobadilla *et al.*, 2006; Muñoz *et al.*, 2007). Despite all these protective factors, parasites continue to develop methods of evading detection by their host (Sitjà-Bobadilla, 2008). These evasive mechanisms can be employed against complement (Nunes *et al.*, 1997) and phagocytic respiratory burst, and can also include antigen masking by the acquisition and incorporation of host molecules into their surface as a means of immunological disguise (Sitjà-Bobadilla, 2008).

In Atlantic salmon aquaculture, numerous parasites that have caused serious concerns for the industry over the years. Two protozoan parasites of concern to salmon aquaculture are *Ichthyobodo necator* and *I. multifiliis*, the aetiological agents of costiasis and white spot, respectively. These parasites are associated with the thickening of skin mucus, lethargy and respiratory distress with both ultimately causing high levels of mortality (60 – 100 %, if untreated) (Francis-Floyd *et al.*, 2009; Rodger, 2010; Roberts, 2012; Shinn *et al.*, 2015). In addition, *I. necator* is also associated with necrosis of the

epidermis while *I. multifiliis* causes the appearance of white spots on the skin and gills (Rodger, 2010). There is no effective prevention of these parasites but the treatments include bathing with formalin or salt baths (Rodger, 2010). A further parasite of key interest is *Kudoa thrysites* (Rodger, 2010; Henning *et al.*, 2013). It is recognised that there are no external clinical signs associated with this myxosporidean parasite, instead the main clinical sign is myoliquefaction, or flesh softening, at post-mortem (Rodger, 2010; Henning *et al.*, 2013). This ultimately results in the fish becoming unmarketable and is the cause of the high economic losses associated with this parasite. In addition, the marine amoebae *Neoparamoeba perurans*, causative agent of Amoebic Gill Disease (AGD) has represented a significant problem for the global marine salmonid industry (*e.g.* Tasmania, Ireland, Scotland and Norway) since the mid-1980's (Young *et al.*, 2008a; Crosbie *et al.*, 2012; Oldham *et al.*, 2016). Clinical signs include respiratory distress, flared opercula and lethargy, and macroscopic manifestation of white mucoid patches on the gills (Munday, 1986; Munday *et al.*, 1990). Histological examination shows profound changes to gill architecture including extensive hyperplasia and lamellar fusion (Munday, 1986; Roubal *et al.*, 1989; Adams and Nowak, 2003). With losses incurred through mortalities, reductions in growth and condition as well as treatment costs, the economic losses associated with this parasite can range between 5 and 80 million USD worldwide per year (Norwegian Scientific Committee for Food Safety (VKS), 2014; Shinn *et al.*, 2015). As such, it is now recognised that AGD is the most significant disease caused by gill parasites (Shinn *et al.*, 2015).

However, in recent years, one group of parasites has overtaken all others in terms of negative effects and is now considered the most important parasitic disease problem for Atlantic salmon aquaculture (Costello, 2006). Known generally as “sea lice”, this group of ectoparasitic marine copepods includes species such as *Lepeophtheirus salmonis* and *Caligus elongatus* and represents economic losses in excess of 430 million USD worldwide per year (Costello, 2009; Fast, 2014). Large enough to be visible, sea lice attach to their host during the copepodid life stage after which they go through two to four chalimus stages, depending on the species, while embedded into host tissues (Hamre *et al.*, 2013). If part of the *Lepeophtheirus* genus, the lice will undergo two pre-adult transformations following the chalimus stages (Hamre *et al.*, 2013). Finally, the lice will go through their one adult transformation, at which point they are motile and able to do the most damage to their host. This damage comes as a direct consequence of feeding on the skin tissue of their host, causing extensive skin abrasion and ultimately resulting in the exposure of bone particularly over the skull, the occurrence of secondary infections, osmoregulatory failure and death. As

with other aquatic parasites, treatments and mitigation strategies are the only available methods for the control of this parasite including cypermethrin, deltamethrin, azamethiphos and hydrogen peroxide; the implementation of cleaner fish and genetic selection. The absence of a vaccine for aquatic parasites can come as no surprise though as only a handful of vaccines are available for commercial use in mammals (Morrison and Tomley, 2016). However, with respect to sea lice, a vaccine would prove a great commodity and work continues to develop and produce a vaccine against this devastating parasite (Raynard *et al.*, 2002; Monaghan *et al.*, 2014).

1.4.6. Disease treatment and management

The vast array of pathogens representing problems for the salmon aquaculture industry highlights the requirement for effective disease management (Scarfe *et al.*, 2006; Pietrak *et al.*, 2014). Efficient biosecurity, defined as the “prevention, control and eradication of economically important diseases” (Pruder, 2004; Gudding, 2014), is therefore essential for the continued success of the aquaculture industry. Meyer (1991) indicates that the first steps in disease biosecurity are preventing the introduction of pathogens, maintaining good water quality and avoiding environmental stressors. On aquaculture sites, these steps are put into place through the daily monitoring of parameters such as quality of incoming water, appearance of dead/moribund fish to ensure safe removal and potential environmental stressors such as low dissolved oxygen or temperature extremes (Subasinghe, 2009; Scottish Salmon Producers Organisation, 2015). Fish farms can further ensure good biosecurity measures by quality checking incoming stocks (*e.g.* eggs, fish), regularly disinfecting entering vehicles and fish-handling equipment, avoiding the transfer of equipment between sites and ensuring good employee hygiene (especially those working directly with the fish) (Subasinghe, 2009; Scottish Salmon Producers Organisation, 2015). Subsequently, Meyer (1991) highlights further steps in disease management as providing adequate nutrition, ensuring disease treatment occurs promptly and using vaccination where possible.

1.4.6.1. Chemotherapy

Chemotherapy is defined as “the treatment of a disease using chemicals or drugs” (Collins Dictionaries, 2017f). Chemical treatments for disease have now been applied in aquaculture for more than 50 years, with early work assessing sulphonamides as a treatment for *A. salmonicida* in rainbow trout (Inglis, 2000). The use of chemotherapeutics has grown vastly over the last decades as has the number of chemotherapeutics available (Inglis,

2000). Some of the approved chemotherapeutants that have been of key use in salmonid aquaculture include florfenicol (control of RTFS), oxytetracycline (control of furunculosis) and hydrogen peroxide (control of fungus and parasites such as *Saprolegnia* sp., sea lice and *N. perurans*) although nowadays these are marketed by pharmaceutical companies under a variety of names (Meyer, 1991; U.S. Food and Drug Administration, 2011). While the use of these chemicals has been highly successful and heavily relied upon for the control of disease, it is recognised that their increased use has caused numerous problems. It has been shown that some of the chemical treatments used in the aquaculture industry have the potential to be toxic to fish and that toxicity is affected by environmental conditions (Srivastava *et al.*, 2004). In an early study, Foster and Woodbury (1936) tested copper sulfate as a treatment for fungus. After a concentration of 1 in 2000 was found to be ineffective, they raised the concentration to 1 in 1000 and found that it also had little effect on the fungus as well as causing mortalities in their fish population. Alderman (1985) conducted a review of malachite green and the author highlights a relationship between environment and toxicity. It is stated that, at a single concentration, the median Lethal Time (LT₅₀; exposure time producing 50% mortality) in rainbow trout decreased as the water temperature increased, with good chemical tolerance at low temperatures (8 °C). In addition, malachite green was found to change the behaviour and blood parameters in catfish (*Heteropneustes fossilis*), with erratic swimming and loss of equilibrium observed along with depletion of serum calcium and protein levels (Srivastava *et al.*, 1995). However, further studies into the effects of malachite green found it to be more toxic in certain fish species as well as being carcinogenic to mammals (including humans), and its use is now banned in numerous countries (Srivastava *et al.*, 2004).

Further supporting the suggestion of environmental modification on toxicity, Reardon and Harrell (1990) assessed the toxic effect of formalin and copper sulfate concentrations at varying salinities in striped bass (*Morone saxatilis*) fingerlings. They observed that salinity had an impact on the toxicity of formalin and copper. However, it was also found that, regardless of salinity, increasing concentrations of formalin and copper sulphate had increasing toxic effects on fingerlings, with 100 % mortality observed at the highest concentrations (formalin: 60 and 120 mg L⁻¹; copper sulphate: 8 and 16 mg L⁻¹). In a further study, Rach *et al.* (1997) found that, as well as environmental conditions, species and life stage can also affect chemical toxicity. All species assessed coped well with 15 minute treatment except walleye (*Stizostedion vitreum*), while 45 minute treatments affected more species (walleye, brown trout and fathead minnow (*Pimephales promelas*)). Increased

mortality occurred in the later life stages of rainbow trout during 15 minute treatments with longer treatments amplifying this trend. In terms of temperature, hydrogen peroxide toxicity increased as temperature did in all three tested species: rainbow trout, channel catfish (*Ictalurus punctatus*) and bluegill (*Lepomis machrochirus*). As such, it is recognised that there are a number of important considerations to make when treating fish with chemicals including exposure time, chemical concentration, temperature, salinity, life stage and species. In aquaculture, these factors need to be considered carefully to achieve successful treatment and avoid unnecessary costs through chemical overuse and mortalities (Piper and Smith, 1973; Gaikowski *et al.*, 1999). Furthermore, the application of chemical treatments in bath form could also cause a stress response, due to the crowding associated with this type of treatment (Corner *et al.*, 2008).

Several studies have been undertaken with findings showing increased stress response (*e.g.* cortisol, glucose, lactate) in response to crowding (Benfey and Biron, 2000; Sadler *et al.*, 2000b; Ortuño *et al.*, 2001; Caipang *et al.*, 2009; Erikson *et al.*, 2016). In another study, Jørgensen and Buchmann (2007) assessed formalin bath treatment, parasite infection and crowding on the stress response of rainbow trout. The authors found that, while all three conditions increased the cortisol response, significantly higher cortisol levels were elicited by the crowding and formalin treatments. However, the study did not assess the effect of chemical treatment and crowding together but it could be assumed that the effects on the stress response would have been additive. This is an important consideration for aquaculture when undertaking chemical treatments as there may be potential for stress to amplify chemical toxicity. However, the most concerning of the problems associated with high levels of chemical use is drug resistance in pathogens (Inglis, 2000; Subasinghe, 2009; Buschmann *et al.*, 2012; Miranda *et al.*, 2013). It is now recognised that pathogens have developed resistance to many successful treatments. For example, Inglis, (2000) highlights oxolinic acid, an efficient treatment for furunculosis during the 1980's, with 50 % of isolates showing resistance by the 1990's (Inglis *et al.*, 1991) and the same patterns were observed for amoxicillin (Inglis *et al.*, 1993), oxytetracycline (Miranda and Zemelman, 2002; Schwarz *et al.*, 2004) and florfenicol (Schwarz *et al.*, 2004; Miranda and Rojas, 2007). In order to reduce the threat of drug resistance for aquaculture, it is essential that research continues to fully elucidate the mechanisms associated with drug resistance and to ensure management strategies are in place to minimise the emergence and spread of resistance in aquaculture, which may include drug rotation through a production cycle (Park *et al.*, 2012; Miranda *et al.*, 2013).

1.4.6.2. Probiotics

The term “probiotic” is used to define a cultured product or live microbial feed supplement which beneficially affects the health of a host by improving its intestinal (microbial) balance (Irianto and Austin, 2002; Kesarcodi-Watson *et al.*, 2008). Probiotics are recognised to work by enhancing colonisation resistance and/or directly inhibiting pathogens as well as by stimulating the immune responses (Irianto and Austin, 2002; Balcazar *et al.*, 2006). With the increasing interest for environmentally friendly alternatives to chemotherapeutics, the research relating to the application of probiotics in aquaculture has greatly expanded over the years (Gatesoupe, 1999; Martinez Cruz *et al.*, 2012). According to Kesarcodi-Watson *et al.* (2008), the selection of a probiotic bacterium for aquaculture requires screening of the bacterial flora of a specific fish species followed by testing the anti-microbial activity against fish pathogens and the pathogenicity in the host fish along with assessing the effects on growth and survival of larvae or challenged fish. Indeed, several probiotics have now been developed for fish and have already been shown to inhibit bacteria both *in vivo* and *in vitro* (Balcazar *et al.*, 2006). Robertson *et al.* (2000) reported that feeding Atlantic salmon fry and fingerlings with an isolate of *Carnobacterium* sp. reduced mortality in response to challenge with *A. salmonicida*, *V. ordalii* and *Y. ruckeri*. Following up this work, Kim and Austin (2006) found feeding rainbow trout with the probiotics *Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33 improved survival during *A. salmonicida* and *Y. ruckeri* challenges (80 % and 76.5 %, respectively) and also enhanced respiratory burst and lysozyme activities. Sharifuzzaman and Austin (2009) used the probiotic *Kocuria* SM1 in the feed of rainbow trout and found reductions in mortality when challenged with *V. anguillarum* as well as increased activity of phagocytes, peroxidase and lysozyme. In addition, Korkea-aho *et al.* (2011) demonstrated that the probiotic *Pseudomonas* M174 inhibited the growth of *F. psychrophilum* *in vitro*, significantly reduced mortalities during an *in vivo* challenge and increased respiratory burst activity. As a result, numerous commercial probiotic products, such as Bactocell®, MacroGard®, and Ergosan™, are now commercially available for use in aquaculture (AquaFeed, 2009; Magnadóttir, 2010). Considering these developmental successes, the use of probiotics is clearly an important tool in protection against diseases but it is also recognised that research needs to continue to fully understand modes of action (Gatesoupe, 1999; Pandiyan *et al.*, 2013).

1.4.6.3. Vaccination

The concept that “prevention is better than treatment” is fundamental to the maintenance of a healthy stock of fish (Shaoqi, 1989; Gudding, 2014). Due to its preventative rather than curative nature, vaccination is becoming increasingly important in the control of infectious diseases and pathogens in the aquaculture industry (Roberts, 2012; Gudding, 2014). The first report of disease prevention through vaccination in fish appears to have taken place in the late 1930’s (Snieszko *et al.*, 1938; Gudding and Goodrich, 2014). The first report of successful vaccination in salmonids occurred in the early 1940’s and found that feeding cutthroat trout with chloroform-killed cultures of *A. salmonicida* (*Bacterium salmonicida*) reduced mortality levels from 75 % in the unvaccinated groups to 25 % in the vaccinated groups (Duff, 1942; Gudding and Goodrich, 2014). Further research into fish vaccination then declined until the 1960’s and 70’s, when extensive studies were undertaken. Ross and Klontz (1965) found that rainbow trout fed a diet supplemented with phenol-killed *Yersinia ruckeri*, the causative agent of enteric redmouth (ERM), had increased survival (90 %) compared to the control group (20 %) following *Y. ruckeri* challenge. Supporting this, Anderson and Ross (1972) prepared *Y. ruckeri* with either phenol, chloroform or by sonication to be supplemented into feeds. They found that all orally immunised groups had increased survival compared to the control groups and that groups immunised with chloroform-prepared bacteria performed best against *Y. ruckeri* challenge. Subsequent to this research, the first product license for a fish vaccine was issued in 1976 for a vaccine against *Y. ruckeri*. In the following decades and well into the 2000’s, work to produce effective vaccines has continued and vaccines are now available against a range of bacterial and viral diseases such as vibriosis, edwardsiellosis (*Edwardsiella tarda*), furunculosis, IPN and ISA (Roberts, 2012). The currently available vaccines have been developed to be delivered in a number of ways, including injection, bath immersion and orally (Roberts, 2012). However, despite these successes, it should be noted that there are still many infectious pathogens, particularly parasites, currently lacking effective vaccines and, as a result, developmental work is still ongoing (Evensen, 2016).

1.4.7. Triploid disease resistance and immune function

Disease and resultant health issues are considered one of the largest single causes of economic losses in aquaculture and, as such, represent a significant constraint to the continued development and success of the industry (Subasinghe, 2005; Weber *et al.*, 2013). Understanding how triploid fish cope with health challenges is therefore crucial and it is

important to characterise their robustness. While triploid Atlantic salmon have been considered a solution to pre-harvest sexual maturation, there continues to be scepticism regarding their use in the industry due to historical field reports of poorer performance, which has included inferior disease resistance (Taylor *et al.*, 2007). However, these reports would have been recorded before the knowledge that triploids have different environmental tolerances than diploids and, as these were field challenge reports, there is the potential for much more variable environmental conditions than in a laboratory setting. As such, it may be suggested that while under optimal conditions both ploidy exhibit similar disease tolerance but the addition of a further environmental stressor may impair the disease tolerance of triploids. However, no studies have yet assessed the interaction between suboptimal environmental conditions and disease challenge. Despite the overall importance of disease in the aquaculture industry, limited studies have been carried out to assess disease resistance and immune function in triploid fish, with conflicting results found in the few studies that have been undertaken (Dunham, 2011).

In the early 1990's, Bruno and Johnstone assessed the susceptibility of diploid and triploid Atlantic salmon to bacterial kidney disease (BKD) following a commercial observation of higher mortalities in diploids than triploids during a BKD outbreak. Fish were challenged with the causative agent of BKD, *Renibacterium salmoninarum*, on four separate occasions. The first challenge found increased disease resistance in triploids thus confirming the earlier commercial observation. However, in the remaining three challenges, diploids and triploids showed no difference in susceptibility to BKD suggesting comparable disease susceptibility between ploidy. In support of this, Kusuda *et al.* (1991) immunised diploids and triploid ayu (*Plecoglossus altivelis*) with formalin-killed *V. anguillarum* cells to assess immune function. No significant differences were found between diploids and triploids in terms of agglutinating antibody titres, complement activity, phagocytic activity (PA) and phagocytic index (PI), suggesting that triploids and diploids have similar immune response to disease. In a further vaccination trial, Johnson *et al.* (2004) found that vaccination against *Listonella anguillarum* caused a comparable serum antibody response between diploid and triploid Chinook salmon. Budiño *et al.* (2006) assessed the general activity of innate immune system components in diploid and triploid turbot. Respiratory burst showed that, while the activity per cell was significantly higher in triploids, the lower number of neutrophils counted in triploids ultimately resulted in no significant difference between ploidy for total respiratory burst activity per microlitre of blood. Total phagocytic activity per microlitre of blood was also found to not differ significantly between ploidy,

again, due to the reduced number of neutrophils found in triploids. They suggest that fewer cells are compensated for by higher cell activity. Additionally, serum complement and lysozyme activities were not significantly different between ploidies. The authors concluded that diploids and triploids showed similar innate cellular and humoral responses, suggesting similar disease resistance in diploids and triploids.

In their assessment of immune function following seawater transfer, Taylor *et al.* (2007) measured plasma and mucus lysozyme activity as well as respiratory burst in rainbow trout. Seawater transfer elevated plasma lysozyme activity in both ploidy and at 12 hours post-transfer with triploids showing significantly higher activity. As with Budiño *et al.* (2006), triploid macrophages exhibited significantly higher respiratory burst compared to diploids. The authors suggest that respiratory burst may be a major indicator of innate immunity which ploidy can influence and agree with the suggestion made by Budiño *et al.* (2006) that fewer cells are compensated for by greater activity. A study by Frenzl *et al.* (2014) showed similar infection levels between ploidy in both experimental and natural challenges with the ectoparasite sea lice, *L. salmonis*. A recent study by Moore *et al.* (2017) experimentally infected diploid and triploid Atlantic salmon with salmonid alphavirus subtype 3 (SAV3) and found no difference in the viral load between ploidy. They did, however, find that the prevalence of the virus accumulated more slowly in triploids although had reached 100 % in both ploidy by the point of termination. While the authors state that the reasoning behind this slow accumulation is not yet clear, they hypothesise that differences in number and size of cells in head kidney between diploid and triploid may play a role. They also suggest that the slow accumulation could have implications for aquaculture as it has the potential to delay or even avoid the onset of disease. In an earlier study of Atlantic salmon by Langston *et al.* (2001), they found that following injection with LPS, alternative complement activity (ACH50) and hypoferraemic response did not differ greater between ploidy. However, they did find ploidy differences in the kinetics of these two responses. The authors found that while there was an initially rapid decline in ACH50 in both ploidy, it took longer for ACH50 levels to return to normal in triploid Atlantic salmon. For the hypoferraemic response, there was again an initial rapid decrease in total iron with the LPS-injected diploids exhibiting lower total iron than triploids, suggesting a faster hypoferraemic response in the diploid. The authors suggest that, although differences between the ploidies are minor, the longer time for triploids to recover ACH50 and to activate a hypoferraemic response may increase their susceptibility to pathogens and put them at a disadvantage in terms of defence. Therefore, while overall findings support

previous studies (little/no difference between ploidy), the final conclusion is contradictory and would require further investigation.

Disease resistance is one of the most important physiological aspects to consider when selecting a species for commercial aquaculture production. While the majority of studies would indicate similar disease resistance and immune response between diploids and triploids, specific studies would suggest that there are certain aspects for triploids that could have a serious effect on their ability to combat pathogen efficiently, such as delayed response time (Langston *et al.*, 2001). As such, it is essential that further investigations be carried out to elucidate the full effect of ploidy on disease resistance and immune function.

1.5. STUDY AIMS

The overall aim of this thesis was to investigate the health and immune response of triploid Atlantic salmon and assess any differences in relation to the response of diploids. The specific aims of the study were:

- To assess the immune response of triploid Atlantic salmon following experimental challenge with a commercially significant bacterial disease;
- To assess the immune response of triploid Atlantic salmon following experimental challenge with a commercially significant parasitic disease ;
- To investigate the effect of a routine on-farm health treatment on the stress and immune response of triploid Atlantic salmon;
- To assess the response of triploid Atlantic salmon to commercial vaccination and investigate the development of the adaptive immune response and the occurrence of side-effects

Chapter 2

A comparison of the response of diploid and triploid Atlantic salmon (*Salmo salar*) siblings to a commercial furunculosis vaccine and subsequent experimental infection with *Aeromonas salmonicida*.

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2.1. INTRODUCTION

In recent years, the Atlantic salmon aquaculture industry has paid particular attention to the issues associated with sexual maturation prior to harvest and escapees (Friars *et al.*, 2001; Oppedal *et al.*, 2003; Fraser *et al.*, 2012a; Preston *et al.*, 2013). Sexual maturation in fish causes the transfer of energy from normal somatic growth to gonadal development. This is known to have adverse effects on body growth rates and flesh quality, and may increase incidences of disease and mortality (Ojolick *et al.*, 1995; Benfey, 2001; Leclercq *et al.*, 2010). Sexually mature fish that escape from production sites also have the potential to interact with wild fish, impacting on the genetics and fitness of the wild population (Glover *et al.*, 2013). Triploid salmon are the only commercially available and acceptable means of achieving sterility in fish, and are increasingly being used as a method to control sexual maturation (Oppedal *et al.*, 2003; Taylor *et al.*, 2011; Fraser *et al.*, 2012b; Preston *et al.*, 2013).

Triploidy can be readily induced through the application of a hydrostatic or temperature ‘shock’ to newly-fertilised eggs and the process has been optimised for several commercially important species in aquaculture including Atlantic salmon, rainbow trout, brown trout, turbot and grass carp (Tiwary *et al.*, 2004; Maxime, 2008; Piferrer *et al.*, 2009; Preston *et al.*, 2013). This ‘shock’ treatment prevents second meiotic division and causes the retention of the second polar body, which results in three sets of chromosomes rather than two, and in turn sterility in triploid fish (Tiwary *et al.*, 2004; Maxime, 2008). Importantly, only females appear to be fully sterile as males have been found to develop gonads, although sperm is aneuploid and as a result not functional (Benfey *et al.*, 1986). However, despite the clear advantages of being sterile, if triploid Atlantic salmon are to be considered for commercial production, they must perform equally as well as diploids in all aspects of their biology and physiology.

Many studies over recent years have investigated triploid salmon physiology and performance, including effects on egg incubation (Taylor *et al.*, 2011), smoltification (Taylor *et al.*, 2012), growth (O’Flynn *et al.*, 1997; Oppedal *et al.*, 2003; Leclercq *et al.*, 2010; Taylor *et al.*, 2013, 2014) and deformities and cataracts (Fraser *et al.*, 2013; Taylor *et al.*, 2013). There are, however, very few studies that have focussed on triploid salmon health and immunity. Disease and resulting health issues are considered one of the largest single causes of economic losses in aquaculture and, as such, represent a significant constraint to the continued development and success of the industry (Subasinghe, 2005; Weber *et al.*, 2013). Understanding how triploid fish cope with health challenges is therefore crucial and

it is important to characterise their robustness. Empirical evidence suggests no differences in mortality of triploid salmon compared to diploid siblings when challenged with disease in commercial settings, although few scientific assessments have been carried out. A study by Frenzl *et al.* (2014) showed similar infection levels between ploidy when Atlantic salmon were challenged with sea lice. However, there is a clear lack of data on triploid salmon response to bacterial and viral challenges and conflicting results have been reported in other fish species (Jhingan *et al.*, 2003; Budiño *et al.*, 2006).

Furunculosis, caused by the bacterium *A. salmonicida* subsp. *salmonicida*, is recognised as one of the most commercially significant infectious diseases of Atlantic salmon (McCarthy, 1975; Toranzo *et al.*, 2005). Furunculosis was first described in farmed brown trout in Germany by Emmerich and Weibel (1894) who designated it *Bacterium salmonicida* (Austin and Austin, 2007; Roberts, 2012; Midtlyng, 2014). It was later reclassified as *Aeromonas salmonicida* in the 1950's (Griffin *et al.*, 1953), with the *Aeromonadaceae* separated into its own family in the 1980's (Colwell *et al.*, 1986). Following the first isolation, the disease and associated bacteria were found in fresh and brackish waters throughout North America, England and Scotland (Midtlyng, 2014) with subsequent seawater outbreaks occurring in the 1970's (Novotny, 1978) and in Scotland and Norway throughout the 1980's (Roberts, 2012; Midtlyng, 2014). It has now been reported in all areas with wild or farmed salmonids, excluding Australia (Midtlyng, 2014). Furunculosis is an acute to chronic condition, with a variety of clinical signs. Clinical signs of acute infection can include darkening, anorexia, lethargy and small skin haemorrhages as well as mortalities. Internal examination reveals hyperaemia and diluted blood vessels as well as swollen spleen and kidney (Bullock *et al.*, 1983; Roberts, 2012; Midtlyng, 2014). More sub-acute or chronic furunculosis can cause the characteristic “furuncle-like” lesions on the musculature as well as tissue necrosis, haemorrhaging and mortality (McCarthy and Roberts, 1980; Roberts, 2012; Midtlyng, 2014).

Due to the severity of this pathogen, effective disease prevention has been essential to protect growing salmon aquaculture industries. It is recognised that Duff (1942) undertook the first *A. salmonicida* vaccination trial with cutthroat trout (*Salmo clarkii*). The author found that supplementing feed with chloroform-killed cultures of *A. salmonicida* improved survival in the experimental groups (75 % survival) compared to control groups (25 % survival), as well as increasing antibody response in the experimental groups. Extensive research was undertaken over the next few decades to fully describe an efficacious furunculosis vaccine with research breakthroughs occurring throughout the

1980's and 90's (Krantz *et al.*, 1964; Spence *et al.*, 1965; Michel, 1979; McCarthy *et al.*, 1983). In the late 1980's, Paterson *et al.* (1985) undertook a trial to assess the efficacy of a commercial injectable furunculosis vaccine and found that vaccination significantly reduced mortality from 74 % in the unvaccinated controls to only 10 % in the vaccinated groups. The 1990's saw further successful testing of adjuvanted furunculosis vaccines with experimental laboratory and field based trials yielding increased survival and protection against furunculosis, as well as a reduction in the use of antibacterial drugs (Lillehaug *et al.*, 1990, 1992; Erdal and Reitan, 1992; Midtlyng *et al.*, 1996a; Markestad and Grave, 1997; Munro and Gauld, 1997). As a result of this extensive research, effective commercial vaccines against furunculosis are now widely used in salmon aquaculture (Adams *et al.*, 1997). However, the application of vaccines can be stressful for the fish, and can result in side-effects, compromising fish welfare. In a study by Fraser *et al.* (2014a), it was shown that vaccination with Norvax MINOVA 6 Vet (Norvax®, Intervet International B.V., Boxmeer, Netherlands), a multivalent vaccine against furunculosis, classical vibriosis, coldwater vibriosis and IPN, induced higher abdominal adhesions in triploid salmon, compared with diploid fish in out-of-season (S0+) smolts (Fraser *et al.*, 2014a). Further studies are clearly required to fully elucidate the response of triploid salmon to infectious agents and vaccination, and to determine if their responses are significantly different from their diploid counterparts.

2.1.1. Aims

The aim of this Chapter was to investigate the response of triploid Atlantic salmon to vaccination against furunculosis using a commercially available vaccine and assess levels of protection elicited by the vaccine through experimental infection with *A. salmonicida* compared with their diploid siblings.

2.2. MATERIALS AND METHODS

2.2.1. Fish stock and husbandry

Eggs and milt were obtained from commercial Atlantic salmon broodstock stripped in October 2012 (Aquagen® Atlantic QTL-innOVA® IPN/PD strain Norway). Following fertilisation, half of each egg batch was subjected to a pressure shock (655 bar for 6.25 min, 37.5 min post-fertilisation at 8 °C) to induce triploidy. Eggs were then incubated at 6.0 ± 0.5 °C until eyeing. Eyed eggs (21st December 2012, 372 degree days, °D) were supplied to the University of Stirling facilities (Howietoun Fish Farm) and incubated in complete

darkness at 7.1 ± 0.3 °C until hatching (7th January 2013, ~470-500 °D). First feeding commenced on 25th February 2013 (~880 °D) and temperature gradually increased (8.4 ± 1.3 °C). On 28th May 2013, fry were transferred to the Niall Bromage Freshwater Research Facility (NBFRF), Buckieburn and maintained on constant light until late August and simulated natural photoperiod thereafter, under an ambient water temperature (range: winter, 3 °C – summer, 15 °C) to produce in-season (S1+) smolts. Fish were fed a commercial diet (BioMar Inicio Plus), distributed by automatic feeders (ARVOTEK). Specific feeding rates (% tank biomass per day) were adjusted automatically according to predicted growth and daily temperature, and pellet size (0.5 to 2.0 mm) increased with fish size. Mortality between first feeding and vaccination was 1.18 % for diploids and 1.99 % for triploids. On 21st January 2014, fish were transferred from the NBFRF to 0.1 m³ tanks (100 L; 1 L min⁻¹ flow rate) at the Aquaculture Research Facility (ARF), Institute of Aquaculture, University of Stirling where water temperature was maintained at 9.6 ± 1.1 °C and fish were fed a daily 0.5 % biomass diet.

2.2.2. Vaccination and sampling

Fish were vaccinated on 11th November 2013 (5 °C) using a commercial vaccination gun (FISHJECTOR 0.1 ml, Kaycee Veterinary Products, UK). Initial mean weight (\pm SEM) was 71.4 ± 2.74 g and 58.5 ± 3.57 g for diploids and triploids, respectively. Fish from both ploidy (180 ploidy⁻¹) were divided into three treatment groups in triplicate tanks (60 fish tank⁻¹), sedated using MS-222 (Pharmaq AS, Oslo, Norway), and then injected intraperitoneally (IP) as follows: (1) sham injected (0.02 M phosphate buffered saline, PBS group); (2) injected with adjuvant alone (liquid paraffin adjuvant, PHARMAQ AS, Oslo, Norway) (ADJ group); or (3) injected with commercial vaccine against furunculosis and infectious pancreatic necrosis (ALPHA JECT 2.2® vaccine, PHARMAQ AS, Oslo, Norway) (VACC group). Following injection, fish were transferred into their designated 1 m³ triplicate tank (280 L; 1 L min⁻¹ flow rate) for recovery. They remained on the same feeding regime, photoperiod and water temperature as previously described.

Sampling was undertaken at 5 time-points post-vaccination (50, 250, 450, 600 and 750 °D) with 3 fish sampled per tank at each time-point (9 fish per treatment). Weight was assessed prior to challenge at 750 °D. At all time-points, each fish was assessed for adhesion severity according to the Speilberg Scale (Table 2.1) (Midtlyng *et al.*, 1996b). Blood samples were taken from the caudal vein and serum collected before being stored at -20 °C. Serum from 50 and 750 °D were used to assess complement activity and antibody response,

respectively. A portion of the blood sampled at 50 °D was also removed for blood cell counts. At 50 °D, head kidney was dissected from the fish under aseptic conditions for assessment of macrophage activity.

2.2.3. Immunological assays

All immune parameters were assessed using 50 °D serum samples with the exception of antibody response which was analysed at 750 °D.

2.2.3.1. Blood cell counts

Whole blood was diluted 1 in 100 with phosphate buffered saline (PBS, 0.02 M Phosphate, 0.15 M NaCl, pH 7.2) for white blood cell (WBC) counts and 1 in 1000 for red blood cell (RBC) counts. Cell counts were then performed using a Neubauer haemocytometer (Marienfeld, Germany). Cell counts were adjusted using the following calculation and expressed as number of cells $\times 10^4 \text{ ml}^{-1}$:

$$((\text{total cell number} / 4) \times \text{dilution factor})$$

2.2.3.2. Head kidney macrophage isolation

Isolation of head kidney macrophages was performed according to Secombes (1990), with modifications. Head kidney was homogenised through a 100 µm nylon mesh with 5 ml L-15 medium (Sigma, UK) containing 10 µl heparin (50 mg ml⁻¹) (Sigma, UK). Cell suspensions were layered onto 34 % / 51 % Percoll gradients and centrifuged at 400 xG for 30 min at 4 °C. The band of cells at the 34 - 51 % interface was transferred into 15 ml centrifuge tubes and the volume adjusted to 15 ml with L-15 medium. Suspensions were centrifuged at 600 xG for 7 min at 4 °C. The resultant cell pellets were re-suspended in 1 ml L-15 medium containing 5 % foetal calf serum (Sigma, UK) and 1 % potassium benzyl-penicillin/streptomycin sulphate (Sigma, UK). Cells were counted using a Neubauer haemocytometer and cell concentrations adjusted to $1 \times 10^7 \text{ cells ml}^{-1}$.

Table 2.1: Speilberg scale used to assess intra-abdominal adhesions and lesions post-vaccination (Midtlyng *et al.*, 1996b)

Score	Visual appearance of abdominal cavity	Severity of lesion
0	No visual lesions	None
1	Very slight adhesion most frequently localised close to the injection site. Unlikely to be noticed by laymen during evisceration	No/minor opacity of peritoneum after evisceration
2	Minor adhesion, which may connect colon, spleen or caudal pyloric caeca to the abdominal wall. May be noticed by laymen during evisceration	Only opacity of peritoneum remaining after manually disconnecting the adhesions
3	Moderate adhesion, adhesions including more cranial parts of the abdominal cavity, partly involving pyloric caeca, the liver or ventricle, connecting them to the abdominal wall. May be noticed by laymen during evisceration	Minor visible lesions after evisceration, which may be removed manually
4	Major adhesions with granuloma, extensively inter-connecting internal organs, which thereby appear as one unit. Likely to be noticed by laymen during evisceration	Moderate lesions, may be hard to remove manually
5	Extensive lesions affecting nearly every internal organ in the abdominal cavity. In large areas, the peritoneum is thickened and opaque, and fillet may carry focal, prominent and/or heavily pigmented lesions/granulomas	Leaving visible damage to carcass after evisceration and removal of lesions
6	Even more pronounced than 5, often with considerable amounts of melanin. Viscera cannot be removed without damage to fillet integrity	Leaving major damage to carcass

2.2.3.3. Respiratory burst

Respiratory burst of head kidney macrophages was assessed using the method described by Secombes (1990). The prepared macrophage suspensions (see 2.2.3.2) were added to 96 well microplates ($100 \mu\text{l well}^{-1}$) and left to incubate for 2 h at room temperature (RT) (i.e. 20°C) to allow the macrophages to adhere. The plates were then washed 3 times with L-15 medium to remove non-adherent cells. To respective wells, $100 \mu\text{l}$ of 1 mg ml^{-1} nitroblue tetrazolium (NBT) (Sigma, UK) solution, 1 mg ml^{-1} NBT with $1 \mu\text{l}$ phorbol myristate acetate (PMA) (Sigma, UK) or lysis buffer was added. Microplates were incubated for 1 h at RT. While incubating, the number of adherent cells was determined by counting the released nuclei in the lysis buffer wells with a Neubauer haemocytometer, then applying the following calculations to generate a ‘multiplier factor’:

e.g. Mean cell number = 25.5

Convert to $1 \text{ ml} = 25.5 \times 10^4 \text{ cells / ml}$

Convert to $100 \mu\text{l} = (25.5 \times 10^4) / 10 = 25.5 \times 10^3 \text{ cells / } 100 \mu\text{l}$

Convert to $1 \mu\text{l} = 0.255 \times 10^5 \text{ cells / } \mu\text{l}$

‘Multiplier factor’ = $(1 / 0.255) = 3.92$

Following incubation, the reaction was stopped by fixing the cells with 100 % methanol. The plates were washed three times with 70 % (v/v) methanol then the wells were allowed to air dry. The insoluble formazan was subsequently dissolved by adding $120 \mu\text{l well}^{-1}$ 2M potassium hydroxide (KOH) (Fisher Scientific, UK) followed by $140 \mu\text{l well}^{-1}$ dimethyl sulfoxide (DMSO) (Sigma, UK). The contents of each well were carefully mixed and air bubbles removed. The absorbance of the wells were measured at 610 nm using a microplate reader (Synergy HT, BioTek, USA) and the Gen5 Data Analysis Software. The results were adjusted using the multiplier factor and then expressed as absorbance at 610 nm for $\times 10^5$ cells:

e.g. NBT average absorbance = 0.267

So, $0.267 \times 3.92 = 1.05$ absorbance per 1×10^5 cells

2.2.3.4. Phagocytosis

The phagocytic activity of head kidney macrophages was assessed according to the method described by Thompson *et al.* (1996), with modifications. Two circles were marked on glass slides using an ImmEdge pen (Vector Laboratories Inc, UK). Cell suspensions were added ($100 \mu\text{l circle}^{-1}$) and slides incubated for 1 h in a humid chamber. Non-adherent cells were then removed with L-15 medium. A 0.5 % (w/v) yeast (*Saccharomyces cerevisiae*) suspension was then added to one circle ($100 \mu\text{l}$) and L-15 medium ($100 \mu\text{l}$) to the other as a negative control. Slides were incubated as previously described and washed with L-15 medium. Cells were fixed for 5 min with 100 % methanol ($100 \mu\text{l circle}^{-1}$) and then washed five times with 70 % methanol. Slides were stained with a Rapid Romanowsky staining series (TCS Biosciences Ltd, UK). Two hundred macrophages were examined by strategically scanning the slide from one side to the other using light microscopy (100x) and counted, along with the number of yeast per macrophage. Phagocytic activity, (PA), phagocytic index (PI) and phagocytic capacity (PC) were determined according to Findlay and Munday (2000), using the following calculations:

- PA = (number of phagocytising macrophages / total number macrophages) x 100;
- PI = (total number of yeast cells consumed / number of phagocytising macrophages);
- PC = (total number of macrophages containing a given number of yeast cells / total number of macrophages containing any yeast).

2.2.3.5. Alternative complement pathway

Spontaneous haemolytic activity was determined by adapting the method described by Langston *et al.* (2001). Briefly, serum samples (in duplicate) were doubly diluted in 0.1 % gelatine-complement fixation buffer (G-CFB) (1 complement fixation tablet (Oxoid, UK), 0.1 g gelatine) in a U-well microplate (Fisher Scientific, UK) (final volume $25 \mu\text{l well}^{-1}$). A 5 % (v/v) sheep red blood cell (SRBC) suspension was then added to all wells ($10 \mu\text{l well}^{-1}$). Each microplate also contained control wells. As a positive control, producing 100 % SRBC lysis, 0.1 % (w/v) anhydrous Na_2CO_3 was added to wells in place of serum samples. As a negative control, eliciting 0 % lysis of SRBC, 0.1 % G-CFB replaced serum samples. All samples were incubated for 90 min at RT with constant shaking, after which the reaction was stopped by adding G-CFB containing 20 mM ethylenediaminetetraacetic acid (EDTA) (Sigma, UK) ($140 \mu\text{l well}^{-1}$). The microplates were centrifuged at 750 g for 6 min before

supernatants were transferred into flat-well microplates (Fisher Scientific, UK) ($100 \mu\text{l well}^{-1}$). The absorbance was measured at 450 nm. The percentage lysis for each sample and dilution was calculated using control values. The dilution that produced 50 % lysis was determined using PROBIT analysis and the reciprocal expressed as spontaneous complement haemolysis (ACH50%).

2.2.3.6. Enzyme linked immunosorbent assay (ELISA)

The specific antibody response of diploid and triploid Atlantic salmon to *A. salmonicida* was measured using the methods described by Erdal and Reitan (1992) and Romstad *et al.* (2012) using sonicated antigen to coat the ELISA plates as described. Briefly, 96 well microplates (Immulon 4HBX, Fisher Scientific, UK) were coated with sonicated whole cell *A. salmonicida* MT423, diluted to $20 \mu\text{g ml}^{-1}$ in coating buffer (carbonate-bicarbonate solution; C-3041, Sigma-Aldrich, USA) ($100 \mu\text{l well}^{-1}$). The plates were incubated overnight at 4°C , washed with low salt wash buffer (LSWB: 0.02 M Tris, 0.38 M NaCl, 0.05 % Tween 20) and post-coated for 2 h at RT with 3 % (w/v) casein ($250 \mu\text{l well}^{-1}$). Doubling serum dilutions were added to the microplates along with PBS as a negative control ($100 \mu\text{l well}^{-1}$) and incubated overnight at 4°C . The microplates were then washed with high salt wash buffer (HSWB: 0.02 M Tris, 0.5 M NaCl, 0.1 % Tween 20), incubating for 5 min on the last wash. Microplates were incubated for 1 h at RT with rabbit anti-trout polyclonal antibody (Aquatic Vaccine Unit, University of Stirling, Stirling, UK), diluted 1:1000 with PBS ($100 \mu\text{l well}^{-1}$). After washing with HSWB, conjugate (anti-rabbit-Horseradish peroxidase, Sigma-Aldrich, USA) diluted 1:2000 with conjugate buffer (1g bovine serum albumin (BSA) (Fisher, UK) in 100ml LSWB), was added for 1 h ($100 \mu\text{l well}^{-1}$). The reaction was developed by the addition of chromogen (3'3'5'5'-Tertamethylbenzidine dihydrochloride (TMB)) in substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4) ($100 \mu\text{l well}^{-1}$) and, after 10 min, was stopped with 2 M sulphuric acid (H_2SO_4) ($50 \mu\text{l well}^{-1}$). Absorbance was measured at 450 nm and is expressed as optical density (OD).

2.2.4. Vaccine efficacy testing

The strain of *A. salmonicida* used for the experimental infection ('Hooke') following vaccination was kindly provided by Dr D. A. Austin, Heriot Watt University. The challenge dose was pre-determined using three doses (6×10^3 , 6×10^2 & 6×10^1 colony forming units (CFU) fish $^{-1}$) with the two highest doses resulting in 100 % mortality and the lowest giving

83 % mortality. The latter dose was subsequently used in the infection trial. At challenge (870 °D post-vaccination, 24th March 2014), the mean weights (\pm SD) of diploids and triploids were 74.6 ± 13.2 g and 61.7 ± 12.5 g, respectively. Twenty fish from each triplicate ploidy/treatment tank were anaesthetised and injected IP with a 0.1 ml dose of *A. salmonicida* suspension (4×10^2 CFU ml⁻¹; final concentration 4×10^1 CFU fish⁻¹). Following injection, fish were immediately transferred into their designated 0.1 m³ experimental challenge tanks (100 L; 1 L min⁻¹ flow rate) and allowed to recover. As mortalities occurred, swabs from head kidney and spleen were sampled onto brain heart infusion agar (BHIA) (Oxoid, UK) plates for identification to confirm specific mortalities. Following termination of the challenge (11th April 2014), all surviving fish in the diploid and triploid PBS and ADJ groups (diploid PBS: 3; triploid PBS: 1; diploid ADJ: 7; triploid ADJ: 6), along with 15 randomly sampled survivors (5 tank⁻¹) from the diploid and triploid VACC groups, were sacrificed and sampled to confirm bacterial recovery or clearance.

2.2.5. Statistical analysis

Minitab software version 16 (Minitab Inc., Pennsylvania) was used to perform basic descriptive statistics and comparisons using a significance level of 5 % ($p = 0.05$). Prior to analysis, datasets were checked for normality using the Anderson-Darling test. Where appropriate log transformations were performed to normalise the data. Post-hoc analyses were carried out using Tukey's multiple comparison tests with values considered significantly different at p -values < 0.05 . General linear model (GLM) manipulated to three-way analysis of variance (ANOVA) was used to analyse adhesion score, with ploidy, treatment and time (sampling time-point) considered fixed factors. Further two-way ANOVAs were carried out for weight at 750 °D, blood cell counts, respiratory burst, phagocytosis, complement activity, antibody response and challenge mortalities using only ploidy and treatment as fixed factors.

2.3. RESULTS

2.3.1. Growth

At 750 °D post vaccination, there was no significant difference in the weight of fish between the vaccinated and unvaccinated groups. For the diploid fish, their mean weight (\pm SD) was 79.5 ± 12.4 g, 74.7 ± 15.2 g and 69.4 ± 10.2 g for the PBS, ADJ and VACC groups, respectively. The mean weight of the triploid groups (PBS, ADJ and VACC) was 66.1 ± 12.5 g, 57.1 ± 11.5 g and 62.3 ± 12.3 g, respectively.

2.3.2. Adhesion score

Ploidy did not have a significant effect on adhesion score throughout the course of the trial (Figure 2.1). Treatment had a significant effect on adhesion score with adhesions found in fish injected with adjuvant and vaccine, as early as 50 °D. Differences between treatments (PBS, ADJ, VACC) were significant from 250 °D onwards, with adhesion scores reaching a mean peak value of 2.7 ± 0.2 at 600 °D in the VACC groups (Figure 2.1).

