Nutritional and environmental effects on triploid Atlantic salmon skeletal deformity, growth and smoltification

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Declaration

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

Marie A. Smedley

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Species list

Astyanax Mexicanus	Cave fish
Caligidae	Sea louse
Danio rerio	Zebrafish
Dicentrarchus labrax	European seabass
Gadus morhua	Atlantic cod
Ictalurus punctatus	Channel catfish
Lactobacillus rhamnosus	
Lepisosteus oculatus	Spotted gar
Mus musculus	Mouse
Neoparamoeba perurans	Amoebic gill disease
Onchorynchus mykiss	Rainbow trout
Oncorhynchus masou	Masu salmon
Oncorhynchus tshawytscha	Chinook salmon
Oreochromis niloticus	Nile tilapia
Osmerus mordax	Rainbow smelt
Pediococcus acidilactici	Bactocell™
Salmo salar	Atlantic salmon
Salmo trutta	Brown trout
Salvelinus fontinalis	Brook charr
Saprolegnia	Cotton mould
Squalius alburnoides	Sea bream
Tetradon nigroviridis	Spotted green puffer fish
Umbrina cirrosa	Shi drum
Vibrio anguilliarium	

List of Abbreviations

1,25(OH)2D	1,25-dihydroxyvitamin D
2N	Diploid
3N	Triploid
AA	Amino acid
AGD	Amoebic gill disease
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
BM	Bone mineral
BP	Boosted phosphorous
BW	Body weight
Ca	Calcium
$[Ca_{10}(PO_4)_6(OH)_2]$	Hydroxyapatite
cDNA	Complementary DNA
Cl^-	Chloride
cm	Centimeter
CpG	Cytosine-phosphate-guanine
CSI	Cardio-somatic index
DNA	Deoxyribonucleic acid
DNAme	DNA methylation
DNMT	DNA methyltransferase
˚DPF	Degree days post fertilisation
dV	deformed vertebra
˚*Dws*	Degree days post winter solstice
EAA	Essential amino acid
EC	European Commission
ECM	Extracellular matrix
eFCR	Food Conversion Ratio
EST	Expressed Sequence Tags
EU	European Union
FAA	Free Amino Acid
FGF23	Fibroblast growth factor 23
FL	Fork Length
FW	Freshwater
g	gram
GH	Growth Hormone
GI	Gastrointestinal tract
GLM	General Linear Model
GSI	Gonadosomatic index
H	High

His	Histidine
HP	High phosphorous
HPLC	High Performance Liquid Chromatography
HSMI	Heart and Skeletal Muscle Inflammation
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IGF-I	Insulin-like Growth Factor
IgM	Immunoglobin
K	Condition factor
Kg	Kilogram
KJ/g	Gross energy
L	Litre
L: H	Length to height ratio
LD	Light: Dark
LJC	Lower jaw curvature
LL	Constant light
LP	Low phosphorous
m	meter
mg	Milligram
Mg	Magnesium
miRNAs	micro RNAs
mJ	Total work
MP	Medium phosphorous
mRNA	Messenger RNA
MS222	Tricaine methanesulfonate
mya	Million years ago
N	Force (Newton)
NaCl	Sodium chloride
NKA	Na^+, K^+ - ATPase
NM	Nitrogenous metabolite
Npt2a	Sodium-phosphate cotransporter
NQC	Norweigen Quality Cut
OPN	osteopontin
P	phosphorous
PO_4^{3-}	Inorganic orthophosphate
ppm	Parts per million
psi	Pounds per square inch
PST	Parr-Smolt Transformation
PTH	Parathyroid hormone
qPCR	Quantitative polymerase chain reaction
R1	vertebrae 1-8
R2	vertebrae 9-30
R3	vertebrae 31-50
R4	vertebrae ≥ 51

RNA	Ribonucleic acid
S	Standard inclusion
S0+	Underyearling
S1+	Yearling
SAM	S - Adenosyl methionine
SBC	Standard inclusion with Bactocell™
SNP	Simulated Natural Photoperiod
SP	Standard nutrient package
SW	Saltwater
t	Tonnes
TGC	Thermal Growth Coefficient
UK	United Kingdom
V	Vanadium
v	Vertebra
WD	Work Done
WGD	Whole Genome Duplication
WGS	Whole-genome shotgun contigs
Zn	Zinc
µl	Microlitre
µm	micrometre
τ_s	tau somite

Glossary

Acellular bone	Bone mineral devoid of osteocytes specific to teleost fish
Alevin	A newly hatched salmonid still carrying yolk
Agnostic behaviour	Social behaviour related to fighting
Amphicoelous centra	Single vertebra with concave end plates
Anadromous	Fish born in freshwater, migrating to sea and returning to freshwater to spawn
Androgen	Male sex hormone
Aneuploid	Abnormal number of chromosomes
Ankylosis	Abnormal adhesion of skeletal joints
Blastulation	Early embryonic developmental stage of the blastodisc where morphogenic cell movements to form the germ ring
Chordacentrum	Early mineralisation of the vertebral body around the notochordal sheath
Chorion	The very outer embryo membrane
Chrondrogenesis	Process by which cartilage is developed
Cleavage	Early embryonic developmental stage where rapid cell division occurs and initial formation of blastodisc
Conspecific	A member of the same species
Cranio-caudal axis	The central region from which the vertebral column is formed in teleosts
Desmoltification	The reversal of the smoltification process in fish from a hypo-osmoregulatory competent state to hyper
Diploid	Paired cell organism
DNA methylation	Epigenetic mechanism that restricts DNA expression through SAM and DNMTs
DNA remethylation*	The process by which embryonic DNA is remethylated following demethylation
DNA reprogramming*	The process by which maternal embryonic DNA methylation is reprogrammed to paternal methylome
Epiboly	Spreading of cell layers across the embryo in embryogenesis
Epigenetic	Stably heritable phenotype resulting from changes in a chromosome without alterations in a DNA sequence
Escapee*	Domesticated salmonid unintentionally released from a farm
Essential Amino acid	An amino acid that cannot be produced by the body
Eyeing	Stage of embryogenesis where the eye pigmentation appears
Free Amino Acid	Metabolically available form of an amino acid
Fry	Start feeding juvenile salmonids post yolk-sac absorption

Gastrulation	Stage of embryogenesis from germ ring development to the formation of the axial organ complex
Gene dosage compensation*	Equalisation of expression in triploids to diploid expression levels
Gene dosage effect*	Maintained expression in triploids of additional chromosome
Genetic introgression*	Movement of a gene from domesticated salmonids to wild conspecifics through repeated backcrossing which may lead to a fitness depression
Gigantism*	Increase in phenotypic size of polyploids relative to the increase in genomic material and cell size
Haemal	Situated on the ventral side of the body
Haematology	Morphology and physiology of the blood
Heritability	How much phenotypic variation of a trait is attributed to genetic variation in a population
Heterozygosity	How many alleles are available at a given locus
Hydroxyapatite	The main mineral component in bone formed primarily and calcium and phosphate
Karyotype	The number and appearance of chromosomes in a cell
Kyphotic	Dorsoventral concave curvature of the spine
Lordotic	Dorsoventral convex curvature of the spine
Mariculture	Cultivation of marine organisms
Maternal	Relating to the female parent
Maternal - zygotic transition	Degradation of maternal products in early embryogenesis and transfer to zygotic regulation
Meristic	Counting quantitative fish features
Methylome	Heritable DNA methylation pattern
Microsatellite	Nucleotide tandem repeats in DNA sequences
Myogenesis	Formation of muscular tissue
Notochord	a stratified epitheloid tissue enclosed by an acellular fibrous sheath
Ongrowing	Saltwater period of farmed Atlantic salmon rearing
Ontogeny	Life cycle development and origination of an organism
Oocyte	Female immature gamete
Operculum*	Anatomical structure that covers the gills
Osteoblast	Osteoid secreting cell
Osteoclast	Bone remodelling cell
Osteocyte	Former osteoblast encased in mineralised osteoid
Osteogenic	Relating to mineralisation of bone
Osteoid	Not yet mineralised organic matrix of bone
Ova	Female mature gamete
Parental	Referring to both / either maternal and paternal origin
Parr	Post-fry freshwater developmental stage in salmonids prior to smoltification
Paternal	Referring to male parent
Photoperiod	Period of time in a day in which an organism receives light

Phytate	Phosphorous rich plant-based compound
Platyspondyly	Flattened vertebral bodies
Polyploid	Organisms containing more than two paired chromosomes
Probiotic	Microorganisms that provide health benefits upon consumption
Scoliotic	Severe curvature of the spine
Septum transversum	Mesenchyme formed in the embryo dividing thoraic and ventral internal development.
Sibling	With common parents
Smoltification	Physiological adaptation enabling movement from a freshwater to saltwater environment
Somitogenesis	Process by which somites form, the embryonic segmentation in animals
Stenothermal	The thermal range capacity of which an organism is capable to live
Sterile	Incapable of producing viable offspring
Tetraploid	Organisms containing three chromosome pairings
Triploid	Organisms containing four chromosome pairings
Whole genome duplication	Nondisjunction within meiosis which produces an additional copy of the genome within an organism
Zygotic gene activation	The stage of embryogenesis alongside degradation of maternal transcripts of which the zygote controls further development

* Definitions that are specific for this thesis

Abstract

The Atlantic salmon (*Salmo salar*) is an iconic species that dominates the global finfish production sector with increasing market demand. The Scottish industry and government alone aspires for expansion of the sector to 210,000 t by 2020 with 154, 000 t produced in 2013. As such, there are pressures to improve sustainable development in particular to minimise the genetic impact of escapees on wild populations and reduce sea lice infection which are required for the granting of "green licenses" in Norway. The use of triploidy has been tested in the 1980's with little success owing to suboptimal rearing conditions leading to elevated mortalities, poorer growth and a higher prevalence of deformities, in particular of the skeleton. Collectively: recent success of triploid trout farming, expansion to the salmon production sector and potential resulting pressure on wild stocks through escapee increases have reinstated interest to implement artificially induced triploid Atlantic salmon in commercial production. As diploid Atlantic salmon have undertaken extensive domestication to achieve the high quality production and welfare standards observed to date, triploid conspecifics too require husbandry optimisation to realise potential. In particular, industrialisation requires that higher observations of deformities and inconsistent growth trajectories during seawater ongrowing be resolved through optimisation of rearing regimes and subsequent standardization of husbandry protocols.

Triploids possess additional genomic material and increased cell size yet reduced frequency that reflects known differences in physiology and supports that, in effect, triploids should be considered as a new species relative to diploid conspecifics. Therefore, this doctoral thesis aimed to study nutrition and temperature effects on triploid Atlantic salmon traits throughout the production cycle from 'egg to plate'. Nutrition trials aimed to improve growth potential and mitigate skeletal deformities both in freshwater (FW) and saltwater (SW) whilst attempts were made to define a window of smoltification to ensure optimal ongrowing performance. Finally, impacts of embryonic temperature regimes that are known to impact long term performance and deformity development in triploids, were examined in relation to DNA regulation and yolk composition in an attempt to underpin potential mechanisms for the environmental impact of temperature on developmental phenotype.

One of the main restrictions to triploid Atlantic salmon implementation is the increased prevalence and severity of skeletal deformities, particularly after the maring phase. The work performed in this thesis first demonstrated that protein and / or phosphorous (P) supplementation throughout SW ongrowing not only reduced the level of severely deformed (≥ 10 deformed vertebrae observable by x-radiography) individuals by 30 % but also sustained 6.8 % faster growth and improved harvest grade compared to triploids fed a standard grower diet (chapter 2). Comparison of x-radiography and

severely deformed individuals between harvest and sea transfer highlighted that protein and P supplementation arrested deformity development whereas prevalence increased in triploids fed a standard grower diet. This implied that severe deformities were of FW origin and strongly suggest requirement for improved nutrition in FW to optimise SW performance. Therefore investigation of higher dietary P inclusion in FW was investigated and results showed significantly reduced number of deformed vertebrae and no severely deformed individuals in those fed 19.7 g total P Kg^{-1} compared with those fed 13.0 & 16.7 g total P Kg^{-1} (chapter 3). Most deformities were localised in the central (vertebrae 27 – 31) and caudal (vertebrae 52 – 57) regions for all treatments. However, triploids fed lower dietary P displayed a particular increase in prevalence within the tail region (vertebrae 32- 47) which is consistent with SW ongrowing reports and results from chapter 2, further highlighting FW origin of higher vertebral deformities reported in SW ongrowing in triploids. Higher P supplementation in FW also significantly improved growth in triploid parr compared to diploids and lower supplementation. However, this effect did not transpire in later FW smolt stages where weights were significantly higher in triploids fed lower compared to higher P supplementation. Expression of target genes involved in osteogenesis and bone P homeostasis in vertebrates were then analysed and a ploidy effect of osteogenic genes *alp, igf1r* and *opn* as well as a dietary effect on P homeostasis gene *fgf23* was apparent in the parr stages but not smolt. In addition, stronger

ploidy-diet effects were also observed in parr stages for whole body mineral concentrations. Collectively, growth, gene expression and whole body mineral content results indicate these earlier parr life stages may be more sensitive to P supplementation. This pronounced effect may be a consequence of seasonal accelerated growth associated with this period, where higher temperatures were also observed. The potential for shorter P supplementation windows in commercial production was addressed in chapter 4 with hope to cut economic cost to raw mineral inclusion in feed and also mitigate potential anthropogenic eutrophication on the environment that may be induced by P leached through uneaten feed and faeces. Triploids were fed higher dietary P (17.4 g total P Kg^{-1}) until either early (5 g) or later (20 g) parr stages, or smolt (83 g) and monitored for performance throughout freshwater (FW) development. During later parr development (30 g), x-radiography assessment demonstrated that increased dietary P reduced the number of deformities and severely deformed individuals with no indication that feeding P for shorter windows improved skeletal integrity. Hence, P supplementation may be required throughout FW development for optimal skeletal performance. In addition, no differences in deformities were observed between triploid treatments at smolt. An effect of dietary P supplementation on whole body mineral concentration was observed in the early and later parr stages that was not as pronounced as smolt, which is consistent with results in chapter 3. Together, these results indicate that skeletal assessment during early

developmental stages may not reflect smolt performance most likely as a consequence of seasonal effects of improved linear growth in the cooler winter temperatures prior to smolt where reversible deformities observed at parr may also be alleviated. In the same study (chapter 4), the inclusion of the probiotic *Pediococcus acidilactici* (Bactocell™) was also tested as a means to enhance gut assimilation as suggested in previous studies and therefore reduce the levels of P supplementation. Results clearly indicate superior skeletal performance in parr (30 g) as well as significantly less deformed vertebrae and no severely deformed individuals. However, at smolt (~83g), no effects of the dietary probiotic treatment were observed which may also be attributed to seasonal effects. Overall, nutritional research clearly indicate triploids require higher dietary P for optimal growth and skeletal development, which although is not consistent between life stages, is ultimately required throughout FW for optimal skeletal development at smolt. The use of probiotics offer a promising avenue for reduced P requirement in FW feed and further research should verify results and assess long-term performance.

Timing of SW transfer according to correct parr-smolt transformation (PST) is essential for survival and growth performance in ongrowing where feeding and growth rate accelerate post-transfer. So far, SW transfer regimes and in particular the smoltification 'window' remains loosely defined in triploid Atlantic salmon and it is crucial that this be addressed to ensure optimal ongrowing survival and performance.

Results in chapter 5 show that triploid Atlantic salmon reared under an ambient photo-thermal regime (S1+) have a wider smoltification window within 155 – 365 degree days as well as an earlier onset by 48 degree days. This was confirmed through raised Na^+, K^+ - ATPase (NKA) activity that was maintained for a longer duration and earlier skin silvering compared to diploid siblings. In addition, reduced plasma chloride (Cl^-) levels alongside improved survival following SW challenge compared with diploid siblings strongly suggest that triploids had improved hypo-osmoregulatory capacity and a wider smolt window. Although other studies have demonstrated that triploid salmonids may have earlier onset of PST none to date have investigated the window duration. Results in this study need to be verified against other photo-thermal regimes; a wider smolt window may be of great benefit to industry as there is potential for reduced FW rearing periods, earlier onset of ongrowing and increased sea-transfer flexibility compared to diploid conspecifics.

Suboptimal egg incubation conditions, in particular higher temperatures, are one of the primary causes of deformity in triploid Atlantic salmon. This may be associated with embryogenesis being stenothermal and also where the critical process of somitogenesis and the underlying changes in DNA regulation occur. Hence, diploid and triploid embryos were reared at temperature regimes known to be optimal and suboptimal for development (5.9, 7.9 and 10.7 ºC) from fertilisation until the eyeing stages and then

at 7.8 ºC until hatch. Temperature / ploidy associated mechanisms that may induce phenotypic variation were analysed comprising of global DNA methylation (DNAme), as an indicator of DNA regulation, as well as changes in Nitrogenous metabolites (NM) including Free Amino Acid (FAA) concentrations. Differences in genomic weight between diploids and triploids may potentially impact DNA regulation and the availability of maternally provided resources such as NMs for the dramatic process of reorganisation of the methylome during embryogenesis. Although changes in NM utilisation were apparent between life stages and influenced by temperature, no impact of ploidy was evident. In addition, no impact of temperature was observed on DNAme levels. This indicates availability of maternally provided NMs and DNA programming may not necessarily be a factor in temperature induced deformities in triploids and phenotype assessment in later life-stages would verify this conclusion. In addition sequence specific DNAme results and analysis of other epigenetics process such as hitstone modification would verify or reveal other epigenetic effects. However, results did reveal interesting ploidy differences in DNAme levels post gastrulation where triploids maintained lower DNAme levels relative to diploids throughout somitogenesis indicating a delay in the DNA remethylation or reprogramming process. This is the first study to identify potential triploid specific differences in DNA reprogramming in

salmonids and so verification as well as an understanding of the impact on epigenetics and long-term phenotype must be assessed.

This doctoral work adds significantly to existing knowledge on improved husbandry practice of triploid Atlantic salmon through: improved nutritional regimes and understanding of PST that have potential for improved production traits including growth, reduction of skeletal deformities and reduced rearing periods. This work also pioneers study of DNA regulation in triploid embryogenesis that pose important future questions to explain fundamental differences associated with altered cellular and genomic make-up in triploids. This research will assist in enabling triploid salmon as a tool for sustainability in global aquaculture production of Atlantic salmon as demonstrated by the development of patented triploid feed in relation to these trials and optimised protocols for SW transfer. Ultimately, this additional knowledge highlights the potential for triploids to perform equally well if not better than diploid conspecifics

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Chapter 1: General Introduction

1 Atlantic salmon and aquaculture production

The Atlantic salmon (*Salmo salar* L.) is an anadromous teleost fish native to the temperate and subarctic regions of the North Atlantic Ocean (Aas *et al.,* 2010). The life history is complex and initially involves a freshwater (FW) juvenile phase commencing from egg incubation into alevin, fry and parr stages. Depending on body size and seasonal cues, parr undergo smoltification, a dramatic physiological and morphological transformation that enable migration into saltwater (SW) for feeding and substantial growth (McCormick *et al.,* 1997). Upon sexual maturation adults return to their native river to spawn. For reproductive success, spawning populations rely on the ability to return to their native stream making them vulnerable to genetic interference from potential cross-breeding with domesticated conspecifics (Fleming *et al*., 2000). Farmed Atlantic salmon populations dwarf wild populations by 92 - 95% (Gross, 1998) and are a prominent species in aquaculture that are produced extensively both within and out-with their native geographical habitat.

Aquaculture is presently the fasting growing animal food production sector with global food fish production increasing at an average rate of 6.2% from 32.4 million tonnes in 2000 to 66.6 million tonnes in 2012 whereas capture production statistics have remained static (FAO, 2014). Mariculture represents only 12.6 % of the global finfish

production, yet accounts for 26.6 % of production by value. Atlantic salmon is the dominant finfish species produced with Norway, Chile and Scotland leading production (Fig. 1.1) and export. In Europe, Atlantic salmon is the leading aquaculture species by volume and market value. In Scotland, Atlantic salmon represents the leading export with significant plans to increase the market to China (The Scottish Government, 2012). In addition to the economic weight of production, the Atlantic salmon is a highly iconic species and efforts are being made to preserve local populations and farm responsibly. For instance the leading producer Norway recently issued "green licenses" through the Norwegian Ministry of Fisheries and Coastal Affairs which can only be achieved if producers reduce occurrence of sea lice (Caligidae) outbreaks and risk of escapees (Forskrift om løyve til havbruk med Matfisk, 2013).

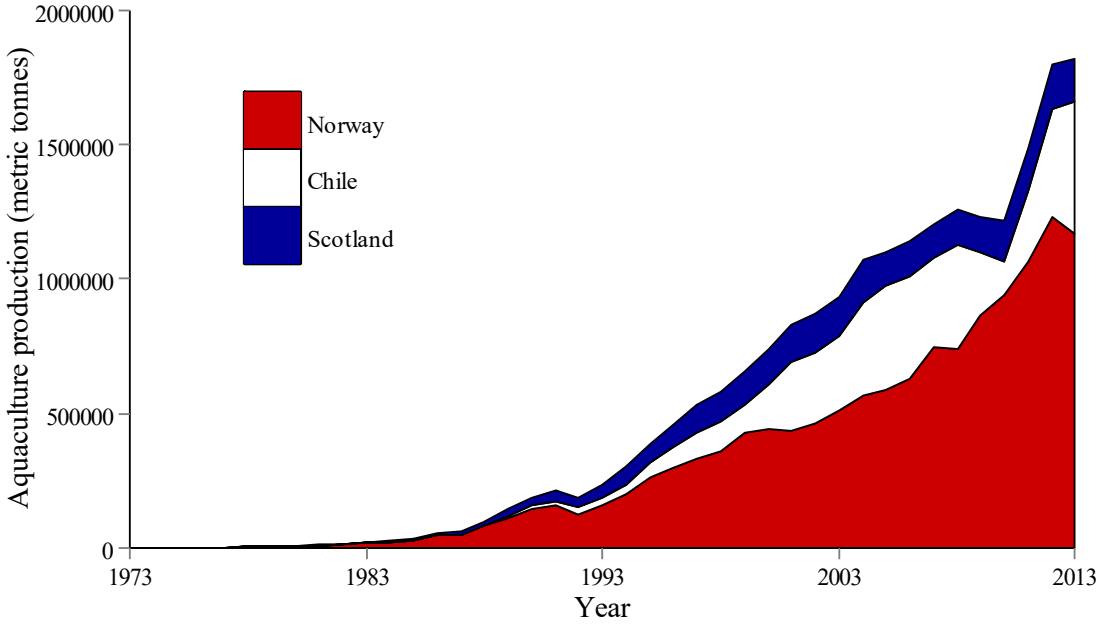

Figure 1.1 Aquaculture production of within dominant producing nations: Norway, Chile and Scotland from 1973 – 2013 (FAO 2014).

Escapees may impact on native salmon populations and minor incidents may occur through routine husbandry procedures whereas major accidents may involve damage to net pens. Owing to differences in geographical origin (Gjedrem *et al*., 1991), genetic drift (Fleming & Einum, 1997) and domestication (Skaala *et al.,* 1990; Gjedrem *et al.,* 1991; Fleming *et al.,* 2002), farmed Atlantic salmon are genetically different to wild populations. Breeding of escapees with wild conspecifics may lead to introgression (Glover *et al.,* 2013) and cumulative fitness depression (McGinnity *et al.,* 2003) that could threaten vulnerable populations. Genetic impact of farmed escapees may vary between native populations: recent research on Norwegian salmon populations suggest that genetic structure of native populations has largely been retained (Glover *et al.,* 2012) whereas Bourret *et al.,* (2011) attributed a loss of local genomic adaptation in a severely threatened Canadian population to genetic introgression.

2 Artificial induction of triploidy in cultured salmonids

Production of functionally sterile fish will not only suppress reproductive interaction with wild populations but also poses additional production benefits. Cessation of sexual maturation may: improve growth performance through diversion of reproductive resources into somatic growth; mitigate the associated reduction in disease resistance and flesh quality in mature fish; and provide the means for breeding companies to protect

their intellectual property. Both female and male Atlantic salmon devote 60 % of their energetic resources during reproduction (Aas *et al.,* 2010) resulting in reduced somatic growth (McClure *et al.*, 2007), reduced flesh quality (Aksnes *et al.*, 1986; Johnston *et al*., 2006; Martin *et al*., 1993), increased disease susceptibility (St-Hilaire *et al*., 1998) and an overall economic loss (McClure *et al.*, 2007). Presently, the only publically and commercially acceptable means of achieving sterility in teleost, including and salmonids, is through the artificial induction of triploidy (EEC, 1990) which is typically achieved through administration of thermal or hydrostatic pressure shocks post fertilisation. These methods are the most consistent techniques to produce large commercial egg batches without the use of chemicals (Benfey, 2001). Research into aspects of triploid Atlantic salmon production potential and performance traits has been ongoing since the 1990's in: America (Galbreath *et al*., 1994; Galbreath & Thorgaard, 1995), Canada (Mcgeachy *et al*., 1995; O'Flynn *et al.*, 1997), France (Quillet & Gaignon, 1990), Scotland (Johnstone *et al.*, 1992; McCarthy *et al.*, 1996) and Tasmania (Jungalwalla, 1991). High mortality in the egg incubation and marine phase has led to little interest for triploidy adoption in America, Canada and Europe (Benfey, 2015). Conversely, Tasmania benefits from reduced incidence of early sexual maturation, which is associated with higher temperature regimes relative to Europe (Jungalwalla, 1991; Hughes, 1992; Sadler *et al.,* 2001) and current reports claim 20 % (7000 t) representation of total Atlantic salmon

production (35,000 t; J. Taylor; personal communication). Although recent reports have shown comparable survival (Oppedal *et al.,* 2003; Leclercq *et al.,* 2011; Taylor *et al.,* 2011; Taylor *et al.,* 2012), other reports of inconsistent growth in the marine phase (Cotter *et al*., 2002; Oppedal *et al.*, 2003; Taylor *et al*., 2014) and higher incidents of malformations compared to diploid siblings particularly within the marine phase (Leclercq *et al*., 2011; Fraser *et al*., 2013), constrain commercial implementation of triploid Atlantic salmon in production areas outside of Tasmania. A comprehensive understanding of the impact of triploidy on production traits is required for husbandry protocols and production regimes to then be optimised. Current research on triploid Atlantic salmon is mainly driven by industrial support. Hence research topics are primarily focused on optimising commercial production through: nutritional requirement studies, investigation of performance traits and optimising environmental rearing conditions, in particular oxygen and temperature requirements (reviewed in: Benfey, 2015).

2.1 Triploid induction and ploidy verification

Polyploid induction is widely performed in plants (Chen & Ni, 2006) and also in shellfish (Piferrer *et al.,* 2009). Normally, oocyte development in fish is arrested during metaphase of meiosis II and resumed upon fertilization, after which meiosis II continues and the second maternal chromosome set is extruded via a polar body. Thus, a diploid embryo is

produced with a single maternal and paternal chromosome contribution. It is at this point where an artificial shock may be applied to prevent extrusion and induce triploidy retaining 2 maternal and 1 paternal chromosome sets (Figure 1.2). In salmonids thermal shocks (Benfey & Sutterlin, 1984b; Johnstone 1985; Quillet & Gaignon, 1990), hydrostatic pressure (Benfey & Sutterlin, 1984b; Taylor *et al.,* 2011) or chemicals such as Freon and nitrous oxide (Johnstone *et al.,* 1989) have been used successfully to induce triploidy. Refinement has advanced over the past few decades and to date the most commonly administered technique for Atlantic salmon is hydrostatic pressure shocking owing to the simplicity of application, greater consistency across egg batch, higher egg survival than thermal shocks and resulting appropriate commercial applicability (Benfey, 2001). Standards dictate that for optimum survival and absolute induction success that a 50 º minutes treatment at 9500 psi be administered 300 º minutes post – fertilisation at 8 °C (Taylor *et al.,* 2011).

Alternatively fertilisation of diploid ova with tetraploid sperm in rainbow trout (*Oncorhynchus mykiss*; Myers & Hershberger, 1991; Weber *et al.,* 2013; 2014) has also been successfully used to induce triploidy although the low viability of tetraploids has so far prevented large scale commercial adoption of this method in salmonids. Recent research comparing interploid crossing and pressure shock induced rainbow trout indicated that interploid cross induced triploids show improved growth performance and

comparable disease resistance (Weber *et al.,* 2013, 2014). However, past attempts at artificially induced Atlantic salmon tetraploids produce low viability and must be addressed before verifying potential for triploid induction through interploid crosses

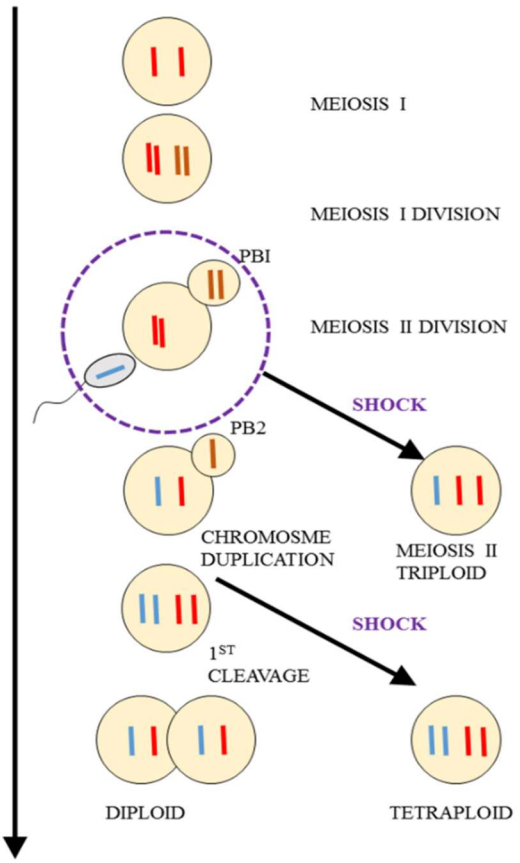

Figure 1.2. Schematic of ploidy manipulation in fish redrawn based on (Piferrer *et al.,* 2009) where each coloured bar represents a set of chromosomes. Ova are released at metaphase of meiosis II, which resumes upon fertilization. Shocking applied at the appropriate time suppress release of the polar body and maternal chromosome creating (reviewed in: Piferrer *et al.,* 2009).

As well as the induction methodology itself, triploidisation success in fish may also be affected by family and genetic stocks (Devlin *et al*., 2010; Johnson *et al*., 2004) as well as egg quality (Aldridge *et al.,* 1990; Devlin *et al.,* 2010; Taylor *et al.,* 2011), which may also impact on performance during later development (Taylor *et al.,* 2011).

Specific combinations of maternal – paternal genomes may tolerate induction better than others (Johnson *et al.,* 2004) and induction stress may interfere with maternally provided substances such as mRNA and immunoglobin (IgM) during early development emphasising the importance of stock and egg selection for induction.

Successful implementation of triploidy in aquaculture requires that induction success rate be 100% hence, accurate and practical verification procedures are essential. Procedures used to date include: karyotyping (Johnson & Wright, 1986), microsatellite genotyping (Glover *et al.,* 2015), nucleolar organizing region analysis (Lozano *et al.,* 1992), and cell size measurements (Benfey *et al.,* 1984a; Lecommandeur *et al.,* 1994; Preston *et al.,* 2013). Cytogenic techniques such as karyotyping are appropriate and almost undisputable ploidy verification procedures providing direct chromosome information, however, they are too time consuming and complex for industrial application (reviewed in: Maxime, 2008; Piferrer *et al.,* 2009; Mable *et al.,* 2011). The most common procedures used to date are cytometric procedures that determine cell and nucleur size differences through either flow cytometry or measurement of meristic counts, typically on erythrocytes which are the most easily obtained. Flow cytometry is regarded as the fastest and most accurate technique determining DNA content based on fluorescence of individual cells (Lecommandeur *et al.,* 1994; Preston *et al.,* 2013) and in addition such methodology can be used on early stage embryos enabling early

verification of ploidy status (Lecommandeur *et al.,* 1994). Refinement of microsatellite genotyping techniques have potential for commercial protocols (Glover *et al.,* 2015) as extraction protocols may be simpler and also enable earlier verification. Early verification of triploidisation success in commercial batches is important not only for customer assurance and the ability to generate replacement batches but also for legislation and auditing purposes, for instance green licensing in Norway (Forskrift om løyve til havbruk med Matfisk, 2013).

3 Impact of triploidy on cell size and gene regulation

3.1 Larger cell size and reduced frequency

Polyploidy may induce morphological differences in non-vertebrates, for instance gigantism in shellfish (Guo & Allen, 1994) whereas in vertebrates including salmonids, similar size and appearance is maintained between ploidy conspecifics (reviewed in: Piferrer *et al*., 2009; Mable *et al*., 2011). Morphological similarities are largely thought to be attributed to the possession of larger cell size and reduced frequency compensating for the increase in nucleur volume and genetic material (Swarup, 1959; Small & Benfey, 1987; Aliah *et al.,* 1990; Johnston *et al.,* 1999; Flajšhans *et al.,* 2011). Surprisingly little research has investigated differential cellular physiology between tissues in artificially induced triploid and diploid fish including salmonids which has largely been restricted to

brain (Small & Benfey, 1987; Fraser *et al*., 2012b), muscle fiber (Johnston *et al*., 1999), retina (Small & Benfey, 1987) and haematology (Small & Benfey, 1987; Cogswell *et al.,* 2002; Hyndman *et al.,* 2003 a,b; Flajšhans *et al.,* 2011) research. Increased cytosol: plasma membrane ratios in triploids may increase intracellular distance between the membrane and nucleus, which may impact processes such as signal transduction from the cell surface to the nucleus as well as intra and extracellular movements of RNA and transport proteins (reviewed in: Benfey, 1999). These costs may be offset by reduced membrane maintenance and smaller relative surface area over which osmotic gradients must be maintained. However, cellular processes in triploids still remain relatively unknown.

3.2 Impact on gene regulation

One of the least comprehensively researched areas of artificial induction of polyploidy in fish is the impact on gene regulation (Maxime, 2008; Piferrer *et al.,* 2009;. Fraser *et al.,* 2012a) which is also the case for naturally occurring polyploid fish (Mable *et al.,* 2011). Polyploidy increases genomic material, alleles per locus and interactions between loci (Chen & Ni, 2006; Jackson & Chen, 2010). The reaction between species to this genomic alteration varies dramatically. In plants induced polyploidy results in rapid epigenetic alterations (Matzke *et al.*, 1999; Rodin & Riggs, 2003), increased genomic plasticity (Jackson & Chen, 2010) and increased phenotypic variation (Paun *et al.,* 2007) whereas

in mammals triploidy has devastating consequences for health as they lack homeostatic mechanisms to compensate for additional genomic material (Jiang *et al.,* 2013a). Polyploidy in teleosts and anurans is relatively more common and is thought to occur in teleosts naturally as a consequence of being ectothermic in extreme climatic spawning conditions in FW (Mable *et al.,* 2011). Salmonid fishes have historically undergone whole genome duplication (WGD) and autotetraploidisation 25 – 100 mya (Johnson *et al.,* 1987; Koop & Davidson, 2008) and an additional teleost specific WGD has also occurred in the teleost lineage itself (Berthelot *et al.,* 2014). Mechanisms to cope with this dramatic genomic adjustment in artificially induced polyploid salmonids and teleosts has been suggested to be predisposed from this evolutionary pathway (reviewed in: Mable *et al.,* 2011). Polyploids theoretically have an advantage over diploid conspecifics through increased heterozygosity, divergence of duplicate genes and increased expression of key physiological proteins (Hawkins *et al.,* 2000). Indeed, naturally occurring polyploid fish are deemed to have improved potential for environmental tolerance (Mable *et al.,* 2011) and duplicate genes in salmonids have been associated with improved genetic fitness (Wang *et al.,* 2002) as well as the evolution of anadromous transformation (Ohno, 1970; Alexandrou *et al.,* 2013). This is inconsistent with performance observed in artificially induced salmonids indicating naturally evolved polyploids are better able to exploit duplicate genes (Comai, 2000).

The reduction in cell frequency as a consequence of increased cell size compensating for increased nuclear material in polyploid fish (Leggatt & Iwama, 2003) implies gene dosage effects be crudely maintained that would otherwise require compensation where cell frequency is sustained, such as gigantism in plants (Mable *et al.,* 2011). Gene dosage is regulated by epigenetic processes including: changes in DNA methylation, degrees of histone modifications and non-coding RNAs such as micro RNAs (miRNAs; Tollefsbol, 2011). Epigenetic processes are particularly important in embryogenesis where gene expression regulates potential for pluripotent cells to differentiate into a cell-type specific state (Cheng *et al*., 2011) and are known to be impacted by environemental factors such as temperature with long-term developmental implications (Feil & Fraga, 2011). Methylated sequences of DNA are considered transcriptionally inactive as methyl donors (S - Adenosyl Methionine; SAM) bind to repeated cytosine –guanine residues in the DNA sequence through the catalytic action of DNA methyltransferases (DNMT; Cheng *et al.,* 2011). In particular, during embryogenesis large changes in DNA methylation levels occur where the embryonic methylome moves from hypomethylation to complete methylation (Santos *et al*., 2002). Teleosts are collectively known to be stenothermic during embryogenesis (Pörtner & Farrell, 2008) and are relatively prone to polyploidisation in nature compared with other vertebrates (Mable *et al*., 2011). Nonetheless, little research has investigated epigenetic

mechanisms in teleost embryos or polyploids. In addition, none to date have investigated the role of epigenetics in regulatory factors such as temperature in salmonids, let alone the impact of artificially induced triploidy. Albeit, elements of gene dosage compensation in triploids have been verified through: observations of ploidy similarities in mRNA expression levels in chinook salmon (*Oncorhynchus tshawytscha*; Ching *et al.,* 2009) and rainbow trout (Suresh & Sheehan, 1998); DNA methylation levels in diploid and triploid rainbow trout (Covelo-Soto *et al.,* 2015); increased phenotypic variance attributable to additive and maternally heritable effects in brown trout (*Salmo trutta*), rainbow trout and chinook salmon (Blanc *et al.,* 2001; 2005; Johnson *et al.,* 2007). Conversely, Pala *et al.,* (2008) attributed reduced RNA:DNA ratios to gene dosage compensation however, cell frequency was not considered. In addition, Ching *et al.,* (2009) found variations in gene expression levels when chinook salmon were infected with *Vibrio anguilliarium* suggesting a loss of genetic regulatory homeostasis in triploids under immunological stress. Research implies that triploids maintain dosage effects however, the stability of regulation and consistency between tissues remains to be elucidated. In particular, environmental stressors such as chronic higher egg incubation temperatures are known to induce long-term developmental cardiac and vertebral deformities (Fraser *et al.*, 2013a, 2015a). The role of ploidy and environmental stressors such as temperature in

determining phenotypic response through epigenetic processes in Atlantic salmon remain to be elucidated.

4 Impact of triploidy on production traits

4.1 Survival

Historically triploid salmonids have demonstrated relatively poorer survival to their diploid conspecifics (Galbreath & Thorgaard, 1995; McCarthy *et al.,* 1996; Cotter *et al.,* 2002). This has been attributed to a number of factors relating to ploidy-specific inadequate husbandry practices. The predominant impact occurs in the embryonic and larval stages where survival following pressure induction in triploid salmonids has been reported to be lower compared to diploid siblings (Mcgeachy *et al.,* 1995; O'Flynn *et al.*, 1997; Johnson *et al.,* 2004; Cotter *et al.,* 2002; Fjelldal & Hansen, 2010; Fraser *et al.,* 2013a) although survival rates generally improve post eyeing (Withler *et al.,* 1998; Cotter *et al.,* 2002). Taylor *et al.,* (2011) observed comparable survival between ploidy up to first feeding and attributed family-instances of reduced survival to over-ripened ova known to impair viability in diploids (Bromage *et al.,* 1992; Aegerter & Jalabert, 2004). Fish are more stenothermal within the embryonic developmental stages (Pörtner & Farrell, 2008) and Fraser *et al.,* (2013a) attributed reduced survival of triploid and diploid Atlantic salmon embryos and fry to egg incubation temperatures when rearing at 6, 8 and

10 ºC indicating mortality can be reduced with lower rearing temperatures. Myers & Hershberger (1991) show comparable survival between diploid and triploid rainbow trout induced through interploid crossing indicating pressure shocking may account for the increased mortality as opposed to triploidisation *per se*. In vertebrates acute environmental shocks may reduce embryo viability (Rivera & Hansen, 2001) and sensitivity to temperature shocks in salmonid embryos (Rombough, 1997) highlight the importance of environmental shocks within this life stage although the impact of pressure shocking alone on survival has not yet been verified. Despite this, it has been shown through tailoring shock induction treatments that triploidisation success can be maintained whilst improving survival rates (Benfey & Sutterlin, 1984b; Hussain *et al.,* 1991; Johnstone *et al.,* 1992; Teskeredžić *et al.,* 1993). Triploid Atlantic salmon generally have comparable post-larval survival rates to diploids both in FW and SW development (Oppedal *et al.,* 2003; Leclercq *et al.,* 2011; Taylor *et al.,* 2011; Taylor *et al.,* 2012). Increased mortality in triploids compared to diploid siblings at post-larval stages have been attributed to temperature induced low-oxygen saturation levels (Myers & Hershberger, 1991; Ojolick *et al.,* 1995; Hansen *et al.,* 2015), communal ploidy rearing of diploids and triploids (Quillet & Gaignon, 1990; Galbreath *et al.,* 1994; Galbreath & Thorgaard, 1995; Ojolick *et al.,* 1995;O'Flynn *et al.*, 1997;) and inadequate nutrition (Fjelldal *et al.,* 2015). Historically it was perceived that increased mortality following

seawater transfer was due to a failure to smolt (McCarthy *et al.,* 1996; Cotter *et al.,* 2002). However, factors known to compromise triploid Atlantic salmon performance such as combinations of suboptimal temperature and oxygen (Hansen *et al.,* 2015) as well as communal rearing (Taylor *et al.,* 2014) were present and may have compromised survival and post-transfer feeding ability. More recent research show comparable transfer survival rates especially when transferred earlier to diploid siblings (Leclercq *et al.,* 2011; Taylor *et al.,* 2012).

Triploids have been suggested to be immunocompromised, however, literature that has reported increased disease susceptibility and mortality has been associated with simultaneous higher water temperatures (Myers & Hershberger, 1991; Ojolick *et al.,* 1995; Cotter *et al.,* 2002; Ching *et al.,* 2009). The reduced cell: size: frequency ratios of immune cells in triploids (Budiño *et al.,* 2006; Fraser *et al.,* 2012c) are in effect compensated by maintained respiratory burst, complement and neutrophil activity (Yamamoto & Iida 1995; Budiño *et al.,* 2006) as well as immune-related gene expression (Larsen *et al.,* 2013). Nonetheless the implications that triploids may have altered immunocompetence under conditions of stress (Ching *et al.,* 2009) requires further research both *in vitro* and *in vivo* to fully determine the impact of disease challenge on survival, particularly under periods of environmental stress.

4.2 Growth

Diversion of energy from reproductive development into somatic growth has been observed in triploid salmonids in periods of sexual maturation relative to diploids (Bonnet *et al.,* 1999; Schafhauser-Smith & Benfey, 2001). However, pre-sexual maturation developmental stages have more varied growth response of improved (Oppedal *et al.,* 2003; Leclercq *et al.,* 2011; Taylor *et al.,* 2011, 2012; Fjelldal *et al.,* 2015), comparable (Quillet & Gaignon 1990; Galbreath *et al.,* 1994; O'Flynn *et al.*, 1997; Leclercq *et al.,* 2011) and reduced (Friars *et al.,* 2001; Cotter *et al.,* 2002; Taylor *et al.,* 2014) growth relative to diploid siblings. Improved growth in triploid Atlantic salmon are primarily occurs in FW and is sustained within the first year at sea (Jungalwalla 1991; Cotter *et al.,* 2002; Oppedal *et al.,* 2003; Burke *et al.,* 2010; Leclercq *et al.,* 2011; Taylor *et al.,* 2012; Fraser *et al.,* 2012c). Oppedal *et al.,* (2003) is the only study to date that reported sustained accelerated growth rate throughout the production cycle and this may be due to a combination of lower FW rearing temperatures (< 7 °C) and increased feed intake relative to diploid siblings during ongrowing. However, other research reveals little difference or a reduction in feed intake between diploid and triploid salmonids (O'Keefe & Benfey 1999; Carter *et al.,* 1994; Tibbetts *et al.,* 2013).

Studies have shown significant ploidy-family interaction on weight, length, growth rate and condition factors (K) in salmonids (Withler *et al.,* 1998; Bonnet *et al.,*

1999; Oppedal *et al.,* 2003). This may have implications for strain selection as in this case diploid families may not have the same heritability of growth traits as triploids from the same family. However, despite the initial family effects observed in brown and rainbow trout in the 'pan-size' stages, no effect of ploidy-family interaction was seen at harvest (Bonnet *et al.,* 1999). Increased variance in growth traits of maternal origin compared to paternal in triploid rainbow trout (Blanc *et al.,* 2001) and brown trout (Blanc *et al.,* 2005) indicate the importance of dams in strain selection. Conversely, Wagner *et al.,* (2006) observed comparable performance between diploid and triploid rainbow trout across families, however, only three strains were compared. Friars *et al.,* (2001) analysed 20 Atlantic salmon families and showed that superior growth performance in diploids did not translate into triploids, further supporting the importance of family selection in triploid culture. The lack of translation from diploid to triploid traits may be attribute to the increased maternally inherited phenotypic variance in triploids owing to the additional maternal chromosome (Blanc *et al.,* 2001; 2005; Johnson *et al.,* 2007). Conversely, Taylor *et al*., (2013) observed comparable growth within families between ploidy in Atlantic salmon indicating selection of growth traits in diploids may reflect into triploid growth performance.

Triploid salmonids consistently show lower K than their diploid siblings in instances of comparable (O'Flynn *et al.*, 1997; Cotter *et al.,* 2002; Leclercq *et al.,* 2011)

and superior (Fjelldal & Hansen 2010; Taylor *et al.,* 2012) weight gain and may be impacted by smolt regime (Taylor *et al.,* 2012; Fraser *et al.,* 2014). Repeated observations of lower K in triploid salmonids indicate deposition of skeletal components at a greater rate than muscle and the reduced frequency of hypertrophic muscle cells observed in triploid salmon may in part explain this difference (Johnston *et al.,* 1999). This has important implications for commercial adoption of triploid salmon as Fulton's K is strongly correlated with whole body lipid content and fillet yield in diploids (Herbinger & Friars 1991; Leclercq *et al.,* 2010). This is concurrent with Tibbetts *et al.,* (2013) where increased lipid gain and retention was observed in triploid Atlantic salmon with no difference in nitrogen, energy and other growth parameters. Manor (2015 a,b) found greater lipid stores in triploids as well as reduced fatty acid mobilization and β-oxidation alongside increased deposition under periods of sexual maturation. Collectively lipid research suggest triploids may have increased potential to synthesise and retain lipids compared to their diploid conspecifics and this may be reflected in lower Ks.

Some studies have shown communally reared diploid and triploids to perform equally well (O'Keefe & Benfey, 1997; Withler *et al.,* 1998; Fraser *et al.,* 2013b) however, generally triploids reared communally with diploids show a reduced performance including growth (Mcgeachy *et al.*, 1995; McCarthy *et al*., 1996; Taylor *et al*., 2014), survival (McCarthy *et al.*, 1996; O'Flynn *et al.*, 1997) and deformity (O'Flynn

et al., 1997) prevalence that is generally associated with comparably reduced agnostic behavior and resulting feed availability. This has now been associated with the historically poorer performance in the 80's where communal rearing was commonly practiced. Studies that have directly compared communal and isolated rearing of triploid and diploid salmonids have shown improved growth and survival under isolated conditions, but also a decrease in deformity prevalence under communal rearing hypothesized to be related to a lower growth rate (Taylor *et al.,* 2014). Triploids may be less aggressive due to lower circulating levels of sex steroid hormones in triploid females that are known to induce territorial dominance (Piferrer *et al.,* 2009) and when reared separately can be seen to perform equally well by comparison with similar agnostic behavioural levels (Garner *et al.,* 2008; Preston *et al.,* 2014). Differential brain morphology between triploid and diploid Atlantic salmon including a smaller olfactory bulb and a larger cerebellum and telencephalon may explain the reduced aggression and potentially foraging ability and spatial cognition (Fraser *et al.,* 2012b).

Overall, in order for triploidy to be commercially accepted, growth performance must be superior or comparable to diploid counterparts to the point of harvest. Implementation of refined husbandry protocols should incorporate understanding of potential impacts of communal ploidy rearing, family effects as well as potential nutritional and environmental requirements. In particular reduced growth typically

observed in the later marine-phase must be resolved. Collectively, research highlight ploidy differences in K with associated lipid metabolism, feeding and nutritional response, and response to environmental temperature and oxygen. Ultimately, research aiming to improve growth performance in triploids should prioritise nutritional and environmental (temperature and oxygen) requirement studies.

4.3 Sterility and flesh quality

As triploid salmonids possess three chromosome sets they are rendered functionally sterile as the homologous chromosome is unable to synapse correctly during the first meiotic division (reviewed in: Piferrer *et al.,* 2009). Gonad functionality and gametogenesis is determined by gender in triploids as oocyte maturation is preempted by meiosis whereas in spermatogonia it is sequential (Piferrer *et al.,* 2009). Hence, females display the most reduced gonad development with no primary oocyte development and suppressed characteristic sexual development (Lincoln & Scott, 1984; Benfey *et al.,* 1989). Males may still develop sexual characteristics due to circulating androgen (Oppedal *et al.*, 2003) but sperm is aneuploidy, lower in content and largely unviable (Benfey *et al*., 1986). In addition, captive male triploid Atlantic salmon have been shown in instances to stimulate spawning in diploid females and thus industrial adoption of triploids may favour all-female stocks (Fjelldal *et al.,* 2014).

The Tasmanian industry favours the adoption of triploid Atlantic salmon to suppress early sexual maturation which reduces flesh quality and growth. Lower consequential Gonadal Somatic Index (GSI) by 43.0 – 88.2% has been observed in triploids compared to diploid siblings (Johnson *et al.,* 1986; Galbreath & Thorgaard, 1995; Bonnet *et al.,* 1999; Segato *et al.,* 2006). How gonad development may translate into flesh quality attributes around the time of sexual maturation is unclear and results are conflicting. Higher gaping scores and soft texture have been reported (Bjørnevik *et al.,* 2004) converse to other reports of superior cooking texture (Poontawee *et al.,* 2007) in triploid rainbow trout. Increased fat storage is now thought to be associated with softer fillet texture compared to maturing diploids (Aussanasuwannakul *et al.,* 2011). Gutted yields of triploids are found to be 5.3 % higher (Lincoln and Scott, 1984). In addition, K is associated with lipid storage (Herbinger & Friars, 1991) and is generally lower in triploid salmonids at harvest, which may be associated with their known tendency for lipid synthesis and storage compared to diploid siblings (Manor *et al.,* 2014, 2015a,b). However, out-with the sexual maturation period, triploid rainbow trout are reported to have a significantly lower fillet fat content (diploid: 2.9 %, triploid: 3.6) yet higher fillet weight (diploid: 177.0 g, triploid: 211.7 g) alongside fewer and larger muscle cell fibres (Werner *et al.*, 2008). Triploid muscle flesh maybe altered as a consequence of reported greater rates of hypertrophic growth alongside a lower density of satellite cells and

proportional decrease in fiber recruitment (Johnston *et al.,* 1999). Results are also variable for proximate analysis in diploid and triploid Atlantic salmon. Similar proximate analysis levels reported for energy (Oliva-Teles & Kaushik, 1990b), protein (Bjørnevik *et al.,* 2004; Poontawee *et al.,* 2007; Werner *et al.*, 2008), lipid (Sheehan *et al.,* 1999; Bjørnevik *et al.,* 2004), moisture (Bjørnevik *et al.,* 2004; Segato *et al.,* 2006) and ash (Poontawee *et al.,* 2007) in diploid and triploid salmonids. Other research has shown inconsistent reports of higher or lower levels of protein, lipid, ash and moisture with little uniformity (Segato *et al.,* 2006; Poontawee *et al.,* 2007; Werner *et al.*, 2008; Burke *et al.,* 2010). Hence, overall flesh quality attributes in triploid salmonids are seemingly inconsistent, which may in part be attributed to differences between species of salmonids, differences in feeds used between trials as well as the historical lack of standardisation in triploid rearing protocols such nutritional and environmental regimes. However, there is evidence for a degree of improved lipid storage potential within periods where diploids would normally undertake sexual maturation.

4.4 Parr-smolt transformation and seawater transfer

As an anadromous fish Atlantic salmon parr undergo physiological and morphological transformations, particularly in the gills, gut and kidney, that enables them to migrate from FW into SW pelagic environments as smolts (McCormick *et al.,* 1997). Key to the monitoring of this transformation: chloride cells proliferate in the gill lamellae with raised

activity of both freshwater (α1a) and marine (α1b) isoforms of Na^+, K^+-ATPase (NKA) (McCormick *et al.*, 2013, 2009), K factor decreases as a result of caudal elongation in preparation for pelagic swimming (Fjelldal *et al*., 2006; Winans & Nishioka, 1987), and skin colouration becomes silver with a loss of parr marks and darkening of the fin edges (Sigholt *et al.*, 1995). This PST is an energetically costly and physiologically stressful event that is crucial for survival and development during ongrowing. Smolt status is maintained for a period of time and referred to as the "smolt window" before desmoltification occurs (Stefansson *et al.,* 1998; Handeland *et al.,* 2004), which is marked by a reduction in gill Na^+, K^+-ATPase (NKA) activity (McCormick *et al.*, 1997). It is now believed that triploids may have a tendency to smolt earlier to diploid siblings owing to an earlier observation of skin silvering and raised NKA activity in out-of-season smolts leading to transfer 4 weeks earlier than diploids (Leclercq *et al.,* 2011; Taylor *et al.,* 2012;) with some studies showing similar smoltification patterns (Johnson *et al.,* 1986; Boeuf *et al.,* 1994; Cotter *et al.,* 2002; Fraser *et al.,* 2013b; Taylor *et al.,* 2014). However, there are no comprehensive studies characterising PST or desmoltification in triploid Atlantic salmon. Historically, triploids have shown reduced survival following transfer relative to diploids which has been attributed to failed smolt syndrome where fish fail to resume feeding (Galbreath & Thorgaard, 1995; McCarthy *et al.,* 1996). However, both of these studies involved communal rearing on transfer, which means

potential differences in competition for feed resources between ploidy (Fraser *et al.,* 2012b; Preston *et al.,* 2014) was unaccounted for. In addition, failure to smolt has also been reported post-transfer in separately reared triploid Atlantic salmon (Cotter *et al.,* 2002) although water temperature (>19 °C) and oxygen saturation (7 mg / L) levels were beyond that of which is known to be suitable for triploid Atlantic salmon (Hansen *et al.,* 2015).

Known physiological aspects of triploid salmonids that are different to diploid conspecifics may have an impact on smoltification and hypo-osmoregulatory ability. Reduced Ks are associated with PST (Fjelldal *et al.*, 2006; Winans & Nishioka, 1987) as well as lipid content (Myers & Hershberger, 1991) in diploid salmonids. Hence additional energetic consequence may be expected for triploid salmonids which have frequently been observed to have reduced K compared to diploid siblings (O'Flynn *et al.*, 1997; Cotter *et al.*, 2002; Fjelldal & Hansen, 2010; Leclercq *et al.*, 2011; Taylor *et al.*, 2012; Fraser *et al.*, 2013b, 2014). Indeed, triploid salmonids are shown to have altered lipid dynamics in terms of synthesis and storage (Manor *et al.,* 2014; 2015 a,b) however, this is only verified for sexual maturation and PST remains undefined for triploid salmonids. In addition, triploids appear to have a reduced density of longer gill filaments (Leclercq *et al.,* 2011) and fewer pyloric caeca (Peruzzi *et al.,* 2014), and the altered surface area may alter osmoregulatory potential in both organs. Further research is required to define

the onset of PST, the smoltification window and the underlying physiological mechanisms in order for optimised seawater transfer regimes and subsequent long-term performance in commercial production of triploid Atlantic salmon.

4.5 Deformities

One of the main preventing factors of full commercial adoption of triploid salmon in aquaculture is the known higher deformity prevalence of triploids relative to diploids. For instance the percentage of triploid Atlantic salmon individuals with ≥ 1 deformed vertebrae ranges from 30 – 80 % compared to their diploid siblings (8 – 65 %; Fjelldal & Hansen, 2010; Leclercq *et al.,* 2011; Fraser *et al.,* 2013b). Deformities are particularly prevalent in cultured fish where the artificial environment lacks selective pressures present in nature that afflicted fish may not likely survive from (Boglione *et al.,* 2001). Deformities raise economic and welfare concerns to production as they hinder: swimming, respiration and recovery (Lijalad & Powell, 2009; Powell *et al.,* 2009); harvest grading (Michie, 2001); and general public and customer perception. It is therefore of paramount importance that deformities be minimised in cultured triploid Atlantic salmon in order to meet ethical standards and improve industrial feasibility. Typical deformities reported in triploid Atlantic salmon include: skeletal of the jaw and vertebrae, opercula, aplasia of the septum transversum ocular cataract, gill and abdominal adhesion (Table 1.1). The aetiologies of these deformities are multifactorial and similar

Table 1.1. Instances of increased deformity prevalence in triploid Atlantic salmon compared to diploid siblings along with reported aetiology.

