Norwegian College of Fishery Science
Faculty of Biosciences, Fisheries and Economics

# Skeletal anomalies and shape variation in diploid and triploid Atlantic salmon (*Salmo salar* L.) fed different diets

**Kevin Salbuvik**

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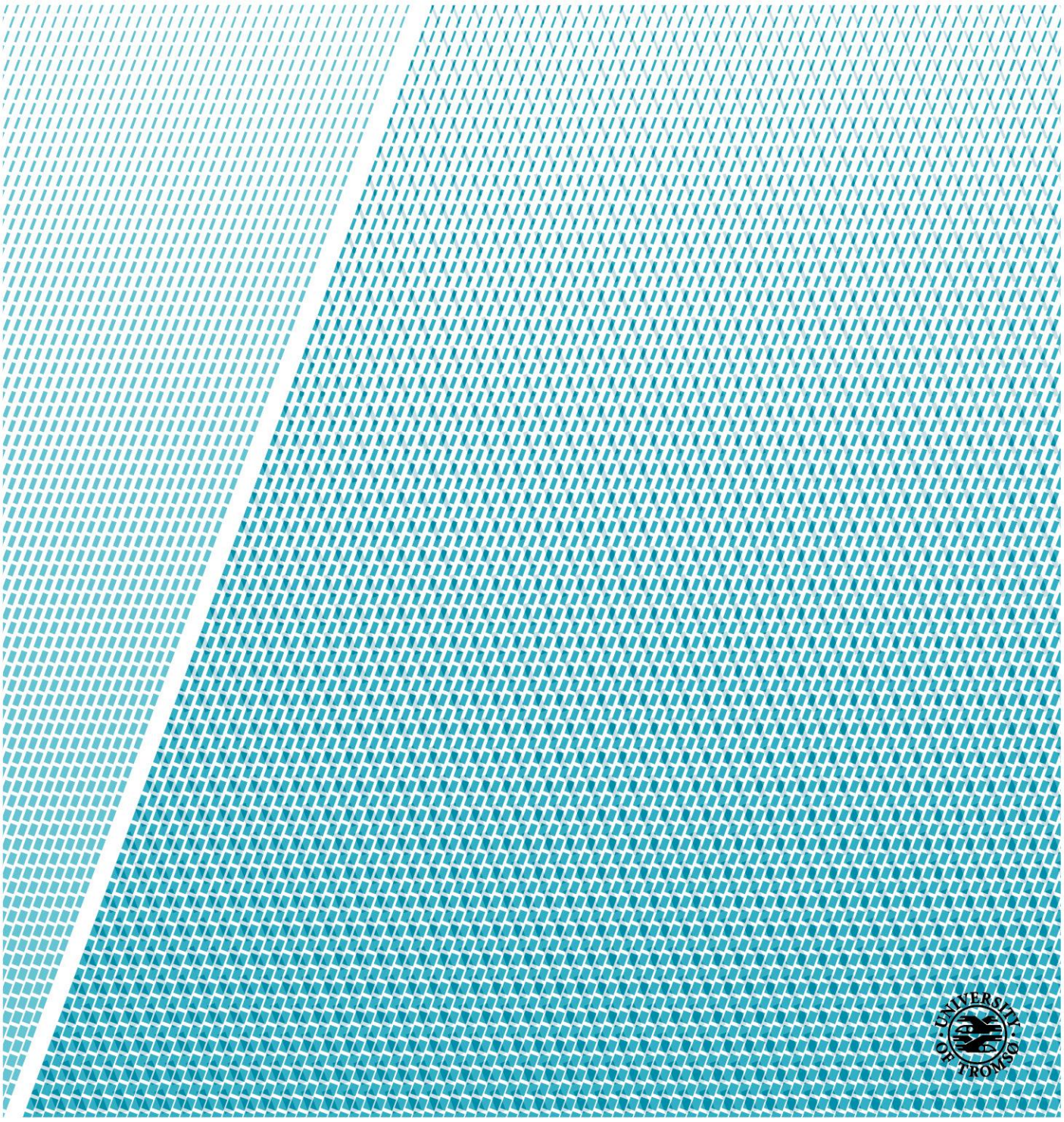

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# Abstract

Usage of triploid Atlantic salmon (*Salmo salar* L.) have been of interest for salmon farmers to mitigate interbreeding between farmed and wild population, and to prevent early sexual maturation. Triploids tend to be more prone to develop skeletal deformities than diploids. This may result from inadequate rearing temperatures and/or diet formulation (i.e. low dietary phosphorus). The main objective of this study was to examine for differences in the occurrence of skeletal anomalies between diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a commercial high-protein phosphorus-rich fishmeal-based diet (56-60% protein; ca. 18g phosphorus $kg^{-1}$; STD) and an experimental diet where 45% of fishmeal was replaced with hydrolyzed proteins (EXP). Shape analysis was applied to see if any group differed morphological relative to the control group (undeformed 2n STD).

Diploids and triploids were divided into twelve tanks (initially 3000 fish per tank; tank biomass ca. 620 g) and were fed either STD or EXP diet (3x2n STD, 3x2n EXP, 3x3n STD and 3x3n EXP) from start-feeding until parr-smolt transformation. At the end of the feeding experiment, the fish were euthanized with an overdosed of anaesthetic (Benzocaine, 120 mg $L^{-1}$) and then stored frozen (-20°C). A total of 594 (2n STD, 123; 2n EXP, 171; 3n STD, 138; 3n EXP, 162) post-smolt were thawed and stretched before being measured, visual inspected and x-rayed. Five fish were placed on a digital plate at a time for each picture. ImageJ was used to analyze x-ray pictures for skeletal anomalies and to plot xy-coordinates for shape analysis. Shape analysis was carried out in the statistical software R with the package "geomorph".

Both diploids and triploids on the EXP diet had a significant higher incident of spinal deformities (diploid, 11.77 ± 4.22; triploid, 24.58 ± 0.99) compared to the groups on STD diet (diploid, 5.83 ± 1.01; triploid, 22.12 ± 2.47). Triploids had slightly but significantly fewer vertebrae (STD diet, 57.82 ± 0.45; EXP diet 57.78 ± 0.42) than diploids (STD diet, 58.03 ± 0.43; EXP diet, 58.00 ± 0.48). The cranial and caudal trunk (R1 and R2) was the most affected area with vertebral deformities amongst all groups. Five main axes of morphometry explained more than 5% of the shape variation, and these accounted for 73.3% of the total variability among groups. Shape analysis did not reveal any major shape differences between diploids and triploids in both diets, except for a slight bend in the cranial trunk and elongation of the caudal trunk region in triploids, that could be caused by a ploidy effect or underlying deformities in that region. Overall, the incidents of skeletal deformities were reduced compared to previous studies done on triploid Atlantic salmon (Diploids 20+%, Triploids 40+%), and could be a result

of a combination of low rearing temperature and phosphorus-rich diets. The EXP diet with hydrolyzed protein will potentially increase the incidents of skeletal deformities compared to phosphorus-rich standard diet.

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# 1 Introduction

Pre-harvest sexual maturation and farmed escapees represent threats to the economic and environmental sustainability of the world salmon farming industry. As the industry grows the risk of interaction between farmed and wild fish becomes increasingly significant. Farmed escapees have long been a problem for the Atlantic salmon (*Salmo salar* L.) production, and accidental escapes have a significant impact on ecological and genetic interaction between farmed and wild population (Glover *et al.* 2017). Another problem in many cultured finfish species is the onset of early sexual maturation that results in energy allocation from somatic growth to reproductive development that causes reduced flesh quality and losses for farmers (Taranger *et al.* 2010). Use of sterile fish has been suggested as a solution for reproductive control and genetic containment of farmed stocks to meet both industrial and environmental criteria. Triploidy is seen as the only current feasible method to produce sterile fish for commercial production (Benfey 2016), and sterile triploid fish have been used in Tasmanian salmon aquaculture for the past 25 years (Sadler *et al.* 2001). Their performance is currently being investigated and evaluated under large-scale commercial production in Norway and elsewhere (Hansen & Fjelldal 2015).

Triploids have been studied since the early 1940s (Maxime 2008), but despite this their general culture and nutritional requirements are still poorly understood. If triploids are to be used by the industry, a thorough understanding of the functional consequences (physiological and morphological) need to be established. In general, triploid Atlantic salmon have shown reduced growth in the seawater phase and higher incidence of skeletal anomalies than diploids. Severe skeletal deformities reduce harvest weight, results in down-grading losses, and constitute an ethical dilemma with regards to fish welfare (Fjelldal *et al.* 2016). Triploid Atlantic salmon have been shown to have a higher dietary phosphorus requirement than sibling diploids, but triploids' nutritional requirement and digestion capacity are still poorly understood as well as their effects on body deformities.

This thesis aims to compare the incidence of skeletal anomalies (opercular, jaw and vertebral) in diploid and triploid Atlantic salmon smolts fed a commercial high-protein phosphorus-rich fishmeal-based diet and an experimental diet with high proportions of hydrolyzed proteins while reared at low-temperatures. By studying external visual deformities combined with x-ray observation of cranial and vertebral deformities, the goal is to assess the incidence of skeletal anomalies across groups and to investigate if the combination of

conditions employed in the present study may lead to a reduction of skeletal anomalies in triploid salmon. There were also employed advanced geometric morphometric tools to see if there were any shape differences amongst ploidy and diet groups, as well as the effect from skeletal deformities.

# 2 Atlantic Salmon

The Atlantic salmon is an anadromous finfish species belonging to the Salmonidae family and it is naturally distributed in the Atlantic Ocean, lakes and connected rivers systems. It spawns in the autumn after a growth period of 1-4 years in the sea, and the adults returns to the river to spawn (Figure 1.1). After hatching, the juveniles spend between 1-5 years in the river before migrating to the sea. They start migrating when they reach a specific size and receive cues from the environment (stimulated by photoperiod, temperature and other factors) when to start the smoltification process. Before reaching the sea, they go through physiological changes, particularly the conversion from hyperosmotic to hypoosmotic ion regulation and increased seawater tolerance (Hoar 1976, Folmar & Dickhoff 1980). The smoltification process is an important process for the survival of anadromous species in general, but it is not only physiological changes that occur, but also its appearance changes of various morphological characteristics. When the smoltification process begin the parr (juvenile salmon) transforms into a longer and slimmer body shape (change in condition factor) to increase it hydrodynamics in the sea (Figure 1.1). It will also lose its parr marks (fingerprint like marks on the sides) and change its color from brown (river bottom camouflage) and become silver coated on the sides. From a dorsal perspective the salmon will reflect the color of its surroundings like the sea's blueish green color, but from a ventral perspective it will have a white abdomen to match the surface reflection (Luna & Kesner-Reyes 2018).

