Triploid Atlantic salmon, temperature, early development, and the potential for epigenetic programming

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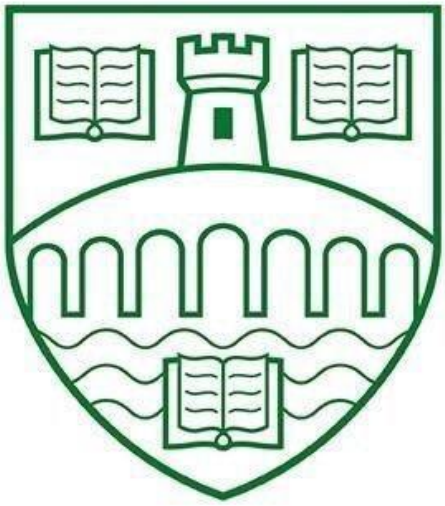

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Declaration of originality

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.

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SPECIES LIST

Abalone	*Haliotis midae*
Atlantic cod	*Gadus morhua*
Atlantic salmon	*Salmo salar*
Baboon	*Simia hamadryas*
Brine shrimp	*Artemia franciscana*
Broiler chicken	*Gallus gallus domesticus*
Brook trout	*Salvelinus fontinalis*
Brown rat	*Rattus norvegicus*
Brown trout	*Salmo trutta*
Carp	*Cyprinus carpio*
Domestic pig	*Sus scrofa domesticus*
European sea bass	*Dicentrarchus labrax*
Gilthead sea bream	*Sparus aurata*
Gold fish	*Carassius auratus*
Goldeneye	*Hiodon alosoides*
Guinea pig	*Cavia aperea*
House mouse	*Mus musculus*
Marine amoeba	*Neoparamoeba perurans*
Marine sticklebacks	*Gasterosteus aculeatus*
Migratory locust	*Locusta migratoria*
Mosquito fish	*Gambusia holbrookia*
Olive flounder	*Paralichthys olivaceus*
Pacific oyster	*Crassostrea gigas*
Rainbow trout	*Oncorhynchus mykiss*
Salmon louse	*Lepeophtheirus salmonis*
Sea louse	*Caligus elongatus*
Sheep	*Ovis aries*
Sheepshead minnow	*Cyprinodon variegatus*
Spiny damsel fish	*Acanthochromis polycanthus*
Turbot	*Scophthalmus maximus*
Whiteleg shrimp	*Litopenaeus vannamei*

Wild turkey	*Meleagris gallopavo*
Yellow perch	*Perca flavescens*
Zebra fish	*Danio rerio*

LIST OF ABBREVIATIONS

µg	Microgram
2n	Diploid
3n	Triploid
AGE	additive genetic effect
CT_{max}	Critical thermal maxima
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAme	DNA methylation
DNMT	DNA methyltransferase
g	Gram
GE	genetically engineered
GM	genetically modified
HAT	histone acetyltransferases
HDAC	histone deacetylases
HIF	hypoxia inducible factor
K	Condition factor
PGC	Primordial germ cell
Psi	Pounds per square inch
RMR	resting aerobic metabolic rate
RNA	Ribonucleic acid
SAM	S-adenosyl-methionine
SBM	Soybean-meal
TGC	Thermal Growth Coefficient
µL	Microlitre
ʈs	tau somite

GLOSSARY

Alevin	A newly hatched salmonid still carrying yolk
Anadromous	Fish born in freshwater, migrating to sea and returning
Aneuploidy	The condition of having an abnormal number of chromosomes in a haploid set
Anti-sense RNA technology	One of the approaches that are used for the inhibition of gene expression or downregulation of a gene. This technology works on the principle that an antisense nucleic acid sequence base pairs with its complementary sense RNA strand and prevents it from being translated into protein
Apoptosis	The death of cells which occurs as a normal and controlled part of an organism's growth or development.
Bulbous arteriosus	A pear shaped chamber that functions as a capacitor, maintaining continuous blood flow into the gill arches
Cardiac arrhythmia	An abnormality of the heart's rhythm
Cataract	A condition in which the lens of the eye becomes progressively opaque, resulting in blurred vision
Cerebellum	The part of the brain at the back of the skull in vertebrates, which coordinates and regulates muscular activity
Chromosome	A threadlike structure of nucleic acids and protein found in the nucleus of most living cells, carrying genetic information in the form of genes
Clearing solution*	Solution used to make an opaque fish egg transparent
Condition factor (K)	Condition of the fish as determined by weight (W) and fork length (L)
Cytoskeleton	A microscopic network of protein

	filaments and tubules in the cytoplasm of many living cells, giving them shape and coherence
Degree days*	A value used, especially during egg incubation, to estimate and predict the various stages of development. Calculated by multiplying the average temperature by the number of days.
Diploid	Paired cell organism
DNA methylation	Epigenetic mechanism that restricts DNA expression through SAM and DNMTs
Epigenetic	Stably heritable phenotype resulting from changes in a chromosome without alterations in a DNA sequence
Erythrocyte	A red blood cell
Eyed stage	Stage of embryogenesis where the eye pigmentation appears
Feed conversion ratio	Ratio between feed eaten and weight gained
Flow cytometry	The determination of the number, size, and nucleic acid content of cells using a flow cytometer
Gaussian distribution	A bell-shaped curve, and it is assumed that during any measurement values will follow a normal distribution with an equal number of measurements above and below the mean value
Gene dosage compensation*	Equalisation of expression in triploids to diploid expression levels
Gene dosage effect*	Maintained expression in triploids of additional chromosome
Genetic drift	Variation in the relative frequency of different genotypes in a small population, owing to the chance disappearance of particular genes as individuals die or do not reproduce
Gigantism*	Increase in phenotypic size of polyploids

	relative to the increase in genomic material and cell size
Haploid	A cell or nucleus having a single set of unpaired chromosomes
Heterozygosity	How many alleles are available at a given locus
Heterozygous	Having multiple different alleles of a particular gene or gene
Homozygous	Having identical alleles of a particular gene or genes
Hydrostatic pressure	Pressure exerted by or existing within a liquid at rest with respect to adjacent bodies
Introgression	The transfer of genetic information from one species to another as a result of hybridization between them and repeated backcrossing
Karyotype	The number and appearance of chromosomes in a cell
Karyotyping	The process of pairing and ordering all the chromosomes of an organism, thus providing a genome-wide snapshot of an individual's chromosomes
Maternal	Relating to the female parent
Meiosis	A type of cell division that results in four daughter cells each with half the number of chromosomes of the parent cell, as in the production of gametes and plant spores
Methylome	Heritable DNA methylation pattern
Microsatellite	Nucleotide tandem repeats in DNA sequences
Mitosis	The process by which a cell replicates its chromosomes and then segregates them, producing two identical nuclei in preparation for cell division

Morpholinos	A type of oligomer molecule used in molecular biology to modify gene expression. Its molecular structure contains DNA bases attached to a backbone of methylenemorpholine rings linked through phosphorodiamidate groups
Oocyte	A cell in an ovary which may undergo meiotic division to form an ovum
Oogonia	An immature female reproductive cell that gives rise to primary oocytes by mitosis
Operculum*	Anatomical structure that covers the gills
Osmoregulation	The maintenance of constant osmotic pressure in the fluids of an organism by the control of water and salt concentrations
Osteoblast	A cell which secretes the substance of bone
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
Poikilothermic	An organism with a variable body temperature that tends to fluctuate with and is similar to or slightly higher than the temperature of its environment : a cold-blooded organism
Polar body	Each of the small cells which bud off from an oocyte at the two meiotic divisions and do not develop into ova
Primordial germ cell	The primary undifferentiated stem cell type that will differentiate towards gametes: spermatozoa or oocytes
Pyloric caeca	Tubular pouches opening into the alimentary canal in the pyloric region of most fishes provide additional digestive

	areas to the intestine
Septum transversum	Mesenchyme formed in the embryo dividing thoracic and ventral internal development
Smoltification	Physiological adaptation enabling movement from a freshwater to saltwater environment
Somitogenesis	Process by which somites form, the embryonic segmentation in animals
Spermatogonia	Heterogeneous group of proliferative cells in the testis that are the precursor cells for mature sperm
Sterile	Incapable of producing viable offspring
Telencephalon	The most highly developed and anterior part of the forebrain, consisting chiefly of the cerebral hemispheres
Transcriptome	The transcriptome is the set of all RNA transcripts, including coding and non-coding, in an individual or a population of cells
Transgenic	Relating to or denoting an organism that contains genetic material into which DNA from an unrelated organism has been artificially introduced
Triploid	Organisms containing three chromosome pairings

*****Definitions are specific for this thesis

ABSTRACT

Triploidy is the only technique approved for inducing sterility in fish intended for human consumption. To increase the environmental sustainability and public perception of the aquaculture industry, scientific understanding of triploids must increase, assumptions must be challenged, and the additional costs linked with triploid production must be reduced. The gap between triploids and diploids in terms of performance is shrinking, but temperature tolerance remains a significant hurdle. In this thesis we aimed to tackle some of these issues. A suite of microsatellites was developed to verify triploidy, a protocol was established for extracting usable DNA (Deoxyribonucleic acid) from embryos between 26-78 degree days. In combination, these tools can reduce the cost and resources related with triploidy validation. The held assumption that triploids develop at the same rate as diploids during embryogenesis was validated; although differences in the timing of hatch were observed, with triploids hatching earlier. Optimal cleaning solution was determined, providing a useful tool for further studies. Triploidy was shown to have no significant negative impact on health or performance after thermal shocks at around 360 degree days, providing more support for the acceptance of triploids. Triploids performed well incubated at 4°C till eyeing and 6°C until hatch, with only a slight differential in radiological vertebral abnormalities found and no ploidy difference in mortalities or other malformations. Transcriptomic analysis after embryonic thermal shock and during thermal challenge later in life improved scientific understanding of triploids, revealing slight the differences between ploidy. Epigenetic analysis revealed triploids increased variability in the case of DNA methylation patterns. The potential of thermal programming to improve temperature tolerance was first demonstrated in a fish species, with shocked triploids experiencing increased growth under thermal stress. Raising the exciting prospect of not only removing a major barrier in triploid acceptance, but also providing a vital tool as the aquaculture industry faces rising temperatures worldwide.

Table of contents

List of Figures

List of Tables

Chapter 1: General Introduction

1.1 The Atlantic salmon aquaculture industry

There is archaeological evidence that aquaculture may date back as far as 6,000 BC with the managed fish farming of carp in Neolithic China (Nakajima *et al*. 2019), whilst historical records show aquaculture of tilapia and carp occurring in Egypt and China as far back as 1,500 bc (Nakajima *et al*. 2019). The culture of Atlantic salmon (*Salmo salar*) is a much more recent development, with the first experimental farming beginning in the 1960s (Paisley *et al.* 2010). In 1980 total production was 12,000 tonnes (Asche, 2013), by 2021 this figure reached 2,905,395 tonnes (FAO, 2021). Norway dominates production of Atlantic salmon at just over 1.5 million tonnes per annum, followed by Chile (725,000), Scotland (205,000), and Canada (120,000) (FAO, 2021).

The production of Atlantic salmon begins at the hatchery. Selected broodstock have their eggs and milt (sperm) stripped, and mixed together in freshwater (Kilcaid and Stanley, 1989). The fertilised eggs are then incubated for around 480-520 degree days before they hatch (Benchmark, 2022). Atlantic salmon hatch with a yolk-sac attached (Benchmark, 2022). This yolk-sac provides the hatched alevin with nutrients for around 370-440 degree days after hatch during which time they grow and develop the ability to feed independently . Unlike most marine species, Atlantic salmon can be fed a pellet diet at first feeding rather than requiring live feed. As the fish continue to grow, they may be transferred to freshwater water bodies or remain in land-based flow through or recirculating systems.

As with other *r*-selected species, which place fecundity over individual off-spring survival, not all Atlantic salmon embryos that hatch will make it to adulthood (Reznick, 2014). In the wild, large numbers of salmon will be eaten by predators or perish in a wide variety of manners before making it to adulthood. In aquaculture premature mortalities still occur, as do unviable individuals. These unviable individuals are often called pins and runts. Pin fry, also called pin heads, are fry which have been unable to consume appropriate amounts of food at first feeding and develop with large heads and small, undersized, bodies (European Food Safety Authority, 2008). Runts are similarly undersized individuals but at stages from parr onwards. In nature they would eventually perish but given the higher level of

protection afforded by the farming system these individuals survive much longer. Due to farming practices such as the transition to larger feed pellet sizes, these individuals will eventually starve to death. It is unethical and uneconomical to try and keep these unviable individuals alive and instead and they must be humanely culled.

The grow-out stage of the Atlantic salmon production cycle occurs at sea. In order to avoid predation and tolerate the change in osmotic conditions the salmon undergo a number of physiological changes in a process known as smoltification (Martinez *et al*. 2023). Smoltification can occur from 8 months onwards and in captivity this process is usually controlled using a mixture of lighting regime, temperature, and salinity so as to stock sea cages at the optimal time (Bergheim *et al.* 2009). There is a period of time when these physiological changes result in the most effective transfer to sea, this is known as the “smolt-window”, outside of this window transfer can result in “failed smolt syndrome” a condition where the fish fail to tolerate the change in environmental conditions which results in increased mortality, emaciation, disease, and stress (van Rijn *et al*. 2021). Failed smolt syndrome is just one example where proper understanding of the physiological requirements and genetic condition of the stock can help improve farming success. The same can be said for fully understanding the differences between triploid and diploid Atlantic salmon, and this lack of knowledge is one the major limiting factors in the acceptance of triploids culture.

1.2 Triploid Atlantic salmon

1.2.1 What is a triploid?

A triploid is an organism that has 3 sets of chromosomes (Tiwary et al., 2004), rather than, with in animals at least, the more typical 2 sets (diploid) (Otto & Gerstein, 2008). To accommodate for the 50% increase in genetic material, the cells of triploids are larger than those of diploids (Guo & Allen, 1994; Mable et al., 2011; van de Pol et al., 2020). Triploidy is a natural phenomenon that occurs in a wide range of species from plants to animals. Triploidy in mammals is fairly common, in humans it occurs in 1-2% of all clinically recognised pregnancies (McWeeney et al., 2009). However, mammals lack a mechanism to cope with an additional set of

chromosomes, making triploidy a lethal condition (Jiang et al., 2013; Niebuhr, 1974). If triploidy occurs in humans, it most often results in spontaneous abortion in the first trimester (Gainer, 2018; McWeeney et al., 2009). In rare instances where the foetus survives beyond the first trimester, it is often stillborn or dies shortly after birth (Gainer 2018; McWeeney et al. 2009). The ability of different classes of animals to cope with triploidy differs in varying degrees. Shellfish for example often exhibit gigantism when triploid, whereas triploid fish, at least superficially, appear very similar to their diploid conspecifics (Guo & Allen, 1994; Mable et al., 2011). The reason for these size differences is related to the larger cells of triploids (Guo & Allen, 1994; Mable et al., 2011; van de Pol et al., 2020). Whether diploid or triploid, shellfish (as well as plants and insects) appear to have the same number of cells overall, they therefore show increased size as triploids (Guo and Allen 1994; Mable et al. 2011). Fish and amphibians appear to compensate for the increased genetic material by having a reduced number of cells, this results in little to no change in overall size or morphology (Aliah, 1990; Bogart, 1980; Johnston et al., 1999; Mable et al., 2011; Small & Benfey, 1987; van de Pol et al., 2020). It is suggested that the ability of salmonids to tolerate triploidy is due to gene dosage compensation mechanisms balancing out gene expression (Ching et al., 2009; Christensen et al., 2019; Devlin et al., 2010; Odei et al., 2020; Pala et al., 2008; Ren et al., 2017). Compensation is a regulatory mechanism that results in triploids exhibiting gene expression at a diploid level, despite the differences in gene dosage levels (Shrimpton et al. 2007). The mechanisms behind gene dosage compensation have yet to be fully elucidated, but what is known suggests that the balancing of gene expression is less to do with allele or chromosome silencing and more to do with transcriptomic or epigenetic regulation (Christensen et al., 2019; Ren et al., 2017). It is also thought that historic whole genome duplication may contribute to this tolerance (Mable et al., 2011). Recent studies support the theory that strong selection for the maintenance of genome stability and therefore gene dosage compensation follows events of whole genome duplication (Gillard et al. 2021).

1.2.2 Why use triploid salmon?

There is a drive in the aquaculture industry to commercialise the use of sterile salmon (Benfey, 1999, 2016). Currently, the only accepted method of producing sterile salmon for consumption purposes is through the use of triploidy (Madaro et

al., 2021). One of the main reasons why there is a push to use sterile salmon is the risk of escapes. Getting an accurate figure on the number of escapes is difficult but yearly estimates put it in the hundreds of thousands (Glover et al., 2017). Escapes can occur in large numbers following net failure caused by bad weather or mismanagement, but “drip-leakage” during routine farm operations or through small tears in the net are also a major source (Glover et al., 2017). If a farmed salmon escapes, there is a risk that it will eventually “run” a river and mate with wild salmon (Fleming et al., 2000). The breeding of wild and farmed fish could potentially result in introgression and cumulative fitness depression (Bolstad et al., 2021; Diserud et al., 2022; Glover et al., 2013; McGinnity et al., 2003). One study of 109 Norwegian rivers found that 51 rivers showed significant farmed genetic introgression with some rivers showing introgression at high at 42% (Karlson et al., 2016). Introgression occurs due to the fact that farmed salmon are often genetically distinct from the local wild salmon, this is a result of a mixture of factors including different geographical origin, genetic drift, domestication, and selective breeding (Bolstad et al., 2021; Diserud et al., 2022). Whilst not all escaped salmon will end up in a position to breed with wild salmon, the risk is considered large enough, and the consequences severe enough, that there are efforts to protect wild populations with the use of triploidy (Fjelldal et al., 2014; Skaala et al., 2019).

Female triploid salmon are sterile, and triploid male salmon are effectively sterile, i.e. successful fertilisation of diploid egg is unlikely and survival of off-spring is very low). Triploidy inhibits the first meiotic division, as the homologous chromosome is unable to correctly synapse (Piferrer et al., 2009). This affects males and females differently. In female’s meiosis marks the transition from oogonia to oocyte, and with meiosis impeded females fail to produce any oocytes (Piferrer et al., 2009). In male’s meiosis occurs with the onset of puberty and therefore the testis can reach similar sizes to diploids, with spermatogonia undergoing many rounds of cell division through mitosis (Piferrer et al., 2009). Male triploid salmon can produce sperm, although lower in number than in diploids, and there is evidence that they may be able to mate with diploid females and fertilise eggs (Fjelldal et al., 2014). However, fertilisation success is lower than diploid-diploid pairings and the offspring are thought to have very low survival, this helps protect wild populations (Fjelldal et al., 2014). The reason behind the low survival of successfully fertilised diploid eggs by triploid sperm is that the

sperm from a triploid fish (3n) is an intermediate between haploid and diploid (1.46n), this results in aneuploidy offspring (2.42n) with abnormal physiology (Peruzzi et al., 2009). Even if the offspring of a triploid male are unviable, mating with a diploid female would still reduce the availability of eggs for diploid males to fertilise, for this reason all female populations of triploids are likely to become the industry standard in the future (Benfey, 2016).

The fact that female triploids do not mature can also have welfare and economic benefits. Maturation in salmon can reduce growth performance, flesh quality and increase mortality (Aksnes et al., 1986; Gjerde, 1984). Triploidy may also be a way to protect intellectual property, with no way to get viable offspring from a female triploid even if it escapes or is stolen. There is evidence that triploids will divert resources that would otherwise be used for reproductive development into somatic growth (Bonnet et al., 1999; Schafhauser-Smith & Benfey, 2001) although this is yet to be shown to translate reliably to improved overall growth rate to harvest. In summary, the interest in triploids centres around the benefits that sterility bring, namely, lower risk to populations should they escape, prevent premature maturation, and protect intellectual property.

There is a downside to triploidy, however. Triploids have different husbandry requirements and different thermal and oxygen tolerances to diploids, as such it has been suggested that they be treated as a new “species” with regards to aquaculture development (Benfey, 2001). There has recently been put in place a ban on the use of triploids in Norwegian salmon aquaculture due to questions regarding their performance and welfare (Bortoletti et al., 2022), this follows one of the first commercial scale comparisons of triploid and diploid performance to harvest in which welfare issues such as higher rates of deformity, emaciation, and mortality were observed in most of the triploid groups, with the triploids typically scoring lower on the FISHWELL morphological welfare indicator (WI) scoring scheme (Madaro et al., 2021).

The reasons for the differences in performance will be discussed below, as well as some evidence that triploids can perform as well as diploids in the right environment. Triploids are still used in certain circumstances, such as in areas where premature maturation is a particular issue (Tasmania) or where the effects of introgression are

particularly high (Genetically engineered salmon - AquAdvantage, and Eastern Canada).

1.2.3 Alternative methods of sterility

Whilst there are other methods of producing sterile fish, these are not viable for use in commercial aquaculture. Methods such as hormonal, chemical or radiation treatments have been shown to be affective but are unlikely to be approved for use in fish sold for human consumption (Benfey, 2001). A more targeted approach uses anti-sense technology to target *Deadend* (*dnd*) an essential gene involved in primordial germ cell (PGC) development (Fujimoto et al., 2010; Wong & Zohar, 2015a). A bath treatment can deliver the compound into the eggs, which prevents the primordial germ cells migrating to the genital ridge (Wong & Zohar, 2015b). Sterilisation success of 100% in zebra fish (*Danio rerio*) has been shown (Wong & Zohar, 2015b) and whilst success is lower in other species (85% in trout and 50-60% in Atlantic salmon, *Salmo salar*) there is hope that this will increase with further optimisation (Y. Zohar, personal communication). This method has a drawback that a chemical bath (morpholinos) is needed to take the treatment into the eggs (Wong & Zohar, 2015a). If considered safe by the relevant legislative bodies it could be approved for use in aquaculture, but issues surrounding consumer acceptance may remain, especially in species such as salmon which maintains an image of a “natural” product. An alternative to this treatment is the use of transgenic fish. Transgenic fish can be created to have inducible expression of important genes involved in primordial germ cell migration or ovarian termination (Hsu et al., 2010; Hu et al., 2010; Li et al., 2018; Wong & Collodi, 2013). Most of these treatments rely on a chemical treatment to induce gene expression (Hsu et al., 2010; Hu et al., 2010; Li et al., 2018), more recently injection of Atlantic salmon embryos with CRISPR-Cas9 has been shown to knockout *dnd* (Güralp et al., 2020; Wargelius et al., 2016). Follow-up studies showed that none of the CRISPR-Ca9 dnd knockout salmon entered puberty (Kleppe et al., 2022; Kleppe et al., 2014). There are difficulties with the commercial application of this procedure if all fish eggs require injection (Li et al., 2017; Wargelius et al., 2016), a more economic and large-scale approach is to create genetically sterile broodstock, which through germ cell rescue, can produce

off-spring who are themselves fully sterile this has been proven effective in a recent paper (Güralp et al., 2020).

There are issues surrounding legislative and consumer acceptance of transgenic livestock and although steps forward are being made with legislation (Waltz, 2016), the EU still fails to see a legislative difference between GM (genetically modified), genetically engineered, and gene-edited organisms (European Commission/SWD, 2021). Genetic engineering and genetic modification are often used interchangeably, and both can be used to classify an organism that is manipulated through the use of molecular biology, this can include the introduction of genes from another species (Zahry and Besley, 2019). Gene editing technology, such as CRISPR-Cas9, is also defined as precision breeding and does not allow for the introduction of novel genetic material (Briefing paper No CBP 9557, House of Commons Library, 2023). Consumer acceptance of gene edited products still lags behind the science in Europe, with the strongest objections being held against gene edited animals and animal products, rather than plants (Busch et al., 2022). There may also be ethical issues, some groups are fundamentally opposed to all forms of genetic editing, modification, or engineering products whilst advocates may feel that the real-world benefits of alleviating hunger, and increasing nutritional availability outweigh the negatives.

Currently, triploids are the only method to produce sterile fish for human consumption, and whilst the benefits of triploidy are obvious, they are not without their issues. Historically high mortality during egg incubation, inconsistent growth during the seawater stage, and increased rates of deformity have limited industry acceptance (Benfey, 2016; Fraser et al., 2013; Leclercq et al., 2011; Oppedal et al., 2003). One of the issues is that until recently all research on triploids treated them the same as diploids. It is now clear that they have different requirements, and research into these differences has helped alleviate some of the issues related to triploidy. Changes to husbandry practices such as the temperature of incubation have been shown to have an effect on survival and fitness of triploid salmon (Fraser et al., 2015; Fraser et al., 2013). Other issues that have plagued triploid research have been communal ploidy rearing and inadequate nutrition, these issues negatively affect triploid growth, survival and deformity prevalence (Fjelldal et al., 2016; Taylor et al., 2014, 2015). Mismatched transfer times may also impact

seawater survival with new evidence suggesting that triploids may have a different natural smoltification regime than diploids and this is likely to have been a factor behind the reduced survival of triploids at seawater transfer (Taylor et al., 2012). One of the issues with research into triploidy is that it is difficult to distinguish between problems caused by triploidy itself or by the method used to induce triploidy. Therefore, it is possible that many issues attributed to triploids are in fact a result of imperfect methods to induce triploidy. Whilst similar to diploid salmon on the surface, a myriad of differences between triploids and diploids have been found to the extent that, at least with regards to their husbandry, they can be considered a different species.

1.2.4 Methods of triploidy induction

Triploidy can be induced through the use of a shock precisely timed after fertilisation. Before ovulation, fish eggs possess 4n chromosomes (Rottmann et al., 1991). Upon the initiation of ovulation, the first meiotic division occurs and a small cell containing half of the homologous chromosomes is released from the egg, this is the release of the first polar body and it reduces the number of chromosomes in the egg to 2n (Liu, 2012; Rottmann et al. 1991). Upon fertilisation a sperm cell containing 1n chromosomes will enter the egg (Rottmann et al. 1991). A short time after this the second meiotic division will occur and with it the second polar body containing half of the sister chromatids, will be released from the egg reducing the chromosome number in the egg from 3n to 2n (Liu, 2012; Rottmann et al. 1991). If the second polar body is prevented from budding off, then the egg will remain at 3n and a triploid fish will result (Rottmann et al. 1991). The second polar body can be prevented from budding off through the use of a shock. The shock can be thermal (either hot or cold), hydrostatic pressure, or chemical, with success depending on the time after fertilisation, and the length and intensity of the shock (Felip et al., 2001). Pressure shock is the preferred method of triploidy induction in salmon aquaculture with cold shock (Lincoln et al., 1974), heat shock (Benfey & Sutterlin, 1984), and chemical shock (Allen & Stanley, 1979), all having more limited success. Whilst pressure shock does require specialist equipment, the system does allow for rapid, universal, and consistent application of required pressures across large numbers of eggs. In Atlantic salmon, triploidy can be induced by a hydrostatic pressure shock of 9500

PSI (655 BAR/65,500,000 pascal)applied for 50 degree-minutes, 300 degree-minutes post-fertilisation (Benfey, 2016. This is reported to have a >98 % success rate at inducing triploidy (Benfey, 2016). This was initially conducted at 10 °C (Johnstone & Stet, 1995), but the procedure has been refined in recent years, and it is now common to induce at 8 °C (Smedley et al., 2016) or 6 °C with no apparent decrease in efficacy (M. Mommens, personal communication).

Triploidy can be induced intentionally with a high rate of success, but it is not a man-made phenomenon and occurs naturally at low levels in the wild and unintentionally in salmon aquaculture. Interestingly, there are 2 orders of magnitude difference between the numbers of triploid individuals in wild Norwegian and Russian Atlantic salmon populations (0.017 %) (Jørgensen et al., 2018) compared to the number of triploids that occur inadvertently in Norwegian aquaculture (2 %) (Glover et al., 2015), with some farms being reported to show up to 28 % triploidy in supposedly “diploid” fish (Glover et al., 2015). The reasons for this are not clear, one possibility is that triploids in the wild suffer higher rates of mortality compared to diploids as they are more protected if grown in aquaculture (Jørgensen et al., 2018). Whilst this may be part of the reason, such a large difference in the frequency of triploidy is likely due to other reasons as well. One of these reasons may be that in the aquaculture industry, it is not uncommon for eggs to over-ripen and age before fertilisation whereas wild fish are more likely to spawn at the optimal time (Jørgensen et al., 2018). Indeed, triploidy is more likely to occur in over-ripened eggs and eggs at higher temperatures (Aegerter & Jalabert, 2004), higher temperatures also being common in aquaculture. These factors coupled with perhaps too vigorous mechanical disturbances are thought to be the likely reasons behind the increased occurrence of triploids in aquaculture compared to the wild (Aegerter & Jalabert, 2004).

1.2.6 Triploidy verification

Despite the high efficacy of pressure induced triploidy, the procedure is not faultless, and batches of triploids must be verified before they can be sent to farmers. Currently, the most common method for triploidy verification is flow cytometry. Flow cytometry uses a DNA (Deoxyribonucleic acid) specific fluorescent dye and can determine the DNA content of individual cells and erythrocyte nuclear size can be

observed, with triploids having larger nuclei due to the fact that they possess more genetic material (Benfey et al., 1984; Preston et al., 2013). Other methods can also be used, including karyotyping (Johnson & Wright, 1986), and nucleolar organising region analysis (Lozano et al., 1992). Cytogenetic approaches are the most definitive methods of triploidy verification as they provide information on chromosomes, these approaches are also expensive, time-consuming and more complex than cytometric methods. Cytometric methods lack this level of observation and therefore fail to establish if all the cells in the animal are triploid, allowing for the possibility that mosaicism could be occurring (Benfey, 2016). Cases of mosaicism have been noted following triploidy induction (Ewing et al., 1991; Goudie et al., 1995; Teplitz et al., 1994), however these cases are thought to be rare and mosaics are thought to suffer from increased rates of deformity and mortality increasing the likelihood that mosaics will fail to reach adulthood (Benfey, 2016; Fox et al., 1986). Even in rare instances that a mosaic is viable, the production of viable gametes is unlikely, although it is possible (Yamaki et al., 1999, 2006). One method that may be commercially viable in the future is the use of genomic markers. Through the use of highly polymorphic microsatellites, triploids can be identified by the presence of three alleles at multiple loci. This method has been shown to achieve up to 100% ploidy verification in plants (Esselink et al., 2004), frogs (Pruvost et al., 2015), and important aquaculture species such as pacific oyster (*Crassostrea gigas*) (Kang et al., 2013), and abalone (*Haliotis midae*) (Slabbert et al., 2010). This method has also proven successful in Atlantic salmon (Glover et al., 2016; Glover et al., 2015; Jacq, 2021; Jørgensen et al., 2018), Turbot (*Scophthalmus maximus*) (Hernández-Urcera et al., 2012), and Goldfish (*Carassius auratus*) (Jakovlić & Gui, 2011). Whilst a cheaper method of ploidy verification than using flow cytometry (Hernández-Urcera et al., 2012) the majority of the work in Atlantic salmon has been done in juveniles or adults (Glover et al., 2016; Glover et al., 2015; Jørgensen et al., 2018), and suites of a large number of microsatellites (18 in each of these studies). The higher the number of microsatellites, the higher the cost and the more difficult it is to run in house on systems with smaller capacity. Jacq (2021) validated their suite of 12 microsatellites on Atlantic salmon at the eyed stage, this is the same stage as current conventional methods measure triploidy. Triploidy validation at this stage requires additional resources both in terms of space, expense, and number of broodfish. This is because current practice is to accelerate a batch of fish to the eyed stage using

increased temperatures so that triploidy can be verified (M. Mommens, personal communication). Without acceleration, the stripping season could be over before bad batches are detected, removing the possibility that orders can be remade. To be able to use microsatellites to verify triploidy in eggs before the eyeing stage would be highly beneficial, but experimental work is needed to make this a reality.

For microsatellites to be successfully used to verify triploidy then microsatellites that are both highly polymorphic and also exhibit high levels of chromosomal cross over are required. Crossover is essential as without it the alleles inherited from the mother will be identical (figure 1.1). Chromosomal crossover occurs during meiosis, when matching regions on matching chromosomes break and then reconnect to the other chromosome. Crossing over followed by stopping the 2nd polar body budding off results in the possibility that 3 different alleles could be present at a specific locus. Without crossover, the entirety of one set of the maternal chromosomes will be lost during the extrusion of the 1st polar body and the offspring will be either homozygous or double heterozygous. Polymorphism is important because if the mother is homozygous or the father supplies one of the same alleles as the mother then it will be impossible to determine between a triploid and diploid using fragment analysis even if crossing over has occurred (figure 1.2).

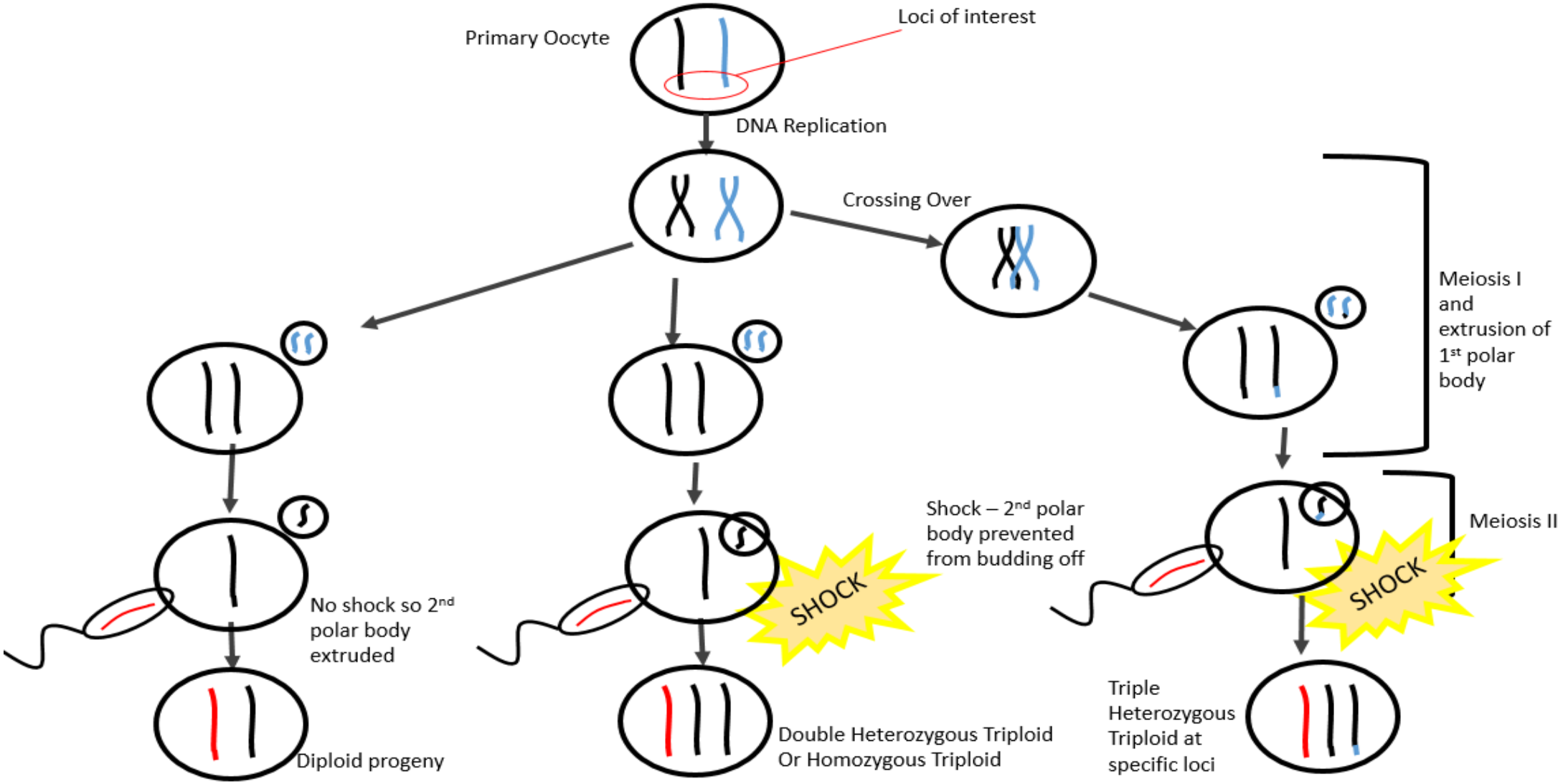


Figure 1.1 Visualisation of the process of how chromosomal crossover and followed by the triploidisation procedure can result in a triple heterozygous individual

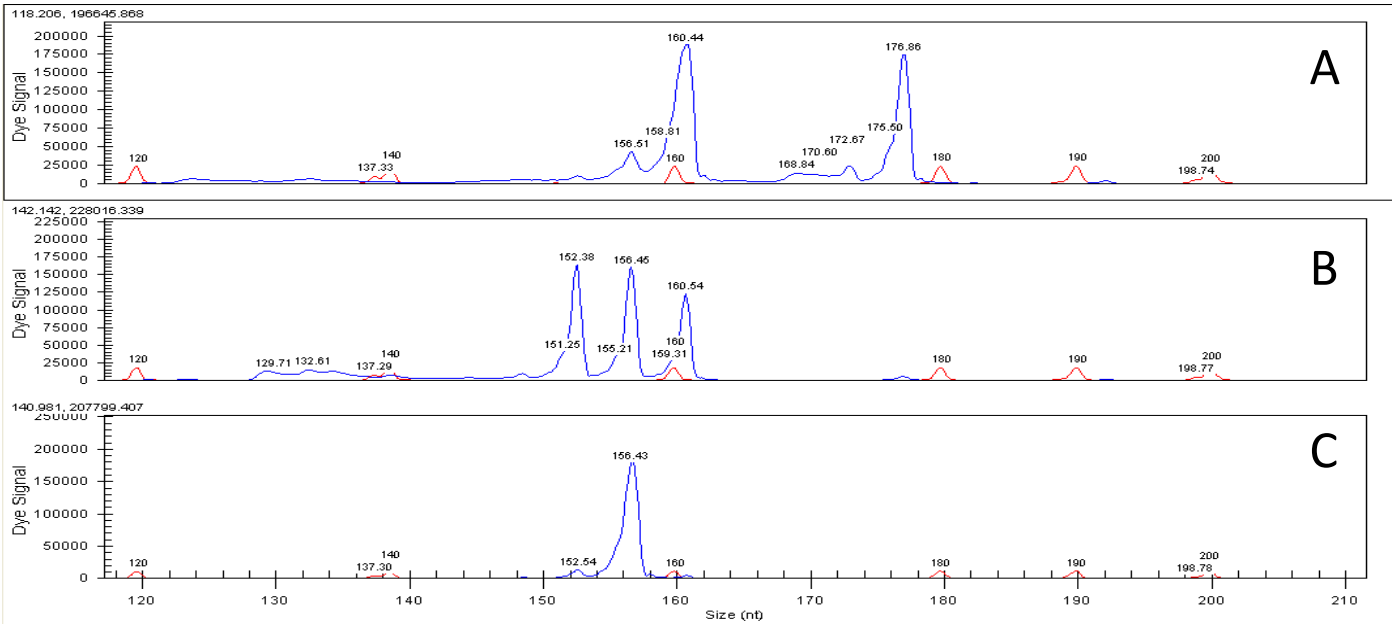


Figure 1.2 Examples of the three expected genotype patterns from electropherograms generated from raw data by the CEQ software. Results shown are for the microsatellite locus Ssa197. Panel A heterozygous diploid or triploid; Panel B triploid; Panel C homozygous diploid or triploid.

1.3 The differences between diploid and triploid Atlantic salmon

1.3.1 Survival

As mentioned above triploids were initially reported as having fundamentally lower survival than diploids (Cotter et al., 2002; O'flynn et al., 1997) and this is considered one of the factors that limited the uptake of triploids within the European aquaculture sector (Taylor et al., 2013). More recent studies have also shown an increased mortality associated with triploidy although these generally occur between fertilisation and first feeding (Amoroso, 2016; Fjelldal & Hansen, 2010; Fraser et al., 2013) or at temperatures that are supraoptimal for triploids (Madaro et al., 2021; Sambraus et al., 2017). Multiple studies have shown that under the correct conditions triploids can survive as well as diploids (Fjelldal & Hansen, 2010; Madaro et al., 2021; Oppedal et al., 2003; Peruzzi et al., 2018; Sambraus et al., 2017; Sambraus et al., 2017, 2020; Smedley et al., 2016, 2018; Taylor et al., 2012; Taylor et al., 2013; Vera et al., 2019).

The reason for the historic differences in ploidy is thought to be down to higher than optimal(supraoptimal) incubation temperatures, over-ripened eggs, and inadequate husbandry (Taylor et al., 2011; Taylor et al., 2013). It is also possible that the choice of shock used in early experiments, rather than the state of triploidy itself, may have an impact on survival, with some types of shock resulting in more mortalities than others (Benfey et al., 1984; Allen & Stanley, 1979; Lincoln et al., 1974). It is now thought that given optimised husbandry including separate ploidy rearing, adequate nutrition, correct incubation and grow out temperatures, and correctly timed transfer to sea, that the survival of triploids is no different to diploids in the seawater and freshwater stages (Leclercq et al., 2011; Taylor et al., 2012, 2014; Taylor et al., 2013).

1.3.2 Disease susceptibility

There are suggestions that triploids are more susceptible to disease but often these instances correspond with increased temperatures (Ching et al., 2010; Cotter et al., 2000; Myers & Hershberger, 1991; Ojolick et al., 1995). The tolerance to high temperatures is an issue in and of itself and will be mentioned later, and there is evidence that under conditions of stress the ability of triploids to regulate gene expression and immune function may suffer (Ching et al., 2010). Triploids have a lower number of cells involved in immune function, but these cells are larger and show higher levels of activity and are thought to make up for the lower abundance (Budiño et al., 2006). In addition, exposure challenges to salmonid alpha virus and *Neoparamoeba perurans* (the causative agent of amoebic gill disease) suggest no ploidy differences in severity or resistance to the diseases whilst virus loads were lower in triploids, possibly due to differences in cell number and metabolism (Chalmers et al., 2017; Herath et al., 2017; Moore et al., 2017).

In terms of disease prevention and treatment, triploids have also been found to have a similar tolerance to hydrogen peroxide treatments (Chalmers et al., 2018), a common method to treat sea lice (*Lepeophyheirus salmonis* and *Caligus elongatus*), and vaccination against furunculosis (Chalmers et al., 2016) and *Aeromonas salmonicida* (Chalmers et al., 2020) work just as well on both ploidies.

1.3.3 Growth

In addition to triploidy increasing the amount of genetic material in cells, the larger size of triploid cells results in a smaller surface area to volume ratio, with an increased intracellular distance between the nucleus and the membrane (van de Pol et al., 2020). This may increase the costs of maintaining membranes and osmotic gradients (van de Pol et al., 2020), a process that uses approximately 25-30% of the energy budget of the cell in diploids (Hulbert & Else, 2000), but it also may impact the movements of RNA (Ribonucleic acid) and transport proteins (Kozłowski et al., 2003; Szarski, 1983; van de Pol et al., 2020). To date, very little is known about the cellular processes involved in triploid development.

Theoretically, the fact that triploids don't mature should mean that they are able to allocate resources to somatic rather than reproductive growth, resulting in improved growth performance. This does seem to occur at times when diploids are maturing (Bonnet et al., 1999; Schafhauser-Smith & Benfey, 2001) but there is yet to be a consensus as to the overall growth performance compared to diploids. Whilst some studies have shown lower growth (Cotter et al., 2002; Friars et al., 2001; Taylor et al., 2014), a larger number of studies have shown improved growth rates (Burke et al., 2010; Fjelldal et al., 2016; Fraser et al., 2012; Galbreath & Thorgaard, 1995; Leclercq et al., 2011; O'Flynn et al., 1997; Oppedal et al., 2003; Taylor et al., 2011, 2012; Taylor et al., 2013), although these are generally over a short period of time and in freshwater. Only a few studies have shown comparable (Fjelldal et al., 2016; Galbreath & Thorgaard, 1995; Leclercq et al., 2011; Taylor et al., 2013) or improved (O'Flynn et al., 1997; Oppedal et al., 2003; Smedley et al., 2016) growth at the end of a seawater trial.

Common across growth trials is the fact that triploids have a lower condition factor (Cotter et al., 2002; Fjelldal & Hansen, 2010; Friars et al., 2001; Leclercq et al., 2011; O'Flynn et al., 1997; Taylor et al., 2012; Taylor et al., 2013). Condition factor (K) is the relationship between weight and size as determined by the calculated using the equation $K = 100W/L^3$, where W is the weight of the in grams and L is the length of the fish in cm. Condition factor has commercial implications as it is linked to fillet yield and fat content (Einen et al., 1998, 1999; Margeirsson et al., 2007; Rørå et al., 1998). Whilst reductions in yield have obvious drawbacks, it has been suggested

that depending on the market a fillet with high or low fat content is preferable (Einen et al., 1998; Robb et al., 2002; Rørå et al., 1998). The reason for the lower condition factor in triploids is thought to be the fact that they deposit skeletal components at a greater rate than muscle components (Johnston et al., 1999). Further evidence that the increased growth rate is behind the lower condition factor was evidenced by the fact that triploids reared communally with diploids grew slower, likely due to decreased food availability, but they also had lower levels of deformity and a higher condition factor (Taylor et al., 2014).

The slower growth rate of triploids when reared communally with diploids is thought to be due to a lower ability to compete for food. Current evidence suggest that triploids are less aggressive with lower levels of sex steroid hormones (Piferrer et al., 2009), and also have a smaller olfactory bulb, which influences foraging ability (Fraser et al., 2012).

It is clear that advances in the husbandry of triploid salmon are being made, but further work is needed to establish a consistent and comparable growth performance over an entire production cycle, work which is likely to include nutritional, environmental, genetic, and epigenetic work.

1.3.4 Flesh quality

There is no clear consensus as to the effect of triploidy on flesh characteristics, but a few factors do seem to be altered in salmonids. As with other types of cells, triploid salmonids have fewer and larger muscle fibre cells (Aussanasuwannakul et al., 2011; Bjørnevik et al., 2004; Poontawee et al., 2007). Triploid salmonids also appear to have less fat generally (Werner et al., 2008) but more fat when diploids are undergoing sexual maturation, a time at which the protein and fat content of diploids decreases (Aksnes et al., 1986; Poontawee et al., 2007). Sexual maturation negatively affects the odour, flavour, and texture of salmon flesh, something which can be avoided by using all female triploid populations (Aksnes et al., 1986; Gjerde, 1984). Outside of sexual maturation triploids have shown comparable flesh quality to diploids (Lerfall et al., 2017; Taylor et al., 2013).

1.3.5 Deformities

One of the major hurdles for the commercial acceptance of triploid salmon is the significantly higher rate of deformities. Triploids suffer from a higher rate of deformities of vertebrae, jaw, opercular, gill, eye, heart, and abdominal adhesion. These issues and their aetiologies will be discussed below.

Vertebral deformities

The issue of vertebral deformities is one of the major hurdles for the commercial acceptance of triploid salmon (Fjelldal & Hansen, 2010; Fraser et al., 2013; Leclercq et al., 2011; Taylor et al., 2013). The presence of deformities to the vertebral column is an obvious welfare and ethical issue with deformities negatively affecting swimming performance, therefore increasing the metabolic costs of swimming, restricting access to feed, and decreasing disease resistance (Castro et al., 2011; Herbert et al., 2011; Powell et al., 2009). The issue is also an economic one, with triploids being reported as showing higher rates of downgrading due to deformity (Fraser et al., 2013) and deformed fish having lower slaughter weights than healthy fish (Aunsmo et al., 2008). Both factors lead to a lowering of the sale price per fish (Fraser et al., 2013). Downgrading results in farmers getting a lower price for their fish and can be caused by a range of physiological issues such as deformities, softer flesh, maturation, and signs of disease or injury. The issue of downgrading does not always arise however, suggesting that with proper husbandry triploids can be farmed at the same quality as diploids (Taylor et al., 2013). Deformities also cause problems with public perception, and a large scale changing of production focus to triploids before this issue is resolved is likely to negatively affect the image of the salmon industry.

There are 3 main reasons that might explain why triploids suffer more vertebral deformities than diploids. One reason is that they grow faster, at least during the freshwater stage. Fast growth has been linked to vertebral deformities in both triploids (Leclercq et al., 2011) and diploids (Fjelldal & Hansen, 2010). Further evidence is provided by the fact that when triploids are subject to decreased feed intake, due to competition during communal rearing, growth rate decreased as did the prevalence of vertebral deformities (Taylor et al., 2014). Another reason behind the difference in deformities is nutritional. The increased growth rate of triploids

alongside the fact that compared to diploids they deposit skeletal components at a greater rate than muscle components (Johnston et al., 1999) results in triploids having different dietary requirements. By increasing the amount of phosphorous in the diet of triploid Atlantic salmon the severity of vertebral deformities can be significantly reduced (Fjelldal et al., 2016; Peruzzi et al., 2018; Sambraus et al., 2020; Smedley et al., 2016, 2018). Whilst suboptimal egg incubation temperatures have been shown to cause issues in diploids (Ytteborg et al., 2010), the issue is more acute in triploids. If triploids are incubated at temperatures suitable for diploids (10°C) they will suffer a range of pathologies including increased rates of vertebral deformity (Fraser et al., 2013, 2014, 2015; Sadler et al., 2001; Takle et al., 2005). If incubated at <8°C however these issues reduce (Fraser, Hansen, et al., 2015), and reduce further again if the temperature is lowered to 6°C (Clarkson et al., 2021), there was no significant difference in the level of either externally visible or radiologically assessed deformities between ploidy at this temperature. These are examples of how treating diploids and triploids in the same manner can have a negative impact on the fitness of triploids. One area that has not been researched in triploids is the effect of a short-term shift in temperature during incubation.

Jaw deformities

In addition to vertebral deformities, triploids also suffer deformities to the lower jaw (Fjelldal et al., 2016; Leclercq et al., 2011; Sadler et al., 2001). Lower jaw curvature, also known as “screamers disease”, has obvious welfare implications, reducing swimming ability (Lijalad & Powell, 2009) and possibly the ability to feed properly. Lower jaw curvature also leads to downgrades in affected fish and negatively affects the public image of the salmon industry. High temperature during Smoltification is thought to exacerbate the issue, whilst increasing dietary phosphorus can reduce instances of the pathology (Fjelldal et al., 2016; Roberts et al., 2001). Recent data has revealed that the issue is likely driven by cartilage impairment (Amoroso et al., 2016).

Cataracts

Triploid Atlantic salmon have been found to suffer from higher rates of cataracts. This is a pathology that was also a significant issue in diploid culture during the 90s (Waagbø et al., 2010). Whilst higher temperatures have been linked to increased

rates of cataracts (Bjerkås et al., 2001; Sambraus et al., 2017), the increase in cataracts in diploids in the 90s was primarily of dietary origin. When blood meal was banned as feed ingredient in 1990, Atlantic salmon started to suffer suboptimal levels of histidine and this caused cataracts (Breck et al., 2003; Waagbø et al., 2010; Wall, 1998). Like diploids, increasing dietary histidine decreases the prevalence of cataracts in triploids (Sambraus et al., 2017; Taylor et al., 2015). The reason as to why triploids require more histidine than diploids is not clear, but it may be linked to the faster growth rate of Triploids during freshwater. Gene expression differences between diploid and triploid salmon with cataracts are minor, with mechanisms related to protein turnover, NO-induced oxidative stress, cytoskeleton stability, and lipid metabolism being put forward as possible reasons for increased prevalence of cataracts (Olsvik et al., 2020).

Gill deformities

Triploids have longer gill filaments than diploids, but the density of gill filaments is significantly lower (Leclercq et al., 2011; Sadler et al., 2001). Triploids are also more prone to gill filament deformity syndrome (GFD) (Sadler et al., 2001) which is characterised by the absence of primary gill filaments, possibly caused by environmental disturbances during embryogenesis. Both these factors result in reduced gill surface area. Being one of the primary respiratory and osmoregulatory organs in fish, reductions in the surface area of the gill can cause a reduction in the ability of the animal to osmoregulation, or cope with physical exertion or hypoxia (Sadler et al., 2001).

Heart deformities

Triploids have been shown to have a more acute angle of the bulbous arteriosus (Fraser, Mayer, et al., 2015; Fraser et al., 2013) a condition more similar to wild type fish (Poppe et al., 2003). It is thought this condition may arise from an increase in cardiac workload (Leclercq et al., 2011; Poppe et al., 2003) in the face of reduced arterial O_2 loading (Verhille et al., 2013). Triploids also suffer from increased instances of aplasia of the *septum transversum* (Fraser et al., 2014) a condition thought to be induced by suboptimal incubation temperatures (Takle et al., 2005, 2006). This condition is thought to reduce cardiac function and has been linked to increased mortalities in times of stress (Brocklebank & Raverty, 2002; Poppe et al.,

2003; Takle et al., 2006). Reduced cardiac function is also observed in triploids with un-deformed hearts, with triploids more prone to cardiac arrhythmia if subject to high temperatures (Verhille et al., 2013). These issues are part of the reason as to why triploids suffer increased mortalities at times of stress or high temperature compared to diploids.

1.3.6 Vaccine side effects

Triploids are thought to be more susceptible to vaccine side effects (Fraser et al., 2014; Larsen et al., 2014). Vaccination can lead to melanin spots and abdominal adhesions which can have a welfare and economic impact, with fillet quality being negatively affected (Fraser et al., 2014; Larsen et al., 2014).

1.3.7 Nutritional requirements

The differences between diploids and triploids also expand to the digestive system. Triploids have fewer pyloric caeca, a reduced relative gut length and less compensatory mucosa cells than diploids (Peruzzi et al., 2015). Seeing as these organs are vital for digestion and nutrient uptake, a reduction in their capacity could impact growth performance and dietary requirements. This theory is supported by research on trout with fewer pyloric caeca showing reduced growth performance (Blanc et al., 2001). Reports on feed conversion ratio (FCR) are not consistent with some studies showing inferior FCR (Fraser et al., 2013; Smedley et al., 2018), whilst others find no difference between ploidy (Burke et al., 2010; Hansen et al., 2015; Sambraus et al., 2017). There is evidence that triploids can grow as fast as diploids in seawater and faster in freshwater when fed the same quantity of the same diet as diploids (Leclercq et al., 2011; Taylor et al., 2013) suggesting equal or lower FCR. There is also evidence that triploids can outperform diploids in seawater if fed *ad libitum* and reared at optimal temperatures (Oppedal et al., 2003). In addition, a number of nutritional parameters seem to show no ploidy variation including balance of energy use (Wiley & Wike, 1986), protein efficiency (Oliva-Teles & Kaushik, 1990; Pechsiri & Yakupitiyage, 2005), feed utilisation (Burke et al., 2010; Oliva-Teles & Kaushik, 1990; Pechsiri & Yakupitiyage, 2005) and feed intake (Burke et al., 2010; Nuez-Ortín et al., 2017; O'keefe & Benfey, 1999; Tibbetts et al., 2013). There is a suggestion that at lower temperatures (≤9 °C) triploids eat more than diploids (Sambraus et al., 2017). Differences in the turnover and deposition of proteins and

lipids suggest that given increased supplementation, triploids may have the potential for greater deposition of proteins and lipids (Cleveland & Weber, 2013, 2014; Manor et al., 2015; Segato et al., 2006). Indeed, diets with increased protein and phosphorus can increase growth rates of triploids to above that of diploids fed a standard diet (Smedley et al., 2016).

As mentioned earlier the phosphorous and histidine requirements of triploids are elevated and this is likely linked to increased growth rates (Fjelldal et al., 2016; Roberts et al., 2001; Smedley et al., 2016; Taylor et al., 2015). The complex nature of nutritional requirements combined with the faster growth of triploids suggest that it is unlikely that all the requirements are currently acknowledged with deficiencies in vitamins and minerals being possible (Fjelldal et al., 2006; Fjelldal & Hansen, 2010; Lall & Lewis-McCrea, 2007). Research into probiotics may also help avoid deficiencies by aiding the uptake of essential nutrients (Aubin et al., 2005; Ferguson et al., 2010; Lamari et al., 2013; Merrifield et al., 2010).

1.3.8 Smoltification

Smoltification is a key stage in the life cycle of anadromous salmon. Smolting salmon undergo a series of physiological changes to prepare for the transition from fresh water to seawater (Fjelldal et al., 2006; McCormick et al., 2009, 2013; Sigholt et al., 1995; Zydlewski, 1997). These changes are energetically demanding and physiologically stressful (Zydlewski, 1997). The correct timing of the parr-smolt transformation is crucial for survival and development during on-growing. Salmon can only tolerate being in the transition stage for a certain amount of time, this is known as the “smolt window” (Zydlewski et al., 2005). Historically triploids were treated as diploids and transferred to sea at the same time, this led to the theory that they had reduced survival at seawater transfer and may suffer increased rates of failed smolt syndrome (Galbreath & Thorgaard, 1995; McCarthy et al., 1996). Whilst it is true that triploids have physiological differences that may alter osmoregulatory potential, such as reduced density of longer gill filaments (Leclercq et al., 2011), fewer pyloric caeca (Peruzzi et al., 2015), and a reduced relative gut length (Peruzzi et al., 2015), it is not clear as to the effect of triploidy on smoltification. Some authors have found that triploids will smolt earlier and have a longer smolt window (Leclercq et al., 2011; Taylor et al., 2012), whilst other studies have found smoltification to

occur at the same time as diploids (Fraser et al., 2013; Taylor et al., 2014; Taylor et al., 2013). One reason for this difference is possibly size related, with experiments that show early smoltification also having significantly larger triploids (Leclercq et al., 2011; Taylor et al., 2014; Taylor et al., 2013). When subjected to low temperatures and fed a diet high in phosphorous and protein, triploids have been shown to transfer to sea as well as diploid counterparts, with no stress response and no difference in growth (Bortoletti et al., 2022).

1.3.9 Brain morphology

Neurologically, triploids are different from diploids. Triploids possess a smaller olfactory bulb than diploids but larger cerebellum and telencephalon (Fraser et al., 2012). The size of the olfactory bulb has been directly linked to foraging ability, which would help explain the observed reduced foraging ability in triploids (Fraser et al., 2012; O'Keefe & Benfey, 1997). That being said, a larger cerebellum and telencephalon have been linked to an increased foraging ability (Huber et al., 1997; Kolm et al., 2009; Salas et al., 1996; Wilson & McLaughlin, 2010). It is not clear to what extent neurological differences are the cause of the reduced foraging ability in triploids.

1.3.10 Gene expression

A number of studies investigating differences between diploid and triploid Atlantic salmon have of course looked at gene expression differences. These studies have improved our understanding of triploidy and revealed mechanisms behind some of the differences between ploidy such as lower jaw deformities (Amoroso et al., 2016), vertebral deformities (Smedley et al., 2018), and the impact of supraoptimal incubation temperatures (Clarkson et al. 2020). These studies focus on genes established to be linked to specific pathologies in diploids. What is generally overlooked in comparisons between ploidy are transcriptomic differences, with only four known transcriptomic studies to date (Odei et al., 2020; Olsvik et al., 2020; Vera et al., 2017, Taylor et al., 2019). Three of these looked at quite specific criteria. The effect of different diets (Taylor et al., 2019) or nutritional programming (Vera et al. 2019) on the transcriptome and the differences in diploid and triploid fish with and without cataracts (Olsvik et al., 2020). Given the time spent above listing the many ways in which triploids and diploids differ it is perhaps surprising how closely their

transcriptomes match. Diet was seen to be a larger predictor of gene expression than ploidy (Taylor et al., 2019, Vera et al., 2017), whilst the transcriptomes of triploids and diploids at the same stage of development showed fewer differentially expressed genes that within ploidy differences between stages (Odei et al. 2020). Triploids and diploids with cataracts showed a larger difference in gene expression than those without (Olsvik et al., 2020). The lack of differences are thought to be due to gene dosage compensation mechanisms. The exact mechanisms within salmon are still to be elucidated, but the result is that under normal conditions the expression levels of genes are remarkably similar between triploids and diploids (Ching et al., 2010; Christensen et al., 2019; Odei et al., 2020). It is thought that under stressful conditions the homeostasis of dosage compensation mechanisms breaks down (Ching et al., 2010). More general transcriptomic studies at different stages of development and under different environmental conditions and stressors are lacking currently and these would provide information on the differences between ploidy and areas in which research could be conducted to improve husbandry.

1.3.11 Temperature tolerance after hatch

Poikilothermic animals such as salmon cannot regulate their internal body temperature and must therefore regulate their temperature through their behaviour, such as by moving to warmer or colder waters (Berman & Quinn, 1991). This is not possible for salmon in aquaculture or during the egg stage and as such correct temperatures are vital for the survival and fitness of the fish. As mentioned earlier incorrect egg incubation temperatures can result in increased rates of deformity (Fraser et al., 2013, 2014, 2015; Sadler et al., 2000; Takle et al., 2005) as well as causing mortality (Gunnes, 1979). Correct rearing temperatures are also important for Atlantic salmon after hatch in aquaculture, and this appears to be more of an issue for triploids.

There is evidence that triploids suffer more at higher temperatures and lower oxygen concentrations when compared to diploids, with triploids thought to have a lower thermal optimum than diploids (Myers & Hershberger, 1991; Ojolick et al., 1995; Quillet & Gaignon, 1990). Atkins & Benfey (2008) looked at routine metabolic rate over a 2-month period between triploids and diploids of 2 different species. Atlantic salmon were reared at 12, 15, and 18 °C, whilst Brook trout (*Salvelinus fontinalis*)

were reared at 9, 12 and 15 °C. It was shown that at lower temperatures triploids of both species had a higher routine metabolic rate than diploid conspecifics, whilst this trend was reversed at higher temperatures with triploids showing the lower routine metabolic rate (Atkins & Benfey, 2008). The theory that triploids have a lower thermal optimum than diploids and therefore will suffer in temperatures that a diploid will not, has been shown in a number of other experiments. Hyndman et al. (2003) looked at the differences between ploidy in the Brook trout during exhaustive exercise at high temperatures. It was found that triploids used less phosphocreatine but more glycogen, this was evidence that they had difficulty utilizing anaerobic pathways and meant that they took longer to recover from metabolic disturbance (Hyndman et al., 2003). The experiment also resulted in a high mortality rate in triploids but not in diploids (Hyndman et al., 2003). Riseth et al. (2020) showed that triploids had significantly lower maximum metabolic rates at higher temperatures, which resulted in a lower aerobic scope. A lower metabolic optimum was also shown by Altimiras et al. (2002) who looked at triploid brown trout (*Salmo trutta*) and found that maximum in vivo cardiorespiratory performance reached a plateau between 14 and 18°C. This plateau coincided with increases in standard and routine metabolic rates, as well as resting heart beat and ventilation frequency. This combination of factors resulting in a reduced factorial metabolic scope and was the reason attributed to the significantly higher mortality rate observed in triploids compared to diploids when held at higher temperatures (Altimiras et al., 2002). An increase in mortality was also observed in triploid rainbow trout (*Oncorhynchus mykiss*) when they were kept at chronic high temperatures (Ojolick et al., 1995) although the exact reason for the high mortality rate in this experiment was likely multifaceted.

