The effects of temperature, egg quality and nutrition on the early development of triploid Atlantic salmon (*Salmo salar* L.)

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Dedicated to the memory of

James Clarkson Heggie

DECLARATION OF ORIGINALITY

This thesis is the result of my own work and composed solely by myself except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

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ABSTRACT

This thesis has successfully advanced understanding regarding potentially sub-optimal early life stage triploid culture. Further investigation of egg incubation temperature requirements elucidated that a constant temperature of 6 °C from fertilisation to first feeding would improve survival and performance of triploids compared to the diploid standard of 8 °C or any temperature alterations throughout embryogenesis. Triploids were more sensitive to post-ovulatory ageing as shown with significantly higher mortality and larger size variation compared to their diploid siblings. Quality indicators suggested that ageing resulted in increased lipid peroxidation and decreased antioxidant activity in unfertilised eggs, suggesting fertilising eggs within five days post-ovulation is beneficial for triploid culture. Nutritional programming was successfully demonstrated in *Salmo salar* of both ploidy. A nutritional intervention at the point of first feeding resulted in better acceptance and utilisation of vegetable-rich diets in later life. Ploidy generally responded the same, however triploids showed a higher variation in nutrient retention and LC-PUFA biosynthesis, highlighting the need to understand further the difference between nutritional requirements. Fundamentally, the impact of the second set of maternal chromosomes present in triploids is not understood. A panel of expressed microsatellite loci was identified and exploited to assess allele expression and results confirmed codominant expression of these genes in diploid fish. There was clear evidence that all three alleles present in triploids were expressed at both the offspring whole animal level and in blood samples, however poor amplification of RNA from single cells prevented further understanding. Collectively, these studies highlight differences in early life culture requirements of diploid and triploid *Salmo salar*.

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ETHICS STATEMENT

All experimental procedures were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice. HMSO: London January 1997) under project licence PPL70/7916 “Environmental Regulation of Fish Physiology” H. Migaud) in accordance with EU regulation (EC Directive 86/609/EEC). All experimentation performed at the Institute of Aquaculture (IoA) was subject to an ethical review process carried out by the University of Stirling Animal Welfare and Ethical Review Board (AWERB) prior to the work being approved.

LIST OF ABBREVIATIONS AND ACRONYMS

2N	diploid
3N	triploid
6DMAP	6-dimethylaminopurine
ADO	allelic drop-out
ALA	α-linolenic acid, 18:3(n-3)
alp	alkaline phosphatase
ANF	anti-nutritional factor(s)
ANOVA	analysis of variance
AS	Alsever's solution
ATP	adenosine triphosphate
bar	metric unit of pressure
bFCR	biological feed conversion ratio
BHT	butylated hydroxytoluene
BLAST	basic local alignment search tool
bmp2	bone morphogenic protein 2
bmp4	bone morphogenic protein 4
bp	base pairs
BPC	bean protein concentrate
BW	body weight
BWa	body weight of alevins
c. (italicised)	circa
Ca	calcium
CB	cytochalasin B
cDNA	complementary DNA
CLT	central limit theorem
cm	centimetre(s)
col1a1	collagen type I alpha 1
col2a1	collagen type II alpha 1
CPD-A1	citrate-phosphate-dextrose-adenine
d	day(s)
DAG	diacylglycerol

DAPI	4’,6-diamidino-2-phenylindol
DHA	docosahexaenoic acid, 22:6(n-3)
DI	distal intestine
DM	dry matter
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DPO	days post-ovulation
dV	deformed vertebrae
EAA	essential amino acid(s)
EC	electrical conductivity
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EG	eosinophilic granulocytes
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
elf-α	elongation factor 1-alpha
EPA	eicosapentaenoic acid, 20:5(n-3)
EST	expressed sequence tag(s)
FA	fatty acid(s)
fads2d6	delta-6 fatty acyl desaturase
FAME	fatty acid methyl ester(s)
fas	fatty acid synthase
FCM	flow cytometry
FCR	feed conversion ratio
FE	feed efficiency
Fe	iron
FFN	final fibre number
FI	feed intake
FL	fork length
FSC	forward scatter
FM	fish meal
FO	fish oil
FW	freshwater
g	gram(s)
g (italicised)	relative centrifugal force

GDH	glutamate dehydrogenase
gDNA	genomic DNA
GIT	gastrointestinal tract
gphb5	glycoprotein hormone subunit beta 5
H&E	haematoxylin and eosin
H_2O_2	hydrogen peroxide
het	heterozygous
His	histidine
HNO_3	nitric acid
hom	homozygous
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
hr(s).	hour(s)
hsp	heat shock protein
hsp70	heat shock protein 70
ICP-MS	inductively coupled plasma mass spectrometry
IEL	intra-epithelial lymphocytes
IGF	insulin-like growth factor(s)
igf1	insulin-like growth factor 1
igf1r	insulin-like growth factor 1 receptor
igf2	insulin-like growth factor 2
igfbprp	insulin-like growth factor binding protein related protein
IoA	Institute of Aquaculture
IQR	interquartile range
K	potassium
k strategist	few offspring, high probability of survival
KCl	potassium chloride
K_f	Fulton's condition factor
kg	kilogram(s)
kJ	kilojoule(s)
L	litre(s)
LA	linoleic acid, 18:2(n-6)
LC-PUFA	long chain – polyunsaturated fatty acid(s)
kV	kilovolt(s)

LP	lamina propria
LJD	lower jaw deformity
LT	long term egg incubation regime
lxr	liver X receptor
M	molar mass
m	metre(s)
mA	milliampere(s)
MDA	multiple displacement amplification
MFBMA	mucosal fold base mitotic activity
Mg	magnesium
mg	milligram(s)
min(s).	minute(s)
mL	millilitre(s)
mM	millimolar(s)
mm	millimetre(s)
mmp13	matrix metalloproteinase 13
mOsm	milliosmole(s)
mRNA	messenger RNA
myf5	myogenic factor 5
myod	myogenic differentiation 1
N	nitrogen
n =	number of samples
Na	sodium
NaCl	sodium chloride
NAH	N-acetylhistidine
NCBI	National Centre for Biotechnology Information
ng	nanogram(s)
NH	nutritional history
nm	nanometre(s)
NQC	Norwegian Quality Cut
ns	not significant
O_2^-	superoxide anion
O.C.T.	optimal cutting compound
ocn	osteocalcin

$ONOO^-$	peroxynitrite
opn	osteopontin
P	phosphorous
p (italicised)	probability value
PCR	polymerase chain reaction
PepT1	peptide transporter 1
PI	propidium iodide
POA	post-ovulatory ageing
PPC	pea protein concentrate
ppm	parts per million
psi	pounds per square inch
PST	parr-smolt transformation
PUFA	polyunsaturated fatty acid(s)
qPCR	quantitative polymerase chain reaction
r strategist	many offspring, low probability of survival
r^2	coefficient of determination
RAS	recirculating aquaculture system
RNA	ribonucleic acid
RNase	ribonuclease
RO	rapeseed oil
ROS	reactive oxygen species
SBM	soybean meal
SD	standard deviation
SDS	sodium dodecyl sulphate
sec(s).	second(s)
SUBEM	sub-epithelial mucosa
SEM	standard error of mean
SFR	specific feed rate
SLS	sample loading solution
sparc	osteonectin
SPC	soy protein concentrate
srebp1	sterol regulatory element binding transcription factor 1
SS	size standard
SSC	side scatter

SSR(s)	simple sequence repeat(s)
ST	short term egg incubation regime
SW	seawater
T_A	annealing temperature
TAG	triacylglycerol
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TGC	thermal growth coefficient
TL	total lipid
totRNA	total RNA
UV	ultraviolet
V	vanadium
v:v	volume : volume
w:v	weight : volume
WG	wheat gluten
WGD	whole genome duplication
WTA	whole transcriptome amplification
YSW	yolk-sac weight
Zn	zinc
β-actin	beta-actin
µg	microgram(s)
µL	microliter(s)
µM	micromolar
µm	micrometre(s)
µS	microsiemen(s)
Δ6D	delta-6 desaturase
°C	degrees (Celsius)
°day(s)	degree day(s)
°min(s)	degree minute(s)

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Figure 1.3. Schematic representation of how hydrostatic pressure shock at 300 °min post-fertilisation results in the retention of the second polar body in the oocyte, affecting normal meiosis II and subsequent cell divisions, creating a triploid organism. Image adapted from Tom Hansen, IMR, Norway.

Figure 1.4. Hydrostatic pressure vessel used to triploidise *Salmo salar* eggs commercially sold from TRC hydraulics.

Figure 1.5. Erythrocytes from blood smears of (**a**) diploid and (**b**) triploid siblings stained with 6 % Giemsa.

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Figure 1.13. Representation of increasing severities of cataracts in *Salmo salar* according to the cataract score scale reported in Wall and Bjerkås (1999).

Figure 2.1. (**a**) Egg incubation temperature experimental design including LT temperature regimes represented by solid lines (11LT; black circles, 8LT; grey circles and 6LT; white

circles) and ST temperature regimes represented by dashed lines (11ST; black circles and 6ST; white circles) and (**b**) average daily temperatures during subsequent grow on from first feeding until smolt.

Figure 2.2. Cumulative mortality from fertilisation to smolt of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Breakdown of stage-specific mortality is reported in Table 2.2.

Figure 2.3. (**a**) Body weight (BWa), (**b**) yolk sac weight (YSW) and (**c**) yolk sac total lipid (TL) (n = 3, 5 alevins ploidy^{-1} treatment^{-1}) at different stages through embryogenesis. Data are expressed as means ± SEM and significant differences are denoted in Table 2.3.

Figure 2.4. Growth summary including (**a**) body weight in first feed alevins; BWi (g), (**b**) final body weight in smolts; BWf (g) and (**c**) thermal growth coefficient (TGC) calculated from first feeding to smolt of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Data are expressed as tank value ± SD ($p < 0.05$, Two-Way ANOVA). Superscripts denote significant differences between treatments.

Figure 2.5. Prevalence (%) of external jaw and vertebral deformities in diploid and triploid *Salmo salar* smolts (50 fish ploidy^{-1} treatment^{-1}) incubated as eggs under different temperature treatments. Breakdown of deformity is reported in Table 2.5.

Figure 2.6. Average number radiological deformed vertebrae (dV) per deformed individual in diploid and triploid *Salmo salar* smolts incubated as eggs under different temperature treatments. Data are expressed as means ± SD.

Figure 2.7. Prevalence (%) of X-ray radiologically assessed deformities presented according to severity index (Mild, 1-5 dV; Moderate: 6-9 dV; Severe ≥10 dV) in diploid and triploid *Salmo salar* smolts (50 fish ploidy^{-1} treatment^{-1}). Breakdown of deformity is reported in Table 2.6.

Figure 2.8. mRNA expression of *fas*, *lxr* and *srebp1* expressed as fold change relative to 8LT within each ploidy at respective developmental stage. Data were normalised using the geometric mean of *β-actin* and *elf-α* housekeeping genes. Data show expression prior to ST transition at 200 °days in (**a**) diploids and (**b**) triploids, at eyeing in (**c**) diploids and (**d**) triploids, and after inclusion of ST regimes at first feeding in (**e**) diploids and (**f**) triploids. Data are expressed as means ± SD (6 fish ploidy^{-1} treatment^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$). ns = not significant.

Figure 2.9. mRNA expression of *igf1*, *igf1r*, *igf2*, *igfbprp*, *myf5* and myod expressed as fold change relative to 8LT within each ploidy at respective developmental stage. Data were normalised using the geometric mean of *β-actin* and *elf-α* housekeeping genes. Data show

expression at first feeding in (**a**) diploids and (**b**) triploids, and at smolt in (**c**) diploids and (**d**) triploids. Data are expressed as means ± SD (6 fish ploidy^{-1} treatment^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$). ns = not significant.

Figure 2.10. mRNA expression of *alp*, *bmp2*, *bmp4*, *col1a1*, *col2a1*, *mmp13*, *ocn*, *opn* and *sparc expressed* as fold change relative to 8LT within each ploidy at respective developmental stage. Data were normalised using the geometric mean of *β-actin* and *elf-α* housekeeping genes. Data show expression at first feeding in (**a**) diploids and (**b**) triploids, and at smolt in (**c**) diploids and (**d**) triploids. Data are expressed as means ± SD (6 fish ploidy^{-1} treatment^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$). ns = not significant.

Figure 2.11. Predicted smolt weight of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Predicted weights were calculated using the respective TGC data and applied to the number of feeding days (399 days) and temperature profile that 11LT groups would have experienced based on the same first feeding date as the 11LT group.

Figure 3.1. Variance of final weight of diploid and triploid *Salmo salar* after (**a**) 0 DPO, (**b**) 5 DPO, (**c**) 10 DPO and (**d**) 15 DPO. IQR = interquartile range.

Figure 3.2. Variance of final length of diploid and triploid *Salmo salar* after (**a**) 0 DPO, (**b**) 5 DPO, (**c**) 10 DPO and (**d**) 15 DPO. IQR = interquartile range.

Figure 3.3. Impact of partial stripping and milt motility on fertilisation success and egg survival in egg groups from experimental (5 dams) and control (5 dams) broodstock at 20 DPO. Data are presented as means ± SD and significant differences are denoted by different superscripts ($p < 0.05$; One-Way ANOVA). Control A = control eggs fertilised with original sire. Control B = control eggs fertilised with second sire.

Figure 3.4. Vitamin E (α-tocopherol) concentration in eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model).

Figure 3.5. TBARS concentration in eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model).

Figure 5.1. An example chromatogram of locus BG934281 showing allele peaks in (**a**) maternal, (**b**) paternal and (**c**) triploid offspring whole individual samples.

Figure 5.2. Spectrophotometer ratios of (**a**) 260/280 and (**b**) 260/230 describing RNA quality of blood samples (n = 3) from blood after being held for eight hours in one of each of the

holding solutions; AS, EDTA, or CPD-A1. Red dashed lines represent values taken from fresh blood (260/280, 1.74 ± 0.26; 260/230, 1.81 ± 0.12). Data are means ± SD and significant differences are denoted by different superscripts ($p < 0.05$; Two-Way ANOVA). ns = not significant.

Figure 5.3. Blood smears from *Salmo salar* from (**a**) fresh sample and then held in (**b**) AS, (**c**) CPD-A1, and (**d**) EDTA after four hours of storage. Smears were stained with Giemsa and images analysed using Zen software (Zeiss).

Figure 5.4. An example chromatogram of locus BG934281 showing allele peaks in (**a**) maternal, (**b**) paternal and (**c**) diploid offspring single erythrocyte.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. The Atlantic salmon

1.1.1. Overview

The Atlantic salmon (*Salmo salar*) is one of 226 species of the Salmonidae family. Natural geographic distribution is determined by the temperature in which they acquire optimal development and these ray-finned species can be found across the northern part of the globe, with *Salmo salar* being native to the rivers of Northern Europe, West Coast of North America and the North Atlantic Basin between (Fig. 1.1.).

Figure 1.1. Geographical distribution of *Salmo salar* in the natural environment. Modified from Nature Table, www.animalcaseprofile.wordpress.com.

Salmo salar have the ability to switch osmoregulatory function in order to migrate between fresh water and saline environments, characterising them as diadromous. Whilst the salmon lives during the majority of its adult life in seawater, the early stages are spent in freshwater until the fish adapt to the saline environment in a process called smoltification. This journey is driven by the requirement to source more food. It has been suggested that

there is a social dominance when it comes to migration to sea, with the larger or earliest first feeders from a population migrating in the first sea winter, rather than after two or more years as in the later first feeders (Metcalfe *et al*., 1989; Metcalfe and Thorpe, 1992). Once mature, salmon are able to revert back to their original physiology and return to freshwater to reproduce. These physiological switches depend on environmental triggers i.e. temperature and day length, meaning that salmon will both smolt and return to freshwater in nature at particular times of the year. This evolutionary life strategy permits an understanding of the optimal environments salmon require at each life stage.

1.1.2. Life cycle and development

1.1.2.1. Embryogenesis

Life begins as an egg; a package full of nutrients which the mother has invested in each of her offspring to see them through until they can source their own food exogenously. Fish are ectothermic, and as such post-fertilisation egg developmental rate is regulated by external temperature. Rate of development can be described using the term "degree days" (°days) which is based on the accumulation of average daily temperatures units over a period of days. Degree days are used to understand and predict fundamental stages throughout their life span. During embryogenesis, bones will begin to form, gastrointestinal tract will develop and at around 250 °days post-fertilisation, eyes will form (Gorodilov, 1996). In *Salmo salar*, hatching then occurs at 500 °days post-fertilisation (Webb *et al*., 2007). At this stage, the yolk-sac fry (alevins) leave the egg as the chorion breaks and remain in the gravel substrate until the nutrient reserves are exhausted and becomes reliant on actively sourcing exogenous food at around 850-900 °days. At this stage, the fish are termed "swim-up fry", and in nature, they would leave the safety of the gravel substrate to begin their life independently (Fig. 1.2.).

1.1.2.2. Juvenile stage

Fry swim up to the water's surface to take in air and inflate the swim bladder which allows for neutral buoyancy in the water column. Now able to hunt for food, the fish must learn how to feed by themselves. This generally occurs during periods of dawn and dusk in which they can evade predation, identifying them as a crepuscular species. As the fry grow larger, they develop into parr which can be identified by their defined "parr marks" (Fig. 1.2.). It is likely that these markings are useful for camouflage against predation. The fish will remain in this developmental stage in their home river until they are ready to undergo parr-smolt transformation (PST). This transformation is initiated by the decrease in day-length and temperature associated with entering the winter months and completes with the increasing day-length and temperature during spring (Murphy and Houston, 1977; Clarke *et al*., 1978; Jonsson and Ruud-Hansen, 1985; Saunders *et al*., 1985). Parr undergo physiological, morphological and behavioural changes which prepare them for their migration to sea. Physiologically, osmoregulatory organs must adapt to tolerate a saline environment. Gill ionocyte function adapts to allow appropriate salt secretion. This change involves an increase in Na^+/K^+-ATPase (NKA) activity which in turn provides a negative charge and promotes Na^+ and Cl^- secretion. Conversion of the NKAα1a isoform to NKAα1b occurs in the gills during PST which is routinely used to monitor smolt development (McCormick *et al*., 2009). The gut adapts also to be able to take up saline water from drinking and the kidneys evolve to excrete less dilute urine to maintain osmotic balance (McCormick, 2013). An obvious morphological adaptation occurs as the fish turns into a smolt with the body turning silver as a result of deposition of guanine and hypoxanthine crystals and the edge of the fins appear black (Mills, 1989; Webb *et al*., 2007). PST also alters the behaviour of the fish, making

them more accustomed to swimming with the flow, initiating the seaward migration (McCormick, 2013).

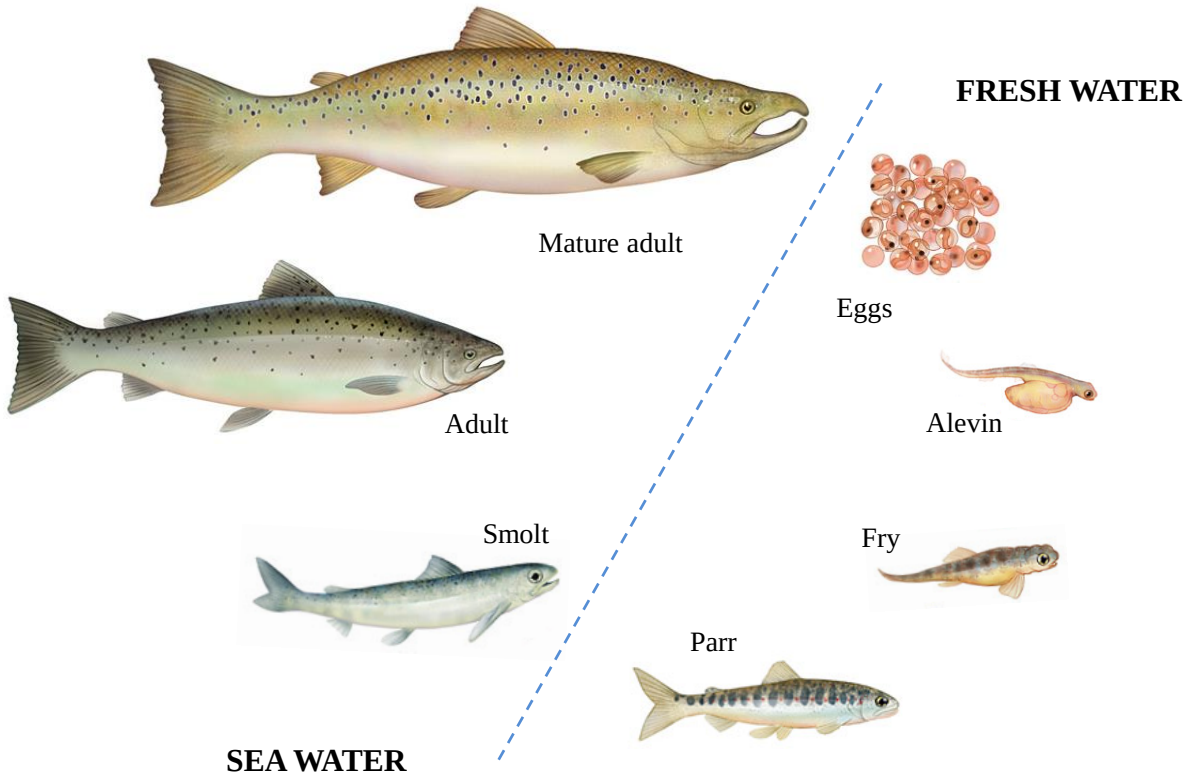


Figure 1.2. Full life cycle of *Salmo salar* encompassing both freshwater and sea water stages. Modified from Barbara Harmon, www.harmon-murals.blogspot.com.

1.1.2.3. Adult stage

Salmon migrate to the sea where there is improved food availability to meet an increased energy demand and can remain there for up to 4 years (Jacobsen and Hansen, 2000; Webb *et al*., 2007). Their diet consists of crustaceans (i.e. shrimp and prawns) and some fish species such as herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) (Hislop and Shelton, 1993) which are rich in omega-3 fatty acids.

Once they have reached a genetic threshold for adult body size and energetic status, they find their way back to native rivers at some point between spring and autumn with a

homing behaviour they have developed (Hansen and Jacobsen, 2000). This may be a result of sensitivity to the Earth's magnetic field (Quinn and Brannon, 1982), the ability to recognise pheromones from conspecifics in their natal river through a sophisticated olfactory mechanism (Nordeng, 1977), or an imprinting of the route from their earlier journey to sea as smolts (Hansen *et al*., 1989). The homing trait has been shown to be highly heritable, while influenced by environmental changes in food availability, temperature, and water flow (Jonsson and Jonsson, 2009; Milner *et al*., 2012).

Following return to their natal rivers, and in response to the decreasing day-length during autumn and early winter, the maturing adults will spawn (Fig. 1.2.). The female will create a gravel nest ("redd") in the river bed using her tail and lay her eggs which are fertilised by the male. *Salmo salar* can spawn between 2,000 and 15,000 eggs depending on body size, with larger females depositing more eggs (Mills, 1989). Due to the extreme nature of the environment the eggs are deposited in, namely its unpredictability and the abundance of opportunistic predators, salmon produce a large mass of eggs in the hope that the numbers will overcome the probable low survival. This reproductive strategy is known as "r", where a larger number of offspring are created with the quality equally shared, whereas in mammals, "k" strategists, more attention is given to fewer offspring of higher quality (Marschall *et al*., 1998). In addition to the energetic costs associated with return to their natal streams, reproduction is associated with high energy expenditure as the brood fish will invest a lot of nourishment into the offspring. Consequently, most *Salmo salar* only spawn once as they fail to survive after this ordeal (Marschall *et al*., 1998).

In order to produce salmon in aquaculture, it is common sense to replicate their natural life cycle to ensure optimal development. This includes the requirement of freshwater for juvenile stages and reproduction, and then seawater for the adult development. It is clear that environmental triggers are what drive the adaptive changes in the salmon necessary to

complete their life cycle and this must be translated into commercial protocols for successful and sustainable aquaculture.

1.1.3. The Atlantic salmon aquaculture industry

The global human population is expanding and creating growing pressure on the industry to meet demands for farmed *Salmo salar* as a protein source. Prior to maturity and the associated relocation of provisions, *Salmo salar* is a major beneficial food source for human consumption. They offer high valuable protein and high levels of omega-3 fatty acids like EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) which are beneficial for human health (Covington, 2004; Lee *et al*., 2009; Calder, 2014). Overfishing has previously been a major problem and quotas have been put in place to preserve the natural fish stocks, as fishing pressure on the few species of interest can lead to near or complete depletion (UK sea fisheries statistics, 2016). In light of these events, aquaculture has been rapidly growing as an alternative method to source fish, alleviating pressures on wild fisheries. Farmed *Salmo salar* production has significantly increased in recent decades. Since the industry began in the 1970's, the expansion of *Salmo salar* farming activity has led to a global supply in 2016 of in excess of 2.24 million tonnes with a value of over 14.3 billion USD, predominantly coming from Norway, Chile, Scotland, Canada and Tasmania (FAO, 2018). *Salmo salar* aquaculture is, at present, one of the fastest growing and most successful food industries that benefits man today (Asche *et al*., 2013).

The industry is constantly improving production and welfare standards as it moves towards more intensive systems. With growing societal pressure on the issues associated with aquaculture and environmental impact, the industry has had to carefully adapt in a sustainable manner. Improved technologies, such as the development of breeding programs, and optimising methods of culture and care, are the tools allowing the industry to succeed and

progress. Since the 1970's, breeding programs were designed in Norway in order to produce better growing and disease resistant fish (Gjedrem and Baranski, 2009) and European production of *Salmo salar* eggs today can be traced back to one of four original strains; Jakta, Bolaks, Mowi and Aquagen (Janssen *et al*., 2015).

Any form of anthropogenic developments will affect the environment in some way. Albeit beneficial for feeding the human population, fish farming can be a disturbance on the natural ecosystem and the sustainability of practices is essential to relieve pressures it may present. It is important to acknowledge that, on a global scale, compared to other human activities such as construction developments and agriculture, the aquaculture industry has a relatively low impact on the environment with *Salmo salar* being one of the most efficient sources of protein production (Froehlich *et al*., 2018; Fry *et al*., 2018). One sustainability issue that the industry faces is the dependence on wild caught fish used for salmon feed production. *Salmo salar* aquaculture currently acquires about one third of these species for feed manufacture, whilst the remaining mass is used in the agricultural industry (Naylor *et al*., 2000). Fish meal and fish oils are extracted from low-value oily fish species and used to manufacture optimal diets to feed the salmon (Tacon and Metian, 2008; Naylor *et al*., 2009). With the expansion of the industry, amongst several other modern uses for the marine raw materials, these are ultimately finite resources. In order to maintain the drive for a sustainable industry, significant research has been directed towards alternative feed ingredients as to marine raw materials (Gatlin *et al*., 2007; Tacon *et al*., 2011). Another significant sustainability concern the industry has faced for decades is the escape of domesticated fish into the natural environment. These domesticated stocks are selectively bred and inter-breeding with wild populations could ultimately narrow the genetic pool and reduce fitness of wild-living offspring with the potential of complete extinction in more vulnerable populations (McGinnity *et al*., 2003; Skaala *et al*., 2012). Despite efforts to combat escapes, i.e. improved

containment through increasing the robustness of cages and improved predation defence, escape events in the Scottish *Salmo salar* industry cannot be fully prevented. In 2017, Scotland lost ~30,000 (0.07 % of 46.1 million transferred to sea cages) to the sea due to unforeseen escapes (Scottish Government Natural Scotland, 2018). These concerns are driving developments in industry and one such technology under investigation is the use of triploidy as a method of reproductive containment.

1.2. Triploidy in aquaculture

The potential of triploid *Salmo salar* in the aquaculture industry is of great interest for several reasons. In one study, flesh quality analysis showed no difference in fillet colour between diploid and triploid harvest fish, although triploids did have significantly higher fat content and levels of polyunsaturated fatty acids (PUFAs) which could have direct benefits to consumer health (Taylor *et al*., 2013). Polyploidy is not a new technology in food production, as utilised for the growth of seedless fruits (Hancock, 1997; Yemets and Blume, 2008) and maturation control in shellfish in order to supply a year-round product (Piferrer *et al*., 2009). Triploid fish are rendered sterile, and growing sterile individuals can prevent the negative attributes associated with pre-harvest maturation including growth depression, a decreased condition, poorer flesh quality, and increased disease susceptibility (Mazeaud *et al*., 1977; Martin *et al*., 1993; Kadri *et al*., 1997; Michie, 2001; Leclercq *et al*., 2010), which all ultimately impact the quality and marketability of the end product. Triploidy was first investigated in the 1970's as a mitigation strategy to control pre-harvest sexual maturation. However, the concept was abandoned due to concerns of inefficiency, low survival, increased deformity and poor growth, and photoperiod regimes were instead established to control maturation (Fraser *et al*., 2012). With a better scientific understanding, triploidy has recently been revisited. Also, the potential of the interbreeding with wild stocks is avoided as no

functional reproductive organs are developed (Benfey, 2001). By developing a better quality triploid, the benefits of sterility may be realised. Energy which would normally be used for gonadal development should be redirected into overall somatic growth so there is also a potential to increase yield through their ability to grow larger and harvest earlier. Less time at sea has secondary benefits such as a decrease in exposure time to disease and the potential for less associated treatments. Although initial results with triploid *Salmo salar* showed high mortalities and poor performance, prior knowledge gaps have been addressed by recent research and the industry should recognise that diploids and triploids may have different culture requirements.

1.2.1. Artificial triploid induction

In teleost species, a spawning female will ovulate during a halted metaphase stage of meiosis II (Piferrer *et al*., 2009). Development of the oocyte will remain fixed until it is fertilised, whether it be in a natural environment or manually orchestrated in a commercial brood stock site. Shortly after fertilisation, triploidy can be artificially induced (Fig. 1.3.). The concept behind triploid induction is relatively straight forward and consists in artificially inducing the retention of the second polar body as oocyte development continues post-fertilisation, thus duplicating the maternal genome and leaving the individual with three sets of chromosomes. This extra set of homologues is what prevents normal pairing of chromosomes, interrupting the development of gonads from the earliest stages in oogenesis, however males have shown to develop functional testis but produce aneuploid sperm (Benfey, 1999). The physical change in the cell is thought to be a result of spindle breakdown during the normal cell division (Piferrer *et al*., 2009) and this can be instigated through one of several methods.

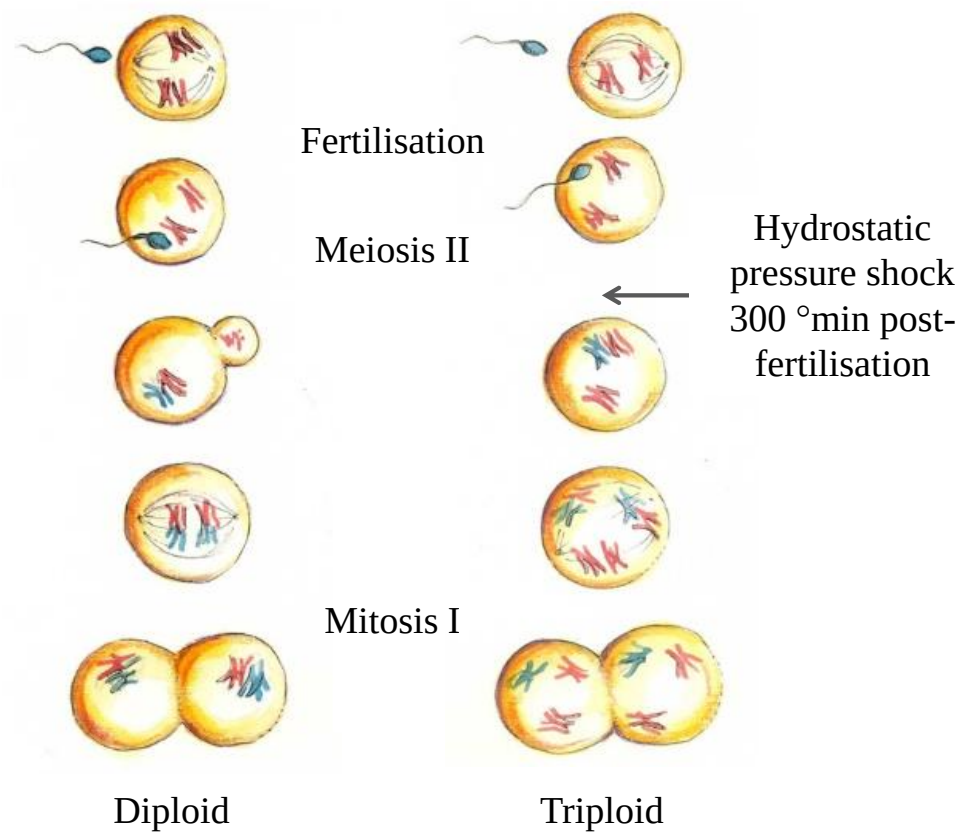


Figure 1.3. Schematic representation of how hydrostatic pressure shock at 300 °mins. post-fertilisation results in the retention of the second polar body in the oocyte, affecting normal meiosis II and subsequent cell divisions, creating a triploid organism. Image adapted from Tom Hansen, IMR, Norway.

Several forms of polyploidy are regularly accomplished in botany to improve heterosis (Comai, 2005). Induction of polyploidy in shellfish has also been developed commercially using diploid x tetraploid crosses however a range of chemicals including cytochalasin B (CB) and 6-dimethylaminopurine (6DMAP) are also efficient methods of producing triploid shellfish (Guo *et al*., 2009). This idea was attempted with salmonids, however producing viable tetraploids for parental crossing and subsequent fertilisation with diploid sperm proved very difficult in fish, primarily caused by low yields and survival of offspring (Piferrer *et al*., 2009). For decades, temperature has been used to induce triploidy with both cold shock (Piferrer *et al*., 2000; Dias de Silva *et al*., 2007) and heat shock (Solar *et*

al., 1984; Johnstone, 1985; Don and Avtalion, 1986; Sutterlin *et al*., 1987; Rougeot *et al*., 2003), providing some success in a range of teleosts.

In *Salmo salar*, amongst other salmonids, the most effective method to date is the use of a hydrostatic shock using a pressure vessel (Fig. 1.4.). This is favoured over the relatively successful heat shock method as it is easier to apply and has more consistent effects throughout the entire egg batch. Furthermore, previous comparisons in rainbow trout (*Oncorhynchus mykiss*) have shown a greater survival compared to heat-shock protocols (Haffray *et al*., 2007). Developed in the 1980's from success in amphibians (Benfey and Sutterlin, 1984a), triploidy induction was attempted in several species and now, a species-specific method has shown great success in *Salmo salar*, generally producing upwards of 98 % triploid offspring (O'Flynn *et al*., 1997; Oppedal *et al*., 2003; Taylor *et al*., 2011; Benfey, 2015). This involves a shock of 9,500 psi, or 655 bar, of pressure beginning at 300 °mins. after fertilisation for a duration of 50 °mins. (Table 1.1.).

Figure 1.4. Hydrostatic pressure vessel used to triploidise *Salmo salar* eggs commercially sold from TRC hydraulics.

Table 1.1. Example of triploidy pressure shock protocols for *Salmo salar* at 6, 8 and 10 °C incubation temperatures post-fertilisation. Adapted from Benfey *et al*. (1988).

Pressure (psi / bar)	Temperature (°C)	Begin pressure shock post-fertilisation	Duration of pressure shock
9,500 / 655	10	30 mins.	5 mins.
9,500 / 655	8	37 mins. 30 secs.	6 mins. 15 secs.
9,500 / 655	6	50 mins.	8 mins. 20 secs.

The treatment duration is temperature-dependant and this was long practised at 10 ºC (Johnstone and Stet, 1995; Taylor *et al*., 2011). However, more recent reports showed an improved survival and development of the fish when triploidised at 8 ºC (Fjelldal and Hansen, 2010; Hansen *et al*., 2015; Smedley *et al*., 2016; 2018).

1.2.2. Ploidy determination

In order to confirm successful induction of triploidy in the eggs, ploidy levels must be determined. Several methods can be used, but the most common approaches include; erythrocyte nuclei measurements (Benfey *et al*., 1984; Benfey, 1999), flow cytometry (FCM, Thorgaard *et al*., 1982; Lecommandeur *et al*., 1994; Peruzzi *et al*., 2005) and the use of microsatellite markers for allelic profiling (Glover *et al*., 2015; Harvey *et al*., 2017; Feng *et al*., 2018).

1.2.2.1. Erythrocyte measurements

In order to work with erythrocytes, fish must be of adequate size to acquire a sufficient volume of blood to sample. This means there is a disadvantage of waiting for some time before ploidy identification and potentially rearing unsuccessful triploid stock. Erythrocyte measurements (either manually or semi-automated) can be used as a ploidy determination tool due to a larger size and increased nucleic mass in triploids compared to diploids (Fig.

1.5.). In brief, the erythrocytes are removed in a heparinised syringe and smeared onto a microscope slide. Dried samples are stained (6 % Giemsa), allowing an easy measurement of cell or nuclei size (Benfey *et al*., 1984). The size of nuclei increases with the addition of an extra set of chromosomes, although this increased cell size is generally correlated with a reduction in cell number to maintain an overall cell mass (Benfey and Sutterlin, 1984b).

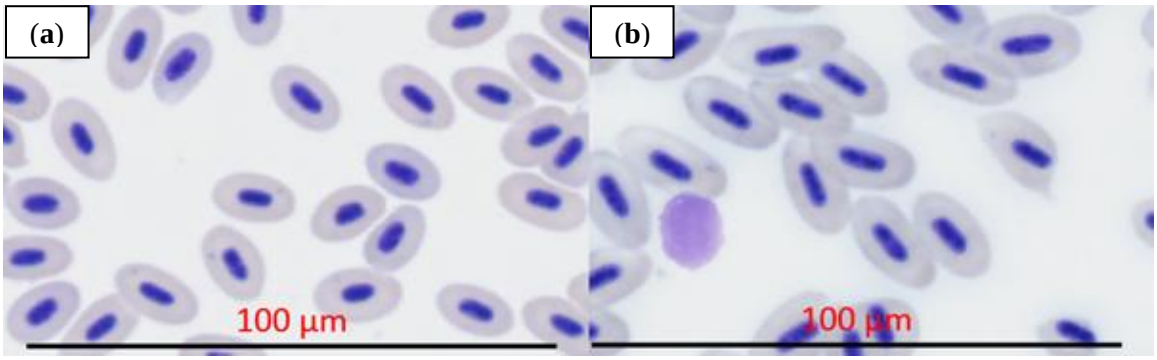


Figure 1.5. Erythrocytes from blood smears of (**a**) diploid and (**b**) triploid siblings stained with 6 % Giemsa.

Determining ploidy by comparing the nuclei major axis (Fig. 1.6.) has been a common approach in a range of teleost species (Woznicki and Kuzminski, 2002; Haniffa *et al*., 2004; Peruzzi *et al*., 2005; Normala *et al*., 2016) and regularly practised in recent *Salmo salar* studies (Taylor *et al*., 2011; 2014; Smedley *et al*., 2016; 2018).

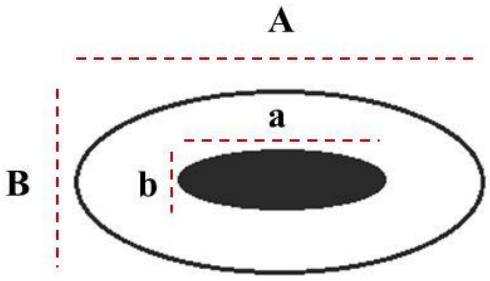


Figure 1.6. Measurements of erythrocyte (**A**) cell major axis, (**B**) cell minor axis, (**a**) nucleus major axis, (**b**) nucleus minor axis used for ploidy verification.

Erythrocyte shape and size will differ depending on the maturity of the cells. This poses a degree of uncertainty in using this method for ploidy identification and there have been some reports of erythrocyte dimensions overlapping when comparing diploid and triploid cells, likely caused by different life stages of the cells e.g. in African catfish (*Clarias gariepinus*) (Normala *et al*., 2016). It has not yet been established if all cells in a triploid individual are consequently triploid as there have been reports of ploidy mosaicism between cells in other teleost investigations post-triploidy induction (Teplitz *et al*., 1994; Goudie *et al*., 1995; Arai, 2001). This may lead also to a difference in cell size, although this scenario is relatively uncommon (Benfey *et al*., 2016). Nonetheless, and although this method remains labour intensive without the use of automated image analysis, measuring erythrocyte nucleus diameter is an inexpensive and simple approach to ploidy identification at the whole animal level.

1.2.2.2. Flow cytometry

A well-established rapid test to analyse a large number of cells that is more direct and very accurate for ploidy assessment is the use of flow cytometry (FCM) (Thorgaard *et al*., 1982; Lecommandeur *et al*., 1994). Erythrocytes can also be used for this method (Peruzzi *et al*., 2005) however the requirement to wait until the fish are large enough still remains. For an earlier analysis, DNA from fin clips and eyed eggs can be used (Lecommandeur *et al*., 1994; Lamatsch *et al*., 2000). There are several ways to exploit FCM but the fundamental concepts are relatively simple (Ochatt, 2006; Rahman, 2006). In all cases, cells are extracted from the tissue into suspension and fed through a fluidics system, using laminar flow to draw the samples through. The cells are then passed through an assembly of monochromatic laser beams, exciting fluorescence which is detected through a succession of dichroic filters (Fig. 1.7.).

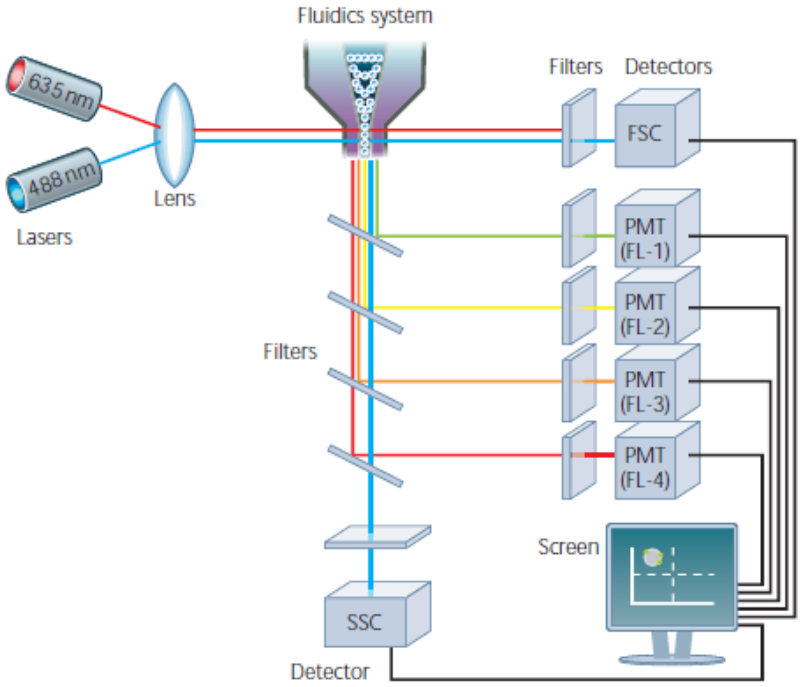


Figure 1.7. Schematic diagram of a flow cytometry process. Image obtained from Rahman (2006).

Each detector translates specific colour intensities into data points which are plotted onto a graph illustrating the characteristics of each particle. For this to be possible, the DNA must be pre-labelled with a fluorescent tag i.e. DAPI (4′,6-diamidino-2-phenylindol) or PI (propidium iodide). The use of side scatter (SSC) detection is the more intricate use of FCM. Fortunately, these fluorescent tags are stoichiometric, where the detection data produced is completely parallel with the volume of DNA present. Therefore, cells with increased numbers of chromosomes i.e. triploids, will show a higher level of fluorescence than a diploid. Forward scatter (FSC) is also important for each particle. However, this detection is simpler and determines a rough estimate of the particle size, which is useful to confirm particles or cells of interest. A fundamental process in data interpretation is criteria selection with the use of gating as highlighted with pink “gates” in Fig. 1.8. This allows assortment and isolation of desired data points before quantification, eliminating unwanted cell types, dead cells and

debris. FCM is a fairly rapid method once a sufficient amount of cells can be extracted from tissue of choice, although the equipment required is more expensive than following erythrocyte measurement protocols. However, there is capacity to use this method much earlier than measurements of blood cells and the current industry protocol involves cell extraction from eyed eggs (~250 °days).

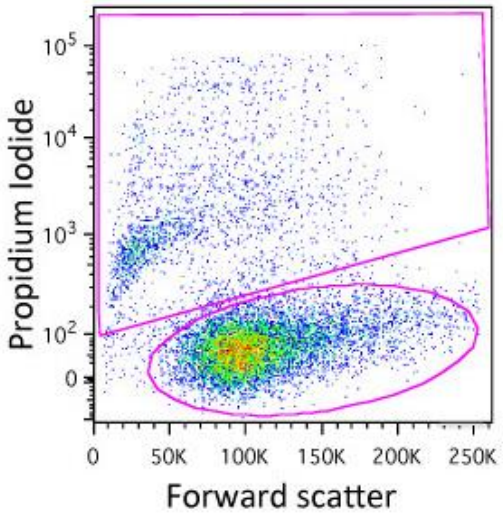


Figure 1.8. Example of gating to select cells of interest processed through flow cytometry. Image obtained from www.bitesizebio.com.

1.2.2.3. Microsatellite markers

The science of genomics and genetics (knowledge, awareness, instrumentation and practical application) has evolved dramatically over recent decades, giving rise to technologies such as the use of microsatellites for individual analysis and parentage assignment (Castro *et al*., 2006; Hernández-Urcera *et al*., 2012). Microsatellite markers, otherwise known as simple sequence repeats (SSRs), are simply short repeated genomic sequences of 1-5 nucleotides. SSRs are useful tools for genotyping biological samples because they are highly informative. Allelic profiling using microsatellite markers is theoretically a logical approach to identify the ploidy status of an organism as demonstrated in *Salmo salar* (Glover *et al*., 2015). As triploids possess three sets of chromosomes, they will show three different alleles in the

output of this method. However, this relies on the polymorphism of the marker, meaning that it is necessary to design the markers based on having multiple alleles available for that given marker. Ideally, if both maternal and paternal copies of the markers contain two distinct alleles each, screening of the offspring will show two distinct allelic peaks in a diploid and three in a triploid (Fig. 1.9.).

This technique can be used early with hatchlings as reported in Senegal sole (*Solea senegalensis*) (Castro *et al*., 2006) and is relatively simple and cost efficient when working with large sample numbers, ideal for commercial practice. Moreover, the ability to assess triploidy success using microsatellite markers during the egg stage would further benefit the industry. As with flow cytometry, verifying ploidy in eggs allows practical decisions to be made early. This early confirmation of ploidy status allows replacement of a potentially unsuccessful triploid egg batch as the spawning season may well still be ongoing.

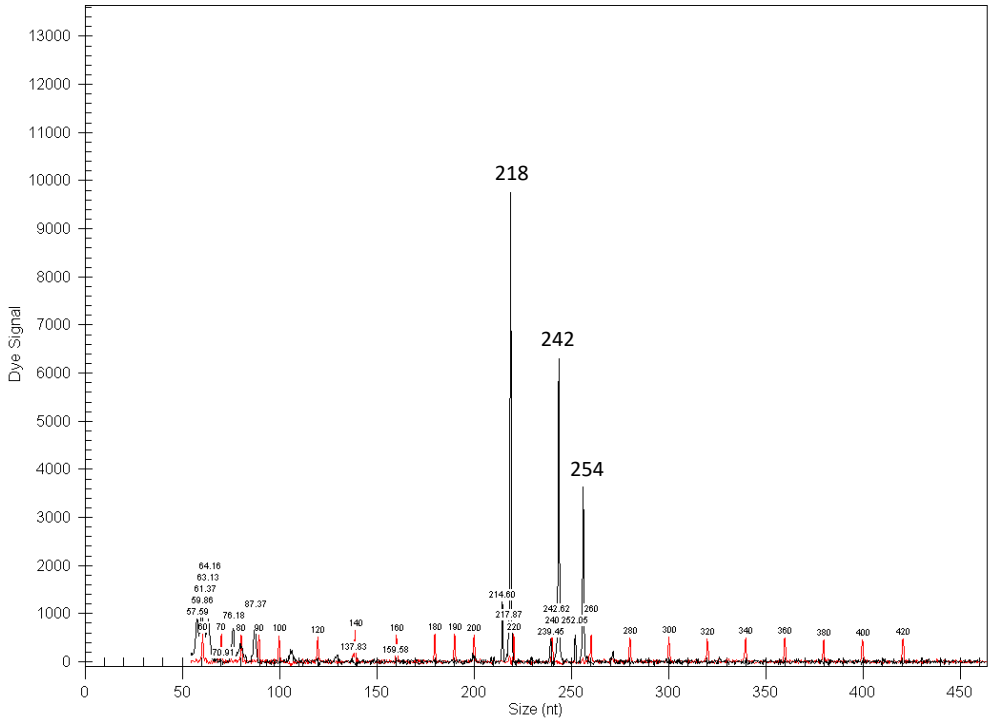


Figure 1.9. An example chromatogram showing three distinct allele peaks in a triploid *Salmo salar* erythrocyte.

Each approach has its advantages and disadvantages when considering time investment, cost and reliability. Identifying the ploidy status of the offspring is important as culture requirements are probably different between the ploidies. More importantly, one key requirement for triploid production is that triploid success is as close as possible to 100%. The method used must be robust and economically viable and an early ploidy verification method would be advantageous in order to replace any unsuccessful attempts at triploid induction prior to considerable investment of rearing time and resources.

1.3. Cellular and genomic impacts of triploidy

1.3.1. Cellular architecture

Triploid cells clearly differ in cellular architecture compared to diploid counterparts (Benfey, 1999). Although generally phenotypically similar, the assemblage of cells is rather distinct with triploids having larger cells to house the 50 % increased abundance of nuclear material. Therefore, to maintain a similar tissue and overall somatic mass, there are much fewer cells in all corresponding tissue types (Small and Benfey, 1987; Benfey, 1999; Piferrer *et al*., 2009). The mechanisms behind this arrangement are unknown, but may result in some of the physiological and behavioural differences observed between the ploidy i.e. thermal tolerance, oxygen requirements and feeding behaviour. An interesting point of discussion is the consequential decrease in cell surface to volume ratio (Benfey, 1999). Furthermore, cytoplasmic mass will increase causing a greater distance between the nucleus and the cell membrane. These structural alterations are likely to pose a challenge for normal transport and diffusion processes which may potentially limit nutrient absorption and retention, metabolite and ion exchange and messenger molecule function. Ytteborg *et al*. (2010a) outlines the ontogeny and growth of the vertebral column in diploid *Salmo salar* and how the regulatory mechanisms (i.e. signalling molecules) are responsible for its development. It is as yet

unknown how a triploid cell can maintain this function due to their different design. The reduced surface area to volume ratio of triploid cells also likely affects gas exchange which may impact on aerobic functions (Benfey, 1999). Also, the ability to restore metabolites in muscle cells after stressors such as elevated temperature was reduced in triploid brook trout (*Salvelinus fontinalis*) (Hyndman *et al*., 2003) and Leggatt *et al*. (2006) suggested a compromise in the antioxidant capacity of triploid *Oncorhynchus mykiss* in response to the stress involved with hyperoxia. It is possible that anaerobic pathways in triploid salmonids are also challenged as they appear to have difficulty excreting metabolic waste. Homeostasis appears to be maintained at the whole animal level (Ching *et al*., 2010), but there may be drastic impact at the cellular level, specifically with changes to the increased genomic content and subsequent transcriptional activity.

Determining this fundamental difference between diploid and triploid cellular mechanisms may prove useful in elucidating the precise environmental and nutritional requirements of triploid *Salmo salar*.

1.3.2. Impact of extra maternal chromosomes

The effects of ploidy on gene regulation are not fully understood. Two forms of polyploidy exist. Allopolyploidy occurs when an individual has sets of chromosomes coming from different species. The tiger trout is an allotriploid, hybridised from the brown trout and the brook trout (*Salmo trutta* x *Salvelinus fontanalis*) and in which triploidy is induced (Scheerer and Thorgaard, 1983). This sterile hybrid is bred predominantly for recreational fisheries for their desirable growth and visual pattern.