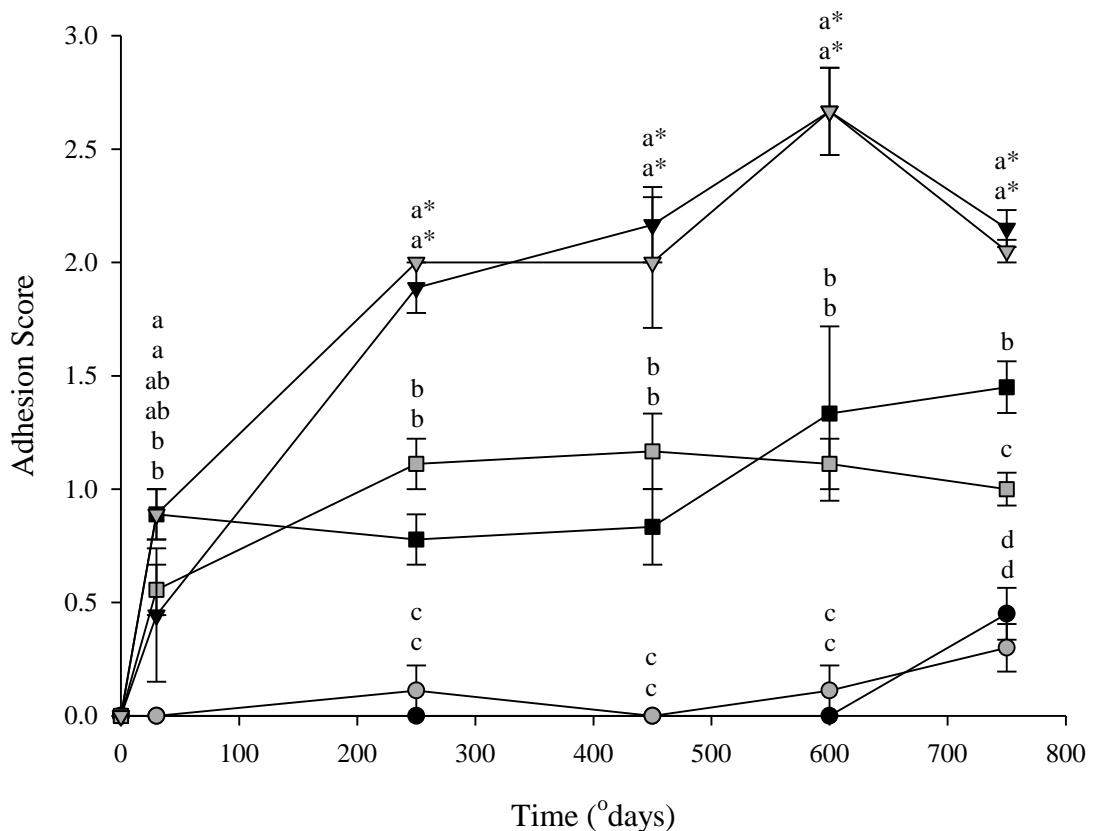


Figure 2.1: Adhesion score (mean \pm SEM, $n = 9$) in diploid (black) and triploid (grey) Atlantic salmon injected with PBS (circles), ADJ (squares) or VACC (triangles). Significant differences between ploidy/treatments at a given time-point are indicated by different superscripts. Significant differences relative to 50 °D are indicated by asterisk (*).

2.3.3. Immune response

Diploid fish had higher WBC counts than their triploid counterparts in all treatment groups, but only significantly in the VACC fish (Figure 2.2 A). A similar pattern was observed for RBC counts, with diploids showing significantly higher numbers than triploids in the ADJ group (Figure 2.2 B). For diploid fish, WBC counts in the ADJ group were significantly lower than the PBS or VACC groups, while the triploid PBS group showed significantly higher WBC counts than the other two groups. In contrast, no significant treatment differences were observed for RBC counts.

Triploid fish exhibited significantly higher respiratory burst activity by head kidney macrophages compared to diploids for all groups (Figure 2.2 C). Treatment did not have a significant effect on respiratory burst.

No differences in complement activity were observed between ploidy and this was not influenced by vaccination (Figure 2.2 D).

Macrophages of PBS-injected triploid fish had a significantly higher PI than their diploid counterparts, while the opposite was found for macrophages from the VACC groups (Figure 2.2 E). Within the triploid groups, the VACC group was found to have significantly lower PI than the PBS and ADJ groups, while no significant differences were evident between the diploid groups. Ploidy was not found to have a significant effect on PA (Figure 2.2 F). Within the triploid groups, the VACC group showed significantly lower PA than the PBS group. No significant effect following vaccination was observed in the diploid fish.

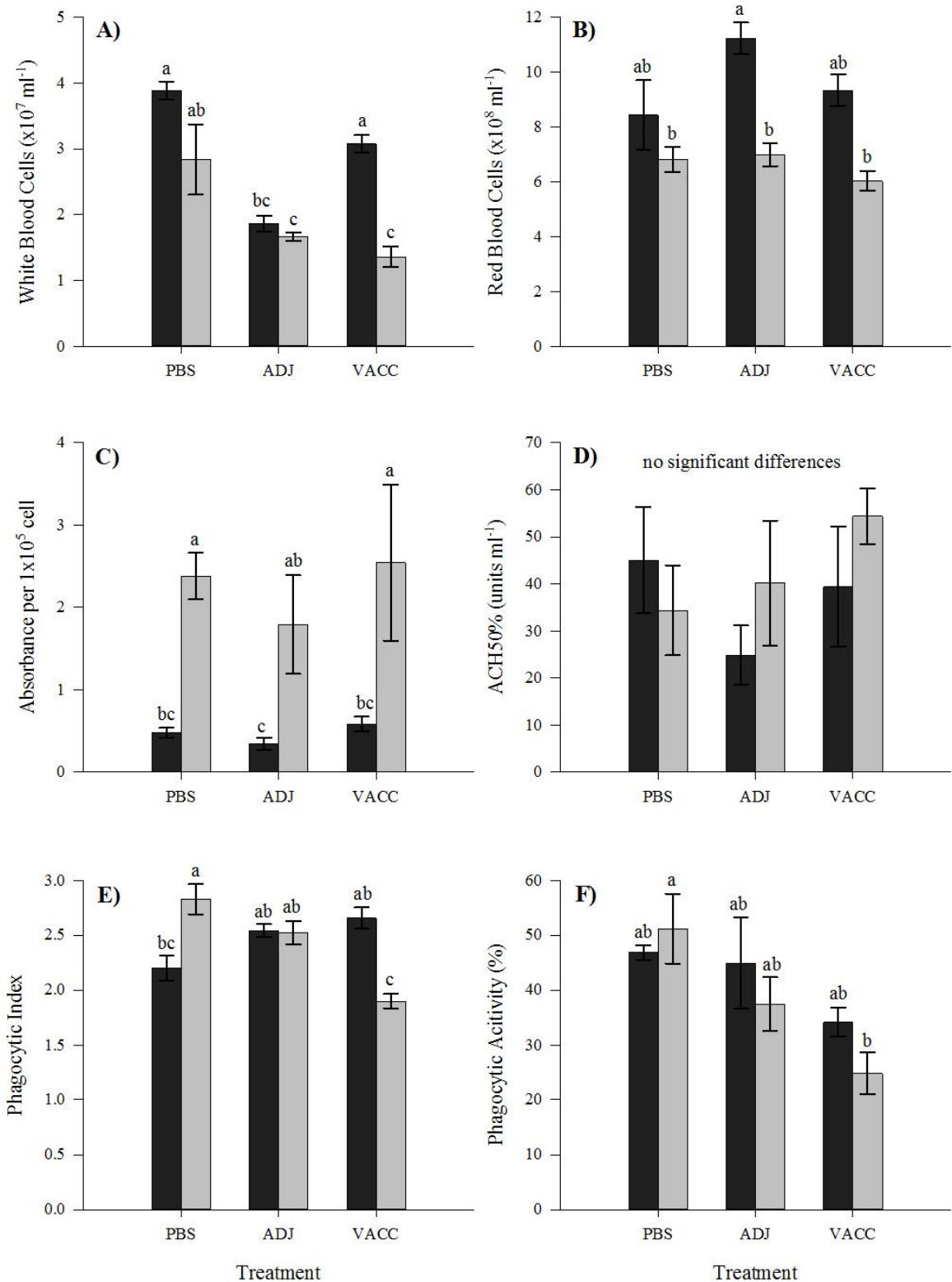


Figure 2.2: Comparison of (A) white and (B) red blood cell counts, (C) respiratory burst activity, (D) complement activity (ACH50 %), (E) phagocytic index and (F) activity (%) between diploid (black) and triploid (grey) Atlantic salmon injected either PBS, adjuvant (ADJ) or vaccine (VACC) at 50 °D. Values expressed as means \pm SEM (n = 9). Significant differences are indicated by different letters.

Significant differences in PC were noted between ploidy (Table 2.2). In the PBS groups, diploid macrophages phagocytosed a significantly higher percentage of low yeast numbers (*e.g.* 1, 2), while triploid macrophages were found to consume significantly more high yeast numbers (*e.g.* 5, 6, 7, +). No statistical significance was noted between ploidy in the ADJ groups, but a similar pattern to that of PBS groups was observed. For the VACC groups, the opposite pattern was noted, with triploids consuming a higher percentage of low yeast numbers (1 & 2) and diploid macrophages consuming a significantly greater percentage of high yeast numbers (4, 6 and 7).

Table 2.2: Comparison of phagocytic capacity between diploids and triploids in each treatment group.

Treatment	Ploidy	Active macrophages consuming a given number yeast cells (%)								
		1	2	3	4	5	6	7	+	
PBS	Diploid	39.3 ^a	24.7 ^a	16.8	8.7	4.9 ^b	2.4 ^b	1 ^b	2.2 ^b	
	Triploid	25.7 ^b	19.2 ^b	16	12.9	10 ^a	5.4 ^a	5.2 ^a	5.6 ^a	
ADJ	Diploid	33.5	20.2	18	9.9	9	3.1	3.4	3	
	Triploid	35.7	19	13.6	8.9	6.8	5.5	5	4.5	
VACC	Diploid	31.2	15.8	14.4	10.2 ^a	8.9	5.6 ^a	5.8 ^a	8.1	
	Triploid	46	17.3	10.6	5.7 ^b	5.4	2.3 ^b	2 ^b	10.8	

Significant differences between ploidy, within a particular treatment, are indicated by different lowercase superscripts

The antibody responses of all diploid and triploid groups were assessed at 750 °D. There was no effect of ploidy on antibody response in any of the treatment groups examined (Figure 2.3). Significantly greater antibody responses were elicited in both VACC groups compared to their respective ADJ and PBS controls.

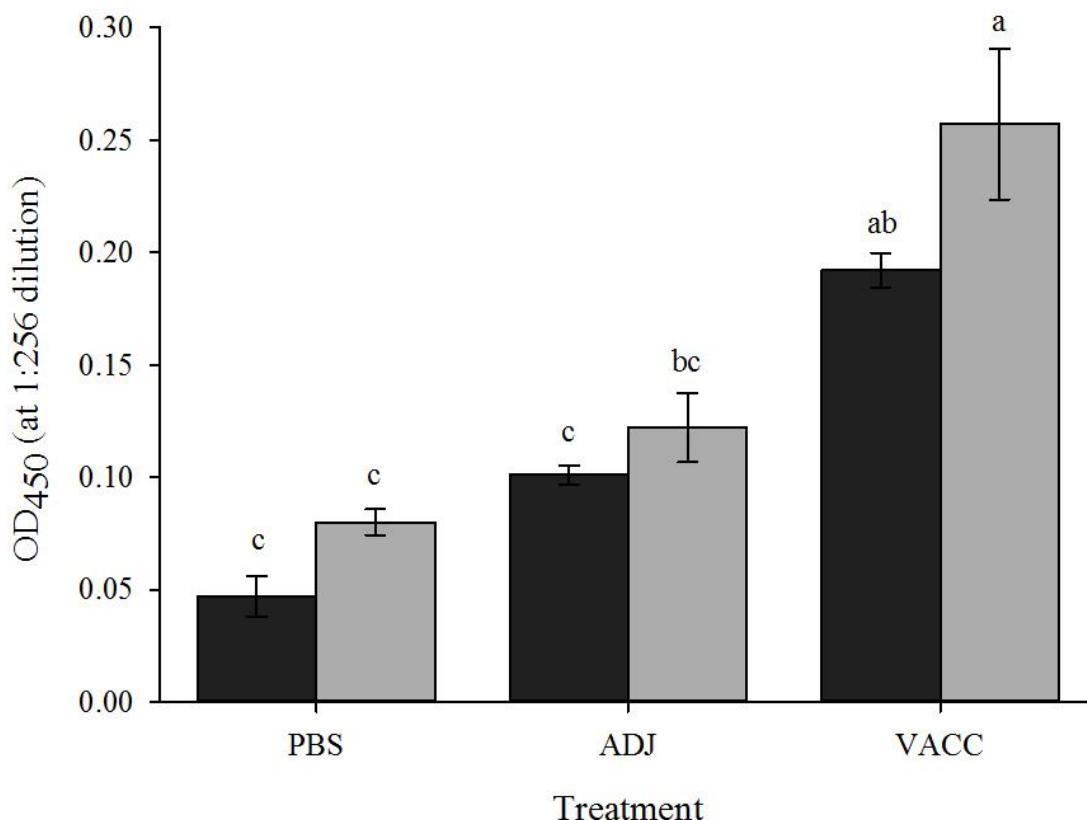


Figure 2.3: Comparison of antibody response ($OD_{450\text{nm}}$) between diploid (black) and triploid (grey) Atlantic salmon injected either PBS, adjuvant (ADJ) or vaccine (VACC) at 750 °D. Values expressed as means \pm SEM ($n = 9$). Significant differences are indicated by different letters.

2.3.4. Vaccine efficacy testing

Following challenge with *A. salmonicida*, mortalities increased over time in both the PBS and ADJ groups while it remained low in the VACC groups (Figure 2.4). Final mortality was not significantly different between ploidy for any of the treatment groups (Diploid PBS 95 %, ADJ 88.3 %, VACC 1.7 %; Triploid PBS 98.3 %, ADJ 90 %, VACC 5 %). Both diploid and triploid VACC groups had significantly lower mortalities compared to the PBS and ADJ groups. Relative percent survival (RPS) for the diploid and triploid VACC groups was 98.3 % and 94.9 %, respectively. Specific mortalities were confirmed by the presence of *A. salmonicida* in swabs taken from the kidney and spleen of infected fish. *Aeromonas*

salmonicida was isolated from all fish that died during the challenge and was confirmed by the appearance of dark/brown pigmented agar and by Gram stains. No *A. salmonicida* was recovered from the challenge survivors from any of the ploidy/treatment groups.

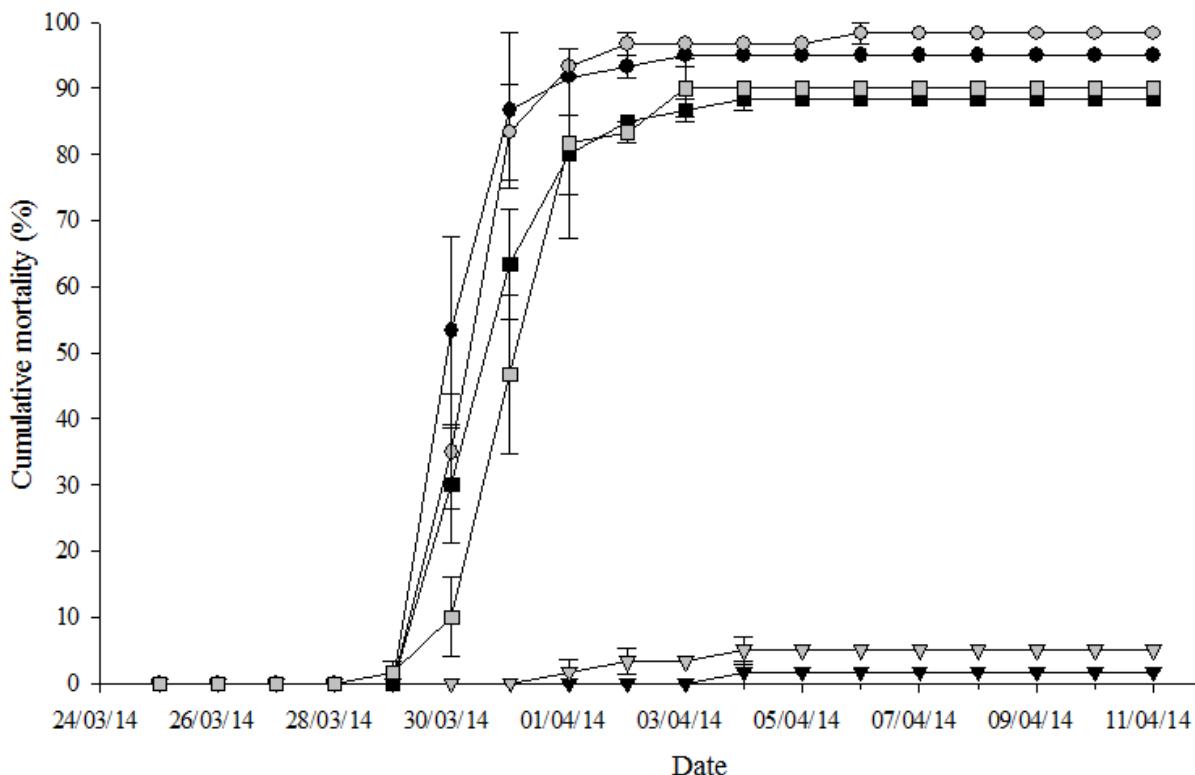


Figure 2.4: Cumulative mortality (%) of diploid (black) and triploid (grey) Atlantic salmon injected with PBS (circles), ADJ (squares) or VACC (triangles) then experimentally infected with *Aeromonas salmonicida*. Values expressed as mean treatment group mortality (%) \pm SEM ($n = 3$). Significant differences between groups are indicated by different letters.

2.4. DISCUSSION

The response of diploid and triploid Atlantic salmon siblings to vaccination with a commercial furunculosis vaccine was compared in the present study, as well as to injection with either PBS or adjuvant alone, used as control groups. Innate immune responses were examined in these fish at 50 °D and antibody response was assessed at 750 °D, together with the level of protection elicited by the furunculosis vaccine. Side-effects from vaccination e.g. growth and adhesions were examined throughout the trial as previous reports indicate poorer growth and more severe adhesions in triploids as a result of vaccination (Fraser *et al.*, 2014a). The main findings from this study indicate that triploid Atlantic salmon respond equally as well to vaccination as diploids, and ploidy does not

significantly affect the severity of the adhesions that develop. Vaccine efficacy was not significantly affected by ploidy, with similar RPS values obtained for both diploid (98.3 %) and triploid (94.9 %) fish.

The growth performance of triploids is much debated (Tiwary *et al.*, 2004; Taylor *et al.*, 2012, 2013; Fraser *et al.*, 2012b), with triploid growth following vaccination relatively uncharacterised. Vaccination did not have a significant effect on the weight of diploids or triploids in this study, however, there are reports of a reduction in weight in triploids post-vaccination from other studies (Fraser *et al.*, 2012a, 2014a, 2015b). It should be noted that our study was only performed for 4 months in freshwater, while the other studies cited lasted for considerably longer in both fresh- and seawater (Fraser *et al.*, 2012a, 2014a). While non-significant effects of vaccination were observed on growth in this study, supporting the use of commercial vaccines in triploid Atlantic salmon, it is recommended that vaccination should be assessed over a full production cycle.

Vaccination is known to produce other side-effects including internal abdominal adhesions (Lund *et al.*, 1997; Berg *et al.*, 2006; Fraser *et al.*, 2014a). This has serious implications at harvest, as adhesions can lead to downgraded carcass quality (Poppe and Breck, 1997; Colquhoun *et al.*, 1998). Due to the limited assessment of the vaccination process in triploid Atlantic salmon, much is still unknown about the relationship between vaccines, their side-effects and triploidy. In this study, ploidy did not have a significant impact on the severity of adhesion scores observed in diploid and triploid fish. This finding is supported by previous vaccination studies using S1+ triploid smolts, which, in fact, were conducted over a longer time scale (Fraser *et al.*, 2013, 2014a). Vaccination did result in adhesions in both ploidies, with adjuvant alone also inducing adhesions. The PBS group of both ploidy groups consistently showed little evidence of adhesions, as expected. From 250 °D onwards, adhesion scores in the VACC groups were significantly higher than both the PBS and ADJ groups, with adhesion scores in the ADJ group significantly higher than those of the PBS group. These results agree with previous studies, which found that while adjuvants alone could cause adhesions, vaccines and thus the inclusion of an antigen increased the severity of adhesion scores (Midtlyng *et al.*, 1996a; b; Mutoloki *et al.*, 2004; Berg *et al.*, 2012). Significant difference was noted in the ADJ groups between ploidies at 750 °D, where triploid adhesion scores were significantly lower than that of diploid and may indicate that triploids recover more quickly from the effects of adhesions than diploids, but a longer trial would help elucidate this. From the results of this study, however, it can

be suggested that triploid Atlantic salmon experience the same degree of side-effects as diploids.

Before being able to produce triploid fish commercially, it is essential that their health and immunity be carefully assessed compared to diploid counterparts (Budiño *et al.*, 2006). Several studies have now been undertaken to investigate this, with reports of greater or equal disease resistance in triploids compared to diploids (Bruno and Johnstone, 1990; Langston *et al.*, 2001; Budiño *et al.*, 2006; Fraser *et al.*, 2012a). As such, much remains to be elucidated about the effects of triploidy on health and immunity. In this study, ploidy appeared to have an effect on some of the immune parameters measured.

Ploidy had an effect on WBC and RBC counts, with triploids showing lower cell numbers compared to diploids. It is recognised that the third set of chromosomes possessed by triploids is compensated for by increased cell size and reduced cell number and since the late 1950's, numerous studies have supported this, with reduced cell numbers continually observed in triploids (Small and Benfey, 1987; Cogswell *et al.*, 2002; Peruzzi *et al.*, 2005; Taylor *et al.*, 2007). In terms of treatment effects, WBC counts were the lowest in both the ADJ groups and the triploid VACC group. Following vaccination, it would be expected that WBC numbers in the VACC groups increase over that of the controls. From vaccination to the 50 °D time-point, the water temperature was low (4.9 ± 0.2 °C) and so, it could be suggested that low temperature had suppressive effect on the WBC's of the innate immune response and the levels remain basal. In a previous study of diploid rainbow trout, similar cell numbers were recorded at the same temperature observed in this study (Morgan *et al.*, 2008). Variations between treatment groups were also found in RBC counts, with similar levels previously exhibited in rainbow trout (Morgan *et al.*, 2008). As such, with RBC's not playing a major role in the immune response, it could be suggested that the numbers observed are of normal population variation.

Ploidy had a significant effect on the activity of head kidney macrophages. Respiratory burst in triploids was significantly higher than in diploids, with the triploids consistently showing more than double their activity. This finding may reflect the compensatory mechanism employed by triploid fish to deal with the reduced number of cells present and is supported by previous studies which found increased activity in triploid cells compared to diploid counterparts (Budiño *et al.*, 2006; Taylor *et al.*, 2007). Treatment was not found to have a significant effect on respiratory burst. Given previous research which showed increased respiratory burst following vaccination or exposure to an antigen (Secombes, 1994; Solem *et al.*, 1995; Chin and Woo, 2005), it was expected that the

respiratory burst activity of the VACC groups would exceed that of the controls. However, it could again be suggested that the low temperature experienced during this study was having a suppressive effect on the respiratory burst activity in the VACC groups. This is supported by a previous study in diploid rainbow trout which showed similar respiratory burst activity to that recorded in this study, at the same temperature (Morgan *et al.*, 2008).

In terms of phagocytosis, varied patterns of activity were recorded. Considering previous studies, triploids would be expected to show increased phagocytic activity (Kusuda *et al.*, 1991; Budiño *et al.*, 2006) but neither diploid nor triploid head kidney macrophages consistently showed increased activity. It would also be expected that PI and PA would be higher in the VACC group due to antigen stimulation of the immune response. In terms of triploid phagocytosis (i.e. PI and PA values), lower values were recorded in the VACC groups compared to the controls. It could be suggested that while vaccines stimulate the fish's immune response, the overall reduction in cell number in triploids may have had an effect on the overall number of yeast being consumed. Further research is needed to understand the compensatory mechanisms that may occur in triploid leucocytes, particularly macrophages, in relation to genome regulation and gene expression.

In addition, a greater percentage of triploid macrophages had a higher PC, consuming greater numbers of yeast cells (i.e. between 5 and 7) compared to diploid fish in the PBS and ADJ groups. Budiño *et al.* (2006) also showed that the lower cell numbers observed in triploids may be compensated for by increased cellular activity, and due to their larger size, the cells have higher membrane surface and volume than diploid cells, thus increasing their ability to engulf particles.

No significant differences in ploidy were observed in complement activity (ACH50%). The results obtained concur with the findings of a 19 day study undertaken by Langston *et al.* (2001), in which a decrease in ACH50 was observed for both ploidies at 2 and 3 days post-injection with LPS. In our study, complement activity was only measured at 50 °D (8 days post-injection), which suggests that the early changes observed by Langston *et al.* (2001) may have been missed, and the results obtained reflect recovering activity levels, and further vaccination trials should be undertaken to assess ploidy differences in complement activity at earlier time-points. Complement activity in the triploids was lowest in the PBS injected group, followed by ADJ group, with the VACC group exhibiting the highest activity, although not significantly different. Other studies have found increased complement activity in vaccinated fish compared to unvaccinated controls (Harikrishnan *et al.*, 2010; Dan *et al.*, 2013).

In this study, antibody response was assessed at 750 °D post-vaccination, and ploidy was not found to have a significant effect on the antibody response obtained. There was, however, a trend for triploids to have greater antibody response than the diploids. This is supported by the results from Kusuda *et al.* (1991) which revealed a non-significant trend for greater agglutinating antibody titres in triploid ayu (*Plecoglossus altivelis*). It is encouraging that triploids are able to produce similar levels of antibodies to diploids. There was a significant treatment effect on antibody response within the ploidy groups, with higher antibody responses recorded in vaccinated fish compared to the non-vaccinated groups, in accordance with other studies (Erdal and Reitan, 1992; Secombes, 1994). The antibody response obtained in vaccinated fish was lower than expected, from other studies examining antibody responses in fish vaccinated against furunculosis (Erdal and Reitan, 1992; Romstad *et al.*, 2012). This is possibly linked to the low temperature at vaccination temperature (Lillehaug *et al.*, 1993; Lillehaug, 2014), but this ultimately did not affect the efficacy elicited by the vaccine.

Vaccine efficacy was confirmed by comparing the level of mortalities in each ploidy/treatment group when experimentally infected with *A. salmonicida*. While much is still unknown about the effect of ploidy on disease resistance (Piferrer *et al.*, 2009), studies investigating this issue have demonstrated equal or greater disease resistance in triploid (Bruno and Johnstone, 1990; Langston *et al.*, 2001; Budiño *et al.*, 2006; Fraser *et al.*, 2012a). This study clearly supports this as no significant effect of ploidy on mortality was evident.

2.5. CONCLUSION

In conclusion, this study showed that a commercial furunculosis vaccine was equally effective in protecting diploid and triploid Atlantic salmon from mortality caused by *A. salmonicida* infection, and ploidy did not affect the severity of the adhesions that occurred in vaccinated fish. Ploidy differences were observed in the immune response, however, with increased respiratory burst observed in triploids. It has been suggested that the differences seen may be due to a mechanism to compensate for the reduced number of cells present in triploids. Ploidy also did not have a significant effect on levels of mortalities or vaccine efficacy during an experimental infection with *A. salmonicida* post-vaccination. Overall, the findings of this study contribute to knowledge that triploid salmon appear to be as robust as diploid siblings and provides a base for further research into the immune response of triploid Atlantic salmon.

Chapter 3

A comparison of disease susceptibility and innate immune response between diploid and triploid Atlantic salmon (*Salmo salar*) siblings following experimental infection with *Neoparamoeba perurans*, causative agent of amoebic gill disease

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3.1. INTRODUCTION

Sexual maturation in fish is known to have adverse effects on body growth and flesh quality, due to the transfer of energy to gonad development (Ojolick *et al.*, 1995; Leclercq *et al.*, 2011). Sexually mature escapees from production sites also pose a threat to wild fish genetics (Glover *et al.*, 2013). Therefore, pre-harvest sexual maturation is of serious concern for the salmonid aquaculture industry and triploidy has been proposed as a method to control sexual maturation (Oppdal *et al.*, 2003; Taylor *et al.*, 2011). Despite the advantages of sterility, to be accepted for aquaculture production, triploids must be proven to perform as well as diploids in all aspects of their biology and physiology, with recent studies continuing to overcome the previously reported issues of deformity and poorer performance (growth and survival) (Burke *et al.*, 2010; Fjelldal and Hansen, 2010; Leclercq *et al.*, 2011; Taylor *et al.*, 2011, 2012). However, few studies have been undertaken to investigate triploid Atlantic salmon health and immunity.

Understanding how triploid fish cope with health challenges is an important milestone to characterise their robustness, especially as disease and subsequent health issues continue to restrict the development and success of the aquaculture industry (Weber *et al.*, 2013). Anecdotal evidence suggests no differences in mortality of triploid salmon compared to diploid siblings when challenged with disease in commercial settings although no scientific assessments were carried out. Frenzl *et al.* (2014) showed similar infection levels between ploidy when Atlantic salmon were challenged with sea lice, both naturally and experimentally. In contrast, higher *Gyrodactylus salaris* infection levels in triploids were reported by Ozerov *et al.* (2010). Additionally, triploid goldfish (*Carassius auratus*) showed higher loads of *Metagonimus* sp. than diploids (Hakoyama *et al.*, 2001) and findings from rainbow trout showed triploids were more susceptible to infection by *Ergasilus sieboldin* (Tildesley, 2008). However, with an overall lack of conclusive data, the response of triploid Atlantic salmon to economically significant parasites remains to be fully elucidated.

One such parasite is *Neoparamoeba perurans*, a free-living, marine amoebae and the causative agent of Amoebic Gill Disease (AGD) (Young *et al.*, 2008a; Crosbie *et al.*, 2012). Amoebic Gill Disease has represented a significant problem for the marine salmonid industry since the mid-1980's, particularly in Tasmania, and has now become a persistent problem for a growing number of countries including Ireland, Scotland and Norway (Oldham *et al.*, 2016). Amoebic Gill Disease was first described in Tasmanian salmonids by Munday (1986), although no causative agent was identified at the time. Using morphological analysis, the aetiological agent was later described as *Paramoeba* sp. and

then subsequently as *Paramoeba pemaquidensis* (reclassified to *Neoparamoeba pemaquidensis* (Page, 1987)) (Kent *et al.*, 1988; Roubal *et al.*, 1989). For many years after this, *N. pemaquidensis* was considered the only aetiological agent of AGD, having been consistently isolated from outbreaks and diseased fish (Kent *et al.*, 1988; Oldham *et al.*, 2016). However, attempts to elicit an experimental infection with laboratory cultured amoebae were consistently unsuccessful (Findlay, 2001; Morrison *et al.*, 2005). However, cohabitation trials and infection using amoebae isolated directly from infected fish elicited the disease (Findlay *et al.*, 1995; Akhlaghi *et al.*, 1996; Findlay and Munday, 1998; Zilberg and Munday, 2000; Findlay, 2001; Zilberg *et al.*, 2001). Despite the issues of producing the disease with cultured amoebae, further morphological and molecular studies continued to support *N. pemaquidensis* as the aetiological agent of AGD (Dyková *et al.*, 2000; Wong *et al.*, 2004). It was not until the late 2000's that the true aetiological agent was described. It began with the isolation of *Neoparamoeba branchiphila* together with *N. pemaquidensis* from gills harvested from Atlantic salmon with clinical AGD and this indicated that AGD may have a mixed aetiology (Dyková *et al.*, 2005). Then, Young *et al.* (2007) used PCR to amplify the 18S rRNA gene of non-cultured, gill-derived amoebae from AGD-affected Atlantic salmon using *N. pemaquidensis* and *N. branchiphila* specific oligonucleotides. While *N. pemaquidensis* and *N. branchiphila* were both PCR amplified, the amplification was variable and this led the authors to compare the 18S rRNA and 28S rRNA gene sequences from non-cultured, gill-derived and clonal cultured, gill-derived *N. pemaquidensis* and *N. branchiphila*. Phylogenetic analyses inferred from 18S and 28S rRNA gene sequences explicitly separated a lineage consisting of non-cultured, gill-derived amoebae from the other members of *Neoparamoeba*. The authors then designed, validated and used species-specific oligonucleotide probes on AGD-affected gill tissue and determined that neither *N. pemaquidensis* nor *N. branchiphila* were associated with AGD-lesions. This indicated that the non-cultured, gill-derived amoebae were a new species and this was assigned *Neoparamoeba perurans* (Young *et al.*, 2007). Further work by Young *et al.* (2008b) assessed archived samples from AGD outbreaks and consistently found that, in all species and geographical regions assessed, *N. perurans* was actually the only detectable species associated with AGD gill lesions. Subsequently, Crosbie *et al.* (2012) successfully fulfilled Koch's postulates for AGD by culturing *N. perurans* on agar plates and using the cultured amoebae to effectively induce AGD in naïve Atlantic salmon in a laboratory setting, confirming undeniably that *N. perurans* is the true aetiological agent of AGD in Atlantic salmon.

Clinical signs of AGD include respiratory distress, flared opercula and lethargy, and macroscopic manifestation of white mucoid patches on the gills (Munday, 1986; Munday *et al.*, 1990). The appearance of these white patches is regularly used as a preliminary indicator of disease occurrence and can be scored to determine disease severity and progression (Adams *et al.*, 2004; Taylor *et al.*, 2009; Bustos *et al.*, 2011). Histological examination shows profound changes to gill architecture including extensive hyperplasia and lamellar fusion (Munday, 1986; Roubal *et al.*, 1989; Adams and Nowak, 2003). It is now recognised that AGD is the most significant disease caused by gill parasites in terms of economic impact (Shinn *et al.*, 2015). While numerous studies have been undertaken to elucidate the interaction of *N. perurans* with the host fish immune system (Gross *et al.*, 2005; Pennacchi *et al.*, 2014, 2016), further research is required to fully explain the overall immune response of Atlantic salmon to AGD. This is particularly important in relation to triploids, with a previous study showing reduced survival in triploids when challenged with *N. perurans* but fewer gill lesions than diploids and no ploidy differences in immune response (Powell *et al.*, 2008).

3.1.1. Aims

The aim of this Chapter was to investigate and compare the response of triploid and diploid Atlantic salmon to an experimental cohabitation challenge with *N. perurans* and to assess their susceptibility and immune response to this parasite.

3.2. MATERIALS AND METHODS

3.2.1. Fish stock and husbandry

Fish used in this study were obtained from two commercial breeding companies (Stock A and Stock B) and supplied as eyed ova (Stock A: 372 °D, 21st December 2012; Stock B: 380 °D, 4th January 2013) to the University of Stirling Freshwater Research Facility (56 °N 4 °W). Triploidy was induced at both company sites using the same protocol, whereby post-fertilisation, half of each egg batch (Stock A, 26 unrelated dams and 6 sires; Stock B, 20 unrelated dams and 5 sires) was exposed to a hydrostatic pressure shock of 655 bar applied for 6.25 min, 37.5 min post-fertilisation at 8 °C. Ova (3 tanks ploidy⁻¹ stock⁻¹ with 2500 ova tank⁻¹) were incubated in complete darkness at 7.1 ± 0.3 °C until hatching, where temperature was gradually increased to 10 °C for first feeding (Stock A: 25th February 2013; Stock B: 4th March 2013). At first feeding, fish were reared under constant light and fed a commercial diet (diploids - Inicio Plus; triploids – Inicio-TriX, BioMar UK), distributed by

automatic feeders (Arvo-Tec Oy, Finland). Specific feeding rates (% tank biomass per day) were adjusted automatically according to predicted growth and daily temperature, and pellet size (0.5 to 2.0 mm) increased with fish size. From August 2013, fish were maintained under ambient water temperature (minimum: 3 °C, maximum: 15 °C) and photoperiod to produce S1+ smolts for sea transfer on 22nd April 2014 (Marine Environmental Research Laboratory (MERL), Institute of Aquaculture, Machrihanish, UK, 55.4 °N 5.7 °W). Fish were routinely vaccinated in November 2013 with ALPHA JECT 2-2 (PHARMAQ, UK) to protect against *A. salmonicida* and IPNV. Mortality between first feeding and sea transfer was 1.18 % and 1.99 % in Stock A, and 2.8 % and 3.5 % in Stock B, respectively for diploids and triploids. Following sea transfer, fish were maintained under ambient temperature (min: 9 °C, max: 11 °C) in 1 m stock tanks (400 L; 1 L kg biomass⁻¹ min⁻¹ flow rate) with aeration provided by air stones for 24 days until allocation to challenge tanks. During this period, mortality was 1.32 % for diploids and 1.79 % for triploids in Stock A, and 4.9 % for diploids and 0.3 % triploids for Stock B.

To verify ploidy status in each stock, smears were prepared from blood collected by tail ablation from euthanised fish at 5 g (100 ploidy⁻¹). After air drying, slides were fixed in 100 % methanol and then placed into 6 % Giemsa stain (6 ml Giemsa in 94 ml distilled water) for 10 min. Erythrocyte length and diameter were measured at 40× magnification using image capture software (Image-Pro Premier, MediaCybernetics, Rockville, USA). All erythrocytes were numbered then selected using a random number generator. A total of 20 randomly chosen nuclei per slide were measured to the nearest 0.01 µm. Diploid control groups had significantly smaller erythrocyte nuclear lengths with no overlaps with the pressure shock triploid groups (2N 6.8 – 7.7 µm; 3N 9.0 – 10.2 µm) confirming that fish subjected to hydrostatic pressure shock were triploids.

3.2.2. Amoebic gill disease cohabitation challenge

On 12th May 2014, fish were randomly allocated into twelve 1 m tanks (400 L; 1 L kg biomass⁻¹ min⁻¹ flow rate), with each ploidy held separately (6 tanks ploidy⁻¹). Thirty fish from each Stock (Stock A: 2N 93.2 ± 8.9 g, 3N 85.8 ± 9.8 g; Stock B: 2N 98.8 ± 13.6 g, 3N 83.1 ± 13.4 g) were added to appropriate ploidy tanks (60 fish tank⁻¹) before being randomly allocated to a challenge group in triplicate: diploid uninfected and infected; triploid uninfected and infected (3 tanks group⁻¹). Stock B fish were fin-clipped to distinguish them from Stock A. Fish were maintained under ambient temperature (min: 11 °C, max 13°C). The challenge facility was flow-through in configuration, supplied with 100 µm filtered

natural seawater. Water quality was regularly checked and maintained at $> 7 \text{ mg L}^{-1}$ DO, pH 8.3, $< 0.25 \text{ mg L}^{-1}$ NH₃-N, $< 0.15 \text{ mg L}^{-1}$ NO₂-N and $< 5 \text{ mg L}^{-1}$ NO₃-N.

The cohabitation challenge was undertaken according to methods developed at the Institute of Aquaculture, Stirling, Scotland. Seeder fish for the challenge were produced using a stock of infected Atlantic salmon held at the MERL facility as part of an *in vivo* amoebae culture. Four of these pre-infected fish were added to a separate stock of 40 naïve Atlantic salmon smolts. Gills were grossly assessed until the appropriate gill score for cohabitation infection (approximately 1.5 – 2 gill score) was achieved. The seeder fish were adipose fin-clipped, marked with panjet (0.0652 g alcian blue mL⁻¹, Sigma-Aldrich, UK) and added to the appropriate challenge groups (6 seeders tank⁻¹). A group of uninfected seeder fish were also produced using the same method, with 4 uninfected Atlantic salmon added to another stock of 40 naïve smolts. No clinical pathology was observed in uninfected seeders after two weeks. Sampling occurred at 7, 14, 21 and 28 days post infection (d.p.i), after which the challenge was terminated. At each sampling time-point, 5 fish stock⁻¹ tank⁻¹ were removed from the tanks and culled by lethal anaesthesia (10 % Benzocaine, Sigma-Aldrich, UK) before being sampled. Gills were visually assessed and scored for gill lesion severity according to Taylor *et al.* (2009) (Table 3.1). Blood was then sampled from the caudal vein using a non-heparinised needle and syringe. Blood samples were kept overnight at 4 °C before being centrifuged at 3000 xG for 10 min to collect serum. Finally, the left 2nd gill arch was excised into paraformaldehyde for histological processing.

Table 3.1: Gross gill score system to estimate the severity of AGD (Taylor *et al.*, 2009).

Infection level	Gill score	Gross description
Clear	0	No sign of infection and healthy red colour
Very light	1	1 white spot, light scarring, undefined necrotic streaking
Light	2	2 - 3 spots/small mucus patch
Moderate	3	Established thickened mucus patch or spot groupings up to 20 % of gill area
Advanced	4	Established lesions covering up to 50 % of gill area
Heavy	5	Extensive lesions covering most of gill surface

3.2.3. Histopathology

After 24 hours fixation in paraformaldehyde, gills were transferred into 70 % ethanol. Gills were dehydrated (Thermo Shandon Citadel 2000), embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. To assess changes in pathology severity, gill sections from 7 and 28 d.p.i were analysed. The total number of filaments on each gill section were counted along with the number of filaments presenting AGD-associated lesions. These lesions were defined according to Adams *et al.* (2009) with hyperplastic fusion of the secondary lamellae in close proximity to *N. perurans* trophozoites. The percentage of filaments affected by AGD lesions was subsequently calculated.

3.2.4. Histochemistry

The second left gill arch from all fish, fixed and processed as above, were stained for the detection of mucous cells using a combined alcian blue-PAS technique according to Mowry (1956), with modifications. Briefly, sections were de-waxed, rehydrated and immersed in alcian blue solution (pH 2.5) for 5 min. The residual stain was removed by washing in water. Sections were oxidised in 1 % (aq) periodic acid (5 min), washed (5 min) and immersed in Schiff's reagent (20 min). Finally, sections were washed in running tap water (10 min) and counterstained with haematoxylin Z (2 min) before being washed, dehydrated, cleared and mounted. To assess changes in mucous cell populations, mucous cells from fish at 7 and 28 d.p.i were counted and measured. Sections were first scanned using a slide scanner (Pannoramic 250 Flash III, 3DHISTECH Ltd, UK). To then assess changes in cell number, mucous cells were counted in five regions across a gill section image using ImageJ (Maryland, USA). Mucous cell size was also assessed by measuring the diameter (µm) of 20 random cells on the gill arch (CaseViewer, 3DHISTECH Ltd, UK).

3.2.5. Immunohistochemistry

Chloride cells were targeted for immunohistochemical identification according to the methods of Adams and Marìn de Mateo (1994). Chloride cell staining used gill tissue fixed as previously described. Sections were de-waxed in xylene (10 min) and rehydrated through a graded ethanol series (100 % for 5 min and 70 % for 3 min). Sections were then blocked for endogenous peroxidase activity (3 % H₂O₂ in methanol – 20 min), washed with PBS (5 min) and incubated with 10 % normal goat serum (20 min) to block non-specific binding sites. Sections were blotted dry and incubated overnight in a humid chamber with a mouse

monoclonal antibody to Na⁺/K⁺-ATPase (1:200, IgG_a5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa, USA). Sections were washed with PBS and incubated (1 h, room temperature) with 1:200 goat anti-mouse IgG (A4416, Sigma, USA) before being washed again. ImmPACT DAB Peroxidase (HRP) Substrate (SK-4105; Vector Laboratories, Burlingame) was applied as per manufacturer instructions and sections were incubated for 5 min. Slides were then immersed in tap water (2 - 5 min) to stop the reaction, counterstained with haematoxylin Z (3 min), dehydrated, cleared and mounted.

3.2.6. Immunological assays

All immune parameters were assessed using the 7, 14 and 21 d.p.i samples. The 28 d.p.i were contaminated and could not be used for analysis.

3.2.6.1. Lysozyme

Lysozyme activity in serum samples was measured turbidimetrically according to Morgan *et al.* (2008). A 0.04 M sodium phosphate buffer (0.04 M NaH₂PO₄.2H₂O, 0.04 M Na₂HPO₄.2H₂O, pH 5.8) was incubated at 25 °C for 30 min. Lyophilised *Micrococcus lysodeikticus* was then added to the buffer at a concentration of 0.2 mg ml⁻¹. Serum samples were added in duplicate to 96 well microplates (10 µl well⁻¹) before 190 µl of the *M. lysodeikticus* suspension was added to all wells. This was with the exception of the control wells, to which 200 µl of sodium phosphate buffer was added. Immediately after the addition of the *M. lysodeikticus* suspension, the reduction in absorbance at 540 nm was measured at 1 minute intervals for 5 minutes using a microplate reader (Synergy HT, BioTek Instruments, USA) and the Gen5 Data Analysis Software. One unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance at 0.001/min. Activity was expressed as units min⁻¹ ml⁻¹.

3.2.6.2. Alternative complement pathway

Spontaneous haemolytic activity was determined according to the method described in Chapter 2 (Section 2.2.3.5)

3.2.6.3. Anti-protease activity

Detection of anti-protease activity was undertaken according to the methods of Ellis (1990) and Thompson *et al.* (1996), with modifications. A 100 µl ml⁻¹ trypsin solution was prepared

by adding 1 ml of 25 mg ml⁻¹ of trypsin stock solution to 249 ml 0.1 M Tris.HCl (pH 8.2). In round-bottomed 96 well microplates, serum samples were diluted two-fold in Tris.HCl buffer (20 µl well⁻¹) then 60 µl of trypsin solution was added. Plates were incubated for 5 min at RT. Finally, chromogen solution in distilled water (0.1 % Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA)) was added to each well. Controls were also added to each microplate: As a zero reference, only BAPNA solution and Tris.HCl buffer were added to wells while wells containing BAPNA solution, Tris.HCl buffer and trypsin solution were used as positive controls. All plates were incubated at RT for 30 min before being centrifuged at 750 g for 6 min. One hundred µl from each well was transferred into new flat-bottomed microplates and then absorbance (410 nm) was measured using a microplate reader. Trypsin inhibition (%) was then calculated as ((positive control OD – sample OD) / positive control OD) x 100.

3.2.7. Statistical analysis

Minitab software version 16 (Minitab Inc., Pennsylvania) was used in this study to perform basic descriptive statistics and comparisons using a significance level of 5 % ($p = 0.05$). Prior to analysis, datasets were checked for normality using the Anderson-Darling test. Where necessary, log transformations were performed to normalise the data. Post-hoc analyses were carried out using Tukey's multiple comparison tests with values considered significantly different at p -values < 0.05 . Non-parametric Kruskal-Wallis test was used to assess gross gill score. General linear model (GLM) manipulated to four-way ANOVA was used to analyse histopathology severity, mucous cell parameters, lysozyme, complement and anti-protease activities, with ploidy, infection, time (d.p.i) and stock considered fixed factors. Three-way ANOVA was also used to assess the previously mentioned parameters with ploidy, infection and, time (d.p.i) as fixed factors. Further three-way ANOVA was used to analyse total mortality (%) with ploidy, infection and stock as fixed factors. In addition, survival was analysed by Kaplan-Meier survival analysis, with differences between groups determined by log-rank tests (MedCalc, MedCalc Software, Belgium).

3.3. RESULTS

3.3.1. Challenge mortality

For Stock A, total mortality was $6.7 \pm 1.9\%$, $3.3 \pm 1.9\%$, $13.3 \pm 10\%$ and $8.9 \pm 2.2\%$ for the diploid uninfected, triploid uninfected, diploid infected and triploid infected groups respectively. In the diploid uninfected, triploid uninfected, diploid infected and triploid

infected groups respectively groups from Stock B, total mortality for the challenge was $3.3 \pm 3.3\%$, $7.8 \pm 2.9\%$, $11.1 \pm 2.9\%$ and $7.8 \pm 4.8\%$, respectively. Statistical analysis found no significant effect of ploidy, infection or stock on total mortality. Kaplan-Meier survival analysis was also performed and similar survival probability was exhibited in all groups from both stocks (0.9 ± 0.0 to 1.0 ± 0.0). No significant effect of stock, ploidy or infection was observed (data not shown).