Oxygen levels, in addition to high temperatures alone, have been shown to be one of the reasons why triploids may be less tolerant of higher temperatures. There is an intrinsic link between water temperature and oxygen content, with dissolved oxygen decreasing as temperatures increase (Wetzel, 2001), it can therefore be difficult to design an experiment that looks at these factors separately. This was managed however in an experiment by Hansen et al. (2015). The experiment raised triploid and diploid Atlantic salmon at 10 and 19°C. After an acclimation period, O_2 concentrations of 100 % air saturation and 70 % air saturation were maintained using different quantities of hyper-oxygenated water. Whilst weight did not differ

significantly between ploidy, blood samples showed significant differences. Plasma levels of Cl−, TAG, ALP and bilirubin were lowered in triploids. Plasma Cr levels trebled and plasma K+ levels dropped in triploids subjected to 70 % O2 for 29 days. There was also a significant difference in mortalities, with the triploid group subjected to hypoxia suffering significantly higher mortalities than all over groups. The authors concluded that based on the results of the experiment that production performance was highest in both the diploid groups (100 % O_2 and 70 % O_2) followed by the triploid groups, with the 100 % O_2 group performing better than the hypoxia group for each ploidy (Hansen et al., 2015). An interesting observation made during the experiment was that triploids at both temperatures would actively ventilate, swimming against the current and ram ventilating, as opposed to moving along with the current in the case of the diploids (Hansen et al., 2015). This experiment provides evidence that as well as triploid salmon performing worse at higher temperatures, they also perform worse during moderate hypoxia events independent of temperature.

One of the reasons for this difference may be the heart morphology of triploids being different to diploids (Fraser, Mayer, et al., 2015; Leclercq et al., 2011). Verhille et al. (2013) suggested that the different heart morphology was limiting the ability of triploids to deliver oxygen to the body, and was this evidence by higher rates of cardiac arrhythmia in triploids at high temperatures during the experiment. Other differences such as the size and frequency of erythrocytes may contribute to a lower hypoxia tolerance (Peruzzi et al., 2005), the impact being that triploids have a lower haemoglobin - oxygen loading ratio and blood oxygen content (Bernier et al., 2004). There are no ploidy related difference in total haemoglobin, haematocrit and corpuscular haemoglobin (Benfey & Sutterlin, 1984; Bernier et al., 2004; Sadler et al., 2000; Taylor et al., 2007). Cell size is also likely to play a part, with the larger cells of the triploid increasing intracellular diffusion distances and the reduced surface area to volume ratio providing a lower capacity for transmembrane transport of resources including oxygen (van de Pol et al., 2020).

Some studies have stated that there is no difference in the thermal tolerance between ploidy (Bowden et al., 2018). This experiment looked at Atlantic salmon and found that whilst there was an initial difference in resting (aerobic) metabolic rates

(RMR) at 10, 14, and 18°C, with triploids showing a significantly lower RMR, this difference decreased and disappeared past week 7 of the experiment (Bowden et al., 2018). Critical thermal maxima (CT_{max}) trials were undertaken and whilst it was found that triploids had a lower metabolic rate during the temperature ramp, the CT_{max} did not differ between ploidy (Bowden et al., 2018). Galbreath et al. (2006) studied the time to chronic *lethal* maximum in triploid and diploid rainbow trout and brook trout, it was found that overall performance did not differ between ploidy for either species. Benfey & Sutterlin (1984) found that in landlocked Atlantic salmon there was no difference in oxygen consumption at asphyxiation and this was used to suggest that triploids should be able to tolerate low oxygen conditions as well as a diploid, although the temperature used in this experiment was 15°C, which is lower than most other experiments that found lower triploid fitness or survival. Benfey et al. (1997) looked at the upper tolerance limit of triploids, in this case looking at the CT_{max} of brook trout. It was found that there was no ploidy difference in CT_{max} although there was a difference based on age and size, with the younger and smaller fish having a high CT_{max} (Benfey et al., 1997). No ploidy difference in CT_{max} was also found in rainbow trout in an experiment by Scott et al. (2014), but there was a significant ploidy difference in the time to loss of equilibrium when hypoxia coincided with elevated temperatures, with triploids suffering before diploids.

The lack of difference in CT_{max} seems counterintuitive given the evidence that suggests triploids are less tolerant of higher temperatures. Differences between experiments may in part be due to the different developmental stages being studied, with smaller juvenile fish being used by Bowden et al. (2018) and Galbreath et al. (2006). Smaller fish have been shown to be more thermally tolerant than larger fish, with smaller fish having a higher CT_{max} than larger fish (Clark et al., 2012; Messmer et al., 2017), and smaller fish having been shown to recover and regain homeostasis faster after exhaustive exercise (Clark et al., 2017). Ploidy differences may therefore manifest at later stages, as seen in experiments that found differences using larger fish (Altimiras et al., 2002; Hansen et al., 2015). It is possible that a difference between ploidy is still present, but that higher temperatures than the ones used in these experiments are needed before the difference appears in smaller fish. Different strains being used may also play a part in explaining why there are discrepancies between studies with Scott et al. (2014) finding that whilst CT_{max} did not differ

between ploidy in the study, there was a significant difference due to strain. This is an important consideration if developing a breeding program focused on temperature tolerance. When considering Atlantic salmon it is important to consider that when using fish at different stages one is also using fish in different environments, juvenile fish being a freshwater animal and later stages growing in seawater. Therefore, when comparing the upper tolerance of triploids in different studies it should be considered that salinity affects dissolved oxygen, with seawater having around 20% less oxygen than freshwater at the same temperature (Wetzel, 2001). This fact alongside the fact that adult salmonids can face more energetically costly physiological processes such as osmoregulation in a hypertonic environment, may also help explain the difference in results between studies. The ploidy related differences of upper temperature tolerance may be relatively minor in some cases (as seen by the results of CT_{max} experiments) but triploids appear to have a reduced thermal optimum and a reduced capacity to deal with multiple stressors simultaneously or recover from stress events. In a comparison of triploids at different farm sites, the performance of triploids was considerably worse in the sites where high summer temperatures were reached (Madaro et al., 2021). This can be seen in the experiments by which alongside chronic high temperatures also involved disease problems (Ojolick et al., 1995) or experiments which used exhaustive swimming alongside high temperatures (Altimiras et al., 2002; Hyndman et al., 2003).

Evidence for this theory is provided by the fact that Hyndman et al., (2003) ran a mirror experiment of the above-mentioned exhaustive swimming trial but at 9 °C rather than 19 °C and found triploids to recover faster than diploids. Further evidence for this was provided by an experiment by Sambraus et al. (2017). It was found that as well as optimal food consumption occurring at lower temperatures in triploids (12 °C vs 15 °C), triploids also suffered more mortalities than diploids when high temperatures and low oxygen events were combined (Sambraus et al., 2017). It was shown that the upper thermal tolerance limit of triploids was >18 °C when O_2 was not limited, and that they performed as well as diploids up to 15 °C (Sambraus et al., 2017). Plasma ion loss was also shown to be an issue, but only significantly when high temperatures coincided with anoxia (Sambraus et al., 2017). Across all experiments the largest ploidy differences in metabolism and mortality occur when high temperatures coincide with another stress condition such as hypoxia,

exhaustive swimming, or disease. A conclusion shared by Riseth et al. (2020) who stated that triploid Atlantic salmon have the aerobic capacities to thrive in most aquaculture settings so long as very high temperatures, severe hypoxia, and strong water currents can be avoided. The data suggests that triploids do have a lower thermal optimum, but it is in conjunction with other stress events that it becomes a major welfare and economic concern. If this issue cannot be resolved, it could have real world applications and result in triploids being farmed having a more limited geographical distribution than diploids.

It is clear by now that there are a host of differences between diploid and triploid Atlantic salmon, and it may be pertinent to treat these animals, in terms of their husbandry, as separate species. The more work that is done on refining the husbandry of triploids and establishing the requirements of these animals, the better they perform and this will increase the chance that they will be a viable alternative to diploid salmon in the future.

To improve husbandry further, more research is needed into the differences between triploid and diploid Atlantic salmon. Assumptions are often made, such as the idea that there is no difference in the rate of embryogenesis between triploids and diploids. Triploids have been shown to hatch smaller than diploids (Taylor et al., 2011), but other than a study that looked at one very specific stage of development (Johnston et al., 1999) there has been no investigation to determine if the rate of embryogenesis differs. This could mean that a treatment conducted on both ploidy at a specific time is actually conducted at different developmental stages. Further exploration into transcriptomic differences will increase our understanding of triploids and reveal pathways which can be focused on when trying to improve the performance of these animals.

1.4. Potential for thermal programming

The tolerance of triploid Atlantic salmon to high temperatures at sea is a major hurdle for the widespread acceptance of these fish. Unlike other historic issues that triploids have faced, this issue is unlikely to be solved by feeding a more refined diet or by reducing incubation temperatures.

Two ways to increase temperature tolerance would be through genetic engineering or a conventional breeding program. Both these approaches would be expensive and take many years before gains would be seen. Breeding programs have the problem that heat tolerance is not currently recorded as a trait and adding it to the list of selected traits in a current breeding program would be difficult. As mentioned above, GE fish are banned over almost the entire globe, and there is still widespread public mistrust and an unwillingness to eat GE/GM fish.

A third potential way to increase temperature tolerance would be through the use of thermal programming. This process would involve the use of early life stimulation to induce changes to the epigenome which would persist through life, these changes would influence gene expression and prepare the fish to tolerate stressors of the same nature as the stimuli later in life. The mechanism behind this process, as well as evidence of programming in fish, and thermal programming in other species will be described in the next section of this chapter.

1.5. Epigenetics

The term “Epigenetics” was first proposed by Waddington (1942) as a way to signify how genotype is interpreted into phenotype through a series of developmental stages rather than being directly translated. Since then, the term “epigenetic” has been used to signify changes in gene expression and function that occur without a change to the DNA sequence, initially a heritable change (Holliday, 1987), and now days regarded more generally as a long-term and stable change, though not necessarily heritable (Aguilera et al., 2010; Wang et al., 2017). Epigenetic mechanisms whilst not altering the DNA sequence, instead control gene activity at transcription, translation, and post-translation levels (Jaenisch & Bird, 2003), they are generally stable, although they can be influenced by the environment (Aguilera et al., 2010; Granada et al., 2018). There are 3 main classes of epigenetic mechanism, based on DNA modifications, histone modifications, and non-coding RNA (figure 1.3).

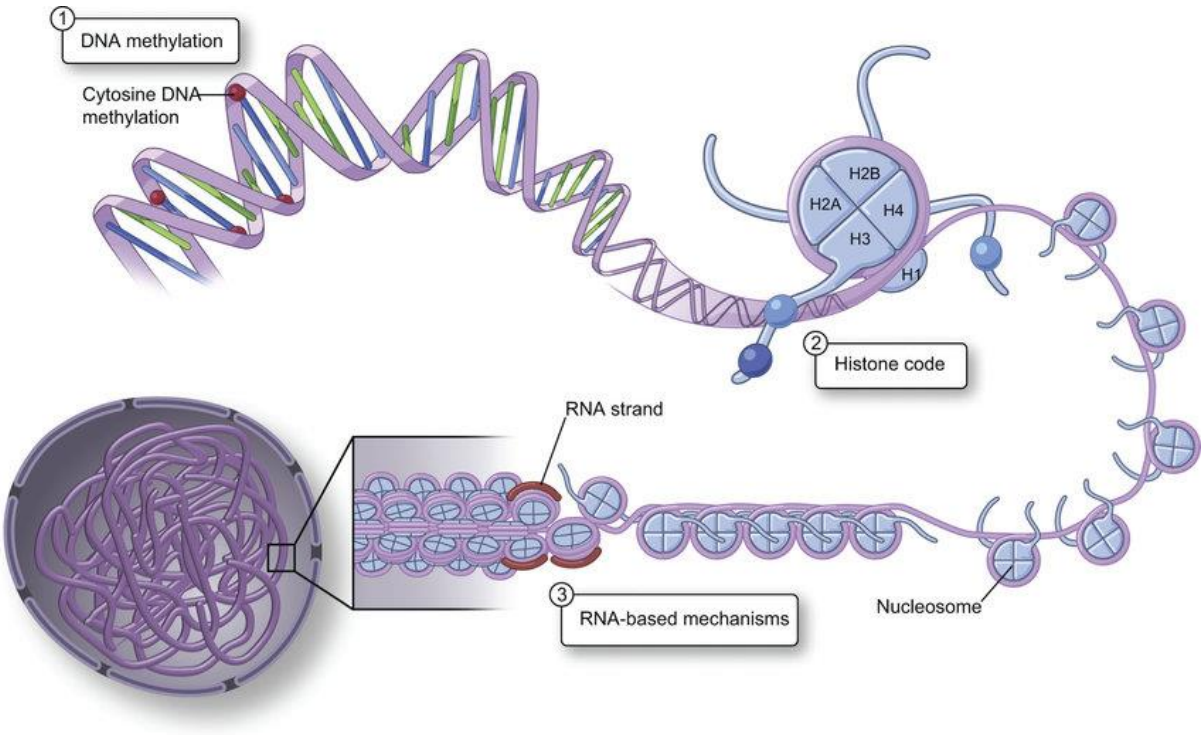


Figure 1.3. The three main classes of epigenetic mechanism, DNA methylation, Histone modifications, and non-coding RNA-based mechanisms. Highlighting the level on compaction the DNA strand undergoes. DNA is wound around histone which group to form nucleosome, which in turn form chromatin. Taken from Yan et al. (2015).

1.5.1 DNA modifications

DNA can be modified by the addition of a methyl (-CH3) or hydroxymethyl (-CH_2-OH) group to the carbon 5 position of cytosine residue to form 5-methylcytosine (5mC) or 5-hydroxymethylcytosine (5hmC) (Gosselt et al., 2020; Sadakierska-Chudy et al., 2015). Methylation of cytosine generally occurs within CpG dinucleotides (a region in which a cytosine nucleotide is followed by a guanine nucleotide in the direction to 5' to 3') although there is evidence that cytosine in other positions can be methylated (Granada et al., 2018; Metzger & Schulte, 2016; Sadakierska-Chudy et al., 2015). CpG dinucleotides do not appear symmetrically along the genome and are instead distributed in GC-rich and GC-poor regions (Sadakierska-Chudy et al., 2015). GC-rich regions (containing more than 50% GC) are termed CpG-islands. Almost all housekeeping genes and many tissue specific genes possess CpG islands at the 5' ends with around ½ of all genes in humans having a CpG island (Antequera & Bird, 1993). CpG islands can be highly methylated in the gene body or intergenic regions, but when these islands occur at promoters they are generally unmethylated, with the level of methylation being inversely correlated with the transcriptional activity of the

gene (Antequera & Bird, 1993; Sadakierska-Chudy et al., 2015). There is some disagreement as to the correct nomenclature to describe this phenomenon. Some authors describe DNA methylation (DNAme) as a switch, turning a gene on when removed or turning a gene off when present (Domann & Futscher, 2004; Suzuki & Bird, 2008). Other authors describe this phenomenon as more akin to a dial, with DNAme and other epigenetic modifications having a quantitative impact on gene expression, working together to regulate the level of expression of genes between totally “on” or “off” and various levels of expression in-between (Bjornsson et al., 2004; Pai et al., 2011; Sadakierska-Chudy et al., 2015).

Methylation patterns are generally stable, but can change during specific developmental stages and due to environmental factors. In mammals (on which the majority of epigenetic research has been conducted), the spermatozoa are highly methylated with oocytes less so (McCarrey, 2014). Upon fertilisation there is a rapid and active loss of methylation inherited from the sperm and a more gradual passive loss of the methylation inherited from the oocyte, this continues until the blastocyst stage at which point the gamete is largely demethylated (McCarrey, 2014). De novo methylation follows this point, and somatic cells will increase in methylation until reaching the adult profile. Another large-scale demethylation event occurs in primordial germ cells (PGCs) upon migration to the genital ridges. PGCs maintain a state of hypomethylation for several days before a rise in methylation levels to that of mature gametes (McCarrey, 2014). This is not seen in Atlantic salmon, however, with an increase in methylation through somitogenesis (M. Smedley, PhD thesis, 2016). Another difference seen in salmon is that triploids and diploid seem to show a difference in both overall methylation and as to the speed at which remethylation occurs (M. Smedley, PhD thesis, 2016). The reason for the ploidy differences may be related to the higher maternal inclusion in the genome of triploids, with the maternal methylome in zebra fish being reprogrammed whilst the paternal methylome is maintained (Jiang et al., 2013; Potok et al., 2013).

In addition to changes during development, epigenetic profiles can be altered by the external environment. In Atlantic salmon factors such as captivity (Wellband et al. 2021), embryonic temperature (Burgerhout et al. 2017), high temperatures later in

life (Beemelmanns et al. 2021), diet (Irvine et al. 2019), and disease (Mukiibi et al. 2021) have all been shown to alter methylation profiles.

1.5.2 Chromatin and histone modifications

The basic unit of chromosome folding is chromatin (Dixon et al., 2016). Chromatin is a complex of DNA and proteins that allows DNA to be highly compacted so that it may fit inside the nucleus of a eukaryotic cell (Dixon et al., 2016). To be able to achieve this incredible level of compaction whilst also allowing the DNA to be accessible requires a complex system of proteins, enzymes and RNA's. Chromatin can take the form of 2 different structures; euchromatin and heterochromatin (Labbé et al., 2017). Euchromatin is gene rich and its open structure allows for active gene transport (Labbé et al., 2017). Heterochromatin is the area in which silenced genes lie, it is denser and more gene poor than euchromatin (Labbé et al., 2017). The basic unit of chromatin is the nucleosome, which is a segment of DNA (145-147 base pairs) wound around 2 sets of 4 different histones (Labbé et al., 2017; Lämke & Bäurle, 2017). The pairs of 4 core histones that make up a nucleosome are H2A, H2B, H3, and H4 (Labbé et al., 2017; Lämke & Bäurle, 2017). A 5^{th} histone, H1, acts as a "knot" at the end of the nucleosome and maintains the chromosome structure (Labbé et al., 2017; Lämke & Bäurle, 2017). Histones possess N-terminal and C-terminal tails rich in lysine and arginine, although the chains also include serine, tyrosine, threonine, glutamate and proline (Sadakierska-Chudy & Filip, 2015). Whilst most histones across all chromosomes are the same, there exists a number of histone variants which consist of different numbers of amino acid residues (Sadakierska-Chudy & Filip, 2015). Histone variants have been linked to changes in gene expression, as well as being linked to DNA repair, the regulation of chromatin states, and driving the initiation and the progression of cancer (Kamakaka & Biggins, 2005; Malik & Henikoff, 2003; Pusarla & Bhargava, 2005; Vardabasso et al., 2014).

The two most studied histone modifications are methylation and acetylation. Histone methylation occurs at lysine and arginine bases, generally on histones H3 and H4 (Kouzarides, 2007; Sadakierska-Chudy & Filip, 2015). The level of methylation is thought to affect chromatin folding via an electrostatic mechanism (Völkel & Angrand, 2007). The effect of methylation, as well as other histone modification, on gene expression is however complex. The methylation of different lysine molecules

on the same histone can have different effects, the trimethylation of lysine 4 promotes transcription (to the extent that it is used as a maker of euchromatin) and trimethylation of lysine 36 supports elongation by interaction with RNA polymerase II (Labbé et al., 2017). The trimethylation of lysine's 9 and 27 of histone 3 are however linked to heterochromatin formation and silencing of gene expression (Sadakierska-Chudy & Filip, 2015). Acetylation only occurs at lysine, but it can occur at all 4 core histones (Sadakierska-Chudy & Filip, 2015). Acetylation effects gene expression by removing the positive charge of lysine, this causes chromatin to relax to facilitate access of transcription factors to gene promotors (Sadakierska-Chudy & Filip, 2015).Less well studied modifications include phosphorylation (Perez-Cadahia et al., 2010), glycosylation (Sakabe et al., 2010), carbonylation (Goto et al., 2007), ubiquitylation (Zhang, 2003), biotinylation (Kobza et al., 2005), sumoylation (Nathan et al., 2003), citrullination (Denis et al., 2009), ADP-ribosylation (Hottiger, 2011), N-formylation (Jiang et al., 2007), crotonylation (Tan et al., 2011), propionylation (Chen et al., 2007; Zhang et al., 2008), butyrylation (Chen et al., 2007; Zhang et al., 2008), as well as proline- (Nelson et al., 2006) and aspartic acid- isomerization (Doyle et al., 2013). These modifications occur on different amino acid residues, although the most commonly modified site is lysine, and can either be associated with transcriptionally active or repressed states, depending on the modification and the location.

The large number of possible modifications that can occur across a number of sites makes establishing the phenotypic impact of specific modifications incredibly difficult. To make matters more difficult, there is strong evidence that post-translational modifications of histones can interact to varying degrees and this can increase or decrease transcription activity (Delcuve et al., 2012; Lo et al., 2000). Whilst work is still very much just beginning on the subject of the effects of histone modifications, very real changes to gene expression can result from modification to histones, and it is important that attempts are made to link phenotypic changes to possible epigenetic mechanisms. Diet has been shown to cause changes to histone acetylation in sea bass (Terova et al. 2016) and histone acetylation and methylation in rainbow trout (Marandel et al. 2016, Panserat et al. 2017). Whilst in Atlantic salmon behavioural fever and the temperature changes associated with that resulted in changes to histone methylation (Boltana et al. 2018). Behaviour fever is an

evolutionary conserved response and the physiological and transcriptomic response to behavioural fever differ from those of fish involuntarily exposed to those temperatures (Boltana et al. 2018). Behaviour fever can influence immune response and this response appears to be closely linked to histone modification (Boltana et al. 2018).

1.5.3 Non-coding RNA

Large portions of the eukaryotic genome transcribe non-coding RNA (ncRNA), initially these non-coding regions were thought to be “junk DNA” or “transcriptional noise” but now it is thought that ncRNAs play important roles in epigenetic regulation (Sadakierska-Chudy & Filip, 2015; Zhou et al., 2010). ncRNAs can be either structural or regulatory. Structural ncRNAs are involved in the normal function and viability of the cell, whereas regulatory RNAs are involved in the alteration of the epigenome and gene expression (Sadakierska-Chudy & Filip, 2015). Micro-RNAs (miRNAs) can cleave or repress translation of targets of mRNA whilst also inhibiting enzymes involved in epigenetic modification including DNMTs and histone modifiers (Iorio et al., 2010). Small-interfering RNAs (siRNAs) are involved in the activation and repression of transcription and are involved with histone and DNA methylation (Li et al., 2006). Anti-sense RNAs (asRNAs) have been found to reduce DNA methylation (Mattick & Makunin, 2005). Piwi-interfering RNAs (piRNAs) and Long ncRNAs (lncRNAs) are involved in guiding DNA methylation and chromatin modifying complexes respectively (Lin, 2007; Ponting et al., 2009). Promoter-associated RNAs (paRNAs) are thought to be involved in transcriptome regulation and chromatin organisation (Sana et al., 2012). Centromere repeat associated small interacting RNA (crasiRNA) is involved in centromere establishment and segregation as well as heterochromatin formation (Carone et al., 2009). Telomere-specific small RNAs (tel-sRNAs) are also thought to be subject to epigenetic regulation, although the exact mechanisms are unclear (Cao et al., 2009).

miRNAs have been shown to play an important role in immune response in Atlantic salmon (Andreassen et al. 2017), with lncRNAs also playing a role in both Atlantic salmon (Boltaña et al. 2016) and rainbow trout (Paneru et al. 2016). Whilst high temperatures during embryonic development alters the miRNA profile in Atlantic cod

(Bizuayehu et al. 2015). miRNAs are also thought to play an important role in the biosynthesis of long-chain polyunsaturated fatty acids in teleosts (Xie et al. 2021).

1.5.4 Importance of considering epigenetics in breeding programs

The importance of epigenetics on eventual phenotypes is rapidly becoming recognised and there are potential ramifications for the estimation of breeding values and the implementation of genomic selection. One of the reasons that epigenetics may be so useful to aquaculture is the fact that most of the traits of interest in current aquaculture breeding programs are quantitative (Gjedrem & Akvaforsk, 2005; Moghadam et al., 2015). This means that phenotypes generally show a typical gaussian distribution due to the additive effects of many genes combined with environmental effects (Gjedrem & Akvaforsk, 2005; Moghadam et al., 2015). The key to a successful breeding program is an accurate estimate of additive gene effects (Gjedrem & Akvaforsk, 2005). Whilst there is some controversy over whether or not epigenetic effects are stable across multiple generations the general consensus is that if gametes are exposed to a varied environment then the epigenetic profile and, importantly for breeding programs, the phenotype, of the fully-grown adults can be altered. Epigenetic factors that alter phenotype could mask additive gene effects due to the fact that they can be altered by changes in environmental conditions and potentially inherited by offspring (Moghadam et al., 2015). If epigenetic effects are stable of many generations, then they can be considered as part of the true additive genetic effect (AGE) (Moghadam et al., 2015) but if they are more temporary, lasting only within generations or over one generation, then they would cause a layer of noise and lead to inaccuracies in breeding values. The simple pedigree structures used in aquaculture breeding programs make this issue more likely, increasing the possibility that all offspring could inherit epigenetic marks from the parents giving the impression that they have inherited superior additive genetic variation when in fact the trait in question may be influenced by short term epigenetic changes (Moghadam et al., 2015). Genotype-by-environment interactions should also be considered when altering environments to induce epigenetic change or when testing the success of potential programming. By only testing the performance of “programmed” fish in certain environments you introduce to possibility that GxE interactions are going unnoticed (Sae-Lim et al.,

2016) and given the potential for large GxE interactions in Atlantic salmon on important traits such as growth (Gonzalez et al., 2022) and thermal tolerance (Debes et al., 2021), this interaction should be considered to maximise gains. Determining what phenotypic changes are purely down to genotype or changes to the epigenome is difficult with rearing environment having a large effect on the epigenome of an individual (Christensen et al., 2021) as well as influencing DNA methylation profile of gametes (Wellband et al., 2021), with DNA methylation patterns determined by an interaction between both genotype and environment (Berbel-filho et al., 2019).

There are many potential avenues to alter the epigenetic profile of production fish, which are basically based on the effect of different environments in the population. The embryonic environment and diet at first feeding are both options, but also options are the broodstock environment and diet. Care should be taken with altering diets however, as a range of bioactive compounds can also alter DNA methylation and histone modifications (Choi & Friso, 2010). Bacteria is also known to alter the epigenetic markers (Bierne et al., 2012) and this should be something that is taken into consideration, especially with broodstock that have suffered and survived an infection and are still used as broodstock. The best way to get an idea of the true extent of epigenetic effects in aquaculture would be the production of inbred or homozygous double haploid fish, but this work involves resources that are often prioritised for other areas of research (Moghadam et al., 2015) and if inbreeding is too intense there can be large effects on DNA methylation patterns (Venney et al., 2016).

Another factor that should be considered within breeding programs is the fact that cryopreservation can alter the epigenome of sperm (Bøe et al., 2021, Riesco & Robles, 2013, Cheng et al., 2014). One suggestion is that environmental changes such the freezing and thawing induce the epigenetic changes, with environmental changes known to alter the epigenome at other stages (Chatterjee et al., 2017). Another possibility is that epigenetic changes are caused by the chemicals used in the process. Cryopreservation involves multiple chemicals including cryoprotectants, which commonly contain a methyl group (Cabrita et al., 2010; Muchlisin, 2005). DMSO (Dimethyl sulfoxide) a common cryoprotectant has been shown to induce epigenetic changes to mouse cells *in vivo* independent of freezing (Iwatani et al.,

2006). Cryopreservation could potentially affect the epigenome of the offspring due to the fact that unlike in mammals, the epigenome of the offspring in fish (zebra fish) matches the paternal rather than maternal epigenome (Jiang et al., 2013; Potok et al., 2013).

1.5.5 The effect of the environment on the epigenome

The genome, environment, and epigenome all interact to help determine the phenotype of an individual (Heim & Binder, 2012). There is now good evidence that many of the differences in gene expression, phenotype, and behaviour that accompany changes to the early environment are epigenetic in origin (Heim & Binder, 2012; McGowan et al., 2009, 2011; Murgatroyd et al., 2009; Weaver et al., 2004). Some of the most in depth experimental evidence suggest that it is possible to change phenotypic traits through early life experiences, and that this is linked to changes to the epigenome, has been shown in rats exposed to different levels of pup licking behaviour from their mothers. Rats which received high levels of maternal licking were less fearful and also had hormonal differences (Weaver, 2007). As well as behavioural and hormonal changes, pup licking also altered the DNA structure at the glucocorticoid receptor gene promoter in the hippocampus, this change persisted even after pups were swapped between mothers to account for genetic differences (Meaney & Szyf, 2005; Weaver, 2007). The authors of the paper concluded that differences in the DNA structure observed were the result of differences in DNA methylation, histone acetylation and chromatin structure changes (Weaver, 2007).

As well as changes to the environment of an individual, there is also evidence that the environment of the parents or even further generations can result in inherited changes to the epigenome. Termed “transgenerational inheritance” the best evidence also comes from work in rats and mice. Several experiments have examined the effects of exposure to the commonly used fungicide vinclozolin across multiple subsequent generations (Anway et al., 2005, 2006; Crews et al., 2007). Changes to multiple phenotypic traits were observed after vinclozolin exposure including male fertility (Anway et al., 2005), susceptibility to a number of diseases (Anway et al., 2006) and mate choice (Crews et al., 2007). A benefit of the experimental design for the above experiments that looked at disease (Anway et al., 2006) and fertility (Anway et al., 2005) was the use of gestating females (F0) as the

initial test subjects. This allowed for the effects of different degrees of exposure to be examined across multiple generation's starting with the F1 offspring which were exposed in utero and F2 which were exposed as the germ cells of F1. The effects were also observed in generations F3 and F4 which had no direct exposure to vinclozolin even as germ cells. Interestingly, in these 2 experiments, F2 generations suffered the adverse effects of exposure to a greater degree than generation F1. In the case of male fertility, F3 and F4 had similar levels of sperm forward motility and a greater number of sperm than F1 although lower than controls (Anway et al., 2005). With regards to sperm apoptosis and disease relating to the kidneys, testis, immune system or tumours, F3 and F4 suffered more than F1 (Anway et al., 2005, 2006). These experiments suggest that the transgenerational inheritance of epigenetic states is possible and can have large effects generations after exposure. Whilst the experiment that studied mate preference did not look at intermittent generations, there was a clear difference in mate preference with F3 female rats with a history of exposure preferring male rats that had no history of exposure (Crews et al., 2007). It has been suggested therefore that not only can epigenetic effects have transgenerational effects but also potentially "transpopulational" with epigenetic states having potentially evolutionary effects (Crews et al., 2007). Unfortunately much of the work regarding methylation inheritance focuses on mammals and specifically mice and rats. The mechanisms behind this process are not yet well understood in salmon.

There is good evidence in mammals that the environment of the parent or during the early development of the individual can alter the phenotype of an individual, and that these changes can be epigenetic in origin. There is also evidence that these changes can have negative effects, such as in the cases of rodents exposed to vinclozolin (Anway et al., 2005, 2006; Crews et al., 2007). What is less clear is how these changes could have potentially positive effects. Theoretically in the case of juvenile rats exposed to different levels of maternal licking behaviour (Meaney & Szyf, 2005; Weaver, 2007), the fact that the rats who received lower levels of maternal care (a situation that could occur in the wild following predation of the mother) were more fearful could be beneficial in an environment with high levels of predation, but at the same time this would likely make them less competitive in finding food or mates in environments with lower levels of predation. This is the

concept of environmental mismatch (Gluckman et al., 2005a, 2005b) and it suggests that the plasticity of the epigenome in early development, combined with its stability later on, can provide a "predictive adaptive response" (Bateson et al., 2014; Gluckman et al., 2005b) or "program" (Barker, 2002; Ozanne & Hales, 2002; Weaver et al., 2004) an individual to anticipate a certain environment in later life.

Benefits can be seen if the predicted environment matches that of the actual adult environment, but negative effects can be seen if there is a mismatch. Some work in this area focuses on the Dutch famine (1944-1945). The Dutch famine caused over 20,000 deaths from malnutrition alone and affected many millions more (Lumey & Poppel, 1994). Whilst undoubtedly a humanitarian disaster, the famine provides a unique opportunity to study the effects of reduced calorie intake during gestation with average rations falling below 1,000 calories a day toward the end of 1944 and between 400-800 calories between February and May 1945 (Roseboom, 2017). Individuals in utero during the famine are more likely to die from cancer and to develop psychological problems such as addiction or schizophrenia (Roseboom, 2017). People who were at a stage of early development during the famine are thought to show a preparedness for an adult environment lacking in available nutrition (Ekamper et al., 2014; Roseboom et al., 2000; Roseboom, 2017; van Abeelen et al., 2011). As adults these people show increased retention of fat (Lumey et al., 2009; Stein et al., 2009). These phenotypic changes combined with an environment in which food is virtually unlimited result in an increased risk of cardiovascular disease, obesity, diabetes, and mortality (Lumey et al., 2009; Stein et al., 2009). There are 3 theories as to why a mismatch between the environment at gestation, or even during previous generations, and the environment during adult life has a negative impact. This is thought to be due to a "thrifty phenotype" (Hales & Barker, 1992; Wells, 2007), "thrifty gene" (Neel, 1962), or bridging the two hypotheses and supported by recent studies on the Dutch famine (Heijmans et al., 2008; Roseboom, 2017; Tobi et al., 2009) a "thrifty epigenome" (Genne-Bacon, 2014; Stöger, 2008). Whilst negative effects are shown if there is a mismatch, this plasticity also shows how animals can adapt to environmental changes and the evidence suggests that were the famine to have continued or to reoccur then these same people, who now suffer from increased rates of cardiovascular disease, obesity, diabetes would be the best prepared to survive in an environment with low

food availability (Roseboom, 2017). This predictive adaptive response will confer an adaptive advantage if the prediction is accurate, and therefore could potentially be used for early life programming (Khan et al., 2005). This theory is the basis for experiments into nutritional programming in aquaculture species.

1.6. Potential uses of epigenetic programming

1.6.1 Nutritional programming

Nutritional programming is based on the idea that early exposure (or even maternal exposure) to diets lacking in one or more specific nutrients or composed of compounds that would normally have a low digestibility to the animal, can result in adults being more tolerant of these diets in the future. Nutritional programming has been shown in a range of species including Whiteleg shrimp (*Litopenaeus vannamei*) (Lage et al., 2018), Baboons (*Simia hamadryas*) (Mott et al., 1995), Sheep (*Ovis aries*) (Knox et al., 2003), Rats (*Rattus norvegicus domesticus*) (Heywood et al., 2004; Khan et al., 2005), and Pigs (*Sus scrofa domesticus*) (Guilloteau et al., 2010).

In aquaculture there is an interest to move towards alternative diets such as those higher in carbohydrate or diets that mainly consist of plant-based, rather than animal-based, meals and oils. If these alternative diets could be commercialised then it would have a significant impact in terms of sustainability, public image, and profits. Nutritional programming attempts in fish have focused on different areas, depending on the species. The possibility to program fish to high carbohydrate diets has been shown in zebra fish (Fang et al., 2014). In this experiment, the ability of adult zebra fish to utilise and digest a diet high in carbohydrate was improved using early nutritional programming. A suite of genes involved in carbohydrate digestion, including glucokinase (GK), increased in expression and overall growth performance was not negatively affected in the fish fed the altered diet. It was concluded that nutritional programming was indeed possible, but that programming should commence at as early a stage as possible to ensure the best results (Fang et al., 2014).

Other attempts to program fish include the use of soybean-meal (SBM) based diets. In an experiment on Yellow Perch (*Perca flavescens*) success was limited with numerically but not statistically higher growth once reintroduced to the diet, but

success was found attempting to avoid the negative effects typically associated with SBM diets including reduced fecundity, egg quality or fertilization rate (Kemski et al., 2018). In Zebrafish, nutritional programming changed the gut microbiota and increased tolerance to SBM later in life (Patula et al., 2021).

Some of the most successful attempts at nutritional programming in fish involve programming carnivorous fish to tolerate diets rich in plant-based products. Rainbow trout have been shown to tolerate plant-based diets after programming, showing higher growth rates, feed intake and feed efficiency (Geurden et al., 2013). Feeding Rainbow trout vegetable diets over the long term can still result in viable offspring, although ova are reported as being smaller and survival at first spawning being lower (Lazzarotto et al., 2015). Improved growth rate was shown in gilthead sea bream (*Sparus aurata*) when programmed to a diet low in fish oil and fish meal but high in linseed oil (Izquierdo et al., 2015; Turkmen et al., 2017). It was shown that that 4-month-old sea bream better utilised the vegetable-based diet if their parents had been subjected to the altered diet during the preconception stage (Izquierdo et al., 2015; Turkmen et al., 2017). This increased utilisation extended to 16-month-old fish if 4-month-old fish were fed for 1 month on the vegetable diet (Turkmen et al., 2017). These studies show the potential for programming by altering the environment of early life stages as well as the maternal environment. The potential of feeding vegetable diets has also been shown in Atlantic salmon. Both triploid and diploid salmon showed higher growth rates and feed efficiency when reintroduced to a vegetable diet 15 weeks after initial programming at first feeding (Clarkson et al., 2017). There were no differences in feed intake, indicating that this increased growth was due to increased utilisation of the vegetable diet. This initial study was expanded on and how the nutritional programming affected the liver transcriptome also investigated (Vera et al., 2017). This study found that a wide suite of genes were altered. Several pathways were up-regulated in response to the vegetable diet including oxidative phosphorylation, pyruvate metabolism, TCA cycle, glycolysis, and fatty acid metabolism (Vera et al., 2017). Also altered were pathways involving endoplasmic reticulum, RNA transport, endocytosis and purine metabolism (Vera et al., 2017). Whilst global DNA methylation did not change in diploids, it increased during the challenge phase in triploids (Vera et al., 2017). No specific link was found between changes in vegetable tolerance and changes in the epigenome although a

more focused approach that looked at gene specific changes of DNA methylation and studies on histone modifications may have provided more insight. A similar expression profile of molecular pathways was also found in rainbow trout fed a vegetable diet, again suggesting epigenetic changes but DNA methylation and changes to histone modifications were not investigated (Balasubramanian et al., 2016).

Whilst the majority of nutritional programming studies fail to investigate any epigenetic changes that might explain the phenotypic changes that are reported, a handful of studies have shown how diet can influence gene expression and epigenome together. Changes in DNA methylation and histone modifications have been shown in rainbow trout, gilt-head sea bream and sea bass (*Dicentrarchus labrax*) fed altered diets with these changes being linked to changes in gene expression (Marandel et al., 2016; Panserat et al., 2017; Perera & Yúfera, 2017; Terova et al., 2016). Whilst these studies do suggest that the increases in tolerance to certain diets that have been observed in many other studies could be explained by changes to the epigenome, tolerance to alterative diets was not investigated, and research that links increased tolerance with epigenetic changes is needed.

Not only can nutritional programming be used to increase the tolerance of fish to cheaper and more sustainable ingredients such as vegetable based or those high in starch and carbohydrates, but programming can also be used to avoid the negative effects of otherwise potentially useful ingredients such as soy bean meal. It has also been shown that gains can be achieved through both programming at first feeding or through broodstock diet. That being said proper broodstock nutrition is incredibly important (Izquierdo et al., 2001; Migaud et al., 2013) and whilst giving vegetable diets to broodstock can increase growth of juveniles fed the same diet they can also reduce fecundity, spawn quality, and growth of juveniles (Izquierdo et al., 2015). Care should be taken to balance the nutritional requirements of the broodstock with the possible benefits of nutritional programming.

Care should also be taken to consider the potential for certain nutrients and bioactive compounds in feeds to alter the epigenome. This can occur through directly inhibiting enzymes that catalyse DNA methylation or histone modifications, or by altering the availability of substances necessary for these enzymatic reactions

(Moghadam et al., 2015). DNA methylation and histone modifications can be affected through natural compounds that act as methyl donors or inhibitors of methyltransferases (Choi & Friso, 2010). Compounds including, but not limited to, Folate, Vitamin B-12, and methionine can alter DNA methylation whilst other B vitamins, including B3, B5, and B7 can alter histone modifications (Choi & Friso, 2010). Enzymes involved in epigenetic mechanisms can also be affected directly. DNA methyltransferases (DNMT) can be affected by genistein and tea catechin, whilst resveratrol, butyrate, sulforaphane, and diallyl sulfide inhibit histone deacetylases (HDAC) and curcumin inhibits histone acetyltransferases (HAT) (Choi & Friso, 2010).

Both Folate and genistein alter DNA methylation profiles and both of these compounds are found in soy beans (Fukutake et al., 1996; Mo et al., 2013), a common ingredient in Atlantic salmon feeds (Refstie et al., 2000). The nutritional requirement estimates for folate in Atlantic salmon range from 0.6-1.1 mg kg^{-1} (National Research Council, 2011) to 3.3 mg kg^{-1} (Hemre et al., 2016). Whilst no research has been done onto the effect of folate on the epigenetics in fish, this level is much higher than the 400µg a day that has been shown to alter the methylome of 17-month-old children (Steegers-Theunissen et al., 2009). Folate contains a methyl group and there is evidence that methylation levels increase as dietary folate increases in mice (Keyes et al., 2007), this would suggest that diets with different levels of folate will alter the methylome to different degrees. Genistein is not required in the diets of salmon, but is an isoflavone present in soy beans that has been linked to off flavours in fish (D'Souza et al., 2005). Genistein inhibits DNMT and therefor alters the levels of DNA methylation (Choi & Friso, 2010; Olsvik et al., 2017; Qin et al., 2009). The levels of genistein in the salmon diet containing 8% soy bean meal (Wang et al., 1990) are higher than the levels that can affect the methylome in salmon liver cells (Olsvik et al., 2017) or adult women (Qin et al., 2009), therefore it is possible that soy bean meal diets can alter the epigenome. Different levels of soy bean meal will likely alter the epigenome to different degrees as genistein, as well as folate, has been shown to have dose-specific effects (Qin et al., 2009). Even when discussing just these two bioactive compounds, it is clear that different diets could have a large impact on the results of an epigenetic investigation, but the fact that certain dietary compounds can affect the epigenome is not necessarily positive or

negative, but it should be a consideration. Choosing an unsuitable diet could make the results of a study in epigenetic changes unreliable, whilst the use of different diets in different experiments could cause problems when comparing the results. If these dietary compounds are found to have a large effect on the fish epigenome it would be wise to start including details of diets used in epigenetic studies. This is also evidence that as diets change and new compounds and ingredients are introduced these should be investigated for their potential to change different epigenetic processes.

It is clear that nutritional programming has the potential to greatly improve aquaculture, but the potential benefits of epigenetic programming in fish go beyond nutrition.

As mentioned earlier in this review, triploids struggle with environmental conditions to a greater extent than diploids, especially a lower tolerance to high temperatures and hypoxia. As a result, heat tolerance and robustness are seen as some of the major hurdles that must be overcome. Increased knowledge of epigenetics and epigenetic programming could potentially help increase the triploids tolerance and therefore increase their viability both biologically and economically.

1.6.2 Thermal programming in other species

The majority of attempts of thermally program animals have come from poultry. Thermal manipulations during egg incubation has been shown to produce improved thermoregulation and thermal tolerance in Turkey (*Meleagris gallopavo*) (Piestun et al., 2015) and broiler chicken (*Gallus gallus domesticus*) (Collin et al., 2005; Loyau et al., 2016; Morita et al., 2016; Piestun et al., 2008; Yahav et al., 2004).

There is some evidence that fish can also be thermally programmed, with zebrafish being subjected to a variable temperature regime being more tolerant to higher temperatures than those in stable incubation regimes (Schaefer & Ryan, 2006). Whilst there is also evidence that parental rearing temperatures can alter characteristics in the offspring that are related to the thermal optimum, such as growth rate and metabolism (Donelson et al., 2012, 2018; Salinas & Munch, 2012; Seebacher et al., 2014; Shama, 2015, 2017; Shama et al., 2014, 2016; Shama & Wegner, 2014). Sheepshead minnows (*Cyprinodon variegatus*) showed increased

growth rates when reared at the same temperatures as the parents (Salinas & Munch, 2012). The maternal temperature in marine sticklebacks (*Gasterosteus aculeatus*) has been shown to alter the body size (Shama, 2015, 2017; Shama et al., 2014; Shama & Wegner, 2014), and mitochondrial respiratory capacity (Shama et al., 2016) of offspring, with the best performance being observed when offspring and maternal temperatures matched. Similar results were found in studies looking at aerobic scope in spiny damsel fish (*Acanthochromis polycanthus*) (Donelson et al., 2012) and the metabolic scope and locomotor performance of mosquito fish (*Gambusia holbrookia*) (Seebacher et al., 2014). These studies fail to determine if the effects observed are epigenetic in origin, and whilst the differences are not thought to be due to egg size (Donelson et al., 2012; Shama et al., 2014), it is possible that differences in proteins, hormones or other somatic factors could also influence these characteristics (Munday, 2014). That being said there is some evidence that these characteristics can be influenced by grandparent as well as parental environment (Shama et al., 2016; Shama & Wegner, 2014), with the potential stability of epigenetic modifications suggesting that in this case epigenetic inheritance is more likely a cause than inheritance of hormones or proteins.

Within Atlantic salmon, studies are beginning to examine the role of early life stress on development and potential preparation for future stress events. Kelly et al. (2020) showed that hypoxia during embryogenesis changes DNA methylation and the upregulation of hypoxia response genes. Whilst embryos (250-450DD) subjected to a 5x repeated shock of 7°C to 0.2°C for 1 min followed by air exposure (15°C) for 1 min before returning to 7°C, showed differential expression of a large number of genes related to development as well as changes to the methylome (Moghadam et al., 2017; Robinson et al., 2019). Fish treated with shocks during embryogenesis and post-hatch showed differential expression of genes related to stress response immediately after being faced with stress of a similar nature later at 800DD (Robinson et al., 2019). These results are promising and shows strong evidence of the potential of early life experiences to change the epigenome and potentially the response of Atlantic salmon to stress. Unfortunately, these experiments failed to investigate if these transcriptomic and epigenetic changes resulted in improved performance when faced with a stress event. However, Uren Webster et al. (2018) showed how cold shock could increase tolerance to a pathogenic challenge and

there is evidence that zebra fish can be programmed to be more resistant to hypoxia following parental exposure (Ho & Burggren, 2012). Whilst this study did not show an increased temperature tolerance alongside increased hypoxia tolerance, there can be an interaction between tolerances to both these stressors. The interaction between heat shock and hypoxia tolerance was first observed in the increased tolerance of mice against drowning after thermal challenge (Hiestand et al., 1955). Numerous subsequent (and more refined) experiments have provided evidence that both hypoxia inducible factor (HIF) and heat shock proteins (HSPs) interact and that they can both be activated by thermal or hypoxic shock, the result of this being that it is possible to build a cross tolerance to these stressors (Airaksinen et al., 1998; Banti et al., 2008; Burleson & Silva, 2011; Michal Horowitz, 2007; Rissanen et al., 2006; Sørensen & Loeschcke, 2001; Tetievsky et al., 2017; Treinin et al., 2003; Wen et al., 2002; Wu et al., 2002; Zambonino-Infante et al., 2013).

1.7. Considerations

When doing work into thermal programming in fish, it is worth noting that tissue specific differences have been observed in fish with regards to HSP stress response (Dyer et al., 1991). Another consideration when conducting a thermal programming experiment is that the capacity to express heat shock proteins can undergo natural selection (Chen et al., 2018). Populations of migratory locusts (*Locusta migratoria*) showed large differences in HSP expression depending on the latitude of the population, HSP90 expression only increasing after a heat shock in high latitude locusts and cold shock only inducing HSP90 in low latitude populations (Wang & Kang, 2005). This is an important consideration when setting up an experiment, and even more so if one was to attempt to commercialise thermal programming. One factor that makes experiments on thermal programming easier is the fact that handling and sampling are not thought to increase HSP expression (Vijayan et al., 1997; Washburn et al., 2002; Zarate & Bradley, 2003), this allows for responses to shocks to be observed without the worry that procedure-based stress will affect results.

1.7.1 Factors affected by a short-term shock

It is clear that long term exposure to suboptimal incubation temperatures can have negative effects on triploid salmon. Less is known about the effects of short-term

shocks such as those that could be used in a thermal programming regime. There is evidence that medium duration heat shocks can also cause significant increases in spinal deformities. Wargelius et al. (2005) exposed diploid salmon embryos incubated at 6 °C to a 12 °C shock for 24 hrs and found a 27-34 % increase in caudal vertebral column condensations depending on the timing of the shock. Developing osteoblasts were also shown to be highly sensitive to increasing temperatures at early stages of development (Ytteborg et al., 2010). Takle et al. (2004) showed that by increasing the temperature from 8 °C to 12 °C for 2.5 days in Atlantic salmon, heterogenous nuclear ribonucleoprotein A0 (hnRNP A0), acylcoenzyme A binding protein (ACBP), and atrial natriuretic peptide (ANP) mRNAs increased in expression. hnRNP A0 is involved in splicing and posttranscriptional regulation, and ACBP likely indicated changes in metabolism and cell signalling. Of these proteins, ANP is the most obvious candidate for the increase in mortalities that follows increased temperature exposure (Takle et al., 2004). ANP is thought to be a negative regulator of cardiac cell growth (Horio et al., 2000; Loretz & Pollina, 2000; Takle et al., 2006; Wu et al., 1997) and is involved in the development of the embryonic cardiovascular system, regulating blood pressure, body fluid homeostasis as well as regulating growth and development of cardiovascular tissues and bone (Cameron & Ellmers, 2003). Further studies in fish on long term exposure to elevated incubation temperatures show significantly decreased relative heart weight, and increased instances of *Septum transversum* and *Situs inversus* (Takle et al., 2005, 2006). ANP is not only increased after prolonged exposure to high temperatures but also significantly changed in expression after just 1 hour at 16 °C, decreasing during the 1st somite stage and increasing after the 15th somite stage (Takle et al., 2006). Further evidence for the potential adverse effects of increase ANP expression has been shown in transgenic mice. Overexpression of ANP leads to decreased blood pressure (Steinhelper et al., 1990) and a 30-40 % reduced heart mass at birth (Klinger et al., 1993).

Another factor that is involved in formation of the embryonic heart and could be a factor in the increased instances of cardiac malformation in triploids are executioner caspases. Caspase 6 was shown to increase in the heart of embryonic Atlantic salmon that had been subjected to a shock, with this increase not being observed in other organs. (Takle et al., 2006). Caspase 3 was increased in the heart of Olive

Flounder (*Paralichthys olivaceus*) after heat shock (Yabu et al., 2003). These caspases induce apoptotic activity and an overexpression of caspase-3 has been linked to abnormal cardiac formation and function (Condorelli et al., 2001; Yamashita, 2003). The fact that cardiac cells seem particularly vulnerable to caspase induction after heat exposure could help explain why this is a major pathology in triploid salmon exposed to high temperatures. This should be considered when attempting to use heat shocks to create a thermal programming response in salmon especially triploids. Care should be taken to look at the health and performance of the shocked fish as well as their potential thermal tolerance.

1.8. Aims of the thesis

The overall aims of the thesis are to improve scientific understanding of early triploid development, explore the potential for thermal programming in Atlantic salmon, and to overall improve the production potential of triploid Atlantic salmon. We also aim to improve triploidy verification and reduce industry costs associated with this. To assess assumptions about triploidy development, increasing scientific understanding of these animals as well as increasing the confidence of embryonic studies in triploids, and to investigate the impact of temperature shocks during embryogenesis, providing useful information to the industry about the sensitivity of triploids at this vital stage of development, as well as increasing scientific understanding through the use of transcriptomic analysis. We hypothesise that microsatellite markers can be used to verify triploidy in recently fertilised eggs and that the rate of embryogenesis may differ between ploidy. We also hypothesise that a thermal shock during early development may change the epigenome of Atlantic salmon to the extent that they are more tolerant to thermal stress later in life, and that transcriptomic and epigenetic differences between ploidy can be used to explain some of the differences between diploid and triploid Atlantic salmon.

Specifically, we aim to identify and validate a suite of microsatellites that can be used to verify triploidy earlier than current methods of verification **(Chapter 2)**. To achieve this we will need to establish a protocol through which we can extract usable DNA from recently fertilised salmon embryos **(Chapter 2)**. Together this will provide the industry with a cheaper, quicker, and less resource intensive method of triploidy verification. In addition, we aim to establish if the assumption that triploids and

diploids develop at the same rate during embryogenesis is in fact valid **(Chapter 3)**. Without this validation experiments focusing on embryogenesis may not be comparable between ploidy, validating this will strengthen both scientific understanding and confidence. Finally, we aim to establish if thermal shocks at the eyed stage impacts the growth and performance of Atlantic salmon during the freshwater phase, and to explore the potential for thermal programming by assessing temperature tolerance when thermally challenged later in life **(Chapter 4).** Better understanding of the impact of thermal shocks will increase scientific understanding whilst allowing the industry to better anticipate the effect of unintentional temperature fluctuations. Exploring thermal programming has the potential to tackle a major hurdle in triploid acceptance and also provide the industry with a tool that can allow for continued culture of animals in areas which may become unviable with rising temperatures. Understanding of the physiological impact of the shocks will be supported by transcriptomic analysis. Our knowledge of the differences between triploids and diploids, and the reasons behind the reduced thermal tolerance of triploids, will be increased through transcriptomic analysis during thermal challenge **(Chapter 5)**, whilst our understanding of the impact of the shocks will be improved through transcriptomic analysis 24hrs after the shocks **(Chapter 6)**. These two chapters will use the same experimental set-up as chapter 4 and support the physiological analysis undertaken there, together these last three chapters will not only increase scientific understanding of both triploids and diploids but also provide the industry with increased justification whether or not triploids are viable alternatives to diploids.

Chapter 2. Microsatellite validation of triploidy in young Atlantic salmon (*Salmo salar*) eggs

Abstract

Triploid Atlantic salmon are typically produced using a hydrostatic pressure shock precisely timed after fertilisation. Whilst this approach is generally reliable, it is not infallible. Accordingly, successful triploidisation of a given batch of eggs requires being ascertained by a suitable protocol. Traditional methods of testing such as flow cytometry can be costly and require that embryos be incubated at around 350 degree-days before they can be tested. Genotyping tests for microsatellite markers provide an alternative option. In this chapter we tested multiple suites of microsatellites and demonstrate their suitability to verify triploidy. This method is cheaper and requires less specialist training than traditional methods. We also report protocols for DNA extraction from embryos aged between 26 and 78 degree-days, which allows verifying triploidy through microsatellite genotyping at an earlier developmental stage than possible with traditional methods.

2.1. Introduction

A variety of methods can induce sterility in Atlantic salmon, with varying levels of success and legislative restrictions. Hormonal (Komen et al., 1989) and chemical (Twohey et al., 2003) treatments can be effective but are not permitted on fish for human consumption. Modern approaches have focused on preventing successful germ cell migration or development, both these approaches have seen some success in salmon and other species. Morpholinos have been shown to be effective though both injection (Wong & Zohar, 2015b) and bath treatment (Güralp et al., 2020) in knocking out *nos1/nanos3* and *dnd* (dead-end) (Škugor et al., 2014; Wong & Zohar, 2015b), genes responsible for germ cell migration and development respectively. CRISPR-cas9 has also been used to remove *dnd* function and result in sterile fish (Wargelius et al., 2016). Both the morpholino and CRISPR-cas9 approach have their issues, and it remains to be seen if the use of these chemical treatments

(morpholinos) and gene editing (GE) technologies such as CRISPR-cas9 would be approved for food production in many countries around the world. Whilst the USA is leading the way in terms of acceptance for GM and GE crops and animals for food production, many other countries including the entire EU currently ban their sale, and legislatively currently fail to see a difference between GE species and GM species. The only currently accepted method for producing sterile salmon is through the use of triploidy.

Triploidy can be induced through thermal (Benfey et al., 1984; Lincoln et al., 1974), chemical (Allen & Stanley, 1979), and pressure treatments (Benfey, 2001), although both thermal and chemical treatments have issues regarding their reliability and increased mortalities (Benfey, 2001). Conventional triploidy production employs the use of a precisely timed hydrostatic pressure shock to prevent the release of the 2nd polar body during the second meiotic division, this stops the release of the second set of maternal chromosomes and the egg remains in the 3n state (Rottmann et al., 1991). Whilst this method is generally reliable, it is not infallible and there have been examples of companies releasing “triploid” eggs that were mistakenly still diploid. Verification of the triploid process is important not only for maintaining consumer trust, but in cases where sterility is a requirement for production licenses such as in the case of transgenic salmon production or previously the case of green licenses in Norway. In addition, diploids and triploids require different rearing conditions, and it is important to know which ploidy you have on your farm. Due to these facts, each batch of eggs that undergoes the triploidy procedure must be validated for triploidy.

Verification of triploidy can be achieved through karyotyping (Johnson & Wright, 1986) and nucleolar organising region analysis (Lozano et al., 1992), but these are expensive, time-consuming, and complex. Flow cytometry is the most common method currently used and is both cheaper and quicker than the above methods, although due to the large number of eggs needed to be tested for results to be reliable, this method is not cheap, it also requires specialised sample preparation. Flow cytometry relies on the fact that the erythrocyte size in triploids is larger and contains more genetic material than in diploids (Benfey et al., 1984; Preston et al., 2013). Flow cytometry can only be used once the eye is sufficiently formed, around 350 degree days (DD). To be able to make use of the verification information and

reproduce any failed batch within the stripping window, a portion of each batch eggs need to be developmentally sped up (M. Mommens, personal communication). The length of the stripping window depends on the individual fish and the regime under which they are held but it is often as short as 4 weeks (M. Mommens, personal communication). This requires the incubation of small batches of eggs at higher temperatures than the rest of the eggs, this requires additional space and resources and the subsequent disposal of this batch of eggs due to the supraoptimal incubation temperatures at which they have been raised (M. Mommens, personal communication). This is a further use of resources and increases the overall number of broodfish required over the years' production.

One of the downsides of using a cytometric approach such as flow cytometry is that by only looking at erythrocytes it fails to establish if all the cells in the animal are triploid, allowing for the possibility that mosaicism could be occurring (Benfey, 2016). Cytogenetic approaches such as karyotyping are the most definitive methods of triploidy verification as they provide information on chromosomes, these approaches are also expensive, time-consuming, and more complex than cytometric methods. Mosaicism is also thought to be a rare occurrence, although no study has thoroughly investigated its prevalence.

One other possible method of verification is the use of microsatellites, this method of verification is not only half as cheap as flow cytometry, it also offers a cytogenetic approach and potentially at a much earlier stage of development. Through the use of highly polymorphic microsatellites, triploids can be identified by the presence of a trisomic state at multiple loci. This method has been shown to achieve up to 100% ploidy verification in plants (Esselink et al., 2004), frogs (Pruvost et al., 2015), and important invertebrate aquaculture species such as pacific oyster (*Crassostrea gigas*) (Kang et al., 2013), and abalone (*Haliotis midae*) (Slabbert et al., 2010). This method has also proven successful in a number of fish species including Goldfish (*Carassius auratus*) (Jakovlić & Gui, 2011) and Turbot (*Scophthalmus maximus*) (Hernández-Urcera et al., 2012).

In Atlantic salmon, microsatellite suites have already been used to determine rates of spontaneous triploidy in adult fish (Glover et al., 2015; Jørgensen et al., 2018), and there has also been some use of microsatellites to verify triploidy in industry after

350DD. One published study looking at microsatellites in salmon eggs was able to determine triploidy in 436DD Atlantic salmon eggs, but this study had a <99% success, with a number of fish failing to show a trisomic state in any loci but being determined to be triploid through other methods (Glover et al., 2020). Whilst work was underway for this current study, another study was published also investigating microsatellites as a method of validation for triploidy. Jacq (2021) used 12 highly variable microsatellites on eyed Atlantic salmon eggs. This study achieved 94.6% reliability when using the criteria of three unique alleles at two or more loci and 97.9% when the criteria was one or more loci. Improving this reliability will improve consumer confidence and reduce the potential that batches of triploids will need to be remade due to false negatives. In addition, if the stage of which the microsatellite panel can be employed can be reduced this will reduce the need to incubate eggs for extended periods of time before testing can be carried out.

The method through which one determines whether a trisomic state is present at each microsatellite loci is PCR followed by capillary electrophoresis to determine the fragment sizes of PCR products obtained. If three peaks are present at a specific locus then we can say that, at least at that location, the animal is triploid. If a trisomic state (three different alleles) is present at multiple loci on separate chromosomes, then we can say that the animal is almost certainly a full triploid. Without looking at all chromosomes, e.g. through karyotyping, we cannot say with 100% certainty that the animal in question is 100% triploid and there will remain a slight possibility that tests may have failed to spot mosaic triploid. However, mosaicism is thought to be rare (Benfey, 2016), and given that fact that karyotyping is prohibitively expensive and the current method of verification, flow cytometry, by using only erythrocytes provides even less protection against mosaicism, this should not be considered as an obstacle towards the acceptance of the use of microsatellites for triploid verification.