The other form of polyploidy is autopolyploidy, when an individual has sets of chromosomes coming from the same ancestral species. For example, Glover *et al*. (2015) observed up to 50 % of naturally occurring triploids in response to over-ripened oocytes,

which may occur in both aquaculture and the natural environment. Another potential cause is oocytes being spawned in areas of extreme temperature fluctuations (Mable *et al*., 2011). In *Salmo salar*, there has been great success in autopolyploidy through artificial induction. This restructure of the genome and the cell poses several complications for fundamental process i.e. cell cycling, metabolism and gene regulation (Wertheim *et al*., 2013). Comai (2005) describes how the differential growth of internal versus nuclei surface components may result in a gene dosage inequality.

In order to maintain a degree of normality, one of two suggested complex chromosomal restructurings occurs to stabilise the genome. Gene stability in polyploidy has been reviewed (Birchler and Veitia, 2007; 2012) and it appears that gene expression is correlated with increased copies of a particular gene, therefore greater in higher ploidy levels. There have been reports of increased gene expression in polyploid maize, suggesting that there is a positive dosage effect in the numerous loci analysed (Guo *et al*., 1996). This phenomenon is exploited for improved heterosis as an increase in phenotypic traits is observed (Comai, 2005). Conversely, other loci have shown up- or downregulation in response to polyploidy e.g. in cotton (Adams and Wendel, 2004). This capacity is termed "dosage compensation", where the positive gene dosage effect is compensated for through gene silencing, resulting in re-diploidisation of the cell (Soltis and Soltis, 1999; Birchler *et al*., 2001). Furthermore, both gene dosage and dosage compensation were found to be gene-specific in an investigation on the silkworm, *Bombyx mori* (Suzuki *et al*. 1999), highlighting the complexity of polyploid cell arrangements.

Similarly, salmonids have been shown to exhibit both responses to polyploidy. In two separate studies investigating gene dosage in Chinook salmon (*Oncorhynchus tshawytscha*), triploids showed no difference in expression levels compared to diploids in most genes, however, there was a difference found in expression of genes associated with immune

response when triploids experienced stress and reduced performance (Shrimpton *et al*. 2007; Ching *et al*. 2010). These results suggest a degree of gene compensation overall, however, this may be affected under sub-optimal conditions. Moreover, Pala *et al*. (2008) observed gene dosage compensation in *Squalius alburnoides* allopolyploids with one of three alleles silenced, but not consistently for all genes investigated. These results support the previous assumption that the whole haplome is not silenced and that allelic-specific patterns likely differ between genes and tissues.

Increased genomic material in autopolyploid salmonids causes greater diversity and number of alleles (Leary *et al*. 1985). The additional set of maternal chromosomes in triploid cells means that they have a higher chance to be heterozygous than a normal diploid cell. Although this may be beneficial for desirable traits i.e. fitness levels (Wang *et al*. 2002), there is possibility that a negative effect can be caused through the alteration of gene interaction, depending on the trait alleles present in the fish. It is therefore logical to assume that family performances may be affected differently by the gene dosage effect of triploidy (Friars *et al*. 2001). Also, Harvey *et al*. (2017) showed the interaction of ploidy and parent-origin to be significant in differing dosage effects, suggesting that strain of *Salmo salar* should also be considered during investigations.

This highlights the difficulty of incorporating triploids into a breeding program, a necessity if they were to be implemented in aquaculture production. Due to their sterility, triploids cannot be selected based on their performance over generations, as is normally practised in diploids. The unpredictability of allelic expression is the limitation. A better understanding of genome and transcriptome response to triploidy is needed before industry can begin to understand how to select for triploid-specific traits within a breeding program.

1.4. Current challenges of triploid *Salmo salar*

1.4.1. Performance characteristics

Traditionally, triploids have been cultured under standard diploid conditions, including nutrition, health management and environmental conditions, which has seen conflicting reports on survival, growth and deformity prevalence. Diploids and triploids appear phenotypically identical, but the difference in physiology results in a higher sensitivity in triploids. Producing triploid eggs has previously resulted in a reduced survival rate compared to diploid siblings (McGeachy *et al*., 1995; O'Flynn *et al*., 1997; Fraser *et al*., 2013). Also, growth performance of triploids has been conflicting between reports, with inferior (McGeachy *et al*., 1995; O'Flynn *et al*., 1997; Friars *et al*., 2001), comparable (Cotter *et al*., 2000; Fraser *et al*., 2015) and superior growth (Oppedal *et al*., 2003; Fjelldal and Hansen, 2010; Taylor *et al*., 2011) when compared to diploid siblings. This may be a result of missing the optimal window for triploid success on occasions (Hussain *et al*., 1991) or likely caused by the physical shock applied to eggs. Also, investigations in triploid *Salmo salar* have generally used the last egg batches of the year when the quality of the oocytes are likely sub-optimal (Taylor *et al*., 2011). This has been a result of egg suppliers using premium eggs for routine diploid production and supplying excess stock for triploid investigations. Moreover, triploid induction requires further handling of the eggs during water hardening, a stage where mechanical handling acts as an additional stressor. If egg quality is reduced as a result of end of season spawning or biochemical changes as a result of post-ovulatory ageing, the triploid induction process may only further exacerbate effects of poor quality eggs.

1.4.2. Deformity of the vertebral column

The occurrence of skeletal deformities is not uncommon in *Salmo salar* aquaculture, likely a result of the absence of natural causes of mortality in weaker fish present in the wild e.g.

predation. Deformities of the vertebral column reported in *Salmo salar* can be categorised into deformity types according to Witten *et al*. (2009). The divisions encompass malformations ranging from minor vertebral shifts (Fig. 1.10a) to the more severe compressions (*platyspondyly*) and fusions (*ankylosis*) (Fig. 1.10b). Hansen *et al*. (2010) suggested the number of deformed vertebrae (dV) may influence the growth and the welfare of the individuals but also that the speed of growth may be a cause of increased dV. Harvest fish with <6 dV did not appear to have impinged growth or welfare, while those with >10 dV were considered as severe cases as growth was significantly reduced. Although these more severe cases of deformed individuals were observed in the sea water stage, it is likely these manifest prior to smoltification and are exacerbated with the faster growth post-seawater transfer (Fjelldal *et al*., 2012). It is not yet fully understood exactly why such deformities arise, but a contributing factor is likely poor mineralisation during development as an increased prevalence of skeletal deformities has been linked to nutritional deficiencies, of phosphorus (P) in particular, throughout the life cycle (Fjelldal *et al*., 2007; 2009; 2012; 2015). In teleosts, vertebral formation becomes visible at roughly 700 °days post-fertilisation as mineralisation of the notochord begins (Grotmol *et al*., 2005) and continues to grow as bone cells are differentiated around the notochord (Ekanayake and Hall, 1988). Development of strong vertebrae (57-60 in *Salmo salar*) involves efficient mineralisation and production of collagen fibres (Fig. 1.11.). Wargelius *et al*. (2010) reported a significantly higher expression of *mmp13* (matrix metalloproteinase 13) in vertebrae from fish with severe deformities. Moreover, Ytteborg *et al*. (2010a) reported downregulation of *col1a1* (collagen type I alpha 1 chain), *osteocalcin* and *osteonectin* correlating with vertebral deformities induced by elevated water temperature. These genes encode for important developmental stages in bone formation respective to remodelling of the extracellular matrix (ECM) and growth of hydroxyapatite crystals.

Poor vertebral development may result in skeletal deformities, posing serious welfare implications and potential growth depression which results in downgrading of harvest fish and ultimately unwanted economic loss (Fjelldal *et al*., 2012; Fraser *et al*., 2012; Benfey, 2015). Triploid *Salmo salar* have been reported to have increased prevalence of vertebral deformities (Sadler *et al*., 2001; Lijalad and Powell, 2009; Fjelldal and Hansen, 2010; Leclercq *et al*., 2011; Taylor *et al*., 2013; Fraser *et al*., 2015; Amoroso *et al*., 2016a; 2016b). As triploids have the potential for greater growth, this comes as no surprise as faster growing diploid *Salmo salar* also appear to result in an increase of deformity prevalence (Hansen *et al*., 2010; Fjelldal *et al*., 2012). The faster growth suggests an increased nutritional demand to reduce skeletal deformities as seen with increased P supplementation in triploid diets (Fjelldal *et al*., 2015; Smedley *et al*., 2016). Also, Fraser *et al*. (2015) identified sub-optimal egg incubation temperatures as being a risk factor in the development of vertebral deformity in later life. Furthermore, the influence of a third set of chromosomes on the regulation of skeletal health must be further elucidated.

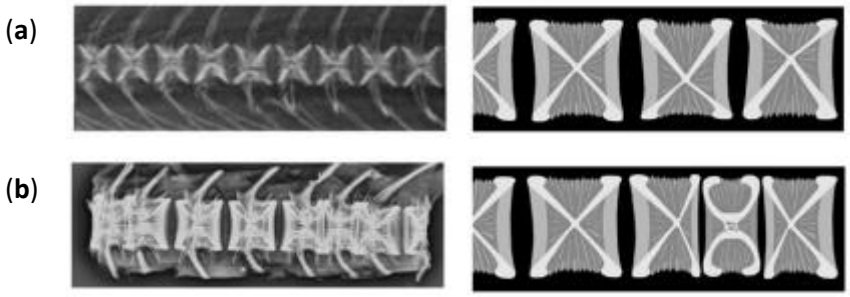


Figure 1.10. Radiograph images of (**a**) internal ventral shift and (**b**) compression and fusion of *Salmo salar* vertebrae. Images obtained from Witten *et al*. (2009).

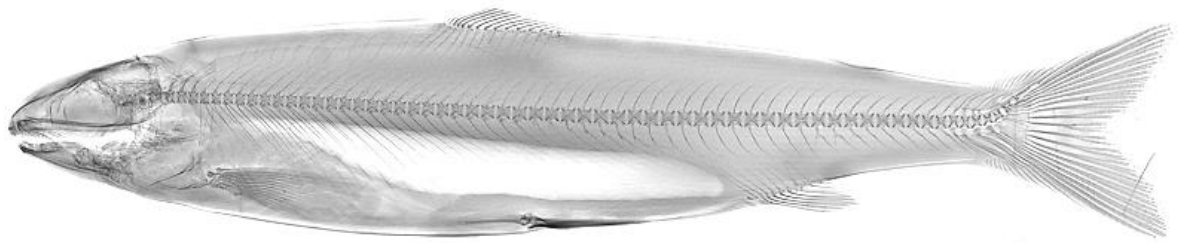


Figure 1.11. Radiograph image of *Salmo salar* smolt.

1.4.3. Deformity of the jaw

Lower jaw deformity (LJD) has also been associated with domesticated *Salmo salar* (Bruno, 1990; Quigley, 1995; McGeachy *et al*., 1996). The aetiology of LJD remains unknown but it may result from increased respiration rates alongside phosphorous and vitamin C deficiencies (Roberts *et al*., 2001). Increased respiration may be necessary with sub-optimal environmental conditions such as increased temperature which coincides with decreased dissolved oxygen (DO) availability. This specific skeletal anomaly involves shortening and / or downward curvature of the jaw (Fig. 1.12.) which can inhibit normal feeding and thus prevent optimal growth as well as a decreased respiratory efficiency (Lijalad and Powell, 2009). An increased prevalence of LJD has been found in triploid *Salmo salar* compared to diploid siblings (Sadler *et al*., 2001; Leclercq *et al*., 2011; Smedley *et al*., 2016) which has coincided with lower body weights and poorer condition (Taylor *et al*., 2013). Furthermore, LJD has been shown to affect respiration and swimming ability (Lijalad and Powell, 2009). Elevated temperature (Amoroso *et al*., 2016a) and P deficiency (Fjelldal *et al*., 2015; Smedley *et al*., 2016) have been linked to the development of LJD in triploids during the fresh water period. Elevated egg incubation temperatures have also been associated with an increase in LJD, more pronounced in triploids, suggesting that jaw pathologies likely manifest during embryogenesis and prior to any exogenous feeding (Fraser *et al*., 2015).

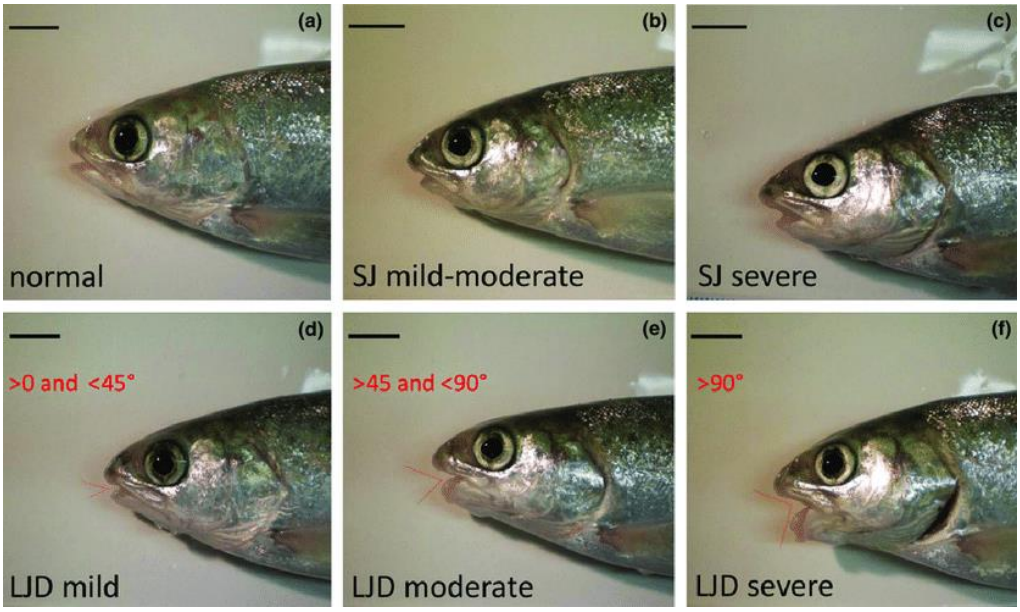


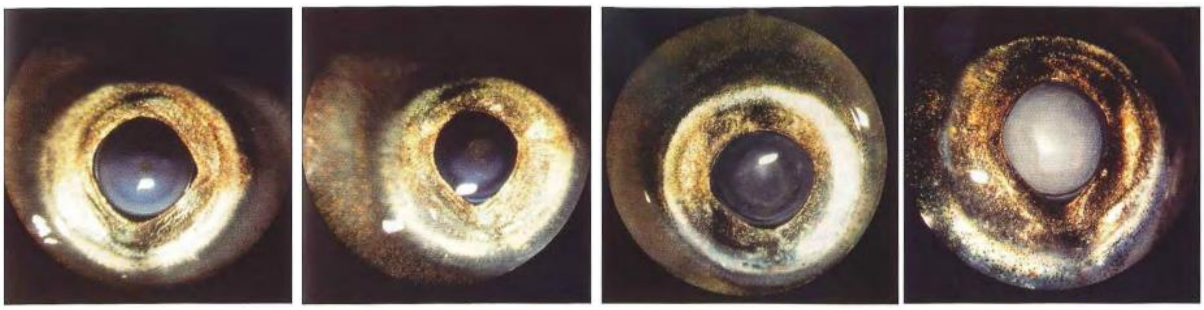
Figure 1.12. Examples of jaw deformities in *Salmo salar* individuals; (a) normal lower jaw, (b) moderate shortened lower jaw, (c) severe shortened lower jaw, (d-f) lower jaw deformity (LJD) with increasing severity described by angle of jaw curvature. Scale bars are 1 cm. Images taken from Amoroso *et al*. (2016c).

Amoroso *et al*. (2016b) were the first to suggest potential biomarkers for specifically LJD cases in *Salmo salar*, as *col2a1* (collagen type II alpha 1 chain) and *gphb5* (glycoprotein hormone subunit beta 5) were consistently downregulated in affected individuals. If collagen formation is disturbed, the jaw may still mineralise however with an incorrect structure. Whilst *col2a1* is known to be directly associated with collagen formation, the function of *gphb5* is not fully understood but has been linked with bone formation (Bassett *et al*., 2015). Similar to the reports of vertebral deformities, LJDs are more common post-seawater transfer but are also found in fresh water production stages (O'Flynn *et al*., 1997; Sadler *et al*., 2001) supporting the theory that jaw deformities also likely manifest in an early life stage.

1.4.4. Cataract formation

The development of cataracts is another concern in *Salmo salar* aquaculture. This eye pathology causes the lens to become cloudy (Fig. 1.13.) and therefore distorting vision which

likely impacts on normal feeding (Hargis, 1991; Ersdal *et al*., 2001). Cataract formation is known to occur during the freshwater (Rhodes *et al*., 2010; Sambraus *et al*., 2017) and also during the seawater (Waagbø *et al*., 2010) life stages. However, manifestation of cataracts is particularly prevalent during the smoltification process (Waagbo *et al*., 1996; Bjerkås and Sveier, 2004). During these stages, cataracts have been associated with elevated temperatures and accelerated growth (Taylor *et al*., 2015; Sambraus *et al*., 2017), parasitic occurrence (Seppänen *et al*., 2008) and histidine (His) deficiency (Breck *et al*., 2003; Waagbø *et al*., 2010; Remø *et al*., 2014; Taylor *et al*., 2015). Increased prevalence of cataracts in diploid *Salmo salar* corresponded to the exclusion of mammalian blood meal in the diets (Wall, 1998), suggesting that this histidine-rich source of protein suppressed cataract formation. His supplementation has been successful at controlling cataract formation in diploid *Salmo salar* and Taylor *et al*. (2015) concluded higher levels of His are required in triploid-specific diets to suppress the formation of cataracts. Like skeletal deformities, these results suggest that triploids are more prone to cataract formation. This may be a result of the increased cell size in triploids and the consequent diffusion implications. For example, N'-acetylhistidine (NAH), the histidine imidazole known to prevent cataract formation, aids water balance in the ocular lens (Remø *et al*., 2014) and normal function may be impeded with extended diffusion migration in triploid cells. Such abnormalities pose major welfare implications and equally cause reduction in product value (Menzies *et al*., 2002).



Score 1	**Score 2**	**Score 3**	**Score 4**
<10 % cover	10 – 50 % cover	50 – 75 % cover	>75 % cover
"slight"	"moderate"	"severe"	"intense"

Figure 1.13. Representation of increasing severities of cataracts in *Salmo salar* according to the cataract score scale reported in Wall and Bjerkås (1999).

Although genetic inheritance of the aforementioned anomalies should not be discounted (Gjerde *et al*., 2005), these studies suggested that triploidisation of *Salmo salar* eggs increases the probability of unpredictable performance characteristics, cataracts and skeletal deformities. Understanding the causative (environmental, nutritional, genetic) factors is a priority in order to develop preventive measures.

1.5. The importance of gamete quality

Embryogenesis is the most important life stage for development and therefore high quality reserves from the yolk-sac are essential. Optimal egg quality is a fundamental biological requirement for adequate development of fish, however, this tends to be highly variable (Kjörsvik *et al*., 1990). A mature female will spawn eggs containing nutrients for each individual to absorb throughout embryogenesis until they become fry and initiate exogenous feeding. A concern, particularly in aquaculture, which can affect the quality of the oocytes, is the increased ripening of the eggs. This can result from eggs remaining in the body cavity of the spawning dam for prolonged periods, otherwise known as post-ovulatory ageing (POA). This has been investigated in *Oncorhynchus mykiss* (Craik and Harvey, 1984; Springate *et*

al., 1984; Lahnsteiner *et al*., 2000; Aegerter and Jalabert, 2004; Rime *et al*., 2004) and more recently in *Salmo salar* (Mommens *et al*., 2015) and has been shown to induce morphological and biochemical changes to the oocytes which consequently results in decreased survival and growth performances. However, no studies to date have investigated the interaction of POA and triploidy in *Salmo salar*.

Given that triploids have higher dietary requirements, it is logical to assume that the yolk sac may be deficient for optimal triploid development throughout embryogenesis as this evolved to meet the needs of diploid development. Although phenotypically similar, the physiological characteristics between diploid and triploid *Salmo salar* are significantly different i.e. triploids appear to have a higher sensitivity to stressors. Taylor *et al*. (2011) suggested that a good egg quality is vital to triploid success. The triploid induction protocol involves increased handling of the oocytes during a sensitive life stage which is likely an increased stressor. Furthermore, it is common for triploids to be produced at the end of the stripping season in order to preserve normal diploid production, which may result in greater post-ovulatory ageing (Taylor *et al*., 2011). If using oocytes of sub-optimal quality for triploid induction, the consequences will likely be exacerbated with this increased physical handling involved. Therefore, there is a requirement to understand the impact of egg quality on the future survival and development of triploid *Salmo salar*.

1.6. Environmental conditions

Temperature and oxygen availability are important environmental factors for the performance and welfare of ectothermic animals, determining physiology and behaviour of *Salmo salar*. With the improvement of hatchery systems and recirculation aquaculture system (RAS) technology for juvenile culture, these factors can be easily controlled. Temperature is

regularly controlled in aquaculture at all life stages for several reasons, including manipulating rate of egg development, exploiting greater growth and altering PST timing.

1.6.1. Sub-optimal environment during embryogenesis

Understanding temperature requirements during embryogenesis is critical as suboptimal conditions during early ontogeny may have life cycle long impacts on performances and welfare (Elliot and Elliot, 2010). Temperature is known to impact on survival of eggs and hatchlings, as well as utilisation of the yolk sac (Klimogianni *et al*., 2004; Mueller *et al*., 2015). Hayes *et al*. (1953) described the temperature range for diploid *Salmo salar* eggs ranging from 0 to 16 °C, however, other reports suggested an increase in mortalities in diploid eggs incubated below 4 °C and above 8 °C (Peterson *et al*., 1977; Gunnes, 1979). Thus, the *Salmo salar* aquaculture industry has adopted the higher end of this optimal range to maximise production. However, incubation temperatures are regularly manipulated and used as a tool to exploit developmental rates to coincide with production planning, egg intake and stock management.

Gorodilov (1996) described the embryonic states of *Salmo salar*, including development of anatomical features from fertilisation to first feeding using somite pair formation as development milestones. In *Salmo salar*, the first 60 somite pairs form at the same speed under a constant temperature. Embryogenesis is temperature-dependant, as muscle differentiation in later life has been shown to vary when exposed to different egg incubation temperatures in *Salmo salar* (Stickland *et al*., 1988; Usher *et al*., 1994; MacQueen *et al*., 2008). Although initially causing a slower developmental rate, lower egg incubation temperatures have been shown to produce higher numbers of white muscle fibres and ultimately larger adult fish (Bjørnevik *et al*., 2003; MacQueen *et al*., 2008). Development of the teleost skeletal complex also begins during embryogenesis as the notochord sheath starts

to mineralise into segmented vertebrae after approximately 700 ºdays which coincides with increased *alp* (alkaline phosphatase) activity (Grotmol *et al*., 2003; 2005). From this point throughout life, the bones undergo constant remodelling which involves mature bone resorption and replacement with new bone (Lall and Lewis-McCrea, 2007). New bone tissue development undergoes organic and mineralisation development phases (Totland *et al*., 2011), ultimately comprising of type 1 collagen fibres, hydroxyapatite crystals which host calcium (Ca) and P, and other proteins such as osteocalcin, osteopontin and osteonectin (Lall and Lewis-McCrea, 2007; Ytteborg *et al*., 2012). Elevated incubation temperatures appear to compromise optimal skeletal development in *Salmo salar*, likely due to increased production of notochord tissue (Ørnsrud *et al*., 2004a; 2004b). Also, acute heat shocks during embryogenesis have resulted in an increased prevalence of vertebral deformities in later life (Wargelius *et al*., 2005). Expression of *hsp70* appeared to be developmental stage dependent when developing embryos were exposed to prolonged elevated temperature (12 ºC) and an increase of heart and swim bladder anomalies was observed (Takle *et al*., 2005). Moreover, *hsp70* expression was upregulated in heat shocked (16 ºC, 1 hr.) and cold shocked (1 ºC, 1 hr.) *Salmo salar* eggs at several embryogenic stages with the 45th somite stage showing highest expression (+12 fold and +4 fold, respectively). Furthermore, the cold shock induced vertebral deformities (14 %) at the 45th somite stage indicating increased sensitivity to temperature shocks during these particular developmental stages.

Hypoxia can also prevent efficient development during embryogenesis, therefore optimal oxygen conditions in the early life stages is of great importance (Hamor and Garside, 1976; Matschak *et al*., 1995). Hypoxia is defined as when the consumption of oxygen exceeds the supply. The impact of the relationship between hypoxic conditions and elevated temperatures remains relatively undiscovered, but in high temperatures, the affinity of oxygen to blood decreases (Irving *et al*., 1941). Sánchez *et al*. (2011) observed that a chronic

exposure of hypoxic conditions in *Salmo salar* alevins resulted in vertebral deformities, with longer exposure correlating with a higher intensity of anomalies including poorer bone mineralisation and notochord structure, ultimately reducing the body length of the fish.

In light of the importance of egg incubation temperature in *Salmo salar* aquaculture, recent results showed that triploid survival and later performances are compromised when eggs have been incubated at the commercially accepted temperature of 8 °C or above (Fraser *et al*., 2015). Irrespective of life stage, these results suggest that triploids have a significantly lower thermal optima than their diploid siblings. Generally, salmonids experience accelerated growth when exposed to elevated temperatures. This may be a result from temperature altering trypsin isozyme expression which is common in early life stages and has been shown to affect feed conversion and subsequent growth efficiency (Rungruangsak-Torrissen *et al*., 1998). However, high temperatures have shown to induce LJD (Amoroso *et al*., 2016b) and vertebral deformities (Grini *et al*., 2011) in adults and during embryogenesis in *Salmo salar* (Wargelius *et al*., 2005; Ytteborg *et al*., 2010a). Sub-optimal egg incubation temperatures have also resulted in deformities for both ploidy in later life stages with triploids being more prone. This further supports there is a difference in thermal tolerance between ploidy (Ørnsrud *et al*., 2004a; 2004b; Fraser *et al*., 2015). Therefore, it is evident that triploid-specific rearing conditions during embryogenesis need to be established for optimal production of triploid *Salmo salar*.

1.6.2. Sub-optimal environment impact during on-growing

Understanding the optimal rearing temperature for salmonids at different life stages is important to maximise productivity and welfare in aquaculture. Diploid adult *Salmo salar* exposed to temperatures of 15 to 19 °C displayed a reduction in feed utilisation and ultimately growth and a thermal optima of 13 °C was reported (Hevrøy *et al*., 2012; 2013).

However, Elliot and Elliot (2010) concluded that the optimum temperature in the UK was 15.9 °C and in Norway was 16.3 – 20 °C, suggesting that defining this requires multifactorial analysis as several synergistic factors may play a role i.e. genetic stock, fish size and other environmental parameters such as water quality. Interestingly, it has been shown that *Salmo salar* will avoid the upper end of their temperature range if given the choice (Stehfest *et al*., 2017). Similarly, fish in their natural environment have the ability to detect hypoxic areas and evade such conditions (Wu, 2002; Stehfest *et al*., 2017). However, in culture, adult *Salmo salar* are reared in pens which are suspended in a natural seawater environment, and conditions are thus dictated by the prevailing ambient environment and depth of the pens. Temperature fluctuations of less than 0.5 °C are recognised by fish and can impact on basic physiological processes such as respiration and metabolic rate (Murray, 1971; Kieffer *et al*., 1998; Farrell, 2002). DO levels in the environment can be unpredictably low due to pollution or eutrophication (Sánchez *et al*., 2007) and high temperatures can be a contributing factor to low DO content in a water body (Oppedal *et al*., 2011). Reduced oxygen availability can cause reduced feed intake (Remen *et al*., 2012) and an increase in disease susceptibility (Fisk *et al*., 2002; Sundh *et al*., 2010; Lund *et al*., 2017). Elliot (1981) reported a higher susceptibility to water temperature fluctuations in small *Salmo trutta* compared to larger *Salmo trutta*, concluding that the higher tissue mass in the larger fish offers a buffer for thermal equilibrium. This supports the theory that earlier life stages are more vulnerable to sub-optimal environmental conditions.

The environmental requirements of triploid *Salmo salar* are not yet fully understood, although they appear to differ significantly from that of diploids. Ultimately, the rearing conditions of both ploidy in recent studies have generally been the same and the results collated over the years have shown that the triploids require much different environmental conditions for optimal growth and welfare. Although some investigations have shown no

difference between ploidy in salmonids reared at elevated temperatures (Benfey *et al*., 1997; Galbreath *et al*., 2006), most reported a significantly poorer response from triploids (Ojolick *et al*., 1995; Altimiras *et al*., 2002; Hyndman *et al*., 2003; Hansen *et al*., 2015; Sambraus et al., 2017). These contrasting results remain unexplained, however the use of different genetic strains may be influential. The cause of deformity development is likely multifactorial and temperature is likely not the sole factor. The rapid growth itself as a response to high temperatures may be a physiological pressure and several other potential reasons have been explored. Fraser *et al*. (2012) described how a combination of low oxygen and sub-optimal temperature conditions were detrimental to triploid *Salmo salar*, preventing efficient oxygen delivery and/or utilisation. Similar to temperature tolerance, there are contrasting reports of survival and performance when comparing diploid and triploid salmonids in hypoxic environments (Ellis *et al*., 2013; Hansen *et al*., 2015). Optimal temperature for development correlates with the maximum aerobic scope, the highest temperature in which the fish can utilise available oxygen and perform basic functions optimally (Breau *et al*., 2011). At extremely low or high temperatures, this aerobic scope is compromised as oxygen utilisation becomes difficult. In response, energy is created through anaerobic metabolism through utilisation of carbohydrate such as muscle glycogen, or energy phosphates i.e. phosphocreatine or adenosine triphosphate (ATP) (Pörtner and Farrell, 2008). Ultimately, these forms of reactions result in depletion of important chemicals and an accumulation of unwanted metabolites such as lactate from glycolysis (Brett, 1971). When considering the aerobic scope of salmonids, it is likely that triploids possess a narrower capacity and have a lower tolerance to sub-optimal temperatures. Verhille *et al*. (2013) reported no difference in heart performance between adult diploid and triploid *Oncorhynchus mykiss* reared at 10 °C, but when held at 22 °C, 100 % of triploid fish showed evidence of cardiac arrhythmia, whereas this was only observed in 30 % of diploids. Conversely, Benfey and Bennet (2009)

did not report a difference between ploidy in egg and alevin stages although heart rate did increase in parallel, suggesting a comparable demand and delivery of oxygen when exposed to a given temperature.

1.7. The development of *Salmo salar* aqua-feeds

1.7.1. *Salmo salar* nutritional requirements

Salmo salar are a naturally fast-growing carnivorous species. Through intensification strategies including selective breeding, photoperiod manipulation and temperature regimes, the potential for this rapid growth is enhanced for better yield with a reduction on production duration. In order to sustain this growth, a nutritionally complete diet is required to support healthy muscle, skeletal and energetic developments supporting physiological and biochemical activities (Barton and Iwama, 1991). According to the National Research Council (2011), during the freshwater stage, *Salmo salar* require 42-50 % protein containing essential amino acids (EAAs). Protein is essential for muscle growth in *Salmo salar* (Grisdale-Helland and Helland, 1997; Bjørnevik *et al*., 2003) and vital for tissue repair, reproduction and as an energy source (Tibbetts *et al*., 2013). If protein availability is not optimal, *Salmo salar* will acquire additional energy from dietary lipids, commonly referred to as "protein sparing". Optimal lipid inclusion for *Salmo salar* diets is 16-24 % with an emphasis on n-3 long chain polyunsaturated fatty acids (LC-PUFAs); EPA and DHA (National Research Council, 2011). Considering optimal skeletal development, the most abundant minerals in *Salmo salar* bones are P and Ca. As P is a limiting factor in the environment, this must be delivered to the fish through the diet, whereas Ca is readily available and abundant in water (Lall and Lewis-McCrea, 2007). Aqua-feeds are developed with this in mind and in order to sustain rapid growth, levels of bioavailable dietary P are considered to ensure optimal development of bone in the fish (Bæverfjord *et al*., 1998;

Vielma and Lall, 1998). In addition to bone formation, P is vital for normal cellular function, i.e. for membrane, nucleic acid and ATP composition, further highlighting its importance (Skonberg *et al*. 1997).

1.7.2. Alternative aqua-feed ingredients

Demand for farmed *Salmo salar* heavily outweighs the availability of the raw materials, fishmeal (FM) and fish oil (FO), historically used to formulate feeds. The expansion of the *Salmo salar* aquaculture industry is a telling sign that alternative ingredients need to be sourced to sustain this positive growth. Traditionally, FM and FO sources have come from wild caught fish which are a finite and limited resource. Thus, the use of vegetable-derived proteins and oils has been considered due to their ease of production, low costs and greater availability. Numerous studies have detailed the utilisation difficulties surrounding the transition of salmonids onto plant based diets due to their carnivorous nature. Reduced palatability and utilisation of such ingredients ultimately reduces growth performance and currently has welfare implications (Francis *et al*., 2001; Gatlin *et al*., 2007). Also, a reduced digestibility of these ingredients can reduce growth ability (Refstie *et al*., 1998; 2000; Aslaksen *et al*., 2007; Wacyk *et al*., 2012). Consequently, several health implications have been associated with the inclusion of vegetable-based ingredients. Enteritis of the intestine has been observed in several studies resulting from increased inflammation (Bæverfjord *et al*., 1996; van den Ingh *et al*., 1996; Knusden *et al*., 2008; Urán *et al*., 2009; Penn *et al*., 2011; De Santis *et al*., 2015). Anti-nutritional factors (ANFs) have been highlighted as potential causes for these detrimental effects (Francis *et al*., 2001; Gatlin *et al*., 2007), however, developments in raw material processing, production of refined protein concentrates and amino acid balance have overcome many of these issues. The increased usage of such ingredients likely reduces the nutrient bioavailability compared to FM/FO

diets. The micronutrient profile of vegetable-based diets also differs greatly from a FM/FO diet. For example, B-vitamins are abundant in FM/FO diets however are low when using vegetable protein sources and this has consequences for the performance of the fish, as B-vitamin deficiencies are associated with reduced appetite and anorexia (Hansen *et al*., 2015). Therefore, in addition to supplementation of n-3 LC-PUFAs that are lacking in vegetable oils, a particular micronutrient package needs to be established when considering vegetable-based aqua-feeds (Hemre *et al*., 2016). An alternative strategy to promote acceptance and efficiency of such diets is using the concept of nutritional programming. This concept involves a nutritional intervention during sensitive developmental stages to stimulate particular metabolic processes (Lucas, 1998). This concept has shown success with acceptance of soybean meal (SBM) in *Oncorhynchus mykiss* (Geurden *et al*., 2013) and should be explored further when considering implementation of vegetable-based aqua-feeds.

1.7.3. Triploid *Salmo salar* nutritional requirements

It has been suggested that triploid *Salmo salar* may have different nutritional requirements to diploids. There have been contrasting reports on whether ploidy affects feed intake with some concluding no difference (Tibbetts *et al*., 2013) while others observed a higher feed intake in triploids (Oppedal *et al*., 2003; Burke *et al*., 2010). However, utilisation of the nutrients is of equal if not greater importance. No ploidy difference was found in metabolic and digestive utilisation of feeds in *Oncorhynchus mykiss* juveniles (Olivia-Teles and Kaushik, 1990). Burke *et al*. (2010) also reported no ploidy difference when assessing P utilisation in *Salmo salar* parr, however this particular feeding trial began when the fish were 45 g and may have failed to identify an early requirement of P supplementation in triploids. Recent studies have shown improved P utilisation and improved growth in parr when supplementation began from first feeding (Fjelldal *et al*., 2015; Smedley *et al*., 2018). Also, a greater requirement for

dietary His was observed in triploid *Salmo salar* to reduce the prevalence of cataracts (Taylor *et al*., 2015; Sambraus *et al*., 2017). Prior to the supplementation, the triploid fish had lower basal levels of N'-acetylhistidine (NAH) in the ocular lens, which is known to prevent the formation of cataracts suggesting they have a greater requirement (Taylor *et al*., 2015). With the capacity for faster development, it is logical that triploids may require more nutrients to support faster tissue growth as observed with improved growth and reduced skeletal deformities when dietary phosphorous bioavailability was increased (Peruzzi *et al*., 2018). The greater nutritional requirements may be a result from the different physiological and morphological characteristics of tissues and organs in triploids. Peruzzi *et al*. (2015) described a difference in the *Salmo salar* digestive tract between ploidy, with triploids having a shorter gut length and possessing fewer pyloric caeca, which has likely implications for nutrient absorption. Similarly, this may also be a result from the alteration to the cellular architecture observed with larger cells (Piferrer *et al*. 2009). It is therefore likely that triploids do have a greater nutritional requirement and would benefit from nutritional supplementation. To date, most triploid – diploid comparative performance studies have fed both ploidy a "standard" diploid diet which may be a contributing factor to the increased deformity prevalence that has been observed. More recent investigations have shown that with supplementation of particular nutrients i.e. P and His, these deformities can be reduced significantly (Fjelldal *et al*., 2015; Taylor *et al*., 2015; Smedley *et al*., 2016; 2018).

Given that triploids are generally not yet adopted widely in commercial practice, studies on plant-based alternative diets are scarce. However, with the potential of commercial triploid implementation, research must keep pace with the advancements in *Salmo salar* aqua-feeds. This includes the adoption of alternative feed ingredients such as vegetable-derived proteins and oils and the capacity of triploid *Salmo salar* to utilise them efficiently.

1.8. Aims of thesis

It is clear that there are several advantages of using triploid *Salmo salar* in aquaculture, namely their growth potential and reproductive containment. Before triploidy can gain widespread commercial acceptance and can be fully implemented, the key issues associated with welfare (increased mortality, deformity prevalence and growth variation) must be addressed. Although advances in research have reduced mortality and deformity prevalence and subsequently improved triploid yield and productivity, results highlight that the remaining increased mortalities, unpredictable growth performances and increased skeletal deformities compared to diploids may be originating from the earliest stages of life which is not yet fully understood. The principal aim of this thesis is to understand and refine early life stage environmental conditions for triploid *Salmo salar* in order to address sub-optimal traits reported later in life and produce more robust individuals. This research will consider a range of factors including egg incubation temperatures and egg quality. Also, early nutritional programming in first feeding diploids and triploids will be investigated to assess for the first time if *Salmo salar* can be successfully conditioned to better accept a vegetable-based diet in later life. Furthermore, understanding the impact of the second set of maternal chromosomes is a necessity to recognise how the combination of triploidy and early life culture conditions affects the development of the fish.

This thesis is organised around four experimental chapters with specific scientific objectives:

TEMPERATURE OPTIMA DURING EGG DEVELOPMENT DIFFERS BETWEEN PLOIDY IN ATLANTIC SALMON (*Salmo salar* L.).

To (i) investigate the impact of different egg incubation temperatures on growth, muscle development and skeletal deformities, and (ii) determine if a change in temperature during egg incubation affects this development (**Chapter Two**).

THE IMPACT OF POST-OVULATORY AGEING ON THE DEVELOPMENT OF DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.).

To (i) define any interaction between post-ovulatory ageing and subsequent survival, deformity prevalence and growth variation, and (ii) characterise any biomarkers as potential candidates of ovarian fluid or egg quality change in response to post-ovulatory ageing (**Chapter Three**).

EARLY NUTRITIONAL INTERVENTION CAN IMPROVE UTILISATION OF VEGETABLE-BASED DIETS IN DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.).

To determine if the concept of nutritional programming functions in both diploid and triploid *Salmo salar* fed a vegetable-based diet (**Chapter Four**).

DEVELOPMENT OF A ROBUST SINGLE ERYTHROCYTE ISOLATION TECHNIQUE FOR ANALYSIS OF ALLELIC-SPECIFIC PATTERNS OF INHERITANCE IN TRIPLOID ATLANTIC SALMON (*Salmo salar* L.).

To determine the feasibility of identifying and measuring triploid allele-specific expression using polymorphic microsatellite markers to understand gene regulation post-triploidisation (**Chapter Five**).

CHAPTER TWO

TEMPERATURE OPTIMA DURING EGG DEVELOPMENT DIFFERS BETWEEN PLOIDY IN ATLANTIC SALMON (*Salmo salar* L.)

2.1. Introduction

Fish are ectothermic and their metabolism and development is directly influenced by their external temperature profile. For example, temperature regimes are routinely adjusted in aquaculture to promote growth and manipulate production windows. The ontogeny of embryonic stages, previously described for *Salmo salar* (Gorodilov, 1996), is directly influenced by environmental conditions, in particular, water temperature. Higher incubation temperatures lead to accelerated development (Hayes *et al*., 1953; Gorodilov, 1996) in relation to an increased metabolic rate (Clarke and Johnston, 1999). For example, eggs incubated at 5 °C can take twice as long to develop than if incubated at 10 °C (Usher *et al*., 1994). Diploid *Salmo salar* eggs can tolerate temperatures from 0-16 °C (Hayes *et al*., 1953), however, early reports suggested an increase in mortalities in diploid eggs incubated below 4 °C and above 8 °C (Peterson *et al*., 1977; Gunnes, 1979). Thus, an upper thermal maximum egg incubation temperature of 8 °C has been adopted in commercial *Salmo salar* hatcheries. Despite this, incubation temperatures are regularly manipulated in commercial brood stock facilities to meet the requirements of customers requesting stock at particular times of particular developmental stages. Furthermore, incubation temperatures during the hatchery phase post-supply may also be manipulated in order to align batches with stock management plans. Elevated temperatures outwith the tolerable range of *Salmo salar* eggs and/or manipulation of temperatures to fit within a production strategy may be useful for increasing developmental rate short term, however, subsequent negative consequences must be considered. A previous study showed that incubating diploid *Salmo salar* eggs at an elevated temperature (i.e. 10 °C) until the eyeing stage can produce larger fish throughout the freshwater cycle (MacQueen *et al*., 2008). However, by contrast those incubated at 2 and 5 °C in the same study had higher growth rates post-sea water transfer and the 5 °C group finished the trial with the same weight as those from the higher temperature group. Somatic

growth can be associated with altered muscle development resulting from early exposure to particular temperatures. Lower temperatures appear to promote muscle fibre recruitment in fish with preference towards hyperplasia over hypertrophy as reported with higher myotome counts (Hempel and Blaxter, 1961; Stickland *et al*., 1988; Usher *et al*., 1994; Nathanailides *et al*., 1995; Johnston and McLay, 1997; Johnston *et al*., 2003). A tendency towards hyperplasia explains the initial smaller size in the 5 °C fish and suggests potential for these fish to growth faster and larger at a later life stage (MacQueen *et al*., 2008). Lower temperatures may equally reduce skeletal abnormalities in *Salmo salar*. Recent studies have shown a correlation between higher temperatures and low survival and increased prevalence of jaw (Fraser *et al*., 2015; Amoroso *et al*., 2016a; 2016b) and vertebral (Vågsholm and Djupvik, 1998; Wargelius *et al*., 2005; Ytteborg *et al*., 2010a) deformities. There is little known about the underlying mechanisms of skeletal abnormalities as a result of elevated temperatures, however it is likely that increased muscle mass is poorly supported by under-mineralised bone found in faster growing *Salmo salar* (Fjelldal *et al*., 2006).

Triploid *Salmo salar* have been reported to be initially smaller than diploid siblings but have increased growth potential in later life (Benfey, 2015). This may be a result of a higher occurrence of hyperplasia. However, an increased prevalence of skeletal deformities has also been reported which is likely associated with faster growth (Benfey, 2015). In order to support skeletal reinforcement for this enhanced growth, it is likely that triploids have higher nutritional requirements. Such results have elicited investigations into comparative nutritional requirements between ploidies, with particular focus on phosphorous (Fjelldal *et al*., 2015; Smedley *et al*., 2016). Supplementation of nutrients and the development of triploid-specific diets are certainly reducing prevalence of deformities, however, while dietary approaches have been effective in reducing the occurrence of malformation in triploids, associated pathologies still remain. As such, many of these malformations are proposed to originate

from early developmental stages prior to first feeding and have been hypothesised to be related to egg incubation temperature regimes. This is likely a result from the thermal stress during a sensitive and vulnerable life stage. An upregulation of heat shock protein, *hsp70*, was observed in eggs with both a prolonged incubation temperature outwith the tolerable range (12 °C) and when exposed to an acute temperature shock (1 °C or 16 °C) for one hour (Takle *et al*., 2005). This defence mechanism suggests a particular sensitivity to thermal change during embryogenesis.

Thermosensitivity of triploid eggs during embryogenesis may therefore be of particular importance. Fraser *et al*. (2015) showed that incubating eggs at 6 °C, rather than 8 or 10°C, from fertilisation to first feeding can reduce the prevalence of skeletal deformities in triploids, supporting a potential increased thermosensitivity in triploids compared to diploid siblings. No studies to date have investigated how temperature changes during embryogenesis may affect survival and growth performance of triploid *Salmo salar*. Further, bone regulatory mechanisms specific to triploid *Salmo salar* have not been studied. However Ytteborg *et al*. (2010b) described upregulation of genes associated with bone formation (e.g. alkaline phosphatase, *alp*; collagen type 1 alpha 1 chain, *col1a1*; osteocalcin; *ocn*; bone morphogenetic protein 2, *bmp2;* and bone morphogenetic protein 4, *bmp4*) in diploids in response to elevated temperatures during development. Furthermore, Wargelius *et al*. (2005) found that gene expression associated with vertebral development was altered in *Salmo salar* embryos exposed to acute and chronic sub-optimal temperatures. With increased sensitivities, it is likely that triploids require lower incubation temperatures to promote optimal skeletal development. This said, the adoption of lower hatchery temperatures to ensure optimal performance later in production will inevitably slow down fish development and extend current production cycles.

The objective of the present study was to compare the growth, muscle development and skeletal deformities in diploid and triploid *Salmo salar* incubated at different temperatures during the egg / alevin incubation window prior to first feeding. Response to different temperatures was monitored throughout embryogenesis in diploid and triploid siblings and later during freshwater rearing under ambient conditions. Furthermore, each experimental temperature was investigated as a constant temperature (fertilisation to until first feeding) or a temperature switch (at 400 °days post-fertilisation until first feeding thereafter) to compare the impact on development and growth.

2.2. Materials and methods

2.2.1. Fish stock and culture conditions

On 8th December 2014, green eggs from three unrelated dams (2-sea winter) and milt from three unrelated sires (2-sea winter) were provided by Landcatch Ltd. (Ormsary, UK) and transferred to the Institute of Aquaculture (University of Stirling, UK). Eggs were fertilised (30 secs. mixing milt, 60 secs. rinse with 8 °C freshwater) creating three full-sib families and then divided (50 : 50 from each of the three full-sib families) for ploidy differentiation and placed into a water bath at 8 °C prior to triploid induction. Triploidy was induced in one group (655 bar of hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. post-fertilisation) according to Smedley *et al*. (2016), while the others did not receive a hydrostatic shock and were maintained as diploid controls. Post-water hardening, eggs (~3,000 ploidy^{-1} treatment^{-1}) were further divided into three incubation temperatures (5.8 ± 0.7, 8.3 ± 0.1 or 10.8 ± 0.1 °C, referred to as 6, 8 and 11) and incubated in triplicate in aluminium egg trays within temperature-specific trough systems (15 mL sec^{-1} flow) in darkness. At 400 °days, sub-groups from 6 and 11 °C (~800 eggs ploidy^{-1} temperature^{-1}) were transferred to 0.3 m^2 recirculation system (RAS) tanks and incubated at a constant temperature of 8 °C until 1 g

(Fig. 2.1.). These groups are termed short-term (ST) incubation. The remaining eggs were kept at their respective incubation temperatures until first feeding before transferred to 0.3 m^2 tanks at a constant temperature of 8 °C until 1 g and were termed long-term (LT) incubation. A total of five treatments per ploidy (6LT, 6ST, 8LT, 11ST, and 11LT) were created, with 8LT being the control temperature as routine in commercial practice. All temperature treatments were triplicated during the incubation up until first feeding, however for the long term performance assessment, fry were pooled and single treatment groups were monitored. At 1 g, all fish were transferred to the Niall Bromage Freshwater Research Unit, Stirling, UK and reared in 10 x 1.6 m^3 (5 $ploidy^{-1}$) circular tanks under ambient conditions until smolt (24th April 2016). Due to the egg incubation temperature differences and subsequent change in developmental speed, the number of feeding days from first feeding until the end of the trial differed between treatments (6LT, 331d; 6ST, 364d; 8LT, 386d; 11ST, 395d; 11LT, 399d). Diploids were given a standard commercial diet (INICIO Plus) and triploids were given a triploid-specific diet (INICIO TRI-X) in accordance with the manufacturer's guidelines (BioMar Ltd, UK).

2.2.2. Verification of ploidy

To confirm ploidy status, red blood smears were prepared from samples taken from the caudal peduncle of euthanised fish (n = 20 fish $treatment^{-1}$ $ploidy^{-1}$; 49.9 ± 24.6 g). Air dried slides were fixed in 100 % methanol and then placed into Giemsa stain for 10 mins. Slides were digitised using a slide scanner at 20x magnification (Axio Scan Z1, Zeiss) and erythrocyte length and diameter was determined by Fiji software (ImageJ). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm and a total mean taken for presumed diploid and triploid fish. Diploid control groups had significantly smaller erythrocyte nuclear lengths, with no overlaps with the triploid groups (2N, 8.0 ± 0.6 μm; 3N,

10.4 ± 0.9 µm) confirming that all fish that were subjected to hydrostatic pressure shock were likely to be triploids.

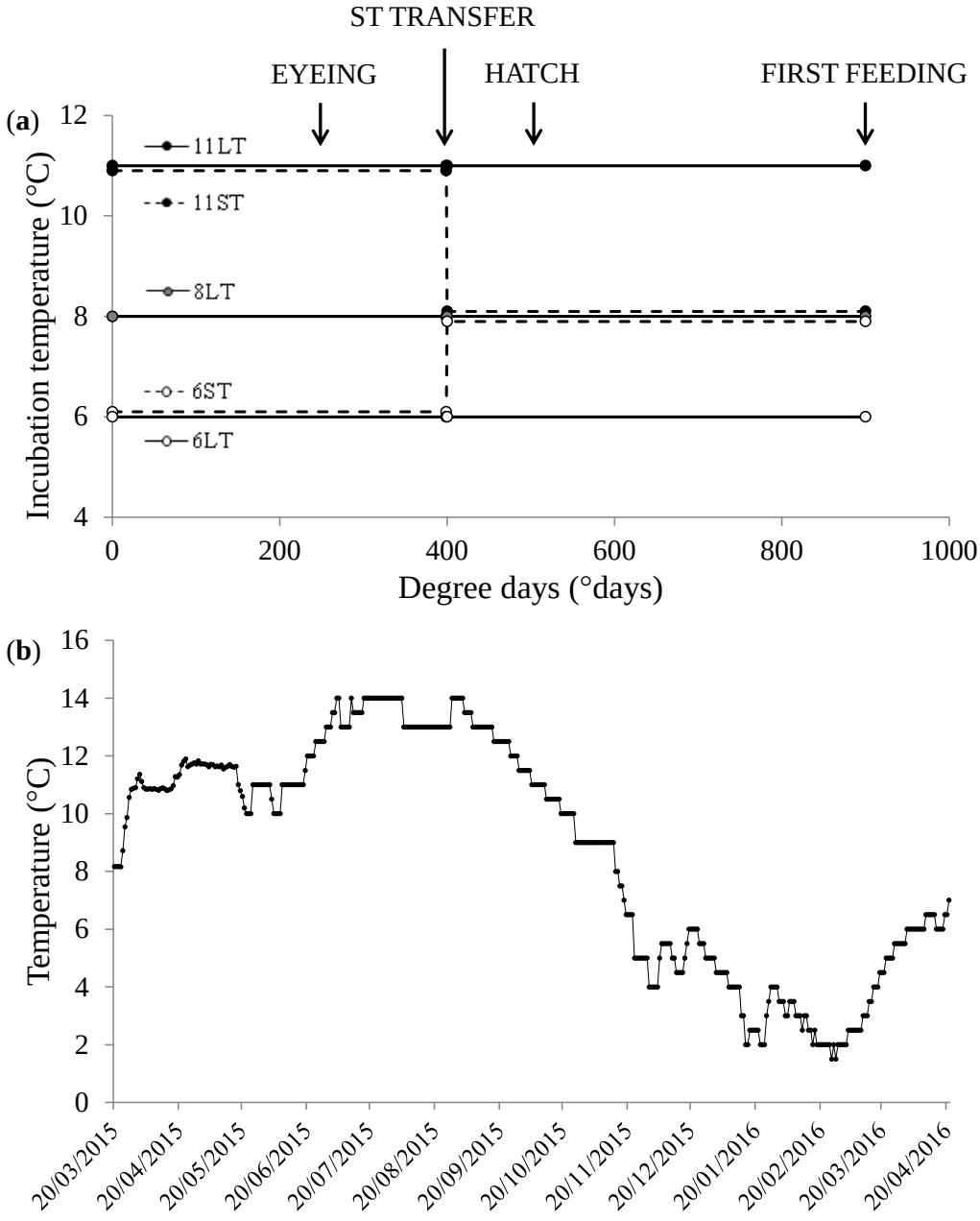


Figure 2.1. (**a**) Egg incubation temperature experimental design including LT temperature regimes represented by solid lines (11LT; black circles, 8LT; grey circles and 6LT; white circles) and ST temperature regimes represented by dashed lines (11ST; black circles and 6ST; white circles) and (**b**) average daily temperatures during subsequent grow on from first feeding until smolt.

