Solving bottlenecks in triploid Atlantic salmon production

Temperature, hypoxia and dietary effects on performance, cataracts and metabolism

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Scientific environment

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Contents

Abstract

In salmon aquaculture, fish occasionally escape from net pens. These domesticated salmon are genetically maladapted for living in natural environments however they still manage to interbreed with wild fish, resulting in severe levels of genetic introgression of farmed salmon in some Norwegian rivers. The use of sterile triploid farmed salmon, with three complete chromosome sets, would avoid further genetic introgression. Initial studies comparing diploids and triploids show reduced performance and higher mortality of triploid salmon that impeded their adoption to commercial farming. With advances in fish husbandry and further knowledge on triploid salmon biology, some of the farming related issues have been mitigated. However, to date, there are still challenges remaining in order to farm triploid Atlantic salmon profitably, sustainably and without jeopardizing fish welfare. Triploids perform poorly at high water temperatures and hypoxic periods, often associated with reduced growth and higher mortality compared to diploids. Further, triploid Atlantic salmon are more prone to develop ocular cataracts that can affect vision, feed intake and welfare. Additional supplementation of the amino acid histidine to the diet successfully mitigated cataract outbreaks and progression in diploid Atlantic salmon. However, the interactive effect between water temperature and dietary histidine level on cataract development in triploid salmon during the risk period of smoltification has not been studied.

In order to investigate the temperature threshold for satisfactory performance and the physiological mechanisms behind reduced or poor performance at suboptimal environmental conditions, diploid and triploid Atlantic salmon post-smolts and adult fish were exposed to water temperatures between 3 and 18 °C (3 °C steps) and to hypoxic periods at cold (6 °C) and warm (18 °C) temperatures. Feed intake, growth and mortality were monitored as well as oxygen consumption, white muscle energy phosphate and carbohydrate storages, blood haematology and plasma parameters. Triploids of both age classes had higher feed intake than diploids at ≤ 9 °C, similar to diploids between 9 and 12 °C, but lower at higher water temperatures. Triploid post-smolts had higher mortality during reduced oxygen saturations at high water

temperature compared to diploids and adult triploids, respectively. Adult triploid salmon had consistently lower white muscle energy phosphates and lower haemoglobin and hematocrit at high temperatures compared to diploids. Over the course of the experiments adult triploids also developed a higher cataract score. The results suggest that triploids have a lower temperature optimum for feed intake and a reduced metabolic scope at higher water temperatures. In the production of triploid post-smolts, periods of low oxygen saturation and particularly during high water temperatures should be avoided.

To assess cataract development in one of the major risk periods, around smoltification, both ploidies were reared at medium and high water temperatures (10 vs 16 °C) and fed with two diets differing in the level of histidine (10.4 vs 13.1 g kg^{-1}) in the weeks before and after seawater transfer. All groups at 16 °C developed significantly more cataracts than groups at 10 °C with additional effects of higher cataract scores due to triploidy and the diet with the lower level of histidine. Furthermore, groups reared at 16 °C experienced severe mortality (triploid > diploid) in the early seawater period due to insufficient hypo-osmoregulatory ability. When reared at 10 °C and fed the diet with more histidine, triploid salmon only developed mild cataracts, well within the level of commercial acceptance. The results suggest that triploids have a higher requirement of dietary histidine compared to diploids in order prevent cataract development and are more sensitive to environmental conditions around seawater transfer.

The main conclusion from this thesis is that water temperature is one of the main drivers in the production of triploid Atlantic salmon. In areas with low or moderate water temperatures, triploids should be a viable option. However, triploid salmon are more sensitive to high water temperatures typically resulting in reduced appetite, increased cataract development, and if combined with low oxygen saturation, increased mortality. Nevertheless, when fed diets with sufficient histidine supplementation and reared at cold or moderate water temperatures, triploid Atlantic salmon can perform better or as well as to diploids.

List of publications

Paper I

Sambraus, F., Fjelldal, P.G., Remø, S.C., Hevrøy, E.M., Nilsen, T.O., Thorsen, A., Hansen, T.J. and Waagbø, R. Water temperature and dietary histidine affect cataract formation in Atlantic salmon (*Salmo salar* L.) diploid and triploid yearling smolt. Submitted the Journal of Fish Diseases.

Paper II

Sambraus, F., Olsen, R.E., Remen, M., Hansen, T.J., Torgersen, T. and Fjelldal, P.G. Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. Under review at Aquaculture.

Paper III

Sambraus, F., Remen, M., Olsen, R.E., Hansen, T.J., Waagbø, R., Torgersen, T., Lock, E.-J., Imsland, A., Fraser, T.W.K. and Fjelldal, P.G. Water temperature and oxygen: The effect of triploidy on performance and metabolism in adult farmed Atlantic salmon (*Salmo salar* L.). Manuscript.

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1. Introduction

1.1 General introduction

Aquaculture closes the gap between the global demand for seafood and stagnating yields from fisheries (FAO, 2014). The production of Atlantic salmon, *Salmo salar*, in aquaculture began in the 1970s and grew rapidly, now producing annually over 2 billion tons worldwide (www.fao.org). Norway has always been the leading country in Atlantic salmon aquaculture and today produces over 50 % of global production (Asche et al., 2013). Other significant salmon producers include Chile, the UK, Canada, and Australia. The production of aquaculture continues to rise rapidly, with Norway seeing an 8 % yearly increase over the last 10 years (www.fiskeridir.no[a]). However, concerns around the environmental impact of salmon farming have begun to restrict the further expansion of the industry in Norway.

One of the greatest challenges regarding the environmental impact of salmon farming is farmed fish escaping from the facilities. As nearly all of the seawater grow-out production of Atlantic salmon takes place in open net-pens in fjords or coastal areas, farmed salmon can escape into the wild. This usually happens during handling, due to holes in the nets, or cage damage from environmental extremes (Jensen et al., 2010). In Norway, on average 354,800 farmed salmon have been reported as escaped annually between 2001 and 2015 (www.fiskeridir.no[b]). Moving from culture to nature, the escaped farmed salmon can mature in seawater and migrate into rivers to complete sexual maturation and spawn (Butler et al., 2005; Glover et al., 2012). This allows escaped farmed salmon to interbreed with wild salmon. Farmed salmon are selectively bred for commercially desired traits (Gjedrem et al., 1991) and have therefore become less well adapted to live in the wild (Skaala et al., 2012), while they are as fertile as their wild counterparts (Yeates et al., 2014). In severe cases, more escaped farmed than wild salmon have been documented on spawning grounds (Fiske et al., 2006). The offspring not only interfere in the wild gene pool with an introgression level of up to 47 % in some Norwegian rivers (Glover et al., 2013), but also show reduced performance and survival compared to wild salmon (McGinnity et

al., 1997, 2003). This could result in declining wild fish stocks and hence end in ecological and economical consequences (Liu et al., 2013).

The most effective method to prevent genetic exchange between domestic escapees and wild salmon is to farm sterile fish. Sterile products in agriculture (e.g. triploid fruit cultivation: bananas, apples, citrus fruits; castrated male animals: ox, barrow, mutton) are widely available, and sterilisation is a common and ubiquitous tool for both quality improvement and non-spreading of seeds in farming. In fish, sterilization can be achieved through surgical castration, hormone treatments or gonadal autoimmunity (Donaldson et al., 1993; Donaldson and Devlin, 1996). A new method for sterilization is CRISPR/Cas9 technology that enables the knockout of a vital protein for gamete production (Wargelius et al., 2016). However, the induction of triploidy in fish is to date the most established, practically and ethically accepted method to induce sterility on a commercial scale (Pifferer et al., 2009; Taranger et al., 2010; Benfey, 2015).

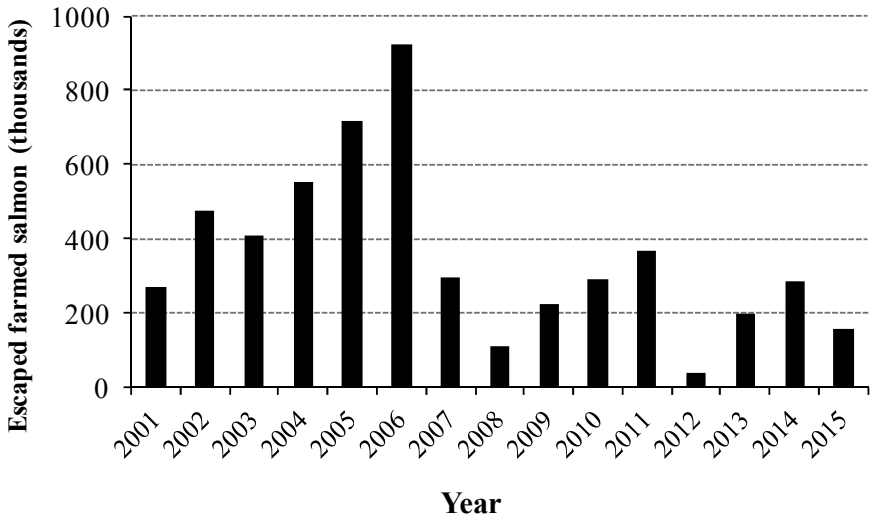

Figure 1 *Number of escaped farmed Atlantic salmon in Norway per year. Data from the Norwegian Directorate of Fisheries (www.fiskeridir.no[b]).*

1.2 Triploidy

Triploidy describes the state of individuals in which somatic cells contain three complete chromosome sets (3N) instead of the common two chromosome sets (2N). In farmed Atlantic salmon, triploidy can occur spontaneously with an average frequency of 2.0 % (Glover et al., 2015), and triploidy has been found in wild fish (Leggatt and Iwama, 2003).

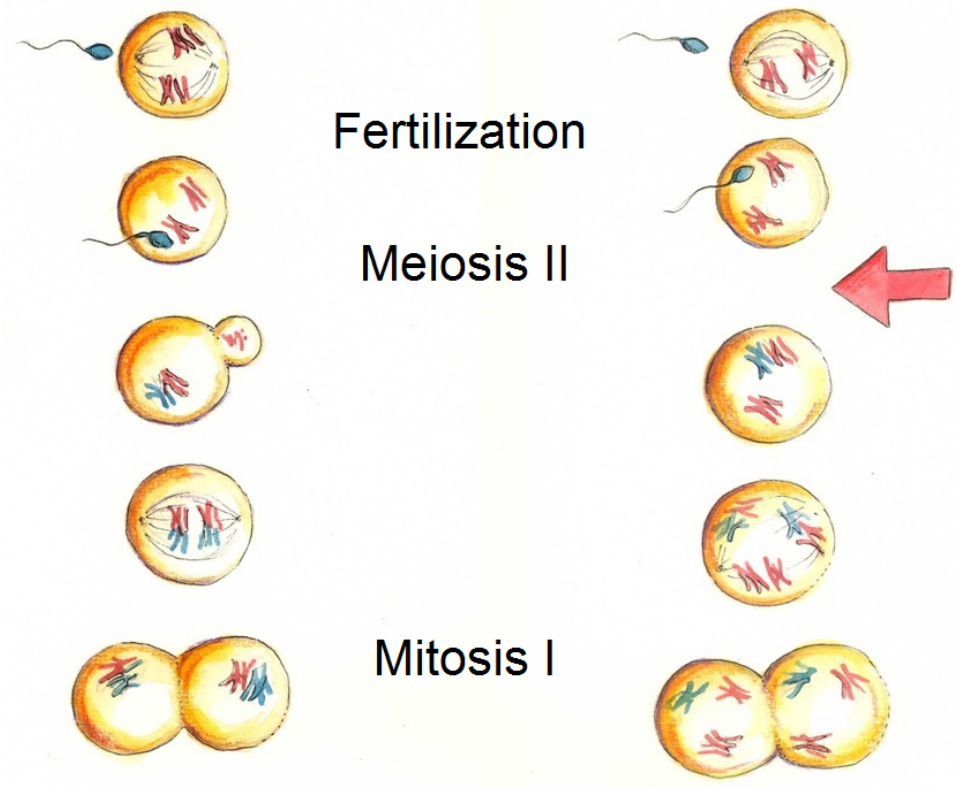

Figure 2 *Fertilization, meiosis II and mitosis I in a diploid (left) and triploid (right) organism. A pressure shock applied during meiosis II (red arrow) leads to the retention of the second polar body inside the egg. Subsequently chromosome duplication and cell division result in three complete sets of chromosomes in each cell. Adapted from Tom Hansen, IMR, Norway.*

The induction of triploidy in salmonids is commonly done in the egg soon after fertilization by either a pressure (e.g. Benfey et al., 1988), temperature (cold shock: Felip et al., 1997; heat shock: Rougeot et al., 2003), or chemical treatment (Thorgaard, 1983), whereby pressure treatments have been identified to be the most successful and efficient (Haffray et al., 2007). The specific moment in time of the shock treatment is dependent on the water temperature and carried out during meiosis II to prevent the extrusion of the second polar body, although allowing subsequent chromosomal division (Piferrer et al., 2009). Furthermore, the duration and intensity of the shock treatment influences the triploidization success (Felip et al., 1997) and differs between species (Piferrer et al., 2009). Another option to produce triploid offspring is by interploid crossing where the eggs of a diploid female are fertilized with the sperm of a tetraploid (4N) male (Francescon et al., 2004). This method is commonly practiced in oysters, but due to general difficulties in the production of tetraploid fish and reduced fertilization rates with diploid sperm, this method is considered unsuitable for commercial purposes (Blanc et al., 1993; Piferrer et al., 2009).