Deformity type	Life Stage	Aetiology	Reference
Abdominal adhesion	SW	Vaccination	(Fraser *et al.,* 2014)
Cardiac	FW	Egg incubation temperature	(Fraser *et al.,* 2013a)
Cataracts	Post-smolt	Dietary deficiency	(Wall & Richards, 1992; Taylor *et al.,* 2015) (Leclercq *et al.,* 2011)
Gill	FW		(Sadler *et al.,* 2001)
	SW		(Jungalwalla, 1991)
Opercula	FW		(Sadler *et al.,* 2001)
Jaw	FW	P deficiency	(Fjelldal *et al.,* 2015)
		Environmental / growth	(Sadler *et al.,* 2001)
	Post-smolt	Accelerated growth	(Leclercq *et al.,* 2011)
	SW	Environment / growth	(Sadler *et al.,* 2001)
		P deficiency	(Fjelldal *et al.,* 2015)
Vertebral	Post-smolt	Accelerated growth	(Leclercq *et al.,* 2011)
	FW	P deficiency	(Fjelldal *et al.,* 2015)
		Accelerated growth	(Fjelldal and Hansen, 2010)
		Egg incubation	(Fraser *et al.,* 2015a)
	SW	P deficiency	(Fjelldal *et al.,* 2015)

to those observed in diploids indicating that induced triploidy exacerbates existing causes and rearing regimes may be inappropriate. For instance, in diploids, accelerated growth correlates with higher frequency of deformations (Fjelldal *et al.,* 2010) and similarly accelerated growth rate in triploids has also been found to be associated with higher frequencies of deformities (O'Flynn *et al.*, 1997; Leclercq *et al.,* 2011). Selection for deformity traits has not been explored as a potential tool to mitigate deformities in triploids and research indicate selection for other triploid performance traits is not synonymous to diploid siblings (Blanc *et al.,* 2001; Friars *et al.,* 2001; Blanc *et al.,* 2005). Gjerde *et al.,* (2005) found an additive genetic component in diploid Atlantic salmon on the prevalence of deformities in domesticated populations. Comparable deformity prevalence between ploidy in some instances (Taylor *et al.,* 2011, 2012) highlight the

potential for equivalent performance to diploids with improved knowledge and understanding. However, these studies were not followed throughout the ongrowing period until harvest where further deformities may evolve and, as end performance could not be verified, findings may not be commercially representative. In order to minimise deformities in triploids, culture conditions must be refined including definition of dietary and environmental (mainly temperature and oxygen) requirements on the basis of understanding the underlying mechanisms for ploidy differences (Benfey, 2015).

4.5.1 Skeletal deformities

Vertebral and jaw deformities are of particular welfare concern in fish and salmonid production (Noble *et al.,* 2012) and are the most commonly reported deformities in Atlantic salmon. Skeletal deformities are known to impede swimming performance in triploid Atlantic salmon (Lijalad & Powell, 2009; Powell *et al*., 2009), which may impact other performance aspects. In diploid Atlantic salmon swimming performance is known to influence: growth rate and ability to access feed (Herbert *et al.,* 2011), vertebral strength (Totland *et al.,* 2011) and disease resistance (Castro & Grisdale-Helland, 2011). Hansen *et al.,* (2010) found Atlantic salmon with higher numbers of deformed vertebrae (>10 deformed vertebrae) exhibited lower growth rates in SW, which suggests low occurrence of verterbral malformations at post-smolt have little impact on long-term growth performance. Skeletal deformities in triploids have been shown to occur primarily

as a consequence of higher egg incubation temperatures of ≥8 °C (Fraser *et al.,* 2015a), accelerated growth (Leclercq *et al.,* 2011; Taylor *et al.,* 2012*,* 2014) and dietary deficiency (Fjelldal *et al.,* 2015). Similar factors are observed in diploid conspecifics (Fjelldal *et al.,* 2009b; Fjelldal & Hansen, 2010; Ytteborg *et al.,* 2010a), however, ploidy specific differences in gene regulation, accelerated growth and gut morphology may exacerbate known aetiologies. Where triploids may be exposed to environmental stress a loss of gene regulatory homeostasis may occur (Ching *et al.,* 2009), which may be influenced by epigenetic factors, and impact processes such as skeletal remodeling that lead to deformity development. This may in part explain the particular susceptibility of triploid Atlantic salmon to high egg incubation temperature – induced malformations (Fraser *et al*., 2013a, 2015a) where during embryogenesis teleosts are more stenothermic (Pörtner & Farrell, 2008) and the DNA methylome is reorganised (Jiang *et al*., 2013b). However, no literature to date has addressed this concept, hence, the collective role of epigenetic processes and temperature on deformity development in triploid Atlantic salmon remains to be elucidated. In addition accelerated growth in combination with inadequate nutrition may increase malformation development (Fjelldal *et al*., 2009b). Overall, further research on deeper mechanisms of the aetiology of deformities in relation to these growth and environmental factors is required to improve welfare standards, facilitate performance and ultimately enable commercialisation.

4.5.1.1 Bone mineralisation and skeletal development in fish

Skeletal development differs greatly in teleosts compared to other terrestrial and avian vertebrates in that as aquatic animals, they are not weight bearing and the skeleton is pivotal for locomotion (Grotmol *et al.,* 2005). The fish skeleton or sclerotome is formed early in embryonic ontogeny through outward segmental mineralisation of the notochordal sheath from the central cranio-caudal axis (Grotmol *et al.,* 2003). Eventually, four layers mineralise: the chordacentrum, a thin layer of collagen fibers, the autocentrum and the arcocentrum (Nordvik *et al.,* 2005; Fig. 1.3). The resulting amphicoelus vertebrae are open structures which enable outward growth with minimal remodeling. Bone remodeling depends on the presence of osteoblast (bone mineralising), osteoclast (multinucleated bone resorbing) and osteocyte (entrapped within bone structure) cells to mineralise and reshape bone (Peavy, 2003). The bone structure is comprised primarily of collagen and hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] which acts as a reservoir for Calcium (Ca) and P that requires constant remodelling (Lall & Lewis-McCrea, 2007). As a phylogenetically more primitive species, Atlantic salmon is known to have acellular bones (Nordvik *et al.,* 2005; Krossøy *et al.,* 2009) as opposed to other teleosts that may have cellular bone, which lack osteocytes (Lall, 2003).

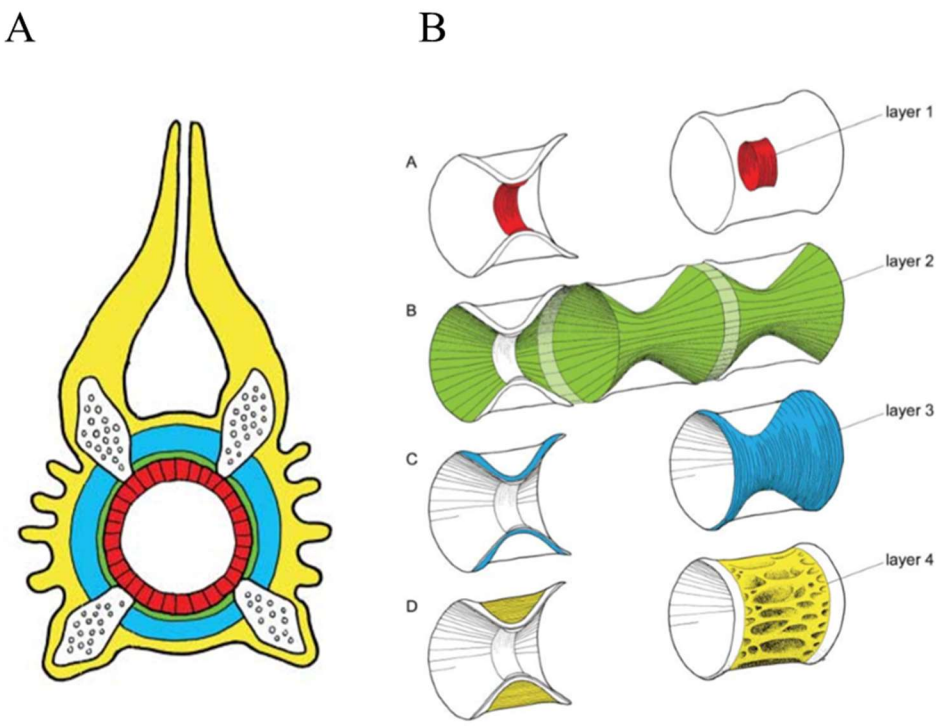

Figure 1.3. The four layers that mineralise in the salmon vertebral column: the chordacentrum, a thin layer of collagen fibers, the autocentrum and the arcocentrum from a cross section of the amphicoelus vertebrae (A) and the individuals layers from a cranio-caudal lateral perspective (B). Images taken from (Nordvik *et al.,* 2005).

4.5.1.2 Vertebral pathology

As salmonids display continuous vertebral growth (Witten & Huysseune, 2009) localisation and characterisation of vertebral deformity development in diploid Atlantic salmon varies depending on ontogeny (Fjelldal *et al.,* 2006*,* 2007a, 2009 a, b; Sullivan *et al.,* 2007a). Witten *et al.,* (2009) classified vertebral deformities observable by x-radiography into twenty pathology types that were broadly defined by five categories: platyspondyly (compressive) or ankylosis (fusion) factors; radio-translucent or radio-

opaque indicative of hyper and hypodense vertebrae; symmetry deviations and displacement of vertebral bodies; and severe multiple deformities (Fig. 1.4). For the purpose of spatial and ontogenetic assessment the Atlantic salmon vertebral column, typically comprised of 58 – 60 vertebrae, is divided into four regions according to morphometry where: the cranial region (R1: v1-8 & R2: v9-30) is devoid of haemal spines; the caudal region (R3: v31-50 & R4: v51-60) has two connecting haemal arches; and the end regions are subject to stark morphological variability (R1 & R4; Kacem *et al.,* 1998; Figure 1.4). Increased deformities in the trunk region (v9-30) in triploid Atlantic salmon (Fjelldal & Hansen 2010; Fjelldal *et al.,* 2015) are associated with FW development as observed in diploids (Fjelldal, *et al.,* 2007b, 2009a; Sullivan,*et al.,* 2007b) in particular the very central vertebrae. The area immediately beneath the dorsal fin is also the cranio-caudal axis for vertebral formation and mineralisation where the chordacentrum is initially formed (Grotmol *et al.,* 2003), and may in part explain the higher prevalence of deformities seen in earlier developmental stages. SW deformities of the spinal column are largely found in the caudal region in triploid Atlantic salmon (Leclercq *et al.,* 2011; Fjelldal *et al.,* 2015) again similar to those observed in diploid conspecifics (Fjelldal *et al.,* 2009b). This area may be more affected by pelagic swimming and increased lateral muscular activity inducing mechanical strain (Fjelldal *et al.,* 2009b; Totland *et al.,* 2011) as well as vaccination induced inflammation (Berg *et al.,*

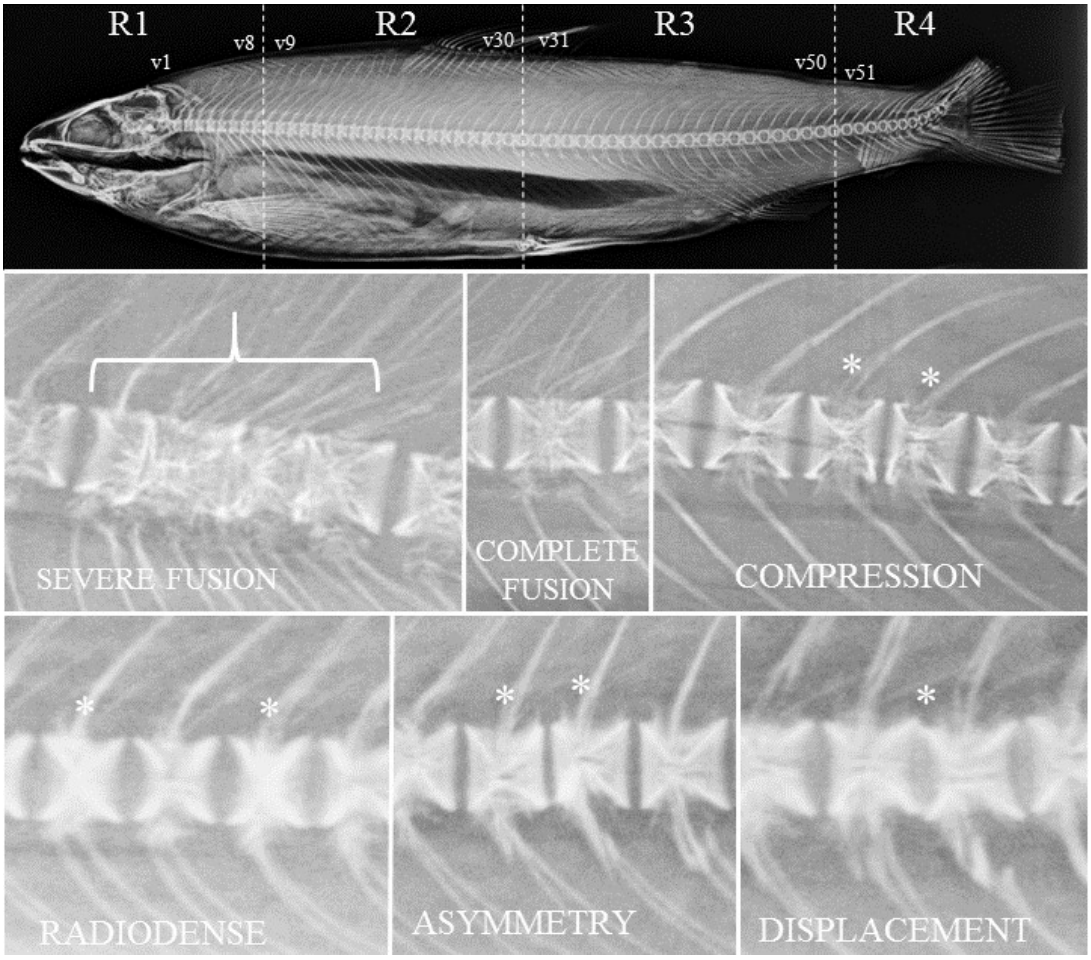

Figure 1.4. Visual summary of regional definition of the vertebral column in salmonids according to (Kacem *et al.,* 1998) denoting the vertebral number (A) and examples of typical deformities observed in salmonids inclusive of compression and fusion, radiodense, asymmetrical and severe pathology as indicated by an asterisk (Witten *et al.,* 2009).

2006; Gil Martens *et al.,* 2012). Triploids are also predisposed to higher levels of vaccine-induced melanin deposits and abdominal adhesion scores (Larsen *et al.,* 2014; Fraser *et al.,* 2014) which may be exacerbated by their known reduced tolerance to higher water temperature (Atkins & Benfey, 2008) which is typical at the time of vaccination. It was recently identified in triploids and diploids that vertebral deformities observed in SW may largely be of FW origin (Hansen *et al.,* 2010; Fjelldal *et al.,* 2015). Thus, optimal

skeletal development in FW is crucial for later somatic and skeletal growth where deformity factors such as increased temperature and accelerated growth are compounded.

Suboptimal egg incubation temperatures (≥ 8 ºC) are primarily known to reduce performance and induce developmental defects later in life in triploid Atlantic salmon (Fraser *et al.,* 2013a, 2015a). In diploids, egg incubation temperature may influence axial patterning and subsequent skeletal development (Wargelius *et al.*, 2005b), as well as muscle fiber growth (Macqueen *et al.,* 2007) and 8 ºC is deemed to be optimal for vertebral development conversely to triploids (Fraser *et al*., 2013a, 2015a). Occurrence of vertebral deformities may also be largely attributed to accelerated growth (Fjelldal & Hansen, 2010; Leclercq *et al.,* 2011) alongside nutritional deficiency (Fjelldal *et al*., 2015) which has also been observed in diploid Atlantic salmon (Fjelldal *et al.,* 2012a). Reduced jaw and vertebral deformity development between diploid and triploid seabass (*Dicentrarchus labrax*) has been observed following periods of slower growth (Felip, 2001; Peruzzi *et al.,* 2004). However, triploid Atlantic salmon have altered temperature requirements (Atkins & Benfey, 2008) and the use of higher temperatures in accelerated smolt regimes had been implicated to impede growth and subsequently lead to reduced deformity prevalence compared to yearling (1+) smolt (Fraser *et al.,* 2014). In diploid Atlantic salmon accelerated growth in hyperthermic hatchery conditions has been shown to disrupt vertebral tissue formation and induce malformations (Ytteborg *et al.*, 2010a)

and out of season smolt production has increased skeletal malformation prevalence after SW ongrowing in another study, although temperature profiles were similar post-transfer between S1+ and S0+ populations (Fjelldal *et al*., 2009b).

4.5.1.3 Jaw pathology

One of the predominant deformities found in triploids are lower jaw curvatures (LJC) otherwise known as 'screamer's' disease (Jungalwalla, 1991; Sadler *et al.,* 2001; Leclercq *et al.,* 2011; Fjelldal *et al.,* 2015) which reduces the ability of triploid Atlantic salmon to cope with exhaustive swimming (Lijalad & Powell, 2009) and may compromise ability to feed efficiently. Observations of jaw deformities in diploid Atlantic salmon has been associated with decreased weight gain and increased mortality (Venegas *et al.,* 2003). Recent research in diploid and triploid Atlantic salmon confirmed that low dietary P inclusion increased prevalence of LJCs relative to diploid siblings both in FW and SW (Fjelldal *et al.,* 2015). Analysis of husbandry practice against outbreaks of LJC in diploid Chilean Atlantic salmon farms not only identified risk factors as diets deficient in P and vitamin C but also smolting in higher water temperatures (>20 °C; Roberts *et al.,* 2001). LJC may also arise as a consequence of excessive buccal-opercular pumping under respiratory strain where triploid salmonids are known to have reduced performance and altered respiratory behaviour within an environment of higher

temperature and reduced oxygen saturation (Hansen *et al.,* 2015). This may in part explain the increased susceptibility in triploids (reviewed in Fraser *et al.,* 2012a).

4.5.2 Cardiac performance

In diploids, heart deformities including irregular shape and aplasia of the septum transversum, have been linked with increased mortality during stressful handling procedures (Brocklebank & Raverty, 2002) and reduced disease tolerance to salmonid alphavirus in Atlantic salmon (Rodger & Mitchell, 2011). Occurrence of aplasia of the septum transversum in triploids is strongly associated with high egg incubation temperatures but not in diploids (Fraser *et al.,* 2013a), although incidents of ventricular hypoplasia incidents have also been associated with high egg incubation temperatures in diploids (Poppe & Taksdal, 2000). Triploids have larger hearts with a reduced angle of the bulbous arteriosus compared to diploid siblings more akin to wild conspecifics (Leclercq *et al.,* 2011; Fraser, *et al.,* 2015b) and higher routine metabolic rates which result in an increased cardiac workload through respiratory demand (Atkins & Benfey, 2008). Hence, increased cardiac workload and reduced circulatory resistance associated with increased metabolic rates (Poppe *et al.,* 2003; Atkins & Benfey 2008) are suggested as a primary cause of heart malformations in triploids.

4.5.3 Cataracts

Ocular cataracts has been reported at higher incidence in triploid Atlantic salmon relative to diploid conspecifics (Wall & Richards, 1992; Oppedal *et al.,* 2003; Leclercq *et al.,* 2011) and has not been reported for any other cultured triploid fish species. One of the main causes of cataract is thought to be oxidative damage to the lens, which may be induced through environmental, nutritional or disease-related factors and subsequently alter lens permeability causing it to swell or shrink (Williams, 2006). Cataract development in diploid Atlantic salmon has been primarily induced through faster growth associated with high water temperature (Bjerkas *et al.,* 2001), and nutritional deficiencies associated with these growth and environmental stressors (Waagbø *et al.,* 2008; Trösse *et al.,* 2009; Waagbø *et al.,* 2010). Removal of the now banned blood meal component in animal feed, which is rich in Histidine (His), results in higher prevalence of cataracts especially given the rapid growth of farmed Atlantic salmon in modern regimes (Breck *et al.,* 2003). As triploids have shown accelerated FW and initial post transfer growth alongside increased nutritional requirements in His (Taylor *et al*., 2015) and P (Fjelldal *et al.,* 2015) the increased cataracts susceptibility is unsurprising. Supplementation of dietary His has been shown to prevent cataract formation in triploid Atlantic salmon post-smolts when fed at higher levels to conventional diploid diets (Taylor *et al.,* 2015).

Alongside other instances of deformity prevalence, this suggests that triploids may generally have different nutritional requirements to their diploid conspecifics.

5 Welfare Considerations and husbandry requirements

5.1 Environmental Temperature and Oxygen

Triploid fish have historically had increased mortality when reared under higher temperatures (Quillet & Gaignon, 1990; Myers & Hershberger, 1991; Ojolick *et al.,* 1995) which in light of recent research has been attributed to reduced oxygen delivery under higher temperatures (Hansen *et al.,* 2015). Anttila *et al*., (2013) has shown that critical thermal maxima is positively correlated with hypoxia tolerance in diploid Atlantic salmon families (n = 41) which supports oxygen and capacity limited thermal tolerance. Triploid salmonids are now known to have comparable critical and chronic thermal maxima to diploid siblings assessed through a loss of equilibrium (Benfey *et al.,* 1997; Galbreath *et al.,* 2006). It is thought that reduced triploid performance under higher temperatures may be due to a lower haemoglobin – oxygen loading ratio and blood oxygen content (Bernier *et al.,* 2004) owing to the alterations in erythrocyte frequency and larger cell size observed in triploids (Peruzzi *et al.,* 2005). This could be exacerbated in warmer temperatures where higher routine metabolic rates are observed in triploid salmonids (Atkins & Benfey, 2008). Cardiac acclimation according to environmental

temperatures in diploid Atlantic salmon (Anttila *et al*., 2014) and known altered heart morphology in triploids (Leclercq *et al*., 2011; Fraser *et al*., 2015b) suggest triploid Atlantic salmon may have acclimated cardiac structure according to their different thermal and oxygen hypoxic tolerance. Despite this, heart rate Q_{10} is comparable between diploid and triploid brook charr (*Salvelinus fontinalis*) embryos and larvae indicating cardiac performance may not adjust to altered cellular respiratory demand and may not be impacted by ploidy (Benfey & Bennett, 2009). The reduced frequency of erythrocytes is offset by a higher cellular volume and haemoglobin content per cell. This reflects the lack of difference observed in total haemoglobin, haematocrit or mean corpuscular haemoglobin concentration between diploid and triploid salmonids (Benfey & Sutterlin 1984a; Sadler *et al.,* 2000; Bernier *et al.,* 2004; Taylor *et al.,* 2007). Hence, the majority of elements of oxygen delivery are deemed to be similar in diploid and triploid salmonids (Fraser *et al.,* 2012a) including resting oxygen consumption rate (Oliva-Teles & Kaushik, 1990a; Hyndman *et al.,* 2003 a, b; Bernier *et al.,* 2004). However, triploid rainbow trout suffered higher mortality with stress confinement attributed to lower oxygen levels indicated by marked increases in cellular hepatic glutathione suggestive of a compromised antioxidant system (Leggatt *et al.,* 2006). This indicates that anaerobic pathways and the ability to clear cells of metabolic waste may be compromised in triploids relative to diploid conspecifics.

However, lower post-exercise oxygen consumption rates and increased excretion of ammonia following exercise (Hyndman *et al.,* 2003a,b) suggests triploid salmonids may have a higher ability to clear metabolic process and may be able to recover faster from anaerobic exercise. Brook charr show no ploidy difference in time taken to fatigue during increasing water currents indicating that triploids have a similar aerobic capacity to diploids (Stillwell & Benfey, 1997). Another study found triploids were more likely to experience fatigue at higher swimming speeds reflected by lower arterial oxygen content (Bernier *et al.,* 2004). Aerobic activity is impeded at higher temperatures where increased lactate and sustained phosphocreatine levels imply increased recovery time post-exercise (Hyndman *et al.,* 2003b). When triploid Atlantic salmon were reared at a combination of high temperatures and low oxygen they were seen to display increased ram ventilation behaviour compared to diploids reflective of reduced oxygen delivery under metabolic strain (Hansen *et al.,* 2015). Triploid Atlantic salmon performance traits are clearly impacted by environmental factors of temperature and oxygen. Investigations have largely focused on impacts of temperature on metabolic and cardiac performance. Recent literature has also associated high temperatures during egg incubation (Fraser *et al*., 2013a, 2015a,) and FW (Fraser *et al*., 2014) with deformity development. In order to improve performance traits in triploid Atlantic salmon, the role of temperature in

phenotypic response and potential underlying mechanisms must be investigated, for instance gene regulation pathways and epigenetics in early development stages.

5.2 Nutrition

Fast growing species such as salmonids require high energy marine lipid and protein diets to realise growth potential, which may be greater in triploids. Triploid Atlantic salmon have: fewer pyloric caeca, a shorter relative gut length and compensatory mucosa cells (Peruzzi *et al.,* 2014). Other studies in rainbow trout and brown trout found reduced growth performance in triploids alongside fewer pyloric caeca (Blanc *et al.,* 2001, 2005) that indicate compromised nutrient assimilation that hinder growth. Reduced protein retention in triploid shi drum (*Umbrina cirrosa*; Segato *et al.,* 2006) and fractional protein synthesis rates in triploid rainbow trout (Fauconneau *et al.,* 1990) would indeed support greater nutritional requirement to diploid conspecifics. Increased feed intake in triploid salmonids compared to diploid siblings may be associated with periods of increased growth rates (Oppedal *et al.,* 2003; Burke *et al.,* 2010). In addition, the ability to access feed may be reduced in triploid salmonids through reduced pellet retrieval success (O'Keefe & Benfey, 1997) and anecdotal evidence of delayed dispersal upon first feeding (Mcgeachy *et al.,* 1995). Reduced feeding as a consequence of impaired foraging ability is supported by results of Fraser *et al.,* (2012b) where triploids have a reduced olfactory bulb and brain sensory organs associated with foraging ability. Conversely, other studies

show similar nutritional processing capabilities between ploidy including: protein efficiency (Oliva-Teles & Kaushik, 1990a; Pechsiri & Yakupitiyage, 2005), standardised energy balances (metabolism, egestion and excretion pathways; Wiley & Wike, 1986), apparent digestibility coefficients (Oliva-Teles & Kaushik, 1990a; Burke *et al.,* 2010), feed utilisation and conversion (Oliva-Teles & Kaushik 1990a; Sajjadi & Carter, 2004) and feed intake (O'Keefe & Benfey, 1999; Carter *et al.,* 1994; Tibbetts *et al.,* 2013). Diversion of reproductive energy into somatic growth is reflected in reduced gonads alongside an increase in net protein utilisation and percentage gutted weight in triploid Nile tilapia (*Oreochromis niloticus*; Pechsiri & Yakupitiyage, 2005). In addition, triploid rainbow trout have reduced protein catabolism and increased anabolism relative to maturing diploids attributable to a combination of reduced autophagy, gene dosage of growth recovery genes as well as different feeding strategies (Cleveland & Weber, 2013, 2014), although differences are likely to be an effect of sexual maturation rather than just ploidy. Triploids also tend to have higher levels of fat deposition particularly in females (Lincoln & Scott, 1984; Henken *et al.,* 1987; Peruzzi *et al.,* 2004; Segato *et al.,* 2006) and differential fatty acid turnover rates associated with storage and synthesis in muscle, viscera and liver are associated with reduced gonadal growth in rainbow trout (Manor *et al.,* 2015a). Overall triploids may have potential for greater protein and lipid storage to their diploid counterparts and may benefit from improved nutritional supplementation.

Of particular interest is the role of nutrition in mitigating deformity development in triploid Atlantic salmon. Research has identified triploid Atlantic salmon to have higher dietary His (Taylor *et al.,* 2015) and P (Fjelldal *et al.,* 2015) requirements to support correct ocular and skeletal development compared to their diploids siblings that may have implications for other nutritional needs. Further investigation is required into the role of nutrition in skeletal development and known nutritional risk factors in diploids include dietary P (Baeverfjord & Shearer, 1998; Fjelldal *et al.,* 2012a), various vitamins (Lall & Lewis-McCrea, 2007; Lewis-McCrea & Lall 2010), lipids and protein sources (Fjelldal *et al.,* 2010), and probiotics (Aubin *et al.,* 2005).

5.2.1 Phosphorous

As bone mineral is formed primarily from hydroxyapatite, of which P and Ca are the primary constituents, it is obvious to see why P may be an important nutritional risk factor for skeletal development (Lall & Lewis-McCrea, 2007). P is an essential mineral for other processes such as components of RNA, DNA, ATP and cell membrane phospholipids (Peavy, 2003) and is available in the plasma circulating as an inorganic orthophosphate PO_4^{3-}. Ca is readily available from the aquatic environment both in FW and SW whereas P requirements must be met by the diet in marine and FW teleosts (Lall, 2003). PO_4^{3-} and calcium Ca^{2+} homeostasis is systemically regulated through circulating parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25(OH)2D] and fibroblast growth factor

23 (FGF23; Martin *et al.,* 2012; Figure 1.5). These endocrine factors coordinate uptake and absorption in the gut, retention in the kidney and remodelling in bone according to demand and availability. External elevation of PO_4^{3-} around osteoblasts increase osteogenesis through increased expression of alkaline phosphatase (ALP) and osteopontin (OPN; Beck *et al.,* 2000). In turn, hyperphosphatemic conditions increases FGF23 production by osteogenic cells: osteoblasts and osteocytes, which inhibit renal PO_4^{3-} reabsorption by suppressing circulating [1,25(OH)2D] (Takeda *et al.,* 2004; Martin *et al.,* 2012) and reduce kidney sodium-phosphate cotransporter (Npt2a) activity (Sugiura *et al.,* 2003).

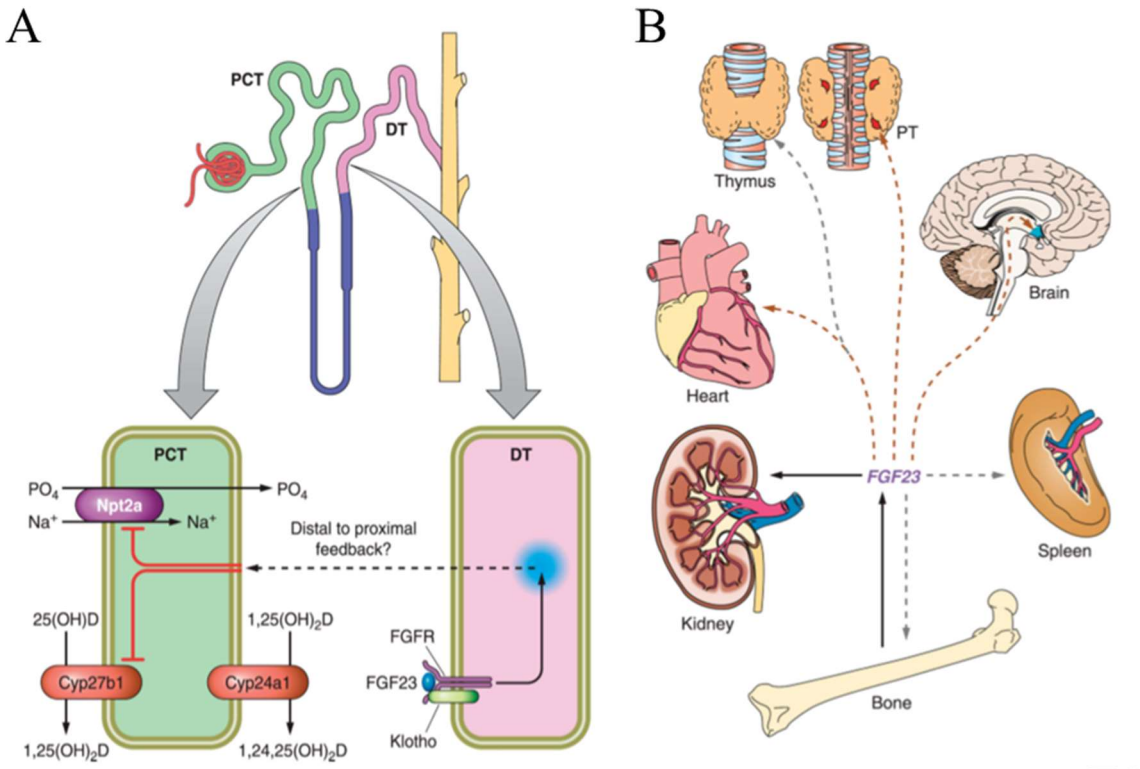

Figure 1.5. Schematic taken from (Martin *et al.,* 2012) depicting the renal (A) and extrarenal (B) regulation of P in mammals. FGF23 activates FGFR/Klotho complexes in the renal distal tubules (DT) which inhibit Npt2a and Cyp27b1 which reduces reabsorption of phosphorous and production of 1,25(OH)2D (A). Extrarenal targets are depicted in B.

According to the National Research Council (NRC, 2011) the minimum requirement for dietary P in salmonids is 8 g total P kg^{-1}. However, other research indicate that Atlantic salmon require at least 10 g available P kg^{-1} (Asgard & Shearer, 1997) and may be greater in periods of faster growth (Helland *et al.,* 2005) which is a point of interest given the higher growth potential of triploid Atlantic salmon. Feeding Atlantic salmon below levels of 10 g available P kg^{-1} can result in long term effects of poor spinal development (Baeverfjord *et al*., 1998), reduced bone strength (Fjelldal *et al*., 2009b), increased skeletal deformities (Fjelldal *et al.*, 2009b, 2012a) and a subsequent reduction in whole body Ca and P levels (Baeverfjord *et al.*, 1998). Radiography reveals that lower levels of P result in radiodense vertebrae indicative of cartilage formation and incomplete mineralisation (Helland *et al.,* 2006). Conversely an excess of dietary P may also lead to decreased survival in rainbow trout fry (Fontagné *et al.,* 2009) which may be induced through a reduction in net availability of other essential nutrients and altered gut integrity (Boersma & Elser, 2006). P digestibility and availability is impacted by the source material and is generally administered in feed in the form of animal by-products, plant by-products, and organic and inorganic PO_4^{3-} supplements (Hua & Bureau, 2006). Bone P is acquired from the mineral hydroxyapatite and is fairly digestible whereas phytate from plant sources is poorly digested by salmonids. Organic PO_4^{3-} is considered to be highly digestible whereas inorganic PO_4^{3-} digestibility is dependent on solubility. In

addition, the collective digestibility within feed is not additive and is dependent on interacting components for instance bone-P becomes less digestible in the presence of higher levels of Ca monobasic / Na / K inorganic phosphate supplements.

Feeding higher dietary P to triploid Atlantic is associated with increased skeletal deformity prevalence of the spine and jaw both in SW and FW (Fjelldal *et al.,* 2015). Furthermore, increased dietary P in triploids both improved growth when fed throughout FW (Fjelldal *et al.,* 2015) and reduced growth when fed within the later parr- smolt stages. (45 – 75 g; Burke *et al.,* 2010). Subsequently, when high P was fed throughout both in FW, impeded end growth performance in the marine phase was observed (Fjelldal *et al.,* 2015). This indicates increased dietary P may not necessarily be required throughout development in triploids. For instance, lower levels of plasma P are observed in triploid parr (< 45 g) indicative of a reduced intake or increased requirement for active processes which was not observed in later stages (> 75 g; Burke *et al.,* 2010). As a final consideration with respect to increasing dietary P supplementation, there is additional interest to minimise the use of higher dietary P in aquafeeds as higher inclusion can increase leaching into the water column through the faeces (Phillips *et al.,* 1993). Anthropogenic P input is a primary contributor to aquatic eutrophication in FW bodies (Conley *et al.,* 2009) and salmon farming is a contributor (Folke *et al.,* 1994). In particular, Scottish Environment Protection Agency regulations state that anthropogenic

P input should not alter the trophic and subsequent ecological status of a FW loch (SEPA, 2014). Definition of triploid specific aquafeeds will therefore require careful consideration and tailoring to suit both economic and environmental requirements.

5.2.2 Probiotics

Dietary probiotics may be used to improve production traits such as growth (Lamari *et al*., 2013; Merrifield *et al*., 2011), survival (Ferguson *et al*., 2010), immune function (Ferguson *et al*., 2010) and skeletal development (Lamari *et al.*, 2013) in commercially cultured teleosts. The use of probiotics such as *Lactobacillus rhamnosus* and *Pediococcus acidilacticit* have been shown to reduce vertebral compressions in rainbow trout (Merrifield *et al.,* 2011), improve skeletal conformation in European seabass (*Dicentrarchus labrax*; Lamari *et al.,* 2013) and increase bone deposition alongside upregulation of osteogenic genes in zebrafish (*Danio rario*; Maradonna *et al.,* 2013). The mechanisms are largely unknown but may be through: a reduction in pro-inflammatories (Picchietti *et al.,* 2009), bacteriostatic effects of pathogenic bacteria (Aubin *et al.,* 2005) and improved mineral solubility through increased gut acidity (Scholz-Ahrens *et al.,* 2007). The use of probiotics is of particular interest to triploid Atlantic salmon owing to differences in gut morphology (Peruzzi *et al.,* 2014) and increased species-specific gut microbial concentrations that may alter susceptibility to probiotics (Cantas *et al.,* 2011). In the case that assimilation of essential nutrients such as P can be improved with

inclusion of probiotics, then overall industrial and environmental costs may be reduced compared to supplemented feed.

6 Aims of the thesis

The artificial induction of triploidy is currently considered the only feasible means of inducing sterility in salmonids, in particular Atlantic salmon, for which there are potential economic and environmental benefits. With the expanding industry and the known threat of sea lice on production success and wild salmonids, it is important to reduce time spent at sea for ongrowing and mitigate escapee impact for which triploids may potentially be used. Owing to their differing physiology to diploid conspecifics, it is understood that triploid salmonids should be treated as a separate species in order to aid refinement of rearing regimes essential for optimal performance (Benfey, 1999, 2015). Research has identified suboptimal regimes that have historically reduced performance and survival primarily through the combined effect of low temperature and oxygen, communal ploidy rearing, nutritional deficiencies and suboptimal sea transfer protocols. These areas can be successfully addressed as demonstrated by the effects of segregation of diploid and triploid stocks and the use of dietary His to arrest cataract formation. Hence, successful implementation of triploidy in aquaculture depends on the mitigation of historical issues in production traits outlined in this review including the increased levels of deformities,

variable growth performance and definition of unknown paradigms such as nutrition, smoltification, and gene regulation.

The specific objectives and hypotheses tested of the thesis were:

1. To investigate the impact of protein and P supplementation on diploid and triploid performance in terms of: growth, final-harvest quality attributes and skeletal performance during the SW rearing period. The hypothesis tested here was that feeding triploids a diet with increased dietary protein and P would sustain faster growth and mitigate vertebral deformities by harvest compared to diploids and triploids reared on a standard commercial diet. (**Chapter 2**).
2. To examine the impact of dietary P supplementation throughout FW rearing on growth performance and skeletal development in diploid and triploid Atlantic salmon. The hypothesis tested here was increasing dietary P in triploids would benefit growth and skeletal development where the same benefits may not necessarily be seen for diploids. More specifically, vertebral development and whole body mineral content would be assessed at the fry, parr and smolt stages to assess differences in ontogenetic requirements (**Chapter 3**).
3. To explore the potential to reduce higher P supplementation in triploid Atlantic salmon through feeding for shorter windows or improving assimilation through the use of probiotics. The hypothesis tested here was that the same benefits observed from higher

P supplementation could be achieved through feeding for shorter periods associated with a higher risk to deformity development. Or alternatively, feeding probiotics may enhance P or other nutrient uptake beneficial to growth and skeletal development (**Chapter 4**).

4. To define the smoltification window in triploid Atlantic salmon reared under natural photo-thermal regime compared to diploid siblings. The hypothesis tested here was that triploids would smolt and desmolt earlier relative to their diploid siblings (**Chapter 5**).
5. To investigate the impact of temperature and ploidy on ontogeny of DNA regulation (DNAme) and endogenous energy reserves (Nitrogenous Metabolites (NMs) & Free Amino Acids (FAAs)). The hypothesis tested here was that triploids would demonstrate altered DNAme reflective of the altered genome as well as associated NM and FAA concentrations. This may be exacerbated by temperature (**Chapter 6**).

Chapter 2: Dietary phosphorous and protein supplementation enhances seawater growth and reduces severity of vertebral malformation in triploid Atlantic salmon (*Salmo salar* L.)

Chapter is edited based on published work:

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Abstract

Diploid (2N) and triploid (3N) sibling post-smolts were divided between six sea pens and fed: a standard commercial nutrient package diet (2 x 2N SP, 2 x 3N SP), or an iso-energetic nutrient boosted (higher dietary protein and P) package (2 x 3N BP) until market size. 3N groups initially grew significantly faster than 2N, and by harvest, 3N BP weighed significantly more (3210 ± 87g) than 2N SP and 3N SP (3007± 64g; 2965 ± 88g), while there was no significant difference in weight between ploidy in SP diet. Higher visible vertebral (9.6 ± 0.4%) and jaw deformities (10.6 ± 1.2%) were observed in 3N compared to 2N (0.9 ± 0.1%; 1.3 ± 0.5%). However, x-ray radiography revealed that 3N BP and 2N SP had comparable levels of severely affected (≥ 10 deformed vertebrae) individuals compared with 3N and 2N at point of sea transfer respectively. Whereas 3N SP showed a 3 fold increase in the severity of deformed individuals since point of sea transfer. The caudal region (R3-4) in 3N SP fish had both the lowest vertebral strength and stiffness (R4), and the highest number of deformed vertebrae. Fillet quality attributes were comparable between diet and ploidy. These findings show that by increasing dietary P and protein triploid growth rate can be sustained in the marine phase until harvest and more importantly, the progression of spinal deformity beyond that at sea transfer can be stabilised.

1 Introduction

Commercial adoption of triploid Atlantic salmon (*Salmo salar*) is being considered in Europe to remove the risk of interbreeding between escapees and wild populations, which is shown to reduce genetic fitness (McGinnity *et al.,* 2003). In addition, triploids are shown in some instances to have faster growth compared to diploids (Taylor *et al.,* 2012; Fraser *et al.,* 2013b). However, although growth in freshwater is generally superior than diploids (Fjelldal & Hansen, 2010; Taylor *et al.,* 2012), it is the decelerated growth in the later marine phase (Fraser *et al.,* 2013b; Taylor *et al.,* 2013) and increase in skeletal deformity (Fjelldal & Hansen, 2010; Leclercq *et al.,* 2011; Taylor *et al.,* 2011) and cataract (Taylor *et al.,* 2015) that have hindered full scale uptake. These traits reduce harvest weight (Hansen *et al.,* 2010), increase downgrading in production (Michie, 2001), and raise welfare concerns (Noble *et al.*, 2012). Aetiologies of skeletal deformities in diploid Atlantic salmon are well documented and include high egg incubation temperatures (Wargelius *et al.,* 2005), genetic factors (Gjerde *et al.,* 2005), vaccination (Berg *et al.,* 2006), S0+ smolt regimes (Fjelldal *et al.,* 2006) and nutritional deficiencies (Lall & Lewis-McCrea, 2007) in particular dietary P (Baeverfjord *et al*., 1998; Fjelldal *et al.,* 2009*,* 2012a). Aetiology of skeletal deformities in triploids have primarily been attributed to high egg incubation temperature (Fraser *et al.,* 2015a) and accelerated

growth (Leclercq *et al.,* 2011; Taylor *et al.,* 2012*,* 2014) in association with dietary deficiency (Fjelldal *et al.,* 2015) in triploids. Definition of nutritionally complete aquafeeds will be essential in triploid salmon culture in order to fully meet their nutritional requirements for somatic growth and metabolic function. However, to date, virtually all studies exploring production traits of triploids have used standard commercial diets formulated for diploids, and specific experiments on triploid nutritional requirements are limited (Burke *et al.,* 2010; Fjelldal *et al.,* 2015; Taylor *et al.,* 2015).

Triploid salmonid nutritional research has shown comparable feed utilisation and conversion compared to diploids (Oliva-Teles & Kaushik, 1990b; Carter *et al.,* 1994) but some morphological differences suggest higher requirements compared to diploids including fewer pyloric caeca with compensatory mucosa cells and a relatively shorter gut length (Peruzzi *et al.,* 2014). In addition, altered metabolic function and differential gene regulatory pathways in triploid fish may contribute to a higher nutritional requirement for growth compared to diploids (reviewed in: Maxime, 2008; Benfey, 2015). Triploid rainbow trout have reduced catabolism and increased anabolism of protein in comparison to diploids (Cleveland & Weber, 2013, 2014) and differential fatty acid turnover rates associated with storage and synthesis in muscle, viscera and liver (Manor *et al.,* 2015a,b). Differences may be linked to less gonadal growth (Manor *et al.,* 2015b), reduced autophagy (Cleveland & Weber, 2013), growth recovery gene dosage

(Cleveland & Weber, 2014) and different feeding strategies (Cleveland & Weber, 2014). In diploids, protein accumulation rate in skeletal muscle largely determines growth rate (Bureau *et al.,* 2006) and a positive correlation exists between amino acid consumption and rate of protein synthesis (Houlihan *et al.,* 1995). Collectively, reports of higher growth rate, comparable feeding performance and differences in protein metabolism suggest triploids may have higher protein requirements where feed intake and formulation cannot be increased to meet demand. To date no commercial feed charts exist for recommended feeding rates of triploid Atlantic salmon. In addition specific dietary Essential Amino Acid (EAA) and general protein requirement studies have yet to be conducted in triploid Atlantic salmon. However, evidence exists to show triploids require higher dietary histidine (His; 17 vs. 12g kg^{-1}; Taylor *et al.,* 2015) compared to that of diploid salmon (14.4 g kg^{-1}; Remø *et al.,* 2014). In addition, it is well established in diploid salmon that EAA deficiencies such as methionine can lead to growth depression and increased protein catabolism (Belghit *et al.,* 2014). Thus other protein and EAAs requirement studies for triploids are essential.

Nutritional supplementation is known to mitigate skeletal deformity in diploid salmonids (Lall & Lewis-McCrea, 2007) and may have potential for improvement of triploid skeletal health (Benfey, 2015) particularly dietary P supplementation (Fjelldal *et al.,* 2015). In diploid post-smolts, reduced skeletal deformity, higher mineral content and

increased vertebral strength was observed in fish fed high dietary P (9.3g available P kg^{-1}) than those without supplementation (6.3g available P kg^{-1}) when fed for 17 weeks immediately following sea transfer (Fjelldal *et al.,* 2009b). By contrast a similar study using comparable dietary P levels but at later stage (>200g) post-sea transfer found no beneficial effect on deformity, suggesting a stage specific requirement (Gil Martens *et al.,* 2012). More recently, Fjelldal *et al.,* (2015) fed 9.4g total P kg^{-1} to triploid Atlantic salmon from first feeding until smolt and thereafter a standard commercial diet (7.1g total P kg^{-1}) throughout SW ongrowing and observed minimised skeletal deformities and improved final weight by harvest. However, feeding high P diets during hatchery rearing raises environmental sustainability concerns due to the potential for eutrophication of FW bodies by increased P discharge (Phillips *et al*., 1993; Folke *et al*., 1994; Wang *et al*., 2013). As yet triploid dietary P requirements for optimal skeletal development in SW are yet to be defined and may provide a means to stabilising skeletal deformity whilst minimising environmental impacts.

Thus the aim of the present study was to investigate whether a diet supplemented with increased dietary P and protein during SW grow-out of triploid Atlantic salmon could reduce vertebral deformities whilst sustaining growth in comparison to triploids fed a standard commercial diploid diet. As triploids must perform equally or better than

diploid counterparts in order to be considered commercially viable, a reference diploid group was also fed the standard diet.

2 Methods and Materials

2.1 Fish stock and husbandry

On 26th November 2010, fish eggs from the Aquagen strain were induced for triploidy at the Aquagen Broodstock Site, Hemne, Norway. Triploidy was induced using a hydrostatic pressure shock of 9500 psi applied 300 ° minutes post fertilisation for 50 ° minutes at 8°C (Taylor *et al*, 2011). Eyed ova (~380 °days) were transferred to Marine Harvest Inchmore Hatchery, Glenmorriston, Scotland (57°N, 5°W) on 13th of January 2011 and on-grown under commercial protocols (Thermal regime: eye-hatch, 4.4 ± 0.8˚C; hatch-1st feed, 5.9 ± 1.6˚C). First feeding fry were reared under constant light (LL) and ambient water temperature (12.0 ± 2.2˚C). On the 9th of August 2011, parr (~5g) were transferred to the Glenfinnan cage site and raised in two separate pens 10 x 10 x 5m (1 / ploidy) under ambient photoperiod (LD) and water temperature (9.9 ± 3.1˚C) and fed a standard diploid salmon feed (Skretting, UK) according to manufacturer's guidelines until sea transfer. Fish were vaccinated on the 16th November 2011 with Birnagen Forte. Completion of smoltification was verified in house by gill Na^+,K^+ ATPase activity

(McCormick, 1993) and skin silvering (Sigholt *et al.,* 1995). Red blood smears were prepared from blood collected from the caudal vein using heparinised syringes from fish at 5g (n = 100 / ploidy). After air drying, slides were fixed in 100% methanol and then placed into Giemsa stain for 10min. Erythrocyte length and diameter were measured at 100× magnification using image capture (ImagePro Software). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm. Diploid control groups had significantly smaller nuclear lengths with no overlaps than pressure shock triploid groups (2N 6.9–7.8 μm; 3N 9.1–10.2 μm) confirming that all fish that were subjected to hydrostatic pressure shock were likely to be triploids. All experimental procedures and husbandry practices used in the present study were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice) in accordance with EU regulation (EC Directive 86/609/EEC) and approved by the Animal Ethics and Welfare Committee of the University of Stirling.

2.2 Experimental design

On 5th April 2012, triploid smolts (mean weight 79.0 ± 17.4g) were transferred to SW at Marine Harvest Ardnish Farm Trial Unit, Lochailort, Scotland (57°N, 6°W) and divided into four 10 x 10 x 15m pens, (n = 6625 / pen). Diploids (mean weight 88.0g ± 20.8g) smolted later and were transferred on 28th of April into two pens (n = 6625 / pen). All fish were fed a standard commercial feed (Biomar, CPK) up until the 20th of June after

which duplicate pens of diploid and triploid smolts were fed a standard nutrient package (SP), while a further two pens of triploids were fed a boosted nutrient package (BP) containing additional P and increased BioFish premix. Feed formulations for the experimental period are provided in Table 2.1. Fish were handfed three times daily in accordance with manufacturer feeding table recommendations. Due to the scale of the study no feed collection devices were used and satiation was observed visually. Mortality, environmental data including water temperature and dissolved oxygen were recorded on a daily basis (Fig 2.1).

Table 2.1. Composition (%) of the standard nutrient diet (SP) and boosted nutrient diet (BP) fed during the experimental period.

	SP	**BP**
Diets as formulated (%)		
Fish/Crustacean meal	25.1	28.3
Pea protein	1.2	1.2
Soy Protein Concentrate	3.8	4.6
Corn gluten	8.3	8.2
Sunflower expeller	10.0	5.7
Wheat	7.0	5.4
Wheat gluten	7.6	6.6
Dehulled beans	5.8	7.1
Fish oil	13.4	13.9
Rape oil	14.6	14.9
Additives*	3.1	4.2
Nutritional content (Analysed)		
Oil (%)†	32.8	32.8
Protein (%)‡	37.6	40.1
Gross Energy (KJ/g) ‡	24.8	24.8
Total Phosphorus (g kg^{-1})§	9.9	12.0

*BioFish premix (not commercially available) with additional Essential Amino Acids
†Nutritional Analytical Service, University of Stirling, UK
‡BioMar, Grangemouth, UK
§Eurofins, Denmark

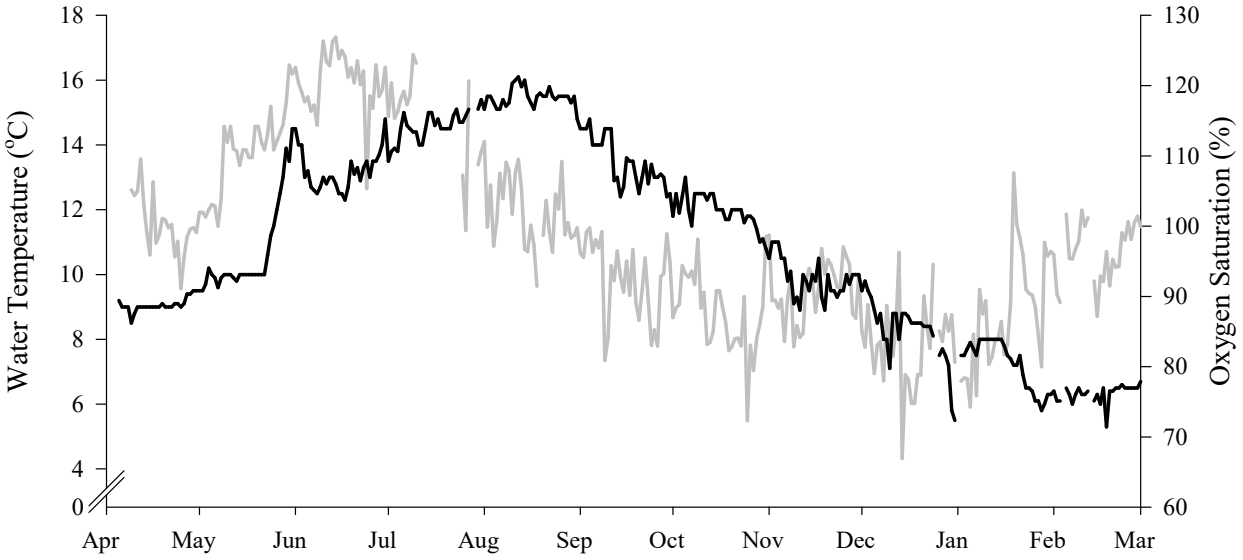

Figure 2.1. Water temperature (°C; black line) and oxygen saturation (%; grey line line) for the cage site during the trial period. Oxygen saturation has been corrected for salinity and temperature.

2.3 Sampling protocol

In June, July, September and November 2012 a total of 100 fish / pen were anaesthetised (50ppm Tricaine methanesulfonate (MS222), Pharmaq, UK) and individual body weight (BW ± 10g) and fork length (FL ± 0.5cm) recorded. Each fish was independently assessed for cataracts using a handheld ophthalmoscope according to Wall & Richards, 1992 and externally assessed for vertebral and jaw deformities in accordance with Taylor *et al.,* (2014). Weight data were used to calculate thermal growth coefficient (TGC) and economic feed conversion rate (eFCR) for each sampling period until harvest where TGC was calculated as: $(W_f^{1/3}-W_i^{1/3})$ x $(\sum D^o)^{-1}$, where W_f is the final body weight, W_i is the initial body weight and D^o is the cumulative sum of water temperature in degrees per day. eFCR was calculated as: $F/(B_f-B_i+B_m)^{-1}$ where F is the food fed (kg) B_f is the final

biomass (kg), B_i is the initial biomass (kg) and B_m is the mortality biomass for the period (kg). Feed intake could not be calculated due to size and use of commercial pens, therefore eFCR provides a crude estimate of feed conversion.

On the 7th February 2013 a final sampling was carried out prior to harvest. From the 100 fish anaesthetised / pen, terminal samples were collected (10 and 20/pen for 2N and 3N respectively) using a percussive blow to the head and severing of the gill aorta in accordance with schedule 1 UK Home Office procedure. Triploid fish were subjectively selected according to normal/no visible deformity (n = 10 / pen) or the appearance of externally observable lower jaw deformity (n = 5 / pen) or vertebral deformity (n = 5 / pen), which was assessed visually and through palpatation. The heart was dissected out from each fish and preserved in 10% neutral buffered formalin. Fish were number tagged using a cable tie, placed in polystyrene boxes, packed flat with ice and left for 72 hours to achieve rigor prior to fillet quality analysis.

From Feb 25th one pen per day was harvested according to commercial protocol. 500 fish per pen were individually assessed for externally visible deformities on each harvest day to determine overall deformity prevalence within each cage population. All harvested fish were classified as superior, ordinary or rebate according to Marine Harvest Quality standards.

2.4 Parameters analysed

2.4.1 Fillet quality

Of terminal samples collected at harvest per pen (2N = 10 / pen; 3N = 20 / pen), the left hand side fillet was carefully removed for flesh quality analysis carried out with the assistance of Biomar (Grangemouth, UK). Fillets were assessed for pigmentation inside a light box using Roche SalmoFan Lineal Card (Hoffman-La Roche, Basel, Switzerland) and scored by two independent observers. A Norwegian quality cut (NQC) was removed from each fillet and frozen for later fatty acid composition analysis using near-infrared NIR analysis and additional pigment analysis (Marine Harvest SOP adapted from Folkestad *et al.,* (2008).

2.4.2 Texture analysis

Texture analysis was carried out according to Johnston *et al.,* (2004). Briefly, two cuboid sections of flesh were removed from the side fillet below the dorsal fin measuring 20mm x 40 mm x 40 mm and chilled to 4°C before analysis was carried out using a texture analyser (TA-HDi Texture Analyser, Stable Micro Systems, Haslemere, UK) with a steadily advancing Warner-Bratzler blade set to travel at 1 mm second^{-1}. The cutting load was continuously recorded and used to calculate the maximum force (N) required and the total work done (WD).

2.4.3 Heart morphology

Sample hearts were pinned and photographed with the cranio-ventral surface facing uppermost before being turned and photographed from a side view according to the method of Poppe *et al.,* (2003). Image analysis was carried out on each using Fiji (version 1.47b, NIH, USA). Heart width and height was measured along with the angle of the bulbous arteriosis. The heart was squeezed to remove excess fixative and weighed to calculate the cardio-somatic index (CSI) such that CSI = (100 x Heart Weight (HW)) / Body Weight (BW)).

2.4.4 Vertebra radiological assessment

After careful removal of the side fillet, two radiographs (anterior and posterior) were taken of each fish using a portable x-ray unit (Celtic SMR PX40 HF) with an extremity plate measuring 24 X 30 cm, and each plate exposed for 32 mAs at 40kV. Images were then digitized (AGFA CR-35X) and radiographs examined using Adobe Photoshop CS 6 (version 13.0.1, Adobe system Incorporated, California, USA). Total number of vertebrae were recorded for each fish and the spine was divided into four regions (R1, 2, 3, and 4) as per Kacem *et al.,* (1998) for which deformities were classified based on Witten *et al.,* (2009). In addition, the length: dorsoventral diameter ratio (L:H) for each individual vertebrae was also analysed.