2.2.3. Sampling procedures

Throughout the embryogenesis period, samples were taken from each LT incubation temperature treatment at hatch, 600 °days and 750 °days to investigate yolk-sac lipid utilisation in alevins. Only LT regimes were used to assess lipid utilisation as this parameter was not originally included in the sampling plan. Each individual alevin had the yolk sac carefully removed and both body weights (BWa) and yolk sac weights (YSW) were measured. Separate pools of body and yolk sac (n = 3; 5 individuals $pool^{-1}$ $treatment^{-1}$) were created within each treatment and stored in 5 mL chloroform: methanol (2:1, v:v) with addition of antioxidant butylated hydroxytoluene (BHT; 0.01 %) at -20 °C until lipid analysis.

At smolt, fish were randomly selected and sacrificed using an overdose of anaesthetic (Tricaine, Pharmaq; 1000ppm) and frozen flat at -20 °C for later deformity analysis (50 individuals $treatment^{-1}$) and to assess whole carcass mineral composition (n = 3; 3 individuals $pool^{-1}$ $treatment^{-1}$). Additional smolts were selected and sacrificed (Tricaine, Pharmaq; 1000ppm) for myogenic morphology assessment (6 individuals $treatment^{-1}$). A 6 mm thick cross section was excised from the trunk immediately anterior to the dorsal fin. Steaks were then mounted onto cork using optimal cutting temperature (O.C.T.) compound and frozen in isopentane cooled to -170 °C in liquid nitrogen and subsequently stored at -70 °C until processing.

To investigate expression of genes associated with lipid utilisation, samples were taken as whole individuals during embryogenesis at 200 °days, eyeing, and first feeding (6 individuals $treatment^{-1}$ developmental $stage^{-1}$). Samples for expression of genes associated with muscle and bone formation were collected as whole individuals at first feeding (6 individuals $treatment^{-1}$). At smolt, muscle-related genes were analysed in dissected muscle from the Norwegian Quality Cut (NQC) region and the vertebral column under the dorsal fin

(~10 vertebrae) was sampled for bone associated genes (6 individuals treatment^{-1}). All samples for gene expression analysis were collected into 'RNA Later' until processing.

2.2.4. Lipid analysis

Total lipid (TL) was extracted according to Folch *et al*. (1957). Approximately 0.25 g of eggs were homogenised in 10 mL chloroform: methanol (2:1, v:v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough). Lipids were separated from contents by adding 5 mL of potassium chloride (KCl; 0.88% w:v) and left on ice for 1 hr. The upper layer was aspirated and the lower layer was dried under nitrogen. TL content of each sample was determined gravimetrically after 12 hrs. in a vacuum desiccator. Unless stated otherwise all reagents were analytical grade purchased from Sigma-Aldrich, USA, or Fisher Scientific, UK.

2.2.5. Growth assessment

Growth performance was assessed at first feeding (i; initial) and smolt (f; final). Following 24 hrs. of fasting, 30 individuals per tank were sedated (Tricaine, Pharmaq; 50ppm) for body weight (BWi and BWf) and fork length (FL) measurements. Growth rate was calculated using the thermal growth coefficient (TGC, % BW °C d^{-1}).

2.2.6. Myogenic morphology assessment

Muscle fibre analysis was investigated according to Johnston *et al*. (1999). A cryostat (Leica CM1860 UV, Leica Biosystems, Nussloch, Germany) cooled to -18 °C was used to process 7 µm sections which were mounted onto charged microscope slides and stained with haemotoxylin and eosin (H&E). Slides were digitised (Axio Scan Z1, Zeiss) and subsections (2 x 1 mm areas) were selected from seven different myotome blocks to ensure analysis of

~1000 muscle fibres from different areas across the cross section. Morphometric analyses were carried out using Fiji Software (ImageJ).

2.2.7. Radiological deformity analysis

Right lateral radiographs were taken of smolts from each treatment group (50 individuals treatment^{-1}) using a Faxitron UltraFocus Digital Radiography System (Faxitron Bioptics LLC., Arizona, USA) exposing individuals for 1.8 mA at 26 kV. Radiographs were digitalised (AGFA CR35-X) and subsequently examined using ClearCanvas Workstation (Personal Edition, Synaptive Medical, Toronto, Canada) by two independent blind evaluations. Severity of deformities was classified according to Hansen *et al*. (2010).

2.2.8. Mineral composition

Mineral compositions were determined from whole smolt carcass using the nitric acid (HNO_3) digestion technique. Briefly, pools of fish were homogenised and oven dried at 75 °C for 24 hrs. and subsequently powdered using a mortar and pestle. Samples were digested in Kheldal digestion tubes with 69 % nitric acid using a MARS microwave digestion system (CEM MARSXpress, CEM ltd., Buckingham, UK) using the following program: 10 mins. heating phase to 190 °C, maintain 190 °C for 20 mins., cooling phase to 21 °C for 60 mins. Samples were then diluted with distilled water to 2 % HNO_3 and analysed for mineral content via Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo X series II; Collision cell technology). Due to a technical error, data for 11ST diploid and triploid groups could not be determined.

2.2.9. Gene expression analysis

2.2.9.1. RNA extraction and cDNA synthesis

Whole individuals at the first feeding stage and dissected vertebrae and muscle tissue from smolts were kept in 'RNA Later' until processing. Samples were added to TriReagent® (Sigma-Aldrich, Gillingham, UK) at a ratio of 100 mg mL^{-1} reagent according to the manufacturer's protocol. Total RNA (totRNA) concentration was determined using a Nanodrop spectrophotometer (ND-1000; Labtech Int., East Sussex, UK) and quality of samples was confirmed by assessing the integrity of 28S and 18S ribosomal RNA (rRNA) with agarose gel electrophoresis (1 %). To eliminate genomic DNA (gDNA) contamination, samples were treated with DNA-free™ (Applied Biosystems, UK) as per the manufacturer's guidelines. cDNA was subsequently synthesised using 1 µg total RNA and a high capacity reverse transcription kit (without RNase inhibitor) (Applied Biosystems, UK). Final cDNA 10 µL reactions were diluted 1:10 in nuclease-free water to a total volume of 100 µL and 2.5 µL was used for each 10 µL (2.5 ng μl^{-1}) qPCR reaction.

2.2.9.2. Sequence information and primer design

Sequence-specific primers for all genes analysed were based on registered sequence data in *Salmo salar* from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) (Table 2.1.). Sequence information was then subjected to BLAST analysis against a *Salmo salar* genome and transcriptome (NCBI). Primer pairs for lipid biosynthesis, muscle formation, bone formation and development were manufactured by MWG Eurofins Genomics (Ebersberg, Germany) and sequences with associated information are detailed in Table 2.1. Each primer product was purified by GeneJET PCR Purification Kit (Thermo Scientific, UK) according to manufacturer's instructions. Products were then cloned using the pGEM®-T Vector System (Promega) and plasmids were harvested using a

GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, UK) according to manufacturer's instructions. Each resulting plasmid was subsequently sequenced via LIGHTrun™ sequencing (GATC, Cologne Germany) to confirm identity. Plasmid was then linearised by enzymatic digest and standards for qPCR assays were generated using a serial dilution from 10^8copies to 10 copies of each gene investigated.

Table 2.1. Primer sequences of target genes and associated information used for real time qPCR mRNA expression investigation in diploid and triploid *Salmo salar*. T_A: annealing temperature.

Gene name	ID	Forward sequence 5' – 3'	Reverse sequence 5' – 3'	T_A °C	Accession no.
Lipid utilisation					
Fatty acid synthase	*fas*	GTGAGCCCTGCCTCTTTTCT	AGAGCTTGCTTGCCTGTGAT	59	CK876943
Liver X receptor	*lxr*	GCCGCCGCTATCTGAAATCTG	ATCCGGCAACCAATCTGTAGG	58	NM_001159338
Sterol regulatory element binding transcription factor 1	*srebp1*	CCAACATGGCTACCGTCACT	ACCGCTCGGAAAGTGTTCAA	56	NM_001195818.1
Myogenesis					
Insulin-like growth factor 1	*igf1*	CAAAACGTGGACAGAGGCAC	TCCCTGTCCGTTAGCTTCTG	56	M81904
Insulin-like growth factor 1 receptor	*igf1r*	GTCGGCCAGCATGAGAGAGA	ACGGGTCTTTAGCCCGTAGT	58	EU861008
Insulin-like growth factor 2	*igf2*	TTGCGCCGGACTTTTAACTG	ATCTTGCATCGACCCTCACA	56	AY049955
Insulin-like growth factor binding protein related protein	*igfbprp*	GTGCGTTAAGAGCGACAAGA	CAATGACAGGTGTTGGG	56	EF432866.1
Myogenic factor 5	*myf5*	GCCTAAGGTGGAGATCCTGC	AGTCAACCATGCTGTCGGAG	57	DQ452070
Myogenic differentiation 1	*myod*	ACTCCAAATGCTGATGCCAGA	CTACCCTCCTGAACTGATAAC	56	NM_001123601.1
Bone homeostasis and mineralisation					
Alkaline phosphatase	*alp*	ATCCTGCTCATCTGCTCCTGC	AGTATTCGTGCTGCCGTCACT	56	FJ195609
Bone morphogenic protein 2	*bmp2*	TCTCATATCGCTGCTGG	TCCGAACATATTGAGCAGCC	56	BTO59611
Bone morphogenic protein 4	*bmp4*	GAACTCTACCAACCACGCCA	CGCACCCTTCCACTACCATT	56	FJ195610
Collagen type 1 alpha 1 chain	*col1a1*	TGGTGAGCGTGGTGAGTCTG	TAGCTCCGGTGTTTCCAGCG	56	FJ195608
Collagen type 2 alpha 1 chain	*col2a1*	TGGTCGTTCTGGAGAGACT	CCTCATGTACCTCAAGGGAT	56	FJ195613
Matrix metallopeptidase 13	*mmp13*	CCAACCCAGACAAGCCAGAT	GCTCTGAGAGTGGATACGCC	56	DW539943
Osteocalcin	*ocn*	GACTCCTCTACCTCCACTGC	AATGATCCCAGCTGTGTCCA	60	FJ195611
Osteopontin	*opn*	CTTACTGAGGTGGCCCCTGT	GCTGTCCGATGTTGGGTCTG	57	AF223388.1
Osteonectin	*sparc*	TCCTGCCACTTCTTTGCCCA	CAGCCAGTCCCTCATACGCA	56	FJ195614

2.2.9.3. Quantitative PCR (qPCR)

Absolute quantification qPCR assays were designed for genes described in Table 2.1. and performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009). qPCR was performed in duplicate on individual samples using a Lightcycler® 480 II (Roche Diagnostics, West Sussex, UK) with relevant primer pairs, cDNA template, nuclease-free water, and Luminaris™ Color HiGreen qPCR Master Mix (Applied Biosystems, UK) in a total reaction volume of 10 µL. Amplification was achieved in 384-well plates and conducted in a thermo cycling programme consisting of a pre-incubation of 95 °C for 10 mins. followed by 40 cycles of; 95 °C for 15 secs., T_A °C for 30 secs. and 72 °C for 30 secs. This was followed by a temperature ramp from 60 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. Quantification was achieved by a parallel set of reactions containing standardised plasmids described above.

Results from cDNAs were normalised by relating expression data to geometric mean of reference genes; *β-actin* and *elf-α*. Gene activity was expressed as a fold change from 8LT (current industry incubation temperature) specific to each ploidy.

2.2.10. Statistics

Data were analysed using Minitab (Version 17.0, Minitab Inc., Pennsylvania, USA) statistical analysis software. Normality and homogeneity of variance in the data were confirmed using Kolmogorov-Smirnov and Levene's tests, respectively. Two-way ANOVAs were used to test ploidy, temperature treatment and their interaction. Post-hoc tests were determined by Tukey's multiple comparisons. When necessary, data were logarithmically transformed and percentage data were arcsine transformed to achieve normality. If required, non-parametric

Kruskal-Wallis with Dunn's multiple comparisons tests were used to compare gene expression data within each ploidy. Where there was single replicates, approximate 95% confidence intervals were estimated according to the central limit theorem (CLT; within 1.96 standard deviations of the mean) and overlapping standard deviations between treatments were considered not significantly different. Use of this approach is referred to as "Analysis of CLT" throughout this chapter. The relationships between final fibre number (FFN) and fork length (FL), and fibre area and FL for each ploidy was determined using linear regression analysis. All significance was accepted at $p < 0.05$.

2.3. Results

2.3.1. Mortality

The cumulative mortality throughout the duration of the trial shows the net effect of the egg incubation temperatures (Fig. 2.2.). Within all temperate treatments, triploids had a higher mortality than their diploid counterparts. Irrespective of ploidy, there was an increase of mortality in response to increasing egg incubation temperatures and duration of temperature (6LT < 6ST < 8LT < 11ST < 11LT).

Stage specific mortality data are shown in Table 2.2. There was no ploidy or temperature effect on mortality from fertilisation to 400 °days.

During the following period (400 °days to hatch), with the inclusion of the ST regimes, there was no ploidy difference under 6LT, 6ST and 11ST, however triploids had a higher mortality than diploids under 8LT and 11LT. In diploids, mortality from 6LT, 6ST and 8LT was comparable, however was significantly increased in 11ST and again in 11LT (6LT = 6ST = 8LT > 11ST > 11LT). In triploids, mortality at 6LT and 6ST were comparable however was significantly increased in 8LT, 11ST and 11LT. Mortality in 11LT was also significantly higher than in 8LT and 11ST (6LT = 6ST > 8LT = 11ST > 11LT).

During the hatch to first feeding period, the only ploidy difference was observed in 11LT with triploids having significantly greater mortality than diploids. Within diploids, there was a significant increase in mortality from 6LT to 6ST to 8LT. Diploid alevin mortality in 11ST was comparable to 6ST and 8LT, and was significantly higher at 11LT compared to all other temperature treatments. Triploids showed a similar trend with mortality significantly increasing from 6LT to 6ST to 8LT. Triploid alevin mortality was comparable in 8LT and 11ST, and was significantly higher at 11LT compared to all other temperature treatments.

During the final period (first feeding to smolt), triploids had a higher mortality than diploids under 6ST, 8LT and 11ST regimes, but comparable under 6LT and 11LT. Both ploidy showed an increase in mortality from 6LT to 6ST to 8LT and had comparable mortality between 8LT and 11ST. There was a decrease in mortality in 11LT in both ploidy, comparable to that in 6ST.

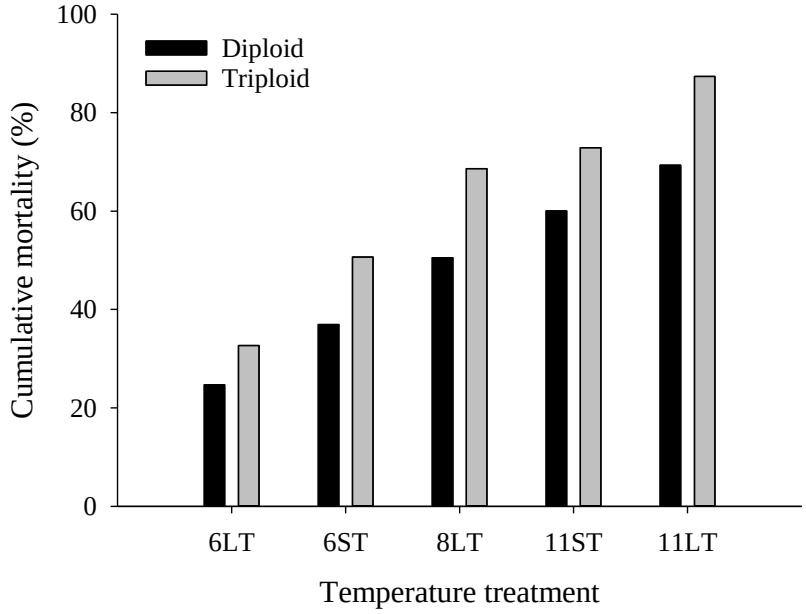


Figure 2.2. Cumulative mortality from fertilisation to smolt of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Breakdown of stage-specific mortality is reported in Table 2.2.

Table 2.2. Mortality during specific development stages of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Where there was replication (between fertilisation and 400 °days), data are expressed as means ± SD ($p < 0.05$, Two-Way ANOVA). Where there were single groups thereafter, data are expressed as tank value ± SD ($p < 0.05$; Analysis of CLT). Superscripts denote significant differences between treatments and ploidy within specific developmental stages.

		Stage of Development			
Temperature regime	**Ploidy**	**Fertilisation to 400 °days**	**400 °days to Hatch**	**Hatch to First feeding**	**First feeding to Smolt**
6LT	Diploid	16.4 ± 19.2	1.8 ± 0.4^{d}	2.6 ± 0.5^{f}	6.7 ± 1.6^{f}
	Triploid	22.3 ± 21.9	2.8 ± 0.6^{d}	2.3 ± 0.6^{f}	6.3 ± 2.3^{f}
6ST	Diploid	·	3.0 ± 1.2^{d}	10.6 ± 2.2^{de}	13.8 ± 2.6^{e}
	Triploid	·	2.8 ± 1.1^{d}	8.5 ± 2.0^{e}	26.7 ± 3.3^{bc}
8LT	Diploid	18.7 ± 20.5	2.1 ± 0.5^{d}	14.5 ± 1.2^{c}	27.4 ± 1.7^{b}
	Triploid	26.4 ± 23.1	7.1 ± 1.1^{c}	14.4 ± 1.5^{c}	46.6 ± 2.5^{a}
11ST	Diploid	·	6.4 ± 1.7^{c}	14.1 ± 2.5^{cd}	32.0 ± 3.6^{b}
	Triploid	·	7.8 ± 1.9^{c}	16.6 ± 2.7^{c}	46.5 ± 4.1^{a}
11LT	Diploid	27.0 ± 19.9	13.4 ± 1.2^{b}	41.4 ± 1.9^{b}	17.3 ± 2.0^{de}
	Triploid	34.1 ± 24.0	43.1 ± 2.3^{a}	57.5 ± 2.6^{a}	20.8 ± 3.3^{cd}

2.3.2. Yolk-sac utilisation

No ploidy difference was found in body weight (BWa) of alevins at hatch or 600 °days, however, diploids had an overall significantly higher BWa than triploids at 750 °days ($p = 0.009$) (Fig. 2.3a; Table 2.3). An overall temperature effect was observed with decreasing BWa at hatch ($p = 0.017$), 600 °days ($p = 0.004$) and 750 °days ($p < 0.001$) in which both ploidy responded the same.

Triploids had an overall greater yolk sac weight (YSW) than diploids at hatch ($p = 0.039$), however, neither ploidy was affected by increasing temperature (Fig. 2.3b; Table 2.3).

At 600 and 750 °days, YSW did not differ between ploidy, however, an overall effect of temperature treatment was observed (p = 0.002 and p = 0.001, respectively) in which both ploidy responded the same (6LT > 8LT = 11LT).

Total lipid (TL, %) was overall higher in diploid yolk sac then triploids but only at hatch (p = 0.005) (Fig. 2.3c; Table 2.3). No significant difference was found between ploidy at 6 or 8 °C, however diploids had a significantly higher TL % at 11 °C. Diploids showed no difference in TL % at hatch in response to increasing temperature, however triploids had a significantly lower TL % in 11LT compared to 6LT and 8LT. At 600 and 750 °days, TL % did not differ between ploidy however an overall effect of temperature treatment was observed (p = 0.002 and p < 0.001, respectively) in which both ploidy responded the same (6LT > 8LT = 11LT).

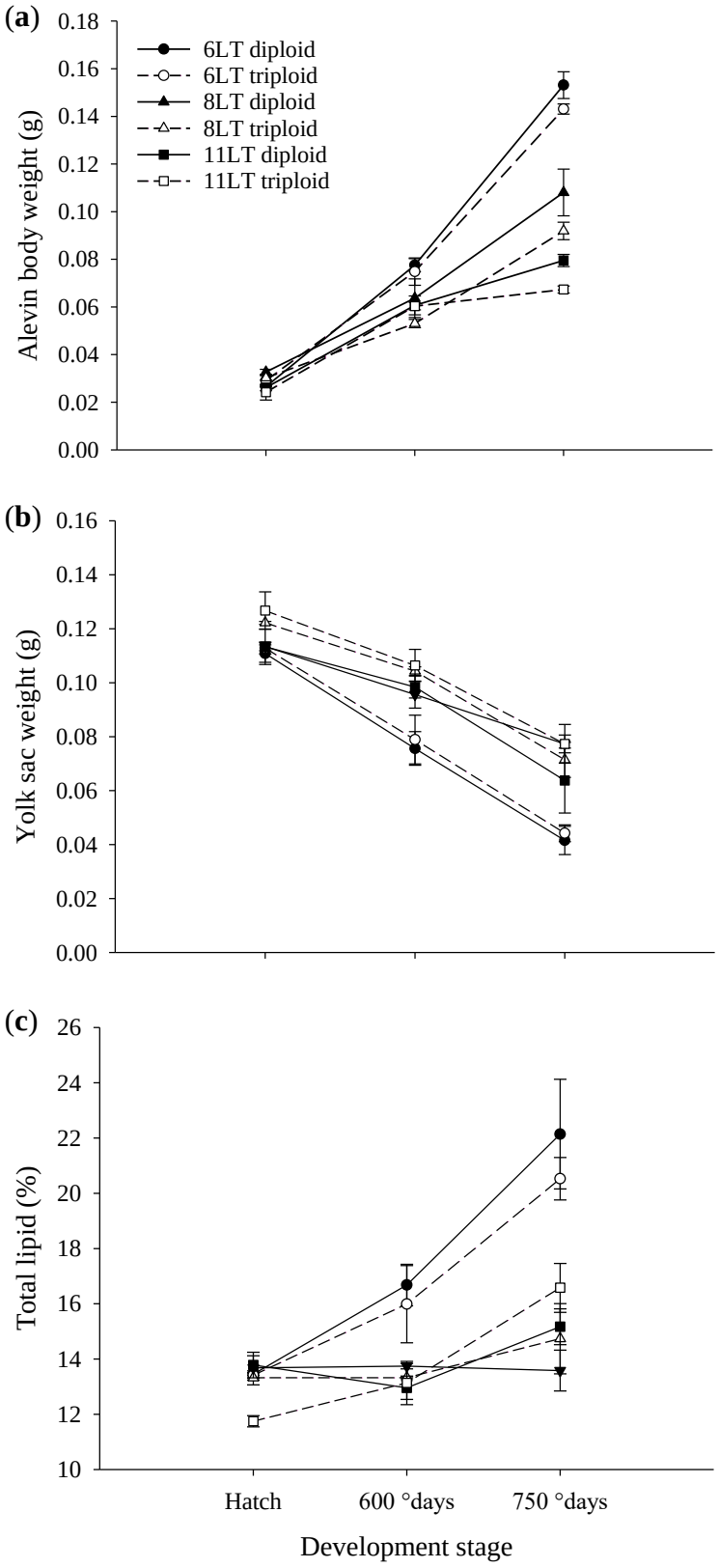


Figure 2.3. (a) Body weight (BWa), **(b)** yolk sac weight (YSW) and **(c)** yolk sac total lipid (TL) (n = 3, 5 alevins ploidy^{-1} treatment^{-1}) at different stages through embryogenesis. Data are expressed as means ± SEM and significant differences are denoted in Table 2.3.

Table 2.3. Body weight of *Salmo salar* alevins (BWa), yolk sac weight (YSW) and yolk sac total lipid (TL) at different stages through embryogenesis (n = 3, 5 alevins ploidy^{-1} treatment^{-1}). Data are expressed as means ± SEM and significant differences are denoted by different superscripts ($p < 0.05$, Two-Way ANOVA). ns = not significant.

	6LT		**8LT**		**11LT**		***p* value**		
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	ploidy	temp	ploidy*temp
BWa (g)									
Hatch	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	ns	**0.017**	ns
600 °days	0.08 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	ns	**0.004**	ns
750 °days	0.15 ± 0.01	0.14 ± 0.00	0.11 ± 0.01	0.09 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	**0.009**	**< 0.001**	ns
YSW (g)									
Hatch	0.11 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.11 ± 0.01	0.13 ± 0.01	**0.039**	ns	ns
600 °days	0.08 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	ns	**0.002**	ns
750 °days	0.04 ± 0.01	0.04 ± 0.00	0.08 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	ns	**0.001**	ns
TL (%)									
Hatch	13.45 ± 0.05^{a}	13.41 ± 0.10^{a}	13.68 ± 0.44^{a}	13.32 ± 0.25^{a}	13.78 ± 0.46^{a}	11.75 ± 0.20^{b}	**0.005**	**0.046**	**0.010**
600 °days	16.68 ± 0.75	15.99 ± 1.40	13.74 ± 0.10	13.32 ± 0.04	12.96 ± 0.42	13.13 ± 0.78	ns	**0.002**	ns
750 °days	22.14 ± 1.98	20.53 ± 0.77	13.58 ± 0.74	14.73 ± 1.27	15.17 ± 0.65	16.58 ± 0.89	ns	**< 0.001**	ns

2.3.3. Growth performances

There was a significant effect of both ploidy ($p < 0.001$, 2N > 3N) and temperature ($p < 0.001$, 11LT < 11ST = 8LT = 6ST < 6LT) on first feeding weight (BWi) and a significant interaction between ploidy and temperature ($p < 0.001$). No difference between ploidy was observed in BWi in 6LT and 6ST, however, diploids had a significantly higher BWi than triploids in 8LT, 11ST and 11LT (Fig. 2.4a). Within diploids, BWi was significantly higher in 6LT compared to 8LT and 11LT, however, no significant differences were observed compared to 6ST and 11ST. In triploids, BWi in 6LT was significantly greater than 8LT, 11ST and 11LT. Further, triploid BWi was significantly greater at 6ST compared to 11LT.

There was a significant effect of both ploidy ($p < 0.001$, 2N > 3N) and temperature ($p < 0.001$, 11LT < 6ST < 11ST = 6LT = 8LT) on final smolt weight (BWf) and a significant interaction between ploidy and temperature ($p < 0.001$). BWf was comparable between ploidy in 6LT and 6ST, however, diploids had significantly higher BWf in 8LT, 11ST and 11LT (Fig. 2.4b). Within diploids, there was an increase in BWf with increasing temperature up to 8LT and then comparable thereafter (6LT > 6ST > 8LT = 11ST = 11LT). Conversely, BWf of triploids increased from 6LT to 6ST and 8LT however a significant decrease in BWf was observed in 11ST and 11LT.

Growth rate, as measured by thermal growth coefficient (TGC) from first feeding to smolt is shown in Fig. 2.4c. Although statistical differences could not be determined, TGC in 6LT and 6ST showed little difference between ploidy. However, triploids had lower TGC values in 8LT, 11ST and 11LT compared to diploids. Within diploids, a similar TGC was observed irrespective of incubation temperature. In contrast, TGC in triploids was affected by higher temperatures as shown by lower TGC in treatments 8LT, 11ST, and 11LT.

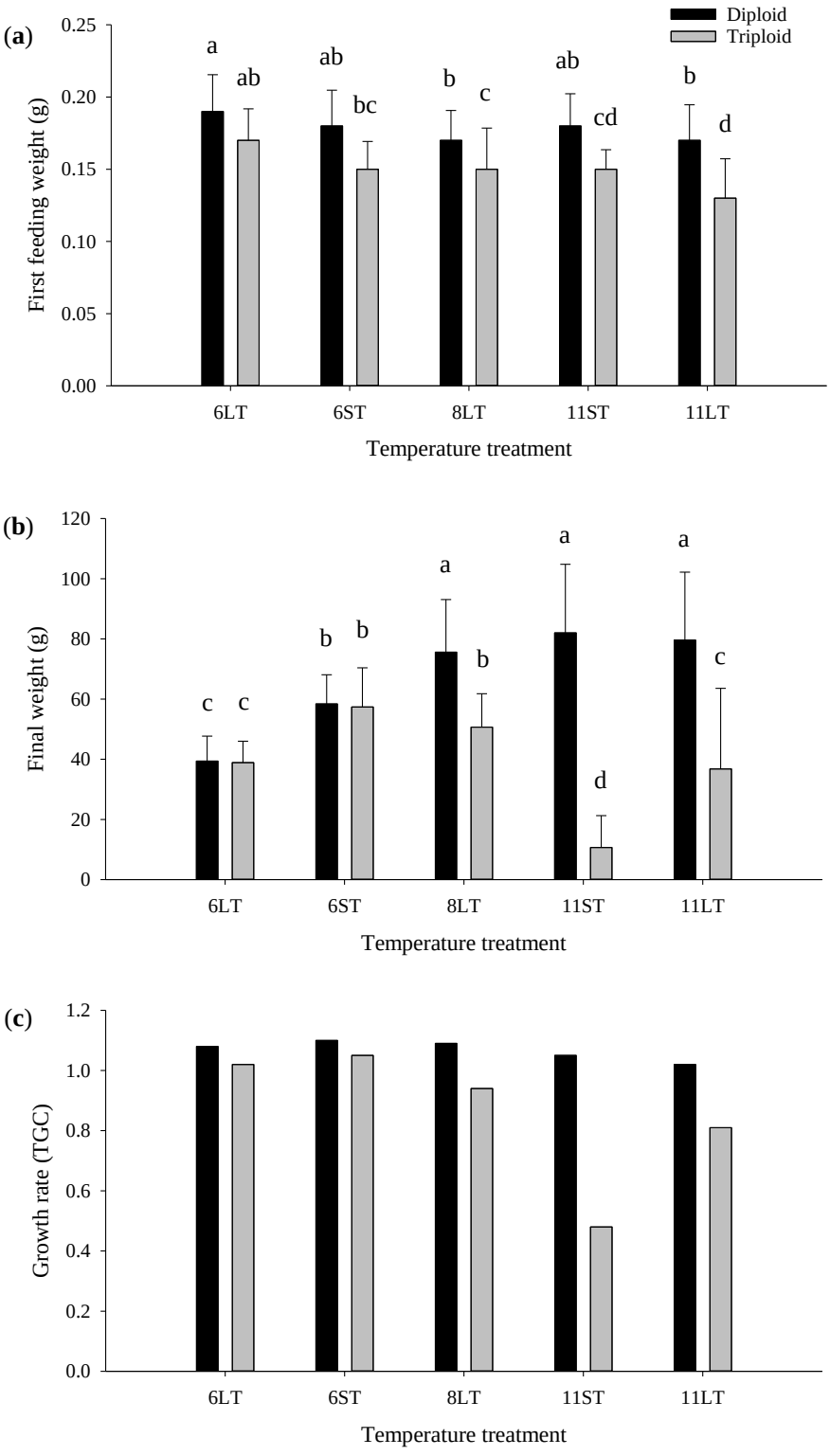


Figure 2.4. Growth summary including (**a**) body weight in first feed alevins; BWi (g), (**b**) final body weight in smolts; BWf (g) and (**c**) thermal growth coefficient (TGC) calculated from first feeding to smolt of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Data are expressed as tank mean ± SD ($p < 0.05$, Two-Way ANOVA). Superscripts denote significant differences between treatments.

2.3.4. Muscle analysis in smolts

Triploids in the 6LT treatment displayed a significantly higher (+35 %) final fibre number (FFN) than 6LT diploids (Table 2.4). No other significant effect of ploidy was observed within any other temperature treatment. An overall temperature effect was observed (p = 0.009) with FFN with 6LT significantly greater than 11ST, however, there was a significant interaction with ploidy ($p < 0.001$). In diploids, there was no significant difference in FFN between any temperature treatments, while in triploids, 6LT fish had significantly more fibres per mm^2 compared to 6ST, 11ST and 11LT but comparable to 8LT.

Diploids in 6LT had a significantly larger (+35 %) muscle fibre area than triploids, but significantly lower (-23 %) muscle fibre area in 6ST (Table 2.4). No difference was found between ploidy in any other treatments. There was a significant interaction between ploidy and temperature ($p < 0.001$) on muscle fibre area whereby no differences were found within diploids, while triploids had significantly lower muscle fibre areas in 6LT compared to 6ST, 11ST and 11LT but comparable to 8LT.

Table 2.4. White muscle fibre morphometrics of diploid and triploid *Salmo salar* (6 fish ploidy^{-1} treatment^{-1}) at smolt including final fibre number per mm^2 (FFN, mm^{-2}) and fibre area (μm^2). Data are expressed as means ± SD and significant differences are denoted by different superscripts ($p < 0.05$, Two-Way ANOVA). ns = not significant.

Temperature regime	Ploidy	FFN (mm^{-2})	Fibre area (μm^2)
6LT	Diploid	403.3 ± 21.8^{b}	2485.2 ± 134.3^{ab}
	Triploid	544.9 ± 44.8^{a}	1844.7 ± 154.6^{c}
6ST	Diploid	474.4 ± 32.0^{ab}	2115.0 ± 137.9^{bc}
	Triploid	386.3 ± 36.7^{b}	2608.1 ± 269.0^{a}
8LT	Diploid	466.7 ± 44.5^{ab}	2157.4 ± 205.9^{abc}
	Triploid	476.3 ± 37.4^{ab}	2109.2 ± 167.1^{bc}
11ST	Diploid	427.0 ± 11.1^{b}	2343.2 ± 60.1^{ab}
	Triploid	398.9 ± 38.6^{b}	2522.3 ± 241.5^{ab}
11LT	Diploid	437.4 ± 69.7^{b}	2328.0 ± 350.2^{abc}
	Triploid	406.4 ± 26.2^{b}	2468.3 ± 161.5^{ab}
***p* value**	ploidy	ns	ns
	temp	**0.009**	**0.016**
	ploidy*temp	**< 0.001**	**< 0.001**

2.3.5. Deformity analysis

No visible external deformities were observed in either ploidy in the 6LT treatment and very few (6 and 2 %, respectively) were found in both ploidy from 6ST (Fig. 2.5.; Table 2.5.). Triploids had greater visible external deformities compared to diploid counterparts at higher temperatures and duration (8LT: 8 *vs*. 0 %; 11ST: 50 *vs*. 18 % and 11LT: 38 *vs*. 10 %, respectively), significantly different at 11ST and 11LT. In diploids, visible external deformity prevalence was significantly greater at 11ST and 11LT compared to 6LT and 8LT but not

different from 6ST. Triploids had a significantly higher prevalence of visible external deformities at 11ST and 11LT compared to all other temperature treatments.

These deformities were mainly comprised of jaw malformation as vertebral deformities were negligible. Triploids had higher jaw deformity prevalence in 8LT and 11ST compared to diploids but no ploidy effect was found in any other temperature treatment. In diploids, jaw deformity prevalence was significantly higher in 11ST compared to 6LT and 8LT. However, in triploids, jaw deformity prevalence was significantly higher in 8LT, 11ST and 11LT compared to 6LT. Also, triploid jaw deformity prevalence was significantly higher in 11ST compared to 6ST and 8LT. Albeit not significant, there was an increase in jaw deformity prevalence in 6ST and 11ST compared to their respective LT regimes irrespective of ploidy.

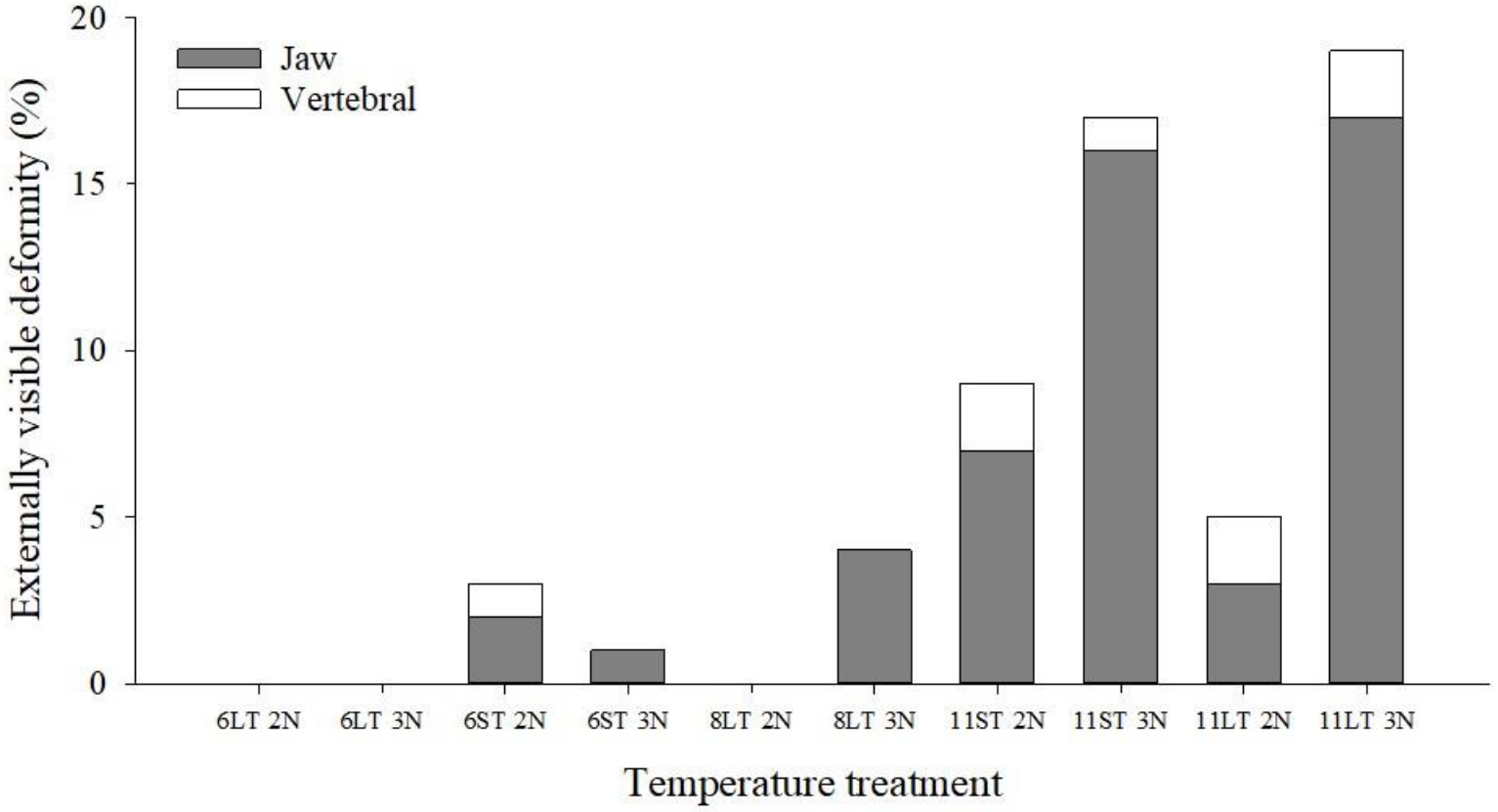


Figure 2.5. Prevalence (%) of external jaw and vertebral deformities in diploid and triploid *Salmo salar* smolts (50 fish ploidy^{-1} treatment^{-1}) incubated as eggs under different temperature treatments. Breakdown of deformity is reported in Table 2.5.

Table 2.5. Prevalence (%) of external jaw and vertebral deformities in diploid and triploid *Salmo salar* smolts (50 fish ploidy^{-1} treatment^{-1}) incubated as eggs under different temperature treatments. Data are expressed as means ± SD and significant differences are denoted by different superscripts ($p < 0.05$, Analysis of CLT).

Temperature regime	Ploidy	Deformed Individuals (%)	Jaw (%)	Vertebral (%)
6LT	Diploid	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0
	Triploid	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0
6ST	Diploid	6.0 ± 6.6^{cd}	4.0 ± 5.5^{bc}	2.0 ± 3.9
	Triploid	2.0 ± 3.9^{d}	2.0 ± 3.9^{bc}	0.0 ± 0.0
8LT	Diploid	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0
	Triploid	8.0 ± 7.6^{cd}	8.0 ± 7.6^{b}	0.0 ± 0.0
11ST	Diploid	18.0 ± 10.8^{bc}	14.0 ± 9.7^{b}	4.0 ± 5.5
	Triploid	50.0 ± 17.1^{a}	47.1 ± 17.0^{a}	2.9 ± 5.8
11LT	Diploid	10.0 ± 8.4^{c}	6.0 ± 6.6^{bc}	4.0 ± 5.5
	Triploid	38.0 ± 13.6^{ab}	34.0 ± 13.3^{ab}	4.0 ± 5.5

There was no difference in the number of vertebrae per fish, assessed radiologically, seen between ploidy or treatments (58.3 ± 0.2; $p < 0.05$). There were no significant differences observed in number of deformed vertebrae (dV) in deformed indiviudals between treatments or ploidy (Fig. 2.6.). Nevertheless, mean number of dV remained low for both ploidy in 6LT, 6ST and 8LT (2N, 1.9 – 2.8 dV; 3N, 2.8 – 3.5 dV), however there was an increase in 11ST and 11LT (2N, 3.7 – 6.2 dV; 3N, 5.6 – 5.9 dV).

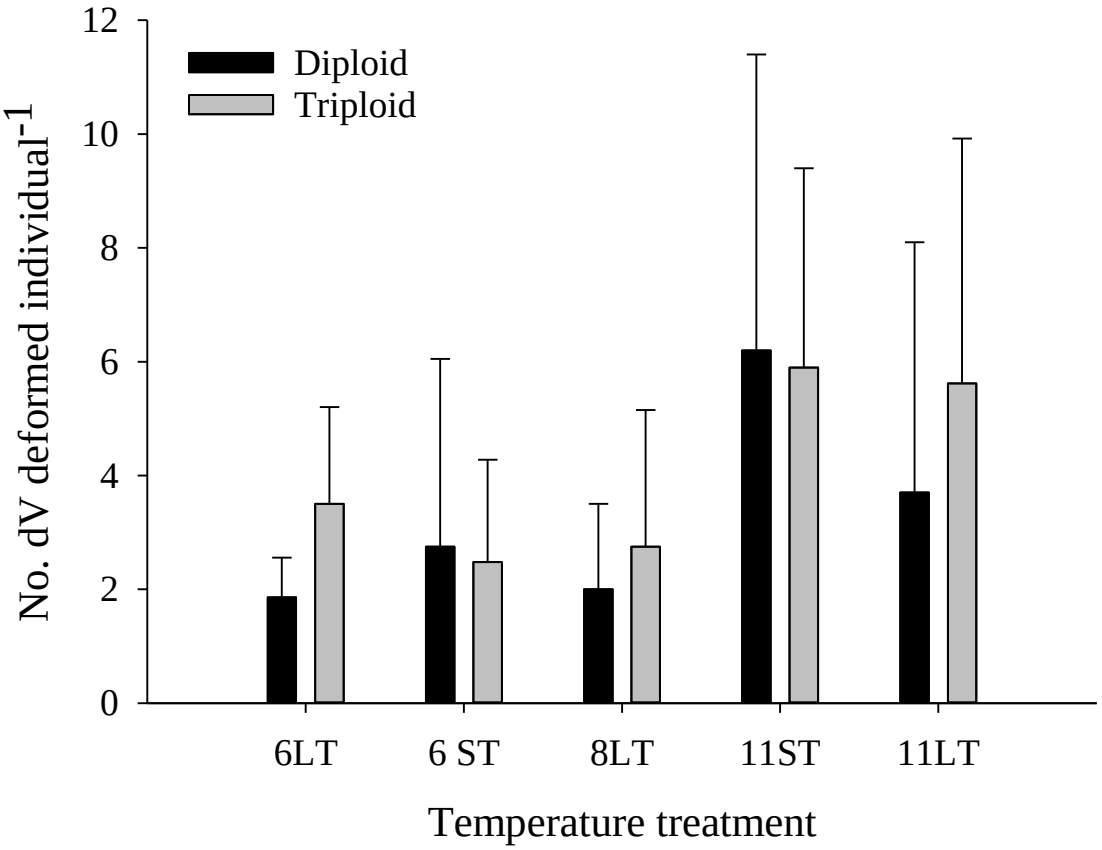


Figure 2.6. Average number radiological deformed vertebrae (dV) per deformed individual in diploid and triploid *Salmo salar* smolts incubated as eggs under different temperature treatments. Data are expressed as means ± SD.

Triploids had a significantly higher deformity prevalence than diploids in 8LT (72 *vs.* 24 %), 11ST (88.2 *vs.* 40 %) and 11LT (90 *vs*. 60 %) treatments, but prevalence was comparable between ploidy in 6LT and 6ST (Fig. 2.7.; Table 2.6.). The prevalence of radiologically deformed individuals increased with increasing incubation temperatures and durations in both ploidy. Within diploids, prevalence was significantly higher in 11ST and 11LT compared to 6LT. Further, prevalence at 11LT was significantly greater than 6ST and 8LT. In triploids, deformity prevalence was significantly greater in 8LT, 11ST and 11LT compared to 6LT and 6ST.

Deformed individuals were categorised by severity bands (Mild: 1-5 dV, Moderate: 6-9 dV or Severe: ≥10 dV). Triploids displayed a significantly higher prevalence of 1-5 dV than diploids in 6LT (25 *vs.* 14 %) and 8LT (64 *vs.* 22 %) treatments; while no differences between ploidy were found in other temperature treatments. Within diploids, 11LT fish (52 %) showed greater 1-5 dV prevalence than other treatments (≤22 %). However, in triploids, the only significant difference was found between 8LT (64 %) and 6LT (25 %).

In the 6-9 dV category, only triploids from 11ST (38.2 %) and 11LT (32 %) had a significantly greater prevalence than diploids (10 and 2 %, respectively) with no difference between ploidy evident in other treatments. Diploids from 11ST (10 %) had greater prevalence than 6LT and 6ST only (0 %), whereas in triploids both 11ST (38.2 %) and 11LT (32 %) had greater prevalence than all other groups (4-5 %).

Finally, for ≥10 dV, ploidy effects were only found in 6ST (2n: 2 % > 3n: 0 %) and 8LT (3n: 4 % > 2n: 0 %). In diploids, prevalence of fish with ≥10 dV was significantly greater in 6ST, 11ST and 11LT (2-8 %) compared to 6LT and 8LT (0 %). However, in triploids, prevalence of fish with ≥10 dV was significantly greater in 8LT, 11ST and 11LT (4-10 %) compared to 6LT and 6ST (0 %).

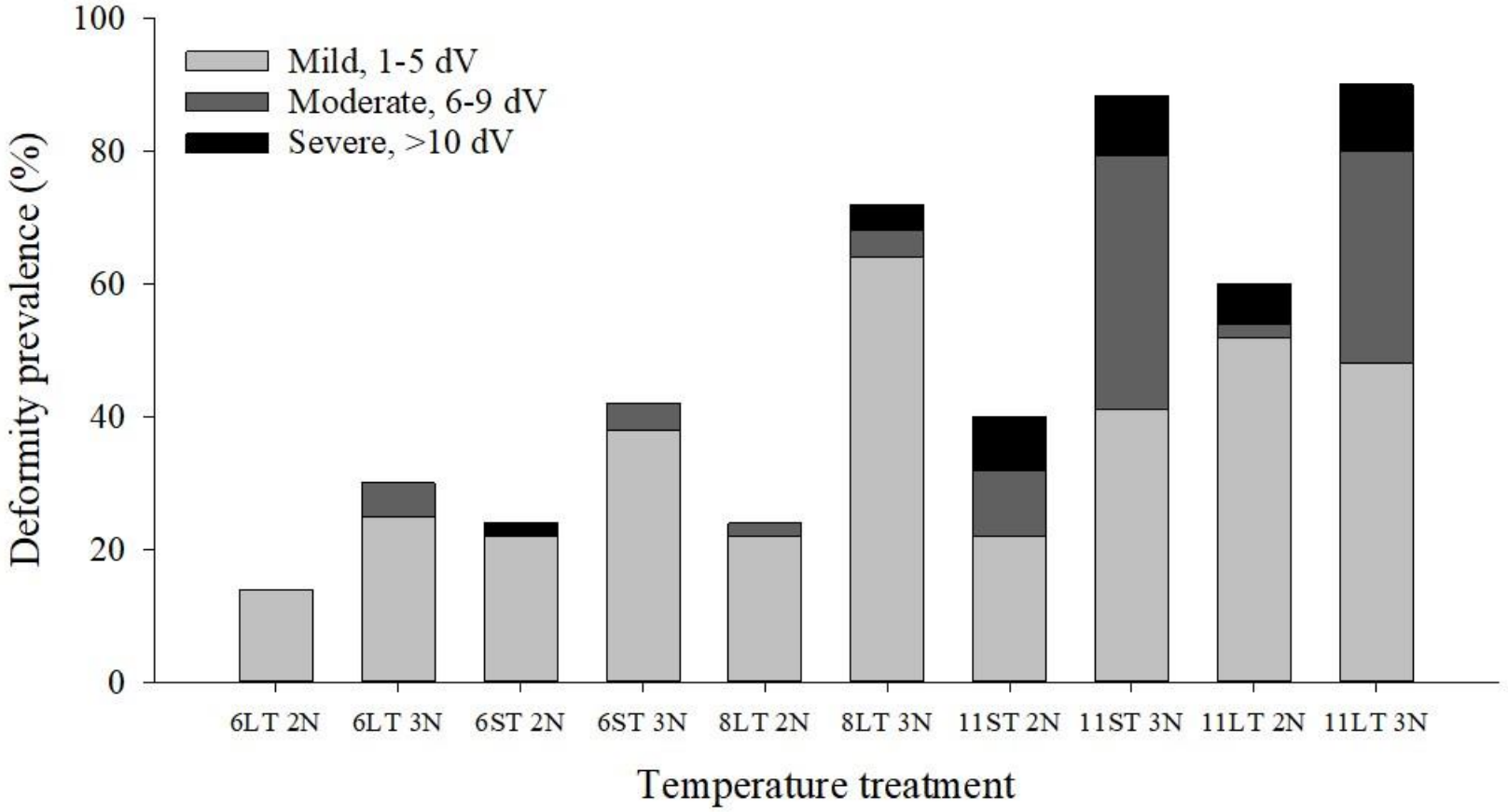


Figure 2.7. Prevalence (%) of X-ray radiologically assessed deformities presented according to severity index (Mild, 1-5 dV; Moderate: 6-9 dV; Severe ≥10 dV) in diploid and triploid *Salmo salar* smolts (50 fish ploidy^{-1} treatment^{-1}). Breakdown of deformity is reported in Table 2.6.

Table 2.6. Prevalence (%) of X-ray radiologically assessed deformities presented according to severity index (Mild, 1-5 dV; Moderate: 6-9 dV; Severe ≥10 dV) in diploid and triploid *Salmo salar* smolts (50 fish ploidy^{-1} treatment^{-1}). Data are expressed as means ± SD and significant differences are denoted by different superscripts ($p < 0.05$, Analysis of CLT). dV: deformed vertebrae.