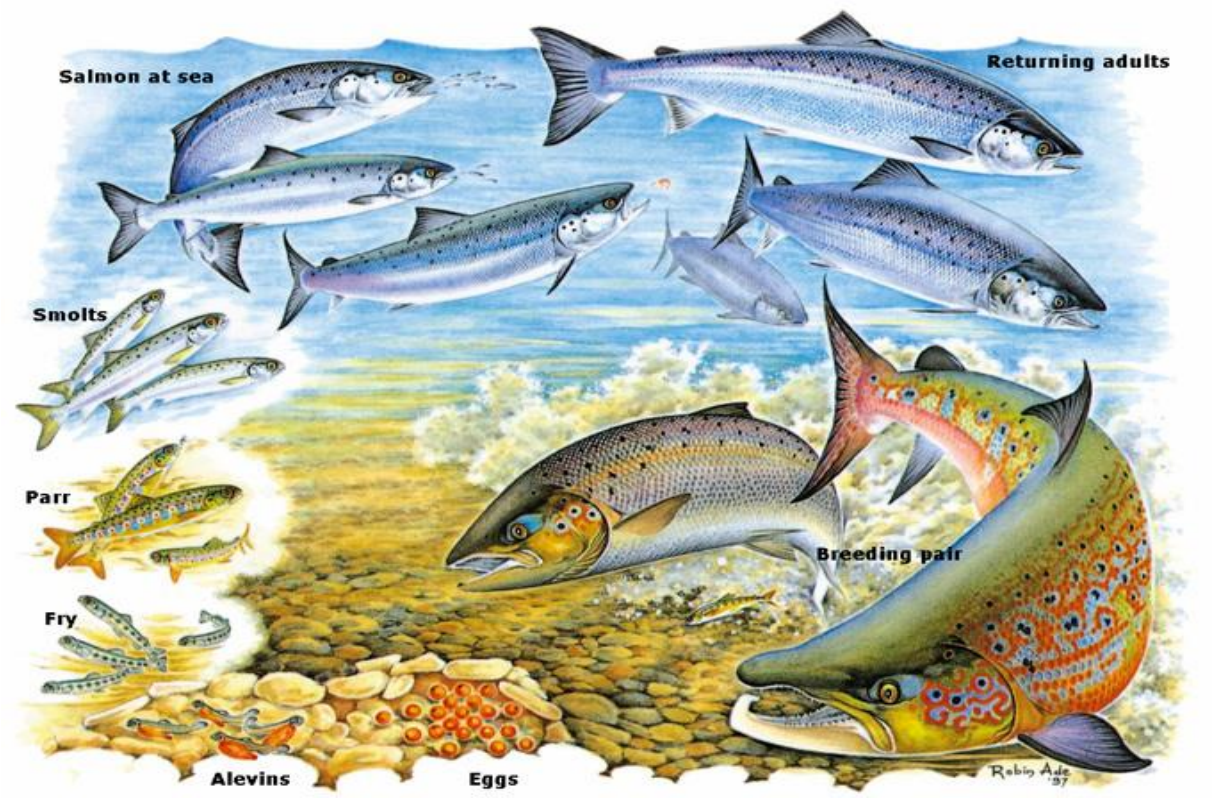


Figure 1.1. Life cycle of Atlantic salmon (*Salmo salar* L.) from egg stage to sexual maturation. Adapted from (NASCO 2018).

## 2.1 Status of Atlantic Salmon farming

The Norwegian aquaculture industry has rapidly expanded since the early attempts at commercial production in the early 1970s, and worldwide aquaculture production has followed the same trend. Global aquaculture production is expected to reach 109 million tons in 2030 from 80 million tons in 2016, corresponding to an 36.7% increased production over the course of 15 years (FAO 2018b). The growing popularity of seafood is putting more pressure on production areas on land and at sea to satisfy the growing demand of a growing world population.

Several cold-water finfish species have been the object of cultivation attempts in the past two-three decades (e.g. rainbow trout (*Oncorhynchus mykiss* Walbaum), Arctic charr (*Salvelinus alpinus* L.), Atlantic cod (*Gadus morhua* L.), Atlantic halibut (*Hippoglossus hippoglossus* L.), spotted wolffish (*Anarchichas minor* Olafsen)), but none has been as successful as the Atlantic salmon, especially in Norway. The industry came about due to declining captures of wild Atlantic salmon and its farming success has ensured that the demand is met the entire year without endangering the wild populations by overfishing (Benfey 2016). Atlantic salmon farming dominates the aquaculture production in the North Atlantic. With a

worldwide production of 2.25 million tons of Atlantic salmon in 2016, Europe stood for 1.5 million tons with Norway as the leading production country with 1.23 million tons, followed by United Kingdom and Faroe Islands with an production of 163 000 tons and 83 000 tons respectively (FAO 2018a). The Atlantic salmon are also produced outside its native habitat, and in South America where Chile has adopted the species for its own production cycles has become the second largest Atlantic salmon producer in the world with a production of 532 000 tons. Atlantic salmon are also produced in Asia and Oceania, but at relatively small quantities compared with Europe and America. With the increased production, more areas had to be utilized to maintain growth and to supply the demand, and with more fish at sea, problems started to arise. In the 1980s, problems like bacterial (i.e. furunculosis (*Aeromonas salmonicida*) and cold-water vibriosis (*Aliivibrio salmonicida*)) and viral (i.e. infectious salmon anemia or ISA) diseases became larger issues in many countries, as well as more farmed fish were observed in rivers and lakes. The largest problems Norwegian Atlantic salmon industry are facing today are the salmon lice (*Lepeophtheirus salmonis* Krøyer), pancreas disease (PD) epidemic and accidental farm escapes (Hjeltnes *et al.* 2018). The latter have probably been an issue since the first successful commercial production was carried out and genetic selection started, meaning that wild salmon populations have been exposed for introgression (introduction of genes from one species genepool to another or domesticated genes over to wild populations genepool) for almost 50 years (Glover *et al.* 2017). Although continued improvement in system design and operational management as the industry has matured have reduced the risk of farmed escapees, there are still significant escapes that have great consequences for the wild population (Benfey 2016, Glover *et al.* 2017). Production of Atlantic salmon today is cost effective, with a short production cycles and the species are one of the most researched finfish species in the world (Mather *et al.* 1998, Glover *et al.* 2017).

## 2.2 Farmed salmon escapees

In the last decade there has been a lot of discussion around the environmental consequences from salmon production, especially from the seawater stage of the production cycle. As long as the farming practice are placed in open waterflow systems (sea cages) in the sea, the risk of interaction between wild and farmed finfish species are always going to be an issue. In Norway where the Atlantic salmon is a native species, the risk of genetically polluting the native populations are high, but in other countries where the Atlantic salmon are an exotic species (e.g. Chile, Tasmania etc.), the genetic interaction alone is not an issue. But if farmed

salmon escapes, they can cause other negative effects in several ways to the natural habitats; escapees can transmit cross-species pathogens to native populations, compete for the food sources and disrupt spawning habitats along the coasts. The effects from escapees can be observed all over the world where salmon is produced (e.g. Faroe Islands, Chile, Scotland etc.) (Glover *et al.* 2017). Because of unnatural high density of fish placed in sea cages, the risk of epidemics from different pathogens are also higher than in natural habitats. Just one sea cage of salmon in Norway has approximately 200 000 individuals and that makes it almost half of the total population of the wild Atlantic salmon along the Norwegian coast (Forseth *et al.* 2017). As a result of high density, the pathogens are far more likely to cause epidemics, and farmed salmon are one of the largest reservoirs of known pathogens. In worst cases, the salmon can act as a vector for transmission of diseases into new geographical areas (Johansen *et al.* 2011). There have been studies around the transmission of ISAV (infections salmon anemia virus) from herring (*Clupea harengus* L.) between salmon farms. Herring may be an natural reservoir for the virus and can be transmitted between species (Nylund *et al.* 2002). This risk of epidemics and transmission within and between species must be taken into consideration before starting any farming practice of any species in any area.

### 2.2.1 Genetic consequences of escapees

The impact of farmed salmon escaping and mixing with wild populations are considered a threat to the natural populations (Fleming *et al.* 2000). In 2017, about 530 000 wild Atlantic salmon were estimated to return to Norway for spawning, which was an increase from the year before, but under half of the returns observed 30 years ago (Forseth *et al.* 2017). There are concerns about the negative impacts farmed escapees have had on the wild population over the last decade, especially the genetic interaction between them (Benfey 2016). With the tremendous amount of salmon in sea cages in relations to the wild populations along the coast, a large escape event could be potentially catastrophic for the wild population when considering the genetic consequences.

In Norway, domestication of the Atlantic salmon was initiated by Mowi A/S and Grøntvedt Brothers in 1969. After the first harvest was cleared as a great economic success in 1971, the interest around the farming potential of the Atlantic salmon spread quickly and the Norwegian breeding program was established (Gjedrem *et al.* 1991). Today, there are mainly four strains used to supply most of the commercial used ova, and the strains are different in terms of genetic, phenotypic, behavior and reproductive capability than their predecessors from

the wild population (Einum & Fleming 1997, Fleming *et al.* 1997, Glover *et al.* 2009). Over 10 generations of salmon breeding for economical important traits (i.e. growth, flesh quality, late sexual maturation) has changed its genetical architecture and have made it unfit to survive in the wild throughout a whole life-cycle, due to lose of fitness and behavior related traits (Solberg *et al.* 2013).

Farmed and wild salmon have different phenotypical and genetic expression, but wild salmon are structured into populations and meta-population also (Jensen *et al.* 2010), and the evidences for local adaption in salmonids are clear and well investigated (Taylor 1991, Garcia de Leaniz *et al.* 2007, Fraser *et al.* 2011). Their ability to return to the same river they originally came from (also referred to as "homing" ability) isolates them from reproductive diversity unfavorable for their local habitats, but also makes them more sensitives to interbreeding with escapees. Interbreeding between farmed and wild salmon can cause populations to loss their local adaption, and possible reduce survival of the hybrid offspring between them (Taylor 1991). Farmed offspring has been shown to have lower survival and fitness in the wild, which can lead to extermination of vulnerable population (McGinnity *et al.* 2003).

There are several studies on the interaction between farmed and wild salmon, and that interbreeding have occurred over a long period of time (Gausen & Moen 1991, Carr *et al.* 1997, Skaala *et al.* 2012, Fjelldal *et al.* 2014, Karlsson *et al.* 2016, Glover *et al.* 2017). Escaped smolt or post-smolts tend to return to the fjord system they originally escaped from at a later stage making the flow of escapees to river system a constant problem (Heggberget *et al.* 1993, Karlsson *et al.* 2016), and it has been documented in an experimental setting that escapes at an earlier life stage can increase the survival of the escapees (Fleming *et al.* 1996, Fleming *et al.* 1997). Adults escapees can travel 10s if not 100s kilometers from the farms they escaped making them unpredictable to which river system they could turn up in under the spawning season, meaning any river are potentially accessible (Jensen *et al.* 2010). Especially exposed rivers are those that are inside fjords or just outside of fjords with farms. In Scotland, Youngson *et al.* (1997) studied the distribution of escaped salmon in eastern and western Scotland. They showed that increased activity of salmon farming on the west coast has led to an increased frequency of escaped salmon in the river system relative to the east coast where there is little farming and the river systems were not likely effected by introgression. The major problem with escapees is genetic introgression from farmed to wild salmon. With a constant supply of artificial selected genes put into the gene pool, the concern of wild population to lose their adapted edge in their environment are high. The concern is based on the lower survival of

progenies from farmed and especially hybrid salmons relative to pure wild salmon (Skaala *et al.* 2012). Hybrids can interact with the wild descendants and continually expose wild population with farmed genes over many generations. Norway has over 200 rivers and Karlsson *et al.* (2016) reported in their study that out of 147 different wild salmon populations, all were affected in some degree of introgression from farmed salmon. The amount of introgression was significantly associated with the average proportion of escaped salmon in the river over the last 25 years.

Number of salmon escapes have declined the last decade (Figure 2.1) due to improved procedures, system designs and operational management, but with the increased production there are still significant escapes from sea farms (Benfey 2016). It should also be considered that the amount of escapes is higher than reported, although Norway has the most comprehensive national record of escaped farm fish (Jensen *et al.* 2010). Studies done by the Institute of Marine Research during 2005-2011 has shown that the numbers of escapes potentially are 2-4 times greater than reported (Forseth *et al.* 2017). Jensen *et al.* (2010) investigated the different reason for escapes in Norway and showed that 68% of salmon escapes at sea was caused by structural failures during 2006-2009, followed by operational failures and external failures with 8% and 8% respectively. Structural failures are mostly caused by storms and in their data most of the large escapes event happened in the autumn when Norway has most of its bad weather.