3.3.2. Gross gill scores

Gross gill scores of all diploid and triploid infected groups increased over time, while those of uninfected groups remained low (Figure 3.1). Neither stock nor ploidy had a significant effect on gill score. For both stocks, time and infection had a significant effect. Gill scores in the infected groups from 14 d.p.i onwards were significantly higher than at 7 d.p.i, and all infected groups had significantly higher gill scores compared to their respective uninfected group.

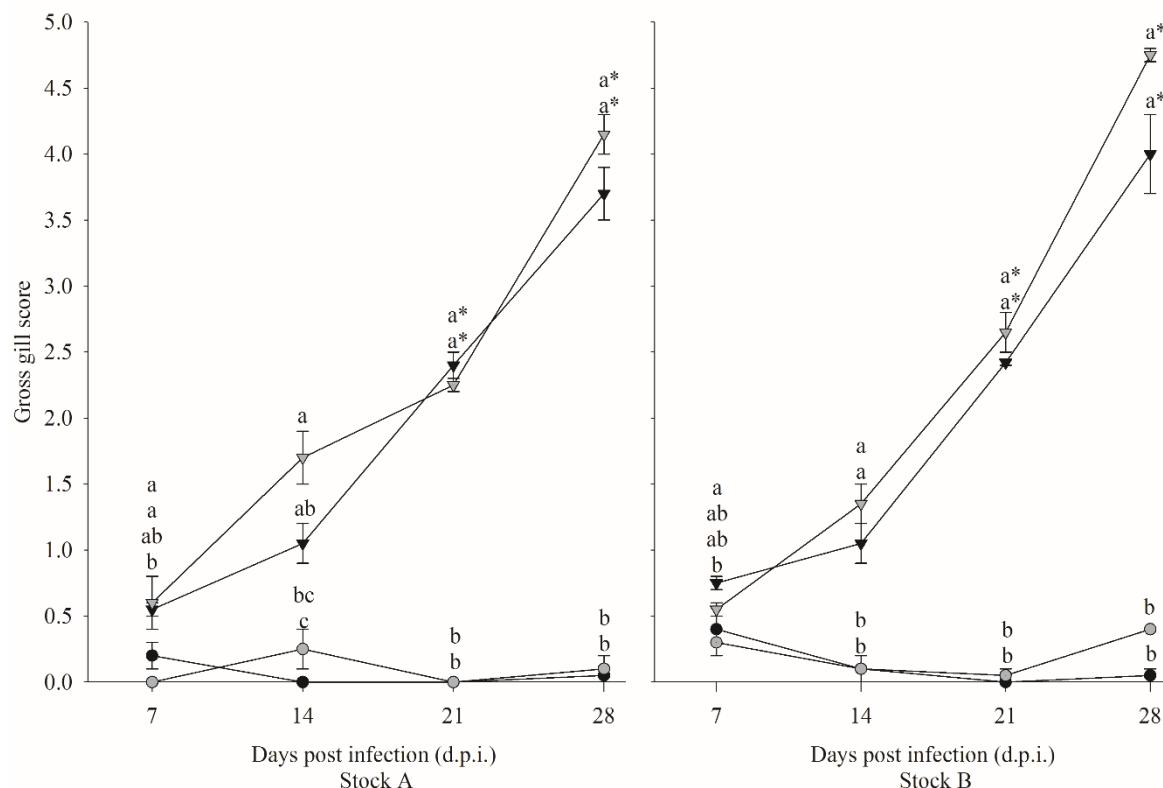


Figure 3.1: Progression of gill scores (mean \pm SEM) over time in infected (triangle) and uninfected (circle), diploid (black) and triploid (grey) Atlantic salmon from Stock A and B. Statistically significant differences between groups at each time-point are indicated by

different lowercase letters. Asterisks (*) indicate significant time differences relative to 7 d.p.i.

3.3.3. Histopathology severity

Gills at 7 d.p.i appeared normal in all ploidy groups from both stocks, with individual filaments and lamellae clearly visible (Figure 3.2 A - B; Figure 3.3 A - B). Analysis of diploid and triploid gills at 28 d.p.i revealed that, while the gill structure of uninfected fish remained unchanged over time (Figure 3.2 C; Figure 3.3 C), noticeable structural changes had occurred in the infected groups. In all diploid and triploid infected groups, severe hyperplasia and hypertrophy were clearly evident as well as lamellar fusion resulting in lacunae formation (Figure 3.2 D - E, Figure 3.3 D - E). Within the lacunae, amoebae were often visible (Figure 3.3 E). To further support visual analyses, the percentage of gills showing AGD-associated lesions was analysed (Table 3.2). Time had a significant effect in the infected groups with a significantly higher percent of affected gill filaments recorded at 28 d.p.i (Stock A: 2N 38.3 %, 3N 49.8 %; Stock B: 2N 34.6 %, 3N 41.8 %) than at 7 d.p.i (Stock A: 2N 0.0 %, 3N 0.0 %; Stock B: 2N 0.0 %, 3N 0.0 %) (Table 3.2). Infection significantly affected both stocks at 28 d.p.i, with the infected groups showing higher affected gill filaments (%) than their respective uninfected group. In addition, ploidy had a significant effect on the Stock A infected groups at 28 d.p.i, with triploids showing a higher percentage of affected filaments than their diploid counterparts.

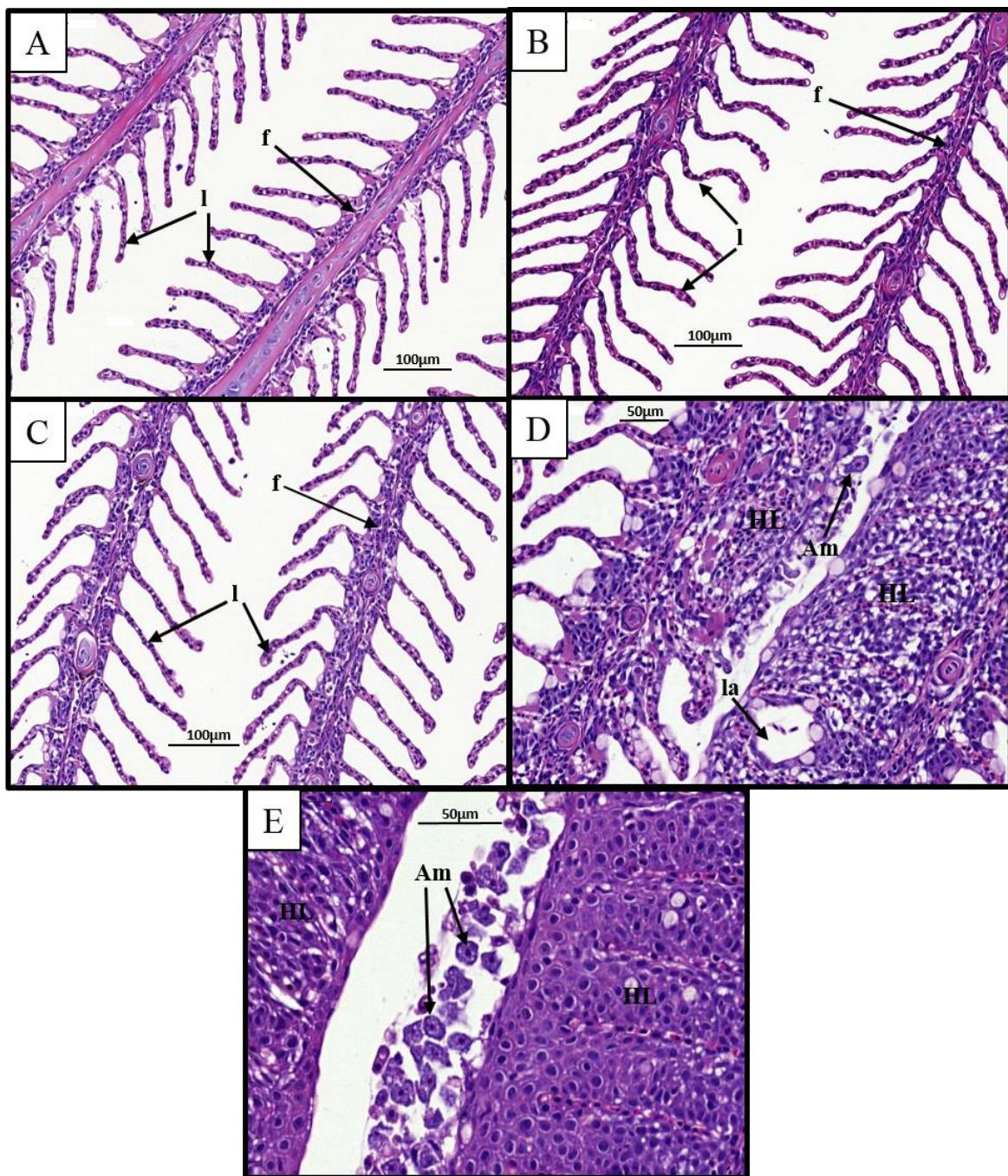


Figure 3.2: Time changes in histological pathology in the gills of infected and uninfected diploid Atlantic salmon. (A) Uninfected fish at 7 d.p.i. Individual filaments (f) and lamellae (l) visible. (B) Infected fish at 7 d.p.i. (C) Uninfected fish at 28 d.p.i. Individual filaments and lamellae remain visible. (D) Infected fish at 28 d.p.i. Hyperplastic AGD lesions (HL) clearly visible with lamellar fusion causing lacunae formation (la). Amoeba (Am) visible next to lesion. (E) Infected fish at 28 d.p.i. Numerous amoebae associated with hyperplastic gill tissue.

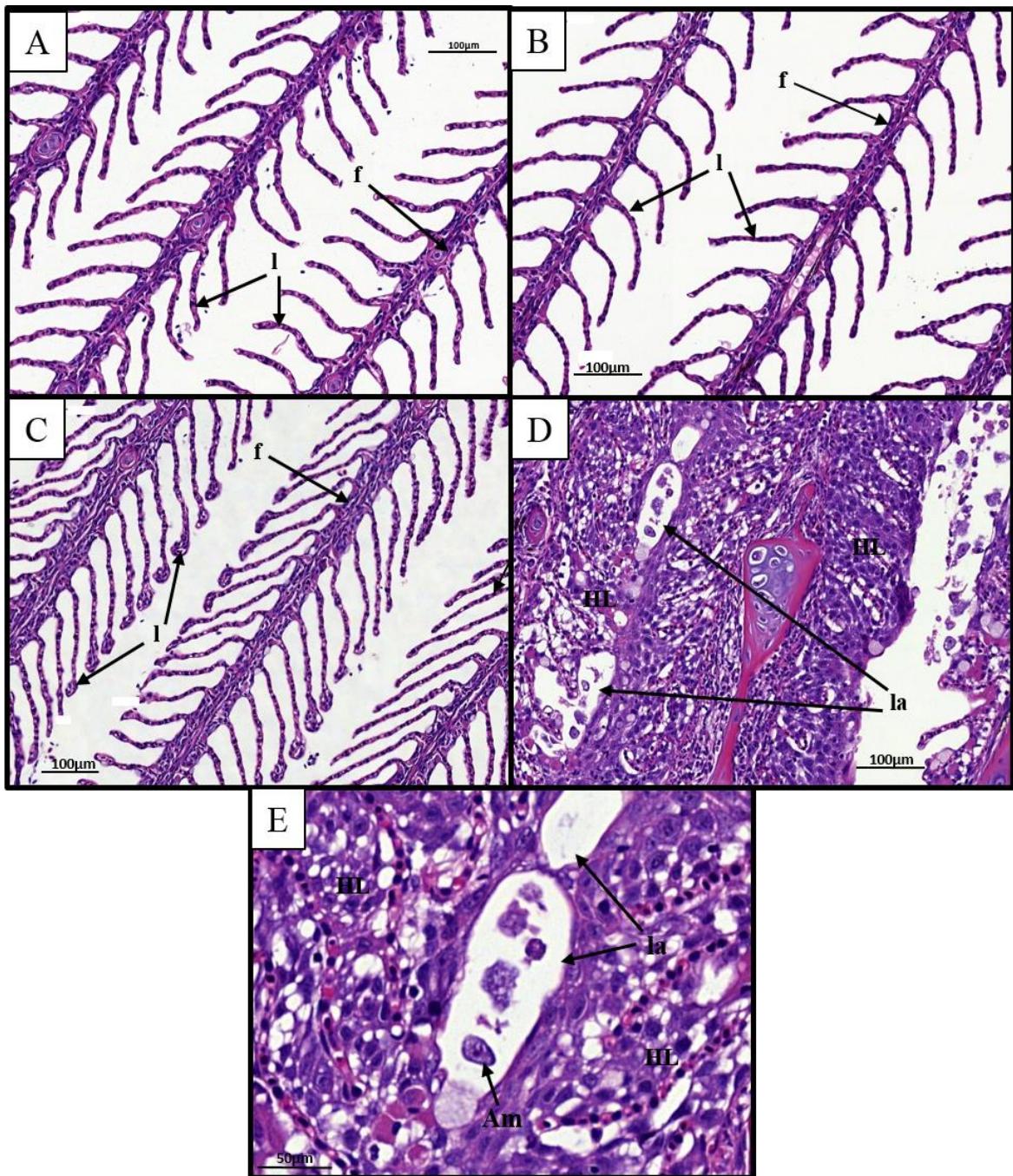


Figure 3.3: Time changes in histological pathology in the gills of infected and uninfected triploid Atlantic salmon. (A) Uninfected fish at 7 d.p.i. Individual filaments (f) and lamellae (l) visible. (B) Infected fish at 7 d.p.i. (C) Uninfected fish at 28 d.p.i. Individual filaments and lamellae remain visible. (D) Infected fish at 28 d.p.i. Hyperplastic AGD lesions (HL) visible with lamellar fusion causing lacunae formation (la). (E) Infected diploid at 28 d.p.i. Single amoeba contained within a gill lacunae.

Table 3.2: Comparison of AGD affected filaments (%) (mean \pm SEM) in uninfected and infected diploid (2N) and triploid (3N) Atlantic salmon from Stocks A and B at 7 and 28 days post-infection (d.p.i).

Time (d.p.i)	2N uninfected	2N infected	3N uninfected	3N infected
Stock A				
7	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c	0.0 \pm 0.0 ^c
28	0.0 \pm 0.0 ^c	38.3 \pm 4.5 ^b	0.0 \pm 0.0 ^c	49.8 \pm 1.3 ^a
Stock B				
7	0.0 \pm 0.0 ^b	0.0 \pm 0.0 ^b	0.0 \pm 0.0 ^b	0.0 \pm 0.0 ^b
28	0.0 \pm 0.0 ^b	34.6 \pm 1.6 ^a	0.0 \pm 0.0 ^b	41.8 \pm 6.2 ^a

Lowercase superscripts denote significant differences within each Stock

3.3.4. Mucous cells analysis

Histological examination of gill mucous cells at 7 d.p.i revealed small mucous cells in low numbers near or at the bases of secondary lamellae in all groups (Figure 3.4 A - D). At 28 d.p.i, analysis of diploid and triploid uninfected gills revealed little change in the appearance and position of mucous cells (Figure 3.4 E - F). However, in all infected groups, noticeable changes had occurred in the mucous cell populations (Figure 3.4 G - H). Mucous cells were found distributed along the entire length of the lamellae. Additionally, there appeared to be an increase in mucous cell number and diameter. In terms of cell number, no significant effect of stock, ploidy, nor time was observed (Table 3.3). In the Stock A groups at 28 d.p.i, there was a significant effect of infection on diploid and triploid groups, exhibiting significantly higher numbers than their respective uninfected group. For cell diameter, stock did not have a significant effect. Time had a significant effect on cell diameter in all infected groups and in the Stock B triploid uninfected group, with greater cell diameter recorded at 28 d.p.i than at 7 d.p.i. Infection was found to have a significant effect on cell diameter at 28 d.p.i, with significantly higher size recorded in the infected groups compared to their respective uninfected group (Table 3.3). Ploidy had a significant effect on the uninfected groups of Stock A, with larger cell diameter recorded in triploids.

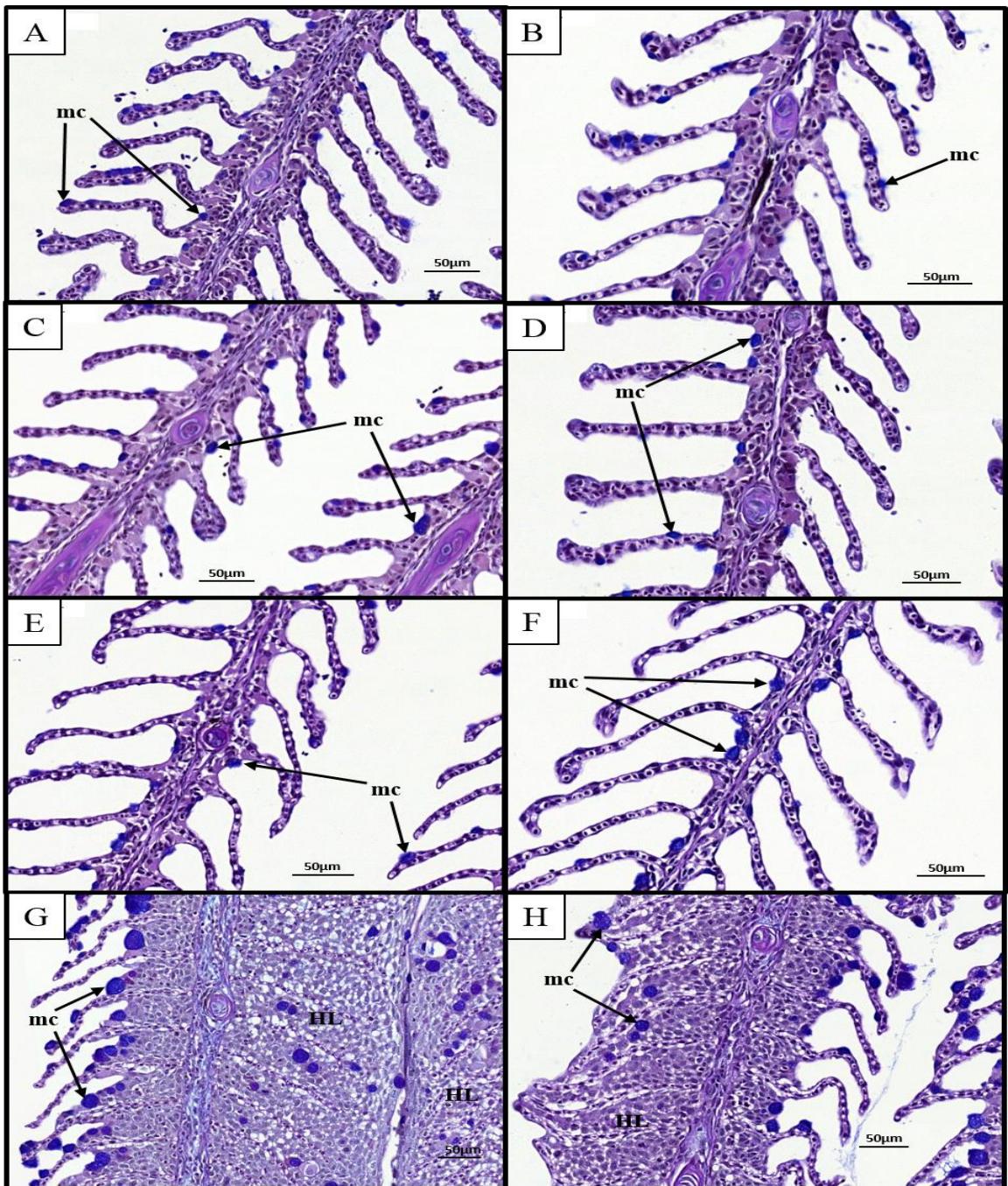


Figure 3.4: Time changes in mucous cells in the gills of infected and uninfected diploid and triploid Atlantic salmon. (A) Uninfected diploid at 7 d.p.i. Individual filament (f) and lamellae (l) visible. Small mucous cells (mc) observed on lamellae. (B) Uninfected triploid at 7 d.p.i. (C) Infected diploid at 7 d.p.i. (D) Infected triploid at 7 d.p.i. (E) Uninfected diploid at 28 d.p.i. Mucous cell remain unchanged (F) Uninfected triploid at 28 d.p.i. (G) Infected diploid at 28 d.p.i. Hypertrophied mucous cells visible on and around hyperplastic gill lesions (HL). (H) Infected triploid at 28 d.p.i.

Table 3.3: Comparison of mucous cell number and diameter (μm) (mean \pm SEM) in uninfected and infected diploid (2N) and triploid (3N) Atlantic salmon from Stock A and B at 7 and 28 days post-infection (d.p.i).

Time (d.p.i)	Mucous cell counts				Mucous cell diameter (μm)			
	2N uninfected	2N infected	3N uninfected	3N infected	2N uninfected	2N infected	3N uninfected	3N infected
	Stock A							
7	256.0 \pm 36.0	288.0 \pm 38.0	249.0 \pm 21.0	281.0 \pm 35.0	8.5 \pm 0.1 ^b	9.5 \pm 0.2 ^{ab}	10.7 \pm 0.4 ^a	9.9 \pm 0.4 ^{ab}
28	181.0 \pm 44.0 ^b	343.0 \pm 2.0 ^a	187 \pm 3.0 ^b	329.0 \pm 19.0 ^a	10.1 \pm 1.0 ^b	15.8 \pm 0.4 ^{a*}	11.6 \pm 0.2 ^{ab}	15.8 \pm 1.2 ^{a*}
Stock B								
7	204.0 \pm 31.0	259.0 \pm 8.0	282.0 \pm 24.0	219.0 \pm 41.0	7.9 \pm 0.3	7.8 \pm 0.3	9.2 \pm 0.1	9.2 \pm 0.3
28	217.0 \pm 18.0	356.0 \pm 38.0	195.0 \pm 19.0	354.0 \pm 67.0	10.4 \pm 0.2 ^c	15.4 \pm 0.7 ^{ab*}	13.1 \pm 0.6 ^{bc}	17.5 \pm 0.7 ^{a*}

Lowercase superscripts denotes significant differences between groups at each time-point within each Stock. Asterisk (*) denotes significant difference between 7 and 28 d.p.i for each group within each Stock.

3.3.5. Chloride cell

An assessment of chloride cells in infected groups at 7 d.p.i revealed chloride cells in abundance along the bases of secondary lamellae (Figure 3.5 A - B). Time dependent changes became evident at 28 d.p.i, with cells observed towards the tips of lamellae and an apparent reduction in cell number along advanced AGD lesions (Figure 3.5 C - D).

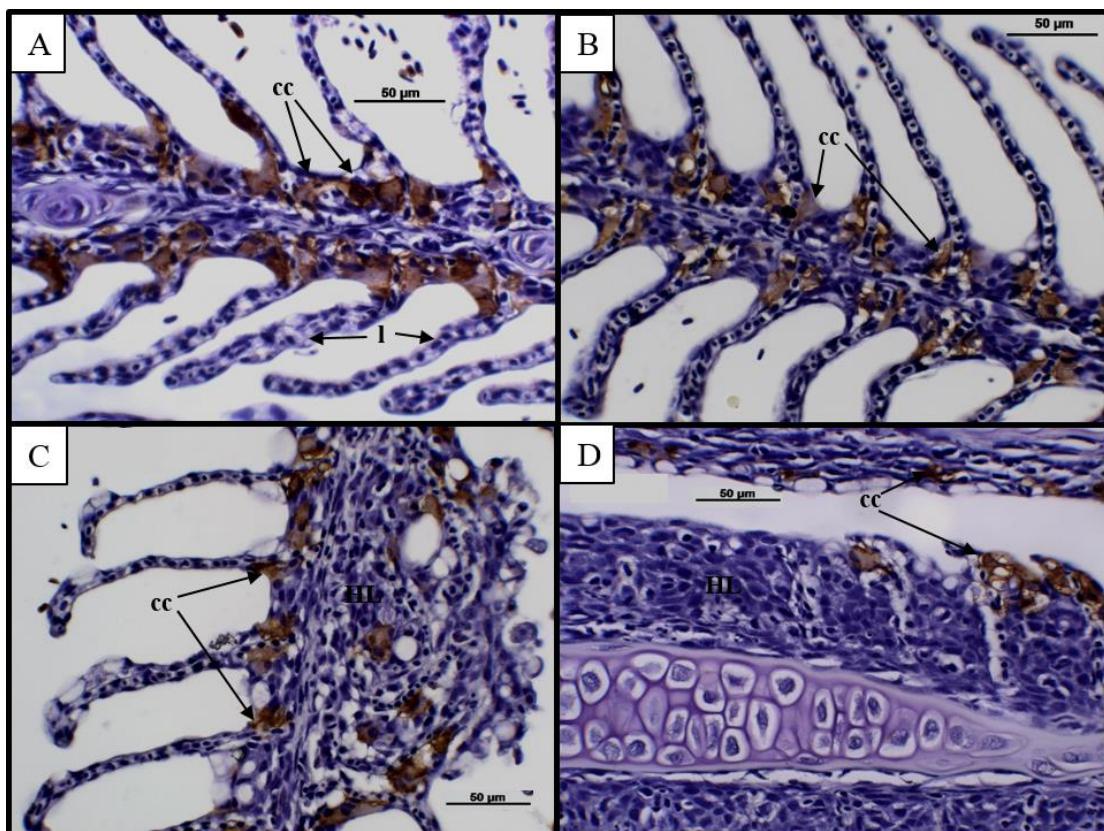


Figure 3.5: Time changes in chloride cells in infected diploid and triploid Atlantic salmon.
 (A) Diploid at 7 d.p.i. Chloride cells (cc) visible in abundance along bases of lamellae (l).
 (B) Triploid at 7 d.p.i. (C) Diploid at 28 d.p.i. Chloride cells found at the tips of lamellae. Chloride cells absent along advanced hyperplastic AGD lesions (HL). (D) Triploid at 28 d.p.i.

3.3.6. Immune response

Over time lysozyme activity increased in the infected and uninfected diploid groups of both stocks while it decreased in all the triploid groups (Figure 3.6). Stock and infection did not have a significant effect on lysozyme activity. Time had a significant effect on the infected diploid groups of Stock A and B, with higher lysozyme activity recorded at 21 d.p.i compared to at 7 d.p.i. Additionally, significantly lower lysozyme at 21 d.p.i than 7 d.p.i

was recorded for the Stock B infected triploid group. Ploidy had a significant effect on lysozyme activity. In Stock A, significantly higher activity was recorded for both diploid groups at 21 d.p.i, while for Stock B, both diploid groups showed significantly higher activity than the triploid groups at 14 and 21 d.p.i.

Variable levels of complement activity ($\text{ACH}50\%$, units ml^{-1}) were recorded in all groups from both stocks (A 29.0 ± 4.9 ; B 26.0 ± 5.3) but stock, ploidy, infection and time did not have a significant effect on complement activity (data not shown).

Similar levels of anti-protease activity (trypsin inhibition, %) were exhibited by all groups from both stocks (A 61.0 ± 5.3 ; B 57.0 ± 6.2) and no significant effect of stock, ploidy, infection or time were observed (data not shown).

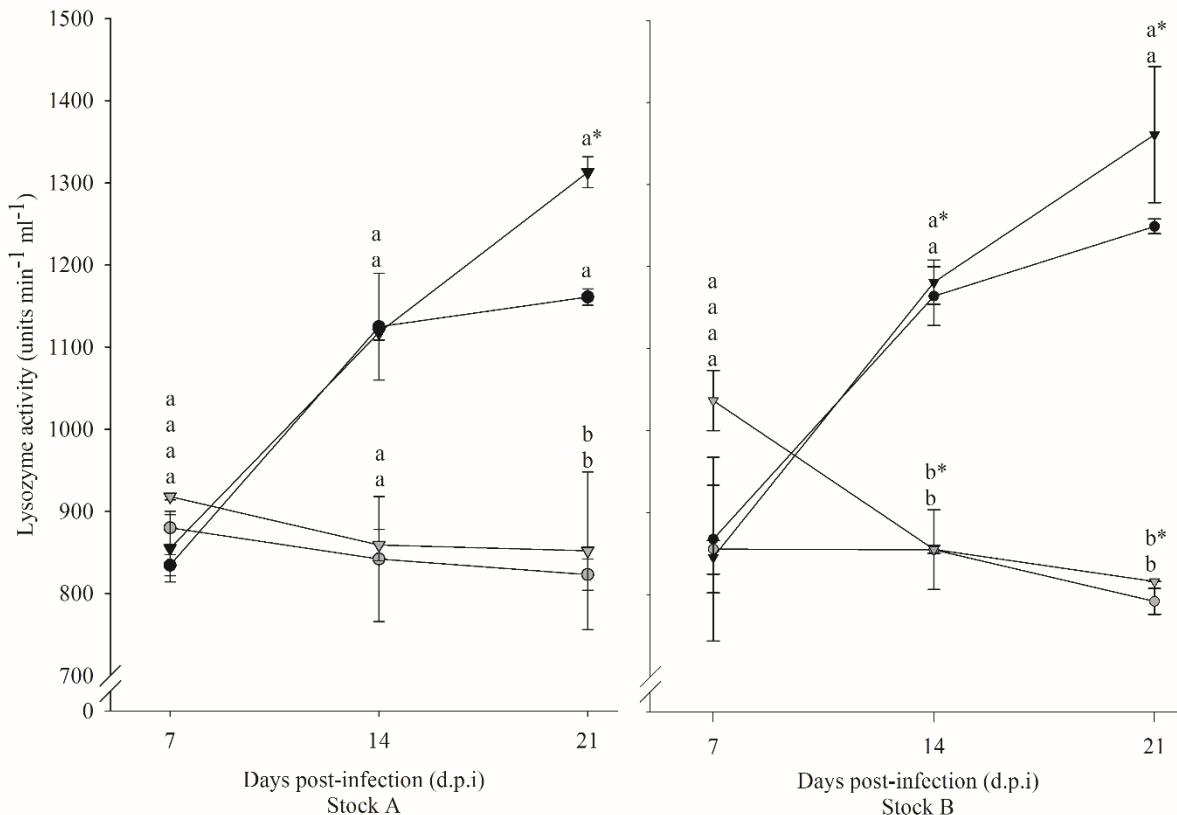


Figure 3.6: Lysozyme activity (unit $\text{min}^{-1} \text{ml}^{-1}$) (mean \pm SEM) over time in infected (triangle) and uninfected (circle), diploid (black) and triploid (grey) Atlantic salmon from Stocks A and B. Statistically significant differences between groups at each time-point are indicated by different lowercase letters. Asterisks (*) indicate significant time differences relative to 7 d.p.i.

3.4. DISCUSSION

As triploid Atlantic salmon are now being used more extensively, elucidating how they cope with AGD is important in determining their robustness and, therefore, suitability for commercial aquaculture practices (Weber *et al.*, 2013). This study compared the response of diploid and triploid Atlantic salmon to infection with *N. perurans*, the causative agent of Amoebic Gill Disease.

Throughout the 28 day challenge, mortality remained low in all groups. Ploidy did not have a significant effect on challenge mortality, and this concurs with findings of a Scottish field-based study in which sibling groups of diploid and triploid Atlantic salmon experienced a natural AGD challenge (temperature range: 11 – 15 °C, comparable to this current study), yet mortality was comparable between ploidy (Smedley *et al.*, 2016). However, these results are in contrast to an experimental infection study with *N. perurans* performed in Tasmania, in which triploids were shown to have lower survival than diploids (Powell *et al.*, 2008). Unfortunately, details of challenge model were not described in Powell *et al.* (2008), as such contrasting results between their study and the current trial may arise from differences in challenge severity, water temperature, other environmental factors, origin of fish, and location. Infection did not significantly affect mortality. While infected fish could have been expected to experience higher mortalities, considering the levels of mortality associated with AGD outbreaks (Oldham *et al.*, 2016), it is not possible to make study comparisons at this time due to the limited experimental cohabitation or bath challenge studies presenting mortality data. As such, studies should be planned to fully assess the occurrence and progression of mortalities in response to experimental AGD.

A key feature of AGD pathology is the occurrence of white mucoid patches on the gills, and these are used to grossly assess and score infection level (Taylor *et al.*, 2009). In this study, gross gill scores increased over time in the diploid and triploid infected groups while they remained consistently low in the uninfected groups. This is consistent with previously published 28 day AGD cohabitation studies (Findlay *et al.*, 1995; Zilberg and Munday, 2000) as well as bath challenge studies of the same duration (250 amoebae trophozoites L⁻¹) (Norte dos Santos *et al.*, 2014) or shorter (10 days) with a higher amoebae load (2000 trophozoites L⁻¹) (Pennacchi *et al.*, 2014). Additionally, non-significant differences in the levels of grossly observable pathology between groups for each time-point indicate that the cohabitation challenge utilised provided a consistent approach to inducing AGD under experimental conditions. In the present study, ploidy did not have a significant

effect on gill score severity which is consistent with a previous study indicating that ploidy did not affect the severity of infection with sea lice (Frenzl *et al.*, 2014).

In addition to gross gill scores, histological gill examination is also utilised to indicate both pathogen presence and host response (Adams *et al.*, 2004; Taylor *et al.*, 2009). Taking into account the increase in gross gill score severity over time, it was considered that the gills from the 7 and 28 d.p.i sampling events would show the most severe pathological changes and were, therefore, chosen for histological processing and examination. Clear alterations to gills were evident in the infected groups. While normal gill structure was observed at 7 d.p.i, extensive epithelial hyperplasia as well as complete lamellar fusion and lacunae formation was found at 28 d.p.i. Amoebae were also found associated with areas of the infected gill tissue and within the lacunae. These gill changes are amongst the key pathological features of AGD and are supported by a 19 week study collecting samples from natural AGD outbreaks as well as experimental bath challenges ranging from 10 (250 trophozoites L⁻¹) to 16 days (10,000 trophozoites L⁻¹) in length (Adams and Nowak, 2003; Leef *et al.*, 2005; Peyhan and Powell, 2006). When assessing histopathological changes, the percentage of gill filaments exhibiting AGD-associated lesions was analysed. The percentage of AGD-affected filaments recorded for the uninfected groups remained at 0 % for the duration of the study, while it increased significantly in the infected groups. This is consistent with another cohabitation study which found more severe pathology in infected fish at 28 d.p.i than at 7 d.p.i (Zilberg and Munday, 2000). In the present study, ploidy had a significant effect on the percentage of AGD-affected gill filaments at 28 d.p.i in Stock A, with triploids showing a higher percent affected than diploids. In contrast, the opposite was observed by Powell *et al.* (2008) with the diploids showing more affected filaments. In their study, Powell *et al.* (2008) also observed increased mortality in triploids and so it could be suggested that the most severely affected triploids had died before day 28. However, as an assessment of lesion severity in the mortalities was not performed either by Powell *et al.* (2008) or in this study, it is difficult to confirm whether lesion severity had an impact on mortality.

As well as alterations to overall gill structure, infection by *N. perurans* has also been shown to cause changes in mucous cells (Roberts and Powell, 2003; Adams and Nowak, 2003). At 7 d.p.i, small mucous cells were found distributed along the length of the lamellae. At 28 d.p.i, while mucous cells remained unchanged in uninfected fish, they were found nearer the tips of secondary lamellae in the diploid and triploid infected groups. This finding concurs well with previously published research and is suggestive of a link to the excessive

mucus production associated with AGD (Roberts and Powell, 2003; Morrison and Nowak, 2008; Mitchell and Rodger, 2011). Additionally, increases in mucous cell number and size were observed. Infection had a significant effect on the mucous cell numbers of the Stock A groups at 28 d.p.i, with infected fish showing significantly higher numbers than the uninfected groups. This is consistent with results from previous studies using various infection methods and time-frames, all of which found increased mucous cell numbers in response to infection by *N. perurans* (Zilberg and Munday, 2000; Roberts and Powell, 2003; Adams and Nowak, 2003). Mucous cell diameter increased significantly from 7 to 28 d.p.i in the infected fish for both stocks and ploidy. As such, larger cells were observed in infected fish at 28 d.p.i compared to uninfected counterparts. Such hypertrophied mucous cells have been commonly reported with AGD (Adams and Nowak, 2004). The lack of clear and significant differences in mucous cell number and size between ploidy is surprising. Indeed, many studies have reported significantly reduced cell numbers and greater cell size in triploids as a compensatory mechanism for having three sets of chromosomes. This mechanism has been found in a number of other fish species and cell types, particularly erythrocytes (Small and Benfey, 1987; Stillwell and Benfey, 1996; Budiño *et al.*, 2006). However, for both mucous cell counts and measurements, it should be noted that the depth of gill section in which the cells were observed was not constant. It is recognised that this difference may have impacted on the counts and measurements reported and, as such, it is recommended that further work on the impact of AGD on diploid and triploid Atlantic salmon mucous cells be conducted with key attention paid to histological processing and gill section depth.

Along with changes in mucous cells, other AGD associated gill changes include the movement or location of chloride cells (Adams and Nowak, 2003) which was also evident in the current study. It was found that, prior to the appearance of clinical signs in diploid and triploid Atlantic salmon, chloride cells were abundant along the bases of secondary lamellae. By 28 d.p.i, chloride cells were observed at the tips of secondary lamellae and were missing completely from advanced AGD lesions. These findings are consistent with a 10 day experimental bath challenge as well as studies collecting samples from natural AGD outbreaks over a longer time-frame (Adams and Nowak, 2003; Bermingham and Mulcahy, 2006; Peyhan and Powell, 2006). Fish with extensive AGD lesions have previously been shown to suffer osmoregulatory failure and it has been suggested that reductions in gill chloride cells during AGD may cause increased blood sodium levels (Findlay, 2001; Adams and Nowak, 2003). In contrast, studies have also shown comparable plasma ion and enzyme

activity levels in the gills of infected and uninfected fish, suggesting that AGD does not cause osmoregulatory dysfunction (Powell *et al.*, 2001, 2008). However, these differences in findings could be attributed to the method of infection used, with the studies collecting samples from bath and cohabitation challenges as well as natural in-field challenges. As such, while AGD is known to damage the gills, having the potential to compromise the function of chloride cells (Findlay, 2001; Adams and Nowak, 2003), it is recommended that additional studies on the impact of AGD on chloride cells be carried out to fully clarify these effects.

Previously published data regarding the immune response of Atlantic salmon to AGD has generally focussed on gene expression, antibody production and histopathology (Valdenegro-Vega *et al.*, 2014). As such, studies have continued to describe and elucidate the host innate and adaptive immune responses when infected with *N. perurans* (Gross *et al.*, 2005; Young *et al.*, 2008c; Valdenegro-Vega *et al.*, 2014). In this study, lysozyme activity increased over time in both diploid groups while it decreased slightly in the triploid groups. Although ploidy differences were significant at 21 d.p.i, the lysozyme activity recorded was found to be in the range previously reported for Atlantic salmon and other salmonids (Saurabh and Sahoo, 2008; Korkea-aho *et al.*, 2011; Dick, cited in Nowak *et al.*, 2014; Metochis *et al.*, 2017). In terms of the infected groups, this significant ploidy difference is in contrast to previous results in which no ploidy differences in serum lysozyme activity were observed (Powell *et al.*, 2008). As the study duration was the same, it could be suggested that this difference may be due to the challenge model utilised or parasite load but with no details presented, it is difficult to elucidate. For the uninfected groups, this ploidy difference is also contrasting to previous studies as comparable lysozyme was observed in response to seawater transfer and seasonal changes (Budiño *et al.*, 2006; Taylor *et al.*, 2007; Tolarová *et al.*, 2014). As such, it is unclear why these ploidy differences occurred and it is recommended that studies be carried out to determine ploidy differences in lysozyme in response to AGD. Within ploidy, infection did not have a significant effect on lysozyme activity and this is supported by Gross *et al.* (2005), who found comparable lysozyme activity between infected and uninfected diploid Atlantic salmon throughout an 11 day study. While lysozyme has been shown to change in response to other parasites such as *I. multifiliis* and *Ceratomyxa shasta* (Alvarez-Pellitero, 2008), the current results suggest that serum lysozyme may not play a major role in AGD. This was also suggested by Gross *et al.* (2005) who proposed that, as lysozyme levels are regulated by peripheral blood leucocytes, infection by *N. perurans* may not stimulate these cells in the serum to elicit a

response. In contrast, a study by Cook *et al.* (2012) found a marker encoding a novel g-type lysozyme, the expression of which suggests its involvement in the innate immune response against AGD.

Complement, a group of serum proteins known to assist the killing properties of antibodies (Yano, 1992), has been shown to protect against other parasites including *Cryptobia salmositica*, *I. multifiliis* and *G. salaris* (Harris *et al.*, 1998; Alvarez-Pellitero, 2008), as well as kill *G. salaris* *in vitro*. However, limited knowledge is available on its involvement in AGD. Variable patterns of complement activity were observed in this study but with no apparent significant effect of ploidy, infection, time or stock. This is again consistent with previous studies which found no significant change in serum alternative complement activity following infection with *N. perurans* (Powell *et al.*, 2008; Dick, cited in Nowak *et al.*, 2014). Additionally, Gross *et al.* (2005) reported on a pilot study showing that normal serum had no killing effect on *Neoparamoeba* sp. As such, it could be suggested that complement in the serum does not play a key role in providing protection against AGD.

In terms of anti-protease activity, trypsin inhibition varied little between the groups, with no significant effect of ploidy, infection, time or stock observed. A previous study investigating the effects of AGD on diploid and triploid Atlantic salmon also found no significant effect of ploidy on anti-proteases (Powell *et al.*, 2008). However, due to the limited data regarding the involvement of anti-proteases in AGD and, more so, the lack of data concerning anti-protease activity in triploids, it is difficult to confirm whether the patterns observed in this study are expected. While anti-proteases are known to be involved in other parasitic infections such as *C. salmositica* and *Enteromyxum leei* (Sitjà-Bobadilla *et al.*, 2008; Alvarez-Pellitero, 2008), these are endoparasites and it could be suggested that, as an ectoparasite, *N. perurans* does not elicit a response from anti-proteases in the serum.

3.5. CONCLUSION

In conclusion, this study found that the severity of AGD was not affected by ploidy and that both diploids and triploids exhibited a similar degree of gill pathology. However, considering the previously reported ploidy differences in AGD associated pathology, it is recommended that further studies be undertaken to fully elucidate ploidy differences in the development of AGD associated pathology. Complement and anti-protease activities were not influenced by ploidy or infection while lysozyme activity was significantly affected by ploidy but was not found out-with normal ranges. This could suggest that the serum innate

immune response has a limited role in protecting against infection with *N. perurans*. Overall, the findings of this study would suggest that triploid salmon appear to have similar susceptibility to diploid siblings in response to disease challenge with *N. perurans* and provides a base for further research into the immune response of triploid Atlantic salmon.

Chapter 4

Comparison of stress and immune responses between sibling diploid and triploid Atlantic salmon (*Salmo salar*) following experimental treatment with hydrogen peroxide

4.1. INTRODUCTION

As the global human population continues to grow, so will the demand for aquaculture food products, with the industry aiming towards the intensification and expansion of production (Bostock *et al.*, 2010; The World Bank, 2013). However, increasing intensification is not without risks as it is considered that fish in intensive aquaculture systems can experience higher pathogen infection pressures than their wild counterparts (Ernst *et al.*, 2002; Bondad-Reantaso *et al.*, 2005). As a result, disease and the resultant health issues are recognised as one of the largest single causes of economic losses for aquaculture, representing a significant constraint to the industry's continued development and success (Meyer, 1991; Subasinghe, 2005; Defoirdt *et al.*, 2011).

Over the years, bacterial and viral diseases have caused significant problems for the Atlantic salmon aquaculture industry including those caused by *A. salmonicida*, *M. viscosa*, *V. salmonicida*, IPNV, SAV and ISAV (Rodger, 2016). Effective vaccines have been developed to prevent and control many of these diseases, particularly for those caused by bacterial pathogens (Meyer, 1991; Erdal and Reitan, 1992; Rimstad, 2014; Rodger, 2016). Currently, the issues associated with parasitic diseases pose the most significant threat for Atlantic salmon aquaculture. In particular, sea lice and, more recently, AGD are considered two of the most damaging parasites for the salmonid industry, with losses equating up to 430 million and 80 million USD worldwide per year, respectively (Costello, 2009; Whelan, 2010; Fast, 2014; Shinn *et al.*, 2015). With no vaccine available for the prevention of these parasites and mitigation often difficult, a large proportion of the associated economic losses can be attributed to chemotherapeutic treatments (Costello, 2009). One such treatment, now regularly employed by the aquaculture industry for the control of both sea lice and AGD, is a bath application of hydrogen peroxide (H_2O_2) (Adams *et al.*, 2012; Ruane and Jones, 2013).

Hydrogen peroxide has long been used in aquaculture as a disinfectant for eggs (Yanong, 2014). Its use in the control of sea lice infestations began in the early 1990's and it has been implemented to control AGD since 2012 (Adams *et al.*, 2012; Helgesen *et al.*, 2015). In current aquaculture practices, the recommended H_2O_2 concentration for the treatment of these parasites is 1500 ppm (Kiemer and Black, 1997; Treasurer and Grant, 1997; Rodger, 2014) although it is recognised that concentrations of up to 2000 ppm are also used (Kiemer and Black, 1997). Several factors make this product suitable for application in aquaculture. It has a highly reactive nature which makes it ideal for combatting external parasites (Yanong, 2014). Hydrogen peroxide is a strong oxidising

agent that causes mechanical paralysis, peroxidation of lipids and cellular membranes, inactivation of enzymes and inhibition of DNA replication. In sea lice, this compound appears to induce mechanical paralysis when bubbles form in the gut and haemolymph, causing the parasite to release from the host and float to the surface (Burridge *et al.*, 2010). Additionally, H₂O₂ breaks down into water and oxygen when in the aquatic environment, therefore leaving no toxic by-products and making it reasonably environmentally friendly (Yanong, 2014; Arvin and Pedersen, 2015). However, concerns have been raised regarding the issue of fish welfare during exposure to this chemical as it has been reported to cause stress in Atlantic salmon in the first 24 h post-exposure (Bowers *et al.*, 2002; Vera and Migaud, 2016).

Triploid Atlantic salmon have long been considered as a solution for the issues associated with pre-harvest sexual maturation and escapees in the aquaculture industry (Benfey, 2015). While many similarities have been observed, differences have also been documented, with poorer growth and increased deformities reported in triploids (McGeachy *et al.*, 1995; Withler *et al.*, 1995; O'Flynn *et al.*, 1997; Sadler *et al.*, 2001). With the expansion of triploid research over the years, advances in triploid nutrition and rearing have now shown triploids performing equally or better than their diploid counterparts (Taylor *et al.*, 2011, 2012, 2013, 2015, Fraser *et al.*, 2013, 2015a; Fjelldal *et al.*, 2016). The findings presented in the earlier chapters of this thesis (**Chapters 2 and 3**), along with previous studies, continue to reveal no significant difference between diploids and triploids in response to disease challenge and vaccination. However, the response of triploid Atlantic salmon to chemical treatments such as H₂O₂ is still a relatively unexplored subject. Considering the potential to apply triploid salmon in full commercial production, it is crucial to understand their physiological response when exposed to aquaculture medicines in order to optimise health management strategies without compromising fish welfare.