Temperature regime	Ploidy	Deformed Individuals (%)	1-5 dV (%)	6-9 dV (%)	≥10 dV (%)
6LT	Diploid	14.0 ± 9.7^{e}	14.0 ± 9.7^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}
	Triploid	30.0 ± 14.4^{de}	25.0 ± 13.6^{b}	5.0 ± 6.8^{bc}	0.0 ± 0.0^{b}
6ST	Diploid	24.0 ± 12.0^{de}	22.0 ± 11.6^{cd}	0.0 ± 0.0^{c}	2.0 ± 3.9^{a}
	Triploid	42.0 ± 13.8^{d}	38.0 ± 13.6^{abc}	4.0 ± 5.5^{bc}	0.0 ± 0.0^{b}
8LT	Diploid	24.0 ± 12.0^{de}	22.0 ± 11.6^{cd}	2.0 ± 3.9^{bc}	0.0 ± 0.0^{b}
	Triploid	72.0 ± 12.6^{ab}	64.0 ± 13.4^{a}	4.0 ± 5.5^{bc}	4.0 ± 5.5^{a}
11ST	Diploid	40.0 ± 13.7^{cd}	22.0 ± 11.6^{cd}	10.0 ± 8.4^{b}	8.0 ± 7.6^{a}
	Triploid	88.2 ± 11.0^{a}	41.2 ± 16.8^{abc}	38.2 ± 16.6^{a}	8.8 ± 9.7^{a}
11LT	Diploid	60.0 ± 13.7^{bc}	52.0 ± 14.0^{ab}	2.0 ± 3.9^{bc}	6.0 ± 6.6^{a}
	Triploid	90.0 ± 8.4^{a}	48.0 ± 14.0^{ab}	32.0 ± 13.1^{a}	10.0 ± 8.4^{a}

2.3.6. Mineral composition

Ploidy had a significant effect on all whole body composition minerals analysed, while only Ca:P ratio and zinc (Zn) were significantly affected by temperature (Table 2.7.). Overall, triploids had higher levels of calcium (Ca), phosphorous (P), magnesium (Mg) and zinc (Zn) and a higher Ca:P than diploids ($p < 0.001$). In contrast, diploids had overall higher levels of iron (Fe) than triploids ($p < 0.001$). However, there was also a significant interaction between ploidy and temperature for whole body Fe and Zn. Fe was significantly greater in diploids compared to triploids in 6LT. Diploids showed no change in Fe levels in response to increasing temperature, however, triploids had higher levels at 11LT compared to 6LT. Zn was significantly greater in triploid compared to diploids in 6LT and 11LT. In diploids, Zn was significantly higher in 6ST compared to 6LT and 11LT while no temperature effect was observed in triploids.

Table 2.7. Whole body mineral levels (µg mg^{-1} wet weight) and Ca:P ratio in diploid and triploid *Salmo salar* smolts (n = 3; 3 fish $pool^{-1}$ $treatment^{-1}$ $ploidy^{-1}$). Data are expressed as means ± SEM ($p < 0.05$; Two-Way ANOVA) and different superscripts denote significant differences. 11ST diploid and triploid samples excluded due to analytical error. ns = not significant.

Temperature regime	Ploidy	Calcium (Ca)	Phosphorous (P)	Ca:P	Magnesium (Mg)	Iron (Fe)	Zinc (Zn)
6LT	Diploid	3947.0 ± 146.5	4065.7 ± 104.8	0.97 ± 0.01	267.7 ± 4.1	13.2 ± 1.4^{a}	32.8 ± 0.5^{bc}
	Triploid	4471.8 ± 201.9	4476.2 ± 61.3	1.00 ± 0.03	283.7 ± 4.3	8.4 ± 0.4^{b}	42.2 ± 1.5^{a}
6ST	Diploid	3852.1 ± 116.8	4067.7 ± 102.2	0.94 ± 0.01	269.3 ± 11.0	13.4 ± 0.6^{a}	42.7 ± 2.5^{a}
	Triploid	4765.2 ± 256.6	4521.2 ± 186.2	1.05 ± 0.02	286.0 ± 9.5	11.2 ± 0.5^{ab}	40.9 ± 1.7^{a}
8LT	Diploid	3897.4 ± 338.8	4078.0 ± 249.1	0.95 ± 0.02	273.9 ± 6.2	11.3 ± 11.2^{a}	35.2 ± 2.0^{abc}
	Triploid	4581.8 ± 543.1	4548.0 ± 311.0	1.00 ± 0.05	297.0 ± 10.9	10.8 ± 0.26^{ab}	39.8 ± 1.5^{ab}
11ST	Diploid	.	.	.	.	.	.
	Triploid	.	.	.	.	.	.
11LT	Diploid	3282.8 ± 115.9	3775.2 ± 24.9	0.89 ± 0.03	265.1 ± 8.0	11.7 ± 0.4^{a}	28.8 ± 2.3^{c}
	Triploid	4203.5 ± 307.0	4340.0 ± 200.8	0.97 ± 0.03	285.2 ± 11.9	11.8 ± 1.1^{a}	39.0 ± 2.2^{ab}
***p* value**	ploidy	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	temp	ns	ns	**0.012**	ns	ns	**< 0.001**
	ploidy*temp	ns	ns	ns	ns	**0.003**	**0.009**

2.3.7. Gene expression analysis

2.3.7.1. Genes associated with lipid utilisation

At 200 °days, there was no significant difference in *fas* mRNA expression between temperature treatments in diploids, but triploids showed a downregulation under 6ST/LT and 11ST/LT (~-0.5-0.6 fold) compared to 8LT (Fig. 2.8a, b). This stage was prior to the inclusion of specific ST regimes, hence why 6ST/LT and 11ST/LT are grouped. The same effect was found for *lxr* expression levels in triploids, whereas in diploids, upregulated expression was seen in 6LT/ST (~+ 2.2. fold) and 11LT/ST (~+1.5 fold) compared to 8LT (Fig. 2.8a, b). Diploids showed an upregulation of *srebp1* 6LT/ST (~+2.3 fold) compared to 8LT and 11ST/LT (~+1.2 fold) groups however no difference was found between temperature treatments in triploids.

At eyeing, no significant differences in *fas, lxr* or *srebp1* expression were found between temperature treatments within each ploidy (Fig. 2.8c, d).

Conversely, differences were found at the first feeding stage with *fas* upregulation in 6ST (~+2 fold) and 11ST (~+2 fold) diploids compared to 8LT and 11LT, but comparable with 6LT (Fig. 2.8e, f). No differences were found in *fas* expression in triploids at first feeding. Similarly, diploids showed no differences in *lxr* expression at this stage, however in triploids, 6ST was significantly downregulated compared to 6LT and 8LT. Expression of *srebp1* was upregulated in 6ST (~+7.5 fold) and 11ST (~+5.5 fold) diploids compared to 6LT, 8LT and 11LT at this stage and this was also true in triploids (~+4 fold and ~+4.5 fold).

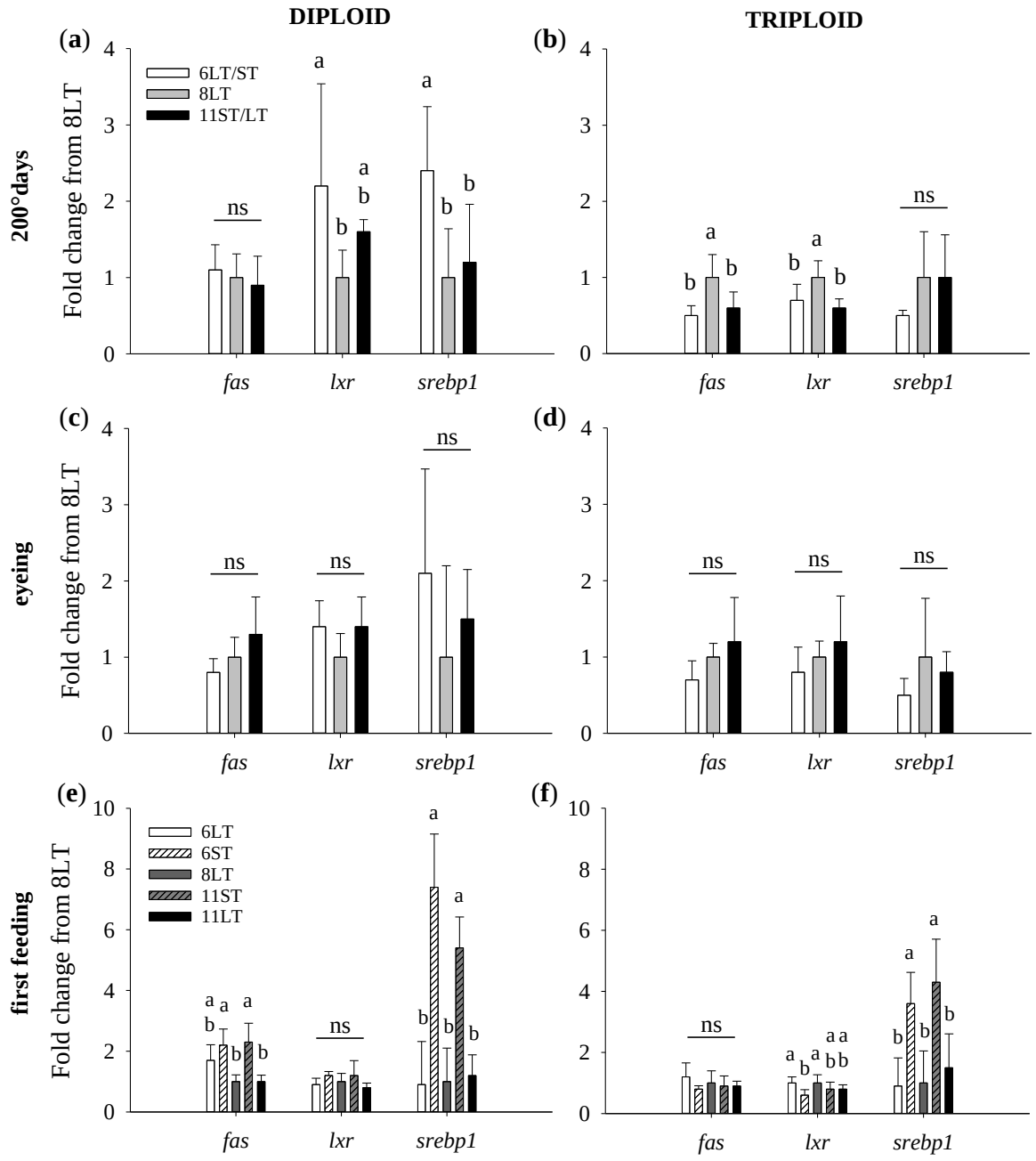


Figure 2.8. mRNA expression of *fas*, *lxr* and *srebp1* expressed as fold change relative to 8LT within each ploidy at respective developmental stage. Data were normalised using the geometric mean of *β-actin* and *elf-α* housekeeping genes. Data show expression prior to ST transition at 200 °days in (**a**) diploids and (**b**) triploids, at eyeing in (**c**) diploids and (**d**) triploids, and after inclusion of ST regimes at first feeding in (**e**) diploids and (**f**) triploids. Data are expressed as means ± SD (6 fish ploidy^{-1} treatment^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$). ns = not significant.

2.3.7.2. Genes associated with myogenesis

At first feeding, no difference was found in *igf1* expression between any temperature treatments in diploids (Fig. 2.9a). Conversely, *igf1r* showed lower expression in diploids under 6ST and 11ST compared to 6LT and 8LT (Fig. 2.9b). The expression of *igf2* and *igfbprp* in diploids under 6ST and 11ST showed a significant upregulation (7-9 fold) compared to 6LT, 8LT and 11LT. In triploids sampled at first feeding (Fig. 2.9b), *igf1* had significantly greater expression in 11ST (~+8 fold) compared to 6LT, 8LT and 11LT, and 6ST (~4 fold) had significantly greater expression than 8LT.

Similar to diploids, triploids exhibited a downregulation in *igf1r* expression in 6ST and 11ST compared to 8LT at first feeding (Fig. 2.9b). *igf2* expression was significantly greater in 6ST than 8LT and also 6LT and 11LT treatments whereas *igfbprp* expression was significantly greater in 6ST and 11ST (~4 fold) compared to 8LT and 6LT (Fig. 2.9b).

At smolt, there were no differences found in expression between temperature treatments in diploid groups for any of the genes except for *igf1* which showed an upregulation (~+2.5 fold) in 6LT compared to all other treatments (Fig. 2.9c). In triploids, no differences were found between temperature treatments for any of the genes except for *igfbprp* which showed a significant increased expression under 6LT and 6ST compared to 11LT which showed a downregulation compared to 8LT (Fig. 2.9d).

Irrespective of ploidy, there were no differences found between temperature treatments in mRNA expression of *myf5* and *myod* at both the first feeding and the smolt stage (Fig. 2.9a-d).

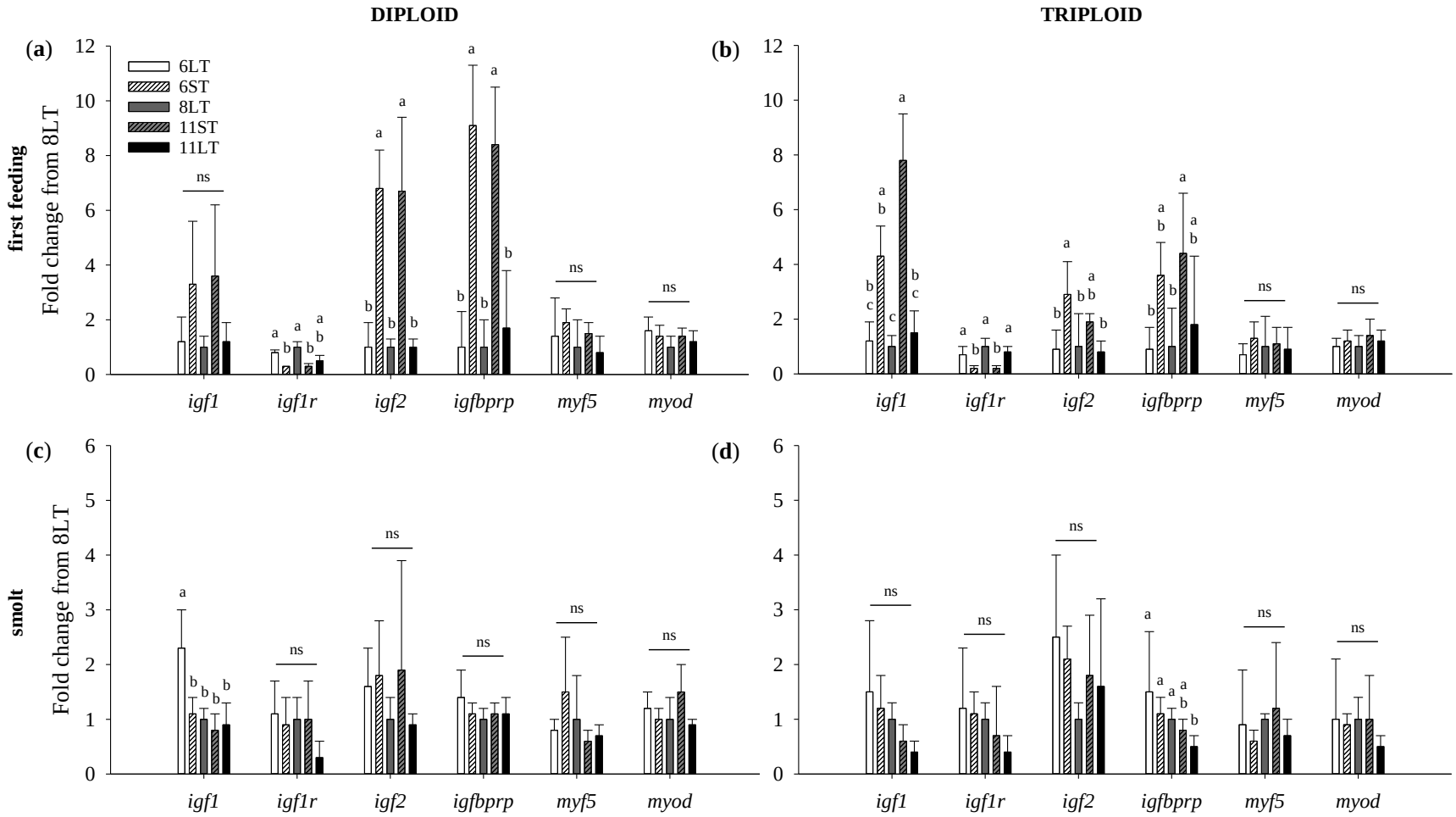


Figure 2.9. mRNA expression of *igf1*, *igf1r*, *igf2*, *igfbprp*, *myf5* and *myod* expressed as fold change relative to 8LT within each ploidy at respective developmental stage. Data were normalised using the geometric mean of *β-actin* and *elf-α* housekeeping genes. Data show expression at first feeding in (**a**) diploids and (**b**) triploids, and at smolt in (**c**) diploids and (**d**) triploids. Data are expressed as means ± SD (6 fish ploidy^{-1} treatment^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$). ns = not significant.

2.3.7.3. Genes associated with bone homeostasis and mineralisation

At first feeding, diploids under 11ST showed a significant upregulation of *alp* compared to 11LT (Fig. 2.10a). *bmp2* expression was significantly greater in 6ST (+5.5 fold) compared to 6LT, 8LT and 11LT. *bmp4* expression was significantly greater in 11ST (~4 fold) compared to 8LT and 11LT, and 6LT also had higher expression than 11LT. The only difference found for *col1a1* expression was that 11ST was greater than 6LT. However, for *col2a1*, 11ST had significantly greater expression (~13.5 fold) than all other treatments and 6ST also showed greater expression (~7 fold) that 6LT, 8LT and 11LT. Similarly, 11ST showed significantly greater expression (~11 fold) in *mmp13* compared to 6LT, 8LT and 11LT. *ocn* had significantly greater expression in 6LT compared to 6ST and 11ST only. *opn* showed greater expression (~5.5 fold) in 6ST compared to 6LT, 8LT and 11LT. Further, 11ST had greater expression of *opn* than 6LT. Both 6ST and 11ST had significantly greater expression (~6.5 fold) of *sparc* than 6LT, 8LT and 11LT.

In triploids at first feeding, *alp* showed increased expression (~2.2 fold) in 11ST compared to 6LT, 8LT and 11LT (Fig. 2.10b). Also, 11ST had a significantly higher *bmp2* expression (~3.2 fold) compared to 8LT. Both 6ST and 11ST had greater expression (~2.2 fold) of *bmp4* compared to 6LT, 8LT and 11LT. No difference was found between any triploid treatments in *col1a1* expression, however expression of *col2a1* was greater (~4.5 fold) in 11ST compared to 6LT, 8LT and 11LT. Moreover, 6ST had a higher expression of *col2a1* compared to 6LT. 11ST had a significantly greater expression (~4.5 fold) of *mmp13* compared to all other temperature treatments and 6ST had higher *mmp13* expression compared to 6LT and 11LT. As in diploids, 6LT had greater expression in *ocn* compared to 6ST and 11ST. There was a higher expression (4.2-4.5 fold) of *opn* in 6ST and 11ST when compared to 6LT, 8LT and 11LT. Finally, 11ST had a higher expression (~3 fold) of *sparc*

compared to 6LT, 8LT and 11LT, and 6ST had higher expression (~2 fold) than in 6LT and 8LT.

At smolt, there were no differences found in expression between temperature treatments in diploid groups for *bmp2*, *mmp13*, *opn* and *sparc* (Fig. 2.10c). 6LT had a greater expression of *alp* than 11LT. Also 6LT and 6ST both had greater expression of *bmp4* than 11LT. 8LT had a higher expression of *col1a1* than 11LT and 6LT had a higher expression of *col2a1* than 11LT. Both 6LT and 8LT had a greater expression of *ocn* when compared to 11LT. By contrast, in triploids sampled at smolt no significant differences in any genes were observed between temperature treatments (Fig. 2.10d).

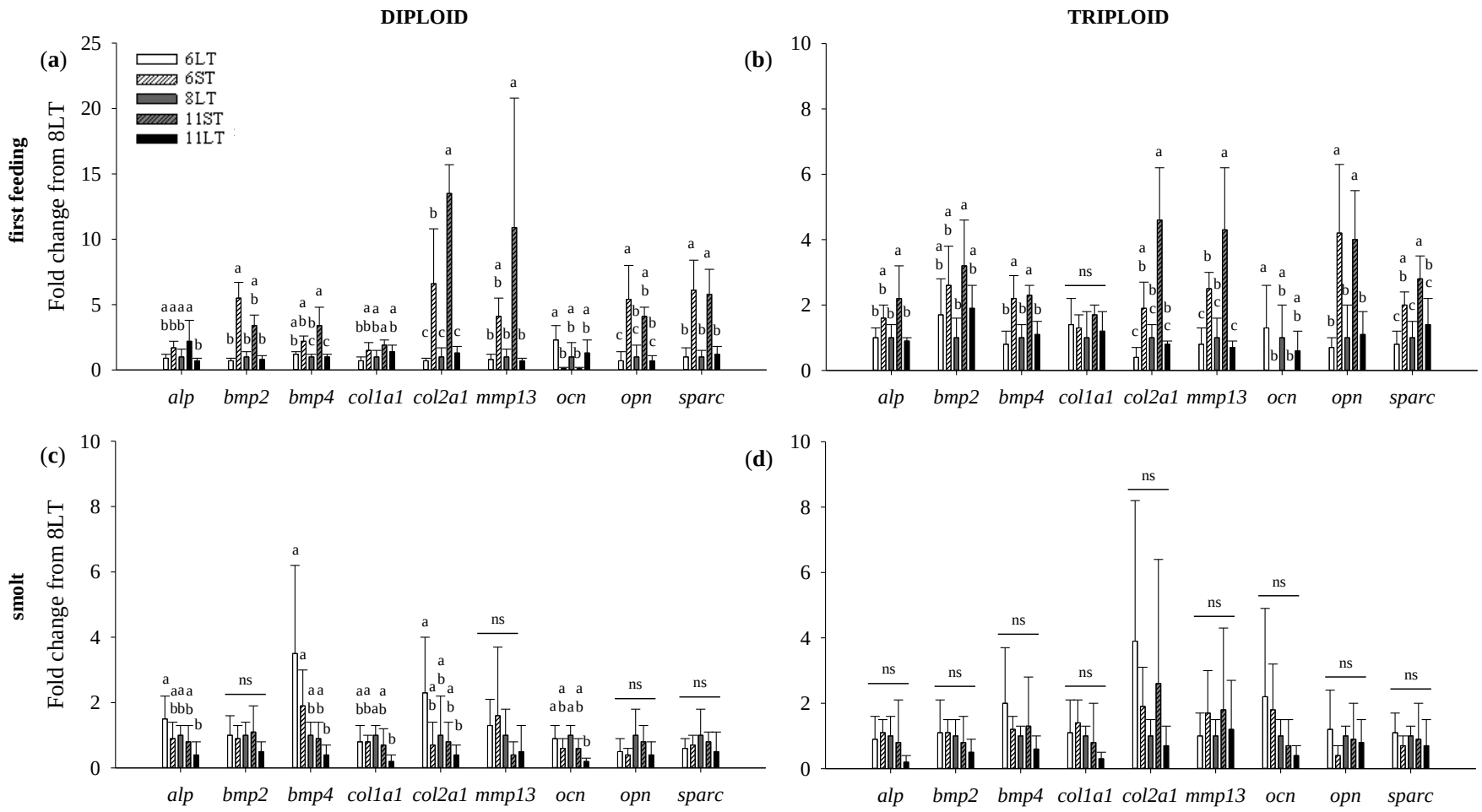


Figure 2.10. mRNA expression of *alp, bmp2, bmp4, col1a1, col2a1, mmp13, ocn, opn and sparc* expressed as fold change relative to 8LT within each ploidy at respective developmental stage. Data were normalised using the geometric mean of *β-actin* and *elf-α* housekeeping genes. Data show expression at first feeding in (**a**) diploids and (**b**) triploids, and at smolt in (**c**) diploids and (**d**) triploids. Data are expressed as means ± SD (6 fish ploidy^{-1} treatment^{-1} sampling point^{-1}) and different superscripts denote significant differences ($p < 0.05$). ns = not significant.

2.4. Discussion

The present study showed that egg incubation temperature has an important effect on both diploid and triploid *Salmo salar* development and skeletal health. The results suggest that triploids are more thermosensitive, however they show an improved survival and growth rate when incubated at ~6 °C during embryogenesis. This reduced temperature from the conventional temperature of ~8 °C also improves somatogenesis and reduces the prevalence of skeletal malformations which is supported with associated gene expression. However, when switching temperature during embryogenesis, which may be routine in a commercial setting, negative traits such as increased mortality and deformity prevalence can be experienced. This poses problems for aquaculture production, particularly with triploids, as both broodstock and ongrowing hatchery sites will regularly manipulate rearing temperatures to meet customer demands. Evidently, a balance must be met and optimisation of triploid-specific hatchery temperature conditions further contributes to the growing knowledge of triploid culture requirements to promote optimal performance and welfare and support commercial implementation.

As reported previously in other trials, the mortality rate during embryogenesis was higher in triploids compared to diploid siblings (Sutterlin *et al*., 1987; O'Flynn *et al*, 1997; Benfey, 2001; Fraser *et al*, 2015; Amoroso *et al*, 2016a). With recent knowledge on the thermosensitive nature of triploids throughout the full life cycle, it has also been reported that triploids have increased survival when incubated at a lower (~6 °C) incubation temperature during embryogenesis (Fraser *et al*, 2015). This agrees with the present study in which triploid survival was comparable to diploids at the lower end of the temperature spectrum. The survival rate in both ploidy improved in 11ST compared to 11LT for the remainder of embryogenesis, suggesting that prolonged exposure to 11 °C was detrimental irrespective of ploidy. Conversely, a reduced diploid and triploid survival was shown in 6ST compared to

6LT which indicates that the handling of the eggs coupled with the change in temperature from 6 to 8 °C at 400 °days may have induced negative consequences. The analysis during freshwater ongrowing up to smolt also showed mortality correlating with incubation history, with those exposed to a higher temperature during egg incubation having the highest mortality rates. An exception to this was the 11LT group which showed a lower mortality rate than expected. This is likely caused by the much lower number of fish in this treatment resulting from earlier high mortality rates leaving the most robust individuals. During the ongrowing period, there was no replication and only single treatment groups were used to monitor from thereon. This was due to the number of treatments under study including control groups, two experimental temperature groups each having two thermal windows in both diploid and triploid fish. Unfortunately, to determine the response of all treatment groups, the replication had to be compromised.

The current study showed egg incubation history had no effect on the diploid growth rate during the on growing period. In triploids however, there was a significant impact of the temperature history with triploids showing a lower TGC than diploids in the higher temperature treatments (8LT, 11ST, and 11LT). This agrees with the theory that triploids have a lower thermal tolerance than diploids, however triploids have also been reported to have better growth rates than diploids under different experimental incubation temperatures (Taylor *et al*., 2011; Fraser *et al*., 2015). These contrasting results are difficult to explain at this stage but may result from an impact of triploidy on differences in egg quality which led to the objectives in Chapter 3. Also, a difference in genetic strain should not be disregarded and attempts were made to investigate this in Chapter 5. There were no differences in growth rate between ploidy in 6LT and 6ST groups. Due to fertilisation of all treatments occurring on the same day (to control for parental effect) and each treatment experiencing different thermal regimes, the first feeding dates differed. As a result, treatments experienced a

different number of feeding days up to the point of smolt. This resulted in different final body weights (BWf) at smolt between temperature groups that could not be compared directly. However, the BWf was impacted by ploidy within these groups. BWf in higher temperature treatments (8LT, 11ST, and 11LT) was reflective of the growth rate and resulted in triploids with lower weights. The weights were comparable between ploidy in 6LT and 6ST treatments. 6ST fish were ~33 % larger than 6LT in both ploidy due to short term window and an earlier shift to 8 °C. There was a decrease in first feeding body weight (BWi) in both ploidy with increasing incubation temperatures. This initial difference would certainly impact the subsequent growth trajectory of the groups. Using the growth rate of fish from each treatment group, the final smolt weight was predicted assuming all had experienced the same duration of feeding (Fig. 2.11.). Diploids show an increasing smolt weight with increasing egg incubation temperatures except when a lower weight was observed in 11LT. In contrast, triploid smolt weight was expected to be lower in 8LT, 11ST and 11LT suggesting the increased thermosensitivity of this ploidy. Also, the predicted smolt weight in 11ST is lower than in 11LT further supporting that the temperature switch this group experienced at 400 °days further disrupted normal development, which agrees with other results reporting thermal impact during embryogenesis (Takle *et al*., 2005). Similar to other previous studies (Galbreath *et al*., 1994; McGeachy *et al*., 1995; Taylor *et al*., 2011), BWi of all temperature groups in this study were higher in diploids than in triploids. As this is prior to any exogenous feeding, it is clear that temperature and triploidy have an impact on utilisation of the yolk sac.

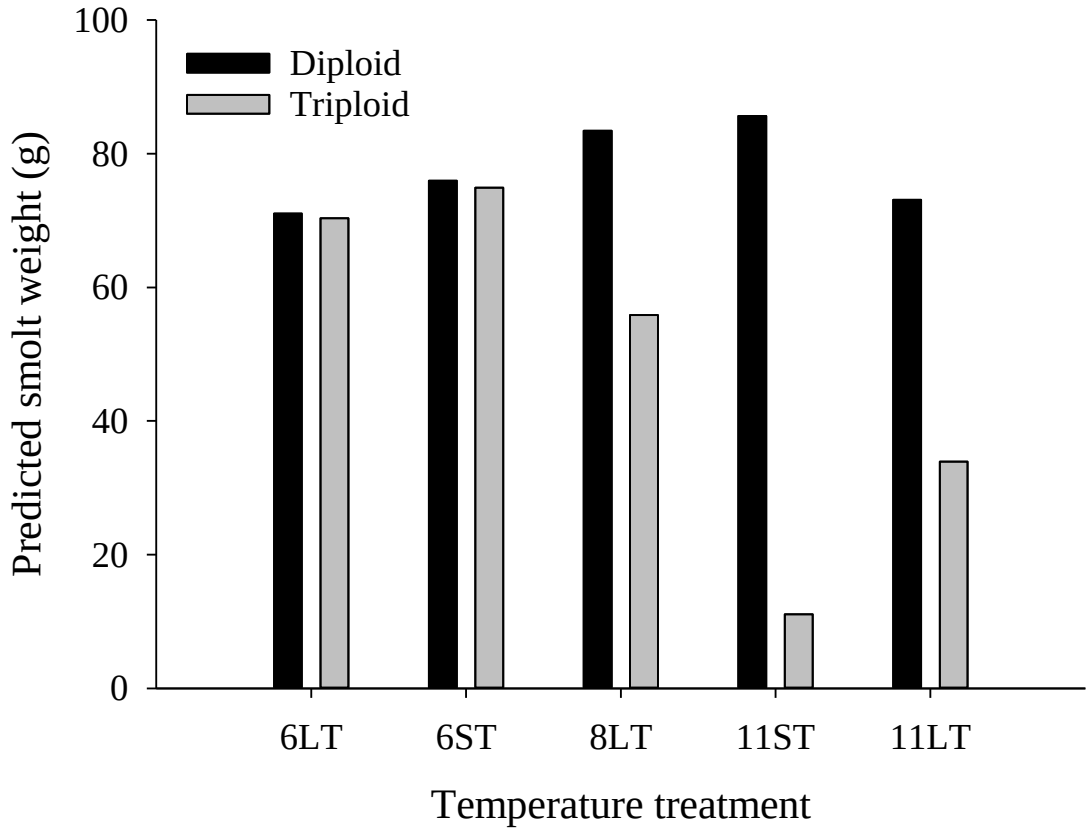


Figure 2.11. Predicted smolt weight of diploid and triploid *Salmo salar* incubated as eggs under different temperature treatments. Predicted weights were calculated using the respective TGC data and applied to the number of feeding days (399 days) and temperature profile that 11LT groups would have experienced based on the same first feeding date as the 11LT group.

Both diploids and triploids incubated at 6 °C showed a different utilisation of the yolk sac to those at 8 and 11 °C. From 600 °days, a higher alevin BW was observed in the 6 °C fish. This coincided with a lower YSW in these groups, suggesting that the improved growth in the 6 °C fish was a result of more efficient utilisation of the yolk sac. Teleost eggs are supplied with a package of nutrient reserves, predominantly lipids and proteins, deposited by the dam to support development until exogenous feeding starts in a reproductive strategy known as lecithotrophy. Fagotto (1995) described how pH and enzymatic latency are the most likely determinants of yolk degradation and it is well established that temperature has a

direct impact on the activity of an enzyme and the ionisation of a solution, which subsequently alters the pH. Therefore, it is highly likely that temperature not only affects somatic metabolism, but more fundamentally, the utilisation of the yolk sac during embryogenesis. Previous studies in salmonids suggested that temperature during egg incubation plays a major role in the catabolism of these reserves (Marr, 1966; Peterson *et al*., 1977; Heming, 1982; Peterson and Martin-Robichaud, 1995; Ojanguren *et al*., 1999), which is likely to be linked to thermosensitive enzymes. If metabolism is stimulated under a high temperature incubation, more energy will be required resulting therefore in a faster utilisation of the yolk sac. As hypothesised by Matschak *et al*. (1997), an increased demand for oxygen in also likely towards the later stages of embryogenesis to support the elevated metabolic activity. With higher incubation temperatures and therefore an increase in metabolic rate, an oxygen deficiency could limit the aerobic metabolism due to an increase in hypoxia. Furthermore, increased temperatures will likely decrease the affinity of oxygen-haemoglobin binding. In the present study, the fish incubated at 6 °C may have consumed the yolk slower, but when comparing to the other temperatures directly against developmental stage, this group showed increased utilisation efficiency. Total lipid (%) was determined from the yolk sac at each developmental stage analysed. At 6 °C there was a higher percentage of total lipid when compared to the other treatments. This suggests that there was a preference of protein uptake at this lower temperature and is likely factor contributing to the higher weights of the first feeding fry (BWi) of both ploidy in the 6 °C groups. Diploids and triploids showed differential patterns of expression in lipid genes at 200 °days, suggesting that at 6 °C, diploids had a higher activity of *lxr* and *srebp1* and that at 8 °C triploids had higher activity of *fas* and *lxr*. This confirms that the two ploidy show different metabolism at different temperatures from an early life stage. Conversely, no differences at all were observed in either ploidy at the eyeing stage. However, at first feeding, there was a higher expression of *srebp1* in the ST

regimes compared to the LT regimes in both ploidy supporting the theory that a switch in temperature during embryogenesis affects normal development. No difference was found in *lxr* expression in diploids but there was significantly lower expression in 6ST triploids compared to 6LT. The acute temperature change clearly influences the ability to utilise the yolk sac during this life stage. *fas*, a gene encoding for fatty acid biosynthesis, was influenced by temperature with 6 °C incubated fish having an increased expression than the higher temperature groups in the diploids. The reduced activity of fatty acid biosynthesis at the higher temperatures may be linked to the change in aerobic scope and therefore the hastened utilisation of the lipid. No differences were found in triploids suggesting that fish were equally synthesising fatty acids irrespective of egg incubation temperature. Higher protein absorption has been previously recorded in triploid carp compared to their diploid siblings and an upregulation of glutamate dehydrogenase (GDH) and oligopeptide transporter (PepT1) were likely possibilities of their better growth (Liu *et al*., 2012; 2014). In the present study, triploids at the first feeding stage were significantly smaller than diploids when incubated at 8 °C or above, but there was no difference between ploidy in both 6LT and 6ST. This further supports the hypothesis that triploids have a lower thermal optima.

The protein from the yolk will be metabolised and used to build muscle through a combination of hyperplasia (fibre recruitment) and hypertrophy (growth of present fibres). Previous studies in diploid *Salmo salar* have reported a preference for muscle growth through an increase in hypertrophy over hyperplasia when embryos are incubated at 10 or 11 °C compared to 1.6 or 6 °C, respectively (Stickland *et al*., 1988; Usher *et al*., 1994). This may be a result of the need to grow more rapidly with the most efficient use of energy available through increased metabolism and activity of the fish. Moreover, nuclear division (hyperplasia) requires more energy expenditure than hypertrophy. This theory coincides with reports of fewer, yet larger fibres and fewer nuclei present in muscle samples of fish

incubated at high temperatures (Usher *et al*., 1994). In the present study, samples for muscle fibre analysis were taken at the smolt stage. It is important to note that each temperature treatment group were at different developmental stages at this sampling point, yet this would be the transition to seawater in a commercial setting (i.e. end of parr-smolt transformation). In triploids, only the 6LT showed a significantly greater FFN than other treatments. Furthermore, it was within the 6LT treatment that had the smallest fibre area which was significantly smaller than all other triploid treatments with the exception of 8LT. There was no such significant variation in the diploid groups, suggesting that muscle development was similar irrespective of incubation temperature. However, when ploidy are compared, this phenotypic difference in higher numbers of smaller fibres is only significant in triploids from 6LT. Johnston *et al*. (1999) discovered a lower density of satellite cells in triploids which are important precursors to muscle fibre recruitment. At smolt, those with a higher proportion of newly recruited fibres are likely to have a greater potential for growth through subsequent hypertrophy post-seawater transfer however the present trial could not be continued into seawater stages to determine this. During embryogenesis, there were no notable differences in expression of *myf5* and *myod* between all treatments, both of which are genes involved in the initial stages of muscle development. This suggests that the increased muscle fibre recruitment may begin at a later life stage. However, there was an altered activity in genes associated with the IGF system in both ploidy in the ST groups. *igf1*, *igf2*, and *igfbprp* all generally showed an increased expression at the first feeding stage (after the temperature change), which supports the idea of direct thermal influence on gene expression. There was a significant downregulation of *igf1r* in the ST regimes for both ploidy. This receptor binds and mediates the insulin-like growth factors (IGFs) and having a lower expression may account for an over expression in the IGFs. At the smolt stage, these differences in expression levels between LT and ST groups from each temperature were no longer present. However, diploids

from 6LT had a higher expression of *igf1* than all other groups. Also, triploids from 6LT, 6ST and 8LT all had an increased expression of *igfbprp* compared to 11LT. The increase of hyperplasia in the 6 °C groups is likely to be linked to this elevated activity in growth hormone genes.

A higher prevalence of externally observable deformities was found with increasing incubation temperature, with jaw malformations being the most common. Visible vertebral deformity prevalence was generally low and mainly found in 11ST and 11LT in both ploidy. Jaw malformations increased with increasing temperatures and duration in both ploidy. This suggests that the pathogenesis of jaw malformation manifests during the embryogenesis stage. Also, there was an increase in jaw malformation occurrence in 6ST and 11ST of both ploidy compared to their LT equivalent. This is likely due to the change in temperature these groups experienced when transferred to 8 °C during this period of embryogenesis. Amoroso *et al*. (2016b) observed a significant downregulation of *col2a1* in smolts with jaws affected with LJD suggesting a cartilaginous impairment. In contrast, in the present study there was a significant upregulation of *col2a1* observed at first feeding in fish that experienced a temperature switch at 400 °days compared to those from a constant temperature. Results reported from Amoroso *et al.* (2016b) were obtained from smolts with known LJD whereas the samples in the present trial were whole body individuals at first feeding and vertebral dissections of smolt with deformity status unknown. These differences likely explain the contrasting results between the studies. Despite this, it is clear that this gene, associated with cartilage formation, is likely associated with LJD and a temperature change during embryogenesis alters normal cartilage development. The prevalence of LJD in 6ST compared to 6LT was still very low in comparison to the difference observed between 11ST and 11LT. Similar to previous studies (O'Flynn *et al*., 1997; Benfey, 2001; Fraser *et al*., 2015), there was a higher occurrence of jaw malformations in triploids when compared to diploids,

however this was only evident in 8LT, 11ST and 11LT. In recent years, deformity results in triploid trials have sparked investigation into nutritional requirements and triploid specific diets have been trialled with supplementation of bone building blocks, in particular phosphorous (Fjelldal *et al*., 2015; Smedley *et al*., 2016). In the present trial, all triploids were fed a high phosphorous diet and a deformity response was still observed in the higher temperature groups, suggesting that the triploids are likely to also have particular environmental requirements during embryogenesis.

Using mammography, deformities were radiologically assessed at smolt. Again, a clear correlation between prevalence and increasing temperature was found, and in all temperature groups, triploids had a higher prevalence of deformed vertebrae compared to diploids. This further supports the increased thermosensitivity of triploids. When assessing deformity prevalence, all malformed vertebrae were considered although these are not necessarily detrimental to the health and welfare of the fish. With that in mind, severities of deformities are a more relevant parameter for comparison. According to Hansen *et al*. (2010), a low prevalence of deformed vertebrae (<6 dV) at harvest would not have detrimental effect on the performance and welfare of the fish. In the present study, deformity assessment was done at smolt, therefore how these deformities would manifest in sea water is unknown. However, there is a clear link between deformity severity and increasing incubation temperature in both ploidies which concurs with results from Fraser *et al*. (2015). Severe deformities (≥10 dV) were absent in the 6 °C groups, with the exception of one single diploid fish in 6ST. Both diploid and triploid fish showed increased prevalence of severe deformities under 11ST and 11LT and this coincides with a growth depression. Further, triploids had an increase of severe deformities in 8LT compared to 6LT and 6ST whereas diploids had 0 % prevalence supporting the hypothesis that triploids have a higher thermosensitivity. It can therefore be assumed that an increase in severe skeletal malformations associated with the sub-optimal

incubation conditions have significant implications for fish welfare and growth performance, with triploids in particular (Grini *et al*., 2011; Fraser *et al*., 2012; 2015; Amoroso *et al*., 2016a). The cause of this is likely linked to thermal effects on bone formation during embryogenesis where mineralisation and ossification occurs. Moreover, accelerated growth observed at higher temperatures has been linked with altered gene transcription, particularly those coding for osteoblast development and chondrocyte growth (Ytteborg *et al*., 2010a).

Samples were collected for mineral analysis to assess the composition against skeletal deformities. Ca and P are both key minerals for the bone mineralisation process (Fjelldal *et al*., 2007). No significant differences were found in P, Ca, and Ca:P between temperature groups within either ploidy despite the obvious impact on bone deformity prevalence. P and Ca levels observed appeared to reflect good bone mineralisation (Bæverfjord *et al*., 1998) suggesting that increased incubation temperature was impairing normal cartilage development in this study as observed in Amoroso *et al*. (2016b) rather than disrupting the process of ECM mineralisation directly. Diploids showed no difference in Fe with increasing temperature, but 11LT had greater Fe levels than 6LT in triploids. Contrastingly, Zn levels did not change in triploids at different temperatures, but diploids at 6ST had greater levels of Zn than at 6LT and 11LT. These results suggest that the causes of skeletal malformations are more complex than deficiencies of Ca and P and investigation into different dietary packages are necessary.

Temperature had little or no impact on the expression of genes associated with bone homeostasis and mineralisation in both ploidy at the first feeding stage. However, differential expression between LT and ST regimes were more apparent. Although not always significant, both 6ST and 11ST showed higher expression in both ploidy for all genes except one, suggesting the temperature change they experienced when moved to 8 °C at 400 °days influenced upregulation of gene expression. These groups developed the highest prevalence

of severe vertebral deformities. Contrastingly, Amoroso *et al*. (2016b) found a downregulation of *col2a1* in individuals possessing a lower jaw deformity (LJD), suggesting that differential expression of these genes may be pathology-specific. At the smolt stage, selected genes (*alp*, *bmp4*, *col1a1*, *col2a1*, *ocn*) showed downregulation in diploids under 11LT when compared with 6LT, suggesting a better capability of bone formation under 6LT. Ytteborg *et al*. (2010c) observed downregulation of *col1a1*, *ocn* and *bmp4* in vertebrae that were in the process of fusion, or had already fused. Still, no differences were found in the triploid smolts in the present study, however the aforementioned studies involved samples taken from fish with known deformities which likely explains the contrasting results. Another explanation for this contradiction may be a result of the extra set of chromosomes that triploids possess. How this dosage or dosage compensation influences gene expression remains unclear. Amoroso *et al*. (2016b) concluded *col2a1* and *gphb5* to be reliable biomarkers of LJD by analysing relative expression in individuals who had these malformations. The clear differences in expression between the LT and ST regimes in our study further support the ability of temperature to influence the expression of genes involved with bone and cartilage formation. Further investigations into biomarkers for specific malformation aetiology using individuals which possess particular vertebral deformities should be explored.

2.5. Conclusions

The present study confirmed that an egg incubation temperature of 6 °C is favourable to maintain good development of diploid and triploid *Salmo salar*. This lower temperature during embryogenesis reduced mortality rates historically associated with triploids. Furthermore, yolk-sac utilisation and subsequent muscle development was improved in diploids and triploids. Also, optimal somatogenesis and skeletogenesis are permitted with an

egg incubation temperature of 6 °C in both ploidy. This investigation suggests that early rearing of triploids at 6 ˚C until 400 °days was sufficient in achieving the improvement of these physiological parameters, whilst minimising the impact on production cycle duration. The 6ST regime only adds ~2 weeks to the period of embryogenesis under a production cycle, but has major advantages on triploid *Salmo salar* health compared to the conventional 8 °C regimes. Despite this, the temperature switch at 400 °days still resulted in increased mortality and deformity prevalence compared to an incubation period of a constant 6 °C to first-feeding, particularly in triploids. This supports the theory that triploids are likely more sensitive to temperature alterations that may be experienced during commercial egg rearing. Egg producers may overcome this by using earlier spawning tactics (i.e. advanced photoperiod regimes) to produce triploid eggs earlier in the season. This in turn would allow for a constant 6 °C incubation temperature prior to transfer of triploid eggs into the hatchery phase. Furthermore, the farms receiving the eggs could receive triploid eggs earlier allowing for longer egg incubation at a constant 6 °C. Such alterations would need to be carefully assessed in terms of the economic expenditure associated with longer incubations against the potential benefits in triploid productivity, yield and welfare. Despite the positive results observed when incubated at a constant 6 °C, there is still a degree of ploidy difference with higher mortality, reduced growth and increased deformity prevalence in triploids. Sub-optimal egg quality may be a factor as reduced quality and additional handling associated with triploid induction have been reported to impact on survival and performance (O'Flynn *et al*., 1997; Taylor *et al*., 2011). This was investigated in Chapter 3.

There were two main criticisms of the experimental design. Firstly, three full-sib families were used and eventually pooled into groups to assess the response to ploidy and temperature treatment in smolts. This may play a role in masking significant differences between groups as there is a likely a difference in performance between families and

therefore increasing the error from the means. Also, the lack of replication during this ongrowing period was a limitation. This will further reduce the confidence of the results having only single groups to compare.

Application of a triploid-specific egg incubation temperature, in combination with other triploid specific culture requirements, has the potential to negate the historical issues surrounding growth, deformity and welfare in triploid *Salmo salar*. This study further contributes to the growing knowledge of triploid *Salmo salar* aquaculture conditions and further studies should determine the effect of egg incubation temperature on triploid *Salmo salar* throughout the seawater phase up to harvest size fish.

CHAPTER THREE

THE IMPACT OF POST-OVULATORY AGEING ON THE DEVELOPMENT OF DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.)

3.1. Introduction

Historically, triploid *Salmo salar* have shown highly variable performance with low survival and poor growth in some cases (Galbreath *et al*., 1994; McGeachy *et al*., 1995; O'Flynn *et al*., 1997). However, with the implementation of triploid-specific rearing conditions, more recent trials have reported survival and growth rates comparable to, or better than their diploid siblings (Oppedal *et al*., 2003; Burke *et al*., 2010; Taylor *et al*., 2011). Despite their larger growth potential observed throughout the life cycle, triploids can display a higher variation in growth which raised concerns regarding stock management (Taylor *et al*., 2011). Also, survival rates in triploid eggs during early embryogenesis generally remain lower than diploids (O'Flynn *et al*., 1997; Cotter *et al*., 2002). Furthermore, a high prevalence of skeletal deformities was observed in early investigations of triploid fish (Sutterlin *et al*., 1987; Sadler *et al*., 2001; Fjelldal and Hansen, 2010). Although not completely resolved, more recent research has shown that the prevalence of deformities can be significantly reduced, supporting the concept that culture requirements of triploids are different than their diploid siblings. For example, nutritional studies have demonstrated that triploids have a greater requirement for dietary phosphorous (Fjelldal *et al*., 2015; Smedley *et al*., 2016; Smedley *et al*., 2018) and dietary histidine (Taylor *et al*., 2015; Sambraus *et al*., 2017) to reduce the occurrence of spinal malformation and cataracts, respectively. Furthermore, the incidence of jaw and spinal deformities in triploids has been clearly demonstrated to be positively correlated with increasing egg rearing temperature as shown in Chapter 2 and previously published literature (Fraser *et al*., 2014; 2015; Amoroso *et al*., 2016). This highlights the sensitivity of triploids especially during the embryogenesis period which may result in the increased mortality and poorer growth performances reported. Lowering the egg incubation temperature from 8 to 6 °C was shown to reduce skeletal deformities in triploids, however prevalence still remains higher than in diploids (Fraser *et al*., 2015).

Triploid induction involves increased handling of the eggs and this additional stressor may impact on egg quality. Furthermore, if the eggs used for triploid induction are already of sub-optimal quality, the process of triploidisation will likely exacerbate the subsequent problems reported. Investigations in triploid *Salmo salar* have generally used egg batches collected towards the end of the stripping season when the quality of the oocytes are usually reduced (Taylor *et al*., 2011). Another important factor that has not been studied yet is the effects of egg over-ripening on triploid survival, yield and future development. In diploids, an increase in mortality and prevalence of abnormality has been reported from over-ripe eggs (Mommens *et al*., 2015). Triploidisation of eggs undergoing over-ripening would further reduce yield.

Unlike in the wild, domesticated *Salmo salar* do not spawn naturally as environmental and social cues are lacking. In culture, female broodstock are held in tanks until the farmers manually strip them to collect the eggs. While farmers regularly check fish for ovulation, if the number of dams to assess is high, it is likely that some ovulated dams may not be stripped on a particular day, and as a consequence, the ripening process will begin. Environmental conditions can also be a factor as temperature is an important cue to trigger the ovulation process (Taranger and Hansen, 1993; Vikingstad *et al*., 2016). When temperature is not controlled, many dams may ovulate at the same time causing further difficulty with more broodstock ready for stripping simultaneously. Under these circumstances, the eggs are held in the body cavity for longer durations until they can be stripped. Delayed stripping may lead to over-ripening of the eggs and a reduction in egg quality (Aegerter and Jalabert, 2004).

Optimal egg quality is a fundamental biological requirement for survival and adequate development of fish. Oocytes are provided with maternal reserves that are used for embryogenesis until they can acquire exogenous food sources. The process of egg degradation from prolonged ageing is also known as post-ovulatory ageing (POA) and can

lead to decreased viability of salmonid oocytes (Craik and Harvey, 1984). Compositional changes of the oocyte in response to POA have been previously described in *Oncorhynchus mykiss* (Craik and Harvey, 1984; Springate *et al*., 1984; Lahnsteiner, 2000; Aegerter and Jalabert, 2004; Rime *et al*., 2004), lake trout (*Salmo trutta lacustris*) (Lahnsteiner *et al*., 1999) and more recently in *Salmo salar* (Mommens *et al*., 2015). It is important to note that the results collated in any trial of this nature will depend greatly on the dams as well as any other factors impacting on egg quality (Aegerter and Jalabert, 2004). The optimal stripping time for *Salmo salar* remains unknown in aquaculture and the synchronization of ovulation can be challenging. Unlike other species, *Salmo salar* eggs are able to remain in the body cavity with little impact on survival, growth and deformity for up to two weeks depending on the incubation temperature (Mommens *et al*., 2015) and up to three weeks in *Oncorhynchus mykiss* depending on the broodstock holding temperature (Aegerter and Jalabert, 2004). The physiological changes associated with ripening in fish eggs can lead to a decrease in fertilisation and hatch success (Bromage *et al*., 1992; Aegerter *et al*., 2005; Mommens *et al*., 2015), increased deformities (Mommens *et al*., 2015) and spontaneous occurrence of triploidy (Nomura *et al*., 2013; Glover *et al*., 2015). To date, there has been no study looking at the impact of POA on the subsequent performances of triploid *Salmo salar*.