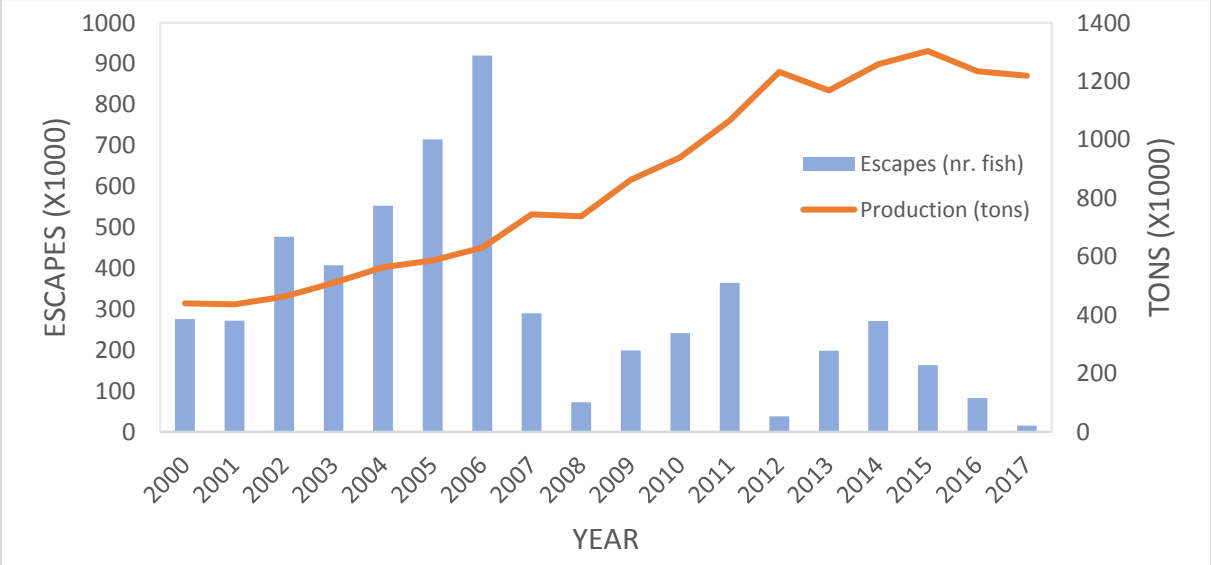


Figure 2.1. Total production in tons (x1000) and escapes (x1000) of Atlantic salmon (*Salmo salar* L.) in Norway between 2000 to 2017. Production data adapted from Fiskeridirektoratet (2018a), and fish escape data adapted from Fiskeridirektoratet (2018b).

With the steady growth of the industry, raising popularity of seafood and decreasing area availability for fish farming, the likelihood of farms being placed in more exposed waters are high (i.e. offshore ocean farms), even following thorough investigations of location and environmental factors (e.g. streams, waves, wind, icing). Reducing the impact of escapees on the wild population relies primary on effective physical barriers, but this can never be fully effective (Benfey 2016). In the event of accidental escapes, genetic containment of farmed fish can be achieved by the use of gametically sterile fish, notably triploids. Other fish sterilization methods do exist or are under investigation, but the induction of triploidy represent by far the most common and reliable method for production of sterile fish under commercial settings (Benfey 2016).

# 3 Triploidy in fish

The simplest term for a triploid is an organism that has an additional set of chromosomes to the two sets normally found in the somatic cells of higher organisms and as a consequence the individual is sterile or unable to reproduce. Inducing polyploidy (having more than two homologous sets of chromosomes in the genome) have been used in agriculture for many generations to produce plants with disease resistance, superior growth, seedless fruits and more. But inducing polyploidy in higher vertebrates like terrestrials has proven to be difficult to nearly impossible, thus the induction of polyploidy has been restricted to use on lower vertebrates (i.e. fish, amphibians) and plants (Piferrer *et al.* 2009). Most vertebrates have two sets of chromosomes in the genome inherited from each parent (referred to as diploids), but triploids have a condition which there are one extra set of homologous chromosomes, usually from maternal origin (Piferrer *et al.* 2009). Because of the extra set of chromosomes, the nucleus requires more space that often results in larger, but fewer cells (Small & Benfey 1987). The number of cells decrease proportional to the increased cell size to maintain normal organ and body size (homeostasis), as well maintaining normal hematocrit value (Benfey 1999, Tiwary *et al.* 2004).

Manipulation of ploidy in fish has been carried out since the early 1940s, and in 1959, Swarup successfully managed to rear triploid threespine stickleback (*Gasterosteus aculeatus* L.) to adulthood, and could compare growth and sexual maturation differences in relation to diploids (Maxime 2008). Triploids differ from their diploid counterpart in three fundamental ways; their cells are larger, they are generally more heterozygous (more crossover of alleles at any given locus), and gonadal development are disrupted to some extent or completely (Benfey 1999). The latter is of great interest for the aquaculture industry because of mainly two issues; genetic containment of farmed fish escapees that may attempt to reproduce with the wild population, and possible improvement of culture performance as sexual maturation before market size may lead to decreased body growth and flesh quality.

Energy allocation from somatic growth to reproduction is a well-known issue that causes loss for farmers in terms of reduced growth, higher incidence of disease and change in organoleptic properties. The reason for those changes are that maturing fish are chronically stressed (Mazeaud *et al.* 1977, Piferrer *et al.* 2009). Grilsing (salmon that sexual mature after one winter at sea) are one of the major issues in salmonids, and McClure *et al.* (2007) did a study on which factors affected early sexual maturation on Atlantic salmon and how they could

predict the risk of grilsing in a specific cage or farming site, to strategically harvest before the onset of maturation. In short, their study concluded that higher average weight in the second August in seawater and fluctuation of sea temperature between the first February to second September in seawater, caused a higher risk of grilsing. They also concluded that a change in feeding intensity and feed type could reduce grilsing, as well as earlier studies have shown that manipulation of photoperiod influence grilsing (Hansen *et al.* 1992, Oppedal *et al.* 1997, Taranger *et al.* 1998, Porter *et al.* 1999). But predicting maturation on a commercial scale with all environmental variables and different management practice would take years to develop a feasible method and grilsing would always be a factor in the production cycle, and farmers still would be in need to harvest before the stock reaches marketable size. Research around sterile salmonids was initiated as a response to the grilsing problem and how the grilsing caused consequent losses to the industry each year. Later on, it became an alternative solution to reduce interbreeding between wild and farmed populations in the event of accidental escapes (Cotter *et al.* 2002).

Sterility observed in triploids is due to the extra set of chromosomes in the genome interfering with meiotic division. The interference is a direct consequence of the odd number of chromosomes or unpaired chromosomes leading to a failure of normal pairing and crossing-over between homologous chromosomes during meiosis I (Benfey 2011), resulting in reduced gonadal development and/or aneuploid gametes, meaning they are unable to produce viable offspring (Benfey 1999, Tiwary *et al.* 2004). However, there are differences between triploid male and female gonadal development, as well as level of secondary sexual characteristics (Benfey 2011).

Triploid male testis grows to nearly the same size as diploids testis, but females on the other hand have greatly suppressed ovarian development (Figure 3.1A). This is due to how normal diploids produce germ cells. When females undergo vitellogenesis, the oocytes are held at the first meiotic metaphase before they undergo rapid growth, whereas males produce post-meiotic spermatozoa that are a little bit smaller than pre-meiotic spermatogonia. In a triploid state, most of the germ cells do not proceed through the first meiotic prophase in either sexes, resulting in small ovaries with small numbers of previtellogenic oogonia and primary oocytes, whereas males testis have a large number of spermatogonia and primary spermatocytes, resulting in testis almost as large as diploid testis (Benfey 1999). In short, female's ovarian growth is almost completely suppressed and will not be able to produce viable eggs, whereas male develop testis as normal, but milt is heavily diluted with aneuploid spermatozoa, resulting

in aneuploid embryos that will not survive beyond hatching (Figure 3.1C). Males also develop secondary sexual characteristics whereas females do not (Figure 3.1B/D). That is because male triploids steroidogenic cells are present and active, resulting in normal endocrine changes like diploids, while female diploids have specialized cells that synthesis and secrete steroids from enclosed follicles that develops from primary oocytes. Triploid female's oocytes do not go through meiosis, thus do not reach that stage of gonadal development where the eggs are covered in hormone producing cells and will stay in a juvenile endocrine stage (immature or pre-pubertal stage) throughout their lives (Benfey 1999, Cotter *et al.* 2002, Benfey 2011).

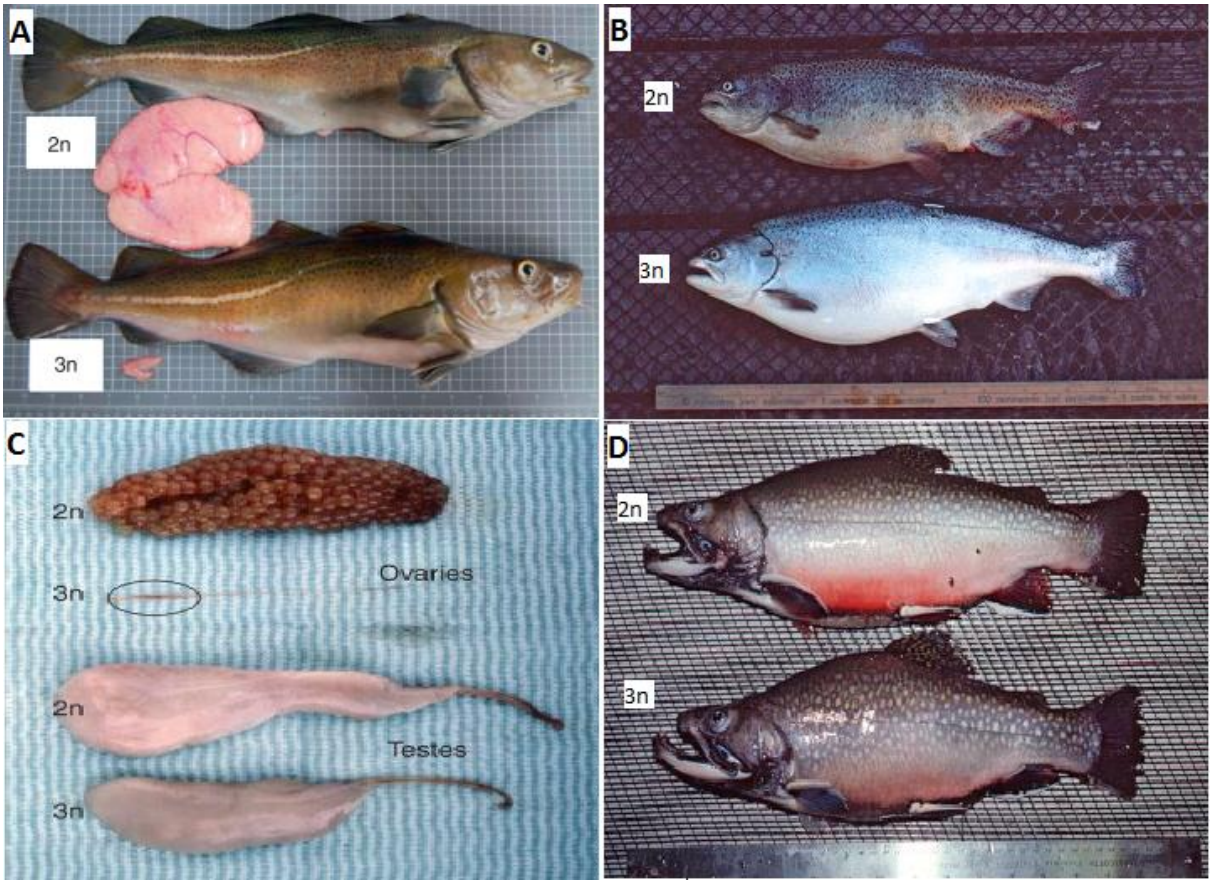


Figure 3.1. Different reproductive development in diploids (2n) and triploids (3n) sibling of different species; A, ovarian development in Atlantic cod (*Gadus morhua* L.) siblings in diploid and triploid status; B, differences in secondary sexual characteristics in female chinook salmon (*Oncorhynchus tshawytscha* Walbaum) siblings where triploid female are held back in a juvenile stage/appearance whereas diploid have started maturing; C, differences in gonadal development in pink salmon (*Oncorhynchus gorbuscha* Walbaum) siblings; D, differences in secondary sexual maturation in male brook charr (*Salvelinus fontinalis* Mitchill) between diploid and triploid siblings. Adapted from (Benfey 2011).

## 3.1 Artificial production and determination of triploidy in fish

One way to induce polyploidy in fish are physically suppressing the meiotic spindle within the egg, thus retaining the second polar body. Most fish species have an oviparous reproductive strategy, meaning they lay eggs without an internal fertilization process, thus the egg is arrested at the prophase of meiosis II. Entry of fertilizing spermatozoon (male haploid cell carrying a paternal chromosome set) results in completion of meiosis II with extrusion of the second polar body (Figure 3.2). The chromosomes then duplicate and get ready for the first cell division (first cleavage) (Benfey 2011). To induce triploidy, a shock is used between the entry of the spermatozoon and extrusion of the second polar body is applied to block the completion of meiosis II. This will give the organism two sets of homologous maternal chromosomes (one destined to become the pronucleus and other to become the second polar body that will be retained) and one set of paternal chromosomes (also referred to as maternal triploids) (Piferrer *et al.* 2009).