To determine the triploid state at a specific locus requires the presence of 3 different alleles. Therefore, important considerations during the creation of a viable microsatellite suite are the level of polymorphism at the chosen loci and the level of chromosomal crossover. Polymorphism is essential as if the mother is homozygous or the father supplies the same allele as one of the maternal chromosomes, then we

will be unable to determine the difference between a diploid and a triploid. Without crossover, the entirety of one set of the maternal chromosomes will be lost during the extrusion of the 1st polar body and the offspring will be either homozygous or double heterozygous. The level of polymorphism and the location on the chromosome determine the likelihood of this. Another important consideration is the possibility of null alleles. Null alleles result inconsistent amplification, and the presence of null alleles at certain loci can vary between populations (Banks et al., 1997). This can result in microsatellites that are informative for one population being unusable in other populations.

If microsatellites can be shown to produce 100% reliable verification of triploidy from 350DD onwards, it will provide a simpler and cheaper method than the use of flow cytometry, but it will still require separate incubation of small batches of eggs.

If DNA could be successfully extracted from younger eggs and used in tandem with a reliable suite of microsatellites, this would allow for bad batches to be reproduced within the stripping season, as well as decreasing the cost of triploidy verification significantly.

Extracting usable DNA from Atlantic salmon eggs can be difficult. Early on during development the amount of embryonic DNA is low, whilst the lipid-rich nature of many species of fish eggs, can hinder DNA recovery and inhibit PCR (Cary, 1996; Rehbein et al., 1997). One approach is to dissect the embryo from the rest of the egg, this takes skill, time, and therefore money to perform. Kits can be used such as the Qiagen DNEasy kit (Glover et al., 2020; Yang et al., 2011) but these are expensive if large numbers of eggs are to be analysed, and it is still recommended that the embryo be dissected out before use. More traditional approaches to DNA extraction can be used such as salting out or phenol-chloroform. Both these approaches are laborious and, despite generally working well on most tissues, can require fine-tuning when using fish eggs. Quicker methods of crude DNA extraction such as HotSHOT and celex can be used to extract DNA rapidly and cheaply, but with the downside that the quality and level of contamination is likely to be worse (Aranishi, 2006; Yang et al., 2011). Depending on the downstream applications of the DNA extraction these can be acceptable especially for simple gel electrophoresis and PCR (Aranishi, 2006).

The aims of this chapter were to identify and validate a suite of microsatellites that can be used to verify triploidy, and to develop a method of DNA extraction that can extract DNA, usable for microsatellite verification, from recently fertilised eggs.

2.2. Materials and Methods

2.2.1 Sample origins

Initial testing was conducted on Triploid Atlantic salmon (*Salmo salar*) eggs already present and stored at the IoA for a year by the time the experiment began. These eggs were sourced from Landcatch Natural Selection (Ormsary, Scotland) in November 2017. Atlantic salmon fin clips were sourced from Aquagen (Norway) from a previous study in 2017. The samples consisted of 5 maternal broodfish and 100 triploid offspring (from a random mix of the maternal broodfish and a single male broodfish). In both the above cases triploidy was ascertained through erythrocyte measurement. The final batch of Atlantic salmon eggs also came from Aquagen (Norway). These eggs were 26 and 78DD. All eggs were placed into 100% ethanol for at least 24hrs at 4-8°C in a 10:1 ethanol:egg volume ratio. Prior to shipping the ethanol was poured off, the eggs were shipped on ice to the IoA and the ethanol was re-filled upon arrival.

2.2.2 DNA extraction

Eggs

Triploid Atlantic salmon (*Salmo salar*) eggs already present and stored at the University of Stirling were used to test at which age DNA could reliably be extracted using the HotSHOT (hot sodium hydroxide and Tris) method (Magalhaes *et al.* 2017). The eggs were of the ages 26, 44, 61, and 78 degree days (DD) (approximately 5, 9, 12, and 16% of time from fertilisation to hatch). Multiple variations of the method outlined by (Montero-Pau et al., 2008) were made using the 78DD eggs. Variation in the quantity and concentration of reagents, timing and duration of stages, as well as handling of eggs were trialled, with the optimal method outlined below.

Individual eggs were placed on clean tissue to remove excess ethanol. Eggs were then placed into an Eppendorf tube with 500µl of Tris-HCL 5mM, pH 8.0, for 15

minutes. The eggs were then removed, dried on clean tissue, and placed into a 1.5ml screw cap tube. It was determined that the most efficient method was to use a volume of 400µl alkaline lysis buffer (NaOH 25mM, EDTA 0.2mM, pH 12.0) per egg, this was added to each tube and a hypodermic needle used to pierce the eggs. The tubes were then sealed, inverted 5 times, and placed in a heat block at 90°C for 30 minutes. After this time the tubes were removed and placed on ice for 5 minutes before an equal amount (400µl) of neutralising buffer (Tris-HCl 40mM, pH 5.0) was added. DNA extraction was still successful without the cooling or soaking in Tris-HCL, but it was found that the efficacy of the extraction was improved with the addition of these steps. Extending the heating stage did not improve DNA yield. After the neutralising buffer was added, the tubes were rapidly inverted 10 times and then spun down briefly. Samples were stored at 4°C for use in PCR/QPCR reactions, or at -20°C for a longer storage period.

To test the efficiency of the DNA extraction, a combination of PCR followed by gel electrophoresis and QPCR were used to establish if DNA extraction was successful and usable.

To assess the success of the DNA extractions, a gene coding for a fragment of malic using malic enzyme 2 (exon 3, 472 bp) was amplified using primers previously designed and validated by Taggart *et al*. (2022), Primer sequence 5' – 3' Forward: AGGCCTAATAAAGGAATGCTAAAGA, Reverse: TCGTGCAGTGATTAGTGATAATGAG. this gene chosen due to it being well established within the laboratory in question and it being within the size range of the microsatellites that we would select. Tubes containing the egg and HotSHOT mixture were spun for 30 seconds at 14,000 RPM (~20,000 xg). The middle layer was used for the PCR, with the bottom layer containing the egg and solid contaminants and the top layer containing lipid contaminants. PCR reactions were performed in a total volume of 6µl using 0.5µl sample DNA, 3µl MyTaq HS mix (Bioline), 0.6pM of each primer (0.12µl) and 2.26 ultrapure water. The thermal profile consisted of 38 cycles of 95°C for 15 seconds, 59°C for 15 seconds and 72°C for 40 seconds. 2.5µl of the PCR product placed into a 1.25% agarose gel with 5µl of 1.5x loading dye (ThermoFisher Scientific) in 0.5x TAE buffer. Gels were stained with ethidium

bromide and visualised under UV in a transilluminator for the quality of bands and the presence of smear or primer dimer.

Once the effectiveness of the DNA extraction method was confirmed though the use of PCR and gel electrophoresis, QPCR was used to assess the impact of potential contaminants and egg age. QPCR reactions were run on a QTower 3 [Analytik Jena, Jena, Germany] in accordance with the manufacturer's instructions and performed in total volume of 10µl and using the same primers as above, 5µl Sensifast SYBR No-ROX kit [Bioline, Memphis, Tennessee], 1pM of each primer (0.2µl), 3.6µl ultrapure water and 1µl sample DNA. Tubes containing the egg and HotSHOT mixture were spun for 30 seconds at 14,000 RPM (~20,000 xg). The middle layer was used for the QPCR. The thermal profile consisted of 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 30 seconds. The CT values from these reactions was used as a qualitative proxy for extraction efficiency in the absence of qualitative measures. CT values were compared between egg ages (n=10 per age). To understand the impact of potential contaminants, the expression values and melt curves were also compared with pure salmon DNA.

Fin clips

DNA extraction from the fin clips was achieved using an SSTNE/salt precipitation method (Grant et al., 2016). A section of fin approximately 0.5cm^2 was added to a 225µl solution containing 200µl of SSTNE buffer (0.30 M NaCl; 0.04 M Tris; 200µM EDTA; 0.199 mM EGTA [E3889, Sigma Aldrich, St Louis, MO, USA]; 4.89 mM spermidine [SO266, Sigma Aldrich, St Louis, MO, USA]; 1.4 mM spermine [S1141, Sigma Aldrich, St Louis, MO, USA], 20µl 10% SDS [L3771, Sigma Aldrich, St Louis, MO, USA] and 5µl proteinase K (10 mg/mL) [P2308, Sigma Aldrich, St Louis, MO, USA]). This solution was mixed well and incubated overnight at 55°C to dissolve all visible tissue. Following digestion, the samples were incubated at 70°C for 15 minutes to inactivate the proteinase K. 5µl of RNase A (2mg/ml) [R6148, Sigma Aldrich, St Louis, MO, USA] was added to each tube. Tubes were then incubated at 37°C for 1 hour. After this, 161µl of 5mM NaCl was added, samples were stored on ice for 10 minutes and then centrifuged at 10,000 xg for protein precipitation. 280µl of the supernatant was retained, transferred to fresh tubes, and an equal volume of isopropanol was added and the tube vigorously mixed. This was then centrifuged for

10 minutes at 10,000 xg to produce a pellet. The supernatant was removed, and the pellet washed using 75% ethanol, dried, and re-suspended in 25µl 5mM Tris. DNA was quantified using a Nanodrop 1000 Spectrophotometer [ThermoFisher Scientific, Waltham, MA, USA] and all samples diluted to 20ng/µl. Aliquots of the DNA were made, and a 96 well plate was sent to Queen's University, Belfast. The rest was stored at -20°C and retained.

2.2.3 Microsatellite suites

The attempt to identify a suitable suite of microsatellites involved the assessment of multiple combinations of microsatellites and the use of DNA extracted from both eggs and fin clips. Initial attempts to identify microsatellites began at the university of Stirling and were conducted on DNA extracted from eggs, this was suite one (table 2.1). To select highly polymorphic microsatellites with high chromosomal crossover regions of the chromosome some distance away from the centromere were chosen as the sites for the microsatellites. The first and last 500,000 bases of each of the 29 salmon chromosomes were taken in the form of FASTA files from the whole genome assembly of the S. salar genome (ICSASG_v2, GCF_000233375.1) on NCBI (National Center for Biotechnology Information). These sequences were then run through step 3 of the program QDD and also Primer 3, looking for microsatellites with the following criteria; 2-4 base repeat, repeating 15-40 times, and primers with a PCR product size of <400bp, primers beginning ~20 bp from the microsatellite and having an annealing temperature of as close to 60°C as possible. With these parameters, around 400 likely candidate microsatellites were identified. These were then manually sorted and the 4 that were decided to be most likely to succeed were ordered [Eurofins, Ebersberg, Germany] for verification in the lab (table 2.1). The Criteria for success were higher repeat number, good position on chromosome, and GC content below 60%. The primers were used for PCR on DNA extracted from the 78DD triploid individuals using the above-mentioned DNA extraction. PCR reactions were performed in a total volume of 6µl using 0.5µl sample DNA, 3µl MyTaq HS mix [Bioline, Memphis, Tennessee], 0.6pM of each primer (0.12µl) and 2.26 ultrapure water. Each primer set was tested at different annealing temperatures 57, 58, 59, 60, 61, 62 and 63°C. These PCR products were then run on a 1.25% agarose gel and the quality of bands and presence of smearing or primer dimer was observed. For each set, 62°C was chosen as the optimal temperature. The thermal profile

consisted of 35 cycles of 95°C for 15 seconds, 62°C for 15 seconds and 72°C for 40 seconds. 2.5µl of the PCR product placed into a 2.25% agarose gel with 5µl of 1.5x loading dye [ThermoFisher Scientific, Waltham, MA, USA] in 0.5x TAE buffer. The gels were made in the absence of ethidium bromide with ethidium bromide instead being used as a wash after the electrophoresis step. 5µl EtBr /100ml buffer was used to wash the gel for 1 hr after which the gel was washed in fresh buffer for 5 minutes to remove excess EtBr, the gel was then visualised under UV in a transilluminator.

Table 2.1 Suite one of microsatellite markers designed and tested at the University of Stirling.

Microsatellite	Chromosome	Repeat region	Primer sequence (5' – 3')	Annealing temperature
ssa01A:651	1	GT	F: CAGGTATATGTGCCGACGCT R: CAGGTCCCAGTGGTATGCTG	62°C
ssa06A:8971	6	TG	F: GATGACCTAGCGACAGTGGG R: AGATGTCTACCGTGGACCCA	62°C
ssa20B:31670	20	CTGA	F: GACTGGCTGGCTGACTGAAT R: AGCCAGTCAGTCATTCAGCC	62°C
ssa06B:9868	6	AC	F: AGCGGTAACAACAGGTAGGC R: CCTCAGGATGGACGAGAACG	62°C

Whilst the novel microsatellites in suite one were being validated, DNA extracted from fin clips was sent to Queen's University Belfast for the validation of suite two (table 2.2). This suite consisted of microsatellites identified from the literature as highly polymorphic or that had been shown in other studies to be potential candidates for triploid identification.

Table 2.2 Suite two of microsatellites analysed at Queen's University, Belfast.

Microsatellite Name	Reference sequence Ascension number	Chromosome	Forward Primer	Reverse Primer	Citation
Ssosl438	Z49134.1	17	TAGTGTAGCGCCG ATACGTCATA	GAACCAGGGTGTT TCAGAATGCT	(Slettan et al., 1996)
Ssleer15.1	U86708	20	ACAACAGCGTCAC CT GTC	ACTGACTTGAAGG AC ATTAC	(Schill and Walker, 1997, unpublished)
Ssp2201	AY081807	13	TTTAGATGGTGGG ATACTGGGAGGC	CGGGAGCCCCATA ACCCTACTAATAAC	(Paterson et al., 2004)
SSsp2210	AY081808	12	AAGTATTCATGCAC ACACATTCACTGC	CAAGACCCTTTTTC CAATGGGATTC	(Paterson et al., 2004)
SsaA124	AF525202.1	16	GAGCCTGTTCAGA GAAATGAG	CAGAGGTGTTGAG TCAGAGAAG	(King et al., 2005)
SSsp1605	AY081812.1	23	CGCAATGGAAGTC AGTGGACTGG	CTGATTTAGCTTTT TAGTGCCCAATGC	(Paterson et al., 2004)
Ssf43	U37494.1	16	AGC GGC ATA ACG TGC TGT GT	GAG TCA CTC AAA GTG AGG CC	(Clabby, 1995, unpublished)
Ssa202	U43695.1	2	CTT GGA ATA TCT AGA ATA TGG C	TTC ATG TGT TAA TGT TGC GTG	(O'Reilly et al., 1996)
Sssp3016	AY372820	11	GGGCAGGCTAGGA CAGGGCTAAGTC	AGTAAGCCAGGGC AATAGCCTGCTTG	(Paterson *et al.,* 2003, unpublished)
Ssa171	U43693.1	6	ATTATCCAAAGGG GTCAAAA	GTTTGAGGTCGCT GGGGTTTACTAT	(O'Reilly et al., 1996)
Ssa289	N/A	19	CTTTACAAATAGAC AGACT	TCATACAGTCACTA TCATC	(McConnell et al., 1995)
SLEEI53	U86704	3	TGATTTGTTGCCTG CTGCTTCC	TCCTGCTGCCCAC ATCATCC	(Schill and Walker, 1997,

unpublished)

Ssos311	Z48597.1	10	TAGATAATGGAGG AACTGCATTCT	GTTTCATGCTTCAT AAGAAAAAGATTGT	(Slettan et al., 1996)
Ssd30	N/A	29	AGC AGT AAA GAG AGA GAC TG	TGT TGA CTT CCT TCC CCA AG	(Sanchez et al., 1996)
Ssosl25	Z48581.1	19	ATCTACACAGCTC CTGGTGGCAG	CATGTAATGGGTC GAGAGAAGTG	(Slettan et al., 1996)
Ssa98	AF019195	15	GTTTGCTGTTGTCA TTTGCAGTCC	GGCATCTGTAGTT GGGCAAG	(O'Reilly et al., 1996)
EST405	AJ402722.1	4	CTGAGTGGGAATG GACCAGACA	GTTTACTCGGGAG GCCCAGACTTGAT	(Cairney et al., 2000)
SsaD144	AF525203.1	3	TTGTGAAGGGGCT GACTAAC	TCAATTGTTGGGT GCACATAG	(King et al., 2005)

Testing of additional microsatellites was then conducted at Stirling. In suite three, these microsatellites were designed in the same manner as outlined in the 1st attempt were identified with the addition of microsatellites that are located near to microsatellites that from the work at Queen's appeared to be in regions of high crossover but were themselves not polymorphic enough. The list of microsatellites chosen or designed here can be seen in table 2.3.

These were validated at Stirling using PCR and Gel electrophoresis, and those that produced useable products were analysed for their ability to determine triploidy using capillary electrophoresis as described above.

Table 2.3 Suite three of microsatellites tested at the University of Stirling.

Microsatellite Name	**Reference sequence Ascension number**	**Chromo some**	**Forward Primer**	**Reverse Primer**	**Citation**
Ssa197	U43694.1	15	GGG TTG AGT AGG GAG GCT TG	TGG CAG GGA TTT GAC ATA AC	(O'Reilly et al., 1996)
Ssa403UOS	AJ402720.1	3	CTTTAGAAGACGG CTCACCCTGTA	: GCTACTTCGTACTGAC TGCCTCA	(Cairney et al., 2000)

Ssa404UOS	AJ402721	17	ATGCAGTGTAAGAGGGGTAAAAAC	CTCTGCTCTCCTCTGACTCTC	(Cairney et al., 2000)
Ssa410UOS	AJ402727.1	1	GGAAAATAATCAATGCTGCTGGTT	CTACAATCTGGACTATCTTCTTCA	(Cairney et al., 2000)
co2-277	n/a	2	TGTCACATGGCGATAGGTGG	GGCAGGAGGTTAGGAAGTGC	n/a
co16-64	n/a	16	GCTCTCCTGCACCTGACTTC	ATTTCTGGAGAGGCAGGCAG	n/a
co3-55	n/a	3	ACACCTGCATCCAGTCAGTG	TCAGTGCCACTCAGAACACC	n/a
c16-57	n/a	16	TGAATCATTTCCAGCATTCGC	AGGTTGCATCTCCATTAACAACA	n/a
c02-280	n/a	2	TCACATGACGTTAGGTGGTCA	GTTGCTGTCCTGGGACTCTG	n/a
c03-57	n/a	3	TGATGTGAGCTTCAAGGAATGA	CCCAGGCAAGGTTACCCATT	n/a
c15-5952	n/a	15	ACCATCATGGACTGGGAGGA	ACCGTGTGATGCGTATGTGT	n/a
c16-70	n/a	16	CTAGGGGACGGCACAGATG	TCTCAGGGAGGTTGCATCTC	n/a
ssad157	AF525204	26	ATCGAAATGGAACTTTTGAATG	GCTTAGGGCTGAGAGAGGAATAC	(King et al., 2005)
sssp2216	AY081811	24	GGCCCAAGACAGATAAACAAACACGC	GCCAACAGCAGCATCTACACCCAG	(Paterson et al., 2004)
sssp1605UOS	n/a	23	GTCTCTCTTCATCCACTGAGGT	ACGCAATGGAAGTCAGTGGA	n/a
ssspG7	AY081813	29	CTTGGTCCCGTTCTTACGACAACC	TGCACGCTGCTTGGTCCTTG	(Paterson et al., 2004)
sssp2215	AY081810	24	ACTAGCCAGGTGTCCTGCCGGTC	AGGGTCAGTCAGTCACACCATGCAC	(Paterson et al., 2004)
sssp2001UOSNB	n/a	13	ATCGTCGTGACACAGACAGG	GGCCTGAGATCAGAACAGACC	n/a
ssa01AUOS	n/a	1	CAGGTATATGTGCCGACGCT	CAGGTCCCAGTGGTATGCTG	n/a

ssa06AUOS	n/a	6	GATGACCTAGCGACAGTGGG	AGATGTCTACCGTGGACCCA	n/a
ssa06BUOS	n/a	6	AGCGGTAACAACAGGTAGGC	CCTCAGGATGGACGAGAACG	n/a

Suite four contained a combination of microsatellites from the previous suites. This suite was analyzed at Queen's University. 5 batches of eggs were sampled at 26 and 78DD, 50 eggs per batch at 26DD (250 total) and 96 egg per batch at 78DD (480 total). Delays meant that it was only possible to test the 4 microsatellites from suite one (table 2.4) on 3 batches of 78DD eggs (288 total). The DNA was extracted from these eggs using the HotSHOT before being shipped to Queen's University in 96 well plates.

Table 2.4 Suite four of microsatellites tested at Queen's University, Belfast

Microsatellite Name	**Reference Sequence Ascension number**	**Chromosome**	**Forward Primer**	**Reverse Primer**	**Citation**
sssp1605UOS	n/a	23	GTCTCTCTTCATCCACTGAGGT	ACGCAATGGAAGTCAGTGGA	n/a
sssp2215	AY081810	24	ACTAGCCAGGTGTCCTGCCGGTC	AGGGTCAGTCAGTCACACCATGCAC	(Paterson et al., 2004)
ssad157	AF525204	26	ATCGAAATGGAACTTTTGAATG	GCTTAGGGCTGAGAGAGGAATAC	(King et al., 2005)
ssspG7	AY081813	29	CTTGGTCCCGTTCTTACGACAACC	TGCACGCTGCTTGGTCCTTG	(Paterson et al., 2004)
sssp2216	AY081811	24	GGCCCAAGACAGATAAACAAACACGC	GCCAACAGCAGCATCTACACCCAG	(Paterson et al., 2004)
sssp2001UOS	n/a	13	ATCGTCGTGACACAGACAGG	GGCCTGAGATCAGAACAGACC	n/a
EST405	AJ402722.1	4	CTGAGTGGGAATGGACCAGACA	GTTTACTCGGGAGGCCCAGACTTGAT	(Cairney et al., 2000)
Ssosl25	Z48581.1	19	ATCTACACAGCTCCTGGTGGCAG	CATGTAATGGGTCGAGAGAAGTG	(Slettan et al.,

					1996)
Ssos311	Z48597.1	10	TAGATAATGGAGGA ACTGCATTCT	GTTTCATGCTTCATA AGAAAAAGATTGT	(Slettan et al., 1996)
SsaD144	AF525203.1	3	TTGTGAAGGGGCTG ACTAAC	TCAATTGTTGGGTGC ACATAG	n/a

2.2.4 Fragment analysis

To determine the size of the fragments replicated and therefore the possible presence of a triploid the microsatellites were analysed using capillary electrophoresis [Beckman Coulter CEQ 8000, Beckman Coulter, Brea, California, USA]. PCR was conducted on DNA from both fish fin samples (extraction protocol below) and eggs at 78DD. PCR reactions were performed in a total volume of 10µl using 1µl sample DNA, 5µl MyTaq HS mix [Bioline, Memphis, Tennessee], 0.188µl of 1µM universally tailed forward primer, 0.313µl of 10µM pig-tailed primer, 0.313µl of 10µM fluro tag [WellRED dyes D2,D3, or D4. Eurofins, Ebersberg, Germany], 3.188µl ultrapure water. The forward primers were labelled at the 5' end with a sequence that allowed for the fluro tags to be attached to the PCR product this is more cost effective than direct labelled locus specific primers, pig-tailed reverse primers ensures. Pig-tailing the reverse primer on the 5' end improved the adenylation of the forward strand (Brownstein et al. 1996).The thermal profile consisted of 32 cycles for fin clip DNA and 40 cycles for egg extraction DNA of 95°C for 15 seconds, 58°C for 15 seconds and 72°C for 40 seconds. To test for quality and to estimate the quantity of the PCR product 2.5µl of the PCR product placed into a 1.25% agarose gel with 5µl of 1.5x loading dye [ThermoFisher Scientific, Waltham, MA, USA] in 0.5x TAE buffer. Gels were stained with ethidium bromide and visualised under UV in a transilluminator for the quality of bands and the presence of smear or primer dimer.

The strength of the band was used to determine the amount of DNA to be added to the capillary electrophoresis (between 0.5 and 1 µl). The plate allowed for up to 3 different dyes [WellRED dyes D2,D3, or D4. Eurofins, Ebersberg, Germany] to be added to each well, along with the before mentioned quantity of DNA for each primer set, 30 µl of sample loading solution (SLS), and 0.35 µl of size standard [WellRED size standard, Eurofins, Ebersberg, Germany]. Each well was topped off with a drop

of mineral oil. The limitation of the system to read 4 different dyes meant that alongside a size standard only 4 different PCR products could be read in each well at once. This meant that for suites larger than 3 multiple runs had to be made.

2.2.5 Statistical analysis

CT values between ages of eggs (n= per age) were compared using One-way ANOVA once Levene's test of equality had confirmed equal variance. If One-way ANOVA determined a significant difference (p=>0.05)then a Bonferroni post hoc test was used to determine between which groups a significant difference occurred.

2.3. Results

2.3.1 DNA extraction

Initial attempts at DNA extraction had varying success. After adjusting the method by altering reagent volumes, as well as the addition of a cooling and pre-soak step, the modified HotSHOT protocol proved to be quick, simple, and reliable, and we were able to extract DNA that was suitable for our purposes. The protocol worked well for eggs preserved in ethanol for over a year, the strength of the bars shown using gel electrophoresis where stronger when using fresher eggs. The quantity of DNA could not be determined using nanodrop, therefore CT value obtained using QPCR of the above mentioned primers was also used as a proxy of DNA concentration, and it was shown that the age of the embryo correlated directly with the average CT values from 10 eggs of each age, stored in ethanol, can be seen in figure 2.1. One way ANOVA showed significant difference was found between the groups ($F(39) = 43.78$, p= <0.001). Post hoc analysis showed that groups significantly differed in CT value in all pairwise comparisons. Older eggs had significantly lower CT values than younger eggs.

Initial testing of suite one at the University of Stirling showed that the DNA extracted through the modified HotSHOT extraction without clean-up was compatible with fragment analysis testing and the microsatellites tested were capable of exhibiting 3 distinct peaks, the signature of trisomy at that loci and therefore evidence of triploidy (figure 2.2).

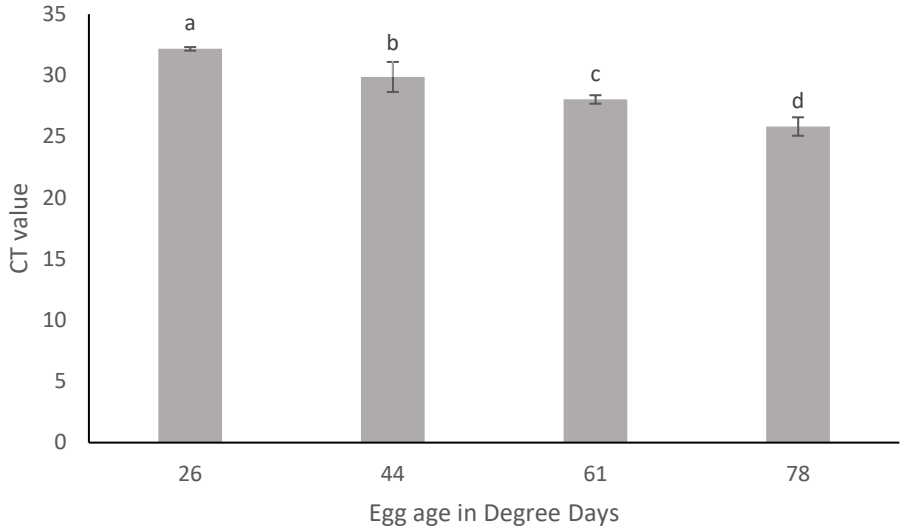


Figure 2.1 Mean CT values for malic enzyme 2 (exon 3) following qPCR for HotSHOT extracted DNA from eggs incubated for 26,44,61, and 78 degree days. n=10. Error bars deonte standard error. Superscript denotes significant (*p*= <0.05) differences.

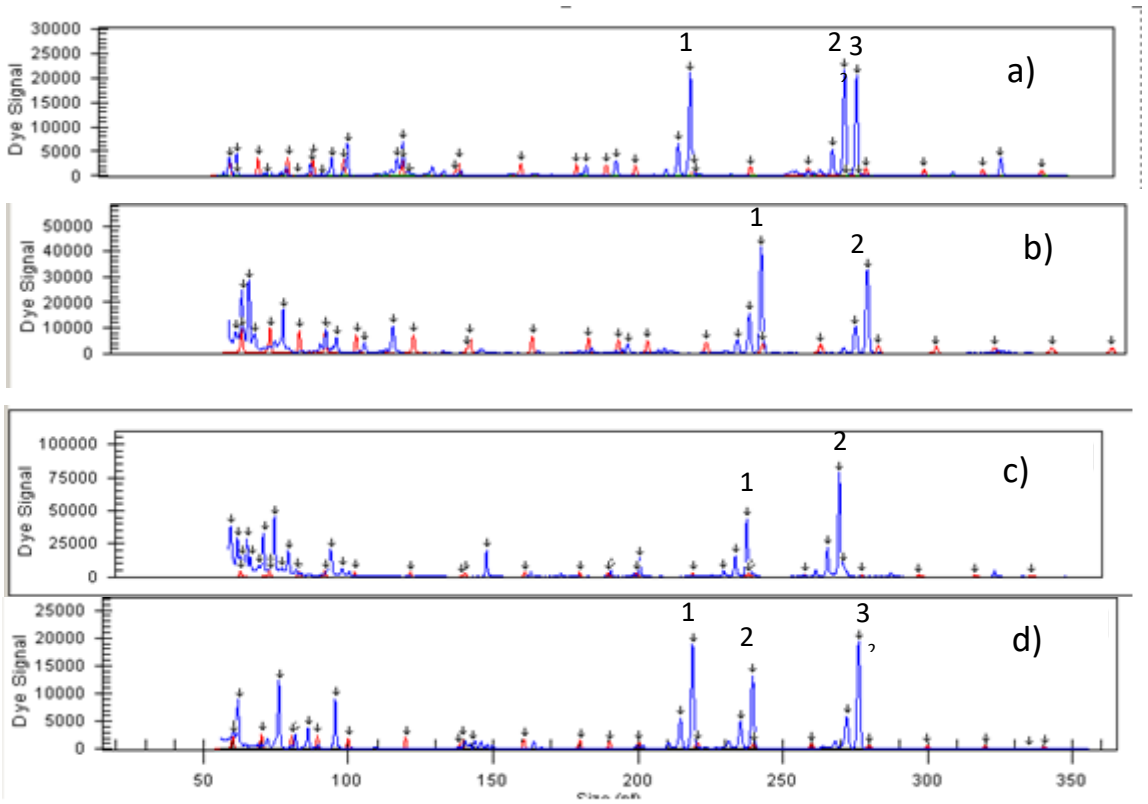


Figure 2.2 Fragment analysis electropherogram. Peaks show peaks of fluorescent activity at specific fragment sizes. The early peaks around 50-100 base pairs show unspecific background amplification. Panels A and D show 3 distinct peaks, providing evidence for triploidy. Results in panels B and C, which are characterised by two peaks, are indicative of diploidy or homozygous triploidy.

2.3.2 Microsatellite suite two

The 18 microsatellites of suite two which was tested at Queens detected a trisomic state at 2 or more loci in 94% of 89 individuals. 100% of individuals tested showed 1 or more trisomic loci. The percentage of individuals trisomic at each locus can be seen in figure 2.3. Not all microsatellites work as well as others, with microsatellites EST404 and Ssosl25 showing much higher rates of trisomy than all other microsatellites and 5 microsatellites failing to detect any trisomic individuals. These microsatellites were successfully amplified but showed high rates of homozygosity, with 2 microsatellites exhibiting only 1 fragment size (ssleer15.1 and sleei53) and the remaining 2 only 2 different fragment sizes (ssa289 and sssp3016), suggesting limited potential of these specific microsatellites to identify triploidy, at least within a population of similar genetic origin.

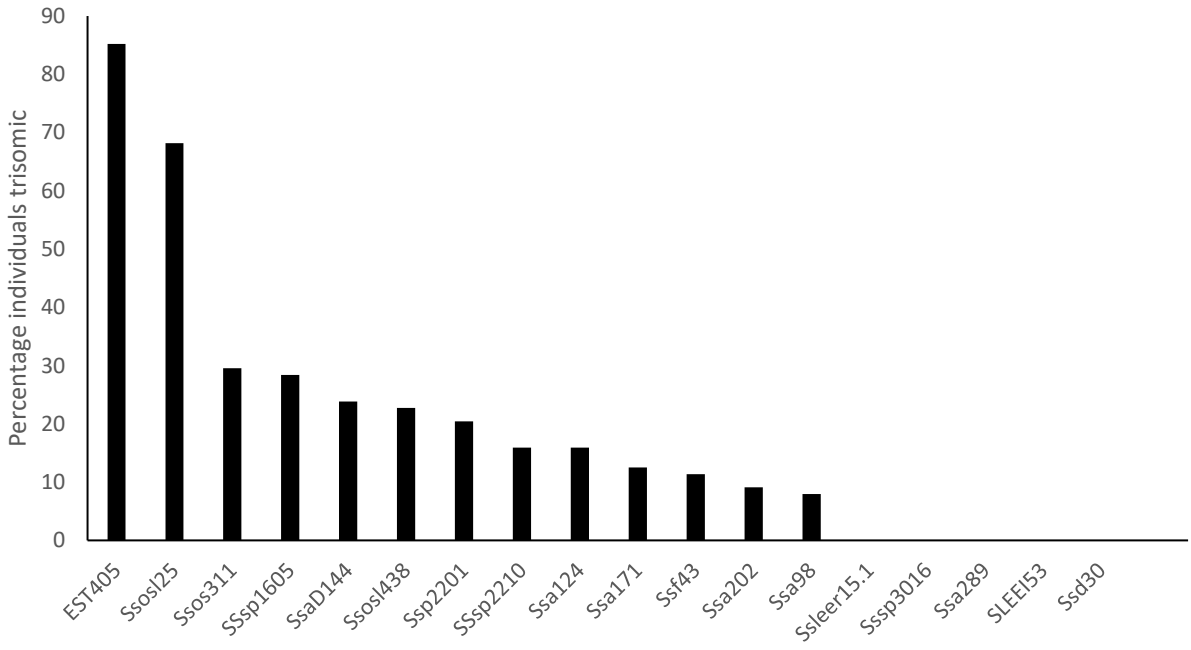


Figure 2.3 Percentage of individuals showing a trisomic state at each locus from suite two of microsatellites. (N=89). 100% individuals showed ≥1 trisomic loci, 94% showed ≥2.

Despite the success of some microsatellites, a suite of multiple microsatellites is still required to get the requisite coverage. Analysis involving removal of the worst performing microsatellites from the results data showed that 2 reduced suites of 7 and 5 microsatellites can be formed with no increase in individuals showing 0

trisomic loci (table 2.5) although as the number of microsatellites are reduced the number of individuals showing 2 or more trisomic loci also decreases.

Table 2.5. Two reduced microsatellite suites capable of producing ≥1 trisomic loci in every individual

Microsatellites in suite	Number of microsatellites	Percentage of individuals with ≥2 trisomic loci	Percentage of individuals with ≥1 trisomic loci
Ssa124 SSSp1605 Ssa202 Ssos311 Ssos125 EST404 SsaD144	7	89.8	100
Ssa202 Ssos311 Ssos125 EST404 SsaD144	5	69	100

Some of the less successful microsatellites, namely sssp1605 and ssp2001, did however show that crossing over was occurring, as seen by the presence of 3 alleles when the mother was a heterozygote. New microsatellites near these locations as well as novel microsatellites were tested at the University of Stirling (table 2.3) to determine a new suite to be tested again at Queen's University.

2.3.3 Microsatellite suite four

The 7 microsatellites tested were chosen to form suite four (table 2.4). This suite achieved a triploidy verification of 75.78% at 2 or more loci and 93.31% at 1 or more loci. The eggs used in this analysis were the 480 78DD eggs. The 26DD failed to produce reliable results when using the higher throughput approach, although eggs of this age did work at the IoA on the smaller scale. The high throughput approach was had a greater level of automation than the work completed at IoA and used more dyes per well allowing for more microsatellites to be assessed per run, it was completed in 96 well plates. The aim was to complete this suite with 4 of the best microsatellites from the suite two (table 2.4), 2 of the microsatellites produced too much stuttering despite working well in the initial test and as such could not be used in this analysis. The 2 additional microsatellites that were tested were EST405 and ssaD114. Of the 288 eggs tested alongside the rest of suite four, produced an

82.98% verification at 2 or more loci and 94.09% at 1 or more loci, these 288 eggs had slightly lower verification just using suite 2 at 68.75 and 92% verification at the 2 respective criteria. Assessment of triploidy at each loci was determined by the presence of three different sized alleles, this was in the form of specific sizes of base pairs rather than number of peaks. The percentage of individuals trisomic at each locus from suite four can be seen in figure 2.4, the 2 additional microsatellites and the results from the 288 individuals tested can be seen in figure 2.5. The average percentage of individuals per loci showing a trisomic state is 37.5% this compares to 27% for suite two (figure 2.3), not including the 5 loci with no trisomic individuals (if they were included the average is 20%). All microsatellites in this suite have a higher percentage of individuals in a trisomic state than all but the first two microsatellites from the 1st suite. SSSP1605UOS and SSSP2001UOS were microsatellites designed to be nearby to microsatellites in suite two which showed crossover but were not heterozygous enough (SSSP1605 and SSSP2001). SSSP1605UOS and SSSP2001UOS showed an improved percentage of trisomic individuals by 2.92% and 9.82% respectively.

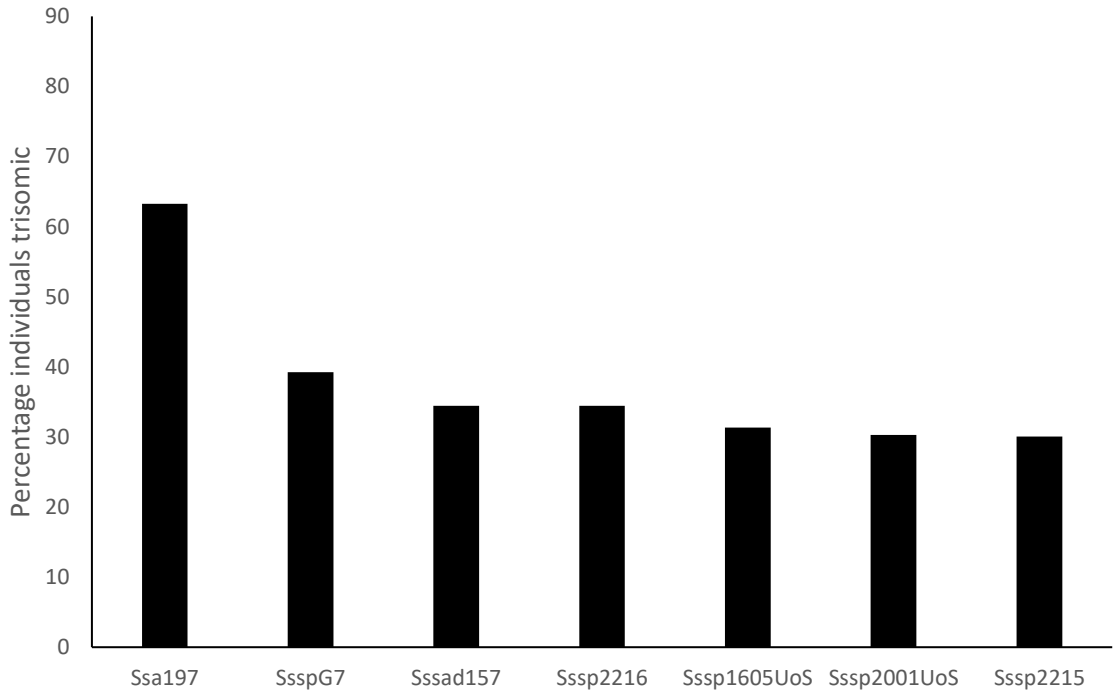


Figure 2.4. Percentage of individuals showing a trisomic state at each locus from suite four of microsatellites. (N=480). 93.31% of individuals showed ≥1 trisomic loci, 75.78% showed ≥2.

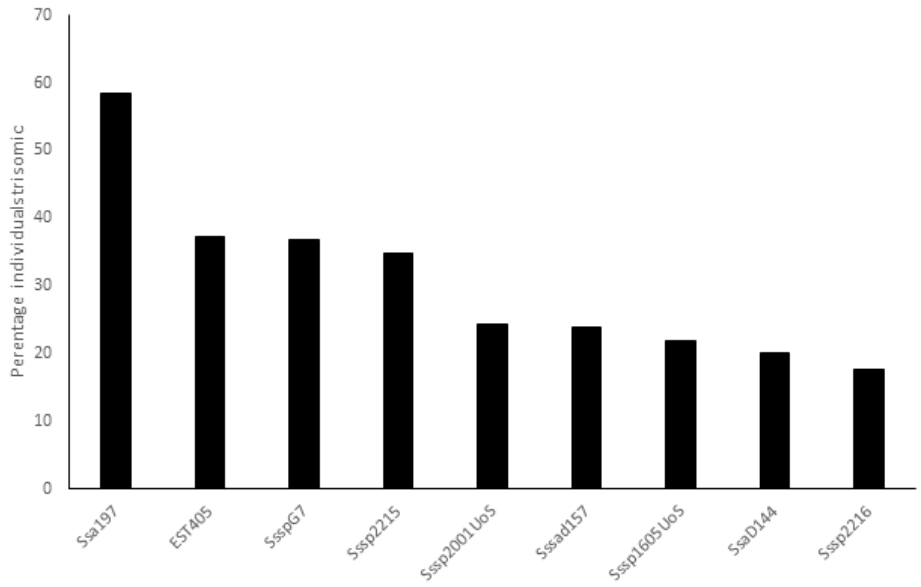


Figure 2.5. Percentage of individuals showing a trisomic state at each locus from suite four of microsatellites with the 2 additional microsatellites. (N=288). 94.08% of individuals showed ≥1 trisomic loci, 82.98% showed ≥2.

2.4. Discussion

Extracting usable DNA from fish eggs, especially younger eggs, is not easy (Cary, 1996; Rehbein et al., 1997). The procedure developed here can extract DNA from young Atlantic salmon eggs that is usable for the analysis of microsatellite loci using PRC and fragment analysis. This provides laboratories with a rapid and simple method of DNA extraction to yield extracts suitable for PCR based genotyping methods. If used in conjunction with an informative suite of microsatellites, this method of DNA extraction can provide information on ploidy in less than half a day. The current work increases the number of available microsatellites applicable for verifying triploidy. In addition, the HotSHOT method of extraction moves the point at which the method of verification can be used to earlier time points in development. Not only are microsatellites cheaper and easier to use than traditional methods of ploidy verification, the use of HotSHOT removes the need to hold additional testing populations which are developmentally sped-up. This decreases the space and time needed for ploidy verification, as well as reducing the number of brood fish of the course of a production cycle.

In particular, the HotSHOT technique can extract useable DNA in much younger fish than are tested using methods such as flow cytometry. Eggs as young as 26DD provided useful data using fragment analysis at the IoA, but when analysis was scaled up for use on the automated system the results were unreliable. It is possible that with more refinement this DNA would be useable, but when 78DD eggs produced such good quality results, the use of these slightly younger eggs may be unnecessary. The ability to use eggs as young as 78DD is a huge boost and is much younger than the 350DD eggs used in the previous microsatellite study (Jacq, 2021). Unfortunately, the resources were not available to test the suite at eggs of intermittent ages, but it is likely that eggs younger than 78DD also produce useable results.

Not all microsatellites were as successful as others. Indeed, a number of Atlantic salmon microsatellites shown in the literature to be highly polymorphic were not overly useful in this study. Ssa202 for example was used to show triploidy in paper by Jørgensen *et al.* 2018 but in the current study only showed a trisomic state in 9% of the triploid individuals. There are two reasons as to why this is the case. One reason is that some of these microsatellites have been shown to be heterozygous in wild populations, but this investigation involved the use of farmed and selectively bred populations, likely decreasing the rate of heterozygosity at these loci. This would be especially an issue if these microsatellites are linked to traits that have been selected for. That being said the results of this study and those of Jacq (2021) show that microsatellites can be used to verify triploidy in farmed populations. This study looked at 12 microsatellite markers across 9 chromosomes and found a low false negative rate, a rate of zero false positives, and an overall allelic error rate of 0.3%. This study looked at 18 independent triploid batches over 3 years showing the robustness of the technique.

A study by Glover *et al*. 2015 also looked at triploidy in farmed populations but this suite was used to identify cases of spontaneous triploidy in “diploid” populations. Some of the microsatellites that were used in this study showed reduced effectiveness in the current experiment. Two microsatellites which showed trisomic states in a number of individuals in the cited experiment, Ssa289 and SSsp3016, failed to show a single trisomic state in the current experiment.

This shows the importance of choosing the correct suite for a specific population and that care should be taken to validate the microsatellite suites every few generations as changes in the level of inbreeding or heterozygosity could affect the efficacy of the suite. We did not explore this possibility or the role of the regions we selected for use in triploid verification. Another reason for the lower success than Jacq (2021) is that some of these microsatellites have not been used in Triploid studies. It is likely that whilst they have high rates of heterozygosity, they are located too close to the centromere and that crossing over is not occurring. This results in two homozygous maternal alleles being retained, no matter how heterozygous the microsatellite.

Attempts to design more polymorphic microsatellites in regions near to microsatellites that showed high rates of crossover were successful. Both SSSP1605UOS and SSSP2001UOS showed improved rates of triploidy identification compared to the microsatellites they were based off, this suggests this approach can be used to find other microsatellites based on those which show high rates of cross over.

At a triploidy verification rate of 75.78% at 2 or more loci and 93.31% at 1 or more loci the 2nd suite of microsatellites did not achieve results comparable with traditional methods of verification, the addition of 2 of the best microsatellites from the 1st suite increased the triploidy at 1 locus slightly (up to 94.09%) and increased the number with 2 or more trisomic loci by 7.2% (up to 82.98%). Unfortunately, external validation was not possible in this case, and whilst it is not necessarily as high as 99% it is unlikely to be as low as 93%, once these results are available it will become possible to truly validate the accuracy of this suite. The differing success of microsatellites between samples of different genetic origin is highlighted by the fact that EST405 showed a trisomic state in 85.2% of individuals tested in suite 1 whilst only showing a trisomic state in 37.15% of individuals when tested on the eggs for suite 2.

Given more time, this suite would be further refined and updated. Unfortunately, due to the amount of time it took to receive results, further analysis was not possible. Despite this, the microsatellites identified here increase the number of microsatellites for triploidy verification, they increase the arsenal of tools available for triploidy work and may prove more useful in fish of different genetic origin than those for which a

suite already exists (Jacq, 2021). It is not quite clear the minimum number of microsatellites that a suite would ideally use. The higher the number the higher the coverage and the chance of finding a triploid but the higher the costs as well. Due to the variation in the efficacy of some microsatellites compared to others a suite could theoretically be as low as three or four the heterozygosity and crossover was high enough, but this number would require significant effort to achieve and the savings compared to using larger suites may make the exercise uneconomical.

Microsatellites provide an advantage over conventional methods of triploidy verification in multiple ways. As seen above they can be used to determine triploidy at much earlier stages of development. Testing eggs using this method is also significantly cheaper, which given the larger number of eggs that require testing, can result in large savings. Microsatellites also provide a cytogenetic method of verification. Allowing a triploid state to be verified across multiple chromosomes rather than one specific cell type. This provides a more comprehensive verification of the triploid state and reduces the risk that mosaicisms may have occurred.

In conclusion, we have provide a suite of microsatellites will provide a useful tool in the validation of triploidy at the chromosomal level. Delays in external validation has meant that a comparison of this suite to conventional methods has not been possible but at the very least this suite adds to the tool kit available to future studies. Likely more importantly we have established a protocol that allows for the extraction of DNA from salmon eggs as young as 78DD, if not younger, that can be used for the verification of triploidy. This will remove the requirement of incubating additional batches of eggs and save the industry money, space, and time.

Chapter 3. Comparison of diploid and triploid Atlantic salmon (Salmo salar) physiological embryonic development

Abstract

Diploid and triploid Atlantic salmon show distinct physiological differences including heart, brain, and digestive system morphology, propensity for certain deformities, temperature tolerance as eggs and once hatched, and different nutritional requirements. Whilst several studies have looked in detail at the rate of embryogenesis in diploid salmon no study has compared the rate of embryogenesis between ploidy across the entire time from fertilisation to hatch. This study based its assessment on a previous seminal paper and used the same techniques to compare the rate at which triploid and diploid embryos developed morphological characteristics. No significant difference was found in the rate of embryogenesis between ploidy. This provides well needed evidence for the assumption that both ploidies develop at the same rate and gives scientific weight for studies which involve manipulation at these stages of development. One factor that did differ how ever was the timing of hatch. Triploids hatched more quickly than diploids and reached 50% hatch at a significantly earlier point.

3.1. Introduction

Triploid Atlantic salmon are produced by the aquaculture industry due to the fact that they are effectively sterile (Benfey, 1999). This sterility helps to mitigate the damage that escaped fish can cause (Bolstad et al., 2021; Diserud et al., 2022; Glover et al., 2013; McGinnity et al., 2003). If a farmed salmon escapes there is a risk that it will eventually “run” a river and mate with wild salmon (Fleming et al., 2000), this can potentially result in introgression and cumulative fitness depression (Glover et al., 2013; McGinnity et al., 2003). Despite the industrial production of triploids salmon, the process of triploidy is a natural phenomenon and occurs in a variety of fish species including rainbow trout (*Oncorhynchus mykiss*) (Aegerter & Jalabert, 2004),

tench (*Tinca tinca*) (Flajšhans et al., 1993), coho salmon (*Oncorhynchus kisutch*) (Devlin et al., 2010), and European catfish (*Silurus glanis*) (Váarkonyi et al., 1998).

Environmental conditions can result in a meiotic non-disjunction of chromosomes (Jørgensen et al., 2018), if the second polar body is prevented from budding off during the 2nd meiotic division the egg will remain in a 3n state rather than the more typical 2n state (Rottmann et al., 1991). It is thought that environmental conditions such as shifts in temperature (Glover et al., 2015), ageing of eggs both post- and pre- stripping (Glover et al., 2015; Jørgensen et al., 2018), as well as mechanical disturbance (Jørgensen et al., 2018) can all cause triploidy to be induced. Triploidy has been observed in wild Atlantic salmon populations at (0.017%) of the population (Jørgensen et al., 2018) and about 2% in farmed “diploid” populations (Glover et al., 2016).

The differences between triploid and diploid Atlantic salmon stretch further than just cell size and number. Physiologically triploids differ in a variety of ways from their diploid counterparts, including gill filament density (Leclercq et al., 2011; Sadler et al., 2001), and gut (Peruzzi et al., 2015), brain (Fraser et al., 2012; O’Keefe & Benfey, 1997), and heart morphology (Fraser et al., 2013, 2015). Triploids are more susceptible to a range of deformities including vertebral (Fjelldal et al., 2016), lower jaw (Amoroso et al., 2016), ocular (Taylor et al., 2015), and heart (Fraser et al., 2015) deformities. They require lower incubation temperatures (Clarkson et al., 2020; Fraser et al., 2014, 2015) and ploidy specific diets with increased histidine (Sambraus et al., 2017; Taylor et al., 2015; Waagbø et al., 2010), phosphorus (Fjelldal et al., 2016; Smedley et al., 2016, 2018), and protein (Smedley et al., 2016). Research into the differences between triploid and diploid salmon has been far from exhaustive, and we are likely to find more ways in which husbandry can be optimised in the coming years.

One area that has not been widely studied is the difference in early life development between ploidy. More investigation into this area and with it an increased understanding of early developmental processes and potential ploidy differences can inform us as to the basic mechanisms of triploidy, as well as possibly helping to explain why there are long term differences later in life.

Gorodilov (1996) looked at the rate of embryogenesis in Atlantic salmon in a pioneering study. This paper has been used by a number of studies to estimate the transition between stages of embryogenesis and to correctly time sampling (Macqueen et al., 2007; Takle et al., 2005, 2006a, 2006b; Wargelius et al., 2005). Gorodilov (1996) tracked the internal development of the embryo visually after clearing the egg using a clearing solution, and provided detailed drawings and descriptions at each stage. The reason for the influence and reliability of this paper is the quality of these drawings and descriptions, as well as the approximate timing after fertilisation that specific stages transition. The timing system used in this study is Tau-somite (τ_S) corresponding to the time it takes to form one somite pair during somitogenesis. This can be converted into degree days using the table in the paper.

Before a photograph can be taken of processes inside the egg, it is necessary to clear the egg. To do this, the eggs must be submerged in clearing solution. Numerous clearing solutions can be found in the literature and also in protocols established at the University of Stirling. These clearing solutions are however only typically used to establish fertilisation, rather than make clear and detailed observations of embryogenesis, so whilst they allow neural tube formation to be observed, many of these solutions are not suitable for use in this experiment. It is also worth noting that whilst some clearing solutions may effectively clear the egg they can also change the stability of the egg and this can make taking a clear photo difficult. A solution that allows the egg to maintain its shape is preferred. A number of solutions were therefore tested on a batch of eggs before the main experiment began.

Restrictions in the availability of Atlantic salmon eggs meant that trials to optimise the clearing solutions were conducted using rainbow trout (*Oncorhynchus mykiss*) eggs. The definitive experiments were conducted using salmon eggs. There are small differences in the size (Gjedrem & Gunnes, 1978) and lipid content between the two species (Berg et al., 2001; Craik & Harvey, 1984; Einum & Fleming, 2000), but overall they are quite similar and the same histological methods typically work for both species (Rainuzzo, 2020).

Only one study has looked at effects of ploidy on the rate of embryogenesis in Atlantic salmon (Johnston et al., 1999). The main purpose of that study was to

investigate muscle growth, but the authors also investigated the rate of somitogenesis (Johnston et al., 1999). In the study, the rate of somitogenesis did not differ between ploidy, but did not investigate earlier and later stages of embryogenesis. There may therefore be ploidy based differences during embryogenesis that have yet to be observed.

There are multiple reasons as to why establishing if there is a difference in the rate of embryogenesis between ploidy is important. One reason is that it increases understanding of the process of triploidy. Increasing understanding of the very earliest processes after fertilisation and the triploidisation procedure can help inform as to how and why triploids differ physiologically from diploids, this has both practical and core scientific benefits. Another reason is practical, most studies investigating aspects of early development used either the study by Gorodilov (1996) or degree day estimates to set the timing of sampling and treatments. These studies make assumptions on the rate of triploids embryogenesis based on diploid research. If embryogenesis does differ between ploidy, then a procedure done at a specific time point on both ploidy could actually be being conducted at two different stages of embryogenesis.

The aim of this study to investigate the development of triploid and diploid Atlantic salmon embryos, using the methodology of Gorodilov (1996), in order to determine whether animals of both ploidies differed in the timing of their external development from fertilisation until hatch. The study was expected to establish if as many studies have assumed, triploids and diploids differ in their rate of development regarding the rate of development hold true, and support scientific study during this stage of development.

3.2. Materials & Methods

3.2.1 Clearing solution

Reflecting the restricted availability of Atlantic salmon eggs, the preliminary investigation into the optimal clearing solution and duration was conducted on rainbow trout. 310 eyed rainbow trout eggs (n=310) were brought from the Niall Bromage freshwater research facility of the Institute of Aquaculture (IOA), University of Stirling (2019-10-14). Six different clearing solutions were tested (table 3.1).

Table 3.1: Clearing solutions tested and the durations tested.

Ingredients	Ratio	Durations (min)	Source
99.8% Methanol : acetic acid : water	1:1:1	30, 40, 50	University of Stirling protocol
Glycerol, glacial acetic acid, formalin	1:1:1	10, 20, 30	University of Stirling protocol
Ethanol: glacial acetic acid	3:1	10, 20, 30	(Gorodilov 1996)
Ethanol: formalin: glacial acetic acid	6:3:1	10, 20, 30	(Stoeckel & Neves, 1992)
Methyl salicylate (wintergreen oil)	Neat	30, 40, 50	(Stelly et al., 1984)
Methyl salicylate (wintergreen oil): ethanol	2:1	10, 20, 30	(Stelly et al., 1984)

During the tests, 5 eggs were placed into a beaker of each solution in triplicate for the 3 different durations. The eggs were then placed into a 35 mm Petri dish and observed under a dissecting microscope and photographed using a Olympus IMT-2 inverted compound microscope fitted with an Olympus SC100 10.6-megapixel digital colour camera while being immersed in a 6:1 solution of ethanol and glacial acetic acid. The effectiveness of the clearing solution and duration was determined by counting the number of fertilised eggs in each batch of 5 in which the gill arches could clearly be seen. After pre-trials were conducted the results were validated using Atlantic salmon eggs. The top three performing solutions were tested at each of the three specified durations. Due to differences in development stage these solutions and durations were simply qualitatively scored.

3.2.2 Triploidisation and husbandry

On 2019-11-12, 6,000 unfertilised Atlantic salmon eggs were stripped from 2 hens at AquaGen Holywood facilities, Dumfries, Scotland. Milt from 1 male was also taken.

The eggs and milt were taken to the IoA where equal volumes of eggs were mixed and split by future ploidy. All eggs were then fertilised with an excess of milt. After fertilisation the eggs were rinsed with 8 °C water and left to harden in an 8 °C water bath for half an hour. The “triploid” batch of eggs were placed into a pressure vessel and shocked 37 minutes post-fertilisation using a hydrostatic pressure of 9500 PSI (655 BAR/65,500,000 pascal) for 6 minutes and 15 seconds (Smedley et al., 2016).

Triploidy was verified using the microsatellite panel developed in the previous chapter “Microsatellite validation of triploidy in young Atlantic salmon (Salmo salar) eggs”. 250 eggs from the triploid batch were collected at 150DD, DNA was extracted and sent for analysis in 96 well plates (see previous chapter for more details on the extraction process).

After shock the eggs were removed from the pressure container and allowed to water harden for 1 hour before 3,000 triploid and 3,000 diploids were laid down across 6 egg trays per ploidy (500 eggs per tray). These eggs were placed into 4 troughs held in an incubator running at 6 °C +/- 0.1 °C. Two troughs were located in the upper portion of the incubator and 2 in the lower portion. Three trays of each ploidy were present at both heights to take into account the possibility of small variations in temperature inside of the incubator. The eggs were checked twice daily and any dead eggs removed. A 30 % water change was conducted 3 times a week using de-chlorinated water at the same temperature as the incubator. Despite this, the bottom 2 troughs in the incubator suffered from a fungal infection midway through the experiment. This was treated with Malachite green and increased water changes.

3.2.3 Photographs

Every 3 days, 20 eggs were taken for each ploidy, cleared using the above-mentioned method and then viewed and photographed using a dissecting microscope. During somitogenesis, photos of 66 eggs per ploidy were taken every 3 days. After somitogenesis, the number of photos decreased back to 20 per ploidy. The photographs were randomly coded and then blindly compared to each other and detailed drawings in Gorodilov (1996) and assigned a stage of development from 1 to 34 in line with the stages defined by Gorodilov (1996) (Table 3.2). The value was then used to determine whether there were any differences in the rate of development between ploidy. This photographs were compared from fertilisation to hatch, at which point the experiment was terminated. Over the period of hatch,

hatched alevin were removed from each trough daily and recorded, this continued until all the eggs hatched or died.

Table 3.2. Stages of development assigned by Gorodilov (1996) and their corresponding time in degree days.

Stage	Description	Age in Degree days
1	Fertilisation and formation of blastodisc	0
2	Blastodisc divided into 2 cells	3
3	Blastodisc divided into 4 cells	
4	Blastodisc divided into 8 cells	
5	Blastodisc divided into 16 cells	
6	Completion of synchronous cleavage, early blastula, there are about 2000 cells in the embryo	20
7	Mid blastula	
8	Late blastula	54
9	Beginning of epiboly	55
10	Formation of embryonic shield	
11	Embryonic shield enlarges and axial structures begin to form	90
12	3 somite pairs - embryo proper begins to form	94
13	6 somite pairs - embryo proper begins enlarges	99
14	~ 10 somite pairs - optic vesicles have formed	106
15	~ 17 somite pairs - pericardiac and oesophageal cavities develop, and optic vesicle appears	117
16	~23 somite pairs - lense begin to form, straight heart tube appears	127
17	~27 somite pairs - lense clearer, anterior border of gill cavity outlined	134
18	~33 somite pairs - stomach anlage and gill segments begin to form, mouth depression	143
19	~40 somite pairs - 1st gill fissure, heart tube bends, responds to tactile stimulation	155
20	~45 somite pairs - 2nd gill fissure, stomach forms, spontaneous heart contractions begin	163
21	~ 52 somite pairs - tail fin fold forms, 3rd gill fissure, olfactory placodes begin forming	174
22	~56 somite pairs - pectoral fin formation begins, first phase of otolith formation	181
23	~65 somite pairs - eye pigment begins, 4th gill fissure,	200
24	Vitelline plexus begins to grow, upper jaw more clear	205
25	Eye pigmentation increases, caudal end of notochord curved upwards	225
26	Formation of the semicircular canals, lower jaw separates from yolk	253
27	Hatching glands seen, concentration of mesenchyme in areas of prospective fins	269
28	Dorsal and anal fins begin to form	293
29	1st caudal fin ray formed, melanophores show, 1st caudal fin ray, operculum begin to form	306
30	3 caudal fin rays, operculum covers first gill arch	326
31	5 caudal fin rays, pelvic fins begin to develop	350
32	8 caudal fin rays, pectoral fins quivering irregularly	383
33	10 caudal fin rays, pectoral fins waving rhythmically	407
34	13 caudal fin rays, vertical posterior edge of dorsal fin, hatching begins	464

3.2.4 Statistical analysis

Each clearing solution and duration the percentage of eggs in which the gill arches could clearly be seen was compared using SPSS statistics for windows, version 26. (IBM corp, Armonk, N.Y., USA). One-Way ANOVA followed Levene's test and a difference was found between the combined solutions and durations in their ability to produce a clear picture (p=0.05). The Post-hoc test Tukey HSD was used to determine between which solutions and durations there was a significant difference (p=0.05). When testing ploidy differences in embryonic development, the assigned embryonic stages at each age were compared. All sampling points were not normally distributed. Therefore, a Mann-Whitney *U*-Test was used to determine if there was a statistically significant difference between the stages of embryogenesis of each ploidy at each sampling point. To account for the increased risk of type 1 error the Holm-Bonferrroni correction was used to adjust for multiple comparisons (p=0.0011) (Holm, 1979). Differences in mortality between ploidy were assessed using Mann-Whitney test on cumulative percentage mortality. SPSS was also used to run a log rank test to determine if there was a difference in the rate of hatch to between diploids and triploids.