4.1.1. Aims

The aim of this Chapter was to investigate the response of diploid and triploid Atlantic salmon to experimental exposure with H₂O₂ and assess susceptibility along with stress, immune and toxicological responses to this widely practiced treatment for parasitic diseases.

4.2. MATERIALS AND METHODS

4.2.1. Fish stock and history

Eggs and milt were stripped from commercial Atlantic salmon broodstock (Landcatch Ltd.) and delivered to the Institute of Aquaculture, University of Stirling in December 2014. Following fertilisation, half of each egg batch was subjected to a pressure shock (655 bar for 6.25 min, 37.5 min post-fertilisation at 8 °C) to induce triploidy. Eggs were then incubated at 8.0 ± 0.1 °C in troughs throughout hatching (5th February 2015). At first feeding (2nd April 2015, 949 °D), fry were transferred into 300 L tanks and reared under constant light. Fry were fed a commercial diet (diploids - Inicio Plus; triploids – Inicio-TriX, BioMar UK), distributed by automatic feeders (Arvo-Tec Oy, Finland). When approximately 1 g (1738 °D), all fry were transferred to the NBFRF, Buckieburn. They were maintained in 1.6 m³ tanks under ambient water temperature (average: 8.3 ± 4.2 °C; range: winter 1.5 °C – summer 14.0 °C) and photoperiod to produce S1+ smolts. Specific feeding rates (% tank biomass per day) were adjusted automatically according to predicted growth and daily temperature, and pellet size (0.5 to 3.0 mm) increased with fish size. Diploid and triploid Atlantic salmon were then transferred from the NBFRF to the Institute of Aquaculture Temperate Aquarium Facilities on 18th February 2016. In preparation for sea water transfer, fish were vaccinated on 14th March 2016 with WINVIL® 3 micro (Elanco Europe Ltd., United Kingdom). Mortality between first feeding and sea transfer was 4.8 % and 5.1 % for diploids and triploids, respectively.

On 14th April 2016, 250 diploid (88.5 ± 2.2 g average body weight) and 250 triploid (78.2 ± 1.0 g average body weight) Atlantic salmon smolts were transferred to the MERL seawater facility (Machrihanish, Institute of Aquaculture, Stirling, Scotland) and stocked into two 2 m diameter stock tanks (3 m³; 0.5 L kg biomass⁻¹ min⁻¹ flow rate). Tanks were maintained under ambient temperature (11.5 ± 1.8 °C) with aeration provided by air stones for 96 days until the trial commenced.

4.2.2. Hydrogen peroxide challenge

4.2.2.1. Dose-response toxicity test

A dose response test was firstly undertaken to determine an appropriate H₂O₂ dose for the stress challenge in diploid and triploid Atlantic salmon. This test was performed in July 2016 at ambient water temperature (14.0 °C) and simulated natural photoperiod set at 17 h light: 7 h darkness. For the dose response test, 21 diploid (183.0 ± 5.6 g body weight and 266.7 ± 2.4 mm body length) and 21 triploid (215 ± 4.9 g body weight and 282.4 ± 2.2 mm

body length) Atlantic salmon were used. Experimental fish were randomly allocated into 14 x 397 L cylindrical tanks ($n = 3$ fish tank $^{-1}$; 7 tanks ploidy $^{-1}$). Following stocking, one tank ploidy $^{-1}$ was allocated to each test concentration (1500, 1700, 1900, 2100, 2300, 2500 and 2700 ppm). Each concentration was then assessed separately. Fish were exposed to each concentration for 20 min before the tanks were flushed and the water refilled. During the treatment, water was aerated ensuring that oxygen levels were above 84 % saturation. After exposure, fish were monitored for 2 h for visual signs of stress *e.g.* flared opercula, increased ventilation, loss of equilibrium, death. While fish at all concentrations coped well during the exposure time, loss of equilibrium and mortalities (diploid: 14.3 %; triploid: 9.5 %) were observed following treatment with concentrations of 2100 ppm and above. As such, 1900 ppm was selected for the acute stress response trial.

4.2.2.2. Acute stress response test

The acute stress response challenge was performed in August 2016 with ambient water temperature (14.0 °C) and simulated natural photoperiod set at 17 h light: 7 h darkness. For the challenge, 224 diploid (183.0 ± 5.6 g body weight and 266.7 ± 2.4 mm body length) and 224 triploid (215 ± 4.9 g body weight and 282.4 ± 2.2 mm body length) Atlantic salmon were used. Experimental fish were randomly allocated into 32 x 397 L cylindrical tanks (1 m diameter, 0.4 m depth, $n = 7$ fish tank $^{-1}$; 16 tanks ploidy $^{-1}$) (Figure 4.1). All fish were then acclimated in trial tanks for 1 week prior to H₂O₂ exposure. On the day of H₂O₂ challenge, 112 diploid and triploid salmon ($n = 16$ tanks ploidy $^{-1}$) were treated with a H₂O₂ concentration of 1900 ppm at 09:00 h and then fish were sampled at 1, 3, 6 and 24 h post-exposure (h.p.e) ($n = 4$ tanks time-point $^{-1}$; 28 fish) (Figure 4.1). Different tanks were sampled at each time-point to avoid repeat netting of the fish. The water in the tanks was turned off and lowered to a set volume (200 L) before fish were exposed to 1900 ppm H₂O₂ for 20 min after which the tanks were flushed and refilled with clean water. During the treatment, water was aerated ensuring that oxygen level was 84 % saturation.

Water samples were collected from each tank following the addition of H₂O₂ and the H₂O₂ concentration was immediately measured by a cerium sulphate titration method (Vera and Migaud, 2016). To this end, 5 ml of 5 N H₂SO₄ and 7.5 ml of cerium IV sulphate solution were mixed in a conical flask. Then, a burette was filled with 50 ml of water sample which was slowly dispensed into the cerium IV sulphate solution, swirling to mix until the solution went colourless. The reading of the burette was then recorded and H₂O₂ concentration calculated:

H_2O_2 concentration = $(7.5 \times 0.1 \times 1000 \times 34) / (2 \times \text{volume of seawater sample used})$.

Where 7.5 is the volume of cerium IV sulphate, 0.1 is the concentration of cerium IV sulphate, 1000 is the conversion factor and 34 is the molecular weight of H_2O_2 . The average measured concentration across all experimental tanks was 1807 ± 83.9 ppm.

At each time-point, all 28 fish per ploidy were culled by lethal anaesthesia (MS-222, 1000 ppm, PHARMAQ, Norway) before being sampled. Fish from each time-point tank ($n = 4$ tanks ploidy $^{-1}$; 7 fish tank $^{-1}$) (Figure 4.1) were sampled within 15 min post-cull. Blood samples were obtained from the caudal vein using a heparinised needle and syringe. An aliquot of blood was used for glucose and lactate measurements *in situ* while the remaining blood volume was kept on ice until centrifugation at 3000 xG for 10 min after which plasma was removed and frozen at -20 °C for cortisol analysis. Finally, small sections of the left second gill arch and liver were excised into RNAlater and kept on ice until further storage at -20 °C prior to gene expression. On completion of H_2O_2 challenge, the tanks were restocked as previously described (Figure 4.1) and fish acclimated for 1 week before the trial and samplings were repeated in full for the diploid and triploid control groups. To this end, at 09:00 h on the sampling day, control fish were subjected to water volume reduction, aeration, water flushing and refilling but H_2O_2 was not added, and then fish were sampled 1, 3, 6 and 24 h later. At each time-point, 28 control diploid and triploid salmon were sampled as described previously ($n = 4$ tanks ploidy $^{-1}$) (Figure 4.1).

4.2.3. Glucose and lactate

Blood glucose and lactate were measured immediately after extraction by means of handheld meters: Contour® USB (Bayer HealthCare, UK) and LactatePro™ 2 (Arkray Europe, The Netherlands), respectively. These had been previously validated for Atlantic salmon samples using enzymatic-colorimetric commercial test kits: Glucose (GO) Assay Kit (Sigma) and Lactate Dry-Fast (Sentinel Diagnostics, Italy) (Vera and Migaud, 2016).

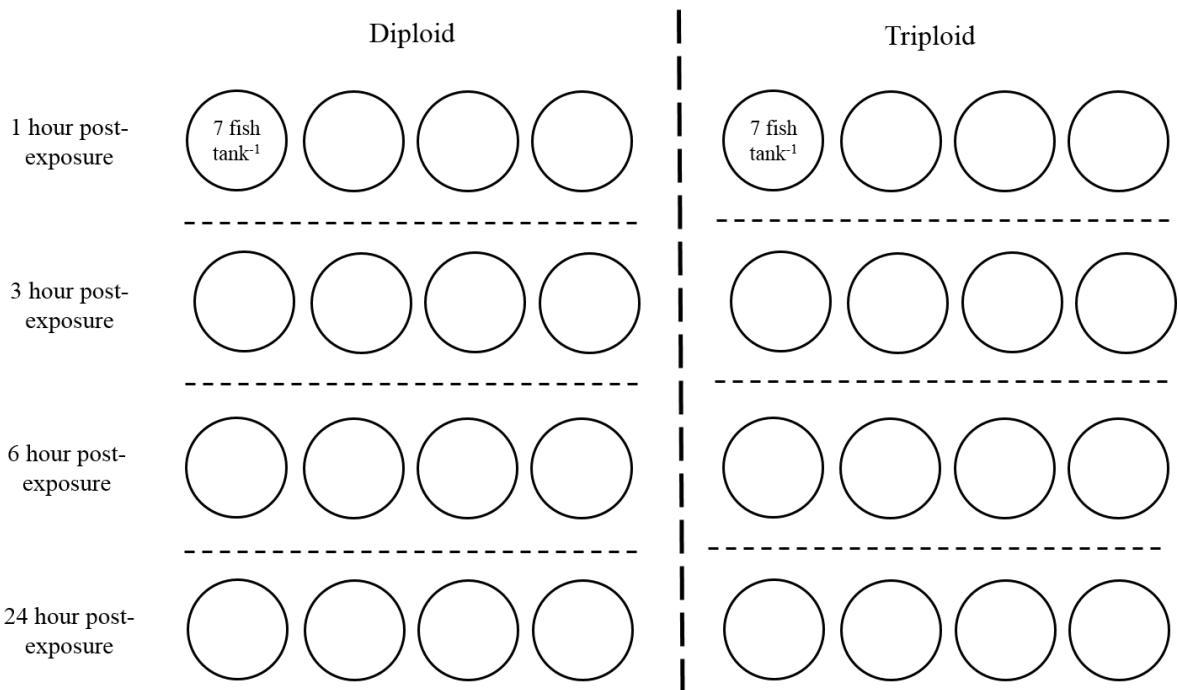


Figure 4.1: Schematic representation of experimental tank set-up for diploid and triploid Atlantic salmon. Indicates 4 tanks allocated to each sampling time-point and 7 fish allocated to each tank. Dotted lines used to differentiate tanks between sampling time-points (hours post exposure) and ploidy

4.2.4. Cortisol

Plasma cortisol levels were measured with a commercial ELISA kit (RE52061, IBL-International, Hamburg, Germany). This kit had been previously used to quantify plasma cortisol in Atlantic salmon (Oxley *et al.*, 2010; Fraser *et al.*, 2014b). Briefly, 20 µl of plasma sample (undiluted plasma for the diploid and triploid control groups and plasma diluted 1/3 for both H₂O₂-exposed groups) was added to a 96 well microplate (provided with the kit) along with standards A - G (0, 20, 50, 100, 200, 400 and 800 ng/mL) and controls (41 – 96 and 146 – 271 ng/ml). Enzyme conjugate was then added (200 µl well⁻¹) and the plate incubated at RT for 60 min. The solution was then discarded and the plate washed 3 times with 300 µl of wash buffer (provided with the kit) after which the excess was removed. TMB substrate solution (provided with the kit) was then added to each well (100 µl⁻¹) and the plate incubated at RT for 15 min. Following this, the TMB stop solution (provided with the kit) was added (100 µl⁻¹ well) and absorbance measured at 450 nm using a plate reader (Synergy HT, BioTek, USA). The Gen5 Data Analysis Software was then used to calculate the concentration of cortisol in each sample based on the known standard curve

concentrations, the dilutions made and the absorbances obtained. The intra- and inter-assay coefficients of variation were 2.6 – 3.5 % and 2.1 – 5.0 %, respectively (n = 20).

4.2.5. Lysozyme

Lysozyme activity was determined according to the method described in Chapter 3 (Section 3.2.6.1)

4.2.6. Gene expression

Liver and gill samples were added to 1 ml of TriReagent (Sigma Aldrich, UK) and incubated on ice for 60 min. Samples were then homogenised using a Mini-BeadBeater 24 (BioSpec Products Inc., Oklahoma, USA) until the tissue was disrupted. The homogenised samples were then incubated at RT for 5 min following which samples were centrifuged at 12,000 xG for 10 min at 4 °C and the supernatant transferred into a fresh tube. Then 100 µl of 1-Bromo-3-chloropropane (BCP) was added to each sample and the tube shaken vigorously by hand for 15 s. Samples were incubated at RT for 15 min after which they were centrifuged at 20,000 xG for 20 min at 4 °C. The aqueous (upper) phase was then slowly and carefully transferred into a fresh tube (approximately 300 – 400 µl). A half volume (per aqueous phase volume; e.g. 150 – 200 µl) of RNA precipitation solution (1.2 M NaCl, 0.8 M sodium citrate sesquihydrate) was added to each sample followed by the same volume of isopropanol before being gently inverted 4 to 6 times. Samples were incubated 10 min at RT and centrifuged at 20,000 xG for 10 min at 4 °C. The RNA precipitate formed a pellet on the side/bottom of the tube. The supernatant was discarded and the pellets washed with 1 ml of 75 % ethanol for 30 min at RT. Then the pellets were centrifuged at 20,000 xG for 5 min at RT and the supernatant discarded. The ethanol wash was repeated for 1 h before re-centrifuging at 20,000 xG for 5 min. The supernatant was discarded and the RNA pellet allowed to air dry at RT for 3 to 5 min to remove all traces of ethanol. RNA pellets were rehydrated in MilliQ water (250 µl liver samples; 75 µl gill sample) and incubated at 55 °C for 5 min and then at RT for 40 min with gentle flicking of the tubes every 10 min to aid resuspension. Total RNA concentration was determined using a ND-1000 Nanodrop spectrophotometer (Labtech Int., East Sussex, UK) and RNA integrity (300 ng in 5 µl) was assessed by electrophoresis. To eliminate genomic DNA contamination, total RNA was DNase treated (DNA-free™ kit, Thermo Fisher Scientific, Waltham, USA), following the manufacturer's instructions. The relative expression of 10 genes (6 oxidative stress markers and 4 immune genes) was determined in liver and gills from fish of all treatments. The

primers used to amplify *cat*, *gpx1*, *gr*, *hsp70* were previously tested and validated for Atlantic salmon (Betancor *et al.*, 2014; McStay *et al.*, 2014; Olsvik *et al.*, 2014; Vera and Migaud, 2016). The primers used to amplify *sod1*, *sod2*, *saa5*, *crp/sap1a*, *crp/sap1b* and *illβ* were designed *de novo* using software PRIMER3 (Untergasser *et al.*, 2012) and their target specificity was checked *in silico* using Blast (NCBI). cDNA was reverse transcribed from 1 µg of DNase-treated total RNA using random hexamer and Oligo (dT) 12-18 primers in a 20 µL total reaction volume (High-Capacity cDNA Reverse Transcription kit, Thermo Fisher Scientific, Waltham, USA). Real-time PCR was performed using Luminaris colour Higreen qPCR Master mix (Thermo Fisher Scientific, Waltham, USA). Reactions were run in duplicate in a LightCycler 480 thermocycler (Roche, UK) programmed to perform the following protocol: 50 °C for 2 min, 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s, annealing at X °C for 30 s (Table 4.1) and extension at 72 °C for 30 s. This was followed by a temperature ramp from 70 to 90 °C for melt-curve analysis to verify that no primer-dimer artefacts were present and only one product was generated from each qPCR assay. The final volume of the PCR reaction was 10 µL: 2.5 µl of cDNA, 5 µl of the qPCR Master Mix, 1.5 µl H₂O and 0.5µl each of forward and reverse primers (Table 4.1). Amplifications were carried out including systematic negative controls containing no cDNA (NTC, no template control). No primer-dimers occurred in the NTC. Gene expression quantification was achieved by including a parallel set of reactions containing serial dilutions from all pooled cDNA experimental samples and assigning each dilution the appropriate value of relative units (RUs). As a result, an estimated number of relative copies, corrected for the efficiency of the reaction, was automatically calculated for each sample.

4.2.7. Statistical analysis

Minitab software version 16 (Minitab Inc., Pennsylvania) was used in this study to perform basic descriptive statistics and comparisons using a significance level of 5 % ($p = 0.05$). Prior to analysis, datasets were checked for normality using the Anderson-Darling test. Total mortality (%) was arcsine transformed for normality then non-parametric Kruskal-Wallis and Dunn's multiple comparison post-hoc test were used to assess ploidy and time differences. For cortisol, glucose, lactate and lysozyme activity, ANOVA manipulated by a GLM was carried out to analyse possible interactions between experimental groups and time-points. For this, ploidy, treatment (control or H₂O₂-exposed) and time (h.p.e) were considered fixed factors and tank considered a random factor. Statistical differences in cortisol, glucose, lactate and lysozyme activity between sampling time-points for a given

experimental group were analysed by a one-way ANOVA. At each time-point, cortisol, glucose, lactate and lysozyme activity were also compared between experimental groups (diploid H₂O₂-exposed, diploid control, triploid H₂O₂-exposed, triploid control) by further one-way ANOVA. Statistical differences in gene expression levels between sampling time-points were analysed by one-way ANOVA. Additionally, for a given ploidy, gene expression levels were compared between treatments at each sampling time-point, using 2-sample t-tests. The normalized expression values were generated by the ΔC_t method (Pfaffl, 2001) and the results expressed as mean normalized ratios (\pm SE) between the RUs of target genes and a reference gene index calculated from the geometric mean of the most stable reference genes (*i.e.* *b2m*, *rpl2* and *rpl1* for diploid liver; β -*actin*, *rpl1* and *rpl2* for diploid gill; *eflα* and *rpl2* for triploid liver; *eflα* and *b2m* for triploid gill). Housekeeping genes stability was determined applying a correction for efficiency to the raw C_t standard deviation (Pfaffl, 2004) using RefFinder (Xie *et al.*, 2012).

Table 4.1: Atlantic salmon primer sequences used for real-time PCR

Gene	Accession	F/R	Primer	Anneal (°C)
<i>β-actin</i>	AF012125	F	ATCCTGACAGAGCGCGGTTACAGT	60
		R	TGCCCATCTCCTGCTCAAAGTCCA	
<i>eflα</i>	DQ834870	F	CACCACCGGCCATCTGATCTACAA	60
		R	TCAGCAGCCTCCTCTCGAACTTC	
<i>rpl1</i>	NM_001140 826.1	F	ACTATGGCTGTCGAGAAGGTGCT	60
		R	TGTACTCGAACAGTCGTGGTCA	
<i>rpl2</i>	BT049591.1	F	TAACGCCTGCCTCTCACGTTGA	60
		R	ATGAGGGACCTTGTAGCCAGCAA	
<i>b2m</i>	BT046451.2	F	TCCCAGACGCCAACGCAG	60
		R	TGTAGGTCTTCAGATTCTCAGG	
<i>cat</i>	BT059457	F	CCCAAGTCTTCATCCAGAAACG	60
		R	CGTGGGCTCAGTGTGTTGA	
<i>gpx1</i>	DW5665563	F	GCCCACCCCTGTTGTGTA	60
		R	AGACAGGGCTCCACATGATGA	
<i>gr</i>	XM0141991 33.1	F	CCAGTGATGGCTTTTTGAACCTT	60
		R	CCGGCCCCCACTATGA	
<i>hsp70</i>	BG933934	F	CCCCTGTCCCTGGGTATTG	60
		R	CACCAGGCTGGTTGTCTGAGT	
<i>sod1</i>	Q3ZLR1	F	GACCCCACCTATCATCGGC	60
		R	AATAACTCCACAGGCCAGGC	
<i>sod2</i>	C0H894	F	CTGGGCTTCGACAAGGAGAG	60
		R	GCTCACGTTCTCCCAGTTGA	
<i>saa5</i>	B9EPA2	F	ACAAGTACTCCACGCTCGG	60
		R	TCCTCATGTCCTCGACCAC	
<i>crp/sap</i> <i>1a</i>	P79905	F	GGGAGCGTCACTGGATTCA	60
		R	AGAATCCTCCGTGCACCTCG	
<i>crp/sap</i> <i>1b</i>	B5X672	F	GTGGATGGAGAAGCTGCTGT	60
		R	GCTTGTCTCGACTGGGATGA	
<i>il1β</i>	Q6IWH5	F	TGAAGTCCATCAGCCAGCAG	60
		R	GGATGGTGAAGGTGGTGAGG	

4.3. RESULTS

4.3.1. Mortality

Mortalities occurred in the diploid and triploid exposed groups within the first 6 h post-exposure to H₂O₂, while no mortalities occurred in the diploid and triploid control groups. Total cumulative mortality in the diploid exposed groups was 34.8 ± 2.1 % (1 h.p.e: 3.6 ± 0.5 %; 3 h.p.e: 13.4 ± 0.7 %; 6 h.p.e: 10.7 ± 0.5 %; 24 h.p.e: 7.1 ± 0.9 %). In the triploid exposed group, total mortality was 35.7 ± 1.3 % (1 h.p.e: 6.3 ± 1.3 %; 3 h.p.e: 8.9 ± 1.3 %; 6 h.p.e: 8.0 ± 0.7 %; 24 h.p.e: 12.5 ± 1.1 %). Statistical analysis found no significant effect of ploidy or time-point tank on total mortality.

4.3.2. Cortisol

A significant effect of treatment and time on cortisol levels was observed throughout the trial ($p < 0.05$). However, no significant effects of ploidy or interaction between ploidy, treatment and time were observed ($p > 0.05$).

In diploid Atlantic salmon, cortisol levels in the H₂O₂-exposed fish were highest at 1 h.p.e before decreasing over time, whereas in the control group cortisol remained fairly constant throughout the study in the control group (Figure 4.2). At 1 h.p.e, cortisol levels in the H₂O₂-exposed group (499.4 ± 5.3 ng/ml) were significantly higher than the control group (274.5 ± 43.9 ng/ml). Within the diploid H₂O₂-exposed group, significantly lower cortisol levels were observed at 6 h.p.e (190.0 ± 81.7 ng/ml) and 24 h.p.e (178.8 ± 44.4 ng/ml) compared to 1 h.p.e, while time did not significantly affect the control group.

In the triploid groups, similar patterns of cortisol to the diploids were observed. Cortisol levels in the triploid H₂O₂-exposed group were highest at 1 h.p.e before decreasing over time, with cortisol levels in the control group remaining relatively stable throughout the study (Figure 4.2). At 1 h.p.e, cortisol levels in the H₂O₂-exposed group (666.4 ± 52.8 ng/ml) were significantly higher than the control group (297.9 ± 29.4 ng/ml). Within the triploid H₂O₂-exposed group, cortisol level was significantly lower at 3 h.p.e (425.6 ± 63.3 ng/ml), 6 h.p.e (183.7 ± 49.1 ng/ml) and 24 h.p.e (250.8 ± 16.3 ng/ml) relative to 1 h.p.e, with cortisol level at 6 h.p.e also significantly lower than at 3 h.p.e.

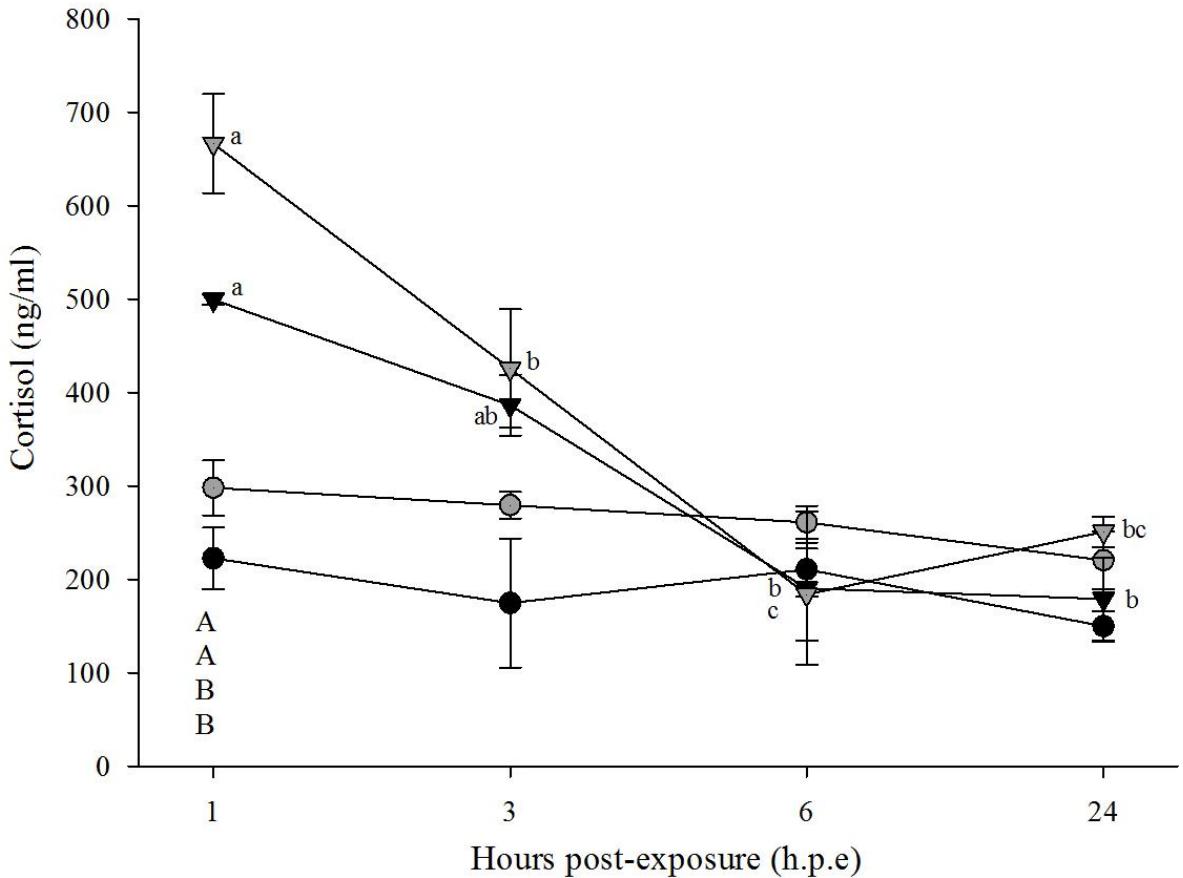


Figure 4.2: Plasma cortisol levels (mean \pm SEM, $n = 4$) over time in control (circle) and H₂O₂-exposed (triangle), diploid (black) and triploid (grey) Atlantic salmon. Statistically significant differences between experimental groups at a particular time-point are indicated by different capital letters (one-way ANOVA $p < 0.05$). Significant differences between time-points (h.p.e) for a particular experimental group are indicated by different lowercase letters (one-way, ANOVA $p < 0.05$).

4.3.3. Glucose

A significant effect of time on blood glucose levels was evident throughout the trial ($p < 0.05$). However, no significant effect of ploidy, treatment or the interaction between ploidy, treatment and time was found ($p > 0.05$).

In diploid Atlantic salmon, glucose levels in the H₂O₂-exposed fish increased over time to peak at 6 h.p.e while glucose remained fairly constant throughout the study in the control group (Figure 4.3). At 6 h.p.e, the glucose levels in the H₂O₂-exposed group (5.3 ± 0.3 mmol/L) were significantly higher than the control group (4.5 ± 0.1 mmol/L). Within the diploid H₂O₂-exposed group, glucose levels were significantly higher at 6 h.p.e compared to 1 h.p.e (4.2 ± 0.3 mmol/L) and 3 h.p.e (4.5 ± 0.1 mmol/L), with glucose level

at 24 h.p.e (4.9 ± 0.1 mmol/L) also significantly higher than at 1 h.p.e. Time did not significantly affect the diploid control group.

In the triploid groups, glucose levels in the H₂O₂-exposed group increased before peaking at 6 h.p.e, with glucose levels in the control group increasing slightly at 3 h.p.e (Figure 4.3). No significant differences were found between H₂O₂-exposed and control triploid groups throughout the study. Within the triploid H₂O₂-exposed group, glucose levels at 1 h.p.e (3.5 ± 0.3 mmol/L) and 3 h.p.e (4.3 ± 0.3 mmol/L) were significantly lower than at 6 h.p.e (5.3 ± 0.1 mmol/L), with levels at 1 h.p.e also significantly lower than at 24 h.p.e (5.0 ± 1.5 mmol/L). In the triploid control group, glucose levels were significantly higher at 3, 6 and 24 h.p.e (approximately 4.6 mmol/L) compared to 1 h.p.e (4.1 ± 0.1 mmol/L).

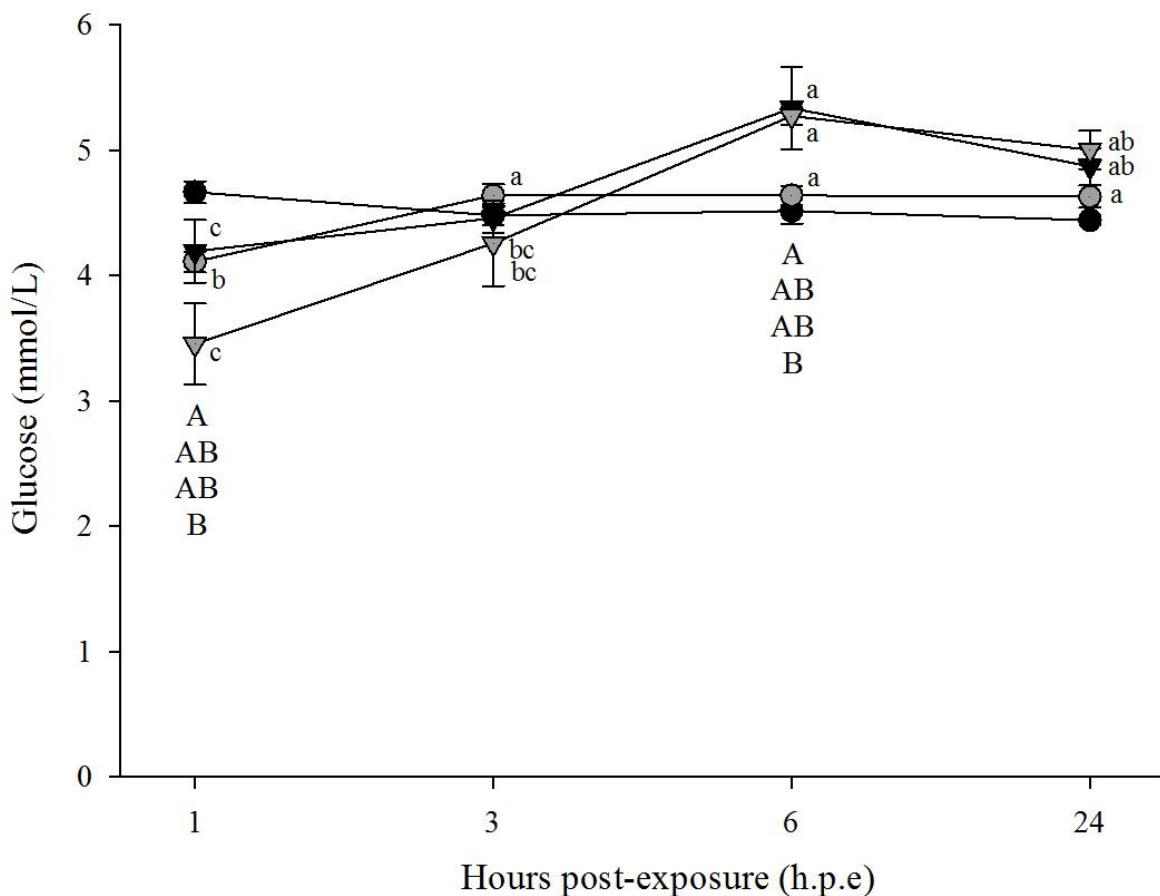


Figure 4.3: Blood glucose levels (mean \pm SEM, n = 4) over time in control (circle) and H₂O₂-exposed (triangle), diploid (black) and triploid (grey) Atlantic salmon. Significant differences between experimental groups at a particular time-point are indicated by different capital letters (one-way ANOVA $p < 0.05$). Significant differences between time-points (h.p.e) for an experimental group are indicated by different lowercase letters (one-way ANOVA, $p < 0.05$).

4.3.4. Lactate

A significant effect of treatment and time on blood lactate levels was evident throughout the trial ($p < 0.05$). However, no significant effect of ploidy or interaction between ploidy, treatment and time was found ($p > 0.05$).

In diploid Atlantic salmon, lactate levels in the H₂O₂-exposed fish were highest at 1 h.p.e before decreasing over time while levels remained fairly constant throughout the study in the control group (Figure 4.4). Lactate levels were significantly higher at 1 and 3 h.p.e in the H₂O₂-exposed group (1 h.p.e: 9.7 ± 1.3 mmol/L; 3 h.p.e: 6.1 ± 1.4 mmol/L) than in the controls (1 h.p.e: 2.7 ± 0.2 mmol/L; 3 h.p.e: 2.6 ± 0.1 mmol/L). In addition, time had a significant effect on the diploid H₂O₂-exposed group with lactate levels at 1 h.p.e significantly higher than at 24 h.p.e (3.2 ± 0.2 mmol/L), while time did not significantly affect lactate in the control group.

In the triploid fish, a similar trend in lactate levels was observed. Lactate levels in the triploid H₂O₂-exposed group were highest at 1 h.p.e before decreasing over time, with lactate in the control group remaining relatively stable throughout the study (Figure 4.4). At 1 h.p.e, lactate level in the H₂O₂-exposed group (10.6 ± 1.3 mmol/L) was significantly higher than the control group (3.3 ± 0.2 mmol/L). Within the triploid H₂O₂-exposed group, lactate levels were significantly higher at 1 h.p.e than at the other three time-points (3.4 – 4.9 mmol/L), while time post-exposure did not significantly affect lactate levels in the control group.

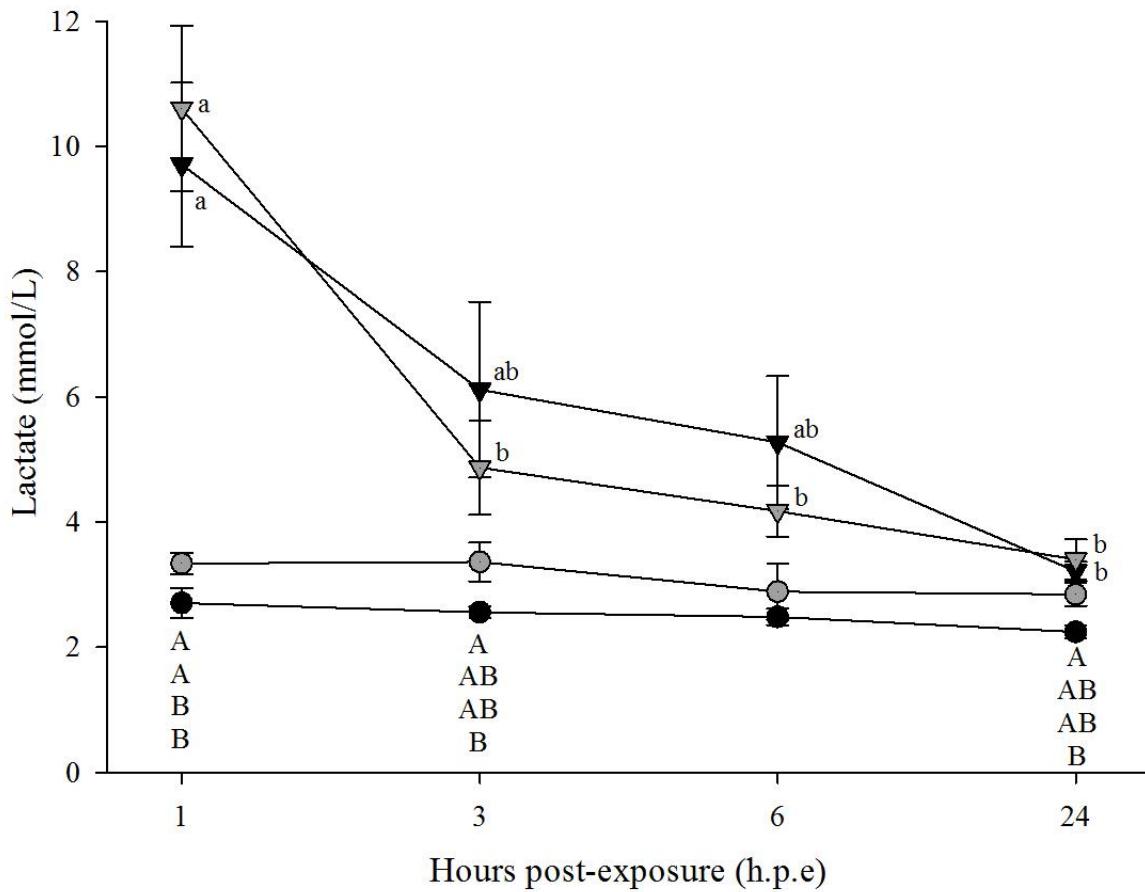


Figure 4.4: Blood lactate levels (mean \pm SEM, $n = 4$) over time in control (circle) and H₂O₂-exposed (triangle), diploid (black) and triploid (grey) Atlantic salmon. Significant differences between experimental groups at a particular time-point are indicated by different capital letters (one-way ANOVA $p < 0.05$). Significant differences between time-points (h.p.e) for an experimental group are indicated by different lowercase letters (one-way ANOVA, $p < 0.05$).

4.3.5. Lysozyme

Variable patterns of lysozyme activity were recorded for each group throughout the study (Figure 4.5). Significant effects of ploidy and treatment on plasma lysozyme activity were evident throughout the trial ($p < 0.05$). However, no significant effect of time or the interaction between ploidy, treatment and time was found ($p > 0.05$).

In diploid Atlantic salmon, lysozyme activity in the H₂O₂-exposed fish increased over time to peak at 24 h.p.e while in the control group it remained fairly constant from 1 to 3 h.p.e and then a decrease was observed at 24 h.p.e (Figure 4.5). Lysozyme activity recorded for the diploid H₂O₂-exposed group at 1 h.p.e (817.1 ± 32.9 units min⁻¹ ml⁻¹), 3 h.p.e (804.3 ± 38.9 units min⁻¹ ml⁻¹) and 6 h.p.e (1015 ± 15.4 units min⁻¹ ml⁻¹) was significantly lower than the diploid control group at the same time-points ($1376 - 1516$ units min⁻¹ ml⁻¹). Time also affected both diploid groups, with the H₂O₂-exposed group showing significantly lower lysozyme activity at 1 and 3 h.p.e compared to at 6 and 24 h.p.e (1167.9 ± 83.9 units min⁻¹ ml⁻¹). The control group showed significantly lower lysozyme at 24 h.p.e (1248.9 ± 39.6 units min⁻¹ ml⁻¹) than at 1 h.p.e (1515.7 ± 38.1 units min⁻¹ ml⁻¹).

In the triploid groups, lysozyme activity in the H₂O₂-exposed groups increased over 24 hours while it decreased in the control group (Figure 4.5). Lysozyme activity in the control group was significantly lower at 6 h.p.e (1185.6 ± 36.3 units min⁻¹ ml⁻¹) and 24 h.p.e (1235.4 ± 58.8 units min⁻¹ ml⁻¹) than at 1 h.p.e (1402.5 ± 47.9 units min⁻¹ ml⁻¹), with no significant effect of time observed in the H₂O₂-exposed group. Treatment did not significantly affect triploid fish, with comparable lysozyme activity recorded for the H₂O₂-exposed and control groups at each time-point.

Ploidy had a significant effect on lysozyme activity in the H₂O₂-exposed groups, with diploids exhibiting lower lysozyme at 1, 3 and 6 h.p.e ($804 - 1015$ units min⁻¹ ml⁻¹) compared to the triploids at the same time-points ($1298 - 1420$ units min⁻¹ ml⁻¹).

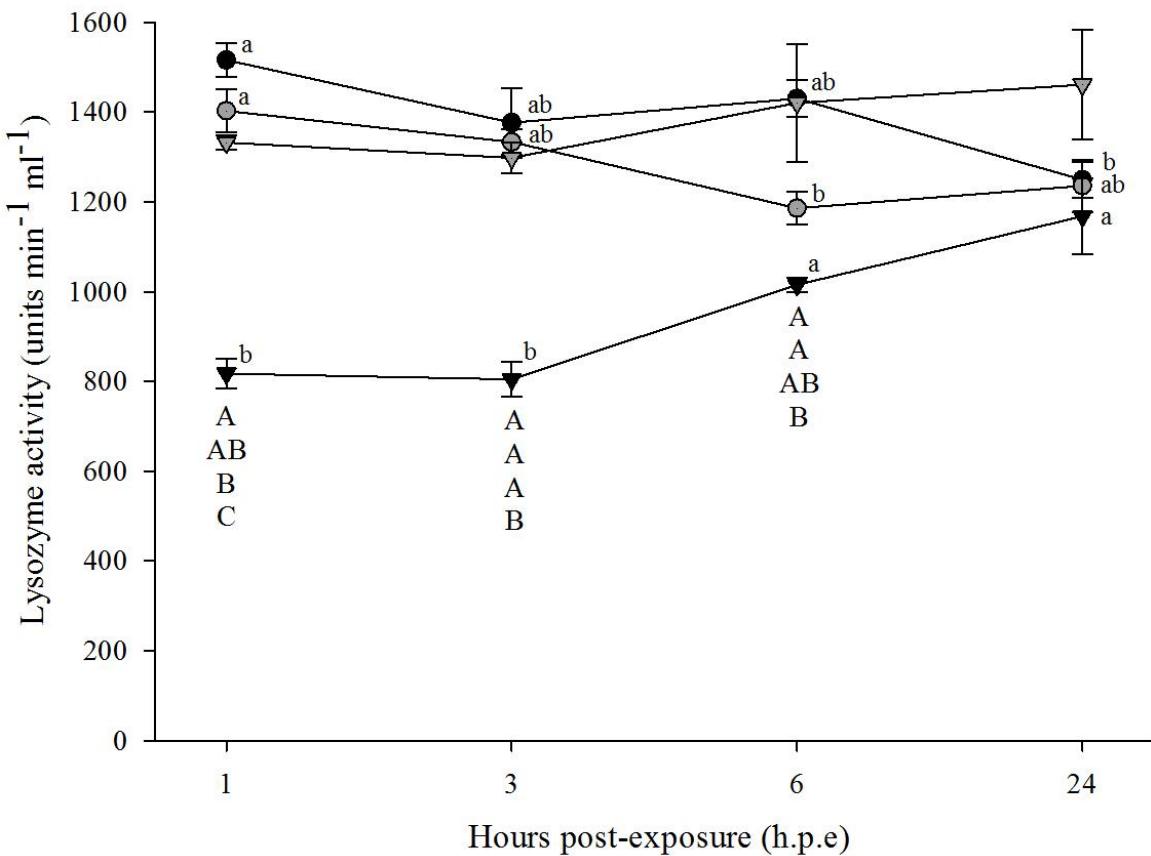


Figure 4.5: Plasma lysozyme activity (mean \pm SEM, n = 4) over time in control (circle) and H₂O₂-exposed (triangle), diploid (black) and triploid (grey) Atlantic salmon. Statistically significant differences between experimental groups at a particular time-point are indicated by different capital letters (one-way ANOVA $p < 0.05$). Significant differences between time-points (h.p.e) for an experimental group are indicated by different lowercase letters (one-way ANOVA $p < 0.05$).

4.3.6. Gene expression

While the effects of polyploidy on gene expression have been well studied in plants, there is an overall lack of information regarding triploid gene expression in fish species (Shrimpton *et al.*, 2007; Ching *et al.*, 2010). In the few studies that have been undertaken, a dosage effect on triploid gene expression has been identified, which appears to compensate for the cell size increase and cell number reduction typical of triploids (Shrimpton *et al.*, 2007; Pala *et al.*, 2008; Ching *et al.*, 2010; Cleveland and Weber, 2014; Correa *et al.*, 2015; Ren *et al.*, 2017). However, the mechanisms underlying dosage effects remain to be fully elucidated and further studies to determine this are necessary (Correa *et al.*, 2015). As such, it was not deemed appropriate to make direct comparisons between diploid and triploid gene expression in this study, and therefore the results are presented individually.

4.3.6.1. Diploid liver

For oxidative stress markers, there was a general trend for H₂O₂-exposed fish to show increased gene expression compared to the control group. *Cat* expression was significantly higher at 1 h.p.e in the H₂O₂-exposed group (74.1 ± 7.6 normalised relative unit (NRU)) compared to the control group (50.6 ± 2.1 NRU) (Figure 4.6 A). However, no significant differences in *cat* expression levels were detected between time-points in either group. Expression of *gpx1* in the control group at 24 h.p.e (91.5 ± 5.1 NRU) was significantly higher than at 1 h.p.e (60.8 ± 6.1 NRU) and 6 h.p.e (54.4 ± 6.1 NRU) (Figure 4.6 B). Expression of *gr* in the control group at 3 h.p.e (80.8 ± 4.8 NRU) and 24 h.p.e (89.7 ± 3.1 NRU) was significantly higher than at 1 h.p.e (57.7 ± 4.9 NRU) and 6 h.p.e (62.9 ± 4.5 NRU) (Figure 4.6 C). No significant differences in the expression of *gr* were detected between the H₂O₂-exposed and control groups. The expression of *sod2* in the H₂O₂-exposed group (443.8 ± 44.8 NRU) at 1 h.p.e was significantly higher than in the control group (303.2 ± 21.5 NRU) (Figure 4.6 F). Regarding *hsp70* and *sod1* expression, no significant differences were found between treatment groups or time-points (Figures 4.6 D - E).

For the immune genes investigated, variable patterns of expression were exhibited and no significant effect of treatment or time was observed for *saa5* (Figure 4.7 A), *crp/sap1a* (Figure 4.7 B) or *crp/sap1b* (Figure 4.7 C).