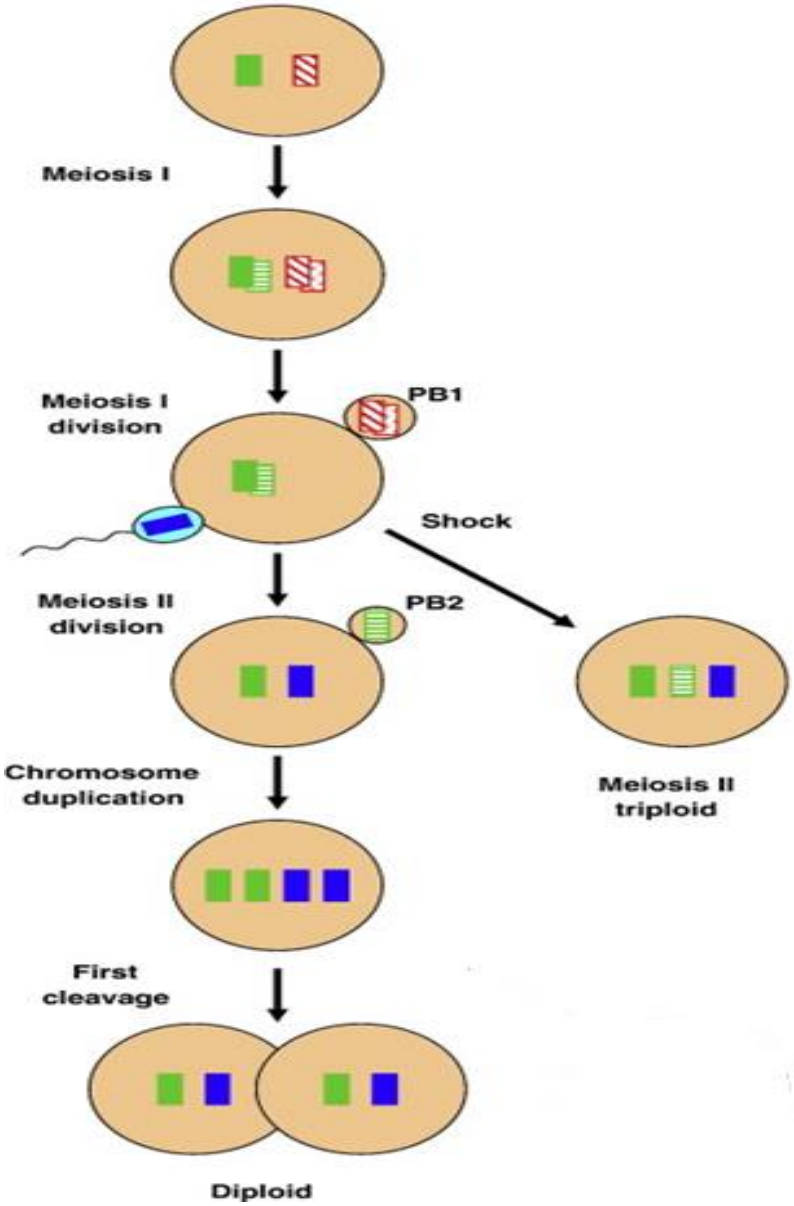


Figure 3.2. Manipulation to achieve triploidy in fish. Inducing triploidy with help of physical shock treatment by suppressing completion of meiosis II after fertilization. Adapted from Piferrer *et al.* (2009).

There are a few methods to induce polyploidy in fish, either as physical or chemical shock treatments, but the most common methods for salmonids are in form of physical shocks. This is because they are easy to apply and control, reliable, and have better consumer acceptation than chemical treatments (Benfey 2001). Physical shock treatments used are either in form of pressure shock or thermal shock, and the latter can be divided into cold shock or heat shock (Piferrer *et al.* 2009). Thermal shocks are applied by lowering eggs into a container of cooled or heated water for a certain period. Thermal shocks have been proven harder to control than pressure shocks because of its difficulty to ensure all eggs in a batch are heated/cooled at

the same rate and to the same temperature, making thermal shocks less reliable. Pressure shocks are applied by using a device that abruptly increases the hydrostatic pressure to a container with fertilized eggs. Pressure shocks have been proven to be less harmful for eggs, easier to design standardized protocols and more efficient in form of success rate and therefore more suited to use for commercial production of triploids (Maxime 2008, Benfey 2009). But there is some limitation to the physical shock treatments. Since they are applied directly to the eggs there is always going to be a possibility for a lower success rate in the induction process based on inexperience and/or inadequate control over the variables (timing, intensity and duration of the treatment). Protocols must be species-specific to achieve high success rate, and treated batches needs to be quality ensured for ploidy (Benfey 2009, Piferrer *et al.* 2009). Devlin *et al.* (2010) demonstrated in their study that suboptimal pressure treatment gave lower success rate (97.6%), and even lower when optimal hydrostatic pressure was used, but eggs were over-ripped (97.0%). Even with an optimal protocol, it cannot be guaranteed that the treatment will provide 100% all-triploid groups, but it does appear that the induction achieved >98% triploids on a commercial scale production.

Methods used to quality ensure ploidy level are classified into direct and indirect methods. The indirect methods measures the size of cells or nucleuses and are a popular low-cost method (Benfey 1999). As stated earlier, the triploid cells are larger than diploid cells due to 50% more DNA content in the nucleus, thus they are easy to separate from each other under a microscope with a blood smear, but a less time-consuming and precise direct method to determine ploidy level are required (Piferrer *et al.* 2009). Some of the direct methods are genotyping with DNA markers (i.e. microsatellites), measuring DNA content and karyotyping (chromosome counting). Karyotyping are recognized as the most precise and are widely used, but may be one of the most time consuming of the direct methods and not suitable to use for commercial determination of ploidy (Tiwary *et al.* 2004). DNA content by flow cytometry are a rapid and accurate method to determine ploidy. Since flowcytometry can analyze several hundred individuals per day, it is more suited for a commercial producer in relations to karyotyping, but as with karyotyping it requires some training and experience to perform (Linhart *et al.* 2001). Flow cytometry estimates the DNA content of thousands of nuclei that are stained with a DNA specific fluorescent dye, thus can calculate which ploidy the cells derived from by analyzing the light spectrum that surrounds the nucleus. It should also be noticed that flow cytometry can be performed at embryo stage, making early screening of ploidy a possibility.

### 3.1.1 Production of all-female triploids

Triploid males follow the same sexual maturation stages as their diploid counterparts, and examination of gonadal development in many species have proven the importance of using all-female triploids for commercial production (Benfey 1999, Maxime 2008, Piferrer *et al.* 2009, Fraser *et al.* 2012a). Triploid males cannot produce viable progenies since their spermatozoa are aneuploid, but they can show mating behavior. Studies on triploid male Atlantic cod and Atlantic salmon has been carried out to document their behavior with ovulating females, and they found that males showed the full range of sexual behavior equally to diploids (Feindel *et al.* 2010, Fjelldal *et al.* 2014). The testis in male triploids follow the same development pattern as their diploid counterparts, meaning they develop to full functional endocrine organs and produce sex steroids, thus they develop the same phenotypic characteristics at the same age/size as diploids (Benfey 2016). To summarize, triploid males that escapes will still sexually mature, eventually reach spawning grounds, be able to fertilize eggs from wild females and compete for food sources. It also contradicts the purpose of using triploids for producers, especially for salmon farmers due to reduced flesh quality.

Production of all-female salmon populations are a fairly simple process, since they have a female homogametic sex determination similar to the one found in mammals (XX-female/XY-male system). To induce gynogenesis (no contribution of paternal genome in embryonic development) in salmon the paternal genome has to be modified or destroyed without affecting the motility of the spermatozoon and its ability to penetrate and activate the egg. This is usually done by using UV-radiation on the spermatozoa and this inhibits the paternal genome, and when eggs are being fertilized it will resume the meiosis as normal yielding all-female salmon (Benfey 2009). To make all-female triploid salmon requires a few extra steps. The all-female salmons are given androgens or aromatase inhibitors to change from female to functional males (referred to as neomales). Neomales yield all-female progenies when paired with normal females (Benfey 2009), and to induce triploidy, physical shocks are used as described earlier. There is limited literature on the production of neomales in Atlantic salmon (Johnstone & MacLachlan 1994, Lee *et al.* 2004), but it effectiveness has been confirmed (Lee *et al.* 2004), and are currently being used in the Tasmanian aquaculture industry in Australia to produce all-female triploid Atlantic salmon.

As mentioned, triploid males display full range of secondary sexual characteristics, but females generally do not reach ovulation, because of little to none develop ovarian follicles that can produce sex steroids. Even the oocytes that do complete vitellogenesis will not be released

due to not receiving the last endocrine signal for final maturation and in the end, ovulation. But there has been reports on female triploids releasing matured and ovulated oocytes. Johnstone *et al.* (1991) estimated that 0.1% of female triploid Atlantic salmon in a population released ovulated eggs, but the eggs that was released varied in size and produced aneuploid embryos when fertilized from diploid males (Benfey 2016).

If all-female triploids are to be used to minimize the impact escapees has on the wild population, there are a chance that one in a thousand could spawn with a wild male, and potentially removing them from the gene pool. But the likelihood of such events could impact the native population structure may be insignificant (Benfey 2016). If a mixed-sex population of triploids are to be used, then there is a theoretical chance that triploid males could spawn with triploid females that do mature and release gametes. Such event could lead to triploid progenies due to both gametes are aneuploid, but this is highly hypothetical since female triploids do not show either courting behavior or have a "homing" ability making them far less likely to reach a suitable spawning ground (Benfey 2016). However, to effective use triploids for commercial production, there should be all-female production, therefore triploid male escapees should not be an issue in the first place.

## 3.2 Morphological and physiological differences in triploid Atlantic salmon relative to diploids

Triploids have many potential useful applications in aquaculture, both related to genetic containment and performance improvement of farmed fish. The expected growth potential in triploids is higher due to sterility and reduced gonadal development in relation to diploids (Tiwary *et al.* 2004, Piferrer *et al.* 2009). However, from a producer's point of view, the potential gain from triploidization in relation to their diploid counterpart must outweigh the potential negative effects to even be considered an alternative. How triploids perform are highly species-specific and research on environmental condition and nutritional requirements are needed to make triploids commercially available, and a great deal of research have been carried out on triploid Atlantic salmon to uncover any unique culture requirement (Benfey 2016), but thus far triploids well-documented at a laboratory scale and far less on a commercial scale (Piferrer *et al.* 2009). The advantages with triploid salmons are known by producers, but they have been reluctant to adopt the practice. Only in the resent years have some companies started experimenting on commercial production of triploids in Norway, while Tasmania have had all-

female triploid salmon production for over 25 years (Amoroso *et al.* 2016b). The producers fear may have basis in reduced consumer acceptance (i.e. misconception from consumers that triploids are genetically modified organisms (GMO)) (Piferrer *et al.* 2009), reports of higher mortality before the onset of first feeding (O'Flynn *et al.* 1997, Cotter *et al.* 2002), and higher prevalence of skeletal deformities (Benfey 2001, Sadler *et al.* 2001).

The assumption that triploid fish would grow larger than diploids as a result of larger cells stems from gigantism that are often seen in polyploid plants or molluscs (Piferrer *et al.* 2009). This is not the case for fish or other polyploid vertebrates. Literature regarding growth in triploid Atlantic salmon are currently inconclusive as earlier studies have shown contradictory results in both freshwater and seawater phase. Galbreath *et al.* (1994) and Fjelldal & Hansen (2010) had better growth in their studies, McGeachy *et al.* (1995) and O'Flynn *et al.* (1997) found no difference in growth rate between groups, while Cotter *et al.* (2002) had better growth in diploids in freshwater phase. For the seawater phase, O'Flynn *et al.* (1997) reported that when triploids and diploids were reared separately, triploids grew significantly better than diploids, but together had no significant difference. The explanation could be that diploids are more aggressive and will be more competitive for space and food. Oppedal *et al.* (2003) also found that triploids grew better or equal to diploids under different light regimes, while Cotter *et al.* (2002) had lower growth in seawater. But many authors have reached the same conclusion that triploid Atlantic salmon reach smolt stage earlier, but when entering the seawater phase have no significant growth advantage over diploids. There is also observed a difference in condition factor where Fjelldal & Hansen (2010) found that triploid smolt had a lower condition factor than the diploids and their findings corresponds with earlier findings of Thrush *et al.* (1994) where all-female triploids reared under a six-month compressed under-yearling smolt production regime also had significant lower condition factor (Fjelldal & Hansen 2010), and Cotter *et al.* (2002) who found a significant lower condition factor in triploids in their 1996 year class, but not in 1995. The occurrence of lower condition factor in triploids could be explained with that they have fewer, larger muscle cells and a lower muscle cross-sectional area than diploids (Fjelldal & Hansen 2010).