Although triploids are functionally sterile, testes can grow in males up to the size normally observed in fertile diploid fish. The reason behind this is the high quantity of small pre- and post-meiotic produced spermatozoa in males including a similar steroid-profile during maturation as in diploids (Benfey et al., 1989). The few sperm cells that become mature by completing meiosis are aneuploid (Benfey et al., 1986) and the offspring of fertilized eggs cannot survive beyond larval stages at the latest (Feindel et al., 2010). Contrary to males, triploid females produce a low quantity of relatively large oocytes and ovarian growth is nearly completely suppressed. The unpaired chromosome set disables a normal progression through meiosis, and the absence of gonadotropin production, estrogen and vitellogenin production stops the females from entering puberty (Benfey et al., 1989).

1.3 Use of triploidy in aquaculture

The benefits of sterile triploid stocks in Atlantic salmon aquaculture became evident soon after significant growth in this industry in the 1970s, but at that time experiments with temperature shock treatments showed limited success in salmon (Lincoln et al., 1974). It was only in the following years that set protocols for heat- and pressure shock treatments transpired to be more successful, especially in salmonids (Benfey and Sutterlin, 1984a). However, the advantages of triploid sterility in salmonids were associated with production disadvantages such as reduced growth performance (Carter et al., 1994), particularly under suboptimal rearing conditions (Ojolick et al., 1995), an increased prevalence of skeletal deformities (Jungalwalla, 1991), as well as a higher occurrence of ocular cataracts (Wall and Richards, 1992) compared to diploids.

Under commercial aquaculture operations, triploid Atlantic salmon have only been adopted in Tasmania as all female stocks due to the high incidence of sexual maturation in diploids (Jungalwalla, 1991). Sexual maturation with the development of secondary sexual characteristics (e.g. reduced flesh composition, change in skin pigmentation and aggression) reduces growth and flesh quality. Hence, pre-harvest maturity is associated with reduced welfare and increased down-grading losses (McClure et al., 2007). Production of all-female triploids resolves this problem, since triploid females do not mature (Benfey, 1999). The production of all-female lines is a common and effective tool where female fish are treated with androgens and become functional males that can develop testes (so called “neo-males”), which, if crossed with common females, give all-female offspring (Benfey, 2009). The production of all-female triploid rainbow trout (*Oncorhynchus mykiss*) is commonly farmed in the United States and Japan, and Europe (Hulata, 2001; Pifferer et al., 2009). Nevertheless, the adoption of triploid Atlantic salmon in European aquaculture has been slow since pilot projects have reported inferior performance compared to diploids. However, research and production of genetically modified (GM) salmon has increased (e.g. Devlin et al., 2004, 2014; Tibbetts et al., 2013; AquaBounty Technologies Inc., Fortune, PE, Canada). These fish must be sterile both in order to

gain production licenses for protection of the wild stocks and for the producer to protect their GM product. Furthermore, there is an increase pressure to prevent interbreeding between farmed and wild salmon, therefore sterile fish production is becoming more of a necessity in countries such as Norway.

There is also some production of triploid fish for release into the wild, such as production of triploid rainbow trout and brown trout (*Salmo trutta*) for restocking purposes in lakes and rivers in the United States (Dillon et al., 2000; Kozfkay et al., 2006; Koenig et al., 2011) and Europe (Environmental agency, 2009; Piferrer et al., 2009), and production of triploid grass carp (*Ctenopharyngodon idella*) in the United States for biological weed control in lakes and reservoirs (Kirk et al., 2014). Also in shellfish aquaculture, triploidy is used to make a beneficial sex ratio (Chinese shrimp, *Fenneropenaeus chinensis*, Li et al., 2003), and ensures consistent product quality (Pacific oysters, *Crassostrea gigas*, Nell, 2002).

Although triploid Atlantic salmon are unable to produce viable offspring, released farmed triploid males can return to freshwater (Cotter et al., 2000), and stimulate wild females to spawn (Fjelldal et al., 2014). Anadromous behaviour is generally suppressed in triploids compared to diploids, and the return rate of hormonally deficient triploid females to freshwater is lower than in males (Cotter et al., 2000). However, Cotter et al (2000) concluded that the substantially reduced return rate of mature triploid males to the coast and to freshwater demonstrates the potential for triploidy as a means of eliminating genetic interactions between cultured and wild populations, and of reducing the ecological impact of escaped farmed fish.

To promote the use of triploid salmon in Norwegian aquaculture and test their potential on a commercial scale, the Directorate of Fisheries has issued triploid research licenses to salmon producing companies; the operations are ongoing and have thus far shown mixed results regarding growth and mortality (Stien and Fjelldal, 2016; www.fiskeridir.no[c]). In addition, in 2014 the Directorate of Fisheries has awarded green licences to farm triploid Atlantic salmon commercially (Hersoug, 2015). In parallel, after years of research and testing triploid Atlantic salmon for

commercial production on the Canadian east coast (Benfey, 2001) there are currently establishments to farm triploid salmon in Atlantic Canada (Fisheries and Oceans Canada, www.dfo-mpo.gc.ca).

However, challenges remain in the sustainable and feasible production of triploid Atlantic salmon. In particular, reduced performance at suboptimal environmental conditions resulting in lower growth rates compared to diploids, as well as welfare issues (reviewed by Fraser et al., 2012) such as higher mortality or higher incidences and severity of skeletal deformities and cataracts.

1.4 Bottlenecks in the production of triploid Atlantic salmon

1.4.1 Poor triploid performance

Under optimal experimental and environmental conditions, triploids grow and survive equally well or superior as diploids during both the freshwater (Fjelldal and Hansen, 2010; Fraser et al., 2013; Taylor et al., 2013) and seawater (Oppedal et al., 2003; Leclerq et al., 2011) life phases. However, numerous studies have highlighted poorer growth and increased mortality in triploids compared to diploids in seawater (Friars et al., 2001; Cotter et al., 2002; Fraser et al., 2013; Taylor et al., 2013). The reasons behind poor triploid performance are unclear, but are generally associated with lower temperature and hypoxia tolerance (Ojolick et al., 1995; Altimiras et al., 2002; Hyndman et al., 2003a; Verhille et al., 2013; Hansen et al., 2015), as well as an increased incidence of production disorders like skeletal deformities (Fjelldal and Hansen, 2010; Fjelldal et al., 2016) and cataracts (Wall and Richards, 1992; Taylor et al., 2015).

1.4.2 Temperature and hypoxia sensitivity

Temperature and oxygen can be considered as the two main abiotic drivers in salmon aquaculture and have a major influence on fish growth and welfare. In contrast to the freshwater rearing of Atlantic salmon that mainly takes place in tanks on land where farmers generally have control over temperature and oxygen levels, the on-growing farming of Atlantic salmon is primarily carried out in floating net pens where environmental conditions fluctuate according to season, time of day, and water current velocity (e.g. Burt et al., 2012; Johansson et al., 2006; 2007; Oppedal et al., 2011, Stien et al., 2012). The environmental conditions within the net pens set the limit for physiological function and production performance of the fish, primarily through their effects on fish metabolism (Fry, 1947, 1971). Temperature is one of the main controlling factors of metabolism and dissolved oxygen (DO, % oxygen saturation) is often the main limiting factor, therefore these are key environmental factors to consider.

1.4.3 Temperature tolerance in diploid salmonids

In salmonids, both the standard and the active metabolic rates (SMR and AMR, the MR of resting, fasted fish and the MR of maximally active fish, respectively) increase with temperature until the thermal optimum (T_{opt}) is reached. Beyond this, SMR continues to increase while AMR falls off and decreases with further temperature increases (reviewed by Pörtner, 2010). The capacity of the fish to perform energy-demanding activities beyond upholding basic physiological functions, termed metabolic scope (MS, defined as AMR-SMR; Fry, 1947, 1971), is highest at the T_{opt} and decreases towards zero levels at the upper and lower critical temperatures (T_{crit}; reviewed by Farrell, 2009). Fish growth rate, considered the principal parameter of production performance is an example of such energy-dependent activity, which is expected to co-vary with the size of the MS (Khan et al., 2014; Claireaux and Lefrançois, 2007). Observations suggest that the lower T_{crit} is ≤ 2 °C (Arnesen et al., 1998), that the T_{opt} is between 13 and 19 °C (Anttila et al., 2014; Handeland et al.,

2008; Hevrøy et al., 2013) and that the upper T_{crit} is ~25 °C (Anttila et al., 2014) for diploid Atlantic salmon post-smolts. Maximal feed intake in Atlantic salmon is usually recorded a few °C past T_{opt} for growth, whereas the lowest food conversion ratio (FCR) is usually a few °C below T_{opt} (Jobling, 1994). With increasing water temperature past the point of maximal feed intake (e.g. in summer or autumn), appetite decreases.

1.4.4 Temperature tolerance in triploid salmonids

Although some studies investigating temperature tolerance in triploid and diploid salmonids detected no significant difference between ploidies (Benfey et al., 1997; Galbreath et al., 2006; Ellis et al., 2013), a majority report reduced triploid performance and survival at high water temperatures in Atlantic salmon (Quillet and Gaignon, 1990; Myers and Hershberger, 1991; Ojolick et al., 1995) and other salmonids (Blanc et al., 1992; Simon et al., 1993). This suggests a lower T_{opt} and T_{crit} in triploids than diploids, as shown for triploid Atlantic salmon and brook trout (*Salvelinus fontinalis*), which have a lower T_{opt} than diploids (Atkins and Benfey, 2008).

An explanation as to why triploids are more susceptible to higher water temperatures may be due to differences in their cellular physiology (Benfey, 1999). As the triploid cell contains a third set of chromosomes, they not only have larger but also fewer cells than similar sized diploids (**Figure 3**; Benfey, 1999). Depending on the shape of the cell, a fundamental consequence of larger cells is a reduced surface to volume ratio. This may not only alter inner transport and diffusion distances and signalling pathways, but also nutrient, metabolite and ion exchanges (Benfey, 1999; Maxime, 2008). It is possible that triploid cells may be limited in oxygen utilization compared to diploids (Small and Benfey, 1987) that may lead to welfare issues if triggered or amplified under changing environmental conditions.

It has been suggested that haematological differences between diploids and triploids underlie their altered response to temperature, but findings are not consistent. Oxygen affinity in blood and haemoglobin was found to be similar in diploid and triploid salmonids (Graham et al., 1985; Sadler et al., 2000a; Verhille and Farrell, 2012), while blood haemoglobin concentration was lower in triploids (Benfey and Sutterlin, 1984b; Graham et al., 1985; Sadler et al., 2000a). Furthermore, the haemoglobin-oxygen loading ratio was reported lower in triploid Atlantic salmon (Graham et al., 1985) and in triploid Chinook salmon (*Oncorhynchus tshawytscha*) at critical swimming speeds (Bernier et al., 2004), and reflects inferior blood oxygen carrying capacity in triploids. Lower erythrocyte pH was attributed to the reduced haemoglobin-oxygen saturation in triploids (Bernier et al., 2004). At high water temperatures triploid fish may be additionally challenged since the blood oxygen affinity decreases with increasing water temperature (Irving et al., 1941). Similarly, in the presence of hydrogen ion or carbon dioxide during or after exercise the haemoglobin affinity for oxygen is also reduced due to Bohr and Root effects. King and Lee (1993) reported higher frequency of damaged erythrocytes (e.g. pinched cells or bisected nuclei) in triploid than diploid salmon, and these cells may contribute to less blood oxygenation. Therefore, triploid fish may more easily suffer from starts of hypoxia than diploids.