4.3.6.2. *Diploid gill*

Variable patterns of expression were observed for oxidative stress markers. *Gr* expression was significantly higher in the H₂O₂-exposed group (97.5 ± 37.3 NRU) than in the control group (34.2 ± 5.6 normalised RU) at 3 h.p.e (Figure 4.8 C). No statistically significant differences in *gr* expression were found between time-points for both the H₂O₂-exposed and control groups. In the other genes investigated (*cat*, *gpx1*, *hsp70*, *sod1* and *sod2*), no significant effects of treatment or time were observed (Figure 4.8 A, B, D-F).

Variable expression patterns were also observed for the immune genes. The control group showed significantly higher expression of *saa5* than the H₂O₂-exposed group at 1 h.p.e (control: 116.5 ± 24.1 NRU; H₂O₂-exposed: 49.8 ± 14.6 NRU) and 24 h.p.e (control: 124.8 ± 20.7 NRU; H₂O₂-exposed: 61.8 ± 12.7 NRU) (Figure 4.9 A). The expression of *crp/sap1a* was also significantly higher in the control group (41.6 ± 10.7 NRU) than in the H₂O₂-exposed group (7.5 ± 4.5 NRU) at 1 h.p.e (Figure 4.9 B). The opposite pattern was observed for *crp/sap1b*, with the H₂O₂-exposed group (42.7 ± 5.7 NRU) showing significantly higher expression than the controls (24.1 ± 4.7 NRU) at 3 h.p.e (Figure 4.9 C). No significant differences between time-points were found for *saa5*, *crp/sap1a* and *crp/sap1b* expression. For *ill1β*, expression was significantly higher in the H₂O₂-exposed group than the control group at 1 h.p.e (H₂O₂-exposed: 85.4 ± 12.2 NRU; control: 28.6 ± 2.7 NRU), 3 h.p.e (H₂O₂-exposed: 106.8 ± 11.9 NRU; control: 29.7 ± 6.4 NRU) and 6 h.p.e (H₂O₂-exposed: 146 ± 20.8 NRU; control: 21.1 ± 1.8 NRU) (Figure 4.9 D). Significant differences between time-points were detected in the H₂O₂-exposed group, with expression of *ill1β* significantly lower at 24 h.p.e (34.6 ± 6.4 NRU) than at 6 h.p.e.

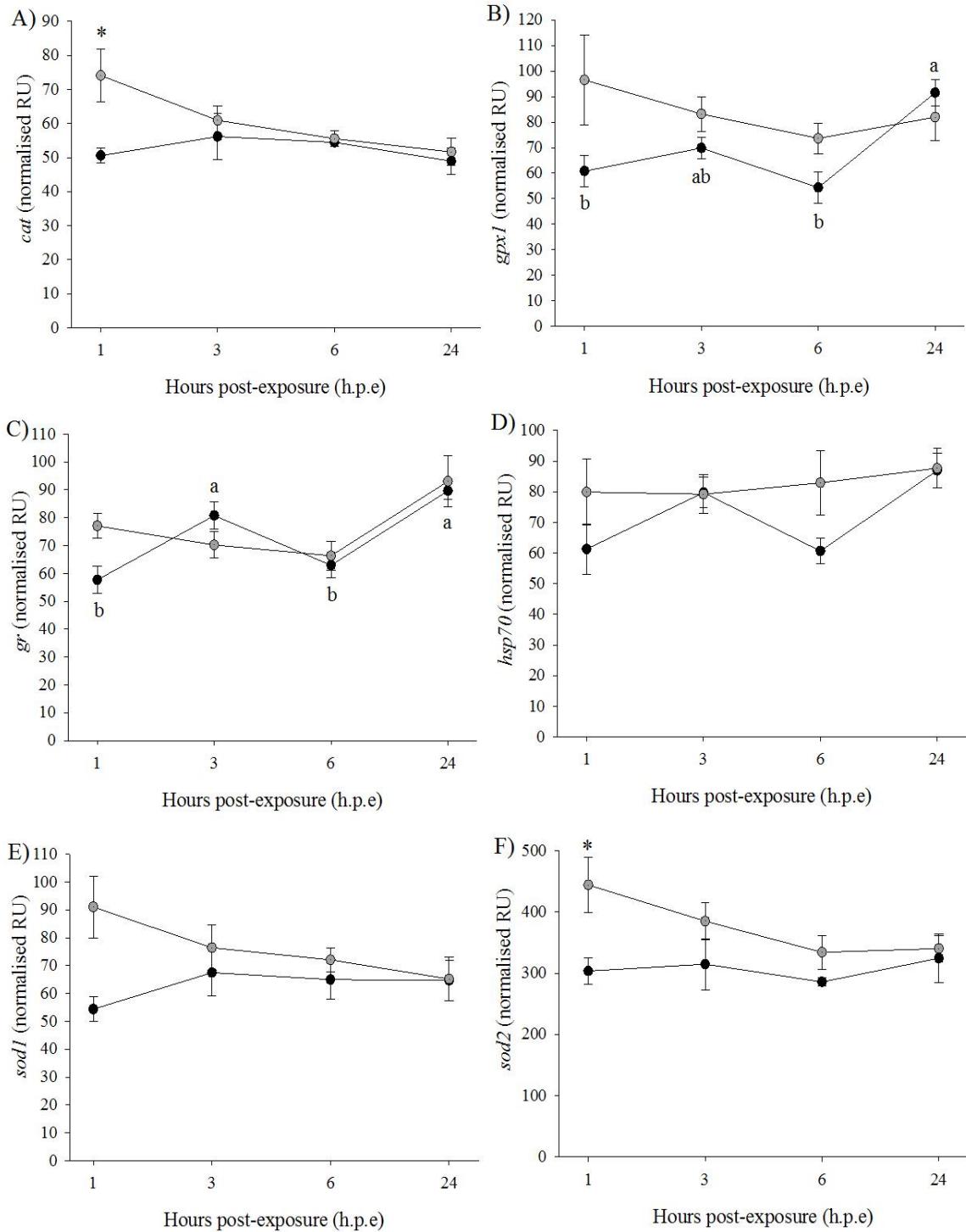


Figure 4.6: Gene expression (mean \pm SEM, $n = 4$) of (A) *cat*, (B) *gpxI*, (C) *gr*, (D) *hsp70*, (E) *sod1* and (F) *sod2* genes in liver of control (black circle) and H₂O₂-exposed diploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, $p < 0.05$). Superscript letters indicate significant differences between time-points (h.p.e) within the control group (one-way ANOVA, $p < 0.05$).

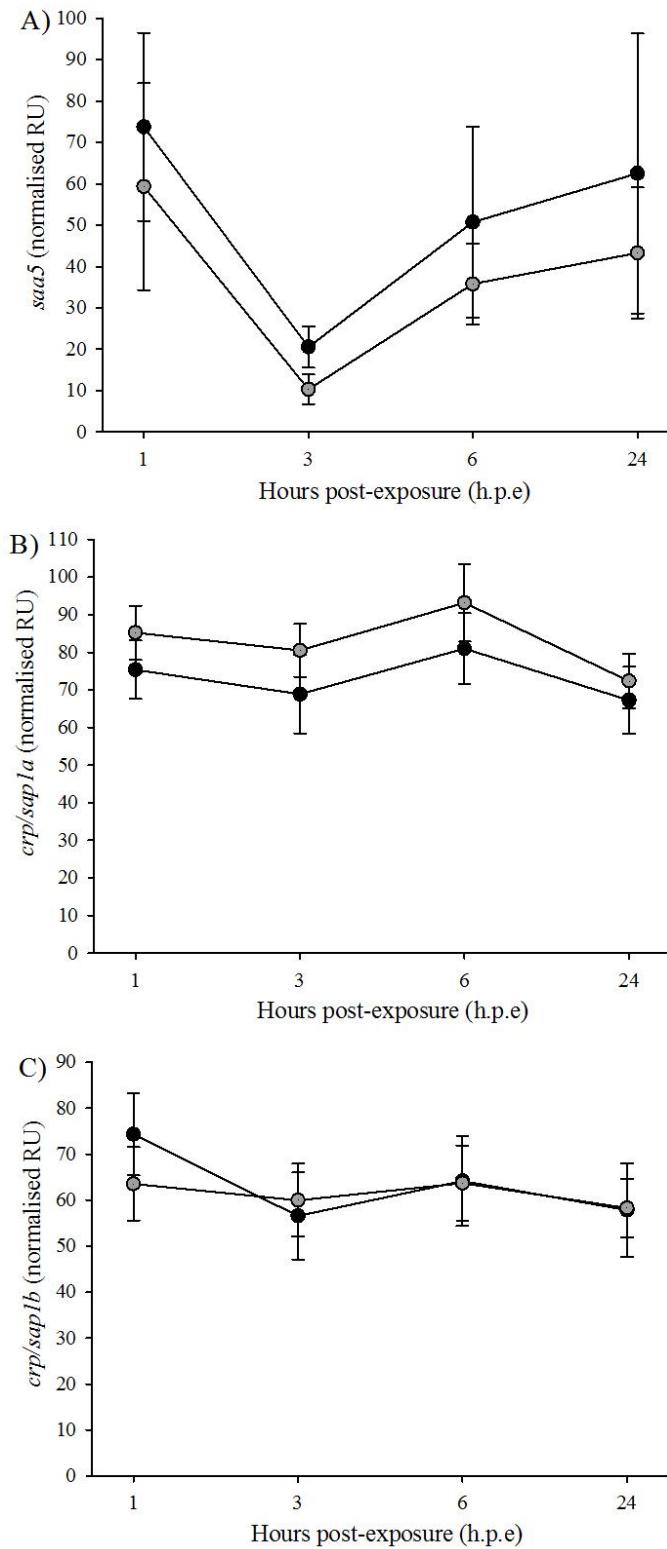


Figure 4.7: Gene expression (mean ± SEM, n = 4) of (A) *saa5*, (B) *crp/sap1a* and (C) *crp/sap1b* genes in liver of control (black circles) and H₂O₂-exposed diploid salmon (grey circle). No significant differences were observed.

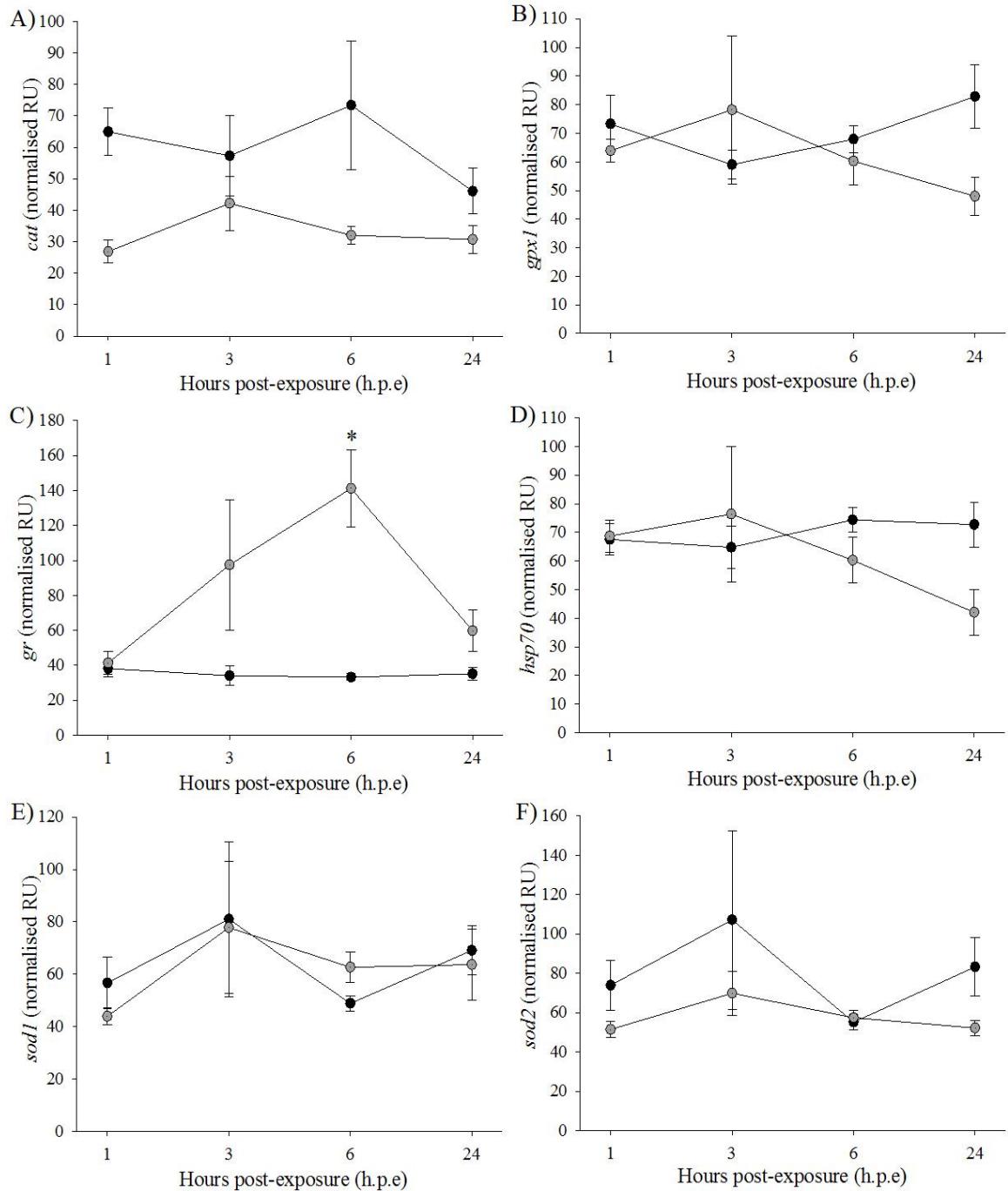


Figure 4.8: Gene expression (mean \pm SEM, n = 4) of (A) *cat*, (B) *gpx1*, (C) *gr*, (D) *hsp70*, (E) *sod1* and (F) *sod2* genes in gill of control (black circles) and H₂O₂-exposed diploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, $p < 0.05$).

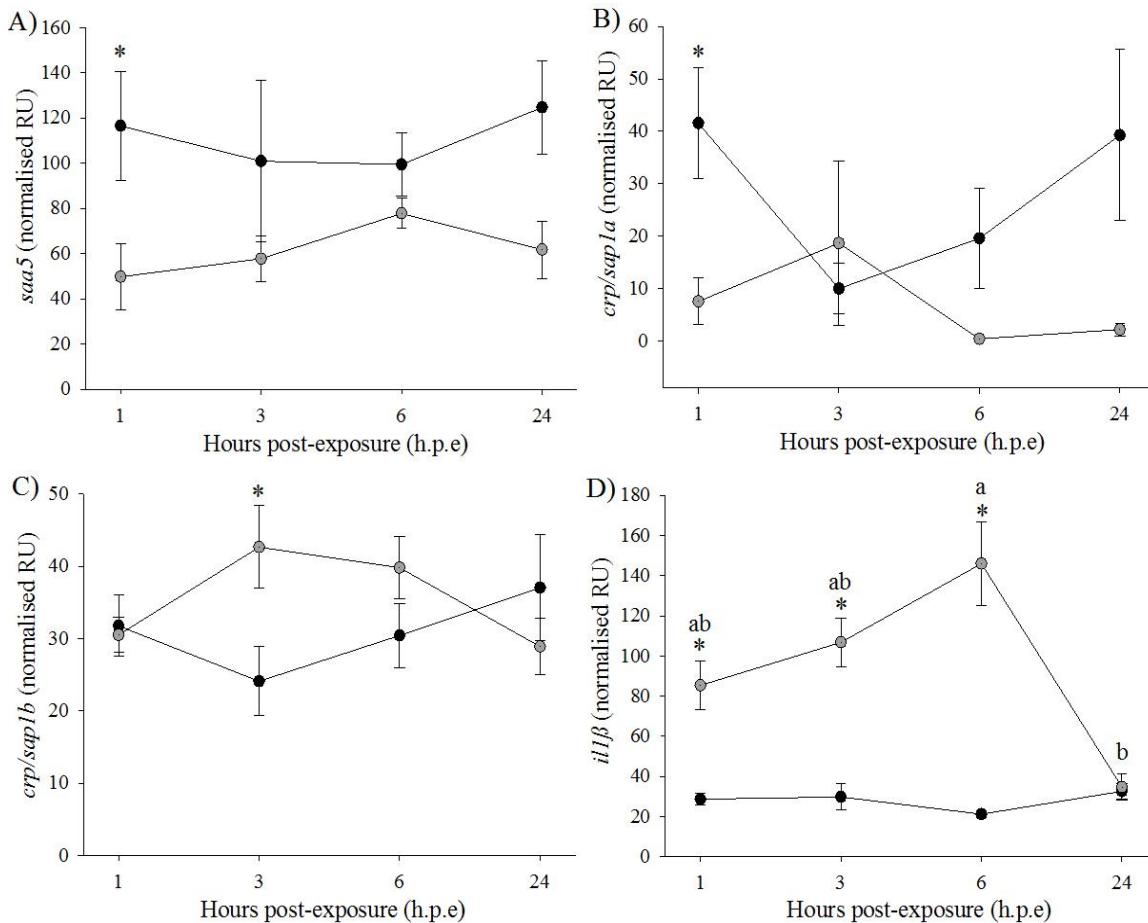


Figure 4.9: Gene expression (mean \pm SEM, $n = 4$) of (A) *saa5*, (B) *crp/sap1a*, (C) *crp/sap1b* and (D) *illβ* genes in gill of control (black circles) and H_2O_2 -exposed diploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, $p < 0.05$). Superscript letters indicate significant differences between time-points (h.p.e) within the H_2O_2 -exposed group (one-way ANOVA, $p < 0.05$).

4.3.6.3. Triploid liver

The expression of *cat*, *hsp70* and *sod1* at 3 h.p.e was significantly higher in the H_2O_2 -exposed group (*cat*: 85.9 ± 6.3 NRU; *hsp70*: 95.1 ± 5.7 NRU; *sod1*: 106.5 ± 10.3 NRU) than in the control group (*cat*: 53.8 ± 6.4 NRU; *hsp70*: 67.0 ± 3.3 NRU; *sod1*: 70.9 ± 6.6 NRU) (Figure 4.10 A, D, E). For these three genes, no significant differences were found between time-points in the H_2O_2 -exposed or in the control group. The expression of *gr* in the control group at 24 h.p.e (81.9 ± 8.5 NRU) was significantly different to the expression recorded at 1 h.p.e (93.5 ± 7.3 NRU) and 3 h.p.e (71.7 ± 5.7 NRU) (Figure 4.10 C). There were no significant effects of treatment or time on *gpx1* or *sod2* (Figure 4.10 B, F).

Differences in the expression of immune genes were also observed. The expression of *saa5* was also significantly higher at 3 h.p.e in the H₂O₂-exposed group (53.1 ± 11.5 NRU) than in the control group (20.8 ± 4.4 NRU) (Figure 4.11 A). This difference was also observed in the expression of *crp/sap1a* with expression levels in the H₂O₂-exposed group at 3 h.p.e (464.9 ± 38.6 NRU) significantly higher than the control group (369.1 ± 49.8 NRU) (Figure 4.11 B). In addition, the expression of *crp/sap1b* at 1 h.p.e was also significantly higher in the H₂O₂-exposed group (82.5 ± 6.0 NRU) than in the control group (51.1 ± 9.6 NRU) (Figure 4.11 C).

4.3.6.4. Triploid gill

The expression of *gpx1* in the control group was significantly lower at 1 h.p.e (87.6 ± 6.2 NRU) than at 24 h.p.e (100.9 ± 7.0 NRU) (Figure 4.12 B). In addition, the expression of *gpx1* at 1 h.p.e in the control group was significantly higher than in the H₂O₂-exposed group (69.1 ± 4.6 NRU). The expression of *gr* was significantly higher in the H₂O₂-exposed group than the control group at 3, 6 and 24 h.p.e (H₂O₂-exposed average: 156.5 ± 14.7 NRU; control average: 46.2 ± 4.9 NRU) (Figure 4.12 C). Within the H₂O₂-exposed group, *gr* expression was significantly higher at 3 h.p.e and 24 h.p.e compared to 1 h.p.e (54.3 ± 3.7 NRU), with expression at 6 h.p.e significantly higher than at the three other time-points. Regarding *sod1*, expression level at 3 h.p.e was significantly higher in the H₂O₂-exposed group (98.4 ± 16.3 NRU) than in the control group (65.9 ± 5.1 NRU) (Figure 4.12 E). Finally, for *cat*, *hsp70* and *sod2* expression, no significant differences were found between treatment groups or time-points (Figure 4.12 A, D, F).

The expression of *crp/sap1b* in the H₂O₂-exposed group was significantly lower at 3 h.p.e (48.2 ± 3.8 NRU) than at 1, 6 and 24 h.p.e ($74.0 - 79.0$ NRU) (Figure 4.13 C). At 1 h.p.e and 3 h.p.e, the expression of *il1β* was significantly higher in the H₂O₂-exposed group (1 h.p.e: 130.6 ± 14.1 NRU; 3 h.p.e: 131.3 ± 20.1 NRU) than in the control group (1 h.p.e: 34.1 ± 3.8 NRU; 3 h.p.e: 35.1 ± 3.2 NRU) (Figure 4.13 D). Regarding the expression of *saa5* and *crp/sap1a*, no significant differences were found between treatment groups or time-points (Figure 4.13 A, B).

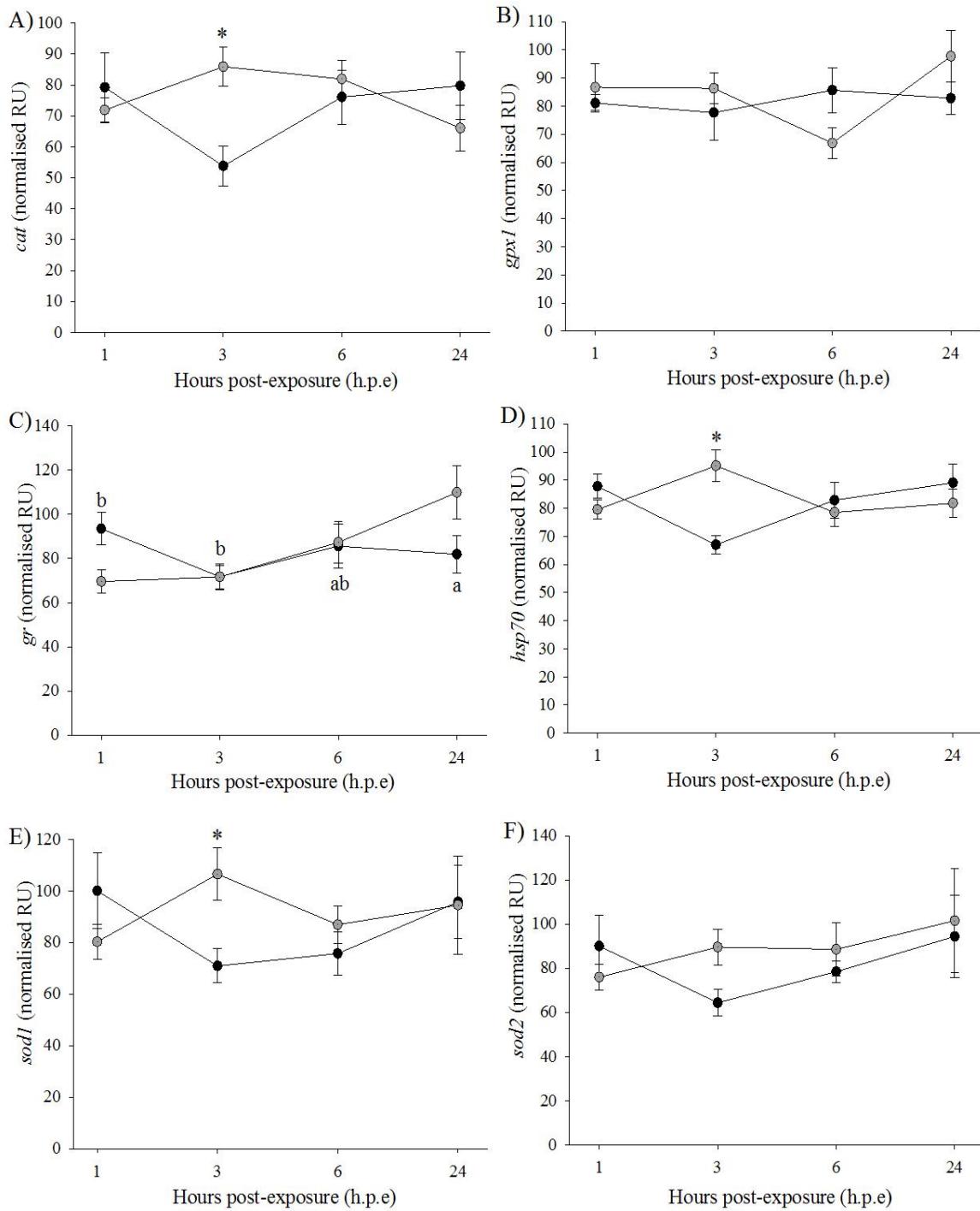


Figure 4.10: Gene expression (mean \pm SEM, $n = 4$) of (A) *cat*, (B) *gpx1*, (C) *gr*, (D) *hsp70*, (E) *sod1* and (F) *sod2* genes in liver of control (black circles) and H_2O_2 -exposed triploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, $p < 0.05$). Superscript letters indicate significant differences between time-points (h.p.e) within the control group (one-way ANOVA, $p < 0.05$).

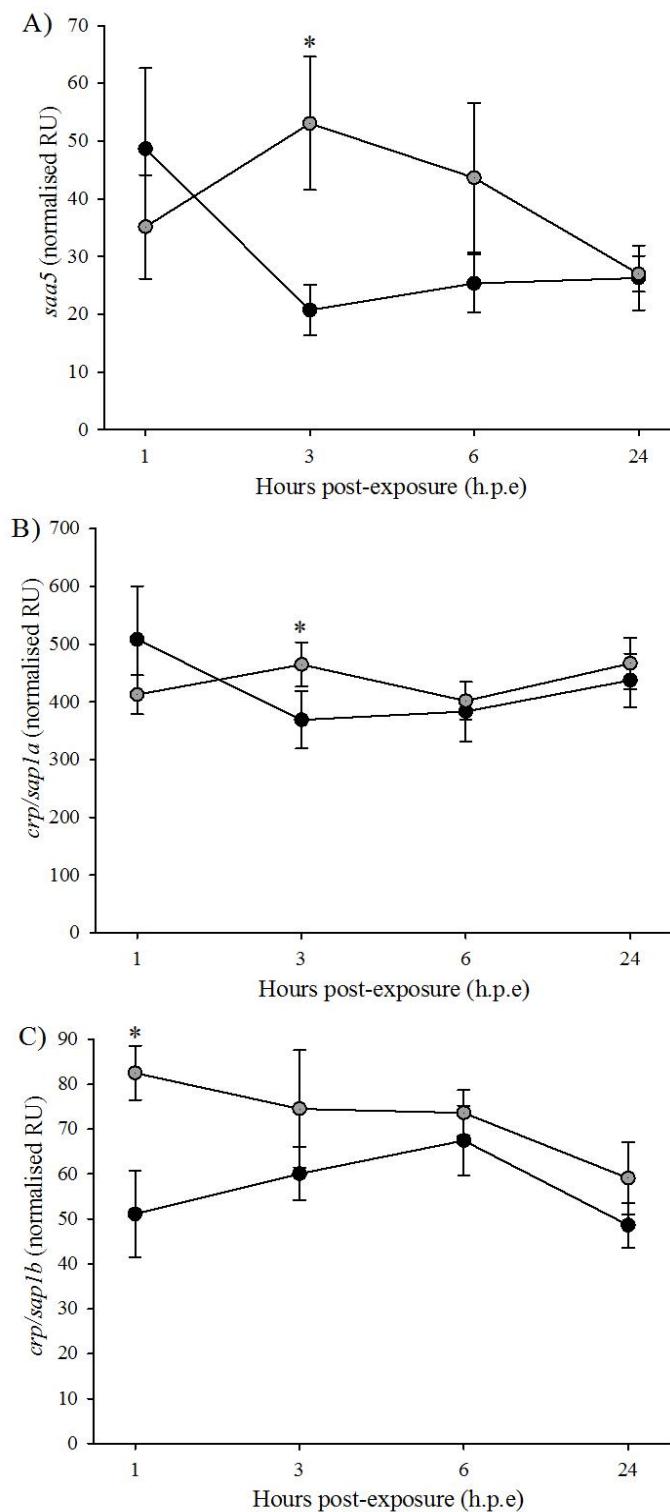


Figure 4.11: Gene expression (mean \pm SEM, n = 4) of (A) *saa5*, (B) *crp/sap1a* and (C) *crp/sap1b* genes in liver of control (black circles) and H₂O₂-exposed triploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, p < 0.05).

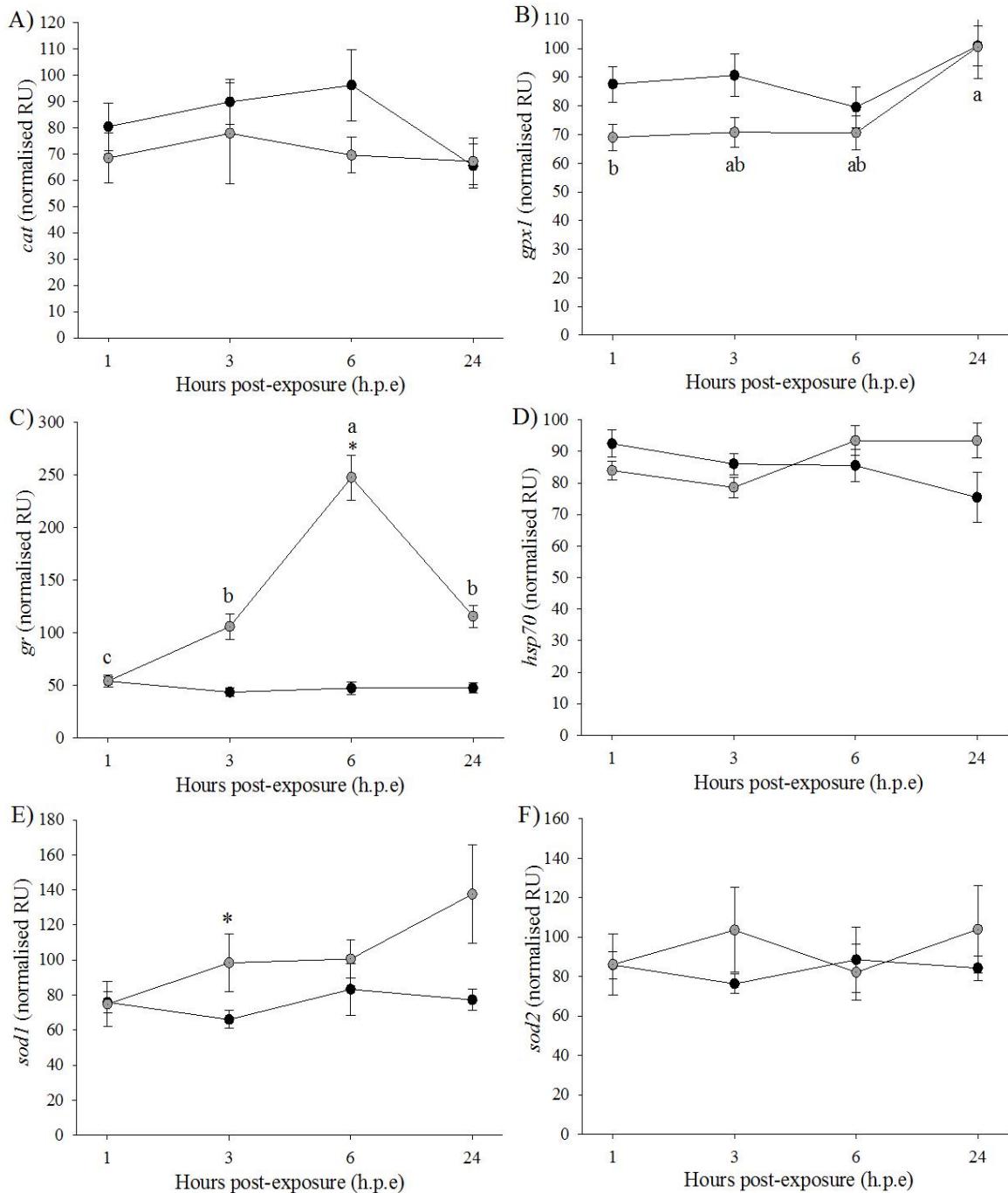


Figure 4.12: Gene expression (mean \pm SEM, $n = 4$) of (A) *cat*, (B) *gpx1*, (C) *gr*, (D) *hsp70*, (E) *sod1* and (F) *sod2* genes in gill of control (black circles) and H_2O_2 -exposed triploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, $p < 0.05$). Superscript letters indicate significant differences between time-points (h.p.e) within the H_2O_2 -exposed group (one-way ANOVA, $p < 0.05$).

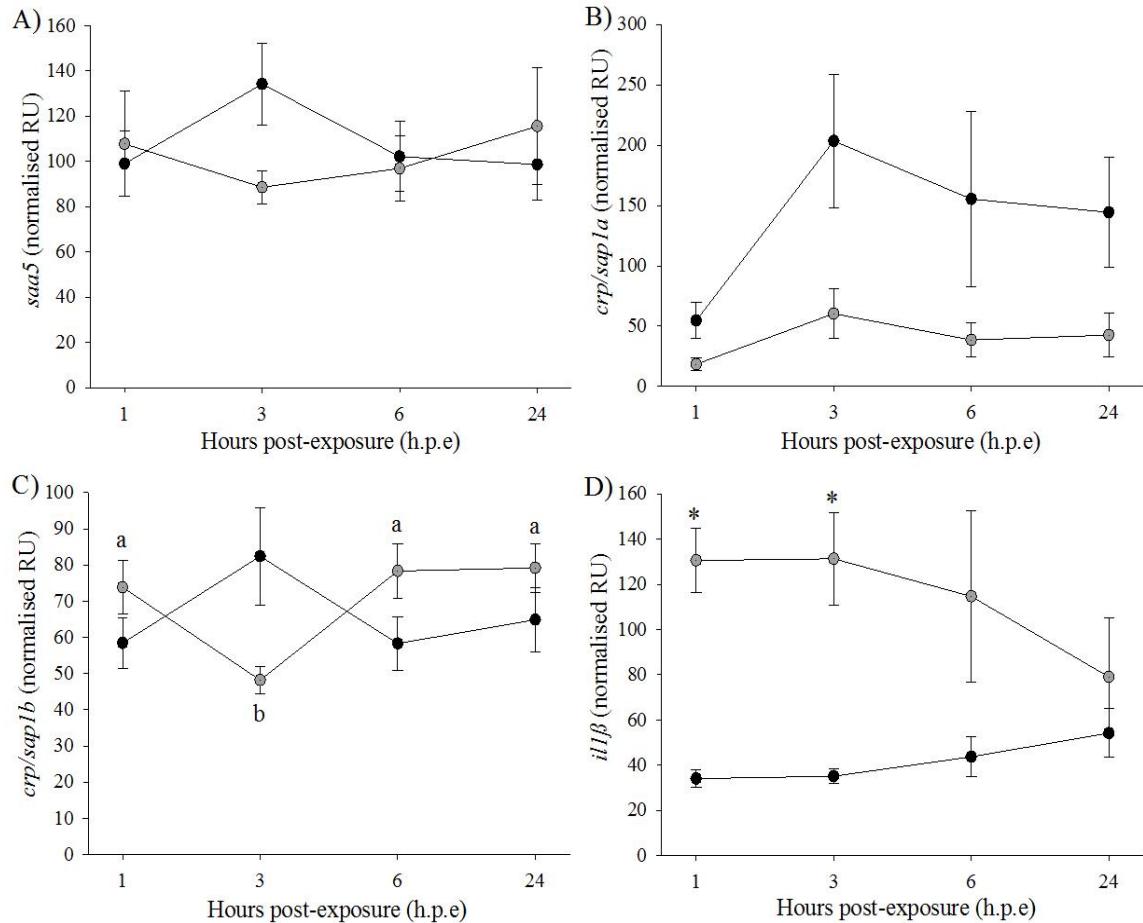


Figure 4.13: Gene expression (mean \pm SEM, n = 4) of (A) *saa5*, (B) *crp/sap1a*, (C) *crp/sap1b* and (D) *illβ* genes in liver of control (black circles) and H₂O₂-exposed diploid salmon (grey circle). Asterisks indicate significant differences between experimental groups at a particular time-point (two-sample t-test, $p < 0.05$). Superscript letters indicate significant differences between time-points (h.p.e) within the H₂O₂-exposed group (one-way ANOVA, $p < 0.05$).

4.4. DISCUSSION

Stress is an intrinsic aspect in the life of fish (Sopinka *et al.*, 2016). In aquaculture, fish can be subjected to higher levels of stress than their wild counterparts often due to husbandry practices such as netting, handling and transport (Varsamos *et al.*, 2006; Tacchi *et al.*, 2015; Sopinka *et al.*, 2016). The treatment of economically significant diseases with chemicals and chemotherapeutics has also been recognised to cause stress to fish in aquaculture (Sanchez *et al.*, 1997; Ortúñoz *et al.*, 2002). Considering the increasing desire to implement triploid Atlantic salmon in full commercial production, it is therefore essential to determine their response to treatments in order to gain a better understanding of their physiological requirements and optimise commercial aquaculture practices accordingly (Weber *et al.*, 2013). This study compared the stress and immune responses of diploid and triploid Atlantic salmon to an experimental challenge with H₂O₂.

Mortalities occurred in both diploid and triploid Atlantic salmon following H₂O₂ treatment. This was in contrast to the dose-response toxicity test, during which no mortalities were observed at 1900 ppm, as well as previously published studies (Bowers *et al.*, 2002; Vera and Migaud, 2016) but concurs with Bruno and Raynard (1994). It is recognised that, while H₂O₂ is an effective treatment for sea lice and AGD, it can become extremely toxic and even lethal at water temperatures of 13.5 °C and above (Thomassen, 1993; Kiemer and Black, 1997; Rodger, 2014). As Bowers *et al.* (2002) and Vera and Migaud (2016) experienced no post-exposure mortalities using temperatures of 10 and 12.3 °C, respectively and with mortalities occurring at 13.5 °C in the study by Bruno and Raynard (1994), it could be suggested that increased mortality in the current study could be linked to the high temperature experienced. However, as no mortalities occurred in the dose-response toxicity test for 1900 ppm at 14 °C, it is recognised that temperature cannot be the only cause and other factors may have contributed, including inter-fish differences in H₂O₂ tolerance and potentially compromised gill function (Rodger, 2014). Due to technical limitations at the experimental facility, it was not possible to control water temperature throughout the trial but it could be proposed that, had temperatures been lower, the use of 1900 ppm would not have caused mortalities but further studies would be required to confirm this suggestion. Ploidy did not have a significant effect on total mortality but, due to the overall lack of data regarding chemical treatments in triploid fish, it is difficult to confirm if this is the expected pattern. Considering these findings, it is recommended that further studies be undertaken to elucidate further the effects of temperature on the efficacy and toxicity of H₂O₂ treatments, particularly for triploid Atlantic salmon.

Cortisol is the principal corticosteroid produced by fish and is thought to have numerous roles in the stress response pathway including energy mobilisation, stimulation of ion regulatory processes and facilitation of oxygen uptake (Olsen *et al.*, 1992; Pickering and Pottinger, 1995; Mommsen *et al.*, 1999; Barton, 2002). As a primary stress biomarker, cortisol is one of the most commonly used indicators of stress in teleost fish (Mommsen *et al.*, 1999; Sopinka *et al.*, 2016). In this study, cortisol was found to be significantly increased in the H₂O₂-exposed groups compared to the control groups at 1 h.p.e before gradually decreasing over time to reach levels comparable to those observed in the control groups. This is consistent with numerous studies which have found elevated levels of cortisol in response to disease treatment with H₂O₂ (Bowers *et al.*, 2002; Roque *et al.*, 2010; Vera and Migaud, 2016) or other chemotherapeuticants (Williams and Wootten, 1981; Sanchez *et al.*, 1997). Ploidy did not have a significant effect on cortisol response and this is in accordance with previous studies investigating the primary stress response of triploid fish which reported similarly elevated cortisol in both diploid and triploid salmonids in response to handling (Benfey and Biron, 2000), confinement (Benfey and Biron, 2000; Sadler *et al.*, 2000a; b), anaesthesia (Fraser *et al.*, 2014c), transport (Leggatt *et al.*, 2006) and sea water transfer (Taylor *et al.*, 2007). However, it should be noted that at 1 h.p.e the cortisol response of triploids appeared to be higher than diploids, although differences were not statistically significant. This is supported by a study by Fraser *et al.*, (2014c) which found that, in response to Finquel (MS-222) and Aqui-S (isoeugenol), triploids showed greater increases in plasma cortisol levels compared to diploids but not significantly. This finding could suggest that, while triploids can cope similarly to diploids when exposed to general husbandry stressors, they may be slightly more sensitive to disease treatments. As such, this would require further study to fully elucidate ploidy differences in stress response induced by chemotherapeutic treatments.

Glucose is an essential carbohydrate involved in the bioenergetics of animals, able to be transformed into both chemical (ATP) and mechanical energy (Martinez-Porcha *et al.*, 2009). Changes in glucose are considered a secondary stress response and this metabolite is regularly measured during stress-associated trials in fish (Pickering and Pottinger, 1995; Sopinka *et al.*, 2016). Glucose levels were similar in all groups at 1 and 3 h.p.e. Glucose levels in the H₂O₂-exposed groups then increased above the control groups at 6 h.p.e, with a significant difference observed between the diploid H₂O₂-exposed and control groups, before returning to similar levels as the control groups at 24 h.p.e. The difference between exposed and control groups is supported by previous studies assessing

the effects of H₂O₂ (Bowers *et al.*, 2002; Roque *et al.*, 2010; Vera and Migaud, 2016). However, it should be noted that the response of glucose appeared delayed. It has previously been stated that secondary stress responses, such as glucose, happen over a slower timescale than primary responses (Sopinka *et al.*, 2016) and the slow response in this study supports previous findings by Bowers *et al.* (2002) who observed a peak in glucose at 6 h.p.e, although this would require further confirmation as no earlier measurements were taken. In addition, ploidy did not have a significant effect on glucose in this study, as observed in other studies assessing handling (Benfey and Biron, 2000), confinement (Sadler *et al.*, 2000a), anaesthesia (Fraser *et al.*, 2014c), transport (Leggatt *et al.*, 2006) and sea water transfer (Taylor *et al.*, 2007) on the stress response of triploid salmonids. This finding further supports the ability of triploids to mount a similar stress response to diploids and is suggestive of similar stress-induced mobilisation of energy reserves between ploidy (Benfey and Biron, 2000).

Another secondary stress response factor that is frequently measured in fish is lactate (Pickering and Pottinger, 1995). Lactate is the product of anaerobic metabolism, produced from pyruvate via the enzyme lactate dehydrogenase (Sadler *et al.*, 2000b; Martinez-Porchas *et al.*, 2009). In this study, there was a significant increase in lactate in the diploid and triploid H₂O₂-exposed fish compared to the control groups at 1 h.p.e, although lactate in the H₂O₂-exposed groups had returned to basal levels by 24 h.p.e. This finding is supported by other studies assessing the effects of H₂O₂ (Roque *et al.*, 2010; Vera and Migaud, 2016) and other chemicals, such as metomidate, Aqui-S™ and clove oil (Iversen *et al.*, 2003), on the fish stress response. Despite the previous statement that secondary responses can occur over a slower timescale than primary responses (Sopinka *et al.*, 2016), the pattern of lactate observed in this study appears more similar to the cortisol response than to the glucose profile, with a peak at 1 h.p.e rather than 6 h.p.e. As increases in lactate are known to occur in response to both exercise and stress (Garcia-Alvarez *et al.*, 2014), it could be suggested that the peak in lactate in this study was induced directly by the stress of H₂O₂ exposure as well as potentially increased swimming activity as a result of the H₂O₂ treatment. Furthermore, it is recognised that lactate production acts as a pathway for glycogenesis, a metabolic pathway causing the production of glucose and so the later peak in glucose may also be related to this mechanism (Mommsen *et al.*, 1999; Garcia-Alvarez *et al.*, 2014; Vera and Migaud, 2016). Considering this, lactate, as well as cortisol, may play an important role in the mobilisation of glucose in the blood of stressed fish and so, it is suggested that studies be undertaken to fully determine the stress response of fish

and the links between cortisol, glucose and lactate (Mommsen *et al.*, 1999; Polakof *et al.*, 2012). Additionally, lactate was not significantly affected by ploidy in this study and this is supported by studies assessing the stress response of diploid and triploid Atlantic salmon following confinement (Sadler *et al.*, 2000a; b) and the effects of exhaustive exercise in diploid and triploid rainbow trout (Preston *et al.*, 2017). However, it should be noted that, following exposure to anaesthetics, triploids showed a greater plasma lactate response than diploids, which may be linked to differences in anaerobic respiration or in the anaerobic pathways during hypoxia (Fraser *et al.*, 2014c). As triploids are known to be less tolerant to low oxygen levels than diploids (Sambrerus *et al.*, 2017), additional studies assessing the development of lactate in triploid Atlantic salmon following a stress event in unfavourable low oxygen conditions are required.

While lysozyme, a bacteriolytic enzyme, is mainly considered as a component of the innate immune response, it is also considered an indicator of stress (Saurabh and Sahoo, 2008). In this study, variable patterns of lysozyme activity were recorded with significant effects of treatment and ploidy observed. The lysozyme activity recorded in the diploid H₂O₂-exposed group was significantly lower than their respective control group at 1, 3 and 6 h.p.e. This is in agreement with previous work by Yildiz (2006) who observed decreased lysozyme levels in groups exposed to Leteux-Meyer mixture (formalin and malachite green) compared to the control groups. However, the present results contrasts with the situation described by Fevolden *et al.* (1994) who found increased lysozyme in response to confinement stress. Significant ploidy effects were observed in the H₂O₂-exposed groups at 1, 3 and 6 h.p.e with diploids showing significantly lower activity than triploids. This concurs with a study by Taylor *et al.* (2007) which found suppressed lysozyme in diploid Atlantic salmon compared to triploids following seawater transfer stress. However, information available on the stress response of triploid Atlantic salmon is very limited and more detailed research will be needed to clarify this point. Nevertheless, it should be noted that lysozyme activity in this study was within the range of 800 to 1600 units min⁻¹ ml⁻¹, similar to that reported in Atlantic salmon infected with *N. perurans* (**Chapter 3**) as well as in previous literature assessing the effects of probiotics and dietary components on the immune response (Korkea-aho *et al.*, 2011; Metochis *et al.*, 2017). It is recognised that lysozyme activity levels can be variable, depending highly on intensity, duration and type of stress, and enhanced or suppressed lysozyme activity has been reported in the literature (Fevolden *et al.*, 1994; Taylor *et al.*, 2007; Saurabh and Sahoo, 2008; Caipang *et al.*, 2009; Easy and Ross, 2010). With such inconsistent and often conflicting evidence available, it

could be suggested that lysozyme is not a strong candidate as an indicator of stress or that further assessment is required to determine the full mechanisms of lysozyme release in response to stress.