Several studies have investigated feed conversion efficiency, digestibility and nutritional retention in triploids, but the majority of studies showed inconclusive results with no difference between ploidy. There have been two separated studies examining nutritional utilization by triploid Atlantic salmon, both using freshwater juveniles; Burke *et al.* (2010) and Tibbetts *et al.* (2013). Burke *et al.* (2010) found no difference in digestibility coefficient (i.e.

dry matter, ash or phosphorus) and protein content, but whole-body lipid and energy levels, as well as nitrogen and energy efficiency ratios were higher in triploids. They also found that triploids growth rate decreased with increasing dietary phosphorus, that could indicate that triploids are less able to process phosphorus. Tibbetts *et al.* (2013) also found no difference in digestibility coefficient, and in relation to Burke *et al.* (2010), found no difference in whole-body lipid and energy levels, as well as nitrogen and energy efficiency ratios, but did observe that triploids had higher lipid retention. Both showed no differences between ploidy with regards to bone ash content and mineral composition (including phosphorus) (Benfey 2016).

Triploid post-smolts are shown to be more vulnerable to cataract formation (Wall & Richards 1992), but Taylor *et al.* (2015) showed that a high concentration of histidine in the diet had a major influence of the prevalence of cataract development. Earlier studies have shown that dietary histidine reduces the incident of cataract in diploid Atlantic salmon, but even higher concentration had to be supplied to triploids to have an effect. The reason for higher histidine requirement in triploids is that they have lower levels of N-acetylhistidine in their eye lenses than diploids. This has a significance because histidine metabolite plays an important role in preventing the formation of cataract (Benfey 2016).

It appears that triploid Atlantic salmon has different nutritional requirement than diploids, but there is little information in general. Fjelldal & Hansen (2010) was the first to actually find a different dietary requirement between ploidy in Atlantic salmon and their study clearly demonstrated different needs in histidine (Benfey 2016).

### 3.2.1 Body shape and gut morphology

There is hard to distinguish the differences between diploids and triploids with regards to morphological and meristic characteristics. But some species have been documented with morpho-anatomical differences like the common carp (*Cyprinus carpio* L.) where differences in scale patterns occurred between diploids and triploids (Gomelsky *et al.* 1992). In triploid grass carp (*Ctenopharyngodon idella* Valenciennes) and bighead carp (*Hypophthalmichthys nobilis* Richardson), facial deformities has been observed (Tave 1993). In triploid tench (*Tinca tinca* L.) there has been documented differences in pelvic fin shape and length in relations to diploids (Flajšhans *et al.* 1993). Tiwary *et al.* (1999) observed nine significant differences in morphological ratios in Indian catfish (*Heteropneustes fossilis* Bloch) and was an effective tool to distinguish between diploids and triploids. However, Fast *et al.* (1995)

observed a reduction in deformities of triploid Asian catfish (*Clarias macrocephalus* Günther) in relations to diploids.

Triploid salmonids of both sexes show a general lower condition factor as juveniles compared to diploids, and the same characteristics can be seen in triploid immature females comparing to maturing diploid females. Besides secondary sexual characteristics, both triploid and diploid salmon cannot be distinguish based on their external appearance (Wilkins *et al.* 1995). One major problem for triploid Atlantic salmon are skeletal deformities in form of jaw, opercular and vertebral abnormalities and will be discussed in-depth later.

Peruzzi *et al.* (2015) did a study on differences in gut morphology on Atlantic salmon post-smolts and found several differences between ploidy. Triploids had approximately 20% less pyloric caeca with 25% reduction in mass, and the gut was approximately 15% shorter compared to sibling diploids. The findings are in agreement with an earlier study on triploid Atlantic cod (Peruzzi *et al.* 2013). The function of pyloric caeca is to secrete digestive enzymes, increase surface area of the intestine and absorption of nutrition. Reduced size could potentially affect the digestive capacity (absorb, metabolize and retain nutrient) of triploid Atlantic salmon, and in the end reduce their growth rate or cause nutrition deficiency from lack of micronutrients.

### 3.2.2 Cardiovascular, metabolic and respiratory

As mentioned, triploids have an increased cellular and nuclear dimension of erythrocytes, but compensate with a decreased total number, and this maintains organs' and tissues' homeostasis, including in the blood. The increase in size does not affect oxygen consumption rate (Lijalad & Powell 2009), aerobic swimming ability (Lijalad & Powell 2009), hemoglobin-oxygen-binding affinity (Sadler *et al.* 2000b) or stress response (Sadler *et al.* 2000a), but could be the reason for a lower performance in prolonged swimming tests caused by lower blood oxygen carrying capacity (Graham *et al.* 1985, Cotterell & Wardle 2004).

Triploid Atlantic salmon have been shown to have lower tolerance for suboptimal water condition in relations to diploids, especially when exposed to high water temperatures (Hansen *et al.* 2015, Sambraus *et al.* 2017). Combined with low oxygen saturation (below 70% $O_{2\ sat}$ of air saturation) in seawater, there have been reports of higher mortality in triploids. It has been proven that triploid Atlantic salmon have a lower thermal optimum than diploids, and could be the reason for the lower performance triploids have under some culture conditions (Atkins & Benfey 2008). Suboptimal water condition could also cause chronical stress that are seen in triploids leading to lower survival (Maxime 2008). But most of the stress are probably caused

by handling procedures, but high sea temperature might be a contributor to higher mortality in the autumn. In addition, recent research has shown that triploid Atlantic salmon are more temperature sensitive to develop aplasia of the septum transversum than diploids, a condition where the embryo develops a larger heart with elongated ventricles (Fraser *et al.* 2014a). A combined effect of lower blood oxygen capacity with a heart condition where metabolic oxygen demand are high, with a lower oxygen availability in the sea could explain why triploid Atlantic salmon have a lower thermal optimum (Atkins & Benfey 2008, Benfey 2016).

Both triploid salmonids and Atlantic cod have been observed with lower jaw deformity and opercular shortening. Those deformities could disrupt the efficient mechanism for moving water across the gills, and under some circumstances may have reduced respiratory efficiency. Shortening of the operculum are a condition where the skin flap is not fully covering the opercular cavity, reducing the efficiency of the buccal-opercular pumping system (Figure 3.3) (Benfey 2011). To be fully efficient, the system needs to be able to seal all compartments within the system. There are no direct studies on the efficiency of the respiratory systems on triploids, but there have been studies on aerobic swimming performance in triploid Atlantic salmon where those with lower jaw deformities performed worse than triploids and diploids without (Benfey 2011).

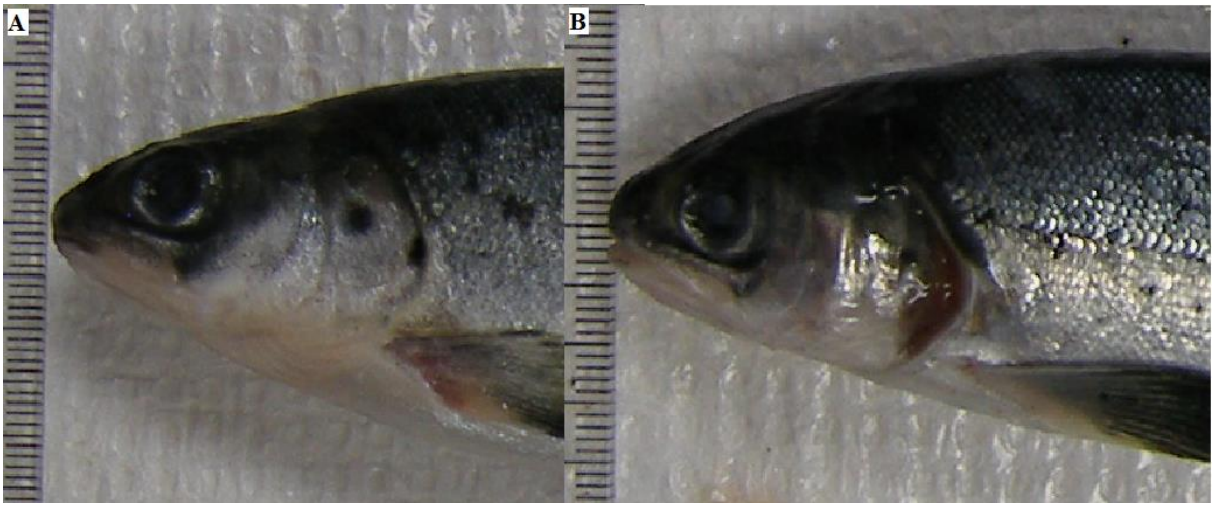


Figure 3.3. Lateral view of the head region of Atlantic salmon (*Salmo salar* L.) at smolt stage. (A) normal operculum and (B) moderate opercular shortening. Photo: Kevin Salbuvik

It has been observed that there are zones of missing gill filaments on the gill arches in triploids Atlantic salmon, as well as lower gill surface area (Benfey 2011). Sadler *et al.* (2001) found that 60% av triploids and 4% of diploids were missing primary gill filaments during freshwater development prior to saltwater transfer, and up to 50% of triploid freshwater smolt and 60% of triploid saltwater smolt suffered some type of gill deformity. This has not been

observed in other species like the triploid tench, but in the latter there have been observed longer and thinner secondary gill lamellae than those of diploids, making their respiratory gas exchange approximately 30% lower (Piačková & Flajšhans 2006, Benfey 2011).

### 3.2.3 Disease resistance

Studies suggest that the immune system is not impaired by triploid state even with fewer but larger immune cells. Challenge studies on salmonids for a range of common disease such as *Vibrio, Aeromonas salmonicida* and infectious hematopoietic necrosis virus showed that ploidy had little to no effect on immune response (Parsons *et al.* 1986, Bruno & Johnstone 1990, Dorson *et al.* 1991, Yamamoto & Iida 1995, Johnson *et al.* 2004, Ching *et al.* 2010), but there are exceptions (Yamamoto & Iida 1994, Jhingan *et al.* 2003). It has been suggested that the immune system in triploids compensate their lower cell numbers with higher activity (Fraser *et al.* 2012c). Fraser *et al.* (2012c) did a study on the effects of triploidy and vaccines on B-cells and neutrophils and found that triploid Atlantic salmon had a lower abundance of B-cells lymphocytes compared to diploids. B-cells are an important part of the acquired adaptive immune system. They have antibodies that binds to antigens on the surface of potentially harmful pathogens and thereby targeting them for phagocytosis (Benfey 2016). Triploids have also been shown to be more prone to develop abdominal adhesions (Fraser *et al.* 2014b) and pigmented (melanized) spots suggesting tissue inflammation (Larsen *et al.* 2014) from commercial oil-adjuvants vaccines. It can be assumed from results of studies that triploid Atlantic salmon may have lower resistance against pathogenic diseases and parasites, and may have worse reaction to vaccines than diploids, however there has only been performed one challenge study on disease resistance in triploid Atlantic salmon. They tested resistance against bacterial kidney disease (*Renibacterium salmoninarum*; BKD) and found no difference between triploid and diploids, but used different families in the study (Bruno & Johnstone 1990). In addition, Cantas *et al.* (2011) did a study on differences in gut microbiota between diploid and triploid Atlantic salmon, that could potentially have consequences for culture performance and health. The microbiota in the gut depends on the structure, mucosal immune system, nutritional absorption and more (Cantas *et al.* 2011). Ploidy also effects these factors, and thereby effect the microbiota. They could not find any differences in diversity, but they found 7.3% more bacteria in total within the whole gut. They concluded that the difference in ploidy may influence the gut by the change in physiology in triploids compared to diploids, and thus providing a different environment for the microbiota in the gut.