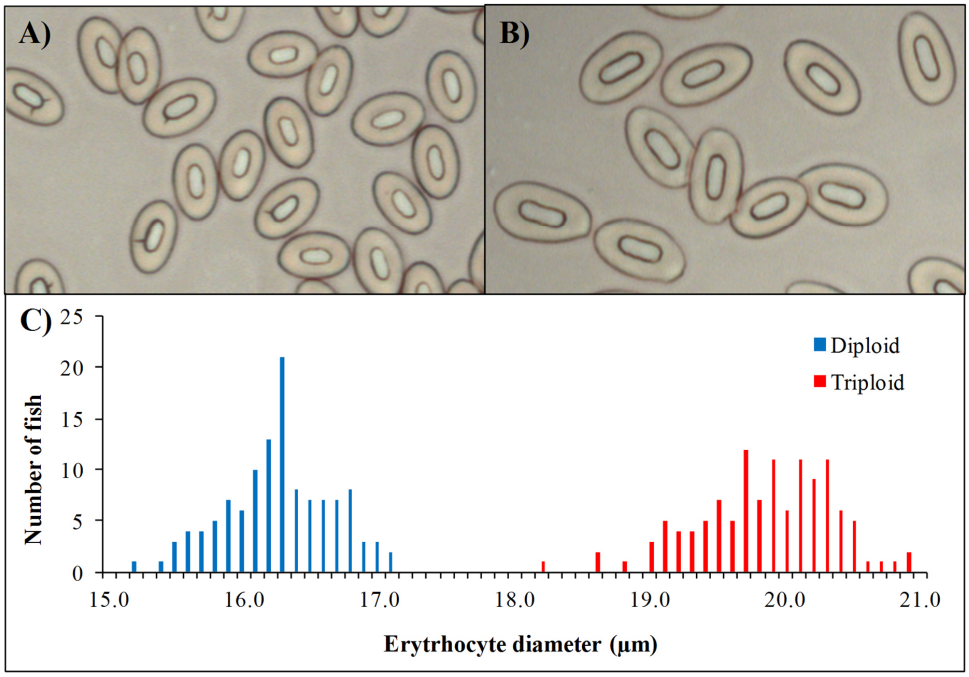

Figure 3 *Blood smears showing erythrocytes of diploid (A) and triploid (B) Atlantic salmon at the same microscopic magnification (40x). No overlap in mean erythrocyte diameter (C) of 80 diploids (blue bars) and 80 triploid (red bars) suggest a triploidization success of 100%. Erythrocyte measurements are a common tool for ploidy verification in salmonids (Benfey et al., 1984).*

The cardiac system is known to play an important role in temperature tolerance in diploid and triploid salmonids (Verhille et al., 2013). Several studies suggest that the triploid cardiac system performs similarly to that in diploids (Altimiras et al., 2002; Mercier et al., 2002; Benfey and Bennett, 2009; Verhille et al., 2013). However, triploid brown trout had unusual high heartbeat frequencies at 18 °C (Mercier et al., 2000), and triploid rainbow trout were more sensitive to increasing temperatures expressed in an earlier onset of cardiac arrhythmia than to diploids (Verhille et al., 2013). It was suggested that triploid salmonids have a reduced aerobic capacity (Bernier et al., 2004) especially at high temperatures (Altimiras et al., 2002). The

oxygen consumption in triploid salmonids is reported to be lower (Stillwell and Benfey, 1995) or similar (Benfey and Sutterlin, 1984c; Yamamoto and Iida, 1994) to diploids. A reduced metabolic scope at higher temperature would also match to the evidence that erythrocyte volume is negatively correlated to the metabolic rate or activity level in teleosts (Glomski et al., 1992; Lay and Baldwin, 1999).

Given physiological differences between diploid and triploid fish, several studies have investigated whether the ploidy effects in temperature tolerance are related to metabolism (Verhille and Farrell, 2012; Verhille et al., 2013). At temperatures near or outside T_{crit} where the aerobic scope is limited, the fish may experience anaerobic metabolism and oxidative stress. The main anaerobic pathways for ATP (adenosine triphosphate) production in the muscles are either through CrP (creatine phosphate) hydrolysis in the cytoplasm or through glycolysis and lactate production. CrP is used as a rapidly mobilizable storage for burst energy activity with ATP production under (i) anaerobic conditions or (ii) when energy utilization is greater than production. Few studies have compared the energy metabolism in white muscle in detail in diploid and triploid salmonids, with respect to energy phosphates (CrP and ATP) and easily accessible carbohydrates (glucose, glycogen), which have been found to be highly dependent on water temperature (Hyndman et al., 2003a, 2003b). After exhaustive exercise at 9 °C, the metabolic response in brook trout was similar between ploidies, while triploids tended to recover faster than diploids with respect to white muscle lactate clearance (Hyndman et al., 2003b). However, at 19 °C triploid brook trout utilized significantly less white muscle CrP, more glycogen and experienced 90 % mortality 4 hours post exercise (Hyndman et al., 2003a). The authors suggested that triploid brook trout have impaired ability to utilize anaerobic pathways at high temperature.

1.4.5 Response to environmental hypoxia

The occurrence of environmental hypoxia in water bodies is a natural phenomenon, caused by stagnant water, ice cover or eutrophication (Nilsson and Östlund-Nilsson,

2008). The severity and frequency of hypoxia in coastal waters has significantly increased within the last half-century (Conley et al., 2007; 2011). This trend may set limits for farming triploid Atlantic salmon with a lower temperature optimum compared to diploids in sea cages in open coastal waters in the years to come. Hypoxia may limit the metabolism through a restriction of the supply of oxygen to the final stage of cellular respiration. If DO is reduced to a level where the MS is restrained, this is termed environmental hypoxia (Farrell and Richards, 2009). The DO in Atlantic salmon net pens has been found to vary between 30 and 130 % DO (Crampton et al., 2003; Johansson et al., 2006, 2007) while feed intake and growth in diploids has been found to be reduced at DO levels below 70 % at 16 °C (Remen et al., 2012). With hypoxia being most prevalent during periods of high water temperatures (Burt et al., 2012; Oppedal et al., 2011), this suggests that low DO may be a considerable hindrance for efficient production in the late summer and autumn period.

Only few studies have focused on the performance (e.g. growth, survival, loss of equilibrium, etc.) of triploid salmonids when exposed to decreasing oxygen concentrations or hypoxic conditions (Benfey and Sutterlin, 1984c; Ellis et al., 2013; Hansen et al., 2015; Scott et al., 2015). This is surprising since several observations indicate poor triploid performance at high temperatures (see above) and the fact that the oxygen solubility of water naturally decreases with increasing temperature. For restocking purposes in ponds and lakes survival of triploids is lower compared to diploids and is also suspected to be related to seasonal changes in temperature and oxygen (Simon et al., 1993; Koenig and Meyer, 2011). Under experimental conditions, results on hypoxia tolerance between the two ploidies show a similar trend as on tolerance to high temperature, with inconsistent outcomes in the literature (e.g. Ellis et al., 2013). Despite diploid and triploid Atlantic salmon reached asphyxiation at a similar low oxygen partial pressure (Benfey and Sutterlin, 1984c) suggesting similar tolerance to hypoxia most studies tend towards reduced tolerance in triploidy (Hansen et al., 2015; Scott et al., 2015).

Yamamoto and Iida (1994) found that triploid rainbow trout lost equilibrium at higher oxygen concentrations than diploids and consumed less oxygen than diploids at 1.6 mg l^{-1} (~24 % O_2 saturation) however oxygen consumption was similar between ploidies at concentrations from 6 to 2 mg l^{-1} (~30-90 %). This is in line with an effect of triploidy on reduced hypoxia tolerance when exposed to low oxygen concentrations by an earlier loss of equilibrium in triploid rainbow trout at 12 (~22 % O_2 saturation, Scott et al., 2015) and catfish hybrids (*Ictalurus punctatus* × *I. furcatus*) (~8 %, Lilyestrom et al., 1999) at 22 °C compared to diploids. Further, in Atlantic salmon, Hansen et al. (2015) reported reduced feed intake, growth and higher mortality in triploid compared to diploid post-smolts when reared at 19 °C and 70 % oxygen saturation for 30 days.

Most haematological (hematocrit, haemoglobin) and physiological (anaerobic metabolism) mechanisms and parameters that relate to inferior performance at high water temperatures also apply to the tolerance during hypoxic periods. Reduced hypoxia tolerance in triploids is not fully understood but suggested to be due to a lower aerobic scope at high water temperatures and reduced environmental oxygen (Hansen et al., 2015). The reason for this assumption are based on larger cell sizes in triploids than diploids, especially erythrocytes, where decreased surface to volume ratio may alter oxygen uptake, blood oxygen carrying capacity, oxygen diffusion and binding characteristics (Benfey and Sutterlin, 1894b). Lower blood haemoglobin concentrations in triploids than diploids and lower haemoglobin oxygen saturation (Graham et al., 1985) would indicate reduced tolerance to hypoxia especially at high water temperatures (Hansen et al., 2015).

1.5 The welfare disorder cataract

Cataracts are opacities of the lens which impact lens transparency and have been observed in both farmed and wild Atlantic salmon (Hargis 1991; Brown and Bron, 1996; Midtlyng et al., 1999; Ersdal et al., 2001; Bjerkås et al., 2003; 2006). In modern Atlantic salmon farming, cataract development has been regarded as a welfare disorder of multiple origin, however mostly related to nutritional insufficiency of the amino acid histidine (Bjerkås et al. 2006). Cataracts were detected as a challenge in commercial triploid fish farming at an early stage (Wall and Richards, 1992).

There are several possibilities to examine cataracts in fish, however for scientific purposes and under practical conditions the use of a slit-lamp biomicroscope (e.g. HEINE® HSL 150 hand-held slit lamp, HEINE Optotechnik, Herrsching, Germany) is most beneficial due to a more detailed view that allows for further characterisation of the opacity (Wegener et al., 2001). Wall and Bjerkås (1999) developed a cataract scoring system, which serves as a uniform grading procedure across species. Depending on the occurrence and degree of opacification the lens is given a score of 0 to 4 for each eye and 0 to 8 for each fish (Figure 4).

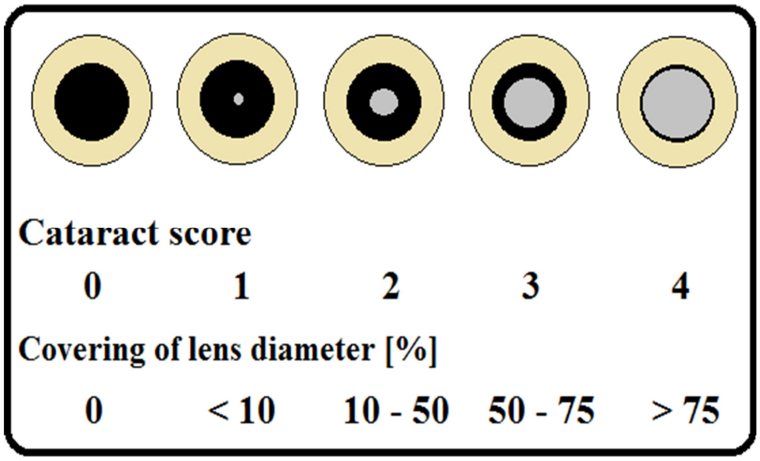

Figure 4 *Schematic drawing of lenses without cataract and cataract infested lenses with different severity (1-4) graded according to the scale of Wall and Bjerkås, 1999*

In fish two main types of cataracts can be distinguished (i) reversible cataract or (ii) irreversible cataract. The reversible type is more distinctive to salmonids as it is induced by osmotic changes in the lens during smoltification (Iwata et al., 1987). After rapid transfer to seawater, the lens may swell due to an increased water influx characterized by vacuoles in the epithelium and cortex (Bjerkås et al., 2003). Although, if the duration or intensity of osmotic stress on the lens is too severe (e.g. long lasting severe swelling or degradation of lens fibres) it may cause irreversible damages to the lens or predispose it to future cataract formation.

Irreversible cataracts are usually associated with severe damage of lens fibres as well as proliferation excrescence of the lens epithelium. Depending on the aetiology, cataract formation can develop in its severity, characteristics and location over time (Bjerkås et al., 2006). The appearance of cataracts in Atlantic salmon in freshwater is rare, but when occurring, is often associated with suboptimal environmental conditions such as chronic high temperatures or rapid temperature fluctuations (Bjerkås et al., 1996, 2001; Bruno and Raynard, 1994).