2.4.5 Vertebra mechanical properties

Vertebra number 6, 7, and 8 from the cranial trunk, v28, 29 and 30 from the centre trunk and v52, 53 and 54 from the tail fin were carefully dissected out post radiography. Each vertebra was crushed individually (n = 3 vertebra / region / fish / pen) using a texture analyser fitted with a 10cm compression plate (TA-HDi Texture Analyser, Stable Micro Systems, Haslemere, UK) to a distance of 4mm at a speed of 0.1mm/s. Yield Load (N), Stiffness (N / mm) and resilience (N x mm) were calculated for each vertebra according to modified protocols of Fjelldal *et al.,* (2004). After mechanical crushing, the three vertebrae from each region were pooled and mechanically stripped of any remaining flesh, defatted in baths of iso-hexane for 24 hours, oven dried at 105°C for 24 hours and incinerated at 600°C for 16 hours. Weights (1 x 10^{-3} mg) of dried and ashed vertebrae were used to calculate the Bone Mineral content (BM%) of each region according to Fjelldal *et al.,* (2006) as Mineral content = (ashed weight / dry weight) x 100. Samples were then digested in 5ml of AristAR® nitric acid (HNO3; VWR International, USA) using a Mars Microwave digestion system (10 min. heating phase to 160˚C, 20 min. at 160˚C, 30 min. cooling phase) and analysed for inorganic elements by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a Thermo X Series II ICP-MS (collision cell model). Percentage concentrations were calculated for P, Ca, magnesium (Mg), zinc (Zn) and vanadium (V).

2.5 Statistics

Results are reported as mean (± SEM). Statistical analysis was carried out using Minitab (Version 16.2.3, Minitab Inc, Pennsylvania, USA). Differences between weight, K and flesh quality parameters were assessed using a general linear model (GLM) and One-Way ANOVA with replicate pens nested within treatment. No replicate differences were observed between pens. Statistically significant differences were considered where $p < 0.05$. Post hoc comparisons were carried out using Tukeys multiple comparisons test. A Two-Way ANOVA manipulated through GLM was used to analyse heart morphology, bone mineral and bone strength attributes. All proportions were transformed using arcsine square root transformation and all data were checked for normality and homogeneity of variance using a Kolmogorov-Smirnov test and Levene's test respectively. Externally visible malformations were analysed for significant differences using a Kruskal Wallis and subsequent Mann Whitney-U comparisons test. X-rays of deformed vertebrae were ranked according to severity and analysed for differences using a PERMANOVA (Version 1.6, University of Auckland, New Zealand).

3 Results

3.1 Growth and mortality

From June to September, both triploid groups maintained a significantly higher weight than diploids (Fig. 2.2a). Furthermore, from July to September, 3N BP attained a significantly higher weight than 3N SP. Greater weight was reflected in a higher TGC and more efficient eFCR (Fig. 2.2a, b) of both triploid diets than diploids during period 1. However, in both 3N dietary groups TGC significantly decreased and eFCR was less efficient during period 2 and 3. As such there was no significant difference in weight between any treatment by mid-November. Diploids also showed a marked reduction in TGC and eFCR between September and November. This period of reduced growth and feeding efficiency (July-November) coincided with a combined outbreak of Amoebic Gill Disease (*Neoparamoeba perurans*: AGD) and heart and skeletal muscle inflammation (HSMI). During this period there was also a concomitant decrease in O_2 saturation and higher water temperature (Fig. 2.1). Cumulative mortality levels were comparable (2N SP: 3.83 ± 0.68; 3N SP: 3.64 ± 0.34; 3N BP: 3.75 ± 1.21) during this period, and overall mortality for the duration of the trial did not differ significantly between any treatment (6.7 ± 1.0, 7.4 ± 0.1 and 6.8 ± 1.8% for 2N SP, 3N SP and 3N BP respectively). November onwards, where water temperature was reduced, fish showed signs of recovery whereby 3N BP achieved a significantly greater final harvest weight

than 3N SP or 2N SP. Irrespective of dietary treatment, triploids maintained a significantly lower K than diploids from September until harvest (Fig. 2.2c).

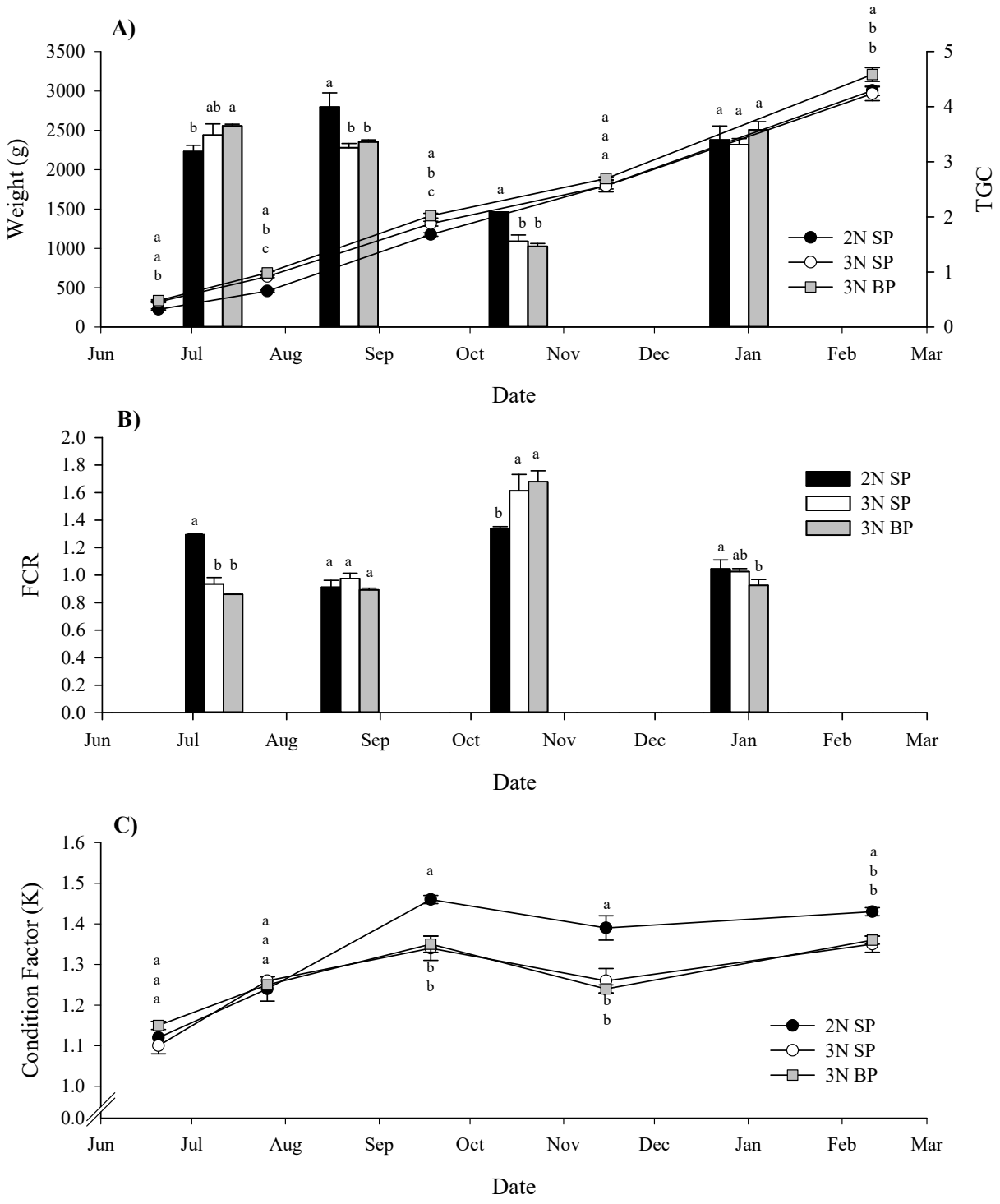

Figure 2.2. A) weight (symbols) and TGC (vertical bars) for each growth period; **B**) eFCR for each growth period; and **C**) K, for diploid and triploid Atlantic salmon fed SP or BP diets during SW (n = 100 x 2 cages x 3 treatments / time point). Values that do not share a common subscript denote significant differences ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

3.2 Deformity

3.2.1 Cataract and externally visible deformity

Cataract prevalence at harvest was very low in this study (incidence of 2.0 ± 1.0%) with a mean score of 2.9 ± 1.6 for affected individuals and did not differ between ploidy (data not shown).

At harvest both triploid dietary groups exhibited similar levels of external deformity (19-21%) in comparison to diploids (~2.2%) with jaw and vertebral pathologies accounting for approximately equal proportions of deformity (9.3-11.4%) within triploid treatments (Table 2.2a).

3.2.2 Radiological deformity

X-ray assessment showed that triploids had on average one less vertebra than diploids (Table 2.2b). At smolt, 37% of diploids and 76.4% of triploids were classified as radiologically deformed, having at least 1 or more deformed vertebra (dV). Triploids also had a significantly higher mean number of dV than diploids at smolt, and only triploids showed individuals with 6-9 dV or ≥10 dV (Table 2.2b).

At harvest, diploids showed a slight increase (+3 %) in radiologically deformed individuals (40 %) compared to that at smolt, while triploids showed a greater increase (+8.6 %), with 85 % of fish classified as radiologically deformed (Table 2.2b). Of note,

in the case of diploids, external jaw or vertebral deformity were mutually exclusive, however, in the case of triploids, of all individuals in 3N SP and 3N BP recorded with external deformities , 5.9 ± 1.1 % (11/184) and 6.8 ± 2.2% (13/196) exhibited both pathologies. 3N SP had a significantly higher average no. dV per deformed fish than 3N BP, with 2N SP having significantly lower average no. dV than either triploid dietary group. Finally, comparing fish with ≥10dV at harvest and smolt showed a small increase in 2N SP (+5%) and 3N BP (+1.1%), but a notable increase (+31.1%) in 3N SP (Table 2.2b). Furthermore, a greater proportion of triploids were classified as having mild deformities (range 1-5dV) in the BP (45 %) compared with the SP diet (10%).

Table 2.2. **A)** Total visible external deformity (%, mean ± SEM) observed at smolt (n = 72-92 ploidy) and at harvest (n = 500 / pen) for 2N and 3N fed SP or BP. **B)** Radiological deformed vertebra (dV) and severity of affected vertebra per deformed fish at smolt (n = 72-92 ploidy) and at harvest (n = 20 / ploidy / diet) for fish exhibiting no externally visible signs of deformity.

	Smolt		Harvest		
	2N	**3N**	**2N SP**	**3N SP**	**3N BP**
A.) External Visible Deformity					
None (%)	n/a	n/a	97.8 ± 0.1^{a}	81.0 ± 1.3^{b}	80.4 ± 1.8^{b}
Jaw (%)*	n/a	n/a	0.9 ± 0.0^{b}	9.7 ± 2.3^{a}	11.1 ± 2.3^{a}
Vertebral (%)*	n/a	n/a	1.3 ± 0.5^{b}	9.3 ± 0.5^{a}	8.5 ± 0.5^{a}
B.) Radiological Vertebral Deformity					
Ave. V No.	59.4^{a}	58.4^{b}	59.2 ± 0.2^{a}	58.4 ± 0.1^{b}	58.4 ± 0.1^{b}
Ave. no. dV	1.9^{b}	5.8^{a}	3.3 ± 0.0^{c}	11.5 ± 1.6^{a}	6.0 ± 1.6^{b}
0dV (%)	63.0	23.6	60.0 ± 0.0^{a}	15.0 ± 5.0^{b}	15.0 ± 5.0^{b}
1-5dV (%)	37.0	43.1	25.0 ± 5.0^{ab}	10.0 ± 10.0^{b}	45.0 ± 5.0^{a}
6-9dV (%)	0.0	19.4	10.0 ± 0.0^{b}	30.0 ± 0.0^{a}	25.0 ± 5.0^{a}
≥10dV (%)	0.0	13.9	5.0 ± 5.0^{b}	45.0 ± 5.0^{a}	15.0 ± 10.0^{b}

^{a,b,c,d} Mean values with different lowercase superscript letters are significantly different with smolt and harvest considered separately ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

Deformed vertebrae were observed in all four spinal regions in triploids, but not in the cranial trunk (R1) in diploids, with the predominate locality of all deformed vertebrae in the tail region (R3), principally v39 - v43, irrespective of ploidy (Fig 2.3a). Triploid

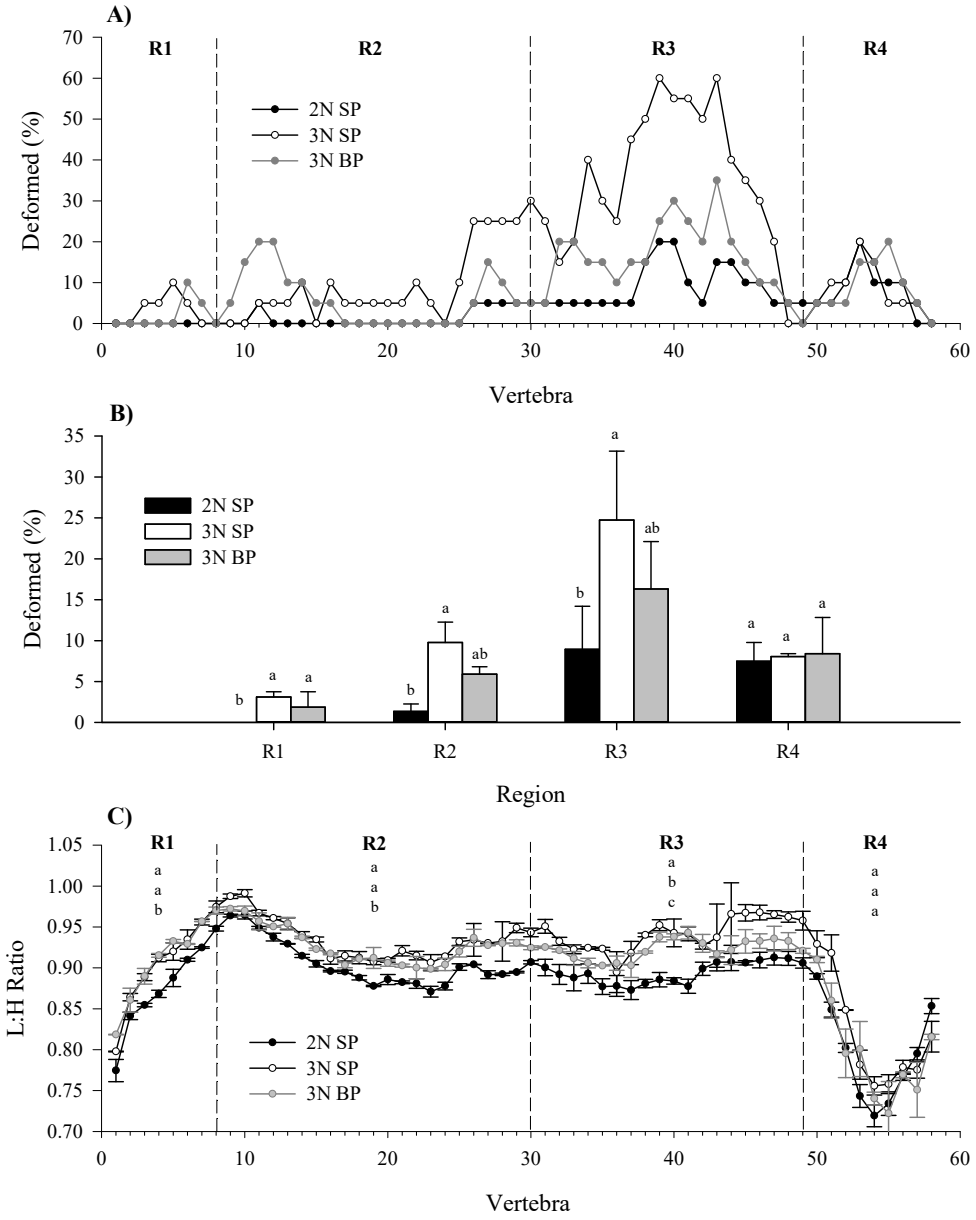

Figure 2.3. A) Percentage of dV along the vertebral column; **B)** Mean percentage of total dV within each spinal region; and **C**) Vertebral length-height ratio (L:H) along the vertebral column in diploid and triploid Atlantic salmon fed SP or BP diets during seawater grow out. Values that do not share a common subscript denote significant differences within region ($p < 0.05$; One-Way ANOVA & Tukey's post hoc). The vertebral column is divided into four regions as defined in Kacem *et al.,* (1998).

dietary groups did not differ significantly in total deformed vertebrae in R1 (Fig. 2.3b. 3N SP had significantly more deformed vertebrae in R2 than 2N SP, with 3N BP not differing significantly from either ploidy on the SP diet. A similar pattern was reflected in the tail region (R3), with 3N BP showing a reduced prevalence to 3N SP, and statistically comparable to 2N SP (Fig. 2.3b). Finally, no significant differences between treatments were observed in the tail fin (R4). Of deformity types observed compression

type pathologies (type 2 compression predominant in diploids and type 5 one-sided compression in triploids) were most common accounting for 43-63% of all deformed vertebra recorded, and symmetry deviations (type 19) accounting for 22-29% of all pathologies irrespective of ploidy (data not shown).

Ploidy and diet had a significant effect on vertebral L:H ratio (Fig. 2.3c). In R1 and R2 triploids had a significantly higher L:H ratio (3N SP: R1- 0.91 ± 0.01, R2 0.94 ± 0.01; 3N BP: R1- 0.91 ± 0.01, R2 0.93 ± 0.01) than diploids (2N SP: R1-0.88 ± 0.01, R2-0.90 ± 0.01) irrespective of diet. In R3, 3N SP (0.95 ± 0.01) had a significantly higher L:H ratio than 3N BP (0.92 ± 0.01), predominantly evident in v45-v49, which were significantly higher than 2N SP (0.89 ± 0.01). Finally, no significant difference in L:H ratio was observed between ploidy or diet in R4.

3.3 Vertebral composition and strength

Total mineral content did not differ between spinal regions in 2N SP or 3N SP groups (Fig. 2.4a). There was no significant difference between cranial trunk and centre trunk within 3N BP, however, the tail fin had a significantly lower mineral content than the centre trunk. The centre trunk had comparable total mineral content between treatments, whilst 2N SP had a significantly higher mineral content than 3N BP in both cranial trunk and tail fin.

As vertebral mineral analysis revealed no significant differences between regions for specific minerals, data were pooled per treatment (Table 2.3). Ca content was significantly higher in 2N SP than 3N BP, but not 3N SP. P content was significantly higher in 2N SP compared to triploids whereas, Ca:P ratio did not differ between any treatment. Mg content was significantly higher in 2N SP than 3N BP, but not 3N SP, although no difference between triploids was observed. V content was significant higher in 3N BP than either ploidy in the SP diet. Both 2N SP and 3N SP had significantly higher

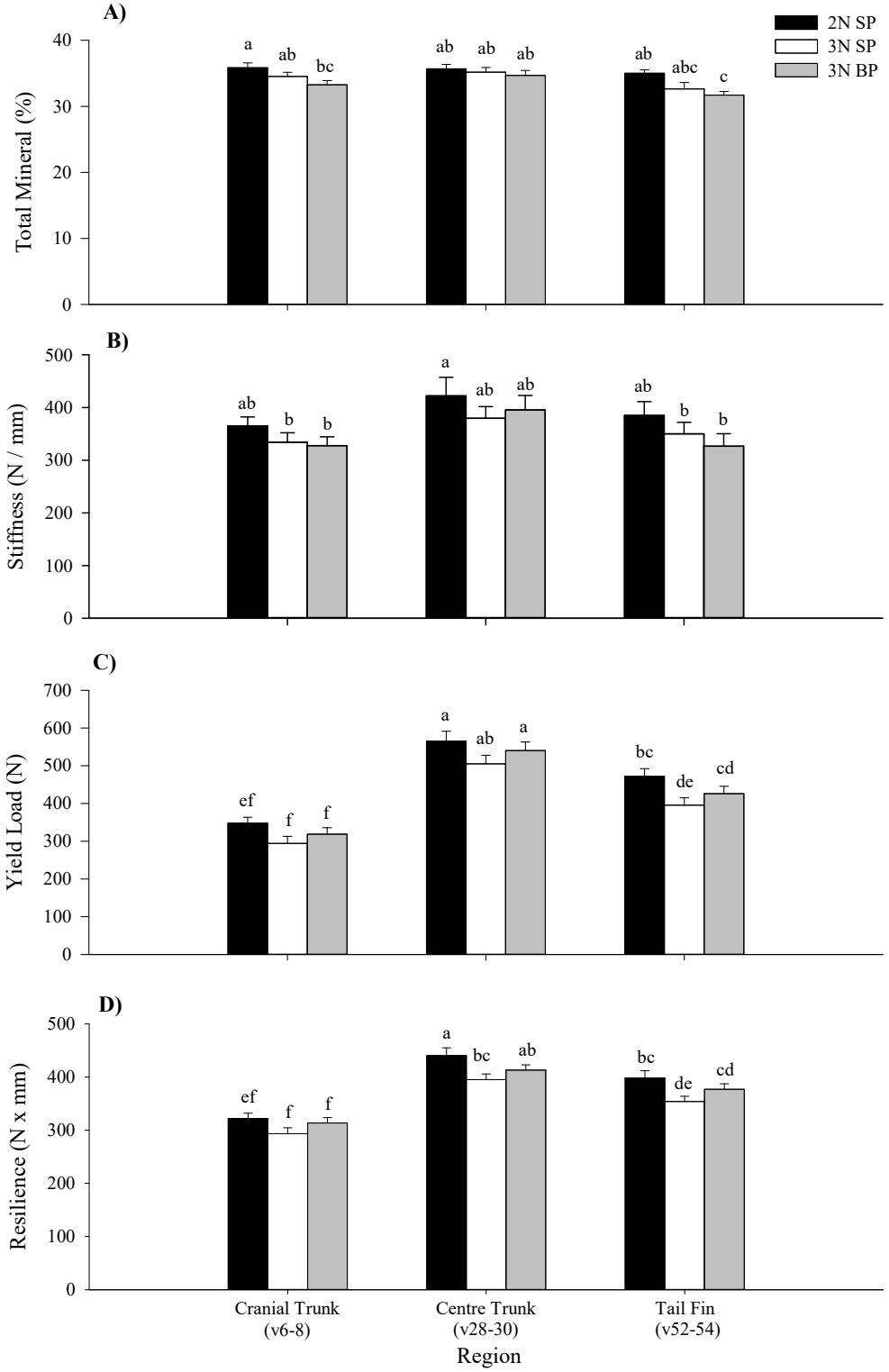

Figure 2.4. A) Total mineral content (% bone dry weight); **B)** Stiffness (N / mm), **C)** Yield load (N) required to crush an individual vertebra; and **D)** resilience (N x mm) as a measure of total energy required to crush a single vertebra for each of the three regions examined (R1 v6-8; R2/3 v28-30 and R4 v52-54) at the end of SW grow out in 2N and 3N Atlantic salmon previously fed an SP or BP diet. Results are the pool of three vertebra from each region per fish analysed. Values that do not share a common subscript denote significant differences within region between treatments ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

Table 2.3. Mineral content (%) of the vertebrae for 2N and 3N fed SP or BP diet.

	2N SP	3N SP	3N BP
Ca	$13{\cdot}10 \pm 0{\cdot}03^{a}$	$12{\cdot}70 \pm 0{\cdot}39^{ab}$	$12{\cdot}18 \pm 0{\cdot}0^{b}$
P	$6{\cdot}76 \pm 0{\cdot}10^{a}$	$6{\cdot}58 \pm 0{\cdot}1^{b}$	$6{\cdot}25 \pm 0{\cdot}0^{b}$
Ca:P	$1{\cdot}94 \pm 0{\cdot}02$	1.93 ± 0.03	$1{\cdot}95 \pm 0{\cdot}01$
Mg	$0{\cdot}172^{a} \pm 0{\cdot}001$	$0{\cdot}169^{ab} \pm 0{\cdot}003$	$0{\cdot}160^{b} \pm 0{\cdot}001$
V	$3{\cdot}26{*}10^{-3}\ 0{\cdot}14^{b}$	$3{\cdot}13{*}10^{-3} \pm 0{\cdot}20^{b}$	$4{\cdot}21{*}10^{-3} \pm 0{\cdot}15^{a}$
Zn	$0{\cdot}0119 \pm 0{\cdot}001^{a}$	$0{\cdot}0118 \pm 0{\cdot}0001^{a}$	$0{\cdot}0105 \pm 0{\cdot}0001^{b}$

[a,b,c,d] Mean values with different lowercase superscript letters are significantly different ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

vertebral Zn content than 3N BP. Vertebral stiffness did not differ significantly between treatments within region (Fig. 2.4b). Lowest stiffness was generally observed in cranial trunk and highest in the centre trunk. Mechanical testing showed significant differences in the yield load (N) between the three regions with the cranial trunk demonstrating the lowest yield load, tail fin then centre trunk (Fig. 2.4c). No significant difference between the three dietary groups was found within cranial trunk and centre trunk. In the tail fin, 3N SP showed a significantly lower value than 2N SP. No significant differences were observed between 3N BP and 2N SP. The centre trunk showed significantly higher resilience (N x mm) than any other region with the lowest resilience observed within the cranial trunk (Fig. 2.4d). No significant differences were found between treatments within the cranial trunk. In the centre trunk and tail fin, 2N SP resilience was significantly higher than 3N SP but there was no statistical difference between 2N SP and 3N BP groups.

3.4 Heart morphology

No significant differences between ploidy or diets were found for CSI (0.17 - 0.18) and H:W ratio (0.09 - 1.12). A significant difference was however found in the angle of the bulbous arteriosis between the 2N SP (35.9 ± 1.6) and 3N SP (30.9 ± 1.4) but not the BP diet (34.7 ± 1.1; data not shown).

3.5 Harvest weight & fillet quality

Size classification at harvest varied between the dietary groups and there was an overall trend towards larger fish in triploids than diploids with triploid BP showing a greater proportion of fish in the 3-4 and 4-5kg grades (Fig. 2.5a). By contrast, 51.6 ± 3.78% of fish harvested in 2N SP weighed in the smaller weight class of 2-3kg compared to 32.9% ± 1.3% in 3N BP and 38.2 ± 2.8% 3N SP.

In both diploids and triploids, fish with jaw deformity showed a lower harvest weight than those without (Table 2.4). In diploids, vertebral deformity did not affect harvest weight, by contrast, harvest weight was significantly higher in triploids with visible vertebral deformity than those without. K was also significantly higher in the fish with vertebral deformities in all ploidy groups, while those with jaw deformity showed a tendency towards a lower K (Table 2.4).

Final harvest saw a greater proportion of fish classed as superior in 2N SP than 3N SP or 3N BP (Table 2.5a). Consequently, the proportion of fish classed as ordinary

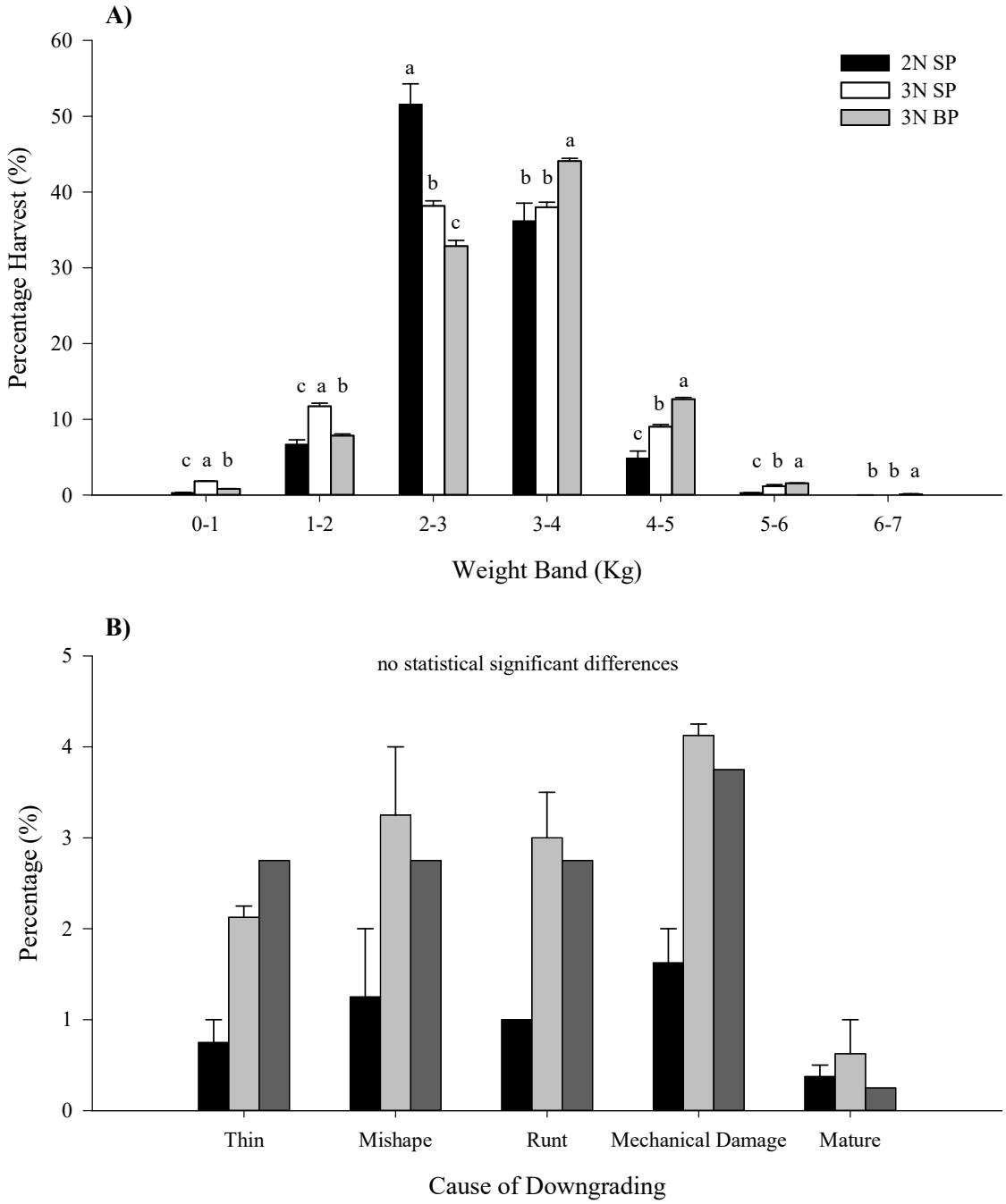

Figure 2.5. Final harvest data showing **A)** distribution of harvested fish weight classification (2N SP: n=13,452; 3n SP: n= 11,075; 3N BP: n= 11854); and **B)** cause of downgrading at final processing according to Marine Harvest Quality Standards. Values that do not share a common subscript denote significant differences ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

Table 2.4 Breakdown of harvest weight and K (mean ± SEM) into fish exhibiting no externally visible signs of deformity (2N SP, n=195; 3N SP, n=160; 3N BP, n=147); those exhibiting jaw deformity (2N SP, n=2; 3N SP, n=21; 3N BP, n=38) and individuals with externally visible vertebral deformity (2N SP, n=3; 3N SP, n=19; 3N BP, n = 12).

	2N SP	3N SP	3N BP
Harvest Weight (g)			
Visibly normal	3010 ± 40^{bA}	2900 ± 50^{bB}	3270 ± 0^{aB}
Jaw	2430 ± 90^{aB}	2830 ± 270^{aB}	2730 ± 0^{aC}
Vertebra	2960 ± 0^{bA}	3680 ± 130^{aA}	3480 ± 30^{aA}
K			
Visibly normal	1.42 ± 0.01^{aB}	1.32 ± 0.02^{bB}	1.34 ± 0.01^{bB}
Jaw	1.38 ± 0.05^{aB}	1.37 ± 0.07^{aB}	1.28 ± 0.01^{aC}
Vertebra	1.51 ± 0.00^{aA}	1.58 ± 0.05^{aA}	1.51 ± 0.02^{aA}

[a,b,c,d] Mean values with different lowercase superscript letters are significantly different within normal, jaw and vertebral ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

[A,B,C,D] Mean values with different uppercase superscript letters are significantly different between normal, jaw and vertebral ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

Table 2.5. Harvest summary of (**A**) percentage grading of harvested fish classified as superior, ordinary or rebate (Fish scored according to Marine Harvest quality standards); **B)** fillet fat content; (**C**) fillet colour and total pigment; and (**D**) Mechanical and textural properties. **NB:** all data presented (B-C) is taken from the fish classed as showing no signs of external deformity (n=20 / ploidy / diet).

	2N SP	3N SP	3N BP
A) Harvest Grade (% Total Harvest)			
Superior	$95{\cdot}0 \pm 1{\cdot}2^{a}$	$80{\cdot}0 \pm 1{\cdot}7^{b}$	$83{\cdot}1 \pm 1{\cdot}3^{b}$
Ordinary	$3{\cdot}7 \pm 0{\cdot}9^{b}$	$13{\cdot}3 \pm 2{\cdot}0^{a}$	$13{\cdot}6 \pm 1{\cdot}3^{a}$
Rebate	$1{\cdot}4 \pm 0{\cdot}3^{c}$	$6{\cdot}7 \pm 0{\cdot}3^{a}$	$3{\cdot}3 \pm 0{\cdot}0^{b}$
B) Fat Analysis (%)			
NQC Fat	$11{\cdot}61 \pm 0{\cdot}43$	$11.72 \pm 0{\cdot}06$	$11{\cdot}84 \pm 0{\cdot}77$
Calculated SQC Fat	$19{\cdot}84 \pm 0{\cdot}68$	$20{\cdot}15 \pm 0{\cdot}73$	$19{\cdot}35 \pm 0{\cdot}20$
DHA	$0{\cdot}93 \pm 0{\cdot}02$	$0{\cdot}90 \pm 0{\cdot}01$	$1{\cdot}04 \pm 0{\cdot}06$
EPA	$0{\cdot}63 \pm 0{\cdot}01^{ab}$	$0{\cdot}68 \pm 0{\cdot}02^{a}$	$0{\cdot}60 \pm 0{\cdot}01^{b}$
Ratio n-3:n-6	$1{\cdot}93 \pm 0{\cdot}02$	$2{\cdot}00 \pm 0{\cdot}03$	$2{\cdot}03 \pm 0{\cdot}17$
C) Fillet Colour			
Pigment (mg/kg)	$5{\cdot}87 \pm 0{\cdot}24^{a}$	$5{\cdot}11 \pm 0{\cdot}07^{b}$	$4{\cdot}81 \pm 0{\cdot}09^{b}$
Roche Average	$26{\cdot}60 \pm 0{\cdot}25$	$26{\cdot}25 \pm 0{\cdot}38$	$25{\cdot}92 \pm 0{\cdot}01$
D) Fillet Texture and Mechanical Properties			
Texture	$2{\cdot}95 \pm 0{\cdot}15$	$3{\cdot}05 \pm 0{\cdot}10$	$3{\cdot}13 \pm 0{\cdot}18$
Gaping	$1{\cdot}30 \pm 0{\cdot}10$	$1{\cdot}05 \pm 0{\cdot}10$	$1{\cdot}28 \pm 0{\cdot}08$
Cutting Force (N)	$17{\cdot}63 \pm 0{\cdot}76$	$17{\cdot}98 \pm 0{\cdot}82$	$17{\cdot}08 \pm 0{\cdot}23$
Total Work (mJ)	$160{\cdot}5 \pm 5{\cdot}6$	$166{\cdot}6 \pm 8{\cdot}0$	$159{\cdot}6 \pm 1{\cdot}6$

[a,b,c,d] Mean values with different lowercase superscript letters are significantly different ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

was higher for the triploid dietary groups. Rebate for 3N SP was significantly higher than 3N BP. 2N SP had the lowest level of rebate at harvest. The major cause of downgrading were mainly attributable to thin, misshapen, runts and mechanical damage, with triploids showing a higher relative proportion than diploids although there was no significant difference between ploidy or diet (Fig. 2.5b). Of fish recorded as mature, only male fish were observed in both ploidy.

Total percentage fillet fat, DHA content, and ratio of n-3:n-6 fatty acids did not differ significantly between ploidy or diet (Table 2.5b). EPA was significantly higher in 3N SP than 3N BP (P = 0.01). Fillet pigment content was significantly lower in both triploid groups relative to diploids, but did not differ between triploid dietary groups (Table 2.5c). Although Roche scores did not show significant difference between treatments and ploidy, scores did correlate with reduced total pigment. Fillet texture, gaping, or mechanical strength showed no significant difference between diet and ploidy (Table 2.5d).

4 Discussion

Triploid Atlantic salmon growth rate can be sustained until harvest when fed a diet enriched with P and protein compared to a conventional diploid diet. Furthermore, supplementation of dietary P and protein in the SW stabilised progression of skeletal

deformities, which is a major contribution towards advancing triploid welfare and subsequent implementation in industry.

Triploids fed the boosted nutrient diet attained a significantly higher weight at harvest, being 7% heavier than triploids reared on a standard commercial diploid diet. This is one of only two studies to show triploids can attain higher weights after the marine phase (Oppedal *et al.,* 2003). By comparison, triploids fed the standard diet had higher weights compared to diploids but only in the first 5 months before weight advantage was lost, which is consistent with previous studies to date (O'Flynn *et al.,* 1997; Friars *et al.,* 2001; Cotter *et al.,* 2002; Leclercq *et al.,* 2011; Fraser *et al.,* 2013b; Taylor *et al.,* 2013; Tibbets *et al.,* 2013). Collectively, these differences in growth potential of triploids between the two diets in the current study suggest that triploids appear to have a higher P and / or protein requirement to support growth. However, as no diploid group were fed the boosted package, the current experimental design does not allow us to conclude ploidy differences in requirement. Nonetheless, growth performance of triploids at least equalled diploid performance when utilising the reference diet. In addition, it has been suggested in the context of husbandry and welfare (Fraser *et al.,* 2012a; Benfey, 2015) that triploids be considered a new species. As such, comparing ploidy differences with respect to nutritional requirement studies in triploids may be obsolete and future nutritional requirement research may only require within triploid comparisons.

Protein accumulation rate in skeletal muscle has been shown to determine growth rate in salmonids (Bureau *et al.,* 2006) and amino acid consumption is positively correlated with protein synthesis rate in fish (Houlihan *et al.,* 1995). Hence, sustained growth rate observed in observed in triploids fed the nutrient boosted package was likely attributed to higher protein inclusion (+ 7%). Non-protein energy sources (oil) were comparable between the standard and nutrient boosted diets. In this respect, increasing dietary protein may have had an energy sparing effect, where non-protein energy can be utilised for metabolism and maintenance, sparing additional protein for growth (Tibbets *et al.,* 2013). In addition, triploid eFCR was lower than diploids during high growth periods in this study. Thus, triploids on the standard diet would be consuming fewer nutrients per kilogram of muscle growth than would be theoretically available through the nutrient boosted package. However, actual feed intake was not measured due to cage size and so improvements in triploid growth cannot conclusively be attributed to either increased feed intake or better feed conversion efficiency under the two different diets. Higher feed intake in triploids may compensate for restricted nutrient availability and resource deficiency (Cleveland & Weber, 2013) that may be associated with deformities. Finally, feeding rates in cages are traditionally assessed in diploids through confirmation of satiation by surface observations. However, observations in this study suggested a deeper feeding behaviour in triploids as previously reported in brown trout, *Salmo trutta*,

(Preston *et al.,* 2014). This could suggest satiation in previous studies may not have been met in triploids. Successful production of triploids will thus rely on developing triploid specific feeding tables in addition to aquafeeds.

From late July to November, growth rate (TGC) in both triploid dietary groups reduced considerably compared to diploids. Water temperatures peaked during this period with a concomitant reduction in oxygen saturation. In a previous study at the same site, Taylor *et al.,* (2013) reported a similar drop in growth performance during the same period with comparable oxygen and temperature profiles. This is consistent with findings that show triploid Atlantic salmon have reduced metabolic rates (Atkins & Benfey, 2008) and lower aerobic metabolic scope in environmental conditions of high temperature (19˚C) and moderate hypoxia (70% O_2 saturation; Hansen *et al.,* 2015). This could set geographical distribution limits to production. In the case of our study the reduced growth performance is likely a result of environmentally-induced metabolic compromise, further exacerbated by a combined outbreak of AGD and HSMI during this period. However, of significant importance is that during this "challenge" period, mortality rates did not differ between ploidy. Following a return to normal environmental conditions, both triploid groups recovered to pre-challenge growth rates, albeit recovery time was longer than diploids. Environmental and disease challenge pressures may place further strain on the cardiac system. In this respect it was evident that triploids under the standard diet had a

more acute angle of the bulbus arteriosus at slaughter consistent with other studies in Atlantic salmon (Leclercq *et al.,* 2011; Fraser *et al.,* 2013b). Altered heart morphology alongside known higher routine metabolic rates at high temperature (Atkins & Benfey, 2008) indicate triploids experience increased cardiac workload from respiratory demand. Indeed, triploid heart morphology has been shown in other studies to be influenced by egg incubation temperature (Fraser *et al.,* 2013a) and vaccination (Fraser *et al.,* 2014). Diet did not affect the angle of the bulbus arterious although a significantly higher angle was found in diploids relative to triploids fed the standard diet. The effect of the angle of the bulbus arteriosus on salmon heart function is yet to be elucidated.

Both triploid groups had lower K than diploids from early autumn, suggestive of increased skeletal growth relative to muscle gain, which is again consistent with previous studies. Fjelldal *et al.,* (2015) suggested that this diverging pattern between skeletal and somatic growth in triploids may have an effect on dietary P demand, as an animal with a rapidly growing skeleton will need a higher mineral input to support normal bone mineralisation. Although, examination of externally visible deformity at harvest did not reveal a dietary difference of prevalence in triploids, x-ray radiography revealed that triploids fed the supplemented diet (+30% dietary P) had three fold less fish (15 vs 45%) with severe spinal deformities (≥ 10 dV) than triploids fed the standard diet. Furthermore, the average number of deformed vertebrae per deformed fish remained was sustained

from the point of sea transfer (smolt) until harvest in the supplemented group (5.8 to 6.0), but had almost doubled in the standard dietary triploid group (from 5.8 to 11.5). Thus the progression of deformity development during SW in triploids appeared to be largely arrested by dietary P supplementation. However, as the effect of increased dietary P and protein cannot be isolated in this study it is not possible to conclude that reduced skeletal deformities are attributed solely to increased dietary P. In addition, the high prevalence of pre-existing deformities observed at smolt emphasise the importance of optimising vertebral development prior to sea-transfer.

Deformity affected both weight and body morphology at harvest. K and body weight was highest in those with compressive spinal deformities in accordance with Hansen *et al.,* (2010), where pathology may be associated with fast growth rates, particularly alongside nutrient deficiency. Fish exhibiting jaw deformities were generally of lower weight (-20%) than non-deformed fish, which may be a consequence of impaired feeding or respiratory ability (Roberts *et al.,* 2001; Venegas *et al.,* 2003; Lijalad & Powell, 2009; Taylor *et al.,* 2013). However unlike spinal deformity and despite P and Ca being suggested as preventative nutritional factors in other research (Roberts *et al.,* 2001), no positive effect of diet on reducing jaw deformity was evident. Jaw deformity may also be caused by mechanical stress and weakening of the lower jaw bones through respiratory-induced excessive buccal-opercular pumping with increasing water

temperature and concomitant oxygen reduction. Reduced egg incubation temperatures (Fraser *et al.,* 2014a) and dietary P supplementation in FW (Fjelldal *et al.,* 2015) have also been shown to reduce occurrence of jaw deformities in triploid Atlantic salmon. Collectively, this suggests that in addition to the role of environmental temperature and nutrition in jaw deformity development during the marine phase, earlier developmental factors such as egg incubation and dietary supplementation in FW may also need to be addressed.

Fish bone strength is highly impacted by mechanical stress (Fjelldal *et al.,* 2009a) with mechanical stimuli further increasing vertebral strength through inducing mineralisation (Lall & Lewis-McCrea 2007; Totland *et al.,* 2011; Ytteborg *et al.,* 2013). In particular, the caudal region undergoes the greatest mechanical strain due to lateral muscular activity and is the region most associated with spinal pathology in SW (Totland *et al.,* 2011; Fjelldal *et al*., 2012a). In our study v52-54 not only displayed the highest occurrence of vertebral deformity, but also the greatest dietary effect on vertebral strength and morphology (L:H ratio). In general, diploids had the strongest bone properties in each region, whilst triploids fed the standard diet generally had the weakest, although differences were generally non-statistically significant. Decreased bone mineralisation and increased vertebral deformities are considered features of a sub-optimal diet (Fjelldal & Hansen, 2010) that manifest as reduced vertebral strength (Fjelldal *et al*., 2009b),

which was clearly evident in triploids fed the standard nutrient package. Furthermore, triploids also had higher L:H ratios than diploids in R1-R3 indicative of more elongated vertebral bodies within these regions, as is observed in triploid yearling (S1+) smolts (Fraser *et al.,* 2014). In addition, L:H ratio was also affected by diet, being significantly higher in triploids fed the standard diet compared to those fed the supplemented diet in R3. Such changes in vertebral morphology could be attributed to inadequate 'thickening' and outward mineralisation of the vertebral column alongside inadequate mineralisation under nutritional deficiency.

Hydroxyapatite is the key mineral structure in bone and its formation is limited through dietary P and Ca, which is non-limiting as it is taken directly from the aquatic environment (Lall & Lewis-McCrea, 2007). NRC (2011) recommendations for dietary P for diploid Atlantic salmon are estimated at 8 g available P Kg^{-1} with other research suggesting 10 g available P Kg^{-1} to be optimal for skeletal development (Asgard & Shearer, 1997). In seawater, Gil-Martens *et al.,* (2012) failed to observe a reduction in vertebral deformity in diploid post-smolts using 6 g or 9 g available P kg^{-1}, whereas Fjelldal *et al.,* (2012a) found a reduction in vertebral deformity in diploid smolts when previously fed 11.7g available P Kg^{-1} as opposed to 6.3g and 8.9g Kg^{-1}. In a more recent study on triploid smolts, vertebral deformity was prevented when fed 12 g available P Kg^{-1} rather than 4 g or 6 g Kg^{-1} (Fjelldal *et al.,* 2015). These observations are in agreement

with Helland *et al.,* (2005) who previously suggested that commercial levels (<10 g total P Kg^{-1}) may be too low for fast growing salmon to maintain skeletal integrity. In the current study we observed clear beneficial effects on improved spinal health in triploid post-smolts by increasing total dietary P by +20% (9.9 vs. 12 g total P kg^{-1}). Available P was not disclosed in this trial owing to a commercial confidentiality agreement and so, although requirement cannot be verified, it is clear that triploid skeletal health may benefit from higher dietary P inclusion.

P is not only important for bone growth but also plays an essential role in many anabolic, catabolic and metabolic processes, and is also a component of important molecules such as ATP, DNA, RNA and cell-membrane phospholipids (Burke *et al.,* 2010). Maintenance processes taking precedence over bone mineralisation (Lall & Lewis-Mcrea, 2007) offer a possible explanation of the high level of deformities in the triploid standard diet at the end of the trial. Although severity of deformity was reduced in triploids fed the nutrient boosted package, vertebral mineral content, P and Ca levels were in general lower than in fish fed the standard diet suggesting that improvement of vertebral integrity through P supplementation is not simply through accumulation. Minerals such as P may be used preferentially to facilitate higher growth rates in triploids fed the boosted diet without compromising bone strength or stiffness. Higher levels of V, a known biometal suppressor of Extracellular Matrix (ECM) mineralisation (Tiago *et al.,*

2008) were also observed in triploids fed the boosted diet compared to the standard package diet. This suggests active suppression of bone mineralisation and therefore increased availability in osteogenic compounds in fish fed the boosted diet, however this needs to be verified. In the case of triploids fed the standard diet, deformed fish had significantly higher weights at harvest suggestive of spinal deformity being a factor of fast growth under nutrient deficient conditions. Collectively these results indicate that the nutrient boosted package appears to facilitate better spinal mineralisation during development which would otherwise be compromised at the expense of accelerated growth under a standard diet.

Irrespective of dietary treatment virtually all triploid flesh quality attributes were comparable to the diploid control and concur with other studies in triploid Atlantic salmon (Taylor *et al.,* 2013). However, in this study we did observe a significant reduction in total pigment in triploids relative to diploids. Differences in pigment and other flesh quality attributes at harvest may also be highly influenced by season. Improved pigment retention through reproductive arrestment has often been cited as a potential benefit for producers of triploid salmon, although this has so far only been shown in rainbow trout (Choubert & Blanc, 1989; Choubert *et al.,* 1997). However, fish in our study were harvested in February and would not be expected to be entering into an active gonadal development at this stage. Therefore we cannot relate these pigment

differences to differing maturation rates between ploidy. A positive relationship between visual colour score and muscle fibre density independent of chemical pigment content has been reported in Atlantic salmon (Johnston *et al.,* 2000), however, Bjørnevik *et al.,* (2004) concluded that differences in muscle fibre structure between ploidy are not a major factor influencing flesh redness. However, it was noted that texture may affect fillet redness, which is concurrent with our study where a decrease in pigment content with increased texture score, albeit non-significant. Reduced pigment deposition may also stem from the decreased surface area to volume ratio and/or binding affinity of triploid cells and requires further study to elucidate differences between ploidy.

In conclusion, feeding increased dietary supplementation of protein and P results in a higher growth rate in triploids compared to feeding a standard SW diet formulated for diploids. Furthermore, development of vertebral deformity from SW transfer (i.e. smolt) until harvest was stabilised by increasing dietary P and / or protein supplementation. However, the incidence of deformity observed at time of sea transfer still remains above ethically acceptable levels and supports reports in other studies (Fjelldal *et al.,* 2015). Origin of deformities should be addressed prior to SW transfer potentially through regulation of egg incubation temperature and FW diets. Finally, this study also provided anecdotal evidence to support similar survival response to disease outbreak between diploids and triploids. However, reduced performance was observed

under simultaneous elevated temperature and reduced oxygen saturation. Collectively, this study makes significant contributions towards improving triploid welfare standards and achieving viable commercial implementation.

Chapter 3: Dietary freshwater phosphorous requirement of triploid Atlantic salmon (*Salmo salar* L.) is different to diploid with reference to early skeletal development.

Abstract

In order to assess the effect of dietary P in reducing vertebral malformations and improving FW performance in triploid Atlantic salmon, both triploid and diploid Atlantic salmon (*Salmo salar*) were fed three different P inclusion levels (low: 13.0, medium: 16.7, and high: 19.7 total P g Kg^{-1}) from first feeding until smolt. Somatic and skeletal response was assessed at fry (~0.5 g), early parr (~5 g) and smolt (~45 g) stages. Triploid parr initially grew faster on the high P diet but low P yielded significantly higher weights at smolt. Low P triploid fry had less well mineralised vertebrae revealed by Ca staining and significantly more deformed vertebrae in the parr and smolt stages evident by x-radiography. Triploid parr fed high and medium P had similar numbers of deformed vertebrae relative to their diploid counterparts but significantly more at smolt. However, triploids fed high P were the only treatment with no severely deformed vertebrae (≥10) in both stages. Low P triploids had the most jaw and vertebral deformities as well as a higher prevalence of deformed vertebrae in the central caudal vertebral region, which was more pronounced at parr than at smolt. Shorter vertebrae dorso-ventral lengths were observed throughout the spinal column in low P parr and only in the caudal region at smolt. Down-regulation of osteogenic factors *alp*, *opn* and *igf1r* expression in triploids and phosphate homeostasis protein *fgf23* in low P diets suggest active suppression of

mineralisation in high P treatments and reduced osteogenic potential in triploids respectively. Comparisons between development stages indicate early P supplementation in triploids is crucial for skeletal development and will aid improvement of dietary regimes in triploid salmon. Ultimately, reducing vertebral deformities observed at smolt with higher P supplementation in triploids would contribute towards improving skeletal performance in the marine phase.

1 Introduction

Sustained occurrence of vertebral and jaw deformity has hindered wide-scale commercial adoption of triploid Atlantic salmon (Fraser *et al.,* 2012a). In chapter 2, it was highlighted that vertebral deformities present at the end of FW rearing may be responsible for high vertebral deformity prevalence in the marine phase. Hence, nutritional requirements in FW rearing of triploid Atlantic salmon must be addressed. Repeated observation of lower K in triploid Atlantic salmon in FW (Taylor *et al.,* 2011 , 2012) indicate increased deposition of skeletal components over muscle which, alongside increased skeletal deformity prevalence, emphasise the importance of nutrition in triploid skeletal development. P, a primary nutritional risk factor for skeletal development in Atlantic salmon (Asgard & Shearer, 1997; Fjelldal *et al.,* 2012a) and fish in general (Lall & Lewis-McCrea, 2007), is also essential for RNA, DNA, ATP and cell membrane phospholipids. Alongside Ca it is the main mineral component of bones, teeth and scales and must be met by diet in fish (Lall, 2003). The minimum recommended requirement for P in salmonids is 8 g total Kg^{-1} (NRC, 2011) and if diploid Atlantic salmon juveniles are fed levels below 10 g available P kg^{-1}, then long term effects of impeded growth, poor spinal mineralisation and increased prevalence of skeletal deformities have been observed (Asgard & Shearer, 1997; Fjelldal *et al.,* 2009b*,* 2012a). Burke *et al.,* (2010)

tested dietary P inclusion (7.6, 9.9 and 13.9 g total P Kg^{-1}) for 12 weeks in Atlantic salmon (+45 g) and showed triploids grew faster on the lower inclusion; however, skeletal development was not assessed. Fjelldal *et al.,* (2015) clearly showed a reduction in skeletal deformities at SW transfer and harvest in triploid Atlantic salmon when fed increased dietary P inclusion in FW (16.3 *vs.* 7.1 g total P Kg^{-1}). Together with high observations of vertebral deformities at sea transfer in triploid smolts in chapter 2, these results suggest that FW developmental stages in triploids require higher dietary P inclusion for skeletal development.

Hydroxyapatite formation is dependent on PO_4^{3-} and Ca^{2+} homeostasis which is systemically regulated primarily through circulating PTH, [1,25(OH)2D] and FGF23 (Martin *et al.,* 2012). These factors coordinate PO_4^{3-} intestinal absorption, remodelling in bone and excretion in kidney according to demand and availability (Fig. 1.5). Little research on these P-bone homeostasis pathways has been conducted in salmonids, however, increased kidney Npt2a activity in rainbow trout is observed under periods of dietary PO_4^{3-} restriction (Sugiura *et al.,* 2003). FGF23 secretions are directly induced through expression of *fgf23* from osteoblasts and osteocytes, the mineralising osteogenic cells in bone, and promote renal PO_4^{3-} reabsorption with coordination of [1,25(OH)2D] (reviewed in: Martin *et al.,* 2012). FGF23, had never been studied in Atlantic salmon at the time of this study and, given its known importance in P homeostasis in bone,

investigation of diploids and triploids fed varying dietary P may indicate how P is regulated in terms of osteogenic activity between ploidy. In addition, ALP and OPN are both markers for extracellular mineralisation around osteoblasts. ALP provide osteoblasts with PO_4^{3-} by dephosphorylating exogenous β-glycerophosphate (Beck *et al*., 2000; Pombinho *et al*., 2004). In turn, the presence of free PO_4^{3-} stimulate OPN secretions to the osteoblast ECM to facilitate osteogenic function (Beck *et al.,* 2000). As such, ALP and OPN are collectively, strong indicators of osteogenic activity in the presence of free PO_4^{3-} and may also be useful markers to determine ploidy differences in response to dietary P.

Vertebral mineralisation can be compromised during temperature induced accelerated growth (Ytteborg *et al.,* 2010a). Thus, vertebral deformities observed in faster-growing triploid Atlantic salmon (Leclercq *et al.,* 2011; Taylor *et al.,* 2012) suggest dietary P requirements may be greater than their diploid counterparts. Faster growth in diploids coincide with increased bone density and expression of *igf-Ir* in bone (Wargelius *et al.,* 2005a), where local expression may be associated with initiating ECM production. Hence, accelerated growth factors anticipated in triploid FW growth may be impacting regulation of mineralisation of the ECM. Environmental factors that induce accelerated growth in diploid Atlantic salmon, such as high temperature, have also led to vertebral fusions and associated upregulation of *MMP-13* (Wargelius *et al.,* 2010) and

downregulation of osteogenic marker *Col1a1* (Ytteborg *et al.,* 2010a). MMP-13 is a matrix metalloproteinase which is involved in the degradation of the ECM associated with chronic inflammatory response characterised by bone remodelling and deformity. These markers may be useful in elucidating mechanisms for deformity in triploids, given their known reduced thermal tolerance (Atkins & Benfey, 2008). In addition, alterations in triploid cell size: frequency ratios, DNA regulation and gene expression owing to additional genomic material alongside accelerated growth, may influence P homeostasis in bone of Atlantic salmon and subsequent correct skeletal development.

The present study aims to further investigate dietary P supplementation in diploid and triploid Atlantic salmon siblings with an emphasis on impacts at three FW life stages including fry, early parr and smolt. Growth performance, skeletal deformity development, mineral concentration and mRNA expression of key bone homeostatic genes were analysed.