Salmon lice infection are considered the most expensive and damaging parasite in marine farming of salmonids (Costello 2006). Frenzl *et al.* (2014) did a series of controlled challenges exposing diploid and triploid Atlantic salmon for salmon lice (within tanks) and found no significant ploidy effect on susceptibility to infection. They also did a study on the abundancy of natural exposed fish (within sea cages) and found no significant ploidy effects. In freshwater, *Gyrodactylus salaris* (Malmberg) are considered the most damaging parasite. Ozerov *et al.* (2010) found that triploid Atlantic salmon had higher counts of *Gyrodactylus salaris* than diploids, but the study design had triploids from unknown origin and were presumed to have "spontaneously" arisen (Benfey 2016).

### 3.2.4 Behavior

Studies have shown that triploids have a disadvantage when reared with diploids in mixed populations, and might be caused by less aggressive behavior from triploids (Carter *et al.* 1994, O'Flynn *et al.* 1997, Taylor *et al.* 2014), but there are exceptions (O'Keefe & Benfey 1997) and it seems to depend on fish size and experimental designs. Recent study from Taylor *et al.* (2014) showed clearly that triploid Atlantic salmon post-smolts performed worse when reared in tanks with diploid compared with all-triploids reared alone. Why triploids show less aggressiveness than diploids are not clear, whether it is due to changes in sensory and central nervous system, or due to reduced levels of androgens, which is known to cause more aggressive behavior (Benfey 1999). Triploidy state does not affect brain size in Atlantic salmon, but Fraser *et al.* (2012b) reported in their study on triploid Atlantic salmon that they had smaller olfactory bulb, but larger cerebellum and telencephalon compared to diploids. This may effect both behavior and welfare in triploids (Fraser *et al.* 2012b). They suggested that a larger cerebellum and telencephalon may enhance triploids cognitive capacity related to foraging and migrating, which could increase their chances to survive in the wild, but also make them more vulnerable to predators. They also suggested that smaller olfactory bulb would make it harder for triploids to find food in the wild. But since the overall brain mass are the same as in diploids, it could simply be a compensation to keep equilibrium in the brain rather than enhancing brain function.

### 3.2.5 Skeletal deformities

Probably the most reported and described anatomical deformity in triploid fishes are the jaw deformity in Atlantic salmon (Figure 3.4) (Sutterlin *et al.* 1987, Jungalwalla 1991, Hughes 1992, Lee & King 1994, McGeachy *et al.* 1996, Lijalad & Powell 2009). In general, the

abundancy of skeletal and jaw deformity, as well as reduced gill surface affects triploid salmon populations more than diploids (Sadler *et al.* 2001, Pepper *et al.* 2004, Powell *et al.* 2009, Fjelldal & Hansen 2010, Leclercq *et al.* 2011, Fraser *et al.* 2013, Taylor *et al.* 2013, Tibbetts *et al.* 2013, Fraser *et al.* 2014b, Taylor *et al.* 2014), but most triploid individuals are not affected by anomalies. Lower jaw deformity has been reported to occur in up to 30% of commercial produced all-female triploids in Tasmania, and causes a significant loss to farmers (Sadler *et al.* 2001). If anomalies are present in either ploidy status, their performance (i.e. swimming ability, recovery from exhaustive exercise and/or metabolic activity), welfare and value are affected (Benfey 2016). The underlaying causes of skeletal anomalies are not fully understood, but recent work suggest that egg quality (Taylor *et al.* 2011) and nutritional requirement (Fjelldal *et al.* 2016) are the most likely factors, but there could also be one or two genotypic or environmental effects caused by their ploidy status (Benfey 2016).

Figure 3.4. Lower jaw deformity in Atlantic salmon (*Salmo salar* L.). Adapted from Amoroso *et al.* (2016b).

Fjelldal & Hansen (2010) did a study on vertebral deformities in triploid Atlantic salmon under-yearling smolts and highlighted that most triploids had normal bone morphology and suggested the higher occurrence of skeletal deformities in triploids was not only related to ploidy but also to external factors. Malformation constitute an ethical dilemma with regards to fish welfare, therefore it is important to focus research on reducing skeletal deformities in triploid Atlantic salmon to make salmon farming more sustainable (Fjelldal *et al.* 2016). Skeletal deformities are also a problem in diploids (Fjelldal *et al.* 2012a) and are associated with production of under-yearling smolts (Fjelldal *et al.* 2006), vaccination with oil-adjuvant vaccines (Berg *et al.* 2006, Aunsmo *et al.* 2008), high temperature during egg incubation

(Wargelius *et al.* 2005), increased water temperature during first period in sea water (Grini *et al.* 2011), dietary persistent organic pollutants (POPs) (Lock *et al.* 2011) and low phosphorus nutrition (Baeverfjord *et al.* 1998, Fjelldal *et al.* 2009, Fjelldal *et al.* 2012b). Several studies has showed that external factors can affect the expression of key endocrine molecules that regulates growth and mineralization of bone in Atlantic salmon (Fjelldal *et al.* 2016). One of those hormones are insulin-like growth factor 1 (IGF-1). IGF-1 has shown an increase in expression level in vertebral bone under continuous light regime together with increased vertebral growth (Nordgarden *et al.* 2006).

Dietary phosphorus requirement is highly species-specific and maintaining a sufficient amount are crucial in fish farming to avoid a deficiency that may result in skeletal deformities. Phosphorus is an essential nutrient that plays a metabolic role in building bone and scale, important component in ATP, nucleic acids and cell membranes. Phosphorus is an essential micronutrient, and levels in freshwater are insufficient to meet the requirement, therefore it has to come from the diet (Fjelldal *et al.* 2016). Commercial salmon feeds contain the required amount of phosphorus and are balanced such at the excess phosphorus do not impact the environment (Mente *et al.* 2006). Several researchers have suggested that triploid and diploid Atlantic salmon should be considered two different species with respect to environmental conditions and nutritional requirements, and recent studies from Fjelldal & Hansen (2010) and Fjelldal *et al.* (2016) have showed different needs in dietary micronutrients histidine and phosphorus respectively, and the effect it could have in different ploidy.

# 4 Utilization of by-products from fisheries and aquaculture

In 2016, 88% of the 90.9 million tons of wild caught fish (including crustaceans and other invertebrates) and 80 million tons of fish (including crustaceans and other invertebrates) produced in aquaculture were estimated used for human consumption (FAO 2018b). The definition "human consumption" are not precise, because it does not include the whole fish and most of the time only counts for the fillet yield. Fish are usually processed in different degrees before being sold to wholesaler or retailers (Olsen & Toppe 2017). The process may consist of deshelling, gutting, beheading, skinning, filleting and trimming, and depending on the species, the usual fillet yield range most often between 30-50% (Rustad *et al.* 2011). Those parts of the fish are called by-products, and are often regarded as low value and used as feed for farmed animals, fertilizers or discarded (Olsen *et al.* 2014). But by-products can also be utilized as fish feed by making hydrolyzed proteins through enzymatic hydrolysis or acidification with silage technology.

Hydrolyzed proteins are proteins that are partially hydrolyzed or broken down to its basic building blocks (amino acids). Hydrolyzed fish proteins contain high concentrations of free amino acids and low molecular peptides, and have been suggested as an alternative approach to overcome the limitations of the digestive capacity in fish larvae amongst others (Kotzamanis *et al.* 2007). The aquaculture industry is also looking for a high-quality feed nutrient that can substitute the use of fish meal in dry feed and makes both fisheries and aquaculture facilities more sustainable with regards to by-products. Effects from dietary protein hydrolysate have been investigated in many species like common carp larvae, Japanese sea bass (*Lateolabrax japonicus* Cuvier), Nile tilapia (*Oreochromis niloticus* L.), rainbow trout, and Atlantic salmon (Kotzamanis *et al.* 2007). Espe *et al.* (1999) showed an increase in growth in Atlantic salmon when 10% of fish meal was replaced by silage protein hydrolysate, but other groups with lower or higher concentrations had reduced growth. Liang *et al.* (2006) did a similar study in Japanese sea bass and reported an increase in growth when 15% of fish meal was replaced with acid silage hydrolysate, but also got reduced growth with higher and lower concentrations. The increased growth may be caused by enzymatic degradation of proteins, making them easier to absorb in the gut. Recent study from Peruzzi *et al.* (2018) on juvenile Atlantic salmon reported that use of high-protein phosphorus-rich diet where 45% of fishmeal were replaced with hydrolyzed fish protein combined with low rearing temperature had a significant reduction in skeletal deformities in triploids compared to triploids fed an phosphorus-rich commercial diet.

Thereby, if the right amount of hydrolyzed proteins is included in the diet, it could be beneficial in terms of reducing skeletal deformities and overall performance in triploids. In addition, Atlantic salmon given fractions of peptides from hydrolyzed muscle and empty stomachs of cod have been showed to stimulate activity in head kidney leukocytes (Bøgwald *et al.* 1996).

# 5 Shape analysis

Geometric morphometrics (GM) analysis is a statistical study of shape and size and their covariations with other variables, and plays an important role in many types of biological studies (Zelditch *et al.* 2012). It is a powerful tool with advantages in visualization of differences of complex shapes almost as easy as visualizing differences in shape of circles or letters in the alphabet. On the basis of a series of morpho-anatomical landmarks (discrete anatomical points that can be recognized as the same point in all specimens), shapes changes can be visualized in deformation grids in form of stretches, compressions and shearing. The numerical outputs from shapes is analyzed with multivariate statistics to see if any areas between landmarks have a significant change (Figure 5.1). GM has become an established method to analyze shape differences in many organisms, including fish, and are more precise and offers better visual expression than traditional morphometrics. The visual expression of shape changes is the key advantage of GM over traditional morphometric analysis. With help of GM, shape changes can be visualized directly as illustrations or computer animations. The various methods of visualization can communicate complex morphological changes much more efficient than the tables of coefficients that results from traditional morphometric analysis (Klingenberg 2013). GM in fish have been used in population studies, biomass estimations, evolution of larvae and fry quality, ploidy differentiating and assessment of environmental and genetic effects (Peruzzi *et al.* 2010).