3.3. Results

3.3.1 Triploidy

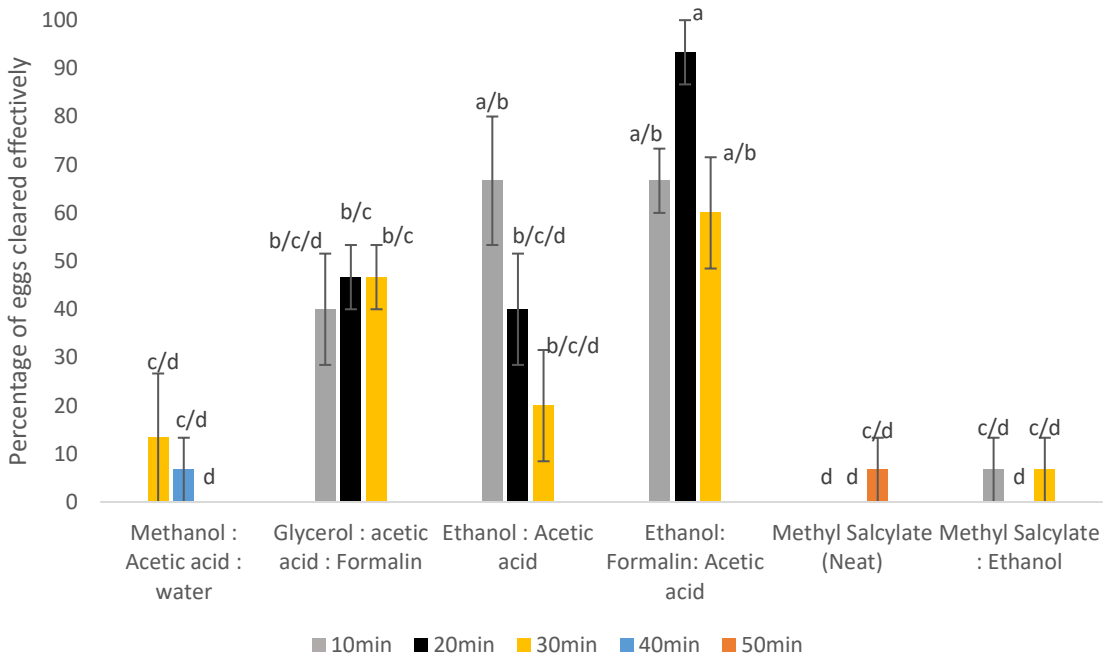
The eggs were analysed with a panel of 7 microsatellite markers. This gave a triploidy confirmation of 90.72%.

3.3.2 Clearing solution

One-Way ANOVA determined that there was a difference between some of the treatments and solution combinations and their ability to produce a clear picture F (17, 53) = 12.328, P= <0.001 (Fig. 3.1). The solution and duration with the highest efficiency was shown to be a 6:3:1 ratio of ethanol: formalin: glacial acetic acid and the optimal time for this solution was 20 minutes (Figure 3.1). This time/solution combination was not significantly more effective than 10 and 30 minutes in the same solution or 10 minute submersion in ethanol: acetic acid (3:1) as used by Gorodilov (1996) A third solution also produced efficiency of around 50 % at two different duration, this was glycerol: acetic acid: formalin (1:1:1) which had been used

previously at the IoA. The other solutions failed to produce many good quality pictures of embryonic development. Dissection did not produce a significantly clearer picture than just using the clearing solution and was considerably more work.

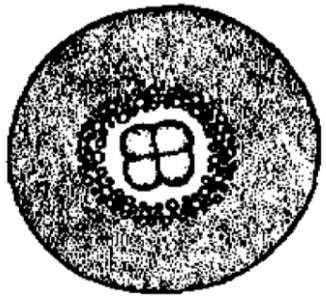
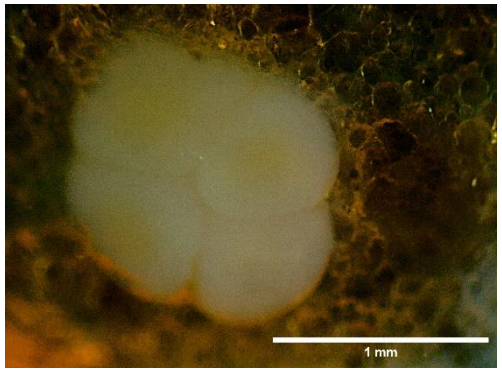
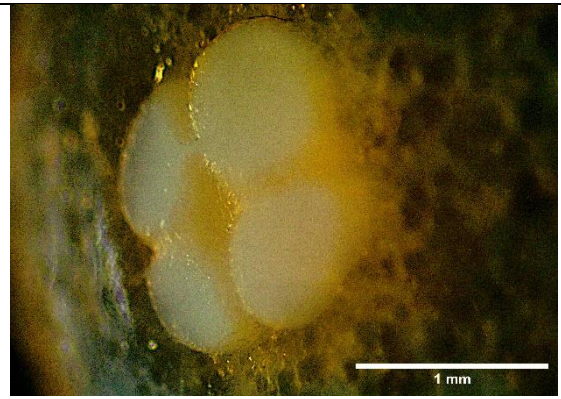
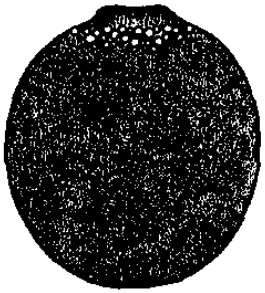
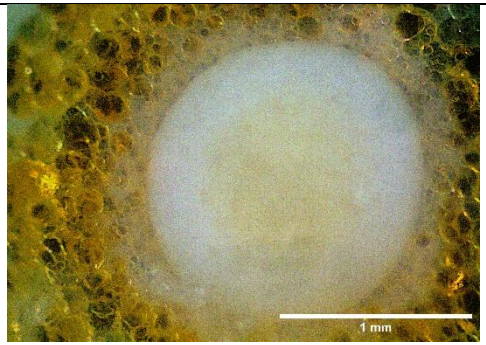
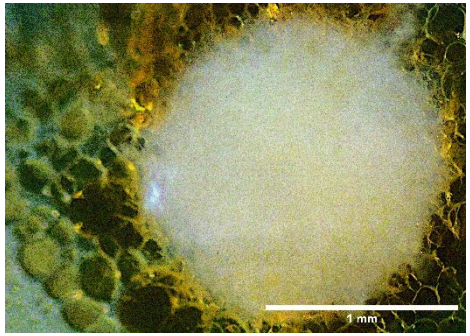
Figure 3.1. Mean percentage of photographs in which a clear image of the gill arch was observed in eyed rainbow trout (*Oncorhynchus mykiss*) n=5 eggs for 6 different clearing solutions and 3 different durations of immersion per solution. Superscript denotes statistical significance (Tukey HSD, *P*=≤0.05). Error bars indicate standard error.



3.3.3 Embryonic development

After all photos were taken they were compared to each other and to the detailed drawings found in Gorodilov (1996), these comparisons were used to determine the stage of embryogenesis from 1 to 34 (Figure 3.2). Examples of diploid and triploid photos can be found in table 3.2, alongside the drawings of the matching stage from Gorodilov (1996).

Table 3.3. Comparison of Diploid and Triploid Atlantic salmon (*Salmo salar*) embryos to the drawings of diploid embryos found in Gorodilov (1996). Degree days state the time at which the photographs were taken. All scalebars represent 1 mm

Degree day	Gorodilov 1996 (Diploid)	Diploid	Triploid
6			
18			

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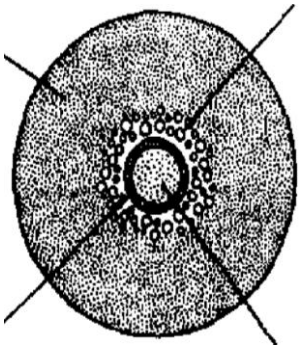

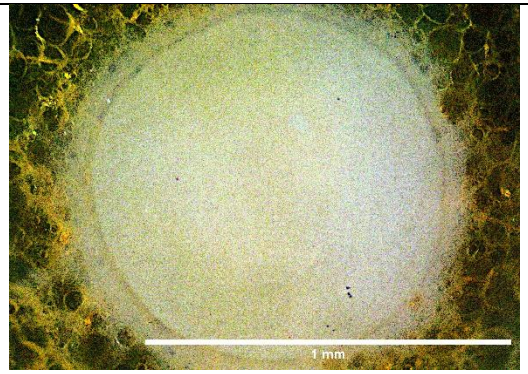

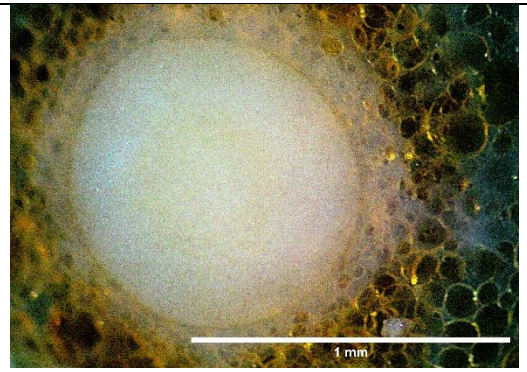

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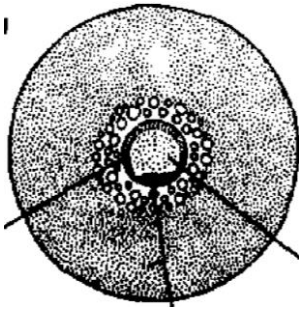

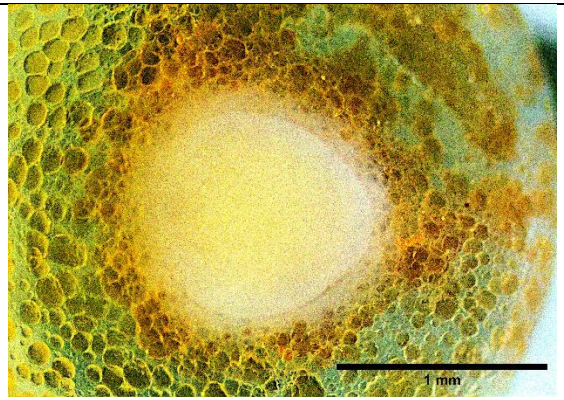

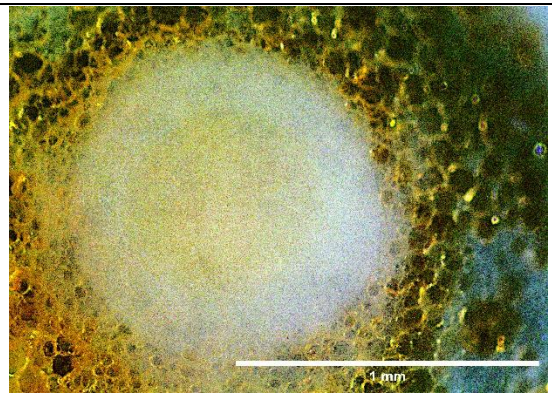

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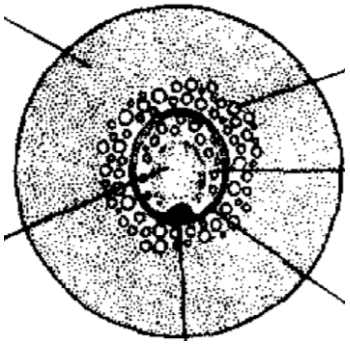

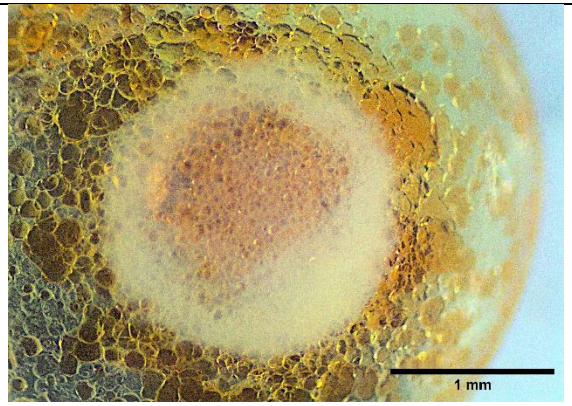

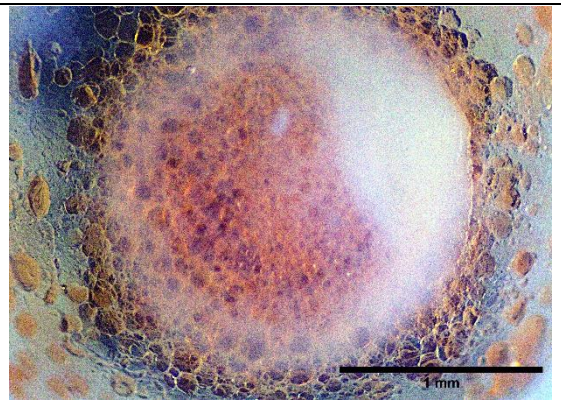

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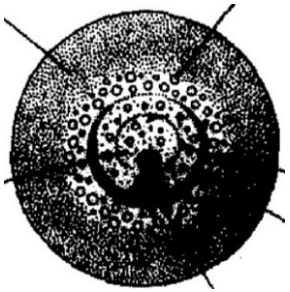

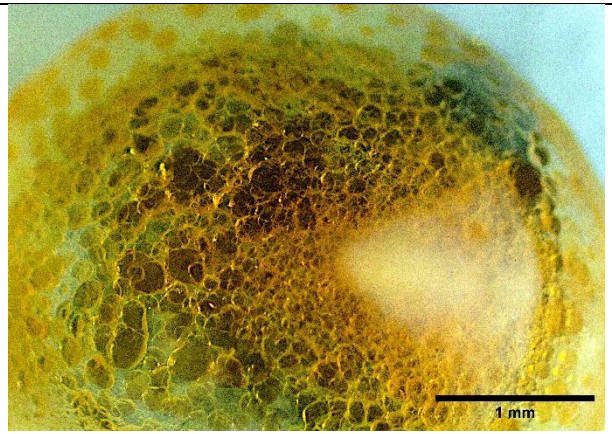

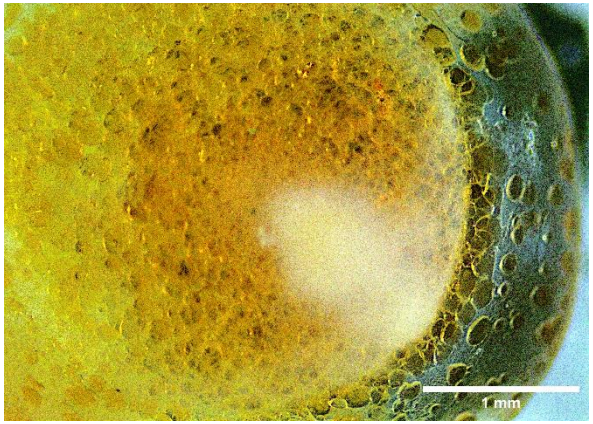

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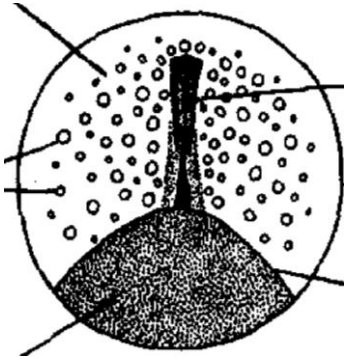

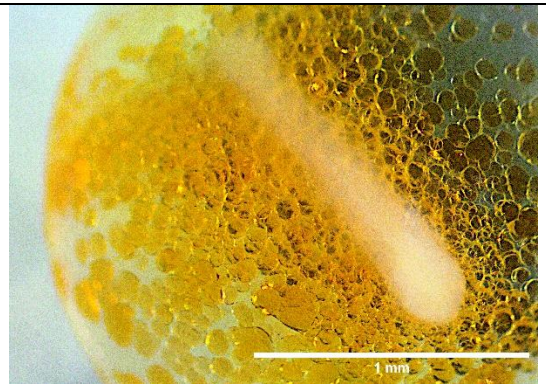

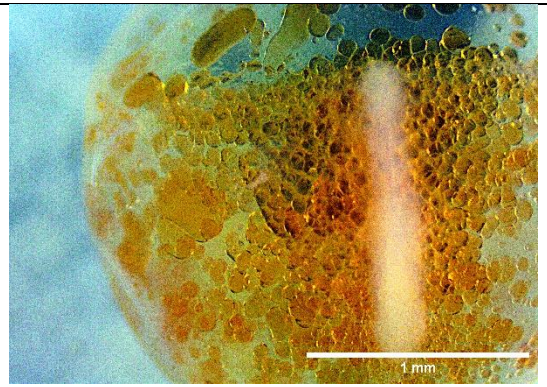

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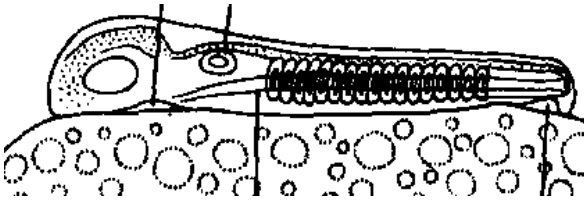

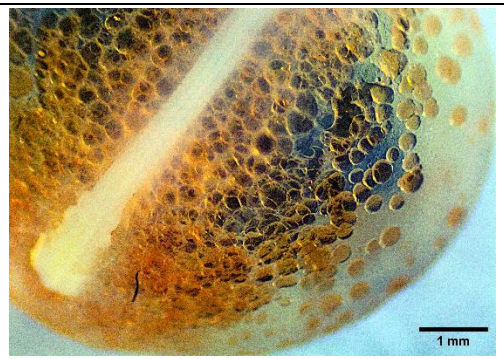

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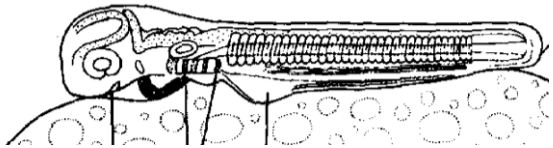

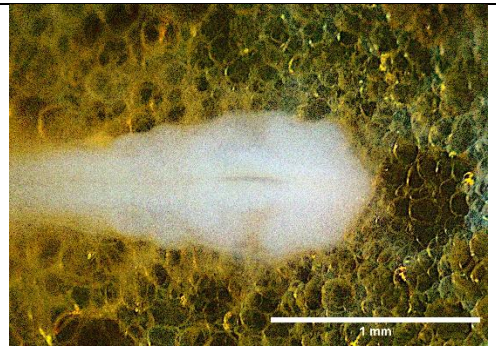

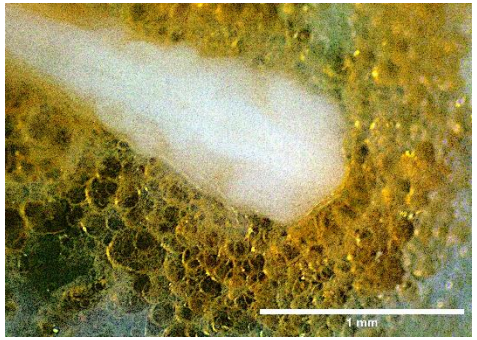

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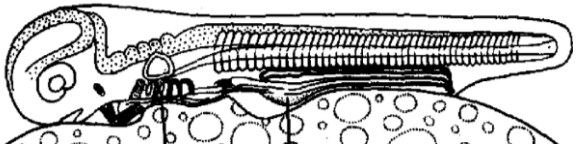

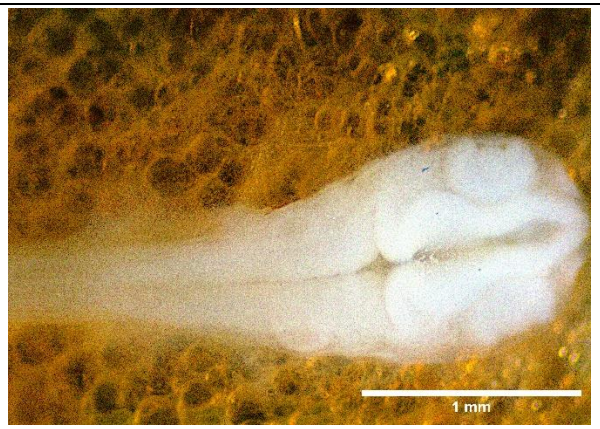

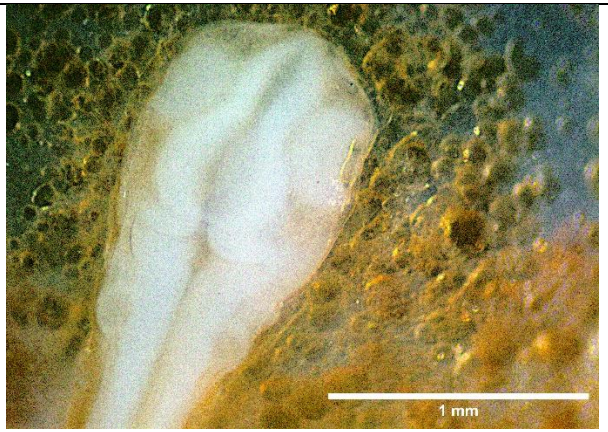

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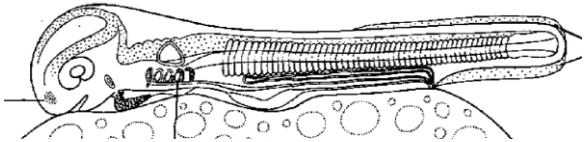

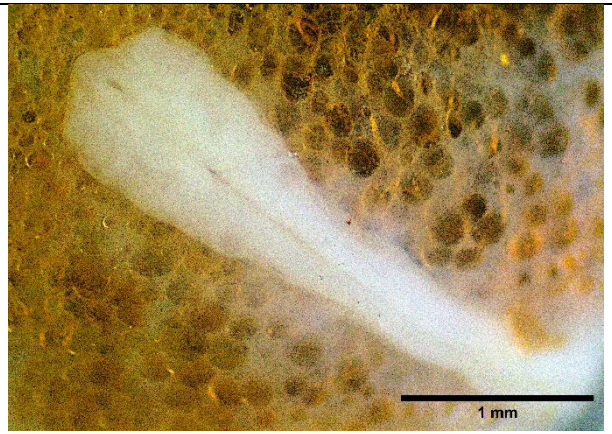

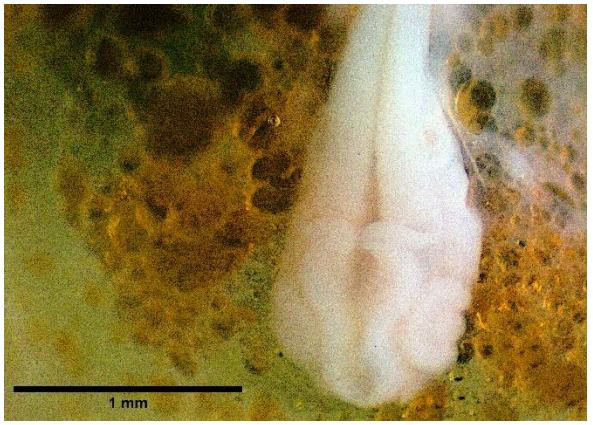

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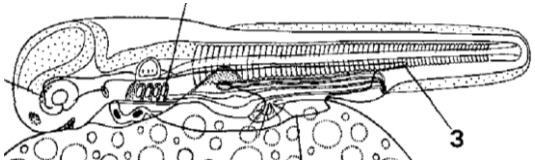

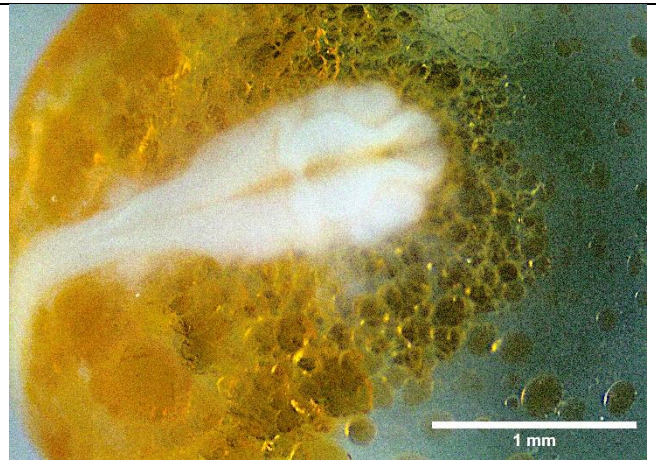

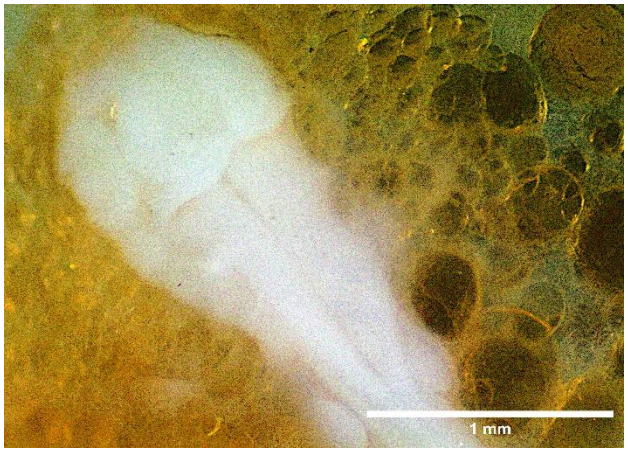

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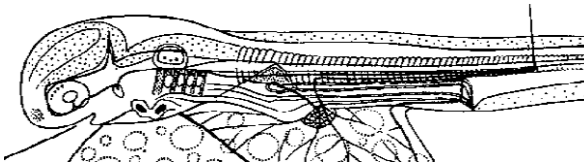

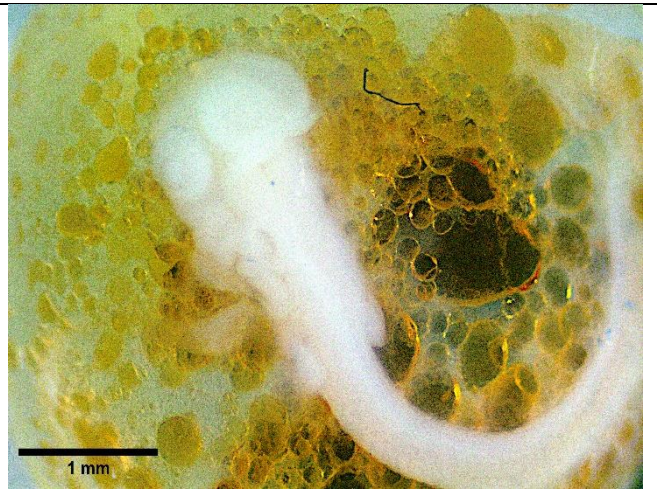

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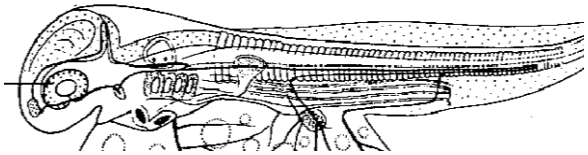

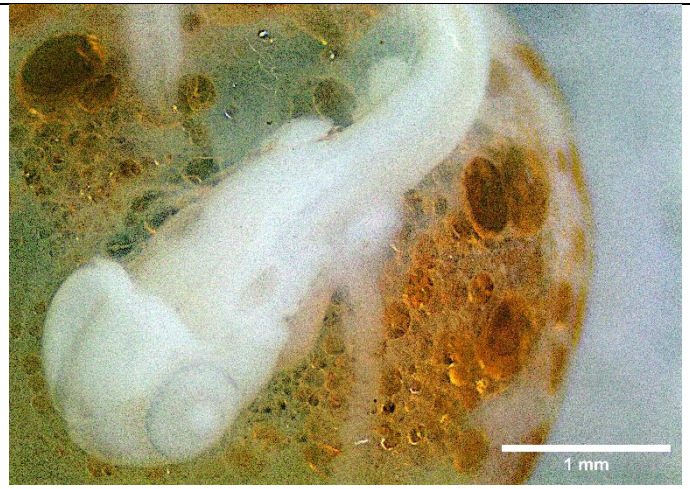

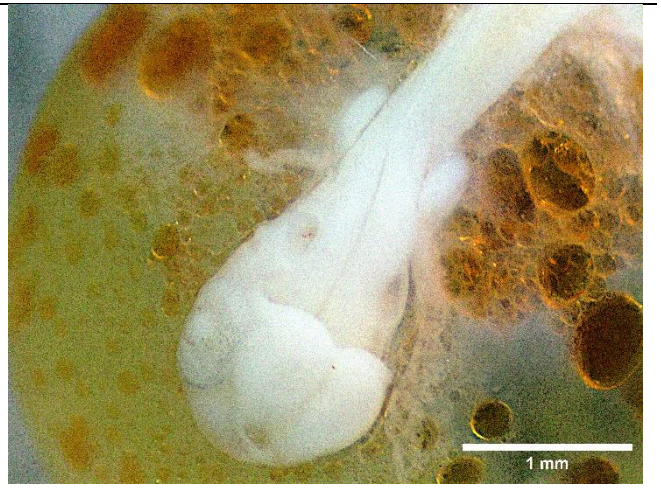

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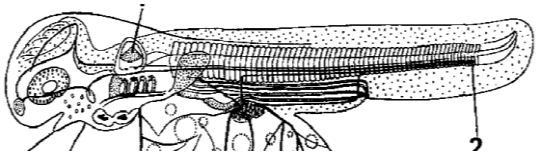

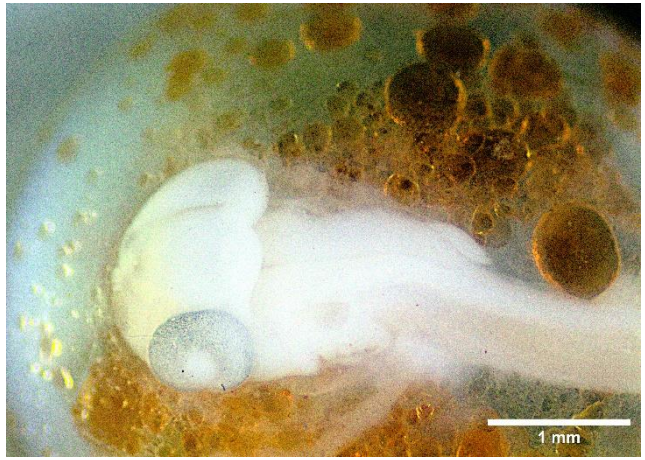

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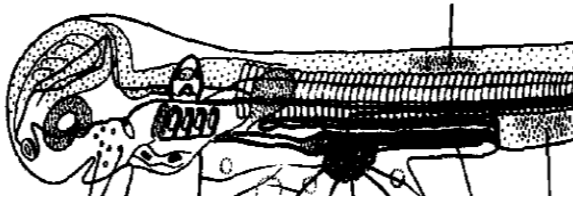

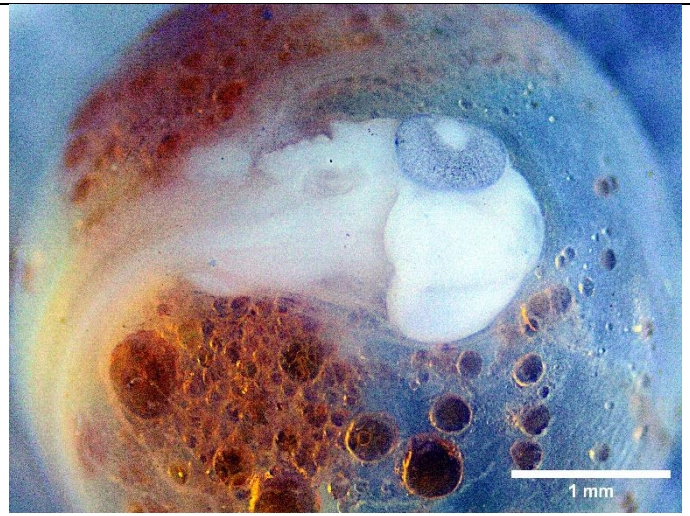

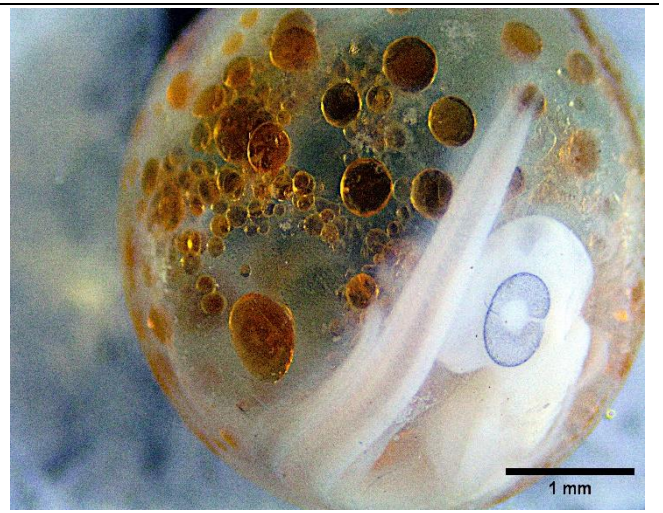

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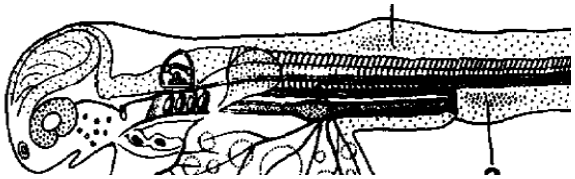

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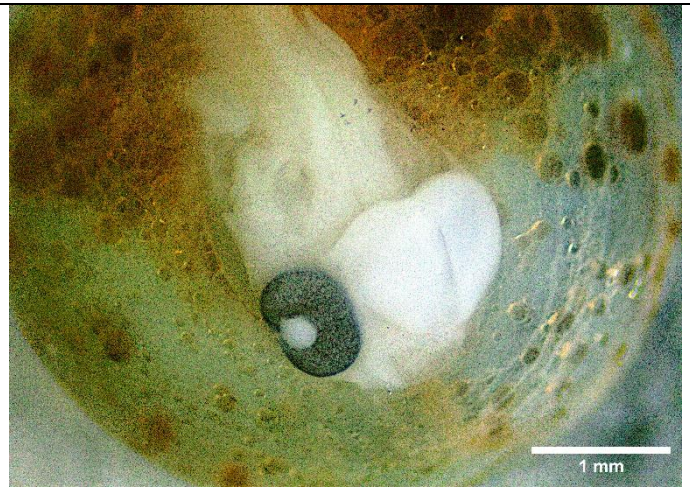

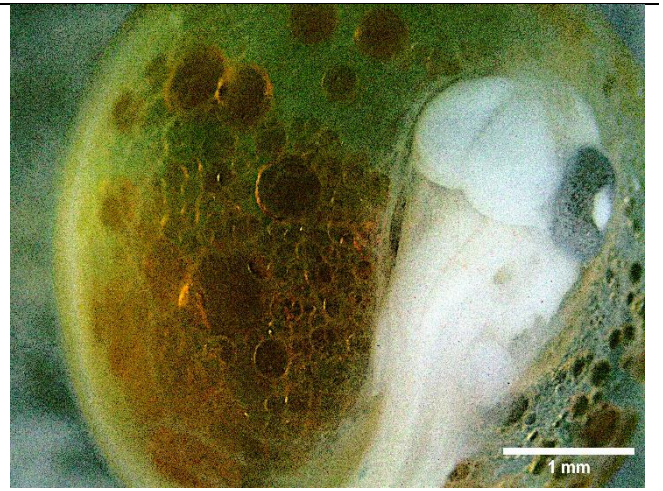

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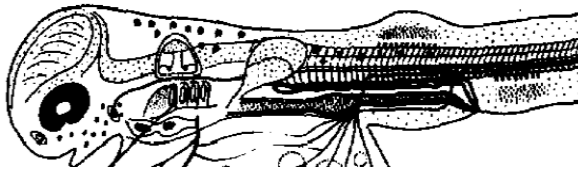

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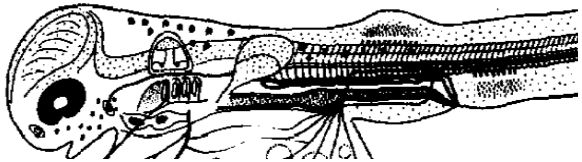

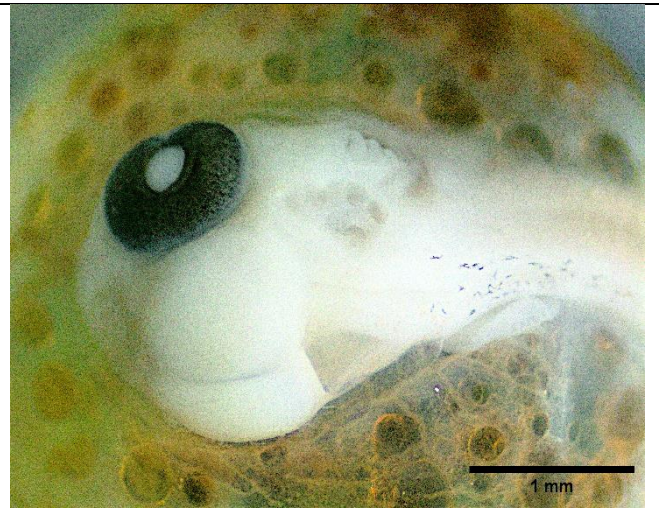

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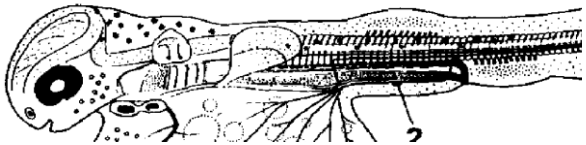

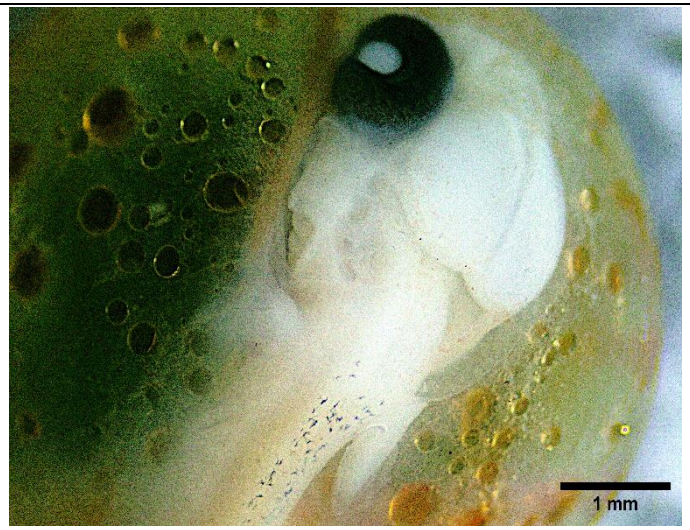

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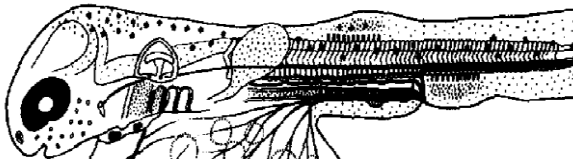

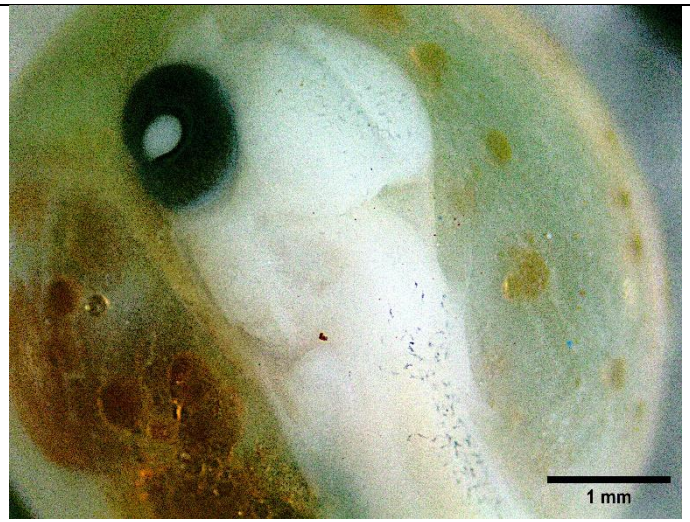

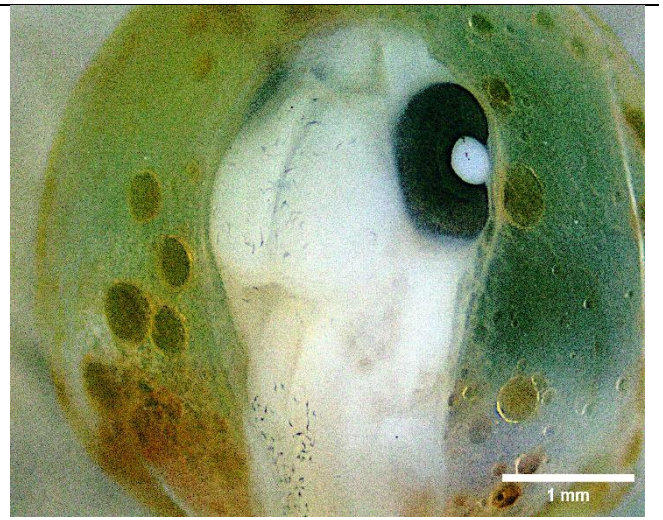

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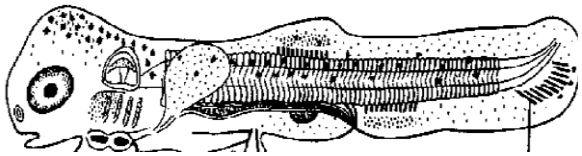

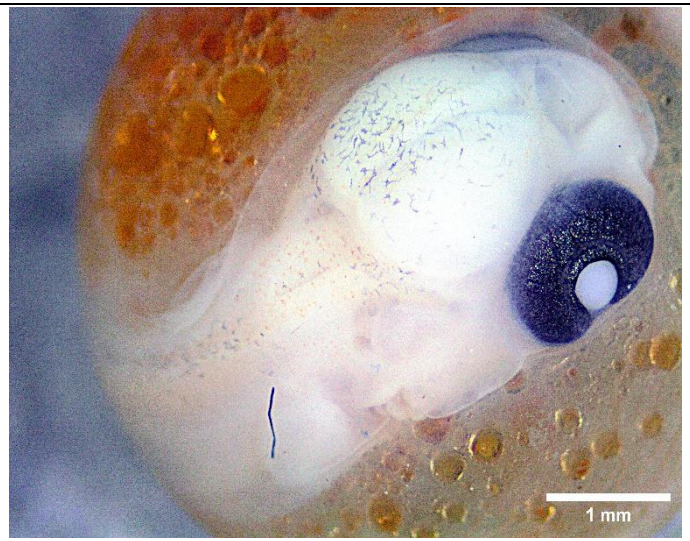

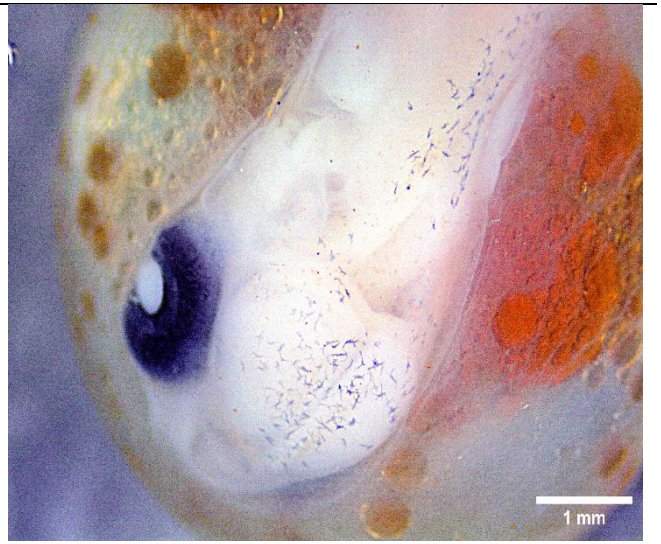

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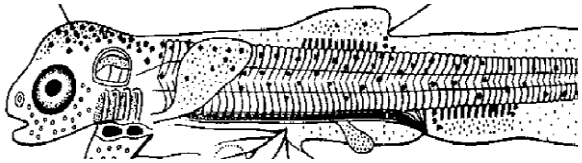

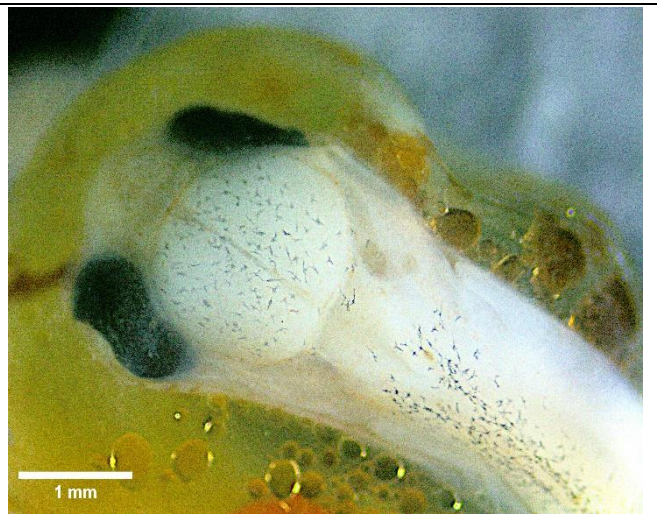

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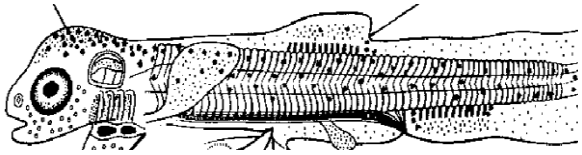


Once all the photographs had been assigned a stage of embryogenesis, they were compared to determine if there was a difference between ploidy. No significant differences were found at any sampling point up until hatching began (figure 3.2).

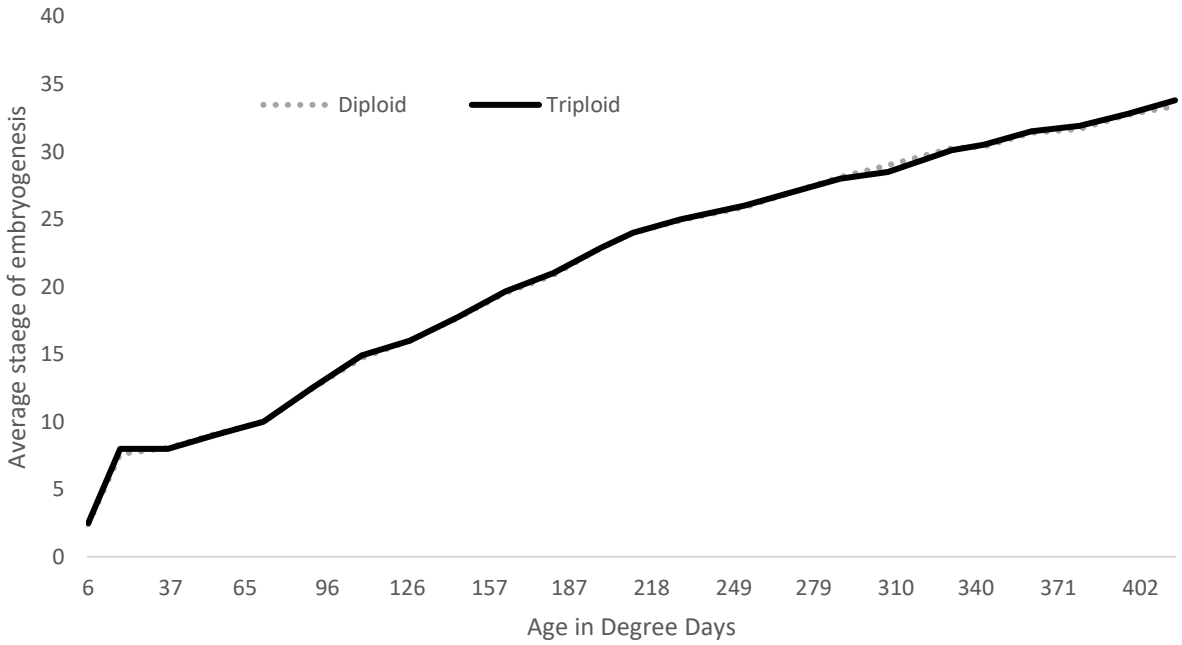


Figure 3.2. Mean stage of embryogenesis between ploidy. Stages as determined by Gorodilov (1999).

There was a significant difference between average cumulative mortality between ploidy (U=1445, p = <0.001). The upper two troughs of eggs within the incubator showed 7.59% and 9.03% end of experiment mortality for diploid and triploid respectively whilst the lower two trays showed 14.27% and 30.76% respectively. The average for diploids was 10.93% and for triploids it 19.89% by the end of the experiment.

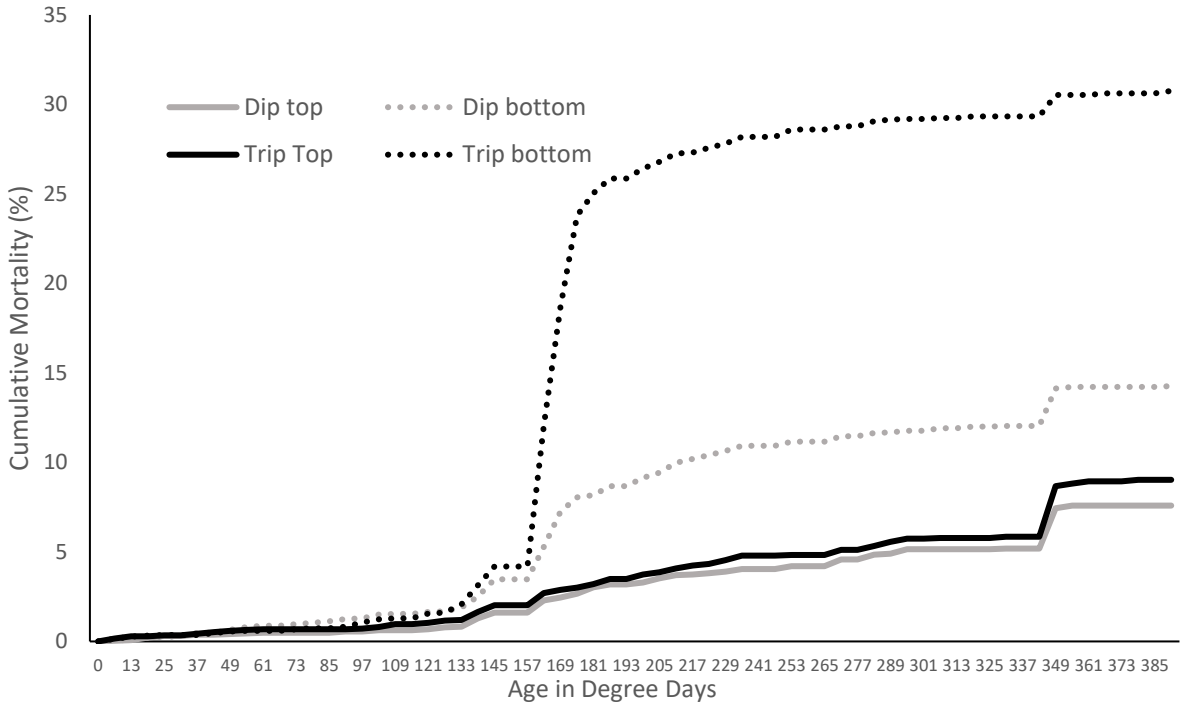


Figure 3.3. Cumulative Mortality (%) by day of triploid and diploid Atlantic salmon (*Salmo salar*). Eggs incubated at 6°C.

The large increase in mortality in the triploid bottom trough corresponds to the fungal infection mentioned earlier. The impact of this can also be seen in the diploid bottom trough, although to a lesser extent. All 4 troughs showed a slight increase in mortality around 133 degree days (DD). The top 2 troughs which did not experience a fungal outbreak showed a gradual increase in mortality from this point until 349DD, this corresponds to the fact that at 337DD all treatments were physically shocked to remove unviable eggs (this is standard practice).

Due to the fungal infection in the lower troughs causing an increase in mortality and pin head embryos, these were not included in the hatching data, and all results pertain only to the upper troughs. The fact that, as alevin, it was impossible to completely separate each of the egg trays in the trough the number of replicates for hatching was reduced from 3 to 1, as the whole trough was counted together. Whilst there was no significant difference between ploidy in their development during embryogenesis, there was a difference in the pattern of hatch. Diploids experienced the first hatch with one hatching on 426DD. After this point the number of triploids hatched rose more rapidly than the diploids, and over the next few days the

cumulative hatch of triploids was higher than that of diploids (Figure 4). On the 4th day of hatch (444DD) 15 % of total triploids had hatched whilst only 4 % of diploids had hatched. Diploids began to catch up after this point, although by the 8th day (468DD) 48 % of triploids had hatched compared to 40 % of diploids. The gap closed after this point, and both ploidy reached 100 % hatch on the same day. The hatching distributions for the two ploidy to 50 % hatch (figure 3.4) were statistically significantly different, $\chi 2$ (1) = 12.141, p < .0005 (n=1). There was no significant difference in time to 100 % hatch.

3.4. Discussion

The aim of this study was to visually compare the rate of embryogenesis between triploid and diploid Atlantic salmon. The first step in this process was validating the most effective clearing solution. We compared the use of 6 different clearing solutions in their ability to effectively clear the egg of a salmonid and allow for a clear photograph to be taken of the embryo inside. Whilst not all solutions allowed for clear photos to be taken, they were all successful to some degree.

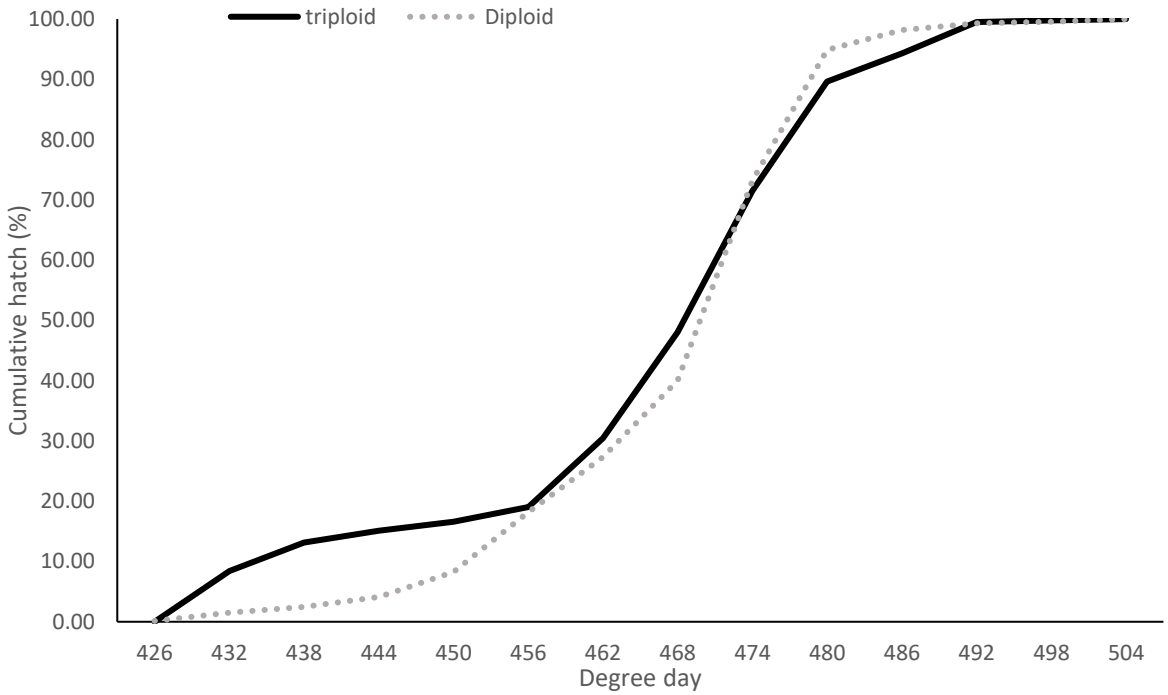


Figure 3.4. Cumulative hatch (%) by day of triploid and diploid Atlantic salmon (*Salmo salar*). Eggs incubated at 6°C.

All solutions whitened the embryo and would most likely all be appropriate for fertilisation confirmation by making the neural tube visible. In this regard, the cheapest or most readily available solution could be used. They were not, however, all appropriate for the purposes of this experiment which was to clearly see changes in embryogenesis. For this aim, the egg must be cleared to a high degree and the integrity of the egg must not be compromised. For example, whilst both of the methyl salicylate solutions did a reasonable job at clearing the egg they also weakened the chorion, this caused the shape of the egg to deform when moved and prevented clear photographs being taken. The solution that produced the clearest photographs was a 6:3:1 ratio of ethanol: formalin: glacial acetic acid. This solution is often referred to in the literature as EFA, AFA, Serr's liquid, Serra, and Serra's fixative and has been successfully used in the past to clear the eggs of Carp (*Cyprinus carpio*) (Brzuska, 1979; Epler et al., 1987), Goldeneye (*Hiodon alosoides*) (Pankhurst, 1985), and nine other freshwater species (Stoeckel & Neves, 1992). EFA was the most effective when it came to clearing the egg whilst also allowing the eggs to remain stable enough that they could be kept up to an hour before photos were taken. Other solutions either failed to properly clear the egg, resulted in cloudiness overtime, or degraded the chorion such that the structure of the egg was no longer viable. The optimal duration for submersion in the clearing solution was 20 minutes, submersion for 10 or 30 minutes produced results comparable to 10 minutes in a solution of only ethanol : acetic acid (3:1 ratio), this solution is less toxic than EFA and requires less in terms of safety measures. Depending on the objective when clearing the eggs this solution may provide a viable alternative to EFA, and indeed was not significantly different in terms of its efficacy, but for the purpose of this experiment clarity of pictures was above all the most important factor and therefore EFA was used. Staining or histology may have produced more precise images of embryogenesis but the aim of the experiment was to compare photos to the drawings created by Gorodilov (1996) and therefore the same methodology was employed.

There was no significant difference between the rate of embryogenesis of triploid and diploid Atlantic salmon (*Salmo salar*) up until hatch. The average stage of development, as based on work by Gorodilov (1999), did not differ significantly between ploidy at any time point. This is evidence towards the fact that despite

triploidy effecting a wide variety of physiological features, it does not seem to affect the rate of embryogenesis.

Even at the early developmental stages of 2 and 4 cells, there was no difference between ploidy, although it should be stated that the resolution with which these early stages were observed could have been improved (samples were taken every 3 day). Johnston (1997) concluded that there was no difference in the rate of somitogenesis between ploidy, and this experiment shows that that is also the case before and after somitogenesis.

The fact that we failed to observe any differences between ploidy during embryogenesis is of interest given the variety of differences seen between ploidy later in life. Transcriptomic analysis during embryogenesis would shed more light onto potential differences during development, but ontogenic development during embryogenesis does not appear to be affected by ploidy.

Whilst the point at which an embryo reaches a specific stage during embryogenesis does not seem to differ between ploidy, there was a difference in the pattern of hatch. Earlier hatch in triploids has also been observed in triploid Rainbow trout (Happe et al., 1988; Quillet et al., 1988), in both cases only by a small number of degree days. This suggests that there may have been subtle differences in development at least at later stages of embryogenesis, although these did not translate into full developmental stage differences. It was suggested that the mitotic rhythm of triploids may be lower due to the larger cell size (Quillet et al., 1988), but that lower number of cells per organ (van de Pol et al., 2020) could reduce the number of mitotic cycles required during embryogenesis and a balance of these two factors could lead to a slight increase in embryonic rate in triploids (Quillet et al., 1988). Whilst this does raise an interesting question, we did not see a difference in embryonic development rate in either the current study or that by Johnston et al. (1999). Another possible explanation is the increase in heterozygosity. Both rainbow trout and Atlantic salmon have shown a positive relationship between heterozygosity and developmental rate, after hatch (Blanco et al., 1998; Koljonen, 1986), pre-first feeding (McCarthy et al., 2003), and timing of hatch (Danzmann et al., 1986; Ferguson et al., 2011). Triploid Atlantic salmon, having inherited an additional set of maternal chromosomes, are more heterozygous than their diploid counterparts

(Allendorf & Leary, 1984; Benfey, 1999). It is not a simple case of greater heterozygosity equals faster growth, and if the genes in question act additively then a homozygote would develop faster but if genes related to developmental rate act dominantly then the heterozygous individuals would develop faster (Danzmann et al., 1986), and that does seem to be the case in these situations.

The mortality data shows a low level of mortality until 133DD (approximately 27 somite pairs) at this point all troughs showed a slight increase in mortality. At 157DD (approximately 40 somite pairs) all troughs once again showed an increase in mortality, this is especially apparent in the 2 lower troughs. The reason for this increase coincides with a fungal outbreak in the lower troughs. What is interesting is that an increase in mortality was also observed in the upper troughs, in which no sign of a fungal outbreak was observed. There are several potential explanations as to why this may be the case. It is possible that fungal spores also killed eggs in the upper troughs even though there were no visible signs of infection were present, and the upper trays were not treated with chemically. Another possibility is that eggs at this stage of development are more prone to mortalities, a previous study found that slightly over-ripe eggs suffered an increase in mortalities at this stage of development (Taylor *et al*. 2011)These stages are key developmental stages during somitogenesis, with the time around 133DD being the time at which neuromeres are formed in the hindbrain, as well as the heart tube appearing and the lenses begin to form (Gorodilov 1996). Minor deviations in these key processes could result in the small increase in mortality observed here. Around 157DD is the point at which the gill region forms segments, whilst the heart tube bends, and tactile stimulation will induce contractions in the pre-heart (Gorodilov 1996). Again due to the important nature of these developments it is possible that it is the reason it is at this stage at which we again see an increase in mortality. That being said, the large increase seen in the bottom troughs is evidently due to the fungal infection, and due to the fact that all troughs were in the same incubator, it is impossible to rule out the impact of the fungal infection on the upper troughs also.