2 Methods and materials

2.1 Fish Stock and husbandry

On January 19, 2012, sibling groups of diploid and triploid Atlantic salmon eggs (Atlantic QTL-innOva® IPN) were supplied from AquaGen (Norway) to Howietoun Fish Farm, Stirling (56˚N, 4˚W) at 395 degree days post-fertilisation (°DPF). Triploidy was induced

using a hydrostatic pressure shock of 9500 psi applied 300 ° minutes post fertilisation for 50 ° minutes at 8 °C (Taylor *et al.,* 2011). From fertilisation to point of shipping ova were incubated at 6.0 ± 0.5 ˚C. 63, 000 eyed eggs were equally split between 12 x 250 L tanks (6 per ploidy) and reared under constant darkness at 8.7 ± 1.0 °C until first feeding (929 °DPF; March 26, 2012). Fry were transferred at 1387 degree Days Post Fertilization (˚DPF; ~0.43 g) to the Niall Bromage Freshwater Research Facility (NBFRF), Stirling (56˚N, 4˚W) where they were maintained in 12 x 980 L covered circular tanks. Fish were reared under LD24:0 until August 31, 2013 followed by simulated natural photoperiod (SNP) and ambient water temperature (Fig. 3.1) until smoltification (~45g 3321 ˚DPF, April 24, 2013). All fish were vaccinated with PHARMAQ Alphaject 2.2 on February 26, 2013. Ploidy status was verified according to protocols in chapter 2 on blood collected from fish weighing 5g (n = 100 / ploidy). Diploid control groups had significantly smaller erythrocyte nuclear lengths with no overlaps with triploid groups (2N 7.3–7.5 μm; 3N 9.2–10.1 μm) confirming the likelihood that pressure shocked fish were triploid, to be absolute.

2.2 Experimental setup and sampling protocol

Duplicate groups of diploid and triploid fish were fed from first feeding until smoltification, (which was confirmed through skin silvering assessment: Sigholt *et al.,* 1995), one of three different diets using clockwork belt feeders according to

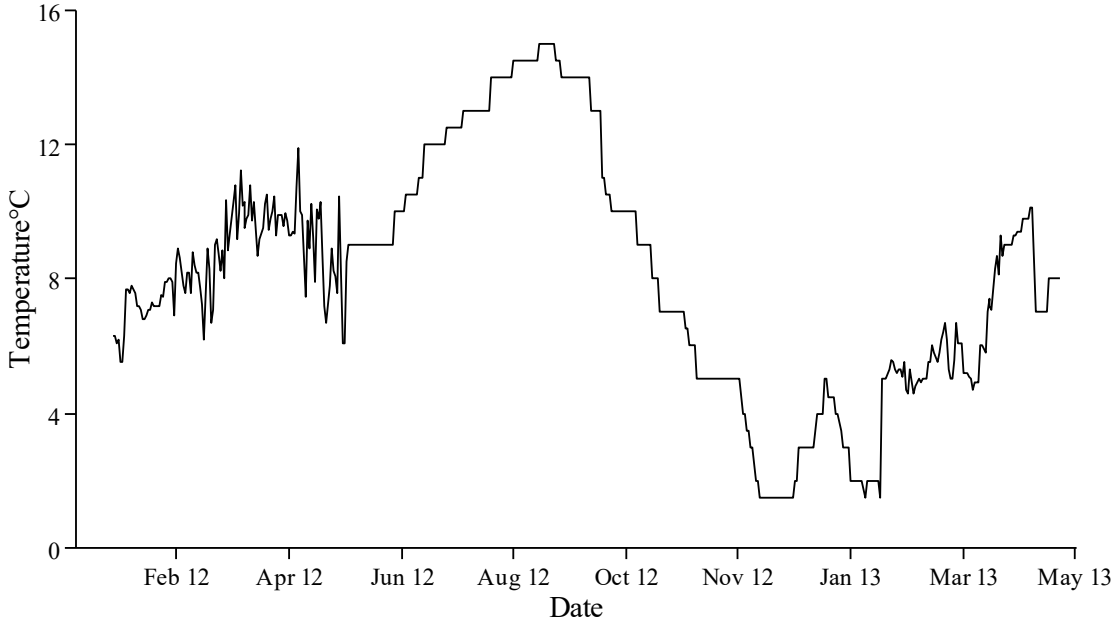

Figure 3.1. Temperature (°C) from the beginning of the trial (January 2012) until smoltification (June 2013).

manufacturer's tables. Each diet was formulated by BioMar UK Ltd (Grangemouth, Scotland) and contained graded levels of dietary P: low P (LP), medium P (MP) and high P (HP; Table 3.1). Growth parameters including: fork length (±1 mm), weight (±0.1 g), K and externally visible deformities (spinal and jaw), were assessed on a monthly basis following anaesthesia (MS222, 0.25 g mL^{-1}) of randomly selected fish (50 / tank, n=2). Whole fish samples were taken at 5 g (parr; n = 50 / tank) and 45 g (smolt; n = 35 / tank) through lethal anaesthesia (>200 mg L^{-1}), and stored at -20 °C until x-radiography and whole body mineral concentration analysis. An additional 10 fish per treatment (n = 5 / tank) were euthanised through lethal anaesthesia (MS222; >200 mg L^{-1}) and fixed in 10 % neutral buffered formalin at 3 and8 weeks post first feeding for whole mount staining. At the parr and smolt stages 12 fish per treatment (n = 6 / tank) were also euthanised

(MS222; >200 mg L^{-1}) and vertebral sections were taken from the central region below the dorsal fin (vertebra 18-32), which is known to have higher susceptibility to deformity development (Fjelldal *et al.*, 2015; Fraser *et al*., 2015a), and stored in RNA later at -20 °C for gene expression analysis. All experimental procedures and husbandry practices used in the present study were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice) in accordance with EU regulation (EC Directive 86/609/EEC) and approved by the Animal Ethics and Welfare Committee of the University of Stirling.

Table 3.1. Formulation and proximate composition of experimental feed pellets.

	LP	MP	HP
Formulation (%)			
Fish/Crustacean meal	51.49	53.68	53.23
Plant protein concentrates	23.83	20.35	19.73
Wheat flour	10.01	9.84	9.22
Fish oil	9.68	9.50	9.43
Rapeseed oil	2.86	2.80	2.76
Monosodium phosphate	0.36	2.01	3.81
Additives*	1.77	1.81	1.82
Proximate analysis (%)			
Moisture†	6.45	6.73	6.42
Oil†	19.34	19.64	18.77
Protein†	52.16	51.51	51.21
Ash†	7.87	9.03	10.20
Phosphorous(total Kg^{-1})‡	13.0	16.7	19.7
Calcium (total Kg^{-1})‡	11.7	11.6	11.7
Calcium: Phosphorous‡	0.89	0.69	0.59

*BioFish Premix - non commercially available: combination of essential vitamins, minerals and amino acids.
†BioMar, Grangemouth, UK
‡University of Stirling, UK.

2.3 Vertebral assessment

2.3.1 Whole mount staining for bone and cartilage

Fry (3-8 weeks post first feeding, ~0.5 g) fixed in 10 % NBF were double stained according to Potthoff (1984) for cartilage (alcian blue, 8GX: Sigma Aldrich, USA) and hydroxyapatite (alizarin red S sodium salt: Alfa Aesar, USA). Stained specimens were photographed using a light tent and table with a Nikon 300S (60 mm F 2.8 lens) and digital images were analysed using Image J (Image J 1.46r, NIH, USA) in which Ca stain as an indicator of vertebral area was also assessed according to four regions (R1, v1-8; R2, v9-30; R3, v31-50; R4, v51-58) as defined in Kacem *et al*., (1998).

2.3.2 Vertebra radiological assessment

Lateral view radiographs were taken at early parr (5 g) and at smolt (45 g). At 5 g, radiographs were taken using mammography x-ray (Bioptics BioVision, Daax Ltd, USA; calibration: kV: 22, Exp: 15s, mAs: 22.52) and at smolt using a standard portable x-ray unit (Celtic SMR PX40 HF; calibration: kV: 40, mAs: 32) with an extremities plate measuring 24 X 30 cm, and subsequently digitised using an AGFA CR35-X Digitizer. Images were analysed for total vertebrae number, deformity classification (Witten *et al.,* 2009) and length: dorso-ventral diameter ratios in DICOM format in Image J (Image J 1.46r, NIH, USA).

2.3.3 Bone mineral

Selected vertebrae were removed from the 4 functional regions at smolt (5 fish / tank): R1 v3-5, R2 v19-21, R3 v39-41, and R4 v53-55 as defined in Kacem *et al.,* (1998) and analysed for bone mineralisation (BM; Deschamps *et al.,* 2008). Neural and haemal arches were removed and individual amphicoelus centra had remaining flesh removed, then defatted in an agitated isohexane bath for 24 hours, rinsed with distilled water, oven dried for 24 hours at 105 °C, weighed (W_{dry}) and incinerated in a muffle furnace at 600 °C for 16 hours and reweighed (W_{ash}). BM (%) was calculated as: W_{dry}/W_{ash}*100. The dentary bone and mandible, or lower jaw, was also processed for BM%. Resulting ash was processed for mineral content by adding 5 ml of AristAR® nitric acid (HNO3; VWR International, USA) to the sample and digested in a MarsXpress digestion system set to 10 min heating phase to 160 ˚C, 29 min. at 160 ˚C and 30 min cooling phase to room temperature. Digested samples were diluted to 2 % HNO3 and elemental concentration analysed with a Thermo X Series II ICP-MS collision cell model for P, Ca, Zn, Mg and V.

2.4 Mineral content and proximate analysis

At parr (~5g) and smolt (~45g) five whole fish were homogenised in triplicate per tank (15 / tank) for 5 minutes in an industrial blender before a subsample (~15 g) was oven dried at 105 °C for 24 hours. Dried samples were divided into 3 technical replicates of

0.1 g for mineral content analysis (section 2.3.3). The same fish homogenate was used for proximate analysis. Moisture (%) was determined through calculating wet weight difference following air-drying in an oven set to 105 ˚C for 24 hours. Ash (%) was calculated through calculating the weight difference after drying in porcelain crucibles in a muffle furnace at 600 ˚C for 16 hours. Oil, or crude lipid, was determined by acid hydrolysis followed by Soxhlet extraction (Woyewoda *et al.,* 1986) and crude protein was determined by combustion using the Dumas process (Association of Official Analytical Chemists, 1990).

2.5 Gene expression

2.5.1 RNA isolation and cDNA synthesis

Only LP and HP diets were assessed for gene expression as they were considered most likely to show differences. Vertebral sections at parr and smolt were manually cleaned of soft tissue using a scalpel blade and total RNA extracted according to the Trizol protocol (Invitrogen, UK) and rehydrated in 12 µl of MilliQ water. Quality and concentration was validated with Nanodrop spectophotometer ND-1000 (Labtech Int., East Sussex, UK) and 1 % agarose denaturing RNA gel electrophoresis. 5 µg totRNA was treated with DNase enzyme (DNA-free™: Applied biosystems, UK), concentrations revalidated with nanodrop and 1 µg of subsequent totRNA was reverse transcribed to cDNA using a high

capacity reverse transcription kit without RNase inhibitor (Applied biosystems, UK). Final cDNA 20 µl reactions were diluted 1:10 in DNA/RNA free water to a total volume of 200 µl and 5µl was used for each 20 µl qPCR reaction.

2.5.2 Sequence information and primer design

Sequence specific primers for *mmp13*, *igf-Ir*, *alp* and *col1a* were based on registered sequence information in Atlantic salmon from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). All available sequence information were subjected to BLAST analysis against an Atlantic salmon transcriptome and genome (NCBI). Forward and reverse primers are outlined in Table 3.2 and were manufactured by MWG Eurofins Genomics (Ebersberg, Germany). Each primer pair was verified by PCR using MyTaq^{TM} Mix (BIO-25041; Bioline) and produced a single band on a 1 % agarose gel indicating a single product was available for sequencing. All products were subsequently purified using GeneJET PCR Purification Kit (KO701; ThermoFisher Scientific) and sequenced using Light Run sequencing service (GATC, Cologne). Resulting qPCR fragment sequences were verified with BLASTn and were suitably aligned with the target sequences.

For *opn,* nucleotide sequence information was available for a number of fish species (Table 3.3), however, T BLAST x in Atlantic salmon Expressed Sequence Tags (EST) and Whole Genome-Sequence contigs (WGS) databases yielded no appropriate

Table 3.2. Primer sequences including housekeeping (HK) gene along with annealing temperatures and accession number used for qPCR.

Primer Name	Forward	Reverse	Annealing Temp (˚C)	Reference	Accession No.
ALPQ1	ATCCTGCTCATC TGCTCCTGC	AGTATTCGTGCT GCCGTCACT	56	(Ytteborg *et al*., 2010b)	Fj195609
Col1a1Q1	TGGTGAGCGTG GTGAGTCTG	TAGCTCCGGTGT TTCCAGCG	56	(Ytteborg *et al*., 2010b)	Fj195608
Fgf23Q1	TCATCCAGCTCC GGCATAGC	AAGAACACGGT GCCACTGGA	57		Not registered
Igf1rQ1	AGCCACCTGAG GTCACTACG	ACATCCCGTCCG CTATCTCC	58	(Wargelius *et al*., 2005a)	AY049954
Mmp13Q1	CCAACCCAGAC AAGCCAGAT	GCTCTGAGAGT GGATACGCC	56	(Wargelius *et al*., 2010; Ytteborg *et al*., 2010a)	NM_001140524.1
HK Gene					
Elf-αq1	TCTGGAGACGC TGCTATTGTTG	GACTTTGTGACC TTGCCGCTTGAG	58	(Ytteborg *et al.,* 2010a)	NM_001123629.1

Table 3.3. Protein and DNA sequence searches for *opn* and DNA sequence searches for *fgf23* from based on searches in NCBI and Ensemble (*; www.ensembl.org).

Species	Accession no. DNA	Accession no. protein
opn		
Danio rerio (Opn)	AY241929.1	AAP72989.1
Oncorhynchus mykiss (Opn like)	AF204760.1	AAG35656.1
Salvelinus fontinalis (Opn like)	Af223388.1	AAG49534.1
Sparus aurata (Opn like)	Ay651247.1	AAV65951.1
*Gadus morhua**	ENSGMOT00000010210	
fgf23		
Danio rerio	BC162711.1	
Astyanax Mexicanus	xm007256048.2	
Osmerus mordax	BT074626.1	
Lepisosteus oculatus	xm006633715.2	
Tetradon nigroviridis	ay753223.1	

alignments. Nucleotide sequences for various species were aligned by ClustalW and revealed little conservation in sequences between species. Hence, sequences were converted to the corresponding protein sequences and then aligned again by ClustalW, which revealed conserved regions with reference to brook trout (Fig. 3.2): Region 1 = Protein 1 – 16 (136 – 183 nucleotide); Region 2 = Protein 150-184 (584-587 nucleotide); Region 3 = Protein 215-232 (780 – 683 nucleotide); Region 4 = protein 310-326 (1065-1113 nucleotide). Four primer pairs were designed in the corresponding nucleotide

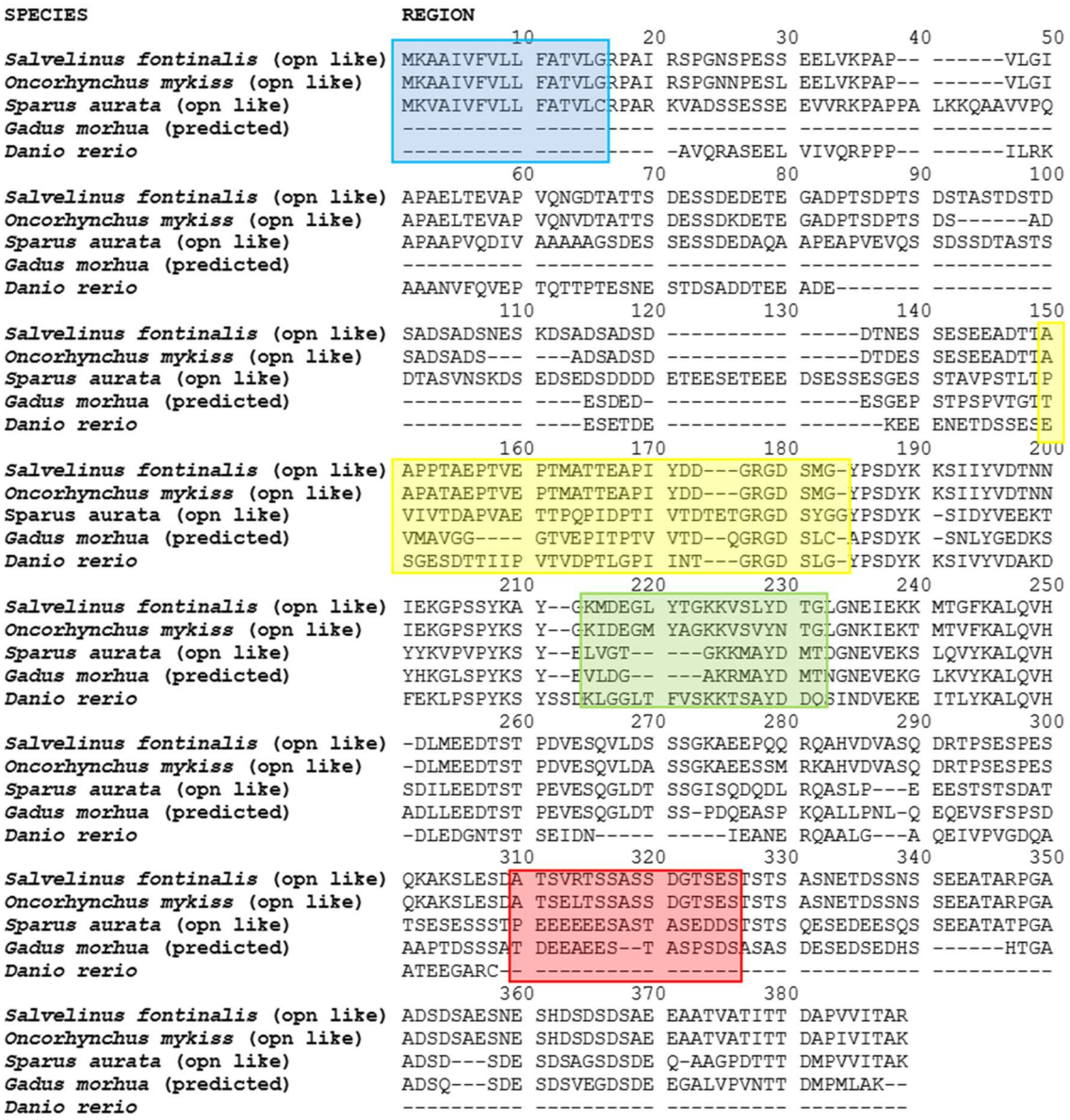

Figure 3.2. Protein sequence alignments with ClustalW based on original nucleotide sequences for brook trout, rainbow trout, seabream, Atlantic cod and zebrafish respectively (accession numbers provided in Table 3.3). The four regions with high protein sequence conservation with reference to brook trout are highlighted accordingly (region 1: blue, region 2: yellow, region 3: green, region 4: red).

sequences in brook trout (Table 3.4). Seven PCR reactions were carried out and sequenced using *LIGHTrun*™ Sequencing (GATC, Cologne, Germany) resulting in a 908 bp consensus sequence being generated for salmon. BLASTn results revealed 83% and 91% identity with brook and rainbow trout sequences. T BLAST x revealed a mean of 68%, protein identity with zebrafish. qPCR primers were then designed within this

Table 3.4. Primer pairs (*opn* and *fgf23*) based on brook trout (Af223388.1) and Atlantic salmon (agkd01034860) nucleotide sequences respectively. Fragment position for *opn* correspond to the brook trout nucleotide sequence.

Primer		Forward	Reverse	Position	Length
***opn* (Af223388.1)**					
1	ost coding 1-4	AGGCTGCAATTG TTTTCGTCC	TGAGTCGCTGT CTGCTGC	140-1110	971bp
2	ost coding 1-2	TGCTCTTTGCAA CGGTCCT	CTTCTTGTAGTC GCTGGGGT	161-595	467bp
3	ost codong 1-3	TGCTCTTTGCAA CGGTCCTC	TGCTGGTGTCCT CCTCCAT	161-805	663bp
4	ost coding 2-3	ACCCCAGCGACT ACAAGAAG	TGCTGGTGTCCT CCTCCATC	608-823	216bp
5	ost coding 2-4	CGTGGAGACAGC ATGGGTT	TGCTGAGTCGC TGTCTGC	589-1113	525bp
6	ost coding 3-4	TTGATGGAGGAG GACACCAG	TGAGTCGCTGT CTGCTGC	802-1110	309bp
7	ost coding 2	GGACGTGGAGAC AGCATGG	AGGCTTTGTAA GAGGAGGGTC	586-685	100bp
***fgf23* (agkd01034860*)**					
1	fgf23	AGACATCTGGGA TGAGGGAC	TGGGGTTTGTTT CCAGACCA	-	-
2	fgf23	TGCTGCAGAATT CAAAGCTACAT	TTGGGGTTTGTT TCCAGACCA	-	-

*Construct has been removed and the current relevant construct is AGKD04000268.1 corresponding to the predicted *fgf23* sequence XM_014153467.1 in the Atlantic salmon transcriptome.

fragment.

Sequence information was available for *fgf23* in a number of fish species (Table 3.3) and BLASTn revealed significant results in Atlantic salmon WGS (agkd01034860) but no salmon EST. Atlantic salmon WGS results were aligned to other teleost sequences (Table 3.3) and displayed the highest identity to rainbow smelt (*Osmerus mordax;* Fig. 3.3). PCR primers for *fgf23* were based on the WGS sequence (agkd01034860), which was trimmed appropriately according to alignments in order to eliminate 5' and 3' regions and introns (Table 3.4). All PCR reactions were sequenced by *LIGHTrun*™ Sequencing (GATC, Cologne, Germany) and results showed the highest identity with rainbow smelt and zebrafish. Resulting qPCR primers designed are found in Table 3.2.

```
                                10         20         30         40         50
AGKD01034860                    GTTTGCAAAC AGTTTGCTGC AGAATTCA-A AGCTACATCC GAAATCTCTG
Salmo salar                     ---------- ---------- ---------- ---------- ----------
Osmerus mordax                  GGGGACACAG AATCAGCTGA AGTTTCCCCA AGCTTGCTGT AGAATTCAAC
Danio rerio                     -----TAAAT AGCCGCGCTC ACGAACTTTT ACGCATCACA ACTTCTTCAG
Astyanax mexicanus              ---------- ---------- ---------- ---------- ----------
Lepisosteus oculatus            ---------- ---------- ---------- ---------- ----------
Tetradon nigroviridis           ---------- ---------- ---------- ---------- -------CAT
                                       60         70         80         90        100
AGKD01034860                    TGATACATTT TCAG------ -TGTTCTTTG GGTAAAA--G ATGGAAATAA
Salmo salar                     ---------- ---------- ---------- ---------- ----------
Osmerus mordax                  TTATACGTTT CTGGATTCAA CCTTCACTCT TTTTAAG--A ATGGAAGGAA
Danio rerio                     CTCTGCACAC CCGG-CTTTA CGCGCTCTGT CAAGATGCGT TGCGCACTTT
Astyanax mexicanus              ---------- ---------- ---------- ----ATGCGT AAAGGCGCTC
Lepisosteus oculatus            ---------- ---------- ---------- ---------- ----------
Tetradon nigroviridis           CCGAAAACTT CCAA---CAG CTCTCACCCG TTTTGCTCCA ATGGACGTAA
                                       110        120        130        140        150
AGKD01034860                    CCAAGACATC TGGGATGAGG GACGCCGTCT TGGTGCTCTT ACTGGCTGTT
Salmo salar                     ---------- ---------- ---------- ---------- ----------
Osmerus mordax                  GAAAGAAATA TGGAATGAGG AACATAGTCC TCATGCTCCT ACTGGCTTTG
Danio rerio                     CCAACCTGCA CATGCTGCAT TCATCCGTCC TCGCGCTGTG GTTCACGGCT
Astyanax mexicanus              CCAGCCTGAA C---CTGCAC TCGC---TGT GTGCGCTGTG GCTCGCGGCG
Lepisosteus oculatus            ---------- ----ATGCAC TCGTGTTTCC TTGCGCTGTG CCTGGCTGTG
Tetradon nigroviridis           ACAGAAGGAT CGGGGTGAAG GACGCCTTGC TGGCGCTCCT GCTCGCCCTT
                                       160        170        180        190        200
AGKD01034860                    CTTCAGGGAT TTCGACTTGT GGATGCCCTT CCAAACCCAT CACCTCTCCT
Salmo salar                     -------GAN TTCGACTTGT GGATGCCCTT NNAA-CCCAT CACCTCTCCT
Osmerus mordax                  CTACAGAGAT TCCGACTTGT GGATTCTCTT CCAAACCACT CTCCACTTGT
Danio rerio                     CTCCAGGGAC TCAGACCTGC AGATGCGGCC CCCAATCCTT CTCCGCTGCT
Astyanax mexicanus              CTGCAGGGAT GTGCCCCCGC GGACGTAGCT CCAAACACAT CTCCTCTGCA
Lepisosteus oculatus            TTGCAGGGTT TGAAGATTGT GCATTCATTC CCTAATCCAT CCCCGCTCCT
Tetradon nigroviridis           CTCCAGGGAT GCCCCCTGGG AGAAACGGCT CCCAACGCGT CACCGCTGGT
                                       210        220        230        240        250
AGKD01034860                    GGGATCCAAC TGGGGGAATC CGAGAAGATA CGTACACCTG CAGACGTCTT
Salmo salar                     GGGATCCAAC TGGGGGAATC CGAGAAGATA CGTACACCTG CAGACGTCTT
Osmerus mordax                  TGGATCCAAC TGGGGGAATC CAAGACGATA CGTGCACCTG CAGACATCGA
Danio rerio                     GGGCTCCAAC TGGGGGAACC CGCGGAGATA CATCCACCTT CAGACCACTT
Astyanax mexicanus              GAGCACCAAC TGGGGCAACC CGAGAAGATT CATCCACCTG CAGACCAGCA
Lepisosteus oculatus            CAGCTCCAAC TGGGGCAACC CGAAGAGATA CGTGCACCTG CAGACGTCTT
Tetradon nigroviridis           CGGTTCCAAC TGGGGGAACC CGAGGAGGTA CGTTCACCTT CAGACATCCA
                                       260        270        280        290        300
AGKD01034860                    CAGACGTGAA CAATTTCTAC CTTGAGATCA GTTTAAATGG CCACGTGCGC
Salmo salar                     CAGACGTGAA CAATTTCTAC CTTGAGATCA GTTTAAATGG CCACGTGCGC
Osmerus mordax                  CGGACCTAAA CAATTTCTAC CTTGAGATTA GTTTGAATGG CCATGTGCGT
Danio rerio                     CAGACTTAAA CAACTACTAC CTGGAGATCA GCCCGAGTGG ACACGTGCGC
Astyanax mexicanus              CAGACCTCAA CAACTTCTAC CTGGAGATCA GTCTGAATGG TCATGTACGC
Lepisosteus oculatus            CAGAAACCAG GAGCTTTTAC CTGGAGATCG ATGAGGATGG GCAAGTGGGA
Tetradon nigroviridis           CAGACATGAG CAACTTCTAC TTGGAGATCA GACTGGATGG AACCGTGCGC
                                       310        320        330        340        350
AGKD01034860                    AAAACTACAC TTCGAAGCTC ATACAGTG-- ATTTTATTGA AGGCGGAAAC
Salmo salar                     AAAACTACAC TTCGAAGCTC ATACAGTGTG ATTTTATTGA AGGCGGAAAC
Osmerus mordax                  AAAACTACCG TTAGAAGTTC TTACAGTGTA ATTTTGATGA AGGCTGAAAC
Danio rerio                     AAAACTACAA ATCGGGGCTC ATACAGTGTA ATCTTATTGA AAACAGAAAG
Astyanax mexicanus              AAAAGTGCGC GGCGAGGTTC TTACAGTGTT GTTCTACTGA AAGCCGAGAC
Lepisosteus oculatus            AAAACAGCCA CCCAGAACGC CTTCAGTGTG TTGCTACTCA AGGCGGAAAC
Tetradon nigroviridis           AAAAGCACAG CCCGGACTTC ATACAGTGTG ATTTTACTGA AAGCCGACAC
                                       360        370        380        390        400
AGKD01034860                    AAGAGAGCGC GTGGCGATAC TTGGAGTCAA AAGTAACCGC TACCTGTGCA
Salmo salar                     AAGAGAGCGC GTGGCGATAC TTGGAGTCAA AAGTAACCGC TACCTGTGCA
Osmerus mordax                  AAGGGAACGT GTTGCAATCC TTGGTGTCAA AAGTAACCGT TACTTGTGTA
Danio rerio                     CAGAGACCGT CTGGCGATAT TTGGAGTGAA AAGTAACCGG TTTTTGTGCA
Astyanax mexicanus              CAGAGACCGC GTGGCAATAT TCGGAGTGAA AAGTAGCCGG TTCTTGTGTA
Lepisosteus oculatus            AAGAGACCGT TTGGCGATCT TCGGAGTGAA AAGTAAACGC TTTCTGTGTA
Tetradon nigroviridis           GAGGGAGCGC ATCGCCATCC TGGGCGTCAA GAGCAACCGT TACCTGTGTA
```

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                          410        420        430        440        450
AGKD01034860              TGGATGCCCT GGGAAACCCT TTCAGCTC-A CCGTTTGCCA CAAGGAAGAC
Salmo salar               TGGATGCCCT GGGAAACCCT TTCAGCTCTA CCGTTTGCCA CAAGGAAGAC
Osmerus mordax            TGGATACAGT GGGTAACCCT TTCAGTTCTC CTATTTGCCA GAAGGATGAC
Danio rerio               TGGATACAGG AGGAACCCTT TTCACATCTA CGATCTGCAA TAAGGAAGAC
Astyanax mexicanus        TGGACGCAGA GGGGAATCTT TACACTTCCA CGGTTTGCAA TAGAGATGAC
Lepisosteus oculatus      TGGATGCAGA AGGAAAGACT TTCACCTCGA CCATCTGCAA CAAAGAGGAC
Tetradon nigroviridis     TGGACCTCGA GGGGAGCCCA TTTAGCTCTC CCACCTGCAT CAGGGACGAC
                          460        470        480        490        500
AGKD01034860              TGTCTTTTTA ACCACAAGTT ATTGGAAAAC CACCGCGACG TGTACTACTC
Salmo salar               TGTCTTTTTA ACCACAAGTT ATTGGAAAAC CACCGCGACG TGTACTACTC
Osmerus mordax            TGTCTTTTCA ACCACAAACT TCTTGAGAAT CACCGAGACG TATACTATTC
Danio rerio               TGTCTTTTCC ACCACAAACT GTTGGAAAAC CATCGTGATG TGTATTACTC
Astyanax mexicanus        TGTCTTTTCC ACCACAAGCT TCTGGAGAAC CATCGCGACG TGTACTACTC
Lepisosteus oculatus      TGCCTATTTC ACCACAAGCT TTTGGAAAAT AACCAAGATG TGTACTATTC
Tetradon nigroviridis     TGCTTGTTCA ACCACAGTCT TCTGGAGAAC AACCGGGACG TCTACTACTC
                          510        520        530        540        550
AGKD01034860              TTGCAGAACT GGTATTCTGC TCAACTTGGA AGGAATAAAG CAAGTGTACA
Salmo salar               TTGCAGAACT GGTATTCTGC TCAACTTGGA AGGAATAAAG CAAGTGTACA
Osmerus mordax            GTGCAGAACT GGCATCTTGT TTAACTTGGA AGGGATAATA CAGGTGTACG
Danio rerio               CACTAAACAC AGCATACTGC TTAATCTGGA CGGGGCCAAA CAGGCGTTTA
Astyanax mexicanus        CCCCAAGACC GGCCTCCTGC TCGACCTGGA CGGCGCGAAG CATCGATACA
Lepisosteus oculatus      CTGCAAGAAC AATCTGGTGC TCAACCTCGA AGGAGTCAAG CACATTTTTG
Tetradon nigroviridis     CAGCCGGACC GGCATTCTCT TCAACCTTGA GGGCTCCCGC CAGGTGTTCG
                          560        570        580        590        600
AGKD01034860              CTGTGGGCCA GAATCTACCG CAAACCTCCC TCTTCCTGTC GGAGAAGAAC
Salmo salar               CTGTGGGCCA GAATCTACCG CAAACCTCCC TCTTCCTGTC GGAGAAGAAC
Osmerus mordax            CCTTTGGTCA GAACCTACCA CAAACGTCCC TTTTCCTCTC GGAGCAGAAC
Danio rerio               TAGCGGGACA AAACCTCCCT CAGTCGTCTC TCTTCTTGTC GGAGAAGAAC
Astyanax mexicanus        CGGCCGGCCA GAACCTCCCC CGCTCCTCTC TGTTCCTGTC GGAGAAGAAC
Lepisosteus oculatus      TCCCTGGACA GAATCTCCCC GCCTATTCCC TGTTTCTGTC TGAAAAGAAC
Tetradon nigroviridis     TGGTGGGCCA GAACGTCCCG CAGACCTCCC TCTTCCTGCC CAGGACGAAC
                          610        620        630        640        650
AGKD01034860              ACGGTGCCAC TGGAGCG--- CCTCTTGCAC CGGGAGAAGA GAAACCGGGT
Salmo salar               ACGGTGCCAC TGGAGCG--- CCTCTTGCAC CGGGAGAAGA GAAACCGGGT
Osmerus mordax            ACGGTGCCGC TGGAGCG--- CCTCCTGCAC AGGGAGAAAA GAAACCGTGT
Danio rerio               ACGGTTCCGC TGGAGCG--- CCTGCAGCAC CGGGAGCGCA GGAACCGGCA
Astyanax mexicanus        ACGGTGTCGC TGGAGCG--- CCTGAAGCAC AGAGAGAGAA AGAACCGGCA
Lepisosteus oculatus      ACCATCCCAC TGGAACA--- CCTGTTGCAC AGGGACAAGA GAAATGGCCA
Tetradon nigroviridis     ACGGTGCCGC TGGAGCGACT CCTTCTGCAC AGGGACAAGC GGAACCAGGT
                          660        670        680        690        700
AGKD01034860              GGTTGACCCT TCTGATCCGT ACAACATGC- --TGGGTCAA ACGGAGGAGG
Salmo salar               GGTTGACCCT TCTGATCCGT ACAACATGC- --TGGGTCAA ACGGAGGAGG
Osmerus mordax            CGTTGATCCT TCCGATCCAC ACAACGTAT- --ATGGGCAG ACCGAAGAAG
Danio rerio               GGTGAACCCA ACAGACCCGC TGAACGCGC- --TCCGGTAC GCGGAGGAG-
Astyanax mexicanus        GGTGGAGCTC TCTGACCCGC TCAGAGCGC- --TCGGCTAT GAGGAGGAG-
Lepisosteus oculatus      TGTCGGTCCT TTGGAGTATA TCAGCCACT- --CAGACCAC GTCGACCCT-
Tetradon nigroviridis     GGTGGACCCC TCTGACCCGC ACCGCGTCGC CGTGGGTCGC GCCGAGGAGG
                          710        720        730        740        750
AGKD01034860              ATGAGGACTC CCGGGCTATG CCGGAGCTGG ATGACACCTT GGAGATGGA-
Salmo salar               ATGAGGACTC CCGGGCTATG CCGGAGCTGG ATGACACCTT GGAGATGGA-
Osmerus mordax            GCTCTGACTC CAAAGCGATG CCGGAG---G AGGACGCC-- -------GA-
Danio rerio               --TCTGATTC CAGAGCCGCG CAGGAG---G ATGATGGA-- -------GA-
Astyanax mexicanus        --TCAGACTC CCGCGCGGTG CAGGAG---G AGGACGCA-- -------GA-
Lepisosteus oculatus      --TCAGATCC CTTAGCTTTA TTCAAGCCCC GCAATATCTA TTACGGGGAA
Tetradon nigroviridis     GCTCGGACTC CCGGGCCTTG CAGGAGGACG ACGCCGACCT GGAGGTGGA-
                          760        770        780        790        800
AGKD01034860              --CCAGGAGG CTGAACTCCC CGAGGGACGC -------AAC ATCTCCCGGG
Salmo salar               --CCAGGAGG CTGAACTCCC CGAGGGACGC -------AAC ATCTCCCGGG
Osmerus mordax            --CCAGGAGG TTGAACTTCC AGAAGGGGCC G----TTAAC GTTTCCAGAG
Danio rerio               --CATGGATT TTGAGCCCTC AGAAGGTCAA -------AAC ATCTCTAGAG
Astyanax mexicanus        --GCAGGA-- ----GCCCTT TGAGGATCGC -------AAT ACGTCCAGAG
Lepisosteus oculatus      ACCTCCGACT CGCGGGCCAT CAACACCAAC CCAGAGCATG GTTTTAACGG
Tetradon nigroviridis     --GACAGAGG TTGAGGTCGG GGACGACGGA C----GCAAC GCGTCCCGGG
```

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                                 810        820        830        840        850
AGKD01034860                AGACCCCTCA GTCTCCCTCC G---ACGACC CGTGGAACG- --TGCATTCC
Salmo salar                 AGACCCCTCA GTCTCCCTCC G---ACGACC CGNGGAACG- --TGCATTCC
Osmerus mordax              AGACACCTCT GTCTCCATCC ACTCACGACC CCTGGAACG- --TGCATTCC
Danio rerio                 AAACCCTTGT TTCCCCTTCC GATGATGATC CATGGGATCT TCTGCACGAC
Astyanax mexicanus          AAGCCCTGCT CTCTCCTTCA GACGATGACC CGTGGGATGC GGTGCACGCC
Lepisosteus oculatus        AGAAGCTCAC GTTGTGTCCC GA--GAGAGC CTTGGACCGA CGTACAATGA
Tetradon nigroviridis       AGCGGCTGCA GGCTCCGTCC GATCACGACC CCTGGGGCG- --TGTTCTCC
                                 860        870        880        890        900
AGKD01034860                CTAAACCCCC CTCCCAGTCC CCGTATCATG AATGCAATGG TGGGATAAGA
Salmo salar                 CTAAACCCNN CTCCCAGTCC CCGTATCATG AATGCAATGG TGGGATAAGA
Osmerus mordax              ACCAACTCCA ACGTTAGCCC GCGTATTAC- --TGGAACGA TGGGATGAGA
Danio rerio                 ACGAGCCCTG GA---AGTCC TCGGATTGC- --AGCAATTG TCGGATAAAT
Astyanax mexicanus          AGGAAACCCA GC---AGCCC GCGCATGTC- --TGCAGTTG CTGGTTAAAG
Lepisosteus oculatus        AGAGGTGGAC CCAAACGATC CCCTGAGGCT TCTGGAACCC AGAGAAAACT
Tetradon nigroviridis       TCCAACCCCG GG---AGCCC CCGCAGCAG- --CGGCACGG TGGGCTGACT
                                 910        920        930        940        950
AGKD01034860                GGACAGGACA CTTGCCGTTG TACACCTGGT TTTAAGAATA TAAGTTGTGT
Salmo salar                 GGACAGGACA CTTGCCGTTG -ACACCTGGN T--------- ----------
Osmerus mordax              GGAC----CA CCTGTAATTG TGTACCCGGA CACAA----- -------TGT
Danio rerio                 CCGC----CA AATGTGACTC TGAGTCTGGA CCCGCCGCT- -------GCT
Astyanax mexicanus          T-GC----CT CATCCATGTC GGA--TCAGA CAGGTGGT-- --------CT
Lepisosteus oculatus        TCAGCCCACG CTACTTCCAG AACATGAGAA CATAA----- ----------
Tetradon nigroviridis       CCGA----CA CTTAGGAG-- ---------- ---------- ----------
                                 960        970        980        990        1000
AGKD01034860                GCGCGTTCAC GTTGGATCTT TTCCAGCTGG TCTGGAAACA AACCCCAACT
Salmo salar                 ---------- ---------- ---------- ---------- ----------
Osmerus mordax              CTGTTTTCAC GTCGGGTCTT TAACAGCTGG TCTGATAAAT ACCCTTAACC
Danio rerio                 TTGCGCAAAG TTAGTTGT-G TGGCA----- ---------- ----------
Astyanax mexicanus          CTGCTGTAGC CCAGTTACAG TGACAAAAAA CACGGTGATC CACCTTAAGA
Lepisosteus oculatus        ---------- ---------- ---------- ---------- ----------
Tetradon nigroviridis       ---------- ---------- ---------- ---------- ----------
                                1010       1020       1030       1040       1050
AGKD01034860                CTT------- ---------- ---------- ---------- ----------
Salmo salar                 ---------- ---------- ---------- ---------- ----------
Osmerus mordax              ATGAAGTGCT GTTGTTCGGT AGCACTGCCT ATGCAAGACC CACTTCTGCC
Danio rerio                 ---------- ---------- ---------- ---------- ----------
Astyanax mexicanus          GATGTGCGTT GATTTAACTG TTTCACTTTG TTTGTAATTT TGCAC-----
Lepisosteus oculatus        ---------- ---------- ---------- ---------- ----------
Tetradon nigroviridis       ---------- ---------- ---------- ---------- ----------
```

Figure 3.3. Nucleotide sequence alignments for *fgf23* with ClustalW based on original nucleotide sequences for agkd1034860, Atlantic salmon (fragment from primers designed on WGS, product not registered on public database), rainbow smelt, zebrafish, cave fish, spotted gar and green spotted puffer fish respectively (accession numbers provided in Table 3.3).

Sequences for *opn* and *fgf23* are phylogenetically compared to protein alignments of like sequences in other teleosts, and mouse (*Mus musculus*) as an outlier in Figure 3.4.

2.5.3 Real time qPCR

Absolute quantification qPCR assays were designed for genes described in Table 3.2 and performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). Each qPCR assay was carried out in a 96 well plate using standardised plasmids of a known concentration

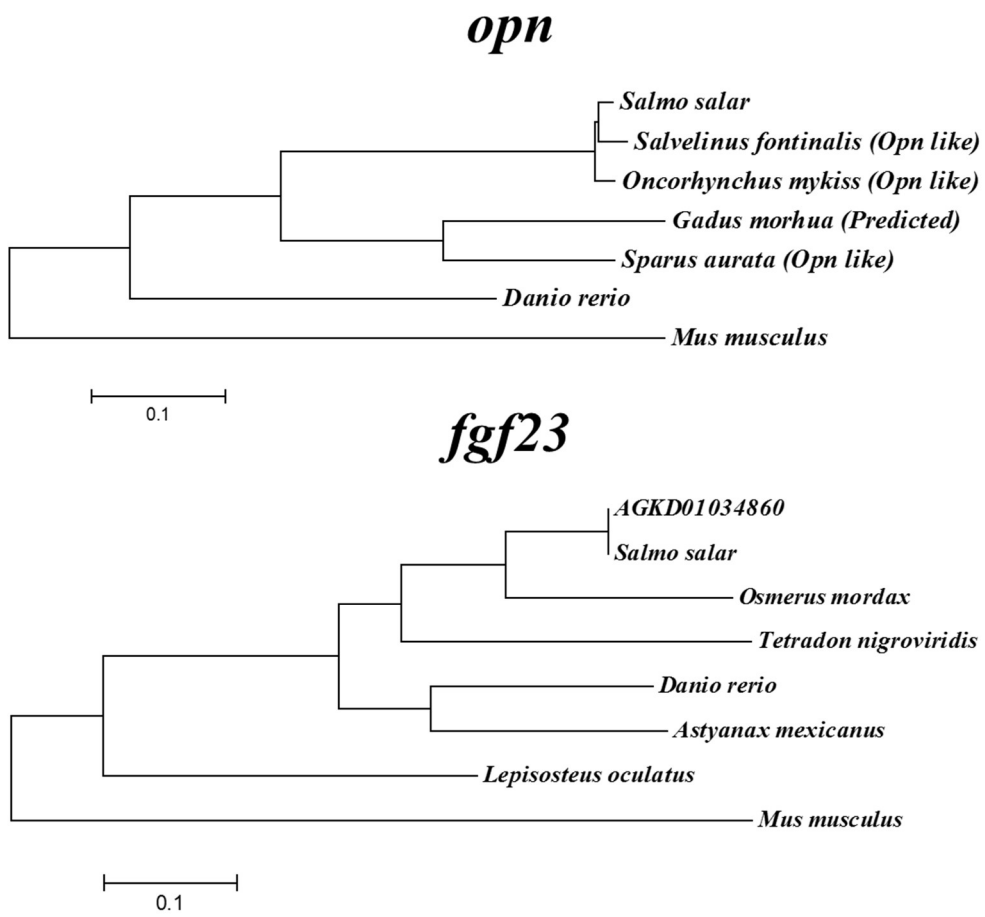

Figure 3.4. Molecular phylogenetic analysis by Maximum Likelihood method for *opn* (AF515708.1) and *fgf23* (NM_022657.4) with mouse (*Mus musculus*) provided as an outlier. Accession numbers for respective species sequences used are provided in Table 3.3. *Salmo salar fgf23* is on cloned *fgf23* fragment based on primers designed on WGS (product not registered on public database). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-4704.7297) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 687 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

with a linear range of 10^6 and 10^2 for all genes except the reference gene which was 10^7 - 10^3, which were generated based on appropriate PCR reactions for each cloned gene. Each 20 µl qPCR reaction consisted of primer pairs at a concentration of 6 pmol/µl, 5 µl of cDNA, 3.8 µl DNA/RNA free H_2O and 10µl of Luminaris Colour HiGreen qPCR Master mix (Thermo scientific). The Luminaris Colour HiGreen qPCR Master mix was made up of: Hot Start Taq DNA Polymerase, UDG and dNTPs (also dUTP) in an optimized PCR buffer with a blue dye; supplied with 40X Yellow Sample Buffer and nuclease-free water. All assays were carried out in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) according to manufacturer's instructions under a thermocycling programme beginning with UDG Pre-treatment of 50 °C for 2 minutes followed by 95 °C for 10 minutes (hot start). This was then followed by 40 cycles of 3 temperature steps; 95 °C for 15 s (melt) annealing at temperatures reported in Table 3.2 for 15 s and 72 °C for 30 s extenstion. This was followed by a temperature ramp from 60 – 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.

Cloning PCR products were purified using a Thermo genejet purification kit (Thermo Scientific, UK). Products were cloned using the pGEM®-T Vector System (Promega) and plasmids harvested using a GenElute™ Plasmid Miniprep Kit (sigma Aldrich). Resulting plasmids (forward and reverse for each, n= 2) were individually

sequenced via SUPREMErunTM sequencing (GATC, Cologne Germany) to confirm identity. Plasmids were then linearised by enzyme digest (HincII; NewEngland Biosciences) and standards for qPCR assays were generated using a serial dilution from 10^8 copies to 10 copies of each gene investigated. Efficacy for all assays was between 1.95 and 2. All samples were run in duplicate and each assay contained non-template controls. Results from vertebral cDNAs were normalized by relating expression data to the reference gene (*Elf-α*) as described in Ytteborg *et al*. (2010a). Stability of the reference gene was verified, as no significant difference in expression was observed between sample points.

2.6 Calculations

K was calculated as: $W^*(L^3)^{-1}$ x100. TGC was calculated as: $(W_f^{1/3}\text{-}W_i^{1/3})\text{x}(\sum^oD)^{-1}$, where W_f is the final body weight, W_i is the initial body weight and ^oD is the cumulative sum of water temperature in degrees per day. eFCR was calculated as: $F/(B_f-B_i+B_m)^{-1}$ where F is the food fed (kg), B_f is the final biomass (kg), B_i is the initial biomass (kg) and B_m is the mortality biomass for the period (kg).

2.7 Statistics

All data were analysed and compared using R language (R Core Team, 2013) and significance was accepted at 5 % ($p < 0.05$). Results are reported as mean (± SEM).

Datasets confirmed to be normal and homogeneous, through Anderson Darling tests, were analysed using the *lme* function in the *nlme* package for Two-Way-ANOVA with replicates nested within ploidy and diet. Datasets including life stage interaction (minerals and gene expression) were analysed with a Three-Way ANOVA also with replicates nested within ploidy and diet. Where no significant three-way interaction was observed Two-Way ANOVAs were performed for each life stage as it was necessary to retain individual life stages. Post-hoc analysis was performed using the *glht* function for Tukey's multiple comparison in the *multcomp* package where significant differences were observed. Radiography deformity datasets showed a negative binomial distribution confirmed with the function *odTest* in the *pscl* package hence, a generalised linear model with over-dispersion was performed using the *glm.nb* function in the *MASS* package. As well as deviance analysis, post-hoc analysis was performed using the *glht* function for Tukey's multiple comparison in the *multcomp* package.

3 Results

3.1 Growth and mortality

Triploids hatched 7% smaller compared to diploids and mean cumulative mortality from first feeding to smolt was 2.8 % ± 0.2 with no significant differences between ploidy and dietary treatment (data not shown). At early parr (2436˚DPF), triploid

fed HP were significantly heavier compared to all other treatments except diploid LP (Fig. 3.5A). By smoltification (4160 ˚DPF) triploids fed HP remained significantly larger than their diploid counterparts and similar in weight to diploid LP (Table 3.5, Fig 3.5B). However triploid LP individuals were significantly larger than triploid HP (Table 3.5, Fig. 3.5B). Final weights were reflected by apparent lower TGC values in diploid MP and HP and higher values in triploid LP (Table 3.5).

Total eFCR was comparable across all treatments (Table 3.5). Triploids overall had a lower K than their diploid counterparts but triploid HP was not significantly different to diploid LP. Diploids MP had the highest K and triploid MP the lowest.

3.2 Vertebral assessment

3.2.1 Whole mount staining

No observable differences were found in cartilage levels indicated by alcian blue staining between diets and ploidies. However there were higher amounts of observable cartilage in the 3 to 8 weeks post first feeding. Calcification, indicated by staining with alizarin red, showed increased levels of ossification of the vertebral column with increasing dietary P inclusion in triploids. By contrast diploids showed comparable levels

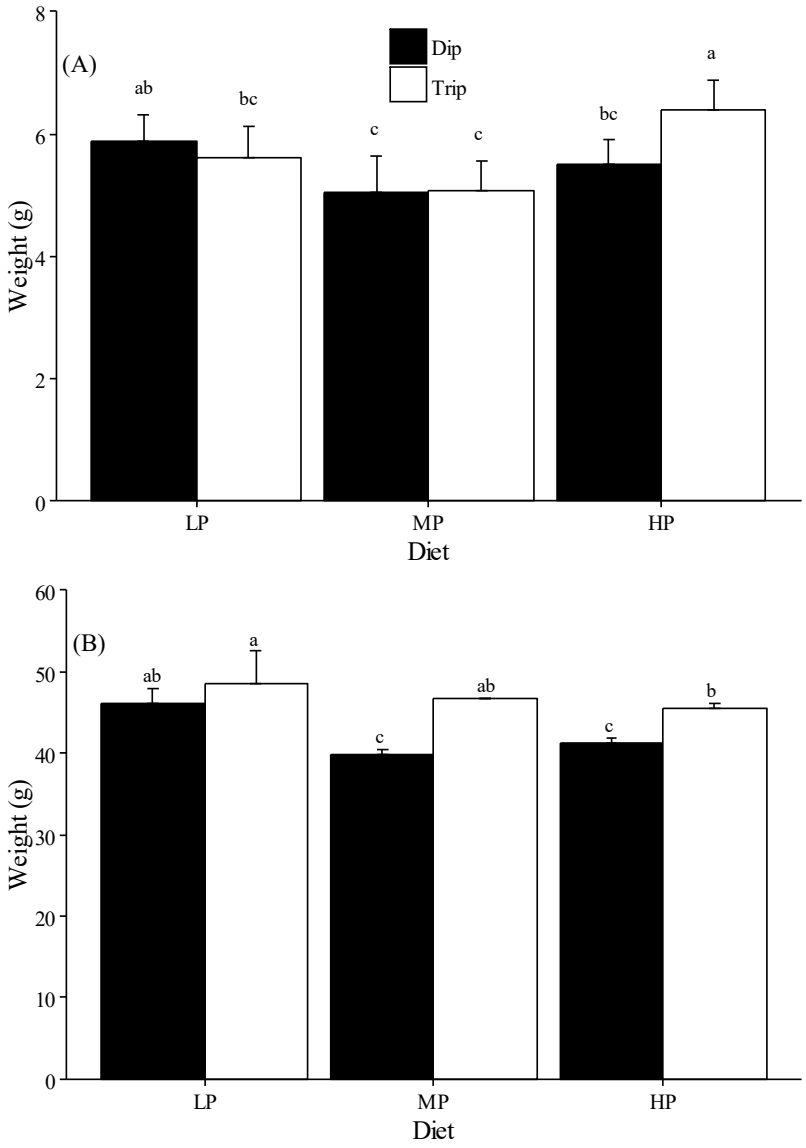

Figure 3.5. Weight performance summary for diploid (black) and triploid (white) fish fed on low (LP), medium (MP) and high (HP) P inclusion (n=2, 50 fish/replicate): (**A**) Mean weight at parr (5 g) (August 13th, 2012; 2436 °DPF) and (**B**) smolt (May 26th, 2013; 4160 °DPF). Values that do not share a common subscript denote significant differences ($p < 0.05$; Two-Way ANOVA & Tukey's post hoc).

Table 3.5 Performance summary including final weight (g), K, TGC and eFCR upon smoltification. (Mean ± SEM, *n* = 2).

	LP						**MP**						**HP**						***P***		
	Dip			**Trip**			**Dip**			**Trip**			**Dip**			**Trip**			**Ploidy**	**Diet**	**Diet x Ploidy**
Weight (g)*	46.2^{ab}	±	1.1	48.6^{a}	±	0.9	39.4^{c}	±	0.7	46.5^{ab}	±	0.7	41.3^{c}	±	0.9	45.6^{b}	±	0.8	**<0.0001**	**<0.0001**	**0.008**
Condition (K)*	1.04^{ab}	±	0.01	1.01^{c}	±	0.01	1.06^{a}	±	0.01	1.02^{bc}	±	0.01	1.04^{a}	±	0.01	1.03^{abc}	±	0.01	**<0.0001**	**<0.0001**	**<0.0001**
TGC (%b.w.˚C d^{-1})*	1.04	±	0.02	1.07	±	0.03	0.98	±	0.22	1.05	±	0.06	0.99	±	0.01	1.04	±	0.01	NA	NA	NA
eFCR*	1.15	±	0.05	1.12	±	0	1.17	±	0	1.11	±	0.13	1.14	±	0.01	1.15	±	0.03	NA	NA	NA

LP, low P diet; MP, medium P diet; HP, high P diet; Dip, diploid, Trip, triploid.

[a,b,c,d] Values that do not share a common subscript denote significant differences ($p < 0.05$; Two-Way ANOVA & Tukey's post hoc)

*50 fish / tank (100 /treatment).

of ossification irrespective of dietary treatments (Fig. 3.6). Individuals were not large enough for accurate image analysis until 8 weeks post first feeding. Significant interactions of diet and ploidy on mean vertebral area (mm^2) were only found in the caudal area where vertebral areas were significantly lower in triploid LP relative to all other treatments (Table 3.6). A significant effect of diet was found in all regions however post hoc analysis revealed no significant differences between diets

3.2.2 External assessment

Entire population assessment upon termination of the trial revealed a larger frequency of individuals with observable jaw deformities in triploid LP (7.7 % ± 0.3) than other treatments (Table 3.6). Frequencies of external skeletal deformities were low overall for all treatments.

3.2.3 Vertebra radiological assessment.

There was no significant effect of ploidy or diet on total number of vertebrae with the mean number of vertebrae being 59 ± 0.1 across all treatments. Triploid parr fed LP had significantly higher numbers of deformed vertebrae (dV) than all other treatments (dV 7.7 ± 1.0) which was maintained at smolt (5.4 ± 0.9 dV; Table 3.6). Triploid parr fed MP had significantly higher mean number of dV (2.8 ± 0.0) than diploid MP (2.0 ± 0.6 dV) which was also maintained until smolt (dV 2.8 ± 0.3 & 1.6 ± 0.2 respectively). MP and

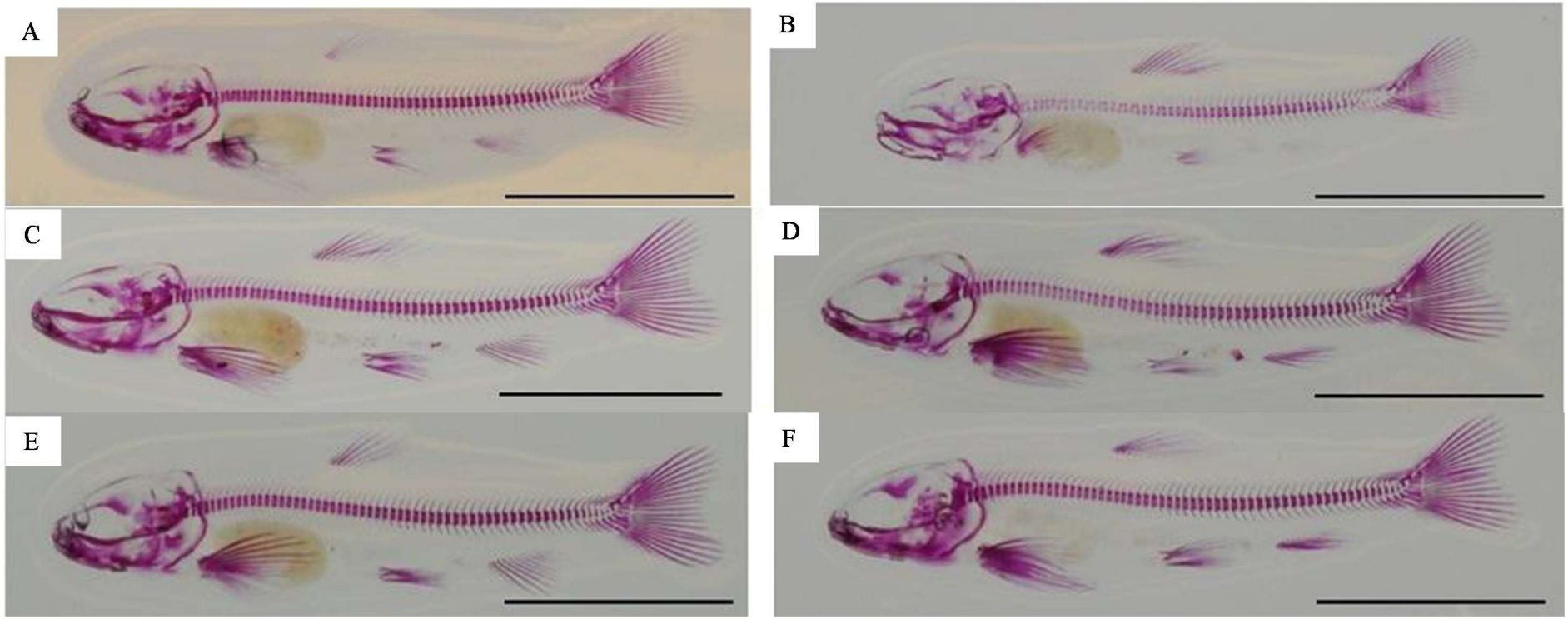

Figure 3.6. Whole mount staining for Ca of diploid (A,C,E) and triploid (B,D,F) fish fed LP (A,B), MP (C,D) and HP (E,F) P inclusion 3 weeks post first feeding (1136˚DPF). Scale bar = 10 mm.