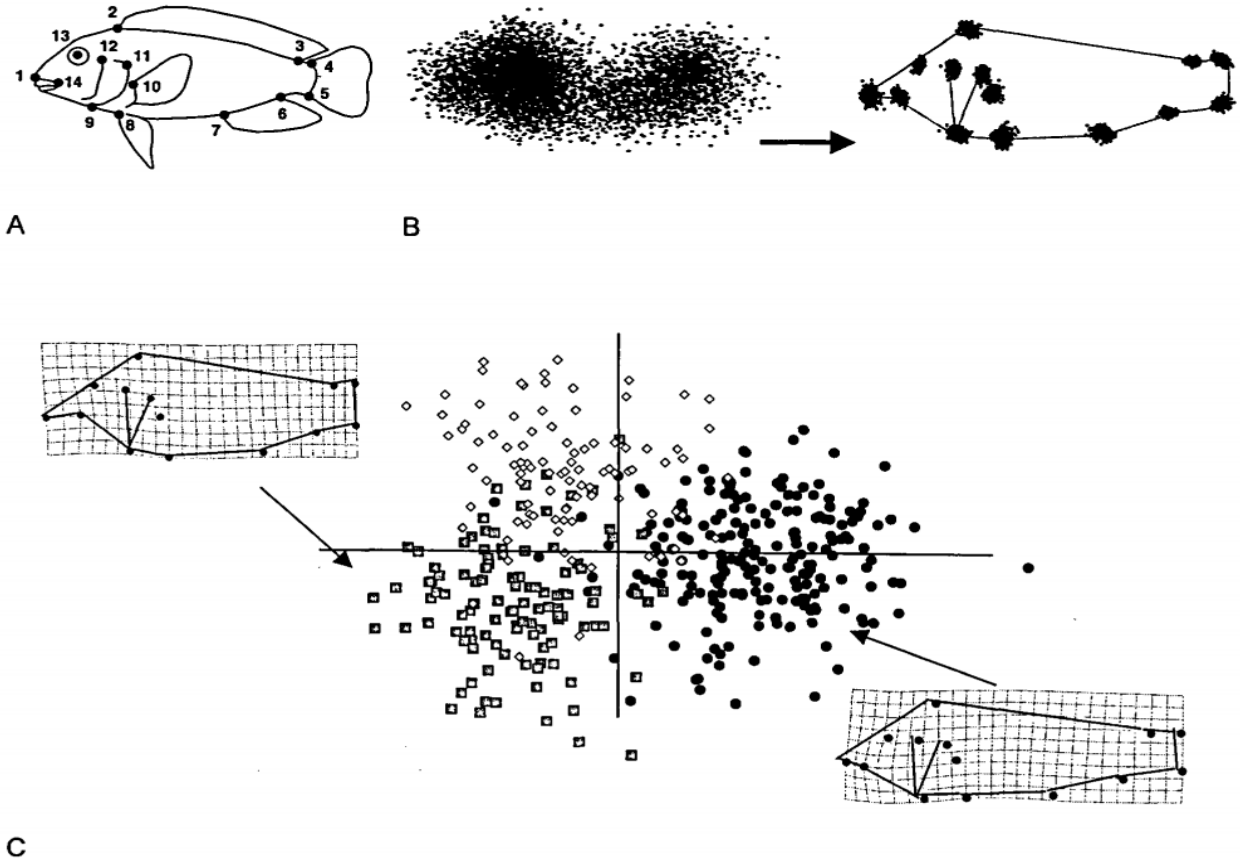


Figure 5.1. Graphical representation of the four-step morphometric protocol. A, quantify raw data (landmark coordinates on body of fish). B, remove non-shape variation (specimens before (left) and after (right) Generalized Procrustes Analysis). C, statistical analysis (e.g. Canonical Variates or Principal Component Analysis) and graphical presentation of results. Deformation grids for mean specimen for (right) Tanganyika Clown (*Eretmodus cyanostictus* Boulenger) and (left) Blue Goby Cichlid (*Spathodus erythroden* Boulenger). Adapted from Rüber & Adams (2001).

# 6 Objectives

By analyzing skeletal anomalies (opercular, jaw and skeletal deformities) in diploid and triploid Atlantic salmon smolts (mean body weight 60-75g), the goal is to highlight the differences in skeletal anomalies between diploid and triploid salmon fed high-protein phosphorus-rich fishmeal-based diet and an experimental diet where some of the fishmeal was replaced with hydrolyzed proteins and see if the triploids have less skeletal anomalies on a diet containing more digestible (hydrolyzed) proteins.

Little is known about the nutritional requirement of triploid Atlantic salmon. The hypothesis is that diploid and triploid salmon fed an experimental diet containing more digestible hydrolyzed proteins combined with low-temperature rearing would reduce the incidence of skeletal anomalies in triploid salmon.

The following objectives were set to analyze skeletal anomalies:

1. Observe difference in external characteristics (i.e. opercular shortening, snout shortening, lower jaw deformities).
2. Analyze and compare cranial and vertebral deformities with help of x-ray pictures
3. Compare body shape characteristics of triploids compared to diploid controls with help of geometric morphometry analyses of landmark coordinates to describe and quantify the nature and extent of morpho-anatomical changes ploidy, diet and skeletal deformities may affect shape.

# 7 Material and methods

## 7.1 Location and radiological examination

Feeding experiment and radiological examination were carried out at the Aquaculture Research Station of UiT and Nofima (Kårvika, Tromsø, Norway). The fish were euthanized with an overdosed of anaesthetic (Benzocaine, 120 mg $L^{-1}$) and then stored frozen (-20°C) at the end of the feeding experiment. A total of 594 (2n STD, 123; 2n EXP, 171; 3n STD, 138; 3n EXP, 162) post-smolts were thawed, measured and x-rayed for this study.

## 7.2 Experimental fish and rearing condition

The Atlantic salmon used in this study originated from the FUNGUT project and the rearing protocol are provided in Peruzzi *et al.* (2018). Briefly, the fish originated from Stofnfiskur's breeding program in Iceland (Stofnfiskur HF, Hafnarfjörður, Iceland). Eggs from 20 females were fertilized with milt from 13 males. Each female was crossed with either one or two males resulting in n=20 families (full-sibs and half-sibs). After fertilization the ova were handled according to standard commercial procedures, then split in halves, where one half was exposed to hydrostatic pressure shock at 9500 psi applied for 5 min, 300° minutes post-fertilization (MPF) at 5°C (Johnstone & Stet 1995). The remaining untreated eggs were used as diploid controls, given 40 groups in total (20 diploid and 20 triploid). At eye-egg stage (ca. 400 day-degree, dd), the ova were shipped by air to the Aquaculture Research Station in Kårvika (Tromsø, Norway). Diploid and triploid families were held in separate incubation trays (n=40) in a flow-through system at an average temperature of 4.8°C (minimum: 3.9°C and maximum: 5.8°C) following standard rearing procedure. Ploidy status were verified by flow cytometry with an in-house method (Peruzzi *et al.* 2007), using 20 and 50 newly hatched fry from each diploid and triploid family, respectively. Three out of the 20 triploid families had a small percentage (2-5%) of diploid fry and were excluded along with their diploid counterparts.

The ova were incubated in 34 hatching trays (n=17/ploidy) at 5°C in darkness. Hatching was completed around 11.12.2015 (570dd) and just prior to start-feeding (ca. 920 dd) the alevins were transferred to twelve 200L circular in-door tanks (ca. 3000 alevins/tank and a biomass of ca. 620g). The tanks were split into ploidy and feed type (Table 7.1). The experimental tanks were placed in two rooms and groups were randomly distributed to minimize the tank effect (n=6 tanks/room). The feed type was a standard high-protein phosphorus-rich fishmeal-based diet (STD) and a modified diet in which 45% of the fish meal

was replaced with hydrolyzed fish proteins (EXP) (Section 7.3; Tables 7.2 and 7.3). The feed was dispensed by electrical driven disc feeders programmed to supply 6-9 meals per day, and the amount of feed provided was consistently more than the consumption.

Table 7.1. Experimental design: two of the groups (Group 1 and 3) were fed a standard diet, and the other two groups (Group 2 and 4) were fed the experimental diet. Each experimental group had three replicates.

| Groups | Feed type | Number of tanks |
|---|---|---|
| Group 1 – diploid | Standard diet | 3 |
| Group 2 – diploid | Experimental diet | 3 |
| Group 3 – triploid | Standard diet | 3 |
| Group 4 – triploid | Experimental diet | 3 |

In the onset of start-feeding the water temperature was gradually increased to 10°C over a period of four weeks and was maintained at this temperature with the use of heated water (10°C ± 0.5), except during the summer (04. July – 02. September) when fish were exposed to ambient water temperature (range 9.5-12.5°C) (Figure 7.1). Standard husbandry condition and procedure were followed for Atlantic salmon and this includes daily removal of dead fish and measuring dissolved oxygen, and levels in outlet water never fell below 80% $O_{2\ sat}$. Fish were fed daily according to which group they belonged either with STD or EXP diet. Fish biomass was re-adjusted when needed to not exceed 45 kg/$m^3$ in each tank. This resulted in four biomass adjustment periods (Period 1-4), where the mean weight of 50 fish/tank was used to estimate the total biomass/tank and number of fish/tank (Figure 7.1). For the first three periods, the total biomass of fish was set to the same level in each tank (data not included in thesis). On 20.05.2016 (1800 dd) the fish were transferred from 200L to 500L tanks. The fish were kept on continuous light regime (24H, LL) throughout the experiment except for the required period of onset to winter stimulation were the photoperiod was gradually reduced to eight hours of light

(8L:16D) to induce parr-smolt transformation. A seawater-challenge test was executed to check if the smoltification process was complete at the end of the experiment.

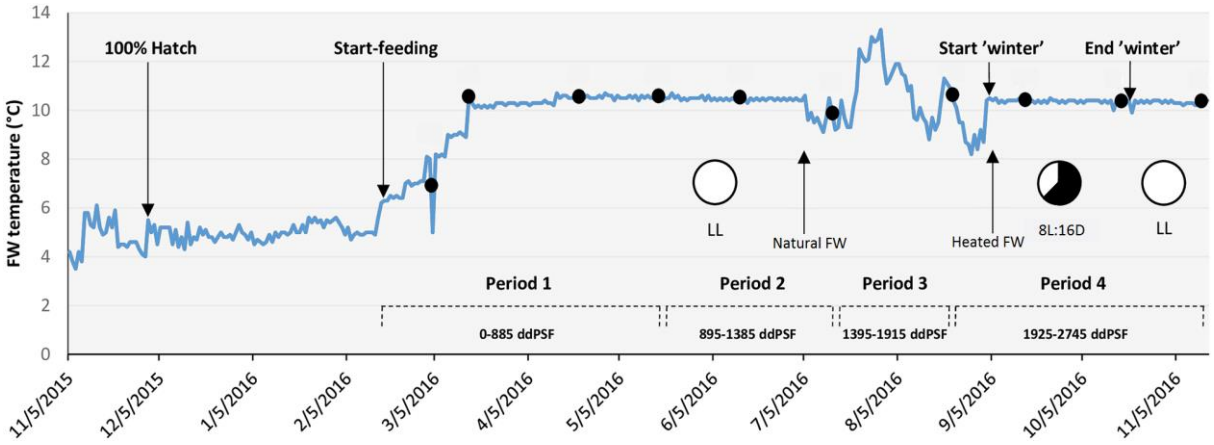


Figure 7.1. Rearing condition and sampling points during feeding experiment. Periods 1-4 cover 0-2745 degree-days post start-feeding (ddPSF). At end of each period, biomass was re-adjusted. Black dots are sampling points during the experiment were weight and length were measured on 25 individuals in each tank. Arrows indicate different events and operation. FW = Freshwater; LL = continuous light; 8L:16D = light regime during "winter" stimulation. Adapted from Peruzzi *et al.* (2018).

## 7.3 Diet

Both the STD and EXP diet were provided by Skretting AS (Stavanger, Norway). The size of the pellets was adjusted with fish growth and started from 0.5 mm pellets at start-feeding and ended with 3.0 mm pellets at the end of experimental period. The experimental diet had 45 % of the fishmeal replaced with hydrolyzed proteins (CPSP Special G – hydrolyzed fishmeal) while lowering the fish oil concentration slightly. The protein concentration was slightly higher in the experimental diet. In addition, the experimental diet contained a larger amount of a premix of different micronutrients added. Full nutritional composition in STD and EXP diet are provided in Tables 7.2 and 7.3. An inert marker (Yttrium) was added in the 3.0 mm feed for measuring feed digestibility (analysis and results of digestibility are not included in the thesis).