It is recognised that exposure to H₂O₂ affects the expression of oxidative stress genes in fish (Vera and Migaud, 2016). However, there is an overall lack of information regarding the effects of triploidy on gene expression in fish species (Shrimpton *et al.*, 2007; Ching *et al.*, 2010). While a few studies would suggest the occurrence of a dosage effect on triploid gene expression (Shrimpton *et al.*, 2007; Pala *et al.*, 2008; Ching *et al.*, 2010; Cleveland and Weber, 2014; Correa *et al.*, 2015; Ren *et al.*, 2017), the full mechanisms underlying dosage effects have not yet been elucidated. As such, it was not considered appropriate to directly compare diploid and triploid gene expression in this study, and so ploidy and tissue were assessed separately.

In diploid liver, there was a general trend for the H₂O₂-exposed group to show higher gene expression at 1 h.p.e, significantly so in *cat* and *sod2*, before returning to levels similar to that of the control group. This is supported by a previous study which found elevated expression of oxidative stress genes in fish exposed to H₂O₂ compared to controls (Vera and Migaud, 2016). However, there was no significant changes in the immune genes assessed in the liver which is in contrast with a previous study reporting that acute phase proteins synthesised in the liver, such as *saa5*, *crp/sap1a* and *crp/sap1b*, are involved in the stress response (Tort, 2011). Other studies in carp, however, have shown that increases in cortisol can have a suppressive effect of the expression of acute phase proteins (Saeij *et al.*, 2003; Nardocci *et al.*, 2014) and so it could be suggested that this may have occurred in diploid liver in the present study.

In diploid gills, *gr* showed significantly higher expression in the H₂O₂-exposed fish than in the controls at 6 h.p.e which agrees with Tort *et al.* (2005) who found increased glutathione in the gill of walleye (*Sander vitreus*) following exposure to H₂O₂. However, no significant effect of H₂O₂ was found in the rest of the oxidative stress genes investigated. This finding contrasts with Vera and Migaud (2016) whose study found elevated expression of a range of oxidative stress genes in the gills of Atlantic salmon. It could be suggested that the differences between the current study and Vera and Migaud (2016) may be linked to the different fish populations used as well as differences in experimental design (H₂O₂ exposures undertaken at different times of the day) or temperature (12.3 ± 0.3 °C) but further studies would be required to confirm this. Regarding the immune genes, *saa5* and *crp/sap1* showed higher expression levels in the control group whereas the H₂O₂-exposed group

showed higher expression levels of *crp/sap1b* and *illβ*. It could again be suggested that cortisol may be suppressing the response of acute phase proteins. The enhanced expression reported for *illβ* is in agreement with previous studies which found increased *illβ* expression following vaccination and short-term handling stress (Fast *et al.*, 2007, 2008).

For oxidative stress genes in triploid liver, the H₂O₂-exposed group showed higher expression of *cat*, *hsp70* and *sod1* than the control group at 3 h.p.e. This finding concurs with a previous study which found increased gene expression in H₂O₂-exposed diploid Atlantic salmon compared to controls (Vera and Migaud, 2016). However, it should be noted that this contrasts with diploids in the current study, where significant differences between treatment groups occurred at 1 h.p.e. While this difference in pattern is difficult to explain due to the lack of previous data on triploid gene expression, other biological responses have been shown to be delayed in triploids compared to diploids. For instance, Langston *et al.* (2001) found that the hypoferraemic response, important in protection against bacteria, was slower in triploid Atlantic salmon than in diploids and suggested that the delay could potentially impact on susceptibility to disease. As such, it may be suggested that a similar effect was observed here, which may impact the ability of triploids to cope with stress. In terms of the immune genes, the H₂O₂-exposed group showed significantly higher expression of *saa5* and *crp/sap1a* at 3 h.p.e and *crp/sap1b* at 1 h.p.e than the control group. While this finding appears to refute the suggestion of suppressive action by cortisol on acute phase proteins, with no other studies with which to compare, it is not clear at this time whether these patterns would be expected.

In triploid gill, the expression of *sod1* and *gr* were significantly higher in the H₂O₂-exposed group compared to the controls at 3 and 6 h.p.e, respectively. This could be supported by the finding from Tort *et al.* (2005) who reported increased glutathione in the gill of walleye (*Sander vitreus*) following H₂O₂ exposure. However, no significant effect of H₂O₂ was found in the rest of the oxidative stress genes investigated (*cat*, *gpx1*, *hsp70* and *sod2*). This finding is again in contrast to Vera and Migaud (2016) who found increased expression of oxidative stress genes in the gills of diploid Atlantic salmon exposed to H₂O₂. As with diploids, *illβ* in the gills was the most reactive gene in response to H₂O₂ exposure, while the remaining three genes showed little changes. This is supported by previous studies which found increased expression of *illβ* following vaccination and short-term handling stress. However, it should be noted that studies regarding the expression of oxidative stress and immune genes in triploid Atlantic salmon, and triploid gene expression in general, are scarce (Shrimpton *et al.*, 2007; Ching *et al.*, 2010; Fraser *et al.*, 2012b). Considering that

mortalities occurred in this trial, it could be suggested that suboptimal temperatures, proposed as a cause of the mortalities, may have also impacted triploid gene expression, however, more detailed studies are required to improve the understanding stress and toxicological responses in triploid Atlantic salmon.

4.5. CONCLUSION

This study found that primary and secondary stress responses were not significantly influenced by ploidy in Atlantic salmon, according to the parameters investigated in the present study (cortisol, glucose and lactate). This suggests that the physiological response of triploids to cope with the stress associated with H₂O₂ treatment would be comparable to that observed in their diploid counterparts. However, with mortalities occurring in this study along with the knowledge that triploids have a lower thermal optimum than diploids and that H₂O₂ can become increasingly toxic at temperatures above 13.5 °C, it is recommended that studies be undertaken to fully assess the effects of triploidy, water temperature and H₂O₂ concentration on the stress and immune responses of triploid Atlantic salmon. Additionally, as fish in this study were not infected by any pathogen during the H₂O₂ “treatment”, as would normally be the case in commercial salmon farming operations, it is also recommended that studies be undertaken to assess the additive effect of pathogen challenge with treatment on both the immune and stress responses. Considering the variations in gene expression between this study and previously published data, additional assessments are necessary to fully characterise the toxicological and immune responses of triploids at a molecular level when fish are exposed to H₂O₂, including exposures during unfavourable conditions. Furthermore, it is essential to improve our understanding of the effects of triploidy on gene expression. Overall, the findings of this study suggest that triploid salmon appear to have similar physiological responses to diploid siblings in response to H₂O₂ but gene expression needs to be further explored in terms of the triploidy effect.

Chapter 5

A comparison of the response of diploid and triploid Atlantic salmon (*Salmo salar*) siblings to intraperitoneal injection with a range of commercially available vaccines.

5.1. INTRODUCTION

In the Atlantic salmon industry, losses can be due to a range of factors including predation and poor environmental quality (Meyer, 1991; Subasinghe, 2005). Disease and the resultant health issues, however, are recognised as one of the most economically significant constraints to the development and success of aquaculture (Meyer, 1991; Subasinghe, 2005; Defoirdt *et al.*, 2011). Fish in intensive aquaculture experience more disease pressure than their wild counterparts due to differences in environmental conditions *e.g.* higher fish densities, temperature fluctuations and increased handling (Meyer, 1991; Bondad-Reantaso *et al.*, 2005; Nicholson, 2006). These conditions can often be stressful for fish and may negatively impact on immunocompetence, which can ultimately lead to increased disease occurrence (Mock and Peters, 1990; Tort, 2011; Turnbull, 2012). Many of the disease-causing pathogens affecting the aquaculture industry are considered part of the ubiquitous and benign microfauna populating the aquatic environment, only becoming infective when the conditions for the fish become unfavourable (Cole, 1982; Bortman, 2003). This change in behaviour clearly highlights the knowledge that disease occurrence is a complex series of interactions between the host, the pathogen and the environment (Carrias *et al.*, 2012; Turnbull, 2012).

Numerous bacterial, viral and parasitic pathogens are recognised to have had or are having significant economic effects on the salmon aquaculture industry such as *A. salmonicida* (furunculosis), *F. psychrophilum* (RTFS) and *M. viscosa* (winter ulcer disease); ISAV (ISA), PMCV (CMS), PRV (HSMI) and SAV (PD); AGD and sea lice. These pathogens can cause significant economic losses for the industry through mortalities, downgrading at harvest and through the use of treatments (Costello, 2009). While antibiotics and chemotherapeutics have been used over the years to treat against many of these diseases, it is recognised that their extensive use has resulted in pathogens developing resistance against them (Leteux and Meyer, 1972; Inglis *et al.*, 1991; Nordmo *et al.*, 1994; Hecht and Endemann, 1998; Inglis, 2000; Michel *et al.*, 2003; Burridge *et al.*, 2010; Noga, 2011; Miranda *et al.*, 2013). As prevention is considered better than treatment, vaccination has now become a crucial factor in controlling infectious diseases in Atlantic salmon aquaculture (Shaoqi, 1989; Gudding, 2014). Since the first report of fish vaccination in the late 1930's (Snieszko *et al.*, 1938), continued developments in fish vaccinology have resulted in the production and commercialisation of many effective vaccines against salmonid aquaculture-significant bacterial and viral diseases such as vibriosis (Antipa *et al.*, 1980; Johnson *et al.*, 1982; Thuvander *et al.*, 1987), furunculosis (Paterson *et al.*, 1985;

Lillehaug *et al.*, 1992; Midtlyng, 1996) and IPN (Leong and Fryer, 1993; Frost and Ness, 1997). It should be noted, though, that the development of vaccines is not straightforward and many infectious pathogens, particularly parasites, still lack effective vaccines and, as a result, developmental work is still ongoing (Roberts, 2012; Monaghan *et al.*, 2014; Hoare *et al.*, 2016).

Vaccination is known to cause side-effects, with many factors having been shown to affect the type and severity of side-effects, including vaccine and adjuvant type, vaccination timing, water temperature and fish life-stage (Midtlyng, 1997; Midtlyng and Lillehaug, 1998; Rønsholdt and McLean, 1999; Berg *et al.*, 2006; Aunsmo *et al.*, 2008; Koppang *et al.*, 2008; Poppe and Koppang, 2014). To reduce side-effect occurrence, vaccine doses have been reduced over the years from the original 0.2 ml to 0.1 ml and recently to 0.05 ml for some vaccines, but side-effects still occur (Lillehaug, 2014; Poppe and Koppang, 2014). Of the known side-effects, intra-abdominal adhesions are the most common (Poppe and Koppang, 2014). Adhesions are formed from fibrinous strands occurring adjacent to the injection site which connect the ventral body wall, spleen and pyloric caeca (Poppe and Koppang, 2014). While these strands will normally detach easily from the peritoneum, they have been found to become severe in some cases, with organs connected tightly together and to the peritoneum (Lillehaug *et al.*, 1992; Poppe and Breck, 1997). The removal of adhesions then becomes difficult and is likely to damage the muscle, resulting in carcass downgrades at harvest (Midtlyng, 1997). This impacts not only on fish health and welfare but also on aquaculture productivity (Lillehaug *et al.*, 1992; Poppe and Breck, 1997; Hastein, 2004). Further to intra-abdominal adhesions, vaccination has also been linked to increased prevalence of vertebral deformities (Berg *et al.*, 2012).

Vertebral deformities can lead to reduced growth and downgrading at harvest, and therefore, represent a serious health and welfare issue (Gil Martens *et al.*, 2012). While some deformities are visible prior to vaccination, it has been suggested that inflammation around/adjacent to the spine, as a result of vaccination and the associated handling *e.g.* grading, can aggravate vertebral deformities post-vaccination (Midtlyng, 1996; Witten and Huysseune, 2009; Gil Martens *et al.*, 2010, 2012; Gil-Martens, 2010). However, the occurrence of vertebral deformities is recognised to be multifactorial, with temperature and size both shown to influence deformities following vaccination (Berg *et al.*, 2006; Aunsmo *et al.*, 2008; Gil-Martens, 2010; Grini *et al.*, 2011; Gil Martens *et al.*, 2012) but the mechanisms linking these factors have not yet been fully elucidated.

Despite numerous advances in triploid research over the years (Taylor *et al.*, 2011, 2013, 2015; Fjelldal *et al.*, 2016), there continues to be a shortage of data regarding the response of triploid Atlantic salmon to diseases and disease treatments (Dunham, 2011). Studies that have assessed the response of triploids to disease have generally found little differences between ploidy (Bruno and Johnstone, 1990; Langston *et al.*, 2001; Johnson *et al.*, 2004; Taylor *et al.*, 2007; Frenzl *et al.*, 2014; Chalmers *et al.*, 2016, 2017; Moore *et al.*, 2017). Few studies have been undertaken to date to investigate the response of triploid Atlantic salmon to vaccination. In **Chapter 2** of this thesis, vaccination with 0.1 ml of a bi-valent vaccine (ALPHA-JECT 2-2, PHARMAQ, Norway) caused similar adhesion scores in diploid and triploid Atlantic salmon and had no significant effect on weight. A study by Fraser *et al.* (2012a) found that IP administration of a 0.1 ml multivalent vaccine (MINOVA 6 Vet, Norvax®, Intervet International B.V., Boxmeer, Netherlands) negatively impacted weight and length in S1+ diploid and triploid Atlantic salmon smolts, with unvaccinated fish being heavier and longer at final smolt. However, other side-effects were not assessed in this study. A further study was carried out by Fraser *et al.* (2014a) using the same vaccine (MINOVA 6 Vet vaccine, Norvax®) to assess side-effects (adhesion & vertebral deformity) and the interaction with ploidy under S1+ and S0+ smolt photoperiod regimes. Again, they found that vaccination negatively affected weight in both ploidy but observed significantly higher adhesion scores in triploid S0+ smolts compared to their respective diploid group, yet found no ploidy differences between adhesions in S1+ smolts. In addition, they found no direct effect of vaccination on vertebral deformities, although triploids had a greater number of deformities than diploids. These studies highlight that vaccination has an impact on the growth and health of triploids (as well as diploids) but are relatively limited in their assessment of adhesions and deformities as side-effects, with focus only on one vaccine type and dose. Considering the positive outcomes associated with applying triploids in commercial aquaculture production, it is essential that studies be undertaken to elucidate the tolerance of triploids to the vaccination process as well as the associated side-effects.

5.1.1. Aims

The aim of this Chapter was to investigate the response of triploid Atlantic salmon to a range of commercially available vaccines, either administered singly or in combination, and assess side-effects in terms of growth, development of abdominal adhesions and vertebral deformities, in addition to antibody response.

5.2. MATERIALS AND METHODS

5.2.1. Fish stock and history

On 23rd October 2015, eggs and milt were stripped (20 dams & 5 sires) from unrelated 2-sea winter Atlantic salmon broodstock (strain PD-Strong, Mowi, Tveitvag, Marine Harvest Norway). Following fertilisation, half of each egg batch was subjected to a pressure shock of 655 bar for 6.25 min, applied at 37.5 min post-fertilisation at 8 °C (TRC-APV; Aqua Pressure Vessel, TRC Hydraulics Inc., Dieppe, NB, Canada) to induce triploidy. Water hardened eggs were then incubated in six upwelling silos (3 ploidy⁻¹) at 6.7 °C (temperature range: 5.4 °C - 7.0 °C) until automatic sorting. Survival at sorting was 88 % and 85 % for diploid and triploid ova, respectively. Eyed ova were delivered to Howietoun Hatchery, Sauchieburn, Stirling on 16th December 2015 (353 °D). Eggs were then incubated in a flow-through system at 7.0 ± 0.3 °C in 6 x 300 L square fibreglass tanks (5000 eggs ploidy⁻¹, 1 m², 0.3 m depth) in darkness until first feeding (26th February 2016). Mortality from egg receipt to first feeding was 5.1 % and 6.5 % for diploids and triploids, respectively. At first feeding, fry were reared under constant light and fed a commercial diet (diploids - Inicio Plus; triploids – Inicio-TriX, BioMar UK), distributed by automatic feeders (Arvo-Tec Oy, Finland). When approximately 0.5 g, fry were divided evenly between 12 x 300 L to reduce stocking density. On 11th May 2016, all fry (~1.22 g) were transferred from the hatchery to the NBFRF, Buckieburn, and stocked into two flow-through 28 m³ holding tanks (1 ploidy⁻¹), exposed to an LL photoperiod (400W BGB submersible Metal Halide) prior to experimental start. Tanks were maintained at ambient temperature (10 – 15 °C). Specific feeding rates (% tank biomass per day) were adjusted automatically according to predicted growth and daily temperature, and pellet size (0.5 to 2.0 mm) increased with fish size. Prior to the start of the experiment (8th August 2016), both stock tanks were graded (5th August 2016) using a box grader (12 mm bar, STERNER AquaTech) to remove the bottom grade.

5.2.2. Experimental design

On 8th August 2016, diploid (18.6 ± 0.3 g) and triploid (25.4 ± 0.4 g) Atlantic salmon were randomly stocked into six lightproof 1.6 m³ circular (2 m diameter) tanks (3 tanks ploidy⁻¹, 750 fish tank⁻¹) and maintained under an LL photoperiod (2 x 28W BioLumen 1500 Nature Perfect LED, TMC, UK). From 18th August 2016, all fish were individually tagged using Trovan® (Trovan Ltd, UK) and Pet ID (Pet ID Microchips Ltd., West Sussex, UK) passive integrated transponder (P.I.T) tags for identification throughout the trial. Mortality between P.I.T tagging and vaccination was 1.67 % for diploids and 1.09 % for triploids. Following

P.I.T tagging (22nd August 2016) all tanks were subjected to a “winter” (LD 12:12) photoperiod for 5 weeks, before being returned to LL (26th September 2016) for ~400 °D to induce S0+ smoltification (7th – 8th November 2016). From 6th to 8th September 2016 (average temperature 15.3 °C), experimental fish were vaccinated (diploids: 44.2 ± 0.7 g; triploids: 57.8 ± 1.6 g; $p = 0.002$).

Four vaccines, all containing liquid paraffin adjuvant, were tested (Table 5.1) with 10 different vaccine treatments created (Table 5.2). These treatments were created by PHARMAQ (Zoetis LLC, New Jersey, USA) using vaccine validation/test data, number of antigens and dose volume to give an increasing scale of severity, according to anticipated adhesion scores (Table 5.2). All fish were anaesthetised with MS-222 (50 ppm, PHARMAQ, Norway) prior to vaccination and then seventy fish per tank were IP injected with one of the ten vaccine treatments. At the point of vaccination, the weight and length of each fish was recorded in addition to corresponding P.I.T tag number. Vaccine injections were performed by Alastair McPhee (NBFRF, Buckieburn, UK) with assistance provided by personnel from Aqualife Services Ltd. (Stirling, Scotland) and PHARMAQ. Vaccine treatments containing one vaccine were delivered using either a FISHJECTOR 0.1 ml or a MICROFISH 0.05 ml vaccine gun (Kaycee Veterinary Products Ltd., West Sussex, UK), depending on the dose. Vaccine treatments containing two vaccines were delivered using a Micro-Matic Twin injector vaccine gun (Henke-Sass Wolf, Tuttlingen, Germany). Following vaccination, all fish were returned to their original trial tank for recovery and were maintained in these tanks for 52 days (622 °D) until sampling.

On 7th and 8th November, following smolt sampling (detailed in Section 5.2.3), all remaining experimental Atlantic salmon (diploids 1290; triploids 1306) were transferred to six sea-cages (triplicate ploidy⁻¹) (5 m height x 5 m width x 5 m depth; net mesh 18 mm) at the Ardnish Feed Trial Unit (FTU) (Lochailort, Marine Harvest Scotland Ltd., Scotland, UK). Fish were maintained at ambient water temperature throughout the freshwater (8th August to 8th November) and seawater (8th November 2016 to 19th May 2017) phases of the trial (Figure 5.1). Specific feeding rates (% tank or cage biomass per day) were adjusted automatically according to predicted growth and daily temperature, and pellet size (0.5 to 2.0 mm in FW, 3.5 to 6 mm in SW) increased with fish size and were distributed by automatic feeders (Arvo-Tec Oy, Finland). In seawater, fish were fed commercial diets (diploids – BioMar CPK; triploids – BioMar CPK-TriX, BioMar UK). Waste feed was collected by siphon uplifts per pen during the seawater phase only.

Table 5.1: Dose and component details for the four commercially available vaccines

Vaccine	Dose volume (μl)	Vaccine components
Micro 1 PD	50	Salmon Pancreas Disease Virus (SPDV) strain AL V405
ALPHA JECT 2-2	100	<i>A. salmonicida</i> subsp. <i>salmonicida</i> ; IPNV serotype Sp
Micro 6	50	<i>A. salmonicida</i> subsp. <i>salmonicida</i> ; <i>L. anguillarum</i> serotype O1; <i>L. anguillarum</i> serotype O2a; <i>V. salmonicida</i> ; <i>M. viscosa</i> ; IPNV serotype Sp
ALPHA JECT 6-2	100	<i>A. salmonicida</i> subsp. <i>salmonicida</i> ; <i>L. anguillarum</i> serotype O1; <i>L. anguillarum</i> serotype O2a; <i>V. salmonicida</i> ; <i>M. viscosa</i> ; IPNV serotype Sp

Table 5.2: Details of experimental vaccine treatments

Vaccine Treatment	Treatment	No. of antigens	Total volume (μl)	Anticipated adhesion score
			Bacterial	Viral
1	Sham (PBS)	0	0	100
2	Micro 1 PD	0	1	50
3	ALPHA JECT 2-2	1	1	100
4	Micro 6	5	1	50
5	Micro 6 + Micro 1 PD	5	2	100
6	ALPHA JECT 2-2 + Micro 1 PD	1	2	150
7	ALPHA JECT 6-2	5	1	100
8	ALPHA JECT 6-2 + Micro 1 PD	5	2	150
9	Micro 6 + Micro 1 PD (double dose)	5	2	150
10	ALPHA JECT 6-2 (double dose)	5	1	200

Vaccine treatment 1 represents an unvaccinated control; vaccine treatments 2 to 8 are combinations used in commercial aquaculture; vaccine treatment 9 and 10 mimic “accidental” double dose scenarios

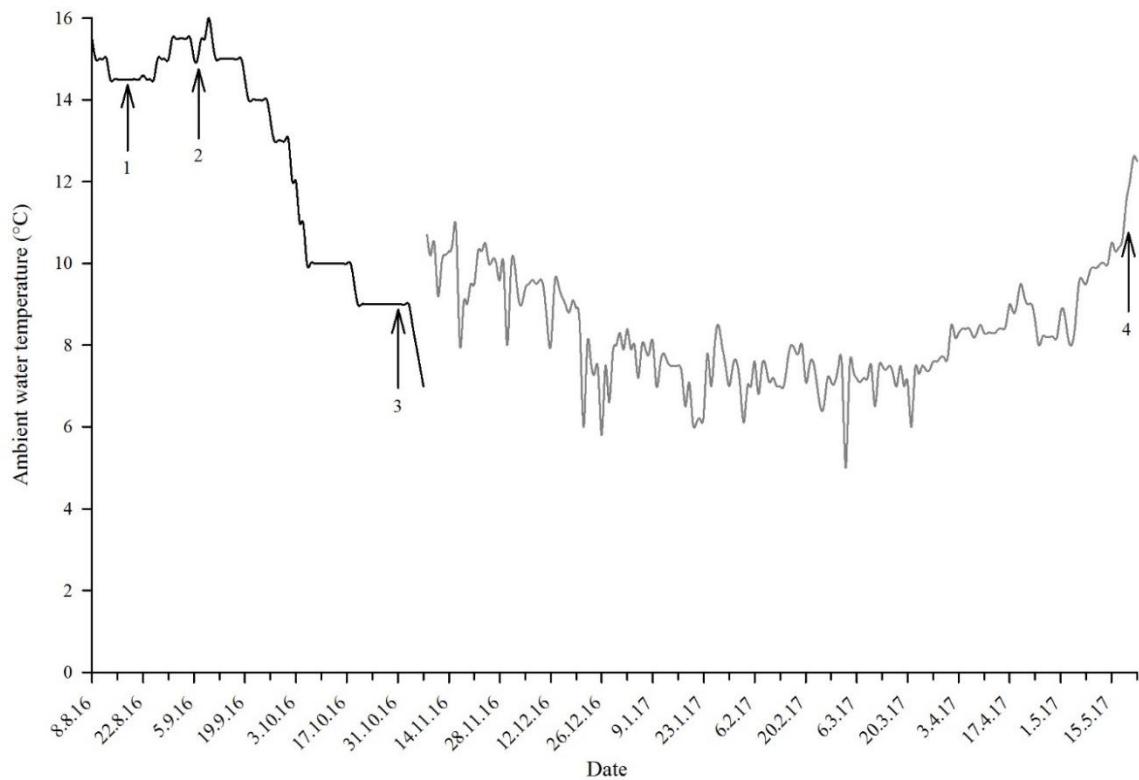


Figure 5.1: Temperature profile for freshwater (black line) and seawater (grey line) phases of the trial. Black arrows indicate 1) P.I.T tagging, 2) vaccination, 3) smolt sampling and 4) post-smolt weight assessment.

5.2.3. Smolt (pre-seawater transfer) sampling

Smolt sampling was undertaken from 31st October to 7th November (excluding 5th and 6th November), with one full tank sampled per day, alternating between triploids and diploids. Fish were removed from the experimental tank and maintained in 300 L holding tanks for the duration of the sampling, with water aerated throughout (average temperature 8.6 °C). All fish were anaesthetised with MS-222 (50 ppm, Pharmaq, Norway) and individually measured for body weight (g) and fork length (mm). Following P.I.T tag identification, 25 fish per vaccine treatment per tank were culled according to Schedule 1 for further sampling. Blood samples were obtained from the caudal vein using a non-heparinised needle and syringe and kept at 4 °C for 24 h. Samples were then centrifuged at 3000 xG for 10 min before serum was removed and stored at -20 °C until determination of antibody response. The severity of intra-abdominal adhesions in the peritoneal cavity was determined visually according to the PHARMAQ scoring system (Table 5.3). To this end, the peritoneal cavity was divided into three regions, detailed below (Figure 5.2), and a score given to each region:

- Region 1: Anterior and anterior-dorsal parts of the abdominal cavity including oesophagus, liver and anterior parts of the swim bladder
- Region 2: Posterior and posterior-dorsal parts of the abdominal cavity including the hind gut
- Region 3: Ventral region of the abdomen close to the recommended injection site

For analysis, the highest score over the three zones was taken as the overall adhesion score. Following sampling, all non-tissue sampled fish (approximately 450 tank⁻¹) were returned to the experimental tank for recovery

5.2.4. X-ray radiography for vertebral deformities

Radiographs of each fish were obtained using a device cabinet x-ray radiography unit (FaxinTron UltraFocus, Daax Ltd, USA; 24 kV, mAs: 5.0) and digital images were generated as dicom files for further analysis. Dicom images were analysed for vertebral deformities in each fish (RadiAnt Dicom Viewer software, Medixant, Poland). The total number of vertebrae (V) per fish was first counted before the location and type of deformity pathology, according to Witten *et al.* (2009), was recorded. To this end, pathologies were grouped together into compressions (type 2, 3, 4 and 5), fusions (type 6, 7 and 8), radiodense (type 12 and 13), symmetry (type 17 and 19) and other (type 1, 9 and 10). Examples of the vertebral deformity pathologies detected in this study are detailed in Figure 5.3. For analysis, the vertebral column was also divided into four regions (R): R1 (cranial trunk, V1 - V8), R2 (caudal trunk, V9 - V30), R3 (tail, V31 - V49) and R4 (tail fin, V50 – end) (Kacem *et al.*, 1998).

Table 5.3: Adhesion score scaling modified from the Speilberg scale (Midtlyng *et al.*, 1996b).

Score	Visual appearance of abdominal cavity
0	No visual lesions
1	Very slight adhesion seen as tiny fibrous tissue most frequently localised close to the injection site. Easily detached
2	Adhesion seen as more clearly defined fibrous threads connecting different organs or viscera to peritoneum in limited areas. The adhesions are easily detached and organs are intact following detachment
3	Firm adhesion connecting some or several organs. Viscera may be firmly attached to the peritoneum but are detached during autopsy without any damage to different organs or peritoneum/muscle tissue. May be observed as a grey-ish fibrous tissue film covering organs. Swim bladder may be attached to viscera
4	Similar to score 3 but more pronounced adhesions in and around organs. Interconnecting organs referred to as an “organ package” may be observed, where the organs appear as one unit, bound together by fibrous connective tissue. Smaller granulomas may be present in or around the organs. Separation of organs attached via fibrous connective tissue will result in organ damage. Viscera cannot be detached from peritoneum without damaging it
5	Extensive lesions affecting several organs in the abdominal cavity. In large areas, the peritoneum is thickened and opaque. Large granulomas in viscera, together with extensive bindings between viscera and peritoneum. The peritoneum and fillet/muscle is damaged when removing the viscera. The side-effects are ethically unacceptable
6	Even more pronounced than 5. Viscera cannot be removed without severe damage to the muscle/fillet. The side-effects are ethically unacceptable

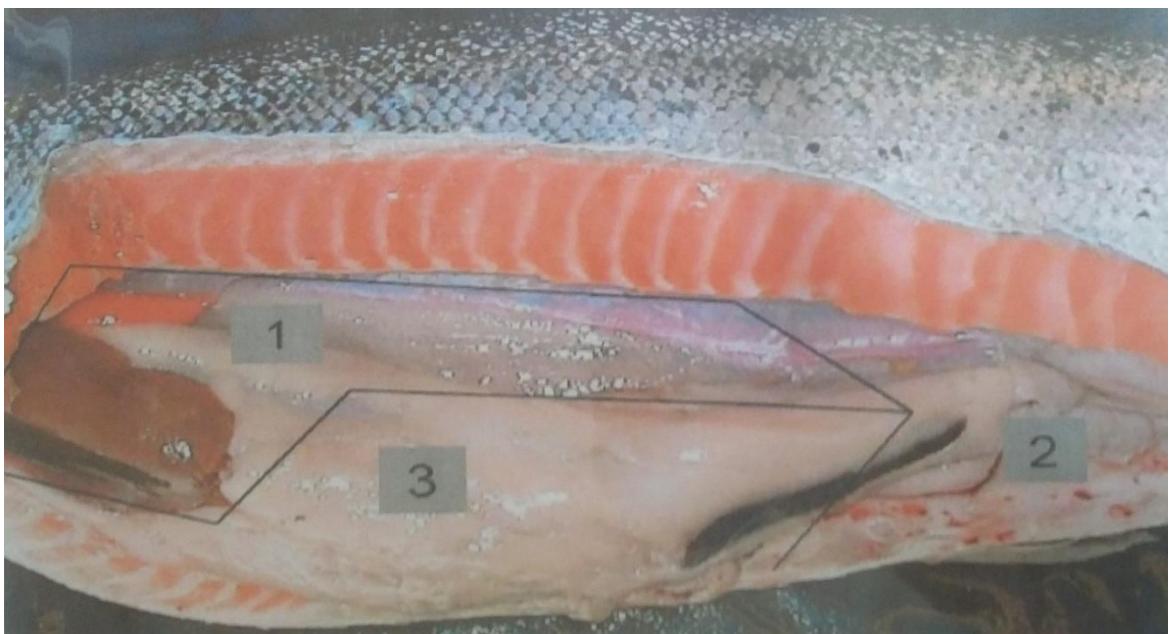
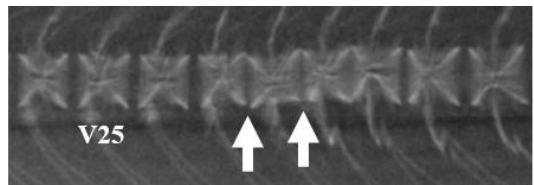
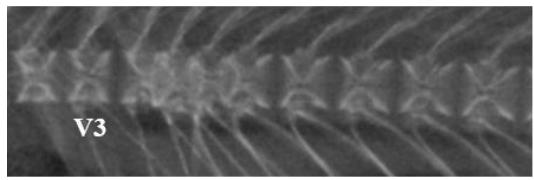


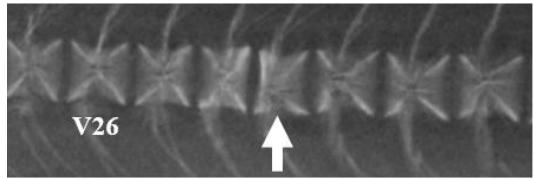
Figure 5.2: Adhesion scoring regions (PHARMAQ, Norway)



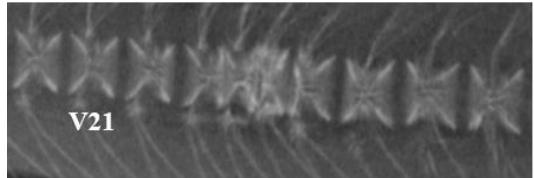
Type 1:
Decreased intervertebral space



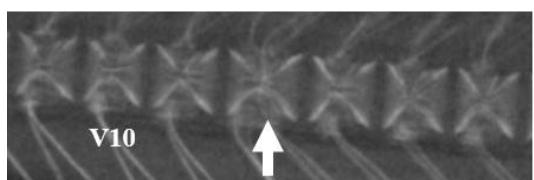
Type 3:
Compression and reduced
intervertebral space



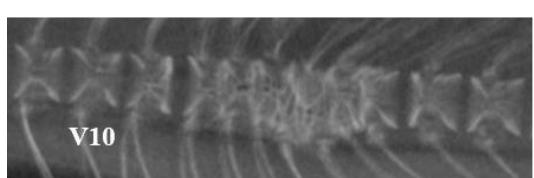
Type 5:
One-sided compression



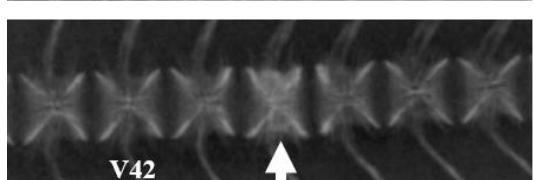
Type 6:
Compression and fusion



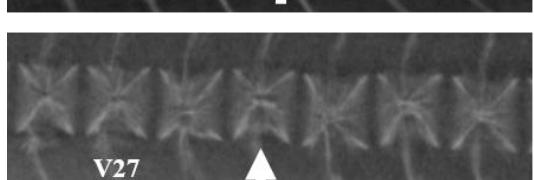
Type 7:
Complete fusion



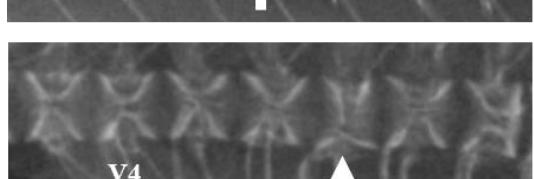
Type 8:
Fusion centre



Type 12:
Hyper-radiodense



Type 17:
Vertically shifted



Type 19:
Internal dorsal or ventral shift

Figure 5.3: Examples of vertebral deformity pathologies detected. Arrows indicate the position of the pathology type

5.2.5. Enzyme linked immunosorbent assay (ELISA)

The specific antibody response (IgM) of diploid and triploid Atlantic salmon to *A. salmonicida* was measured in serum samples according to the method provided by Aquatic Diagnostics Ltd (University of Stirling, Stirling, UK) for particulate antigens. Briefly, 96 well microplates (Immulon 4HBX, Fisher Scientific, UK) were coated with 0.001 % (w/v) poly-L-lysine (P8920, Sigma-Aldrich, USA) in coating buffer ($50\mu\text{l well}^{-1}$) for 60 min before being washed twice with LSWB. Whole cell *A. salmonicida* ‘Hooke’ (1×10^8 bacteria ml^{-1}) was then added ($100 \mu\text{l well}^{-1}$) and the plates incubated overnight at 4°C . Following this, 0.05 % (v/v) glutaraldehyde (G6403, Sigma-Aldrich, USA) in PBS was added to the antigen ($50\mu\text{l well}^{-1}$) and the plates incubated for a further 20 min at RT. Plates were then washed a further 3 times with LSWB and post-coated with 3 % (w/v) casein ($250 \mu\text{l well}^{-1}$) for 2 h at RT to block non-specific binding sites. The post-coat was discarded before the addition of serum samples. Serum samples were diluted 1:50 with PBS, added to the microplates along with PBS in the negative and positive control wells ($100 \mu\text{l well}^{-1}$) and incubated overnight at 4°C . The microplates were washed with HSWB, incubating for 5 min on the last wash. Microplates were then incubated for 1 h at RT with mouse anti-Atlantic salmon IgM monoclonal antibody (F11, Aquatic Diagnostics Ltd., University of Stirling, Stirling, UK), diluted 1:33 with PBS ($100 \mu\text{l well}^{-1}$). This is with the exception of the positive control wells which were incubated with rabbit anti-*A. salmonicida* polyclonal antibody (Aquatic Vaccine Unit, University of Stirling, Stirling, UK), diluted 1:1000 in PBS. The HSWB washes were then repeated before conjugates (anti-mouse IgG-HRP, A4416, Sigma-Aldrich, USA; anti-rabbit IgG-HRP, A6154, Sigma-Aldrich, USA), diluted 1:4000 with conjugate buffer, were added for 1 h ($100 \mu\text{l well}^{-1}$). The HSWB washes were repeated and the reaction was developed with chromogen in substrate buffer ($100 \mu\text{l well}^{-1}$). The reaction was stopped with 2 M H_2SO_4 ($50 \mu\text{l well}^{-1}$) after 10 min then the absorbance was measured at 450 nm and values expressed as optical density (OD). The OD values for negative control wells were then multiplied by 3 and samples were considered positive if higher than this value.

5.2.6. Post-smolt growth assessment

An assessment of growth was undertaken at the Ardnish FTU (Marine Harvest) on 19th May 2017 (193 days, 1586 °D, post-sea transfer). Fish were anaesthetised (MS-222, 50 ppm, PHARMAQ, UK) before individual P.I.T tag numbers and the corresponding weight (± 1.0

g) and fork-length (\pm 1.0 mm) were recorded. Fish were returned immediately to the sea-cage for recovery.

5.2.7. Growth analysis

Condition factor (K) was calculated according to Herbinger and Friars (1991) using the following equation, where W is body weight (g) and L is fork length (mm):

$$K = 100 \times W / L^3$$

Thermal growth coefficient (TGC) was calculated according to Thorarensen and Farrell (2011) using the following equation, where T is temperature in °C and t is time in days:

$$TGC = 1000 \times (W_2^{1/3} - W_1^{1/3}) / (T \times t)$$

5.2.8. Statistical analysis

Minitab software version 16 (Minitab Inc., Pennsylvania) was used to perform basic descriptive statistics and comparisons using a significance level of 5 % ($p = 0.05$). Prior to analysis, datasets were checked for normality using the Anderson-Darling test. For mortality and vertebral deformity data, percentages were arcsine transformed for normality. To analyse antibody response, weight, length, condition factor, TGC, a GLM was manipulated using a two-way ANOVA, with ploidy and vaccine treatment considered fixed factors and tank considered a random factor. Non-parametric Kruskal-Wallis and Dunn's multiple comparison post-hoc test were used to assess mortality, adhesion score and total radiographic deformity (%). A one-way ANOVA was used to assess ploidy differences in total vertebrae number. Statistical differences in the localisation of deformed vertebrae (%) between ploidy for each vertebral region were analysed by a one-way ANOVA. Within ploidy, a GLM manipulated using a two-way ANOVA was used to analyse differences between vaccine treatment 1 (PBS-control) and all other vaccine treatments, with vaccine treatment and vertebral region considered as fixed factors and tank as a random factor. Post-hoc analyses were carried out using Tukey's multiple comparison tests with values considered significantly different at p -values < 0.05 . Regression analysis to detect linearity between adhesion score severity and TGC was carried out using linear regression. Parallelism statistics were also performed using Excel (ANCOVA, Microsoft Office 2013,

Washington, USA) to determine ploidy differences between the gradient of regression slopes.

5.3. RESULTS

The results presented show the effects of vaccination with 10 different vaccine treatments on mortality, antibody response, adhesion score, weight, length, condition factor (K), growth rate (TGC) and radiological vertebral deformity severity (%) in diploid and triploid Atlantic salmon.

5.3.1. Mortality

Low levels of mortality were recorded throughout the trial (Table 5.4). From vaccination to smolt, mortality was less than 3 % in diploids and triploids from each vaccine treatment and was not significantly affected by vaccine treatment or ploidy. In the period from smolt to post-smolt, mortality was less than 8 % in diploid and triploid Atlantic salmon from each vaccine treatment and was not significantly affected by vaccine treatment or ploidy.

Table 5.4: Mortality (%) (mean \pm SEM, n = 3) in diploid and triploid Atlantic salmon from vaccination to smolt (pre-seawater transfer) and from smolt to post-smolt (1586 °D post-seawater transfer) for each vaccine treatment

Vaccine Treatment	Vaccination to smolt		Smolt to post-smolt	
	Diploid	Triploid	Diploid	Triploid
1	1.4 \pm 0.8	2.4 \pm 1.3	5.4 \pm 3.4	0.8 \pm 0.8
2	0.0 \pm 0.0	1.0 \pm 1.0	2.2 \pm 2.2	1.5 \pm 0.8
3	2.4 \pm 1.3	0.5 \pm 0.5	4.5 \pm 2.6	3.0 \pm 2.0
4	2.4 \pm 0.5	1.0 \pm 1.0	3.1 \pm 0.8	4.6 \pm 1.4
5	1.4 \pm 0.8	1.0 \pm 1.0	3.0 \pm 0.7	3.8 \pm 3.8
6	1.0 \pm 1.0	1.9 \pm 0.5	4.5 \pm 3.4	2.3 \pm 1.3
7	1.0 \pm 1.0	1.9 \pm 0.5	1.5 \pm 0.8	3.8 \pm 2.7
8	2.0 \pm 0.5	0.0 \pm 0.0	2.3 \pm 1.3	5.2 \pm 1.5
9	1.0 \pm 0.5	1.0 \pm 0.5	3.9 \pm 2.0	3.8 \pm 1.4
10	2.0 \pm 2.0	1.6 \pm 0.9	3.5 \pm 0.8	8.0 \pm 6.8

5.3.2. Antibody response

The antibody responses (specific IgM to *A. salmonicida*) of fish injected with vaccine treatments 3 to 10 (*i.e.* vaccines containing *A. salmonicida*) were all positive (Figure 5.4, indicated by red dashed line), while antibody responses in vaccine treatments 1 (PBS) and 2 (Micro 1PD – no *Aeromonas* antigen) were negative, as expected. There was no significant effect of ploidy or vaccine treatment in any of the positive samples (Figure 5.4).

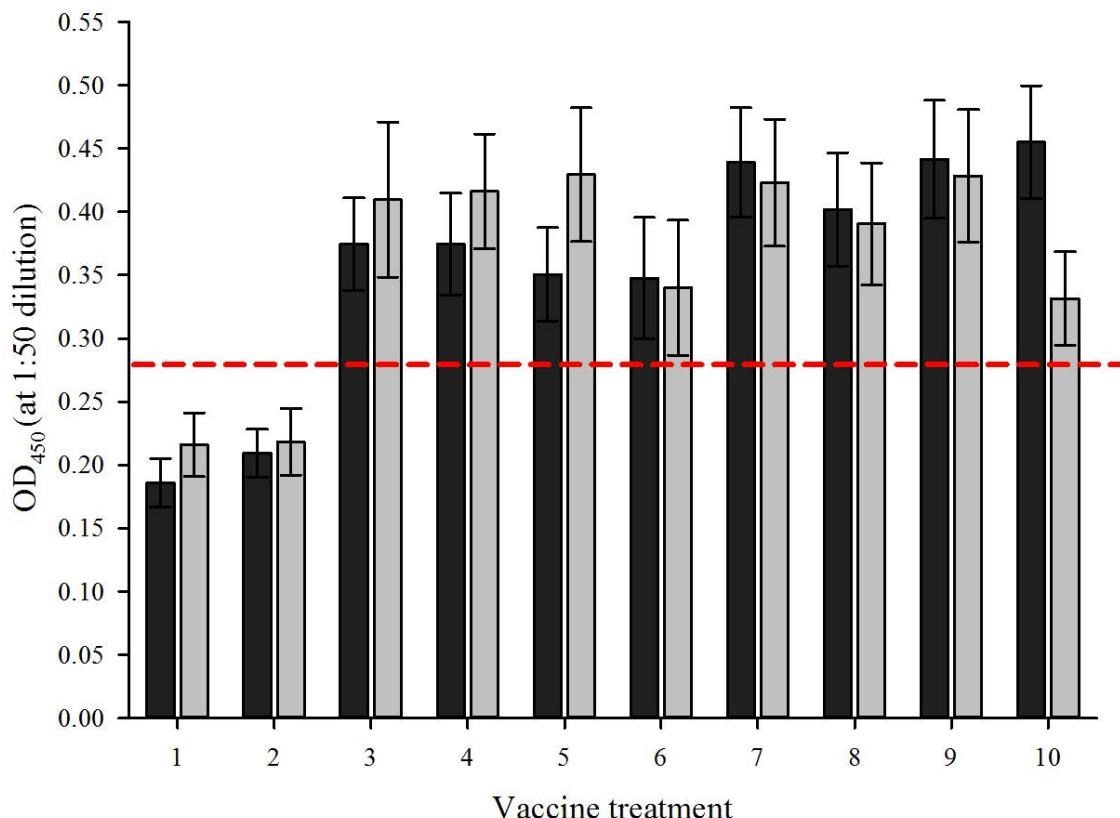


Figure 5.4: Antibody (IgM) response (OD_{450nm}, mean ± SEM, n = 3) against *Aeromonas salmonicida* in diploid (black) and triploid (grey) Atlantic salmon injected with vaccine treatments 1 to 10. Red dashed line represents the value over which samples are considered positive.

5.3.3. Adhesion scores

Adhesion scores in both ploidies increased according to the anticipated adhesion scores of each vaccine treatment (Table 5.5). Adhesion score was significantly affected by vaccine treatment ($p = 0.000$) and ploidy ($p = 0.013$), with the overall adhesion score (all vaccine treatments) significantly higher for triploids than diploids, although within each vaccine treatment no significant difference between ploidy was evident (Figure 5.5).

In diploids, the highest adhesion score was found in vaccine treatment 10 (2.0 ± 0.1) and this was significantly higher than fish in vaccine treatment 1 (0.1 ± 0.0), 2 (0.7 ± 0.1), 3 (1.0 ± 0.1) and 6 (1.1 ± 0.0). In addition, adhesion scores in vaccine treatments 4 (1.7 ± 0.1), 5 (1.6 ± 0.1), 7 (1.8 ± 0.2), 8 (1.8 ± 0.1) and 9 (1.5 ± 0.1) were all significantly higher than those of vaccine treatments 1, 2, 3 and 6.