Table 3.6. Vertebral area (mm^2) at 0.5 g (whole mount Ca staining); externally observable deformities at smolt; non-deformed (0 dV) and deformed (>0 dV) %, average dV% / individual and severely deformed (≥10 dV) % at parr and smolt.

		LP		MP		HP		*P*			
		Dip	**Trip**	**Dip**	**Trip**	**Dip**	**Trip**	**Ploidy**	**Diet**	**Stage**	**Interaction**
Whole mount Ca staining (0.5 g)*											
Area (mm^2)											**(P x D)**
Cranial-Trunk (R1)		0.50 ± 0.02	0.45 ± 0.01	0.53 ± 0.05	0.55 ± 0.01	0.59 ± 0.02	0.53 ± 0.01	0.3	**0.04**	NA	0.4
Caudal-Trunk (R2)		0.43 ± 0.01	0.39 ± 0.01	0.44 ± 0.08	0.51 ± 0.03	0.50 ± 0.03	0.47 ± 0.01	0.9	**0.009**	NA	0.1
Tail Region (R3)		0.73 ± 0.02^{A}	0.59 ± 0.02^{B}	0.75 ± 0.07^{A}	0.72 ± 0.03^{A}	0.77 ± 0.01^{A}	0.77 ± 0.05^{A}	**0.02**	**0.0008**	**NA**	**0.04**
Tail Fin (R4)		0.34 ± 0.01^{A}	0.21 ± 0.04^{B}	0.39 ± 0.04^{A}	0.39 ± 0.01^{A}	0.40 ± 0.01^{A}	0.40 ± 0.01^{A}	**0.02**	**<0.0001**	**NA**	**0.005**
Observable Deformity (%)†											
None		99.4 ± 0.2	92.3 ± 0.3	98.0 ± 0.0	98.0 ± 0.0	99.9 ± 0.1	98.8 ± 0.5				
Jaw		0.6 ± 0.2	7.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	1.2 ± 0.5				
Vertebral		0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0				
Radiological Assessment‡											**(P x D x S)**
Non-deformed (%)	*Parr*	18.0 ± 7.0	4.0 ± 4.0	26.0 ± 12.0	16.0 ± 0.0	24.0 ± 12.0	23.0 ± 2.0			NA	
	Smolt	30.0 ± 1.4	2.9 ± 2.9	35.7 ± 1.4	22.9 ± 5.7	42.9 ± 11.4	20.0 ± 8.6				
Deformed (%)	*Parr*	82.0 ± 7.0	96.0 ± 4.0	74.0 ± 13.0	84.0 ± 1.0	76.0 ± 12.0	77.0 ± 3.0			NA	
	Smolt	70.0 ± 1.4	97.1 ± 2.9	64.3 ± 1.4	77.1 ± 5.7	57.1 ± 11.4	80.0 ± 8.6				
Dv / individual	*Parr*	3.3 ± 0.1^{b}	7.7 ± 1.0^{a}	2.0 ± 0.6^{de}	2.8 ± 0.0^{bc}	2.2 ± 0.6^{bcd}	1.9 ± 0.2^{de}	**<0.0001**	**<0.0001**	**0.0001**	**0.02**
	Smolt	2.5 ± 0.3^{bcd}	5.4 ± 0.9^{a}	1.6 ± 0.2^{de}	2.8 ± 0.3^{bc}	1.2 ± 0.2^{e}	2.3 ± 0.4^{bcd}				
Severely deformed (%)	*Parr*	6.0 ± 0.0	32.0 ± 8.0	0.0 ± 0.0	3.0 ± 1.0	3.0 ± 3.0	0.0 ± 0.0			NA	
	Smolt	1.4 ± 1.4	12.9 ± 1.4	1.4 ± 1.4	7.1 ± 1.4	0.0 ± 0.0	0.0 ± 0.0				

LP, low phosphorous diet; MP, medium phosphorous diet; HP, high phosphorous diet; Dip, diploid, Trip, triploid.

^{A,B,C,D} Mean values with different uppercase superscript letters are significantly different ($p < 0.05$; Two-Way ANOVA & Tukey's post hoc)

^{a,b,c,d} Mean values with different lowercase superscript letters are significantly different ($p < 0.05$; Three-Way ANOVA & Tukey's post hoc)

*3 vertebrae / 5 individuals / tank (10 / treatment)

†Percentage deformed of entire population per tank

‡Parr: 50 fish / tank (100 / treatment); Smolt: 35 fish / tank (70 / treatment)

HP parr treatments showed significantly reduced mean number of dV (diploid MP: 2.0 ± 0.6; triploid MP: 2.8 ± 0.0; diploid HP: 2.2 ± 0.6; triploid HP: 1.9 ± 0.2) relative to LP treatments with the exception of triploid MP which was not significantly different to diploid LP (3.3 ± 0.01). HP triploids had the lowest level of dV at parr, significantly less than all other triploid treatments and diploid LP. Triploid HP smolt was the only treatment to increase in dV, resulting in a significantly higher number than diploid HP and lower than triploid LP. At smolt diploid HP showed significantly lower mean number of dV (1.2 ± 0.2) than triploid HP smolt (2.3 ± 0.4) and was the only treatment to show a significant reduction in dV between life stages. This was reflected in the percentage of non-deformed individuals which remains similar between parr and smolt for triploid HP (23 ± 2 & 20 ± 8.6 %, respectively) and was increased by nearly 20 % in diploid HP (24 ± 12 & 42.9 ± 11.4 %, respectively). Overall higher dietary P appeared to reduce mean number dV in both ploidy which was also reflected in percentage of severely deformed (≥ 10 dV) individuals. A substantially larger percentage of severely deformed individuals were observed in triploid parr fed LP (32.0 % ± 8.0) than at smolt (12.9 ± 1.4) and no severely deformed individuals were observed in triploid parr and smolt fed HP (Table 3.6). The majority of vertebral deformities were observed in the central (v27-31) and the tail region (v52-57; Fig. 3.7). Triploid parr fed LP had notably higher number of dV

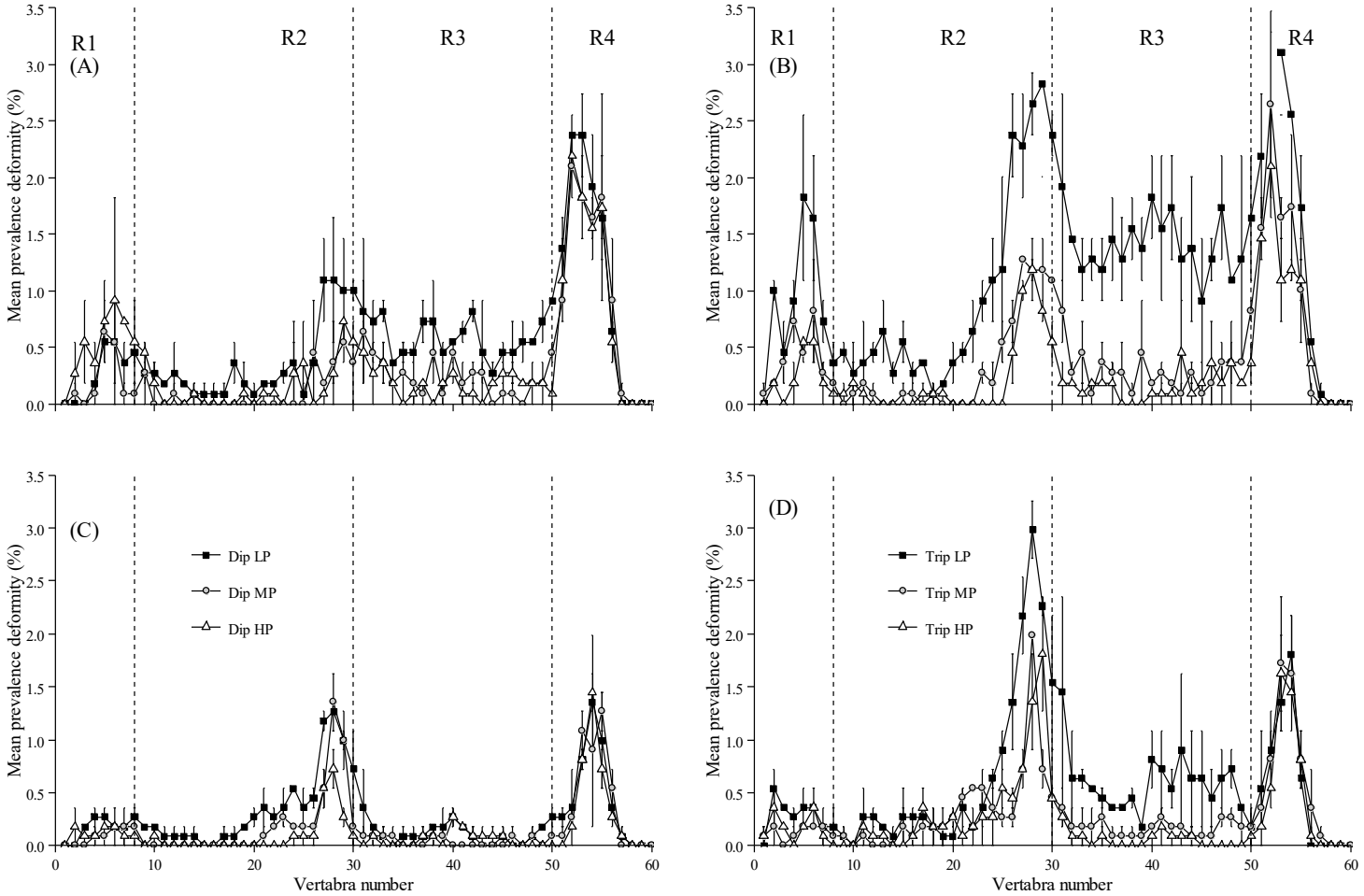

Figure 3.7. Mean prevalence (± SEM) of deformed vertebrae (n=2, 35 fish/replicate) at parr (A, B) and smolt (C, D) for diploid (A, C) and triploid (B, D) fish fed low to high P inclusion (LP, MP and HP). Regions 1(v1-8), 2(v9-30), 3(31-49), and 4(50-60).

relative to the majority of other treatments throughout the vertebral column (Fig. 3.7B) which was evident at smolt particularly from vertebrae 27 - 49 (Fig. 3.7D). Diploid parr fed LP had slightly higher number dV relative to other treatments from vertebrae 27-49 (Fig. 3.7A) but this was largely reduced at smolt (Fig. 3.7C).

Vertebral length to dorso-ventral diameter (L:H) ratios throughout the entire vertebral column, were smaller in parr fed LP compared to the other dietary treatments and this appeared to be more accentuated in triploids (Fig. 3.8A, B). In smolts, L:H ratios were largely similar between treatments however LP treatments maintained lower ratios in region 3 (v32-47; Fig. 3.8C, D).

3.3 Gene expression of bone markers

There was no significant interaction for ploidy, diet and life stage although there was a significant effect of life stage on gene expression for all genes, in which significantly lower mRNA expression levels were observed in parr than at smolt with the exception of *mmp13*, for which there was a significant increase in mRNA expression levels (Fig. 3.9). Significantly higher expression levels of *alp* were observed in diploid than triploid parr. For *fgf23* both diploid and triploid HP parr had significantly higher expression than LP diploids and triploids. Significantly higher expression levels of *igf1r* and *opn* were observed in diploid parr compared to triploid parr, but this did not differ between diets

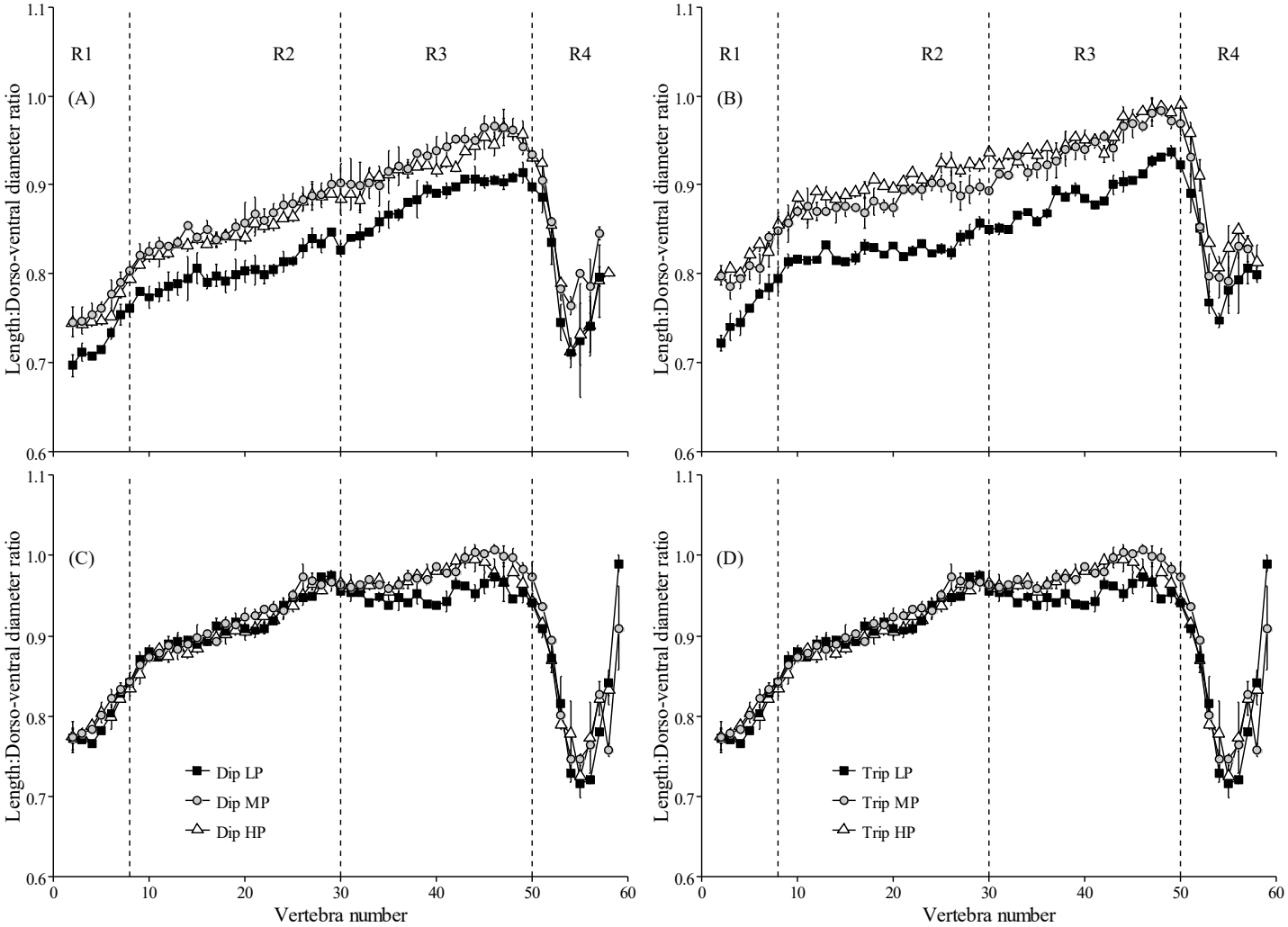

Figure 3.8. Mean length: dorso-ventral diameters ratios (± SEM, n=2, 10 fish/treatment) of vertebrae taken from x-rays at parr (A, B) and smolt (C, D) for diploid (A, C) and triploid (B, D) fish fed low to high P inclusion (LP, MP and HP). Regions 1(v1-8), 2(v9-30), 3(31-49), and 4(50-60).

No significant differences between treatments were observed within the smolt stages (Fig. 3.9).

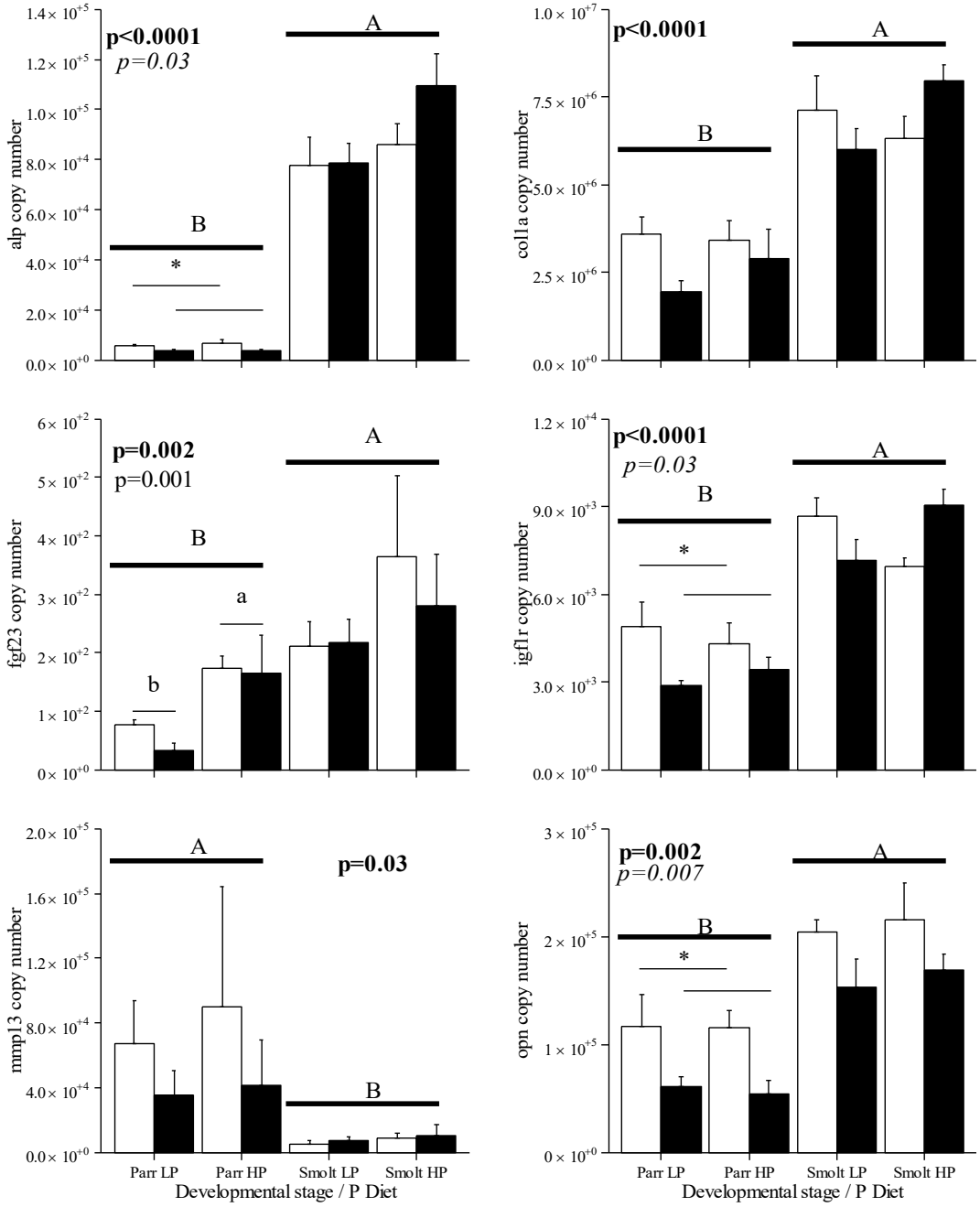

Figure 3.9. Gene expression of *alp, col1a, fgf23, igf1r, mmp13* and *opn* for diploid (□) and triploids (■) fed LP and HP diets (n=2, 6 individuals/replicate) at parr and smolt (copy number per *x* µg total RNA). Capital subscripts denote significant differences between life stages (**$p < 0.05$**; Three-Way ANOVA), asterisks denote significant differences between ploidy ($p < 0.05$; Two-Way ANOVA) and lower case superscripts denote differences between diet ($p < 0.05$; Two-Way ANOVA).

3.4 Whole body mineral content and proximate analysis

Whole body P content was lowest in triploid LP: significant to diploid MP and HP parr, and to all other treatments at smolt (Table 3.7). Triploid HP smolts had significantly higher P levels compared to all other treatments. By contrast Ca content and Ca: P ratio (Ca: P) were significantly lower in triploids (Trip: Ca – 3733 ± 127, Ca:P – 1.03 ± 0.02; Dip: Ca – 4029 ± 150, Ca:P – 1.05 ± 0.02) and LP treatments (LP: Ca – 3143 ± 130, Ca:P – 0.93 ± 0.02; MP: Ca – 3945 ± 127, Ca:P – 1.03 ± 0.02; HP: Ca – 3881 ± 122, Ca:P – 1.02 ± 0.01) in the parr stages. Ca levels were highest in triploid HP at smolt significant to MP treatments and diploid HP. No significant differences in Ca: P ratios were observed between treatments at smolt. In addition, no significant differences in bone P and Ca contents between treatments were observed at smolt (data not shown). V concentrations were lowest in triploid LP parr, significant to diploid HP parr and all smolt treatments with the exception of diploid LP. The highest V concentrations were observed in triploid HP smolt, significant to all other treatments including parr. No significant differences in Zn levels were observed at parr, whereas significantly higher levels were observed in HP triploids than their diploid counterparts at smolt.

No significant differences were observed in bone mineral percentage in smolt vertebral columns between diets or ploidy (Table 3.7). Overall triploids (41.7 ± 0.7) showed significantly lower jaw BM% compared to diploids (43.2 ± 1.2). Diploids were

Table 3.7. Whole body mineral composition (μg/mg) for whole body at parr and smolt stages including bone mineral percentage (BM%) for jaw and vertebrae at smolt. (Mean ± SEM, $n = 2$).

		LP		MP		HP		*P*				
		Dip	**Trip**	**Dip**	**Trip**	**Dip**	**Trip**	**Ploidy**	**Diet**	**Stage**	**P x D**	**P x D x S**
Phosphorous	***Parr***	3460 ± 44^{cd}	3259 ± 129^{d}	3985 ± 17^{bc}	3938 ± 84^{bcd}	4014 ± 52^{bc}	3864 ± 43^{bcd}	0.3	**0.0004**	**<0.0001**		**0.02**
	Smolt	4323 ± 92^{b}	4421 ± 180^{b}	4254 ± 272^{b}	4132 ± 169^{bc}	4193 ± 206^{b}	5119 ± 21^{a}					
Calcium	***Parr***	3368 ± 108	2917 ± 221	4377 ± 44	4229 ± 160	4342 ± 92	4054 ± 131	**0.04**	**<0.0001**	NA	0.7	0.08
	Smolt	4601 ± 121^{AB}	4661 ± 275^{AB}	4296 ± 453^{B}	4193 ± 157^{B}	4331 ± 256^{B}	5425 ± 206^{A}	0.09	0.05	NA	**0.04**	
Ca:P Ratio	***Parr***	0.97 ± 0.02	0.89 ± 0.03	1.09 ± 0.02	1.07 ± 0.02	1.08 ± 0.01	1.05 ± 0.02	**0.03**	**<0.0001**	NA	0.5	0.9
	Smolt	1.06 ± 0.00	1.05 ± 0.02	1.01 ± 0.04	1.02 ± 0.01	1.03 ± 0.01	1.06 ± 0.03	0.6	0.3	NA	0.7	
Vanadium	***Parr***	0.023 ± 0.000^{cd}	0.022 ± 0.001^{d}	0.027 ± 0.000^{bcd}	0.025 ± 0.000^{cd}	0.039 ± 0.000^{b}	0.033 ± 0.000^{bcd}	0.07	**<0.0001**	**<0.0001**	NA	**0.003**
	Smolt	0.031 ± 0.003^{cd}	0.041 ± 0.007^{b}	0.040 ± 0.004^{b}	0.036 ± 0.005^{bc}	0.036 ± 0.011^{bc}	0.058 ± 0.009^{a}				NA	
Zinc	***Parr***	44.03 ± 2.67	44.93 ± 1.86	43.95 ± 1.08	42.42 ± 0.35	42.13 ±1.10	39.98 ± 1.07	0.5	0.2	NA	0.7	0.06
	Smolt	37.90 ± 0.71^{AB}	41.27 ± 3.75^{AB}	40.10 ± 1.81^{AB}	36.88 ± 1.07^{AB}	36.42 ± 1.63^{B}	45.19 ± 1.44^{A}	0.08	0.5	NA	**0.02**	
BM%‡												
Vertebrae		43.34 ± 3.25	40.39 ± 1.23	41.24 ± 1.21	43.70 ± 1.13	41.74 ± 1.12	44.48 ± 1.11	0.3	0.5	NA	0.6	NA
Jaw		29.33 ± 0.71	24.93 ± 1.7	31.63 ± 0.72	27.80 ± 1.29	27.26 ± 1.42	27.27 ± 1.12	**0.01**	0.07	NA	0.2	NA

LP, low P diet; MP, medium P diet; HP, high P diet; Dip, diploid, Trip, triploid.

^{a,b,c,d} Mean values with different lowercase superscript letters are significantly different ($p < 0.05$; Three-Way ANOVA & Tukey's post hoc).

^{A,B,C,D} Mean values with different uppercase superscript letters are significantly different ($p < 0.05$; Two-Way ANOVA & Tukey's post hoc).

†5 fish pooled in triplicate per tank (30 fish / treatment).

‡5 fish per tank (10 / treatment).

found to have significantly higher ash content relative to diploids (Table 3.8). No other significant differences were observed in proximate analysis.

4 Discussion

Feeding high dietary P (19.7g total P kg^{-1}) to triploid Atlantic salmon throughout FW reduced the occurrence of severe skeletal deformities observable by x-ray and externally observable jaw deformities. Triploids fed low P diets (13.0 g total P kg^{-1}) had less well-mineralised vertebrae and a higher prevalence of skeletal deformities including LJCs and severely deformed vertebrae. Higher dietary P initially resulted in improved triploid weight gain but not at smolt, whereas diploid weight gain was consistently reduced by increased dietary P throughout FW development. Radiographs, whole mount staining, gene expression and whole body mineral analysis indicated increased bone mineralisation with higher dietary P in triploids, and that these effects are more pronounced in the earlier stages of FW development.

As is seen in other FW studies, triploids hatched smaller (7%) and showed increased growth rates compared to diploids (Leclercq *et al.,* 2011; Taylor *et al.,* 2011, 2012;) as well as a lower K (Burke *et al.,* 2010; Taylor *et al.,* 2011; Fjelldal *et al.,* 2015). In particular, triploids perform optimally beneath a temperature threshold of 12 ˚C (Atkins & Benfey, 2008) and a higher weight gain relative to diploids was

Table 3.8. Whole fish proximate analysis (moisture, oil, protein, ash) % upon smolification. (Mean values with their standard errors, $n = 2$).

	LP		MP		HP		*P*		
	Dip	**Trip**	**Dip**	**Trip**	**Dip**	**Trip**	**Ploidy**	**Diet**	**Diet x Ploidy**
Proximate Analysis (%)†									
Moisture	72.55 ± 1.07	72.1 ± 0.18	72.44 ± 0.14	72.4 ± 0.15	72.7 ± 0.25	72.23 ± 0.17	0.9	0.1	0.7
Oil	7.9 ± 1.05	8.22 ± 0.42	8.35 ± 0.13	8.76 ± 0.24	7.42 ± 0.76	8.46 ± 1.21	0.3	0.1	0.9
Protein	16.49 ± 0.35	16.87 ± 0.24	16.52 ± 0.75	16.5 ± 0.63	16.63 ± 0.28	16.78 ± 0.11	0.3	0.2	0.4
Ash	2.31 ± 0.28	2.05 ± 0.03	1.98 ± 0.13	1.89 ± 0.05	2.22 ± 0.2	2.14 ± 0.05	**0.03**	0.09	0.6

LP, low P diet; MP, medium P diet; HP, high P diet; Dip, diploid, Trip, triploid.
†5 fish pooled in triplicate per tank (30 fish / treatment)

observed around autumn and winter when temperatures fell below this threshold. The higher weight observed in fed triploids high P was sustained until 30 g, there after they were outperformed by low P counterparts. Burke *et al.,* (2010) fed triploids 13.9 g total P kg^{-1} and 7.6 g total P kg^{-1} from 45 to 80 g and a similar growth reducing effect was observed. High P inclusion impeded diploid growth relative to triploids and diploids fed lower dietary P throughout development and at smolt respectively. This may be related to a reduced net intake of other nutrients or shifts in gut integrity as a result of excess P (reviewed in: Boersma & Elser, 2006). The delayed inhibitory effect of high P in triploids indicates that dietary requirements may be higher particularly for early stages of development. Neither diet nor ploidy had an impact on survival which is concurrent with the majority of recent literature and reflects current knowledge in welfare and survival (Fraser *et al.,* 2012a). Conversely in recent work by Fjelldal *et al.* (2015), dietary P and ploidy increased mortality rates in FW. However, total P inclusion in the lower treatment was overall lower (7.1 g Total P Kg^{-1}) than that used in the current study (13.0 g Total P Kg^{-1}).

Results of the present study showed little occurrence of externally visible vertebral deformities as they do not generally manifest until ongrowing at sea. Increased prevalence of LJC was observed in triploids fed low dietary P. Occurrence of LJCs in diploids has previously been linked to a deficiency in available P in commercial diets

coupled with higher water temperatures (~20 ˚C; Roberts *et al.,* 2001). The absence of jaw deformities in diploids in the current trial suggests the P requirement is lower than for triploids. Conversely, Fjelldal *et al.,* (2015) found no jaw deformities at smolt at a similar size (~56-62 g) when feeding within the ranges of the low P diet in this trial (9.4-16.3 g total P Kg^{-1}) but a large percentage in both diploids and triploids (79-89 %) when fed 7.1 g total P Kg^{1}, which is well below the minimum recommendation for salmonids (8 g available P Kg^{-1};NRC, 2011). In addition, higher temperatures, associated with lower oxygen saturation levels in the summer, may have led to excessive respiratory buccal-opercular pumping and poor mandibular development under nutritional strain (reviewed in: Fraser *et al.,* 2012a), although temperature profiles were not reported in Fjelldal *et al.* (2015). In addition, as suggested by Fraser *et al.,* (2014), deformity prevalence in triploids has been shown to be reduced in underyearling S0+ smolts compared to ambient S1+ smolts. This has been hypothesised to be due to relatively reduced triploid growth under S0+ photo-thermal regime and may explain the higher deformity rate in the current study (S1+ regime) than that of Fjelldal *et al.,* (2015; S0+ regime).

Triploidy is one of several factors shown to result in enhanced growth associated with increased prevalence of vertebral deformities (Fjelldal *et al.,* 2012a). This was confirmed in the current study with triploids showing both higher mean percentage deformed vertebrae and higher TGCs than their diploid counterparts. Vertebral

deformities were largely reduced in triploids fed 16.7 and 19.7 g total P Kg^{-1} inclusion with triploids fed the lowest inclusion showing higher prevalence of vertebral deformities. Other research has also shown a reduction in vertebral deformities in FW salmonids with increasing dietary P inclusion (15, 18 and 21 g total P Kg^{-1}; Fjelldal *et al.,* 2012a) including triploids (7.1, 9.4 and 16.3 g total P Kg^{-1}; Fjelldal *et al.,* 2015). Although mean percentage deformed vertebrae was similar in medium and high P inclusion treatments, the occurrence of severely deformed individuals, as determined by 10 or more dV per fish, was largely reduced with increasing P inclusion resulting in no occurrence in the high P treatment. Hansen *et al.,* (2010) have shown that severe deformities may impede growth indicating that higher P diets may be required in FW to prepare triploids for SW ongrowing. Fjelldal *et al.* (2015) showed triploids fed a FW P inclusion of 9.4 g total P, Kg^{-1}, compared to those fed 7.1 g total P Kg^{-1}, attained significantly higher weights at harvest after feeding standard diets in SW, although severity of vertebral deformities at smoltification was not reported.

Deformities were largely localised in vertebrae numbers 28-30, the cranio-caudal axis for mineralisation along the vertebral column (Grotmol *et al.,* 2003), and 54-57 (R4) where strong variations in vertebral parameters are typically observed (Kacem *et al.,* 1998). Localisation of vertebral deformities are ontogenetic (Fjelldal *et al.,* 2012b) and higher prevalence in the central region has been reported in other FW studies (Fraser *et*

al., 2015a; Fjelldal *et al.,* 2015). Triploids fed a lower P inclusion had a notably higher prevalence of vertebral deformities around the cranio-caudal axis. Given the importance of this region for initial bone formation, these deformities may have stemmed from insufficient outward mineralisation in early development. This is confirmed by the relatively shorter dorso-ventral length ratios relative to their diploid counterparts in parr compared to smolt as well as smaller vertebral areas 3 weeks post first feeding.

Low P triploids also had higher prevalence of deformities in region 3 for which negligible prevalence was evident in other treatments. Deformities within this region are more commonly observed in SW ongrowing life stages and have been associated with a number of factors in diploids including: mechanical strain as a result of lateral muscular activity (Witten *et al.,* 2005), low P levels in combination with accelerated growth in the caudal region at smoltification (Fjelldal *et al.,* 2009a), and vaccination induced inflammation which is not prevented by increasing dietary P (Gil Martens *et al.,* 2012). Increased deformity prevalence and reduced length to dorso-ventral diameter within R3 was observed in triploids fed low P inclusion, and was particularly apparent at smolt. Poor bone development and mineralisation may have been induced through a combination of known risk factors including: potential vaccine-induced inflammation (vaccination performed 8 weeks prior to x-ray assessment), poor mineralisation through observed accelerated growth and restricted P availability.

Abnormal bone development can be associated with lowered mineral content and consequentially reduced structural integrity (Fjelldal *et al.,* 2009b). Significantly higher whole body P and Ca concentration in triploids fed high P inclusion, particularly at smolt, suggest a higher accumulation of the bone mineral hydroxyapatite and $Ca_3(PO_4)_2$ and improved structural integrity. This is also confirmed by x-ray and whole mount staining results. Higher Zn concentrations in triploid smolts fed higher P inclusion may be attributed to metalloenzymes such as ALP important for formation of osteoid and bone mineralisation (Lall, 2003). Higher concentrations of V were found in triploids fed high P at smolt relative to other treatments. Given the known anti-mineralogenic properties of V in fish bone cells (Tiago *et al*., 2008), skeletal mineralisation may be actively suppressed where P availability for bone mineralisation is surplus. In the parr stages, both P and V concentrations in triploids fed high P were not significantly different to other medium and high P treatments. Collectively these results suggest less active suppression in triploid high P parr relative to smolt, therefore, a higher dietary requirement in the earlier stages. Fjelldal *et al.,* (2015) found significantly higher vertebral ash content in triploids smolts fed a 16.3 g total P Kg^{-1} diet throughout FW to their dietary counterparts of 9.4 and 7.1 g total P Kg^{-1}. Burke *et al.,* (2010) found similar results when feeding 13.9 g total P Kg^{-1} as opposed to 7.6 g total P Kg^{-1} for only 12 weeks. In addition, weights of triploids in the current trial fed a high P inclusion were significantly higher at parr

compared to triploids fed a low P diet and vice versa at smolt indicating higher P requirements may be reduced in the later stages of triploid FW development. This may be due to an associated reduction in availability of other nutrients (Boersma & Elser, 2006).

Significantly smaller vertebral areas, indicated through Ca staining, were observed in triploids fed low P inclusion compared to their higher P dietary siblings at 8 weeks post first feeding. Critical formation of the foundations of skeletal development occur at this life stage. Progressive formation of the chordacentra (the templates for vertebral body formation), cancellous bone and trabeculae occurs along the cranio-caudal axis 200 degree days prior and 400 degree days post first feeding (Nordvik *et al.,* 2005). Diploid and triploid parr on low P inclusion showed shorter length to dorsoventral diameters but not at smolt (with exception of slightly shorter vertebrae in region 3) and higher levels of deformities were observed in triploids fed low P throughout development. Bone is formed effectively through the laying down of a primary layer of osteoid which then mineralises into bone. During periods of rapid growth the mineralisation time from osteoid to bone may be extended and reduce mineralisation in areas of growth which in turn may lead to a poorly developed vertebrae. Studies inducing poor mineralisation of vertebrae have indicated linear growth failure resulting in vertebral deformities (Fjelldal *et al.,* 2009b) that may occur through insufficient time to mineralise

osteoid and production of ectopic cartilage in the intervertebral spaces (Witten *et al.,* 2005). Triploids in particular appear to require higher dietary P in these very early life stages to prevent linear growth failure and associated vertebral deformity development. This may also be associated with higher seasonal temperatures that coincide with early developmental stages.

Bone mineralisation is orchestrated by a complex system of bone deposition and remodelling through osteogenic cells in which Ca^{2+} and PO_4^{3-} availability is regulated by circulating PTH, [1,25$(OH)_2$D] and FGF23 (Martin *et al.,* 2012). Expression of *fgf23* in vertebrae of parr fed a low P diet was significantly lower suggesting minimised excretion of renal PO_4^{3-}. Conversely, Fjelldal *et al.,* (2015) found smolts fed with lower P inclusion levels had significantly lower gene expression levels but early life stages were not analysed and in our study no differences were observed at smolt. Higher levels of available PO_4^{3-} are known to upregulate OPN which is associated with activation of ALP and ECM (Beck *et al.,* 2000). Reduced expression of osteogenic factors *opn* and *alp* and skeletal growth factor *igf1r* (Wargelius *et al.,* 2005a) in triploid parr suggest lower osteogenic potential relative to diploids. In addition, upregulation of *fgf23* in the presence of higher P in the parr stages could lead to excretion of renal PO_4^{3-} and lower circulating levels. Lower osteogenic potential in triploids combined with inhibitory factors of mineralisation in the presence of high P indicate triploids may be subject to lower P

availability for skeletal mineralisation. Other studies have shown reduced P levels in plasma of triploids fed lower levels of dietary P than their diploid counterparts (Burke *et al.,* 2010). Lowered PO_4^{3-} availability in triploids may be caused by demand in other systemic processes or reduced digestible efficiency as a results of known altered gut morphology relative to diploids (Peruzzi *et al.,* 2014) which may be accentuated in the parr stage.

In conclusion, feeding higher dietary P inclusion throughout FW development can reduce skeletal deformity in triploid Atlantic salmon and minimise the occurrence of severely deformed individuals to diploid levels. Increasing dietary P was shown to impede growth in diploids whereas improved growth was initially observed in the early stages in triploids but impeded growth in the later stages of FW development. Observation of lowered osteogenic gene expression in the early stages alongside reduced vertebral mineralisation and vertebral elongation suggest that these life stages are particularly sensitive to dietary P inclusion. In addition, the mechanisms for higher dietary P requirement are largely unknown but results indicate a possible reduced availability in triploids may be responsible in conjunction with their accelerated growth. This research therefore not only highlights the importance of high P inclusion in reducing vertebral deformities in triploid Atlantic salmon, but also that earlier life stages are particularly receptive to high P inclusion. Further research is required to investigate

potential to reduce feeding higher P inclusion whilst sustaining the reducing effect on vertebral deformity prevalence. Reducing requirement for higher dietary P would be of environmental and industrial benefit, where associated economic cost of raw feed materials and potential for increased aquatic eutrophication (Conley *et al*., 2009; Folke *et al.*, 1994) would be reduced.

Chapter 4: Dietary probiotics and windows of phosphorous supplementation as potential alternatives to high dietary phosphorous supplementation for prevention of skeletal deformity in triploid Atlantic salmon (*Salmo salar* L.)

Abstract

The use of dietary probiotics and feeding high dietary P supplementation for shorter time periods was investigated with the aim to alleviate known higher P requirement for skeletal development in triploid Atlantic salmon. Diploid and triploid siblings were fed 6 different dietary regimes throughout FW and assessed for growth performance, skeletal development through x-ray assessment and mineral concentration. Dietary groups were as follows: diploid standard (S: 14.3 g total P kg^{-1}), triploid S, triploid S with Bactocell® (SBC, *Pediococcus acidilactici*: 15.2 g total P kg^{-1}) and triploid high P inclusion (H; 17.4 g total P kg^{-1}) fed to 3 different windows. Triploid H dietary windows were fed from first feeding until triploids attained 5 or 20 g in weight, or for the entire experimental period. Radiography results revealed a significant reduction of vertebral deformities at parr (~30 g) in triploid H compared to triploid S with no reducing effect of feeding H only to 5 g or 20 g. Triploid SBC had the least deformities within triploids significant to triploid S and those fed H to 5 and 20 g. In addition, of all treatments, triploid SBC were the only to show no severely deformed individuals (≥10 dV). No significant differences in mean vertebral deformities and number of non-deformed individuals were found within triploid treatments at smolt (~83g) however significantly more deformities were observed relative to diploid S. No significant differences were observed in severely deformed individuals between diploids and all triploid treatments at smolt. Differences in mineral

concentrations important to osteogenesis within the early parr stages reflect increased triploid nutritional requirements for skeletal development in this stage. Differences in skeletal development throughout life stages may reflect higher nutritional requirements of triploids during periods of accelerated growth as no difference was observed in skeletal development in later stages. This is the first study to investigate the use of probiotics in triploid teleost fish in which *P. acidilactici* appears to offer a potential alternative to high dietary P in reducing skeletal deformity during triploid FW development.

1 Introduction

Results of chapter 3 and that of Fjelldal *et al.* (2015) have shown the importance in higher dietary P inclusion for reducing skeletal malformations in FW. Increased effect of dietary P inclusion on skeletal and growth performance was observed in earlier developmental stages in chapter 3 and may have been attributed to reduced availability in periods of accelerated growth and higher temperatures. Accelerated growth in the context of photoperiod (Fjelldal *et al.,* 2006) and temperature (Ytteborg *et al.,* 2010a) can impair skeletal development in diploid Atlantic salmon. In addition, dietary mineral supplementation during such periods can support skeletal development (Fjelldal *et al.,* 2009b). Triploids show accelerated growth and reduced K in FW (Taylor *et al.,* 2011; 2012). Hence, accelerated growth in triploid Atlantic salmon may contribute to increased elongation of the spine relative to diploids and to higher dietary P requirement. P requirement must be met by the diet owing to low environmental availability (Lall & Lewis-McCrea, 2007). However, feeding constant higher levels of P is of higher economic cost to industry and may induce anthropogenic pressures on the environment through eutrophication (Conley *et al.*, 2009). Dietary P retention in triploid salmonids is unknown and shown to be variable in diploid Atlantic salmon (Piedrahita, 2003). Feeding diploid Atlantic salmon higher P inclusion increases leaching through faeces (Phillips *et*

al., 1993) and feeding above 10 g total P kg^{-1} does not lead to further increase in whole body assimilation (Asgard & Shearer, 1997). In countries where intensively farmed salmon aquaculture is prominent, it is a large contributing source to anthropogenic P input and eutrophication risk (Folke *et al.,* 1994). In Scotland, SEPA regulations state that anthropogenic P input into a FW loch should not alter the trophic or ecological status (SEPA, 2014). In light of global concern for sustaining aquatic water bodies it is essential to minimise the use of eutrophication sources such as P. In addition feeding higher levels of P (16.3 g total P kg^{-1}) throughout FW in triploids does not improve growth relative to lower dietary P levels (7.1 g total P kg^{-1}), despite improved vertebral development (Fjelldal, *et al.,* 2015) as is shown in chapter 3 (13.0 vs. 19.3 g total P kg^{-1}). Nutritional needs may need to be further addressed, potentially through restricting duration of higher dietary P supplementation or improving assimilation by other means.

Susceptibility to skeletal deformities in Atlantic salmon ontogeny are typically defined to hatch, FW and SW ongrowing life stages (Fjelldal *et al.,* 2012b). Skeletal ontogeny within FW may be dependent on seasonal factors where photo-thermal regimes impact growth. Large transformations in bone development occur in the early fry stages where the primary layers of cancellous bone and trabeculae become established along the cranio-caudal axis (Nordvik *et al.,* 2005). In addition triploids hatch smaller and typically

exhibit faster growth in FW (Leclercq *et al.,* 2011; Taylor *et al.,* 2011, 2012) and therefore may have a particularly high requirement for P in these early life stages.

Dietary probiotics are known to improve health, growth, body composition, gut morphology and reduce malformations in salmonids (Merrifield *et al*., 2010a). In particular, the effects of the use of the dietary probiotic *P. acidilactici* in commercially cultured fish species is limited (Aubin *et al*., 2005; Ferguson *et al*., 2010; Merrifield *et al.*, 2010a,b, 2011; Lamari *et al*., 2013), but has shown promising results of reduced vertebral column compression syndrome in rainbow trout (Aubin *et al*., 2005) and improved skeletal conformation in European sea bass (Lamari *et al*., 2013). Accelerated bone deposition alongside expression of genes associated with osteogenesis and osteogenic cell differentiation has been observed in zebrafish fed another probiotic, *Lactobacillus rhamnosus* (Maradonna *et al.,* 2013). However, the mechanisms for improved bone formation with dietary *P. acidilactici* and other probiotics remain to be confirmed. Dietary probiotics are of particular interest to artificially induced triploid fish given the increased skeletal malformation prevalence, although no assessment has been conducted to date. Increased species-specific gut microbial concentration in triploid Atlantic salmon indicate potential increased susceptibility to opportunistic pathogens and probiotics (Cantas *et al.,* 2011). In addition, triploid Atlantic salmon show an altered gut morphology relative to diploids with fewer pyloric caeca and shorter gut length (Peruzzi

et al., 2014). Probiotics have shown potential to improve gastric morphology (Merrifield *et al.,* 2009) and introduce phytase producing bacteria enabling digestion of P in the form of phytate (Scholz-Ahrens *et al.,* 2007). Responsiveness to probiotics is valuable when researching commercial viability of triploid Atlantic salmon as probiotics may also hold potential for increased disease resistance, gastric morphology, gastro-intestinal colonisation and reduced use of dietary antibiotics (reviewed in: Merrifield *et al.,* 2010a). Primarily, if dietary probiotics facilitate nutrient assimilation then reliance on high inclusion diets such as P hold potential to be reduced.

Feeding higher dietary P to triploid Atlantic salmon in FW was shown to improve skeletal development but not growth performance at smolt, as reported in chapter 3 and in Fjelldal *et al.* (2015). Higher dietary P is of high cost to industry owing to availability of raw materials and environmental impact. The current study aimed to investigate windows of high P supplementation and dietary inclusion of *P. acidilactici,* with the overall aim to reduce the requirement for higher dietary P whilst maintaining optimal skeletal development. Feeding high P supplementation diets for reduced time periods (~5 g and ~20 g) and throughout entire FW development (smolt: ~83 g) were tested and compared to diploids and triploids fed a standard diet. In addition a standard triploid treatment was fed a probiotic inclusion (*P. acidilactici*) to investigate potential to alleviate the need for higher P supplementation through improving assimilation. Growth

performance, skeletal development and mineral concentration were assessed within the key developmental stages. In addition, the potential environmental impact of different P supplementation and windows of P supplementation on FW P concentrations was modelled for an example case study: Loch Arkaig.

2 Methods and materials

2.1 Fish stock and husbandry

Diploid and triploid Atlantic salmon eggs (Atlantic QTL-innOva® IPN) were supplied from Aquagen (Norway) on December 20, 2012 into Howietoun Hatchery, Scotland (56˚N, 4˚W) at 372 ˚DPF. Triploidy was induced using a hydrostatic pressure shock of 9500 psi applied 300 ° minutes post-fertilisation for 50 ° minutes at 8 °C (Taylor *et al.,* 2011). Prior to transfer diploids and triploids were reared at 6.0 ± 0.05 ˚C and under constant darkness until point of first feeding. Ova were then divided between 18 x 250 L tanks (3 diploid; 15 triploid, 4200 individuals / tank) and reared under constant darkness until first feeding (879.6 ˚DPF) after which fry (~1.33 g) were transferred into 18 x 980 L covered circular tanks at the Niall Bromage FW Research Facility (NBFRF), Stirling (56˚N, 4˚W) at 1638.6 ˚DPF. Fish were maintained under LD24:0 until summer solstice after which they were reared under a Simulated Natural Photoperiod (SNP) and ambient

water temperature (Fig 4.1) until smoltification (~83 g; 4668 ˚DPF, May 16, 2014). Ploidy status was verified according to protocols in chapter 2 on blood collected from fish weighing 5g (n = 100 / ploidy). Diploid control groups had significantly smaller erythrocyte nuclear lengths with no overlaps with triploid groups (2N 7.2–8.1 µm; 3N 10.3–10.9 µm) confirming the likelihood that pressure shocked fish were triploid to be absolute.

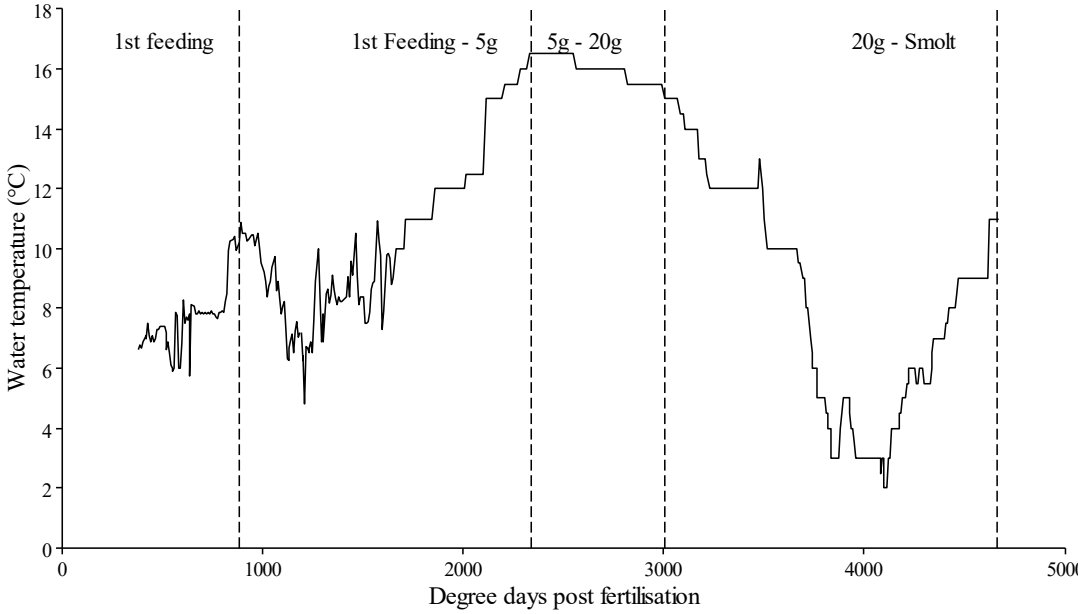

Figure 4.1. Environmental water temperature (˚C) profile throughout experiment (˚DPF).

2.2 Experimental setup and sampling protocol

Six treatments were applied in triplicate from first feeding until smolt (~83 g): diploid standard (Dip S), triploid standard (Trip S), triploid standard with Bactocell® (Lyophilised bacteria: *Pediococcus acidilactici,* MA 18/5M Pasteur institute; SBC)

inclusion and triploid high P (Trip H) inclusion fed to three different temporal windows. Feed composition can be found in Table 4.1. Trip H windows were divided from first feeding until early (~5 g) or late parr (~20 g) stages and thereafter S diet, or up until smolt (~83 g). The aim of this trial was to compare higher and lower P inclusion based on the results of chapter 3. As such, total P levels within the S and H diets are approximations between the LP - MP and MP - HP diets. Feed was manufactured through BioMar UK Ltd (Grangemouth, Scotland) and administered throughout daylight hours using clockwork belt feeders, with ration set according to manufacturer's tables. Smoltification was confirmed through skin silvering assessment (Sigholt *et al.,* 1995). Growth was assessed on anaesthetised fish (MS222, 0.25 g mL^{-1}; n = 30 fish / tank) on a monthly basis including weight (±0.1 g), fork length (±1 mm) and K. Terminal samples were taken for x-ray assessment at 30g and smolt (~83 g; 30 fish / tank, n=3). Additional terminal samples were taken for whole body mineral composition at early (~5 g) and later (~20 g) parr stages and smolt (~83 g; 15 fish / tank, n=3). Individual carcasses were collected following euthanasia following anaesthetic overdose (>200 mg L^{-1}) and stored at -20 ˚C until analysis. All experimental procedures and husbandry practices used in the present study were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice) in accordance with EU regulation (EC Directive

86/609/EEC) and approved by the Animal Ethics and Welfare Committee of the University of Stirling.

Table 4.1. Formulation and proximate composition of experimental feeds

	Diet		
	S	**SBC**	**H**
Formulation (%)			
Fish/crustacean meal	51.01	51.00	51.32
Wheat / wheat gluten	25.90	25.88	24.08
Fish oil	15.85	15.85	15.86
Pea Protein	4.35	4.35	4.35
Soya protein concentrate	3.01	3.01	3.01
Monosodium phosphate	0.33	0.33	1.83
Additives*	1.05	1.05	1.05
Emulthin	0.08	0.08	0.08
Pediococcus acidilactici‡	0.00	0.02	0.00
Yttrium	0.01	0.01	0.01
Drying	-1.58	-1.58	-1.58
Composition (Analysed)			
Proximate (%)			
Moisture†	5.67	5.73	5.10
Oil†	23.08	22.76	22.81
Protein†	51.91	51.86	52.88
Ash†	8.03	8.05	9.16
P (g total Kg^{-1}) †	14.27	15.18	17.36

*Vitamins including BioFish Premix - non commercially available: combination of essential vitamins, minerals and amino acids.
†BioMar, Grangemouth, UK
‡Bactocell®PA10, Lallemand Inc. Lyophilized form.

2.3 Vertebra radiological assessment

Fish were frozen and stored flat (-20 ˚C) prior to analysis to preserve the spine and insure integrity of radiographs. Lateral view radiographs were taken of parr (~30 g) and at smolt (~83 g) using a standard portable x-ray unit (Celtic SMR PX40 HF; calibration: kV: 40, mAs: 32) with an extremities plate measuring 24 x 30 cm, and subsequently digitised

using an AGFA CR35-X Digitizer. Resulting radiographs were analysed for vertebral number, pathology classification and length: dorsoventral diameter ratios in DICOM format using image analysis software Image J (Image J 1.46r, NIH, USA; Fig 4.2).

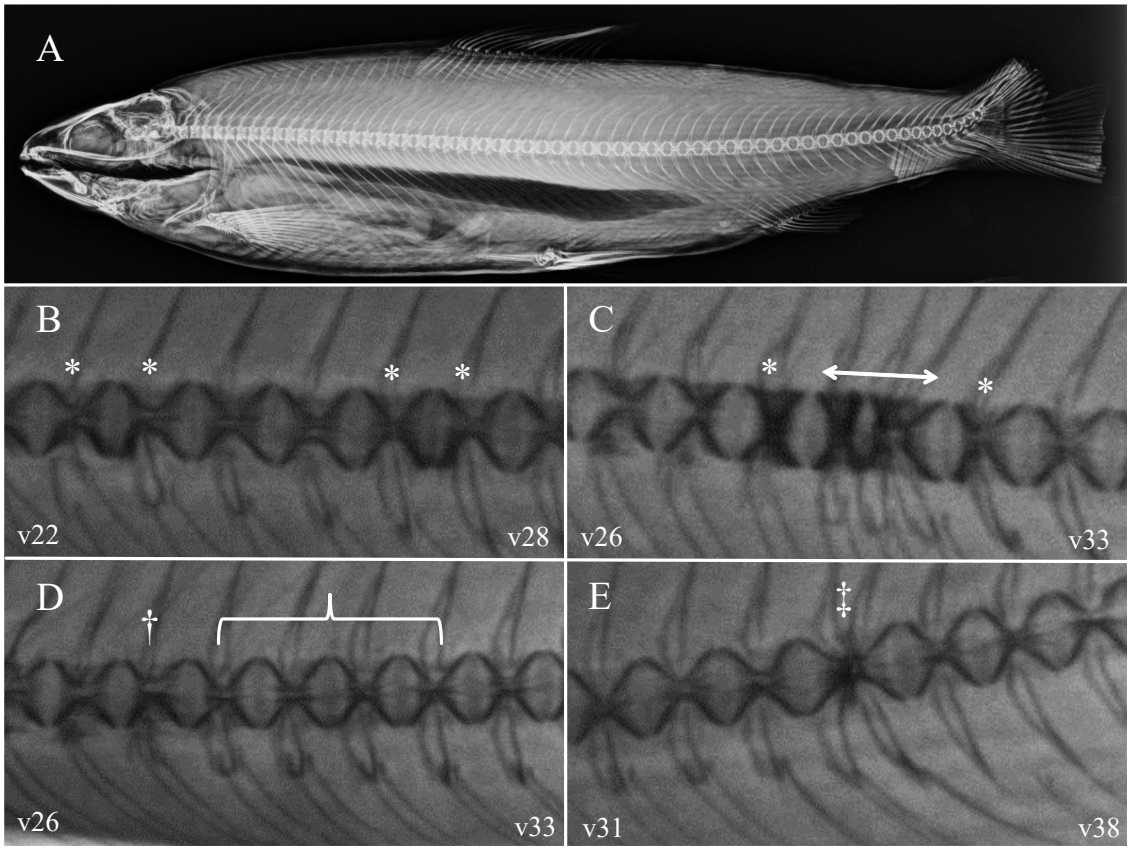

Figure 4.2. X-ray assessment (A) including examples of vertebral pathology (B-E) according to Witten *et al.,* (2009): B) Compression (*); C) fusion (aggravated (double ended arrow); D) radio-opaque (bracket) and asymmetrical (†); and E) complete fusion (‡). Vertebral numbers (v) are also given.