Triploids appear to be more sensitive to production stressors than diploids (Fraser *et al*., 2012). The current protocol for inducing triploidy requires increased handling of the oocytes during a vulnerable developmental stage, which may increase egg drop out and reduce robustness during later development. Furthermore, if eggs are already triploid as a result of POA, subjecting them to triploid induction will likely cause mortality. It is therefore important to understand the ploidy-specific thresholds of eggs exposed to POA at this early stage. Quality indicators have been defined for several species (Samarin *et al*., 2015), but not in *Salmo salar*. Such reliable indicators of quality during egg development are likely species-

specific as the incubation periods and ovulatory processes differ between species (Shields *et al*., 1997). New biomarkers of POA and egg quality would help to define ploidy-specific ripening thresholds and improve stripping protocols for both diploid and triploids and subsequently lead to reduced mortality and enhanced performances.

The main aims of the present study were (i) to investigate the effects of POA on survival, growth and deformity in diploid and triploid *Salmo salar*, (ii) to correlate these traits with any biochemical changes in the eggs (and ovarian fluid) which result from POA and (iii) to determine if any of these compositional changes may be used as potential biomarkers, at very early developmental stages, of egg quality and future performances of diploids and triploids.

3.2. Materials and methods

3.2.1. Fish stock and culture conditions

Broodstock stripping and subsequent incubation in the hatchery was carried out at Landcatch Natural Selection (Ormsary, Scotland). Female broodstock (2-sea winter, 10.9 ± 1.4 kg, n = 10) reared under ambient conditions, that had all ovulated within 24 hours, were isolated on 22nd November 2017 and held in a separate tank. After sedation (Tricaine, Pharmaq; 50ppm), eggs were partially stripped (~300 mL "green-eggs") from five 'experimental' dams on increasing days post-ovulation held in the body cavity (0, 5, 10, 15 and 20 days post ovulation; DPO). Five additional 'control' dams were fully stripped after 20 DPO to assess the impact of the partial stripping relative to the experimental dams. Milt from one sire (to minimise paternal influence on development) was initially collected when the dams first ovulated (0 DPO), aliquoted into 1 mL portions and stored at 4 °C with Ringer solution (0.5 mL Ringer 1 mL milt^{-1}) until use on subsequent stripping dates (Ginsburg, 1963). Milt motility was determined before each fertilisation event. A second sire was stripped at 20 DPO

and used to fertilise sub groups of the control eggs to assess any potential impact of milt quality deterioration over 20 days storage in the original sire. Eggs from the control dams were not triploidised.

Following stripping of the dams at respective time points, every egg group (~300 mL) was fertilised (30 secs. mixing 1 mL milt, 60 secs. rinse with 8 °C freshwater) using the same non-related sire, creating half-sib families. After initial fertilisation, each egg batch was divided volumetrically in two (~150 mL), and placed into a water bath at 8 °C prior to triploid induction. Triploidy was induced in one group (655 bar of hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. post-fertilisation) according to Smedley *et al*. (2016), while the others did not receive a hydrostatic shock and were maintained as diploid controls. The procedure was repeated for each dam and at each stripping date. Following water hardening (~1 hr.), individual egg groups were disinfected (Buffodine, Europharma, 1:100) for 10 mins. before laying down. Egg batches were then incubated separately in 6 L silos (15 mL sec^{-1} flow) in darkness under ambient conditions (3.5 ± 1.4 °C).

Eggs were transferred to the Temperate Aquarium Facilities (IoA, Stirling, UK) at 320 °days where the second phase and remainder of the investigation took place. Egg groups from 0, 5, 10 and 15 DPO were used for continuing investigations however groups stripped after 20 DPO were not transferred for further monitoring due to insufficient numbers resulting from very low survival rates. However, egg and ovarian fluid samples to assess quality parameters were still collected as described later. Eggs (n = 3; 50 $family^{-1}$ DPO^{-1} $ploidy^{-1}$) were pooled and triplicated creating 24 x 0.3 m² tanks of 250 eggs. Again, resulting from a higher mortality, fewer eggs (2N; n = 210, 3N; n = 110) were present in the 15 DPO tanks. Tanks were kept under 24 hrs. light at 6.0 ± 0.2 °C until hatch in a RAS system. The temperature was increased gradually to 7.7 ± 1.6 °C after hatch and then to 13.0 ± 0.9 °C after first feeding and maintained until the end of the experiment at ~1000 °days post first

feeding (~5 g) for final assessment. Fish were fed ploidy-specific diets according to manufacturer's guidelines (BioMar Ltd., Grangemouth, Scotland).

3.2.2. Sampling procedures

Ovarian fluid was collected from each dam at all DPO into 1 mL aliquots and immediately frozen at -70 °C for later analyses of osmolality, mineral composition and proteomics. Green eggs were also sampled from each dam at all DPO. Aliquots of 30 mL were collected for proximate analyses and aliquots of 7 mL were collected for analyses of carotenoid composition, vitamin E content, TBARS, fatty acid and lipid class compositions. Samples were immediately frozen at -70 °C until later processing. Ten eggs from each dam at all DPO were collected in 20 mL Bouin's solution (picric acid: formaldehyde: glacial acetic acid; 15:5:1, v:v) for 24 hrs. and then washed several times in 70 % ethanol to remove picric acid and stored in 20 mL 70 % ethanol for future processing. After 20 DPO, dams were sacrificed (Tricaine, Pharmaq; 1000ppm) and body weight (BW) and length were recorded. Fin clips from all experimental and control parents were taken and fixed in 100 % ethanol.

A random sample of eggs (50 $silo^{-1}$) was assessed for fertilisation success at 120 °days by immersing eggs in clearing solution (methanol: acetic acid: water; 1:1:1, v:v) for 2 mins. Eggs which presented neural tube formation were classified as fertilised. Thereafter, viable eggs were determined after mechanical shock at 300 °days. Dead eggs were counted and removed and the rest used to determine survival rates. Egg size was determined at the point of transfer (320 °days). Photographs were taken and measurements were made using Fiji image analysis software (ImageJ).

3.2.3. Verification of ploidy

To confirm ploidy status of triploidised groups and potential spontaneous triploidy in diploid groups, red blood smears were prepared from samples taken from the caudal peduncle of euthanised fish (20 fish DPO^{-1} $ploidy^{-1}$; 6.5 ± 1.4 g). Air dried slides were fixed in 100 % methanol and then placed into Giemsa stain for 10 mins. Slides were digitised using a slide scanner at 20x magnification (Axio Scan.Z1, Zeiss) and erythrocyte length and diameter was determined by Fiji image analysis software (ImageJ). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm and a total mean taken for presumed diploid and triploid fish.

3.2.4. Ovarian fluid pH, conductivity and osmolality

Ovarian fluid pH (FiveGo F2, Mettler Toledo, Leicester, UK) and electric conductivity (EC; pHOX 52) were measured *in situ* immediately after the dams were stripped. One aliquot of ovarian fluid from each dam at all DPOs was defrosted and osmolality was determined from an average of 3 x 200 μL samples according to manufacturer's guidelines (3250 Single Sample Osmometer; Thermo Fisher).

3.2.5. Green egg and ovarian fluid mineral composition

Mineral compositions were determined on eggs and ovarian fluid from all dams at each DPO (0, 5, 10, 15 and 20 DPO) using the nitric acid (HNO_3) digestion technique. Briefly, egg samples were oven dried at 75 °C for 24 hrs. and subsequently powdered using a mortar and pestle. Ovarian fluid samples were analysed wet. Of eggs and ovarian fluid from each dam at all DPO, three runs of 0.1 g samples were digested in Kheldal digestion tubes with 69 % nitric acid using a MARS microwave digestion system (CEM MARSXpress, CEM ltd., Buckingham, UK) using the following program; 10 mins. heating phase to 190 °C, maintain

190 °C for 20 mins., cooling phase to 21 °C for 60 mins. Samples were then diluted with distilled water to 2 % HNO_3 and analysed for mineral content via an Inductively Coupled Plasma Mass Spectrometry (ICP-MS; iCAP RQ; Collision cell technology).

3.2.6. Green egg proximate composition

Proximate composition of eggs was determined according to standard procedures (AOAC, 2000). Moisture content was determined after drying in an oven at 110 °C for 24 hrs. and ash content determined after incineration at 600 °C for 16 hrs. For protein analysis, eggs were homogenised in a blender (Waring Laboratory Science, Winsted, CT, USA) and crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, U.K). Energy content was measured using bomb calorimetry calibrated with benzoic acid (Gallenkamp Autobomb, Gallenkamp & Co. Ltd., London, UK). Total lipid was extracted from eggs according to Folch *et al*. (1957). Approximately 0.25 g of eggs were homogenised in 10 mL chloroform: methanol (2:1, v:v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough). Lipids were separated from contents by adding 5 mL of potassium chloride (KCl; 0.88% w:v) and left on ice for 1 hr. The upper layer was aspirated and the lower layer was dried under nitrogen. Total lipid content of each sample was determined gravimetrically after 12 hrs. in a vacuum desiccator.

3.2.7. Green egg fatty acid composition

Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 ˚C for 16 hrs. (Christie, 2003), and FAME extracted and purified as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy)

equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME were identified by comparison to known standards and published data (Tocher and Harvie, 1988).

3.2.8. Green egg lipid class

Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC) using 20 × 10 cm plates (VWR, Lutterworth, England). Approximately 2 μL of total lipid alongside polar lipid and neutral lipid standards were applied as single spots. Plates were first left in a polar solvent; methyl acetate: isopropanol: chloroform: methanol: 0.25 % aqueous KCl (25:25:25:10:9, by vol.) until solvent had reached half way up the plate. After drying for 15 mins. the plate was fully developed in a neutral solvent; isohexane: diethyl ether: acetic acid (85:15:1.5, by vol.). The lipid classes were visualised by charring at 160 °C for 25 mins. after spraying with 3 % (w:v) aqueous cupric acetate containing 8 % (v:v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC Scanner (VWR International, Dorset, UK) (Henderson and Tocher, 1992). Scanned images were recorded automatically and analysed by computer using winCATS Planar Chromatography Manager (version 1.2.3).

3.2.9. Green egg carotenoids

To assess total carotenoid content and composition of the samples, 1 g of eggs was homogenised and carotenoids were extracted in three-phases using ethyl acetate: ethanol (1:1, v:v), ethyl acetate, and iso-hexane. After evaporation, samples were re-suspended in iso-hexane and the absorbance measured on a spectrophotometer (CE 2021 2000 series, Cecil

Instruments Ltd., Cambridge, UK) at 470 nm against an iso-hexane blank. All samples were subsequently transferred to auto-sampler vials and processed through high performance liquid chromatography (HPLC; Waters Alliance System, Waters Corporation, MA, USA). Identification of carotenoids present was determined using retention time against a known standard of astaxanthin and canthaxanthin. To quantify the outputs, the area of each peak was measured using Empower 2 chromatographic processing software (Waters Corporation, MA, USA).

3.2.10. Green egg vitamin E content

Vitamin E (α-tocopherol) was extracted and determined by reverse phase HPLC (Waters Alliance System, Waters Corporation, MA, USA) with UV detection at 293 nm (Cowey *et al*., 1981). 1 g of eggs was homogenized in 5 mL of 2 % ethanolic pyrogallol. The homogenates were incubated in a water bath of 70 °C for 5 mins. and then 1 mL of 60 % KOH was added and mixed. Samples were incubated at 70 °C for a further 20 mins. with regular mixing in 5 min. intervals. On removal, samples were cooled on ice and 4 mL distilled water and 6 mL iso-hexane (+ BHT) was added. Each tube was vortexed for exactly 1 min. to allow for extraction of the vitamin E into the iso-hexane layer. Layer separation was encouraged by incubating the samples at -20 °C for 45 mins. 4 mL of the iso-hexane layer was transferred to fresh vials and completely evaporated under nitrogen. Samples were re-dissolved in 1 mL methanol and analysed using HPLC. Vitamin E content of each sample was determined against a known standard using Empower 2 chromatographic processing software (Waters Corporation, MA, USA).

3.2.11. Green egg peroxidative stress

The measurement of thiobarbituric acid reactive substances (TBARS) was carried out using a method adapted from Sørensen and Jørgensen (1996). Briefly, 1 g of eggs was homogenized in 15 mL of 7.5 % trichloroacetic acid (TCA). The homogenate was filtered to remove protein precipitates and 2 mL transferred to a fresh vial. 2 mL of thiobarbituric acid (TBA) was added and mixed before heated at 100 °C for 35 mins. Samples were analysed with a spectrophotometer (CE 2021 2000 series, Cecil Instruments Ltd., Cambridge, UK) at 532 nm against a positive and a negative control.

3.2.12. Fish performance

Assessment of first feeding fry was conducted at ~850 °days post-fertilisation for each DPO. Fish (n = 3, 30 individuals replicate^{-1} DPO^{-1} ploidy^{-1}) were sedated (Tricaine, Pharmaq; 50 ppm) and measured for body weight (BW) and fork length (FL). At 1000 °days post first feeding, all fish were sacrificed following a 24 hrs. fast using an overdose of anaesthetic (Tricaine, Pharmaq; 1000 ppm) and a subsequent blow to the head. BW and FL were measured and growth rate was calculated using the thermal growth coefficient (TGC, % BW °C d^{-1}). Random samples (n = 3, 30 fish replicate^{-1} DPO^{-1} ploidy^{-1}) were selected for mammography assessment and frozen flat at -20 °C until later analysis.

3.2.13. Radiological deformity analysis

Right lateral radiographs were taken of randomly selected parr (n = 30 replicate^{-1} DPO^{-1} ploidy^{-1}) using a Faxitron UltraFocus Digital Radiography System (Faxitron Bioptics LLC., Arizona, USA) exposing individuals for 1.8 mA at 26 kV. Radiographs were digitalised (AGFA CR35-X) and subsequently examined using ClearCanvas Workstation (Personal Edition, Synaptive Medical, Toronto, Canada) by two independent evaluations.

3.2.14. Statistical analysis

All data were analysed using Minitab statistical analysis software (Version 18.0, Minitab Inc., Pennsylvania, USA). Percentage data were arcsine transformed to meet model requirements. A One-Way ANOVA was used to compare the difference in egg viability and fertilisation rate from both experimental and control broodstock. Linear mixed effect (LME) models were used to assess the impact of both ploidy and POA on egg performances, and POA on ovarian fluid and egg quality parameters using the stripped dam as the random effect, accounting for any genetic influence on parameters investigated. Grow-out performance data (mortality, growth, deformity prevalence) were analysed with a Two-Way ANOVA incorporating POA and ploidy and the interaction between them. Normality and homogeneity of variance in the data were confirmed using Kolmogorov-Smirnov and Levene's tests, respectively. Post-hoc tests were determined by Tukey's multiple comparisons. Correlations were determined by Pearson's correlation coefficient and expressed as r^2. All significance was accepted at $p < 0.05$.

3.3. Results

3.3.1. Triploid induction success and spontaneous triploidy

Diploid groups at 0, 5 and 10 DPO had significantly smaller erythrocyte nuclear lengths, with no overlaps with the pressure shocked triploid groups (2N, 6.5 ± 0.6 μm; 3N, 9.6 ± 0.7 μm) confirming that all analysed eggs subjected to hydrostatic pressure shock developed as triploid fish (data not shown). No diploid groups at 0, 5 and 10 DPO had any occurrence of spontaneous triploidy however at 15 DPO, 6.7 % of 'diploids' experienced spontaneous triploidy (no assessment could be done in 20 DPO fish due to high mortalities). Where

spontaneous triploidy did occur in diploid groups (15 DPO), erythrocyte nuclear lengths ranged from 6.6 – 9.5 μm, giving lengths that overlapped both diploid and triploid ranges.

3.3.2. Impact of POA on oocyte survival, fertilisation and size

There was an overall higher survival rate in diploid eggs compared to triploid eggs during the periods between fertilisation and mechanical shock ($p = 0.002$) and between transfer and hatch ($p = 0.034$) (Table 3.1a). There was also an overall POA effect during both periods as survival significantly decreased from 0 to 20 DPO and from 0 to 15 DPO, respectively ($p < 0.001$). Both ploidy responded the same to increasing POA during the period between fertilisation and mechanical shock, however a ploidy and POA interaction was observed between transfer and hatch ($p = 0.020$). In diploids, survival was not affected with increasing POA however triploids had a greater survival in 0 and 5 DPO groups compared to 10 DPO. Also, triploid egg survival during this period was significantly greater at 0 DPO compared to 15 DPO. No dam effect was observed in survival during either period.

Triploids had an overall smaller egg diameter compared to diploid siblings ($p = 0.005$) and an overall significant increase in egg diameter was observed in response to POA ($p < 0.001$) (Table 3.1b). No dam effect was observed. Fertilisation success was also overall significantly reduced in triploids compared to diploids ($p = 0.008$) and an overall significant decrease was observed in response to POA ($p < 0.001$). No dam effect was observed.

Table 3.1. (**a**) Survival between fertilisation and mechanical shock, between transfer and hatch and (**b**) performances including egg diameter and fertilisation success of *Salmo salar* diploid and triploid eggs (5 dams DPO^{-1}) exposed to increasing days of post-ovulatory ageing. Data are presented as means ± SD and superscripts denote significant differences between treatments when a significant ploidy*DPO interaction was observed ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

		(a) Survival		**(b) Performances**	
Days post-ovulation	**Ploidy**	**Fertilisation to shock**	**Transfer to hatch**	**Egg diameter (mm)**	**Fertilisation success (%)**
0 DPO	Diploid	57.1 ± 18.8	92.4 ± 1.4^{a}	6.6 ± 0.2	91.6 ± 7.9
	Triploid	47.1 ± 18.4	91.5 ± 1.4^{a}	6.5 ± 0.2	90.5 ± 5.6
5 DPO	Diploid	36.5 ± 22.6	86.8 ± 2.5^{abc}	6.7 ± 0.2	55.2 ± 21.4
	Triploid	32.3 ± 11.8	90.2 ± 1.1^{ab}	6.5 ± 0.3	52.1 ± 5.6
10 DPO	Diploid	60.3 ± 8.8	87.9 ± 3.6^{ab}	6.8 ± 0.3	75.8 ± 5.6
	Triploid	36.1 ± 17.3	78.5 ± 1.2^{c}	6.7 ± 0.2	54.0 ± 13.5
15 DPO	Diploid	31.7 ± 13.1	87.3 ± 2.2^{abc}	6.8 ± 0.3	49.0 ± 9.7
	Triploid	13.5 ± 11.5	83.9 ± 4.1^{bc}	6.8 ± 0.2	40.7 ± 15.9
20 DPO	Diploid	9.0 ± 6.2	·	7.0 ± 0.1	38.2 ± 12.4
	Triploid	5.6 ± 2.4	·	6.9 ± 0.1	12.4 ± 12.3
***p* value**	ploidy	**0.002**	**0.034**	**0.005**	**0.008**
	DPO	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	ploidy*DPO	ns	**0.020**	ns	ns

3.3.3. Impact of POA on survival, performance and size variation during grow out

Survival between hatch and first feeding was not impacted by ploidy, however an overall significant effect was observed in DPO with 0 and 10 DPO having lower survival compared to 5 and 15 DPO and a significantly lower survival in 0 DPO compared to 10 DPO ($p < 0.001$) (Table 3.2a). Between first feeding and the end of the trial, triploids had an overall lower survival than diploids ($p = 0.001$) however no overall POA effect or interaction was observed.

Diploids had an overall larger first feeding body weight than triploids ($p = 0.012$) however no POA effect was observed in either ploidy (Table 3.2b). Diploids also had an overall greater TGC than triploids ($p < 0.001$) and there was an overall decreased TGC at 5 DPO compared to other DPO groups ($p < 0.001$). This was reflected in the final body weight with diploids having overall significantly greater weights ($p < 0.001$) and an overall DPO effect was observed (0 DPO = 5 DPO < 10 DPO = 15 DPO) ($p < 0.001$). No ploidy or DPO effect was observed in the weight variation expressed by the interquartile range (IQR) (Table 3.2b; Fig. 3.1.). An overall ploidy effect on length was also observed with diploids significantly longer than triploids ($p < 0.001$). Both ploidy showed significantly smaller lengths at 0 and 5 DPO compared to 10 and 15 DPO ($p < 0.001$). An overall greater length IQR was observed in triploids compared to diploids ($p = 0.041$) however no DPO effect was apparent (Table 3.2b; Fig. 3.2.). Condition factor was significantly greater in diploids compared to triploids ($p < 0.001$) and an overall POA effect was observed with condition factor in 0 DPO fish significantly greater than in 15 DPO ($p = 0.046$).

Table 3.2. (**a**) Survival and (**b**) performance summary including weight, IQR of final weight, growth rate, length and condition factor of diploid and triploid *Salmo salar* juveniles exposed to increasing days of post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}). No superscripts are included as ploidy*DPO interactions were not significant ($p < 0.05$; Two-Way ANOVA). IQR = interquartile range; ns = not significant.

	Days post-ovulation								***p* value**		
	0 DPO		**5 DPO**		**10 DPO**		**15 DPO**				
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	ploidy	DPO	ploidy*DPO
(**a**) *Survival (%)*											
Hatch to first feeding	91.8 ± 3.4	92.9 ± 2.0	96.0 ± 1.6	95.8 ± 0.9	87.1 ± 0.9	86.5 ± 3.1	98.1 ± 1.3	95.5 ± 1.1	ns	**< 0.001**	ns
First feeding to end	98.4 ± 1.0	95.3 ± 3.3	97.6 ± 0.4	92.6 ± 2.2	98.8 ± 0.7	95.4 ± 0.9	97.8 ± 1.9	96.1 ± 2.4	**0.001**	ns	ns
(**b**) *Performances*											
First feeding weight (g)	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	**0.012**	ns	ns
Growth rate (TGC)	1.2 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	**< 0.001**	**< 0.001**	ns
Final weight (g)	6.1 ± 0.1	5.0 ± 0.1	5.6 ± 0.3	4.5 ± 0.2	6.6 ± 0.1	5.2 ± 0.2	6.5 ± 0.1	5.8 ± 0.2	**< 0.001**	**< 0.001**	ns
Final weight IQR	1.9 ± 0.3	1.8 ± 0.2	2.2 ± 0.4	2.0 ± 0.2	2.1 ± 0.2	2.3 ± 0.3	2.1 ± 0.2	2.5 ± 0.7	ns	ns	ns
Length (mm)	80.2 ± 0.3	76.5 ± 0.5	78.5 ± 1.2	75.3 ± 0.6	82.6 ± 0.5	78.3 ± 0.9	83.1 ± 0.4	81.2 ± 0.5	**< 0.001**	**< 0.001**	ns
Length IQR	8.7 ± 0.9	8.3 ± 0.9	9.3 ± 0.9	10.5 ± 0.5	8.4 ± 0.5	10.3 ± 0.9	8.3 ± 0.9	11.3 ± 1.4	**0.041**	ns	ns
Condition (K_f)	1.16 ± 0.01	1.08 ± 0.01	1.13 ± 0.02	1.04 ± 0.02	1.14 ± 0.00	1.05 ± 0.01	1.11 ± 0.01	1.05 ± 0.02	**< 0.001**	**0.046**	ns

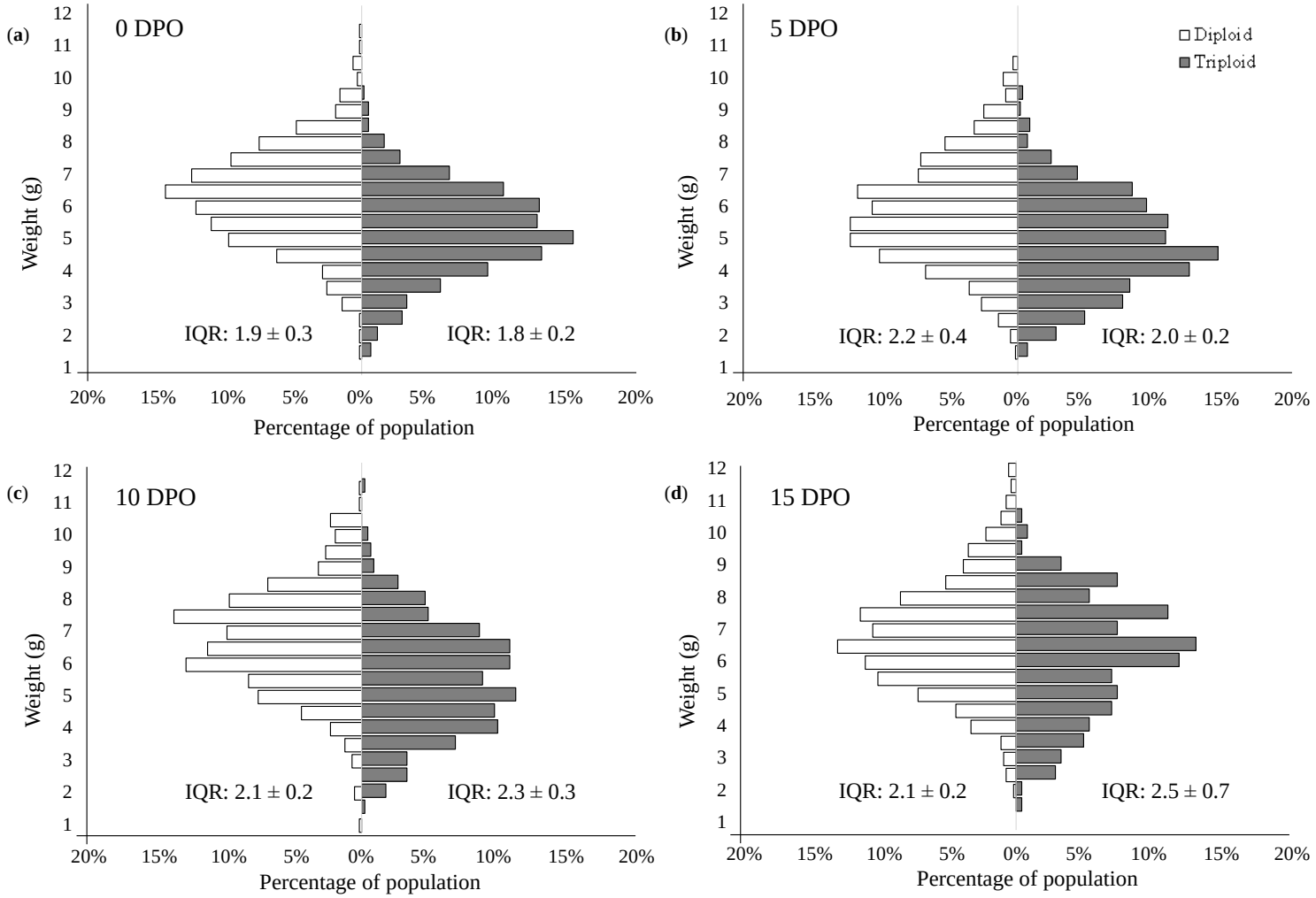


Figure 3.1. Variance of final weight of diploid and triploid *Salmo salar* after (**a**) 0 DPO, (**b**) 5 DPO, (**c**) 10 DPO and (**d**) 15 DPO. IQR = interquartile range.

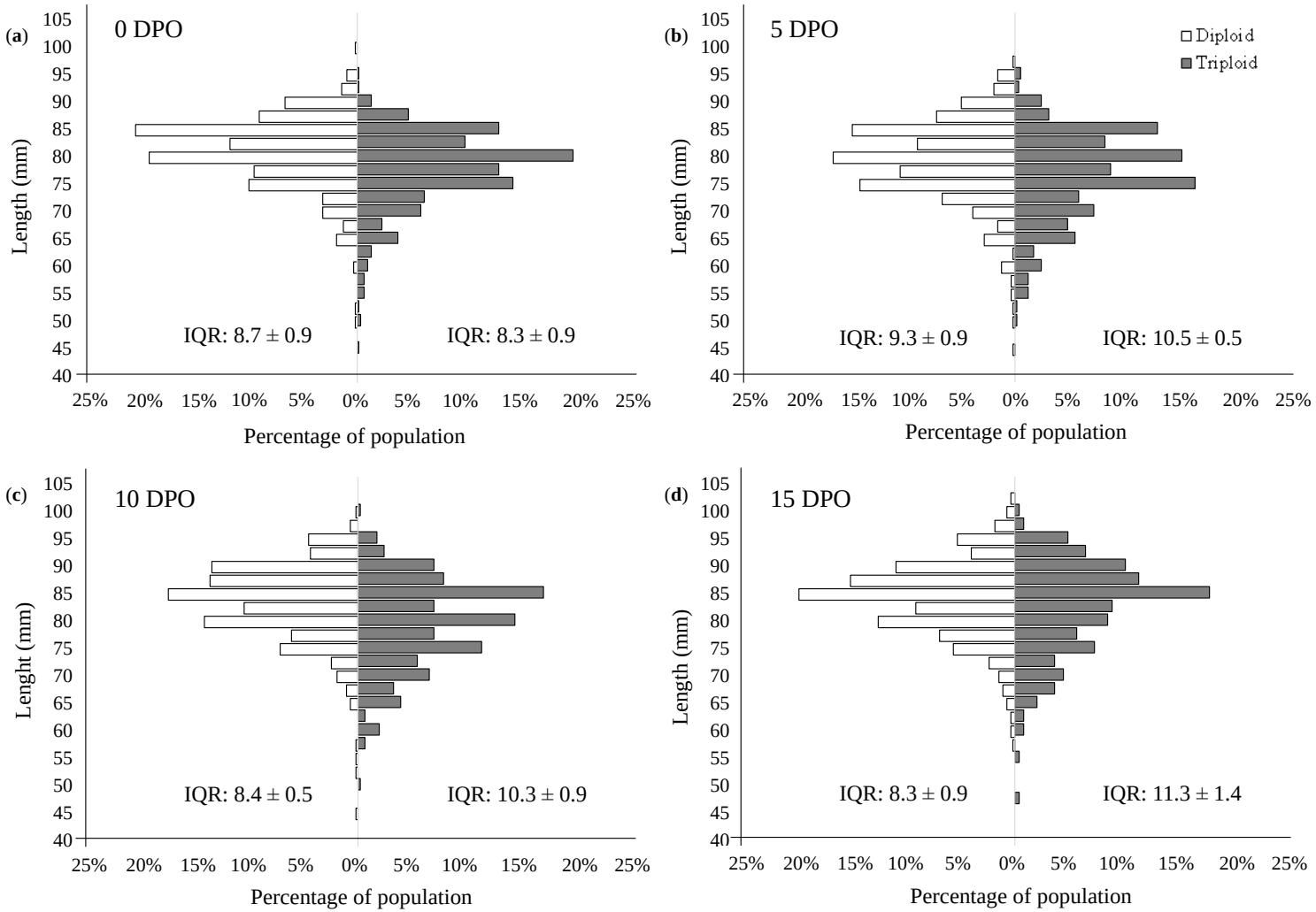


Figure 3.2. Variance of final length of diploid and triploid *Salmo salar* after (**a**) 0 DPO, (**b**) 5 DPO, (**c**) 10 DPO and (**d**) 15 DPO. IQR = interquartile range.

3.3.4. Impact of POA on skeletal deformities

The percentage of deformed (ranging from 0.4 and 2.9 %) or pin (ranging from 0.1 to 2.2 %) alevins were overall significantly greater in triploids compared to diploids ($p = 0.007$ and $p = 0.001$, respectively). No POA effect was observed in either deformed or pin alevins (Table 3.3a).

On a final visual assessment of parr, there was no overall difference between ploidy in the prevalence of externally visible deformities (Table 3.3b). The prevalence of visibly deformed diploid parr significantly increased with increasing DPO (0 > 5 > 10 = 15 DPO), however no DPO effect was observed in triploids. In both ploidy, the visible deformities were largely comprised of opercula shortening (ranging from 0.8 to 11.5 %) while both jaw and vertebral deformities (data not presented) were negligible. Ploidy did not impact the prevalence of opercula shortening however ploidy responded differently as diploids showed a significant increase with increasing DPO (0 > 5 > 10 = 15 DPO) while triploids were not impacted by DPO.

Mammography assessment of the vertebral column showed no ploidy effect on the number of deformed vertebrae (dV) present in deformed individuals (Table 3.3c). Overall, the occurrence of dV was affected by POA with 5 and 10 DPO having a significantly greater number of dV than 0 DPO ($p = 0.015$). There was no impact of ploidy or POA on the prevalence of radiologically deformed individuals (≥1 dV).

Table 3.3. Visible deformity assessment during (**a**) alevin and (**b**) parr stage and (**c**) radiological assessment of diploid and triploid *Salmo salar* parr exposed to increasing days of post-ovulatory ageing. Data are presented as means ± SEM (n = 3). Superscripts denote significant differences between treatments when a significant ploidy*DPO interaction was observed ($p < 0.05$; Two-Way ANOVA). dV: number of deformed vertebrae present in deformed individuals; ns = not significant.

	0 DPO		**5 DPO**		**10 DPO**		**15 DPO**		***p* value**		
	2N	**3N**	**2N**	**3N**	**2N**	**3N**	**2N**	**3N**	**ploidy**	**DPO**	**ploidy*DP**
(a) Alevin abnormalities											
Deformed individuals (%)	0.4 ± 0.2	1.3 ± 0.4	0.7 ± 0.2	1.1 ± 0.3	1.0 ± 0.6	2.9 ± 0.5	1.4 ± 0.5	1.9 ± 0.3	**0.007**	ns	ns
Pin head individuals (%)	0.1 ± 0.1	0.7 ± 0.4	0.7 ± 0.5	1.9 ± 0.5	0.5 ± 0.3	2.2 ± 0.3	0.4 ± 0.2	2.2 ± 1.1	**0.001**	ns	ns
(b) Parr external visible deformity											
Deformed individuals (%)	0.8 ± 0.8^{d}	4.9 ± 1.5^{bc}	3.7 ± 0.5^{c}	4.4 ± 0.8^{bc}	12.6 ± 1.1^{a}	8.8 ± 1.2^{abc}	10.5 ±	4.5 ± 0.5^{bc}	ns	**< 0.001**	**< 0.001**
Opercula shortening (%)	0.8 ± 0.8^{d}	4.1 ± 1.1^{c}	3.0 ± 0.1^{c}	4.0 ± 0.9^{c}	11.5 ± 0.7^{a}	7.3 ± 1.1^{abc}	10.3 ±	4.5 ± 0.5^{bc}	ns	**< 0.001**	**< 0.001**
(c) Parr radiological vertebral deformity											
Deformed vertebrae (dV) (No.)	2.2 ± 0.1	2.5 ± 0.2	5.0 ± 1.0	4.4 ± 0.8	4.5 ± 1.0	5.2 ± 1.0	5.0 ± 0.5	3.9 ± 0.9	ns	**0.015**	ns
Deformed individuals (≥1 dV) (%)	31.1 ± 4.8	35.6 ± 2.9	30.0 ± 5.1	35.6 ± 4.0	32.2 ± 2.9	38.9 ± 4.0	27.8 ± 4.0	27.8 ± 5.9	ns	ns	ns

3.3.5. Impact of partial stripping and milt motility on oocyte viability and fertilisation

No difference was found in fertilisation rates and survival of eggs between the experimental and control dams that had been crossed with the original sire (Control A) (Fig. 3.3.). A significantly higher fertilisation rate was observed in the control groups when using the fresh milt from the second sire (Control B). This coincides with a reduction in milt motility from 100% at 0 DPO to 25% at 20 DPO (data not shown). However, this did not affect the survival as all eggs that had spent 20 days in the body cavity had comparable survival rates.

3.3.6. Ovarian fluid pH, conductivity and osmolality

Ovarian fluid pH was significantly lower at 0 DPO compared to all other DPO (Table 3.4.). No POA effect was observed on EC. Finally, osmolality increased significantly from 0 DPO to 10, 15 and 20 DPO. Also, osmolality at 20 DPO was significantly greater than at 5 DPO. No dam effect was found in any of the three parameters.

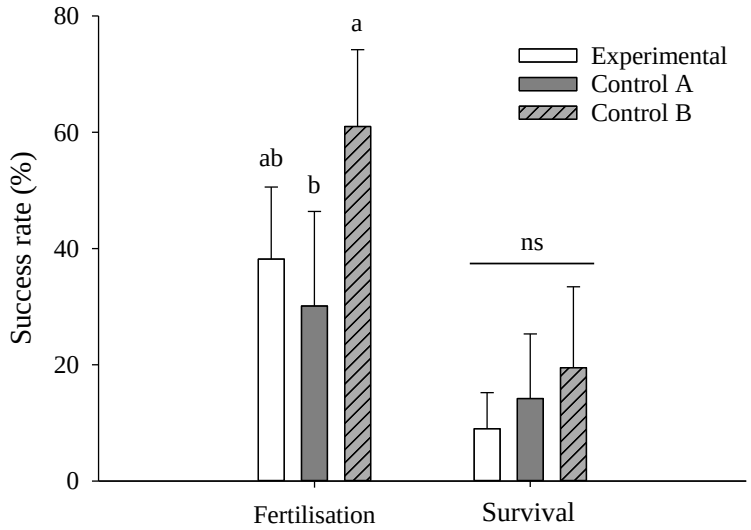


Figure 3.3. Impact of partial stripping and milt motility on fertilisation success and egg survival in egg groups from experimental (5 dams) and control (5 dams) broodstock at 20 DPO. Data are presented as means ± SD and significant differences are denoted by different superscripts ($p < 0.05$; One-Way ANOVA). Control A = control eggs fertilised with original sire. Control B = control eggs fertilised with second sire.

Table 3.4. Ovarian fluid parameters in female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). EC = electrical conductivity; ns = not significant.

	Days post-ovulation					Dam effect
	0 DPO	**5 DPO**	**10 DPO**	**15 DPO**	**20 DPO**	**(*p* value)**
pH	8.3 ± 0.0^{b}	8.5 ± 0.1^{a}	8.4 ± 0.1^{a}	8.5 ± 0.0^{a}	8.4 ± 0.0^{a}	ns
EC (µS; $x10^{-3}$)	7.2 ± 0.1	7.1 ± 0.3	7.4 ± 0.5	7.6 ± 0.4	7.0 ± 0.3	ns
Osmolality (mOsm L^{-1})	268.1 ± 13.8^{c}	277.1 ± 14.3^{bc}	295.2 ± 11.9^{ab}	291.4 ± 11.0^{ab}	301.1 ± 4.3^{a}	ns

3.3.7. Green egg and ovarian fluid mineral composition

No significant differences were found in mineral composition of eggs in response to DPO apart from phosphorous (P) and magnesium (Mg) having higher levels at 15 DPO compared to 10 DPO (Table 3.5a). In ovarian fluid samples, levels of calcium (Ca), sodium (Na), Mg and zinc (Zn) were not impacted by POA (Table 3.5b). However, ovarian fluid P was significantly greater in 5, 10 and 20 DPO compared to 0 DPO and also levels at 20 DPO were significantly higher than 15 DPO. The increase in P gave a corresponding higher Ca:P at 0 DPO compared to all other DPO. Potassium (K) and vanadium (V) significantly increased with increasing POA. Manganese (Mn) was significantly higher at 5 DPO compared to 15 DPO with no other differences observed. No dam effect was observed in any of the minerals analysed in both eggs and ovarian fluid.

3.3.8. Green egg proximate composition

No significant differences were found in dry matter (DM), lipid, ash or energy content of the eggs in response to DPO (Table 3.6.). However, the protein content of eggs at 10 DPO was significantly reduced compared with 5 and 20 DPO and energy content was significantly reduced at 15 DPO compared to 5 DPO. No dam effect was observed in proximate composition analysis.

Table 3.5. Mineral composition of both (**a**) eggs and (**b**) ovarian fluid from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

	Days post-ovulation					Dam effect
	0 DPO	**5 DPO**	**10 DPO**	**15 DPO**	**20 DPO**	**(*p* value)**
(a) *Eggs*						
Calcium (Ca)	1638.1 ± 153.1	1680.6 ± 128.6	1637.1 ± 116.3	1700.8 ± 169.9	1649.5 ± 136.2	ns
Phosphorous (P)	10059.7 ± 440.5^{ab}	10295.4 ± 262.6^{ab}	9871.9 ± 216.3^{b}	10388.0 ± 291.5^{a}	10159.6 ± 356.1^{ab}	ns
Ca:P	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
Sodium (Na)	2010.2 ± 99.4	2025.7 ± 186.6	2214.2 ± 568.6	2355.3 ± 285.9	2012.2 ± 264.6	ns
Magnesium (Mg)	736.9 ± 86.3^{ab}	754.2 ± 62.2^{ab}	734.5 ± 63.7^{b}	776.8 ± 73.7^{a}	761.0 ± 58.3^{ab}	ns
Potassium (K)	3796.9 ± 473.3	3910.2 ± 150.2	3560.9 ± 82.1	3918.3 ± 237.4	3993.8 ± 262.3	ns
Vanadium (V)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	ns
Manganese (Mn)	9.1 ± 1.1	9.2 ± 1.6	9.0 ± 1.4	9.2 ± 1.3	9.4 ± 1.0	ns
Zinc (Zn)	74.6 ± 3.8	76.4 ± 3.7	76.2 ± 7.4	77.0 ± 3.4	74.4 ± 4.8	ns
(b) *Ovarian fluid*						
Calcium (Ca)	86.5 ± 4.4	88.9 ± 9.1	89.2 ± 3.3	83.5 ± 9.2	86.0 ± 9.5	ns
Phosphorous (P)	49.6 ± 4.6^{c}	63.3 ± 8.5^{ab}	62.2 ± 7.0^{ab}	58.0 ± 4.2^{bc}	71.8 ± 3.5^{a}	ns
Ca:P	1.8 ± 0.2^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.2 ± 0.1^{b}	ns
Sodium (Na)	1890.6 ± 105.3	2148.2 ± 220.2	2075.6 ± 205.7	2015.4 ± 48.3	2173.9 ± 173.4	ns
Magnesium (Mg)	9.8 ± 1.0	10.5 ± 1.5	9.9 ± 0.6	9.7 ± 1.1	9.6 ± 0.9	ns
Potassium (K)	55.9 ± 7.0^{b}	53.7 ± 7.6^{b}	60.6 ± 5.4^{ab}	58.0 ± 5.7^{b}	78.4 ± 19.8^{a}	ns
Vanadium (V)	0.002 ± 0.001^{d}	0.003 ± 0.001^{cd}	0.004 ± 0.001^{bc}	0.004 ± 0.001^{b}	0.006 ± 0.001^{a}	ns
Manganese (Mn)	0.102 ± 0.015^{ab}	0.119 ± 0.022^{a}	0.099 ± 0.014^{ab}	0.089 ± 0.016^{b}	0.103 ± 0.029^{ab}	ns
Zinc (Zn)	2.4 ± 3.2	1.8 ± 0.6	3.3 ± 2.1	1.9 ± 1.0	2.4 ± 0.4	ns

Table 3.6. Proximate composition of eggs from female broodstock during post-ovulatory ageing. Lipid, protein and ash results are presented as percentage of sample dry matter (DM). Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

	Days post-ovulation					Dam effect
	0 DPO	**5 DPO**	**10 DPO**	**15 DPO**	**20 DPO**	**(*p* value)**
Dry matter (%)	38.6 ± 0.8	39.1 ± 1.2	38.1 ± 2.4	37.8 ± 1.0	38.9 ± 0.7	ns
Lipid – crude (%DM)	26.2 ± 1.2	26.3 ± 2.4	27.0 ± 0.9	28.0 ± 1.2	26.8 ± 1.6	ns
Protein – crude (%DM)	59.9 ± 1.3^{ab}	61.4 ± 1.7^{a}	58.1 ± 3.1^{b}	59.9 ± 1.2^{ab}	61.8 ± 2.9^{a}	ns
Ash (%DM)	5.5 ± 0.9	5.6 ± 0.9	5.7 ± 0.7	4.5 ± 0.9	5.1 ± 0.8	ns
Energy (kJ g^{-1})	27.0 ± 0.1^{ab}	27.1 ± 0.1^{a}	27.0 ± 0.2^{ab}	26.9 ± 0.1^{b}	27.0 ± 0.2^{ab}	ns

3.3.9. Green egg fatty acid and lipid class composition

There were no significant differences in any of the fatty acids in response to increasing DPO and no dam effect was observed (Table 3.7.). The majority of lipid groups were impacted by increasing DPO (Table 3.8.). The percentage of diacylglycerol (DAG) was significantly greater at 0 DPO compared to 10, 15 and 20 DPO. Cholesterol was significantly highest at 20 DPO compared to all other DPO and levels at 5 DPO were significantly greater than at 0 DPO. The percentages of triacylglycerol (TAG) at 0, 5 and 10 DPO were comparable and all significantly greater than 15 and 20 DPO. There was a significant increase in percentage of sterol/wax esters in 15 DPO compared to 5 DPO with no other differences observed. Total neutral lipid percentage decreased in response to POA from 0 and 5 DPO to 15 and 20 DPO. Sphingomyelin was significantly higher at 15 DPO than all other DPO groups. Phosphatidylcholine was significantly greater in 20 DPO compared to all other DPO groups. The percentage of phosphatidic acid / phosphatidyglycerol was greater at 10 and 15 DPO compared to 0 DPO. The levels observed in 15 DPO eggs were also significantly greater than 5 and 20 DPO. The percentages of phosphatidylethanolamine at 15 and 20 DPO were significantly greater than at 0 DPO. Collectively, the increase of sphingomyelin and phospholipid levels resulted in an increase of total polar lipids in response to POA. This transformation in both neutral and polar lipids resulted in a significant decrease in neutral: polar lipid ratio. The ratio in 0 and 5 DPO eggs was significantly greater than 15 and 20 DPO. Also, a higher ratio was observed at 10 DPO compared to 20 DPO. No dam effect was observed in any of the individual lipid classes.

Table 3.7. Fatty acid composition of eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

	Days post-ovulation					Dam effect
	0 DPO	**5 DPO**	**10 DPO**	**15 DPO**	**20 DPO**	**(*p* value)**
Fatty acid (mg $100g^{-1}$)						
Total saturated	1059.9 ± 71.6	1056.2 ± 170.7	1104.2 ± 171.8	1113.2 ± 68.1	1122.8 ± 179.7	ns
Total monoenes	2607.6 ± 323.7	2521.2 ± 346.0	2724.9 ± 400.5	2620.7 ± 150.4	2758.0 ± 123.2	ns
18:2n-6	629.2 ± 84.5	615.1 ± 98.5	664.6 ± 102.4	629.1 ± 30.8	668.8 ± 27.4	ns
20:4n-6	57.5 ± 6.9	57.9 ± 8.9	62.6 ± 9.7	62.8 ± 7.1	64.6 ± 8.1	ns
Total n-6 PUFA	868.7 ± 128.3	838.6 ± 117.2	911.1 ± 139.0	866.1 ± 49.3	907.4 ± 44.9	ns
18:3n-3	220.8 ± 20.3	224.8 ± 44.2	245.5 ± 47.3	229.5 ± 18.7	246.5 ± 20.4	ns
20:5n-3	454.7 ± 46.5	442.6 ± 58.0	466.7 ± 62.7	458.8 ± 36.7	477.0 ± 27.7	ns
22:6n-3	933.0 ± 68.3	987.0 ± 159.6	1030.3 ± 173.2	1017.0 ± 78.6	1050.2 ± 74.7	ns
Total n-3 PUFA	2112.6 ± 141.2	2162.6 ± 342.4	2285.3 ± 372.5	2237.5 ± 147.2	2325.9 ± 161.3	ns
Total PUFA	2996.4 ± 246.2	3016.0 ± 455.4	3205.1 ± 511.9	3115.9 ± 189.4	3253.2 ± 199.3	ns
Total	6663.9 ± 621.0	6593.4 ± 963.0	7034.2 ± 1079.8	6849.8 ± 386.0	7134.0 ± 442.1	ns

Table 3.8. Lipid class content of eggs from female brood fish during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

	Days post-ovulation					Dam effect
	0 DPO	**5 DPO**	**10 DPO**	**15 DPO**	**20 DPO**	**(*p* value)**
Lipid class (%)						
Diacylglycerol	1.9 ± 1.2^{a}	1.2 ± 0.1^{ab}	0.8 ± 0.1^{b}	0.7 ± 0.2^{b}	0.9 ± 0.2^{b}	ns
Cholesterol	9.9 ± 0.2^{c}	11.1 ± 0.2^{b}	10.0 ± 0.2^{bc}	10.5 ± 0.4^{bc}	13.0 ± 1.4^{a}	ns
Triacylglycerol	55.5 ± 2.0^{a}	55.6 ± 2.8^{a}	54.9 ± 1.4^{a}	49.2 ± 3.6^{b}	45.2 ± 3.2^{b}	ns
Sterol/Wax esters	3.7 ± 2.0^{ab}	3.0 ± 0.4^{b}	4.2 ± 1.1^{ab}	6.8 ± 1.7^{a}	4.2 ± 4.2^{ab}	ns
Total neutral lipids	70.9 ± 2.3^{a}	70.9 ± 2.5^{a}	70.0 ± 1.1^{ab}	67.2 ± 2.6^{b}	63.4 ± 0.5^{c}	ns
Pigmented material	1.5 ± 0.7	0.8 ± 0.5	0.7 ± 0.6	1.3 ± 0.1	1.2 ± 0.3	ns
Sphingomyelin	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	1.4 ± 0.1^{a}	0.6 ± 0.2^{b}	ns
Phosphatidylcholine	22.4 ± 0.9^{b}	21.8 ± 1.5^{b}	22.3 ± 1.0^{b}	21.7 ± 2.1^{b}	28.0 ± 0.7^{a}	ns
Phosphatidic acid/Phosphatidyglycerol	0.7 ± 0.4^{c}	1.0 ± 0.3^{bc}	1.3 ± 0.1^{ab}	1.6 ± 0.3^{a}	1.0 ± 0.2^{bc}	ns
Phosphatidylethanolamine	3.9 ± 1.9^{b}	4.9 ± 0.6^{ab}	5.1 ± 0.3^{ab}	6.8 ± 0.7^{a}	5.8 ± 0.1^{a}	ns
Total polar lipids	29.1 ± 2.3^{c}	29.1 ± 2.5^{c}	30.0 ± 1.1^{bc}	32.8 ± 2.6^{b}	36.6 ± 0.5^{a}	ns
Neutral: Polar	2.5 ± 0.3^{a}	2.5 ± 0.3^{a}	2.3 ± 0.1^{ab}	2.1 ± 0.2^{bc}	1.7 ± 0.0^{c}	ns

3.3.10. Green egg carotenoids

Astaxanthin levels were significantly greater at 0 and 15 DPO compared to 5 DPO (Table 3.9.). Levels in 0 DPO eggs were also significantly greater than 10 and 20 DPO. Canthaxanthin levels were significantly greater in 0 DPO compared to 5 and 10 DPO, however, comparable at 15 and 20 DPO. Adonirubin levels were significantly greater in 0 DPO compared to all other DPO groups. Lutein levels were significantly greater in 0 DPO compared to 5 and 20 DPO only. Collectively, total carotenoid content was significantly greater in 0 and 15 DPO compared to 5 DPO. Also, total content in 0 DPO was significantly greater than at 10 and 20 DPO. There was no dam effect observed.

Table 3.9. Carotenoid levels in eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model). ns = not significant.

	Days post-ovulation					**Dam effect**
	0 DPO	**5 DPO**	**10 DPO**	**15 DPO**	**20 DPO**	**(*p* value)**
Astaxanthin	6.7 ± 1.0^{a}	6.1 ± 0.8^{c}	6.3 ± 0.7^{bc}	6.5 ± 0.8^{ab}	6.3 ± 0.9^{bc}	ns
Canthaxanthin	0.7 ± 0.1^{a}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{ab}	0.6 ± 0.1^{ab}	ns
Adonirubin	3.6 ± 0.5^{a}	3.2 ± 0.4^{b}	3.4 ± 0.4^{b}	3.4 ± 0.5^{b}	3.3 ± 0.5^{b}	ns
Lutein	0.8 ± 0.3^{a}	0.7 ± 0.2^{b}	0.7 ± 0.2^{ab}	0.7 ± 0.2^{ab}	0.7 ± 0.2^{b}	ns
Total carotenoids	11.7 ± 1.7^{a}	10.6 ± 1.4^{c}	11.0 ± 1.3^{bc}	11.2 ± 1.4^{ab}	11.0 ± 1.6^{bc}	ns

3.3.11. Green egg vitamin E content

Vitamin E (α-tocopherol) concentration was significantly reduced in 20 DPO eggs compared to 0, 5 and 10 DPO (Fig. 3.4.). No dam effect was observed.