Several factors have been identified as risk factors for the occurrence, outbreak and development of cataracts in farmed fish such as genetics (strain: Breck et al., 2005a; family: Taylor et al., 2015; triploidy: Wall and Richards, 1992), fast growth rates (Bjerkås et al., 1996; Breck and Sveier, 2001), parasite infestation (Seppänen et al., 2008) and smoltification (Waagbø et al., 1996). Other key elements are water temperature (Bjerkås and Bjørnestad, 1999; Bjerkås et al., 2001; Waagbø et al., 2010) and nutrition (Hughes, 1985; Bjerkås et al., 2006; Waagbø et al., 2010; Remø et al., 2014). Severe cataracts that reduce vision can have implications on welfare, feed intake and growth in Atlantic salmon aquaculture that subsequently lead to economic losses (Breck and Sveier, 2001; Menzies et al., 2002). From the risk factors above, cataracts are a welfare issue of major concern of triploid farming, occurring at fast growth and elevated water temperatures (Bjerkås et al, 2006; Taylor et al., 2015).

1.5.1 Temperature and growth as cataract risk factors

Besides the period of smoltification and seawater adaption the first and second summer with increasing seawater temperatures are key periods for cataract outbreaks when kept under natural conditions (Waagbø et al., 2010). Temperature has in several ways been identified to initiate cataract outbreaks with regards to cold (Bjerkås et al., 2001) and warm exposure (Waagbø et al., 2010), as well as to fluctuations in temperature (Bjerkås et al., 2001). The mechanisms of increased cataract formation in Atlantic salmon during periods with increasing or high water temperature are not fully understood, but are likely based on the interplay between increased oxidative pressure and an altered demand on specific nutritive elements (Waagbø et al., 2010). However, as temperature and growth can be closely correlated it is possible that cataract outbreaks are attributed to multifactorial reasons.

Bjerkås et al. (1996) describe that fast growing Atlantic salmon in freshwater developed more cataracts compared to slower growers and growth rates in fast growing fish decreased after cataracts appeared. The same relationship also accounts for larger fish in the second year in seawater which developed high cataract scores when the water temperature increased >18 °C in the summer. Not only in humans and land animals is oxidative stress responsible for an increase in the formation of cataracts (Ottonello et al., 2000; Williams, 2006), but likewise in fish (Remø et al., 2011). It has been shown that cellular components are negatively affected by oxidative damage with subsequent alterations for physiological functions (Lou, 2003).

1.5.2 Nutritional cataracts

Nutrition has a major role in cataract formation in fish (reviewed by Hughes, 1985; Bjerkås et al., 2006). While in the 1980s a major focus on suboptimal nutrition in salmonids was on zinc (Ketola, 1979; Barash et al., 1982) more recent research

elucidated low levels of dietary histidine to be a risk indicator for cataract development (Breck et al., 2003; Waagbø et al., 2010; Remø et al., 2014).

In farmed Atlantic salmon the prevalence of cataracts increased considerably after the legislative omission of blood meal from the diets in order to reduce the spread of bovine spongiform encephalopathy and the increase in cataracts was presumed to be attributable to dietary deficiencies (Wall, 1998). The amino acid histidine and its derivates function in the fish lens as osmolytes (Baslow, 1998; Rhodes et al., 2010) and antioxidants (Babizhayev, 1989). Histidine is present in high amounts in blood meal and can counteract cataract formation in Atlantic salmon (Breck et al., 2003). However, it has been shown the dietary histidine requirement for growth (8 g histidine kg^{-1}; NRC, 2011) is far below for what is necessary to mitigate cataract outbreaks and severity and finally ensure ocular health at 13.4 g kg^{-1} in Atlantic salmon smolts (Remø et al., 2014).

Unlike the majority of mammals that have the ability to synthesize histidine in the body, the amino acid needs to be supplied with the diet in fish (Espe et al., 2001). The same counts for other non-enzymatic antioxidants (e.g. carotenoids, vitamin C and E) that also need to be supplied with the diet (Lou, 2003). This is in contrast to enzymatic antioxidants (e.g. catalase, glutathione peroxidase, superoxide dismutase) which the metabolism is able to produce itself. Not only does the amino acid histidine itself functions as an antioxidant, but also its derivate N-acetyl-histidine (NAH) and the dipeptides anserine and carnosine. Antioxidants are the counter measure to oxidants and reactive oxygen species produced under oxidative stress and have the ability to mitigate the oxidation of cell components. As antioxidants have a lower oxidative gradient compared to the cellular tissues they have the ability to either being oxidized themselves or by actively reducing the oxidants. Furthermore, they have the ability to neutralise hydroxyl and other radicals to prevent the oxidation of cellular tissues (Wade and Tucker, 1998; Williams, 2006).

The effectiveness of elevated levels of dietary histidine to mitigate cataracts in Atlantic salmon has been demonstrated for various sizes and life stages in seawater

(Breck et al., 2003; Bjerkås and Sveier, 2004; Waagbø et al., 2010; Remø et al., 2014). In Freshwater, histidine supplemented diets had no positive effect on lens NAH, while no increase in cataracts were observed when reared at moderate temperatures ≤ 14.4 °C (Breck et al., 2005a, 2005b). The histidine derivative NAH has a key role as it is the most abundant free amino acid in the salmon lens (Breck et al., 2005a). Its concentration in the salmon lens can be manipulated by the dietary histidine level, and can be used as a lens-specific marker for the histidine status in the salmon (Breck et al., 2005a, 2005b; Remø et al., 2014). Parent substance for NAH synthesis in the lens is histidine and acetyl-coenzyme-A which are synthesized by N-acetyltransferase (Baslow, 1966). However, as NAH is hydrolysed by anserinase (Yamada et al., 2005) which is not found in the lens, NAH needs to be released and can only be hydrolysed when entering the extracellular fluids (aqueous humour) where anserinase is present (Baslow, 1998). After anserinase catalyzes the hydrolysis of NAH to histidine in the external fluid, histidine can subsequently be taken up by the lens and re-synthesized into NAH (Baslow, 1967).

Rhodes et al. (2010) suggested NAH to take the function of an osmolyte in the salmon lens and therefore have an important role to maintain volume regulation and water balance. Similar to plasma osmolality, aqueous humour osmolality and lens NAH is correlated to external medium and thus higher in seawater than freshwater (Breck et al., 2005a; Rhodes et al., 2010).

While the histidine dipetides carnosine and anserine have not been detected in the salmon lens (Breck et al., 2005a), both take an important role in anaerobic metabolism as a buffer in the skeletal muscle of fish during burst swimming activity (Abe et al., 1985). Carnosine is synthesised in skeletal muscle from histidine methylation and β-alanine. Anserine is the major compound in the muscle (Breck et al., 2005a) and is synthesised from either carnosine methylation (carnosine N-methyltransferase) directly to anserine or from histidine methylation (methyltransferase) over 1-methyl-histidine and subsequent β-alanine integration is present at higher quantities than carnosine. The equivalent buffer and antioxidant functions of anserine in skeletal muscle are carried out by NAH in the lens.

1.5.3 Triploidy

The knowledge on cataracts in triploid Atlantic salmon is limited, although the literature suggests they are more susceptible to cataract formation than diploids. The first observation of increased cataracts in triploid compared to diploid Atlantic salmon was that of Wall and Richards (1992). Subsequently, there have been several growth studies that have examined cataracts in both ploidies (Oppedal et al., 2003; Leclercq et al., 2011; Taylor et al., 2013, 2015; Smedley et al., 2016). However, with the exception of Taylor et al. (2015) who focused on the combination of dietary histidine levels (17.4 vs. 12.6 g kg^{-1}) and ploidy in Atlantic salmon, the remaining studies used standard commercial salmon diets for diploids. Importantly, Taylor et al. (2015) suggested that triploid Atlantic salmon have an increased requirement for dietary histidine in order to reduce the occurrence and development of cataracts compared to diploid counterparts. This would explain previous reports of higher cataract scores in triploids in all previous studies, as they utilised diets based on diploid requirements (Oppedal et al., 2003; Leclercq et al., 2011; Taylor et al., 2013; Smedley et al., 2016). However, Taylor et al. (2015) only applied the histidine enriched diets to diploid and triploid Atlantic salmon prior to the second summer in seawater. At that time the fish had experienced several risk periods of cataract development. As triploid salmon often grow faster compared to diploids in freshwater (Taylor et al., 2013), do not always smolt at the same time as diploids (Leclercq et al., 2011; Taylor et al., 2012), and are more susceptible to reduced growth and survival in the early seawater phase (Galbreath and Thorgaard, 1995; Withler et al., 1995; McCarthy et al., 1996), cataract formation in triploids may differ to diploids during these risk periods. It is unknown how sensitive triploid pre- and post-smolts are to histidine supplemented diets to counteract cataract formation and what level of histidine is required.

2. Aims of the study

The main objective of the current thesis was to examine the sensitivity of triploid Atlantic salmon to elevated water temperatures and hypoxia at different live stages on welfare issues like poor smolting physiology and development of ocular cataracts.

The specific aims were:

1. To investigate the effect of elevated water temperature on growth performance, smoltification physiology and cataract development in the fresh- and seawater phase in diploid and triploid Atlantic Salmon, and the possible counteractions by supplementation of histidine in the diet (**Paper I**)
2. To investigate the effect of elevated temperature, hypoxia and their combination on feed intake, overall growth performance and physiology in diploid and triploid Atlantic salmon post-smolts (**Paper II**)
3. To investigate the effect of elevated temperature, hypoxia and their combination on feed intake, overall growth performance and physiology in large adult diploid and triploid Atlantic salmon (**Paper III**)

The thesis aimed to test the hypothesis whether (*i*) cataract development is higher in triploid than diploid Atlantic salmon before and after seawater transfer and if diets supplemented with histidine can mitigate cataract formation equally well in triploids than diploids (**Paper I**) (*ii*) smoltification is similar between ploidies at elevated water temperature (**Paper I**) (*iii*) feed intake and growth performance differs between diploids and triploids throughout different temperatures and when the temperature optima for feed intake is exceeded in Atlantic salmon post-smolts (**Paper II**) and adult fish (**Paper III**) (*iv*) physiological responses are different between ploidies at different temperatures (**Papers I-III**).

3. Abstract of papers

Paper I

The aim of the study was to investigate cataract development in diploid (2N) and triploid (3N) Atlantic salmon smolts and post-smolts at two water temperatures (10 and 16 °C) given either and low (LH, 10.4g kg^{-1}) or high (HH, 13.1g kg^{-1}) dietary histidine before and after seawater transfer. At the start of the experiment triploids (153±1 g) were heavier than diploids (138±1 g), but the cataract was negligible in either ploidy. During the seven week freshwater period, a grave cataract outbreak was recorded in groups reared at 16 °C, with significantly higher mean cataract scores in triploids compared to diploids, although with a reduced severity in both ploidies fed the HH diet. The cataract development at 10°C was negligible. Growth rates in freshwater were higher in 2N at 16 °C, compared to the remaining groups. After transfer to seawater, groups reared at 16 °C displayed osmoregulatory stress in form of insufficient gill Na^+, K^+-ATPase activity, elevated plasma electrolyte concentrations and high mortalities among both ploidies. The respective 10 °C groups performed similarly to each other with respect to growth and mortality. The cataract development was more severe in triploids compared to diploids given the same dietary histidine level, and the cataract score was significantly higher in groups fed the LH diet. Lens histidine and N-acetyl histidine concentrations reflected the dietary histidine level. At the end of the freshwater period, white muscle free imidazoles were lower in groups fed the LH diet compared to the HH diet. The findings of this study demonstrate the importance of environmental conditions in the husbandry of Atlantic salmon, and particularly triploids, with regards to smoltification and the need for adjusted diets to counteract production disorders such as lens opacities.

Paper II

Using sterile triploid fish in Atlantic salmon aquaculture would mitigate the introgressive hybridization between escaped farmed wild and salmon. However, production of farmed triploid salmon is limited due to reports of poorer growth and higher mortality when compared to diploids, in particular under sub-optimal conditions. To address these concerns, triploid and diploid Atlantic salmon post-smolts were monitored at temperatures between 3 and 18 °C and 100 % oxygen saturation (DO), and additional periods of 60 % DO (hypoxia) at 6 and 18 °C, respectively. Feed intake and oxygen consumption rate were monitored throughout the experimental period. Muscle and blood samples were collected at 100 and 60 % DO at 6 and 18 °C for analysis of white muscle energy phosphates (creatine phosphate, adenosine triphosphate) and carbohydrate fuels (glucose, glycogen) as well as blood clinical chemistry (whole blood: hematocrit; plasma: Na^+, K^+, Cl^-, glucose, lactate, pH, triacylglycerol). Mortality was higher in triploids compared to diploids at 18 °C and reduced DO. Compared to diploids, triploids had higher feed intake (% biomass) at ≤ 9 °C, but lower ≥15 °C. Feed intake peaked at 12 and 15 °C for triploids and diploids, respectively. Under hypoxia, triploids had lower feed intake than diploids at 6 °C, while at 18 °C both ploidies nearly ceased feeding. The difference in feed intake was not associated with any ploidy effect on body weight gain or feed conversion ratio, but triploids had greater body length growth compared to diploids. At ≥15 °C triploids consumed less oxygen than diploids. In the white musculature, the only observed difference between ploidies was a lower level of glycogen in triploids compared to diploids at 18 °C and 100% DO. In plasma, the concentration of ions was lower and glucose level higher in triploids compared to diploids at 18 °C and 60 % DO. The results of this study indicate that triploid Atlantic salmon post-smolts are less tolerant compared to diploids at high seawater temperature and low DO. For sea-cage farming of triploid salmon post-smolts, this would favour production areas with moderate maximum temperatures and sufficient DO.