2.4 Whole body mineral content

For each stage (5g, 20g and 83g) groups of 5 whole fish were homogenised in triplicate (15 fish total / tank, n=3) using an industrial blender for 5 minutes. Subsamples of 25 g of homogenate were ashed in porcelain crucibles in a muffle furnace at 600 ˚C for 16

hours. Subsamples were weighed and divided into 3 technical replicates (~0.1 g) for analysis of mineral content. 5 ml of AristAR® nitric acid (HNO3; VWR International, USA) was added to the technical replicates in digestion tubes and placed in a MarsXpress digestion system set to 10 min heating phase to 160 ˚C, 29 min at 160 ˚C and 30 min cooling phase to room temperature. Digested samples were diluted to 2 % HNO3 and elemental concentration analysed with a Thermo X Series II ICP-MS collision cell model for P, Ca and Mg.

2.5 Calculations

K was calculated as: $W^*(L^3)^{-1}$ x100 where W weight and L is length. TGC was calculated as: $(W_f^{1/3}-W_i^{1/3})x(\sum^oD)^{-1}$, where W_f is the final body weight, W_i is the initial body weight and ^oD is the cumulative sum of water temperature in degrees per day. eFCR was calculated as: $F/(B_f-B_i+B_m)^{-1}$ where F is the food fed (kg), B_f is the final biomass (kg), B_i is the initial biomass (kg) and B_m is the mortality biomass for the period (kg). As feed collection data was not taken eFCR can only be a crude estimate.

Resulting whole body P concentrations, eFCR and dietary P concentrations were used to calculate theoretical P discharge into a FW loch in Scotland and impact on existing levels using the Organisation for Economic Cooperation and Development

(OECD) equation (OECD, 1982) adapted from the Scottish Aquaculture Research Forum (SARF, 2010):

$$\Delta TP = a\left[\frac{\Delta TP_{in}}{1+\sqrt{T}}\right]^{b}$$

Where ΔTP = predicted effect of annual fish farm emission on in-loch total P concentration, ΔTP_{in} is the predicted effect of farm emissions on inflow concentration, T = loch residence time (year) and a & b are empirical constants (1.55 & 0.82 respectively) from the OECD combined data set (OECD, 1982). For the sake of this experiment Loch Arkaig was used to model ploidy and dietary effects on P discharge owing to the large existing environmental dataset and appropriate simplicity with this water body. Hence, an existing T value of 1.36 yrs was used. Farm effect on P inflow was calculated as:

$$\Delta TP_{in} = \frac{TP_{load}}{Q}$$

Where TP_{load} is the total P load (kg yr^{-1}) and Q is the loch outflow (m^3 s^{-1}), which is calculated at 5.57 x 10^{-8} for Loch Arkaig. TP_{load} is calculated as:

$$TP = x * TP_{prod}$$

Where x is the increased production (t yr^{-1}) and TP_{prod} is the total P discharged from the farm per tonne produced (Kg total P t^{-1} prod):

$$TP_{prod} = \left[TP_{feed} * FCR\right] - TP_{fish}$$

Where TP_{feed} is the total P content in the feed (Kg) and TP_{fish} is the total P content found in the fish flesh. The maximum value for production (x; t yr^{-1}) in Loch Arkaig was also

estimated based on SEPA requirements where, as an oligotrophic Loch, P concentration should not rise above 8 mg m^{-3} in order to maintain trophic status (SEPA, 2014) and as such, resulting production calculations were adjusted accordingly.

2.6 Statistics

All data were analysed and compared using the R language (R Core Team, 2013) and significance was accepted at 5 % ($p < 0.05$). Results are reported as mean (± SEM). Datasets confirmed to be normal and homogeneous through Anderson Darling's and Levene's statistics (growth, number of vertebrae, length: dorsoventral diameter ratios and mineral concentrations) were analysed using the *lme* function in the *nlme* package for One-Way-ANOVA with replicates nested within dietary treatment. Post-hoc analysis was performed using the *glht* function for Tukey's multiple comparison in the *multcomp* package. Radiography deformity (average number of deformed vertebrae, severely deformed and non-deformed individuals) datasets showed a negative binomial distribution confirmed with the function *odTest* in the *pscl* package so a generalised linear model with overdispersion was performed using the *glm.nb* function in the *MASS* package. As well as deviance analysis, post-hoc analysis was performed using the *glht* function for Tukey's multiple comparison in the *multcomp* package.

3 Results

3.1 Growth and survival

No significant differences in weight were observed between tanks upon first feeding (0.20 ± 0.01 g). Dip S had a significantly higher weight compared to all other triploid treatments within the initial stage (~ 1 g; $p < 0.0001$; Fig. 4.3a). Trip SBC at the first sample point were significantly lower in weight than all other treatments. However, at smolt largely comparable weights were observed between treatments with the exception of Trip H ~20g which was significantly smaller to other Trip H treatments and Trip SBC ($p < 0.001$; Fig 4.3b). Trip SBC held lower K to other treatments at the first and last two time points development with final K factors being significantly lower to Dip S, Trip H and H-5g treatments ($p < 0.0001$; Fig 4.3b). Trip H-5g had a significantly higher K compared to all other treatments at smolt. There were no significant differences between treatments in TGC and eFCRs (Table 4.2). In addition there were no significant differences in survival between treatments.

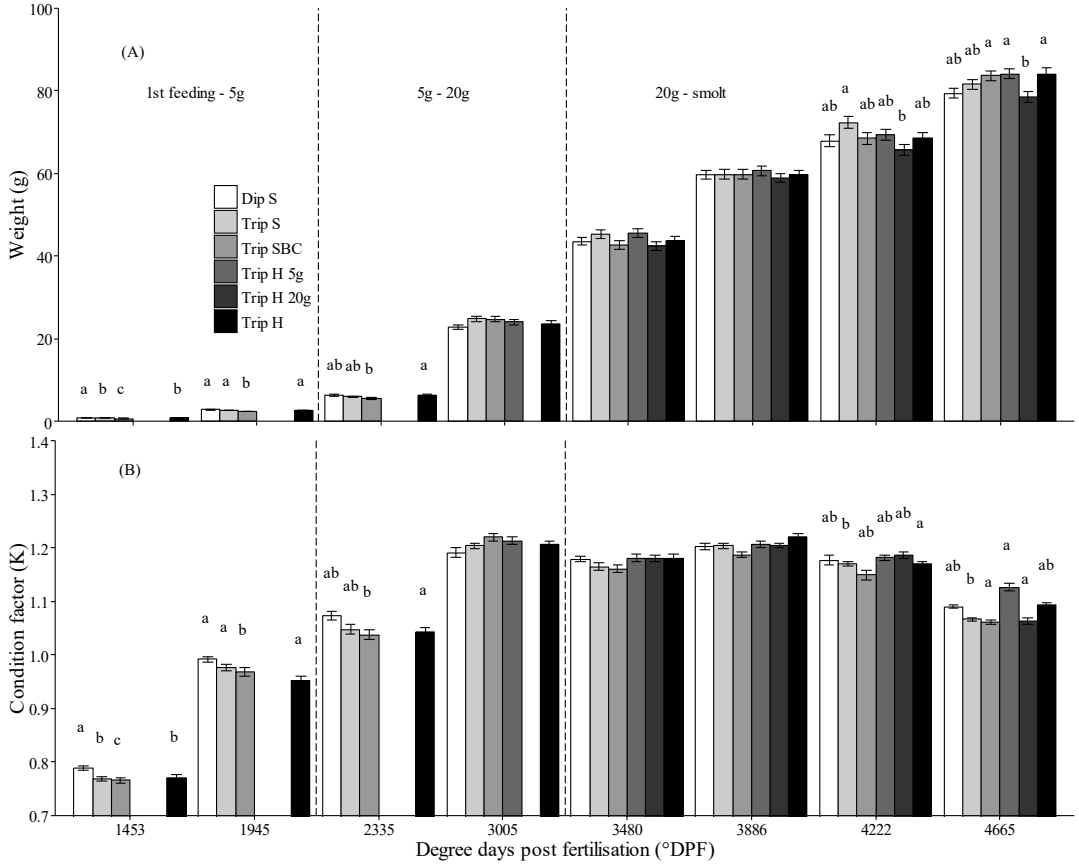

Figure 4.3. (**A**)- Mean weight (**B**) and K (**C**) throughout FW development (˚DPF) including early parr (1st feeding – 5g), later parr (5g – 20g) and until smolt (20g - smolt) for: Dip S, Trip S, Trip SBC, Trip H, Trip H 5g and Trip H 20g. Values are missing for Trip H 5g and Trip H 20g where life stage treatments are not yet present (early parr: Trip H 5g; later parr: Trip H 5 & 20g). Data expressed as mean ± SEM, n = 3, 30 individuals/replicate/time point). Values that do not share a common subscript denote significant differences ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

Table 4.2. Weight (g), fork length (mm) and K at trial termination including cumulative survival (%), TGC and crude eFCR since first feeding (mean ± SEM, n=3 tank means per treatment).

	Dip S	Trip S	Trip SBC	Trip H 5g	Trip H 20g	Trip H
Final weight (g)	79.3^{ab} ± 2.2	81.4^{ab} ± 2.6	83.5^{a} ± 1.4	84.1^{a} ± 1.9	78.5^{b} ± 5.7	84.3^{a} ± 3.4
Final length (mm)	193.2^{b} ± 1.1	196.3^{ab} ± 2.1	198.8^{a} ± 0.8	195.1^{ab} ± 4.6	193.7^{b} ± 3.9	196.6^{ab} ± 2.3
Condition Factor (K)	1.09^{a} ± 0.01	1.07^{c} ± 0.00	1.06^{c} ± 0.01	1.13^{a} ± 0.05	1.06^{c} ± 0.03	1.09^{b} ± 0.01
Cumulative Survival (%) *ns*	98.75 ± 0.11	97.91 ± 0.34	97.45 ± 1.04	97.33 ± 0.79	97.76 ± 0.07	97.33 ± 0.24
TGC *ns*	0.99 ± 0.01	1.00 ± 0.00	1.01 ± 0.01	1.02 ± 0.01	0.99 ± 0.33	1.01 ± 0.02
eFCR *ns*	0.88 ± 0.03	0.93 ± 0.01	0.87 ± 0.02	0.87 ± 0.01	0.91 ± 0.08	0.89 ± 0.03

[a,b,c,d] Mean values with different lowercase superscript letters are significantly different ($p < 0.05$; One-Way ANOVA & Tukey's post hoc).

ns No significant differences ($P < 0.05$; One-Way ANOVA).

3.2 Skeletal assessment

Dip S and Trip H-5g had the most number of vertebrae significant to Trip H ($p = 0.02$) at the parr stages (~30 g) with no differences between number of vertebrae observed at smolt (~83 g; Fig. 4.4).

Trip S parr (~30g) had the highest mean numbers of deformed vertebrae, significantly greater than Dip S, Trip H and Trip SBC ($p = <0.0001$). Trip SBC parr had significantly fewer deformed vertebrae compared to Trip H-5g and Trip H-20g. Dip S had significantly fewer deformed vertebrae compared to triploid treatments, which was maintained until smolt (~ 83g; $p = <0.001$). Trip S and Trip H-5g had significantly higher number of deformed vertebrae at smolt ($p = 0.04$). Dip S had significantly higher numbers of non-deformed individuals both at parr ($p < 0.0001$) and smolt ($p = 0.001$) with no significant differences observed within triploid treatments. In addition, Trip SBC was the only treatment with no severely deformed individuals significant to Trip S within the parr stages ($p = 0.04$). No significant difference between treatments in number of severely deformed individuals was observed in the smolt stages however overall frequency was low with only 1 individual observed per treatment.

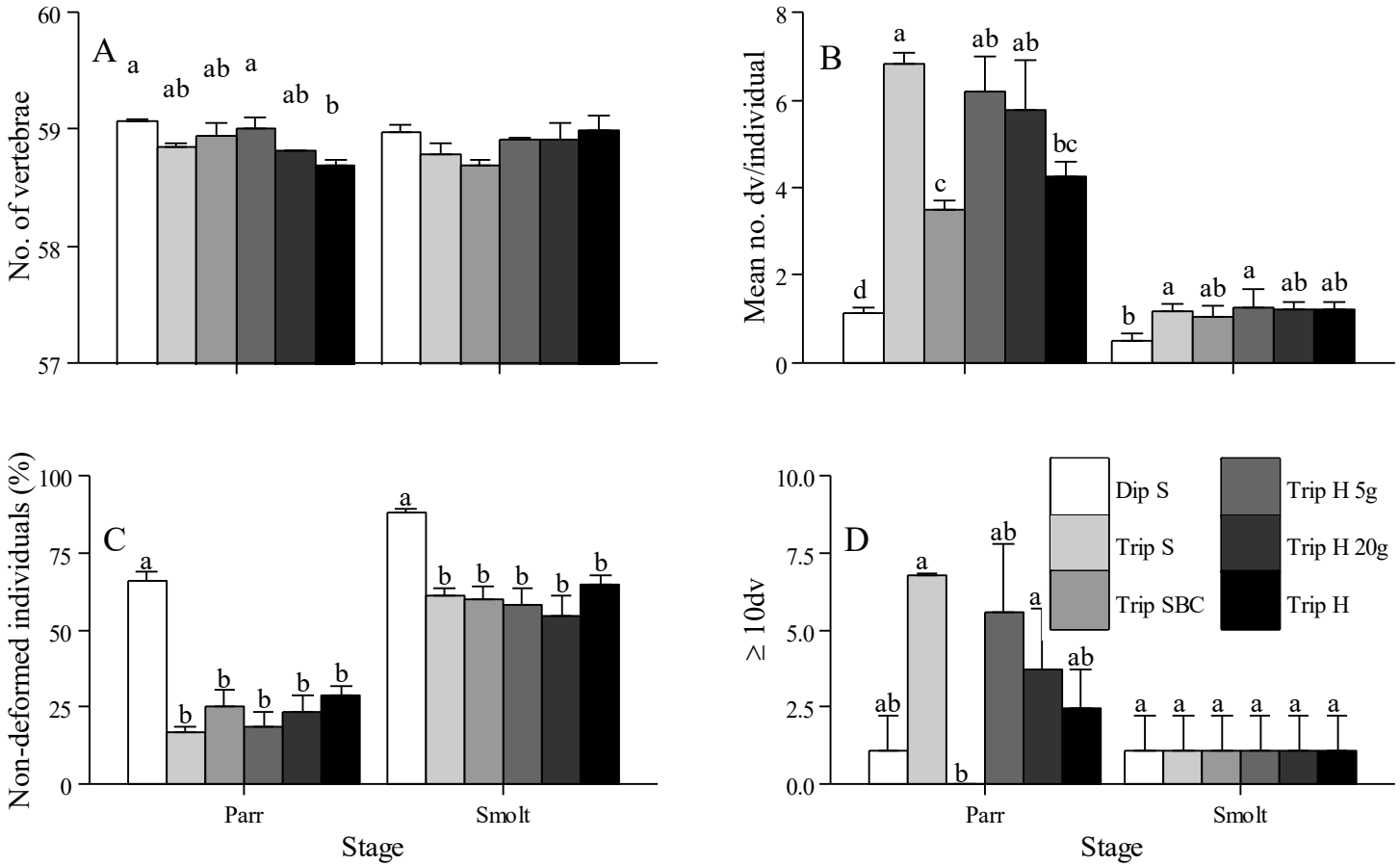

Figure 4.4. Skeletal assessment: mean number of vertebrae (A), mean number of deformed vertebrae (B), percentage non-deformed individuals (C) and individuals with ≥ 10 deformed vertebrae (D) at parr (~30g) and smolt (~83g). Data expressed as mean ± SEM (n=3, 30 fish/replicate/stage). Values that do not share a common subscript denote significant differences (One-Way ANOVA (A), negative binomial GLM (B-D) & Tukey's post hoc analysis).

Of the deformed vertebrae the most frequently occurring pathologies throughout development were asymmetrical / displacement (parr: 13.1 %; smolt 4.4 %) and compression (parr: 9.4 %; smolt 3.8 % Fig 4.5). The largest variation in pathology between treatments occurred in parr. There was a decline from high observations of radiodense / opaque pathology at parr to lower observations at smolt across all treatments (Trip H-5g: -1.4 %, Trip S: -0.9%, Trip SBC: -0.7 %, Trip H-20g -0.5 %, Dip S: -0.1 %, Trip H: -0.1 % reduction respectively). Within treatments, prevalence of fusion pathologies were maintained at similar levels between life stages (parr: 2.1%; smolt: 2.1%). The overall reduction in percentage deformed vertebrae across treatments between parr and smolt was largely represented by a decline in asymmetry / displacement (-8.7 %), compression (-5.6 %) and radiodense / opaque (-3.7 %) pathologies.

Deformity prevalence was largely localised to the central region (vertebrae 26-32) and region 4, and a smaller peak in region 1 (Fig 4.6 a & b). No difference in regional deformity prevalence was observed within triploid treatments but Dip S was noticeably lower in the central region. Overall a larger deformity prevalence was observed at parr (~30g) than at smolt.

At the parr stage, no difference was observed between treatments for vertebral length: dorso-ventral diameter ratio with the exception of region 2 where Trip H-5g had significantly higher values than Dip S and Trip SBC (p = 0.03; Fig 4.6 c & d). At smolt

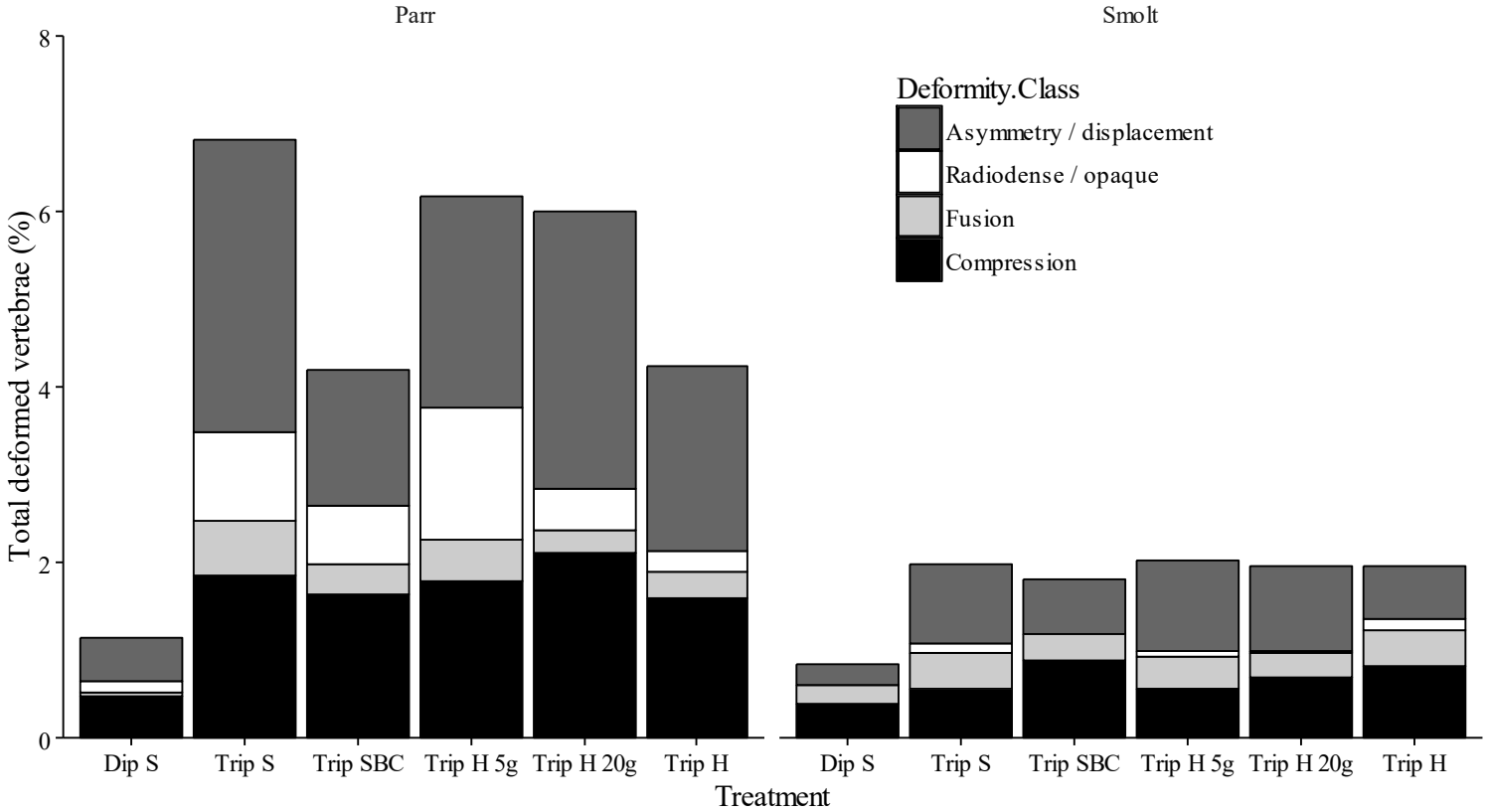

Figure 4.5. Percentage pathology type of total deformed vertebrae (%) grouped according to Witten *et al*.(2009) at parr (~30g) and smolt (~83g; n = 3, 30 fish / replicate/stage; mean ±SEM).

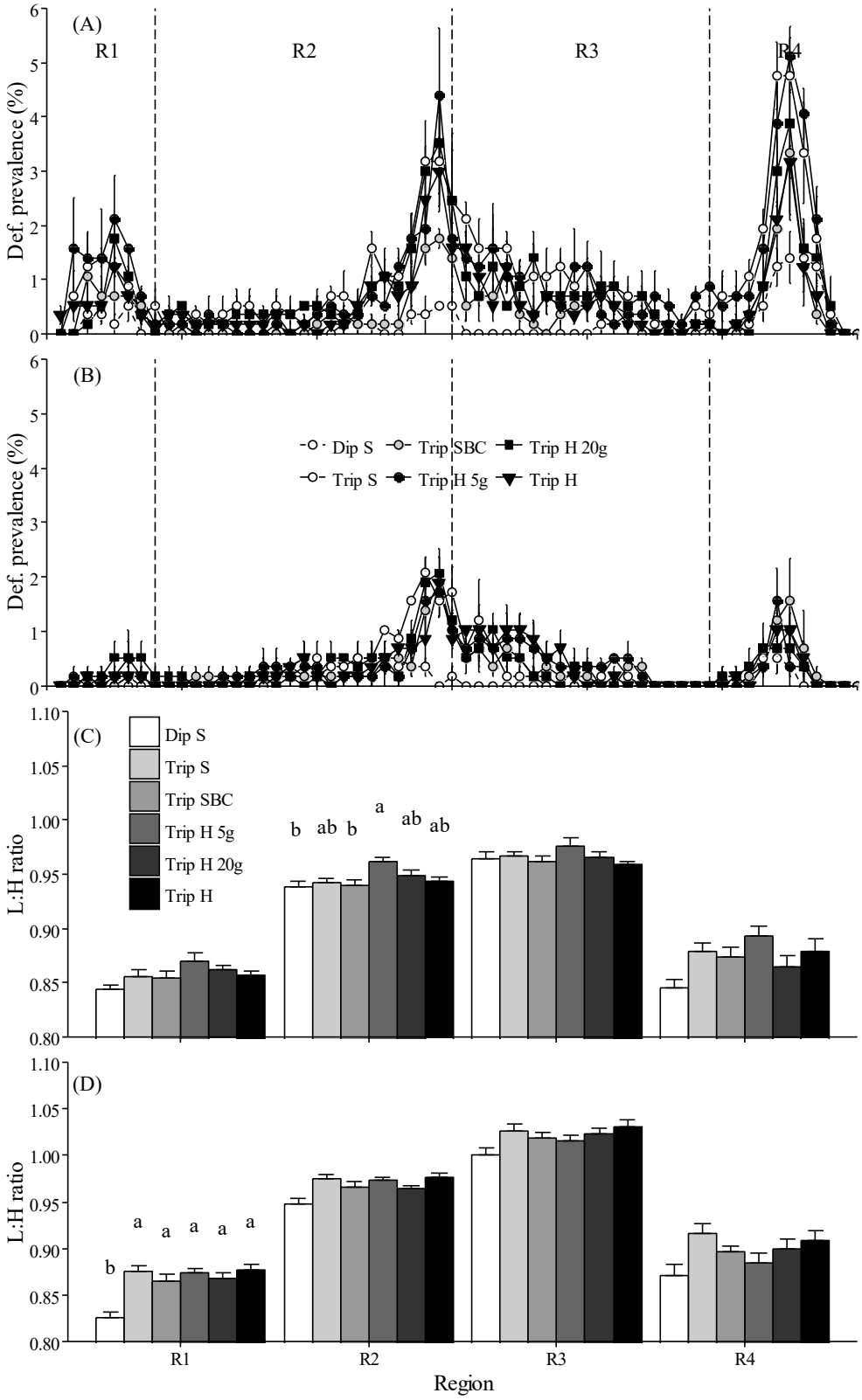

Figure 4.6. Mean prevalence of deformed vertebrae (A &B) and length: dorsoventral diameters (L:H; C & D) at parr (A &C) and smolt (B & D; n=3, 30 fish per replicate). Regions 1(v1-8), 2(v9-30), 3(v31-49), and 4(v50-60). Values that do not share a common subscript denote significant differences (p <0.05; One-Way ANOVA & Tukey's post hoc).

all triploid treatments had significantly higher length: dorsoventral diameters ratios in region 1 than diploid S (p = 0.008) with no difference between ploidy or diet throughout the other vertebral regions.

3.3 Whole body mineral content

In early parr (~5 g, 2335˚DPF) whole body P content was significantly higher in Dip S than Trip S and Trip SBC (p = <0.0001; Figure 4.7a). Trip H was also significantly higher to Trip S but not Trip SBC early parr. In later parr (~20 g; 3005 ˚DPF), Trip H and Trip S had significantly higher P concentrations than Trip SBC (p = 0.04). At smolt (~83 g; 4665 ˚DPF) whole body P concentration in Dip S was significantly higher to all other treatments except Trip H-20g (p = 0.007). Similar results were observed for Ca except Trip H which was not significantly different to any treatment in early parr (p = 0.004; Fig 4.7b) and at smolt Dip S was only significantly higher than Trip H (p = 0.003; Figure 4.7b). Ca and P results reflected the Ca:P ratio except no significant differences were observed at early parr and remained similar to Ca results between treatments within later parr and smolt stages (Fig 4.7c). Mg concentrations were significantly higher in Dip S and Trip H at early parr (p = 0.05; Fig 4.7d) and declined at 20 g where Trip H was significantly higher than Trip SBC and Trip S (p = 0.004; Fig 4.7c). Mg concentrations increased in smolts with no significant differences between treatments.

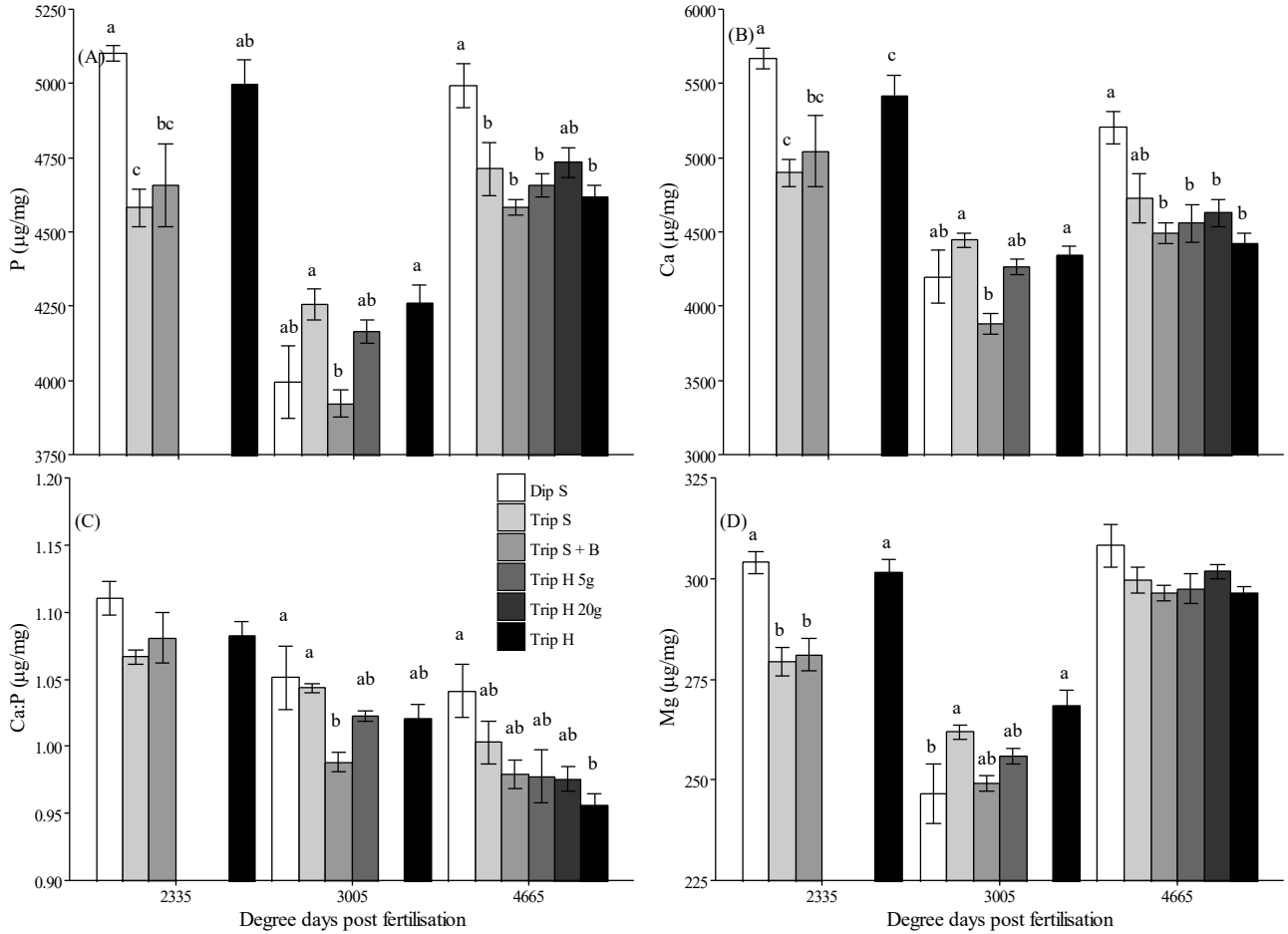

Figure 4.7 Change in whole body mineral composition (µg/mg; 15 fish/replicate/time point) at early and later parr, and smolt (2335, 3005 and 4665 °DPF respectively) stages for P (A), Ca (B), Ca: P ratio (C) and Mg (D). Values are missing for Trip H 5g and Trip H 20g where life stage treatments are not yet present (early parr: Trip H 5g; later parr: Trip H 5 & 20g). Data expressed as mean ± SEM (n=3, 15 fish / replicate / time point). Values that do not share a common subscript are significantly different (p <0.05; One-Way ANOVA & Tukey's post hoc).

3.4 P discharge

There was a clear impact of dietary P inclusion on theoretical P discharge rate where trip H had potential to discharge 20.9 % more from production compared to trip S (Table 4.3). Discharge rates between Trip S and Trip SBC were comparable. Trip H 5g had 7.0 % lower theoretical P discharge compared to Trip S, which was largely attributed to lower eFCRs, whereas Trip H 20g had 6.2 % higher theoretical P discharge. Overall, this translated into a marked higher potential total Loch P concentration with 200 t yr^{-1} of production for Trip H and trip H 20g, alongside reduced production potential to remain within SEPA guidelines (SEPA, 2014), compared to Trip S and Trip H 5g respectively.

Table 4.3. P discharge, Loch P concentration and resulting production potential according to calculations based on the OECD model (OECD, 1982) for each treatment. Theoretical P discharge for each dietary treatment based on eFCR, total whole body and feed P concentration g Kg^{-1} in this trial. P concentration of Loch Arkaig is based on estimations of P discharge, existing P levels in Loch Arkaig and an assumed production value of 200 t yr^{-1}. Production potential is the maximum increase in production (%) that is possible before a Loch P concentration of 8 mg m^{-3} is breached, which would breach the oligotrophic Loch status failing to remain with SEPA guidelines (SEPA, 2014).

	Dip	Trip S	Trip SBC	Trip H 5g	Trip H 20g	Trip H
P discharge (g Kg-1 production)	7.56	8.56	8.64	7.96	9.10	10.82
Loch P concentration (200 t production)	6.35	6.54	6.56	6.43	6.65	6.98
Production potential (%)	116.8	91.7	89.9	106.1	80.3	51.6

4 Discussion

Feeding higher dietary P for reduced time periods from first feeding up until early (~5 g) or later (~20 g) parr stages does not have the same benefit on resulting parr skeletal

performance (~30 g) in triploid Atlantic salmon as feeding until smolt (~83 g). More importantly, inclusion of *P. acidilacticti* significantly improved triploid skeletal performance at 30 g significant to the control diet. However, at smolt, prevalence of skeletal deformities and severity was largely the same within triploid treatments and no difference between ploidy in mean number of severely deformed fish was observed. Similarly, little difference in weight was observed at smolt between diploid and triploid treatments. A stronger effect of P supplementation was observed on whole body content of P and Ca and Mg within the early parr life stages (~5 g) and little effect within triploid treatments at smolt. Results emphasise the sensitivity of triploids to different dietary regimes in earlier life stages or periods associated with faster growth.

Little difference was observed in cumulative mortality between treatments which is similar to results from chapter 3 as well as other studies investigating FW dietary P in triploid Atlantic salmon (Fjelldal *et al.,* 2015), and *P. acidilactici* inclusion in diploid rainbow trout (Aubin *et al.,* 2005). Triploids displayed comparable weight gain compared to diploids by smolt which is similar to results in chapter 3 where triploids had comparable weights at smolt compared to diploids fed 13.0 g total P Kg^{-1}. Collectively these results are converse to other published reports on S0+ (Leclercq *et al.,* 2011; Taylor *et al.,* 2012) and S1+ triploid Atlantic salmon smolts (Taylor *et al.*, 2012). In addition, triploids fed reduced P inclusion in this trial (14.3 g total Kg^{-1}) attained comparable

weights to triploids fed higher P (17.4 g total Kg^{-1}), converse to the apparent growth reducing effect observed in triploid smolts fed HP (19.7 g total Kg^{-1}) compared to LP (13.0 g total Kg^{-1}) in chapter 3. This suggests P levels in this trial were optimal to sustain triploid FW growth. Within triploid treatments, those fed *P. acidilactici* showed reduced growth performance compared to other triploid dietary treatments in early development (< 1g) but attained comparable weights to other treatments at smolt, with exception of triploids fed high P until 20 g which were significantly lower. Previous literature has shown similar improved growth when supplementing *P. acidilactici* to control diets in diploid rainbow trout (Aubin *et al.,* 2005) and European sea bass (Lamari *et al.,* 2013). Reduced growth in the early stages are likely due to a combination of factors including introduction of novel microbes during first feeding and probiotic induced gut alterations that may initially inhibit nutrient assimilation. For instance, reduced GI microbiota species diversity were observed in red Nile tilapia when fed *P. acidilactici* along with potential antagonistic effects (Ferguson *et al.,* 2010), which may have implications for early-stage gut colonisation and growth. In addition, it has been shown in zebrafish that germ-free rearing results in a failure of essential gut epithelial mucosa differentiation (Bates *et al.,* 2006). Triploids supplemented with additional P had similar growth results to triploid control diets both throughout FW and at smoltification. This is consistent with other literature investigating higher dietary P inclusion in triploid FW development where

weight growth differences were observed when low inclusion diets were below 10 g total P kg^{-1} (Burke *et al.,* 2010; Fjelldal *et al.,* 2015).

Whilst triploids fed high P supplementation (17.4 g total P kg^{-1}), including those fed high P only to early parr stage (5 g), displayed significantly higher K at smolt, triploids fed the control diet (14.3 g total P kg^{-1}) and *P. acidilactici* inclusion were significantly lower. In chapter 3 no significant difference in K was observed between triploid treatments and overall K factor in triploids was lower compared to diploid counterparts. Given the lack of difference in weight parameters in this trial it may be assumed that these differences could be attributed to elongation. Similarly, reduced K were also reported in rainbow trout fed *P. acidilactici* with no other parameters affected (Merrifield *et al.,* 2011). In the case of P, higher available circulating P may trigger active suppression of cranio-caudal vertebral mineralisation, important in early development, through bone-P homeostatic pathways, as reviewed in chapter 3. Deformity prevalence was localised to the central (v24-35) and tail region (v52-59) which is similar to results from chapter 3 and previous reports in triploid Atlantic salmon during FW growth (Fraser *et al.,* 2015a; Fjelldal *et al.,* 2015). The central region is the cranio-caudal axis for outward mineralisation (Grotmol *et al.,* 2003), and the tail region has been reported to be more susceptible to morphological variations (Kacem *et al.,* 1998), which explains the nature of deformity prevalence typically observed in these regions. Little difference in

deformity prevalence was observed within treatments with the exception of a generally lower prevalence around the cranio-caudal axis in diploid controls compared to triploids at smolt. In addition, there was no relative increase in deformity prevalence in R3 (known to be associated with pathology in SW) in triploids fed S (14.3 g total P kg^{-1}), unlike in triploids fed LP (13.0 g total P kg^{-1}) in chapter 3. Hence P inclusion in this trial may be adequate for vertebral development during smoltification. However, unlike the current study fish were also vaccinated in chapter 3, and as a known factor of deformity development through inflammation (Gil-Martens, 2010), may have been an exacerbating factor. It has been established that localisation of deformities is ontogenetic (Fjelldal *et al.,* 2012b) and a tendency for higher prevalence in the tail and central region was observed at 30 g and smolt (~83 g) in this study. However, diploid smolts had significantly shorter length: dorso-ventral diameters to triploids despite low and similar deformity prevalence across treatments for this region. This indicates that elongation of the vertebral column during smoltification may indeed be affected by ploidy.

Triploid parr (~30g) fed high P had significantly fewer deformed vertebrae relative to the control diet whereas high P treatments fed up to 5 or 20 g were not significantly different to either high P or control diets. The profile of increased deformity prevalence with decreased high P feeding window in triploids implies that skeletal requirement for higher P is continuous up until 30 g. Triploids fed the control diet and

high P only until 5 g developed the most deformed vertebrae with a larger representation of radiodense / opaque deformities indicative of suboptimal mineralisation (Witten *et al.,* 2009). Our results are consistent with other literature which has shown feeding lower available P in the form of phytic acid results in higher prevalence of radiodense vertebrae in diploid Atlantic salmon (Helland *et al.,* 2006). As a teleost fish, Atlantic salmon skeletal growth and bone remodelling is continuous (Witten & Huysseune, 2009) and as such, nutritional requirement for higher dietary P, may be constant. Conversely, few differences in vertebral deformity occurrence were observable by x-ray at smolt within triploid treatments, which is unlike results in chapter 3 that show a clear reducing effect of vertebral malformations at smolt with dietary P inclusion. Total dietary P levels used in this trial (14.3 - 17.4 g total P Kg^{-1}) were of levels known to minimise vertebral deformities in both triploid (Fjelldal *et al.,* 2015) and diploid Atlantic salmon smolts during FW rearing (Fjelldal *et al.,* 2012a) and may explain the overall good vertebral performance between treatments at smolt. P inclusion levels were lower (13.0 g total Kg^{-1}) in chapter 3 where vertebral deformities were prevalent at smolt. Diploid controls had fewer deformed individuals as well as fewer deformed vertebrae per individual compared to any triploid treatments. Attaining correct skeletal development in FW for sea transfer is crucial in ensuring correct skeletal and somatic performance during later SW ongrowing in Atlantic salmon (Fjelldal *et al.,* 2009b, 2012b) where deformity risk factors

such as accelerated growth and high water temperatures are compounded. In particular high deformity prevalence typically observed at harvest in triploids (Leclercq *et al.,* 2011) may be derived from originally less severe FW pathologies. Hansen *et al* (2010) has shown that post-smolt Atlantic salmon with ≥10 deformed vertebrae may be growth compromised in SW. Occurrence of severely deformed individuals remained similar between diploid and triploid treatments in this trial indicating potential for pathological similarity, which may also have benefits for growth.

Differences in skeletal deformities between triploid treatments in parr (~30 g) but not at smolt highlight higher sensitivity to dietary supplementation during early FW development, which was also indicated in chapter 3. Temperature induced accelerated growth rates throughout parr development may have promoted deformity differences during this period. High water temperature in combination with faster growth is known to disrupt bone and cartilage formation leading to vertebral pathology in diploid Altantic salmon (Ytteborg *et al.,* 2010a). Fraser *et al.,* (2014) showed reduced deformity prevalence in S0+ Atlantic salmon smolts compared to S1+ attributed to prolonged higher temperature and associated growth in S1+ regimes. Cooler environmental water temperatures in the winter in this study may have better enabled mineralisation as temperatures largely remained below 12 ˚C up until smoltification thereby reducing overall vertebral deformities to similar levels between treatments by smolt. In addition

smolt associated elongation of the caudal region in preparation for pelagic swimming (Winans and Nishioka, 1987) may accelerate skeletal growth (Fjelldal *et al.,* 2006). Collectively this indication of increased skeletal growth is supported by a large reduction in compressive and radiodense / opaque vertebrae observed at smolt in the present study. Compressive vertebral pathologies may lead to more severe fusion pathologies (Witten *et al.,* 2006) and radiodense vertebrae, which has been found in diploid Atlantic salmon fed reduced available P, and is attributed to cartilage formation and potential undermineralisation (Helland *et al.,* 2006). These pathologies may have resolved during winter where reduced muscle growth is observed. Pressure from muscle mass and mechanical loading may impede optimal osteogenesis and chrondrogenesis (Witten *et al.,* 2005). This is supported by other research in triploid Atlantic salmon parr with radiodense deformities in warmer temperatures (8-12˚C) which are largely reduced at smolt (<8 ˚C; Fraser *et al.,* 2013a). Levels of severe fusion pathologies were maintained between life stages within treatments. It has been shown in pre-smolts that fusion cannot be resolved and may lead to aggravation of vertebral deformity development later in life (Witten *et al.,* 2006). Results therefore indicate nutritional supplementation is important in facilitating triploid skeletal development in early life stages and periods of faster growth.

This is the first study to date to assess the effect of dietary probiotics on triploid growth and skeletal development in fish. Dietary inclusion of *P. acidilactici* reduced prevalence of vertebral deformities in 30 g fish significant to triploids fed a control diet and those fed high P until 5 g and 20 g. In addition, no severely deformed individuals were observed at 30 g when fed *P. acidilactici* inclusion, which was significantly lower than triploids fed the control diet. *P. acidilactici* has also been shown to reduce vertebral compressions in rainbow trout (Aubin *et al.,* 2005) and improve skeletal conformation in European seabass (Lamari *et al.,* 2013). Triploids have altered gut morphology, including a shorter relative gut length and fewer pyloric caeca (Peruzzi *et al.,* 2014), and so triploid Atlantic salmon may have compromised digestive efficiency that may benefit from dietary probiotics. Longer microvilli have been observed in the proximal intestine of rainbow trout fed *P. acidilactici* (Merrifield *et al.,* 2009). *P. acidilactici* may have therefore contributed to altered gut morphology and enhanced nutrient uptake of key minerals involved in skeletal development such as P. Assessment of gut morphology was not performed in this study, however results indicate further investigation may be required for interpretation of response to dietary probiotics in triploid Atlantic salmon. Other potential mechanisms involved include: reduction of pro-inflammatories (Picchietti *et al.,* 2009); bacteriostatic effects of pathogenic bacteria related to vertebral deformity (Aubin *et al.,* 2005); increased mineral solubility and digestibility through

production of lactic acid and short chain fatty acid production that induce gut acidity (Scholz-Ahrens *et al.,* 2007); potential degradation of the mineral-complexing phytate increasing P availability (Scholz-Ahrens *et al.,* 2007). Response to inducers of inflammation including vaccination in Atlantic salmon (Fraser *et al.,* 2014) and disease challenge in Chinook salmon (Ching *et al.,* 2009) have shown little difference between ploidy rendering it unlikely that the mitigating effect of *P. acidilactici* in this trial is due to a reduction in pro-inflammatories. Investigations into triploid Atlantic salmon gut characteristics show altered community and drug resistance of culturable intestinal microbiotic in triploid Alantic salmon with implications that there may be an increased susceptibility to probiotics driven by differences in gut microbial species concentrations (Cantas *et al.,* 2011). However, further research is required to underpin mechanisms underlying the impact of *P. acidilactici* on triploid Atlantic salmon vertebral development.

More importantly, *P. acidilactici* in triploid Atlantic salmon parr appears to improve skeletal development to further effect than P supplementation. Crude analysis modelling the effects of theoretical P discharge from each of the dietary treatments on aquatic P concentration in an example Loch case study, show that feeding high P throughout FW may markedly increase aquatic P levels and therefore substantially reduce the scope for production. In addition, feeding high P to only 5g increased the scope for

production to further effect than triploids fed a standard diet. However, as also showed in this study, feeding high P to only 5g did not reduce malformation prevalence as observed in trip H. Hence, feeding triploid Atlantic salmon in FW with *P. acidilactici* may be the optimal triploid aquafeed choice to collectively reduce malformation prevalence in FW and mitigate anthropogenic impact of discharge on the environment. However, repeated studies are required to verify dietary effects in the later parr-smolt stages where no impact on vertebral deformity prevalence was observed.

Research has shown that triploid and diploid Atlantic salmon have lower assimilation of P in bone when fed suboptimal levels of 7.6 g total P kg^{-1} as opposed to 9.9 and 13.9 g total P kg^{-1} (Burke *et al.,* 2010). In this study, fish were fed above suboptimal ranges (14.3 - 17.4 g total P kg^{-1}) and whilst whole body P concentration was initially higher in diploid control and triploid fed high P, in the smolt stages triploid treatments had lower overall concentration to the diploid control with the exception of those fed from first feeding to parr stage. The similar observation in Ca levels indicates that less Ca and P may be stored in bone and scales relative to diploids in the later stages. In addition Mg is an important contributor to osteogenesis as a skeletal tissue metabolite (Tiago *et al.,* 2008). Higher levels of Ca, Mg and P in triploids fed higher dietary P relative to their dietary counterparts suggest increased osteogenesis in these early life stages with little effect of diet within later developmental stages in FW.

Overall, triploids require continuous high dietary P to 30g to mitigate skeletal deformities, and may be associated with seasonal factors of high temperatures and accelerated growth in this early developmental stage. It was evident that feeding high P supplementation for specific windows up until 30 g was not sufficient although feeding probiotic *P. acidilactici* inclusion did reduce vertebral deformities. This may benefit future commercial implementation of triploids through reducing phosphate discharge into the environment and mitigating potential environmental impacts. Similarities in vertebral parameters between triploid dietary treatments at smolt unlike parr stages, may potentially be attributed to the cooler water temperature during winter growth in combination with ontogenetic-dependent differences in skeletal growth that may prevent linear growth failure. Further research is required to study the mechanisms of *P. acidilactici* on triploid gut and associated benefits to skeletal growth in order to optimise and ensure future implementation of triploid Atlantic salmon. In particular, ongrowing studies are required to verify the effect of dietary treatments on alleviating skeletal deformities that manifest at sea.

Chapter 5: Parr – smolt transformation windows in diploid and triploid Atlantic salmon (*Salmo salar* L.) under ambient photo-thermal regime.

Abstract

PST was investigated in diploid and triploid Atlantic salmon siblings under SNP with the aim to better define the smoltification window. Growth performance, K, smolt score indexing, gill NKA activity and SW challenge with corresponding plasma Cl^{-1} levels were assessed at intervals from -600 to 1086 degree days post winter solstice (˚*Dws*). Triploids were generally significantly heavier than diploids from 400 ˚*Dws*. Significant differences in K between ploidy were only observed prior to winter solstice and were overall significantly reduced with time until 1086 ˚*Dws*. Triploids showed significantly higher smolt score indexes (skin silvering, parr marks, and darkened fin edges) relative to diploids in the earlier stages –up to 377 ˚*Dws*. Triploids maintained improved survival to SW challenge and corresponding significantly lower plasma chloride (Cl^{-1}) levels relative to diploids from 500 to 750 ˚*Dws*. Triploids also had significantly higher NKA activity levels relative to diploids up until peak of activity at 482 ˚*Dws* and then maintained significantly higher levels between 752 - 914 ˚*Dws*. Together these results imply that triploids may not only have an earlier onset of smoltification by 48 degree days but also a wider smolt transfer "window" compared to diploids, which was estimated to be greater by 155 and 365.5 degree days based on collective results of NKA activity, skin silvering and improved hypo-osmoregulatory ability compared to diploid siblings.

1 Introduction

Literature has suggested that owing to their differential cellular, physiological and morphological assembly, triploid Atlantic salmon (*Salmo salar*) should be treated as a new species (Benfey, 2015). Optimisation of husbandry regimes is therefore required in order to facilitate viable implementation of triploidy, and in this respect, it has already been shown that triploids have different nutritional requirements (Taylor *et al.,* 2015; Fjelldal *et al.,* 2015; chapter 2, 3 & 4) and environmental tolerance than diploids (Fraser *et al.,* 2013a,b Hansen *et al.,* 2015). However, PST and subsequent SW transfer windows remain to be characterised in order to achieve optimal performance in commercial SW on-growing.

PST is one of the most stressful events within the production cycle of anadromous teleosts such as Atlantic salmon. During PST, fish undertake dramatic morphological and physiological changes particularly in the gut, gills and kidney to adapt and cope with transfer from a FW to a SW environment (McCormick *et al.,* 1997). In particular, activity of two isoforms of NKA identified as FW (NKA α1a) and SW (NKA α1b) play a crucial role in the recruitment and activity of gill chloride cells (McCormick *et al.,* 2009) that enable smolts to remain in FW or cope with SW transfer. It has been shown that smolts have a temperature dependent "smolt - window" (Stefansson *et al.*, 1998) primarily indicated by a peak in NKA activity for 250 degree days (Handeland *et al*., 2004) during

which sea transfer is optimal before desmoltification occurs. The onset of PST is mainly triggered through seasonal cues of photoperiod change (Duston & Saunders 1992) and temperature (Handeland *et al.,* 2004) and regulated through endocrine pathways involving primarily growth hormone (GH) and insulin-like growth factor I (IGF-I; McCormick, 2001). Under ambient photo-thermal regimes, the PST window in diploid Atlantic salmon parr occurs in Spring, synchronised by the increasing day length and warming water temperatures (McCormick *et al.,* 1997). Manipulation of photo-thermal regimes can induce early PST and production of out-of-season smolts (Duston & Saunders, 1992), which played an important role in the rapid expansion of the salmon industry by ensuring a year round supply of smolts.

Previous research undertaken has shown earlier SW transfer of triploid Atlantic salmon relative to their diploid siblings as a result of earlier increase in NKA activity and skin silvering for which similar survival post-transfer was observed (Leclercq *et al.,* 2011; Taylor *et al.,* 2012). However, in many studies, SW transfer of triploids was done simultaneously to diploids based on the assumption that triploids smolt comparably to diploids (Cotter *et al*., 2002; Fraser *et al*., 2013b; Taylor *et al*., 2014). Importantly, no research to date has investigated temporal profiles of PST indicators in triploids reared under an ambient photo-thermal regime with reference to diploids and this has a critical

importance in the salmon industry where SW transfer is considered as a key developmental milestone.

Cotter *et al.,* (2002) observed increased mortality post transfer in triploids which was attributed to failed smolting. However, suboptimal environmental conditions, for example temperature <19 ˚C and oxygen saturation levels <7 mg / L, known to impede growth performance (Hansen *et al.,* 2015) in combination with deployment stressors may have largely been responsible. Literature has shown cultured triploid teleosts, especially salmonids, have similar levels of NKA activity to diploid siblings for instance: rainbow trout (Taylor *et al.,* 2007) and European sea bass (Peruzzi *et al.,* 2005). In addition, plasma levels of GH and IGF-I were shown to be similar in other triploid salmonids such as chinook salmon (Shrimpton *et al.,* 2007) and rainbow trout (Taylor *et al.,* 2007) although higher plasma levels of GH were observed during fasting (Cleveland & Weber, 2014). Conversely (Shrimpton *et al.,* 2012) showed that triploid chinook salmon had significantly lower NKA activity than diploids although survival in SW and plasma Cl^{-1} levels were not affected suggesting no compromise on osmoregulatory capacity.

The successful implementation of triploid Atlantic salmon in culture requires the characterisation of the PST window and understanding of stock resilience for optimal SW transfer and performance. This study aimed to define and compare the PST window in triploid and diploid Atlantic salmon siblings using a set of standard indicators (growth,

K, skin silvering, NKA activity and survival following SW challenge with associated plasma Cl^{-1} levels) under an ambient photo-thermal regime.

2 Methods and materials

2.1 Fish stock and husbandry

Diploid and triploid Atlantic salmon eggs (Atlantic QTL-innOva® IPN) were supplied by Aquagen AS (Norway) on January 12, 2012 and transferred to Howietoun Fish Farm (Stirling, 56˚N, 4˚W) at 395 ˚DPF with a historical rearing temperature of 6.0 ± 0.5 ˚C prior to shipping. Triploidy was induced through a standardised pressure shock of 9500 psi for 50 ° minutes at 8 ˚C 300 °minutes post fertilisation (Taylor *et al.,* 2011). Fry were initially ongrown at Howietoun Fish Farm until transfer to smolt rearing facilities (Niall Bromage Freshwater Research Facility, Stirling, 56˚N, 4˚W) on May 15, 2012. Fish were reared under commercial conditions until 19 September 2012 and fed on standard commercial diets (Inicio Plus, BioMar, Grangemouth, UK) provided by automatic feeders. Triploid fish were derived from the same stock as in chapter 3, and therefore are already verified as triploid.

On September 19, 2012, experimental fish (weight range ± SD for dips and trips respectively) were randomly distributed between 6 x 980 L covered circular tanks (3 / ploidy, with 250 fish / tank). Fish were reared under constant light (LL) until October 18,

2012 where they were subsequently reared on a SNP with temperature of 7.8 ± 0.2 ˚C (Fig 5.1). Fish were not vaccinated for this study.

2.2 Sampling protocol

Monthly weight (± 0.1 g) and fork length (± 1.0 mm) assessments were conducted on a randomly netted subsample of 30 fish / tank (n = 3 / ploidy) anesthetised in 0.25 g mL^{-1} of MS222. Approximating every 100 degree days, fish were sampled post winter solstice for smolt score assessment and Na^+, K^+ - ATPase (NKA) activity (15 fish / ploidy (5 / tank) before being euthanised using a lethal dose of MS222 (>200 mg L^{-1}). A gill biopsy was collected from the second gill arch and placed in 100 µl SEI buffer in liquid nitrogen and stored at -80 ˚C for later NKA analysis. A smolt score assessment was then conducted to determine silvering, parr marks and fin colouration (Sigholt *et al.,* 1995). An additional 15 fish / ploidy (5 / tank) were taken for SW challenge and then also euthanised using a lethal dose of anaesthetic (>200 mg L^{-1}; MS222). Blood was immediately taken using heparinised syringes and centrifuged at 2500 g for 15 minutes at 4 ˚C before resulting plasma was aliquoted and stored at -20 ˚C for later Cl^{-1} analyses. All experimental procedures and husbandry practices used in the present study were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice) in accordance with EU regulation (EC Directive 86/609/EEC) and approved by the Animal Ethics and Welfare Committee of the University of Stirling.

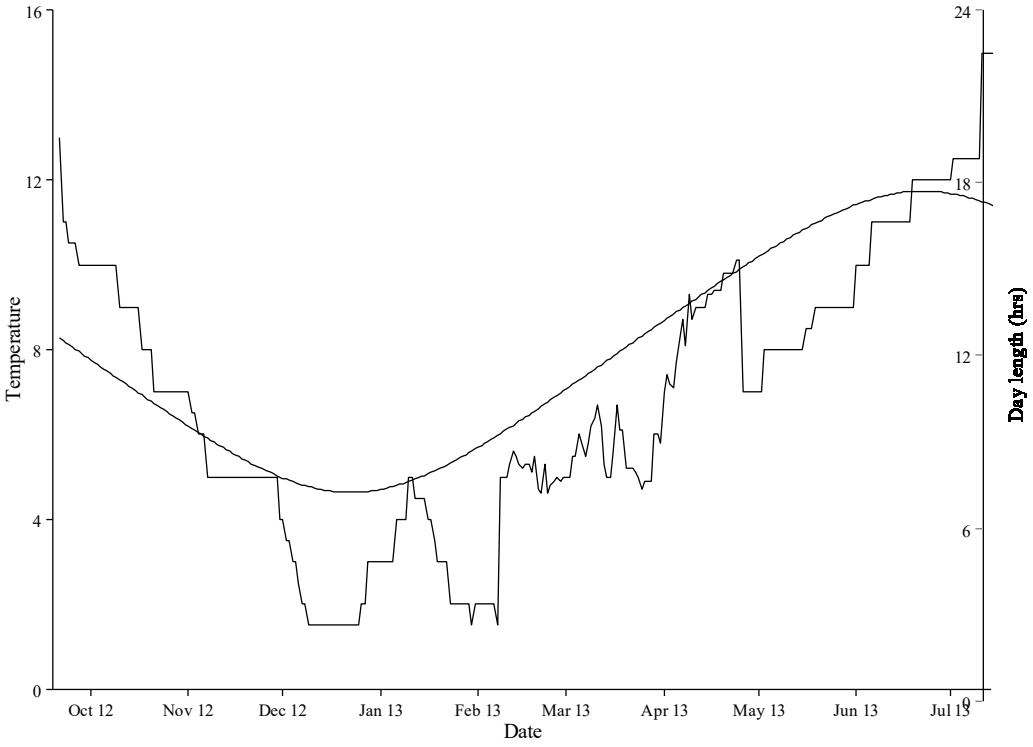

Figure 5.1 Temperature ˚C (solid line) and day length in hours (dotted line) profile from start (September 19, 2012) to end (July 14, 2013) of trial.