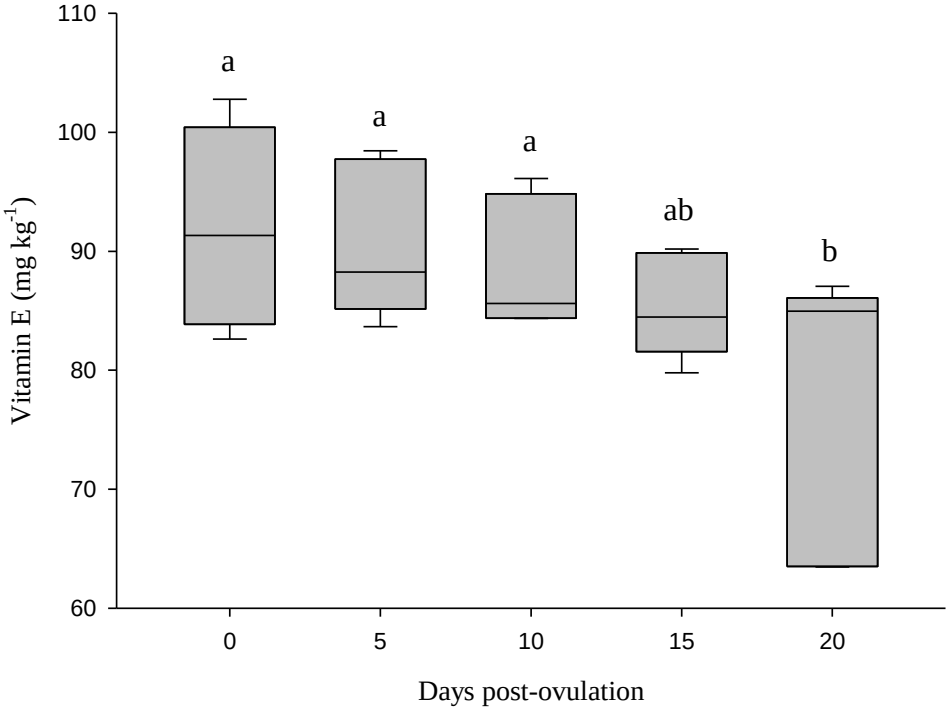


Figure 3.4. Vitamin E (α-tocopherol) concentration in eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model).

3.3.12. TBARS content of green eggs

The level of lipid-peroxidation aldehydes, expressed by TBARS content, was significantly higher in 20 DPO eggs than 0 and 5 DPO (Fig. 3.5.). TBARS levels at 10 and 15 DPO were also significantly greater than at 5 DPO. No dam effect was observed.

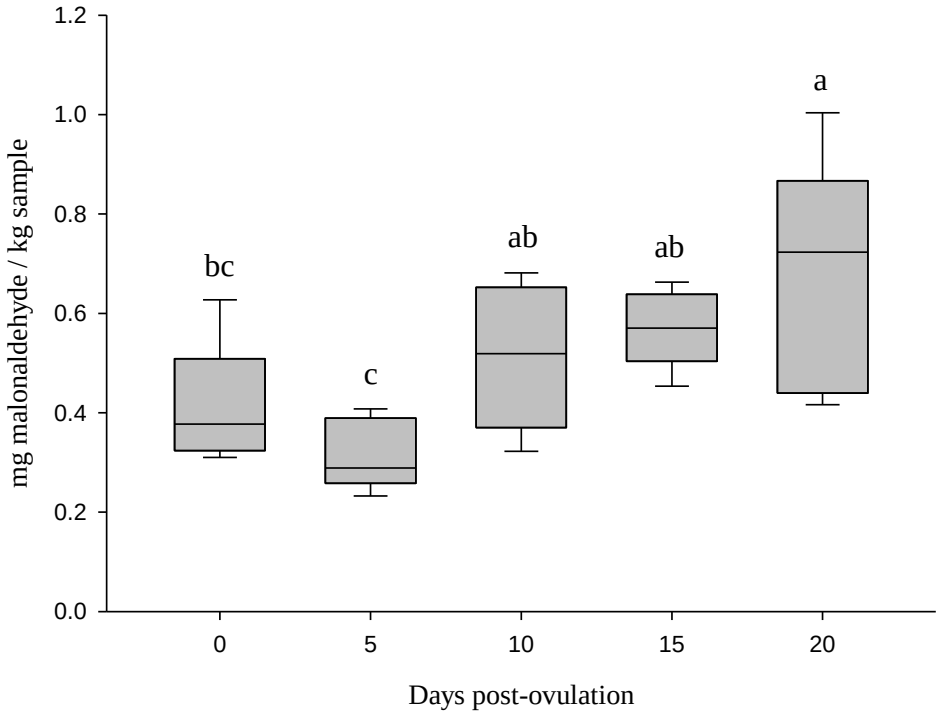


Figure 3.5. TBARS concentration in eggs from female broodstock during post-ovulatory ageing. Data are presented as means ± SD (5 dams DPO^{-1}) and superscripts denote significant differences between treatments ($p < 0.05$; Linear Mixed Effects model).

3.3.13. Correlations

Ovarian fluid osmolality correlated positively with egg diameter in both diploids ($r^2 = 0.954$, $p < 0.05$) and triploids ($r^2 = 0.961$, $p < 0.01$). The change in egg lipid class composition was not correlated with any subsequent trait in diploid fish, however the decreasing neutral: polar lipid ratio correlated positively in triploids with egg survival ($r^2 = 0.929$, $p < 0.05$). The decrease in neutral: polar lipid ratio also correlated negatively with TBARS ($r^2 = 0.918$, $p < 0.05$).

3.4. Discussion

The present study confirmed that post-ovulatory ageing has an impact on the survival and performances of diploid and triploid *Salmo salar*, which can be correlated to several oocyte quality parameters resulting from oocyte ageing. The results suggest that triploids are more sensitive to oocyte ageing as the egg groups exposed to increased POA resulted in an increased variance in individual growth. There is also an increased trend of deformity prevalence in individuals with increasing POA in which triploids appear to be more sensitive to the biochemical changes encountered. Thus, using eggs close to ovulation reduces the likelihood of such undesirable traits. The effect of POA has previously been investigated in diploid *Salmo salar* juveniles (Mommens *et al*., 2015), however, the current study is the first to date which investigated the effects of POA in triploid *Salmo salar*. This knowledge can be applied to industry protocol in improving the quality of egg batches, with triploids in particular, by ensuring the time to stripping post-ovulation is kept to within 10 days, but preferably soon after ovulation for optimal oocytes. Several ovarian fluid and egg quality criteria were assessed as potential biomarkers to determine the future success of a batch in the event of over ripening of eggs. Ovarian fluid osmolality, neutral: polar lipid ratio and TBARS production of green eggs appear to be relevant processes to investigate further.

The reduced fertilisation rate of salmonid eggs observed in the present study in response to POA confirmed previous findings (Bromage *et al*., 1992; Mommens *et al*., 2015). Normally, an egg becomes activated when in contact with freshwater. Water will enter the egg causing swelling and restricting access to the micropyle for spermatozoa (Billard and Cosson, 1992). Osmotic swelling was observed in this study with an increase in egg diameter, albeit not significant. This was correlated with an increase in ovarian fluid osmolality suggesting that water was entering the egg, which is a likely contributing factor to the reduced fertilisation rate. Increased swelling has been shown to decrease chorion thickness in *Oncoryhnchus mykiss* as a result of increased tension within the egg (Samarin *et al*., 2015). No change to the chorion thickness was observed in other studies performed in *Oncorhynchus mykiss* (Lahnsteiner, 2000) and Caspian brown trout (*Salmo trutta caspius*) (Bahre Kazemi *et al*., 2010), however an increase in perivitelline space was evident. Egg samples were collected in the present study to assess chorion morphology and perivitelline space using histology, however, due to time constraints these results could not be included in the thesis. As suggested by Bromage *et al*. (1992), salmonid eggs with a low fertilisation rate generally experience lower survival also during embryogenesis, which was supported by the current study results as egg survival decreased significantly in both ploidy. Therefore, careful production planning with reducing the duration to fertilisation post-ovulation can result in higher numbers of viable eggs for culture. Furthermore, 6.7 % of diploid eggs that were exposed to 15 DPO underwent spontaneous triploidy. Spontaneous triploidy has been previously reported in a large-scale assessment of diploids showing 2 % spontaneous triploidy, confirming that triploidy can occur naturally (Glover *et al*., 2015). Exposing triploid eggs to triploid induction will likely result in mortalities, as suggested in the present study.

Survival from hatch to the end of the trial was comparable between ploidy irrespective of POA status. Moreover, there was no impact of POA on post-hatch survival within either ploidy. Also, POA did not appear to have an impact on first feeding weight of fry although diploids appeared to be overall larger than triploids as previously reported (Galbreath *et al*., 1994; McGeachy *et al*., 1995; Taylor *et al*., 2011). Subsequent growth was affected as triploids exhibited a poorer growth rate than diploids in 0 and 5 DPO groups, but were comparable in 10 and 15 DPO groups. POA had no effect on the growth rate of diploids, but triploids had a greater growth rate in 10 and 15 DPO groups compared to 5 DPO. The growth rates were reflected in the average final weights and lengths of the fish, however, the individual variance appeared to be affected. Although not significant, there was an increase of weight spread in triploid fish in response to increasing POA whereas diploids are only somewhat affected. Furthermore, diploids were significantly longer than triploids overall, however there was a significantly larger variance in triploid length. A decrease in condition factor was observed in both ploidy with increasing ageing. This confirms one of the hypotheses that there is a POA effect resulting in a larger spread of fish size. At the parr stage, fish from five experimental dams were pooled according to ageing status and thus this variation observed may be a result of family influence. Fin clips from all fish were taken and may be used in the future for family assignment. Unfortunately, this could not be done in the time available prior to the submission of this thesis. The increased variation in triploid size poses significant stock management issues. Commercial grading of fish involves categorising individual fish into groups depending on their size usually by passing them over bars spaced at particular distances respective to a size category. A high variation in triploid fish size ("skinny" or "fat") will result in increased numbers falling into the size categories either side of the average and ultimately altering what is considered a normal production observed with diploids. Triploids may be more sensitive to POA, which could potentially be a confounding

factor with the shock associated with triploid induction. This has direct implications in a commercial setting as not only would growth spread within a group be less predictable, but stock management would need to be addressed more regularly to prevent an aggressive hierarchy, thus involving increased handling stress.

As reported in Mommens *et al*. (2015), an increased occurrence of opercula shortening was observed with oocyte ageing in which they concluded stocking density was the likely cause. In the present study, opercula shortening occurrence increased in diploids with POA while in triploids this remained stable irrespective of egg ripening. While the stocking density was comparable for 0, 5 and 10 DPO groups, there was a lower number stocked for 15 DPO due to increased egg mortality prior to stocking. Despite this, there was no difference between diploid and triploid tanks, which suggests that stocking density was not the cause of opercula shortening occurrence. Samarin *et al*. (2016) showed increased abnormalities of Northern pike (*Esox lucius*) larvae in response to POA, however it was only opercula shortening in diploid juveniles in the present study, which appeared to be affected. Mommens *et al*. (2015) also reported an increase in cranial malformations of juveniles in response to POA, however this could not be investigated in the present study. It appears that malformations in juveniles that may have resulted from POA were not associated with jaw or vertebral deformities, as opposed to results reported in Chapter 2 following temperature treatments. Further studies on the impact of POA on specific deformities should be performed.

Broodstock control tests were performed to test if the partial stripping procedure had any detrimental impact on fertilisation rate or egg viability from 20 DPO dams using separate eggs from dams that had also ovulated 20 days prior but with no partial stripping. There was no difference in fertilisation rate or egg viability. However, milt efficiency from the experimental male decreased during the egg stripping period which subsequently led to

reduced fertilisation rates. This suggests that the short term preservation strategy involving Ringer solution and cold storage at 4 °C was not robust enough to keep optimal milt motility for up to 20 days and another approach should be considered if repeated i.e. cryopreservation. Despite this, results confirmed that POA and the associated ovarian fluid and oocyte quality are contributing factors explaining the reduction in fertilisation rate.

Previous investigations have shown a decrease in salmonid ovarian fluid pH in response to increased POA (Lahnsteiner *et al*., 1999; Lahnsteiner, 2000; Aegerter and Jalabert, 2004; Bahre Kazemi *et al*., 2010; Mommens *et al*., 2015). In contrast, pH increased in the present study from 0 to 5 DPO and remained constant thereafter. A decrease in pH is likely a result of the breakdown of the egg membrane which allows the egg content to infiltrate the ovarian fluid (Dietrich *et al*., 2007). The comparable pH between 5, 10, 15 and 20 DPO in this study suggests no cell membrane breakdown occurred during these ageing stages. Mommens *et al*. (2015) found a decrease in pH only between 22 and 28 DPO, which was a longer duration than any of the treatments in the present study. An unexpected low pH at 0 DPO therefore may be likely caused by broken eggs and although precaution was taken, physical damage on the first partial strip should not be discounted. Ovarian fluid osmolality increased linearly with POA and correlated positively with egg diameter in both ploidy. An increase in osmolality was also observed in the study by Mommens *et al*. (2015), who suggested potential leakage of osmolytes from over-ripe eggs, however no osmolality change was observed in some *Oncorhynchus mykiss* investigations (Lahnsteiner, 2000; Aegerter and Jalabert, 2004). However, another assessment of *Oncorhynchus mykiss* coelomic fluid in response to POA suggested that egg protein fragments accumulate in the fluid (Rime *et al*., 2004). Ovarian fluid was collected in the present study for proteomic assessment, however, due to time limitations this analysis could not be completed before the submission of the thesis. Another suggestion could be water ingression into the eggs. As explained earlier,

when eggs are immersed in water, or in this case held in the ovarian fluid for prolonged durations, water will enter the egg via the micropylar canal (Billard and Cosson, 1992), resulting in a higher concentration of solute in the ovarian fluid. An increase in viscosity of the ovarian fluid may further reduce the sperm motility and impact on fertilisation.

Surprisingly, the moisture content of the eggs did not increase with POA. This may be explained by (i) the low volume of water that is able to enter an egg and/or (ii) when processing egg samples for moisture content, if not dried efficiently, it may be possible that ovarian fluid remains. Similarly, no effect was found in energy, total lipid or associated fatty acid composition. Differences were found in protein and energy content between DPO groups, however no trend was observed.

Although total lipid was not impacted by POA, the composition of lipid classes changed. The neutral: polar lipid ratio decreased with egg ripening which was driven by either an increase in phosphate groups or a decrease in diacylglycerol (DAG) and triacylglycerol (TAG). Fraser (1989) discussed how fish larvae catabolise yolk reserves to maintain basal metabolism when experiencing environmental stressors. This energy reserve is predominantly comprised of TAG, which is an important lipid storage utilised for early development in *Salmo salar* alevins (Cowey *et al*., 1985). Therefore, the decrease in TAG observed with POA may be a factor explaining poor survival and growth performance. A decrease in TAG levels was also evident in *Salmo trutta caspius* eggs over 30 DPO (Bahre Kazemi *et al*., 2010). It is likely that increases in reactive oxygen substances (ROS), particularly hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and peroxynitrite ($ONOO^-$), are important factors of POA, inducing oxidative stress and lipid peroxidation (Lord and Aitken, 2013; Samarin *et al*., 2015). Lipid peroxidation has a detrimental impact on cell membranes and enzymatic activity (Lord and Aitken, 2013). Changes to these normal functions are likely causing reduced survival and abnormal development as the decrease of

neutral: polar lipid ratio was correlated with the decrease in egg survival in triploids in the present study. Also, if membrane fluidity is compromised, this may reduce the efficiency of sperm-egg interaction. Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid peroxidation and an increase was observed in response to POA in the current study, which correlated with the oxidation observed in the decrease in neutral: polar lipid ratio. The connection between oxidative stress and oocyte ageing appears to be well-established, however the impact on antioxidant activity is less explored and warrants further studies.

Several antioxidants are present in fish eggs to prevent such oxidative stress. Although not the primary function, carotenoids can serve as an antioxidant as they scavenge and stabilize singlet oxygen and peroxyl radicals (Palace and Werner, 2006). In the present study, individual carotenoids and total carotenoid content were consistently higher at 0 DPO. Vitamin E is also an important antioxidant in teleost eggs (Palace and Werner, 2006). Similar to carotenoid content, vitamin E content decreased in response to POA in the present study. Fish are unable to synthesis their own carotenoids and vitamin E and therefore sufficient levels must be present in the yolk reserves to protect them from ROS experienced with increasing POA. The oxidation process and potentially antioxidant function may serve as useful biomarkers in determining the future health of an egg batch.

3.5. Conclusions

Post-ovulatory ageing appeared to have little effects on the majority of biochemical analyses performed on oocytes. However, between 10 and 15 DPO there appears to be a significant oxidation of lipids with decreased neutral: polar lipid ratio and increased TBARS. These results agree with those of Mommens *et al*. (2015), who suggested that oocyte quality remains stable for up to two weeks post-ovulation. Egg survival and fertilisation were

negatively impacted by POA, however with no differences between ploidy. A difference in performance of juveniles was observed with triploids showing improved average weights and condition factor with increased POA. This may be a result from increased triploid mortality in 10 and 20 DPO during the egg stages subsequently leaving the most robust and better performing individuals. However, variation in size within the triploid groups was greater than in diploids and both ploidy had a decreasing condition factor in response to POA. This would have direct impact on stock management, specifically in the culture of triploids. With this in mind, it would be beneficial to fertilise eggs for triploid production within 5 days post-ovulation to reduce the consequences associated with a higher variation in fish size. For example, an increased range of sizes within a batch will lead to a distinct hierarchy and ultimately increased aggression. Therefore, the need for a reliable biomarker which may predict higher mortalities and increased growth variation would be advantageous for triploid *Salmo salar* aquaculture. Lipid peroxidation and the secondary by-product TBARS levels in unfertilised eggs may be used as an indication of the future survival and performance of a triploid batch. Another factor that may be influencing the differences in traits observed between diploids and triploids is the difference in nutritional requirements they possess and this was investigated in Chapter 4, particularly from first feeding, with the growing demand of alternative feed ingredients.

CHAPTER FOUR

EARLY NUTRITIONAL INTERVENTION CAN IMPROVE UTILISATION OF VEGETABLE-BASED DIETS IN DIPLOID AND TRIPLOID ATLANTIC SALMON (*Salmo salar* L.)

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4.1. Introduction

Demand for farmed *Salmo salar* heavily outweighs the availability of the key raw materials, fishmeal (FM) and fish oil (FO), historically used to formulate feeds. According to the National Research Council (2011), the nutritional requirements for this carnivorous species during the freshwater stages include 42 - 50 % protein, containing essential amino acids, and 16 – 24 % lipid with an emphasis on long-chain omega-3 fatty acids; eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (0.5 – 1 %). Availability of these ingredients from marine resources is finite and alternative protein and lipid sources are required in order to sustain *Salmo salar* aquaculture development. Vegetable-derived proteins and oils are logical alternatives due to their high availability and relatively low production costs.

However, inclusion of plant ingredients in salmonid feeds can result in reduced feed utilisation. This may suggest a digestive and / or metabolic interference, which can cause reduced growth performance and health issues. Reduced digestibility of plant ingredients in salmonid diets has been shown to correlate with reduced retention of protein and energy (Refstie *et al*., 1998; 2000; Aslaksen *et al*., 2007; Wacyk *et al*., 2012), indicating lower metabolic activity and ultimately resulting in lower growth performance. Moreover, health implications such as distal intestinal (DI) enteritis, have been highlighted with some vegetable-based diets (Bæverfjord *et al*., 1996; van den Ingh *et al*., 1996; Knusden *et al*., 2008; Urán *et al*., 2009; Penn *et al*., 2011; De Santis *et al*., 2015). Several anti-nutritional factors (ANFs) have been associated with detrimental effects on growth performance and health when using vegetable-based diets in aquafeeds (Francis *et al*., 2001; Gatlin *et al*., 2007). Advances in feed technology have allowed further enrichment and refinement for several vegetable-based protein ingredients such as the processing of plant meals into protein concentrates i.e. soy protein concentrate (SPC) by alcohol extraction, pea protein concentrate

(PPC) by air classification, and wheat gluten (WG) by physical extraction (Drew *et al*., 2007; Gatlin *et al*., 2007). These processes can reduce or remove ANFs and ultimately reduce the associated health implications on gut morphology posed for salmonids (Rumsey *et al*., 1993; van den Ingh *et al*., 1996; Bureau *et al*., 1998; Burel *et al*., 2000; Drew *et al*., 2007). Regardless, high inclusion of refined ingredients may still cause detriment to salmonids as seen in SPC (Mambrini *et al*., 1999) and PPC (Penn *et al*., 2011) but, at lower levels, such ingredients appear to be successful (Refstie *et al*., 1998; 2001; Carter and Hauler, 2000; Thiessen *et al*., 2003; Øverland *et al*., 2009). Moreover, blending reduced levels of SPC and faba bean protein concentrate (BPC) previously demonstrated improved performance in *Salmo salar* and reduced negative alterations to the gut transcriptome when compared to individual use of each ingredient (Król *et al*., 2016). Continuous refinement of alternative feeds is necessary to maximise benefits and minimise detrimental effects to the fish, with the aim to match the efficiency of traditional feeds optimally designed for carnivorous salmonids.

Nutritional programming has been considered as an option that may help to overcome problems associated with dietary replacement of FM and FO in aquafeeds. This concept involves nutritionally stimulating a physiological function during sensitive, early developmental stages, and has been shown to "programme" or redirect particular metabolic processes in several different mammalian species (Lucas, 1998). The phenomenon has been investigated for several years with studies largely focussed on rodents. Prenatal and postnatal investigations have concluded that a nutritional stimulus can trigger particular cellular development that can impact life development e.g. growth performance (Daenzer *et al*., 2002) and health (Khan *et al*., 2003; 2005; Heywood *et al*., 2004). The idea gained interest in human health studies, and animals such as primates (Mott *et al*., 1995; Mott and Lewis, 2009) and pigs (Guilloteau *et al*., 2010) have been used as models to understand lasting impacts of such nutritional interventions because of their similarities to human physiology. Typically,

investigations have concluded that controlled prenatal or early postnatal nutrition can improve growth and development, and reduce incidence or severity of particular health issues such as obesity and cardiovascular diseases. With regards to agriculture, understanding the consequential importance of the impact of early nutrition will help to (i) improve production and (ii) mitigate potential problems. Evidence suggested that improved performance and increased parasitic resistance in sheep could result from nutritional interventions during the weaning period (Knox *et al*., 2003). To date, there have been only a few similar studies in teleost species. A short exposure of a soybean meal (SBM) diet at first feeding in *Oncorhynchus mykiss* improved the palatability and utilisation of the same diet later in life (Geurden *et al*., 2013). In addition, the programming theory was investigated in zebrafish (*Danio rerio*) and the early nutritional intervention appeared to alter some molecular pathways involved in gut function (Perera and Yufera, 2016).

The overall objective of the present study was to determine if the concept of nutritional programming could operate in *Salmo salar*. The specific aims were, firstly, to determine whether the provision of *Salmo salar* fry with a vegetable-based diet at first exogenous feeding was able to physiologically adapt the fish to accept and more efficiently utilise the same diet at a later life stage without compromising growth performance and health. Secondly, given the growing interest in use of triploid fish in aquaculture, the concept was tested in both diploid and triploid *Salmo salar* in order to establish, not only if there were differences in their performance in response to such changes in raw materials, but also to determine if the concept of nutritional programming was affected by ploidy.

4.2. Materials and methods

4.2.1. Experimental diets

Diets used in this study were formulated by BioMar UK Ltd. and manufactured at the BioMar Tech Centre (Brande, Denmark). Diet formulations and compositions are shown in Table 4.1. Briefly, the marine stimulus diet (Diet $M^{stimulus}$) was a formulation almost exclusively based on fish meal (80 % FM) as protein source and fish oil (4 % FO) as lipid source. The vegetable-based stimulus diet (Diet $V^{stimulus}$) contained only a low proportion of FM (10 % FM) and a mixture of plant protein concentrates (SPC, PPC and WG) as protein sources, whilst rapeseed oil (RO) was the sole added lipid source (0 % FO). The vegetable-based challenge diet (Diet $V^{challenge}$) contained the same FM/FO % and ingredients as Diet $V^{stimulus}$, only a different composition to account for size of pellet.

Table 4.1. Formulation, proximate composition and fatty acid composition of the high marine diet (Diet $M^{stimulus}$) and low fishmeal/fish oil diets (Diet $V^{stimulus}$ and Diet $V^{challenge}$) used in the respective feeding phases**.**

Experimental phase	**Stimulus phase**		**Challenge phase**
Diet	**$M^{stimulus}$**	**$V^{stimulus}$**	**$V^{challenge}$**
Ingredients (g kg^{-1})			
Fishmeal[*]	648.4	50.0	50.0
Crustacean and fish peptones[†]	146.0	50.0	50.0
SPC[‡]	·	163.7	90.2
Wheat gluten[§]	·	214.0	181.7
PPC[‖]	·	210.0	245.7
Wheat[¶]	135.9	139.9	134.4
Fish oil[**]	40.0	·	·
Rapeseed oil[§]	·	60.0	170.6
Vitamins and minerals[††]	22.7	54.8	52.5
Amino acids[‡‡]	7.0	57.6	25.0
Analysed proximate composition			
Lipid – crude (%)	13.3	11.3	21.6
Protein – crude (%)	57.1	56.6	49.6
Energy – gross (MJ kg^{-1})	20.5	20.6	22.7
All fatty acids are % total fatty acids			
PUFA	40.6	37.6	33.3
LA, 18:2n-6	4.8	25.8	22.9
ALA, 18:3n-3	1.3	8.2	8.9
EPA, 20:5n-3	13.0	1.4	0.6
DHA, 22:6n-3	12.1	1.4	0.6

ALA, α-linolenic acid (18:3n-3); DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); LA, linoleic acid (18:2n-6); PPC, pea protein concentrate; PUFA, polyunsaturated fatty acids; SPC, soy protein concentrate.

[*] Feed Services Bremen, Bremen, Germany.
[†] Aker BioMarine, Lysaker, Norway.
[‡] Caramuru, Itumbiara, Brazil.
[§] Cargill, Minnesota, US.
[‖] Agrident, Amsterdam, Netherlands.
[¶] W.N. Lindsay, Tranent, UK.
[**] ED&F Man, London, UK.
[††] DSM, Heerlen, Netherlands.
[‡‡] Evonik, Essen, Germany.

4.2.2. Fish stock and culture conditions

On 8th December 2014, green eggs from three unrelated dams (2-sea winter) and milt from three unrelated sires (2-sea winter) were provided by Landcatch Natural Selection Ltd. (Ormsary, Scotland) and transferred to the Institute of Aquaculture (University of Stirling, Scotland) where the experiment took place in the temperate freshwater recirculation facility. Eggs were divided into two groups (~ 1680 eggs each) for ploidy differentiation. Triploidy was induced in one group (655 bar of hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. post-fertilisation) according to Smedley *et al*. (2016), while the others did not receive a hydrostatic shock and were maintained as diploid controls. Both groups of eggs were incubated at a relatively low temperature of 5.6 ± 0.1 °C to account for the triploids requiring a lower thermal regime for optimal performance (Chapter 2; Fraser *et al*., 2015). Towards the end of the alevin stage (~ 950 degree days, °days), fish were transferred to 12 x 0.3 m² tanks under 24 hrs. light, and water temperature was increased over 11 days prior to first feeding and maintained until the end of the experiment at 12.7 ± 0.5 °C.

4.2.3. Feeding trial

During the first 3 weeks of exogenous feeding, termed the "stimulus" phase, diploid (2N) and triploid (3N) *Salmo salar* were fed either Diet $M^{stimulus}$ or Diet $V^{stimulus}$ using automatic feeders (Arvo-Tec Feeding System, Huutokoski, Finland) for two 4 hr. periods daily. Each of the four treatments (2NM, 3NM, 2NV, and 3NV) was triplicated with 260 fish stocked per tank. During the "marine" phase, all groups were fed a commercial diet for 15 weeks under the same conditions. The "challenge" phase consisted of all groups then being fed Diet $V^{challenge}$ for a further 6 weeks before the trial was concluded. Throughout the experiment, all groups of fish were fed to satiation plus 10 % excess and survival was monitored daily. The only time the fish in the present study were fed different diets was during the stimulus phase

when fish were fed either Diet $M^{stimulus}$ or Diet $V^{stimulus}$. For simplicity, the terms “M-fish” and “V-fish” will be used respectively.

4.2.4. Verification of ploidy

To confirm ploidy status, red blood smears were prepared from samples taken from the caudal peduncle of euthanised fish (n = 20 fish $treatment^{-1}$ $ploidy^{-1}$; 37.0 ± 6.7 g). Air dried slides were fixed in 100 % methanol and then placed into Giemsa stain for 10 mins. Slides were digitised using a slide scanner at 20x magnification (Axio Scan.Z1, Zeiss) and erythrocyte length and diameter was determined by Fiji software (ImageJ). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm and a total mean taken for presumed diploid and triploid fish. Diploid groups had significantly smaller erythrocyte nuclear lengths, with no overlaps with the pressure shocked triploid groups (2N, 8.0 ± 0.6 μm; 3N, 10.4 ± 0.9 μm) confirming that all fish that were subjected to hydrostatic pressure shock were likely to be triploid.

4.2.5. Sampling procedures

For growth assessment, following a 24 hr. fasting period, individuals (n = 3, 30 fish $replicate^{-1}$ $treatment^{-1}$ $ploidy^{-1}$) were weighed (body weight, BW) at the relevant dietary transition periods from the initial (i) to the final (f) sampling point. Fish were anaesthetised prior to weight measurement (50 ppm; Tricaine, Pharmaq, Norway). Growth rate was calculated using the thermal growth coefficient (TGC, % BW °C d^{-1}). For carcass and tissue analyses, fish were randomly selected and killed with an overdose of anaesthetic (1000 ppm; Tricaine, Pharmaq, Norway) and subsequent blow to the head.

4.2.6. Feed intake

Feed intake (FI) was monitored during the final 16 days of the marine phase and for the duration of the challenge phase (41 days). All waste was siphoned out of the tank and uneaten feed was separated from faeces and any detritus. The uneaten pellets were weighed and converted to pellet number and dry weight from earlier calculations. Feed intake was expressed in grams (g) as daily (d) intake per individual (g d^{-1}), and revised for contrasting growth rates between groups; per 100 g average body weight (% BW d^{-1}), and finally to account for the metabolically active tissue, per kg average metabolic body weight (g kg $BW^{-0.8}$ d^{-1}), with 0.8 being accepted as the standard metabolic weight exponent in fish (Lupatsch *et al*., 2003). The response of BW gain to FI during the challenge phase was measured as feed efficiency (FE) and calculated as (BWf - BWi) / FI. Nutrient and energy utilisation efficiency was calculated using determined biochemical compositions (proximate analysis) of whole body fish and feeds with the influence on BW gain.

4.2.7. Proximate composition

Proximate composition of feeds and 24 hr. starved whole fish were determined according to standard procedures (AOAC, 2000). Samples were collected at relevant transition periods after lethal anaesthesia (1000 ppm; Tricaine, Pharmaq, Norway) as described above. Whole fish were homogenised in a blender (Waring Laboratory Science, Winsted, CT, USA) to produce pâtés, and feeds were ground prior to analyses. Moisture contents were obtained after drying in an oven at 110 °C for 24 hrs. and ash content determined after incineration at 600 °C for 16 hrs. Crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, U.K) and crude lipid content was determined after acid hydrolysis followed by Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus, Foss,

Warrington, U.K). Energy content was measured using bomb calorimetry calibrated with benzoic acid (Gallenkamp Autobomb, Gallenkamp & Co. Ltd., London, UK).

4.2.8. Fatty acid composition

Total lipid was extracted from diets, whole fish and tissue pates by homogenisation in chloroform: methanol (2:1, v:v) (Folch *et al*., 1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 ˚C for 16 hrs. (Christie, 2003), and FAME extracted and purified as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME were identified by comparison to known standards and published data (Tocher and Harvie, 1988).

4.2.9. Distal intestine (DI) histology

Fixed DI tissue samples were processed according to standard histological methods. Briefly, the samples were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. Longitudinal cuts (i.e. perpendicular to the macroscopically visible circular folds) of approximately 5 μm were stained with haematoxylin and eosin (H&E). The sections were examined by experienced personnel in three independent blinded evaluations. The following histological characteristics were evaluated according to a previous study (Urán *et al*., 2008): width of the lamina propria (LP) and sub-epithelial mucosa (SUBEM), infiltration of sub-epithelial mucosa by eosinophilic granulocytes (EG), infiltration of the intra-epithelial spaces

by lymphocytes (IEL) and the mitotic activity in mucosal fold base (MFBMA). Details of the histopathological scoring system utilised for the DI samples is given in Table 4.2.

Table 4.2. Description of scoring system covering a range of parameters used to assess severity of enteritis (Urán *et al*., 2008).

Score	Parameter
Lamina propria (LP)	
1	Normal size LP
2	Normal to moderate size LP
3	Moderate size LP
4	Moderate to large size LP
5	Large size LP
Eosinophilic granulocytes (EG)	
1	Few in sub-epithelial mucosa (SUBEM)
2	Increased number in SUBEM (multiple layers)
3	Increased number in SUBEM and migration into LP
4	Diffused number in LP and SUBEM
5	Dense EG in SUBEM and LP
Sub-epithelial mucosa (SUBEM)	
1	Normal SUBEM
2	Normal to moderate size SUBEM
3	Moderate size SUBEM
4	Moderate to large size SUBEM
5	Large size SUBEM
Intra-epithelial lymphocytes (IEL)	
1	Rare IEL (1 per 20 epithelial cells)
2	Mild (focal increase in numbers migrating towards the apical cytoplasm of
3	Moderate (increased numbers often towards the apical cytoplasm of epithelium)
4	Severe (marked increase in IELs)
Mucosal fold base mitotic activity (MFBMA)	
1	Normal (2-3 mitotic epithelial cells)
2	Moderate (5-10 mitotic epithelial cells; some leucocytes might exhibit mitotic
3	High (>10 mitotic epithelial and increment in intestinal leucocyte mitotic

4.2.10. Statistical analysis

Minitab 17 Statistical Software (2010) was used for all statistical analyses. After confirming normality and homogeneity of variance in the data using the Kolmogorov-Smirnov test and Levene's test, a two-way ANOVA was performed on independent parameters considering nutritional history (NH, Diet $M^{stimulus}$ and Diet $V^{stimulus}$), ploidy (2N and 3N), and their interaction. Percentage data were transformed using the arcsine square root function. Significance was accepted at $p < 0.05$ and Tukey's post-hoc test was used to compare significantly different means. To investigate solely nutritional influence, differences shown within a given ploidy were analysed using a Kruskal-Wallis test. Histological scores were analysed statistically using a Kruskal-Wallis test followed by Dunn's post-hoc test for nonparametric, categorical comparison.

4.3. Results

4.3.1. Growth performance

Survival (%) was slightly lower in V-fish and triploids during both the stimulus and marine phases (Table 4.3.). However, during the challenge phase, there were no effects of nutritional history or ploidy on survival. During the stimulus phase, growth rate as measured by thermal growth coefficient (TGC) was higher in M-fish compared to V-fish, and in diploids compared to triploids (Table 4.3.). There was also a significant difference in growth rate during the marine phase with M-fish showing higher TGC than V-fish, although this was only significant in diploids. In general, triploids significantly outgrew diploids ($p < 0.05$) during the marine phase. These effects on growth were observed in the body weights of the fish at the beginning and end of the feed intake trial carried out in the last 16 days of the marine phase, with both initial and final body weights being higher in M-fish than V-fish,

significantly so in both ploidies, and also significantly higher in triploids than in diploids (Table 4.4.). In contrast, during the challenge phase, growth rate for both diploids and triploids was significantly greater in V-fish compared to M-fish as evidenced by the higher TGC values (Table 4.3.). Growth of triploids was also significantly greater than that of diploids during the challenge phase. These growth differences were reflected in final body weights so that, despite the fact that weights of M-fish were higher than those of V-fish at the beginning of the challenge phase, there were no significant differences in final body weights of V-fish and M-fish at the end of the challenge phase (Table 4.5.). The opposite dietary effects on growth rate between the marine and challenge phase were reflected in the respective feed conversion ratios (FCR), with M-fish (both diploid and triploid) showing lower FCR during the marine phase, whereas during the challenge phase, V-fish showed lower FCR values (Table 4.3.). Although the same trend was found in both ploidies, significant differences were only apparent between diploid groups.

Table 4.3. Survival, growth rate and feed conversion ratio of fish during each of the three nutritional phases; stimulus, marine and challenge. Data are treatment means ± SEM (n = 3) based on their ploidy status (diploid or triploid) and their nutritional history during the stimulus phase (Diet $M^{stimulus}$ or Diet $V^{stimulus}$). Percentage data were arcsine transformed for statistical analysis and FCR data in the marine phase was root transformed to achieve normality. Significance was calculated between ploidy, nutritional history (NH) and their interaction (ploidy*NH), and was accepted at $p < 0.05$. Further significant differences within a given ploidy are shown using different superscripts and asterisks indicate a significant difference between phases in a given treatment. ns = not significant.

Ploidy	**Diploid**		**Triploid**		***p* value**		
Nutritional history	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**ploidy**	**NH**	**ploidy*NH**
Survival (%)							
Stimulus phase	98.3	89.2	95.2	79.7	·	·	·
Marine phase	96.7 ± 2.0	92.2 ± 0.4	79.8 ± 1.7	72.9 ± 5.1	**< 0.001**	**0.046**	ns
Challenge phase	99.5 ± 0.3	$99.6 \pm 0.3^{*}$	$98.3 \pm 0.9^{*}$	$98.8 \pm 0.2^{*}$	ns	ns	ns
Growth rate (TGC, % BW °C d^{-1})							
Stimulus phase	0.8	0.5	0.7	0.3	·	·	·
Marine phase	1.4 ± 0.0^{a}	1.3 ± 0.0^{b}	1.5 ± 0.0	1.5 ± 0.0	**< 0.001**	**0.024**	ns
Challenge phase	$1.0 \pm 0.0^{b*}$	1.3 ± 0.1^{a}	$1.1 \pm 0.1^{b*}$	1.5 ± 0.0^{a}	**0.009**	**0.001**	ns
Feed conversion ratio (FCR)							
Stimulus phase	·	·	·	·	·	·	·
Marine phase	0.6 ± 0.0^{b}	0.8 ± 0.0^{a}	0.7 ± 0.1	0.6 ± 0.0	ns	ns	**0.005**
Challenge phase	$0.8 \pm 0.1^{a*}$	$0.6 \pm 0.0^{b*}$	0.9 ± 0.0	0.7 ± 0.0	ns	**0.002**	ns

Table 4.4. Growth parameters, feed intake and feed utilisation during the marine phase. Data are treatment means ± SEM (n = 3) based on their ploidy status (diploid or triploid) and their nutritional history during the stimulus phase (Diet $M^{stimulus}$ or Diet $V^{stimulus}$). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between ploidy, nutritional history (NH) and their interaction (ploidy*NH), and was accepted at $p < 0.05$. Further significant differences within a given ploidy are shown using different superscripts. ns = not significant.

Ploidy	**Diploid**		**Triploid**		***p* value**		
	$M^{stimulus}$	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**ploidy**	**NH**	**ploidy*NH**
Growth parameters (x ind^{-1})							
Initial body weight (g)	11.0 ± 0.2^{a}	9.8 ± 0.2^{b}	13.0 ± 0.3^{a}	10.9 ± 0.7^{b}	**0.006**	**0.005**	ns
Final body weight (g)	17.9 ± 0.0^{a}	14.2 ± 0.0^{b}	19.9 ± 1.2^{a}	16.9 ± 0.6^{b}	**0.007**	**0.001**	ns
Protein gain (g)	1.2 ± 0.0^{a}	0.8 ± 0.0^{b}	1.1 ± 0.1	1.1 ± 0.1	ns	**0.029**	**0.043**
Lipid gain (g)	1.0 ± 0.1^{a}	0.4 ± 0.1^{b}	0.9 ± 0.2	0.5 ± 0.0	ns	**0.009**	ns
Energy gain (kJ)	61.3 ± 3.8^{a}	39.7 ± 3.6^{b}	64.3 ± 7.7	56.3 ± 9.2	ns	**0.046**	ns
Voluntary feed intake (FI)							
FI (g ind^{-1})	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0	0.3 ± 0.0	**0.002**	**0.001**	ns
FI (% BW d^{-1})	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.0	1.9 ± 0.0	ns	ns	ns
FI (g kg $BW^{-0.8}$ d^{-1})	8.1 ± 0.2	8.0 ± 0.2	8.6 ± 0.1	8.2 ± 0.1	ns	ns	ns
Feed efficiency (FE)	1.6 ± 0.1^{a}	1.2 ± 0.0^{b}	1.4 ± 0.1	1.7 ± 0.1	ns	ns	**0.005**
Nutrient and energy utilisation efficiency (% intake)							
Protein retention	50.3 ± 1.4^{a}	37.8 ± 1.1^{b}	41.5 ± 3.2	49.9 ± 3.0	ns	ns	**0.003**
Lipid retention	99.2 ± 9.7^{a}	54.0 ± 13.1^{b}	76.7 ± 11.1	56.3 ± 0.7	ns	**0.022**	ns
Energy retention	71.0 ± 4.6	54.4 ± 4.1	64.2 ± 5.8	68.8 ± 8.5	ns	ns	ns
EPA retention	22.6 ± 1.7	17.1 ± 1.7	20.2 ± 4.2	12.7 ± 6.0	ns	ns	ns
DHA retention	104.9 ± 5.8^{a}	78.9 ± 7.9^{b}	89.7 ± 19.1	55.7 ± 36.0	ns	ns	ns

DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3).

Table 4.5. Growth parameters, feed intake and feed utilisation during the challenge phase. Data are treatment means ± SEM (n = 3) based on their ploidy status (diploid or triploid) and their nutritional history during the stimulus phase (Diet $M^{stimulus}$ or Diet $V^{stimulus}$). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between ploidy, nutritional history (NH) and their interaction (ploidy*NH), and was accepted at $p < 0.05$. Further significant differences within a given ploidy are shown using different superscripts. ns = not significant.

Ploidy	**Diploid**		**Triploid**		***p* value**		
Nutritional history	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**ploidy**	**NH**	**ploidy*NH**
Growth parameters (x ind^{-1})							
Initial body weight (g)	17.9 ± 0.0^{a}	14.2 ± 0.0^{b}	19.9 ± 1.2^{a}	16.9 ± 0.6^{b}	**0.007**	**0.001**	ns
Final body weight (g)	30.0 ± 0.4	28.8 ± 1.3	35.5 ± 1.1	37.1 ± 1.1	**< 0.001**	ns	ns
Protein gain (g)	2.1 ± 0.2	2.3 ± 0.2	2.7 ± 0.1	3.1 ± 0.0	**0.003**	ns	ns
Lipid gain (g)	1.1 ± 0.1^{b}	1.8 ± 0.3^{a}	2.2 ± 0.2	2.7 ± 0.2	**0.004**	**0.029**	ns
Energy gain (kJ)	105.7 ± 1.5^{b}	132.0 ± 14.3^{a}	148.5 ± 10.6	176.3 ± 9.1	**0.005**	**0.040**	ns
Voluntary feed intake (FI)							
FI (g ind^{-1})	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	**0.002**	ns	ns
FI (% BW d^{-1})	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	**0.044**	ns	ns
FI (g kg $BW^{-0.8}$ d^{-1})	5.0 ± 0.4	4.9 ± 0.3	6.0 ± 0.5	6.3 ± 0.2	**0.021**	ns	ns
Feed efficiency (FE)	1.2 ± 0.1^{b}	1.6 ± 0.0^{a}	1.2 ± 0.0	1.5 ± 0.0	ns	**0.001**	ns
Nutrient and energy utilisation efficiency (% intake)							
Protein retention	41.4 ± 3.5^{b}	53.9 ± 0.5^{a}	41.5 ± 2.1	46.4 ± 2.2	ns	**0.011**	ns
Lipid retention	50.4 ± 6.1^{b}	92.4 ± 7.2^{a}	72.8 ± 4.0	87.6 ± 3.2	ns	**0.002**	ns
Energy retention	46.1 ± 2.8^{b}	65.3 ± 2.0^{a}	48.5 ± 1.4	56.6 ± 0.1	ns	**< 0.001**	**0.043**
EPA retention	-8.0 ± 60.8^{b}	165.5 ± 18.9^{a}	-13.9 ± 15.5	121.8 ± 36.4	ns	**0.006**	ns
DHA retention	237.7 ± 152.1^{b}	797.0 ± 50.5^{a}	200.8 ± 34.7	618.5 ± 67.0	ns	**0.002**	ns

DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3).

Table 4.6. Whole fish proximate composition before and after the challenge period. Data are treatment means ± SEM (n = 3) based on their ploidy status (diploid or triploid) and their nutritional history during the stimulus phase (Diet $M^{stimulus}$ or Diet $V^{stimulus}$). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between ploidy, nutritional history (NH) and their interaction (ploidy*NH), and was accepted at $p < 0.05$. Further significant differences within a given ploidy are shown using different superscripts and asterisks indicate a significant difference between phases in a given treatment. ns = not significant.

Ploidy	**Diploid**		**Triploid**		***p* value**		
Nutritional history	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**ploidy**	**NH**	**ploidy*NH**
Pre-Challenge phase							
Dry matter (%)	28.9 ± 0.2	28.3 ± 0.4	28.6 ± 0.0	28.4 ± 0.5	ns	ns	ns
Lipid – crude (%)	10.2 ± 0.5	9.9 ± 0.3	10.6 ± 0.1	10.1 ± 0.6	ns	ns	ns
Protein – crude (%)	15.1 ± 0.2	14.6 ± 0.1	14.5 ± 0.0	14.7 ± 0.1	ns	ns	**0.012**
Ash (%)	2.3 ± 0.1	2.2 ± 0.0	2.0 ± 0.0	2.2 ± 0.1	ns	ns	ns
Energy – gross (kJ $100g^{-1}$)	7.8 ± 0.1	7.7 ± 0.2	7.9 ± 0.0	7.7 ± 0.2	ns	ns	ns
Post-Challenge phase							
Dry matter (%)	30.1 ± 0.3*	30.2 ± 0.4*	31.0 ± 0.3*	30.3 ± 0.1*	ns	ns	ns
Lipid – crude (%)	10.9 ± 0.3	11.8 ± 0.4*	12.0 ± 0.7	12.0 ± 0.2*	ns	ns	ns
Protein – crude (%)	15.8 ± 0.3	15.3 ± 0.0*	15.7 ± 0.3*	15.1 ± 0.1	ns	**0.031**	ns
Ash (%)	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	ns	ns	ns
Energy – gross (kJ $100g^{-1}$)	8.2 ± 0.1	8.4 ± 0.1*	8.6 ± 0.2*	8.4 ± 0.1*	ns	ns	ns

4.3.2. Feed intake

The only significant effect of nutritional history observed on feed intake (FI, g individual d^{-1}) was during the marine phase when M-fish showed higher intake than V-fish, but only in diploids (Table 4.4.). In contrast, triploids consumed significantly more feed than diploids during both marine and challenge phases, irrespective of nutritional history (Tables 4.4. and 4.5.). When FI was corrected (% BW d^{-1}, and g kg met. BW d^{-1}), no impacts of nutritional history or ploidy were observed during either feeding phase.

4.3.3. Feed utilisation

Although feed efficiency (FE) is simply the inverse of FCR, it is perhaps more appropriate when comparing nutrient retentions. Therefore, reflecting the FCR data presented above, during the marine phase FE was higher in M-fish compared to V-fish, significantly so with diploids (Table 4.4.). This resulted in M-fish showing higher protein, lipid and energy retentions and gains in the marine phase compared to V-fish, again significant in diploids with triploids showing identical trends (Table 4.4.). In contrast, the opposite effects on nutrient and energy utilisation were observed during the challenge phase. Thus, FE was higher in V-fish compared to M-fish, significantly so in diploids (Table 4.5.). This resulted in V-fish showing higher protein, lipid and energy gains compared to M-fish, although only significant with lipid and energy gain in diploids. Consistent with these data, protein, lipid and energy retentions in the challenge phase were higher in V-fish than M-fish, significant in diploids with triploids showing identical trends (Table 4.5.). The effects on nutrient utilisation efficiency/retention were therefore consistent across all nutrients, reflected in the fact that there was no effect of nutritional history on whole body proximate composition (Table 4.6.).

4.3.4. Fatty acid retention and composition

The same trend as observed for the macronutrients was also found in EPA and DHA retention in the marine and challenge phases, respectively (Tables 4.4. and 4.5.). Importantly, M-fish lost EPA during the challenge phase, whilst V-fish positively retained EPA (Table 4.5.). In contrast, all fish retained DHA during the challenge phase although retention was far greater in V-fish than in M-fish. Unsurprisingly, the reverse trend was seen during the marine phase, with M-fish have the greater EPA and DHA retentions. As with most parameters, the effects of nutritional history on EPA and DHA retentions were significant for diploids, with triploids showing identical but non-significant trends. Fatty acid profiles of whole body, liver, and pyloric caeca pre-challenge (end of marine phase) and post-challenge phase reflected the dietary fatty acid compositions of the commercial diet (fed in the pre-challenge phase) and Diet $V^{challenge}$ (fed during the challenge phase) in all fish irrespective of nutritional history or ploidy (Table 4.7.). Therefore, total saturated fatty acids and n-3 PUFA (especially EPA and DHA) decreased, and total monoenes and n-6 PUFA (especially linoleic acid [LA], 18:2n-6) increased in all tissues in all fish from pre- to post-challenge. The differences in EPA and DHA retention (based on absolute contents of whole body) between fish of different nutritional history were not reflected in the relative proportions of the fatty acids in whole body.

Table 4.7. Fatty acid compositions (% fatty acid methyl esters) of whole body, liver, and pyloric caeca before and after the challenge phase. Data are treatment means ± SD (n = 3) based on their ploidy status (diploid or triploid) and their nutritional history during the stimulus phase (Diet $M^{stimulus}$ or Diet $V^{stimulus}$). Percentage data were arcsine transformed for statistical analysis. Significance was calculated between nutritional history (NH) within each ploidy and was accepted at $p < 0.05$. Significant differences are shown using different superscripts and asterisks indicate a significant difference between phases in a given treatment.