Apart from these two peaks the level of mortality appears to be fairly stable, increasing at a low, but steady rate until the eggs were manually shocked. This is a trend previously observed in Atlantic salmon (Taylor *et al.* 2011). This shock caused all remaining unviable eggs to whiten. After these eggs were removed, mortality did

not increase until hatch. Despite the problems surrounding the fungal infection, it is unlikely that the experiment was compromised as the mortality observed in the study did not differ significantly from the literature. Clarkson *et al.* (2020) observed a mortality of 16.4% ± 19.2 for diploids and 22.3 ± 21.9 for triploids at 6 °C from fertilisation to 400DD. The highest mortality observed in the current experiment was 30.8 % ± 3.9 for the triploid group in the lower trough, also affected by fungal infection was the lower diploid trough which showed mortalities of 14.3 % ± 3.1. The two top troughs had average mortalities of 9 % ± 3 and 7.6 % ± 0.4, respectively for triploid and diploid, considerably lower than the rate in Clarkson *et al.* (2020).

There is a clear and significant difference between the rate to 50 % hatch in diploids and triploids. Whilst in this experiment both diploids and triploids completed hatching on the same dates, the manner in which they hatched differed, with triploids hatching more rapidly and reaching 50 % hatch earlier than the diploids. What makes the pattern of hatch important, and more specifically; the pattern to 50 % hatch, is that this characteristic is used to predict when first feeding will occur in fish. The onset of exogenous feeding is thought to be one of the most critical stages in the life of a young fish (Jin et al., 2019; Solberg et al., 2014; Yúfera & Darias, 2007). Delaying feeding past the point of yolk-sac absorption can cause an increase in mortalities and decreased size (Koss & Bromage, 1990), that being said, the window in Atlantic salmon is rather large, evidence suggesting that one may be able to delay initial feeding 1-2 weeks without seriously affecting survival or growth rate (Koss & Bromage, 1990). Feeding too early is also linked to increased mortality (Hurley & Brannon, 1969 as cited in Hansen, 1985 and Koss & Bromage, 1990). One limitation is the use of cumulative hatch without replication, in hindsight the tank system would have been designed in a way to prevent embryos moving between trays and the number of replicates could have been increased. Another limitation is that if for some reason both sets of ploidy were developing at abnormal rates then this would not be picked up with out a set of eggs from another source, this is a minor issue.

Whilst a short term delay in feeding may not be enough to impact growth and survival in diploids, this has not been investigated in the historically more sensitive triploids, nor has the effect of short delays in feeding on deformity later in life. Delayed fish have been shown to have a higher specific growth rate once fed (Twongo & MacCrimmon, 1976). Any increase in growth rate within triploids could

result in an increase in vertebral deformities, with faster growth rate being linked to an increase in vertebral deformities (Leclercq et al., 2011; Taylor et al., 2012, 2014).

Whilst the size of the fish at hatch was not investigated in this study, triploids have been shown to hatch smaller than diploids (Taylor et al., 2011). Typically, they subsequently catch up to, and sometimes, overtake the diploids in size before the end of the freshwater phase (Fjelldal & Hansen, 2010; Fraser et al., 2013; Nuez-Ortín et al., 2017; Taylor et al., 2012). Whilst a differential pattern of hatch has not been reported previously experiment, the smaller size at hatch could be linked to the trend seen in this experiment, although it is difficult to say if they would hatch sooner because they hatch smaller, or if they hatch smaller because they hatch sooner. Given the difference in pattern of hatching observed in this experiment, it would be interesting to explore this trend further.

Crips (1981) and Kane (1988) observed the relationship between temperature and the timing to hatch time of Atlantic salmon. It was shown that the use of 500DD as a good estimate of hatch timing is only useful to around 5 °C. Below this temperature it takes less degree days to hatch. This has also been found in industry, with salmon kept at 1 °C taking around 200DD to hatch (S. Kjøglum, personal communication). A difference to 50 % hatched was found in the current experiment but it would be interesting to observe if differences in hatch rate were found between ploidy at lower temperatures, especially given the fact that triploids are more sensitive to suboptimal incubation temperatures.

In conclusion, no differences in the rate of embryogenesis between diploid and triploid Atlantic salmon were observed. However, there was a difference in the pattern of hatch. There was no significant difference in the mortality between ploidy up until hatch. This study expands on previous studies and confirms that the rate of embryogenesis does not differ between ploidy. Results of this nature, whilst grabbing fewer headlines, allows future experiments to be conducted in the confidence that treatments during embryogenesis will be conducted at the same developmental stage on both ploidy. The differential pattern of hatch poses interesting questions as to the reason behind the faster hatch rate in triploids.

Chapter 4. Impact of early temperature regimes on egg development and juvenile performance in diploid and triploid Atlantic salmon (Salmo salar) siblings

Abstract

Triploid Atlantic salmon require lower incubation temperatures than their diploid counterparts to prevent them suffering from a higher range of deformities. Much less studied is the effect resulting from a short term thermal shock during incubation. In this study, triploid and diploid embryos were taken from 6 °C to 10 °C and back again at 360 degree days, with shocks conducted once for 1 hr, once for 6 hrs, or 5 days in a row for 1 hr at a time. There was no significant treatment effect on deformities (e.g. vertebral, aplasia of the *septum transversum*, lower jaw, cataracts) or on mortalities. The triploid group in this study performed better than many previous studies, which is suggested to be related to their incubation at 4 °C until 350 degree days than 6 °C until hatch. One factor that did change due to the thermal shock was growth during thermal challenge. Once the fish reached 50 g in weight, they were subjected to a thermal challenge at 16 °C. The 6 hr triploid group showed a significantly higher weight than the control group after this challenge but not before. The diploid group showed a numerically higher weight and better FCR and specific growth rate, although these were not significant. This experiment shows the first indication that early life shocks can increase thermal tolerance in Atlantic salmon and also shows the ability of triploid Atlantic salmon to tolerate thermal shocks early in life.

4.1. Introduction

Two of the issues yet to be fully resolved regarding the husbandry of triploid salmon are the impact of supraoptimal thermal regimes during incubation and the tolerance of high temperatures and low oxygen after hatch.

There is strong evidence that deformities can occur in significant numbers in triploids that, as eggs, were incubated at temperatures that are suitable for diploids (Clarkson et al., 2021; Fraser et al., 2013, 2014, 2015; Sadler et al., 2000; Takle et al., 2005).

The typical incubation temperatures for diploid Atlantic salmon is 10 °C but at this temperature triploids suffer increased rates of spinal, lower jaw, and heart deformities (Clarkson et al., 2021; Fraser et al., 2014, 2015). These issues decrease in prevalence at temperatures <8 °C, with a further reduction when incubated around 6°C (Clarkson et al., 2020; Fraser et al., 2015). Whilst similar issues also occur in diploids at higher than optimal temperatures (Ytteborg et al., 2010), the reason as to why triploid salmon require lower incubation temperatures is not clear. What is clear, is that more research is needed to determine the optimal temperature at different stages of development and incubation, and to also explore the impact that short term fluctuations may have. All aquaculture systems including modern RAS are fallible, in order to make informed management decisions it is important to know the possible impact that short term fluctuations in incubation temperatures can have upon the future health of the stock. Performance is not just limited to growth and deformities, and immune response is key to the fitness of stocks. Disease is a major threat to salmonid aquaculture, at survival to viral diseases is strongly linked to the performance of type I interferon production (IFN) (Dahle and Jørgensen 2019). Poly I:C, or Polyinosinic:polycytidylic acid, is a type of PAMP (pathogen-associated molecular pattern). Poly I:C is a synthetic analogue of double-stranded RNA (dsRNA) and acts as an immunostimulate. The ability of Poly I:C to promote inflammatory cytokine production and induce IFN activity (Matsumoto & Seya, 2008), has resulted in its use in a wide variety of immune response experiments in Atlantic salmon (Ignatz et al., 2020; Jensen et al., 2002; Lockhart et al., 2004; Reyes-Cerpa et al., 2011; Robertsen et al., 1997). Both the Mx (Ignatz et al., 2020; Jensen et al., 2002; Lockhart et al., 2004; Robertsen et al., 1997) and LGP2 genes (Chang et al., 2011; Ignatz et al., 2020) have been used successfully in studies using Poly I:C to investigate the immune response in salmonids. In combination, these two genes can provide an insight into the mechanisms around IFN immune response. LGP2 (laboratory of genetics and physiology 2) is thought to activate IFN in response to viral PAMPS (Chang et al., 2011), whereas Mx expression is induced by IFN (Collet & Secombes, 2001; Nygaard et al., 2000; Verhelst et al., 2013) and serves the general role of anti-viral activity with the mechanisms of said activity being specific to species and isoform (Lee & Vidal, 2002; Verhelst et al., 2013).

Triploids reduced ability to regulate gene expression under stressful conditions (Ching et al., 2010) may be one of the reasons why they appear to be less tolerant of high temperatures in later life, especially when high temperatures coincide with low oxygen, which typically happens due to the link between water temperature and oxygen saturation levels (Wetzel, 2001). Additional factors such as differences in heart morphology (Fraser et al., 2015; Leclercq et al., 2011), erythrocyte size and frequency (Peruzzi et al., 2005) and gill filament density (Leclercq et al., 2011; Sadler et al., 2001) may also play a part. If the thermal tolerance of triploids cannot be improved, then this may limit the possible geographical distribution where these fish can be farmed, especially as global warming increases the level and duration of the highest summer temperatures (De Silva & Sotto, 2009). Two ways in which it may be possible to increase heat tolerance of salmon include genetic engineering and conventional genetic selection (De Silva & Sotto, 2009; Wang *et al.* 2019). Both these approaches would be expensive and take many years before gains would be seen. Breeding programs have the problem that heat tolerance is not typically recorded as a trait, and adding it to the list of selected traits in a current breeding program would not be without its difficulties. Genetically modified fish are banned over almost the entire globe, in addition, there is still widespread public mistrust and an unwillingness to eat GM/GE fish.

There is increasing evidence to support the possibility that programming may be possible. This comes from the fact that Zebrafish (*Danio rerio*) can be programmed to be more tolerant of hypoxia through parental exposure suggesting an epigenetic component (Ho & Burggren, 2012). Similarly. Atlantic salmon have been programmed to tolerate vegetable based diets by adopting these diets at the earliest possible opportunity (Clarkson et al., 2017; Vera et al., 2017), suggesting that early life experiences can change the epigenome of the fish. Within Atlantic salmon, studies are beginning to examine the role of early life stress on development and potential preparation for future stress events.

Kelly et al. (2020) showed that hypoxia during embryogenesis changes DNA methylation and the upregulation of hypoxia response genes. Whilst embryos (250-450DD) subjected to a 5x repeated shock of 7°C to 0.2°C for 1 min followed by air exposure (15°C) for 1 min before returning to 7°C, showed differential expression of a large number of genes related to development as well as changes to the

methylome (Moghadam et al., 2017; Robinson et al., 2019). Fish treated with shocks during embryogenesis and post-hatch showed differential expression of genes related to stress response immediately after being faced with stress of a similar nature later at 800DD (Robinson et al., 2019). These results are promising and show strong evidence of the potential of early life experiences to change the epigenome and potentially the response of Atlantic salmon to stress. Unfortunately, these experiments failed to investigate if these transcriptomic and epigenetic changes resulted in improved performance when faced with a stress event. Uren Webster et al. (2018) did show however that a cold shock during embryogenesis can improve tolerance to a pathogenic challenge in addition to altering the epigenome.

If early life thermal shocks do indeed increase the tolerance of these fish to higher temperatures later in life, this will not only increase the possibility of industry acceptance of triploid salmon, but also increase the areas in which both triploid and diploid salmon can be grown over the world. It also opens up the fascinating possibility that other forms of programming could be used to alter the phenotype of aquaculture species without the need to alter the genome through GM, editing or expensive breeding programs.

The aim of this experiment is to investigate the effect of fluctuations in temperature during egg incubation on the health and performance of triploid and diploid Atlantic salmon. Specifically, the objectives of the study were 1) to establish if thermal shocks at the eyed stage impacts the 1) growth and performance (including immune response) of Atlantic salmon during the freshwater phase, 2) temperature tolerance of the salmon at a later developmental stage when challenged.

4.2. Methods and Materials

4.2.1 Experimental setup

On 2019-11-04 at AquaGen Holywood facilities, Scotland, 7,200 Atlantic salmon (*Salmo salar*) eggs were stripped from a single female and split into two batches, then fertilised with milt from two males. After fertilisation the eggs were rinsed with 8 °C water and left to harden in a 8 °C water bath for half an hour. Triploidy was induced in one group (9500 PSI/655 BAR/65,500,000 pascal hydrostatic pressure for 6.25 mins. at 8 °C, 37 mins. Post-fertilisation) according to Smedley *et al*. (2016),

while the other batch was handled in the same way but did not receive a hydrostatic shock and were maintained as diploid controls. Eggs were then laid down in two different silos and incubated at 4 °C until the eyed stage reached at 357 degree days (DD) 2020-01-28. At this stage eggs were shipped on ice to the Institute of Aquaculture (IOA), University of Stirling, Scotland.

At the IOA, the eggs were sorted into 12 trays per ploidy (300 eggs per tray), each tray was placed into a 300L tanks running at 6 °C (24 tanks). The experimental shocks started 24 hrs later and consisted of gently moving the egg tray into a Tupperware box whilst still completely submerged in water, and carefully transferring the eggs to a trough of water running at 10 °C ($^{+}$/$_{-}$ 0.1 °C). The Tupperware was lowered into the trough and the 10 °C water allowed to cover the eggs. The eggs were kept in the trough for the allotted time (table 4.1) and then placed back into the tank in the same manner. Three different shocks were applied as described in table 1 including either a single shock of 1 or 6 hours and repetitive daily shocks of 1 hour for five days. There was a maximum of an hour difference in the start times between the first and the last trays. The trays which were shocked over repeated days were shocked at the same time each day. The control group was removed from their tank once in the same manner, and then placed back to replicate the stress of moving the eggs. After all shocks, the eggs were left at 6 °C until hatch.

Table 4.1. Outline of the shocks used – 3 trays per ploidy underwent each treatment + a control. Incubation temperature was 6 °C

Temperature	Duration	Number of days
10°C	1hr	1
10°C	1hr	5 (once per day)
10°C	6hr	1

Low levels of hatch were present from the 2nd day onwards (367 DD). Due to the fact that this consisted of very few individuals per tank per day, this was deemed premature hatch. Considering both welfare concerns regarding time to 1st feeding and concerns over the inclusion of these alevin from a scientific standpoint, they were recorded and removed daily until 427 DD (2020-02-08). From this point, hatched alevin were counted daily until 470 DD when the number of individuals in the tanks made this untenable, and estimates to total hatching were used instead. This was the date at which a jump in the number hatching per day indicated the start

of “true” hatch within the triploid. This occurred a day later in the diploids (476 DD). From this point alevin were incubated at 6 °C until observations on yolk sac absorption indicated that first feeding was imminent, at this point the temperature was increased from 6 °C to 12 °C, by 1 °C a day. From this point, the fish were incubated at 12 °C and grown until around 15 g (2020-04-22 – 2020-08-26). Bulk weighs were performed every month to ensure correct pellet size and feed ration was being given. At 15 g, runts were sorted out using an 8 mm grade in preparation for the feed intake analysis.

Initial feed intake assessment took place at 12 °C between the dates of 2020-09-14 and 2020-09-26. At this point sampling took place and before a set of tanks were sampled, fish were starved for 24 hrs to reduce the risk of regurgitation and welfare concerns, for this reason feed intake assessment was suspended for the 3 days of sampling and the 24hrs before sampling began. A total of 44 fish per tank were culled to maintain appropriate tank biomass in anticipation of increased growth during the upcoming temperature increase.

After all tanks were sampled the temperature was increased from 12 °C to 16 °C (1 °C per day). Feed intake assessment resumed as described above and ran from 2020-10-01 to 2020-10-22. Between 2020-10-26 and 2020-10-28, all tanks were sampled again.

After sampling was complete, the tanks were reduced in number prior to a Poly I:C challenge. All fish, with the exception of 15 per tank, were culled by overdose in benzocaine, death was then confirmed through an approved schedule 1 method. This was conducted over two days with all triploid tanks sampled on the 1st day followed by all diploid tanks on the 2nd day. Fish were then recovered for 48 hrs before the PAMPS challenge began.

Each tank was culled down to 12 fish per tank (the 15 were initially kept to ensure that 12 fish per tank survived until the start of the Poly I:C challenge). 6 fish per tanks were injected with 5 ug/g Poly I:C [Sigma-Aldrich, Burlington, MA, USA], the remaining 6 fish were injected with the same volume of PBS and marked on the abdomen using a PanJet. After 24hrs, fish were sampled.

4.2.2 Sampling

200 eggs were removed at 357DD, flash frozen on dry ice and placed into a -70°C freezer for subsequent triploidy verification (see Section 2.4) .

Feed collection took place twice daily between 2020-09-14 and 2020-09-26 and 2020-10-01 to 2020-10-22.

Between the dates of 2020-09-27 to 2020-09-30 after 13 days of feed intake collection sampling took place (one replicate/treatment/day) with 8 tanks being sampled on each day. 6 fish per tank were randomly removed and euthanized using an overdose of benzocaine, death was then confirmed through an approved schedule 1 method. Each fish was weighed and measured before a section of the frontal lobe of the liver was removed. This was placed into an Eppendorf containing RNA-Later for subsequent RNA extraction (the results of this RNA analysis will be discussed in another chapter). 44 fish were culled and x-rayed individually to assess radiological vertebral deformities. After sampling, the entire tank was bulk weighed.

Between 2020-10-26 and 2020-10-28 all tanks were sampled again (one replicate/treatment/day) as described above.

Following this sampling, all fish with the exception of 15 per tank, were culled in preparation for the Poly I:C challenge, all fish removed were individually weighed and measured. 20 fish per tank were dissected and the condition of the septum transversum was assessed for aplasia.

Following Poly I:C challenge 12 fish per tank were killed by overdose of benzocaine, weighed, measured and the frontal section of the head kidney was removed and preserved in RNALater.

4.2.3 Timeline of experiment

	04-Nov	-	28-Jan	29-Jan	30-Jan	31-Jan	01-Feb	02-Feb	03-Feb	-	16-Feb	-	14-Sep	30-Sep	28-Oct	02-Nov	04-Nov
Time days	0		86	87	88	89	90	91	92	-	105		317	332	359	363	365
Time degree days	0	-	357	363	369	375	381	387	393	399-464	470	-					
Average water temperature (°C)	4	4	6	6	6	6	6	6	6	6	6	12	12	16	16	16	16
	Fertilisation	Incubation	Ship to IoA	Thermal shocks	Thermal shocks	Thermal shocks	Thermal shocks	Thermal shocks	Sampling	Incubation	Hatch	Growth	Feed intake challenge starts	Feed intake challenge	Feed intake challenge ends	PAMPs challenge starts	PAMPs challenge ends
														Temperature increase			
Sampling			x		x				x				x		x		x

4.2.4 Triploid verification using microsatellites

Two hundred eggs from the triploid batch were verified using a microsatellite panel. Full details of the microsatellite panel can be found in the chapter 2.3.3. Due to issues with the external partner and outside of the control of staff at the IoA (described in the previous chapter) these eggs were not tested on the finalised suite and instead only 7 of the final microsatellites. Instead, these eggs were tested using a preliminary suite of microsatellites and returned a triploidy confirmation of 92.7%. That is to say 92.7% of individuals exhibited a trisomic state at, at least one loci. The true value of triploidy is possibly higher, but without the full suite it is not possible to be certain of this.

4.2.5 Feed intake assessment and performance metrics

During feed intake assessment the fish were fed over 2 periods, 00:01-09:00 and 11:00-15:00. A half hour window was left after the end of feed delivery to allow for remaining feed to be consumed. After this point, excess feed was siphoned from each tank, collected in a bucket and then passed through a sieve. Each sieve was rinsed briefly in running water to remove faeces, blotted with blue-roll to remove excess water and then weighed. This value was then converted into weight of given food using a conversion factor. The conversion factor was calculated by submerging 30 g of dry feed in triplicate in system water at system temperature for 8 hrs, this was then weighed and the increase in weight used as the conversion factor. A conversion factor was established at both 12°C and 16°C. Using this conversion factor the weight of uneaten wet food could be converted into weight of uneaten dry food. By subtracting this from the weight of dry food given to the tank it is possible to establish the total weight of feed consumed by the tank per day.

Specific growth rate by weight calculates the percentage increase in body weight (W) per unit time (T), in this case per day. As determined by:

$$[(ln\ W_2 - W_1)\ \times 100]\ /\ (T_2 - \ T_1)$$

Feed conversion ratio calculates the ratio at which feed consumed (F) (as described above) is converted to weight gained (W). As determined by:

$$F\ /(W_2 - W_1)$$

The Thermal growth coefficient is a model which used the relationship between temperature and growth rate to predict future growth with in fish species. As determined by:

$$TGC = [(\sqrt[3]{W_t} - \sqrt[3]{W_0})/(T \times t)] \times 1000$$

Where W_t = final weight, W_0 = initial weight, T=temperature and t= time in days

4.2.6 PAMPs challenge

RNA extraction

RNA was extracted from RNA-Later preserved head kidney samples using the TriReagent method. One millilitre of TriReagent [Sigma, St Louis, MO, USA] was added to approximately 100 mg of Head kidney in a 1.5 ml screw-cap tube [Alpha labs, Hampshire, UK] and incubated on ice for 30 mins, this was then homogenised in 1 ml of TriReagent using a beadbeater [BioSpec products, Bartlesville, OK, USA]. After 5 minutes at room temperature 100 µl of 1-bromo-3-chloropropane (BCP) [Sigma, St Louis, MO, USA] was added and the tube vigorously shaken for 15 seconds. Following 15 minutes incubation at room temperature the tubes were centrifuged at 20,000 xg for 15 minutes at 4°C. Four hundred microlitres of the upper aqueous phase was transferred to a new tube before adding 200 µl each of RNA precipitation solution (1.2 M NaCl and 0.8 M Sodium Citrate Sesquihydrate dissolved in nuclease-free dH_2O) and 100 % isopropanol (propan-2-ol) [Fluka, Buchs, Switzerland]. After inverting 5 times, the samples were incubated for 10 minutes at room temperature before centrifuging at 20,000 xg at 4 °C. The supernatant was removed, and the pellet washed for 30 minutes in 1 ml of 75% ethanol. The ethanol was fully removed before the pellet was re-suspended in 60µl RNase free water at room temperature for 1 hour.

The quality of the RNA was checked using nanodrop [labtech, East Sussex, UK], and gel electrophoresis, with 200 ng RNA being run through a 1 % agarose gel for 45 minutes at 100 V. RNA was quantified using Qubit Broad range kit [Invitrogen, Waltham, MA, USA] according to manufacturer's instructions.

Head kidney RNA was then retained for cDNA synthesis and qPCR. cDNA was synthesised using the QuantiTect Reverse Transcription Kit [Qiagen, Germantown, MD, USA] according to manufacturer's instructions, with 1µg RNA being used as the starting quantity.

RT-PCR

RT-PCR was to assess the immune related genes LGP2 and Mx for their response during thermal challenge, the reactions were performed in 96 well plates [Thermo fisher, Waltham, MA, USA]. Five microlitres of SYBR green Real-Time PCR mix [Thermo fisher, Waltham, MA, USA] was added to each well along with 0.5 µl of each forward and reverse primer (Table 4.2) at 100 pcmol, 2 µl RNase free water and 2 µl cDNA. Each plate contained samples in triplicate plus non-template controls in quadruplicate and a dilution series in duplicate of plasmids at known concentration starting at 2.6^{-8} – 2.6^{-1} copy numbers per µL for LGP2 (laboratory of genetics and physiology 2) and 2.07^{-8} – 2.07^{-1} for Mx (Myxovirus Resistance). Annealing temperature for both sets of primers was 60°C. Following 38 cycles, melt curve analysis was performed. The lack of housekeeping genes was justified through the use of quantitative qPCR using the input of known quantity in dilution series, plus the use of replication to account for variations in input and efficiency. A downside of this approach is the lack of normalisation for extraction efficiency however the use of housekeeping genes would only work well within groups and not between ploidy as was the aim in this case.

Table 4.2. Primers used during qPCR of Head Kidney samples

Primer Target	Forward Sequence	Reverse Sequence	Accession Number	Annealing temperature	Product length
Atlantic salmon LGP2	AACATCTGG AGAATAACC CTGGAG	AAATGAACCTTGT TGACCAGCAC	BT045378. 1	60°C	58
Atlantic salmon MX	GATGCTGC ACCTCAAGT CCTATTA	CGGATCACCATG GGAATCTGA	NM_00113 9918.1	60°C	72

4.2.7 Deformity assessment

Each fish was X-rayed individually using a mammography x-ray machine [Ultrafocus, Faxitron, Tuscon, AZ, USA] (exposure 2.00 mAs, 26kV, 0.4mA, 4.99s) and the image file viewed and manually annotated using MicroDicom software [MicroDicom, Sofia, Bulgaria]. Using the software each vertebrae was counted and visually assessed for abnormalities. When an abnormality was noted this was classified according to the diagrams and descriptions present in Witten et al. (2009), which contains details of the types and morphology of deformities in Atlantic salmon vertebrae. These were then independently verified to validate the findings. Fish were assessed head to tail and the vertebrae number at which deformities occurred was recorded alongside the type of deformity and the total count of vertebrae. Regional assessment was based on Kacem et al. (1998) with four main regions R1 (cranial trunk – Vertebrae (V) 1-8), R2 (caudal trunk – V9-30), R3 (tail – V31-49), R4 (tail fin – V50-60) being used to split the vertebrae into segments.

4.2.8 Statistics

All statistical analysis was performed using SPSS statistical software [IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp]. Results are reported as mean (±SEM). For each comparison triploids and diploids were compared between ploidy and then treatment differences were compared within ploidy separately. This was done to reduce type I error caused by comparing treatment differences multiple times between ploidy. The initial step in processing all subsequently mentioned data was to perform Levene's test for equality of variances. If equal variance was assumed, then in all but one case two-way ANOVA was used to determine if there were any significant ploidy or treatment differences. One-way ANOVA was used to compare deformity prevalence between regions and was followed by the post-hoc test Tukey HSD. If Levene's test for equality of variances determined unequal variance, Kruskal-Wallis was performed. Within ploidy treatment differences were compared to the control were tested using Independent T-test adjusting P value downwards using the Holm-Bonferrroni correction to adjust for multiple comparisons (Holm, 1979).

4.3. Results

4.3.1 Egg quality and early hatch

Before shipping, all eggs were sorted by automatic sorter at 357 DD, morphometrics and quality of eggs can be seen in Table 4.3.

Table 4.3. Results from Automatic egg sorting of Triploid and Diploid Atlantic salmon (*Salmo salar*) at 357DD.

	Triploid	Diploid
Alive (%)	88.08	84.14
Dead (%)	4.14	10.15
Unknown/pin (%)	7.78	5.71
Mean diameter (mm)	5.71	5.72
Max diameter (mm)	6.45	6.41
Min diameter (mm)	5.25	5.21
Eggs per litre	7667	7604

Early hatched alevins were removed until 426 DD. No significant treatment or interaction effects were found between ploidy, but there was a significant ploidy difference $F(1,16) = 13.456$, $p = .002$ (Figure 4.1). The average of each diploid treatment was significantly higher than each triploid counterpart. Within ploidy, there was a significant difference in the early hatch % between diploid 1hr repeated (Mean=7.78 % ±0.4) and the control (Mean=5.56 % ±0.11) with the repeated thermal treatment having a significantly higher instance of premature hatch compared to the control (Independent T-test $t(4) = 5.35$, $p=0.006$).

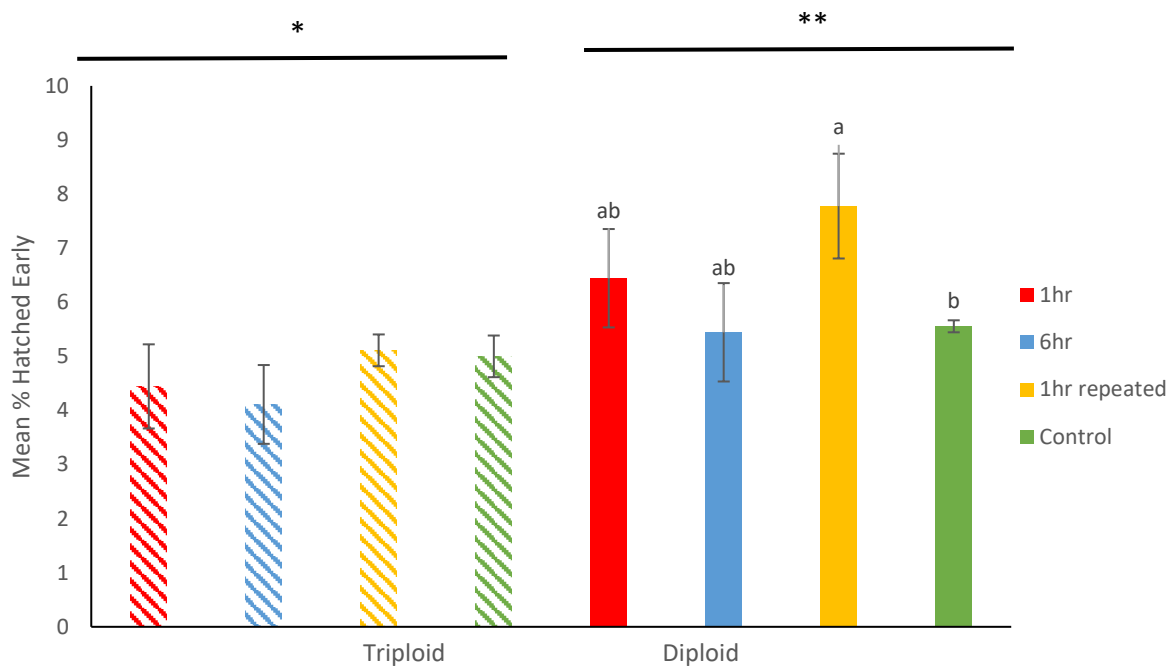


Figure 4.1. Mean hatching rate (up to 426 DD) of Triploid and Diploid Atlantic salmon (*Salmo salar*) exposed to thermal shocks. Data are presented as mean +/- SE (n=3 tanks). Superscripts denote significant differences between treatments within ploidy and not between ploidy (p=<0.05), Asterisks denote significant differences between ploidy (p=<0.05)

4.3.2 Mortality and unviable individuals

In addition to the removal of prematurely hatched alevin from the population, pin and runt fish were also removed. These were fish that were identified as being significantly undersized as alevin (pins) or as parr (runts) relative to the main population. There was no significant treatment effect, but there was a significant ploidy difference in the number of pins and runts removed (Kruskal- Wallis H=17.303, p= <0.001), with triploids having significantly more unviable individuals (Mean=16.72 % ±2.25) than diploids (Mean=3.06 % ±0.7) (Figure 4.2). Despite the high rate of pins / runts, there was no significant ploidy or treatment effect on mortality alone between egg arrival (357DD) 2020-01-28 and the end of the experiment 2020-11-06 (Figure 4.3).

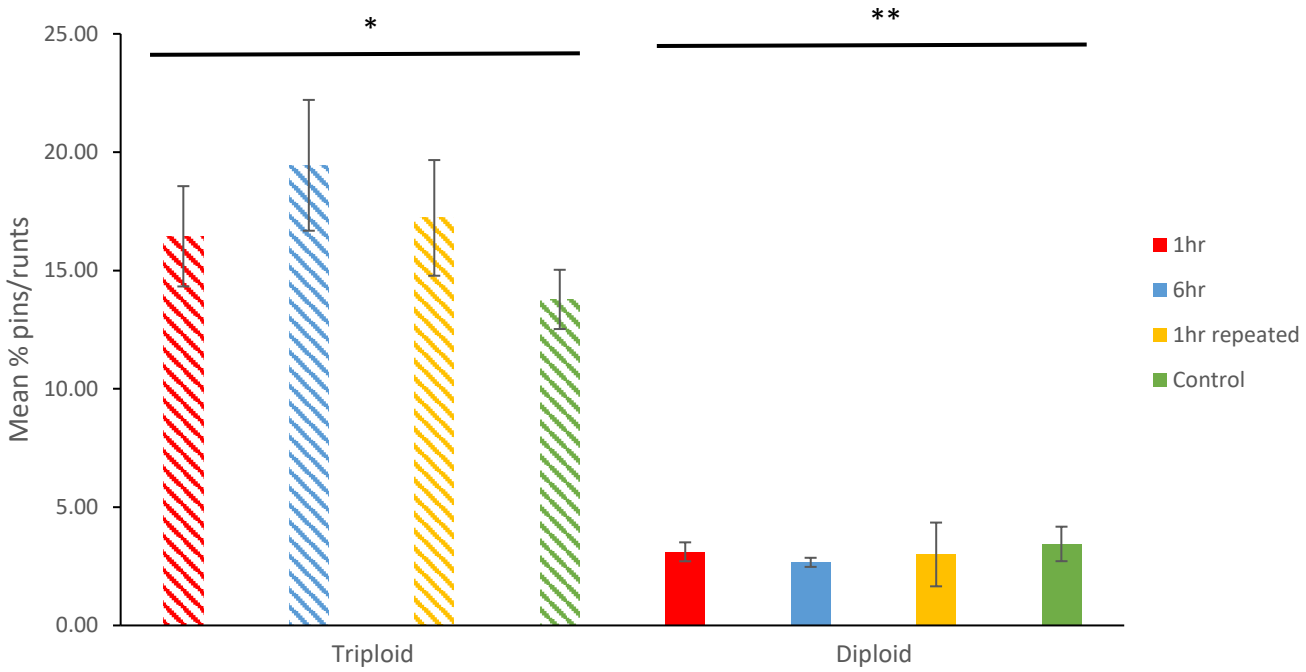


Figure 4.2. Mean percentage of fish removed as pins and runts of triploid and diploid Atlantic salmon (*Salmo salar*) per ploidy and thermal treatment. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (*p*=<0.05).

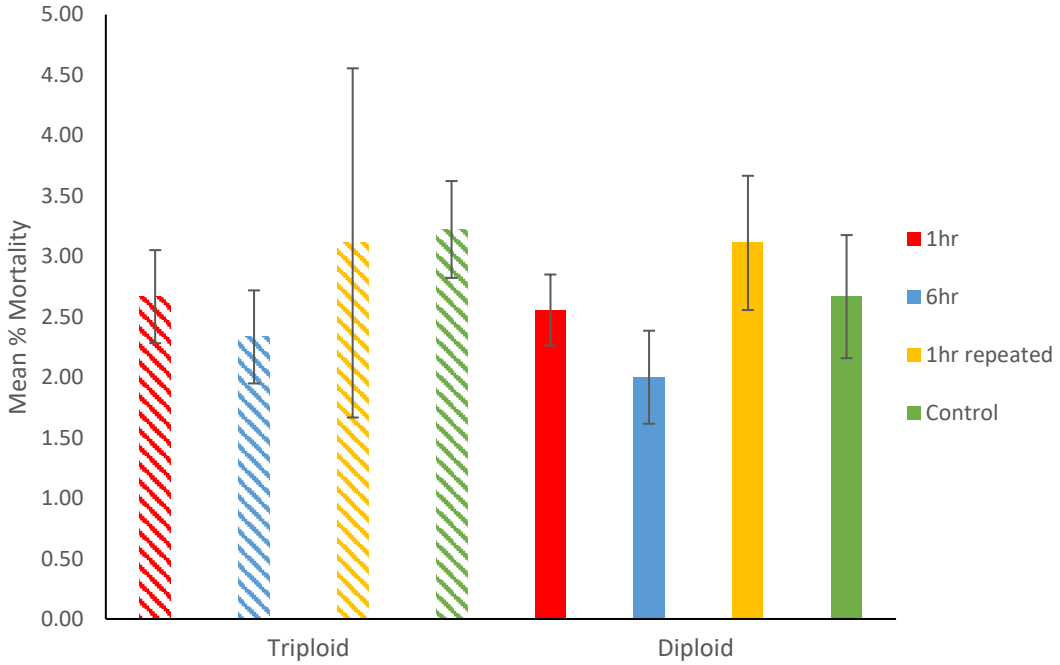


Figure 4.3. Mean percentage mortalities of triploid and diploid Atlantic salmon (*Salmo salar*) per ploidy and treatment between eyeing 2020-01-28 and experiment end 2020-11-06. Data are presented as mean +/- SE (n=3 tanks).

4.3.3 Weight and performance metrics

At first feeding there was a significant ploidy weight difference (Figure 4.4), with triploids (Mean=0.13 g ±0.007) being significantly lighter than diploids (Mean=0.15 g ±0.005) (Two-Way ANOVA: F(1,16) = 75.111, *p* = <.001). No treatment effects were observed on fish weight.

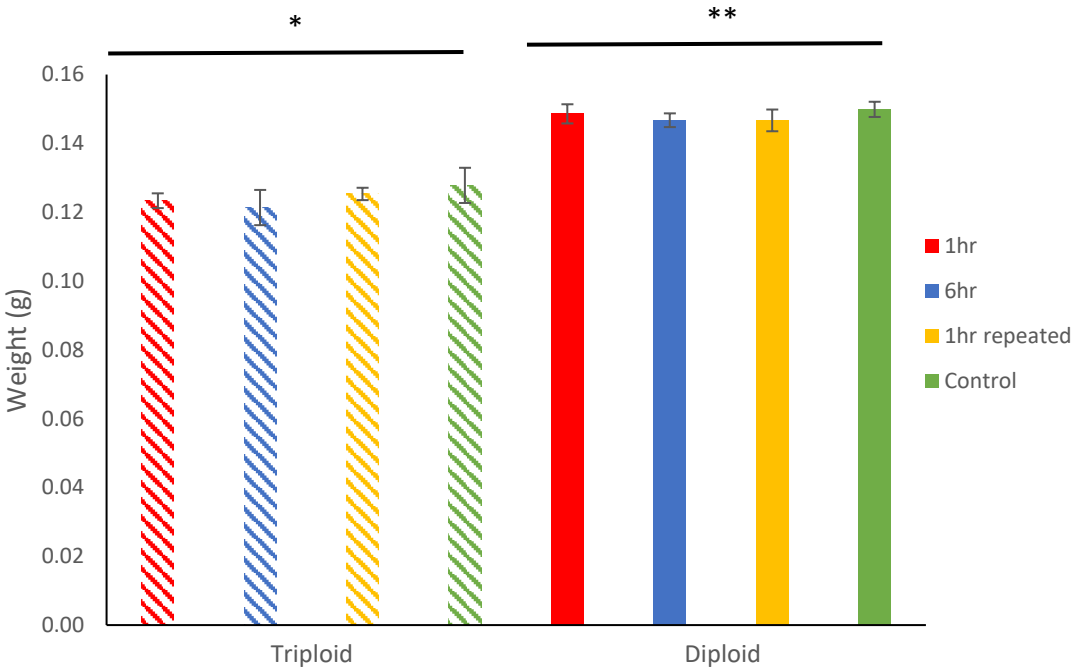


Figure 4.4. Mean weight (g) at first feeding (2020-04-22) of triploid and diploid Atlantic salmon (*Salmo salar*) per ploidy and treatment after bulk weigh. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (*p*=<0.05).

There was a significant weight difference between ploidy until 3 months post-first feeding (Figure 4.5). There was a lack of significant ploidy weight difference for less than 2 months after which point triploids were significantly heavier, this trend continued until the thermal challenge at which point the vast majority of fish were culled (Figure 4.6). There was no significant treatment difference at this stage although both 6hr treatments were numerically lighter than the controls. By the end of the experiment (Figures 4.5 and 4.7), triploids (Mean=64.24 g ±0.89) were on average more than 14 grams (27.96%) heavier than the diploids (Mean=50.21 g ±0.84) (Two-Way ANOVA: F(1,16) = 402.156, *p* = <.001).In both ploidy, the 6hr treatment fish were the heaviest on average at the end of the experiment, this was not significant in the diploids but was significant in the triploids (Independent sample T-test t(4)=5.805, *p*=0.002) this difference over the control was 4.3g (6.96%).

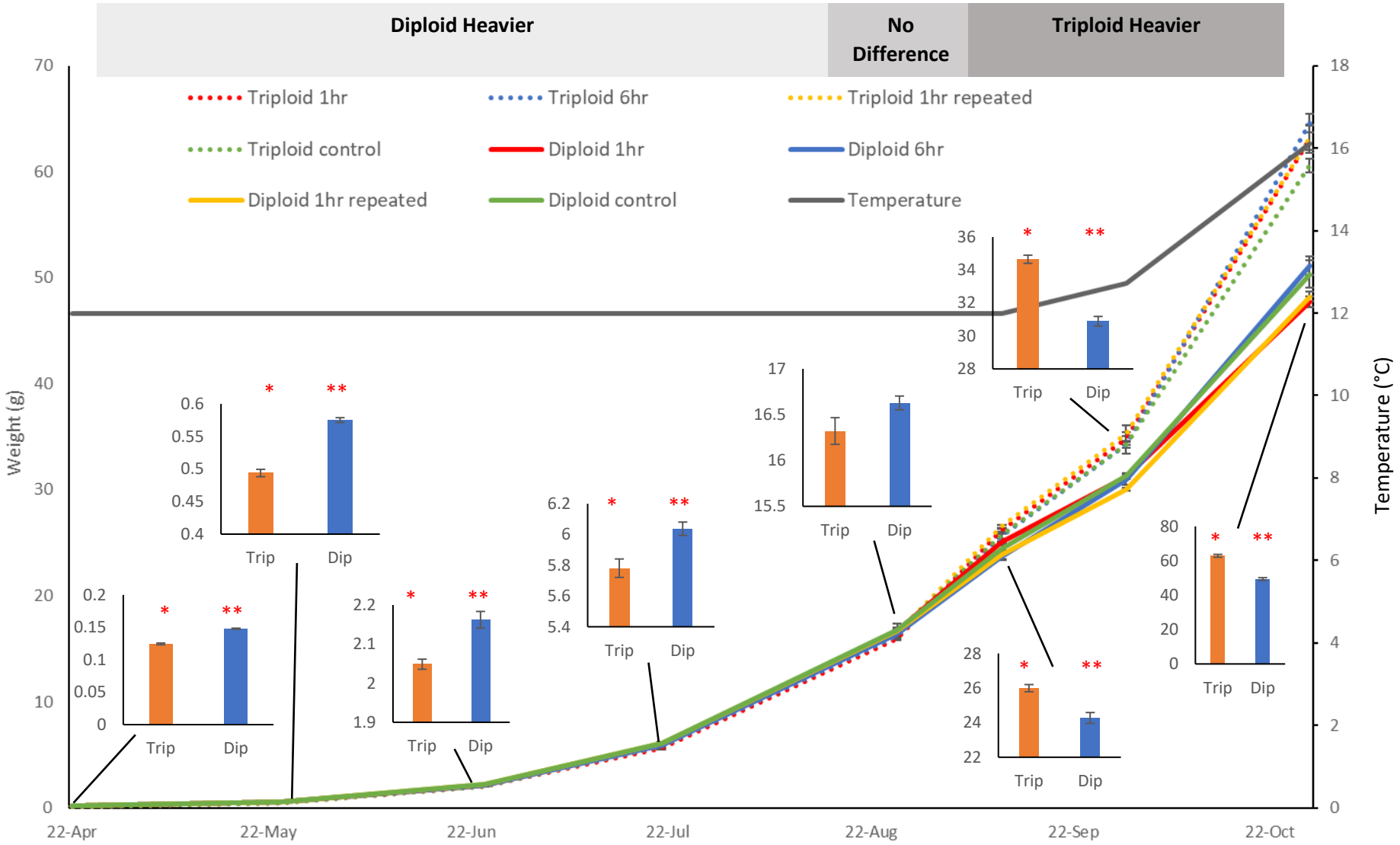


Figure 4.5. Mean weight (g) at first feeding (2020-04-22) until experiment end (2020-11-06) of triploid and diploid Atlantic salmon (*Salmo salar*). Line chart shows differences per ploidy and treatment after bulk weighs, bar charts show ploidy averages at the same time points. N=3 tanks. Significant differences in weight indicated by header (line chart) and superscript (bar chart).

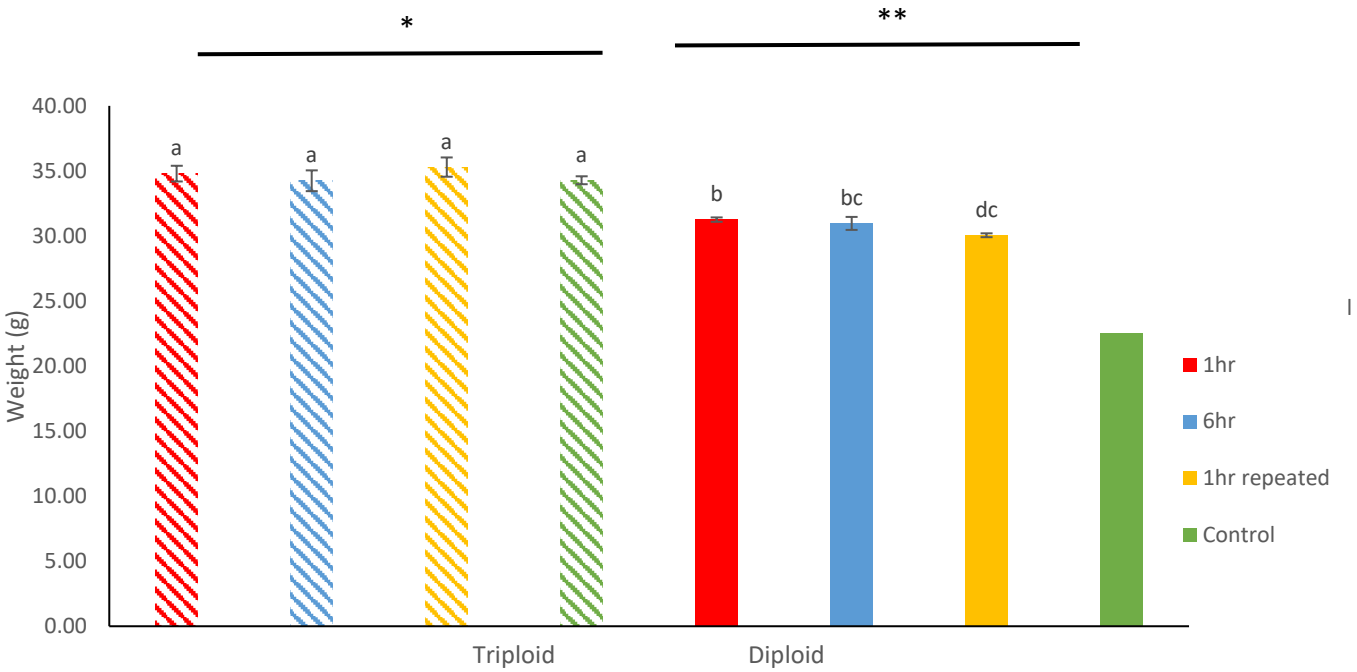


Figure 4.6. Mean weight (g) of triploid and diploid Atlantic salmon (*Salmo salar*) before thermal challenge (2020-09-30) per ploidy and treatment after bulk weigh. Data are presented as mean +/- SE (n=3 tanks). Superscripts denote significant differences between treatments within ploidy and not between ploidy (p=<0.05). Asterisks denote significant differences between ploidy (p=<0.05).

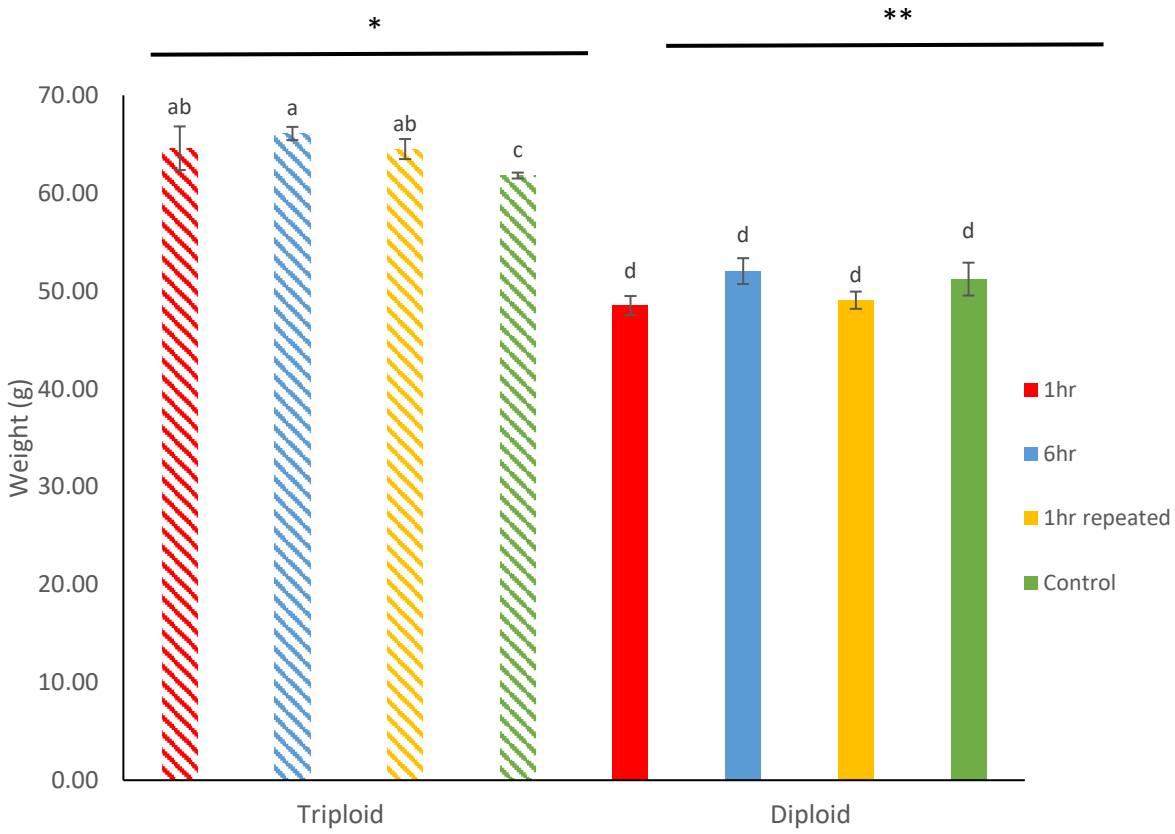


Figure 4.7. Mean weight (g) of triploid and diploid Atlantic salmon (*Salmo salar*) at the end of the thermal challenge (2020-10-28) per ploidy and treatment after bulk weigh. Data are presented as mean +/- SE (n=3 tanks). Superscripts denote significant differences between treatments within ploidy and not between ploidy (p=<0.05). Asterisks denote significant differences between ploidy (p=<0.05).

At both 12 °C and 16 °C stages of the feed intake assessment, triploids (mean=1.45, ±0.16 at 12 °C and mean=2.23, ±0.05 at 16 °C) had a significantly higher specific growth rate by weight (SGRwt) than diploids (Figure 4.8) (mean=1.22 ±0.12 at 12°C and mean=1.75 ±0.06 at 16°C) (Two-Way ANOVA: F(1,16) = 14.466, *p* = .002 at 12°C and F(1,16) = 69.891, *p* = <.001 at 16°C). The 6hr treatment had the highest average SGRwt at both temperatures for both ploidy, but this was not a significant difference compared to the control.

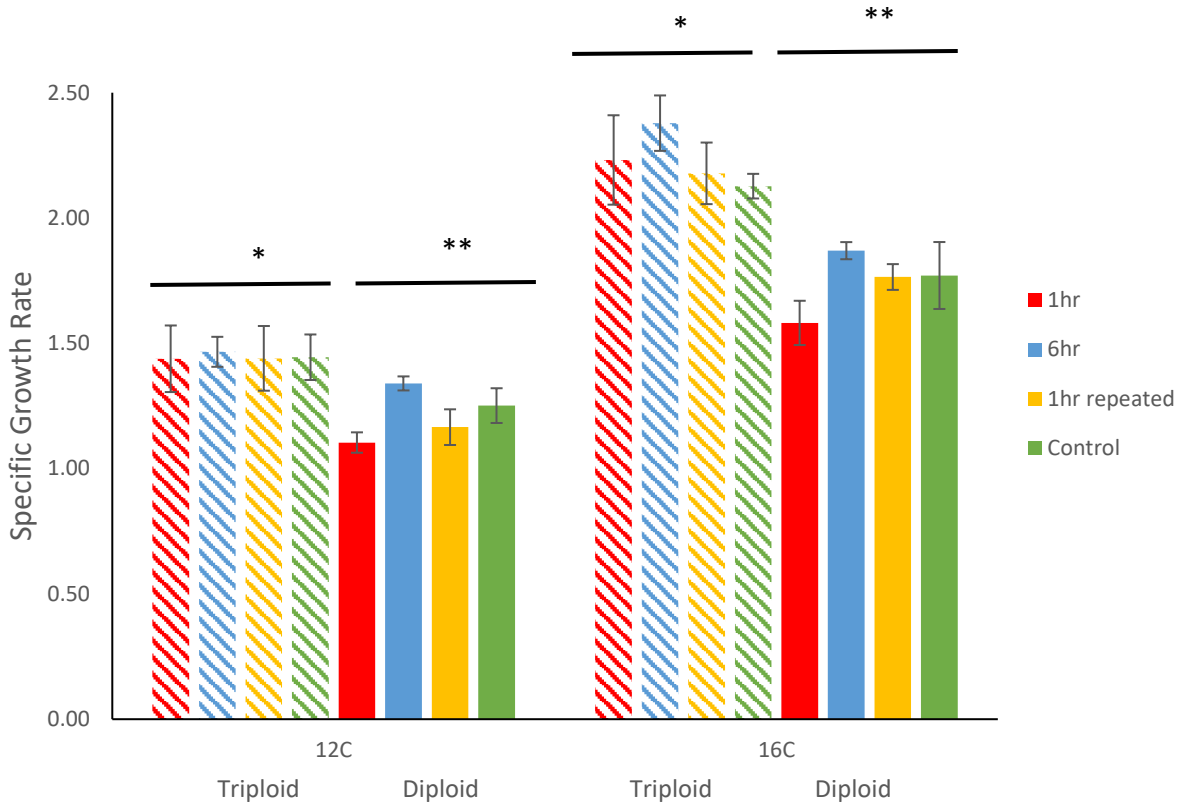


Figure 4.8. Specific growth rate of triploid and diploid Atlantic salmon (*Salmo salar*) by weight during 12°C and 16°C feed intake assessments. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (*p*=<0.05).

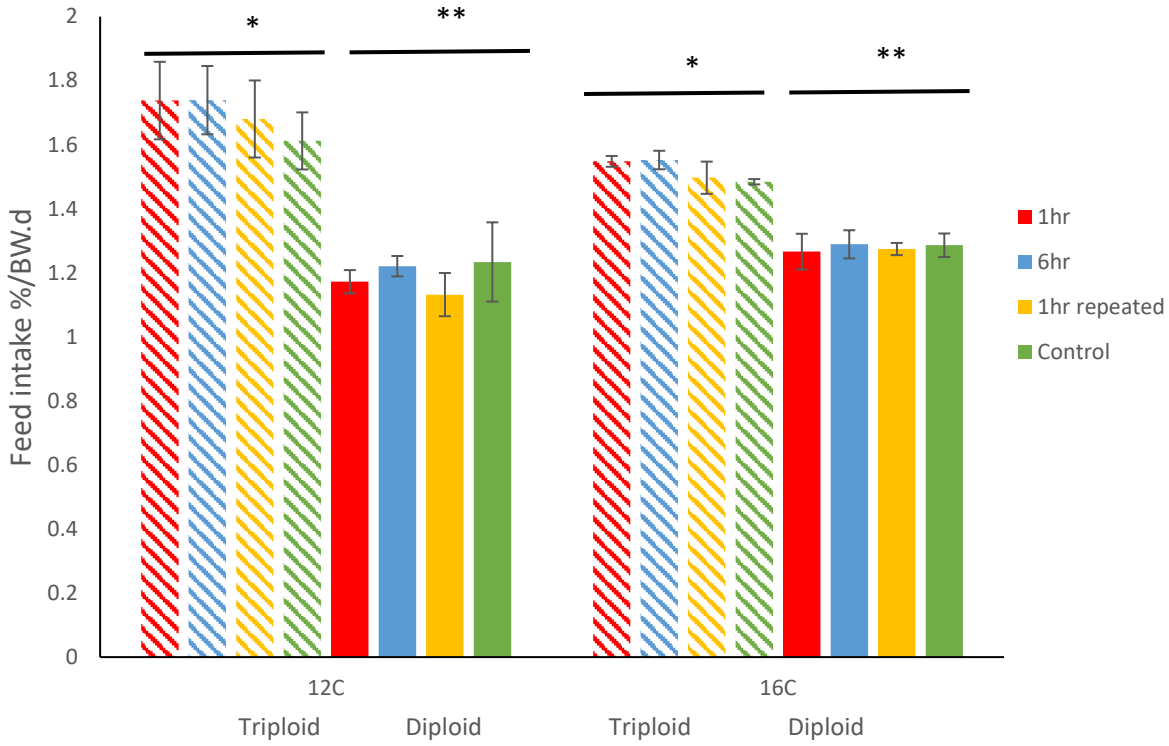


Figure 4.9. Feed intake as a percentage of average body weight of triploid and diploid Atlantic salmon (*Salmo salar*) during 12°C and 16°C feed intake assessments. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (*p*=<0.05).

Triploids (Mean=1.69 ±0.17 at 12 °C and Mean=1.52 ±0.05 at 16 °C) ate significantly more than diploids (Mean=1.19 ±0.12 at 12°C and Mean=1.28 ±0.06 at 16°C) per unit of body weight at both temperatures during the feed intake assessment (Two-Way ANOVA: F(1,16) = 57.214, *p* = <.001 and F(1,16) = 89.008, *p* = <.001 at 12°C and 16°C respectively). (Figure 4.9). This difference disappeared by the end of the 16°C window, with no difference between ploidy in the final days of feed intake assessment (Figure 4.10). On average, the 1hr and 6hr triploid treatments ate more than the control, but not significantly. The difference between the triploid 6hr and control at 16°C was significant being 1-tailed, but not when being 2-tailed.

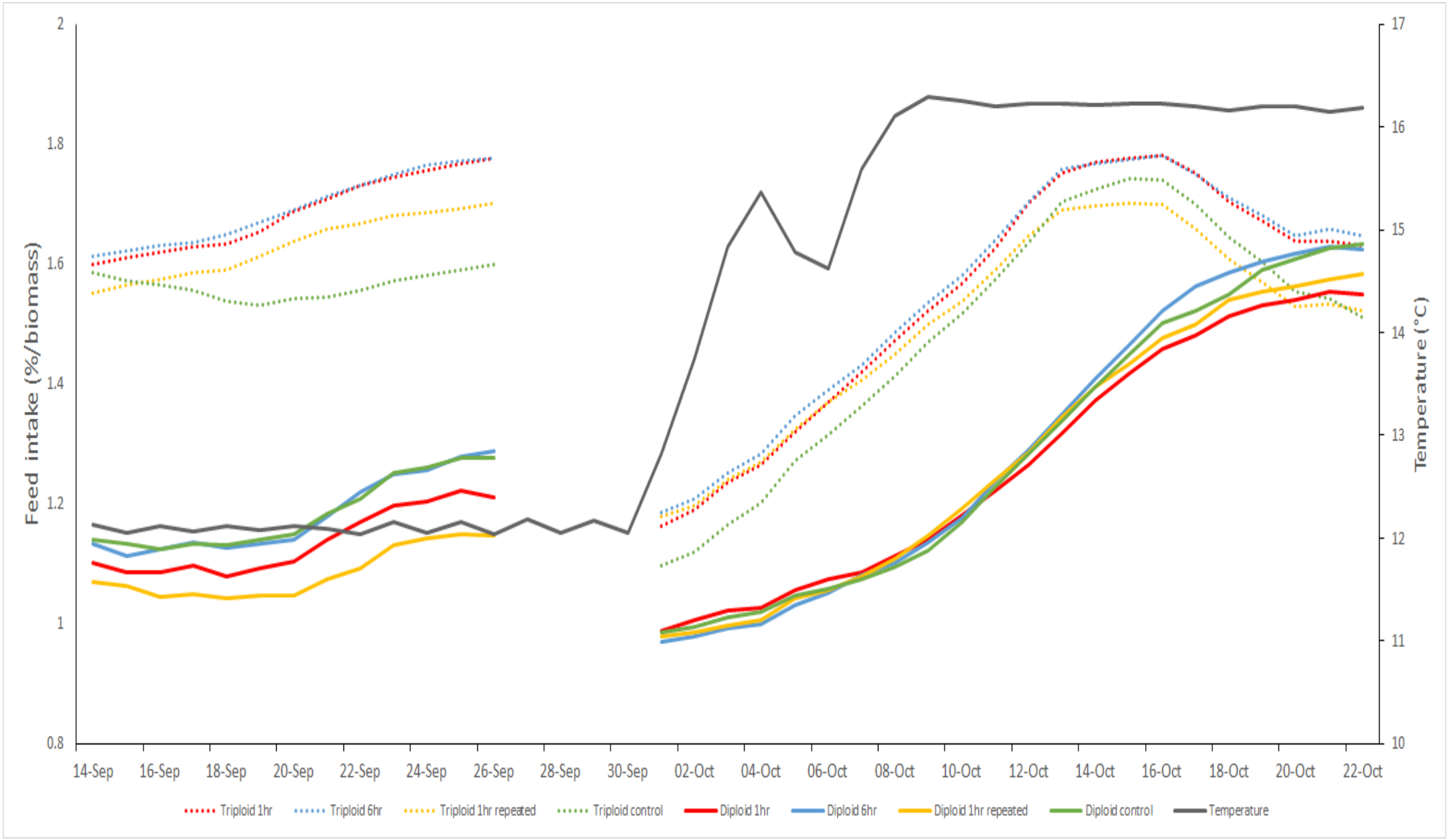


Figure 4.10. Rolling 7-day average of feed intake as percentage of biomass of triploid and diploid Atlantic salmon (*Salmo salar*) during 12°C and 16°C feed intake assessments. N=3 tanks

At both 12°C and 16°C, diploids (Mean=1.085 ±0.048 at 12°C and Mean=0.72 ±0.026 at 16°C) had a significantly lower biological feed conversion ratio (bFCR) than triploids (Figure 4.11) (Mean=1.24 ±0.048 at 12°C and Mean=1.05 ±0.026 at 16°C) (Two-Way ANOVA: F(1,16) = 5.409, *p* = .034 and F(1,16) = 81.753, *p* = <.001 at 12°C and 16°C respectively). Whilst there was no significant treatment effect, bFCR for the 6 hr diploid group appeared to be lower than the control at both temperatures. In the triploids the bFCR of the 6 hr group went from above that of the control at 12 °C to below the control at 16 °C. The difference between the triploid 6 hr and control at 16°C was marginal and just outside of being statistically significant.