Paper III

In commercial salmon farming, the use of sterile triploids (3N) can solve the problem of escapees interbreeding with wild salmon. However, triploid salmonids appear to be less tolerant of high water temperatures and low oxygen levels compared to diploids (2N). In order to investigate how the thermal performance and physiology of adult triploid (2492±80g) Atlantic salmon differs to diploids (2315±38g), both ploidies were acclimated to 9 °C and then subjected to a temperature regime from 9 down to 3 °C and 3 up to 18 °C in steps of 3 °C per week. Furthermore, the fish were also exposed to low oxygen saturation (70 % dissolved oxygen), termed hypoxia, at 6 °C and 18 °C. Triploids had higher feed intake and grew faster between 3 and 9 °C compared to diploids, but fed similar to diploids at 12 °C. At 15 °C, the feed intake significantly dropped in both ploidies, but this drop was more apparent in triploids. During hypoxia, feed intake was higher in triploids at 6 and equal to diploids at 18 °C. The overall feed conversion ratio was similar between ploidies. Mortality was low, not associated with environmental conditions and did not differ between ploidies. Muscle energy phosphates (creatine phosphate, ATP) were generally lower in triploids compared to diploids while muscle glucose, blood haemoglobin and hematocrit tended to be lower in triploids than diploids at ≥ 12 °C. Plasma lactate levels tended to be higher in triploids and increased with increasing temperature and at hypoxia in both ploidies. Plasma cortisol increased in both ploidies at high temperatures and was highest in triploids under hypoxic conditions at 18 °C. Triploids had a higher cataract score at the start of the experiment and developed more cataracts throughout the experiment. The present findings show that adult diploid and triploid Atlantic salmon differ in parts of their energy and physiological metabolism, where triploids perform better at colder water temperatures, however, the response to hypoxic periods were mainly similar between ploidies.

4. Methodological considerations

Triploid induction and fish stock

All fish in the present study originated from AquaGen AS, Norway. Diploid and triploid salmon of Papers II and III originated from the same batch. Triploidy was induced by a hydrostatic pressure treatment thirty-seven minutes and 30 seconds after fertilization of 655 bar for 375 seconds at 8 °C (TRC-APV, Aqua Pressure Vessel, TRC Hydraulics inc., Dieppe, Canada). Ploidy verification was performed by erythrocyte measurements (Benfey et al., 1984) and confirmed as 100 % efficient for all batches. Diploids served as untreated control groups. After fertilisation, the eggs were split in two equal halves, where one half was treated with hydrostatic pressure for triploid induction, and the other half remained as untreated diploid control. Due to efficiency and practicality physical treatments (i.e. hydrostatic pressure) are more common to induce triploidy compared to temperature treatments (Teskeredžić et al., 1993).

Sexual maturation in post-smolts

After terminating the experiment discussed in **Paper I** the remaining fish were euthanized, measured for fork length and body weight, and the gonads dissected for measurement of gonad weight to calculate of gonadosomatic index (GSI = (gonad weight (g) * 100) / body weight (g)) in all fish reared at 10 °C. Only non-maturing fish were included in the material used in **Paper I**. In total, 113 male and 122 female diploids, and 109 male and 113 female triploids were dissected. Maturity was assessed by visual examination of the gonads. Mature males were distinguished from immature males by a GSI > 0.4 % following Endal et al. (2000). Among females, there were no signs of sexual maturation, but diploids had lager ovaries than triploids. Among males, there were individuals with enlarged testis. The data on diet were pooled as there was no effect of dietary level of histidine level and maturation. The occurrence of mature males was 16.8 % among diploid males reared at 10 °C in

contrast to 3 % among triploid males reared at the same temperature (**Table 1**). The fish were sampled 13 weeks after transfer to seawater and were reared under a simulated natural light photoperiod.

Table 1 Gonadosomatic index (GSI) of diploid and triploid Atlantic salmon post-smolts after 13 weeks in seawater reared at 10 °C (**Paper I**)

Ploidy	Immature (GSI < 0.4 %)		Mature (GSI > 0.4 %)		Total n
	Incidence	GSI ± SEM	Incidence	GSI ± SEM	
Diploid	83.2	0.05 ± 0.004	16.8	12.7±0.8	113
Triploid	97.2	0.05 ± 0.004	2.8	8.4±0.4	109

5. General discussion

5.1 Growth and survival in freshwater

After increasing the water temperature from ambient (5.3 °C) to 10 and 16 °C (1 °C day^{-1}), respectively, diploid salmon reared at the higher temperature had the highest growth rates compared to the other groups during the last seven weeks in freshwater. Triploid salmon reared at 10 and 16 °C as well as diploids reared at 10 °C had similar growth rates. In general, triploid Atlantic salmon often grow faster or at similar growth rates compared to diploids in the freshwater phase (McGeachy et al., 1995; Fjelldal and Hansen, 2010; Leclercq et al., 2011; Taylor et al., 2013). At the experimental start triploids were significantly larger than diploids when previously reared under ambient water temperature (5.3 °C). After a temperature increase growth rates at 10 °C were similar between ploidies, indicating higher growth rates in triploids than diploids at colder water temperatures in freshwater (**Paper I**). This is in accordance to Taylor et al. (2013) who recorded higher freshwater growth in 1+ triploid pre-smolts reared at temperatures between 2 and 16 °C. Similarly, Fraser et al. (2014a) compared triploid and diploid Atlantic salmon reared as yearling smolts at ambient temperature (4.5 °C) and underyearling smolts reared at 16 °C during the last six weeks in freshwater. The authors found that the yearling smolt production regime under ambient conditions gave larger triploids than diploids, whereas the underyearling production regime at a high temperature regime resulted in equally sized triploids and diploids. A size effect on specific growth rate (Needham, 1964) may partly compensate the equal SGRs to the advantage of triploids in pre-smolts at 10 °C, but it is also possible that the optimal water temperature for triploids in freshwater is < 10 °C.

The majority of the literature reports longer or thinner triploid than diploid salmon with a lower condition factor in freshwater (O'Flynn et al., 1997; Taylor et al., 2012; Fraser et al., 2013). This was also partly observed in **Paper I** where triploids at 16 °C had a significantly lower condition factor than all other groups. Whereas triploid fish at 10 °C had the same specific growth rate for weight as fish at 16 °C, the latter grew

significantly longer, resulting in a lower condition factor. Besides the condition factor is affected by length growth, it is also in positive correlation to the body lipid storage in salmon (Herbinger and Friars, 1991) which are an important energy source, especially during long lasting periods of high temperatures (Hevrøy et al., 2012, 2013). It appears that growth in length is stimulated similarly in diploid and triploid salmon at high temperature in freshwater independent of weight gain (**Paper I**). However, the fact that diploids at 16 °C grew ~1 cm longer, but triploids at 16 °C grew only ~ 0.5 cm longer compared their respective groups at 10 °C until seawater transfer indicates that length growth was not at its fastest in triploids at 16 °C. The lower specific growth rate in triploids compared to diploids at 16 °C in **Paper I** could indicate that triploidy reduces the scope for the amount of available energy to support muscle growth and lipid deposition due to a high metabolism. Also, triploidy may set limits to the regulation of feed intake due to a reduced metabolic scope at high temperature (Altimiras et al., 2002). In **Papers II and III** triploid post-smolts and adult triploid salmon decreased feeding at increasing water temperatures sooner than the respective diploids. Feed intake in **Paper I** was not measured, but may have confirmed reduced feed intake in triploids reared at 16 °C compared to diploids at the same temperature and triploids at 10 °C, respectively.

From stocking until seawater transfer there were no mortalities in either ploidy. Freshwater survival between diploid and triploid salmon is reported to be similar after first feeding (Taylor et al., 2013) or lower in triploids (Fjelldal et al., 2016) however this has been found to be dependent on water temperature (Fraser et al., 2014b). Here, triploids were also found to have a lower temperature optimum than diploids for hatching and survival until start feeding and rearing until 100 g when incubated at 6 °C compared to 8 and 10 °C, respectively (Fraser et al 2014b).

5.2 Smoltification and initial seawater performance

Fish of both ploidy appeared to successfully complete smoltification after rearing at 10 °C, but rearing at 16 °C led to a notable increase in mortalities following sea transfer (**Paper I)**. Many factors influence the timing of smoltification such as temperature (Solbakken et al., 1994), photoperiod (McCormick et al., 1987) and fish size (Økland et al., 1993). It is possible that the higher degree days from the experimental start of groups at 16 compared to 10 °C (715 vs. 483 °C days) may have induced smoltification earlier in groups at warmer water as demonstrated in previous studies (Solbakken et al., 1994; Shrimpton et al., 2000). Handeland et al. (2004) suggested that peak Na^+, K^+-ATPase (NKA) activity is reached around 350 °C days after the initial increase in NKA activity observed at the end of March. Transferring these data onto **Paper I**, peak NKA activity should have been ten and eighteen days before the actual transfer in groups at 10 and 16 °C, respectively. However, groups reared at 16 °C did not display any elevated NKA levels (< 6 µmol ADP * mg protein^{-1} * h^{-1}). Although fish in **Paper I** were of the same strain as in the study from Handeland et al. (2004), they were significantly larger and smoltification may have thus occurred even earlier.

The most probable reason for the mortality observed in Atlantic salmon shortly after seawater transfer in **Paper I** was that the smolts were not sufficiently adjusted to the hyper-osmotic environment. Several studies have demonstrated a significant effect of temperature on the loss of hypo-osmoregulatory ability during smoltification in Atlantic salmon (Duston et al., 1991; McCormick et al., 1999). Despite the larger cells, triploidy is supposed to be advantageous for osmoregulatory capacity compared to diploids (Maxime and Labbé, 2010). However, there are studies reporting reduced triploid smoltification (Jungalwalla, 1991) and survival after seawater transfer (Galbreath and Thorgaard, 1995; McCarthy et al., 1996). It is possible this may be due to environmental conditions and seawater transfer outside the smolt-window. When transferring both ploidies to seawater, low NKA activity (~3 to 5 vs. 9 to 12 µmol ADP * mg protein^{-1} * h^{-1}) was measured in fish reared at 16 compared to 10 °C. Studies in which 1+ yearling triploid smolts were transferred without significant

mortality state gill NKA activity levels from > 7 to 17. Values of ≤ 5 measured in **Paper I** may not provide sufficient hypo-osmoregulatory ability in 35 ‰ seawater.

Along with gill NKA activity which serves as the most reliable parameter for seawater adaption, osmoregulatory sensitive parameters can give further indication of the physiological state of the fish. After seawater transfer a substantial rise of plasma osmolality and Cl^- concentrations were recorded in groups at 16 °C which mirrored low gill NKA activity and inefficient ability for ion excretion. Plasma osmolality and chloride levels in the respective 10 °C groups were more similar to values of diploid and triploid Atlantic salmon measured by Boeuf et al. (1994) after two days in seawater without osmoregulatory disturbances and mortality.

Previous studies have shown that Atlantic salmon can undergo smoltification at temperatures between 14 and 16 °C (Grini, 2008), but the peak gill NKA activity is rather short and declines quickly thereafter (Johnston and Saunders, 1981). Taylor et al. (2012) mentioned elevated gill NKA activity from the beginning of February to the end of April, thus it is possible that the temperature increase (**Paper I**) from late March to early April (5.3 to 16 °C) has accelerated gill NKA activity in groups reared 16 °C before gill samples were taken. In fact, salmon can also undergo desmoltification in increased water temperatures (Soivio et al., 1988). Due to faster growth in freshwater and higher weights at seawater transfer, triploid Atlantic salmon can smolt about four weeks earlier than diploids when transferred as 0+ underyearling smolts (Leclercq et al., 2011; Taylor et al., 2012). However, these findings are in contrast with significantly larger triploid 1+ yearlings that undergo smoltification concomitantly with diploids (Boeuf et al., 1994; Taylor et al., 2012, 2013).