2.3 SW challenge, plasma Cl^{-1} and Na^+, K^+ -ATPase

Diploids and triploids were anaesthetised (MS222, 0.25 g mL^{-1}) 24 hours prior to challenge and then marked with Alcian blue (VWR) using a panjet. Fish were then placed directly into full strength SW (35 ‰ Instant Ocean sea salts) at 10 ˚C with oxygen saturation >8 mg. L^{-1} and left in complete darkness for 24 hours before checking for survival and plasma Cl^{-1} levels.

Plasma samples were analysed using a clinical range on a Jenway Cl^{-1} meter (model: PCLM3). Twenty µl of each plasma sample was added in triplicate to 15 ml of

acid buffer (Fisher: CHR-102-072S) containing gelatin reagent (300 µl; Fisher: CHR-075M) standardised with clinical Cl^{-1} standard (20 µl; Fisher: CHR-102-090Q). Resulting saturation of Cl^{-1} with silver ions released from silver electrodes was then detected (mg / L).

NKA activity was analysed according to methods developed by McCormick (1993). Gills biopsies were processed using a 96 well plate kinetic assay at 26 ˚C and read at a wavelength of 340 nm for 10 min. Resulting NKA concentrations (mg $protein^{-1}.hr^{-1}$) were determined using a BCA (Bicinchoninc acid) Protein assay kit (Sigma, Aldrich, UK).

2.4 Calculations

K was calculated as: $W^*(L^3)^{-1}$ x100. TGC was calculated as: $(W_f^{1/3}-W_i^{1/3})x(\sum^oD)^{-1}$, where W_f is the final body weight (g), W_i is the initial body weight (g) and ^oD is the cumulative sum of water temperature in degrees per day. eFCR was calculated according to: $F/(B_f-B_i+B_m)^{-1}$ where F is the sum of food fed (kg), B_f is the final biomass (kg), B_i is the initial biomass (kg) and B_m is the mortality biomass for the given period of time (kg).

2.5 Statistics

All data were analysed and compared using R language (R Core Team, 2013) and significance was accepted at 5 % ($p < 0.05$). Results are reported as mean (± SEM). A

Two-Way-ANOVA with tanks nested within ploidy was performed on normal and homogeneous growth datasets and were analysed using the *lm* function in the *nlme* package. Subsequent post-hoc Tukey's multiple comparisons were performed using the *glht* function in the *multcomp* package. For plasma Cl^{-1} and gill NKA results insufficient samples were available for nested ANOVAs attributed to mortalities and a missing tank within certain time points. Hence a t-test using the *t.test* function in the *stats* package was conducted between ploidy at each time point and a One-Way ANOVA using the *lm* function in the *stats* package for overall differences between time points. A Mann-Whitney-Wilcoxon test was performed between ploidy for skin silvering, parr mark and fin colouration scores at each time point using the *wilcox.test* function in the *stats* package.

3 Results

3.1 Growth and mortality

Mortality rates were low throughout the trial and no significant difference was observed in survival between diploids and triploids (Table 5.1). However, one triploid tank was euthanised on June 26, 2013 (937.5 ˚*Dws*) due to an outbreak of *Saprolegnia* (data not shown) and was therefore not included in final stage analysis.

Table 5.1. Cumulative mortality of total population (%), final smolt weight (g), K, total TGC and eFCR of diploids and triploids Atlantic salmon reared under a SNP.

	Diploid			Triploid*		
Mortality (%)	3.03	±	1.20	2.09	±	0.28
Final weight (g)	97.85[b]	±	4.02	105.80[a]	±	3.50
Final K factor	1.01	±	0.03	1.01	±	0.01
TGC	0.99	±	0.04	1.05	±	0.14
eFCR	1.14	±	0.05	1.07	±	0.21

*Final stage missing one triploid tank owing to outbreak of *Saprolegnia.*

a,b Mean values with different superscript letters are significantly different (P < 0.05; One-Way ANOVA & Tukey's post hoc)

Weights remained similar between diploids and triploids throughout development with triploids only attaining a significantly higher weight than diploids at 606 ˚*Dws* (p = 0.03; Fig 5.2) and upon termination of the trial (p = 0.03; 1176 ˚*Dws*; Table 5.1). This was reflected in higher overall TGC and lower eFCR in triploids relative to diploids (Table 5.1). K fluctuated initially with triploids demonstrating significantly higher K at -565 ˚*Dws* (p = 0.03) and significantly lower K at -317 ˚*Dws* (p < 0.0001) relative to diploids (Fig 5.2). Thereafter, no significant difference in K was observed between ploidy. Overall K in both ploidy became reduced significantly throughout development (p < 0.0001) with final K being significantly lower than all other time points (data not shown; Fig 5.2).

3.2 Skin silvering, parr marks and fin colouration

Total mean smolt score index was significantly higher in triploids compared to diploids from 158-377 ˚*Dws* (158 ˚*Dws*: p <0.0001; 206 ˚*Dws*: p = 0.0003; 271 ˚*Dws*: p <0.0001; 377 ˚*Dws*: p <0.0001; Fig 5.3). Thereafter, scores remained similar for the remainder of

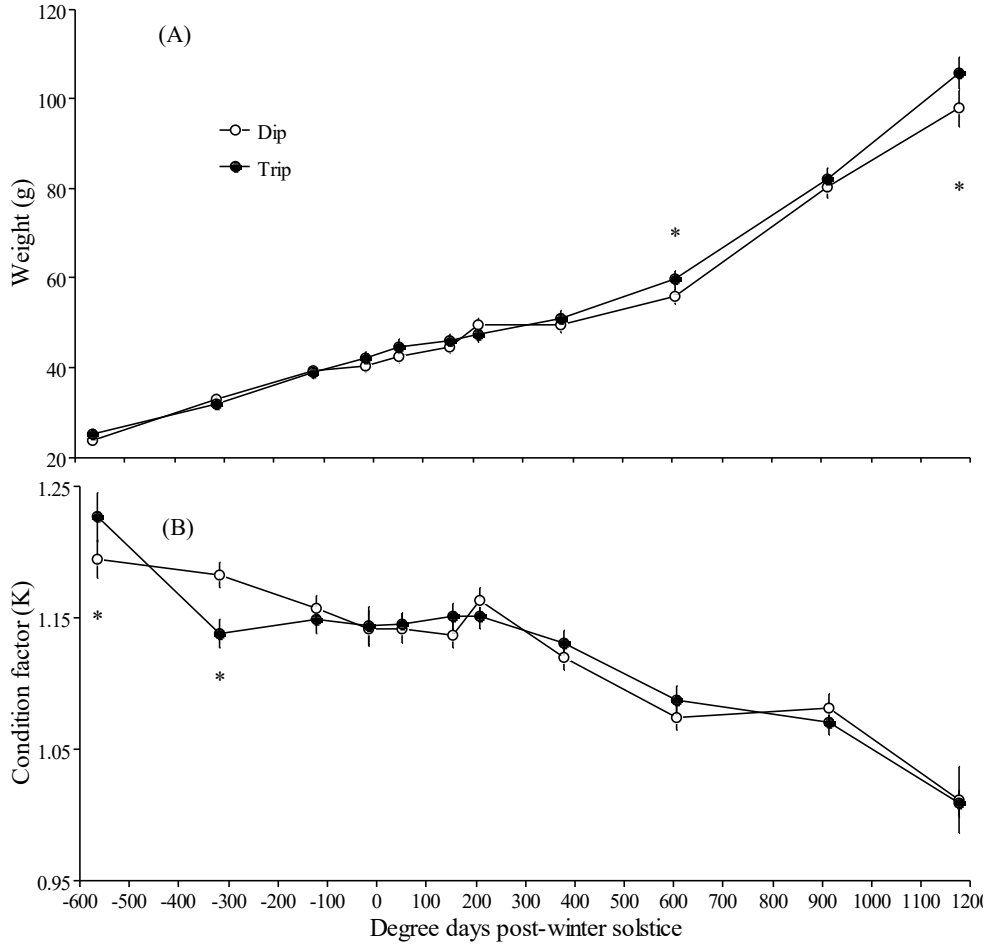

Figure 5.2 Change in weight and K of diploid and triploid Atlantic salmon over time (°*Dws*). Significant differences between ploidy are denoted by an asterisk (Mean ± SEM, n = 30 fish x 3 tanks x 2 ploidy; $p < 0.05$; Two-Way ANOVA & Tukey's post hoc).

the trial. This was reflected in skin silvering scores which were also significantly higher in triploids from 158 - 377 ˚*Dws* (158 ˚*Dws*: $p = 0.03$; 206 ˚*Dws*: $p = 0.05$; 271 ˚*Dws*: $p = 0.0003$; 377 ˚*Dws*: $p = 0.001$). Parr marks were significantly less pronounced (reflected in a higher score) in triploids than diploids at 158 ˚*Dws* ($p < 0.0001$) but did not differ between ploidy thereafter. Fin edge colouration was initially similar between ploidy at 158 - 206 ˚*Dws* and then significantly higher in triploids from 271 - 377 ˚*Dws* (271 ˚*Dws*: $p = < 0.0001$; 377 ˚*Dws*: $p = 0.004$). Thereafter, colouration remained comparable

between ploidy. No difference in skin silvering, parr marks or fin colouration was evident between ploidy from 482 ˚*Dws* as complete in both ploidy.

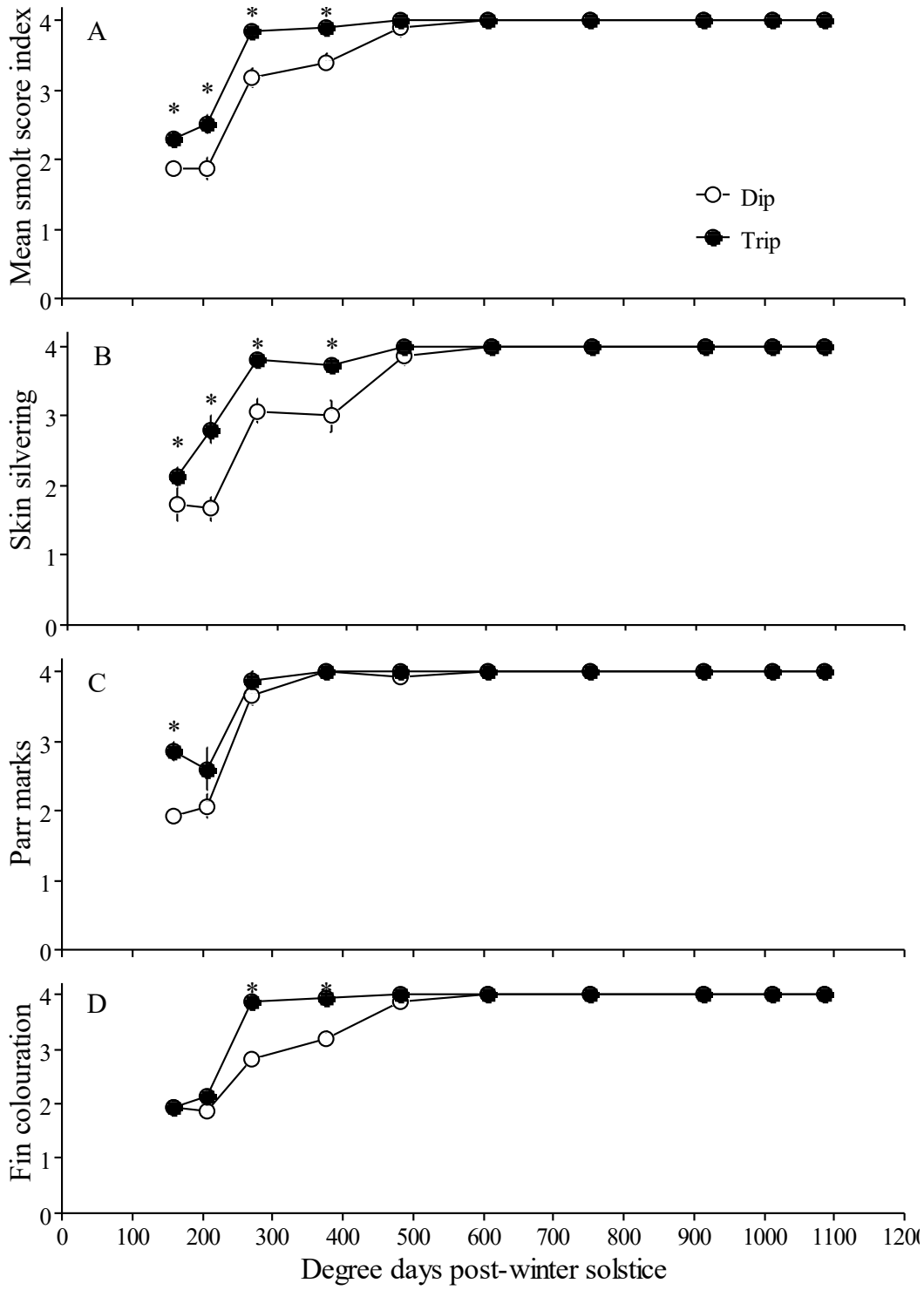

Figure 5.3 Smolt score index (A) of diploid and triploid Atlantic salmon reared under SNP according to degree days post winter solstice: skin silvering (B), parr marks (C) and fin colouration (D). Significant differences between ploidy are denoted by asterisks (Mean ± SEM, n= 5 fish x 3 tanks x 2 ploidy, Mann-Whitney-Wilcoxon test, $p < 0.05$).

3.3 SW challenge, plasma Cl^{-1} and Na^{+}, K^{+} -ATPase

No significant differences between ploidy x time interaction was observed in mortality post SW challenge however, there was an overall effect of ploidy (p = 0.0008). Further analysis within time points were not conducted due to tank mean observations (n = 6) being too few. Mortalities during SW challenge were low throughout development in triploids (Fig 5.4 A) with mortality observed largely originating from the same tank with resulting *Saprolegnia* (data not shown). Diploids initially experienced high mortality (158 ˚*Dws*) after which mortality was low until the later stages of the trial when mortalities peaked at 907 and 1086 ˚*Dws*.

Overall plasma Cl^{-1} levels following SW challenge were higher at 907 (171.4 ± 4.4 mmol l^{-1}) and 1011 ˚*Dws* (180.2 ± 3.5 mmol l^{-1}) significant to earlier (206 - 482 ˚*Dws*; 206 ˚*Dws*: 158.1 ± 3.0 mmol l^{-1}, 271 ˚*Dws*: 162.9 ± 3.2 mmol l^{-1}, 377 ˚*Dws*: 149.5 ± 2.9 mmol l^{-1}, 482˚*Dws* 148.2 ± 2.0 mmol l^{-1}) and later (1086 ˚*Dws*, 159.3 ± 2.5 mmol l^{-1}) time points (p <0.0001). Cl- levels were significantly lower at 482 ˚*Dws* significant to 158 (165.4 ± 7.8 mmol l^{-1}), 271 and 606 - 1011 ˚*Dws* (606 ˚*Dws*: 158.1 ± 3.0 mmol l^{-1}, 752 ˚*Dws*: 179.4 ± 3.3 mmol l^{-1}). Triploid plasma Cl^{-1} levels remained low throughout the trial whereas diploids had higher temporal fluctuations (Fig 5.4 B). At 158 ˚*Dws* diploids had significantly higher plasma Cl^{-1} levels relative to triploids. Thereafter, levels remained similar for a short period and from 482 - 752 ˚*Dws* diploids maintained

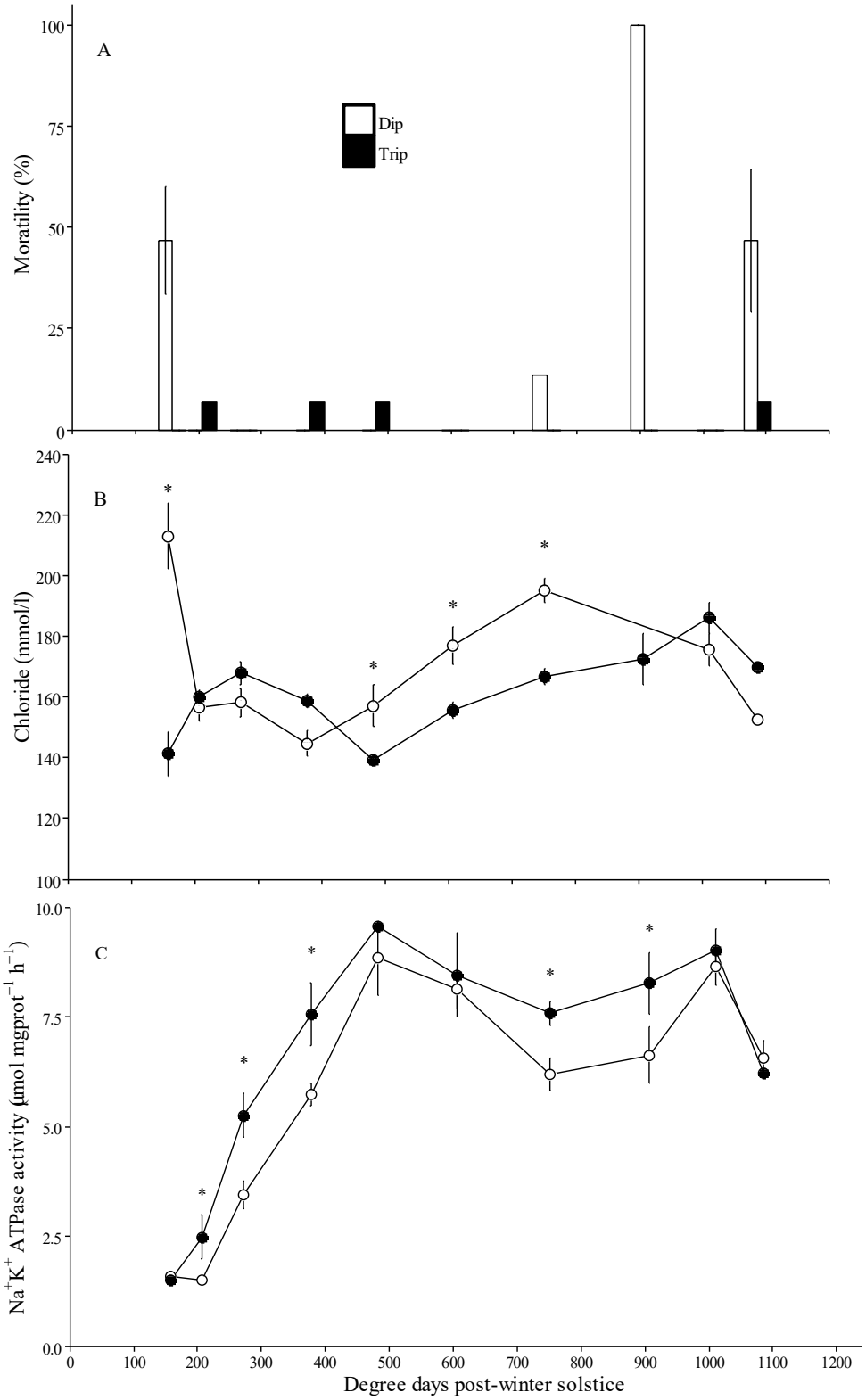

Figure 5.4 Mortality (A) of diploid and triploid Atlantic salmon subjected to SW challenge and corresponding plasma Cl^{-1} levels (B). Gill NKA activity (C) of non-challenged fish according to º*Dws*. Significant differences between ploidy at each time point are denoted with an asterisk (Mean ± SEM, n = 15 fish x 2 ploidy; t-test, $p < 0.05$). Cl^{-1} data is missing from diploids at 906 º*Dws* as 100% mortality was experienced following SW challenge.

significantly higher plasma Cl^{-1} levels than triploids. In the final stages (1011 - 1086 ˚*Dws*) triploids and diploids had similar Cl^{-1} levels following challenge.

Gill NKA overall significantly increased from 158 - 482 ˚*Dws* (158 ˚*Dws*: 1.54 ± 0.06 μmol mg^{-1}, 206 ˚*Dws*: 2.01 ± 0.18 μmol mg^{-1}, 271 ˚*Dws*: 4.33 ± 0.31 μmol mg^{-1}, 482 ˚*Dws*: 9.18 ± 0.53 μmol mg^{-1}) and a significant decrease was observed at the final time point (1086 ˚*Dws*; 6.42 ± 0.37 μmol mg^{-1}; p <0.0001). No significant differences between ploidy were initially observed in NKA activity (Fig. 5.4C). However, triploids showed a significantly higher NKA activity than diploids from 206 - 377 ˚*Dws*. Where NKA activity peaked in both ploidy (482 ˚*Dws*) no significant differences were observed, however, triploids maintained a significantly higher activity than diploids from 752 - 907 ˚*Dws*. No significant differences in NKA activity were observed between ploidy beyond 1000 ˚*Dws*.

4 Discussion

Collectively results indicated that S1 triploid Atlantic salmon exhibited earlier onset of PST by approximately 48 degree days relative to diploid counterparts whilst overall peak NKA activity had a tendency to be similar between ploidy. Triploids had a significantly higher weight at trial termination (8.1 %) relative to diploid counterparts, and K factors fluctuated significantly between ploidy prior to winter solstice. Earlier onset of hypo-

osmoregulatory ability and PST was confirmed in triploids through increased SW challenge survival compared to diploids throughout the experiment and associated reduction in plasma Cl^{-1} levels. This study defined for the first time the temporal profile of PST under ambient photo thermal conditions and potential windows for SW transfer in triploid Atlantic salmon.

K is correlated with whole body lipid content (Herbinger & Friars, 1991) and reductions can be attributable to utilisation of lipid reserves for the PST process (Sheridan, 1989). Both triploids and diploids in this study reduced in K at the onset and throughout PST. Previous research typically reported triploid Atlantic salmon smolts to have lower (O'Flynn *et al.*, 1997; Cotter *et al.,* 2002; Fjelldal & Hansen, 2010; Leclercq *et al.,* 2011; Taylor *et al.,* 2012; Fraser *et al.,* 2013b; Fraser *et al.,* 2014) or in some cases equal (Boeuf *et al.,* 1994; Cotter *et al.,* 2002; Taylor *et al.,* 2012) K relative to diploid siblings and may be impacted by smolt regime (Taylor *et al.,* 2012; Fraser *et al.,* 2014). In addition, triploids had higher weights relative to diploids in the latter part of the study. Wild diploid Atlantic salmon post-smolts had increased K and muscle protein synthesis (measured through RNA: DNA ratios) associated with increased muscle growth and high energy intake through the feeding migration (Stefansson *et al.,* 2012). In addition, increased body lipid concentrations were found in both SW post-smolts and desmolts compared to smolts in masu salmon (*Oncorhynchus masou*; Li & Yamada 1992). Earlier

onset of PST in triploids alongside associated reduced K factors and unrestricted feed availability may explain earlier increase in muscle mass in triploids compared to diploids. Lower muscle fibre recruitment alongside compensatory muscle hypertrophy found in triploid Atlantic salmon (Johnston *et al.,* 1999) may also explain ploidy differences observed in somatic growth during PST.

Reduced K in Atlantic salmon are associated with the onset of PST through elongation of the caudal region (Fjelldal *et al*., 2006) in preparation for swimming in a pelagic environment (Winans & Nishioka, 1987). Triploid K fluctuated prior to winter solstice in this study suggesting differential onset of caudal elongation. Wargelius *et al.,* (2005a) showed that increased plasma levels of GH and mRNA expression of both *gh* and *igf-I* receptor in vertebra of Atlantic salmon during PST were associated with increased vertebral growth and bone density respectively. GH and IGF-I are the primary endocrine regulators of hypo-osmoregulatory ability during PST in salmonids (McCormick, 2001). However, research into triploid Atlantic salmon endocrinology and PST is distinctly lacking. In other salmonid species, research has generally shown triploids to have similar plasma IGF-I levels compared to their diploid siblings although characterisation during PST is lacking (Shrimpton *et al.,* 2007; Taylor *et al.,* 2007; Sacobie *et al.,* 2012; Cleveland & Weber, 2014). In addition, no information appears to

be available on the hormone receptor abundance in triploids hence further investigation is required to confirm mechanisms for ploidy differences at PST.

Triploids in this study had earlier onset of enhanced hypo-osmoregulatory ability through raised NKA activity 48 degree days earlier compared to diploids and reduced mortality alongside associated plasma Cl^{-1} following SW challenge. SW tolerance may be related to differing morphology at the primary osmoregulatory sites including the gills where triploid Atlantic salmon have been shown to have longer gill filaments yet reduced density (Leclercq *et al.,* 2011) and the gut where triploids have a relatively shorter length and fewer pyloric caeca (Peruzzi *et al.,* 2014). Hypo-osmoregulatory ability in the gills is primarily improved through increased size and proliferation of mitochondrion-rich chloride cells (McCormick, 2001) and increased NKA protein activity attributed to both FW and SW isoforms that maintain intracellular homeostasis whilst allowing direct SW transfer or to remain in FW (McCormick *et al.,* 2009). Triploid Atlantic salmon have an increased nuclear volume with a compensatory increase in cell size and reduced frequency although only a few cell types have been studied so far (Swarup, 1959; Small & Benfey, 1987; Aliah *et al.,* 1990; Johnston *et al.,* 1999; Flajšhans *et al.,* 2011). If similar differential patterns are observed within triploid chloride cells then the ability to excrete excess monovalent ions may be altered as a result of the increased cytosol and intracellular distance together with reduced plasma membrane relative to diploids.

Research on the impact of triploid cellular physiology, including chloride cells, would be essential in understanding mechanisms of SW tolerance.

Triploid Atlantic salmon smolts have previously been shown to display earlier increased NKA activity levels to diploid siblings, resulting in transfer to SW 4 weeks earlier (Leclercq *et al.,* 2011; Taylor *et al.,* 2012), or similar patterns (Boeuf *et al.,* 1994; Taylor *et al.,* 2014). The onset of PST indicated through increased NKA activity was 48 degree days earlier in triploids compared to diploids concurrent with previous reports. However, peak in NKA activity was similar between ploidy at 482 ˚*Dws* in this study and were of levels around 9 μmol mg $prot^{-1}$ h^{-1} as reported in Handeland *et al*., (2004) for peak activity in diploid Atlantic salmon. Nonetheless, completion of skin silvering indicate triploids smolted 210 degree days earlier to diploids, and improved survival to SW challenge in triploids at the beginning and end of the trial where diploid survival was relatively poor was also observed. This indicates that factors other than increased NKA activity within chloride cells may be contributing to maintenance of hypo-osmoregulatory ability such as altered gill (Leclercq *et al.,* 2011), cell (Small & Benfey, 1987) and gut morphology (Peruzzi *et al.,* 2014). Increased hypo-osmoregulatory ability in triploids may additionally be attributed in part to known shorter gut lengths (Peruzzi *et al.,* 2014) which may directly interfere with salt uptake and osmoregulation in a more saline environment. However, it has been shown that SW adaptation involves increased

paracellular water flow within the posterior intestine (Sundell *et al.,* 2003) and so further research is required on the triploid gut epithelial cell morphology as well as associated drinking rate (Nielsen *et al.,* 1999).

The window of PST in diploid Atlantic salmon is suggested to be primarily regulated by temperature in which peak NKA activity is maintained for 250 degree days in FW smolts before subsequent reduction (Handeland *et al.,* 2004). Triploid Atlantic salmon are shown to be more sensitive to temperature and have lower thermal optima (Atkins & Benfey, 2008) with respect to blood oxygen carrying capacity and vertebral and cardiac pathology development (Fraser *et al.,* 2013 a,b) although impact on PST has not been reported. Maintenance of higher NKA levels relative to diploids and improved survival to SW challenge in triploids by 155 degree days (752 -907 °*Dws*) in this study suggest that the smolt window may be wider in triploids. Triploid Atlantic salmon had largely comparable survival post SW transfer in previous studies (Boeuf *et al.,* 1994; Leclercq *et al.,* 2011; Taylor *et al.,* 2012; Fraser *et al.,* 2014; Taylor *et al.,* 2014) although instances of increased mortality have been observed that may have been attributed to high temperature (>19˚C) and low oxygen (<7 mg / l; Cotter *et al.,* 2002) as well as communal rearing (O'Flynn *et al.*, 1997). Some studies have transferred triploid Atlantic salmon to SW earlier than their diploid siblings as a consequence of earlier rise in NKA and observable skin silvering (Leclercq *et al.,* 2011; Taylor *et al.,* 2012). Accelerated

appearance of visible indicators of PST: skin silvering, parr marks and fin colouration (Sigholt *et al.,* 1995) and NKA activity in triploids suggest increased SW tolerance observed in this study is attributed to earlier onset of PST.

In conclusion, it is clear that onset of PST occurred earlier in triploid Atlantic salmon than diploids by 48 degree days under an ambient photo thermal regime, although response to artificial photoperiods are also yet to be assessed. Collectively: earlier completion of skin silvering by 210 degree days with immediate improved response to SW challenge at 158 °*Dws*; comparable peaks in NKA activity between ploidy at 482 °*Dws*; and maintained increased NKA activity alongside prolonged improved survival to SW challenge by 155 degree days compared to diploids indicate the smoltification window in triploids may the potential to be wider by 155 – 365 degree days. Wider PST windows may be attributed to multiple factors including earlier onset of physiological and morphological changes as well as a general improved tolerance to salinity changes potentially through altered gut and gill morphology. These findings not only assist implementation of appropriate PST regimes in culture of triploid salmonids but ultimately suggest that triploid smolts hold potential for transfer over a wider smolt window and improved smolt resilience compared to conventional diploid response. Further research is needed, not only to verify the smoltification window, but also to reveal

the underlying mechanisms behind these apparent ploidy differences in particular with regards to endocrine control and long-term smolt performance.

Chapter 6: Embryonic ontogeny of global DNA methylation and utilisation of endogenous energy reserves in diploid and triploid Atlantic salmon eggs incubated at different temperatures

Abstract

Early life stages in fish are known to be sensitive to temperature which can impact development especially muscle growth and deformity. DNA methylation (DNAme) is one of several epigenetic factors that modulate gene regulation and is crucial in early embryonic patterning which impact fish phenotype and coping mechanisms at later life stages. The present work aimed to study known predictors of Atlantic salmon phenotype: temperature (6, 8 and 11 ˚C) and ploidy (diploid and triploid), throughout embryogenesis and the effect on global DNAme profile. Impact of ploidy and temperature on endogenous NM concentration was also assessed to understand utilisation of yolk amino acids and potential associated restrictions on DNAme. Overall, lower incubation temperatures improved embryonic survival rates for both diploids and triploids. Triploids showed reduced survival at 8 ˚C compared to diploids and lower survival between ploidy in the 11 ˚C treatment. Both diploids and triploids reared at 11 ˚C yielded increased prevalence of lethal deformities at hatching. Global DNAme % profiles peaked during somitogenesis, which is later compared with other vertebrate species that peak at gastrulation. This pattern is further emphasised in triploid siblings where DNAme % levels are maintained at relatively lower levels throughout somitogenesis. Similarities between ploidy in DNAme % at eyeing and hatching stages are indicative of dosage

effects and no clear impact of temperature on DNAme was observed. Conversely no effect of ploidy was observed on endogenous NM concentration, however, a strong impact of temperature and developmental stage was observed. Results indicate that long-term effects of poor performance and deformity development typically observed in diploids and triploids under suboptimal embryonic temperatures may not be caused by epigenetic changes to the genome, although verification is required.

1 Introduction

Recent research has begun to identify factors to improve production traits in triploid Atlantic salmon through amended husbandry practices including nutrient enriched diets (Taylor *et al.,* 2015; Fjelldal *et al.,* 2015), increased oxygen saturation levels (Hansen *et al.,* 2015), segregated rearing (Taylor *et al.,* 2014) and lower thermal regimes which have been shown to reduce vertebral and cardiac deformities (Fraser *et al.,* 2013a, 2015a). However, little research has investigated real-time ontogenetic differences in diploid and triploid Atlantic salmon embryogenesis especially in reference to temperature development.

The temperature range or window in which an organism can exist is mirrored through aerobic scope where performance is most optimal at a given temperature and reduces if cooled or warmed. (Pörtner & Farrell, 2008). This thermal window is impacted by organism complexity and life stage. For instance, spawning and embryonic fishes are relatively more stenothermic. The rate of Atlantic salmon embryogenesis increases with water temperature in a non-linear fashion (Gorodilov, 1996). Temperature is known to influence embryonic performance through altered axial patterning and skeletal development (Wargelius *et al.,* 2005b) as well as muscle fibre growth (Johnston, 2006; Macqueen *et al.*, 2008), cellularity (Johnston, 2006; Macqueen *et al.*, 2008),

mitochondrial content (Johnston, 2006), gene expression (Johnston, 2006; Macqueen *et al.*, 2007) and post-embryonic myogenesis (Johnston, 2006; Macqueen *et al.,* 2008). Influence of egg incubation temperature on factors such as skeletal muscle development is especially important as it is irreversible in rapid ontogeny where the cell fate is determined (Johnston, 2006). In particular suboptimal egg incubation temperatures are known to reduce performance and promote developmental defects later in life in diploids and triploids (Fraser *et al.,* 2013a, 2015a). Although triploid salmonids have been shown to display similar acute and chronic thermal maxima to diploid siblings (Benfey *et al.,* 1997; Galbreath *et al.,* 2006), they have shown increased mortality rates (Quillet & Gaignon, 1990; Myers & Hershberger, 1991; Ojolick *et al.,* 1995) which has been attributed to reduced oxygen delivery in an environment of lower oxygen saturation in combination with higher temperatures (Hansen *et al.,* 2015). This may be due to a lower haemoglobin-oxygen loading ratio and blood oxygen content (Bernier *et al.,* 2004) exacerbated through increased metabolism under higher temperature (Atkins & Benfey, 2008).

Normal embryonic development strongly relies on epigenetic processes that regulate the potential for gene expression, it enables cellular differentiation from a pluripotent state to a cell-type specific state (Cheng *et al.,* 2011). The epigenetic processes are changes in DNAme, degrees of histone modifications and non-coding

RNAs such as miRNAs (Tollefsbol, 2011). In particular DNA remethylation is considered to be essential for correct embryogenesis (Martin *et al.,* 1999; Santos *et al.,* 2002; Mhanni & McGowan, 2004). DNA methyltransferases (DNMT) catalyse methyl donors, S - Adenosyl methionine (SAM), to bind to repeated cytosine residues followed by guanines, known as CpG islands, in dinucleotide sequences rendering the sequence transcriptionally inactive (Cheng *et al.,* 2011). In mice, genome wide loss of DNAme and remethylation occurs in three stages: active demethylation of the paternal pronucleus prior to DNA replication, passive demethylation due to the absence of Dnmt1 and restoration of DNAme post – blastulation through the *de novo* methylases Dnmt 3a &b (Santos *et al.,* 2002). A similar pattern has been proposed in zebrafish (Martin *et al.,* 1999; Mhanni & McGowan, 2004). Conversely, more recent research has shown a maintenance of paternal bulk DNA demethylation post-fertlisation in zebrafish and reprogramming of maternal DNAme, suggesting methylomes may be paternally inherited in teleosts (Jiang *et al.,* 2013b; Potok *et al.,* 2013).

To date teleost embryonic DNAme has only been researched in zebrafish and Atlantic cod (*Gadus morhua*; Skjærven *et al.,* 2014) where hypomethylation was identified at blastulation compared to sperm and late gastrulation which is concurrent with literature for other vertebrates. In this instance Atlantic salmon, are of unique interest to study embryonic epigenetic regulation owing to recent whole genome

duplication and the resulting tetraploid state originating from 25-100 million years ago (Johnson *et al.,* 1987; Koop & Davidson, 2008). Polyploidisation in plants is known to induce epigenetic alterations allowing for genomic plasticity and associated phenotypic variation (Paun *et al.,* 2007; Jackson & Chen, 2010). Investigating potential impacts of polyploidy on epigenetic regulation in fish is extremely limited. The resulting duplicate genes have been hypothesized to increase environmental tolerance and evolutionary capacity (Ohno, 1970) such as the complex anadromous migratory behaviour (Alexandrou *et al.,* 2013), and therefore artificially induced triploid Atlantic salmon have potential for increased genomic plasticity. Artificially induced polyploids such as triploid salmonids have shown inconsistent performance (Fraser *et al.,* 2012a) that may be driven by parentally-induced epigenetic instability and may otherwise stabilise in naturally evolved polyploids, as is shown in plants (Comai, 2000). Polyploids have additional genomic material, increased alleles per locus and potentially more complex interactions between loci for which additional transcriptional regulation is required (Chen & Ni, 2006; Jackson & Chen, 2010). In plants there are rapid genetic and epigenetic transformations following polyploidy formation (Matzke *et al.,* 1999; Chen & Ni, 2006). It would therefore be expected that additional gene expression potential in triploid salmonids be reconciled through gene dosage compensation to diploid expression levels. However, triploid Atlantic salmon possess larger cells to compensate for additional nuclear volume

(Small & Benfey, 1987) and so it is likely that there is some degree of maintenance in gene dosage effect from the additional chromosome set to offset the reduced cellular frequency. Ching *et al.,* (2009) verified gene dosage effects with compensatory mRNA expression levels in triploid Chinook salmon that compensate for the increase in cell volume. Similar levels of white muscle tissue RNA concentrations were observed in triploid and diploid rainbow trout siblings suggesting potential dosage effects (Suresh & Sheehan, 1998). Conversely, reduced RNA: DNA ratios alongside similar levels of gene expression in triploid sea bream (*Squalius alburnoides*) to diploid siblings were attributed to gene dosage compensation (Pala *et al.,* 2008). In terms of embryogenesis there may be implications for gene regulation in triploids at the maternal - zygotic transition where the ratio of maternal genome: paternal genome: maternally provided RNA could be greater. This in combination with rapid cell cycle progression may have ramifications to DNA remethylation or reprogramming of the maternal genome.

Environmental stressors such as temperature are known to induce alterations in DNAme and may have long-lasting effects on development (Feil & Fraga, 2011). Of importance, fish are known to be particularly stenothermal during embryogenesis compared to other life stages (Pörtner & Farrell, 2008). Higher egg incubation temperatures in Atlantic cod reduced expression of the DNMTs and inhibition of DNA remethylation at 25% epiboly (Skjærven *et al.,* 2014). In addition, challenge of triploid

Atlantic salmon with *Vibrio anguilliarum* led to changes in relative gene expression in housekeeping genes compared to diploids suggesting that triploid salmonids may experience a loss of gene regulatory homeostasis under periods of stress (Ching *et al.,* 2009). The impact of triploidy on salmonid embryonic development is primarily reported on egg survival and this has been predominantly reduced (Mcgeachy *et al.,* 1995; O'Flynn *et al.*, 1997; Withler *et al.,* 1998; Cotter *et al.,* 2002; Johnson *et al.,* 2004; Fraser *et al.,* 2013a) or comparable (O'Flynn *et al.*, 1997; Johnson *et al.,* 2004; Taylor *et al.,* 2011) respectively, compared to diploid siblings, with egg quality implicated as the cause for poor survival (Taylor *et al.*, 2011). However, higher environmental temperature during embryogenesis has been shown to induce teratogenic effects in both diploid and triploid Atlantic salmon later in life (Fraser *et al.*, 2013, 2015). Given the combined importance of DNAme and temperature to embryo formation and adult phenotype, it is possible that temperature-induced teratogenesis in triploids may be regulated by alterations in epigenetics and DNAme.

Fish embryos rely exclusively on endogenous reserves in the form of yolk as a metabolic fuel and for synthesis of embryonic tissues. Compared to marine pelagic species, the FAA pool in the yolk is considerably reduced where an organic osmolyte pool would be disadvantageous for osmoregulation (Srivastava *et al.,* 1995; Finn & Fyhn, 2010). Hence, supply of FAAs in salmonid embryogenesis is reliant on degradation of

protein bound sources through lysosomes and cathepsins from the yolk proteins (Li *et al.,* 2009; Finn & Fyhn, 2010). Triploid salmonids have lower post-hatch weights compared to diploid siblings indicating relative inefficient yolk utilisation (Happe *et al.,* 1988). In addition, rearing Atlantic salmon at higher temperatures of 10 ºC as opposed to 5 ºC reduced hatch weights by 34 % which may be due to increased metabolism, exhaustion of endogenous reserves and requirement for tissue resorption pre-feeding (Hamor & Garside, 1977). Inefficient yolk utilisation may have a bearing on DNAme which is reliant on the synthesis and remethylation of the methyl donor SAM in the one-carbon metabolism pathway whereby the FAAs methionine and cysteine are required (Niculescu & Zeisel, 2002).

In the present study, genomic DNAme and utilisation of endogenous reserves through FAAs were compared throughout embryonic development in diploid and triploid Atlantic salmon reared at three different constant temperatures: 6 ºC, 8 ºC and 11 ºC. It was hypothesised that elevated temperature may alter DNAme signatures and accelerate exhaustion of FAAs. This is the first study to look at embryonic DNAme in triploid salmon and will therefore provide novel insight into epigenetic processing comparing diploid and triploid embryonic stages of Atlantic salmon.

2 Methods and materials

2.1 Experimental setup and sampling protocol

Atlantic salmon ova were sourced from 2 unrelated dams and inactive milt from 4 unrelated sires on December 5, 2013 at an undisclosed broodstock facility. Roughly 44,000 ova were fertilised at 8.2 ˚C at the temperate aquatic facility at the University of Stirling. Triploidy was induced through a standardised pressure shock of 9500 psi for 50 ° minutes at 300 ° minutes post-fertilisation at 8 ˚C (Taylor *et al.,* 2011). During water hardening fertilized ova were placed within a bucket of 8 ºC water within a trough at the intended experimental rearing temperature. Both diploid and triploid batches were handled identically to exclude interfering factors of handling stress from triploidisation. Ova were then counted out into duplicate aluminium trays per ploidy, per temperature treatment (12 baskets). Three recirculating troughs systems were set to separate environmental rearing temperatures of: 5.9 ± 0.3 ºC, 7.9 ± 0.2 ºC and 10.7 ± 0.2 ºC and then to a common temperature of 7.8 ± 0.0 ºC once embryos were eyed until hatch (Fig 6.1). Oxygen saturation was maintained at above 7 mg L^{-1}. Eggs were monitored daily for mortality and dead eggs carefully removed daily using a siphon pipette. Upon hatching alevins which were deemed to have potentially lethal deformities, according to standard husbandry practice, were removed and recorded according to kyphotic, lordotic, scoliotic, siamese and pin-fry deformities (Fig. 6.1).

Embryos were monitored for temperature dependent development according to the tau somite (τ_s) formation as defined by Gorodilov (1996; Fig. 6.1). Embryos were sampled at cleavage (12 τ_s), blastulation (33 τ_s), gastrulation (55 τ_s), 45th somite (100 τ_s), 65th somite (123 τ_s), eyeing (158 τ_s) and hatch (320 τ_s) for DNAme analysis (9 eggs / temperature / ploidy (triplicate pools of 3)). To ensure accurate embryonic developmental staging, eggs were cleared with 1:1:1 parts acetic acid, methanol and distilled water solution and observed under a microscope. An additional 3 eggs per basket at the temperature extremes of 6 and 11 ºC were sampled for FAA at the early and late stages of embryogenesis: blastulation and eyeing. Excess water was removed from the eggs by placing on clean paper and snap frozen in liquid nitrogen before being stored at -80 ºC until further analysis.

2.2 Nitrogenous metabolite (NMs) and FAA concentration

Individual defrosted eggs were placed into pre-weighed 1.5 ml eppendorfs and homogenised with a bead in 600 µl of 10 % sulphuric acid (33619, Riedel-deHaen), left to stand for 1 hour and centrifuged at 8000 g for 15 minutes. 300 µl of clear supernatant was retained in a fresh tube and an additional 50 µl solely for Taurine analysis. 300 µl of lithium loading buffer (80-2038-10, Biochrom) was then added and 150 µl of the internal standard 2.5 nM Norleucin (N-1398, Sigma). Samples were then filtered through a 0.22 µm Millipore PVDF hydrophilic syringe and stored at -20 ºC until analysis. FAAs were

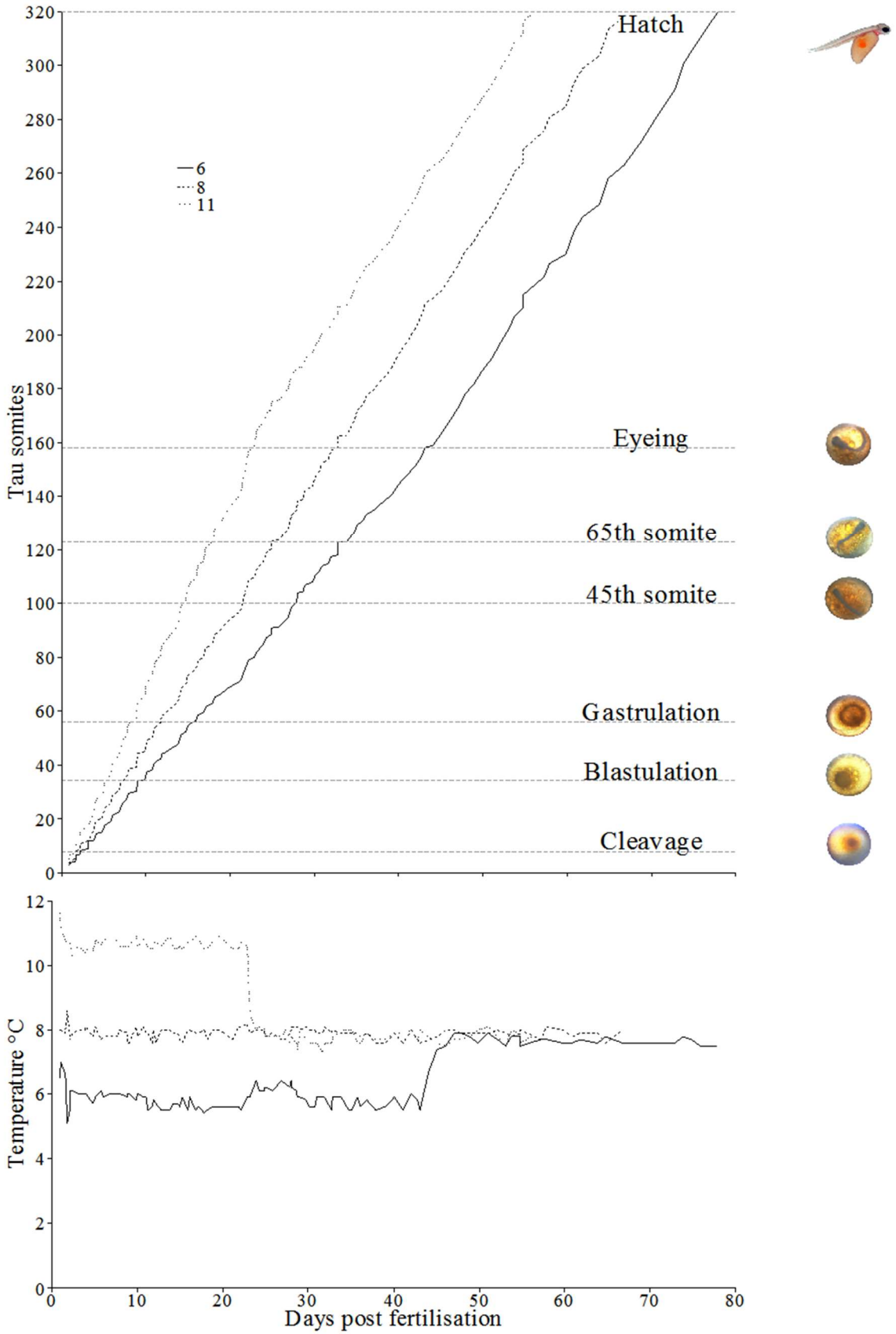

Figure 6.1. Developmental profiles of cumulative somitogenesis (tau somites (τ_s)) and temperature over the time course of the experiment (days post fertilization) for each embryonic thermal regime (6, 8 & 11ºC). Dashed grey lines indicate the stage (τ_s) sampled supported with figures of developmental stage.

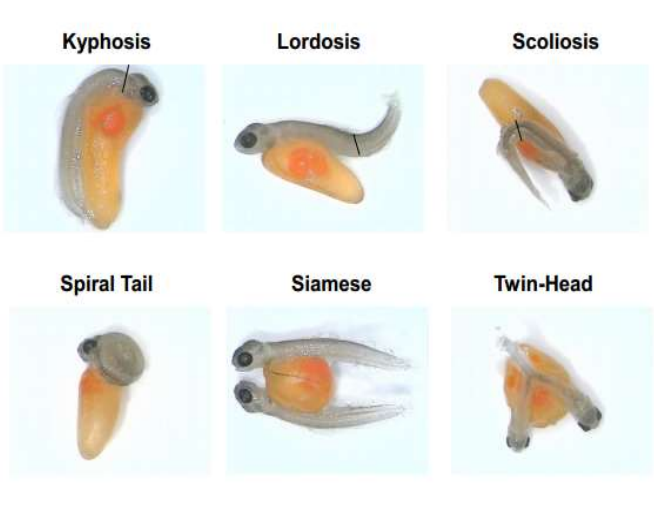

Figure 6.2. Example images of newly hatched Atlantic salmon alevins with lethal deformities: Kyphosis, lordosis, scoliosis, spiral tail (severe scoliosis), Siamese and pin-fry.

then detected with ninhydrin detection using a Biochrom Amino Acid Analyser (Biochrom Ltd, Cambridge, UK). Samples were injected into a heated column whilst ninhydrin reagent (80-2118-30, Biochrom) was simultaneously added and the resulting light reaction read by a spectrophotometer. Resulting concenrations were calculated through quantification against known internal amino acid standards (A-6407 &A-6282, Sigma).

2.3 DNA extraction

To avoid yolk contamination embryos were dissected over dry ice in absolute ethanol chilled to -70 ºC to avoid thawing. For blastulation and gastrulation stages, the disc was carefully separated from the yolk, for somitogenesis and eyeing the embryo could be teased from the yolk and chorion. For hatch the yolk sac was removed. Resulting whole tissue was then place in 200 µl of salt-extraction buffer (0.30 M NaCl; 0.04 M Tris (77-

86-1, Sigma Aldrich); 200 µM EDTA; 0.199 mM EGTA (E3889, Sigma Aldrich); 4.89 mM spermidine (SO266, Sigma Aldrich); 1.4 mM spermine (S1141, Sigma Aldrich) with sodium dodecyl sulphate (10 %; L3771, Sigma Aldrich). 5 µl of proteinase-K (10 mg/ml; P2308, Sigma Aldrich) and 20 µl RNAse(2 mg/ml; R6148, Sigma Aldrich) was added and the solution was incubated for a minimum of 4 hours at 55 ºC until the tissue was completely dissolved. The proteinase-K was then deactivated at 70 ºC for 15 minutes and placed on the bench to cool to room temperature. To precipitate proteins, 200 µl of 5M NaCl was added to the sample, gently mixed through inverting repeatedly and left on ice for 10 minutes before centrifuging at 4 ºC at 16, 000 g for 5 minutes and the supernatant retained. Equal volumes of isopropanol were added, mixed and left on ice for a further 10 minutes for later embryo stages (post gastrulation) where DNA concentrations were higher, and 1 hour in the earlier embryo stages (gastrulation and prior). Samples were centrifuged at 18, 000 g for 5 minutes and supernatant removed to retain the DNA pellet. Ice cold ethanol was added and placed on a rotary mixer overnight to leach out salt impurities before centrifuging again to retain pellet. Pellets were air dried and re-suspended in 20 µl of 5 mM Tris, pH 8. Working concentrations of DNA were made to 50 ng / µl per sample using Nanodrop (Nanodrop 1000 Spectrophotometer; Thermo Scientific, USA) readings through UV-light absorbance at 260 and 280 nm. DNA integrity was assessed using agarose gel electrophoresis where the presence of a high

molecular weight band was considered indicative of a good quality sample (Fig. 6.3). DNA concentration was further confirmed by fluorimetery measurement (Qubit® 2.0 Flurometer; Q32866; Invitrogen). Extracted DNA was stored at -20 ºC until High Performance Liquid Chromatography (HPLC) analysis at the National Institute of Nutrition and Seafood Research, Norway.

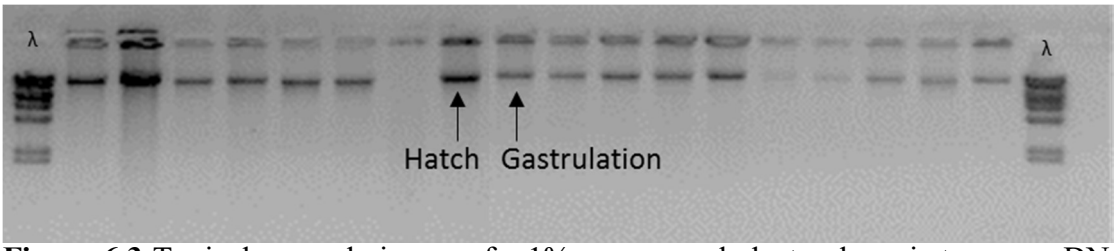

Figure 6.3 Typical example image of a 1% agarose gel electrophoresis to assess DNA sample integrity and concentration showing DNA extracted from hatch and gastrulation stages (indicated by arrows). DNA bands are compared with Lambda Hind III ladder bands (λ; SM0101; ThermoFisher Scientific: molecular weights: 238.4, 97.1, 67.6, 45.0, 23.9, 20.9, 5.8 and 1.3 ng from top to bottom respectively) to assess concentration. Nanodrop spectrophotometer readings for the hatch sample were 47.4 ng / µl with Absorbance ratios for $A_{260:280}$ nm and $A_{260:230}$ nm ratios of 1.95 and 2.09 respectively. Readings for the gastrulation sample were 59.2 ng / µl with Absorbance ratios for $A_{260:280}$ nm and $A_{260:230}$ nm ratios of 1.77 and 0.98 respectively. Qubit® fluorometer readings were of 43.2 and 24.8 ng / µl for hatch and gastrulation respectively.

2.4 DNA methylation level

DNA samples were pooled into 3 groups of 3 per treatment (representing 9 eggs) for digestion to approximately 1000 ng per pool. Samples were digested with appropriate concentrations of DNA Degradase™ and 10X DNA Degradase™ Reaction Buffer (Zymo Research CAT No. E2020 & E2021 respectively) according to manufacturer's instructions. After incubation for 2 hours at 37 °C samples were heat inactivated at 70 °C for 20 minutes and diluted up to 60 µl with 1X TE buffer and checked for appropriate nucleotide concentration with spectophotometry (Nanodrop®ND-1000 UV

Spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA) before storing at -20 °C until high-performance liquid chromatography (HPLC) analysis.

A combination of known standards were used to obtain the standard curve: adenine (dAMP,2'-deoxyadenosine 5'monophosphate, Sigma D6375), cytosine (dCMP, 2'deoxycytidine 5'monophophate, Sigma D7750), guanine (dGMP; 2'-deoxyguanosine 5'monophosphate, Sigma D9500), methylated cytosine (5mdCMP; 5-methyl deoxycytidine 5'-monophosphate, disodium salt, Reliable Biopharmaceutical Corporation (RBC), St. Louis, MO, USA: 61-1979), thymine (dTMP; 2'-deoxythymidine 5'monophosphate,disodium salt, RBC: 61-1925) and additional uracil (U; 2,4-dihydroxypyramidine, Sigma U0750) to validate that the DNA was RNA free. Before combining as standards nucleotides were verified with spectrophotometry (Nanodrop®ND-1000 UV Spectrophotometer).

During HPLC analysis, an auto sampler (Thermo Separation Products TSP, AS 3000) injected 50 µl of sample into the HPLC column (ACE 5 C18-AR 250 × 4.6 mm, Advanced Chromatography Technologies, Aberdeen, Scotland) through the filtered and degassed mobile phase (20 mM orthophospoacid, pH 2.5) using a quaternary pump (Dionex Ultimate 3000, with degasser). The column was maintained at 6 °C by a column compartment (Dionex TCC-3000SD). Eluted concentrations were detected with a combination of a UV-detector (Thermo Separation Products TSP, UV 1000) and a diode

array detector (Thermo Separation Products TSP, UV 6000 LP). The flow rate of the mobile phase was set to 1 ml/min and mobile phase was ran through the HPLC system for 16 hours prior to use. The dilution curve of the standard mix in digestion buffer was set to: 1:10, 1:25, 1:50, 1:100 and 1:200 and ran commencing each sample series and upon termination. Results were converted with an Analog - Digital (A/D) converter (PE Nelson 900 Interface, Perkin Elmer) and read with the Chromeleon software program (Thermo Scientific, CA, USA). At the end of each sample series the HPLC system was washed for 2 hours with 5 % acetonitrile.

3 Results

3.1 Survival and lethal deformity prevalence

Overall, diploid and triploid eggs incubated at 6 ºC maintained higher survival rates than eggs incubated at 8 and 11 ºC until hatch although lower survival was observed in triploids which was attributed to early mortality (Table 6.1). Triploid treatments had 5.8 – 14.7 % lower cumulative survival than diploid treatments immediately following fertilisation (0-12 τ_s; Table 6.1). Thereafter, survival patterns varied between ploidy for each temperature treatment. Both diploid and triploid embryos had reduced survival at 11 ºC from cleavage until eyeing (13 - 158τ) compared to 6 and 8 °C (7.3 – 11.8 %, 6.5 – 10.5 % less respectively). Similar survival between eyeing and hatch (159 - 320τ_s) was

seen in eggs incubated at 8 ºC and 11 ºC with the exception of triploid 8 ºC which was notably lower to its diploid sibling (8.4 % less). Deformity prevalence in hatched alevins was higher in diploids and triploids reared at 11 ºC than 6 and 8 ºC (Table 6.1). In all cases deformity prevalence was largely attributed to lordotic individuals.

Table 6.1. Cumulative survival (%) throughout embryogenesis until hatch (0 - 320 τs) in diploid and triploid Atlantic salmon eggs incubated at 6, 8 & 11 ˚C until eyeing and 8 ˚C thereafter as well as percentage prevalence of deformed individuals within population upon hatch. Initial egg population numbers and population numbers upon hatch (n) are provided.