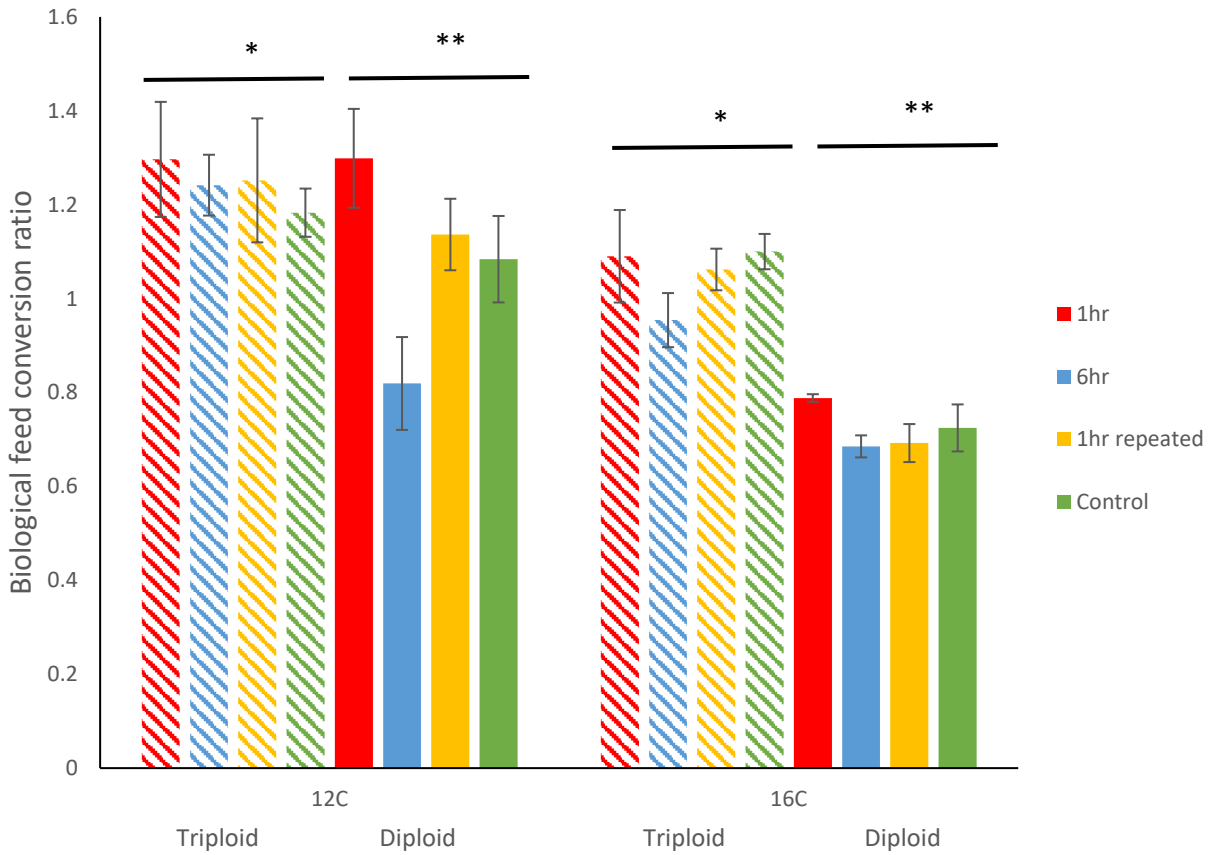


Figure 4.11. Average biological feed conversion ratio of triploid and diploid Atlantic salmon (*Salmo salar*) during 12°C and 16°C feed intake assessments. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (*p*=<0.05).

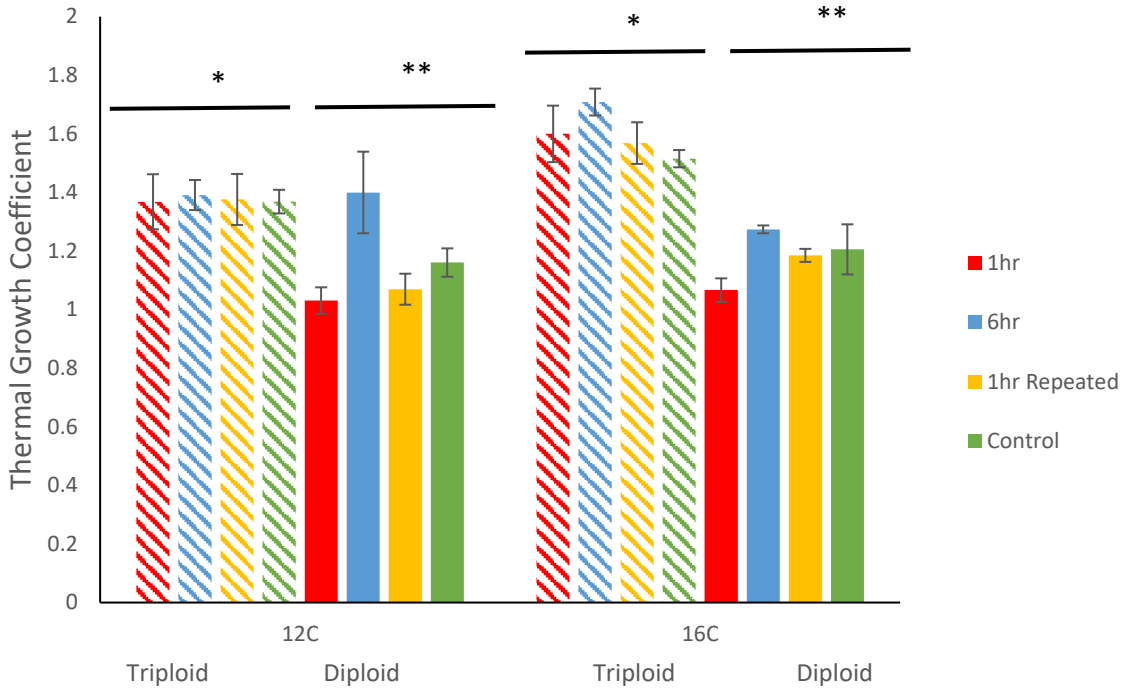


Figure 4.12. Average thermal growth coefficient of triploid and diploid Atlantic salmon (*Salmo salar*) during 12°C and 16°C feed intake assessments. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (p=<0.05).

Triploids (Mean=1.38 ±0.038 at 12°C and Mean=1.6 ±0.029 at 16°C) had a significantly higher thermal growth coefficient (TGC) than diploids (Figure 4.12) (Mean=1.17 ±0.038 at 12°C and Mean=1.18 ±0.029 at 16°C) at both temperatures (Two-Way ANOVA: F(1,16) = 15.01, p = .001 and F(1,16) = 102.4, p = <.001 at 12°C and 16°C respectively). There was no significant treatment effect within or between ploidy. The difference in TGC between triploid 6hr and control at 16°C was significant when tested alone but when adjusted for multiple comparisons this disappeared.

TGC assumes that both sides of the equation $\sqrt[3]{W_t} = \sqrt[3]{W_o} + [\left(\frac{T}{1000}\right) xt]$ are equal. The discrepancy between actual $\sqrt[3]{W_t}$ and actual $\sqrt[3]{W_o} + [\left(\frac{T}{1000}\right) xt]$ can be seen in Figure 4.13. At both temperatures the assumed balance of the TGC equation was violated to a larger extent within triploids (Two-Way ANOVA: F(1,16) =

10.6, p = .005 and F(1,16) = 102.408, p = <.001 at 12°C and 16°C respectively). At 16°C this discrepancy increased within both ploidy.

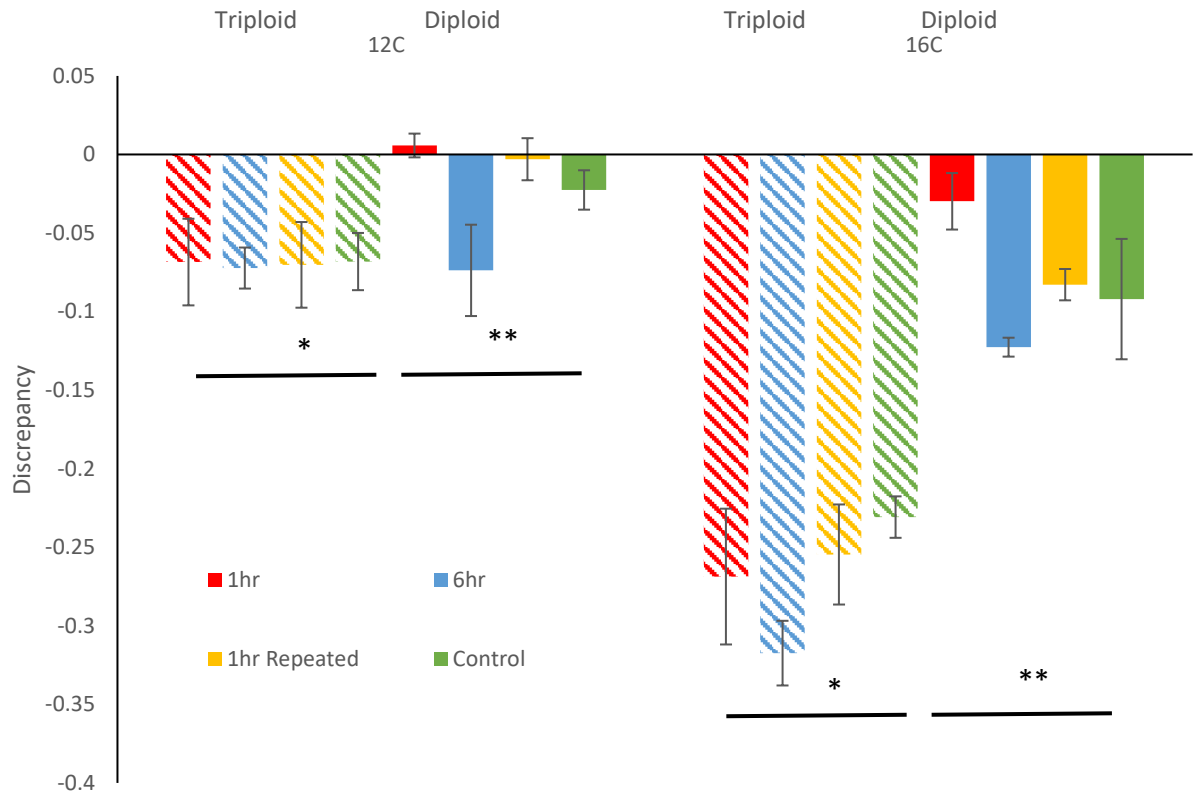


Figure 4.13. Discrepancy between the actual values of the two halves of the TGC equation of triploid and diploid Atlantic salmon (*Salmo salar*) during 12°C and 16°C feed intake assessments. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (p=<0.05).

We also calculated the efficacy of the model to predict future weight at each temperature and ploidy by calculating the discrepancy between a TGC estimate and actual growth performance (Figure 4.14). At 12 °C diploids matched the model more closely than triploids with an average underestimation of growth of 1% ±1.1 compared to an underestimation of 5.4% ±2.1 in triploids. (Two-Way ANOVA: F(1,16) = 113.2, p = .007). The discrepancy between the model and actual weight gain increased with the increase in temperature. The discrepancy was greater at 16 °C within diploids with the TGC model overestimating growth by an average of 19% ±1.2 and 5.8% ±1.9 within triploids (F(1,16) = 108.8, p = <.001).

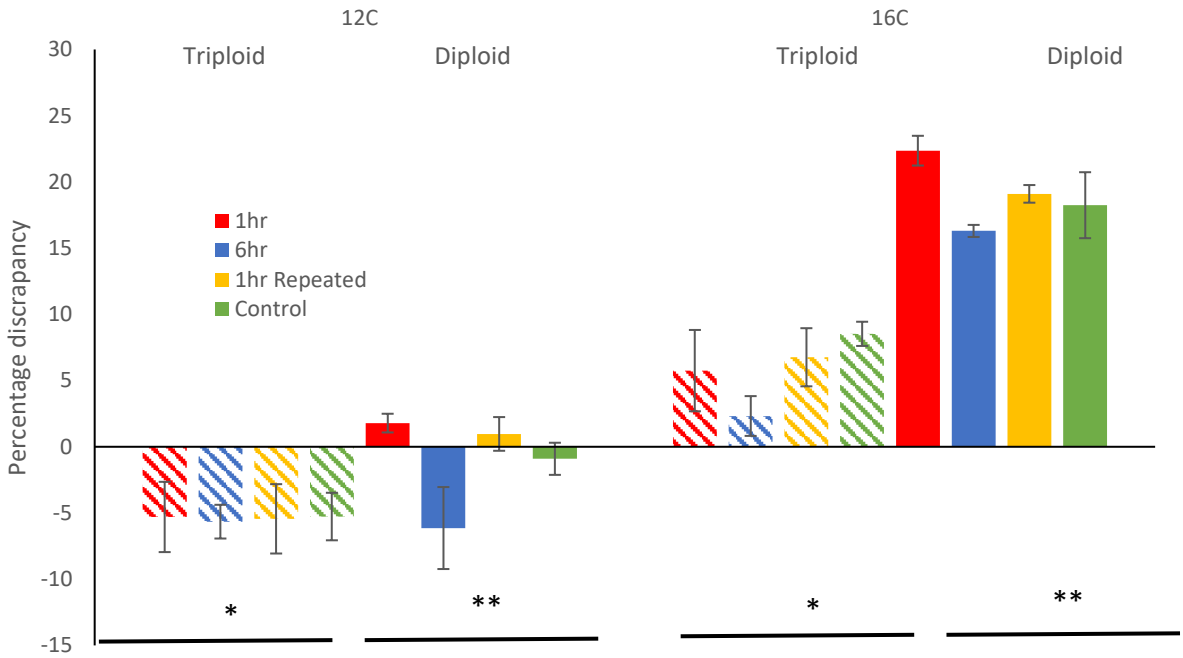


Figure 4.14. Percentage discrepancy between TGC estimated weight gain and actual weight gain during 12°C and 16°C feed intake assessments. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy (p=<0.05).

4.3.4 Deformity assessment

There was no treatment impact on the prevalence of radiological vertebral deformities. There was however a significant ploidy difference. Diploids had significantly more individuals exhibiting no deformed vertebrae (dv), 92.9 % vs 83 % (Figure 4.15) (Two-Way ANOVA: F(1,15) = 30.28, p = <.001). Triploids had significantly more individuals at other severity levels (Figure 4.16). Low severity (1-5dv) 14.1 % triploid vs 6.9 % diploid (Two-Way ANOVA: F(1,15) = 24.20, p = <.001), moderate (6-9dv) 2.1 % vs 0.2 % (Two-Way ANOVA: F(1,15) = 20.45, p = .005), there was no significant ploidy difference in the severe (10+dv) category 0.76 % vs 0% (Figure 4.16).

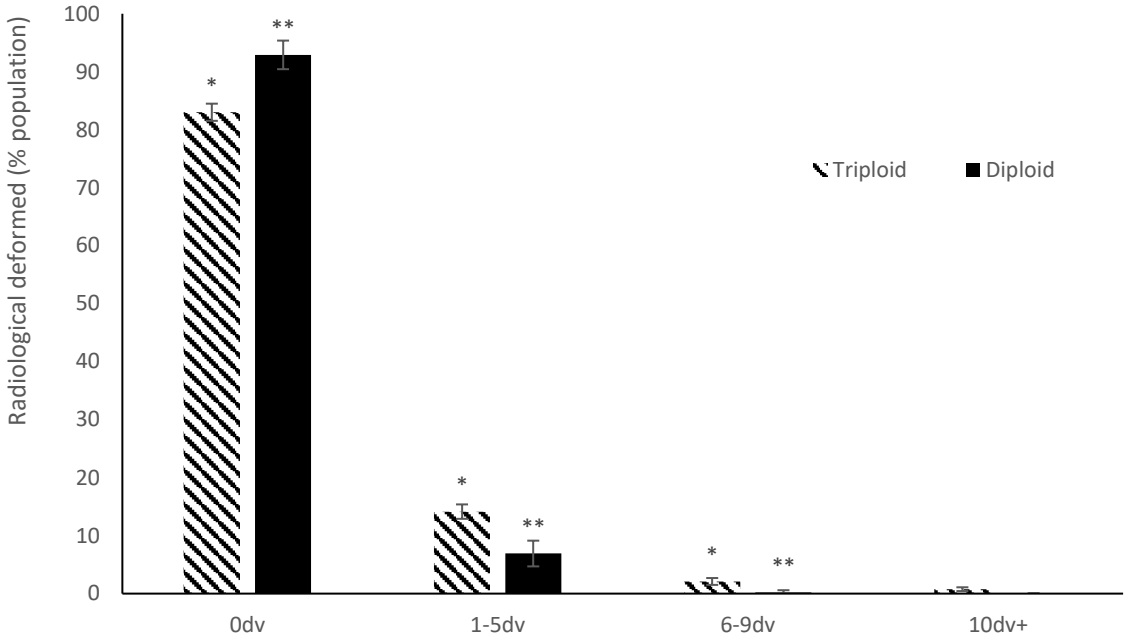


Figure 4.15. Average percentage radiologically deformed individuals of triploid and diploid Atlantic salmon (*Salmo salar*) at 4 severity levels. Deformity of vertebrae determined under criteria defined by Witten et al. (2009). Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between ploidy at each severity level (p=<0.05). 44 individuals per tank.

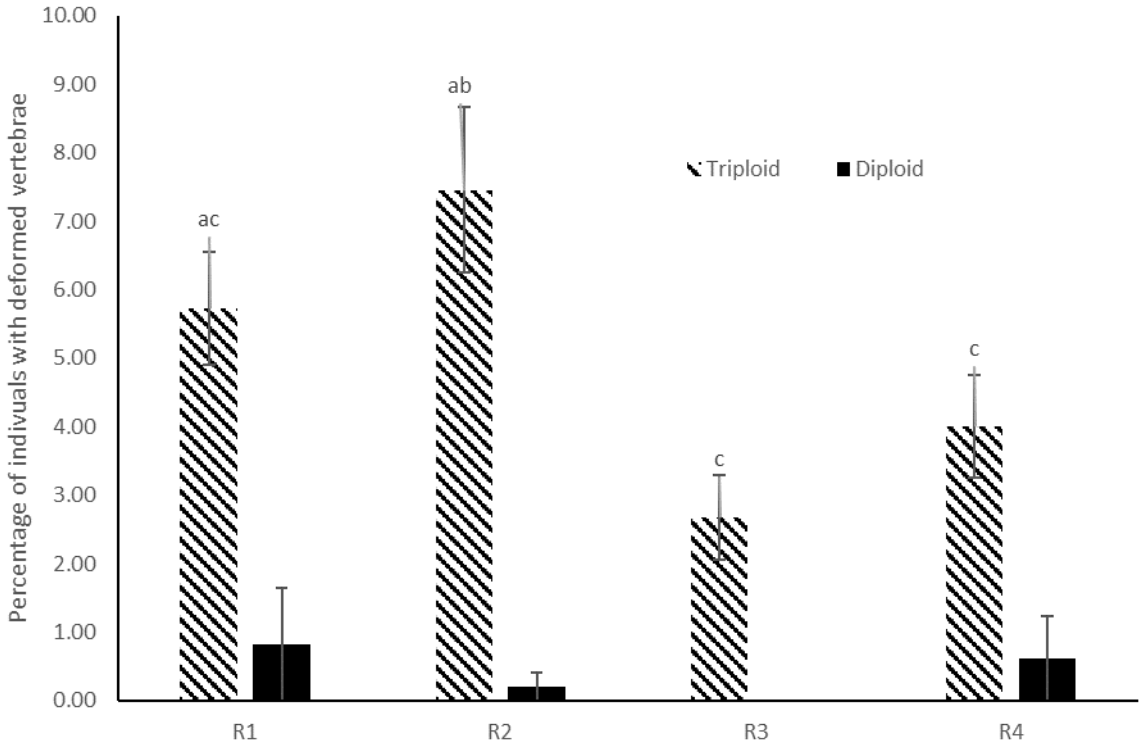


Figure 4.16. Average percentage of individuals with deformed vertebrae of triploid and diploid Atlantic salmon (*Salmo salar*) in 4 regions of the vertebral column. Deformity of vertebrae determined under criteria defined by Witten et al. (2009), regions defined by Kacem et al. (1998). Data are presented as mean +/- SE (n=3 tanks). Superscripts denote significant differences between regions within ploidy and not between ploidy (p=<0.05).

There was no significant difference in the prevalence of deformity between any of the diploid regions. There were significant differences in the percentage of individuals with deformity in triploids (One-way ANOVA: (F(3,45)=5.62, *p*=0.002). A Tukey HSD post-hoc test showed that region 2 had a significantly higher rate of deformity than both R3 (*p*=0.002) and R4 (*p*=0.038).

No cases of aplasia of the *septum transversum* were found in this experiment.

4.3.5 PAMPS challenge

Triploid and diploid control treatment gene expression levels 24hrs after PAMPS challenge were compared using absolute qPCR. In terms of absolute gene expression, there was no significant difference 24hrs following Poly I:C injection for the gene Mx (Figure 4.17). There was a significant difference (Kruskal-Wallis H=17.28, *P*=<.001) for the gene LGP2 with diploids having an average copy number of 43,976 to 5,043 for triploids (Figure 4.17). There was also a difference in response to injection with saline. For both genes (Mx and LGP2) diploids showed significantly higher copy number than triploids; 4,713 vs 1,307 for MX (Kruskal-Wallis H=10.8, *P*=.001) and 1,485 vs 205 for LGP2 (Kruskal-Wallis H=15.87, *P*=<.001).

When saline is used as the baseline, the percentage increase in expression caused by PAMPS injection can be observed (Figure 4.18). For Mx triploids showed a significantly higher increase in expression compared to diploids (Kruskal-Wallis H=8.02, *P*=.005). There was no significant difference for LGP2.

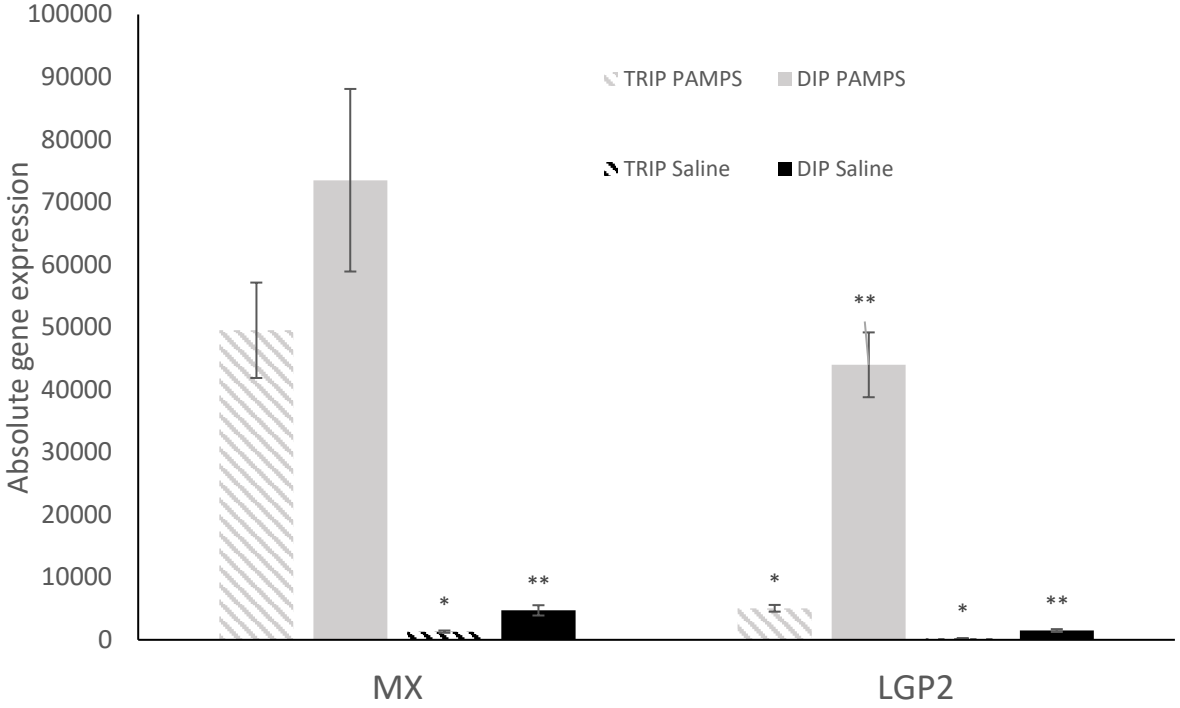


Figure 4.17. Absolute gene expression of genes Mx and LGP2 for triploid and diploid Atlantic salmon (*Salmo salar*) injected with PAMPS and saline solutions. Data are presented as mean +/- SE (n=3 tanks). Asterisks denote significant differences between treatments within ploidy and not between ploidy (p=<0.05)., 6 individuals per tank per treatment.

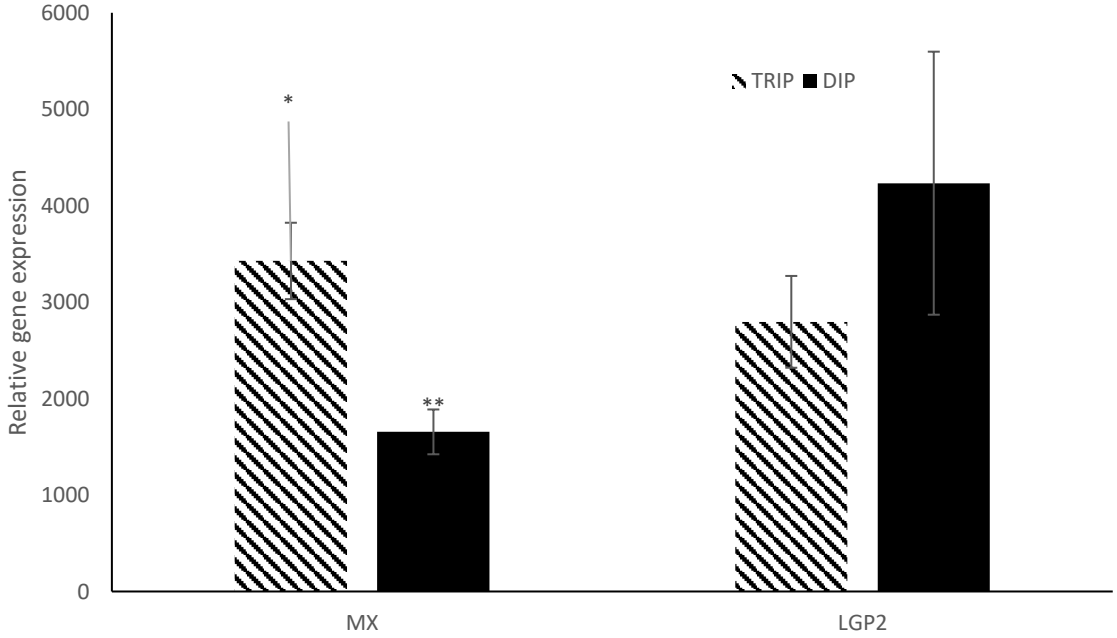


Figure 4.18. Percentage increase in expression of genes Mx and LGP2 for triploid and diploid Atlantic salmon (*Salmo salar*) after injection with poly I:C compared to baseline levels after saline injection. Data are presented as mean +/- SE (n=3 tanks). Asterisks s denote significant differences between treatments within ploidy and not between ploidy (p=<0.05). 6 individuals per tank per treatment.

4.4. Discussion

Despite the wide range of documented differences between diploid and triploid Atlantic salmon (Fraser et al., 2012; Leclercq et al., 2011; Peruzzi et al., 2015; Sadler et al., 2001; Sambraus, 2016; Smedley et al., 2018) the time at which they reach hatch is not reported in the literature. Despite this, a variation in hatch timing based on ploidy was seen in this study, as well as in chapter three in this thesis, with triploids in this case reaching 90 % hatch several days before diploid siblings. In a large commercial hatchery, with plentiful food on site, changes in the timing of hatch would not likely be an issue. Smaller or experimental operations may order food to be delivered in time for first feeding and therefore mistiming this delivery could result in no feed being onsite for triploid alevin.

In this experiment, we also observed a high level of pre-mature hatch, that is individuals that hatched >426DD. On average, 4.6 % of triploids and 6.3 % of diploids placed into the tanks at 350DD hatched prematurely. Both hypoxia and manual handling are known to effect premature hatch in salmonids (Crisp, 1990; Polymeropoulos, 2013; Wood et al., 2020). Water in the 10 °C shock treatment had a lower oxygen carrying capacity than the water in the tanks due to the higher temperature and, whilst all attempts were made to ensure minimal disturbance, the handling may have had an effect on hatching. There is some evidence of treatment effects within diploids with the eggs exposed to repeated shocks having significantly higher premature hatching than the control, this may also be due to the increased level of handling rather than the thermal impact alone (with the repeated shocks being handled on 5 consecutive days rather than just once). It is also possible that the quality of eggs may have been a factor. This batch of eggs was amongst the first commercial batch produced in AquaGen Holywood with broodstock that were generally smaller than normal. There was no significant ploidy impact.

Perhaps surprisingly, there was no significant difference in mortality between ploidy. Supraoptimal incubation temperatures have been found to increase mortality in triploids (Clarkson et al., 2020; Fraser et al., 2014). The short duration of the temperature shock and the low initial incubation temperature is likely the reason that the triploids did not negatively suffer in this regard. Clarkson *et al.* (2020) raised triploids at either 6°C or 8°C to 400DD then raised the temperature of the 6°C batch

to 8°C where this temperature was then maintained. Compared to the current experiment, where eggs were kept at 4°C until eyeing (357DD) and then 6°C until first feeding with short term shocks of 10°C, the mortality in Clarkson *et al.* (2020) to first feeding was much higher (33.6 % and 47.9 % for 6 °C and 8 °C) compared to 13.2 % to first feeding in the current experiment. This shows the importance of lower incubation temperatures for triploid development, and also highlights their robustness against short term thermal shocks.

When temperatures were raised during the final stages of the experiment, and triploids exhibited decreased tolerance in other areas such as feed intake, the decreased tolerance of triploids to high temperatures did not extend as far as mortality. Triploids are thought to be “weaker” than diploids, especially when higher incubation temperatures are a factor, but the shocks had no impact on mortality in either ploidy. Shocks of a higher temperature may have influenced mortality to a greater extent, but the shocks used in this experiment were within the scope of possible fluctuations within the production cycle and were not insignificant with the eggs being exposed to a 66 % increase in temperature immediately and an immediate 40 % drop in temperature once the time in the bath had finished.

Whilst there was no ploidy difference with actual mortality, there was a difference in the number of unviable individuals. That is to say, individuals who were found as “pins” or “runts” and who, being unable to tolerate pellet change increases, were removed for welfare reasons but who had managed to survive up until that stage in the culture system. This difference was significant, both statistically and in terms of sheer number, with 13.8 % of triploid control being removed compared to just 3.4 % in the diploid control. This has obvious implications for triploid production, with more eggs being needed to ensure the same number of individuals reach harvest. One silver lining is that these losses were located early in the production cycle, reducing the resources wasted by each loss.

The final weight is the place where we see some evidence of thermal programming. In Figure 4.7 one can see the weight of the treatments before the thermal challenge began with the control group being the largest group in the diploids and in the triploids the 6 hr group being the smallest. One month of thermal challenge later, both 6 hr groups were the largest of each ploidy and this difference was significant in

the triploid group (Figure 4.6). The triploid 6 hr group had gone from being almost equal weight with the control group to being almost 7 % (4.3 g) heavier. The manifestation of this sudden difference in weight suggests that there is a significant difference in the manner in which these two groups deal with the change in environmental conditions. The 6 hr group, having previously exhibited no fitness advantage over the control group, now showed the ability to grow at a faster rate. As the only change during this period was water temperature one can make the assumption that the 6 hr group had an advantage at higher temperatures due to a programmed ability to better tolerate the increase. In diploids, this difference is less clear, but the 6 hr group went from being 0.34 g lighter to being 0.82 g heavier (Figure 4.6). This trend continues into specific growth rate, at both temperatures and ploidy the SGR was the highest in the 6 hr group, but this difference was notably higher in triploids at 16°C compared to 12°C (Figure 4.8). At both temperatures and ploidy the 6 hr group ate the most food on average (Figures 4.9 and 4.10). The biological feed conversion ratio was the lowest (best) in the 6 hr diploid group at both temperatures, in the triploids the 6hr group was higher than the control at 12 °C but lower by a wide margin at 16 °C Figure 4.11). The thermal growth coefficient was the highest in the 6 hr group at both temperatures and ploidy, the difference between the triploid 6 hr group and the control increased with the temperature (Figure 4.12), the TGC of the 6 hr triploid group was significantly higher than the control before it was adjusted for multiple comparisons.

The above performance metrics all show the same trend, that on average the 6 hr group performed better than the control, although generally not significantly. The consistency of the results however, coupled with the fact that the triploid 6 hr group finished significantly heavier than the control group all suggest that Atlantic salmon can be thermally programmed to improve their thermal tolerance. These results are in line with previous studies on Atlantic salmon which showed that early life exposure to certain stimuli, in that case alternative diets, can increase the tolerance to said stimuli later in life (Clarkson et al., 2017; Vera et al., 2017). The potential of programming has already been shown in zebrafish with hypoxia (Ho & Burggren, 2012) but this is the first indication that thermal programming can potentially improve thermal tolerance in fish. Nevertheless, more research needs to be conducted and, whilst the growth data is promising, data from other performance metrics was less

clear. The major question regards the timing and extent of the shock. The temperature and time period used in this study were chosen due to the relative robustness of the fish at this later stage of embryonic development and the hope that this substantial temperature shift would not be enough to induce pathologies or mortalities. This author suggests that the next aim should be to establish the thermally labile stages of development by looking at a large number of developmental stages across a shorter experimental window.

Between ploidy differences in growth in freshwater is something that has previously been studied, and this experiment reaches the same conclusion as other studies, namely that triploids hatch at a smaller size than diploids (Taylor et al., 2011), but over time in freshwater their growth rate is higher than diploids (Taylor et al., 2013). Diploids were significantly heavier for 3 months post first-feeding (PFF) but by 5 months PFF triploids were significantly heavier and finished the experiment >13 grams or 27 % heavier. During the feed intake assessment period at both 12°C and 16°C triploids had a significantly higher specific growth rate for weight.

This difference in growth is not down to increase capacity to utilise food, with triploids exhibiting a worse FCR than diploids at 16°C in this study and no difference at 12°C. Instead, the difference in growth is down to an increased intake of food. At both temperatures triploids ate significantly more food than diploids on average. It is not clear why triploids eat more food. Triploids differ from diploids in brain morphology possessing a smaller olfactory bulb but larger cerebellum and telencephalon, areas which are linked to foraging ability (Fraser et al., 2012). It is possible that increased foraging ability could help triploids consume more feed, but considering that during the feed intake period the fish were fed 10 % past satiation this seems unlikely. Another possibility is changes in satiation and appetite. More research is needed to fully understand the mechanisms behind satiation in fish, but there is evidence that this process is controlled by the hypothalamus in similar ways to that in humans, with hormones such as leptin, insulin, ghrelin, and CCK being key to feed intake and satiation regulation(Delgado et al., 2017). In terms of size and volume there was no ploidy difference observed for the hypothalamus (Fraser et al., 2012). It is possible that the increased intake of food is linked to altered digestive morphology (Peruzzi et al., 2015). The activity of digestive enzymes which are linked to satiation (Delgado et

al., 2017), have been found to differ in expression between ploidy (Martínez-Llorens et al., 2021).

When calculating growth and predicted growth in Atlantic salmon, one common metric is thermal growth coefficient (TGC). This is the idea that the size of fish at one time point can be used to predict the size of fish at another future time point if the fish are reared at a known temperature (Jobling, 2003). The TGC is based on the model $\sqrt[3]{W_t} = \sqrt[3]{W_o} + [(\frac{T}{1000}) \times t]$, where T is temperature, t is time in days, W_t is final weight and W_o is initial weight.

Using this model one can calculate the expected growth of a population over time. This is incredibly useful when organising healthcare treatments, harvest, and increases in pellet size. The model is not perfect and has three basic assumptions:

- Growth increases in a steady and predictable manner with increasing temperature
- The length (L)^weight (W) relationship is $W \propto L^3$
- Growth in length (for any temperature) is constant over time (i.e. L increases linearly over time).

Taken from (Jobling, 2003)

All of which can be violated depending on abiotic conditions. One major issue is that growth does not increase steadily with temperature, but rather in a “bell-shaped curve” (Jobling, 2003). As a result, the model is only reliable for diploid Atlantic salmon between 4°C and 14°C (Thorarensen & Farrell, 2011). Situations where fish deviate from the TGC model can obliviously have a negative impact on both efficient production and fish welfare. Figure 4.13 shows the extent to which the TGC prediction matched recorded growth in this study. At 12°C diploids matched the model more closely than matched with an average underestimation of growth of 1% compared to an underestimation of 5.4% in triploids. The model, as expected, performed more poorly at the higher temperature of 16°C. Perhaps surprisingly, the model produced a larger discrepancy when for diploids rather than triploids at the higher temperature. The TGC model overestimated growth in both ploidy at the higher temperature. The discrepancy was greater within diploids with the TGC model overestimating growth by an average of 19% and 5.8% within triploids. Whilst the

model is not used at these high temperatures, the degree of discrepancy within the triploid population suggests that they better fit the model at higher temperatures, at least whilst they remain feeding and healthy.

The thermal treatments did not induce an increased prevalence of radiological vertebral deformities. Given previous studies findings that long term exposure to supraoptimal incubation temperatures (≥8°C) causes increased levels of deformity in triploids (Clarkson et al., 2021; Fjelldal & Hansen, 2010; Fraser et al., 2015), this result is interesting. These studies focused on longer term exposure to supraoptimal temperatures, in the case of Clarkson et al. (2021) a shift from an optimal temperature to a supraoptimal temperature for an extended period of time. Shorter term shifts have been shown to cause spinal deformities, with Wargelius et al. (2005) exposing diploid Atlantic salmon incubated at 6°C to a shock at 12°C for 24 hrs at 68, 115, 121, and 160DD. These treatment groups exhibited cases of radiological vertebral deformity in 34, 31, 34, and 27 % of individuals respectively compared to the control at 3 %. The diploids in the current experiment shocked at 10°C at 360DD for 1 hr, 6 hrs, and 1 hr for 5 days repeatedly had 3.17, 11.36, and 9.25 % of individuals exhibiting radiological vertebral deformities compared to 4.6 % for the control. Due to the differences in temperature and duration of shock it is impossible to determine exactly the effect of timing vs severity, although the sensitive stage at which the eggs were shocked (somitogenesis) almost certainly played a part in the level of deformity. The low level of triploid deformity is encouraging especially given the fact that the fish were fed a diploid diet throughout, it has been clearly shown that a triploid diet with increased levels of phosphorus can decrease the level of vertebral deformities (Fjelldal et al., 2016; Smedley et al., 2018). The reason a diploid diet was not used in this experiment is that using a different diet between ploidy would add an additional confounding factor.

There was a significant ploidy difference in vertebral deformities, with triploids having 17 % of individuals with at least 1 radiologically deformed vertebrae, compared to 7.1 % in diploids. That being said, the level of deformity in the triploids was still lower than previous studies (Clarkson et al., 2021; Fjelldal & Hansen, 2010; Fraser et al., 2015), especially since they were fed a diploid diet throughout. The eggs in this study were kept at 4°C until shipping and then 6°C until first feeding, and this low temperature regime may have had an effect. Fish with low levels of radiological

vertebral deformities are not uncommon within Atlantic salmon populations including diploids, and the presence of a low number of deformed vertebrae is not a welfare concern. What is of relevance to welfare, and also of economic concern with fish either being unable to be sold whole or in the worst cases being unable to be processed due to their shape (Fjelldal et al., 2012), are fish that have multiple deformed vertebrae. For the purpose of this experiment we used the same severity criteria as Smedley et al., (2018); No deformed vertebrae, low (1-5dv), moderate (6-9dv) and severe (10+dv). Whilst the triploid groups had a significantly higher number of cases of low and moderate deformities the levels were overall low. Only 0.76 % of all triploids showed “severe” deformity, and a level at which welfare is likely compromised (Fjelldal & Hansen, 2010). This is lower than previous studies on triploids (Clarkson et al., 2021) and shows again the importance of low incubation temperatures early in life. Moderate severity was not thought to be a welfare concern although may lead to problems later in life should the level of deformity increase, this level was also lower than observed in previous studies.

Aplasia of the *septum transversum* is a type of heart malformation linked to supraoptimal incubation temperatures (Fraser et al., 2014; Takle et al., 2006) and thermal shocks during incubation (Takle et al., 2005), with triploids being more prone to this pathology when exposed to the same incubation temperature as diploids (Fraser et al., 2014). The *septum transversum* separates the cardiac and abdominal cavities and aplasia of this tissue results in changes to heart morphology and reduced cardiac function (Fraser et al., 2014; Poppe et al., 1998). Heart pathology such as aplasia of the *septum transversum* has been linked to cases of sudden mortality when stressful conditions present (Brocklebank & Raverty, 2002; Rodger et al., 2011).

Triploid Atlantic salmon without this pathology still suffer from a more acute angle of the bulbous arteriosus (Fraser et al., 2015; Fraser et al., 2013), this is thought to be linked to the higher cardiac workload of triploids (Leclercq et al., 2011), and in triploids with un-deformed hearts arrhythmia is more common than in diploid counterparts when subjected to high temperatures (Verhille et al., 2013).

These factors mean that for the welfare of the triploids it is vitally important that cases of aplasia of the *septum transversum* are avoided. It is therefore encouraging

that no instances of Aplasia of the *septum transversum* were observed in either ploidy. This finding is encouraging and shows that triploids are resistant to short term thermal shocks at this stage in their life with regards to this particular pathology.

There were no treatment-based differences in the expression of either Mx or LGP2. These two immune related genes have been shown to respond well to PAMPS challenge and were used as indicators of overall immune responsiveness. The lack of differences between treatments is encouraging as to the tolerance of triploids to short term thermal shocks and suggests that shocks of this nature do not affect immune response, just as they don't increase deformities, mortalities, or decrease performance.

In terms of absolute gene expression, diploids were significantly higher for the gene LGP2 but not for Mx. Expression of both the genes to the PBS treatment was significantly lower for triploids. Previous studies have shown that triploids are not more susceptible to disease under normal conditions (Moore *et al*. 2017). In general, triploids are thought to possess dosage-compensation mechanisms that result in similar expression levels between diploids and triploids (Christensen et al., 2019; Odei et al., 2020; Ren et al., 2017). There is a suggestion that triploid homeostasis and dosage-compensation breakdown under times of stress, and it is possible that this explains the difference in gene expression between ploidy.

Diploids exhibited a large increase in expression of both genes over PBS, displaying a robust immune response to the PAMPS challenge. Whilst the % increase of triploids for gene Mx was significantly larger than that of diploids, it is likely due to the lack of response to the saline injection. Absolute expression of Mx was lower, though not significantly, for triploids after Poly I:C injection but so was the expression of Mx after PBS injection and significantly so. The lower response of triploids to the mild stressor of injection with saline may suggest that these animals require larger triggers for immune response. Triploids had a significantly higher absolute expression of Mx compared to LGP2 and also a significantly higher increase in expression of the gene compared between PAMPS and PBS. It is not clear as to why the difference in expression between Mx and LGP2 is so much greater for triploids than for diploids. As mentioned above, the expression of LGP2 directly influences the expression of Mx via IFNs. This pattern is observed in diploids but

less so in triploids in this study. One possible explanation is that the expression of Mx has been influenced through alternative pathways. Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) have been shown to activate IFN expression alongside LGP2, and it is possible that these genes may have affected the expression of Mx despite the low level of LGP2 expression.

The overall lower expression is in line with findings that at higher temperatures triploids are more susceptible to disease events and mortalities associated with disease.

In conclusion, short term thermal shocks at the eyed stage of incubation have little to no impact on negative performance traits including but not limited to mortalities, deformity, and growth. The first evidence of thermal programming in a fish species is shown here, with triploids treated with a 6hr shock being 6.5% heavier than the control by the end of the experiment. Sequencing and epigenetic analysis should inform us further on this topic. Triploids performed very well compared to diploids and apart from higher cases of unviable individuals and slightly higher radiological vertebral deformities, and their freshwater growth was significantly higher than that of diploids. This experiment showed that triploids are more robust than previously thought when it comes to thermal shocks, and perhaps provides more evidence for triploids to be a viable alternative to diploids.

Chapter 5. Transcriptomic comparison of diploid and triploid Atlantic salmon (Salmo salar) at optimal and supraoptimal temperatures

Abstract

Triploid and diploid Atlantic salmon differ in terms of many physiological characteristics, but much less studied are their transcriptomic differences. Differences in gene expression can help explain why these ploidy perform differently such as their tolerance to high temperatures. No study has so far looked at transcriptomic differences between ploidy during a thermal challenge. This study compared the transcriptome of triploid and diploid salmon at 12°C and 16°C. Between ploidy there were only 5 genes showing significant differential expression at each temperature. This low number is supported by previous studies and suggest that subtle differences in the transcriptome are responsible for the large differences in physiology and performance. There was a difference within ploidy, with the diploids differentially regulating 37 genes compared to 121 in triploids. Some of the functional groups these genes belong to can help explain why triploids suffer more at high temperatures with the differential regulation of groups such as cardiac function and development, transcriptional regulation, and circadian regulation, and the lack of change in genes relating to cytoprotection providing an insight into the differences between ploidy. Increasing the understanding of the mechanisms behind these differences can help the industry understand ways in which it can improve the performance of both triploid and diploid salmon.

5.1. Introduction

Much has been said over the years about the multitude of physiological differences between triploid and diploid Atlantic salmon (*Salmo salar*). Physiologically, triploids differ from diploids in many ways from the most fundamental such as possessing fewer but larger cells (Benfey, 1999; Goo et al., 2015; Swarup, 1959; Van De Pol et al., 2020), to the more complex such as alterations in gill filament density (Leclercq et al., 2011; Sadler et al., 2001) and changes in gut (Peruzzi et al., 2015), brain (Fraser et al., 2012; O'Keefe & Benfey, 1997) and heart morphology (Fraser et al.,

2013, 2015). These differences have an impact on the husbandry of the fish with triploids requiring higher levels of dietary phosphorus (Fjelldal et al., 2016; Sambraus et al., 2020; Smedley et al., 2016, 2018), histidine (Sambraus et al., 2017; Taylor et al., 2015) and protein (Smedley et al., 2016), lower incubation temperatures (Clarkson et al., 2021; Fraser et al., 2014, 2015) as well as having a reduced tolerance to high temperatures later in life (Sambraus et al., 2017, 2018). Whilst these overarching physiological and requirement-based differences have received the majority of the attention over the years, much less well researched are the mechanisms behind them or specific differences in the transcriptome at different life stages or under different conditions.

The majority of studies investigating gene expression in triploids have selectively focused on a small number of genes. At the time of writing there were 4 known studies involving transcriptomic comparisons between diploid and triploid Atlantic salmon using either RNAseq (Odei et al., 2020; Olsvik et al., 2020) and microarray (Taylor et al, 2019, Vera et al., 2017). These studies investigated transcriptomic differences in the liver after nutritional programming or different diets (Taylor et al, 2019, Vera et al., 2017), the lens with and without cataracts (Olsvik et al., 2020), and the differences in the liver at three different life stages (fry, parr, and smolt) under normal grow out conditions (Odei et al., 2020). Studying the genetic differences between ploidy will increase scientific understanding of the mechanisms behind the above-mentioned physiological differences. Of particular relevance to industry is the reduced tolerance of triploids to higher temperatures during on-growing, this limits the success of the animals in trials and therefore their commercial acceptance (Sambraus et al., 2017, 2018). It is thought to be linked to a breakdown in the homeostasis of gene compensation mechanisms when under stress (Ching et al., 2010), but the mechanisms behind gene compensation breakdown are yet to be elucidated. Investigating transcriptomic differences between triploids and diploids under optimal and stressful conditions, may reveal the mechanism behind the reduced tolerance to higher temperatures seen in triploids. Transcriptomic analysis of the liver can reveal details of a number of key biological processes such as immunity, metabolism, and stress response (Beemelmanns et al. 2021, Martin et al. 2010, Taylor et al 2022, Soengas et al. 1996). This one of the reasons as to why this organ was chosen in three of the four previous transcriptomic studies on triploid

Atlantic salmon (Olsvik et al., 2020, Taylor et al. 2019, Vera et al., 2017). The liver has also been shown to be a good candidate organ for characterising temperature related stress within Atlantic salmon (Beemelmanns et al. 2021, Olsvik et al. 2013, Shi et al. 2019), and therefore may help reveal mechanism behind triploids lower temperature tolerance.

Investigation into the transcriptomic response of triploids to thermal stress would be of relevance to industry and of scientific value at both freshwater and saltwater stages. In this current study the freshwater stage of development was chosen as it is a stage at which manipulation of temperature should be stressful but not critically so for the triploid cohort. Experimentation in seawater would come with additional costs and complexity but with little to no added benefit over conducting the experiment in freshwater.

The aim of this experiment was to investigate transcriptomic differences in the liver between ploidy during the freshwater stage of development under two thermal conditions, a 12 °C optimal temperature and a 16 °C challenge temperature.

5.2. Materials & Methods

5.2.1 Experimental design

For a full description of the experimental design please see the chapter four section 4.2. In brief, and relevant for this chapter: triploid and diploid Atlantic salmon half siblings (1 hen, 2 males) were reared at 4°C from 2019-11-04 until eyeing (357 degree days (DD)) 2020-01-28 at which point they were shipped to the Institute of Aquaculture (IoA). One thousand and eight hundred eggs (900 per ploidy) were split between 6 control 300L tanks (300 per tank) at 6°C. As the controls for the above-mentioned experiment, these eggs were left relatively undisturbed with the exception of 1 period 24 hrs after arrival at the IoA. Each tray was gently moved into a Tupperware box whilst still completely submerged in water, this box was then removed from the tank and carried approximately 10 m across the aquarium and back, the box was then placed back into the tank and the egg tray returned to its original position.

After this point, the eggs remained undisturbed in the tanks. Just before first feeding the temperature in the tank was raised, 1°C a day, up to 12°C. The fish were grown

out until the 2020-09-26. All fish were fed a diploid diet, this was done so as not to add an additional confounding factor. Sampling took place over 3 days, with 1 set of replicates per ploidy being sampled each day. Two fish per tank were sampled for use in this experiment, 6 fish per ploidy per time point. The fish were randomly removed from the tank and euthanized using an overdose of benzocaine, death was then confirmed through an approved schedule 1 method. The frontal lobe of the liver was removed and placed into an Eppendorf containing RNA-Later and stored at -20 °C until subsequent RNA extraction. Once all tanks were sampled, the temperature was increased, 1°C per day, to reach 16°C. The 16°C challenge period ran until 2020-10-26, at which point all tanks were sampled over 3 days as described above. In total, the livers of 24 fish were sampled, 12 per ploidy with 6 per ploidy being taken at each temperature 12 °C and 16 °C.

5.2.2 RNA extraction

RNA was extracted from the liver samples using TriReagent [Sigma, St Louis, MO, USA]. A detailed description of this method can be found in chapter 4.2.6. The quality of RNA was checked using nanodrop [labtech, East Sussex, UK] to compare 260/280 and 260/230 ratios, with respective values of ~2.0 and between 2.0 and 2.2 being considered pure. Additionally, gel electrophoresis was also used to check for integrity and primer dimer, with 200ng RNA being run through a 1% agarose gel stained with ethidium bromide for 45 minutes at 100V. Bands were visualised using a UV transilluminator. RNA was quantified using Qubit Broad range kit [Invitrogen, Waltham, MA, USA] according to manufacturer's instructions. Aliquots of 100ng/µl total RNA in a total volume of 35µl were shipped on dry ice to Novagene UK for illumina RNA-seq sequencing [Cambridgeshire, UK]. Sequencing was performed using NovaSeq 6000 paired end 150bp.

5.2.3 Sequence analysis

Once sequencing had been completed, gene expression was compared within ploidy between 12°C and 16°C and also between ploidy at both 12°C and 16°C. Significance was determined at ≥2-fold expression change with p=0.001.

For transcriptomic assembly clean reads were obtained from the raw reads by filtering ambiguous bases, low quality sequences (< Q20), length (150 nt), absence

of primers/adaptors and complexity (entropy over 15) using fastp v0.23.1 (Chen et al., 2018). rRNA sequences were removed using SortMeRNA v3.0.2 (Kopylova et al., 2012) against the Silva version 119 rRNA databases (Quast et al., 2013). The remaining reads were mapped to the *S. salar* genome (Ssal_v3.1, GCF_905237065.1) using HiSat2 v2.2.0 (Kim et al., 2019). The expression levels were estimated using StringTie2 v2.1.0 (Kovaka et al., 2019) following the workflow: (a) reads were mapped to the genome with HiSat2 and aligned with StringTie2; (b) mapped reads were merged in order to generate a non-redundant set of transcripts observed in all the samples; (c) transcript abundance estimates and read coverage tables were expressed in the fragments per kilobase of exon per million mapped reads (FRKM).

The resulting transcript abundances for each sample were analysed using bioconductor/DESeq2 v3.10 (Love et al., 2014) and visualised using R/pheatmap v1.0.12. Abundance (Kolde, 2019) values were normalised using variance-stabilising transformations and Binomial-Beta models. Differential expression was estimated using the function lfcShrink (Stephens, 2016); thresholds, p-value < 0.001 and fold-change > 2. All *P* values were corrected for multiple testing (Bonferron) to reduce the false positive rate.

KEGG analysis was undertaken to identify functional pathways, Ensembl gene IDs were converted to NCBI using a custom script in R [R Core Team (2020)], these were then input into the pathway browser on reactome.org. No pathway contained a significant number of entries from either the triploid or diploid groups, in addition a reasonable portion of genes remained uncategorized using this approach and the number of pathways identified using KEGG was too large to be useful in a top-down approach. For these reasons, the functional groups were manually assigned.

Ensembl numbers were manually searched on Ensembl.org. For the majority of genes the Ensembl entry included a gene name, whereas genes which were less readily identifiable paralogues were identified using the Ensembl website and assigned a single HGNC. These identifiers were then searched for on zfin.org, uniprot.org, and genecards.org. The entries on these websites generally provided enough consistent information to assign function to each gene, for genes for which

the websites provided incomplete or inconsistent information Google Scholar was used to provide additional information on the functional purpose of the gene.

5.3. Results

5.3.1. Transcriptomic analysis

The sequencing of the 24 samples generated a total of 1,282,714,206 raw paired-end reads, with an average of 53,446,425 per sample. The reads were deposited in the European Bioinformatics Institute (EBI) European Nucleotide Archive (ENA) project ID PRJEB55249. An average of 52,867,750 paired-end reads (98.9%) passed the pre-processing filters and were used during the mapping.

5.3.2 Triploid v Diploid gene expression

At 12°C only 5 genes out of 55819 candidates showed a higher than 2-fold difference in expression between ploidy (p=0.001). Table 5.1 lists the ensembl number of these genes alongside common names, level of difference, and ploidy with the highest expression. Of the 5 genes, 4 were more highly expressed in diploids and 1 was more highly expressed within triploids. The two genes with the highest difference in expression between ploidy (hamp1 and hepc1) are paralogs of hepcidin.

Table 5.1. List of genes differentially expressed between triploid and diploid Atlantic salmon (*Salmo salar*) half siblings at 12°C. Significant change in expression determined as ≥2-fold change in expression with p=0.001.

Ensembl number	Common name	Fold difference	Ploidy with the highest expression
ENSSSAG00000053028|hepc1	hepcidin-like	60.78	Dip
ENSSSAG00000068171|hamp1	hepcidin-like	12.20	Dip
ENSSSAG00000054781|si:dkey-31g6.6	uncharacterised	7.13	Dip
ENSSSAG00000046431|INHBC	Inhibin Subunit Beta C	3.72	Dip
ENSSSAG00000081445|MCF2L	Guanine nucleotide exchange factor DBS	2.77	Trip

At 16°C, the between ploidy difference in gene expression remained low with only 5 genes reaching the criteria of a higher than 2-fold difference in expression at *p*=0.001 (Table 5.2). All of the genes were more highly expressed within the diploids and there was no crossover between the between ploidy DEGs at 12°C and the between ploidy DEGs at 16 °C.

Within ploidy, the number of genes with differential expression at the higher temperature was considerably higher within triploids, with 121 genes being differentially expressed compared to 37 within the diploids (Figure 5.1). Of these genes, there were 15 which were shared between ploidy.

Table 5.2. List of genes differentially expressed between triploid and diploid Atlantic salmon (*Salmo salar*) half siblings at 16°C. Significant change in expression determined as ≥2-fold change in expression with *p*=0.001.

Ensembl number	Common name	Fold difference	Ploidy with the highest expression
ENSSSAG00000005446|hgfa	Hepatocyte growth factor a	4.40	Dip
ENSSSAG00000003944|si:ch73-141c7.1	COQ10 / ubiquinone	3.68	Dip
ENSSSAG00000057844	TSC22 domain family member 3	3.40	Dip
ENSSSAG00000054690	regulator of G-protein signalling 3-like isoform X6	2.93	Dip
ENSSSAG00000006726|arhgef1a	Rho guanine nucleotide exchange factor (GEF) 1a	2.42	Dip

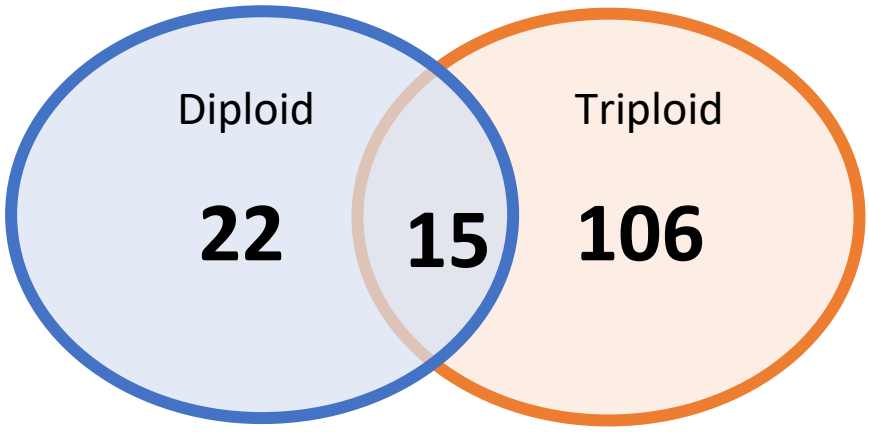


Figure 5.1. Number of genes differentially regulated within the liver between 12°C and 16°C in triploid and diploid Atlantic salmon (*Salmo salar*) half siblings. Significant change in expression determined as ≥2-fold change in expression with *p*=0.001.

5.3.3 Within ploidy functional groups

These 144 distinct genes, that showed differential expression between temperature within ploidy, were organised into one of 20 functional groups (Figure 5.2). This grouping gives a broad overview of the functional differences in gene expression between ploidy. Within triploids, 58 genes were upregulated between 12°C and 16°C, whilst 63 were down regulated. These numbers for diploids were 17 and 20 respectively. Of the 21 functional groups, 13 contained genes from both ploidy (figure 5.2). Twelve of these 13 groups contained a larger number of genes from triploids than from diploids. Seven categories contained only genes differentially regulated in triploids. Only two categories contained more diploid genes than triploid; cytoprotection contained two differentially regulated genes from diploids and none from triploids, whilst epigenetic regulation contained 3 from diploids and 1 from triploids.