In the early seawater phase there was high mortality in diploids, and particularly in triploids, with stagnating or decreasing growth in groups at 16 °C thereafter. After seawater transfer feed intake in Atlantic salmon can be restrained up to several weeks due to adjustments of the new environment (Clarke et al., 1981; Usher et al., 1991; Stradmeyer, 1994). In general, initial seawater performance is highly dependent on the time point of seawater transfer combined with good hypo-osmoregulatory ability

which was not applicable to diploids and triploids at 16 °C. If these factors are considered, smoltification, survival and initial seawater performance can be comparable in diploids and triploids (Leclercq et al., 2011). In order to farm triploid salmon successfully it is essential to further examine the mechanisms behind triploid smoltification and establish protocols in order to ensure seawater transfer in the "smolt-window" that allows for optimal hypo-osmoregulatory ability, high survival and subsequently swift recovery of feed intake, growth and condition factor.

Diploid post-smolt maturation was found to be a significantly affected by ploidy (**Paper I**) In line with the present findings, Fraser et al. (2014a) found between 3.1 and 3.4 % post-smolt maturation among diploid males compared to 0 % among triploid males. Both temperature (Fjelldal et al., 2011, Imsland et al., 2014) and size (Fjelldal et al., 2007) are important risk factors for post-smolt maturation in Atlantic salmon. The fact that up to 93 % of males can mature as post-smolts when reared at 16 °C (Melo et al., 2014), reveals a potentially major down-side of accelerated temperature regimes for early maturation in salmon aquaculture. Based on the industries intention to move parts of the post-smolt production into closed aquaculture systems to avoid the parasitic sea lice, concerns have risen about impaired welfare and mortality caused by early maturation using this strategy (Good et al., 2014). Hence, triploid salmon may be a viable alternative for post-smolt production in closed systems, especially when the rearing temperature is in their optimal range (**Paper II**).

5.3 Feed intake, growth and survival in seawater

Temperature was closely associated with feed intake irrespective of ploidy and life stage, but triploids had a lower optimum temperature than diploids (**Papers II and III**). For example, triploids had higher appetite than diploids at lower temperatures between 3 and 9 °C as both post-smolts (**Paper II**) and adults (**Paper III**). Similar findings were reported from Hansen et al. (2015) who recorded slightly higher feed intake in triploid compared to diploid post-smolts at 10 °C. Feed intake in the present studies was similar between ploidies at 12 °C in both life stages, however significantly reduced with increasing temperature (15 °C) in triploids. In contrast, diploid post-smolts increased feeding after increasing the temperature from 12 to 15 °C and only decreased feeding at a further increase to 18 °C. This indicates an optimum around 15 °C for diploids, that agrees with findings from Handeland et al. (2008), compared to 12 °C in triploids.

The temperature optimum in fish usually shifts with age. In adult triploids (**Paper III)** feed intake was highest between 9 and 12 °C compared to 12 °C in post-smolts. This follows the work of Hevrøy et al., (2013) who found that adult diploids (1.6 kg) had temperature optima of 13 °C. The data on feed intake and growth demonstrate a lower temperature optimum in triploids that is consistent through **Papers I, II and III**. Additionally, the reduction in feed intake in triploid adult salmon with increasing temperature (≥ 15°C) was more pronounced compared to post-smolts and points out the inverse relationship between fish weight (and age) and temperature optimum in salmonids (Pörtner and Farrell, 2008; Elliot and Allonby, 2013). However, despite differences in optimum temperatures for feeding, the highest mean feed intake within ploidy did not differ significantly between diploids and triploids at either life stage (**Papers II and III**).

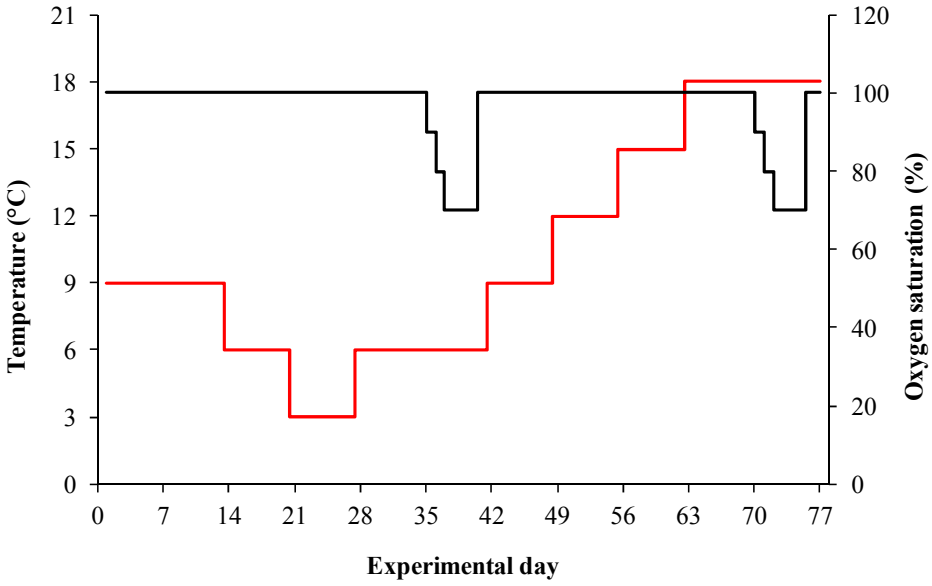

Figure 5 *The experimental setup of* ***Paper III*** *(****Paper II*** *similar). Temperature (red) decreased from 9 to 6 to 3 °C and increased thereafter in steps of 3 °C per week. One additional week 6 and 18 °C were included where the oxygen saturation (black) was reduced in steps of 10 % per day and maintained for 2.5 days at 70 % oxygen saturation, termed hypoxia. In* ***Paper II*** *hypoxia was defined as 60 % oxygen saturation*

Feed intake during hypoxic periods (**Paper II**: 60 % O_2 saturation; **Paper III**: 70 % O_2 saturation) differed between ploidies, temperatures and age. Triploid post-smolts had lower feed intake during hypoxia compared to diploids at 6 °C and also 18 °C when appetite was nearly nonexistent. In contrast, hypoxia only significantly reduced the appetite in diploids at 18, but not 6 °C. Hansen et al. (2015) reared diploid and triploid Atlantic salmon post-smolts at chronic high temperature (19 °C; 100 % O_2 saturation) and in combination with hypoxia (70 % O_2 saturation). Similar to the present study, the authors recorded lower mean daily feed intake (% biomass) in triploids under both regimes (normoxia: 0.25 %; hypoxia: 0.6 %) compared to diploids. While Hansen et al. (2015) reared the fish at 19 compared to a maximum of 18 °C in **Papers II and III** it appears that the additional reduction from 70 (**Paper**

III; Hansen et al., 2015) to 60 % O_2 saturation (**Paper II**) is a crucial threshold for feed intake and growth in triploid post-smolts.

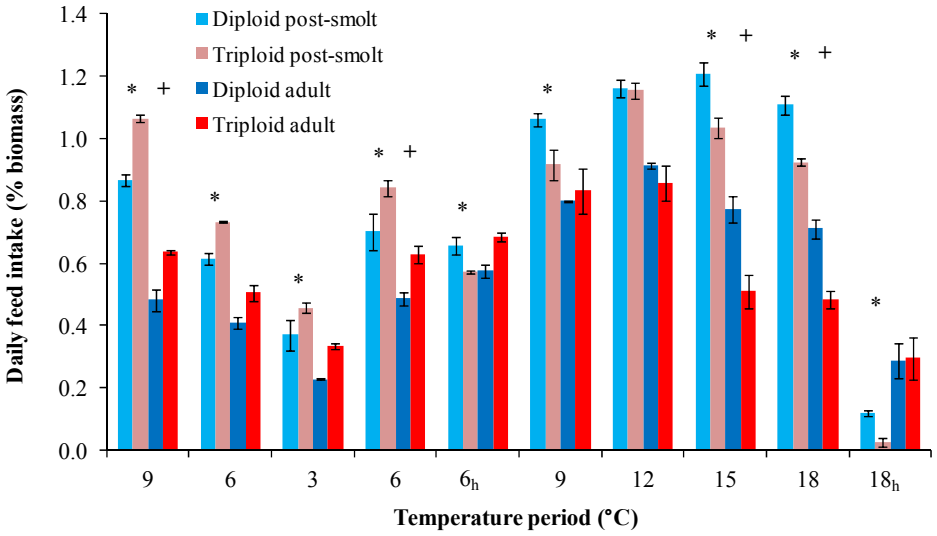

Figure 6 *Mean daily feed intake at different temperatures and hypoxia (60 % oxygen saturation in post-smolts; 70 % in adults) in diploid and triploid post-smolts and adults (**Papers II and III**). Asterisk * indicates a significant difference between diploid and triploid post-smolts at the given temperature. Plus + indicates a significant difference between diploid and triploid adult fish at the given temperature.*

In **Paper III** hypoxia had no negative effect on the appetite of adult diploid and triploid salmon at 6 °C however both ploidies expectedly reduced feeding at the combination of high water temperatures and reduced oxygen saturation. Since the definition of hypoxia was at different O_2 saturations in **Papers II and III** the results may not be directly comparable, but indicate that, with regards to feed intake (0.3 %) and mortality (0 %), adult triploids were able to cope with 18 °C and 70 % O_2 saturation similar to diploids. These results are in contrast to the general trend of reduced triploid performance at suboptimal conditions (**Paper II**; Ojolick et al., 1995; Hansen et al., 2015) however the previous studies have used much smaller fish

compared to **Paper III**. Additionally, Scott et al. (2015) found that juvenile triploid rainbow trout are more sensitive to hypoxia than diploids, whereas there was no significant difference in time to loss of equilibrium between both ploidies of adult fish when exposed to 12 °C and low (~22 %) O_2 saturation. The results suggest that adult triploid Atlantic salmon are similarly tolerant to hypoxia as diploids, whereas triploid post-smolts are less tolerant compared to diploids when exposed to similar environmental conditions.

Feed intake was measured over the course of the temperature curve and some temperatures were measured twice (i.e. 6 and 9 °C) however with inconsistent outcomes. Whereas triploid post-smolts had higher feed intake rates than diploids at the initial 9 °C period, feed intake at 9 °C after hypoxia (6_h °C) was not only lower in triploids than diploids, but also lower compared to the initial 9 °C period. The reasons for this are inconclusive, but it is possible that triploids were under residual treatment effects from previous hypoxia exposure

Diploids on the other hand were less affected of hypoxia at 6 °C and also fed at higher levels at 9 °C thereafter, possibly due to compensatory mechanisms from lower feed intake than triploids at colder temperatures. It has been shown that under farming conditions Atlantic salmon can compensate for reduced feeding during hypoxic periods with elevated feed intake thereafter (Remen et al., 2014). Following hypoxia exposure at 18 °C both ploidies recovered similarly at 15 °C however they failed to reach the previous feeding levels from the same temperature. Compensatory mechanisms would also explain higher feed intake in diploids than triploids at 9 °C after three weeks of lower temperature where diploids fed considerably less than triploids. In contrast, when initially acclimated to 9 °C triploids had significantly higher feed intake than diploids.

The feed conversion ratios from **Papers II and III** were similar between ploidies, as previously reported in Atlantic salmon (Hansen et al., 2015; Taylor et al., 2015) and other salmonids (Habicht et al., 1994). In contrast, Fraser et al. (2013) reported reduced feed conversion in triploid Atlantic salmon reared in sea cages. However, in

those studies the FCR was not measured as food eaten to weight gain but only the offered food was calculated against the weight gain as uneaten pellets could not or were not collected. Therefore the feed intake measurements (Helland et al., 1996) from **Papers II and III** can be considered more accurate. However, as in **Paper II** only the overall FCR throughout a whole range of temperatures was calculated, it is unclear whether triploids utilize feed differently at different temperatures and fish sizes than diploids do (Handeland et al., 2008). Alterations in the gut morphology between diploid and triploid salmon (Peruzzi et al., 2015) and different temperature optima for feed intake would suggest this requires clarification. Handeland et al. (2008) estimated that diploid Atlantic salmon smolts (70-150 g) utilized feed most efficiently at 13.4 °C, but for post-smolts with twice the weight the temperature decreased to 11 °C. As the FCR is one of the most important variables in salmon aquaculture due to the high costs of feed, future research is essential to elucidate potential differences.