	Pre-Challenge phase				**Post-Challenge phase**			
Ploidy	**Diploid**		**Triploid**		**Diploid**		**Triploid**	
Nutritional history	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**
Whole body (%)								
Total saturated	25.3 ± 0.3[a]	24.1 ± 0.3[b]	25.0 ± 1.1	24.2 ± 0.8	19.1 ± 0.7*	18.9 ± 0.6*	18.7 ± 0.5*	18.4 ± 0.2*
Total monoenes	46.5 ± 0.6	47.1 ± 0.6	46.8 ± 0.8	46.7 ± 0.5	50.9 ± 0.6*	51.2 ± 0.6*	52.1 ± 0.6*	52.1 ± 0.1*
18:2n-6	4.7 ± 0.1	5.0 ± 0.2	4.7 ± 0.1[b]	4.9 ± 0.1[a]	11.4 ± 0.7*	11.5 ± 0.7*	11.9 ± 0.6*	11.9 ± 0.3*
20:4n-6	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.0*	0.7 ± 0.0*	0.7 ± 0.0*	0.7 ± 0.0*
Total n-6 PUFA	6.3 ± 0.1	6.7 ± 0.2	6.3 ± 0.2	6.6 ± 0.2	14.7 ± 0.8*	14.8 ± 0.9*	15.4 ± 0.7*	15.3 ± 0.5*
18:3n-3	0.9 ± 0.0	1.1 ± 0.1	0.9 ± 0.0[b]	1.0 ± 0.0[a]	3.0 ± 0.2*	3.0 ± 0.2*	3.1 ± 0.2*	3.2 ± 0.1*
20:5n-3	4.0 ± 0.0	4.0 ± 0.2	4.1 ± 0.2	4.2 ± 0.0	2.0 ± 0.2*	2.0 ± 0.2*	1.7 ± 0.2*	1.8 ± 0.1*
22:6n-3	12.4 ± 0.1	12.4 ± 0.6	12.2 ± 0.4	12.6 ± 0.4	6.8 ± 0.5*	6.7 ± 0.7*	5.7 ± 0.6*	6.0 ± 0.4*
Total n-3 PUFA	21.0 ± 0.1	21.2 ± 0.8	21.1 ± 0.7	21.6 ± 0.4	14.8 ± 0.7*	14.6 ± 0.9*	13.4 ± 0.7*	13.8 ± 0.6*
Total PUFA	28.3 ± 0.3	28.8 ± 0.9	28.2 ± 0.9	29.1 ± 0.6	30.0 ± 0.3*	29.9 ± 0.3	29.2 ± 0.1	29.5 ± 0.1
Liver (%)								
Total saturated	26.8 ± 0.6	26.6 ± 1.5	26.6 ± 0.5	26.3 ± 1.2	16.2 ± 1.1*	17.2 ± 1.2*	18.3 ± 1.1*	17.7 ± 1.6*
Total monoenes	27.5 ± 1.1	29.7 ± 5.2	27.8 ± 3.5	29.5 ± 1.9	50.8 ± 2.1*	50.8 ± 3.0*	47.8 ± 2.4*	50.3 ± 4.8*
18:2n-6	2.4 ± 0.1	2.4 ± 0.2	2.4 ± 0.3	2.4 ± 0.1	9.3 ± 0.9*	9.0 ± 0.4*	8.2 ± 0.7*	9.0 ± 0.8*
20:4n-6	3.1 ± 0.2	2.9 ± 0.4	2.9 ± 0.3	2.9 ± 0.2	4.6 ± 0.8*	4.3 ± 0.4*	5.2 ± 0.9*	4.7 ± 1.1*
Total n-6 PUFA	7.2 ± 0.1	7.0 ± 0.2	7.2 ± 0.1	7.1 ± 0.2	19.6 ± 0.9*	19.4 ± 0.0*	19.2 ± 0.5*	19.4 ± 0.9*
18:3n-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1[b]	0.3 ± 0.0[a]	1.2 ± 0.2*	1.2 ± 0.0*	1.1 ± 0.1*	1.2 ± 1.1*
20:5n-3	5.9 ± 0.2	5.8 ± 0.4	6.0 ± 0.2	6.0 ± 0.1	1.4 ± 0.2*	1.4 ± 0.3*	1.7 ± 0.3*	1.5 ± 0.3*
22:6n-3	30.3 ± 1.4	28.8 ± 3.5	29.9 ± 3.3	28.8 ± 1.2	9.5 ± 0.6*	8.8 ± 1.4*	10.5 ± 1.2*	8.7 ± 2.0*
Total n-3 PUFA	38.4 ± 1.0	36.7 ± 3.8	38.3 ± 3.3	37.1 ± 1.1	13.3 ± 0.7*	12.7 ± 1.8*	14.6 ± 1.4*	12.6 ± 2.3*
Total PUFA	45.7 ± 1.1	43.7 ± 3.9	45.6 ± 3.2	44.2 ± 1.1	32.9 ± 1.5*	32.1 ± 1.8*	33.9 ± 1.3*	32.1 ± 3.2*
Pyloric caeca (%)								
Total saturated	27.9 ± 0.7	27.2 ± 0.4	27.7 ± 0.5	27.7 ± 0.5	19.1 ± 0.5*	18.2 ± 0.4*	17.8 ± 0.8*	17.8 ± 0.*
Total monoenes	45.3 ± 0.1	44.1 ± 0.9	43.2 ± 0.2	42.5 ± 2.7	50.0 ± 0.8*	50.2 ± 0.3*	52.0 ± 0.5[a]*	50.0 ± 0.7[b]*
18:2n-6	4.6 ± 0.2	4.6 ± 0.2	4.6 ± 0.2	4.5 ± 0.1	11.3 ± 0.3[b]*	12.1 ± 0.3[a]*	12.3 ± 0.5*	12.6 ± 0.9*
20:4n-6	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.8 ± 0.2	1.1 ± 0.1*	1.2 ± 0.1*	0.9 ± 0.1*	1.2 ± 0.2
Total n-6 PUFA	6.2 ± 0.2	6.4 ± 0.2	6.4 ± 0.2	6.4 ± 0.3	15.5 ± 0.3[b]*	16.9 ± 0.6[a]*	16.5 ± 0.7*	17.4 ± 1.5*
18:3n-3	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	2.7 ± 0.1[b]*	3.0 ± 0.0[a]*	3.1 ± 0.2*	3.1 ± 0.3*
20:5n-3	3.4 ± 0.3	3.5 ± 0.2	3.5 ± 0.1	3.5 ± 0.2	1.8 ± 0.1*	1.5 ± 0.2*	1.5 ± 0.1*	1.5 ± 0.4*
22:6n-3	12.3 ± 0.3[b]	13.8 ± 0.6[a]	14.2 ± 0.5	15.3 ± 2.3	7.6 ± 0.6*	7.2 ± 0.4*	5.9 ± 0.2*	7.2 ± 0.8*
Total n-3 PUFA	19.8 ± 0.5[b]	21.5 ± 0.7[a]	21.8 ± 0.5	22.7 ± 2.6	15.0 ± 0.4*	14.4 ± 0.9*	13.3 ± 0.3*	14.4 ± 1.0*
Total PUFA	26.8 ± 0.7[b]	28.7 ± 0.8[a]	29.0 ± 0.4	29.9 ± 2.9	30.9 ± 0.4*	31.6 ± 0.6*	30.1 ± 0.5[b]*	32.2 ± 0.7[a]

PUFA, polyunsaturated fatty acids.

4.3.5. Distal intestine (DI) histology

Generally, histological assessment of DI at the end of the marine phase (pre-challenge) indicated that total enteritis scores were low and comparable across the four treatment groups (Table 4.8.). No differences were found in the parameters analysed except for sub-epithelial mucosa (SUBEM), where V-diploids showed increased size compared to M-diploids, and lastly for intra-epithelial lymphocytes (IEL), where M-triploids showed significantly higher prevalence than V-triploids. Similarly, there were generally few significant effects of nutritional history or ploidy on enteritis scores in the distal intestine post-challenge (Table 4.8.). However, in diploids, significantly higher SUBEM in V-fish compared to M-fish, as well as significantly higher total scoring of combined histological characteristics between these groups, was observed post-challenge phase. There were differences in scores within a given treatment between the pre- and post-challenge phases with all overall scores tending to be higher, although only significantly in V-triploids. Furthermore, eosinophilic granulocyte (EG) scoring was significantly higher post-challenge regardless of nutritional history or ploidy. Lamina propria (LP) scoring was generally higher post-challenge, but only significantly so in M-triploids, and SUBEM was significantly higher in triploids post-challenge.

Table 4.8. Total scores and individual scores before and after the challenge phase for the different parameters used to determine severity of enteritis (Urán *et al*., 2008). Data are treatment means ± SD (n = 3) based on their ploidy status (diploid or triploid) and their nutritional history during the stimulus phase (Diet $M^{stimulus}$ or Diet $V^{stimulus}$). Significance was calculated between ploidy, nutritional history (NH) and their interaction (ploidy*NH), and was accepted at $p < 0.05$. Further significant differences within a given ploidy are shown using different superscripts and asterisks indicate a significant difference between phases in a given treatment. ns = not significant.

Ploidy	**Diploid**		**Triploid**		***p* value**		
Nutritional history	**$M^{stimulus}$**	**$V^{stimulus}$**	**$M^{stimulus}$**	**$V^{stimulus}$**	**ploidy**	**NH**	**ploidy*NH**
Pre-Challenge phase							
LP	1.2 ± 0.2	1.8 ± 0.6	1.4 ± 0.3	1.3 ± 0.3	ns	ns	**0.027**
EG	1.1 ± 0.1	1.2 ± 0.3	1.1 ± 0.2	1.1 ± 0.2	ns	ns	ns
SUBEM	1.1 ± 0.3^{b}	1.4 ± 0.2^{a}	1.2 ± 0.2	1.3 ± 0.4	ns	ns	ns
IEL	2.0 ± 0.4	2.0 ± 0.4	2.1 ± 0.2^{a}	1.7 ± 0.3^{b}	ns	**0.017**	ns
MFBMA	1.1 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.2	ns	ns	ns
Total	6.5 ± 0.9	7.8 ± 1.4	7.1 ± 0.4	6.8 ± 1.1	ns	ns	ns
Post-Challenge phase							
LP	$1.6 \pm 0.5^{*}$	1.8 ± 0.2	1.8 ± 0.5	1.6 ± 0.4	ns	ns	ns
EG	$1.3 \pm 0.2^{*}$	$1.5 \pm 0.3^{*}$	$1.5 \pm 0.3^{*}$	$1.5 \pm 0.3^{*}$	ns	ns	ns
SUBEM	1.3 ± 0.3^{b}	1.7 ± 0.2^{a}	$1.8 \pm 0.3^{*}$	1.8 ± 0.3	**0.001**	**0.007**	**0.034**
IEL	1.7 ± 0.5	2.1 ± 0.5	2.0 ± 0.5	2.0 ± 0.4	ns	ns	ns
MFBMA	1.3 ± 0.3	1.5 ± 0.3	1.4 ± 0.4	1.5 ± 0.3	ns	ns	ns
Total	7.2 ± 1.2^{b}	8.5 ± 0.8^{a}	8.5 ± 1.6	$8.4 \pm 0.9^{*}$	ns	ns	ns

EG, eosinophilic granulocytes; IEL, intra-epithelial lymphocytes; LP, lamina propria; MFBMA, mucosal fold base mitotic activity; SUBEM, sub-epithelial mucosa.

4.4. Discussion

The present study confirmed that a short exposure to a vegetable-based diet (Diet $V^{stimulus}$) during first exogenous feeding prepared or adapted *Salmo salar*, irrespective of ploidy, to better utilise a similar diet (Diet $V^{challenge}$) when challenged later in life. This result is consistent with a previous trial which demonstrated a physiological adaptation in *Oncorhynchus mykiss* through nutritional programming (Geurden *et al*., 2013). In addition, the present study showed that while this "nutritional programming" effect was highly significant in diploid *Salmo salar*, the effect was also clearly apparent in triploids albeit the greater variation in the triploid data often reduced the significance of these responses.

Throughout the majority of the trial, there was no significant difference in survival rates between nutritional histories within each ploidy. However, a difference was detected in the stimulus phase. V-fish showed a lower survival rate irrespective of ploidy. The same trend was found for growth during the stimulus phase as V-fish showed a lower growth rate. Reluctance to feed on vegetable-based diets is well documented in *Salmo salar* and other species, especially when first presented (Fournier *et al*., 2004; Soltan *et al*., 2008; Pratoomyot *et al*., 2010) and so it is likely that the greater mortality and lower growth in V-fish was initially due to poor acceptance of Diet $V^{stimulus}$ leading to reduced feed intake, although this could not be accurately measured in the first feeding fry in the present study. Failure or impaired establishment of first feeding of Diet $V^{stimulus}$ may be explained by the physiological characteristics of a carnivorous teleost. Typically, *Salmo salar* develop anatomically according to the type and level of nutrients present in the maternal egg reserves (Palace and Werner, 2006). This has been reviewed in several species concerning important organismal systems including reproductive structure and performance (Izquierdo *et al*., 2001). With regards to the gastrointestinal tract (GIT), morphological and functional differentiation occurs in the very early life stages and is influenced by environment and nutrition (Zambonino-

Infante and Cahu, 2001). Overall, triploids had a lower survival rate during the stimulus and marine phases, which was consistent with previous studies (McGeachy *et al*., 1995; O'Flynn *et al*., 1997; Cotter *et al*., 2002). Similarly, lower growth rates in triploid *Salmo salar* fry during the stimulus phase was also consistent with previous studies (Galbreath *et al*., 1994; Taylor *et al*., 2011). The experimental diets used in the present study were generally based on formulations for diploid *Salmo salar*, and recent studies have indicated that triploids may have different nutritional requirements for certain physiological development, principally during the freshwater stage (Fjeldall *et al*., 2015; Taylor *et al*., 2015; Smedley *et al*., 2016; Sambraus *et al*., 2017). One may speculate that the increased proportions of vegetable-based proteins and oils currently used in commercial salmonid feeds may have reduced nutrient bioavailability compared to FM/FO diets, which could perhaps highlight dietary deficiencies for triploids. However, high quality protein concentrates have shown to be similar or more digestible than FM (Glencross *et al*., 2004; Denstaldi *et al*., 2007). The reduced performance and survival of triploid fry during the stimulus phase may be explained by missing micronutrients in the formulation (Hamre *et al*., 2016; Hemre *et al*., 2016). Also, this drawback may not be solely consequent of nutritional deficiency, but ploidy itself should be considered as a factor.

At the end of the stimulus phase V-fish were ~30 % smaller than M-fish, which was a potential confounding factor during the rest of the trial as fish size itself affects subsequent fish performance. This was taken fully into account in the present study. Ideally, therefore, the stimulus phase should not induce any major phenotypic changes. Very recent data on early nutritional stimuli suggested that three days may be sufficient to prompt a physiological adaptation to diet in zebrafish (*Danio rerio*) (Perera and Yufera, 2016), although species-specific variation should not be excluded. During the marine phase, M-diploids had a higher growth rate than V-diploids. As discussed above, the difference in BW at the beginning of

this phase could have had some impact on growth rate, but another factor could be potentially reduced acceptability of the commercial diet in V-fish. An investigation of nutritional programming in *Oncorhynchus mykiss* concluded that early nutritional intervention can alter transcriptional and physiological characteristics of the olfactory and gustatory systems to suit specific feed formulations (Geurden *et al*., 2013; Balasubramanian *et al*., 2016). Therefore, in this respect we can speculate that M-fish were already adapted for a commercial diet, while V-fish would have required time to adapt to it. Feed intake was not measured at this point but was determined at the end of the marine phase when no difference between M-fish and V-fish was observed.

There was no difference in survival rate between fish during the challenge phase, but V-fish demonstrated significantly higher growth rates than M-fish. The switch in performance in response to Diet $V^{challenge}$ had a positive effect that appeared to be related to the initial dietary stimulus. V-fish adapted to Diet $V^{challenge}$ better than M-fish, suggesting there was a degree of memory to the dietary stimulus. It has been previously described that environmental triggers can influence "multidimensional plasticity" in different organisms, and suggested that transcriptional and physiological changes during early development could significantly affect the resilience of these organisms to different stressors (West-Eberhard, 1989). The weights of V-fish at the end of the challenge phase were not higher than those of M-fish, due to the fact that V-fish were initially smaller due to lower growth during the stimulus and marine phases. However, the weights were comparable between nutritional histories, confirming the greater weight gain and higher growth rate (TGC) in V-fish during the challenge. The higher growth performance was reflected in greater protein, lipid, and energy gains of V-fish compared to M-fish during the challenge phase. Although these differences were generally not statistically significant, with the exception of lipid and energy gain in diploids, every nutrient group showed higher values in V-fish. Moreover, there was a

ploidy effect as triploids appeared to gain significantly more macronutrients than diploids. This may indicate physiological and metabolic differences between ploidies. An earlier study suggested that morphological differences in the GIT, predominantly caused by a reduction in intestinal cell numbers, could hinder the gut's absorptive capability and therefore the intestinal efficiency of nutrient absorption in triploid *Salmo salar* compared to diploid siblings (Peruzzi *et al*., 2015). Therefore, the data in the present study were not consistent with this hypothesis and highlighted the need for further research into the effects of ploidy on GIT morphology, physiology, enzymatic activities and intestinal nutrient absorption.

Due to the lower weight of V-fish, feed intake (FI) measurements during the marine and challenge phases were normalised to enable comparisons between groups, to account for the effect of fish weight on consumption rates. Previously, palatability has been an issue in salmonids fed with feeds containing low levels of marine and high amounts of plant-derived ingredients as fish have shown reluctance to consume these diets. Lower voluntary feed intake was observed in a previous study (Refstie *et al*., 1998) when *Salmo salar* were fed a soybean meal (SBM) diet compared to a FM diet during the initial stages of the trial, although consumption rates were comparable towards the end of the trial suggesting that the fish finally accepted the diet possibly reflecting an adaptation to it. In the present study, there was no difference in FI irrespective of nutritional history or ploidy either at the end of the marine phase or during the challenge phase. However, there was a significant effect of nutritional history on FE and FCR, with V-fish having higher efficiency/lower conversion ratio. Thus, the superior growth performance of V-fish during the challenge phase was likely the result of improved dietary nutrient utilisation. Furthermore, FCRs also confirmed that nutritional history had opposite effects during the marine and challenge phases, as previously discussed for growth performance. Thus, during the marine phase, M-fish exhibited lower FCRs compared to V-fish, consistent with the better growth of the M-fish during this phase,

and the reverse trend was observed throughout the challenge. The FE/FCR data confirmed that the initial, brief exposure of *Salmo salar* fry to Diet $V^{stimulus}$ had a positive impact on these fish when they were challenged with Diet $V^{challenge}$, and further suggested that physiological and metabolic changes and adaptations in these fish were, at least partly, responsible for the observed improvement in the utilisation of Diet $V^{challenge}$ by V-fish. In contrast, a higher feed intake in *Oncorhynchus mykiss* was observed when re-introduced to a vegetable-based diet after an earlier nutritional stimulus (Geurden *et al*., 2013), suggesting that exposure to the diet early in life reduced the aversion of the fish to the challenge diet later in life. However, the fish initially fed the vegetable-based diet also showed improved FE when challenged with this diet later in life. The slight difference in the results between the earlier *Oncorhynchus mykiss* study and the present *Salmo salar* study in terms of palatability may be related to differences in the dietary formulations used. For example, in the *Oncorhynchus mykiss* study the vegetable diet was completely devoid of marine ingredients (0 % FM and 0 % FO) and this may have posed an even more extreme challenge specifically in terms of palatability, but species-specific response should not be excluded. This highlighted the importance of optimising dietary formulations based on both taste preference and utilisation efficiency.

Retention of nutrients and energy was higher in diploid V-fish during the challenge phase, which agreed with data in the earlier trial in *Oncorhynchus mykiss* mentioned above (Geurden *et al*., 2013). As with other parameters, this was the reverse trend from the previous marine phase which showed M-fish having better retentions than V-fish. Although an identical trend for all nutrients was observed in triploids, no significant differences between M- and V-triploid *Salmo salar* were observed in either of the two phases. The present findings, however, could reflect the limited existing knowledge on the precise nutritional requirements of triploid *Salmo salar*, which have been shown in previous studies to be higher

than in diploids specifically in relation to phosphorous (Fjelldal *et al*., 2015; Smedley *et al*., 2016) and histidine requirements (Taylor *et al*., 2015; Sambraus *et al*., 2017). V-fish positively retained EPA (20:5n-3) during the challenge phase whereas M-fish showed negative retention and, although DHA (22:6n-3) retention was positive in all fish irrespective of nutritional history or ploidy, retention was considerable greater in V-fish. This trend was found within both ploidies, but only significantly in diploids. Retention of DHA was consistently greater than that of EPA in all treatments, which is in accordance with previous reports suggesting selective catabolism of EPA over DHA in addition to a possible production of the fatty acids by endogenous biosynthesis pathways (Tocher *et al*., 1997; Torstensen *et al*., 2004). Thus, when considering the EPA and DHA retention data, any value that exceeds 100% will include a proportion of the fatty acids that were biosynthesised from ALA (18:3n-3). Net production of DHA has previously been reported when *Salmo salar* (Sanden *et al*., 2011) and *Oncorhynchus mykiss* (Turchini *et al*., 2011) were fed with high inclusion of vegetable oil, and therefore low levels of dietary EPA and DHA. In the present study, net production of EPA was found in V-fish during the challenge period. This result is consistent with other studies (Turchini *et al*., 2011; Rosenlund *et al*., 2016) where salmonids had received similarly low levels of dietary EPA and DHA (0.7 % of total FA). The selective retention of DHA over EPA, possibly tissue (i.e. neural tissue) specific, has been reported previously in several fish species (Tocher, 2003, 2010; Glencross, 2009). DHA has a greater physiological importance in cell membrane composition and function when compared with EPA and is therefore a higher valued essential fatty acid. The greater retention of both EPA and DHA in V-fish found in this study may reflect one possible and obvious metabolic adaptation in V-fish. It is likely that there is increased long-chain PUFA (LC-PUFA) biosynthesis through upregulation of fatty acyl desaturase and elongase activities (Leaver *et al*., 2008; Tocher, 2010). In an earlier trial in sea bass (*Dicentrarchus labrax*) (Vagner *et al*.,

2009), expression of delta-6 desaturase (*Δ6D* or *fads2d6*) was upregulated in juveniles that had been previously fed an n-3 LC-PUFA deficient diet as larvae. The *Δ6D* enzyme is the reported rate-limiting step in the conversion of ALA to EPA (Bell and Tocher, 2009). However, the early nutritional stimulus had no major effect on the final fatty acid compositions of either whole body or tissues in the present study. Perhaps as expected, fish and tissue fatty acid profiles at the end of the challenge phase reflected the dietary fatty acid compositions and therefore showed increased percentages of plant-derived C18 fatty acids (18:1n-9, 18:2n-6 and 18:3n-3) and decreased proportions n-3 LC-PUFA (EPA and DHA) in all treatment groups with no major influence of nutritional history (or ploidy) (Tocher, 2015). This change in tissue fatty acid profiles is in accordance with many studies on FM and FO substitution with alternative vegetable-based diets in *Salmo salar* (Bell *et al*., 2001; 2002; 2010; Torstensen *et al*., 2004; 2008; Turchini *et al*., 2010). It is well established that, while upregulation of LC-PUFA biosynthesis through increased expression and activity of fatty acid desaturase activities is a consistent response to vegetable diets in *Salmo salar*, it is not sufficient to fully compensate for the lack of dietary EPA and DHA (Torstensen and Tocher, 2010; Tocher, 2015). However, the challenge phase was 6-weeks, which is relatively short compared to most earlier studies on the effects of fish oil replacement with vegetable oils, and the differences in EPA and DHA retention between V- and M-fish recorded in the present study are large, and so it would be interesting to confirm if these would translate into higher levels of these key LC-PUFA in V-fish after longer feeding.

Overall, there were few notable differences in intestinal morphology before and after the challenge phase. Many of the earlier incidences of morphological changes in the gut and enteritis in *Salmo salar* were specifically related with the use of SBM in the diet (Krogdahl *et al*., 2003; Knudsen *et al*., 2008; Urán *et al*., 2009). Aqueous alcohol extraction of proteins from soybeans or soy flour for the manufacturing of SPC, as used in the present study,

reduces contents of specific anti-nutritional factors (ANFs) including saponins, lectins and soy-antigens (Bureau *et al*., 1998; Drew *et al*., 2007), which have been shown to be implicated in intestinal inflammation in fish fed SBM (Francis *et al*., 2001; Hedrera *et al*., 2013). Still, a significant difference in inflammation was found in V-diploids compared to M-diploids after the challenge phase. In a similar study investigating nutritional programming in zebrafish (*Danio rerio*) (Perera and Yufera, 2016), fish previously exposed to SPC were shown to be more prone to intestinal inflammation when refed SPC than groups that had never been exposed to it. Although further processing of diets seem to be reducing incidence of enteritis, the present result suggests that further refinements are needed to equal the responses to current diets. The only treatment that showed an increase in the total scoring of intestinal integrity from pre- to post-challenge analysis was V-triploids. This could suggest that triploid *Salmo salar* may be more sensitive to vegetable-based diets than their diploid counterparts and again highlighted the lack of knowledge regarding effects of ploidy on *Salmo salar* morphological and physiological responses.

4.5. Conclusions

The present study has indicated that nutritional programming can help to improve utilisation of a diet and reduce potential negative impacts associated with the use of alternative raw materials in aquafeeds. In particular, the present study has successfully demonstrated for the first time that both diploid and triploid *Salmo salar* can be adapted to utilise a vegetable-based diet more efficiently after an early nutritional intervention. Ploidy generally responded the same, however triploids showed a higher variation in nutrient retention and LC-PUFA biosynthesis. This supports the difference in nutritional requirements between the ploidy and highlights the need to understand further the impact of the second set of maternal chromosomes which was investigated in Chapter 5. Further optimisation of an effective

stimulus both in terms of diet formulation and duration can further unlock the potential of this strategy. Importantly, the potential of *Salmo salar* to apparently be programmed to be net producers of EPA and DHA should be further investigated. Biosynthesis of these health-promoting omega-3 fatty acids shows promise when considering the limitation of raw material sources. Previous investigations have shown several metabolic pathways and key biochemical and physiological regulators to be influenced in response to consumption of vegetable-based diets in *Salmo salar* (Morais *et al*., 2011; Tacchi *et al*., 2012). The present study was continued and confirmed that early nutritional intervention impacted key genes associated with metabolism which may be associated with the improved nutrient utilisation in programmed fish (Vera *et al*., 2017). Ploidy differences were observed, suggesting differential nutritional requirements and further supporting the urgency to understand how the extra maternal chromosome impacts gene regulation.

CHAPTER FIVE

DEVELOPMENT OF A ROBUST SINGLE ERYTHROCYTE ISOLATION TECHNIQUE FOR ANALYSIS OF ALLELIC-SPECIFIC PATTERNS OF INHERITANCE IN TRIPLOID ATLANTIC SALMON (*Salmo salar* L.)

5.1. Introduction

Artificial triploidy can be induced by subjecting recently fertilised eggs to a physical or chemical shock during the second meiotic division. The most effective method devised to date for salmonids is a hydrostatic pressure shock (Johnstone and Stet, 1995). The shock prevents expulsion of the second polar body which in turn leaves the fertilised egg with cells containing three sets of chromosomes; two of maternal origin and the third of paternal origin (Tiwary *et al*., 2004). In a normal diploid organism, expression of the genes and resulting traits can be achieved by one of two processes. Monoallelic gene expression is the term used to describe when one of the two copies of a gene is expressed and thus the other remains silenced. Otherwise, expression of a gene can be achieved by expression of both associated alleles which is termed biallelic expression. In the event of the latter, the resulting phenotype is determined by the dominance status of the alleles. A well-known case of monoallelic expression is the inactivation of the X chromosome (Singer-Sam, 2010) where most of the genes on one X chromosome of a female are inactivated. This phenomenon occurs in early developmental stages of the zygote and the silencing of one of the chromosome appears to be random, suggesting that an individual will possess a mix of cells; some having one X chromosome activated and some the other. In triploids, however, there is potential for increased heterozygosity and the expression of three different alleles (Piferrer *et al*., 2009). Understanding the outcome of an additional set of chromosomes is important for stock improvement as offspring performances are currently selected based on the outcome of diploid allelic expression. As concluded in Chapters 2, 3, and 4, there remains a degree of difference between the ploidy and is likely an impact of this extra maternal chromosome which is not yet understood.

Artificial triploidy is a form of autopolyploidy, where the additional chromosomes come from that particular species. Also, spontaneous triploidy can occur naturally in response

to sub-optimal environmental conditions during egg development (Glover *et al*., 2015). Irrespective of the method in which triploidy is obtained, in order to regain a balance of the genome, cells must either have to "ignore" the set of retained maternal chromosomes to restore a diploid state or regulate all three sets of chromosomes simultaneously. At present, it is unknown if the dosage of this second set of maternal chromosomes is consistent or if it is expressed in different ratios between families, siblings, tissues or even individual cells and thus giving potential for ploidy mosaicism within an individual. Previous teleost studies have reported ploidy mosaicism within several tissues as a result of triploid induction (Teplitz *et al*., 1994; Goudie *et al*., 1995; Arai, 2001). Gene stability in polyploidy has been reviewed (Birchler and Veitia, 2007; 2012), however it is still unclear exactly how a second set of maternal chromosomes affects resulting phenotypes and development of an individual. Johnson *et al*. (2007) found that the extra set of chromosomes in triploid *Oncorhynchus tshawytscha* does affect variability of growth and survival-related traits. Conversely, Pala *et al*. (2008) reported a level of inactivation in triploid *Squalius alburnoides*, but not of a whole haploid genome, but rather specific genes. Reducing transcript levels to a diploid state suggests a compensation mechanism, with allelic expression profiling suggesting this to be random.

The impact of gene regulation in triploid salmonids is not fully understood. Before modifying a breeding program in order to select desired traits in triploids, it is key to understand if and how regulation of gene dosage is occurring post-triploid induction. In the present investigation, it was firstly necessary to determine the feasibility of identifying and measuring triploid allele-specific expression, specifically if any copy was deactivated to restore a diploid state. This was explored at three key levels; the individual, the tissue and the single cell. Investigation at the single cell level was important to understand if there was ploidy mosaicism present amongst individual cells. If so, allelic expression analysis at the

whole tissue level would likely conceal those with lower ploidy levels. There are several major obstacles to overcome in order to investigate this. Firstly, informative polymorphic markers within expressed genes need to be developed in order to be able to distinguish between all three alleles in a triploid. A promising approach for investigating this is allele-specific profiling mediated by highly polymorphic microsatellite marker screening via fluorescent capillary electrophoresis (Abdul-Muneer, 2014). Vasemagi *et al*. (2005) characterised a number of *Salmo salar* expressed sequence tag (EST) associated microsatellite loci, identifying 75 as having good amplification efficiency. This set of potentially informative markers provided a valuable tool for the current study. When selecting a panel of markers, it cannot be anticipated which would be expressed in particular tissues or a cell, therefore selecting a suite of highly polymorphic microsatellites is necessary.

5.2. Materials and methods

5.2.1. Fish stock and culture conditions

On 3rd December 2015, green eggs and milt from unrelated *Salmo salar* broodstock (2-sea winter, 11.2 ± 1.1 kg, n = 18) were provided by Landcatch Ltd. (Ormsary, UK) and transferred to the Institute of Aquaculture (University of Stirling, UK). Single pair crosses of nine dams and nine sires were performed to create nine potentially informative families. Eggs were fertilised with respective sires (30 secs. mixing milt, 60 secs. rinse with 8 °C freshwater) creating nine full-sib families. Post fertilisation, the eggs were divided into two groups for ploidy differentiation (~2000 eggs family^{-1} ploidy^{-1}). Triploidy was induced in one group (655 bar of hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. post-fertilisation (Smedley *et al*., 2016), while the other half of each egg group did not receive a hydrostatic shock and were maintained as diploid controls. Following water hardening (~1 hr.), individual egg batches were disinfected (Buffodine, Europharma, 1:100) for 10 mins. before

laying down in aluminium egg trays (15 mL sec^{-1} flow) in darkness at a constant temperature (5.8 ± 0.4 °C). Parental DNAs were screened for polymorphism at eleven microsatellite loci (see Table 5.1.). Prior to hatching, six of the nine families were selected based on most informative (heterozygous) crosses at these loci and transferred to separate 0.3 m^3 circular tanks (250 fish $tank^{-1}$) in a recirculation unit (University of Stirling, UK) (15 mL sec^{-1} flow; 5.9 ± 0.5 °C). Diploids were given a standard commercial diet (INICIO Plus) and triploids were given a triploid-specific diet (INICIO TRI-X) in accordance with the manufacturer's guidelines (BioMar Ltd, UK).

5.2.2. Verification of ploidy

To verify ploidy status, red blood smears were prepared from samples taken from the caudal peduncle of euthanised fish (20 fish $family^{-1}$ $ploidy^{-1}$; 57.6 ± 7.3 g). Air dried slides were fixed in 100 % methanol and then placed into Giemsa stain for 10 mins. Slides were digitised by slide scanner at 20x magnification (Axio Scan Z1, Zeiss) and erythrocyte length and diameter were determined by Fiji software (ImageJ). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm and a mean calculated for presumed diploid and triploid fish. Diploid control groups had significantly smaller erythrocyte nuclear lengths, with no overlaps with the pressure shock triploid groups (2N, 6.2 ± 0.4 μm; 3N, 8.8 ± 0.5 μm) confirming that all fish that were subjected to hydrostatic pressure shock were likely to be triploids.

5.2.3. Sampling procedures

At the point of stripping eggs and milt, fin clips from all broodstock were collected into 100 % ethanol for later parental DNA screening. Newly hatched offspring alevins (n = 5 $family^{-1}$ $ploidy^{-1}$; 500 °days) were collected in 'RNA Later' to determine allele expression in offspring

at the individual level. The yolk sac was removed from alevins and remaining body was used for RNA extraction. Whole blood was extracted from parr (n = 5 family^{-1} ploidy^{-1}; 57.6 ± 7.3 g) and stored in 'RNA Later' for later expression analysis at the tissue level.

5.2.4. DNA and RNA extraction from whole tissue

DNA extraction was accomplished using a salt extraction method (Blanquer, 1990). Briefly, *c*. 50 mg tissue was added to 400 µL DNA extraction buffer (0.3 M NaCl, 50 mM Tris, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1 % SDS, 125 mg L^{-1} proteinase K). Samples were incubated overnight at 55 °C with end-over-end mixing. Then 5 µL RNase A (2 mg mL^{-1}) was added to each tube and incubated at 37 °C for 60 mins. Proteins were then precipitated by addition of 0.7 volumes 5 M NaCl, incubated on ice for 10 mins. and centrifuged (25,000 *g*, 10 mins.). The supernatant was removed to a fresh microtube and DNA precipitated by addition of 0.7 volume iso-propanol and brisk mixing. The precipitated DNA was pelleted by centrifugation (21,000 *g*, 2 mins.). The DNA pellet was washed twice in 75 % ethanol over 2-3 hrs., air dried and dissolved in a small volume Tris buffer (5 mM, pH 8.5). Total DNA concentration was determined by Nanodrop spectrophotometry (ND-1000; Labtech Int., East Sussex, UK).

Blood samples were spun (1,200 *g* for 10 mins.), 'RNA Later' and plasma layers removed leaving only blood cells. Total RNA (totRNA) was extracted using TriReagent® according to the manufacturer's guidelines. TotRNA concentration was determined by Nanodrop spectrophotometry. To eliminate genomic DNA (gDNA) contamination, samples were treated with DNA-free™ (Applied Biosystems, UK) as per the manufacturer's guidelines. cDNA was subsequently synthesised using 1 µg total RNA and a high capacity reverse transcription kit (without RNase inhibitor) (Applied Biosystems, UK).

5.2.5. Optimal storage buffer for erythrocyte transportation

Three buffer solutions were selected and tested for the optimal storage buffer based on the ability to maintain the morphology of the cells and the integrity of the RNA for downstream analysis. Alsever's solution (AS; Alsever and Ainslie, 1941), CPD-A1, and EDTA (Sigma-Aldrich, UK) were diluted according to manufacturer's guidelines and regularly tested for efficient blood storage buffers over a 24 hr. period.

5.2.6. RNA extraction from single cells

TotRNA was extracted from single cells and whole transcriptome amplification (WTA) was completed using a single cell amplification kit (REPLI-g WTA Single Cell Kit, Qiagen, Germany) according to manufacturer's guidelines. Following RNA extraction, the kit uses multiple displacement amplification (MDA) technology to synthesise and subsequently amplify cDNA with a reverse transcriptase. Product concentration was determined by Nanodrop spectrophotometry.

5.2.7. Microsatellite selection

In order to establish a sufficient suite of polymorphic loci, eleven microsatellites were initially selected from a previous study investigating microsatellite variability within ESTs in *Salmo salar* (Vasemagi *et al*., 2005) (Table 5.1.). When selecting markers, the distance between each locus and the centromere was assessed by comparison with the published genome (ICSASG_v2, NCBI bioproject PRJNA72713). Marker loci identified as being distal to the centromere were chosen in order to maximise the likelihood of capturing cross-over events. The loci were also selected based on the extent of polymorphism reported by Vasemagi *et al*. (2005), in anticipation of identifying 3-4 alleles per gene per pedigree during

the screening process, such that the fate of individual allele expression could be reliably detected.

5.2.8. PCR amplification

All standard and fluorescently labelled primers were supplied by Eurofins Genomics (Ebersberg, Germany) and hydrated according to supplier's guidelines, with 10 µM aliquots being stored at -20 ºC until use. One oligonucleotide of each primer pair incorporated a 5' unique reporter sequence (M13R, CAG or Godde; Table 5.1.; Fitzpatrick *et al*. 2011) to allow for fluorescent tailing and detection (Oetting *et al*. 1995). The other oligonucleotide of the primer pair incorporated a 5' 'pig tail' (GTTT; Table 5.1.; Brownstein *et al*. 1996) to promote terminal adenylation of the amplified strand.

Initially each primer pair was assessed by gradient PCR (T_A 50, 55, 60, 65 °C) to establish an optimal annealing temperature for the assay. Each reaction (10 µL) comprised 1 µL of cDNA template (*c*. 1-50 ng), 5 µL MyTaq 2× Mastermix (BioLine UK), 1 µL combined forward and reverse locus-specific primers (each at 5 µM concentration) and 3 µL nuclease-free water. The cycling conditions comprised 38 cycles of three temperature steps; 95 ºC for 15 secs. (melt), T_A ºC for 20 secs., and 72 ºC for 30 secs. (extension). Amplification success and relative clarity was assessed by agarose gel electrophoresis (1.5 %), with bands being sized relative to a 100 bp ladder (GeneRuler^{TM} 100 bp; Thermo Scientific, UK).

For subsequent fluorescent-labelling PCR the same cycling conditions were applied, using optimal T_A. Each PCR reaction comprised 10 µL volume: 2.9 µL of Milli-Q water, 5 µL MyTaq 2× Mastermix, 1µl cDNA (*c*. 1-50 ng), 0.3 µL forward (reporter-tailed) locus-specific primer (0.5 µM), 0.4 µL reverse (pig tailed) locus-specific primer (5 µM) and 0.4 µL relevant fluorescently labelled tail (5 µM). A 2 µL aliquot of the product was assessed for quality and quantity by agarose gel electrophoresis (1.5 %). The final panel of microsatellite

loci selected were those that amplified clearly and robustly, were most informative (heterozygous) in the pedigrees and that could be most straightforwardly multiplex screened for cost effective downstream analysis (Table 5.1.).

Table 5.1. Information of eleven expressed sequence tag (EST) linked microsatellite markers considered from Vasemagi *et al*. (2005). Double-underlined sequences indicate the 5' reporter tails added to locus specific designs, while single underline indicates the 5' added 'pig tails'.

Primer set	Forward sequence	Reverse sequence	No. of alleles	Annealing temp. °C	Expected range (bp)	'Reporter' tail
BG935488	GGATAACAATTTCACACAGGTGACCCCACCAAGTTTTTCT	GTTTAAACACAGTAAGCCCATCTATTG	18	60	166 - 234	M13R
BG934281	CATCGCTGATTCGCACATACTGCTTCTCCCCTGCTAOA	GTTTGCGAACCACACATATACCAC	27	60	193 - 267	Godde
CAO48302	CATCGOTGATTCGCACATTTGCCACCTCTAAAGGCTTO	GTTTAAATGAACCCCAGCCATACA	13	60	201 - 255	Godde
CAO55301	CAGTCGGGCGTCATCAAGAACCAAGGGTACCGATCC	GTTTGGGAAATGGGTGGTAAGAAAA	21	60	217 - 263	CAG
CAO48828	GGATAACAATTTCACACAGGGAGGGCTTCCCATACAACAA	GTTTAAGCGGTGAGTTGACGAGAG	24	60	251 - 307	M13R
CAO53480	CAGTCGGGCGTCATCATGGTCACAAACCAAATGGAA	GTTTCCACTCCAGGGTGCTGTAA	16	60	254 - 290	CAG
CB515794	CAGTCGGGCGTCATCACTCAGTGCCATGTCTCCAAC	GTTTCATCCTGTCCTGCTGACTG	16	60	265 - 309	CAG
CAO60177	GGATAACAATTTCACACAGGCGCTTCCTGGACAAAAATTA	GTTTGAGCACACCCATTCTCA	27	60	294 - 374	M13R
CAO59136	CAGTCGGGCGTCATCAAGGGTAGTGAGAAAGCAGCAA	GTTTAACTGGCTGGCCATAGG	27	60	318 - 380	CAG
CAO38592	GGATAACAATTTCACACAGGAAGCATCAAACCAACCTCATT	GTTTCGGGGGTGAAGATGTCTACT	24	60	340 - 426	M13R
CB517044	CATCGCTGATTCGCACATCACCAAGCATGGGAAGCTAT	GTTTGCTGCCACACAGGCTACTTT	28	60	351 - 425	Godde

5.2.9. Allele-specific profiling

Each locus was PCR amplified separately and then products from 3-4 compatible loci were combined for multiplex size detection on the Beckman CEQ8800 sequencer / fragment analyser. Each multiplexed sample comprised loading solution (SLS, 28.6 µL), size standard (SS, 0.38 µL) and a volume of PCR product determined by the fluorescent tag used (Godde, 1.2 µL of 10µM; CAG, 0.75 µL of 10µM, or M13R, 0.55 µL of 10µM). A single drop of mineral oil was layered on the top of each loaded sample. Samples were run according to manufacturer's instructions. Allelic peaks were analysed using CEQ software, with allele sizes being manually called. To control for allele detection error, parental samples were re-amplified and profiled a second time alongside the offspring screening.

5.3. Results and discussion

5.3.1. Microsatellite detection and screening

In total, eleven microsatellite markers were screened in the broodstock fish. These markers were previously identified as having >13 alleles in 196 individuals from eight groups that were surveyed (Vasemagi *et al*., 2005). Using microsatellites for this investigation was beneficial due to their high polymorphism. Having several alleles present for a particular locus / family combination is critical for tracing allele origin to a specific parent. The best case scenario would be to have heterozygous females and males, having four different sized alleles, enabling unambiguous identification. Using a 60 °C annealing temperature, which proved to be optimal for all loci assays, DNA samples were amplified by PCR using fluorescence tagged primers. Three multiplexes were created for parental screening and samples were profiled using the Beckman CEQ8800 sequencer to assess heterozygosity (Table 5.2.). Loci CAO48302 and CB517044 produced chromatograms with a high level of

stutter, which resulted in a low confidence in allele scoring and were therefore discarded from further analyses.

Table 5.2. Assessment of loci heterozygosity in broodstock screening. het = heterozygous, hom = homozygous. Double-underlined loci indicate the final panel that were selected for the investigation. Pedigree crosses that have an asterix represent families that were used in the investigation based on how informative they were and the survival of the offspring.

	Pedigree cross																		Heterozygosity
	1		2		3*		4		5*		6*		7		8		9		
Primer set	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	value
BG935488	het	het	het	hom	het	het	het	het	hom	het	het	het	het	het	het	het	hom	het	83%
BG934281	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	hom	94%
CAO48302	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CAO55301	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	het	100%
CAO48828	hom	het	het	hom	het	het	hom	hom	hom	het	hom	het	het	hom	hom	het	het	het	56%
CAO53480	hom	het	het	hom	hom	het	het	het	het	hom	het	het	het	het	het	het	het	hom	72%
CB515794	het	het	het	hom	hom	het	hom	hom	hom	het	het	het	het	hom	het	het	het	het	67%
CAO60177	het	het	het	hom	het	het	hom	het	het	het	het	het	het	het	het	het	hom	het	83%
CAO59136	het	het	het	het	het	het	hom	het	het	hom	het	het	het	het	het	het	het	het	89%
CAO38592	hom	hom	hom	het	het	hom	hom	hom	hom	hom	hom	hom	het	hom	hom	hom	hom	het	22%
CB517044	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The three most informative pedigree crosses (3, 5 & 6) were used for further analyses. Also, the number of microsatellite markers screened was reduced from eleven to five based on the heterozygosity and discriminatory potential for triploidy detection uncovered during parental DNA screening. The pedigree crosses and microsatellites selected for the analysis are shown (Table 5.3.).

Table 5.3. Genotype results for five markers of interest in parental crosses. Numbers represent allele sizes in base pairs (bp). Parents showing homozygosity have alleles highlighted with an asterix.

	Loci									
Pedigree cross	**BG935488**		**CAO53480**		**BG934281**		**CAO55301**		**CAO60177**	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
3	198	202	273	273*	242	228	256	242	332	348
	246	238	281	273*	254	258	262	262	352	368
5	230	238*	273*	277	218	242	242	258	348	316
	238	238*	273*	281	254	258	256	262	368	364
6	202	202	273	269	246	226	244	230	348	322
	238	238	281	277	256	242	254	262	368	360

5.3.2. Detection of microsatellite markers in offspring at the individual level

The reduction in loci number prior to screening gave an opportunity to reduce to only one multiplex rather than the previous three. However, this meant swapping the fluorescent tag on two loci to prevent overlapping of allele peaks. Offspring samples that amplified successfully were genotyped and alleles were matched with parents (Table 5.4.). One maternal and one paternal allele were expected in the diploid samples. In triploids, three alleles were expected to be observed, one paternal allele and both maternal alleles, assuming normal chromosomal crossover had occurred between the centromere and the marker locus. An example is shown in Fig. 5.1.

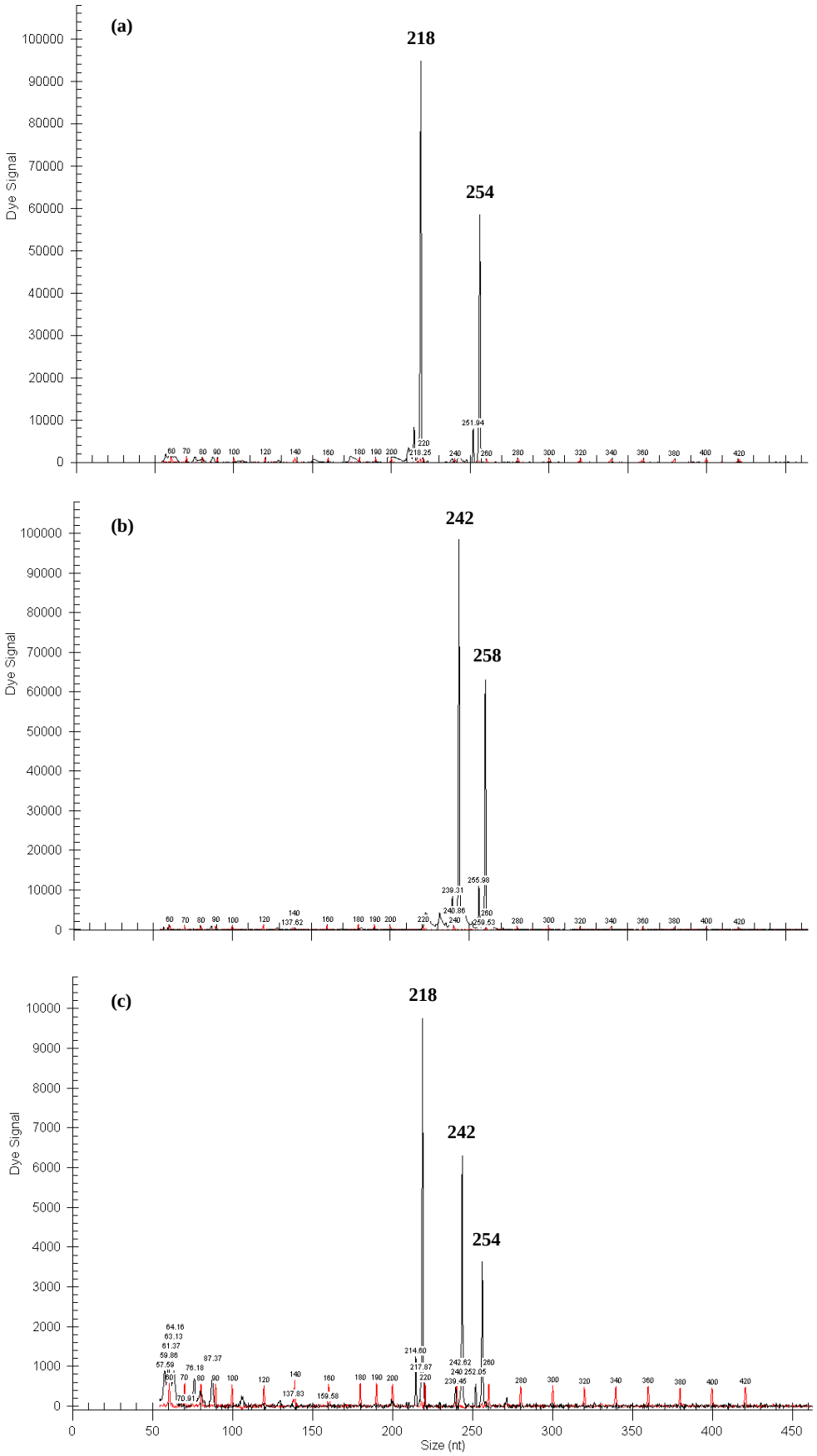

Figure 5.1. An example chromatogram of locus BG934281 showing allele peaks in (**a**) maternal, (**b**) paternal and (**c**) triploid offspring whole individual samples.

Some offspring samples failed to produce any product (Table 5.4.). This likely reflects poor RNA extraction or other issues relating to cDNA synthesis. From all diploid samples that did produce allele peaks, the number and origin of alleles was as expected, i.e. one paternal and one maternal allele. For samples that were subjected to the triploid induction process, the results were less clear cut. From the samples that were able to be genotyped, 82 % of cases where the parental genotypes were expected to be fully informative (i.e. where allele origin was clear and discriminatory) three allele peaks were found, the two maternal alleles and one of the two paternal alleles. However, there were some cases (18 %) where only two peaks were observed – mainly where one of the expected maternal alleles was absent. While this may reflect differential allelic expression there are other explanations for these observations. This likely reflects the absence of crossing over or crossing over distal to the marker for that particular gamete, something that would be expected for a proportion of the gametes. Where this phenomenon was observed at a locus, three alleles were evident at other loci for the same individual, indicating that the offspring was indeed likely to be triploid. Other issues, such as large allele dropout, unequal allelic amplification (Banks *et al*., 1999), may also explain the absence of expected alleles in some of the individual locus assays.

The results clearly illustrate that while PCR profiling of alleles can be a powerful tool in elucidating chromosome set number, it is not 100 % diagnostic. Surveying multiple loci increases the chances of detecting the true ploidy state. Nevertheless the combined panel screened in this study does provide a good proxy for establishing ploidy status. Undoubtedly, with further exploration of EST gene sequences and their position in the genome, a more informative panel may be forthcoming.

Overall, it is clear that in many cases triploid offspring generally showed expression of all three alleles. This was at individual level and thus not indicative of expression at the

tissue level. To investigate this further, whole blood was examined due to its ease of collection and the potential to explore expression to even greater depth later in single cells.

Table 5.4. Genotype results for five markers of interest in three pedigree crosses at the individual offspring level. Numbers represent allele sizes in base pairs (bp). Samples with no PCR product are represented by a hyphen.