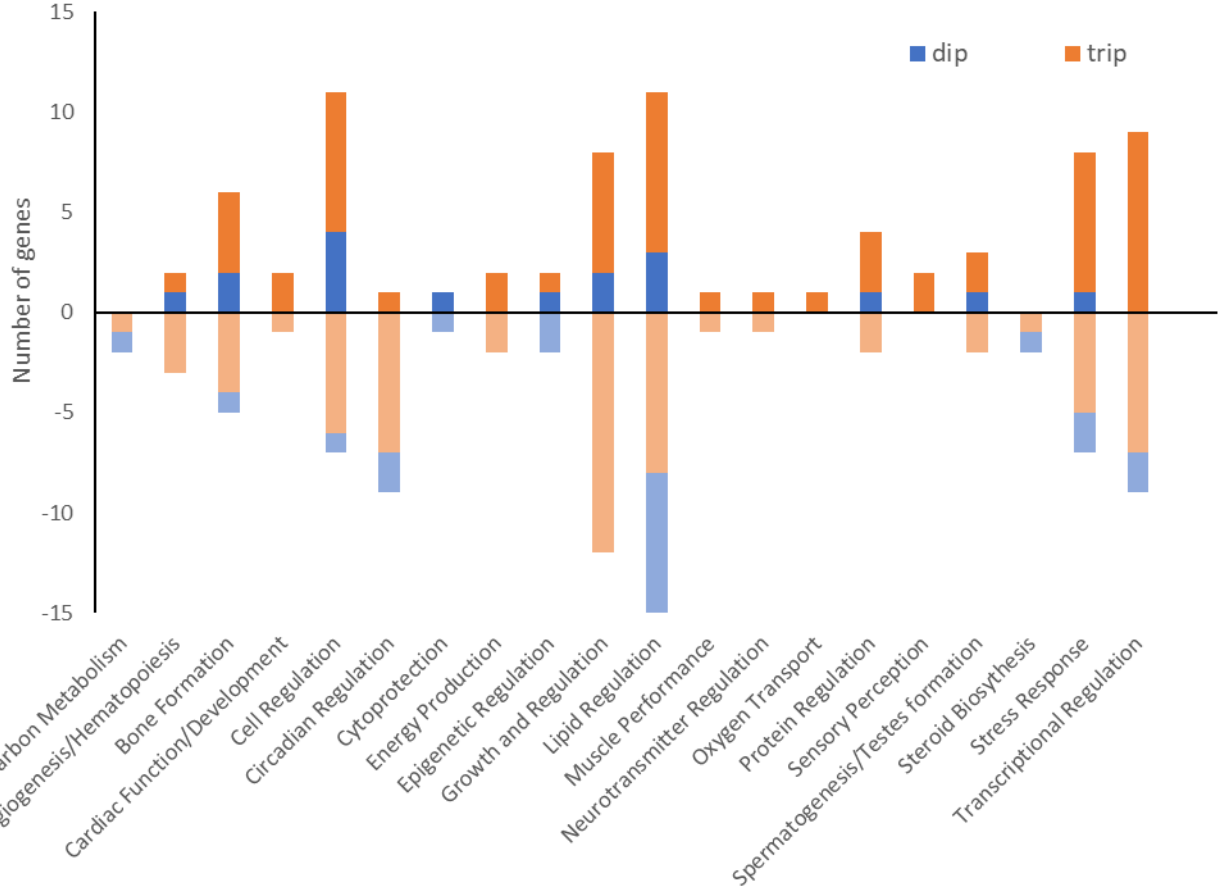


Figure 5.2. Number and functional group of genes differentially regulated within the liver between 12°C and 16°C in triploid and diploid Atlantic salmon (*Salmo salar*) half siblings. Significant change in expression determined as ≥2 fold change in expression with p=0.001.

5.3.4 Largest between ploidy differences

For all 15 shared genes, direction of expression change was consistent between ploidy. Between ploidy, difference in degree of change was limited to <0.5-fold for 11 of the 15 genes. 4 of the genes showed a larger than 0.5-fold difference in their expression change between ploidy (Table 5.3).

Table 5.3. List of the genes with the highest difference between ploidy amongst genes that are mutually differentially expressed between 12 °C and 16 °C in triploid and diploid Atlantic salmon (*Salmo salar*) half siblings. Significant change in expression determined as ≥2-fold change in expression with p=0.001.

Ensembl number	Common name	Direction of change	Fold change triploid	Fold change diploid	Fold difference in expression change
ENSSSAG00000067422	chondroadherin-like protein	neg	3.36	7.13	1.12
ENSSSAG00000064906|PGM1	phosphoglucomutase-1-like	pos	5.42	2.65	1.04
ENSSSAG00000044931|PPP2R2C	protein phosphatase 2 regulatory subunit B gamma	pos	8.11	4.15	0.95
ENSSSAG00000042423|zgc:174917	phytanoyl-CoA dioxygenase domain-containing protein 1-like	pos	17.52	10.78	0.63

5.3.5 Largest within ploidy differences

One hundred and twenty-one triploid genes and 37 diploid genes showed a ≥2-fold change in expression between 12°C and 16°C (p=0.001). Of these genes, 47.4 and 45.9 % showed between 2-3 fold difference in expression for diploids and triploids respectively. These numbers were 81.6 and 77.9 % for2-5-fold difference in expression and 92.1 and 95.1 % for 2-10-fold expression for diploids and triploids respectively. Three genes (from 37) showed a larger than 9-fold increase in expression between 12°C and 16°C for diploids, whilst 6 genes (from 121) showed a more than 9-fold difference in expression in triploids (Table 5.4). Two of the 3 diploid genes and 2 of the 6 triploid genes that showed a greater than 9-fold change in expression were paralogs of the same gene phytanoyl-CoA dioxygenase domain-containing protein 1-like.

Table 5.4. List of the genes with greater than 9-fold change in expression between 12 °C and 16 °C in triploid and diploid Atlantic salmon (*Salmo salar*) half siblings. Significant change in expression determined as ≥2-fold change in expression with *p*=0.001.

Diploid			
Ensembl number	**Common name**	**Direction of change**	**Fold Change**
ENSSSAG00000051749|krt15	Keratin 15	pos	98.17
ENSSSAG00000067386|zgc:174917	phytanoyl-CoA dioxygenase domain-containing protein 1-like	pos	11.76
ENSSSAG00000042423|zgc:174917	phytanoyl-CoA dioxygenase domain-containing protein 1-like	pos	9.50
Triploid			
Ensembl number	**Common name**	**Direction of change**	**Fold Change**
ENSSSAG00000007690	actin alpha 1, skeletal muscle	neg	23.17
ENSSSAG00000076802|bgn	Biglycan	neg	21.17
ENSSSAG00000042423|zgc:174917	phytanoyl-CoA dioxygenase domain-containing protein 1-like	pos	14.84
ENSSSAG00000055428|ciarta	Circadian-associated repressor of transcription a	neg	14.22
ENSSSAG00000055794|ryr2b	Ryanodine receptor 2b protein	pos	12.50
ENSSSAG00000067386|zgc:174917	phytanoyl-CoA dioxygenase domain-containing protein 1-like	pos	10.13

5.3.6 Genes related to diet

A number of DEGs identified within the triploid group are either involved in the regulation of compounds of which triploids have an increased dietary requirement, namely histidine, phosphorous, and protein (table 5.5) or their expression is linked to variable expression of these compounds.

Table 5.5. List of genes with known association to the regulation of histidine, phosphorous, and protein, differentially regulated between 12 °C and 16 °C in triploid Atlantic salmon (*Salmo salar*) half siblings. Significant change in expression determined as ≥2-fold change in expression with p=0.001.

Ensembl number	Common name	Direction of change	Fold Change
Related to histidine			
ENSSSAG00000033265|PPEF2	protein phosphatase with EF-hand domain 2	pos	5.55
ENSSSAG00000065158|rdh12	Retinoic acid receptor RXR-gamma-A-like	pos	6.24
ENSSSAG00000067422	chondroadherin-like protein	neg	3.36
Related to phosphorus			
ENSSSAG00000046835	extracellular serine/threonine protein kinase FAM20C-like	pos	2.9
ENSSSAG00000000328|slc20a1b	sodium-dependent phosphate transporter 1-B-like	neg	2.74
ENSSSAG00000044931|PPP2R2C	serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B gamma isoform.	pos	8.1
ENSSSAG00000076802|bgn	Biglycan	neg	21.17
Related to protein			
ENSSSAG00000051498|rab12	RAB12, member RAS oncogene family	neg	5.47
ENSSSAG00000071192|ctgf	Connective tissue growth factor	neg	4.68
ENSSSAG00000005710|psmb6	proteasome subunit beta type-6-like	neg	2.41
ENSSSAG00000017975|ef2	Elongation factor 2	pos	2.86
ENSSSAG00000075949|psph	Phosphoserine phosphatase-like	neg	2.48
ENSSSAG00000044931|PPP2R2C	serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B gamma isoform.	pos	8.1

KEGG analysis failed to reveal significant pathways of interest. This is likely due to the low number of genes used in the analysis and/or the lack of consistent physiological function between genes.

5.4. Discussion

At 12°C only 5 genes showed more than 2-fold difference in expression between triploid and diploid Atlantic salmon (*Salmo salar*). Despite the large physiological differences between ploidy, this number is not a particular surprise. In a previous study by Odei *et al.* (2020) there were 25 differentially expressed genes (DEGs) at the parr stage between diploids and triploids. This study provided strong evidence of gene dosage compensation in Atlantic salmon and this conclusion is mirrored by the results in the current study. Of the 25 DEGs found in parr by Odei *et al.* (2020) none were present in the current study. When the *p* value in the current experiment was increased to 0.005 and therefore in line with the experiment by Odei *et al.* (2020) the number of DEGs increased to 11.

Two of the 5 DEGs at 12°C, and the 2 with the largest difference in expression, are paralogs of a hepcidin-like gene. These were 60.8 and 12.2-fold higher in diploids than in triploids. Hepcidin (HAMP), also known as LEAP-1 (liver expressed antimicrobial peptide), is a member of a group of molecules known as Antimicrobial peptides (AMPs) (Hilton & Lambert, 2008). HAMP is upregulated in response to iron overload and is in part responsible for iron homeostasis (Fraenkel et al., 2005; Hilton & Lambert, 2008; Pigeon et al., 2001). HAMP is also upregulated during infection and inflammation (Nemeth et al., 2003, 2006), in a variety of species and tissues, from the eye of a human (McIntosh et al., 2005) to the internal organs of a catfish (Bao et al., 2005), this suggests its importance in innate immune defences.

Whilst mammals (with the exception of the mouse) only have a single copy of the hepcidin gene, Atlantic salmon have at least 2 (Douglas et al., 2003). Within Atlantic salmon hepcidin-like genes have been shown to strongly upregulate during infection with *Aeromonas salmonicida* (Douglas et al., 2003; Martin et al., 2006) and it is thought that at least 2 versions of this gene are involved in immune response. It is not known if both HAMP genes are involved in iron homeostasis in Atlantic salmon, two different HAMP genes in the Japanese flounder (*Paralichthys olivaceus*) were found to respond in opposite directions in response to iron injection (Hirono et al.,

2005), but it seems likely that at least one version of this gene will be involved in the process. Whilst HAMP is upregulated in response to infection and iron overload, there is a strong negative response to hypoxia in vitro (using human cells) and in vivo (using mice) (Nicolas et al., 2002).

Hepcidin has been little researched within Atlantic salmon, and without further investigation it is not possible to ascertain the physiological impact upon triploids that having a significantly lower expression of these genes may cause. If basal levels of HAMP are lower in triploids this may impact their immune response and iron homeostasis. Given the difference in expression at normoxia it would be interesting to determine the effect of hypoxia on HAMP expression, especially given the importance of the gene in immune response and the limited tolerance of triploids to hypoxia.

The DEG with the third-largest difference between diploids and triploids at 12°C was unclassified. The fourth was INHBC (inhibin subunit beta C). There was a 3.72-fold difference in expression, with the higher expression being present in the diploid group. Inhibins are proteins first identified as inhibiting the pituitary secretion of FSH (follicle stimulating hormone) (Ling et al., 1986). In mammals INHBC is predominantly expressed in the liver (Rodgarkia-Dara et al., 2006; Schmitt et al., 1996) although there is good evidence that inhibin B may be produced in the testes (Anawalt et al., 1996) and ovaries (Woodruff et al., 1987) and may be used as a biomarker for spermatogenesis in males (Pierik et al., 1998). Little research has been done on the β subunit of inhibin in fish, but there is evidence though the use of gene knockout that INHA can cause infertility in female zebra fish (Lu et al., 2020). The lower expression of INHBC in triploids makes sense given the function of the protein and the sterility of both male and female triploids.

The final DEG at 12°C was MCF2L (Guanine nucleotide exchange factor DBS) the gene had 2.77-fold higher expression in triploids than diploids. MCF2L expression is associated with Osteoarthritis and vertebral pathologies in humans (Day-Williams et al., 2011) and zebrafish (Mitchell et al., 2013). MCF2L has also been observed in the cartilage of developing jaws in zebrafish, suggesting its function in cartilage development (Mitchell et al., 2013). At present, no studies can be found that have investigated the expression of MCF2L in Atlantic salmon, let alone triploids. Given

the propensity for vertebral (Fraser et al., 2013; Taylor et al., 2013) and lower jaw deformities (Amoroso et al., 2016) in triploid Atlantic salmon, and the link between MCF2L and these pathologies, this gene should be considered a likely candidate for further research into triploid deformities.

At 16°C the number of DEGs between ploidy remained low. Only 5 DEGs showed a 2-fold or higher difference in expression between ploidy. This is perhaps more surprising than the closeness at 12°C given the triploids lower tolerance to the higher temperature, as evidenced through the decline in feed intake towards the end of the experiment. It is perhaps the more subtle within ploidy differences that determine the performance of each ploidy, and this will be discussed further down. Of the 5 between ploidy DEGs at 16 °C all were between 4.5 and 2.4-fold differentially expressed. None of the 5 DEGs at 16 °C were consistent with the 5 DEGs at 12 °C. All DEGs at 16 °C showed higher expression within the diploid group. The DEG with the largest difference in expression between ploidy was HGFA (Hepatocyte growth factor a), is an activator of hepatocyte growth factor (Kataoka & Kawaguchi, 2010). It is therefore involved in tissue morphogenesis and homeostasis, regeneration, and tumour progression (Kataoka & Kawaguchi, 2010). HGFA affects epithelial and endothelial target cells and is activated in response to tissue injury (Miyazawa, 2010). HGFA was found to have a negative association to temperature within triploid Atlantic salmon (Ignatz et al., 2022), diploid salmon were not used in this study. HGFA also plays a role in the function of mammalian testes both embryonic and into adulthood (Uzumcu et al., 2009), and is present within the testes of the rainbow trout (*Oncorhynchus mykiss*) (Sambroni et al., 2013). This is one possible explanation for the difference in expression between ploidy.

The DEG with the 2nd largest between ploidy difference at the higher temperature was coq10/ubiquinone. Coq10 is an endogenous antioxidant, present in the inner membrane of mitochondria (Lenaz et al., 2007), that protects tissues and organs from oxidative stress (Hamre et al., 2010). Increasing dietary lipids increase the concentration of coq10 in the liver of Atlantic salmon (Hamre et al., 2010). It is not clear as to why this gene would be expressed higher in diploids than triploids at this temperature, but it may be an interesting candidate for future research into triploid temperature and hypoxia tolerance.

TSC22D3 (TSC22 domain family member 3) is thought to play an important role in immunity, adipogenesis, and renal sodium handling (Suarez et al., 2012), the gene is also thought to regulate the transcription of inflammation related genes (Kanda et al., 2020). In Zebrafish, the gene has been found to play a role in brain development and stimulating the transcription of bone morphogenetic protein 4 (bmp4) (Tse et al., 2013). In Atlantic salmon, TSC22D3 was shown to be down regulated in the liver after infection with the bacterium *Piscirickettsia salmonis* (Tacchi et al., 2011). During infection of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) with the myxozoa *Tetracapsuloides bryosalmonae*, the gene was significantly downregulated in rainbow trout early in the infection whilst it was upregulated in the brown trout later during the infection (Kumar et al., 2015). This suggests that response of TSC22D3 is variable between salmonid species. It should be noted that there was no sign of infection or poor health within either population in the current study. Interestingly, TSC22D3 is thought to play a role in testis development in mammals (Suarez et al., 2012).

RGS3 (regulator of G-protein signalling 3-like isoform X6) is thought to have a role in modulating the luteinising hormone secretory responsiveness to gonadotropin releasing hormone (GnRH) (Neill et al., 1997). In Zebrafish, knockout of RGS3 results in somite patterning defects (Freisinger et al., 2010), and cardiovascular defects (Freisinger et al., 2006). In humans, RGS3 is also thought to play a part in cardiovascular development and physiology (Cho et al., 2003). This is interesting given the differences in heart development between ploidy (Fraser et al., 2013, 2015).

The last DEG between ploidy at 16°C was arhgef1a (Rho guanine nucleotide exchange factor 1a). In humans, arhgef1 has been shown to affect blood pressure (Guilluy et al., 2010) and loss of the gene results in antibody deficiency (Bouafia et al., 2019). There have been no studies on the gene in Atlantic salmon, whilst in zebrafish the gene has been shown to be involved in stress fibre formation (Panizzi et al., 2007). Again due to the limited number of studies, especially regarding adult fish, it is impossible to elucidate as to why this gene shows differential regulation between ploidy at this stage.

As the temperature increased from 12°C to 16°C, the expression of certain genes within ploidy changed in response. The number of genes that change expression was over 3x higher within triploids than diploids, with 121 DEGs between 12°C and 16°C compared to 37 in diploids. Fifteen DEGs were consistent within both ploidy, the remaining 144 genes are distinct to a particular ploidy (these shared genes will be discussed in more detail later in this chapter). The 158 genes can be separated into 1 of 20 functional groups.

The DEGs present in groups such as growth and regulation, protein regulation, and lipid regulation are likely related to increased growth rates at the higher temperature.

As the temperature increased so did the growth rate, feed intake, and the size of the fish (see chapter 4.3.3 for details) to adjust for this genes related growth and regulation, protein regulation, lipid regulation, and angiogenesis/hematopoiesis were differentially regulated (Figure 5.2). Other groups appear to be less related to increased growth rates and, more likely, either the reasons behind, or responses to, the decreased tolerance of triploids to these higher temperatures. Between 12°C and 16°C diploids saw no DEGs involved in cardiac function and development whilst triploids saw 3 DEGs. Triploids have been shown to have a more acute angle of the bulbous arteriosus (Fraser et al., 2013, 2015) a condition more similar to wild type fish (Poppe et al., 2003). It is thought this condition may arise from increase cardiac workload (Leclercq et al., 2011; Poppe et al., 2003) in the face of reduced arterial O_2 loading (Verhille et al., 2013). The reduced capacity of triploid salmonids to supply O_2 alongside increased diffusion distances and reduced comparative cell surface areas (due to larger cells) are thought to be partially responsible for the reduced capacity of triploid salmonids to tolerate higher water temperatures (Altimiras et al., 2002; Hansen et al., 2015; Verhille et al., 2013). In addition, triploids seem to suffer from more rapid on set cardiac arrhythmia (Verhille et al., 2013). It is therefore not particularly surprising that 3 DEGs involved in cardiac function and development were identified in the triploid group compared to none in the diploids.

Triploids also have many more DEGs in categories such as transcriptional regulation and circadian regulation. It is thought that triploid Atlantic salmon, and triploid fish in general, balance gene expression to near diploid levels through genome dosage compensation mechanisms (Ching et al., 2010; Christensen et al., 2019; Devlin et

al., 2010; Odei et al., 2020; Pala et al., 2008; Ren et al., 2017). The exact mechanisms within salmon are still to be elucidated, but the result is that under normal conditions the expression levels of genes are remarkably similar between triploids and diploids (Ching et al., 2010; Christensen et al., 2019; Odei et al., 2020). It is thought that under stressful conditions the homeostasis of dosage compensation mechanisms breaks down (Ching et al., 2010) and this can lead to the problems that triploids face during stress events. The genes in the above-mentioned groups are possibly linked to this breakdown and are useful candidates for studies into this mechanism. The stress response group also saw many more DEGs in triploids than diploids, whilst there were no triploid DEGs associated with cytoprotection and 2 DEGs in the diploid group. The list of DEGs is too extensive to discuss each gene in detail, but should provide a useful resource for future work looking at specific problems with triploid growth and performance.

All 158 DEGs mentioned here showed a ≥2-fold change in expression between 12°C and 16°C. A number of genes showed considerably higher changes in expression. Those above 9-fold change were chosen for further discussion. Within the diploid group, 3 genes from a total of 37 showed a larger than 9-fold change in expression (Table 4). Within the triploid group this number was 6 genes from a total of 121 (Table 4). Two of the largest 3 DEGs in diploids and 2 out of the largest 6 in triploids are paralogues of PHYH (phytanoyl-CoA dioxygenase domain-containing protein 1-like). In all cases this gene was upregulated at 16°C.

PHYH codes for the enzyme phytanoyl-CoA 2-hydroxylase, this enzyme (found in peroxisomes) catalyses the hydroxylation of phytanoyl-CoA (Schofield & McDonough, 2007), this is the 1st step in the α-oxidation of phytanic acid (Jansen et al., 1997). Mutations to PHYH cause a build-up of phytanic acid which causes Refsum's disease in humans (Jansen et al., 1997; Schofield & McDonough, 2007). Phytanic acid is a component of chlorophyll (Schofield & McDonough, 2007). Refsum's disease can be treated through modulation of the diet and as such a number of studies have investigated the dietary sources of phytanic acid (Brown et al., 1993; Roca-Saavedra et al., 2017). Phytanic acid can be present in high concentrations in fish (as well as in ruminant animals and products made from their fats) (Brown et al., 1993; Roca-Saavedra et al., 2017). Phytanic acid is often stored in the fats of animals and as such can reach considerable levels in higher tropic

levels including carnivorous fish (Roca-Saavedra et al., 2017). Thus, farmed Atlantic salmon could quite possibly consume phytanic acid in their the diet (Brown et al., 1993), and depending on the level of inclusion the levels of phytanic acid can be higher in farmed than wild fish, as in the case of the sturgeon (*Acipenser oxyrinchus desotol*) (Chen et al., 1995). The physiological effects of phytanic acid on fish are unknown, and it is possible that high levels could cause retinal problems. Refsum's disease affects the retinal pigment epithelium in humans and causes visual problems (Bernstein et al., 1992) and PHYH was identified as an under-expressed gene in a study on zebrafish bred to be retinal dystrophic mutants (Zhang et al., 2014). The high level of expression found in this study may be down to the increased consumption of feed during the temperature challenge, and the increased need to process the higher levels of phytanic acid.

The remaining DEG with an expression change over 9-fold within the diploid group is KRT15 (Keratin 15). KRT15 showed the largest increase of any gene within the diploid group. KRT15 has been shown to be involved in fin regeneration and in forming wound epithelium in fish (Lee et al., 2020; Murawala et al., 2017). KRT15 is also thought to serve a role in the tooth development of mice and gar (*Lepisosteus oculatus*) (Rostampour et al., 2019) as well as microbial protection in the mucus of the teleost Fugu (*Takifugu ruberipes)* (Shibuya et al., 2019). The fish in this experiment were not seen to suffer from an increase in wounds, abrasions, or fin damage during the temperature challenge.

Within the triploid group, there are 4 remaining DEGs with an over 9-fold change in expression. Ryr2b (Ryanodine receptor 2b protein) showed a 12.5-fold increase in expression between 12°C and 16°C in triploids. Ryanodine receptors mediate the release of intracellular stores of calcium (Darbandi & Franck, 2009). Ryr2b is expressed within predominantly cardiac tissue within fish, from early development into adulthood (Darbandi & Franck, 2009; Wu et al., 2011). In mammals, ryanodine receptors have been shown to have a vital role in the contraction of cardiac muscles (Escobar et al., 2004; Pérez et al., 2005; Rosemblit et al., 1999). As mentioned earlier the cardiac performance of triploids is thought to be inferior to that of diploids, in addition, within salmonids, the maximum cardiac response to temperature seems to be limited by the maximum heart rate (Farrell, 2009; Verhille et al., 2013). Ryr2b

appears to play an important role in the triploid response to high temperatures, and its role in the cardiac function of triploid salmon warrants further investigation.

Ciarta (Circadian-associated repressor of transcription a) also known as Gm129, CHRONO, and CIART is a novel transcriptional repressor. Ciarta was found to be downregulated 14.22-fold at the higher temperature within the triploid groups. Chen *et al*. (2016) found this gene to exhibit the highest rhythmicity within the human cortex, suggesting an important circadian function in the human brain. It is thought to directly interact with BMAL1 to repress CLOCK/BMAL1 function in the liver of mice and fine-tune regulation of rhythmic gene expression (Annayev et al., 2014). Ciarta knockout mice showed a prolonged free-running circadian period (Anafi et al., 2014), and an impaired response of the circadian clock to stress (Goriki et al., 2014), again highlighting the importance of the gene in maintenance of the circadian clock. Ciarta has not been extensively studied, let alone in fish, and it is not clear the response of the gene to temperature stress. What is apparent however is that significant downregulation of this gene could have wide-ranging effects on the circadian clock and beyond.

Bgn (Biglycan) was significantly downregulated between 12°C and 16°C in the triploid group. The role of biglycan in bone development has been known for almost 40 years (Fisher et al., 1983) whilst in more recent years its role as a signalling molecule, playing an important role in inflammatory response, muscle integrity and synapse stability, have become apparent (Nastase et al., 2012). Within Atlantic salmon, BGN has been shown to be down regulated in deformed vertebrae compared to non-deformed vertebrae (Pedersen et al., 2013). Given the propensity of triploids to develop vertebral deformities when they are growing at their fastest (Leclercq et al., 2011), as happens when temperatures increase, the regulation of biglycan may play an important role in the development of vertebral deformities.

ACTA1 (actin alpha 1) was DEG with the largest downregulation in the triploid group. Within humans, ACTA1 is predominantly found in the skeletal muscle and is essential for muscle contraction (Laing et al., 2009). In Zebrafish, ACTA1 is expressed within the cardiac tissue (Ojehomon et al., 2018; Singh et al., 2016). In Atlantic salmon, elevated cardiac mRNA levels were suggested to be linked to cardiomyocyte hypertrophy (Castro et al., 2013; Grammes et al., 2012). As with

Ryr2b this gene could provide useful information on the subject of triploid cardiac development and thermal tolerance.

Four of the 15 shared DEGs showed a >0.5-fold difference in expression between ploidy. The shared DEG with the largest difference in expression between ploidy was chondroadherin-like protein (CHADL) (table 5.3). Between 12°C and 16°C this gene was downregulated in both ploidy. In triploids the decrease in expression was 3.36-fold whilst in diploids it was 7.13-fold, a difference of 1.12-fold, or a 112 % larger decrease in diploid than in triploids. CHADL is thought to have a negative regulatory role in collagen binding and chondrocyte differentiation (Tillgren et al., 2015; Tuerlings et al., 2021). In a study of human cataract patients, CHADL was the protein with the largest down regulation in healthy patients compared to those with ocular disease. Previous studies have shown that triploids are more susceptible to cataracts than their diploid counterparts (Taylor et al., 2015) and that this pathology worsens at 16°C (Sambraus et al., 2017). Increased dietary histidine has been shown to decrease the prevalence of this disease (Sambraus et al., 2017; Taylor et al., 2015). In the current study, a diploid diet was used for both ploidy so as not to introduce an additional confounding factor. CHADL was not identified in a previous transcriptomic analysis of diploid and triploid Atlantic salmon with cataracts (Olsvik et al., 2020).

The DEG with the 2nd largest difference in expression between ploidy was PGM1 (phosphoglucomutase-1-like). In both ploidy this gene was upregulated at the higher temperature with the shift in expression being 1.04-fold greater within triploids than within diploids (Table 5.3). PGM1 is involved in several crucial metabolic pathways including glycogen metabolism, glycolysis, and protein glycosylation (Altassan et al., 2021). Given the role of the gene in energy production, it is not surprising that it was upregulated at a time when the fish were growing faster. It is not clear why triploids would increase their expression of this gene to a larger extent than diploids, but it is possible that the reduced thermal tolerance of the triploids increased the energy demands of the animals.

PPP2R2C (phosphatase 2 regulatory subunit B family) was significantly upregulated in both ploidy (Table 5.3) at the higher temperature but with a fold change in expression of 8.11 for triploids and 4.15 for diploids, the shift in expression was

almost twice as high within the triploid group compared to the diploids. PPP2R2C plays an important role in cell-cycle regulation, controlling cell-growth, as well as cytoskeleton dynamics, and the regulation of multiple signal transduction pathways (Backx et al., 2010; Xu et al., 2006). Suppression of the gene leads to intellectual disability in humans (Backx et al., 2010), whilst the gene also acts as a tumour suppressor gene (Bluemn et al., 2013; Fan et al., 2013). Interestingly, given the above-mentioned performance of the triploid heart, multiple correlation analysis identified PPP2R2C as a candidate gene thought to be linked to an increased susceptibility to coronary heart disease through the (dis)regulation of circadian rhythm pathways (Yan et al., 2018). The gene has not been studied in Atlantic salmon.

PHYH is the DEG with the 4th largest discrepancy between diploid and triploid expression change. As mentioned above, this gene is involved in the breakdown of phytanic acid. PHYH increased in expression to a greater extent in triploids (17.52-fold) than diploids (10.78-fold) (Table 5.3). One possible reason for the greater increase in expression in triploids is simply the fact that triploids were consuming more food than diploids. As triploids ate more they would have consumed a greater level of phytanic acid compared to diploids.

Unfortunately, when designing an experiment involving diploids and triploids, you are faced with a difficult decision. Do you feed each ploidy a ploidy specific diet, therefore introducing an additional confounding factor and accept the problems this will pose when it comes to direct comparisons of gene expression. Or do you feed both ploidy the same diet, if this diet is diploid then you run the risk of an increase in deformities in your triploid group (Fjelldal et al., 2016; Sambraus et al., 2017, 2020; Smedley et al., 2016, 2018; Taylor et al., 2015) and potentially reduced growth potential, at least in the seawater stage (Smedley et al., 2016). If you choose to feed all your fish a triploid diet, the data obtained from diploids becomes less comparable to industry and previous studies, and you run the risk of overexposure to certain nutrients. In this experiment a diploid diet was used for all fish, the reason for this decision was to reduce the noise, and the fact that due to the relatively short duration of the experiment and low incubation temperatures the likelihood of a welfare impact on triploids would be low, this was proven to be correct given the low level of vertebral deformities and the absence of cataracts.

A list of DEGs was however identified from the data whose shift in expression may be linked to the dietary requirements of the triploids (Table 5.5). Why these DEGs were differentially regulated between 12°C and 16°C may be linked to the increased growth rate at this temperature, with faster growth being thought to be linked to an increase in specific deformities (Fjelldal et al., 2016; Smedley et al., 2016; Taylor et al., 2015), there has also been shown a link between higher temperatures and cataract formation with increased dietary histidine reducing this issue (Sambraus et al., 2017). Whilst the diploid diet allows direct comparisons to be made between ploidy, it should be noted that some of the DEGs present within the triploid group may not be present should a triploid diet been fed instead.

In conclusion, it is perhaps surprising how similar the gene expression of triploid and diploid Atlantic salmon is. There are a large number of studies describing the various ways in which triploid salmon differ physiologically from their diploid counterparts yet both this study and work by Odei *et al.* (2020) have shown that the number of genes that differ significantly in their expression between ploidy is rather small. Even at 16°C the between ploidy differences remain minimal. The within ploidy DEGs are where differences start to be seen. Within the triploid group the number of DEGs between 12°C and 16°C was 3x higher than within the diploid group, and the pathways these DEGs relate can help explain why. We have described the most significant DEGs in detail, observing large variation in genes related to known problems such as cardiac function, ocular deformities, cartilage and bone development, we have also observed the significance of less studied genes and areas of neglect within triploid research such as circadian regulation. This work provides the groundwork for future studies looking at specific differences or problems within the culture of triploid Atlantic salmon, especially in studies related to the thermal tolerance of these animals.

Chapter 6. Transcriptomic response of diploid and triploid Atlantic salmon (Salmo salar) eyed embryos after thermal shock

Abstract

Triploid Atlantic salmon (*Salmo salar*) are more sensitive to higher incubation temperatures than their diploid counterparts. Results from Chapter 4 showed how a 6 hr thermal shock at 360 degree days resulted in triploids being more tolerant to a thermal challenge than a control group, with the 6 hr diploid group showing results which suggested, but did not significantly show this same result. This chapter is the first study to explore the transcriptomic and epigenetic differences between ploidy at this stage of development. We saw few genes with a significant difference in expression after thermal shock, but we did see a large change in DNA methylation patterns. Due to the lack of transcriptomic differences, it is impossible to conclude the true impact of the epigenetic differences but previous studies have shown that differences in the DNA methylation can result in changes in tolerance to environmental stress. We also saw differences in the level of methylation between ploidy particularly the fact that the triploid groups showed a larger level of variation between individuals than the diploids. This study adds to the existing body of work and provides a unique insight into the transcriptomic and epigenetic differences between triploid and diploid Atlantic salmon. For triploid salmon to become a viable alternative to diploids then the physiological differences must be addressed and studies which reveal underlying differences or similarities are key to this.

6.1. Introduction

A core pillar of modern genetics is the concept that differences in the genome (the genetic code of an organism), affect the transcriptome (RNA molecules expressed by an organism) and the phenotype of an individual (Lappalainen et al. 2013). There is growing interest in the fact that changes to the epigenome can also alter the transcriptome. Epigenetic differences are “on top of” or “in addition to” genetic differences, and they control gene activity at the transcription, translation, and post-translation levels (Jaenisch & Bird, 2003). Epigenetic mechanisms are described in

detail in Chapter 1 section 5.2 but they include chromatin and histone modifications (Sadakierska-Chudy & Filip, 2015), non-coding RNA (Sadakierska-Chudy & Filip, 2015; Zhou et al., 2010), and DNA modifications (Gosselt et al., 2020; Sadakierska-Chudy et al., 2015). DNA methylation is one of the better understood epigenetic modifications, and reduced reduction Bisulfite sequencing alongside RNAseq have been used to study changed in the transcriptome and epigenome caused by external conditions (Moghadam et al. 2017; Mukiibi et al. 2022; Uren Webster et al. 2018).

Epigenetic changes can be inherited (Aguilera et al., 2010; Wang et al., 2017) and influenced by the environment (Aguilera et al., 2010; Granada et al., 2018). It is the influence of the environment on the epigenome that has promoted recent interest in the idea of epigenetic programming. This is the concept that early life experiences can alter the epigenome, and this can affect the transcriptome later in life, and that these changes may improve an animals' tolerance to stress later in life. It has been explored in fish with nutritional programming (Vera et al., 2017). Thermal shocks have also been used in attempts at programming, and thermal manipulation has been shown to increase thermal tolerance in broiler chickens (Collin et al., 2005; Loyau et al., 2016; Morita et al., 2016; Piestun et al., 2008; Yahav et al., 2004).

In Chapter 4, “Impact of early temperature regimes on egg development and juvenile performance in diploid and triploid Atlantic salmon (*Salmo salar*) siblings” we saw the potential for thermal programming in the growth of triploid Atlantic salmon. Triploid Atlantic salmon that were subjected to a 6 hr thermal shock around 360 degree days (DD) were no heavier than control fish after on-growing at 12°C for 9 months (34.24 g and 34.28 g respectively). However, after spending 23 concurrent days at 16°C, in addition to a 6-day period in which the temperature was slowly increased from 12°C to 16°C, the triploid 6 hr treated fish were significantly heavier than the control group, weighing 4.3 g or 6.96 % more. This is evidence of thermal programming, and the difference in growth during the challenge period shows an increased tolerance to this temperature in the shocked fish.

If thermal programming can be shown to reliably work within Atlantic salmon, this has the potential to massively benefit the industry and improve the welfare of millions of salmon. Triploid Atlantic salmon are particularly susceptible to high temperatures and hypoxia (Riseth et al., 2020), but the farming of diploid counterparts is also

limited geographically by water temperatures and as temperatures continue to rise the range at which they can be safely farmed is likely to shrink (Calado et al., 2021). Increasing the tolerance of Atlantic salmon to high temperatures will not only improve welfare during high summer temperatures, but also potentially allow the continued farming of salmon in areas in which temperatures increase beyond the typical optimum. Increased tolerance of temperature would also go a long way to tackle a major argument against the farming of the sterile triploid salmon (Riseth et al., 2020).

To properly understand thermal programming, it is not enough to just look at the end result. One must also look at the mechanisms behind the change and the effects of the shock. Looking at the transcriptome after a shock can inform us as to which genes are most affected by the shock. If certain genes related to thermal/stress tolerance or known pathologies are differentially regulated, this can shed information of possible positive and negative effects of the shock. Looking at the epigenome such as the methylation patterns at a single nucleotide level (e.g. RRBS) can provide information on genes which may be primed to express differently later in life as a result of the shock. This epigenetic programming can provide an adaptive response when faced with environmental challenges later in life (Bateson et al., 2014; Gluckman et al., 2005).

Few studies have looked into the effect to thermal shock on the transcriptome or epigenome and what recent studies there are have focused on cold stress rather than heat stress.

Two recent studies exposed Atlantic salmon embryos (250-450DD) to a 5x repeated cold shock (7°C to 0.2°C for 1 min followed by air exposure (15°C) for 1 min before returning to 7°C), showed differential expression of a large number of genes related to development as well as changes to the methylome (Moghadam et al., 2017; Robinson et al., 2019). Another study used the same shock but once and this showed very little effect on the transcriptome of the gill after hatch but significant changes to the methylome as well as increased transcriptomic response when faced with a pathogenic challenge (Uren Webster et al., 2018). As far as this author is aware, no studies have looked at the overall transcriptomic or epigenetic response to heat shock within Atlantic salmon embryos and few studies have explored gene response to shocks at this stage of embryogenesis.

The aim of this experiment was to analyse the transcriptome and epigenome of eyed Atlantic salmon embryos that had recently be exposed to a thermal shock. This will not only improve our understanding of the response to diploids and triploids to temperature variations at this stage but also increase our understanding of the transcriptomic and epigenetic reasons behind previously observed thermal programming.

6.2. Materials and methods

6.2.1 Experimental design

The samples used in this experiment were from the same batch of eggs as those used in Chapter 4 and 5 and a more detailed description of the experimental design can be found in Chapter 4.2.1. In brief, triploid and diploid Atlantic salmon eggs where subject to thermal shocks at around 360 degree days. Shocks consisted of a sudden increase in water temperature from 6°C to 10°C and back again after either 1 hr or 6 hrs, with a control being unshocked. 24 hrs after the shocks, the 6 eggs per tank were removed from the egg tray before being instantly frozen using dry ice. A timeline of this experiment can be seen in Figure 6.1. These were then stored at -70°C for subsequent RNA and DNA extraction. The RNA and DNA was extracted from all eggs, with the 2 best eggs from each tank with the best combination of quality RNA and DNA being chosen for sequencing.

Table 6.1. Outline of the shocks used – 3 trays per ploidy underwent each treatment

Temperature	Duration	Number of days
10°C	1hr	1
10°C	6hr	1
No change	Only movement	1

6.2.2 Timeline of experiment

	04-Nov	-	28-Jan	29-Jan	30-Jan	31-Jan	01-Feb	02-Feb	03-Feb	-	16-Feb	-	14-Sep	30-Sep	28-Oct	02-Nov	04-Nov
Time days	0		86	87	88	89	90	91	92	-	105		317	332	359	363	365
Time degree days	0	-	357	363	369	375	381	387	393	399-464	470	-					
Average water temperature (°C)	4	4	6	6	6	6	6	6	6	6	6	12	12	16	16	16	16
	Fertilisation	Incubation	Ship to IoA	Thermal shocks	Thermal shocks	Thermal shocks	Thermal shocks	Thermal shocks	Sampling	Incubation	Hatch	Growth	Feed intake challenge starts	Feed intake challenge	Feed intake challenge ends	PAMPs challenge starts	PAMPs challenge ends
														Temperature increase			
Sampling			x		x				x				x		x		x

Figure 6.1 Experimental timeline showing time in days and average temperatures, key timepoints and sampling. Degree days are shown for until hatch.

6.2.3 Parallel DNA and RNA extraction

Before RNA and DNA could be extracted from the same sampled embryos, the process had to be refined. For this purpose, spare eggs that had been shipped to the IoA, but not laid in the tanks, were used. These eggs were also flash frozen and stored at -70°C.

To extract both DNA and RNA from the same egg, the embryo was first dissected and homogenised. The eggs were removed from -80°C storage and placed into an Eppendorf containing 500µl -20 °C RNA*later*-ICE [Thermo fisher, Waltham, MA, USA]. A syringe was used to pierce the frozen egg multiple times to allow ingress of RNA*later*-ICE and the tubes placed into a -20°C freezer. After 3 hours, the eggs were removed and a syringe used again to further pierce the now defrosted egg. The eggs were stored overnight in the freezer in RNA*later*-ICE solution. The next day, the embryo was dissected out of the egg using a dissection microscope (eggs were stored at room temperature for a maximum of 15 minutes before dissection). The embryo was placed into a pestle stored on ice, 430µl of ice-cold 5mM TE pH8 was added to the pestle and a mortar used to homogenise the embryo. 200µl of the homogenate was added to each of 2 separate tube, one screw cap for RNA extraction and one Eppendorf for DNA extraction. The pestle and mortar were cleaned using RNase free water and 80 % ethanol between samples. The tubes were stored on ice for a maximum of 15 minutes. For RNA extraction 1ml of TRI-Reagent was added to each tube, after 30 minutes of incubation on ice the tube was placed into a -80°C freezer. The remaining steps of the RNA extraction were completed 2-5 days later using the TriReagent method. The tube containing 1 ml TRI-reagent and 200 µl of the homogenate was brought up to room temperature. After 5 minutes at room temperature 100 µl of 1-bromo-3-chloropropane (BCP) [Sigma, St Louis, MO, USA] was added and the tube vigorously shaken for 15 seconds. Following 15 minutes incubation at room temperature the tubes were centrifuges at 20,000 x g for 15 minutes at 4 °C. 400 µl of the upper aqueous phase was transferred to a new tube before adding 200 µl each of RNA precipitation solution (1.2M NaCl and 0.8M Sodium Citrate Sesquihydrate dissolved in nuclease-free dH_2O) and 100% isopropanol (propan-2-ol) [Fluka, Buchs, Switzerland]. After inverting 5 times, the samples were incubated for 10 minutes at room temperature before centrifuging at 20,000 x g at 4°C. The supernatant was removed, and the

pellet washed for 30 minutes in 1 ml of 75 % ethanol. The ethanol was fully removed before the pellet was re-suspended in 60 µl RNase free water at room temperature for 1 hour.

The quality of the RNA was checked using nanodrop [labtech, East Sussex, UK], and gel electrophoresis, with 200 ng RNA being run through a 1 % agarose gel for 45 minutes at 100V. RNA was quantified using Qubit Broad range kit [Invitrogen, Waltham, MA, USA] according to manufacturer's instructions. Aliquots of 100 ng/µl total RNA in a total volume of 35 µl were stored at -70°C until they could be sent for sequencing.

The DNA extraction took place immediately after embryo homogenisation and a modified SSTNE extraction was used. To each tube of 200 µl of homogenate, 136 µl of SSTNE buffer was added along with 16.5 µl SDS, 6.25 µl proteinase K, 12.5 µl EDTA, and 2.5 µl DTT. The mixture was incubated at 55°C overnight in a rotating incubator. The next morning, 3.75 µl Proteinase K was added to each tube and incubated for a further 1 hr. The tube was heated to 70°C for 5 minutes to inactivate the Proteinase K. The tube was allowed to cool to room temperature before 6.25 µl RNase was added and incubated in a rotating incubator at 37 °C. After cooling to room temperature 130 µl of 5M NaCl was added and mixed by inversion for 30 seconds. The samples were incubated on ice for 10 minutes before centrifuge at 21,000 x g for 10 minutes at room temperature. The DNA containing supernatant was transferred to a new tube using a pipette and before the samples were spun again at 21,000 x g for 10 minutes. The supernatant was pipetted into a new tube and an equal volume of room temperature isopropanol was added. This was mixed by 6 rapid inversions and left to stand on ice for 10 minutes, it was then centrifuged at 21,000 x g for 10 min at room temperature and the supernatant removed using a pipette. 1 ml of 70 % ethanol was added and the samples were incubated in a sample rotator at room temperature for 2 hrs. The ethanol was removed and replaced with fresh 70 % ethanol, and the samples incubated overnight in the rotator. The next morning, the samples were centrifuged for 10 minutes at 21,000 x g and the ethanol removed. The samples were resuspended in 35 µl 5mM TE 8pH for 24 hrs at 4°C. The samples were then quantified using Nanodrop and integrity checked using gel electrophoresis.

Aliquots of exactly 100ng in 26µl of water were created and quantified using the Qubit dsDNA BR assay kit [Invitrogen, Waltham, MA, USA]. To assess DNA methylation at the nucleotide level unmethylated cytosines were converted to uracil and then thymine using bisulfite conversion, the methylated cytosines remained cytosines. The Diagenode Premium RRBS (Reduced-representation bisulfite sequencing) kit [Diagenode, Liege, Belgium] was used to bisulfite convert, amplify, and pool the DNA according to manufacturer's instructions. Bisulfite conversion was confirmed using a bioanalyzer 2100 [Aligent, California, USA].

6.2.4 Sequencing

Once Bisulfite conversion was complete, and it was confirmed that the RNA and bisulfite converted DNA from the same embryos would be sequenced, the RNA samples were shipped on dry ice to Novagene [Cambridgeshire, UK] for sequencing. Sequencing was performed using NovaSeq 6000 paired end 150bp. The RRBS libraries were shipped to Genewiz-Azenta [Leipzig, Germany] for sequencing.

For transcriptomic assembly clean reads were obtained from the raw reads by filtering ambiguous bases, low quality sequences (< Q20), length (150 nt), absence of primers/adaptors and complexity (entropy over 15) using fastp v0.23.1 (Chen et al., 2018). rRNA sequences were removed using SortMeRNA v3.0.2 (Kopylova et al., 2012) against the Silva version 119 rRNA databases (Quast et al., 2013). The remaining reads were mapped to the *S. salar* genome (Ssal_v3.1, GCF_905237065.1) using HiSat2 v2.2.0 (Kim et al., 2019). The expression levels were estimated using StringTie2 v2.1.0 (Kovaka et al., 2019) following the workflow: (a) reads were mapped to the genome with HiSat2 and aligned with StringTie2; (b) Mapped reads were merged in order to generate a non-redundant set of transcripts observed in all the samples; (c) transcript abundance estimates and read coverage tables were expressed in the fragments per kilobase of exon per million mapped reads (FRKM).

The resulting transcript abundances for each sample were analysed using bioconductor/DESeq2 v3.10 (Love et al., 2014) and visualised using R/pheatmap v1.0.12. Abundance (Kolde, 2019) values were normalised using variance-stabilising transformations and Binomial-Beta models. Differential expression was estimated

using the function lfcShrink (Stephens, 2016); thresholds, p-value < 0.05 and fold-change > 1.5.

The quality of the raw sequence read data was evaluated using FastQC software version 0.11.9. The data were then cleaned of adaptors and low quality sequences using TrimGalore software version 0.6.6 using the default RRBS paired-end settings. Methylation profile was performed using Bismark software. The Atlantic salmon reference genome was bisulfite converted in silco using bismark_genome_preparation script part of the bismark software pipeline (C- > T forward, G- > A reverse). used to facilitate bisulfite alignment. The cleaned RRBS reads were aligned to this converted genome using bowtie2. The same Bismark script was then used to call the methylation states of each cytosine in the genome from the alignments. The bismark_methylation_extractor script was used to extract the profiled cytosine nucleotides from the resultant bam files.

Differential analysis of methylation was performed using the Bioconductor program in edgeR. CpG sites with low coverage (<8 reads per sample), and those that were always methylated or unmethylated across all samples were removed. The size of the libraries for the samples were averages and the read counts of each CpG site were normalised. Likelihood ratio tests showed differential methylation between groups. The false discovery rate (Benjamini-Hochberg correction) determined that between every comparison no CpG site was significantly differentially methylated. Heatmaps (heatmap.2) however showed clear hierarchical clustering based on treatment. As such it was decided that a comparison could be made between the groups despite the high FDR, a p value of 0.001 was chosen and the groups were compared again.

The identified CpG sites that differed between treatment were functionally annotated using the annotatePeaks.pl tool, in the HOMER software (site). The sites were annotated using the salmon genome (Ssal_v3.1, GCF_905237065.1). Sites were identified as being located at exons, introns, and transcription termination site (TTS) regions (-100 bp to +1kbp around the TTS), intergenic regions, or promoter/transcription start site regions (TSS) (-1kbp to +100bp around the TSS).

A number of genes have yet to be annotated in Atlantic salmon, and for these homologs with high confidence scores were identified using Ensembl. Enrichment

analysis was conducted using the ShinyGo 0.77 comparing genes to pathways identified in Atlantic salmon and with a FDR cut-off of 0.05.

6.3. Results

6.3.1. Transcriptomic analysis

The sequencing of the 36 samples generated a total of 1,633,774,172 raw paired-end reads with an average of 45,382,616 per sample. The reads were deposited in the European Bioinformatics Institute (EBI) European Nucleotide Archive (ENA) project ID PRJEB55249. An average of 40,611,499 paired-end reads (89.5%) passed the pre-processing filters and were used during the mapping. There was a 82.52% mapping efficiency on average.

6.3.2 Transcriptomic between treatment differences

Within the diploids the largest difference was seen between the 1hr and the control groups seeing 3 DEGs under the above-mentioned criteria (figure 6.1). Two of these were listed as non-coding RNA, the third coded for HCN2 (potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2).

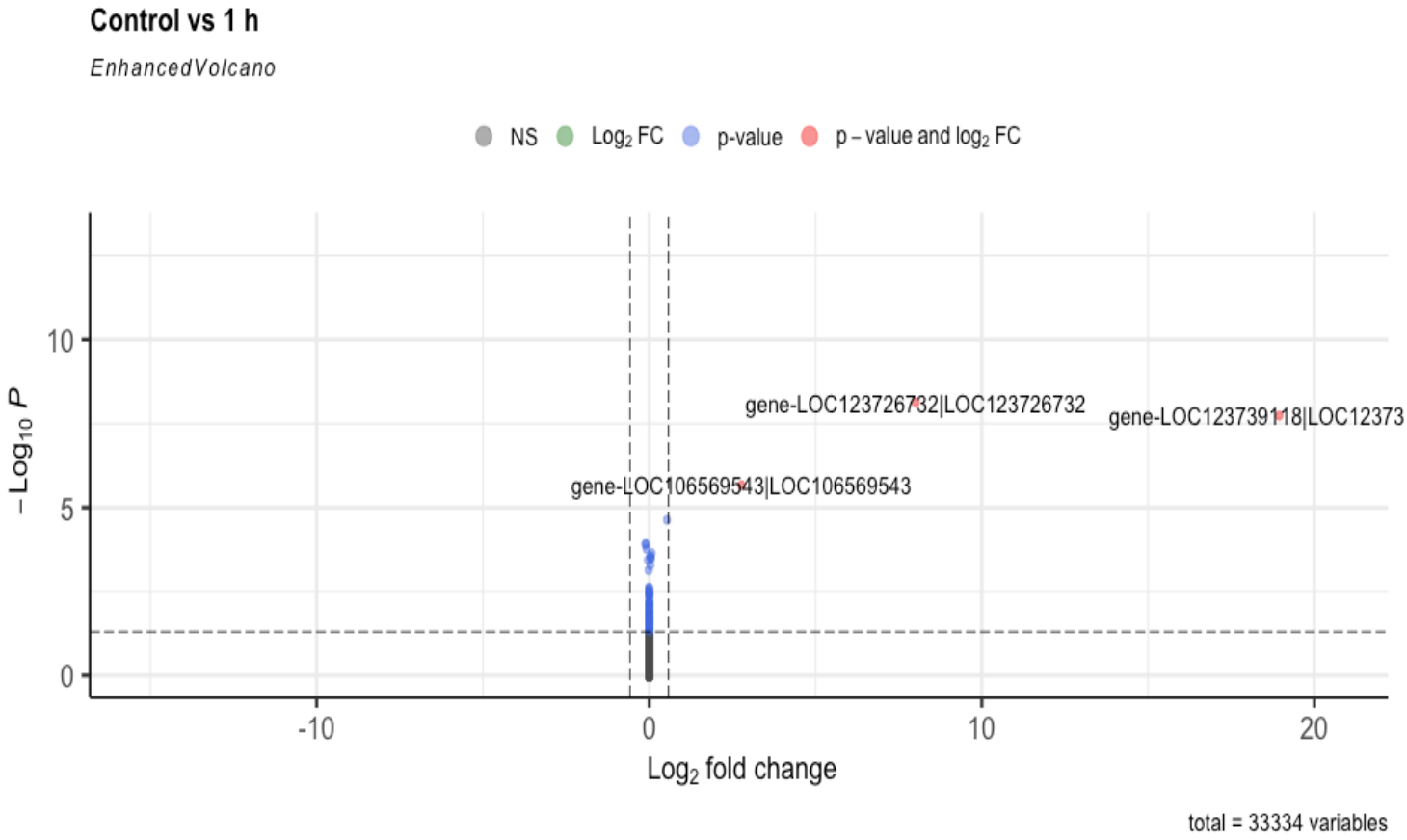


Figure 6.2. Volcano plot showing differentially expressed genes between diploid 1hr and control treatment groups 24hrs after thermal shock. Significance determine by p-value < 0.05 and fold-change > 1.5.

There were no differentially expressed genes between the 6 hr treatment and the control within the diploid group (Figure 6.2), with all treatment groups showing little variation within the diploids.

Within the triploids there was again little variation between the groups with 1 DEG between the 1hr and the control group, this was listed as non-coding RNA (Figure 6.3). The largest difference in gene expression over the control was observed in the triploid 6 hr group, the volcano plot shows a larger variation than the other groups (Figure 6.4) although most of these were below the level of significance with 3 DEGs observed between the 6 hr and the control group (Figure 6.4), these were SYT17 (synaptotagmin-17), DSP (desmoplakin-like), and cldnd (claudin-like protein ZF-A89).

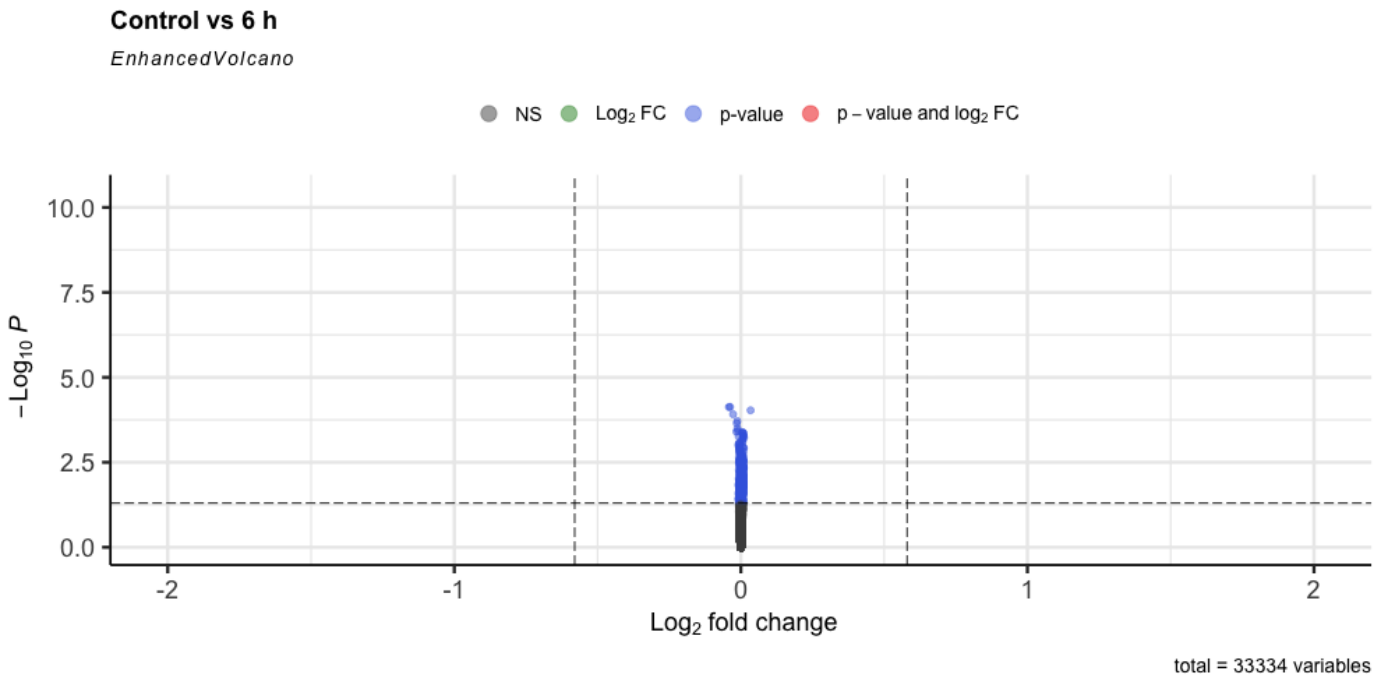


Figure 6.3. Volcano plot showing differentially expressed genes between diploid 6hr and control treatment groups 24hrs after thermal shock. Significance determine by p-value < 0.05 and fold-change > 1.5.

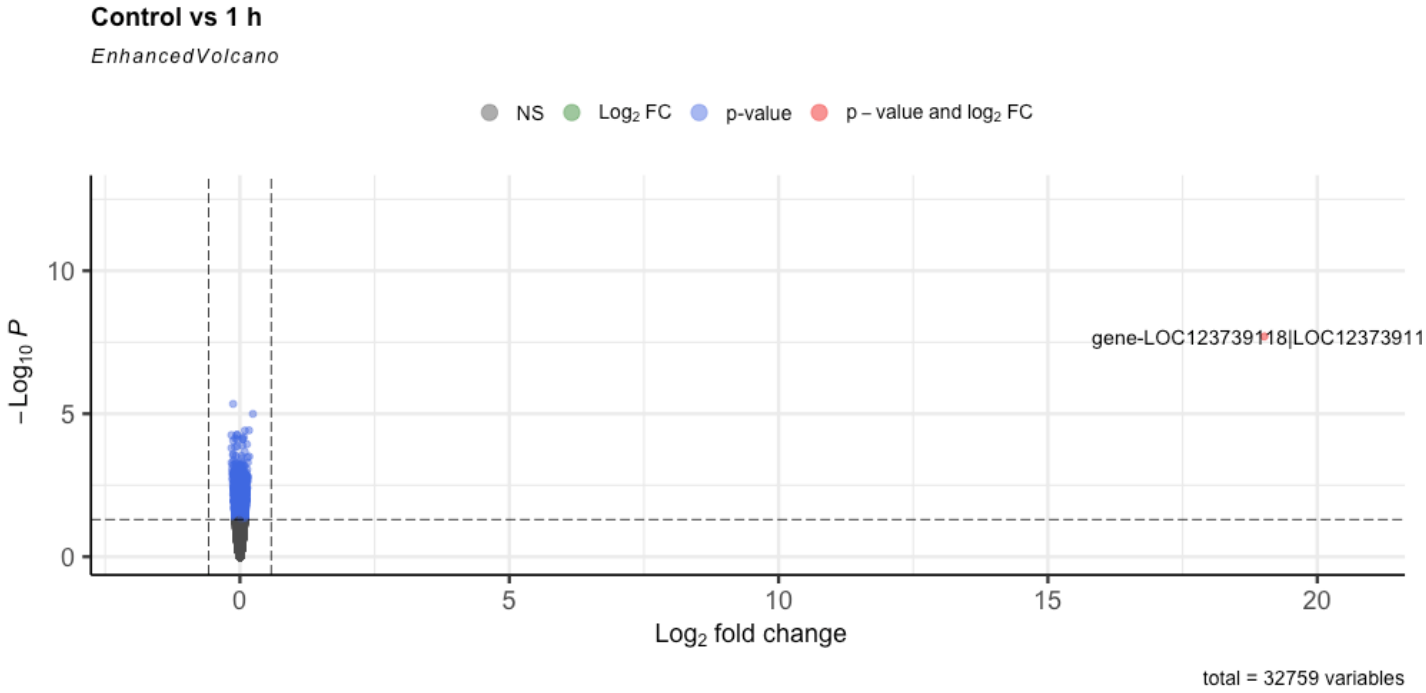


Figure 6.4. Volcano plot showing differentially expressed genes between triploid 1hr and control treatment groups 24hrs after thermal shock. Significance determine by p-value < 0.05 and fold-change > 1.5.

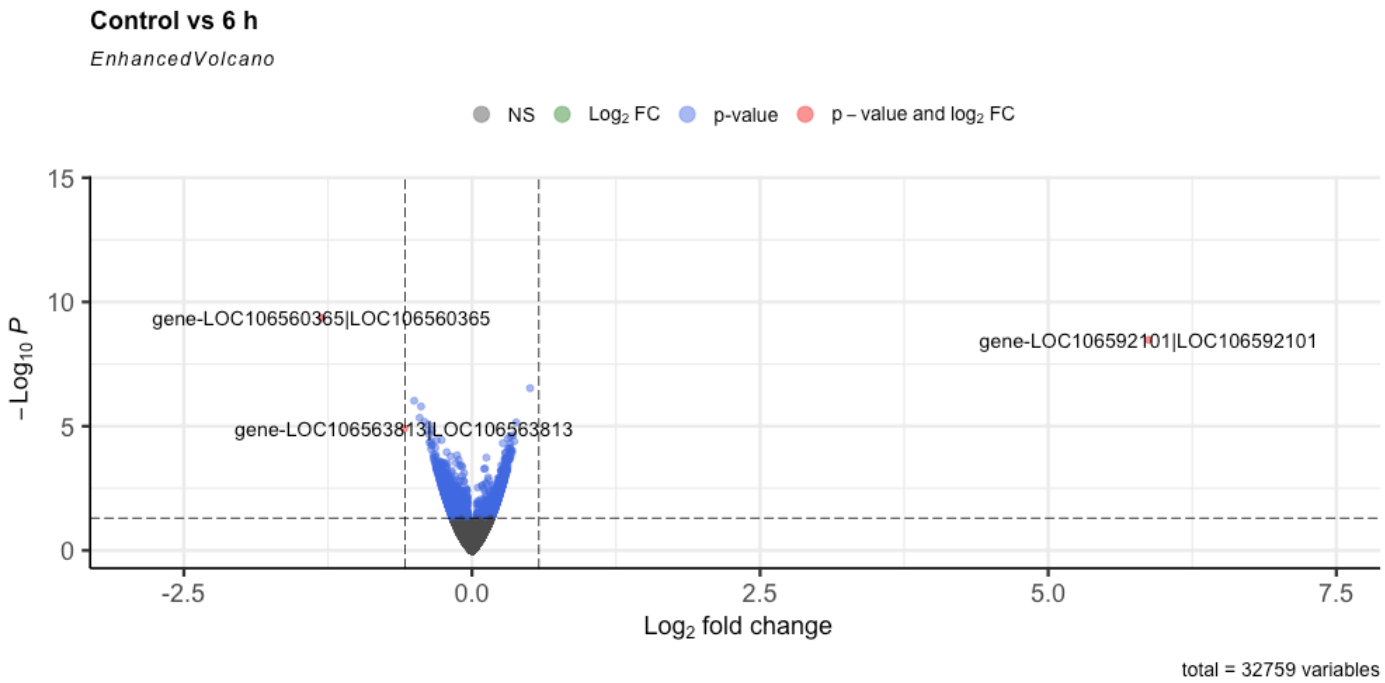


Figure 6.5. Volcano plot showing differentially expressed genes between triploid 6hr and control treatment groups 24hrs after thermal shock. Significance determined by p-value < 0.05 and fold-change > 1.5.