Growth rates in seawater did not differ between diploids and triploids post-smolts (**Paper II**) during either the "cold" (between 3 and 9 °C) or "warm" (between 12 and 18 °C) periods and suggests similar growth performance under these conditions. However, in **Papers II and III** the fish were only exposed to each temperature for a limited period of time (7 days). Previously Hansen et al. (2015) found that triploid post-smolts performed poorly compared to diploids when maintained at chronic high temperatures for 30 days. Therefore, future work may expose adult triploids to chronic high temperatures.

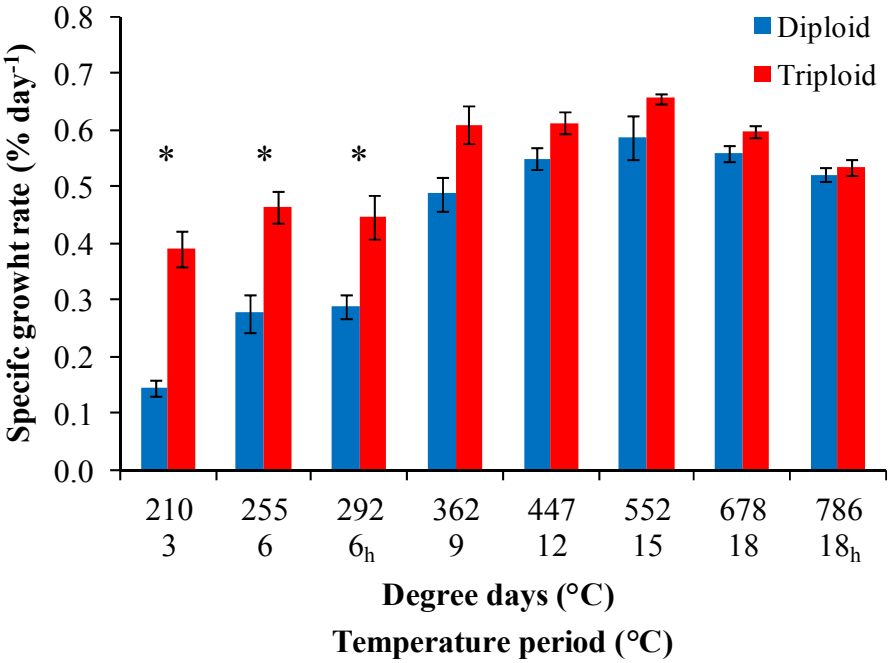

Figure 7 *Specific growth rate of adult diploid and triploid Atlantic salmon* ***(Paper III)*** *exposed to different environmental conditions for one week each. Asterisk * indicates a significant difference ($p < 0.05$) between ploidies at a given temperature period. Interaction of temperature × ploidy ($p<0.05$).*

Growth rates for weight in adult pit tagged diploid and triploid salmon (**Paper III**) were temperature dependent and the difference in mean specific growth rate between ploidies decreased with increasing water temperatures and degree days (**Figure 7**, specific growth rate). However, at cold water temperatures (i.e. 3 to 6 °C, but also ambient freshwater temperature in **Paper I**) in seawater triploid salmon seem to consistently outperform the respective diploids independent of age (**Papers II and III**).

Through **Papers I-III** triploids had greater length growth than diploids, an observation that is consistent throughout the literature (Oppedal et al., 2003; Taylor et al., 2014). The result is that triploids had lower condition factors than diploids, which

has also been documented in several studies (Friars et al., 2001; Taylor et al., 2013; Fraser et al., 2015) and may be associated with photoperiod and temperature. For example, under continuous light salmon vertebrae have increased length growth due to up-regulation of the hormone insulin-like growth factor 1 (IGF-1) (Nordgarden et al., 2006). Further, triploids tend to express higher levels of IGF-1 in the vertebral bone compared to diploids when reared under continuous light (Fjelldal et al., 2016). This may explain increased length growth in triploids and reduced condition factors compared to diploids.

Mortality in post-smolts was low (except in **Paper I**, see smoltification), but higher in triploids at 18 °C and decreasing oxygen levels. The literature may suggest triploid salmonids to be more sensitive to lower oxygen saturations (e.g. Ojolick et al., 1995; Hansen et al., 2015), although studies are inconclusive (Yamamoto and Iida, 1994; Ellis et al., 2013; Scott et al., 2015).

Adult fish on the other hand were hardly affected by mortality, including during periods of high water temperature and low oxygen. The altered mortality observations between age classes in triploid salmon may result from different hypoxia intensity, but since triploid post-smolts from Hansen et al. (2015) had lower survival at 19 °C and 70 % oxygen saturation it may indicate that adult fish are more tolerant to periods of low oxygen saturation. Temperature studies on large Atlantic salmon under controlled conditions are scarce and none have used fish in the size of **Paper III** (>2.5 kg), although these nearly harvest sized fish are economically most important in commercial aquaculture.

5.4 Temperature and hypoxia on haematology and physiology

Haematological parameters are key factors for oxygen distribution in the body and there were few ploidy effects on hematocrit with similar responses to environmental conditions. For example hematocrit in post-smolts (**Paper II**) of both ploidies was higher in the hypoxic (70 % O_2 saturation) compared to the normoxic periods (100 %). In adult fish (**Paper III**), hematocrit generally increased with increasing water temperature in both ploidies, but during hypoxia only at 18 °C. Previous studies reported similar hematocrit, but reduced haemoglobin in triploid salmon at 15 (Benfey and Sutterlin, 1984c), 12.5 (Sadler et al., 2000a), and 6 °C (Graham et al., 1985; Small and Randall, 1989), which suggests no effect of temperature on hematocrit. These studies used fish with mean weights of under 100 g and are more comparable to post-smolts in **Paper II** than adult fish in **Paper III**. Here, diploid salmon increased hematocrit more than triploids and tended to have higher hematocrit levels than triploids between 12 and 18 °C which was concomitant with the same development in haemoglobin. In contrast, haemoglobin in triploids was similar between 3 and 18 °C. The results suggest that increased hematocrit in large triploid salmon (**Paper III**) between 3 and 18 °C (100 % O_2 saturation) was a consequence of a catecholamine stress response causing red blood cell swelling, whereas elevated haemoglobin during hypoxia at 18 °C followed a release of splenic red blood cell reserves.

While the oxygen affinity for triploid blood is reported to be similar to that of diploids (Verhille et al., 2013), triploid haemoglobin carries less (75 %) oxygen (Graham et al., 1985) resulting in lower arterial blood concentrations compared to diploids (Bernier et al., 2004). This may impair triploids with regards to respiratory physiology (i.e. aerobic capacity), especially at high water temperature. In fact triploid post-smolts (**Paper II**) consumed significantly less oxygen compared to diploids at 15 and 18 °C, whereas adult triploid salmon (**Paper III**) only consumed marginally less oxygen than diploids at the same temperatures. Stillwell and Benfey (1996) measured reduced oxygen consumption in triploid brook trout at 13.6 °C and suggested the triploid respiratory system to be inferior in extracting oxygen from the

water. However, several studies comparing metabolic rate between diploids and triploids have found no obvious differences (Benfey and Sutterlin, 1984c, Yamamoto and Iida, 1994; Bernier et al., 2004; Verhille and Farrell, 2012). It may also be that haemoglobin is affected by strain and/or individual, but lower blood haemoglobin concentrations will generally reduce the metabolic scope at high water temperatures, hypoxia, or exercise. This is in line with triploid rainbow trout being more sensitive to increasing water temperature than diploids expressed as an earlier onset of cardiac arrhythmia (Verhille et al., 2013), or generally higher mortality in triploid fish at chronic high temperatures and hypoxia (**Paper II**).

In **Paper II** there were no ploidy effects on the level of energy phosphates (CrP and ATP) at any of the studied temperatures or during hypoxia. In contrast, adult triploid salmon had consistently lower mean values of white muscle CrP and ATP between 3 and 18 °C and lower glycogen concentrations at temperatures ≥ 12 °C (**Paper III**). Previously, Hyndman et al. (2003a) observed 90 % mortality in triploid brook trout (9 out of 10 vs. 0 in diploids) after exhaustive exercise at 19 °C, a result that was associated with highly depleted levels of white muscle glycogen in triploids whereas CrP stores remained high. This led the authors to suggest that triploids have difficulties utilizing anaerobic pathways (Hyndman et al., 2003a). However, as in Hyndman et al. (2003a), lower levels of white muscle glycogen were detected in triploid post-smolt (**Paper II**) and adult (**Paper III**) salmon at 18 °C (100 %), although not during hypoxia at the same temperature. Kieffer et al. (1994) demonstrated that higher acclimation temperature (15 °C) leads to significant lower CrP levels compared to colder temperature (5 °C) in rainbow trout of a similar size as in **Paper II**, while white muscle ATP was unaffected by temperature. One reason may be the higher metabolism in fish with warming water until the upper thermal maximum (Fry, 1971). However, feed intake in **Paper II** was approximately two- to threefold higher at 18 compared to 6 °C and hence supplied more substrate (elevated glucose levels) for ATP production. Adult triploid salmon (**Paper III**) may be impaired in up-regulating CrP directly or triploids may have a reduced mitochondrial density compared to diploids. However, as there was no mortality in large fish of

either ploidy, in particular at 18 °C and hypoxia, the marginal lower white muscle energy levels in triploids are not to the expense of mortality.

Plasma lactate increased at high temperatures, and plasma pH decreased during hypoxic periods, although there was no effect of ploidy (**Papers II and III**). This is in line with similar plasma lactate levels after exhaustive exercise at 9 and 19 °C in diploid and triploid brook trout (Hyndman et al., 2003a, b), chinook salmon at the critical swimming velocity at 9 °C (Bernier et al., 2004), as well as in resting Atlantic salmon post-smolts of both ploidies (Fraser et al., 2014c). This suggests that triploid salmonids have a similar anaerobic and acid-base metabolism compared to diploids.

Welfare is closely related to the stress response in fish. Stress can physiologically be expressed in a variety of parameters (e.g. hematocrit, plasma glucose, osmoregulation), but cortisol is generally referred to as the most reliable indicator for stress (Pottinger, 2008). Plasma cortisol was only measured in adult fish (**Paper III**), and found to be higher in diploid fish at 18 °C. However, under the additional effect of hypoxia at the same temperature, levels were higher in triploids. In parallel, plasma glucose was also significantly elevated in triploid post-smolts and adult fish at the same period. Plasma cortisol is often reported to be similar after stressful events, confinement or exhaustion between diploid and triploid salmonids (Biron and Benfey, 1994; Benfey and Biron, 2000; Sadler et al., 2000a, 2000b; Kobayashi et al., 2009; Fraser et al., 2014c). However, as these studies have exposed the fish to rather acute, but not chronic stressors (e.g. hypoxia over several hours) it may be that the stress response may differ as seen in **Paper III**.

As secondary stress response, plasma glucose concentrations were higher during hypoxia at 18 °C in **Papers II and III**. Biron and Benfey (1994) also found plasma glucose to be elevated in triploid and diploid brook trout after two hours when the fish were previously exposed to five minutes of confinement. In contrast, plasma glucose of triploid Atlantic salmon post-smolts was not elevated compared to diploids after chronic exposure to high temperature (19 °C), and neither in combination with

hypoxia (70 % oxygen saturation) for 30 days (Hansen et al., 2015). It is possible that triploid salmon of **Papers II and III** may have adjusted plasma glucose levels after longer exposure of suboptimal environments. Further, low mean feed intake (~0.25 % of biomass) in the triploid fish of Hansen et al. (2015) may have contributed to slightly lower levels compared to diploids.

Plasma osmolality and ionic composition in Atlantic salmon are known to be relatively stable over a wide range of temperatures (from 3 to 14 °C) (Byrne et al. (1972). **Papers II and III** exceeded this temperature range and there was a general trend of increased plasma ion concentrations with temperature in **Paper II**, and diploid post-smolts also had significantly higher Na^+, K^+ and Cl^- concentrations during hypoxia at 18 °C than diploids. In contrast, there was no effect of ploidy in adult fish. Similarly, Maxime and Labbé (2010) found that erythrocytes of non-maturing diploid rainbow trout were more fragile after a hypo-osmotic challenge compared to triploids. The data may suggest that triploid post-smolts maintained ion regulation better compared to diploids, however it is not clear if other ions in triploid fish have accumulated that were not tested. The effects of larger cells in triploids associated with a reduced surface to volume ratio and potentially longer pathways for signalling are not fully understood.