A similar pattern of adhesion score was exhibited in the triploids. The highest adhesion score was found in vaccine treatment 10 (2.3 ± 0.1), which was significantly higher than all vaccine treatments (range: $0.1 - 1.6$), with the exception of 7 (1.8 ± 0.1) and 8 (1.9 ± 0.1). Moreover, fish injected with vaccine treatments 1 (0.1 ± 0.1), 2 (0.9 ± 0.0) and 3 (1.3 ± 0.1) had significantly lower scores than those in vaccine treatment 7 and 8, with fish in vaccine treatment 1 and 2 continuing to exhibit significantly lower scores than vaccine treatment 4 (1.6 ± 0.2), 5 (1.5 ± 0.2) and 9 (1.6 ± 0.1).

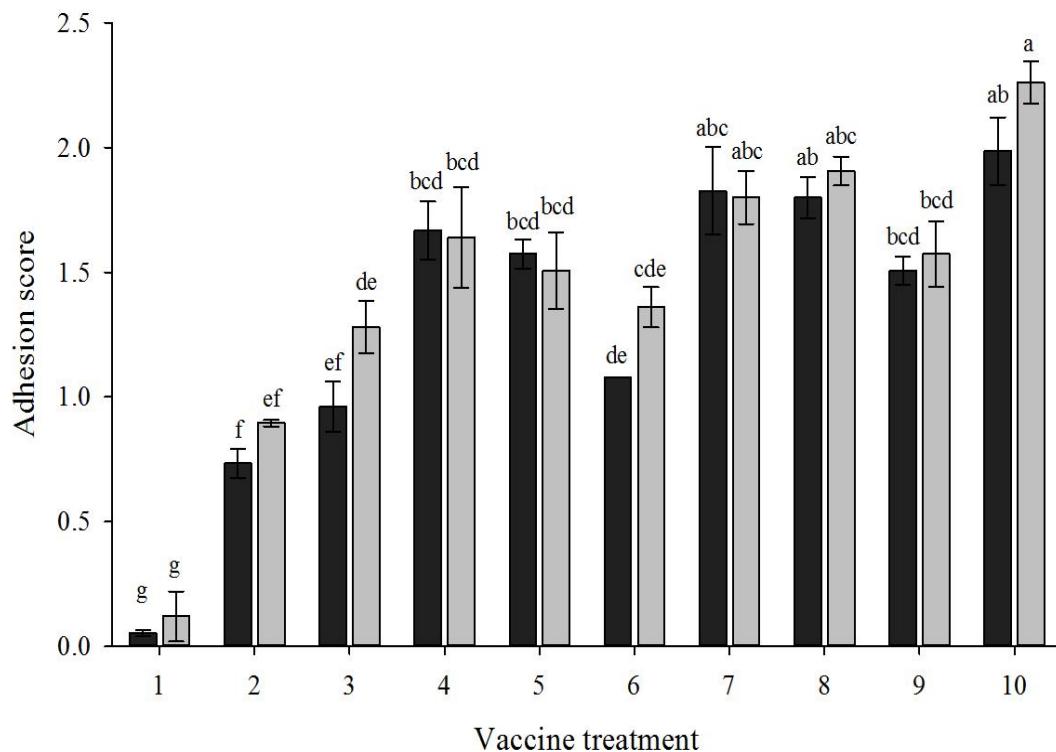


Figure 5.5: Adhesion score (mean \pm SEM, $n = 3$) in diploid (black) and triploid (grey) Atlantic salmon injected with vaccine treatment 1 to 10. Significant differences between vaccine treatment and ploidy are indicated by different letters.

5.3.4. Growth

In diploids and triploids, there was a significant increase in weight and length from the beginning of the trial (initial) to smolt, while there was a significant decrease in condition factor (K) (Figure 5.6). At smolt, weight and length were significantly affected by ploidy while K was not. On average, triploids had a higher final smolt weight than their diploid counterparts in all vaccine treatments (+ 14.1 %), significantly so in vaccine treatments 1 (diploids: 86.4 ± 2.6 g; triploids 102.4 ± 4.9 g), 3 (diploids: 81.2 ± 1.1 g; triploids 101.4 ± 8.3 g) and 4 (diploids: 80.1 ± 2.7 g; triploids 95.3 ± 4.0 g). Triploids also had a significantly greater length than diploids in all vaccine treatments (+ 5.3 %; diploids: 186.9 – 195.9 mm; triploids 195.5 – 206.5 mm), except vaccine treatment 10. Vaccine treatment did not significantly affect growth parameters in diploids, while it significantly affected weight and length in the triploids. In terms of weight, triploids were significantly heavier in vaccine treatment 1 (*i.e.* control fish) than in vaccine treatments 7 (91.3 ± 4.7 g), 9 (92.2 ± 3.9 g) and 10 (86.5 ± 5.0 g). For length, fish in vaccine treatment 1 (206.5 ± 3.8 mm) were significantly longer than in vaccine treatment 10 (195.5 ± 3.7 mm). As reflected by weight, ploidy and vaccine treatment were found to significantly affect TGC from vaccination to smolt (Figure 5.7A). The TGC post-vaccination was higher in diploids in all vaccine treatments but was significantly higher in vaccine treatments 5 (diploids: 1.22 ± 0.02 ; triploids 0.98 ± 0.04) and 9 (diploids: 1.22 ± 0.02 ; triploids 0.96 ± 0.10). In the diploids, TGC was significantly higher in vaccine treatment 1 (1.33 ± 0.03) than in treatment 10 (1.10 ± 0.07), while in the triploids TGC in vaccine treatments 1 (1.18 ± 0.09) and 2 (1.11 ± 0.06) were significantly higher compared to vaccine treatments 9 and 10 (0.89 ± 0.05). The interaction between adhesion score and TGC was examined by linear regression, whereby a significant negative correlation ($p = 0.001$) was found between TGC and increasing adhesion score (Figure 5.7B). Regression analysis showed that the vertical distance between the diploid and triploid slopes was statistically significant ($p = 0.024$), however, parallelism statistics (ANCOVA) revealed no significant difference in gradient ($p > 0.05$) between ploidy, indicating that both ploidy showed the same response to vaccination.

From smolt to post-smolt, there was a significant increase in weight and length in both diploids and triploids, while K was significantly lower at post-smolt (Figure 5.8). At 193 days post-smolt, weight and K were significantly affected by ploidy while length was not. Triploids, on average, weighed significantly more (+ 32.0 %) than their diploid counterparts in all vaccine treatments (diploids: 565.9 – 655.6 g; triploids 730.9 – 884.6 g). While not significant, triploids also remained longer than diploids (+ 8.2 %). The K value

was higher in triploids than diploids in all vaccine treatments with significant ploidy effects evident in vaccine treatments 1, 3, 4, 5, 8, 9 and 10 (diploids: 1.05 – 1.07; triploids 1.11 – 1.13). Vaccine treatment did not have a significant effect on diploid post-smolt weight, length or K, while it significantly affected weight and K in the triploids. In terms of weight, triploids were significantly heavier in vaccine treatment 1 (884.6 ± 32.4 g) than in vaccine treatments 7 (754.8 ± 6.4 g), 8 (780.3 ± 15.4 g) and 10 (730.9 ± 22.4 g). For K, fish in vaccine treatment 10 (1.13 ± 0.01) had significantly higher K than fish in vaccine treatment 6 (1.08 ± 0.01). As reflected in weight, ploidy and vaccine treatment were found to significantly affect TGC at post-smolt (Figure 5.9). At post-smolt, triploids had significantly higher TGC than their diploid counterparts in all vaccine treatments (triploids: 2.86 – 3.12; diploids 2.49 – 2.72). In diploids, TGC was significantly higher in vaccine treatments 2 (2.69 ± 0.07) and 6 (2.72 ± 0.12) compared to vaccine treatment 10 (2.49 ± 0.09). In triploids, the TGC of vaccine treatments 1 (3.09 ± 0.04), 2 (3.07 ± 0.04), 5 (3.12 ± 0.03) and 9 (3.08 ± 0.07) were significantly higher than that of vaccine treatments 7 (2.88 ± 0.04) and 10 (2.86 ± 0.02). In addition, TGC in vaccine treatment 3 (3.06 ± 0.04) was also significantly higher than in vaccine treatment 10.

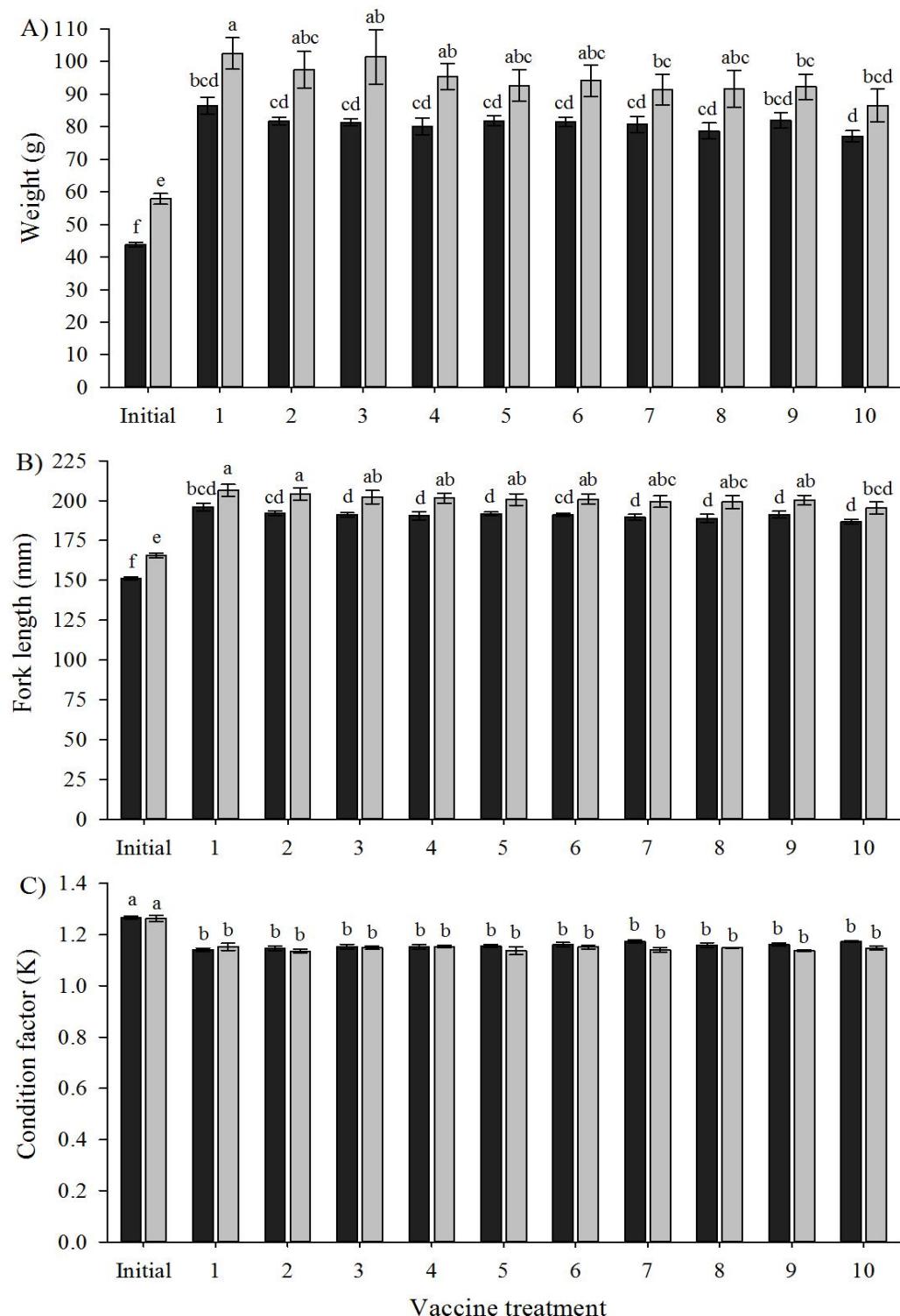


Figure 5.6: Weight (g) (A), fork length (mm) (B) and condition factor (K) (C) (mean \pm SEM, $n = 3$) at smolt in diploid (black) and triploid (grey) Atlantic salmon injected with vaccine treatment 1 to 10. Significant differences between vaccine treatment and ploidy are indicated by different letters.

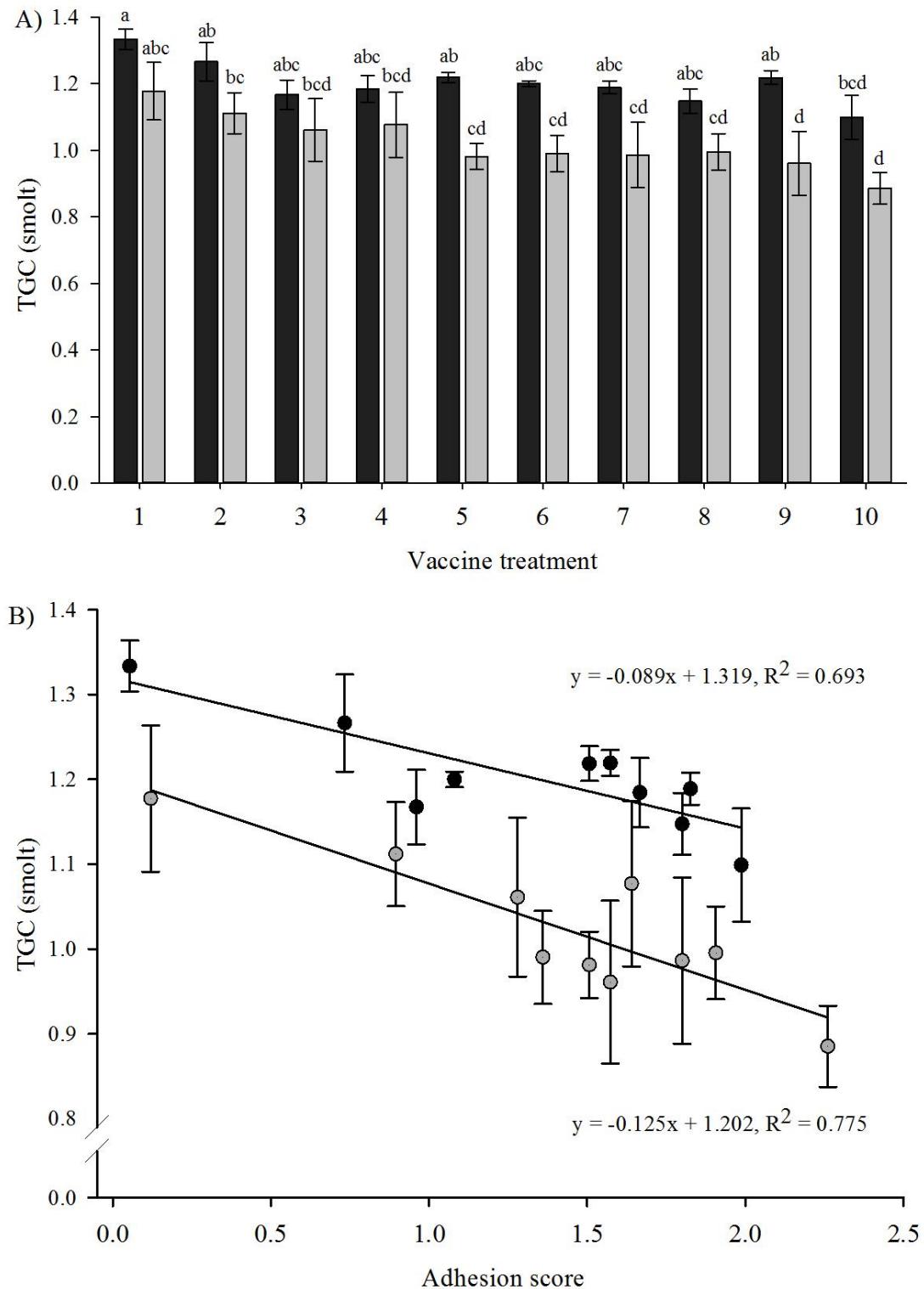


Figure 5.7: A) Smolt TGC (mean \pm SEM, $n = 3$) of diploid (black) and triploid (grey) Atlantic salmon injected with vaccine treatment 1 to 10. Significant differences between vaccine treatment and ploidy are indicated by different letters; B) Linear regression of relationship between adhesion score and smolt TGC in diploid (black) and triploid (grey) Atlantic salmon.

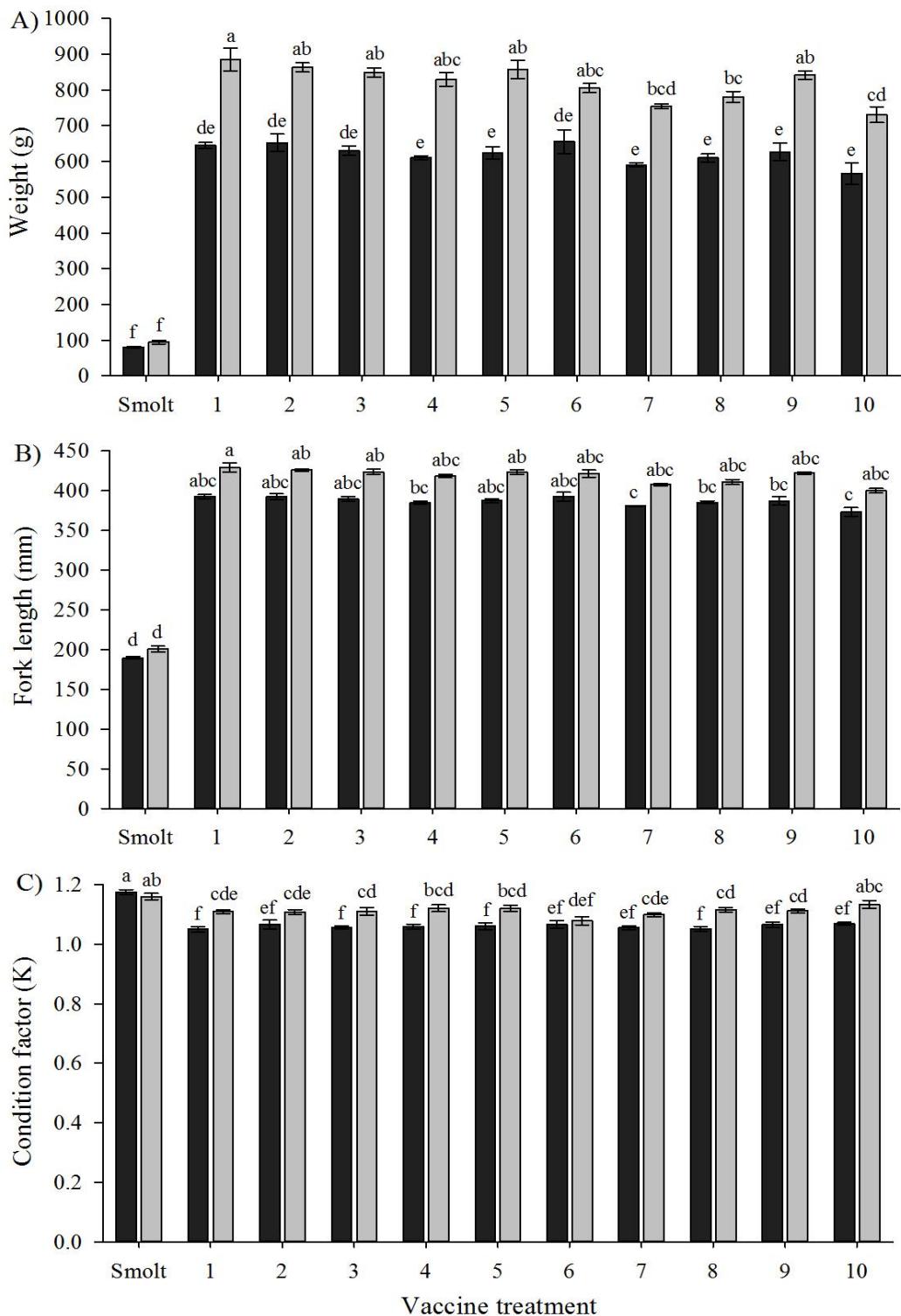


Figure 5.8: Weight (g) (A), fork length (mm) (B) and condition factor (K) (C) (mean \pm SEM, n = 3) at post-smolt in diploid (black) and triploid (grey) Atlantic salmon injected with vaccine treatment 1 to 10. Significant differences between vaccine treatment and ploidy are indicated by different letters

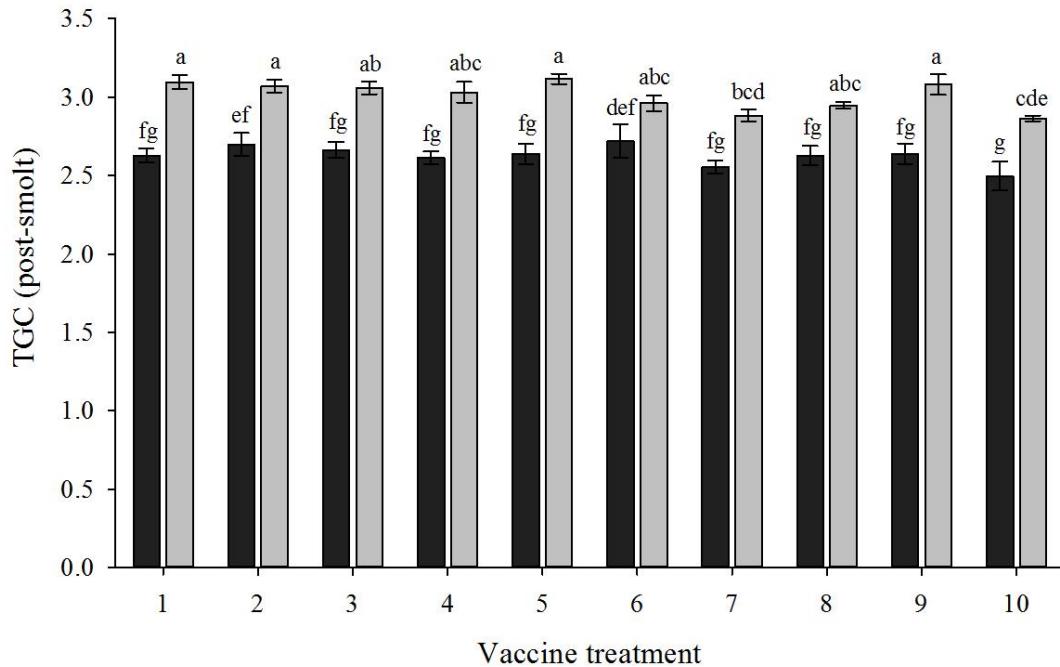


Figure 5.9: Thermal growth coefficient (TGC) (mean \pm SEM, $n = 3$) of diploid (black) and triploid (grey) Atlantic salmon injected with vaccine treatment 1 to 10 at post-smolt. Significant differences between vaccine treatment and ploidy are indicated by different letters.

5.3.5. X-ray radiography for vertebral deformities

Average vertebrae counts for diploid (58.2 ± 0.04) and triploid (58.1 ± 0.06) Atlantic salmon did not differ significantly ($p = 0.492$). Total radiographic vertebral deformity (%) was significantly affected by ploidy ($p < 0.05$) but not by vaccine treatment ($p > 0.05$) (Figure 5.10). The prevalence of deformed vertebrae (dV) did not change significantly from the baseline deformity assessment (prior to trial commencing) to smolt assessment. Triploid Atlantic salmon exhibited significantly more individuals with deformed vertebrae than diploids in all vaccine treatments (diploid: 16.1 – 24.1 %; triploid: 36.0 – 58.7 %), with the exception of vaccine treatment 5. Within deformed fish, the average number of dV per deformed individual was 2.8 ± 0.2 in diploids and 4.1 ± 0.2 in triploids ($p = 0.005$). There was no significant effect of vaccine treatment on average number of dV per deformed individual ($p = 0.422$) or significant interaction between ploidy and vaccine treatment ($p = 0.743$). Within the deformed fish, diploids and triploids showed the highest prevalence for 1 - 5 dV (diploid average: 18.7 %; triploid average: 37.8 %). In all vaccine treatments, triploids were also found to have a higher prevalence of 6 - 9 dV (2.7 – 12.16 %) and > 10 dV (1.3 – 6.7 %) than the diploids (6 - 9 dV: 0 – 3.6 %; > 10 dV: 0 – 3.6 %).

In both diploid and triploid Atlantic salmon, deformed vertebrae were observed in all four spinal regions (Figure 5.11). Triploids were found to have significantly more dV in each of the spinal regions than diploids, except for R4. In triploids, the highest prevalence of dV occurred in the caudal trunk (R2) ($5.5 \pm 0.1\%$), with a peak at V28 - 29. Increased prevalence also occurred in the cranial trunk (R1) ($3.4 \pm 0.1\%$) and tail region (R3) ($1.7 \pm 0.1\%$). In diploids, the highest percentage of dV occurred in the caudal trunk (R2) ($1.7 \pm 0.8\%$) although no discernible peak was observed as in triploids. Similar prevalence was observed in the cranial trunk (R1) ($1.6 \pm 0.2\%$) and tail fin (R4) ($1.4 \pm 0.3\%$).

Within each ploidy, further localisation assessment was undertaken to determine differences between radiological deformity in the PBS control group (vaccine treatment 1) and all other vaccine treatments (Figure 5.12 A – I; Figure 5.13 A – I). In the diploids, the prevalence of deformed vertebrae in each region was variable among vaccine treatments. The prevalence of deformity in each spinal region did not differ significantly between the PBS control group (vaccine treatment 1) and any of the vaccine treatments (Figure 5.12 A – I). In addition, deformity did not vary significantly between regions in each vaccine treatment. In the triploids, while deformity prevalence varied in each region between vaccine treatments, there was no significant difference between PBS and any vaccine treatment. However, it was evident that the greatest prevalence of deformed vertebrae consistently occurred in R2, with increased prevalence also in R1. In vaccine treatment 4, deformity in R2 was significantly higher than in R4, with no significant effect of region exhibited in the other vaccine treatments.

In both ploidy, the type and prevalence of deformity pathologies did not differ greatly from the baseline assessment or between vaccine treatments (Figure 5.14 A - B). Regardless of vaccine treatment, the most prevalent type of deformity pathology in both ploidy was fusions (diploids: 1.8 – 16.4 %; triploids: 16.4 – 30.9 %), followed by compressions (diploids: 0.6 – 10.9 %; triploids: 10.0 – 17.9 %). In diploids and triploids, the most common type of fusion pathology was complete fusion (type 7) (diploids: 1.3 – 11.9 %; triploids: 5.6 – 16.4 %) followed by fusion centre (type 8) (diploid: 0.0 – 6.4 %; triploid: 3.14 – 12.9 %). In terms of compressions, the most prevalent type in both ploidy was one-sided compression (type 5) (diploids: 0.6 – 7.18 %; triploids: 8.1 – 17.3 %) followed by compression without x structure (type 4) in diploids (0.0 – 1.9 %), and compression and decreased intervertebral space (type 3) in triploids (0.0 – 3.5 %).

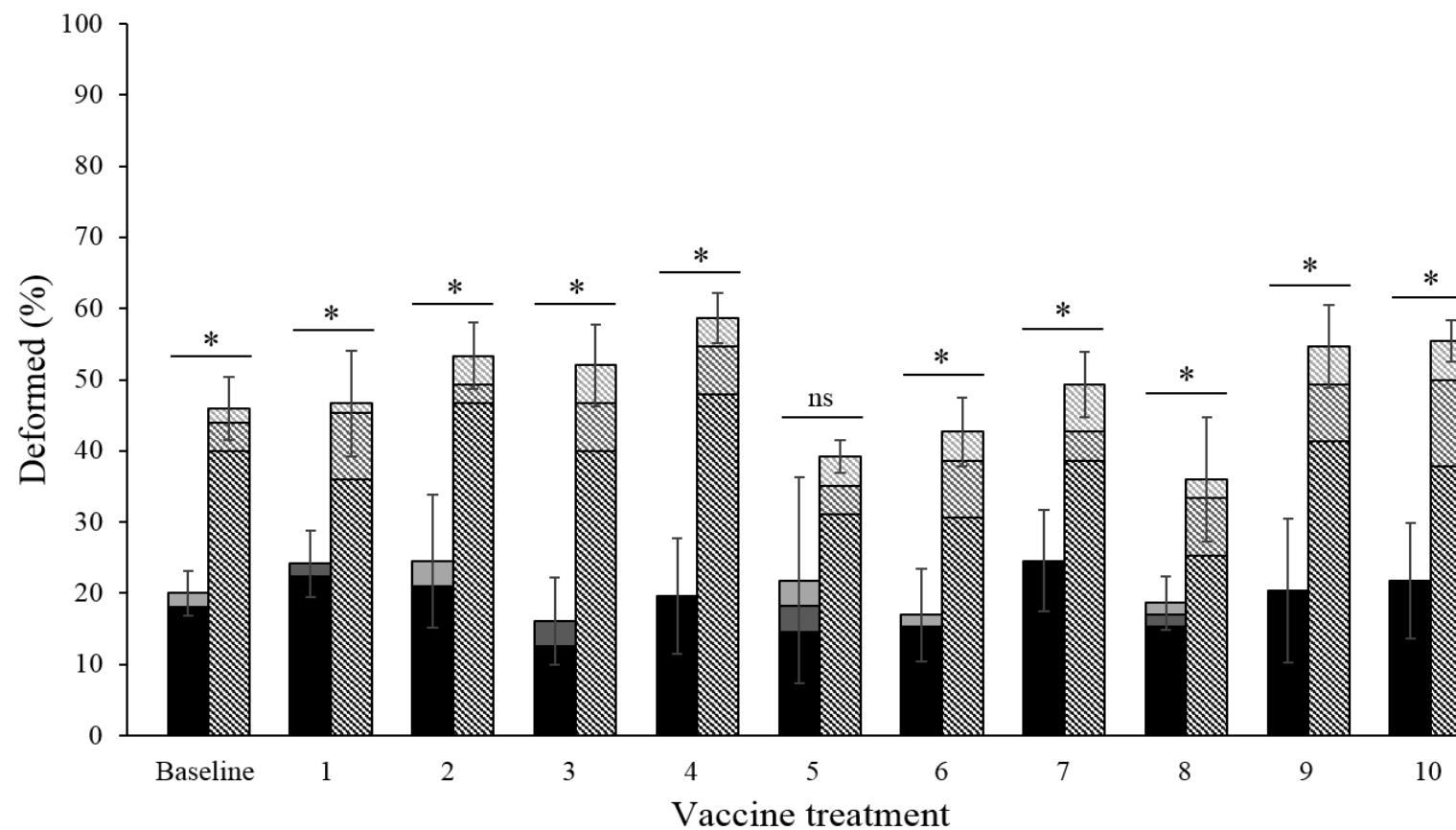


Figure 5.10: Total radiographic vertebral deformity (%) of diploid (solid bars) and triploid (hatched bars) Atlantic salmon at smolt (mean \pm SEM, n = 3) within each vaccine treatment. Bars also indicate prevalence (%) of deformed vertebrae number (1-5 black; 6-9 dark grey; > 10 light grey). Asterisks (*) indicate significant differences between ploidy within vaccine treatment.

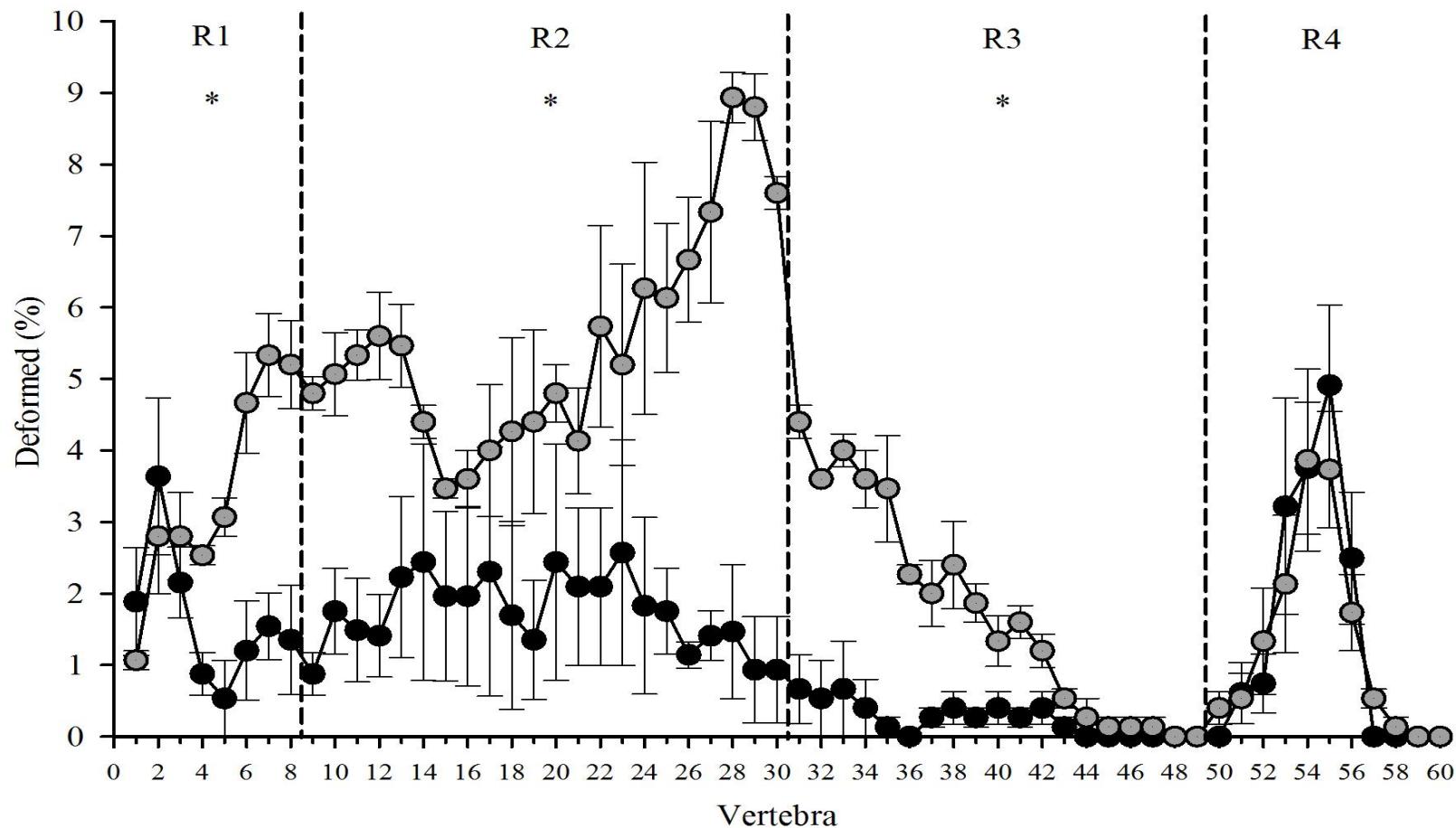


Figure 5.11: Localisation of deformed vertebrae (%) in diploid and triploid Atlantic salmon at smolt (mean \pm SEM, n = 3). Asterisks (*) denote significant differences between ploidy within vertebral regions.

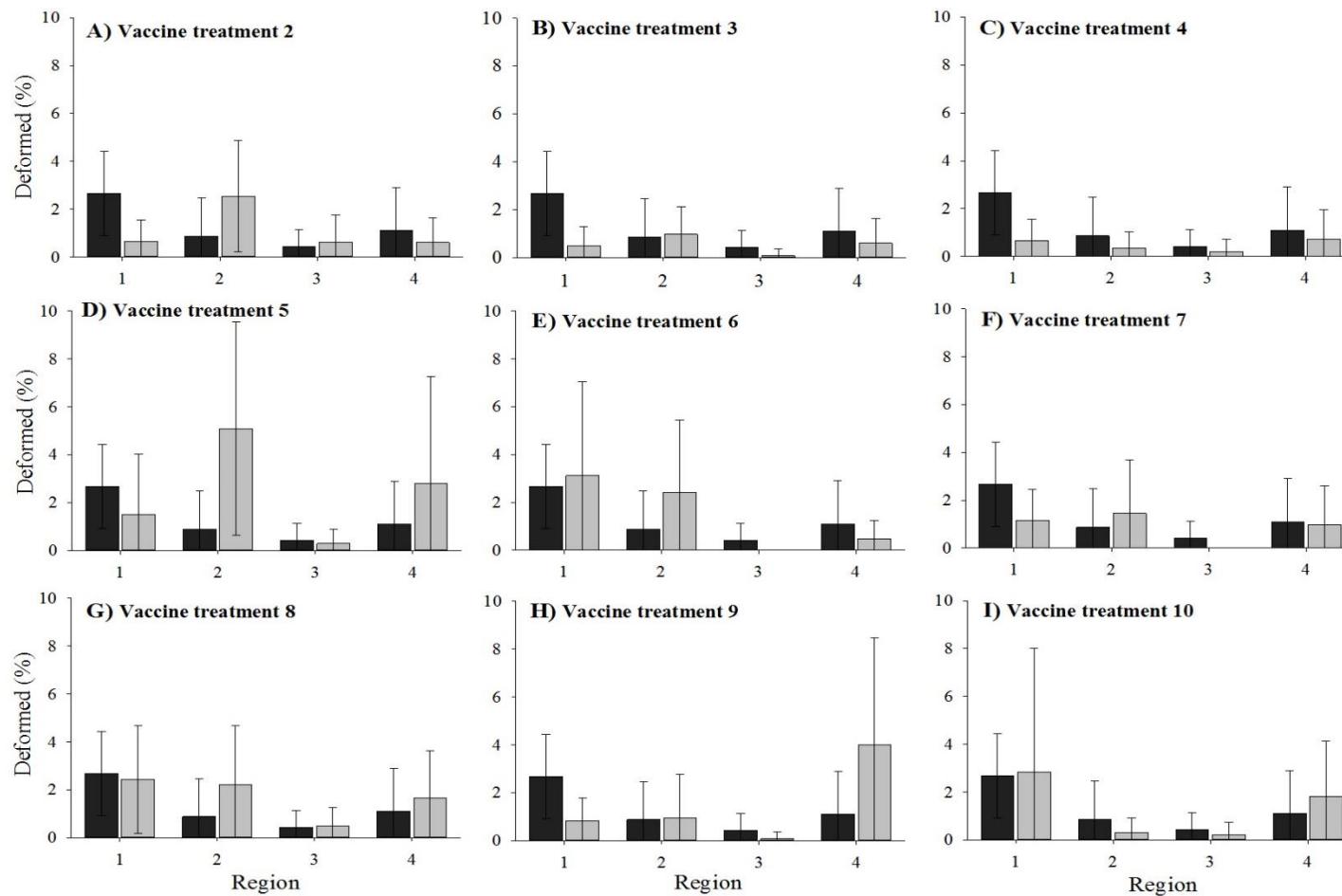


Figure 5.12: Comparison of regional deformed vertebrae prevalence (%) between PBS control (A – I; black) and the other vaccine treatments (grey; A) 2, B) 3, C) 4, D) 5, E) 6, F) 7, G) 8, H) 9, I) 10)) in diploid Atlantic salmon. No significant differences were observed.

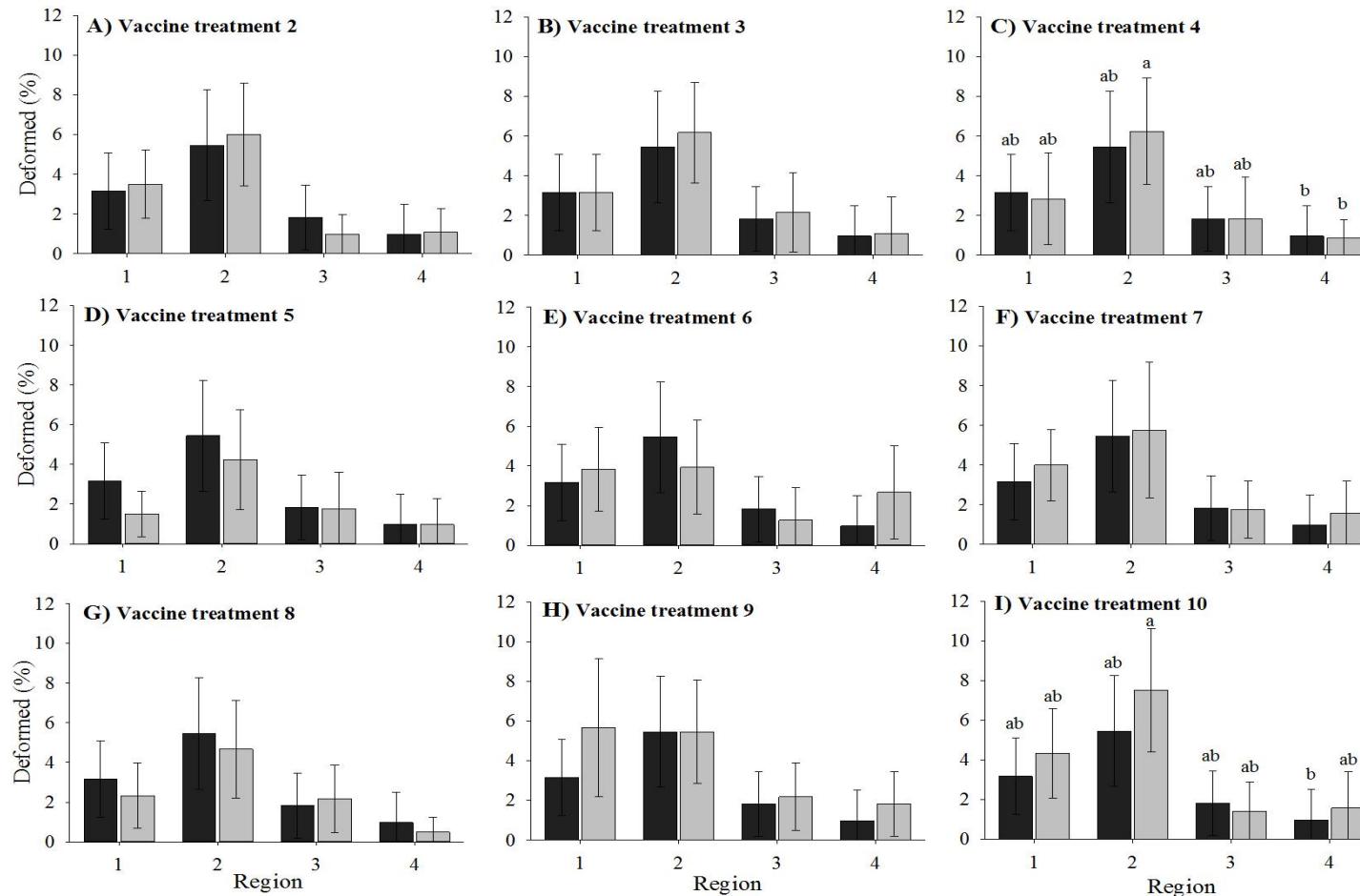


Figure 5.13: Comparison of regional deformed vertebrae prevalence (%) between PBS control (A – I; black) and the other vaccine treatments (grey; A) 2, B) 3, C) 4, D) 5, E) 6, F) 7, G) 8, H) 9, I) 10)) in triploid Atlantic salmon. Significant differences are indicated by different letters

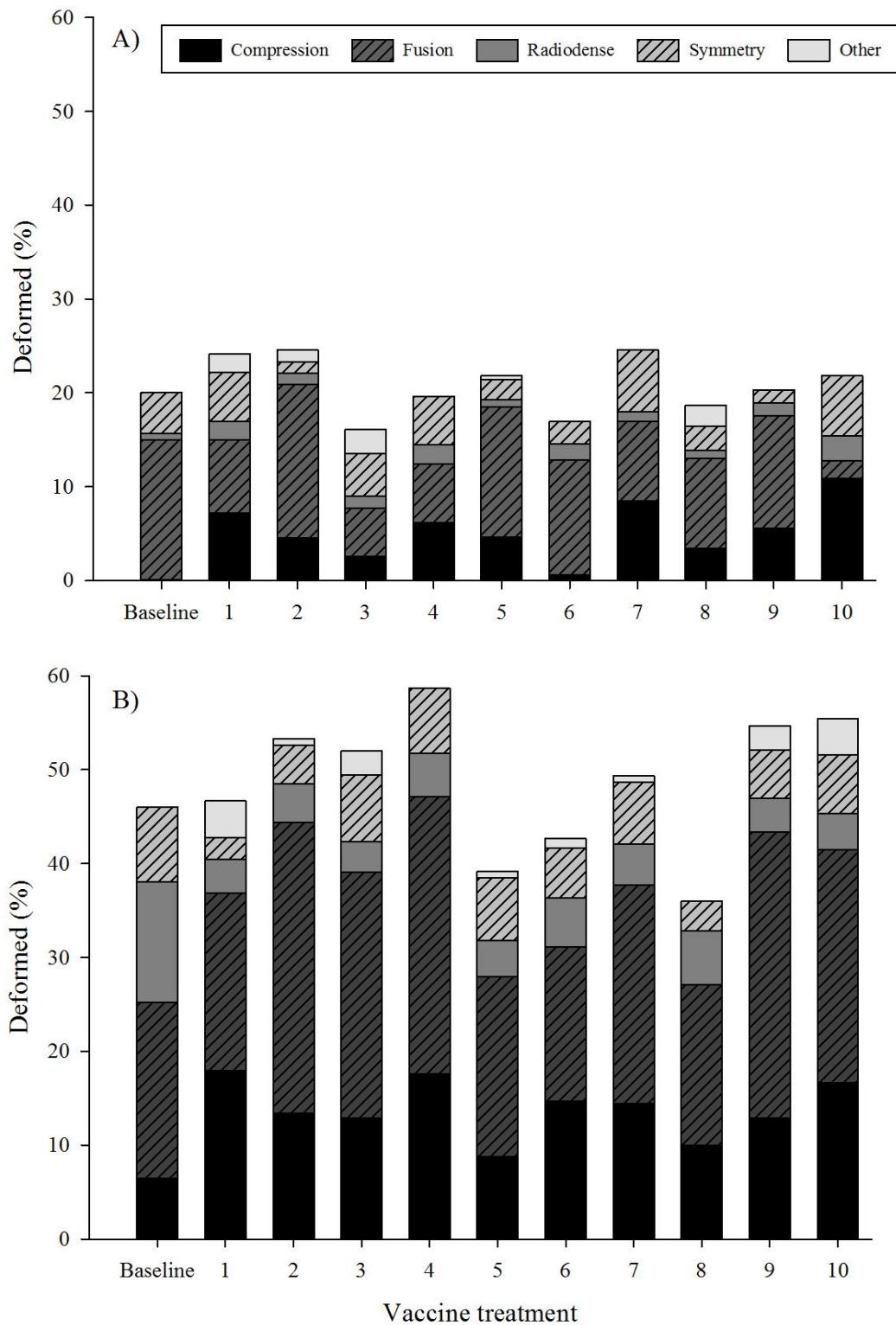


Figure 5.14: Prevalence (%) of different deformity pathology types (compression, fusion, radiodense, symmetry and other) within the deformed population of A) diploid and B) triploid Atlantic salmon at smolt.