			Loci														
			BG935488			CAO53480			BG934281			CAO55301			CAO60177		
Pedigree cross 3	Male		202	238		277	277		228	258		242	262		344	364	
	Female		198	246		277	285		242	254		256	262		328	348	
	Diploid	1	-	-		-	-		-	-		-	-		-	-	
		2	198	238		277	285		254	258		242	262		348	364	
		3	198	202		277	277		242	258		256	262		344	348	
		4	198	238		277	285		-	-		256	262		348	364	
		5	202	246		-	-		254	258		242	262		328	344	
	Triploid	1	198	238	-	277	277	-	242	254	258	242	256	262	328	348	364
		2	198	238	246	277	277	285	228	242	254	242	256	262	328	348	364
		3	198	238	246	-	-	-	242	254	258	256	262	262	328	348	364
		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		5	198	202	-	277	277	285	228	242	254	256	262	262	328	344	348
Pedigree cross 5	Male		238	238		281	285		242	258		258	262		312	360	
	Female		230	238		277	277		218	254		242	256		344	364	
	Diploid	1	230	238		277	285		254	258		242	262		312	360	
		2	-	-		-	-		254	258		242	258		312	344	
		3	230	238		277	281		254	258		256	262		360	364	
		4	230	238		277	281		242	254		256	258		312	344	
		5	238	238		277	281		242	254		256	262		360	364	
	Triploid	1	230	238	238	277	277	281	218	242	254	242	256	-	344	360	364
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	230	238	238	-	-	-	218	254	258	242	256	262	344	360	364
		4	-	-	-	-	-	-	218	242	254	242	256	262	344	360	364
		5	230	238	238	277	277	285	218	254	258	242	262	-	312	-	-
Pedigree cross 6	Male		202	238		273	281		226	242		230	262		318	356	
	Female		202	238		277	285		246	256		244	254		344	364	
	Diploid	1	238	238		-	-		242	-		-	-		-	-	
		2	202	202		-	-		226	246		254	262		344	356	
		3	202	202		273	-		226	256		230	244		344	356	
		4	-	-		-	-		-	-		-	-		-	-	
		5	-	-		-	-		-	-		-	-		-	-	
	Triploid	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	242	246	256	244	254	262	-	-	-
		3	202	202	238	277	-	-	242	246	256	244	254	262	318	344	364
		4	-	-	-	-	-	-	226	246	256	244	254	262	344	356	-
		5	202	202	238	-	-	-	226	246	256	230	244	254	344	-	-

5.3.3. Detection of microsatellite markers in offspring whole blood

Many genes show tissue-specific expression, as such the aim of this analysis was to determine how many of the five loci panel were expressed in whole blood. Fish (n = 5 family^{-1} ploidy^{-1}) were bled and 150 µL whole blood transferred immediately to 'RNA Later' until processing. Samples were spun (1,200 *g*, 10 mins.) and 'RNA Later' was removed. RNA extraction was carried out using the TriReagent® according to manufacturer's instructions and samples were profiled on the Beckman CEQ8800 sequencer. Microsatellite chromatograms from parents and offspring whole blood cDNA assays are shown in Table 5.5.

Expression of all five loci was detected from whole blood, albeit the overall efficiency of successful genotypes was poorer than for whole alevin assays. Chromatogram patterns for expected diploid and triploid offspring were equivalent to those found for whole individual assays, though with lower numbers showing successful PCR (57 *vs*. 67 %, respectively). This may be due to haem inhibitors affecting the various downstream assay steps (Wilson, 1997). One locus in particular (CAO60177) showed a poor response. This probably reflected very low expression in whole blood. Nevertheless, the remaining four loci showed reasonable amplification success and positive identification of triploidy in whole blood. Reliable fluorescence strength from the samples gave confidence that these loci were expressed in blood. This warranted continued exploration this panel at the single cell level.

5.3.4. Detection of microsatellite markers in single erythrocytes

Erythrocytes are a convenient cell type to isolate as they are already in suspension and in ample quantity. Furthermore, erythrocytes make up 98-99 % of whole blood (Fänge, 1994) therefore it was reasonable to assume from the whole blood results that the panel of loci were likely expressed in this cell type. Using single cells in this investigation meant there was a

requirement for flow cytometry (FCM) technology in order to isolate the single cells. There was no such equipment available at the Institute of Aquaculture where the fish were being reared. Therefore collaboration with the Institute of Medical Sciences in Aberdeen was formed to gain access to state-of-the-art FCM cell sorting (BD Influx BSLII Sorter, BD Biosciences). Furthermore, due to Home Office regulations, live fish could not be transported to Aberdeen and thus cell preservation and viability issues had to be addressed prior to single cell isolation.

Table 5.5. Genotype results for five markers of interest in three pedigree crosses offspring whole blood. Numbers represent allele sizes in base pairs (bp). Samples with no PCR product are represented by a hyphen.

			Loci														
			BG935488			CAO53480			BG934281			CAO55301			CAO60177		
Pedigree cross 3	Male		202	238		277	277		228	258		242	262		344	364	
	Female		198	246		277	285		242	254		256	262		328	348	
	Diploid	1	198	238		-	-		228	242		242	262		-	-	
		2	202	246		277	277		242	258		262	262		328	-	
		3	238	246		277	285		228	242		262	262		328	-	
		4	198	202		-	-		228	242		242	256		-	-	
		5	198	238		277	285		242	258		262	262		-	-	
	Triploid	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	198	238	246	277	277	277	242	254	258	256	262	262	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		4	198	238	246	277	277	285	228	242	254	242	256	262	-	-	-
		5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pedigree cross 5	Male		238	238		281	285		242	258		258	262		312	360	
	Female		230	238		277	277		218	254		242	256		344	364	
	Diploid	1	230	238		277	-		218	258		242	262		344	-	
		2	238	238		277	281		254	258		256	258		-	-	
		3	230	238		277	285		218	242		242	262		-	-	
		4	230	238		277	285		254	258		256	262		-	-	
		5	238	238		-	-		245	258		242	258		-	-	
	Triploid	1	230	238	238	277	277	281	218	242	254	256	258	-	-	-	-
		2	230	238	238	277	277	281	218	242	254	242	256	258	-	-	-
		3	230	238	238	277	277	281	218	242	254	256	258	-	-	-	-
		4	230	238	238	-	-	-	218	242	254	242	258	-	-	-	-
		5	230	238	238	277	277	281	218	242	254	242	256	258	-	-	-
Pedigree cross 6	Male		202	238		273	281		226	242		230	262		318	356	
	Female		202	238		277	285		246	256		244	254		344	364	
	Diploid	1	238	238		-	-		226	256		230	254		-	-	
		2	238	238		-	-		226	246		230	244		-	-	
		3	202	238		273	285		242	256		244	262		-	-	
		4	202	238		281	285		242	245		244	262		-	-	
		5	-	-		-	-		-	-		-	-		-	-	
	Triploid	1	202	202	238	-	-	-	242	246	256	230	244	254	-	-	-
		2	202	238	238	277	281	285	226	246	256	230	244	254	-	-	-
		3	202	238	238	-	-	-	226	246	256	230	244	254	-	-	-
		4	202	238	238	-	-	-	226	246	256	230	244	254	-	-	-
		5	202	202	238	-	-	-	242	246	256	230	244	254	-	-	-

5.3.4.1. Erythrocyte storage conditions

Deterioration of blood begins as soon as it comes into contact with air. Coagulation and oxidisation commences, which in turn lowers the quality and viability of the cells. With FCM required to isolate the single cells, changes in erythrocytes' size, shape and physiology needed to be kept to a minimum. Furthermore, the single cell amplification kit selected for extraction and amplification of single cell RNA was designed to work on live cells which excluded the option of fixing the cells before sorting. In order to keep the blood as fresh as possible and the cells viable, an optimal holding buffer had to be established. Heparin has traditionally been used as the go-to anticoagulant when extracting blood from fish (Walenick and Witeska, 2007). However, the use of heparin has been linked to a negative effect on RNA and DNA quality in analyses of human blood (Elliot and Peakman, 2008) and citrate-based anticoagulants are preferred.

Blood from *Salmo salar* parr (n = 3) was collected by venepuncture and pooled into a container. The blood was mixed gently but thoroughly and 100 µL aliquoted into cryovials containing the respective holding buffer (AS, 1:1; CPD-A1, 1:9; EDTA, 1:9). Samples were mixed with the anticoagulants and held on ice until further processing. At each time point post-bleeding (0, 1, 2, 4, and 8 hrs.), blood smears were taken to investigate cell morphology. Slides were left to air dry for 24 hrs. before being stained with Giemsa. Samples were then scanned (Axio Scan Z1, Zeiss) and morphology was assessed. The remaining blood was tested for RNA integrity. Samples were spun down in a centrifuge (1,200 *g*, 10 mins.) and the upper layers were removed leaving only blood cells. TotRNA was extracted using TriReagent® according to the manufacturer's guidelines and concentration was determined by Nanodrop spectrophotometry. RNA quality was verified by the spectrophotometer ratios (Fig. 5.2.).

A 260/280 ratio of 2.0 is considered ‘pure’ for RNA samples. There was no significant difference between each of the holding solutions and the fresh sample which suggests all samples were pure RNA (Fig. 5.2a). The 260/230 ratio is also an indication of sample purity and commonly at a higher level than the former ratio. In this investigation, there was no difference in 260/230 between any of the three holding solutions at any time point except after one hour where AS had significantly higher values than EDTA. Still, all three were noticeably poorer quality than the control (fresh blood sample) (Fig 5.2b). Conversely, there was an obvious difference in the morphology of cells between holding buffers (Fig. 5.3.). Shown are examples from each solution after four hours of storage which was the duration anticipated for the transport of samples to Aberdeen and pre FCM preparation.

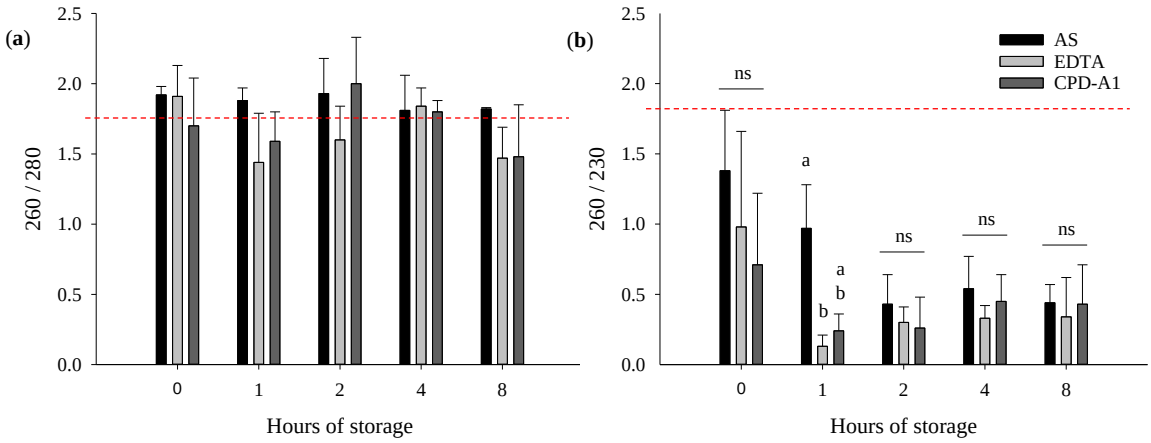

Figure 5.2. Spectrophotometer ratios of (**a**) 260/280 and (**b**) 260/230 describing RNA quality of blood samples (n = 3) from blood after being held for eight hours in one of each of the holding solutions; AS, EDTA, or CPD-A1. Red dashed lines represent values taken from fresh blood (260/280, 1.74 ± 0.26; 260/230, 1.81 ± 0.12). Data are means ± SD and significant differences are denoted by different superscripts ($p < 0.05$; Two-Way ANOVA). ns = not significant.

AS appeared to have the least impact on erythrocyte morphology (Fig. 5.3b), with very similar appearance to the control of fresh blood. The blood samples held in CPD-A1

appeared to be more clustered together (Fig. 5.3c), which may cause problems for isolating single cells using FCM. Finally, the majority of cells held in EDTA had lysed (Fig. 5.3d). There is very little literature available for RNA preservation of fish red blood cells, however, blood preservation in human studies is well-documented. EDTA is commonly used for preservation of human blood and is routine for nucleic acid and protein assessments, however, magnesium concentrations can be impacted. Having EDTA in excess may cause damage to erythrocytes (Korcock *et al*, 1988; Walenick and Witeska, 2007). Hattingh (1975) described a reduction in cell membrane stability and occurrence of lysis as a result of preserving fish blood in EDTA for up to 48 hrs. An increase in rigidity of cell membranes may be the cause for lysis. In addition to damaging the structure of the erythrocytes, EDTA has been linked to a degradation of genetic material in fish red blood cells (Ramsdorf *et al*., 2009). Therefore, AS was selected as the storage buffer to transport blood samples.

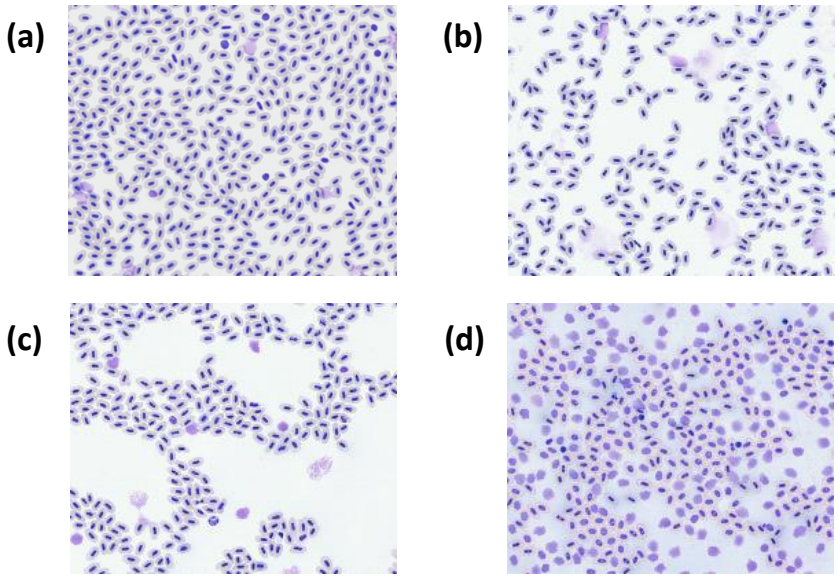

Figure 5.3. Blood smears from *Salmo salar* from (**a**) fresh sample and then held in (**b**) AS, (**c**) CPD-A1, and (**d**) EDTA after four hours of storage. Smears were stained with Giemsa and images analysed using Zen software (Zeiss).

5.3.4.2. Single cell isolation

A pilot study was conducted to first identify and optimise the technique for single cell isolation prior to downstream analysis. Whole blood from both diploid and a triploid *Salmo salar* (n = 1 ploidy^{-1}) was sampled and transported in AS to Aberdeen for single cell isolation. Using the BD Influx^{TM} Cell Sorter (BD Biosciences, NJ, USA), FCM was used to isolate single erythrocytes. There was no need to tag the cells with fluorescence and the simple forward scatter (FSC) was used. This way, erythrocytes were separated from other cell types on the basis of relative size. As mentioned earlier, this is one reason why the cells had to remain live. If fixed, the size and shape of individual cells would change and cause difficulty in the selection process. Erythrocytes were categorised (gated) based on several conditions such as size, auto-fluorescence and granularity. Gating allows setting of thresholds in the sorting process which allows consistent and reproducible isolation of the same cell type.

TotRNA from erythrocytes (n = 5 ploidy^{-1}) was extracted and amplified using the REPLI-g WTA Single Cell kit (Qiagen, UK) as per the manufacturer's guidelines. After this, a set of primer pairs were used to amplify microsatellite locus (BG934281), previously identified as showing significant expression in whole blood, the amplicons being assessed by gel electrophoresis (1.5 %) against a 100 bp ladder. There was little success as only one of ten samples (from a presumed diploid individual) gave a detectable amplification product. This sample was run on the Beckman CEQ8800 sequencer to profile the alleles present. One strong peak at 246 bp was detected from the single erythrocyte (Fig. 5.4.), this allele being present in the dam of the cross. Neither of the two paternal alleles, both of which clearly distinguishable in size from the maternal alleles, were detected.

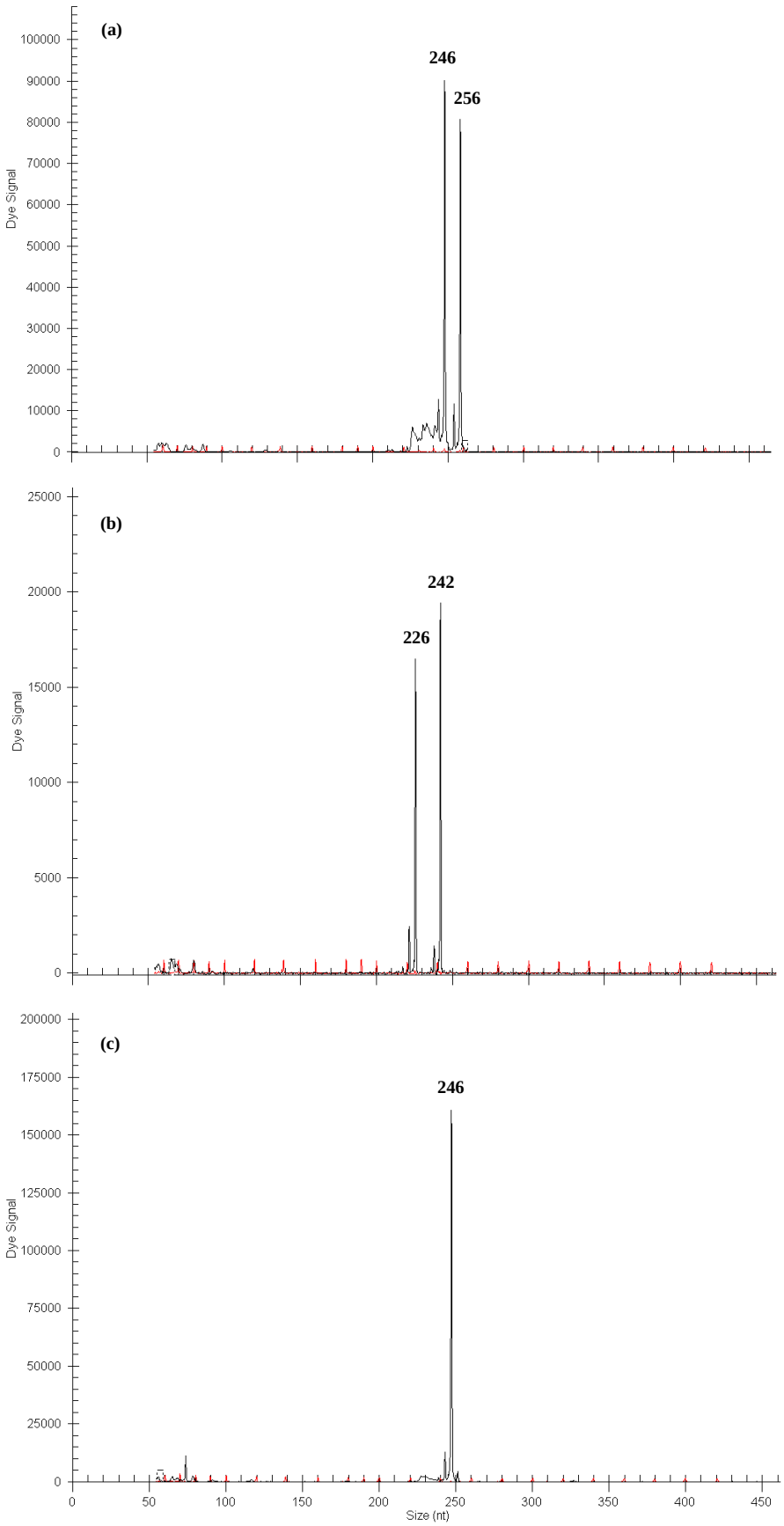

Figure 5.4. An example chromatogram of locus BG934281 showing allele peaks in (**a**) maternal, (**b**) paternal and (**c**) diploid offspring single erythrocyte.

The offspring was a diploid fish therefore having only one peak present may suggest allelic drop-out (ADO), though given the similarity in alleles sizes this may be unlikely. Despite developments of single cell methodologies, ADO has on occasion been a problem when using PCR of limited template material in human studies (Gagneux *et al*., 1997; Whitaker *et al*., 2001; Piyamongkol *et al*., 2003). Failure to amplify both alleles can give a heterozygous cell a homozygous appearance in diploids. Furthermore when considering triploids, if there is ADO in one maternal allele, the cell can appear as diploid. Of course from this one observation, single allele expression cannot be ruled out as an explanation. Regardless, preliminary work demonstrated that it was possible to at least partially profile expression from a single erythrocyte within the existing experimental constraints.

The main concern was that product from only one sample was found after extraction and amplification. This success rate was not very promising and thus further quality control tests were run to investigate this. Two considerations that were readily testable were; (i) FCM may have damaged the cells during the sorting process, and (ii) isolated cells have not reached the collection wells after exiting the cell sorter.

5.3.4.3. Immunofluorescence microscopy

To ensure single cells were both viable and reaching the desired destination post-sorting, erythrocytes were tracked using immunofluorescence microscopy. Firstly, separate samples of erythrocytes were stained with propidium iodide (PI; Sigma-Aldrich, Gillingham, UK), a red fluorescent nuclear stain. PI is not permeant to living cells and therefore only will stain the chromosomes which have left the cell (i.e. dead or non-viable cells). PI stained blood samples were passed through the cell sorter and non-stained individuals were determined, confirming very low amount of non-viable erythrocytes (5.3 %). The apparent low mortality rate of the single cells shows that the sorting process had little impact on cell viability.

NucBlue™ (Thermo Scientific, UK) was used to stain another sample of erythrocytes in the next step of quality control. This was used to determine if the cells had reached the desired destination after the sorting process. The cell sorting involves separation of cells into individual droplets and deposition through a 100 µm nozzle under 20 psi flow velocity into a destination of choice based on the characteristics that cell has. Although the distance between the nozzle and the collection well is not considerable, the possibility of static pull to the side of the plastic well was a concern. Five replicate runs of 100 single cell depositions into a collection plate were investigated for efficiency. NucBlue™ penetrates cells and stains the nuclei of live, or fixed, cells. Images were captured by fluorescent microscopy (Axio Observer Z1, Zeiss) using a DAPI filter (Zeiss set 49). Of the 100 deposited, only 20-30 % detection of cells in the collection wells was recorded. A suggestion was that the cells were bursting on impact into the destination buffer and therefore not detected. To investigate this, the size of the output nozzle on the cell sorter was increased from 100 µm to 140 µm, which would subsequently allow larger droplets to exit, thereby giving each cell more "cushion" on impact. Further, the pressure of the flow was reduced from 20 psi to 7 psi to reduce exit velocity and further safeguard the cells. After these alterations, 100 % of stained erythrocytes were recorded post-sorting. This gave confidence that the previously encountered low sorting success rate could be improved upon. However, these tests only informed that chromosomes were present in the single cells and not how the gene expression was impacted.

A further attempt at single cell cDNA microsatellite detection was undertaken using a modified protocol informed by the observations above. Five cells from three fish were analysed. A PCR for locus BG934281 was run as this microsatellite had previously shown some success. 5 µL of neat product from the REPLI-g Single Cell Kit was used instead of the normal 1 µL recommended for the PCR in an attempt to begin with more template DNA. Also, 45 cycles were programmed to further increase the amplification. The resulting gel (1.5

%) showed few very weak bands around 100 bp, but not in the locus range (193-267 bp). Further PCRs were run with the housekeeping gene, *β-actin*, to assess whether the PCR failure was locus specific or due to problems with the cDNA template. Amplicons of roughly expected size were found with *β-actin* PCR, however there was an inconsistency with band sizes suggesting contamination or degradation in some of the samples. Degradation of RNA occurs as a result of ribonuclease activity (Houseley and Tollervey, 2009) and can readily occur in short periods of time. Although the single cell RNA extraction kit ensures stabilisation of cellular RNA during the lysis stage, this degradation may have occurred prior to the cell entering the buffer i.e. during transportation or the cell sorting process.

5.3.4.4. Cell density investigation

The amount of intact RNA extracted from a single cell may be too little to reliably assess gene expression using the current methodology. To briefly investigate this further, an increasing number of cell numbers were analysed to determine if low RNA volume could be problematic in this methodology. Whole blood from diploid *Salmo salar* was sampled and transported in AS to Aberdeen. Using the BD Influx^{TM} Cell Sorter, FCM was used to create multiple wells containing 1, 100 or 200 erythrocytes. TotRNA was extracted and amplified using the REPLI-g WTA Single Cell kit (Qiagen, UK) as per the manufacturer's guidelines. Following, a set of primer pairs were used to amplify the previously successful microsatellite locus (BG934281), the amplicons being assessed by gel electrophoresis (1.5 %) against a 100 bp ladder. The resulting gel showed more intense bands from the wells containing multiple erythrocytes suggesting that the volume of RNA from a single erythrocyte may not be enough to amplify successfully. Unfortunately, using pools of cells will not inform if all cells express three alleles as there may be a degree of mosaicism there, showing three alleles overall but potentially some cells only expressing two. Although this method of single erythrocyte

isolation appears to be efficient, other methods of single cell transcriptome analysis may need to be explored e.g. single cell RNA sequencing.

5.4. Conclusions

The predominant aim of this study was to investigate if all three copies of inherited genes are expressed or if there is any gene silencing occurring. Having a better understanding of this would increase the chances of explaining and predicting phenotypes/traits for use of triploids in a breeding program. A panel of microsatellite loci was identified and exploited to assess allele expression in both diploid and triploid offspring. Screening of this panel showed codominant expression of these genes in diploid fish, while there was clear evidence that all three alleles present in triploid sibs were expressed, when assayed from cDNA derived from whole alevin and whole blood from parr. While investigation of gene expression at the single cell stage was attempted, this proved unsuccessful in the time available. A number of technical issues were identified but further work is clearly needed to satisfactorily resolve these. Reducing the time between removal of blood and subsequent processing of single erythrocytes would certainly improve the chances of obtaining much better quality samples. While the five locus panel was sufficient for resolving expression, expansion of the microsatellite panel would also be advantageous to improve the chance of assaying strongly expressed genes, which is particularly critical for single cell expression study where template cDNA is extremely scarce. Another aspect to consider is the possibility that there is differential expression between individual cells depending on the cell cycle stage. Altogether, it appears very difficult to investigate allele inheritance between individual cells unless common loci are present in all stages. In order to generate a successful breeding program for triploid *Salmo salar*, genetic inheritance and subsequent expression in triploid offspring has to be understood. For example, Johnson *et al*. (2007) concluded that inheritance of

performance related traits are affected by the extra maternal genome in triploid *Salmo salar*. If this could be predicted, better performing triploids may be selected based on the information identified from diploid broodstock. Currently, the inability to determine expression of target genes in single cells prevents further understanding of how the additional set of maternal chromosomes affects offspring traits which would normally be selected for in a selection program. Further, many desirable traits are polygenic which intensifies the complexity of quantitative genetics in triploidy.

CHAPTER SIX

GENERAL DISCUSSION

The overall aims of this research were to further expand on the current knowledge of triploid *Salmo salar,* address key issues associated with welfare (increased mortality, deformity prevalence and growth variation) that are thought to originate from sub-optimal conditions during early developmental stages and generate recommendations to improve triploid-specific commercial rearing protocols. Before triploids can gain wide commercial acceptance, their performance and productivity must match or better that of diploid counterparts alongside minimising any welfare implications. Although investigations to date have demonstrated major differences in culture requirements between diploid and triploid *Salmo salar*, many reports suggest that undesirable traits such as reduced survival, poor growth performances, increased growth variation and increased deformity prevalence likely manifest during embryogenesis which is not yet fully understood. Three main aspects of early life triploid culture were investigated in this thesis; (i) egg incubation temperature impact on subsequent survival, development and deformity prevalence of *Salmo salar* exposed to both chronic and acute temperature manipulations (Chapter 2), (ii) the impact of post-ovulatory ageing on survival, performance and growth variation (Chapter 3) and (iii) the potential of nutritional programming in triploids to allow the acceptance and utilisation of vegetable-based diets to coincide with the search of alternative ingredients in aqua feeds (Chapter 4). Finally, this research investigated the feasibility of measuring allelic-specific inheritance using microsatellite markers in order to elucidate ploidy differences observed in such traits (Chapter 5). These investigations addressed fundamental gaps in scientific knowledge of triploid *Salmo salar* and generated results which will be beneficial to commercial application, further improving the positive outlook and potential implementation of triploid *Salmo salar* in aquaculture. The main hypothesis of this research is that undesirable production traits (increased mortality, deformity prevalence and size variation) seen in triploid *Salmo salar* are caused by sub-optimal conditions during the early developmental stages (egg incubation

temperature profile, egg quality, nutrition) and cellular/genome differences. The main findings from these investigations are discussed including a critical appraisal of the experimental approaches, potential improvements and commercial applications, remaining knowledge gaps and future directions for research.

6.1. Thermal optima of triploids during embryogenesis

Temperature affects gestation in all animals. Mammals are endothermic and therefore can regulate their internal body temperature allowing protection against temperature fluctuations during sensitive offspring development. In contrast, fish are poikilothermic and development is directly influenced by the prevailing ambient temperature. Temperature experienced by *Salmo salar* eggs during embryogenesis is routinely manipulated in aquaculture to alter developmental speed in order to align with customer demand for receiving eggs. The temperature regime applied during egg incubation, including potential temperature changes, may have short and long term negative consequences such as increased malformations as reported in diploid *Salmo salar* exposed to both chronic and acute temperature changes (Takle *et al*., 2005). This may be further amplified in triploid stocks which appear to be more sensitive to prolonged elevated egg incubation temperatures (Fraser *et al*., 2015). This was confirmed in the present research as triploid *Salmo salar* traits were improved (increased survival and reduced deformity prevalence) when incubated at 6 °C from fertilisation to first feeding compared to the industry norm of 8 °C based on diploid thermal preference. Also, such investigations need to keep pace with advancements in industry protocols. For example, "super-cooling" of eggs at 1 or 2 ˚C for prolonged durations is gaining interest as it brings more flexibility to meet customer demands. To my knowledge, the effect of this egg cooling strategy has not been investigated in either ploidy and should be considered in future investigations. The impacts of temperature changes, which are routine in a commercial

environment during embryogenesis, have also not been previously investigated. Considering this, the present study investigated the impact of a change in temperature during embryogenesis on survival and subsequent development of triploid *Salmo salar* (Chapter 2).

A key finding in the present thesis was the increase in survival and improved yolk sac utilisation observed with decreasing egg incubation temperature and duration. Triploids incubated at 6 °C showed improved survival and yolk sac utilisation compared to other temperatures. A constant incubation temperature was compared against a switch in temperature at 400 °days, which is routinely experienced in commercial hatcheries. Commercially, eggs at this stage will likely experience temperature differences. The survival of triploid eggs incubated at 6 °C were comparable to diploid survival under the current industry norm of 8 °C. This supports the hypothesis that triploids have a higher thermosensitivity than diploids during embryogenesis and therefore alterations to the current temperature regimes in both broodstock and hatchery facilities should be made to address the increase in triploid mortality previously reported. Unfortunately, the original sampling plan did not include yolk sac utilisation assessment and as a result taking samples from eggs that experienced the temperature switch at 400 °days were missed. Consequently, the impact of the temperature shift on yolk utilisation could not be determined, although all other parameters assessed (growth performances, muscle fibre, deformity prevalence, gene expression) included the short-term temperature treatments. Triploids were found to be larger at first feeding when incubated at 6 °C compared to other temperature regimes with no effect observed on diploid weight. This reinforces the difference in thermosensitivity between the ploidy. Importantly, triploids incubated at 6 °C had comparable first feeding weights to diploids. Previous studies (Galbreath *et al*., 1994; McGeachy *et al*., 1995; Taylor *et al*., 2011) have reported significantly smaller triploids at first feeding, however the present study

showed that lowering the temperature can overcome this, allowing for an improved start in triploid growth.

Overall, the results suggested that embryogenic rearing of triploids at 6 ˚C until switching to 8 ˚C at 400 °days improved production traits of smolts compared to a constant 8 ˚C with minimal effect on the production cycle duration (~129 *vs*. ~112 days, respectively). However, an increase in mortality (2N: 12 %; 3N: 18.0 %) and deformity prevalence (2N: 10 %; 3N: 12 %) was observed in these fish, particularly triploids, compared to when incubated at a constant 6 ˚C, however long term exposure at this temperature would result in a prolonged incubation period (~150 days). The change in temperature applied at 400 °days clearly altered normal development as supported by differences in gene expression patterns, particularly for those involved in bone formation. However, gene expression results must be interpreted carefully in triploids as the impact of an extra gene copy on gene expression and genome regulation remains unknown. This was investigated in Chapter 5. Furthermore, future investigations of temperature effects on gene expression should include heat shock protein pathways as increased activity of *hsp70* has been reported following acute temperature shocks during embryogenesis (Takle *et al*., 2005). Due to limitations in egg and incubator numbers, the experimental design did not include a control treatment to assess the effects of egg handling (i.e. moving eggs from 8 ˚C to 8 ˚C). Therefore, the results may not only be associated to the temperature treatment but may have also been influenced by the additional handling stress.

Egg incubation at a constant 6 ˚C was shown to have a beneficial impact on the subsequent development of parr. Although no temperature effect was found on number or size of muscle fibres in diploid fish, triploids had significantly higher numbers of muscle fibres of smaller areas suggesting an effect towards hyperplasia over hypertrophy. The recruitment of muscle fibres in the freshwater phase is likely to lead to increased growth

potential post-sea water transfer as reported by Johnston *et al*. (2000). While temperature had no clear impact on diploid growth performance, triploids were negatively affected when incubated at 8 or 11 ˚C. In addition, deformity prevalence and severity increased in both ploidy with increasing temperatures. Importantly, predicted smolt weight and deformity prevalence in triploids incubated at a constant 6 ˚C was comparable to diploid counterparts. A temperature change during embryogenesis which is very common in commercial facilities resulted in increased deformity prevalence in both ploidy. This strongly supports the importance to fully control temperature regimes in commercial facilities when rearing triploid eggs in order to optimise triploid traits during the production cycle. Given the number of treatments (10) and facility limitations, it was not possible to replicate during the on growing period, and therefore growth and deformity prevalence data were obtained from single tank per treatment. Despite this, results clearly warrant further investigations of the benefits of parr development when incubated at 6 ˚C during embryogenesis.

Overall, the results from the current study confirmed the apparent increased thermosensitivity of triploid eggs as previously suggested in the literature and further extend our knowledge on the mechanisms at play and physiological effects in response to temperature changes during late stages of embryogenesis (i.e. 400-900 °days post-fertilisation) which is routine in commercial practice. Although significant improvements in triploid survival and development were observed when incubated at 6 °C, differences between ploidy were still found with triploids showing slightly higher deformity prevalence. This suggests that other factors may be involved such as other environmental parameters (water quality) and sub-optimal egg quality as shown in Chapter 3.

With the potential adoption of triploid culture, optimal conditions during early rearing of triploid eggs must be defined to increase triploid welfare, yield and productivity. A manageable compromise to both broodstock and hatchery protocols will contribute to

unlocking the potential of ongrowing triploid *Salmo salar*. Egg suppliers in broodstock facilities could aim to produce earlier eggs to allow for a constant incubation of 6 °C through advanced stripping under manipulated photoperiod regimes. Similarly, hatcheries could alter production and receive eggs earlier to allow for a longer incubation at 6 °C. The additional time and investment required for this production alteration may be offset by the return of producing optimal triploids.

6.2. Post-ovulatory ageing impacts survival and development

Building on the positive results from Chapter 2, the premise of this investigation was to ascertain the potential cause of the remaining ploidy difference of residual deformities in triploids observed after improving incubation temperature conditions. Oocytes, when optimal, are produced with high quality maternal reserves sufficient for an individual to develop through embryogenesis until they have the ability to source their own food as free-swimming fry and beyond. Sub-optimal egg quality will reduce favourable development in any species and lead to increased mortalities, irrespective of ploidy status and one cause of reduced egg quality is post-ovulatory ageing as demonstrated in Mommens *et al*. (2015). Egg quality is of great concern when considering triploid culture. Triploid induction requires increased handling of the eggs during a vulnerable stage shortly after fertilisation whereas with diploid culture the eggs would be left untouched. Therefore, changes in oocyte quality and / or cell membrane structure as a result may also lead to higher egg drop out and fish mortalities as reported in triploids. It has been suggested that triploids appear to be more sensitive to stressors (Benfey *et al*., 2015) and results from this investigation supported a higher sensitivity in triploids.

The findings confirmed the hypotheses that post-ovulatory ageing reduces fertilisation and survival during early egg development in both diploid and triploid *Salmo salar* and also

that parr condition factor was negatively impacted. Irrespective of life stage, triploids appear to be more affected by egg ageing as seen with lower survival rates and an increased variation in parr size compared to diploid siblings. This will have direct consequences for triploid-specific stock management practice with a potentially lower final yield or having to increase stocking numbers to maintain production. Also, additional grading would be needed in juveniles to control aggression and competition found with hierarchical dominance in increased growth variation. In large-scale production, it may be challenging to monitor ovulation in broodstock as there would be high numbers of fish to assess regularly and increased broodstock handling would introduce additional welfare issues. If this were to be the case assuming implementation of triploid *Salmo salar* in aquaculture, the survival and development of the fish may thus be impacted. Therefore, having a real-time quality parameter as a useful biomarker would be advantageous in determining the future success of a triploid batch. Also, the ability to assess this immediately after stripping would further benefit the industry as only eggs of sufficient quality would be selected for culture.

The majority of biochemical quality parameters investigated in unfertilised eggs showed no effect related to post-ovulatory ageing, however, there was an increase of lipid peroxidation and decrease of antioxidant activity between 10 and 15 days post-ovulation. Lipid peroxidation has detrimental impacts on cell membranes and enzymatic activities which is likely a cause of higher mortalities and abnormal development observed. Further, a decrease in antioxidant activity lowers the defence capacity against lipid peroxidation. Another study of the effects of post-ovulatory ageing on diploid *Salmo salar* reported some quality parameter changes from 22 days-post ovulation onwards however this duration extended beyond the current study and no lipid peroxidation was investigated (Mommens *et al*., 2015). Despite this, the same study concluded stability in egg quality for up to two weeks

post-ovulation before performance of embryos, alevins and juveniles were affected which concurs with the findings of the present investigation.

Due to time limitations, some parameters that may have strengthened the results could not be included. Samples for chorion morphology and changes to the ovarian fluid proteome were collected, and may have elucidated further the impact of egg membrane degradation. There has been contrasting results regarding changes to chorion thickness in response to salmonid oocyte ageing (Lahnsteiner, 2000; Bahre Kazemi *et al*., 2010; Samarin *et al*., 2015) however an increase in perivitelline space was evident. Moreover, egg protein fragments were observed in *Oncorhynchus mykiss* ovarian fluid that was exposed to increasing post-ovulatory ageing (Lahnsteiner, 2000; Rime *et al*., 2004). Samples collected in the present study for these parameters are currently being analysed and will be included in future publication of this research.

Overall, the results clearly demonstrated that post-ovulatory ageing increases the occurrence of lipid peroxidation and further investigation into the formation of ROS and their subsequent oxygen uptake may provide a better understanding of the consequences of ageing. Increased production of ROS can lead to mitochondrial dysfunction and a reduced production of ATP which is the fundamental energy source of cellular activity (Samarin *et al*., 2015). Thus, the use of an oxidation biomarker to predict the future success of a batch appears to be a logical avenue for further research.

These findings highlight the importance of fertilising eggs after minimal time post-ovulation, particularly in triploids that have shown to have increased size variation in parr from more ripened eggs. Post-ovulatory ageing was only one avenue investigated in this study and there is a requirement to understand other potential causes of reduced egg quality in triploids. The impact of the increased handling and hydrostatic pressure shock associated with triploid induction on the quality of the eggs needs further attention. Although necessary

for successful triploidisation, the disturbance of the oocytes during this sensitive developmental period may impact on egg quality later on. Also, the impact of the extra set of maternal chromosomes, and subsequent gene expression, may likely play a role in the difference of triploid development. This led to the objectives of Chapter 5 which attempted to develop a methodology to investigate gene regulation in three sets of chromosomes in triploid single cells.

It must be acknowledged that this study analysed offspring from only five dams which may have limited the interpretation of the results. For example, ongrowing results were determined from pooled families and likely contributed to the high variation observed. However, the increased variation in triploids compared to diploids suggests a potential ploidy effect and further highlights the need to understand specific gene regulation post-triploidisation. Fin clips from every offspring were collected in this investigation. From this, family differences in survival and growth variation can be elucidated. It has previously been reported that there is a strong family effect on survival and growth response in triploid salmonids (Friars *et al*., 2001; Johnson *et al*., 2004) however the work involved far exceeded the time limitation for this thesis but will certainly be investigated in future work. As these performance parameters are affected by family, introduction of a breeding program incorporating triploid production requires the understanding of gene regulation post-triploidisation.

Ultimately, it is important to remember that eggs are produced assuming they will undergo diploid development. There is already extensive research reporting the increased nutritional demand of triploids regarding exogenous feeding, therefore it is possible that the reserves available during embryogenesis are insufficient, particularly at elevated temperatures as shown in Chapter 2 with a higher thermosensitivity in triploids. Typically, a teleost will develop anatomically according to the type and level of nutrients present in the maternally

deposited egg reserves (Palace and Werner, 2006) therefore, understanding the influence of "diploid-prepared" reserves for triploid development is just as important as the differences suggested with aquafeed requirements. Broodstock nutrition is known to impact the quality of eggs (Bromage *et al*., 1992; Izquierdo *et al*., 2001; Migaud *et al*., 2013). If this were to be a barrier preventing optimal triploid culture, future studies may investigate further the impact of broodstock diet manipulations on the requirements for optimal triploid egg quality.

6.3. Nutritional programming is successful in triploid *Salmo salar*

Another factor that may be influencing the differences in traits observed between diploids and triploids is the difference in nutritional requirements they possess, particularly with the growing use of vegetable-based ingredients in aqua-feeds. Fortunately, an opportunity arose to investigate this and the concept of nutritional programming at the first feeding stage as the H2020 ARRAINA project was running in parallel with this thesis.

Concern over the use of raw materials historically included in salmonid aqua-feeds has been growing for some time now. The oily fish which are currently used as the source of high protein and lipid are finite. With the expansion of the aquaculture industry, amongst other uses for the ingredients, attempts to source alternative ingredients are being made. One idea which has received great attention is vegetable-based protein and oils due to their ease of production and high yield potential. With the potential of triploid *Salmo salar* implementation in aquaculture, the present study set out to understand if nutritional programming could be successful and if there was a difference in response between diploids and triploids.

The results confirmed that a short exposure (3 weeks stimuli) to a vegetable-based diet during first exogenous feeding adapted diploid and triploid *Salmo salar* to better utilise a similar diet when challenged later in life. These findings clearly demonstrated that while the

nutritional programing concept was highly significant in diploids, the effect was also clearly apparent in triploids, albeit with greater variation in the triploid data (i.e. nutrient retention and LC-PUFA biosynthesis). An important point to consider which may explain ploidy differences observed is that the diets were formulated based on diploid *Salmo salar* requirements. Many triploid *Salmo salar* studies, and particularly in this thesis, highlight the difference in requirements (environmental or nutritional) between ploidy and to truly understand the early adaptations to plant-based diets in triploids, their nutritional requirements must be better understood. A recent study investigating micronutrient supplementation in diploid and triploid *Salmo salar* when fed a low FM/FO diet suggested that requirements exceed the current recommendations outlined from the National Research Council (2011) (Taylor *et al*., 2019). Further, a difference between ploidy was evident with triploids showing a higher micronutrient requirement. In the present study, micronutrient levels were not considered in the experimental diets and deficiencies may account for the higher variations observed in between triploid individuals.

The stimulus phase involved the programmed fish feeding on a plant-based diet for three weeks to prompt physiological development towards acceptance of these ingredients. Based on the results obtained, including reduced survival during the stimulus phase and smaller body weights of fish fed Diet V prior to the challenge phase, irrespective of ploidy, it is likely that three weeks of feeding on a plant-based diet in the first instance of exogenous feeding is too long, as reported in other studies (Fournier *et al*., 2004; Soltan *et al*., 2008; Pratoomyot *et al*., 2010). It is possible that a shorter stimulus phase will allow the physiological programming and reduce mortalities during this period.

Of major importance is the finding of a net production of EPA found in programmed fish when they were challenged as parr which agrees with other studies (Turchini *et al*., 2011; Rosenlund *et al*., 2016). Diploid fish showed conclusive results on EPA and DHA retentions,

however due to higher variation between individuals, the triploid response was not significant. This again supports the general hypothesis that triploidy has an impact on variation within a group. Moreover, the challenge phase lasted only six weeks which potentially limited the levels of key LC-PUFA found in programmed fish. On the other hand, assessing the fish after only a short challenge period may have concealed negative responses that may have developed with longer exposure to the diet. *Salmo salar* have the ability to synthesise EPA and DHA from ALA during the freshwater stage as they have fatty acid desaturase and elongase genes (Carmona-Antoñanzas *et al*., 2013). This is likely an evolutionary strategy to allow for low levels present in natural prey. However, this mechanism is lacking post-seawater transfer, likely resulting from the natural supply of LC-PUFA in omega-3 rich prey (Miller *et al*., 2008). Whilst commercial freshwater diets have not experienced the same level of ingredient replacement compared to seawater on-growing diets, this study aimed to prove the concept of nutritional programming. Future studies on nutritional programming in diploid and triploid *Salmo salar* should investigate the concept using seawater on-growing diets and monitor the response up to harvest where high levels of LC-PUFA are a benefit to the consumer but may not be biosynthesised.

Further optimisation of an effective stimulus both in terms of diet formulation and duration can further unlock the potential of this strategy in both ploidy. It is vital to include triploids in diet trials in order to understand better the differences between ploidy before the successful implementation of triploid *Salmo salar* in aquaculture.

6.4. Development of single cell allelic expression methodology

Overall, the research in this thesis has highlighted a repeated observation of higher variation in triploids as seen with parr size (Chapter 3) and utilisation of nutrients (Chapter 4). These findings prompted fundamental investigation of how the extra set of maternal chromosomes

may affect the morphology, physiology and behaviour of triploid *Salmo salar*. Triploids possess two sets of maternal chromosomes caused by prevention of the second polar body release during meiosis II, resulting in a total of three sets of chromosomes in the cells. It is not yet understood how this extra set of chromosomes affects the expression of traits that determine a better, or poorer performing fish, however, it has been suggested that triploids must be considered as a "new species" when defining culture conditions. A fundamental aspect of generating and applying a breeding program for a "new species" is the characterisation of genetic diversity which can be exploited for improving performance over generations. Currently, triploid offspring performances are selected based on the best performing diploid broodstock. However, there is no literature available which outlines the impact of gene regulation post-triploidisation of *Salmo salar* and therefore Chapter 5 set out to develop a methodology to investigate this.

It may be the case that the whole genome from one maternal chromosome is silenced as observed in most mammals described in Singer-Sam (2010), however there may be a degree of expression from all three sets increasing the heterozygosity of the individual (Piferrer *et al*., 2009). In the event of complete inactivation of one X chromosome, selecting for the best performing offspring may be relatively easy as female broodstock with dominant desirable traits can be used and inheritance can be traced. However, in the case of the latter, understanding how alleles from such genes compete for, or share, expression will prove challenging. Gene dosage may be consistent or random (Pala *et al*., 2008; Singer-Sam, 2010) and how this affects different genetic strains, tissues and single cells must be elucidated prior to developing a successful breeding program for triploid *Salmo salar*. No studies to date have attempted to investigate this in *Salmo salar* and before any questions can be answered, a robust methodology has to be defined to be confident in searching for answers. Using allelic profiling with microsatellite markers, this investigation concluded that markers from all three

sets of chromosomes were expressed at the individual offspring level and in their whole blood. Although advances were made in this study, allelic-expression results could not be obtained from single cells and therefore does not answer if all individual cells express each locus investigated, and if expression is equal or random.

Based on results obtained, there appear to be some limitations in the methodology used in this investigation. High RNA/cDNA quality is vital for assessing allelic-expression and some processes (e.g. storage, transportation, cell sorting) in the present study likely contributed to a decrease in the quality. RNA can be easily degraded and therefore samples should be processed immediately after collection, or conditions for storage must be optimised if necessary. The lack of a single cell sorter in Stirling required to perform single cell isolation meant that the samples had to be transported to Aberdeen for downstream analysis. Fish had to be bled in Stirling due to Home Office restrictions regarding transportation of live animals and although precautions were taken to maintain stability and viability of the cells, it is likely there was a degree of degradation during the transportation. Reducing the time between sampling and processing could potentially eliminate the storage period and likely increase the success rate of the investigation. In some cases, assessment of amplified cDNA showed invalid results. This was observed at the whole offspring and whole blood level and became more apparent in single erythrocytes. The dramatic decrease in success observed in single erythrocyte samples may be a result of transportation as this was the only sample type that was processed further in Aberdeen.

However, there was a decrease in expression success in one locus in particular in whole blood compared with whole individual, both of which were determined at Stirling. This is likely a result of poorer expression in this particular tissue. Although this panel was still able to determine ploidy status, there was no such success in single erythrocytes. Therefore, surveying multiple loci would increase the chances of detecting the true ploidy

state in single cells. Erythrocytes are undoubtedly the easiest single cell to obtain however using only one cell type limits the probability of expression from a locus panel as this study suggests a difference in expression between tissue types. These findings highlight the need to analyse other important single cell types (i.e. hepatocytes) and also increase the microsatellite locus panel, ultimately increasing the chances of detecting allele presence.

Quality control tests were conducted to eliminate physical damage caused by the cell sorter prior to RNA extraction in single cells. Despite some positive feedback and subsequent optimisation of the process, the impact of the cell sorter on RNA integrity could not be excluded as a possibility for low amplification success. Furthermore, the single cell kit may not be sensitive enough to analyse single erythrocytes from *Salmo salar*, and this study was the first of its kind. Although this method of single erythrocyte isolation appears to be efficient, other methods of single cell transcriptome amplification may be explored in order to determine allelic expression in individual *Salmo salar* erythrocytes. A global approach of investigating the full transcriptome through RNA sequencing rather than targeted genes may prove beneficial.

In order to generate a successful breeding program which includes triploid *Salmo salar*, genetic inheritance and subsequent expression in triploid offspring has to be understood at the single cell level. A previous investigation concluded that inheritance of performance related traits are affected by the extra maternal chromosome in triploid *Salmo salar* but how these traits are regulated is not yet understood (Johnson *et al*., 2007). If this could be predicted, better performing triploids may be selected based on the information identified from diploid broodstock. Currently, the inability to determine expression of target genes in single cells prevents further understanding of how the additional set of maternal chromosomes affects offspring traits which would normally be selected for in a selection

program. These results clearly warrant further investigations into gene regulation post-triploidisation.

6.5. General conclusions and recommendations

This research has successfully advanced our knowledge on key issues associated with early life development of triploid *Salmo salar*, building on the growing knowledge of their culture requirements. Advancements in ploidy-specific rearing protocols have been made with further understanding on the effects of egg incubation temperature on embryogenesis including a change in temperature commonly experienced in commercial aquaculture. For improved survival and productivity of triploids, the industry would benefit from altering broodstock facility and hatchery protocols to allow for a constant egg incubation temperature of 6 °C from fertilisation to first feeding. For the first time, the impact of post-ovulatory ageing was monitored in triploid *Salmo salar* and confirmed the impact on increased mortality and size variation when compared to their diploid counterparts, highlighting the benefit of fertilising eggs in minimal time post-ovulation. Also for the first time, nutritional programming was successfully demonstrated in *Salmo salar* of both ploidy, however differences in nutritional requirements require further understanding. It is now apparent that alterations to early life stage production conditions can improve the success of a triploid batch and particular scientific knowledge gaps have now been filled and can be applied to commercial practice. However, these findings suggest there is still a degree of difference between the ploidy, in particular a higher variation of responses in triploids which must be considered in future studies. This initiates further hypotheses on fundamental genetic and potential gene regulatory differences between ploidy. Although determination of single cell ploidy status and allelic expression was not successful during this thesis, advancements have been made in the development of robust single cell isolation that should be continued.

Overall, the results presented in this thesis contribute to the growing scientific knowledge of triploid-specific early life stage culture condition requirements which can be aligned with continuous developments in Scottish *Salmo salar* aquaculture.

Recommendations for early life triploid *Salmo salar* culture:

1. Egg incubation temperatures from fertilisation to first feeding should be maintained at 6 °C and fluctuations should be avoided if possible.
2. Female broodstock should be stripped of eggs within five days of ovlation to reduce mortality and increased growth variation as consequences of post-ovulatory ageing.
3. Nutritional requirements of triploids require further understanding, although the potential of using nutritional programming to better accept and utilise vegetable-based diets is possible in both ploidy.
4. Prior to incorporation of triploids into a commercial breeding program, gene regulation should be investigated further to understand methods of selection for desirable traits.

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