5.5 Temperature and diet on cataract formation

Wall and Richards (1992) were the first reporting higher susceptibility of cataracts in triploid Atlantic salmon compared to diploids. When cataracts were assessed in fresh- and seawater (**Papers I and III**) triploids developed a consistently higher prevalence and more severe cataracts than diploids similar to previous studies on this topic (Oppedal et al., 2003; Leclercq et al., 2011; Taylor et al., 2013, 2015; Smedley et al., 2016). Severe cataracts have an impact on fish welfare (Fraser et al., 2012) and can result in reduced growth due to poor vision.

Figure 8 *Atlantic salmon lens without cataract (left) compared to a lens affected by cataract (right) from* ***Paper I****. Source: Havforskningsinstituttet.*

In **Paper I**, the fish were reared at ambient water temperatures (5.3 °C) prior to the experimental start and both ploidies only had minor cataracts that can be considered biologically negligible. Groups at 16 °C experienced a severe cataract outbreak during the last seven weeks in freshwater. In contrast, all groups reared at 10 °C had cataract scores of < 1. Cataract outbreaks in freshwater are less common, but can occur due to fast growth (Bjerkås et al., 1996) or high and fluctuating temperature (Bjerkås et al., 2001). Remø et al. (2014) found that high water temperature influenced the lens's antioxidant defence system in Atlantic salmon smolt reared at 19 °C compared to 13 °C. Fast grow in diploid salmon may have a added to higher cataract score, although it is more likely that the threefold increase in water temperature over eleven days (5.3 to 16 °C; 1 °C day^{-1}) have predisposed both

ploidies to cataracts. The chronic high temperature thereafter may have increased the oxidative pressure in the lenses of diploids and triploids in freshwater that made those groups more susceptible to cataract formation.

One of the major risk periods for cataract formation in Atlantic salmon is during smoltification (Waagbø et al., 1996; Breck and Sveier, 2001). These osmotic induced cataracts can however be reversible depending on the duration and severity (Bjerkås and Sveier, 2004). During the first six weeks in seawater both diploid and triploid post-smolts developed cataracts. Temperature appeared to be the main variable for further cataract development, but also triploidy, that displayed higher cataract scores compared to the respective diploid groups. The fact that both ploidies at 16 °C, but especially triploids, displayed significant osmoregulatory disturbances after seawater transfer (plasma osmolality, Cl^-) may have affected lens homeostasis and promoted cataract progress additionally. Aqueous humour surrounds the lens and interacts with it in terms of ion, antioxidant and nutrient transport (Bjerkås et al., 2006). Aqueous humour osmolality was not measured in **Paper I**, but is closely related to plasma osmolality (Breck et al., 2005a) and may have confirmed this assumption as it quickly increases shortly after seawater transfer (2 h) indicating osmotic stress on the lens (Rhodes et al., 2010). It appears that cataract formation initiated in freshwater continues in seawater (Bjerkås et al., 2001).

Large adult triploid salmon developed more cataracts than diploids at increasing (3-18 °C) water temperature (**Paper III**). It is described that fluctuating water temperature can increase cataract development in diploid Atlantic salmon (Bjerkås et al., 2001). Further, in contrast to **Paper I** fish in **Paper III** were fed the same commercial diet. The majority of studies in the past comparing diploid and triploid performance in Atlantic salmon have usually supplied both ploidies with commercial diets formulated for diploids that often resulted in production disorders (e.g. skeletal deformities, cataracts) in triploids and subsequently in inferior performance compared to diploids (Fjelldal and Hansen, 2010; Fraser et al., 2013; Taylor et al., 2013). In fact, the only studies that formulated diets with regards to mitigating common production disorders in triploids suggest an altered dietary requirement in triploids

(skeletal health: phosphorous, Fjelldal et al., 2016; Smedley et al., 2016; ocular health: cataracts, histidine, Taylor et al., 2015). Taylor et al. (2013) suggested that cataracts, but also skeletal deformities have a major contribution to inferior seawater performance in triploid Atlantic salmon. Therefore the specific reasons for reduced triploid performance have to be elucidated in order to improve future production.

The level of dietary histidine supplementation had a major effect on cataract development within and between ploidies (**Paper I**). Several studies with diploid Atlantic salmon have demonstrated that dietary histidine supplementation above the recommended requirement for growth (8g kg^{-1}, NRC 2011) can mitigate cataract development, especially during risk periods (Bjerkås and Sveier, 2004; Breck et al., 2005a; Waagbø et al., 2010). This is in line with recommendations of 13.4 and 14.4 g histidine kg^{-1} to achieve the lowest severity of cataracts and cataract mitigation, respectively (Remø et al., 2014). The only previous study that combined dietary histidine levels and triploidy suggests that triploid Atlantic salmon have a higher histidine requirement compared to diploids in order to mitigate cataract formation (Taylor et al., 2015). In contrast to the 1+ pre- and post-smolts used in **Paper I**, Taylor et al. (2015) only supplied the histidine enriched diets (12.6 and 17.4 g kg^{-1}) in April prior to the second summer in seawater. Although most risk periods for cataract development had occurred at that point, the authors only detected minor cataracts in both ploidies which may be a result of moderate water temperatures. **Paper I** shows that histidine supplementation is necessary to ensure ocular health also in freshwater at 16 °C, however, this does not appear to be necessary for salmon reared at 10 with similar cataract scores in fish given 10.4 and 13.1 g His kg^{-1}. However, after seawater transfer, histidine supplementation had a beneficial effect also in salmon reared at the lower temperature (10 °C), where cataract development was significantly reduced in fish fed the diet supplemented with more histidine (10.4 vs. 13.1 g kg^{-1}).

Lens NAH levels were higher in diploids than triploids at the experimental start in freshwater and declined in all groups until seawater transfer (**Paper I**). Breck et al. (2005a) also observed decreasing NAH levels in the lens of diploid Atlantic salmon closer to seawater transfer despite a diet with even higher levels of histidine than

what was used in **Paper I** (11.7 and 18.0 g kg^{-1}). As the fish in **Paper I** were only supplied with the histidine enriched diets at the same day the water temperature started to increase it is possible that those fish did not have enough cataract mitigating NAH buffer in the lens (pre-diet: 9.0 g histidine kg^{-1}) or that the temperature increase was too severe. In diploid Atlantic salmon there is a cataract mitigating effect of histidine supplemented diets that are supplied prior to risk periods (Breck and Sveier, 2001; Waagbø et al., 2010).

After two days in seawater lens NAH concentrations remained nearly unchanged (**Paper I**) despite lens NAH synthesis in seawater is reported more efficient than in freshwater (Breck et al., 2005a; Rhodes et al., 2010). After six weeks in seawater lens NAH levels have not reached initial concentrations however increased in groups at 16 °C; stagnated in groups at 10 °C and generally mirrored diet allocation. In contrast, diploid salmon from Breck et al. (2005a) either maintained lens NAH levels from before seawater transfer (11.7 g kg^{-1}) or increased five-fold (18.0 g kg^{-1}) when sampled after seven weeks in seawater at water temperatures similar to our 10 °C groups. The reason for this may be the obviously higher supplementation with histidine compared to **Paper I** (Δ 4.9 g kg^{-1}). Lens NAH is considered an osmolyte and concentrations can be used as a marker for evaluating the risk of cataracts in salmon smolt (Rhodes et al., 2010; Remø et al. 2014), and the results in **Paper I** show that both diploid and triploid salmon with a low NAH status six weeks after seawater transfer and had developed severe opacities after an additional seven weeks.

A general effect of ploidy on lens NAH at samplings in seawater was not evident. However, Taylor et al. (2015) reported lower lens NAH concentrations in adult triploid Atlantic salmon compared to diploids when fed a commercial diet (10.6 g histidine kg^{-1}) until spring. The authors fed histidine supplemented diets thereafter (12.6 and 17.4 g kg^{-1}) and also fish fed the lower histidine enriched diet had increased lens NAH concentrations of about 70 % (>100 % in fish fed the respective other diet). This confirms similar ability to synthesize NAH between ploidies and particularly in seawater where NAH synthesis is more efficiently (Breck et al, 2005a).

6. Conclusions

Triploid Atlantic salmon are more susceptible to cataract development in fresh- and seawater (**Papers I and III**). However, at moderate water temperatures and fed diets with high levels of histidine cataract outbreaks in triploids can be mitigated before and after seawater transfer. A consistent effect of triploidy on higher cataract scores under the same environmental conditions suggests that triploids have a higher requirement for dietary histidine (**Papers I and III**). The causes are unknown but possibly attributable to differences in physiology and metabolism between ploidies.

High water temperatures in the final weeks in freshwater lead to insufficient hypo-osmoregulatory ability in diploids and triploids (**Paper I**). Triploid salmon are more prone to suffer from mortality outbreaks in seawater if smolt production fails to meet the standards required. The smolt-window between diploids and triploid appears to be similar for 1+ yearling smolt however it is considerably influenced by temperature and possibly size. Smoltification at moderate water temperatures is equally successful in triploids as in diploids (**Paper I**).

Triploidy is associated with a lower temperature optimum and tolerance in seawater compared to diploids (**Papers II and III**). At temperatures between 3 and 9 °C triploid post-smolt (**Paper II**) and adult fish (**Paper III**) can grow faster than diploids. The thermal optimum for feed intake was higher in triploid post-smolts compared to adults, but at ≥ 15 °C both age classes feed significantly less than diploids. This indicates a higher sensitivity and greater metabolic limitation to warm temperature in triploidy.

Hypoxic periods were associated with reduced appetite in triploid post-smolts and higher mortality compared to diploids in the context of high water temperatures and

hypoxia (**Paper II**). However, adult triploid salmon performed equally well to diploids during hypoxia at cold and warm temperatures. These results suggest that the oxygen demand in triploid post-smolts could not be adequately compensated by reduced feeding under hypoxia at 18 °C subsequently leading to mortality.

At high water temperatures adult triploid salmon have lower blood haemoglobin and haematocrit compared to diploids (**Paper III**). Lower haemoglobin concentrations directly affect the blood oxygen carrying capacity and could explain reduced performance compared to diploids at the same temperatures.

Adult triploid Atlantic salmon have consistently lower white muscle energy phosphate levels than diploids and lower white muscle glycogen at high water temperatures (**Paper III**). Lower energy storages indicate inferior capacity for prolonged exercise or endurance on burst swimming activity.

7. Future perspectives

The commercial use of farmed sterile triploid Atlantic salmon in Norway has increased in recent years. The most obvious concerns that have been highlighted so far are:

- moderate triploidization success in commercial egg batches
- high early mortalities in some egg batches
- occurrence of lower jaw deformities in some populations
- incidences of high mortality during the early seawater phase

There is a trend of using triploid Atlantic salmon in seawater in Northern Norway where water temperature is lower than southern areas, which is in agreement with the findings in the present **Papers II and III**, where triploids had superior performance at low and moderate temperatures. Also, the finding of massive early seawater mortality in triploids in **Paper I** is highly relevant to the current situation. Generally, high early mortality in commercial aquaculture has been linked to transfer to seawater in late autumn/early winter under harsh climate conditions, but there was also an incidence of sudden massive mortality when fish were transferred to seawater during the summer months. These are all observations from the field, and not from controlled experimental studies, but may still have relevance.

Focused future research and development activity relating to the listed concerns above would help the aquaculture industry in turning towards a more sustainable production, and secure best practice protocols for fish welfare-friendly farming. This would involve optimizing protocols for production of triploid eggs, finding the optimal rearing temperature in freshwater from fertilization to transfer to seawater, finding specific nutritional requirements for phosphorous and histidine, and characterising the smoltification physiology in triploids. Also relevant for triploid Atlantic salmon is the current turn in production towards closed systems during the post-smolt phase to avoid the problematic parasitic sea lice. There are different systems, such as flow through tanks or closed cages, and tanks with recycled water.

In flow through systems, deeper colder water can be used allowing for maximum appetite and growth in triploid post-smolts (**Paper II**). Then transferring triploid post-smolts to open seacages could be beneficial for triploid production since they seem to be more robust (hypoxia in **Paper III**) with increasing size in seawater.

In the scenario of closed production systems during the post-smolt phase, early sexual maturation in males is a realistic problem in conventional diploid salmon farming. **Paper I** and other studies show that triploid farmed males are less likely to mature earlier compared to diploids. With the significant difference between triploids and diploids in male post-smolt maturation in **Paper I** at 10 °C, this is a temperature range suitable for triploid production. Pumped deep seawater would in some areas typically stay stable around 9 °C all year round; here possible interactions between photoperiod and ploidy on early sexual maturation could potentially be used to further optimize production of triploid postsmolts. Although all-female production would completely avoid male sexual maturation, fish farmers tend to use the benefit of superior male growth during late production.

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