6.3.3. DNA methylation

The reduced reduction bisulfite sequencing of the 36 samples generated an average of 44.23 million reads per sample. With an average mapping efficiency of 52.63 %. This is lower than the transcriptome mapping as all none unique hits were removed.

Between ploidy

There were slight between ploidy differences in the genomic distribution of methylation (figures 6.5 and 6.6). 201,058 CpG sites were assessed for methylation status between ploidy. In triploids none of the CpGs located in TSS regions were unmethylated in every individual, they were either always methylated or showed variation between individuals. In diploids TSS sites ranged from always methylated, always unmethylated, or varying between individuals.

There was also a difference in the typical methylation status of CpGs located at promoter sites between ploidy (Figure 6.7). A higher percentage of diploid promoter CpGs were always unmethylated (14.98 % vs 11.73 %). In Triploids a larger percentage of promoter CpGs showed varying methylation between the individuals (81.3 % in triploids and 77.4 % in diploid).

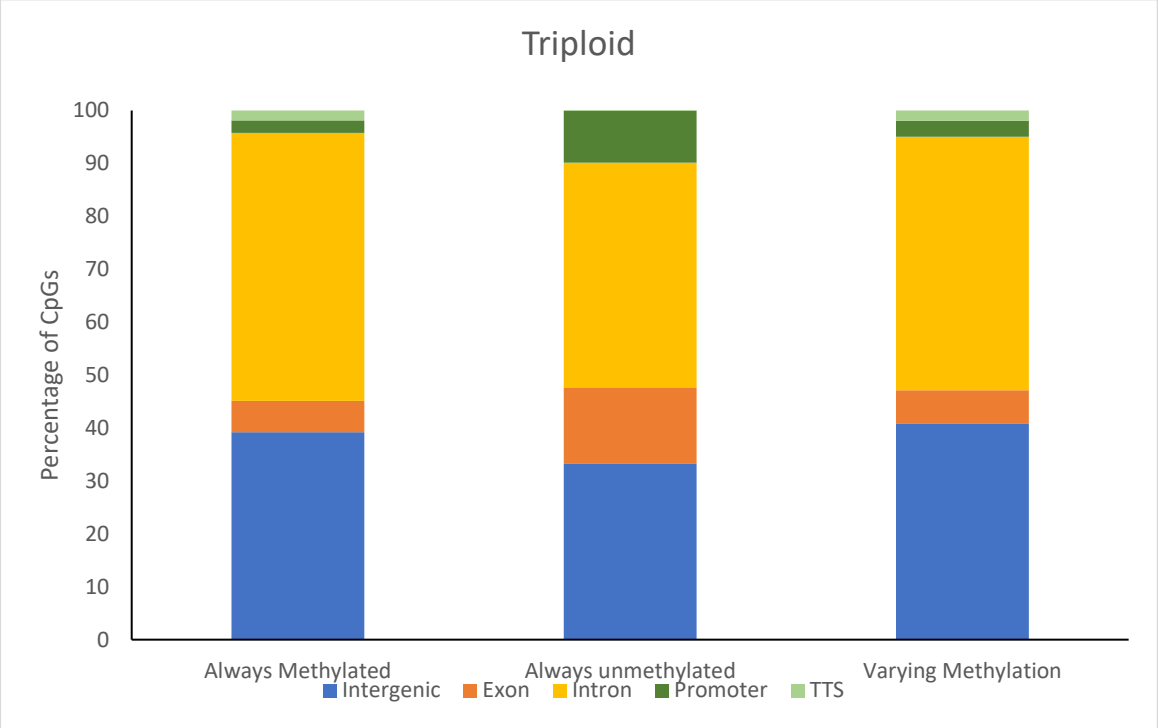


Figure 6.6. Genomic distribution of CpG sites sequenced and their methylations state among 6 triploid Atlantic salmon.

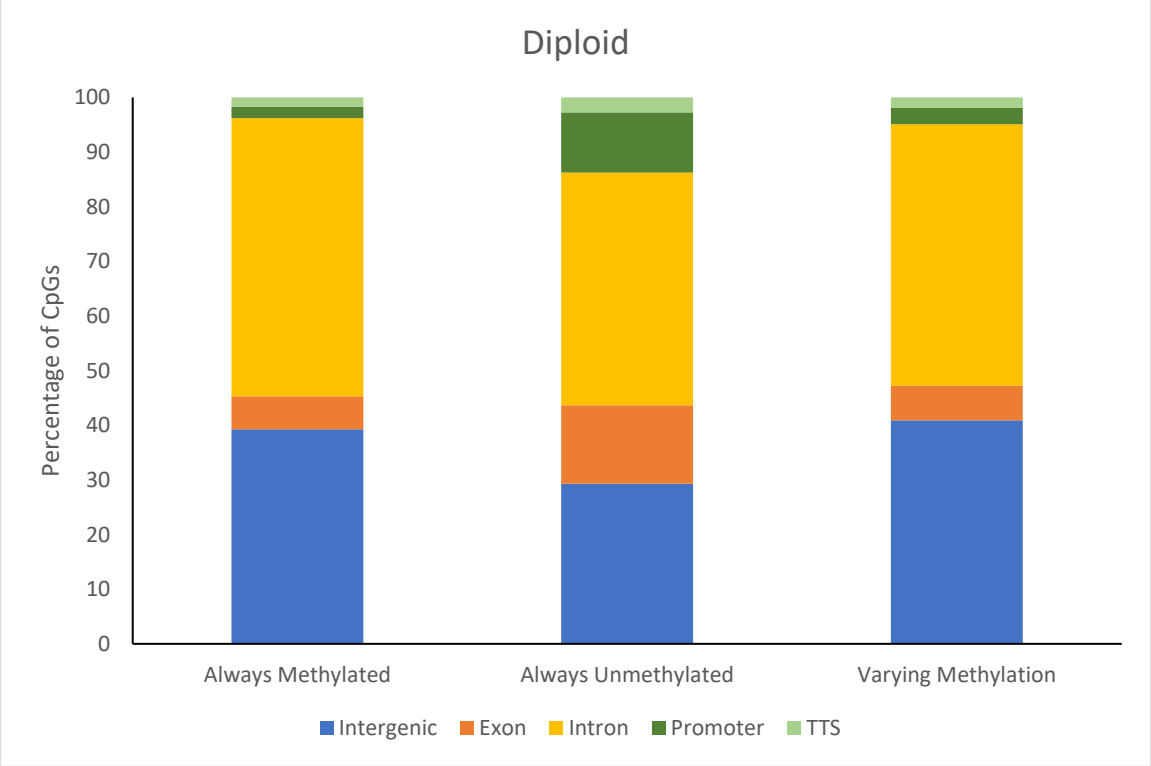


Figure 6.7. Genomic distribution of CpG sites sequenced and their methylations state among 6 diploid Atlantic salmon.

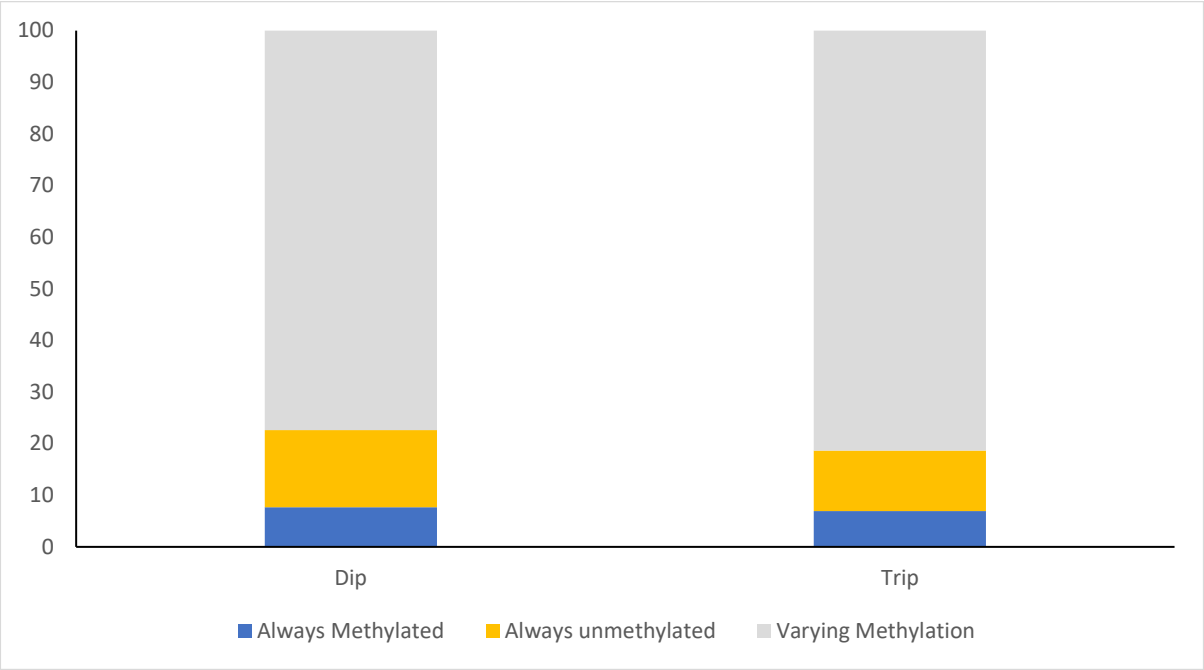
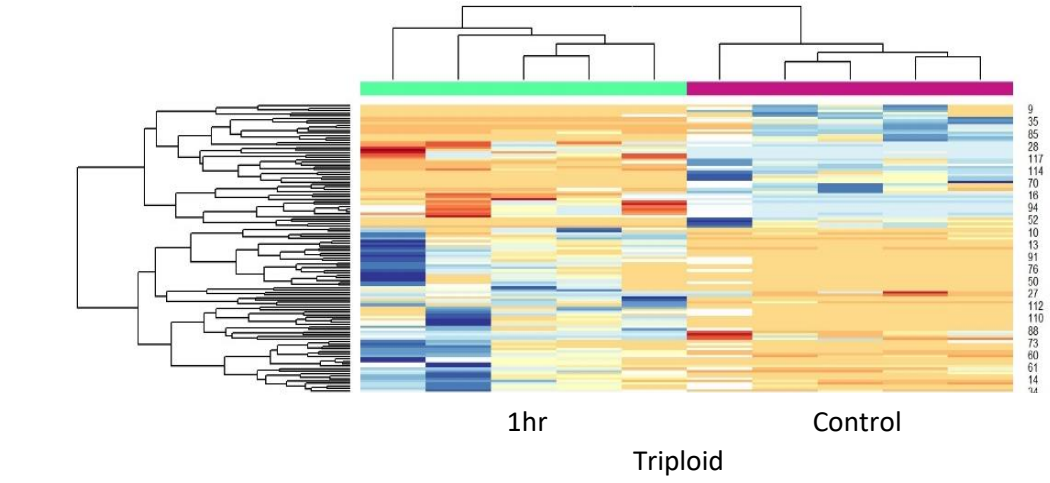
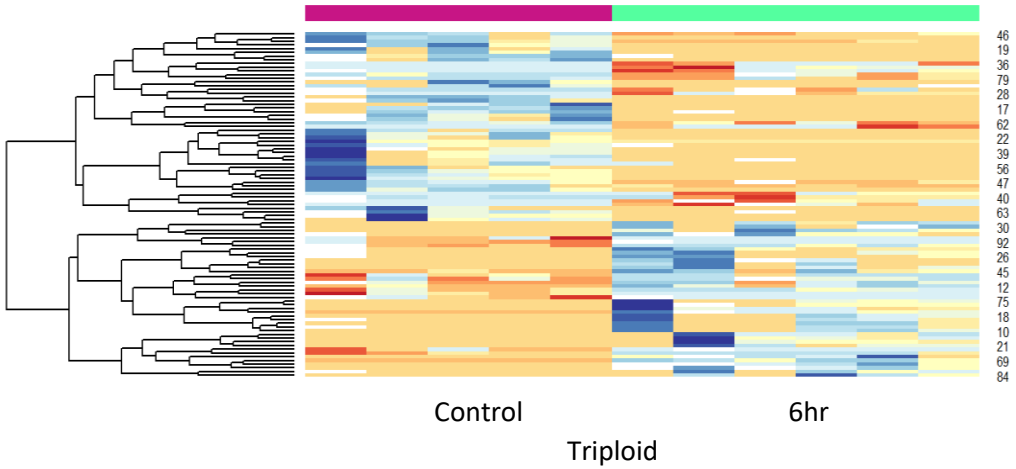


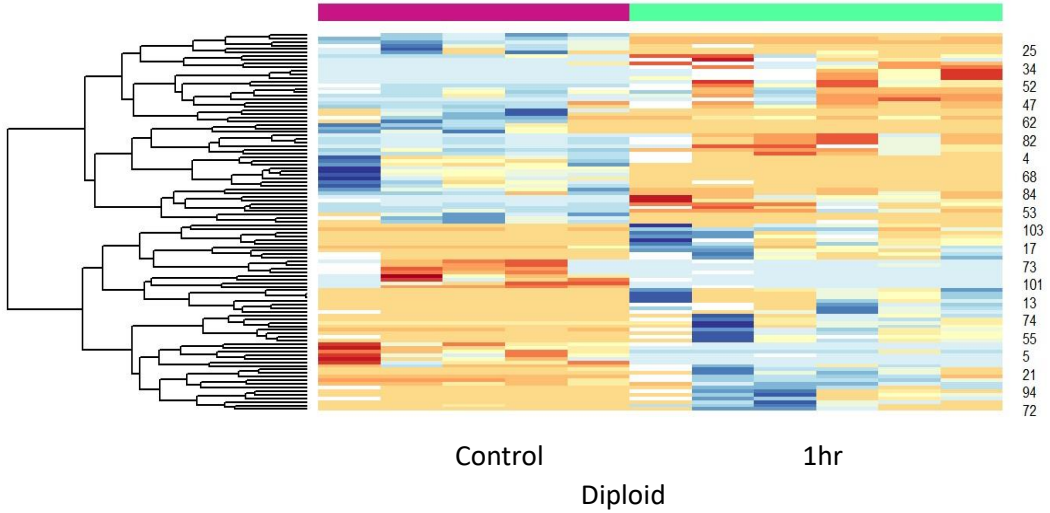
Figure 6.8. Methylation status of CpG sites located in promoter regions in diploid and triploid Atlantic salmon (n=6 per ploidy)

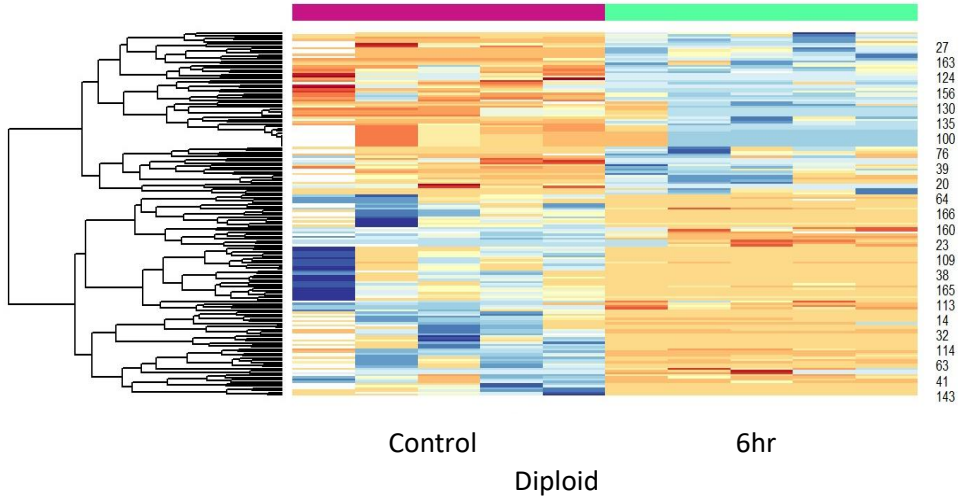
Between treatment

The number of CpG sites that showed a significant difference in methylation status differed between treatment. Figures 6.8 show heat plots of these significantly differentially methylated sites. Compared to the control the triploid 6 hr and 1 hr treatments showed 93 and 117 differently methylated sites respectively (p=0.001). The diploid 6 hr and 1 hr treatments differed in methylation from the control in 168 and 105 CpGs respectively. The location of these sites differed between treatment (Figure 6.9). For all treatments the highest proportion of CpGs were located at intron sites followed by intergenic regions. These regions made up the majority of the variable sites for the diploid 1hr treatment and to a lesser extent the triploid 1 hr treatment. The 6 hr treatments showed a higher proportion of sites also split between the promoter and exon regions.









Figures 6.9 Heat plots showing significantly differentially methylated CpG sites in Atlantic salmon embryos following heat shocks compared to controls (p=0.001).

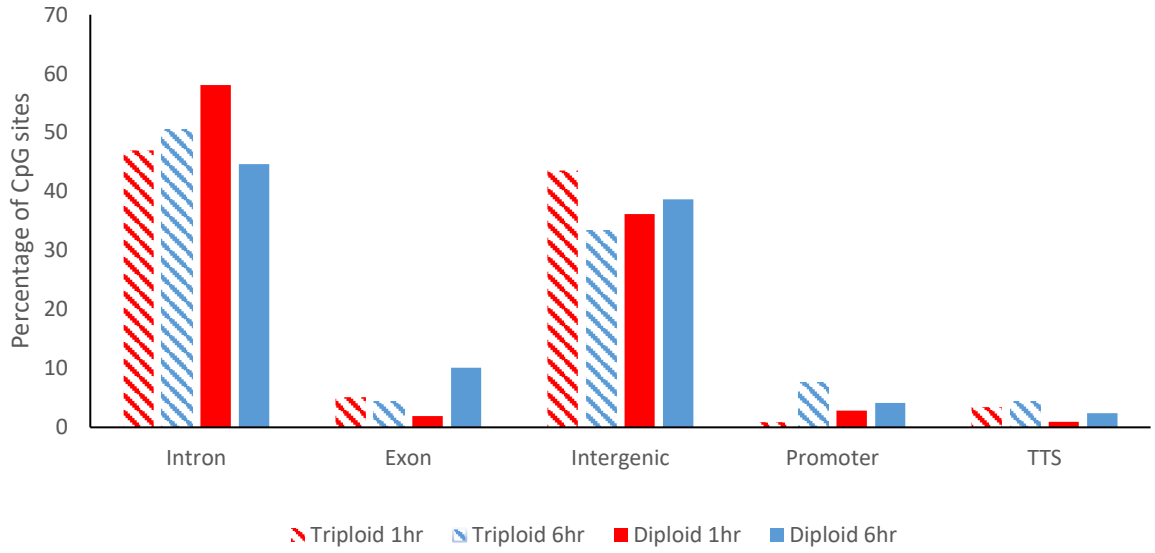


Figure 6.10. The percentage of differentially expressed CpG sites between treatment and control and the genomic location.

The type of the closet gene was identified. The majority of the genes were protein coding followed by lncRNA. A smaller proportion were determined to be rRNA, snRNA, miRNA, and pseudogenes (Figures 6.10-6.14). The triploid 6 hr contained a larger proportion of protein coding related CpG sites (86%) of all differentially methylated site, this was followed by the diploid 6hr treatment (83.3%), the diploid 1 hr (81.9%) and the triploid 1 hr (72.6%).

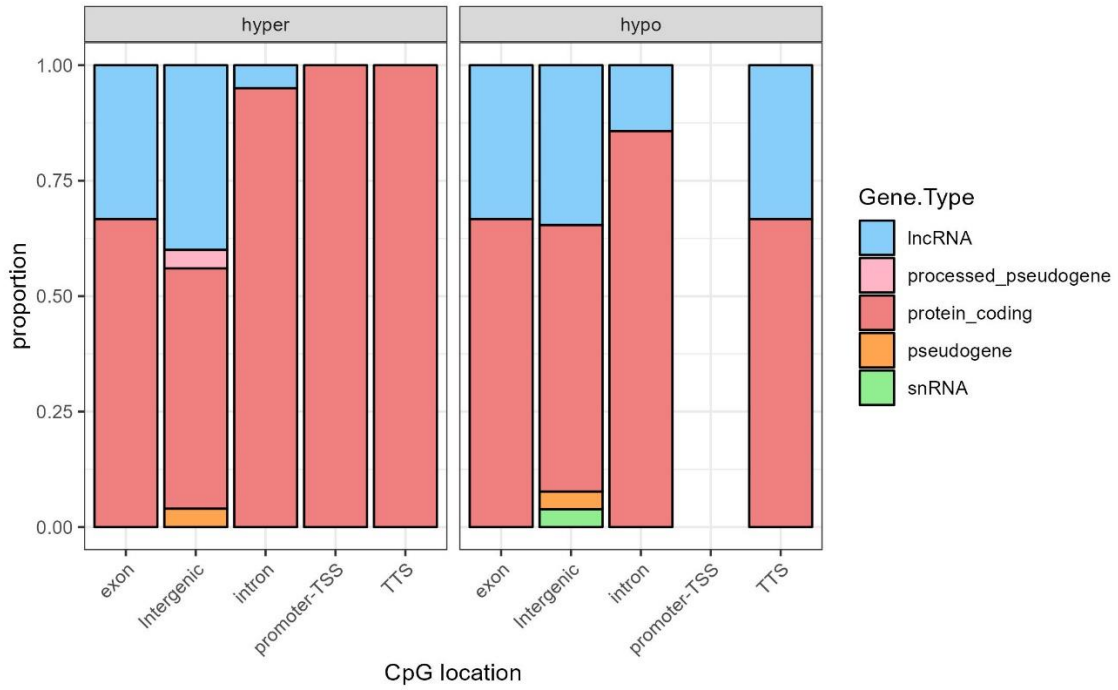


Figure 6.11. Genomic location of differentially methylated CpG sites and the associated gene type in triploid Atlantic salmon embryos treated with a 1hr heat shock compared to an unshocked control.

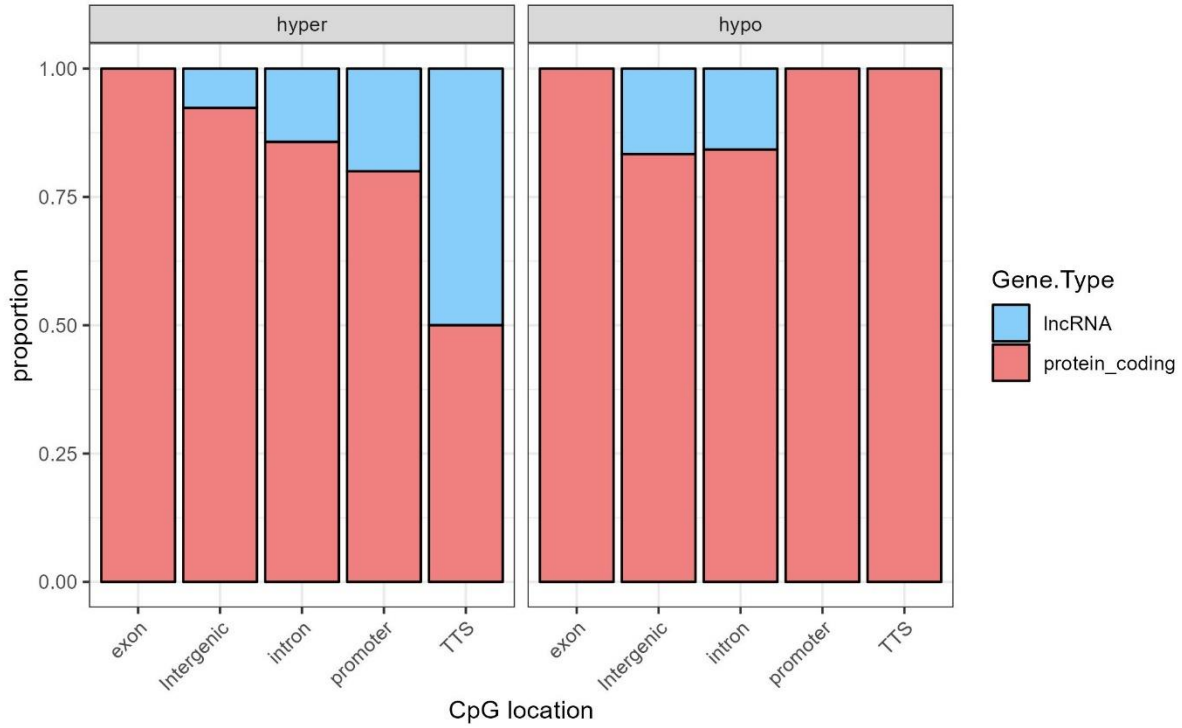


Figure 6.12. Genomic location of differentially methylated CpG sites and the associated gene type in triploid Atlantic salmon embryos treated with a 6hr heat shock compared to an unshocked control.

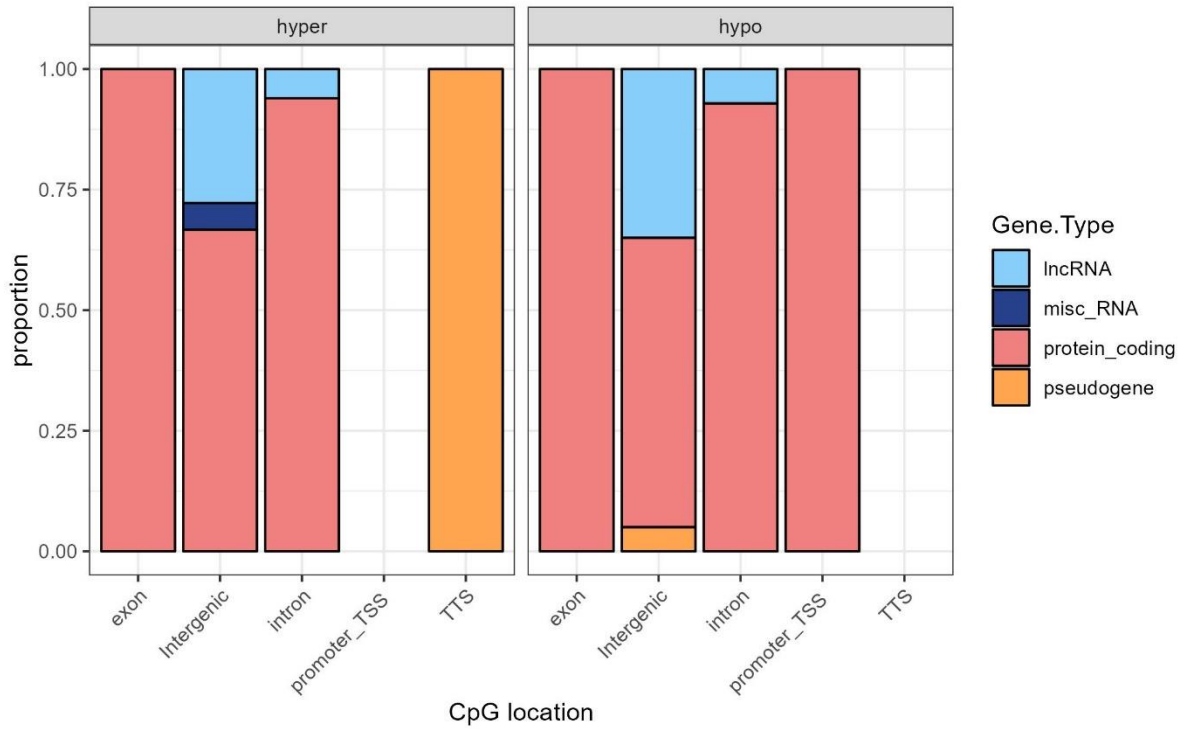


Figure 6.13. Genomic location of differentially methylated CpG sites and the associated gene type in diploid Atlantic salmon embryos treated with a 1hr heat shock compared to an unshocked control.

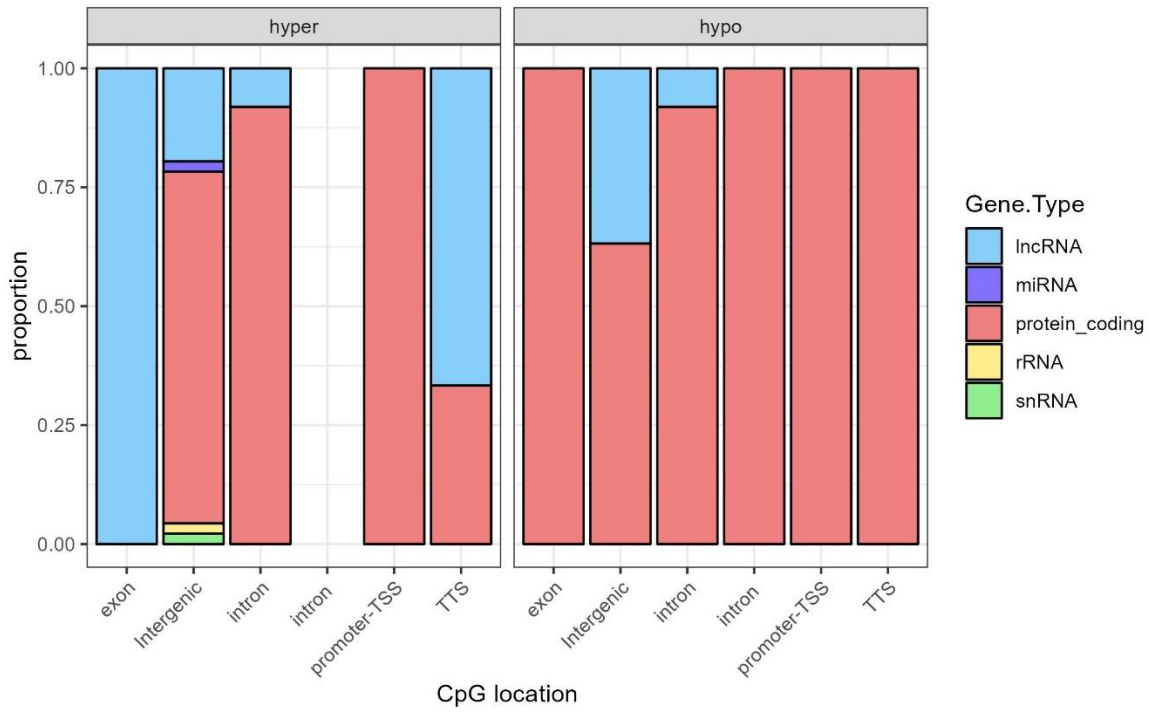


Figure 6.14. Genomic location of differentially methylated CpG sites and the associated gene type in diploid Atlantic salmon embryos treated with a 6hr heat shock compared to an unshocked control.

A small number of pathways contained 2 or more genes significantly associated with a pathway in Atlantic salmon. The triploid 1 hr group had CpG sites with variable methylation from the control at genes associated with propanoate metabolism, cysteine and methionine metabolism, and carbon metabolism pathways. The triploid 6 hr group showed variation in focal adhesion, regulation of actin cytoskeleton, and calcium signalling pathways. Enrichment analysis of the diploid 1 hr treatment highlight pathways involved in oocyte meiosis, progesterone-mediated oocyte maturation, MAPK signalling, MTOR signalling, and adrenergic signalling in cardiomyocytes. The analysis of the diploid 6 hr treatment highlighted only one pathway, glutathione metabolism.

The majority of the CpG sites identified are associated with genes that are distinct to that comparison. However, a number of genes were associated with CpGs that are differentially methylated in multiple treatments (Table 6.2).

Table 6.2. Genes associated with CpG sites with CpGs that are differentially methylated in multiple treatments

Entrez ID	**Gene name /(Homologue)**	**Annotation**	**Functional group**
	Diploid 6hr and Triploid 6hr		
ENSSSAG00000054332	wdr92 - dynein axonemal assembly factor 10	Intron	Cytoplasm function/development
ENSSSAG00000111791	lncRNA	Intron	
	Diploid 6hr and Triploid 1hr		
ENSSSAG00000001994	gpr142 - G protein-coupled receptor	Intergenic	Signalling
ENSSSAG00000082618	lncRNA	Intergenic	
	Diploid 6hr and Diploid 1hr		
ENSSSAG00000100913	LncRNA	Intergenic	
ENSSSAG000000	zinc finger E-	Intergenic	Transcriptional inhibitor

46598	box-binding homeobox 2-like isoform X2		– Cell development
Triploid 1hr and Diploid 1hr			
ENSSSAG00000072543	slc6a5 - solute carrier family 6 member 5	Intron	Glycine transport

Most of the CpG sites that showed differential methylation between treatment and control existed in isolation. However, a number of genes proceeded areas with multiple CpG sites showing differential methylation (Table 6.3)

Table 6.3. Genes that were associated with multiple differentially methylated CpG sites.

Entrez ID	Gene name	Number of associated CpG sites	Predicted functional group/s
Triploid 1hr			
ENSSSAG00000100595	SORBS1	3	Cytoskeleton and cell signalling
Triploid 6hr			
ENSSSAG00000040879	efna2b	2	Neuronal processes and immunity
Diploid 1hr			
ENSSSAG00000069430	GALNTL6	2	Glycosylation
ENSSSAG00000004308	map3k12	2	Apoptosis, nervous system development
ENSSSAG00000108825	dapk1	2	Cell death and autophagy
ENSSSAG00000070643	slc12a5b	3	Neuronal function
ENSSSAG00000066067	slc38a5a	2	Amino acid transport
Diploid 6hr			
ENSSSAG00000093220	MBP	2	Nervous system function/formation

ENSSSAG00000007708	AXIN2	2	wnt signalling pathway regulation
ENSSSAG00000009935	PERCC1	10	Intestine development
ENSSSAG00000078898	POMP	2	Proteasome assembly
ENSSSAG00000057692	vstm2b	4	Membrane processes

6.4. Discussion

Within the diploid group, there were no significantly differentially regulated genes between the 6 hr group and the control. Between the 1 hr group and the control, there were 3 DEGs. Two of these coded for ncRNAs whilst the third coded for HCN2. HCN2 plays a crucial role in regulating cardiac and neuronal rhythmicity (Ludwig et al., 2003) as well an important role in inflammatory and neuropathic pain (Emery et al., 2012). Within fish HCN paralogs have been found in various tissues and are thought to be not only involved in cardiac function but also acid-base and ammonia regulation (Fehsenfeld & Wood, 2020), increasing in conjunction with elevated CO_2 (Williams et al., 2019)

Within the triploid group, the 1 hr was only different to the control in the case of 1 ncRNA.

The group with the largest difference in expression compared to the control was the triploid 6hr group, which differed in the case of 3 genes. SYT17 (synaptotagmin-17) is thought to be a neural plasticity marker in Atlantic salmon (Mes et al., 2020) and is involved in the regulation of neurotransmitter release (Fernández-Chacón et al., 2001). Increased neurological plasticity has been linked to increased tolerance to stress in teleost fish (Sørensen et al., 2013) including Atlantic salmon (Vindas et al., 2017) and rainbow trout (Johansen et al., 2012; Øverli et al., 2005, 2007). Vindas et al. (2017) looked at tank choice during hypoxia and found that proactive fish had showed differential regulation of genes related to neural plasticity and lower post-stress cortisol levels. Studies on rainbow trout found that increased tolerance to

crowding stress was linked to genes involved in neural plasticity (Johansen et al., 2012; Øverli et al., 2005, 2007). Thermal tolerance was not investigated in the above-mentioned studies, but it is likely that an increased tolerance to other stressors, such as crowding, will share functional similarity to thermal tolerance. In mice, SYT17 expression has been linked to cardiac hypertrophy (Mirtschink et al., 2019). In the previous chapter “Impact of early temperature regimes on egg development and juvenile performance in diploid and triploid Atlantic salmon (Salmo salar) siblings” we saw no suggestion of a lack in cardiac function, although deformities other than Aplasia of the *septum transversum* were no examined. As far as this author is aware, there have been no studies investigating SYT17 and cardiac function in fish, but this may be an interesting avenue of investigation given the fact that cardiac function has been purported to be a major factor behind thermal tolerance in Atlantic salmon (Farrell, 2009; Verhille et al., 2013).

DSP (desmoplakin-like) is the major component of desmosomes (Yuan et al., 2021). Desmoplankin is essential for normal myocardial development (Yuan et al., 2021) and cardiac cell-cell interactions (Bowers et al., 2012). Studies in humans have shown that mutations of DSP result in cardiomyopathy (Smith et al., 2020; Yuan et al., 2021). The presence of DSP expression in the cardiac tissue of embryonic and adult zebrafish has been confirmed, in addition to the fact that knockout of the gene results in disruption of desmosome similar to that in humans and a decreased heart rate (Giuliodori et al., 2018). Differential expression of DSP in the 6hr treated embryos may suggest differential cardiac development, the extent to which DSP may impact cardiac function later in life is not known but as mentioned above cardiac development is a major factor in the tolerance of Atlantic salmon to higher temperatures.

Cldnd (claudin-like protein ZF-A89) codes for a member of the claudin family of proteins, these proteins regulate the movement of water and ions into cells (Baltzegar et al., 2013). Cldnd plays an important role in regulating the entry of proteins into cells (Hou et al., 2019). In both zebrafish (Vesterlund et al., 2011) and Atlantic cod (*Gadus morhua*) (Lanes et al., 2013) embryos cldnd is one of the most abundant transcripts. The differential expression of cldnd does not appear to have had a negative effect on the viability of the embryos, with no difference in mortality between the treatment groups. There is no clear link between cldnd expression and

improved temperature tolerance, but one possible link could be the fact that triploids have a lower capacity for transmembrane transport of resources including oxygen (van de Pol et al., 2020) and improvements in the movement of resources across cells could result in improved capacity to tolerate supraoptimal temperatures.

It is perhaps surprising that so few differences in gene expression were observed between treatments groups, but the lack of difference at this stage is supported by the literature. Uren Webster et al. (2018) found only 19 DEGs after a one off cold shock at the same stage of embryogenesis. The duration of the shock was shorter than in the current study, but the change in temperature was much larger with an 8.8°C decrease followed by an 11.8°C increase and finally a 3°C decrease, with the added stressor of exposure to air. This compared to a 4°C increase and 4°C decrease in the current experiment. Larger differences were seen when cold shocks of the same severity were repeated 5 times (Moghadam et al., 2017; Robinson et al., 2019), the period of shocks in these studies meant that the eggs were shocked at both earlier and later developmental stages than the current study, including as early as 250DD which is before the stage at which Atlantic salmon eggs are normally handled. The robustness of Atlantic salmon embryos at the stage which they were shocked by Uren Webster et al. (2018) and in the current study (~360DD) is supported by the fact that this is the stage at which eggs are routinely shipped on ice from broodstock facilities to hatcheries (Jansen et al., 1997). A recent study on brown trout and rainbow trout found no increase in survival in eggs that were taken from just above freezing (1°C) in the transport container to 11°C gradually rather than instantly (Slama et al., 2021). The eggs in this current study were shipped from the broodstock facility to the IoA on ice 24 hrs before the shocks were implemented, on arrival at the IoA the eggs were placed from the ice box into the 6°C tank with no slow increase in temperature, after being manually handled to count the eggs into egg trays. It is possible that the controls in this experiment were somewhat “shocked” already from this process, and this could contribute to the lack of difference seen between the treatments.

In chapter five we saw there was no negative treatment effect with no differences in mortalities, deformities, or number of pins or runts. This suggests that the effect of the shock was relatively mild or that the recovery to a shock of this nature is fairly rapid within embryos of this stage, this is supported by the small number of DEGs

found in this chapter. It remains to be seen what differences in gene expression may have been present sooner after the shock, or indeed what a shock of greater severity or frequency may have produced. It is perhaps possible that gene expression after shock is not a good indicator of programming and the best way to determine molecular memory is to challenge fish and record response.

It is likely however, that the thermal shocks had some impact on the epigenome. This is supported by the fact that in chapter four a significant difference in weight was observed between the triploid 6 hr group and the control after a period of thermal challenge. There was no difference in weight before the increase in temperature, suggesting that the reason the 6 hr group weighed more at the end of the experiment was due to being better able to cope with the supraoptimal temperature. This increased tolerance is likely to be epigenetic in origin, a theory supported by the fact that in a previous study, cold shock at this stage of development produced minimal transcriptional effects but lasting changes to the methylome including enhancing the transcriptional immune response to bacterial challenge (Uren Webster et al., 2018).

The current study found minimal transcriptomic differences between treatment and control but larger differences in loci specific DNA methylation. Despite showing the largest transcriptomic differences the triploid 6 hr treatment showed the lowest number of differentially methylated CpG sites, a higher proportion of these sites where however located near protein coding regions rather than regions that coded for lncRNA (Figure 6.11). There was no clear ploidy effect on the number of CpG sites that differed between treatment and control.

Both of the 6 hr treatments showed a higher proportion of CpG sites in promoter regions (Figure 6.9). The affect of this on gene expression can not be elucidated but given the fact that these two treatments showed the greatest tolerance to higher temperatures later in life this is an interesting finding.

KEGG analysis of the variable CpG sites revealed pathways involved in a variety of developmental or maintenance functions. In the triploid 1 hr group KEGG pathways were focused on metabolism with pathways linked to the metabolism of the amino acids cysteine and methionine being impacted. Carbon and propanoate metabolism pathways were also impacted. Both cysteine and methionine are powerful

antioxidants (Bin et al. 2017), and it is possible that the 1 hr shock may impact the ability of the fish to deal with oxidative stress. The triploid 6 hr group indicated pathways related to mechanical force, namely the actin cytoskeleton and focal adhesion pathways (Raja et al. 2008). Focal adhesion (Wozniak et al. 2004) and the calcium signalling (Bootman et al. 2012) pathways also play a role in the transmission of regulatory signals. This treatment was the only treatment to show a significantly increased growth rate during thermal challenge and these pathways may go some way to explaining the reasons why. The diploid 1 hr group showed the largest number of pathways during enrichment analysis. Pathways involved in oocyte development were highlighted, the reason for this is not clear given the developmental stage of the embryo studied. Pathways involved in cell proliferation signalling (MAPK and MTOR) and signalling in cardiomyocytes were also highlighted. The diploid 6 hr group despite having the largest number of CpG sites represented showed only one pathway during enrichment analysis, this pathway glutathione metabolism, is involved in antioxidant defence and intracellular homeostasis (Wu et al. 2004).

A handful genes were identified in multiple treatments (Table 6.2). The majority of these genes where shared between the diploid 6 hr group and other groups, likely explained by the fact that this group showed the largest number of differentially methylated CpG sites. The diploid 6 hr treatment and the diploid 1hr treatment both showed methylation changes around the gpr142 gene which is involved in the sensing of aromatic amino acids and the maintenance of gut hormones (Rudenko et al. 2019). Trip and dip 6 hr both showed a difference in methylation at CpG sites associated with wdr92. This gene is highly conserved and is involved in apoptosis and the function of motile cilia (Patel-king et al. 2016). The triploid 1 hr and diploid 1 hr group had the gene slc6a5 in common. Slc6a5, also known as glyt2 is a neurotransmitter transporter (Satou et al. 2013), and changes in expression of this gene may influence the nervous system. Both the diploid treatment groups had shared methylation changes at CpG sites associated with ZEB2, a transcriptional repressor thought to aid protection against stress-related apoptosis in Atlantic salmon (Shwe et al/ 2020). This may be linked to diploids increased ability to tolerate stress but the methylation changes mentioned here are limited to only 1 CpG site.

Perhaps having a larger effect on gene expression levels are methylation changes of multiple CpGs per gene. A small number of these genes are found in this study (Table 6.3). With diploids presenting a larger number of genes which proceed multiple differentially methylated CpG sites. Both the triploid groups possessed only 1 such gene, SORBS1 in the case of the 1hr treatment, a gene associated with cytoskeleton and cell signalling, and efna2b in the case of the 6 hr treatment, a gene associated with neuronal processes and immunity. The diploid 1 hr group possessed 5 genes that proceeded multiple CpG sites, these genes were associated with apoptosis and nervous system function (map3k12, dapk1, slc12a5b), as well as glycosylation (galntl6) and amino acid transport (scl38a5a). The diploid 6hr group also contained 5 genes of this type including percc1 which proceeded 10 differentially methylated CpGs. This gene is reported to be involved in digestive tract function and morphogenesis. Other genes were involved in nervous system function (mbp), wnt signalling (axin2), proteasome assembly (pomp), and membrane processes (vstm2b).

In addition to treatment specific differences in methylation the RRBS analysis also presented some ploidy specific differences (Figures 6.5-6.7). The largest difference was the lower proportion of CpGs in the triploid group that were always unmethylated (11.73% vs 14.98%), and sites which showed varying methylation between individuals (81.3% in triploids and 77.4% in diploid). Triploids contained more CpG sites that contained variable methylation between individuals whilst diploids had a more consistent methylation status between individuals. This was particularly true for TSS regions which were never unmethylated in every individual in triploids but were in some cases in diploids. As far as this author is aware no other study has compared loci specific DNA methylation states between ploidy in Atlantic salmon and these results may go some way to explaining some of the differences observed between ploidy. It is not possible to say whether changes in methylation will impact gene expression in a predictable manner. Work by Mukiibi *et al*. (2022) found that whilst methylation was generally negatively correlated with gene expression, a positive correlation was found in a number of genes. What is likely however is that an increase in the variability in DNA methylation patterns between individuals in a population may increase the variability of the population in terms of gene expression and performance.

Whilst the results presented here provide a key insight into the genetic and epigenetic differences between diploids and triploids, and embryonic response to stress, the study has its limitations. It is impossible to say if the epigenetic changes remained consistent later in life and into the thermal challenge period, although evidence suggests that changes of this nature can remain.

In conclusion, Atlantic salmon embryos are relatively robust to short term thermal shocks at this stage of development. Few gene expression differences were found 24 hrs after a moderate thermal shock suggesting either tolerance to the shock or rapid recovery. Despite this the shocks did elicit a response later in life with a 6 hr shock of 6°C to 10°C resulting in triploids that were more tolerant to thermal challenge 9 months after the shock. The epigenetic analysis indicated a larger number of genes that the transcriptomic analysis and highlighted specific genes and pathways which were most sensitive to the thermal shock and may be linked to the increased thermal tolerance observed later in life. The analysis also highlighted ploidy specific differences in DNA methylation patterns further increasing our knowledge of the differences between these animals.

General discussion

The aims of this thesis were to improve scientific understanding of early triploid development, explore the potential for thermal programming in Atlantic salmon, and to overall improve the production potential of triploid Atlantic salmon.

A triploid Atlantic salmon is a functionally sterile Atlantic salmon (Benfey, 2016). Stocking sterile salmon drastically reduces the impact of escapes (Bolstad et al., 2021; Fleming et al., 2000; Glover et al., 2013), prevents the negative side effects of maturation before harvest (Aksnes et al., 1986; Gjerde, 1984), and potentially protects intellectual property. Despite being the only permitted method for producing sterile fish for human consumption (European Commission/SWD, 2021; Madaro et al., 2021), triploid Atlantic salmon have struggled to reach a commercial market. The major reason behind the hesitancy has been historic poor performance of triploids compared to diploids. Over the years triploids have been shown to suffer from increased mortalities (Cotter et al., 2002; O'flynn et al., 1997) and deformities (Fjelldal & Hansen, 2010; Fraser et al., 2013), slower growth to harvest size (Friars et al., 2001; Taylor et al., 2014), and reduced tolerance to high temperatures and hypoxia (Riseth et al., 2020; Sambraus et al., 2017). Improvements in husbandry practices such as lower incubation temperatures (Clarkson et al., 2021; Fraser et al., 2014) and improved understanding of nutritional requirements (Fjelldal et al., 2016; Smedley et al., 2016; Taylor et al., 2015) have reduced the gap between ploidy. Improving our understanding of early triploid development including the role of shifts in temperature during incubation will provide information that can be used both in further research and by industry, and help close this gap further. There is some evidence that epigenetic programming may help fish become more tolerant to stress later in life (Moghadam et al., 2017; Robinson et al., 2019; Uren Webster et al., 2018), no study however has looked at improving the thermal tolerance of a fish. Thermal programming has the potential to remove one of the major obstacles in the acceptance of triploid Atlantic salmon as well as helping the industry prepare for rising seawater temperatures in the years to come. In addition, improving the manner in which the industry verifies triploidy will help reduce the cost of triploid production as well as reduce the number of broodfish required during each production cycle.

During this work we have established a protocol for DNA extraction that will allow triploids to be validated at a much earlier stage than currently possible, and validated microsatellites that can be used in conjunction with recently published suites to provide a comprehensive and reliable method of triploidy verification (**Chapter 2)**. We have investigated a fundamental assumption of triploid biology, namely that embryogenesis progresses at the same rate in both ploidy, allowing embryonic studies between ploidy to be conducted with confidence of comparable results (**Chapter 3**). In this same chapter we also established the most efficient clearing solution, providing a useful tool for future research into Atlantic salmon embryos. In **Chapter 4** we explored the effect of shifts in temperature at a key stage of the Atlantic salmon production cycle as well as investigating the physiological manifestations of thermal programming, providing useful scientific information as to the effects of shifts at this stage as well as further support of decreased incubation temperatures for triploidy culture. This chapter also showed the first known evidence of thermal programming increasing thermal tolerance in a fish species. The effect of temperature on triploids and diploids was explored further in **Chapter 5** with transcriptomic analysis of both ploidy before and during a thermal challenge providing genetic information to help explain the reduced tolerance of triploids to high temperature. The transcriptomic effects of thermal shocks were investigated in **Chapter 6** revealing the tolerance and/or recovery potential of Atlantic salmon embryos heat shocked at this stage of development, it is hoped that epigenetic analysis will provide evidence behind the thermal programming observed in chapter 4.

The process through which triploid Atlantic salmon are produced is a hydrostatic pressure shock of 9500psi applied for 50° minutes, 300°min post-fertilisation (Benfey, 2016). This process is generally reliable with a more than >98% success rate at inducing triploidy (Benfey, 2016), it is not however infallible, and it is important that every batch produced is verified as triploid. Verifying each batch is important for multiple reasons; it is vital for consumer confidence that each batch of triploids bought is truly triploid, different requirements in husbandry make knowledge of ploidy a welfare concern, whilst escape events are likely to have a much larger effect on native populations if the fish are mistakenly diploid. Typically, the method of verification used in industry is flow cytometry (Benfey et al., 1984), using erythrocyte

size as a marker of ploidy, this method of verification is expensive and requires skilled personnel to extract the erythrocytes. An alternative would be the use of microsatellites, these have the potential to be cheaper, faster, and just as reliable. We began work to identify a suite of microsatellites that could be used to verify triploidy reliably. The final suite we were able to test consisted of 9 microsatellites and showed a trisomic state at ≥1 loci at 94.1% of individuals. Whilst the same batch of eggs was validated using conventional methods, these results have not been made available at this point and as such we are unable to determine how far off the true triploidy percentage this value is. The initial plan was to refine the suite until a percentage of verification comparable to flow cytometry was achieved, unfortunately the large amount of time it took to get the results for this final suite made further refinement impossible. During the course of this work, another suite of microsatellites was validated that could verify triploidy up to 97.9% (Jacq, 2021). This suite consisted of 12 microsatellites and has been used in a subsequent study on fish produced by the same breeding company (Madaro et al., 2021). Microsatellites that performed well in studies on wild fish did not perform as well in the current study on farmed fish, likely due to reduced genetic diversity, and it remains to be seen how either suite would fare on fish of different genetic origins. The 9 microsatellites validated in the current study therefore complement the existing suite and increase the size of the arsenal of tools we can use to validate triploidy.

In this chapter, we also refined the HotSHOT extraction procedure and validated its use on Atlantic salmon embryos. The results from this are highly encouraging. Current methods of validation, including the above-mentioned suite of 12 microsatellites, required eggs to be incubated until 350DD before they would be tested. To ensure that the stripping season is not missed and orders can be remade if required, a separate batch of eggs is incubated at a higher temperature to speed up development (M. Mommens, Personal communication). This of course requires additional space, cost, time, and broodfish. The HotSHOT method used in this current study has been shown to produce reliable microsatellite validation in eggs as young as 78DD. Results on the smaller scale suggest that eggs as young as 26DD can be analysed using this method, but due to time restraints we were unable to replicate this when the process was automated. This difference in age would remove the need to incubate separate batches of eggs and ensure that eggs incubated at

standard temperatures can be tested and remade within the stripping season. The method of extraction is cheaper and quicker than most conventional methods, with simple and cheap reagents able to extract usable DNA from large numbers of eggs in a matter of hours. This method alongside an increased suite of microsatellites has provided industry and academia with the tools to improve the cost, resources, and effort associated with triploidy verification.

As well as studying the validation of triploid embryos, we also investigated the rate of embryogenesis. The rate of embryogenesis is well understood within diploid Atlantic salmon and has allowed for different studies to conduct treatments at precisely comparable stages. Despite the importance of ensuring that the stages of development when treatments are conducted are comparable between studies and ploidy, there has been very little work investigating the rate of embryogenesis in triploids. One previous study looked at the rate of somitogenesis over a short period of time (Johnston et al., 1999) but no study has looked at development from fertilisation to hatch. We determined that there was no difference in the rate of embryogenesis between ploidy. Whilst this result does not change our understanding of triploids, it provides assurance that any study that conducts treatments during embryogenesis will be conducting them at the same stage on both ploidy. We did see evidence of a variable pattern of hatch, this was something we saw again in a later experiment, and showed triploids hatching earlier than their diploid counter parts. We also identified the optimal clearing solution for producing clear photographs of embryonic development, the validation of this solution will allow future studies the best available tool for observing embryonic development.

The major findings of the thesis focused on the effects of thermal shocks around 360DD. Minimal negative effects were observed for any of the thermal shocks with no increase in deformity or mortality, and no reduction in performance in terms of growth or immune response. The shocks conducted at this stage consisted of an increase from 6°C to 10°C and back after either 1hr, 6hrs, or 1hr repeated for 5 days. The lack of negative effects is promising when the sensitivity of triploid salmon to supraoptimal incubation temperatures is considered. It appears that longer term exposure to incorrect temperatures is needed to negatively affect the health of triploids, this is of course encouraging for an industry that is still determining to correctly handle these fish. The short term shocks of 1hr and 6hr showed minimal

effect on gene expression of either triploids or diploids 24hrs after the shocks (the repeatedly shocked fish were not tested) showing either a rapid recovery or minimal sensitivity. This is again encouraging and shows the relative robustness of the triploid fish.

What the shocks did influence however, was the subsequent tolerance of the fish to high temperatures. Feed intake assessment was conducted at 12 °C and during a 16 °C challenge period. A trend was apparent from the physiological data of both the triploid and diploid 6hr treated fish, specific growth rate and feed intake were higher and thermal growth coefficient was lower (better) in both diploid and triploid 6hr groups at both temperatures. The biological feed conversion ratio was lowest (better) in the 6hr group at both temperatures in the diploids and at 16 °C in the triploids. These performance metrics suggest a trend that these thermally treated fish are performing better on average than the control fish, this trend was confirmed in the case of the triploid 6hr fish when it came to final weight. Before the thermal challenge the triploid 6hr group showed no difference in weight than the control, after 4 weeks of thermal challenge they weighed 4.3g or 6.96% more. This is good evidence that these fish were more tolerant of the higher temperature. The reason behind this tolerance is likely epigenetic programming. We have unfortunately not been able to conduct the analysis of the RRBS sequencing of the embryos after shock, this would inform us as to epigenetic changes which would result in increased tolerance. As mentioned earlier there were minimal transcriptomic differences 24hrs after the shocks, so the impact of the shock was likely programmed into the epigenome, this change stayed stable for 9 months of growth before revealing itself and allowing the programmed fish to better tolerate the high temperatures. These results show the potential of epigenetic programming to revolutionise the aquaculture industry. There has been some evidence that cold shocks can alter the epigenome of Atlantic salmon (Moghadam et al., 2017; Robinson et al., 2019) and even increase tolerance to pathogens (Uren Webster et al., 2018), but this is the first evidence that a thermal shock can increase temperature tolerance later in life in a fish species. Thermal tolerance a major hurdle in triploid culture and increasing this would increase the acceptance of triploids in the industry. The potential of this development goes beyond triploids and to all aquaculture species, who in the coming years and decades will face higher than ever temperatures due to global warming. More

research is needed to see if this change lasts until sea and potentially improve the shock in terms of its programming ability, but this is certainly encouraging.

The above experiment also showed the importance of low incubation temperatures for triploids with no differences in morality and minimal differences in radiological vertebral deformity between ploidy, this is despite all fish being fed a diploid diet. Previous studies have shown the negative impact of supraoptimal incubation temperatures (Clarkson et al., 2021; Fraser et al., 2015) and the use of 4 °C until eyeing and then 6 °C until hatch resulted in triploids with very low levels of deformity. The triploids performed as expected in this study, starting off smaller than the diploids but overtaking them as time went on. Before the thermal challenge they ate considerably more per unit of body weight than the diploids, but by the end of their time at 16 °C the difference had disappeared. Within the control groups there was little between-ploidy transcriptomic differences at either temperature, with only 5 genes being differentially expressed at 12 °C and 16 °C. There was a difference within ploidy, with the diploids differentially regulating 37 genes compared to the 121 in triploids. Some of the functional groups these genes belong to can help explain why triploids suffer more at high temperatures with the differential regulation of groups such as cardiac function and development, transcriptional regulation, and circadian regulation, and the lack of change in genes relating to cytoprotection providing an insight into the differences between ploidy.

The results presented in this thesis cover a range of areas related to understanding and improving the early husbandry of triploid Atlantic salmon. Naturally, in hindsight there would be things that would be done differently. Aside from making efforts to ensure a faster turnaround of microsatellite work, more could have been done to explore the epigenetic / transcriptomic effect of shocks at different stages of development and of different intensities. Whilst transcriptomic analysis of the treatment groups during the thermal challenge would have helped explain why they were able to grow that much better than the control. In all, though the piece of work covers multiple areas in depth, it provides both practical tools for the industry to improve the culture of triploid Atlantic salmon and increases our scientific understanding of both triploids and diploids, the evidence of thermal programming shows the real potential of this practice to improve aquaculture in general. This work has shown that with the correct incubation temperatures triploids can perform

surprisingly well whilst thermal programming has the potential to remove a major hurdle in their acceptance. Should other methods of sterility remain non-commercially viable, then there is evidence in this piece of work to support continued research into triploid Atlantic salmon.

Future work should build on the concept of thermal programming. The first step would be to finish the RRBS analysis to better understand which genes and pathways have been altered to result in increased tolerance. A factor unexplored in the current work is the gene expression of the shocked fish under thermal challenge, this would help solve the puzzle as to why these fish performed better than the control fish under thermal stress. We failed to see a difference in expression in heat shock protein (HSP) expression 24hrs after shock, suggesting rapid recovery, but it would be interesting to explore the expression of this family of genes during the challenge period. HSPs play an important role in temperature tolerance in fish species, and evidence suggests they are likely to play a role in the improved tolerance observed in the previous experiment. In this authors' opinion, work should also begin into understanding the optimal shock. As seen in the previous chapters the thermal shock caused the triploid group to increase thermal tolerance but not the diploid group, one possible reason for this is the fact that diploids have a higher optimal incubation temperature and therefore the shock was not sufficient to elicit a response. Work should be done to identify appropriate shock temperatures for diploids, shocked fish could be grown out and challenged as per the above experiment or if resources are more limited epigenetic profiles could be matched up to those of the programmed fish in the previous experiment, and increased tolerance inferred from this. Ideally, shocks of multiple temperatures would be tested. Whilst a 6hr shock at this specific stage worked well for triploids, it is not known if a shock at a different stage or of different intensity could have resulted in an even larger difference between treatment groups. To identify potential candidate stages for the shock care should be taken to account for the negative side effects of the shock with shocks of too high a degree, duration, or incorrect stage are likely to result in increased mortalities or deformities.

The programming seen in the previous chapter lasted 9 months before challenge, this is a significant duration, but the major hurdle in triploid temperature tolerance is not in freshwater. It remains to be seen if this tolerance remains until seawater,

where high temperatures pose a hurdle in triploid acceptance. In an ideal world, these shocked fish would be grown out until sea, and growth and performance compared to un-shocked fish during high summer temperatures. There is no reason that this concept will not work in other fish species, and those most susceptible to climate change should be first on the list. If the application of thermal programming can be shown to be reliable and long-lasting then this opens up a host of opportunities for the aquaculture industry, not only would the range at which Atlantic salmon and other species can be grown increase but with the close association between thermal tolerance and tolerance of other stressors it has the potential to make an overall more robust fish. This should be explored in more detail, especially given the susceptibility of triploids to coinciding high temperature and hypoxia.

Outside of thermal programming, it is this author's belief that triploids still have a place within Atlantic salmon aquaculture. The shift in opinions over GM/GE foods is slow, especially when it comes to meat products rather than crops or animal by-products. We have shown how well triploids can perform when given lower incubation temperatures, with the only negative being slightly higher rates of radiological vertebral deformities, and this is whilst being fed a diploid specific diet. There is a lack of studies on triploids grown to harvest, especially under commercial conditions. In the opinion of this author, there is enough evidence to suggest that when farmed on the right sites they can grow well until harvest, and provide environmental and public relations benefits by reducing the impact of escaped salmon, commercial trials are needed to support this. The recent study which influenced the memorandum on Norwegian triploid aquaculture included sites known to be supraoptimal for triploid production, whilst it is unclear which incubation temperatures were used in the trial (Madaro et al., 2021). Additional studies would be useful to determine the success of triploid culture until harvest.

In summation, during this thesis we have shown that triploid Atlantic salmon are not as sensitive to changes in temperature as first thought and with the correct incubation temperatures they can perform much better than previous studies have found. We have improved the verification of triploids as well as validated assumptions about their development, we have improved scientific understanding of the differences between ploidy through transcriptomic analysis. We have also shown

how a heat shock during early development can program triploids to be more tolerant to high temperatures later in life.

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