	6		8		11	
	Dip	Trip	Dip	Trip	Dip	Trip
Initial population (n)	7260	7088	7671	7491	6705	7596
Survival (%)						
Fertilisation - Cleavage(0 - 12 τ_s)	94.39	82.99	94.80	80.14	93.47	87.64
Cleavage – eyeing (13 -158 τ_s)	96.51	97.02	92.03	93.04	84.73	86.55
Eyeing – Hatch (159 - 320 τ_s)	86.64	86.70	60.71	52.27	59.95	63.58
Cumulative	77.54	66.72	47.53	25.46	38.15	37.76
Hatched population (n)	5490	5017	4398	4262	3206	3627
Deformed (%)						
Lordosis	0.08	0.13	0.09	0.09	0.59	0.33
Kyphosis	0.01	0.04	0.01	0.01	0.04	0.16
Scoliosis	0.04	0.08	0.00	0.01	0.02	0.17
Siamese	0.02	0.02	0.00	0.00	0.00	0.00
Pin fry	0.04	0.01	0.02	0.01	0.05	0.03
Total	0.20	0.28	0.12	0.12	0.92	0.70

3.2 Nitrogenous metabolites and FAA concentration

The most common significant differences observed in nitrogenous metabolite (NM) concentrations were found between stages, temperature, temperature by stage, and ploidy by temperature respectively (Table 6.2). Mean concentrations between egg stages for which a significant effect was observed are denoted in Table 6.3. There was an overall significant decrease in FAA concentration with embryonic stages for α – aminobutyric

Table 6.2. Statistical results for FAA concentrations (mg / g) in diploid and triploid Atlantic salmon embryos: gamete, gastrulation and eyeing.

	Stage		Ploidy		Temp		Ploidy x Temp		Ploidy x Stage		Temp x Stage		Ploidy x Temp x Stage	
	2 df		1 df		2 df		2 df		1 df		2 df		2 df	
	p	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F
Alanine**	<0.0001	133	NS		NS		NS		NS		NS		NS	
Ammonia	<0.0001	42.4	NS		<0.0001	27.5	NS		NS		<0.0001	30.1	NS	
Arginine*	0.0003	10	NS		NS		NS		NS		NS		NS	
Asparagine**	<0.0001	24.4	NS		0.007	6.2	NS		NS		0.03	4.3	NS	
Aspartic acid**	<0.0001	29	NS		NS		NS		NS		NS		NS	
Cysteine†	NS		NS		NS		NS		NS		NS		NS	
Ethanolamine‡	<0.0001	159.6	NS		0.004	7	NS		NS		NS		NS	
Glutamic acid**	0.01	4.8	NS		NS		NS		NS		NS		NS	
Glutamine†	<0.0001	69	NS		0.001	9.2	0.05	3.5	NS		NS		NS	
Glycine**	<0.0001	22	NS		0.002	4.6	NS		NS		NS		NS	
Histidine*	<0.0001	17.6	NS		0.002	8.8	NS		NS		0.002	8.5	NS	
Isoleucine*	NS		NS		NS		NS		NS		NS		NS	
Leucine*	<0.0001	37.7	NS		NS		NS		NS		NS		NS	
Lysine*	<0.0001	13.8	NS		NS		NS		NS		NS		NS	
Methionine*	NS		NS		NS		NS		NS		NS		NS	
Ornithine‡	<0.0001	18.6	NS		0.002	8.5	NS		NS		NS		NS	
Phenylalanine*	<0.0001	55.8	NS		<0.0001	19.5	NS		NS		<0.0001	21.9	NS	
Phenylserine‡	<0.0001	72.1	NS		NS		NS		NS		NS		NS	
Polyalanine‡	<0.0001	14.5	NS		NS		NS		NS		NS		NS	
Proline†	<0.0001	73	NS		NS		NS		NS		NS		NS	
Serine**	NS		NS		NS		NS		NS		NS		NS	
Taurine†	0.05	3.3	NS		NS		NS		NS		0.002	8.6	NS	
Threonine*	NS		NS		NS		NS		NS		NS		NS	

	Stage		Ploidy		Temp		Ploidy x Temp		Ploidy x Stage		Temp x Stage		Ploidy x Temp x Stage	
	2 df		1 df		2 df		2 df		1 df		2 df		2 df	
	p	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F
Tryptophan*	0.03	4.1	NS		NS		NS		NS		NS		NS	
Tyrosine**	0.005	6.1	NS		0.004	7.4	NS		NS		0.001	9.5	NS	
Urea‡	<0.0001	57.2	NS		0.001	9.6	NS		NS		0.0003	12	NS	
Valine*	0.04	3.6	NS		NS		NS		NS		NS		NS	
α - Aminobutyric acid‡	<0.0001	93.2	NS		NS		NS		NS		NS		NS	
β Alanine‡	NS		NS		NS		NS		NS		0.01	5.2	NS	

*EAA.
**non-EAA.
†conditional EAA.
‡undefined / other.

Table 6.3. Mean ± SEM NM mg g^{-1} for ova (n = 6, 3 ova / 2 family), blastula (n =12, 3 ova / 2 ploidy/ temperature) and eyed (n = 12, 3 ova / 2 ploidy/ temperature) embryos that had significant differences between stages.

Concentration (mg/g)	Gamete (0 τ_s)	Blastulation (34 τ_s)	Eyeing (158 τ_s)
Alanine	0.142 ± 0.002^{a}	0.134 ± 0.003^{a}	0.071 ± 0.001^{b}
Ammonia	0.0067 ± 0.0007^{b}	0.0059 ± 0.0003^{b}	0.0134 ± 0.0006^{a}
Arginine	0.0332 ± 0.0002^{b}	0.0397 ± 0.0005^{b}	0.0684 ± 0.0022^{a}
Asparagine	0.0263 ± 0.0002^{b}	0.0322 ± 0.0009^{b}	0.0469 ± 0.0005^{a}
Aspartic acid	0.837 ± 0.002^{a}	0.681 ± 0.011^{b}	0.595 ± 0.007^{c}
Ethanolamine	0.0160 ± 0.0005^{b}	0.0126 ± 0.0004^{c}	0.0241 ± 0.0002^{a}
Glutamic acid	0.53 ± 0.01^{a}	0.40 ± 0.02^{b}	0.46 ± 0.01^{ab}
Glutamine	0.154 ± 0.004^{b}	0.170 ± 0.010^{b}	0.230 ± 0.009^{a}
Glycine	0.0257 ± 0.0009^{a}	0.0196 ± 0.0002^{b}	0.0188 ± 0.0002^{b}
Histidine	0.0300 ± 0.0003^{b}	0.0329 ± 0.0015^{b}	0.0469 ± 0.0009^{a}
Leucine	0.084 ± 0.001^{a}	0.069 ± 0.002^{b}	0.049 ± 0.002^{c}
Lysine	0.077 ± 0.001^{a}	0.084 ± 0.002^{a}	0.132 ± 0.003^{b}
Ornithine	0.0015 ± 0.0003^{b}	0.0007 ± 0.0002^{b}	0.0031 ± 0.0001^{a}
Phenylalanine	0.025 ± 0.000^{b}	0.030 ± 0.000^{b}	0.059 ± 0.002^{a}
Phenylserine	0.68 ± 0.02^{a}	0.54 ± 0.01^{b}	0.44 ± 0.01^{c}
Polyalanine	0.046 ± 0.002^{a}	0.045 ± 0.001^{a}	0.039 ± 0.001^{b}
Proline	0.0200 ± 0.0013^{a}	0.0185 ± 0.0007^{a}	0.0106 ± 0.0004^{b}
Taurine	0.154 ± 0.003^{a}	0.139 ± 0.003^{b}	0.141 ± 0.002^{ab}
Tryptophan	0.024 ± 0.003^{ab}	0.020 ± 0.002^{b}	0.028 ± 0.001^{a}
Tyrosine	0.032 ± 0.000^{b}	0.036 ± 0.001^{b}	0.044 ± 0.002^{a}
Urea	0.019 ± 0.005^{b}	0.012 ± 0.001^{c}	0.033 ± 0.001^{a}
Valine	0.075 ± 0.001^{a}	0.059 ± 0.003^{ab}	0.057 ± 0.001^{b}
α - Aminobutyric acid	0.0082 ± 0.0002^{a}	0.0066 ± 0.0001^{b}	0.0033 ± 0.0004^{c}

a,b, c Mean values with different superscript letters are significantly different ($P < 0.05$; One-Way ANOVA & Tukey's post hoc)

acid, alanine, aspartic acid, glycine, leucine, polyalanine, phenylalanine, phenylserine, proline and valine (Table 6.3). Significant increases with embryonic stages were found for ammonia, arginine, asparagine, ethanolamine, glutamine, His, lysine, ornithine, phenylalanine, tryptophan, tyrosine and urea (Table 6.3). Glutamine was found to be overall significantly higher in embryos reared at 8 ºC to those reared at 11 ºC (Fig 6.4). Ammonia was significantly higher in eyed embryos reared at 6 and 8 ºC and to blastula treatments, which were the lowest significantly. Similar results were observed for asparagine except eyed 11 ºC embryos, which were not significantly different to blastula

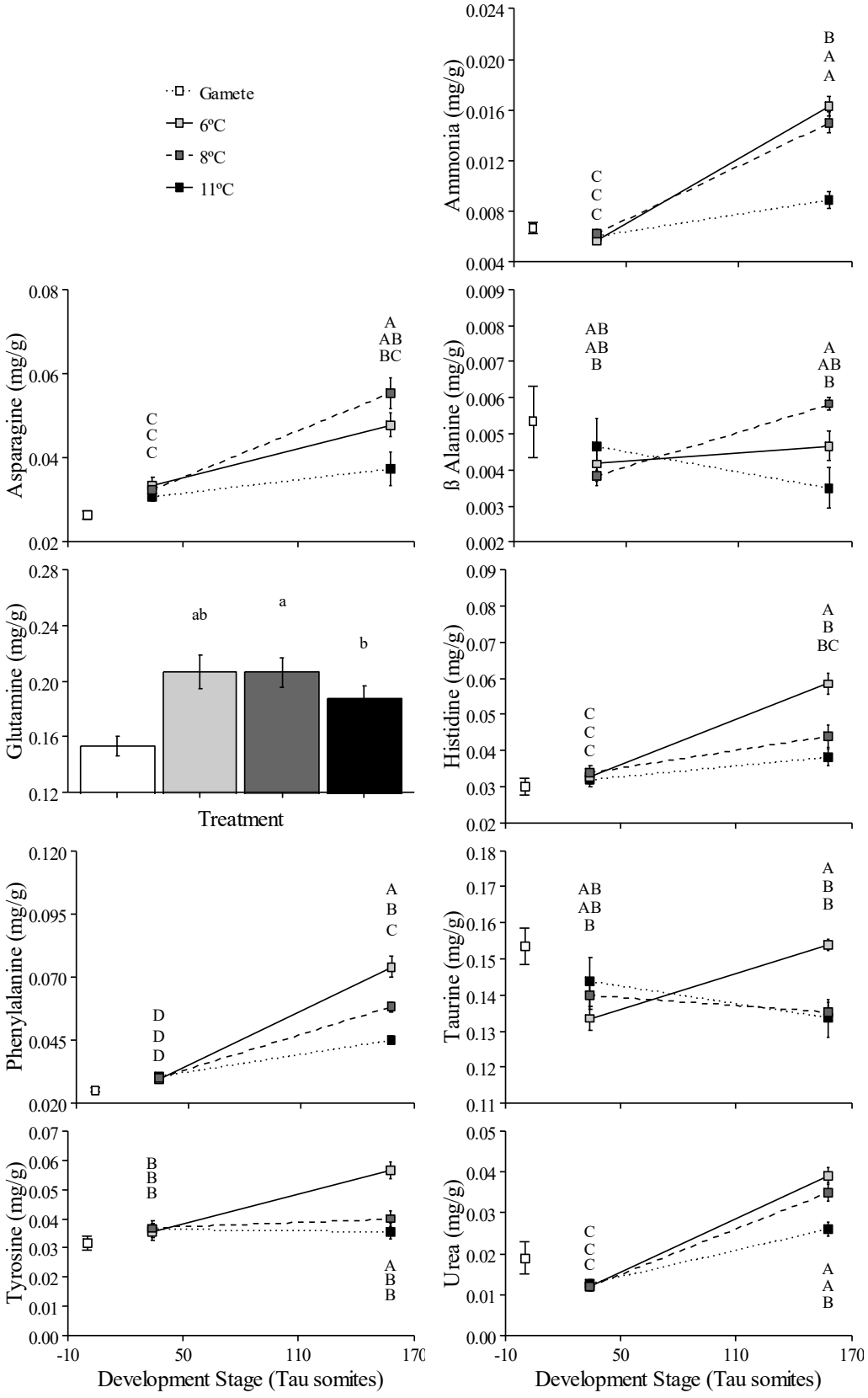

Figure 6.4 FAA (mg / g) in diploid and triploid Atlantic salmon embryos (0, 34, 158τ_s) reared at 6, 8 & 11˚C that had significant interactions for temp x stage and for Glutamine, temperature. Unlike subscripts denote significant differences: (One-Way-ANOVA & Tukey's post hoc; black capital (temperature x stage) and lowercase (temperature).

embryos. His were significantly lower in the blastulation treatments and higher in 6 ºC eyed embryos compared to 8 and 11 ºC eyed embryos. Phenylalanine was significantly lower in blastulation embryo treatments and highest significantly in 6 ºC eyed compared to 11 ºC eyed embryos. Taurine concentrations were significantly higher in 6 ºC eyed embryos compared to 8 and 11 ºC eyed embryos and blastula embryos at 6 ºC. Tyrosine concentrations were significantly higher in eyed 6 ºC embryos relative to all other treatments. Lastly, urea concentrations were significantly higher in 6 and 8 ºC embryos compared to 11 ºC eyed embryos and eyed temperature treatments were overall significantly higher in the eyed stages compared to blastula stages.

3.3 DNA methylation

DNA quality and quantity increased with life stage with DNA in the pre-somitogenesis stages being increasingly predisposed to protein and lipid contamination as implied through spectrophotometer absorbance ratios (example in Figure 6.). Hence, only DNA samples from the late blastulation stages onwards could be used. Global DNAme % results are presented in Figure 6.5. Overall global DNAme % levels were significantly lower in triploids than diploids for all incubation temperatures (Dip: 5.02 ± 0.10 %, Trip: 4.69 ± 0.08 %; p <0.0001). The lowest DNAme was observed at blastulation (3.91 ± 0.15 %; 34 τ_s) significant to 45th (4.78 ± 0.16 %; 100 τ_s) and 65th (5.47 ± 0.09 %; 123 τ_s) somite, and eyeing (5.24 ± 0.09 %; 158τ_s; p <0.0001). DNAme increased until a peak at

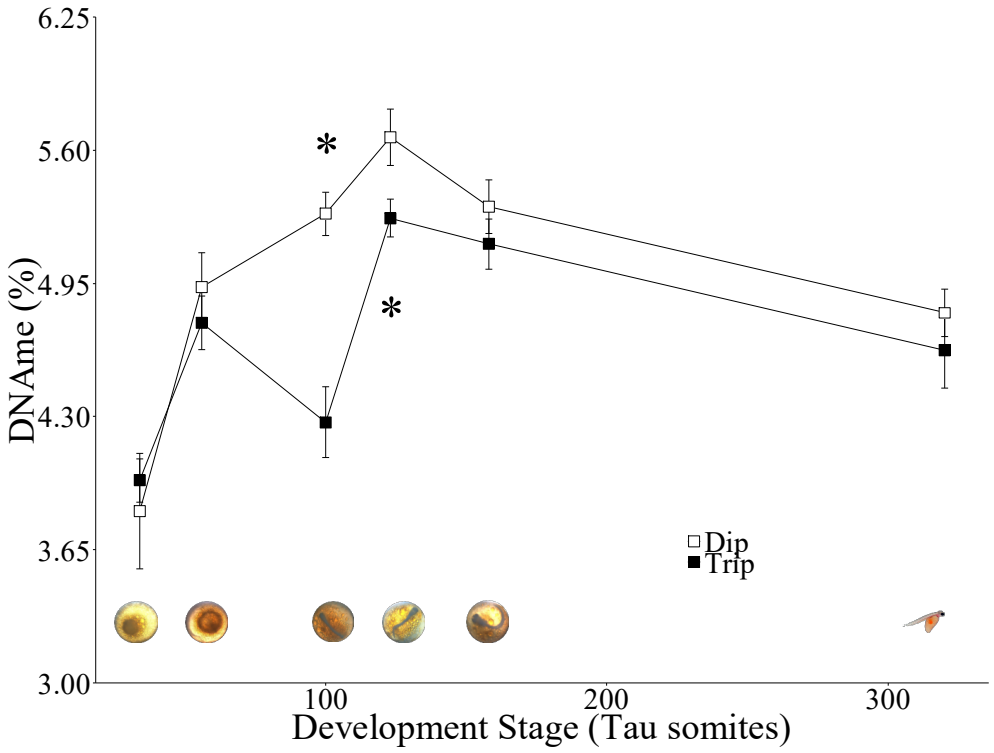

Figure 6.5. Methylated: non-methylated cytosine % in diploid and triploid Atlantic salmon embryonic DNA. Temperature treatments (6, 8 & 11 ˚C from fertilisation until eyeing and 8 ˚C until hatch, 0 - 320 τ_s) were pooled to show overall ploidy effect as there was no significant interaction of temperature x ploidy x development stage. Significant differences between ploidy are denoted by an asterisk ($p < 0.05$; Two-Way-ANOVA).

the 65th somite stage and then became significantly reduced at hatch (4.72 ± 0.11 %). No significant differences were observed between treatments at blastulation. Diploid gastrula reared at 6 ºC had significantly lower DNAme (4.30 ± 0.13 %) compared to diploids reared at 8 (5.21 ± 0.09 %) and 11 ºC (5.28 ± 0.12 %; $p = 0.009$). At the 45th and 65th somite stage diploids had significantly higher DNAme % compared to triploids (45th somite: $p = 0.0001$; 65th somite: $p = 0.03$; Fig. 6.5). Diploid 6 ºC eyed embryos had higher DNAme % at eyeing (5.70 ± 0.23 %) significant to triploid 6 (5.00 ± 0.02 %) and 8 ºC (4.77 ± 0.01 %), and triploid 11 ºC (5.53 ± 0.11 %) were also significantly higher than

triploids reared at 8 ºC at eyeing (p = 0.02;). No significant differences between treatments were observed at hatch.

4 Discussion

Temperature during egg incubation up to eyeing clearly impacted survival with higher temperature (11 ºC) increasing embryonic mortalities and prevalence of lethal deformities post-hatch in both ploidies. Endogenous NMs showed no significant differences between ploidy however, significant differences between incubation temperatures and developmental stages were observed. Increases in DNAme, that imply potential DNA remethylation, was detected post gastrulation and appeared delayed compared to other teleost literature (Jiang *et al.,* 2013b; Skjærven *et al.,* 2014). No impact of temperature was observed on DNAme, which was similar between ploidy with the exception of somitogenesis stages where triploids showed delayed increase in DNAme.

Pressure induced triploid salmonids are known to exhibit lower embryonic survival rates compared to their diploid siblings (Mcgeachy *et al.,* 1995; O'Flynn *et al.*, 1997; Cotter *et al.,* 2002; Johnson *et al.,* 2004; Fraser *et al.,* 2013a) which is concurrent with embryos reared at 6 and 8 ºC in this study. In addition, heat shock during embryogenesis is known to reduce egg viability in diploid Atlantic salmon (Wargelius *et al.,* 2005b) which is in accordance with the overall reduced survival observed at 11 °C in

this study. Fraser *et al.,* (2013a) has shown that reduced egg incubation temperatures of 6 ºC as opposed to 10 ºC marginally reduced egg mortality in diploid and triploid salmon (8.7 & 4.1 % respectively) however, mortality rates were considerably higher in triploids (85.6 - 89.7 %) compared to diploids (18.6 – 27.3 %). Taylor *et al.,* (2011) observed comparable survival between diploid (71.0 – 77.3 %) and triploid (67.5– 75.4 %) Atlantic salmon reared at 8 ºC and attributed instances of lower survival (<40 %) to over-ripened batches known to reduce viability in diploid salmonids (Bromage *et al.,* 1992; Aegerter & Jalabert, 2004). This may offer an explanation to varied triploid egg viability in other research where egg quality is infrequently reported. The immediate increase in triploid egg mortality compared to diploids at the cleavage stage may have been due to the pressure shock rather than an effect of triploidy. Acute environmental shocks are known to reduce viability in vertebrates (Rivera & Hansen, 2001) and Weber *et al.,* (2014) observed improved growth performance in triploid rainbow trout derived from tetraploid crosses compared to pressure shock. Teleost fish are known to be more stenothermal in the embryonic stages (Pörtner & Farrell, 2008) and salmonid embryos are known to be more tolerant to extreme temperatures once epiboly is complete (Rombough, 1997). Here increased mortality was shown from cleavage until eyeing at 11 °C (158 τ_s) although reduced temperatures (6 °C) had the lowest mortality post-eyeing (158 – 320 τ_s). In addition, high egg incubation temperatures are proven to induce vertebral (Fraser *et al*.,

2015a; Wargelius *et al*., 2005b; Ytteborg *et al*., 2010a) and cardiac defects (Fraser *et al*., 2013) both in diploids and triploids later in ontogeny. Long term performance was not assessed, however, increased temperature had a tendency to increase lethal deformity prevalence in diploid and triploid alevins at hatch, which were largely represented by lordosis.

In the present study there was little impact of ploidy on embryonic NMs in either embryonic stages which is concurrent with research on utilisation of endogenous reserves in diploid and triploid rainbow trout embryos (Oliva-Teles & Kaushik, 1987; Oliva-Teles & Kaushik, 1990b), however, there were strong temporal effects. Yolk utilisation in FW fish embryos typically involves catabolism of protein sources into FAAs and so detection will largely depend on developmental stage (Kamler, 2007; Finn & Fyhn, 2010). EAAs e.g. His, isoleucine, leucine, valine; non-EAAs e.g. alanine, aspartate, glycine and polyamine and conditional amino acid e.g. proline decreased in concentration by eyeing indicating utilisation in the earlier developmental stages. This is concurrent with their known functions in embryogenesis: alanine and aspartic acid as primary glucogenic precursors in fish (Li *et al.,* 2009); branched-chain amino acids: leucine, isoleucine and valine muscle as protein synthesis stimulators and proteolysis inhibitors in mammals (Nakashima *et al.,* 2007); glycine for gluconeogenesis (Fang *et al.,* 2002); His for DNA and protein synthesis and as an energy source during starvation (Li *et al.,* 2009);

polyamines in cell proliferation and differentiation in mammals (Wu *et al.,* 2008); and proline as an early life stage conditional EAA in fish (Zhang *et al.,* 2006; Li *et al.,* 2009).

Conversely, whilst low in the earlier stages, essential FAAs e.g. arginine, lysine, phenylalanine, tryptophan; non-essential FAA e.g. tyrosine and conditionally essential FAAs e.g. glutamine and also ammonia, ornithine and urea increased significantly by eyeing indicating that they may be released from protein-bound AAs or synthesised during later embryo development (Kamler, 2007; Li *et al.,* 2009). Notably, glutamine may be synthesised by the branched–chain amino acids (leusine, isoleucine and valine; Li *et al.,* 2009) which were shown to decrease at eyeing in this study. Arginine is an important stimulator of release of hormones such as insulin, GH and glucagon (Mommsen *et al.,* 2001) and is also associated with production of ornithine and urea (Gouillou-Coustans *et al.,* 2002) which is consistent with this study. Lysine is required for the transport of long chain fatty acids to the mitochondria (Harpaz, 2005) and may therefore also be required for further energy utilisation of lipids following depletion of initial readily available endogenous reserves (Kamler, 2007). Phenylalanine and tyrosine are important for production of thyroxine (T4), melanin, epinephrine and norepinephrine and are subsequently important for fish metamorphosis (Pinto *et al.,* 2009), growth (Garg, 2007), pigmentation (Boonanuntanasarn *et al.,* 2004) and neurotransmitters that regulate stress response (Damasceno-Oliveira *et al.,* 2007) explaining the higher concentrations

in the later stages. As the increased or decreased presence of AAs was not fully dependent on being essential or non-EAAs, it can be stipulated that there are specific requirements depending on embryo developmental stage as shown for other teleosts (Kamler, 2007).

Temperature differences in FAA concentrations occurred almost exclusively in the eyeing stages as a consequence of increase in FAAs and was largely attributed to NMs associated with ammonia and urea production and indicators of oxidative stress. This further suggests a restriction in metabolic expenditure in the early embryonic stages with large increases in ammonia excretion found towards the end of embryogenesis and hatch (Kamler, 2007). The significant increase in ammonia and urea at lower temperatures in the eyeing stages may be a consequence of additional time required for development and associated increase in protein catabolism for energy purpose as opposed to direct temperature differences. Ammonia quotients decrease in rainbow trout alevins alongside less metabolised protein in embryos reared at a temperature of 5 ºC as opposed to 10 ºC (Oliva-Teles & Kaushik, 1990b). In addition embryos may have been under higher thermally induced hypoxic stress indicated by lower relative levels of His in the higher temperatures which is known to protect fish against pH changes as a result of hypoxia and lactacidosis in rainbow trout (Mommsen *et al.,* 1980; Li *et al.,* 2009). Higher levels of β-alanine, associated with stress in Senegalese sole (Aragão *et al.,* 2008), in eyed embryos reared at 8 ºC as opposed to 11 ºC also indicate temperature differences in

protein utilisation under oxidative stress. Higher concentrations of phenylalanine and tyrosine were observed at lower temperatures at eyeing and are known to act as precursors to neurotransmitters regulating stress response (Damasceno-Oliveira *et al.,* 2007). High rearing temperatures have historically been associated with decreased protein utilisation in alevins and smaller hatching weights associated with reduced energy availability and yolk absorption period (Heming 1982; Oliva-Teles & Kaushik, 1990b). However, Atlantic salmon embryos reared between a range of 4 – 22 ºC demonstrated a linear increase in yolk absorption (R^2 = 0.93 Ojanguren *et al.,* 1999) and similar patterns have also been observed in other cultured fish species such as Atlantic cod (Pepin *et al.,* 1997) and seabass (Pelosi *et al.,* 1993) attributed to yolk exhaustion through increased metabolic activity (Ojanguren *et al.,* 1999). In this study, temperature had little impact on utilisation of reserves in early development however some temperature related differences in NMs were indicative of impacted protein utilisation, metabolism alongside and oxidative stress at eyeing.

This is the first study to report embryonic DNAme levels in any salmonid species. Zebrafish and Atlantic cod DNAme increases from blastulation, to levels similar to adult or sperm by gastrulation (Jiang *et al.,* 2013b; Potok *et al.,* 2013; Skjærven *et al.,* 2014). This is attributed to maternal reprogramming of the hypomethylated oocyte to the maintained paternal methylation patterns at zygotic gene activation (Jiang *et al.,* 2013b;

Potok *et al.*, 2013). Zygotic gene activation typically occurs at the late blastula stage. In other vertebrates, DNA remethylation occurs, as opposed to DNA reprogramming (Santos *et al.*, 2002; Reik *et al*., 2003). The lowest DNAme levels were observed at late blastula in this study however, DNA could not be extracted in early stages which may have been due to yolk contamination alongside low DNA yields. Converse to reports in zebrafish and cod, Atlantic salmon embryos in this study increased in global DNAme throughout somitogenesis. The reason for this is unknown, although factors of additional genomic material in Atlantic salmon due to recent WGD may be involved. Low levels of gene deletion or inactivation and pronounced conservation of miRNAs since the salmonid WGD are observed in rainbow trout (Berthelot *et al.,* 2014) however it is not understood how this may translate into dosage effects or organisation of the methylome in naturally evolved polyploids. In addition, there was a lack of clear impact of temperature on global DNAme, which is unlike the increased observations and lower levels of gene expression in post-gastrula embryos in Atlantic cod reared at higher temperature (Skjærven *et al.,* 2014). As salmonids spawn in FW it is possible that that they have evolved a genetic resilience to cope with environmental fluctuations which is also associated with polyploid formation as reviewed in Mable *et al.*, (2011). Overall, results indicate that the Atlantic salmon may have starkly different regulatory processes of the genome during embryogenesis relative to other teleosts and vertebrates. This may

be attributed to polyploidisation, although further research is required verify this hypothesis and to underpin the more specific epigenetic mechanisms involved. In particular, significantly lower DNAme observed at the 45th and 65th somite stage in triploids further support that alterations to DNAme in these remethylative stages are attributed to polyploidisation. In addition, the ratio of the paternal to maternal genome is smaller in triploids and so it is anticipated that the known degradation of maternal transcripts around blastulation (Martin *et al.,* 1999) as well as the remodelling process to the paternal genome (Jiang *et al.,* 2013b; Potok *et al.,* 2013) may be impacted. Nonetheless, artificial induction may here also be liable for alterations in DNA regulation and thus cannot be accounted for.

At hatch similar levels of DNAme were observed across treatments, supporting dosage effects in triploids where expression of the additional chromosome may be sustained alongside the presumed increased cell size: frequency ratio that is seen in other tissues in triploid fish (Swarup, 1959; Small & Benfey, 1987; Aliah *et al.,* 1990; Johnston *et al.,* 1999; Flajšhans *et al.,* 2011). Dosage effects are concurrent with recent literature that has shown similar levels of DNAme in diploid and triploid brown trout (Covelo-Soto *et al.,* 2015) and genome-wide expression indicative of dosage effects in Chinook salmon (Ching *et al.,* 2009). However, dosage compensation in specific genes following *V. anguillarum* challenge in Chinook salmon indicate triploids may suffer a loss of

homeostasis under stress. Conversely, Pala *et al.,* (2008) showed similar relative gene expression levels in diploid and triploid sea bream and decreased RNA:DNA ratios in triploid livers which they attributed to gene dosage compensation. However, cell size to frequency ratios were not considered. This study supports that artificially induced triploid salmonids may regulate their genomes with dosage effects in the face of additional genomic material with reduced cell frequency.

As embryonic temperature is an important factor in triploid performance and development of deformities later in development, it was hypothesised that embryonic DNA regulation may be altered. Overall, there was a clear lack of temperature effect on ploidy response of DNAme and NM concentration, despite a clear impact on overall NM concentration in the later eyeing stages. Hence DNAme and potentially yolk utilisation may not be factors in higher temperature – induced deformity prevalence of triploids although long-term phenotype and other epigenetic processes such as histone modifications and miRNAs were not assessed. At hatch, similar global DNAme was observed between ploidy supporting previous reports of dosage effects in triploid fish compared to diploid siblings. Of particular interest, Atlantic salmon show increased DNAme, indicative of DNA remethylation / reprogramming, up until the 65th somite stage which is delayed compared to other teleosts species. This was particularly apparent in triploids where DNAme remained significantly lower compared to diploids throughout

somitogenesis. This supports that ancestral and induced polyploidisation with additional genomic material may in some way decelerate the DNA remethylative / reprogramming process although this cannot be verified at this stage. Collectively, these results propose interesting avenues of investigation into potential differences in gene regulation, dosage and epigenetic processes during early development alongside further investigation of cell size: frequency ratios in a period where cytosol synthesis is known to be compromised by rapid cell cycle replication. In particular, other embryonic mechanisms may be responsible for the temperature – dependent effect ploidy has upon phenotype and deformity development which remain to be elucidated.

Summary of Conclusions

Chapter 2: Dietary phosphorous and protein supplementation enhances seawater growth and reduces severity of vertebral malformation in triploid Atlantic salmon (*Salmo salar* L.)

- Dietary P and protein supplementation improved growth performance, harvest grading and reduced skeletal deformity in triploid salmon during ongrowing
- Dietary supplementation arrested development of severely malformed vertebrae in triploids compared to those fed a standard diet from point of sea transfer until harvest
- Severely malformed vertebrae observed at harvest may be of FW origin.

Chapter 3: Dietary freshwater phosphorous requirement of triploid Atlantic salmon (*Salmo salar* L.) is different to diploid with reference to early skeletal development.

- Feeding higher P supplementation throughout freshwater mitigates development of severely malformed vertebrae in triploid Atlantic salmon.
- Growth performance and skeletal development in triploid Atlantic salmon is impacted more by P supplementation in earlier parr stages compared to smolt.
- Diploid growth is impeded under higher P supplementation throughout freshwater whereas triploid fed high P show superior growth at parr however this effect is reduced at smoltification.

Chapter 4: Dietary probiotics and windows of phosphorous supplementation as potential alternatives to high dietary phosphorous supplementation for prevention of skeletal deformity in triploid Atlantic salmon (*Salmo salar* L.)

- High P supplementation reduced deformity prevalence and severity within the parr stages however reducing effects were not transpired when feeding to shorter windows of P supplementation.

- Probiotic inclusion yielded the fewest skeletal deformities in the parr stages relative to other triploid treatments and mitigated severely malformed vertebrae.
- No dietary effect on subsequent triploid skeletal development or growth performance was observed at smolt.

Chapter 5: Parr – smolt transformation windows in diploid and triploid Atlantic salmon (*Salmo salar* L.) under ambient photo-thermal regime.

- Triploids demonstrated earlier onset of PST by 48 degree days and wider smoltification windows within 155 – 365.5 degree days confirmed through: increased NKA activity, skin silvering and survival to SW challenge alongside reduce chloride levels, compared to diploid conspecifics.

Chapter 6: Embryonic ontogeny of global DNA methylation and utilisation of endogenous energy reserves in diploid and triploid Atlantic salmon eggs incubated at different temperatures

- No impact of temperature was observed on ploidy specific FAA or DNAme levels or prevalence of lethal malformations.

- DNAme levels in Atlantic salmon continue to increase past the blastula developmental stages relative to other species which is further emphasised in triploids indicative of delayed embryonic DNA remethylation / reprogramming.
- Triploids demonstrate similar DNAme % to diploid siblings at hatch implying gene dosage compensation mechanisms may be apparent in fully formed triploid fish.

Chapter 7: General Discussion

The overall aim of this thesis was to further understand production traits of artificially induced triploid Atlantic salmon in order to improve management practice and subsequent fish welfare and productivity. Such knowledge and improved protocols are critical for decision making regarding the implementation of sterile stocks in the salmonid aquaculture industry. Although the concept of triploid salmon has historically been tested in the 1980's, interest has returned over recent years mainly due to increasing concerns over the potential genetic impact of escapees (McGinnity *et al.,* 2003; Bourret *et al.,* 2011; Glover *et al.,* 2013) and need for sustainable expansion of the production sector. The overall scope for investigation is vast given the many gaps in knowledge on triploid salmon physiology (Fraser *et al.,* 2012a; Benfey, 2015). In particular, artificially induced triploid salmonids have historically performed poorly when compared to their diploid siblings in terms of survival (Galbreath & Thorgaard, 1995; McCarthy *et al.,* 1996; Cotter *et al.,* 2002), growth (Cotter *et al.,* 2002; Friars *et al.,* 2001) and deformity prevalence (Jungalwalla, 1991; O'Flynn *et al.*, 1997; Sadler *et al.,* 2001; Leclercq *et al.,* 2011). The main factors shown to impact on performance include suboptimal rearing conditions such as low oxygen saturation (Hansen *et al.,* 2015), too high egg incubation temperatures (Fraser *et al.,* 2013, 2015) and nutritional deficiencies (Taylor *et al.,* 2015; Fjelldal *et al.,* 2015). These studies show that scope for improvement that equals if not betters performance relative to diploid conspecifics can be achieved in triploids through refined

rearing regimes in consideration of triploids as a functionally a new species (Benfey, 2001; Fraser *et al.,* 2012a). Hence, the thesis aims were to assist in mitigation of skeletal deformities and sustain long term growth through investigating influencing factors of nutrition, smoltification windows and egg incubation temperatures. More specifically: to study the effects of supplemented diets (P, protein and probiotics) on triploid growth and skeletal deformity prevalence throughout FW rearing and SW ongrowing (**Chapter 2 – 4**); to characterise the smoltification window in triploids in order that sea transfer protocols be optimised and subsequent performance be sustained in SW ongrowing (**Chapter 5**); to study the impact of egg incubation temperature, a primary factor subsequent development of deformities in triploids, on embryonic DNAme and yolk utilisation (**Chapter 6**). Findings are discussed below alongside the associated broader implications which remain to be defined for successful adoption in commercial production.

Nutritional supplementation sustains growth and mitigates skeletal deformities

Nutritional requirements of triploid fishes are conceivably different to diploid conspecifics, which has been attributed to reported differences in aspects of digestion (Fauconneau *et al.,* 1990; Blanc *et al.,* 2001, 2005; Peruzzi *et al.,* 2014), behaviour associated with feeding (Mcgeachy *et al.,* 1995; O'Keefe & Benfey, 1997; Preston *et al.,*

2014) and higher growth potential (Oppedal *et al.,* 2003; Leclercq *et al.,* 2011; Taylor *et al.,* 2011, 2012; Fjelldal *et al.,* 2015). This thesis investigated the impact of dietary P, protein and probiotic inclusion (*P. acidilactici*) with the aim to optimise overall growth performance and minimise skeletal deformities. At the start of this doctoral work, no research had investigated nutritional requirements of triploid Atlantic salmon. However, during the timeframe of the project, other studies have demonstrated triploids to have higher dietary P requirements to facilitate correct development of the skeleton and reduce deformities throughout FW and SW development (Fjelldal *et al.,* 2015) which was confirmed for FW in this doctoral thesis. In addition higher His inclusion was shown to prevent cataract formation in post-smolts (Taylor *et al.,* 2015). One of the main limitations of the nutritional trials in this thesis is the undisclosed availability of supplemented nutrients, mainly P that was protected for intellectual property reasons. The availability of P within the diet varies depending on the source of raw feed materials and is generally categorised according to: animal, plant, organic or inorganic origin (Hua & Bureau, 2006). For instance plant based P is less available relative to animal based. Hence, total P disclosed in this thesis is not necessarily reflective of actual availability, rendering determination of baseline requirements for P against known standards of 8 g available P kg^{-1} (NRC, 2011) or to conclusively compare levels to other literature unfeasible. Thus, levels will not be stated in the discussion and emphasis will be drawn

solely on phenotypic response to different nutritional treatments. In addition, it is worth acknowledging that experimental designs of chapters 2 and 4 only included a single control dietary treatment for diploids as a reference due to the commercial objectives of the trials which aimed to optimise triploid feed. Therefore comparison of within triploid performance between dietary treatments was preferred. Hence, although it cannot be conclusively stated in either trial that differences in performance of supplemented dietary treatments are owing specifically to higher requirements compared to diploids, literature states that triploid salmon should be functionally considered as a new species rendering the need for need for systematic comparison with diploids for the sake of nutritional requirements void (Benfey, 2001; Fraser *et al.,* 2012).

The repeated observation of triploid specific dietary effects between all first three chapters strongly show that nutritional supplementation improves triploid performance. The overall commercial aim of triploid optimisation is to sustain the accelerated growth that is frequently observed in FW and post-transfer until harvest (Leclercq *et al.,* 2011; Taylor *et al.,* 2011, 2012; Fjelldal *et al.,* 2015) as well as mitigate deformities that impede development and marketability. It is believed that triploids may have a greater potential for growth however, such potential has not consistently been achieved. The SW ongrowing study in this doctoral project (chapter 2) is one of two studies to show sustained superior growth to harvest (Oppedal *et al.,* 2003) and in this instance this was

attributed to both protein and P supplementation. Conversely, Fjelldal *et al.,* (2015) observed accelerated growth when solely supplementing P throughout FW which did not transpire to improved growth in further SW rearing with P supplementation implying increased P requirement is not continuous through ongrowing and that additional protein supplementation may be required to sustain improved growth. In addition, nutritional supplementation of P and protein reduced severity of vertebral pathologies during ongrowing at sea. Comparison between x-radiographs at the time of sea transfer and harvest show that not only did supplementation cease further development of severely deformed individuals but that pathology was predominantly of FW origin and must be primarily addressed to resolve vertebral pathologies observed in SW. P supplementation during the FW phase prevented the development of severely deformed (>10dV) individuals (chapter 3) and this implies that there is potential for improved subsequent ongrowing performance in SW (Hansen *et al.*, 2010). In contrast, overall lower prevalence of severely deformed individuals were found in triploids regardless of dietary treatment in chapter 4 indicating other factors may be influencing higher P requirement. For instance, fish were not vaccinated prior to smoltification in chapter 4, which may explain the greater dietary effect on spinal deformity prevalence in chapter 3 where fish were vaccinated. Other published research investigated impact of vaccination in triploids (Larsen *et al.,* 2014) or P supplementation in conjunction with vaccination (Gil-Martens

et al., 2012) and show no effect of either factors on the prevalence of vertebral deformities following vaccination. By contrast, Fraser *et al.,* (2014) found vaccination increased prevalence of abdominal adhesions in triploid Atlantic salmon although no impact on vertebral deformity was observed. However, no research has investigated both P supplementation and vaccination impacts on skeletal deformity prevalence in triploids.

Both chapters 3 and 4 would suggest that triploids have increased sensitivity to dietary P deficiency in the earlier parr stages owing to the observations of improved growth and reduced prevalence of vertebral deformities with higher P supplementation. Shorter vertebrae in relation to vertebral height were also observed in individuals fed lower dietary P indicating suboptimal mineralisation and subsequent strength and integrity (chapter 3). However, where higher dietary P initially improved triploid parr growth rates those fed comparably lower levels grew faster by smoltification (chapter 3). Similar observations were observed in Fjelldal *et al.,* (2015) at smoltification that indicate higher P supplementation may not be beneficial for growth beyond certain developmental stages and physiological requirements. Nonetheless, overall deformity prevalence was significantly reduced at smoltification compared to those fed the lower dietary P levels which also confirms observations by Fjelldal *et al.,* (2015). It was therefore anticipated that feeding higher dietary P solely within the earlier life stages (chapter 4) would enable correct skeletal development whilst sustaining the faster growth observed in the later

stages. However, little impact on growth was observed between supplemented and standard dietary P within treatments in the subsequent FW trial (chapter 4) and whilst vertebral deformity prevalence was minimised in the parr stages no apparent differences were observed at smoltification. It is therefore apparent that dietary P requirement to reduce deformity prevalence in triploids may be based on a mixture of factors other than developmental stage. Temperature profiles were similar between trials and therefore differences in end performance between trials may not be attributed to this factor. Other reported factors known to impact vertebral deformities in diploids that were not accounted for between chapters 3 - 4 were: different family effects (Sullivan *et al.,* 2007a), the lack of vaccination in chapter 4 (Aunsmo *et al.,* 2008), the unknown availability of dietary P in both trials (Fjelldal *et al.,* 2010) and increased growth rates in chapter 4 (Fjelldal *et al.,* 2010). Regardless, a higher sensitivity of skeletal development and growth to nutritional treatments was apparent within the earlier life stages in both chapters (3 &4). This was evident through reduced Ca deposition and vertebral areas in triploid fry fed lower dietary P that was not apparent in diploid siblings. Also, decreased gene expression of osteogenic factors in triploids and PO_4^{3-}homeostasis in higher dietary P solely within the early parr stages further support a higher P requirement for earlier development (chapter 3). Additionally, there was a higher accumulation of Ca, P, Zn and V in high P in triploids at smolt fed high P reflective of increased osteogenic activity and

lower levels of V within the earlier life stages indicating enhanced mineralisation (chapter 3; Tiago *et al.,* 2008). In both trials earlier development also coincided with increasing water temperature and may additionally explain this increased sensitivity where accelerated growth stressors may be compounded. Subsequent cooler temperatures up until smoltification facilitate skeletal mineralisation and may be why little difference is observed at smolt in chapter 4. This is supported by the observation of a reduction in radiodense pathologies by smoltification which are indicative of osteoid failing to mineralise correctly and linear growth failure (Witten *et al.,* 2005; Fjelldal *et al.,* 2009b). In future research, the use of recirculation systems as a means to control temperature and reduce variability associated with seasonal ambient water temperatures may be preferred for standardised investigation.

Whilst research mainly focused on vertebral deformities, jaw deformity was also investigated. Prevalence at sea was not prevented by dietary treatments unlike the positive effect on vertebral deformities indicating alternative aetiologies were responsible or other potential nutritional deficiencies such as vitamin C as suggested in Roberts *et al.,* (2001) and egg incubation temperature (Fraser *et al.,* 2013a). Alternatively mechanical stress of the jaw owing to increased respiration and buccal-opercular movements during high temperature and lower oxygen saturation conditions alongside an outbreak of AGD and HSMI in this thesis may have been liable. In addition, triploids

had a more acute angle of the bulbous arteriosus consistent with other published studies (Leclercq *et al.,* 2011; Fraser *et al.,* 2013b) further supporting increased cardiac workload in triploids that may have compounded factors of increased jaw deformity prevalence. This alongside the reduced growth regardless of dietary treatment during the adverse conditions suggests that triploids are not as robust to diploid conspecifics when challenged by several stressors. This is clearly an area of research that should be further investigated as most studies assess single challenge or stressor effects that do not necessarily reflect the reality of salmon farming in open cage systems. Nonetheless, similar cumulative mortalities were observed between diploids and triploids.

Localisation of vertebral deformity prevalence was shown to vary with life stage according to that of previously reports in diploid and triploid Atlantic salmon (Fjelldal *et al.,* 2012a). Overall nutritional supplementation was shown to reduce these localised life-stage dependent deformities in this thesis. In ongrowing, supplemented diets appeared to reduce deformities in the caudal region of the spine where deformities are typically reported at this stage (Fjelldal *et al.,* 2009b), which was reflected in subsequent strengthening of the spine as well as increased L:H ratios (chapter 2). Conversely, during FW development, increased prevalence of deformities were found in the central and caudal regions which is concurrent with other published literature (Fraser *et al.,* 2015a; Fjelldal *et al.,* 2015; chapter 3 and 4). However, triploids fed lower levels of dietary P

had increased prevalence of vertebral deformities in the caudal region unlike diploids, where deformities in this region are more common during SW ongrowing. Factors that may be exacerbated here include caudal elongation during PST alongside vaccination induced inflammation and faster growth at this stage within this treatment.

Probably the most striking nutritional effect was within the early stages of FW development where *P. acidilactici* inclusion significantly reduced the number of vertebral deformities and no severely deformed individuals were observed. Triploids therefore have potential for improved skeletal performance than with higher P supplementation. This may not only cut economic cost of dietary raw mineral inclusion and formulation but also poses significant environmental benefits through mitigating discharge and anthropogenic eutrophication (Folke *et al.,* 1994; Conley *et al.,* 2009). The strong impact of *P. acidilactici* indicates that the altered gut morphology observed in triploids may be having an impact on ability to digest or absorb nutrients in the gut and alterations in gut microflora between ploidy have also been observed (Cantas *et al.,* 2011). It is essential that investigations into the use of *P. acidilactici* in triploid FW diets be repeated and effects confirmed and characterised in terms of gut microflora, nutrient assimilation and gut immunity and inflammation. In addition potential long term performance must also be verified and the potential to replace ongrowing diets required in triploids.

Although fish were fed to satiation, nutritional trials in this study were limited by a lack of information regarding feed intake and digestibility due to tank restrictions. Therefore, eFCRs are crude and it cannot be ascertained that triploids were fed the exact same levels as diploids. This is important when considering other literature where triploids may have altered feeding behaviour related to their spatial distribution as shown in brown trout (Preston *et al.,* 2013) and cognitive ability in Atlantic salmon (Fraser *et al.,* 2012b). However, studies have also shown that an initial reduced ability to retrieve feed pellets at first feeding in triploids is overcome (Mcgeachy *et al.,* 1995; O'Keefe & Benfey, 1997). In addition, observations of deeper feeding behaviour in triploids during ongrowing confirm behavioural previously reported differences. Deeper feeding behaviour has also been observed in triploid brown trout (Preston *et al.,* 2014). Producers may rely on the observation of reduced surface feeding activity when hand feeding to determine satiation and if deeper feeding behaviour is not considered a risk of reduced growth performance may be observed. Further studies should aim to understand these differences and determine potential rearing standards and develop triploid specific manufacturer feeding tables and guidelines. Potential factors include increased environmental sensitivity to surface variables including ambient light as well as haloclines or thermoclines and associated oxygen saturation levels.

This research has confirmed and expanded on different aspects within the field of triploid nutrition with emphasis on defining the nature of ontogeny and localisation of skeletal deformities. The aetiology of increased nutritional demands in triploid salmonids are potentially multifactorial and require deeper investigation to confirm results, understand underlying mechanisms at cellular and molecular levels as well as tailoring specific triploid feeds. Altered gut physiology, differential growth and tolerance to environmental temperature have been highlighted as potential factors for the differences in requirement observed in this thesis. Hence, with consideration of these factors and the preliminary research outlined here, triploids may perform equally well if not better relative to their diploid conspecifics with refined nutritional supplementation.

Wider smoltification windows are observed in triploids

It is believed that poorer survival and growth in earlier triploid salmon research in the 80's may have also been attributed to suboptimal SW transfer. The importance of optimal SW transfer in diploids for future growth and development is reflected in poorer performance associated with SW transfer at inappropriate stages of the smoltification process. In this thesis, assessment of smolt indicators demonstrated an earlier onset of PST in triploids by 48 degree days relative to diploids which confirms observations of earlier onset reported in other trials (Leclercq *et al.,* 2011; Taylor *et al.,* 2012). This was

confirmed through: rapidly reduced appearance of parr marks, skin silvering and darkening of the fin edges relative to diploids. Furthermore, improved survival following SW challenge throughout the experimental period was observed in triploids where diploids demonstrated poorer survival at the earlier and later smoltification stages. These changes in smolt indicators and survival were evident in association with earlier increase in NKA activity in triploids which was sustained for longer relative to diploids. This also indicates triploids may have a wider smoltification window compared to diploids within 155 – 365.5 degrees which could have many industrial benefits such as reduced FW rearing periods and increased sea transfer flexibility relative to diploids where SW transfer under ambient photothermal regimes are restricted by the late summer. Unlike other studies which show superior growth performance in FW (Leclercq *et al.,* 2011; Taylor *et al.,* 2011, 2012; Fjelldal *et al.,* 2015), in chapter 5 comparable growth was observed between diploids and triploids at the onset of PST. This indicates that earlier onset of PST is most likely a triploid specific effect and cannot be attributed to factors such as accelerated growth known to advance smoltification (Stefansson *et al.,* 1991). A deeper understanding of the endocrinology in triploid Atlantic salmon during the PST is essential to understand how ploidy effects such as altered cell physiology, gene regulation and anatomy as well as accelerated growth and photo thermal regimes impact the smoltification process. In addition, triploids typically demonstrate lower K to diploid

siblings (O'Flynn *et al.*, 1997; Cotter *et al.,* 2002; Fjelldal & Hansen, 2010; Taylor *et al.,* 2012). Hence, the presence of similar K between ploidy in this study indicate that the population is not entirely representative to other literature although reasons are unknown. Comparative research is needed between different photo-thermal regimes in triploids to verify the nature of the apparent wider smoltification window as a triploid specific effect.

Improved hypo-osmoregulatory ability confirmed with SW challenge needs to be verified with long term performance. A failure to smolt has been observed in other triploid research which have also been largely due to suboptimal environmental conditions and communal rearing (Galbreath & Thorgaard, 1995; McCarthy *et al.,* 1996). Hence, assessment of long term performance and potential failure to smolt must be verified at the far reaches of the smoltification window observed in this study to ensure commercial success. Owing to the fact that consistently superior survival was observed throughout the trial it cannot be discounted that triploids may be more tolerant to increased salinity without true SW readiness and why it is imperative to assess long term performance before making conclusions of wider smolt windows. Triploids are known to have different gut and gill morphology to diploid conspecifics (Leclercq *et al.,* 2011; Peruzzi *et al.,* 2014) and so the hypo-osmoregulatory ability may be derived from these sources. If triploids do sustain a wider smoltification windows in subsequent research this

holds a large potential benefit for commercial adoption through reduced FW rearing periods and increased flexibility for sea transfer.

Temperature impacts on DNA regulation and yolk utilisation

Suboptimal egg incubation temperatures are known to be one of the predominant determining factors of future triploid performance resulting in increased vertebral and cardiac deformity prevalence (Fraser *et al.,* 2013a, 2015a) and this may be largely associated with the importance of somitogenesis in these stages and stenothermal characteristics (Pörtner & Farrell, 2008). Higher temperatures were associated with increased mortality and prevalence of skeletal deformities in this study (chapter 6). However, overall there was a large increase in mortalities immediately following fertilisation in triploids compared to diploids indicating mortalities are attributed to the actual physical shocking procedure as opposed to triploidy in this instance. No research has investigated the impact of triploidy in Atlantic salmon induced through tetraploid crossing on subsequent performance but improved growth was observed in rainbow trout compared to those induced through pressure shocking (Weber *et al.,* 2014). The use of tetraploids has been largely unsuccessful as a tool for triploid induction in salmonids to date, largely attributed by the lack of viability in tetraploid Atlantic salmon. However interploid induced triploids may hold potential for improved performance through

alleviation of any adverse effects induced by environmental shock. It cannot be discounted that subsequent ploidy differences in global DNAme may also be attributed to the pressure shock as opposed to fundamental triploidisation and comparison with interploid cross triploids would verify this. There was no impact of ploidy on the concentration of NMs however, it may be of relevance in other studies to investigate other components of yolk utilisation for instance fatty acids and AAs. FAAs and NMs in this study reflect active metabolic processes whereas AAs reflect storage and potential for energy utilisation in triploids. As triploids hatch smaller it is possible that potential for yolk utilisation is not met which is not necessarily reflected in FAA concentrations. Ongoing research is being conducted at the Institute of Aquaculture, University of Stirling upon yolk lipid utilisation and the impact of temperature in diploid and triploid Atlantic salmon.

As triploids possess additional genomic material there are implications that altered DNA regulation and epigenetics may play an important part in the differential phenotypes observed in comparison to diploids. Within the field of epigenetics DNAme is the best studied process and is known to be additionally impacted by environmental factors such as temperature. Hence, global DNAme was used here to attempt to underpin crude differences in DNA regulation between diploids and triploids. DNAme results appeared to indicate that diploids and triploids may generally have similar percentage of

methylated DNA indicative of potential dosage effects in triploids, although verification is required. This is presuming the typical increased cell size: frequency alterations observed in triploids (Small & Benfey, 1987) are synonymous to this life stage. Cell numbers and size were not quantified in this study and have not been previously defined in the literature for these earlier stages where there are rapid duplications and alterations in the cytosolic ratio. However, gene dosage compensation is concurrent with other salmonid literature demonstrating similar DNAme levels in rainbow trout (Covelo-Soto *et al.,* 2015) and genome wide expression levels in chinook salmon (Ching *et al.,* 2009). Research on gene regulation in triploid salmon is in its infancy and even more so in the realm of epigenetic regulation. In addition this research solely defines global DNAme profiles and it is therefore not possible to conclusively demonstrate how DNA is being regulated or infer directly that there are gene dosage effects or compensation from these results. Given the complexity of regulatory processes, further sequencing of the methylome would verify how triploids adopt either of these two gene homeostatic pathways. Epigenetic regulation also implies that alterations to the maintenance of the genome through processes such as DNAme will be heritable between cell cycles (Tollefsbol, 2011). Hence the 'epigenetic' effect cannot be verified without analysis of resulting phenotypes and attributing them to epigenetic modifications. The intention of this study was to observe this phenotype in the later parr stages as well as epigenetic

response to environmental challenge however, due to technical failure the trial was terminated early.

DNA remethylation, or reprogramming, was demonstrated to be delayed in diploids compared to that of zebrafish (Jiang *et al.,* 2013b; Potok *et al.,* 2013) and cod (Skjærven *et al.,* 2014) and in triploids even further so. It is therefore possible that the effect of polyploidisation in its own right impacts on the remethylation process. Hence, further research would benefit through the exploration of the contribution of paternal and maternal DNA methylomes specifically in this embryonic process. Further work is also required to successfully recover DNA from pre-gastrulation stages to verify the demethylation or remethylation of the maternal genome prior to the zygotic gene activation phase.

Although research here does not necessarily provide solutions for improved production performance, it does suggest important novel differences in embryonic DNA regulation in salmonids. In particular naturally occurring polyploid in fish are thought to arise from temperature fluctuations in the environment suggesting that salmonids may be evolutionarily predisposed to coping with additional genomic material under embryonic temperature stress and this poses interesting avenues for research into other temperature-induced epigenetics in triploid salmon embryos.

Concluding remarks

The optimisation of triploid Atlantic salmon production is dependent on the resolution of unsustained growth rates and increased skeletal deformities. This thesis clearly demonstrated that supplementation of P, protein and *P. acidilactici* in triploid dietary regimes reduced vertebral deformities and in some instances facilitated improved growth. Results indicate that a higher growth potential and associated linear growth failure under dietary deficiency may be the predominant aetiology for this ploidy specific requirement which may be exacerbated by vaccination in FW. Hence future research may benefit from FW recirculation systems that may better control linear growth through regulated photothermal regimes. Future research should focus on the use of *P. acidilactici* which demonstrates promise to improved triploid performance beyond that of P supplementation whilst simultaneously cutting costs to feed supplements and reducing the environmental impact of P. Optimal growth performance dictates that SW transfer periods be correctly performed according to standardised protocols. This trial is the first to investigate smoltification windows in triploid Atlantic salmon and results indicate that windows may be wider within 155 – 365.5 degrees, than previously thought. Although further verification is required, this has great potential for commercial production where reduced rearing times in freshwater and increased flexibility for SW transfer may be apparent. Finally, investigation into embryonic development demonstrated strong ploidy

differences in DNAme that suggest there may be a ploidy-specific delay in remethylation and this may have implications for overall differences in somitogenesis and patterning in triploids. Collectively, nutritional solutions are apparent and differences in PST as well as DNA regulation in important stages of somitogenesis are highlighted. The result of this thesis show that triploids have the potential to be reared to equal if not better standards to diploid conspecifics and highlight areas for potential refinement of nutritional and PST protocols in triploid Atlantic salmon aquaculture as well the need to further research into how performance is impacted by DNA regulation in embryogenesis.

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