5.4. DISCUSSION

It is considered that, with the intensification of the salmon aquaculture industry, the pathogen infection pressure experienced by farmed fish will also increase (Ernst *et al.*, 2002; Bondad-Reantaso *et al.*, 2005). As such, vaccination has become established as an essential tool for disease prevention in farmed fish and is now common practice in commercial salmonid aquaculture (Gudding *et al.*, 1999). The majority of commercial vaccines are multivalent, contain an adjuvant and are administered by injection, but there have been reports of negative side-effects, in particular adhesions (Poppe and Koppang, 2014). Considering the desire to employ triploid Atlantic salmon in full-scale production to reduce potential environmental impacts on wild populations and overcome the issue of pre-harvest maturation, elucidating their response to vaccination is essential in determining their robustness and, therefore, suitability for commercial aquaculture (Weber *et al.*, 2013). This is not only important from the immune response and disease protection point of view, but also with regard to any negative side-effects following vaccination and the associated health and welfare impacts. This Chapter compared the response of diploid and triploid Atlantic salmon to commercially available vaccines (administered singly or in combination) in terms of growth, side-effects and specific antibody (IgM) response to *A. salmonicida*.

Mortality from vaccination to smolt and from smolt to post-smolt was generally low and did not differ significantly between ploidy. This finding concurs with research investigating vaccination in triploids (Fraser *et al.*, 2012a, 2014a) and more recent grow out studies in fresh- and seawater which reported similar survival between ploidy (Fjelldal and Hansen, 2010; Leclercq *et al.*, 2011; Taylor *et al.*, 2012, 2013, 2014; Smedley *et al.*, 2016). It is suggested that the low mortality in this trial and the improved survival in triploid Atlantic salmon previously reported is linked to advances made in triploid nutritional requirements, particularly phosphorus and histidine (Taylor *et al.*, 2015; Smedley *et al.*, 2016; Fjelldal *et al.*, 2016), lower egg incubation temperatures (Fraser *et al.*, 2014d, 2015a) and optimal environmental conditions (Hansen *et al.*, 2015; Sambraus *et al.*, 2017) as well as refined smoltification regimes and smolt transfer timing (Taylor *et al.*, 2012, 2013).

Antibodies are a class of proteins called immunoglobulins which are produced by plasma cells and utilised in the adaptive immune system (Roberts, 2012; Secombes and Wang, 2012). Antibodies act to eliminate invading pathogens by neutralising bacteria and viruses through specific binding and initiating the action of phagocytic cells (Roberts, 2012; Secombes and Wang, 2012). Measurement of IgM levels following vaccination of Atlantic salmon with an *A. salmonicida* vaccine provides an indication of positive immune response

to the vaccine (Midtlyng *et al.*, 1996b). In this study, although vaccines containing a combination of antigens were used, *A. salmonicida* was used as the antigen to measure immune response as it was a common antigen in vaccine treatments 3 to 10, and is known to elicit a positive IgM response that is related with protection from disease (Erdal and Reitan, 1992; Midtlyng *et al.*, 1996a; Romstad *et al.*, 2012). Ploidy did not have a significant effect on antibody response to *A. salmonicida*. This is consistent with the findings presented in **Chapter 2** of this thesis as well as previous studies in ayu and Chinook salmon, which found comparable antibody responses between diploids and triploids (Kusuda *et al.*, 1991; Johnson *et al.*, 2004). Vaccine treatment did not significantly affect the antibody response detected and all the vaccines containing *A. salmonicida* (*i.e.* vaccine treatments 3 to 10) successfully induced positive antibody responses in fish serum when analysed by ELISA, and these results were higher than those from vaccine treatments 1 and 2, which showed a negative response by ELISA. This was to be expected as fish in vaccine treatments 3 to 10 all received an *A. salmonicida* antigen, while fish injected with vaccine treatments 1 and 2 were injected with PBS and Micro 1 PD, respectively. This supports the findings presented in **Chapter 2** as well as previous research, which found that vaccination elicited a higher antibody response than that of a negative or sham-injected controls (Erdal and Reitan, 1992; Johnson *et al.*, 2004; Villumsen *et al.*, 2014). Furthermore, this finding highlights that bivalent and multivalent vaccines are equally efficient in eliciting a positive antibody response and that the addition of more antigens (as in multivalent vaccines) does not negatively affect vaccine efficacy. The antibody response obtained in fish receiving an *A. salmonicida* antigen was perhaps lower than expected. The study by Erdal and Reitan (1992) found that, using a whole cell *A. salmonicida* ELISA and 1/50 serum dilution, fish sampled at 7 weeks post-vaccination (as in the current study) exhibited an average antibody response of 1.16, three times greater than the average antibody response recorded in this study. This could possibly be explained by the timing of sampling ($^{\circ}\text{D}$) and water temperature. For instance, the onset of protection for the vaccines utilised in this study is recognised to occur between 520 and 600 $^{\circ}\text{D}$ post-vaccination so, as sampling occurred at 622 $^{\circ}\text{D}$ post-vaccination, it could be that the response exhibited was still in the early stages of development. In terms of water temperature, Erdal and Reitan (1992) used a constant temperature of 12 $^{\circ}\text{C}$, while water temperature in this trial decreased from 16 $^{\circ}\text{C}$ at vaccination to 9 $^{\circ}\text{C}$ at sampling. So, it could be suggested that this decrease slowed the development of the antibody response and that a greater increase may have been observed had water temperature been constant (Lillehaug *et al.*, 1993; Lillehaug, 2014).

The most common side-effect associated with the vaccination process is the occurrence of intra-abdominal adhesions (Lund *et al.*, 1997; Berg *et al.*, 2006; Fraser *et al.*, 2014a; Poppe and Koppang, 2014). These fibrous strands often connect the ventral body wall and organs and have the potential to cause severe muscle damage and carcass downgrading at harvest (Lillehaug *et al.*, 1992; Poppe and Breck, 1997; Colquhoun *et al.*, 1998; Hastein, 2004; Poppe and Koppang, 2014). With limited assessment of vaccination in triploid Atlantic salmon, it is recognised that the relationship between vaccines, side-effects and triploids is still to be fully explored. In this trial, ploidy did not have a significant impact on the severity of adhesion scores observed. This finding concurs with results for S1+ smolts, as presented in **Chapter 2** and from a previous triploid vaccination study (Fraser *et al.*, 2013) but is in contrast to a previous study assessing vaccination in S0+ smolts, which found significantly higher adhesion scores in triploids (Fraser *et al.*, 2014a). However, a number of factors may be proposed to explain this difference. Firstly, sampling events in the Fraser *et al.* (2014a) study were performed from 800 °D post-vaccination onwards and therefore, if the sampling event in the current study (622 °D) had been performed later, the same significant difference may have been observed. Additionally, it could be suggested that the use of a 0.1 ml dose multivalent vaccine (MINOVA 6 Vet, Norvax®, Intervet International B.V., Boxmeer, Netherlands) in the Fraser *et al.* (2014a) study may have caused worse adhesions in triploids as studies have found increased adhesion scores with multiple antigens (Midtlyng and Lillehaug, 1998). However, considering that mono-, bi- and multivalent vaccines at varying doses were utilised in the current study, with no difference between ploidy, this suggestion seems unlikely. Although considering this, post-vaccination temperatures in the Fraser *et al.* (2014a) study increased from 4.5 to 16 °C and were then maintained at 16 °C for approximately 42 days, while decreasing ambient temperature was experienced in the current trial. With the knowledge that more severe adhesions occur at higher temperatures and with increasing antigen number in Atlantic salmon (Midtlyng and Lillehaug, 1998; Berg *et al.*, 2006; Grini *et al.*, 2011), along with the knowledge that triploids have a lower thermal optimum than diploids (Atkins and Benfey, 2008; Hansen *et al.*, 2015; Sambraus *et al.*, 2017), it could be suggested that the combination of increased temperature and multivalent vaccine in Fraser *et al.* (2014a) culminated in more severe adhesion scores in triploids. Taking into account these suggestions, it is recommended that further study is required to assess the effect of temperature and vaccine type on the occurrence of adhesion scores in triploids, and elucidate any relationship that may be present between triploids, adhesion score severity and

suboptimal temperatures. Time post-vaccination is also important and it should be noted that the fish utilised in this trial will be taken through to harvest size when side-effects will be assessed further.

The vaccine treatments utilised in this study were based on four individual vaccines (PHARMAQ, Zoetis LLC, New Jersey, USA) and were combined in such a way to produce an increasing scale of anticipated adhesion severity according to vaccine treatment number, with 1 representing the least severe and 10 representing the most severe. Vaccine treatment had a significant effect on adhesion score, with an increasing pattern of adhesion score according to vaccine treatment number, as anticipated. The highest adhesion scores for both ploidy were found in the “most severe” vaccine treatment (10), with the lowest scores occurring in the PBS-injected control groups (vaccine treatment 1). This demonstrates the negative effect that can be caused by improper vaccine administration (e.g. double injection) and highlights the necessity for optimal vaccination strategies and practices (Lillehaug, 2014). In both ploidy, the adhesion scores were higher in the “more severe” vaccine treatments (7, 8, 10) than in the “least severe” treatments (1, 2, 3). This concurs with a previous study which found that adhesion score severity increased with increasing antigen number (Midtlyng and Lillehaug, 1998). Furthermore, there did not appear to be a significant effect of vaccine volume on adhesion scores, for example, vaccine treatments 4 (Micro 6, 0.05 ml), 7 (ALPHA JECT 6-2, 0.1 ml) and 10 (ALPHA JECT 6-2 double dose, 0.2 ml) contained the same components at different volumes and exhibited statistically similar adhesion scores. It could, again, be suggested that if temperature had been higher post-vaccination, significant differences between these vaccine treatments could have occurred. This highlights that, despite efforts to lessen adhesions through reduced vaccine volume, they can still occur and indicates the need for further studies to fully elucidate the impact of vaccine volume and temperature on adhesion occurrence. It should be noted, however, that while adhesion scores did show the expected increasing pattern, the actual adhesion scores were considerably lower than the estimated scale. This may have been due to the decreasing water temperature or sampling time post-vaccination, as mentioned previously. There were also exceptions to the expected patterns in vaccine treatments 6 (ALPHA JECT 2-2 + Micro 1 PD) and 9 (Micro 6 + Micro 1 PD double dose). As the adhesion scores of diploids and triploids in vaccine treatment 6 did not differ significantly from vaccine treatment 3 (ALPHA JECT 2-2) and those in vaccine treatment 9 did not differ from vaccine treatments 4 (Micro 6) and 5 (Micro 6 + Micro 1 PD), it is suggested that the addition of Micro 1 PD (even in a double dose) did not impact adhesion scores as negatively

as anticipated. The results at fish harvest size (outwith the time-frame of this thesis) will be important to determine if this holds true.

The growth performance of triploids continues to be debated (Tiwary *et al.*, 2004; Taylor *et al.*, 2012, 2013; Fraser *et al.*, 2012b), with triploid growth following vaccination relatively uncharacterised. In this study, triploids vaccinated with all 10 vaccine treatments were heavier and longer than the respective diploids at both the smolt and post-smolt stages. This concurs with the findings of Fraser *et al.* (2012a) which found that, post-vaccination, triploid Atlantic salmon were heavier and longer than their diploid counterparts. In addition, it agrees with triploid performance studies which found that triploids were significantly heavier and longer than diploids in both fresh- and seawater (Taylor *et al.*, 2007, 2012, 2013; Frenzl *et al.*, 2014; Smedley *et al.*, 2016; Sambraus *et al.*, 2017). Condition factor was similar between diploids and triploids at smolt, which supports previous research (Taylor *et al.*, 2007, 2011), while at post-smolt, triploids had significantly greater K than diploids, contrasting with previous studies reporting lower K in triploids (Taylor *et al.*, 2013, 2014; Fjelldal *et al.*, 2016; Sambraus *et al.*, 2017). Due to the sizeable weight difference between ploidy at post-smolt (triploids + 32.0 %), the increased K observed in triploids may simply be explained by their larger body weight at the time. Vaccine treatment also had an effect on weight and length in diploid and triploid Atlantic salmon. Decreasing patterns of weight (and reflected in reduced TGC) and length were observed in both ploidy according to increasing vaccine treatment severity, significantly so in the triploids. Fish in the “more severe” vaccine treatments at smolt and post-smolt (7, 8 and 10) had a lower body weight and shorter length than the respective control fish (vaccine treatment 1, PBS-injected), with fish in vaccine treatment 10 (ALPHA JECT 6-2, double dose) consistently lighter and shorter than all other treatments. This is supported by previous vaccination studies which found reduced growth in vaccinated fish compared to unvaccinated controls (Lillehaug *et al.*, 1992; Midtlyng and Lillehaug, 1998; Berg *et al.*, 2006; Fraser *et al.*, 2012a, 2014a, 2015b). Combined with adhesion scores, the results that fish in vaccine treatment 10 were the lightest and shortest at both smolt and post-smolt highlight the negative effects that incorrect vaccine administration can have for production.

In terms of growth rate, the TGC of triploids was lower than diploids in freshwater post-vaccination but higher at post-smolt following sea transfer. Lower TGC in triploids at smolt could be indicative of faster growth rate in diploids between vaccination and smolt sampling which would agree with previous studies showing poorer growth rate in triploids during freshwater (Cotter *et al.*, 2002; Shrimpton *et al.*, 2007; Chiasson *et al.*, 2009) but

contrasts with studies showing improved freshwater growth (Oppedal *et al.*, 2003; Leclercq *et al.*, 2011; Taylor *et al.*, 2013). However, with the knowledge that triploids were heavier than diploids at vaccination, which is suggestive of initial faster growth rate from first feeding, and that high temperatures (14 – 16 °C) were experienced in the period just before vaccination as well as post-vaccination, it could be suggested that the increased temperatures impeded TGC in triploids for the period between vaccination and smolt sampling. This is supported by a previous study which found that, at increased temperatures (> 15 °C), physiological and biological parameters *e.g.* feed intake, in triploids were negatively affected, and subsequent growth reduced (Sambraus *et al.*, 2017). At post-smolt assessment, TGC was significantly higher in the triploids than in diploids for all vaccine treatments. This indicates significantly faster growth in the post-smolt stage and is in agreement with previous studies reporting improved growth in triploids during the early seawater stage (O’Flynn *et al.*, 1997; Leclercq *et al.*, 2011; Taylor *et al.*, 2015; Smedley *et al.*, 2016). However, it should be noted that the ability of triploids to maintain a growth advantage throughout seawater is still debated, with many studies showing that improved triploid growth in the early seawater stage is lost later (Cotter *et al.*, 2002; Leclercq *et al.*, 2011; Fraser *et al.*, 2013; Taylor *et al.*, 2013; Tibbetts *et al.*, 2013) and only a few studies showing that triploid growth can be sustained at a higher rate through to harvest (Oppedal *et al.*, 2003; Smedley *et al.*, 2016). With the fish in the current study being monitored through to harvest, it will be interesting to see whether or not triploids continue to maintain their weight advantage. In terms of vaccine effect, the TGC of vaccine treatment 10 was consistently significantly lower than the PBS control group (vaccine treatment 1) in both diploids and triploids at smolt and post-smolt, while differences between the other treatments were generally non-significant. When analysed by regression, TGC was found to be significantly correlated to adhesion score severity in both ploidy at smolt, with increasing adhesion score having a negative effect of TGC. This could have consequences at harvest as fish may need to be downgraded due to inadequate size, which would result in lost revenue. With the knowledge that the highest adhesion score occurred in vaccine treatment 10 (“accidental double dose scenario”), it further highlights the negative impacts of incorrect vaccine administration (double injection). As parallelism statistics (ANCOVA) also revealed no significant difference in slope gradient ($p > 0.05$) between ploidy, this indicates a similar response to vaccine in both ploidy, albeit that the lower TGC observed in triploids relative to diploids could again be linked to a negative effect of high temperatures on triploid growth.

Vertebral deformities are a common problem in salmonid aquaculture and represent both economic and welfare issues for the industry (Gil Martens *et al.*, 2012). In this study, total radiological vertebral deformity (%) was significantly affected by ploidy, with triploids consistently exhibiting a higher level of deformity than diploids. This is consistent with previous studies using S0+ Atlantic salmon smolts which found a higher prevalence of deformed fish in triploids compared to their diploid counterparts (Taylor *et al.*, 2014; Smedley *et al.*, 2016; Fjelldal *et al.*, 2016). However, it contrasts to the study by Fraser *et al.* (2014) which observed that, in S0+ smolts, triploids had equal or lower deformity than diploids. The authors hypothesise that the elevated temperatures utilised in the production of S0+ smolts (16 °C for 42 days) impeded growth rates in triploids compared to diploids and as such, alleviated the risk of deformity development. This is further supported by a study in diploid Atlantic salmon which proposed a possible relationship between growth rate and prevalence of skeletal malformation (Hansen *et al.*, 2010). Considering the earlier suggestion that triploid growth in the current trial was negatively affected by initially high temperatures (14 – 16 °C for 21 days), it could be suggested that, had temperatures been elevated for a longer period of time, the same pattern could have been exhibited. To fully elucidate this, it is recommended that research be undertaken to assess any combinatory effect that high temperatures and smolt regime may have on the development of vertebral deformities in triploid Atlantic salmon. Vaccine treatment did not significantly affect radiological deformity (%) in both ploidy and this concurs with the study by Fraser *et al.* (2014a) which found that unvaccinated and vaccinated fish in both diploid and triploid Atlantic salmon exhibited a similar level of deformity. Moreover, in further agreement with Fraser *et al.* (2014a), the finding would appear to refute the suggestion that the development of vertebral deformities can be aggravated through the vaccination process (Gil Martens *et al.*, 2012). Within deformed fish, the average number of dV per deformed individual was significantly higher in triploids than in diploids. This is in contrast to the study by Fraser *et al.* (2014a) which found that, in both S0+ smolts, there was no significant difference between ploidy in terms of the number of deformed vertebrae per deformed fish. This may again be linked to temperature differences between this study and Fraser *et al.* (2014a) and the suggestion that elevated temperatures can hinder growth rates in triploids and reduce the risk of deformity development, but further studies would be necessary to clarify this. Vaccine treatment did not have a significant effect on the average number of dV per deformed individual. This supports Fraser *et al.* (2014a) and, again, refutes the suggestion

that the development of vertebral deformities can be aggravated by vaccination (Gil Martens *et al.*, 2012).

In both ploidy and all vaccine treatments, the greatest proportion of fish had 1 - 5 dV, with smaller proportions having 6 to 9 or > 10 dV. This finding is in agreement with a previous comparison of deformities in diploid and triploid Atlantic salmon (Taylor *et al.*, 2013). In terms of localisation, dV were found in the four regions of the vertebral column (R1 (cranial trunk, V1 - V8), R2 (caudal trunk, V9 - V30), R3 (tail, V31 - V49) and R4 (tail fin, V50 - end)) in both ploidy. Within R1, R2 and R3, triploids were found to have significantly higher levels of deformed vertebrae than diploids and this supports previous research which observed greater levels of deformities in triploids (Taylor *et al.*, 2014; Smedley *et al.*, 2016; Fjelldal *et al.*, 2016). In both ploidy, the region with the highest prevalence of deformed vertebrae was R2 which concurs with previous studies indicating that this vertebral region experiences the highest levels of deformity during freshwater (Sullivan *et al.*, 2007; Fjelldal *et al.*, 2009a, 2016; Fjelldal and Hansen, 2010). However, it should be noted that triploids also exhibited increased prevalence of dV in the tail region (R3), which is unusual as deformities in R3 are known to occur more frequently following seawater transfer (Gil Martens *et al.*, 2010; Fraser *et al.*, 2014a; Smedley *et al.*, 2016; Fjelldal *et al.*, 2016). While it could be suggested that the occurrence of deformities in this region may be linked to problems with temperature during egg incubation or with the diet, this would require further clarification. Considering that the fish in this study are being taken through to harvest, it will be interesting to see whether the R3 deformities are aggravated in seawater, as would be expected, or whether R2 deformities will continue to prevail. When vaccine treatments were compared against the PBS-control (vaccine treatment 1) in both ploidy, vaccine treatment did not have a significant effect on the deformity prevalence in each vertebral region. This is in agreement with Fraser *et al.* (2014a) who found similar levels of deformity between unvaccinated and vaccination diploid and triploid Atlantic salmon.

The types of dV pathology within the deformed population were also assessed as part of this study. The types of pathologies observed did not differ greatly between vaccine treatments in both ploidy with the most prevalent type of deformity pathology being fusions followed by compressions. This finding is supported by the knowledge that these types of pathologies regularly develop in the freshwater phase (Sullivan *et al.*, 2007) and concurs well with previous research assessing deformities in fresh- and seawater (Sullivan *et al.*, 2007; Taylor *et al.*, 2013; Fraser *et al.*, 2014a, 2015a; Fjelldal *et al.*, 2016). Within fusions,

the most common pathology type was complete fusion (type 7), with the most prevalent compression pathology as one-sided compression (type 5). It has been shown that one-sided compressions (type 5) act as a precursor for the development of complete fusions (Witten *et al.*, 2009; Leclercq *et al.*, 2011). The initial fusion of 2 to 3 vertebrae as part of complete fusion (type 7) pathology may lead to the continuous fusion of further vertebrae culminating in the creation of a fusion centre (type 8) which can shorten the spine and result in compromised welfare and performance of the fish (Witten *et al.*, 2006). However, the fusion of 2 to 3 vertebrae may also result in the remodelling of bones into one relatively normally shaped vertebrae. As such, this “new” reshaped vertebra is considered to “contain” the deformity and not cause further spinal deformities (Witten *et al.*, 2006). As the fish in this study are being taken through to harvest, it will be interesting to see how the deformity pathologies will progress whilst in seawater and whether compression and fusion type pathologies will prevail as the most common pathologies. Collectively, results from the current study would suggest that ploidy in conjunction with other factors, rather than vaccine, is predisposing triploids to increased risk of spinal malformation.

5.5. CONCLUSION

The results presented in this Chapter suggest that triploids respond equally as well as diploids to vaccination. Similar specific antibody responses to *A. salmonicida* in both ploidy following vaccination concurs with findings from **Chapter 2** and suggests that all the vaccines assessed would be as effective in protecting triploids as diploids following disease challenge. Furthermore, ploidy did not affect adhesion score within each vaccine treatment indicating that side-effects following vaccination appeared to be no worse in triploids than diploids, although samples taken at harvest are required to confirm this. Vaccine treatment affected adhesion score with the “most severe” treatment eliciting the highest adhesion scores, thus highlighting the need for efficacious vaccination strategies. Triploid growth rate (TGC) appeared to be significantly affected by high temperatures in freshwater, but improved and accelerated post-seawater transfer. This supports better triploid performance and supports their application in aquaculture. As in previous studies, vertebral deformities remained higher in triploids than in diploids indicating the requirement for further studies to fully clarify triploid nutritional requirements and alleviate this issue. Finally, the findings support the use of commercial vaccines in triploid Atlantic salmon, and continue to support the suitability of triploids in commercial-scale salmon production.

Chapter 6

General Discussion

The overall aim of this thesis was to expand the knowledge and understanding of health and robustness of triploid Atlantic salmon relative to diploids, through investigations of disease susceptibility and immune and stress responses to routine commercial fish farm practices. To this end, four key aspects of health and immunity were investigated, focussing on the response of triploid Atlantic salmon to: 1) vaccination against, and challenge with a commercially significant bacterial disease (furunculosis), 2) challenge with a commercially significant parasitic disease, 3) treatment with a widely-used chemotherapeutic and 4) a range of commercially available vaccines. The research conducted was both fundamental, presenting basic information regarding triploid health and immunity, and applied, providing data which may directly influence the decision of the commercial aquaculture industry to implement triploid Atlantic salmon in production. This general discussion will consider the main findings and the wider implications from the research work, discussing the limitations of the experimental studies performed as well as areas for future research.

6.1. VACCINATION AND CHALLENGE WITH *AEROMONAS SALMONICIDA*

As *Aeromonas salmonicida* represents one of the most economically significant pathogens for Atlantic salmon aquaculture, effective vaccines have been developed for diploid Atlantic salmon, with studies showing good protective responses (Lillehaug *et al.*, 1990, 1992; Erdal and Reitan, 1992; Adams *et al.*, 1995; Midtlyng *et al.*, 1996a). Considering this, an assessment of the response of triploid Atlantic salmon to vaccination (ALPHA JECT 2-2, 0.1 ml dose) against, and challenge with *A. salmonicida* was the first key aspect of this thesis (**Chapter 2**).

Triploids were not negatively affected by vaccination in terms of known side-effects, with weight and adhesion scores not significantly affected by ploidy. This is supported by previous studies (Fraser *et al.*, 2012a, 2013, 2014a, 2015b) and suggests that triploid Atlantic salmon experience the same degree of side-effects as diploids. Certain parameters of the immune response, however, were significantly affected by ploidy, particularly immune-related cells. Red and white blood cell counts were significantly lower in triploids than in diploids, which concurs with the existing knowledge that the third set of chromosomes possessed by triploids leads to increased cell size and reduced cell number (Small and Benfey, 1987; Cogswell *et al.*, 2002; Peruzzi *et al.*, 2005; Taylor *et al.*, 2007). Furthermore, triploid macrophages were found to exhibit significantly greater respiratory burst than diploids as well as the ability to phagocytise a larger number of yeast cells. This

is suggestive of the ability of triploids to compensate for reduced cell number through increased cellular activity (Budiño *et al.*, 2006; Taylor *et al.*, 2007). However, it is recognised that very few studies have been undertaken to fully understand the effects of triploidy on cellular processes. For instance, increase in cell volume is known to cause a decrease in surface area to volume ratio and it has been previously suggested that this could affect nutrient exchange and osmoregulation, which are limited by surface area (Benfey, 2001; Hyndman *et al.*, 2003a). Furthermore, the altered cell volume and shape, such as in red blood cells, has been suggested to increase internal transport distances and as a result, may impact negatively on signal transduction pathways (Benfey, 2001; Goo *et al.*, 2015). As such, studies are required to determine the full effects of triploidy on cellular activities, *e.g.* respiratory burst in macrophage cells, and the impact it may have on wider bodily functions. Furthermore, while triploids are known to have fewer but larger cells, it should be stated that no general histological assessment of all cell types in triploid Atlantic salmon has been performed and, as such, this type of investigation should be undertaken. Similar levels of specific antibody (IgM) response against *A. salmonicida* were detected in both ploidy and vaccination elicited equal protection in diploid and triploid Atlantic salmon following vaccination. These findings are supported by previous studies which demonstrated similar antibody responses in diploid and triploid ayu and Chinook salmon following vaccination against *V. anguillarum* and *L. anguillarum*, respectively (Kusuda *et al.*, 1991; Johnson *et al.*, 2004). The results from **Chapter 2** would suggest that the commercial furunculosis vaccine is equally effective in protecting diploids and triploids against infection by *A. salmonicida* and that the vaccine would be suitable for use in triploid Atlantic salmon, should they be applied commercially.

6.2. AMOEBOIC GILL DISEASE CHALLENGE

Building on the findings from **Chapter 2**, and the knowledge that *N. perurans*, the causative agent of AGD, has been causing major problems during the seawater phase of Atlantic salmon production for many years all over the world (Young *et al.*, 2008a; Crosbie *et al.*, 2012; Shinn *et al.*, 2015; Oldham *et al.*, 2016), it was considered important to next assess the response of triploids to this commercially significant parasite (**Chapter 3**).

Mortalities following AGD challenge remained low in both ploidy throughout the trial and did not differ significantly between ploidy. This concurs with a study experiencing a natural AGD outbreak (Smedley *et al.*, 2016) but is in contrast to an experimental infection study with *N. perurans* performed in Tasmania (Powell *et al.*, 2008). Amoebic Gill Disease

outbreaks are known to occur at temperatures ranging from 10.5 to 20 °C and it has been suggested that mortalities increase dramatically when temperatures are above 16 °C while they are prevented below 13 °C (Douglas-Helders *et al.*, 2001). As the study by Smedley *et al.* (2016) occurred at similar temperatures to this trial (11 - 13 °C) and with temperature not detailed by Powell *et al.* (2008) (although it may be assumed that temperatures were higher considering the experiment was undertaken in Tasmania), it could be suggested that if temperatures had been higher during the current trial, increased mortalities would have been observed in both ploidy, with potentially higher levels in the triploids due to their known lower thermal optimum (< 15 °C) (Sambrerus *et al.*, 2017). As such, it is recommended that studies be carried out in future to assess the effects of varying temperatures on the occurrence and progression of mortalities in Atlantic salmon, particularly in triploids, in response to experimental challenge with AGD. Furthermore, Tasmanian triploid Atlantic salmon have been shown to suffer from increased incidences of lower jaw deformity (LJD) and gill filament deformity syndrome (GFDS), a condition in which primary gill filaments are absent from some or all of the branchial arches (Sadler *et al.*, 2001). It is known that LJD negatively impacts on the ability of fish to pump water across the gills and can affect oxygen uptake (Lijalad and Powell, 2009). In addition, GFDS is recognised to reduce further, the already decreased gill surface area in triploids, which has negative implications for ionic regulation and respiratory processes such as gas exchange, particularly during suboptimal conditions (Sadler *et al.*, 2001; Maxime, 2008). As Powell *et al.* (2008) performed their study in Tasmania, it could be suggested that the fish utilised were potentially compromised by LJD or GFDS and that this may also have contributed to the mortalities observed in their study.

While ploidy did not have a significant effect on overall gross gill score, ploidy differences were observed in terms of percentage of affected gill filaments, with triploids showing less affected filaments. This, again, contrasts with Powell *et al.* (2008) who observed more affected filaments in diploid Atlantic salmon. While it could be suggested that, due to the mortalities observed in their study, the most severely affected triploids had died before the end of the trial, this cannot be confirmed as no assessment of lesion severity in the mortalities was performed by Powell *et al.* (2008). Unfortunately, due to limitations with facilities and technical staff, it was not possible to gill score mortalities as part of this trial and, as such, it is recommended that gill scores from mortalities should be assessed in all future work, both macroscopically and by qPCR, to determine if increased gill score and parasite load is a direct cause of death.

While mucous cells were found to increase in number and size in response to experimental AGD infection, ploidy did not significantly affect number or size. This finding is surprising considering the knowledge that triploids have reduced cell numbers and larger cell volume (Small and Benfey, 1987; Stillwell and Benfey, 1996; Budiño *et al.*, 2006). This further highlights the requirement for an overview study to be performed regarding cell structures in triploid Atlantic salmon. The findings presented in **Chapter 3** suggest triploids can cope as well as diploids to infection with AGD but highlights that further data needs to be gathered to fully determine the response of triploids to AGD. In particular, studies should investigate AGD infections in triploids using conditions that would be experienced by the aquaculture industry but which may be suboptimal for triploid Atlantic salmon. These conditions could include extreme temperature fluctuations, variations in oxygen and, with the knowledge that parasitic disease may facilitate or be facilitated by the occurrence of bacterial disease (Embar-Gopinath *et al.*, 2005), combined disease challenges. Furthermore, defining disease challenge robustness of triploids to any pathogen in relation to suboptimal environments will be vital to help identify fish farm sites which are suitable or not for rearing triploid fish in the future.

6.3. HYDROGEN PEROXIDE CHALLENGE

Following on from the results obtained in **Chapter 3**, alongside the knowledge that H₂O₂ has been used as a chemotherapeutic treatment for parasitic diseases such as sea lice and, more recently AGD, the next step in this thesis was to gain an understanding of the response of triploid Atlantic salmon to this chemical treatment (**Chapter 4**).

Mortalities occurred in both diploid and triploid Atlantic salmon following H₂O₂ treatment. This result was surprising considering that no mortalities occurred while undertaking a dose-response test at the same concentration (1900 ppm) or in previously published studies (Bowers *et al.*, 2002; Vera and Migaud, 2016). Current knowledge indicates a clear link between water temperature and the toxic effects of H₂O₂, with studies showing that the toxicity of H₂O₂ increases rapidly as temperatures exceed 13.5 °C (Johnson *et al.*, 1993; Thomassen, 1993; Kiemer and Black, 1997). As such, with temperatures in this trial at 14 °C, the mortalities exhibited may be related to this known link. While it would have been preferable to perform this trial at a lower temperature, it was necessary to perform the trial under the described conditions as the experimental facility did not have a water temperature control system and the trial could not be delayed due to potential welfare issues with increased tank stocking density. Considering this, further study is required to fully

understand the effects of water temperature and H₂O₂ concentration on the efficacy and toxicity of H₂O₂ in both diploid and triploid Atlantic salmon. Furthermore, as mortalities in this trial were not significantly different between diploids and triploids, it would be interesting to assess whether suboptimal conditions *e.g.* temperature above 15 °C, or reduced O₂ /increased O₂ demand during crowding as could be experienced in a “normal” aquaculture setting, would impact on the ability of triploids to tolerate H₂O₂ exposure.

Ploidy did not have a significant effect on the primary and secondary stress responses assessed in this study (cortisol, glucose and lactate). This would suggest that the physiological response of triploids to cope with the stress associated with H₂O₂ treatment is comparable to diploids. However, it would be interesting to determine if triploid Atlantic salmon could cope as well as diploids to H₂O₂ treatment if addition stressors such as increased temperature were also involved. Many previous studies have indicated that triploids respond similarly to diploid in response to acute stress (Sadler *et al.*, 2000b; Wagner *et al.*, 2006; Taylor *et al.*, 2007). For example, Hyndman *et al.* (2003a) found that diploid and triploid brook trout showed similar storage of energy metabolites, utilization of energy stores and recovery patterns following exhaustive exercise at 9 °C. This was in contrast to their hypothesis that the larger cells and reduced cell surface area to volume ratios exhibited by triploids would negatively impact metabolic recovery post-exercise. However, with triploids appearing more sensitive to chronic stress *e.g.* elevated temperatures, low O₂ (Hansen *et al.*, 2015; Sambraus *et al.*, 2017), studies have been undertaken to assess the effects of combined stressors on the stress response of triploids. Hyndman *et al.* (2003b) assessed the response of triploid brook trout to exhaustive exercise at a constant high temperature (19 °C). In terms of the plasma parameters, glucose and lactate both pre- and post-exercise did not differ significantly between ploidy and that concurs well with the findings presented in this thesis. However, this was not the case for muscle parameters: Pre-stress, diploids and triploids exhibited similar levels of PCr, ATP, glycogen and lactate in the muscle but exhaustive exercise was found to induce varying patterns of metabolite utilisation, accumulation and recovery. Muscle PCr decreased significantly in diploids post-exercise, as expected, but did not decrease in triploids, and muscle ATP recovered more slowly in triploids than diploids. These findings could be suggestive of the inability of triploids to utilise anaerobic pathways at high temperatures. Furthermore, diploids accumulated more muscle lactate than triploids but showed significant signs of recovery by 2 h post-exercise while triploids did not. It could be suggested that the potentially increased internal transport distances in cells, as a result of

altered cell volume and shape, is impacting negatively on the ability of triploids to clear lactate and other waste metabolites from cells, thus, influencing their ability to recover from stress. This is supported by a recent study by Preston *et al.* (2017) which found that, following exhaustive exercise at 10 and 19 °C, triploid brown trout accumulated more lactate than their diploid counterparts, and took longer to recover. As such, it further highlights the negative impact of elevated temperature on triploid responses, as well as the need for studies assessing additive effects of stressors on triploid health and immunity. In addition, it supports the requirement for studies to assess and understand the effects of triploidy, and suboptimal conditions, on cellular functions and processes. Moreover, defining triploid robustness to handling and treatment under environmental extremes would, again, help identify suitable sites for triploid salmon farming, and develop triploid-specific handling protocols for treatment (*e.g.* increased O₂) in such environments to help minimise losses.

While the expression of oxidative stress genes in Atlantic salmon is known to be affected by H₂O₂ exposure (Vera and Migaud, 2016), there is an overall lack of information regarding gene expression in triploids (Shrimpton *et al.*, 2007; Ching *et al.*, 2010). Differences in gene expression were exhibited between this study and previously published data. In terms of diploids, Vera and Migaud (2016) found that the expression of oxidative stress genes such as *cat* and *hsp70* was elevated in H₂O₂-exposed fish compared to the controls, while that was not the case in this study. Considering study differences, a number of factors can be proposed as potential explanations for this including time of H₂O₂ exposure, time of sampling post-exposure and temperature, and so, further studies are required in Atlantic salmon to elucidate these differences. While not compared directly, similarities and differences were observed in gene expression between ploidy, with higher, similar or lower levels of expression exhibited in triploids compared to diploids. For the expression of stress genes in the liver, the diploid H₂O₂-exposed group tended to show higher gene expression than the control group at 1 h.p.e, while the triploid H₂O₂-exposed group showed higher expression than controls at 3 h.p.e. This could be suggestive of delayed gene expression responses in triploids, as suggested for other biological responses. For instance, Langston *et al.* (2001) showed that the hypoferraemic response in triploids was slower than in diploids which could impact negatively on disease susceptibility and so, it may be suggested that a similar effect was occurring here which may impact on the ability of triploids to cope with stress. Stress and immune genes in the gill were variable in both diploids and triploids, with control fish tending to show higher expression of most of the

target genes. This could suggest that elevated water temperature and potentially increased H₂O₂ toxicity were negatively impacting on gene expression in the gills but this would require further study. However, this is with the exception of il1 β , which was significantly higher in H₂O₂-exposed groups than controls in both ploidy. This supports studies in carp and Maraena whitefish (*Coregonus maraena* L.) which found upregulations in il1 β in response to acute stress, suggesting that this gene plays a significant role in the acute stress response (Metz *et al.*, 2006; Korytář *et al.*, 2016). However, with no other studies having assessed the effects of H₂O₂ exposure on gene expression in triploids, it is difficult to determine whether the patterns observed were to be expected and, therefore, these suggestions require further elucidation. In recent years, a few studies have been undertaken to assess the effects of triploidy in different fish species, which have proposed that gene dosage effects and/or dosage compensation are occurring in the expression of triploid genes. This could either mean that gene expression is correlated directly with the gene copy number, with triploids exhibiting higher levels of expression than diploids, or that the third gene copy will be silenced and expression levels will mirror that of diploids (Shrimpton *et al.*, 2007; Maxime, 2008; Harvey *et al.*, 2017). In addition, a more recent study of triploid Atlantic salmon by Harvey *et al.* (2017) revealed that a dosage effect was occurring on the growth performance of triploids and this dosage effect was significantly related to the second maternal chromosome set. As such, they found that the growth exhibited by triploids appeared to mimic that of the maternal-parent, and suggest that this may explain differences in triploid performance during family studies (Friars *et al.*, 2001; Johnson *et al.*, 2004; Shrimpton *et al.*, 2007; Weber *et al.*, 2013; Taylor *et al.*, 2014). The authors highlight the need for aquaculture breeding programmes to focus on maternal performance as a method to potentially achieve the full growth potential of triploids. However, with the full mechanisms underlying this dosage effect and impact of the second maternal chromosome set requiring full elucidation, much work is still required to determine ploidy effects on gene expression at a molecular level.

Further to the earlier suggestions for additional studies, the fish were not infected with any pathogen throughout the present trial and, considering that treatments would not be applied in commercial aquaculture without first indication of disease occurrence, it is recommended that studies combining treatments and pathogen infections be performed. These could be further combined with temperature-interactions to determine if triploids could cope as well as diploids to the effects of these combined parameters. Overall, this Chapter provides the first experimental evidence of the effects of H₂O₂ treatment on triploid

Atlantic salmon, and supports the ability of triploids to cope with chemical treatments and continues to strengthen the proposal for the application of triploid Atlantic salmon into commercial aquaculture production.

6.4. RESPONSE TO COMMERCIALLY AVAILABLE VACCINES

Building further on the findings from **Chapters 2** and **4** that triploids respond equally as well as diploids to both vaccine and chemotherapeuticants, and considering that the response of triploids to vaccination had previously been assessed, with some contrasting findings (Fraser *et al.*, 2012a, 2014a), it was important to investigate the response of triploids to a range of commercially available vaccines (**Chapter 5**).

Low mortality in both ploidy during the fresh- and seawater phases of the trial concurs with previous research and is indicative of improvements in triploid nutrition, rearing and environmental tolerances as a result of recent research advances (Fraser *et al.*, 2012a, 2014a, 2015a; Hansen *et al.*, 2015; Taylor *et al.*, 2015; Smedley *et al.*, 2016; Fjelldal *et al.*, 2016; Sambraus *et al.*, 2017).

Ploidy did not affect specific antibody (IgM) response, and this is in agreement with the findings from **Chapter 2**. Furthermore, all vaccine treatments containing the *A. salmonicida* antigen (vaccine treatment 3 - 10) exhibited a positive antibody response while those that did not contain the antigen (vaccine treatment 1 and 2) gave a negative antibody response. This again concurs with **Chapter 2** which found increased antibody response in vaccinated fish compared to sham-vaccinated controls.

Adhesion scores were not significantly affected by ploidy. This concurs with **Chapter 2** but is in contrast to the study by Fraser *et al.* (2014a) which found higher adhesion scores in S0+ triploid smolts. A number of factors may be proposed to explain this difference including the timing of sampling (800 °D post-vaccination in Fraser *et al.* (2014a) and 622 °D in the current trial) and water temperature post-vaccination (increased to 16 °C in Fraser *et al.* (2014a) and decreased to 9 °C in the current trial). As such, it is necessary to assess the effects of temperature on the occurrence of adhesion scores in triploids. Moreover, a preliminary investigation undertaken in **Chapter 2** of this thesis (data not presented) revealed that body wall thickness in triploids was thinner than that of diploids and, as a result, shorter needles were utilised in subsequent vaccinations. Considering this, it could also be suggested that the increased adhesion scores observed by Fraser *et al.* (2014a) in triploid Atlantic salmon may be linked to incorrect needle size and that the needle may have interfered with the viscera. As such, additional studies should be undertaken to

assess further the impact of needle size on vaccination and the occurrence of adhesions in triploids in order to fully optimise vaccination strategies for triploid Atlantic salmon. The severity of adhesion scores was significantly affected by vaccine treatment and this was to be expected considering the increasing score of vaccine treatment severity created at the outset of this trial. Following the use of ALPHA JECT 2-2 (vaccine treatment 3), adhesion scores in diploid and triploid Atlantic salmon at 622 °D post-vaccination in the current trial were lower (average 1.1) than those exhibited at 600 °D post-vaccination in **Chapter 2** (average 2.7). As previously indicated, triploids have a thinner body wall than diploids and it could be proposed that, as the body wall investigation was undertaken post-vaccination, incorrect needle length led to increased adhesion scores in **Chapter 2**. Moreover, following vaccination in **Chapter 2**, temperature was rapidly increased to 9.6 ± 1.1 °C and fish were maintained at this temperature until sampling at 600 °D (35 days post-transfer to ARF), and this could be suggested as another cause for the increased adhesion scores observed in **Chapter 2**. Furthermore, vaccine treatment 10 (ALPHA JECT 6-2, double dose) was found to elicit the most severe adhesion scores in both ploidy. These findings highlight the need for further studies to elucidate the response of triploids to vaccination, particularly when exposed to suboptimal conditions, and indicates the need for accurate vaccine injection in both diploid and triploid Atlantic salmon.

Triploids were significantly heavier and longer than diploids throughout the study which, again, is supportive of research advances to optimise rearing conditions and diets. However, in terms of growth rate, TGC was lower in triploids than in diploids in freshwater post-vaccination and it is suggested that this is linked to high temperatures (14 – 16 °C) just before and post- vaccination having a negative effect on triploids. This is supported by a previous study which found that high temperatures (> 15 °C) reduced feed intake and growth in triploid Atlantic salmon (Sambräus *et al.*, 2017). It should be noted that this early decrease in TGC did not negatively impact on the continued growth of triploids, with higher TGC than diploids observed in seawater. This highlights the ability of triploids to grow faster and larger than diploids. However, much work remains to be done with regards to triploid growth in seawater, with numerous studies indicating that, after the first few months at sea, triploids can lose their growth advantage over diploids. The recent study by Smedley *et al.* (2016) showed that when triploids were fed a diet containing a boosted nutrient package (higher phosphorus and protein), the fish maintained a higher growth rate over the entire seawater period. As such, it also highlights the requirement for further studies into triploid nutrition to continue to optimise diets and reach the full growth potential in triploids.

Considering that the fish in this study are being monitored through to harvest size, it will be interesting to see how their growth progresses and whether triploids maintain their growth advantage. Vaccine treatments had an effect on growth parameters at both smolt and post-smolt stages with the most severe vaccine treatment (10) continually showing the lowest weight, length, K, and, with the high adhesion scores observed in this treatment negatively affecting TGC. This further indicate the need for accurate and efficient commercial vaccination procedures. It should be noted that vaccine treatment 10 is not a commercial vaccine or combination of vaccines but was used to demonstrate the negative effects that accidental “double-dosing” could have on the fish.

Vertebral deformities were found in both diploid and triploid Atlantic salmon, with triploids consistently showing higher levels of deformity than diploids but this was to be expected considering previous studies. However, the magnitude of difference observed between diploids and triploids (approximately 50 %) was expected to be less considering that the triploids were fed a triploid-specific diet containing a boosted package of nutrients and minerals throughout the current trial. Findings from previous research would suggest that, following supplementation of key minerals such as phosphorus, levels of vertebral deformities were comparable between diploids and triploids (Smedley *et al.*, 2016; Fjelldal *et al.*, 2016). As such, this could be suggestive of errors with the formulation of the diet or suboptimal temperatures during the early egg rearing stages but these would require further clarification. Previous studies have also found that the supplementation of vitamins and minerals in triploids during seawater can stabilise the deformities exhibited in freshwater (Fjelldal *et al.*, 2009b; Smedley *et al.*, 2016). This, therefore, highlights the importance of the fish in this trial being taken through to harvest size. However, it is clear studies are required urgently to further advance the current knowledge of triploid nutrition and to fully elucidate the occurrence of more severe vertebral deformities in triploids. Overall, this Chapter would suggest that triploids respond as well as diploids to the process of vaccination and indicates that the vaccines tested in diploids are suitable for commercial use in triploids. Moreover, results indicates better triploid performance and, thus, continues to supports their application in commercial aquaculture.

6.5. FINAL CONCLUSIONS

This thesis presents novel research investigating the response of triploid Atlantic salmon to pathogens of significance in aquaculture and on-farm treatment practices, thus advancing the current understanding of health and immune response in triploids. The results would suggest that triploid Atlantic salmon can cope as well as diploids with bacterial and parasite challenges as well as during chemical and vaccine treatments, under experimental conditions. However, it is recognised that further studies are required to fully characterise triploid health and immune response, particularly in response to conditions that would be experienced in a commercial aquaculture setting, such as fluctuating temperatures and varying O₂ availability. Environmental conditions play a key role in pathogen occurrence and treatment efficacy and, as triploids appear more sensitive to environmental extremes, one of the next key steps in triploid research would be to assess the additive effect of environmental conditions and disease and/or husbandry challenges on health and immunity. Overall, the research presented provides an excellent base for the continuation of triploid health research and supports the use of triploid Atlantic salmon in commercial aquaculture

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