Table 7.2. Nutritional composition of standard diets. *Marker Yttrium used in 3.0 mm diet only. Adapted from Peruzzi *et al.* (2018).

| | **Standard diet** | | |
|---|---|---|---|
| **Pellet size (mm)** | **0.5 – 1.0** | **1.2** | **1.5 – 3.0** |
| **Wheat (g)** | 7.2 | 6.1 | 6.9 |
| **Wheat gluten (g)** | 10 | 10 | 10 |
| **Soy Protein Concentrate (SPC) (g)** | 14.4 | 16.7 | 17.9 |
| **NA Fishmeal (g)** | 55 | 55 | 50 |
| **CPSP Special G – hydrolyzed fishmeal (g)** | 0 | 0 | 0 |
| **Fish oil Nordic (g)** | 11 | 10.8 | 11.6 |
| **Water (g)** | 0 | 0 | 0.4 |
| **Yttrium premix* (g)** | 0 | 0 | 0.1 |
| **Premix (Minerals, Vitamins, Amino acids) (g)** | 2.4 | 1.4 | 3 |
| **TOTAL (100/g)** | 100 | 100 | 100 |
| **Chemical composition (%)** | | | |
| **Moisture** | 7.9 | 7.5 | 7.2 |
| **Protein** | 55.9 | 56.8 | 56.0 |
| **Fat** | 17.7 | 18.3 | 19.1 |

Table 7.3. Nutritional composition of experimental diets. *Marker Yttrium used in 3.0 mm diet only. Adapted from Peruzzi *et al.* (2018).

| | **Experimental diet** | | |
|---|---|---|---|
| **Pellet size (mm)** | **0.5 – 1.0** | **1.2** | **1.5 – 3.0** |
| **Wheat (g)** | 5.4 | 5.5 | 6.9 |
| **Wheat gluten (g)** | 10 | 10 | 10 |
| **Soy Protein Concentrate (SPC) (g)** | 14.0 | 16.2 | 16.7 |
| **NA Fishmeal (g)** | 30 | 30 | 27.5 |
| **CPSP Special G – hydrolyzed fishmeal (g)** | 25 | 25 | 22.5 |
| **Fish oil Nordic (g)** | 9.4 | 9.2 | 10.2 |
| **Water (g)** | 1.6 | 0.9 | 1.5 |
| **Yttrium premix* (g)** | 0 | 0 | 0.1 |
| **Premix (Minerals, Vitamins, Amino acids) (g)** | 4.7 | 3.3 | 4.6 |
| **TOTAL (100/g)** | 100 | 100 | 100 |
| **Chemical composition (%)** | | | |
| **Moisture** | 7.9 | 7.5 | 7.1 |
| **Protein** | 60.3 | 59.0 | 56.9 |
| **Fat** | 17.3 | 18.8 | 19.6 |

## 7.4 Radiography and morphometric analysis

Fish was thawed and stretched fully before visual inspection and radiography was performed. Each fish was weighed and fork length measured to the nearest 0.1 cm before being placed on the digital plate. Fish were then inspected for visual external deformities like operculum shortening (Figure 3.3) and fish condition factor (K) was calculated as:

$$K = 100(WL^{-3})$$

where W is weight of fish in gram (g) and L are fork length in centimeters (cm). Five fish were placed on their right side on a digital plate (35x43 cm) with a GP flexible phosphorus screen (Direct View CR500, Carestream Health Inc., USA) before exposed twice for 4 seconds at 3

mA and 60 kV using a Nanodor 2 x-ray apparatus (Siemens, Germany). The plates were then placed in a digital scanner (Direct View CR500, Carestream Health Inc., USA). The picture was given other numbers and randomly selected from a folder before they were analyzed with help of an image analysis program (ImageJ v1.51, Wayne Rasband, National Institutes of Health, USA). A total of 15 landmarks were chosen and measured, where 13 was used for shape data analysis similar to Von Cramon-Taubadel *et al.* (2005) study and three was used for calculating the lower jaw index (LJI) (Figure 7.2). All landmark coordinates (x, y) from each individual fish were imported into text files before loaded into the statistical package R for analysis. The spinal column was divided into four regions (R) following the classification of Kacem *et al.* (1998) as illustrated in Figure 7.2. R1 covering vertebrae (V) V1-V8, R2 covering V9-V30, R3 covering V31-V49 and R4 covering V50-57 to V60, respectively.

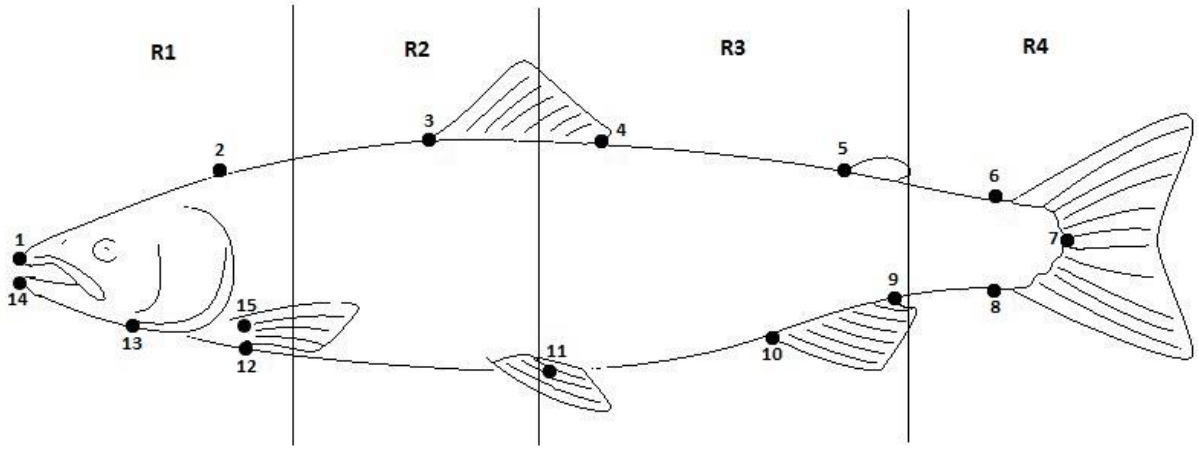


Figure 7.2. Location of the morphometric landmarks on Atlantic salmon (*Salmo salar* L.). Landmark refer to: (1) anterior tip of snout at upper jaw; (2) posterior part of neurocranium (start of scale nape); (3) origin of dorsal fin; (4) posterior insertion of dorsal fin; (5) origin of adipose fin; (6) anterior attachment of dorsal membrane from caudal fin; (7) posterior end of vertebrate column; (8) anterior attachment of ventral membrane from caudal fin; (9) posterior insertion of anal fin; (10) origin of anal fin; (11) posterior insertion of pelvic fin; (12) ventral side below origin of pectoral fin; (13) posterior end of maxillary; (14) tip of snout at lower jaw; (15) articulation point of the pectoral fin; (R1) cranial trunk; (R2) caudal trunk; (R3) tail; (R4) tail fin.

Classification used to identify the type of deformities followed Witten *et al.* (2009) and individuals with at least one deformed vertebra were classified as a deformed fish. The numbers of vertebrae were counted for each individual fish and if there were two or more fused vertebrae, they were counted separately. Only 4 categories of vertebral body malformations were used to

classify spinal deformation, but other categories were considered. The categories are illustrated in Figure 7.3.

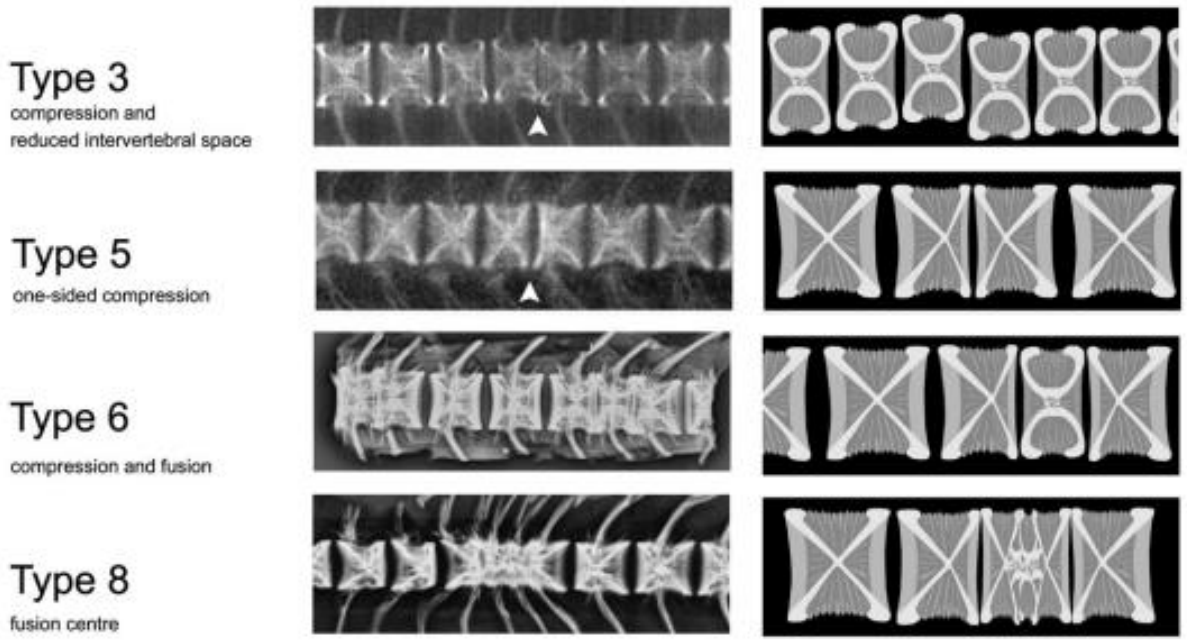


Figure 7.3. Radiographs and schematic representation of spinal deformations in Atlantic salmon (*Salmo salar* L.). Type 3 = Two-sided compression (with reduced intervertebral space indicated with white arrow); Type 5 = One-sided compression (indicated by white arrow); Type 6 = Compression and fusion (two-three vertebrae); Type 8 = Multiple side compressions and fusions. Adapted from Witten *et al.* (2009).

Lower jaw deformities (LJD) were measured using the formula for lower jaw index (LJI) (Lijalad & Powell 2009, Amoroso *et al.* 2016b) as:

$$\text{Lower jaw index (LJI)} = \frac{L2}{L1}$$

Where L1 distance (in pixels) are measured between landmark 1 and 15, and L2 measured between landmark 14 and 15 (Figure 7.2). Fish were also visually examined for LJD both externally and on x-ray pictures.

Ninety-nine of the 594 individuals were taken out of the analysis because of singular or multiple different reasons (e.g. poor picture quality, not calcified bones, too small to properly perform analyses). Measurements from a total of 120 diploids fed STD diet (control group; T1, 38; T2, 42; T3; 40), 153 diploids fed EXP diet (T4, 41; T5, 56; T6; 56), 104 triploids fed STD diet (T7, 42; T8, 34; T9; 28), and 118 triploids fed EXP diet (T10, 39; T11, 45; T12; 34) were transferred into the statistical package R for analysis. The analyses were performed on post-smolt and followed a multi-step geomorphic morphometric protocol (Figure 5.1) (Adams *et al.*

2017). Original positions, orientation and scale were translated and standardized to unit Centroid Size (CS), which is a measure of spread of the landmarks around their centre of gravity (or the centroid). They were then optimally superimposed through a Generalized Procrustes Analysis (GPA), such that all specimens have the centroid in common, rotated to optimal fit according to a least-squares criteria and projected to a linear shape tangent space. This results in shapes with coordinates that can be analyzed with multivariate statistics (i.e. Principal Component Analysis (PCA)). It also ensures that sized-based effects are removed, and only shaped-based differences remain. The influence of size on shape can be investigated by analyzing the correlation between the extracted size residuals (configuration centroid size) and the remaining shape residuals. Outliers were assessed using Procrustes-aligned coordinates and if any specimens were digitized wrong, they were removed from the analysis. To access the variation between groups, PCA was applied to calculate the main axes of morphometric variation (Bookstein 1997). The ordination results were summarized using scatterplots with body shape variations (mean ± 1 standard deviation (SD)) drawn along the associated ordination axis. The mean shape deviations among the groups were summarized by superimposing shape configuration of control group (undeformed fish from 2n STD) and compared against the experimental groups in a landmark-based vector plots and visualizing the differences in shape between the groups by plotting them into thin plate spline (TPS) deformation grids. The groups were then split into deformed and not deformed individuals and visualized in TPS deformations grids to assess the differences deformities have on shape. Only components that explained at least 5% of the variation were taken into consideration when assessing predicted shapes across the axes.

## 7.5 Statistical Analysis

Weight, length and vertebrae data were checked for normality (Shapiro-Wilk test), independence and homogeneity of variances (Levene's test) to satisfy the assumption of ANOVA. When the parameter condition was not met for linearity, the length data were logarithmically-transformed and counting data were arcsin-transformed to improve the distribution. Ploidy and diet differences in vertebrae number was analyzed with non-parametric Kruskal-Wallis test to check for significant differences. Ploidy and diet effects on weight, length and condition factor was tested using nested ANOVA, with tanks as random factors nested in ploidy and diet groups. If a significant difference appeared, Tukey's post-hoc test was applied for pairwise comparisons between groups. Data for opercular shortening, jaw and

skeletal deformities from x-ray inspections were analyzed using two-way ANOVA with ploidy and diet as fixed factors. A Procrustes ANOVA was used to check for size differences between group shapes, as well as shape differences among groups. All significant testing on shape measurements employed a nonparametric 1000-round randomized residual permutation procedure (Adams *et al.* 2017). If a significant difference appeared, a post hoc test of pairwise differences between least square means was applied to compare groups. Size data was recorded and processed in Microsoft Excel 2016 and statistical analyses were performed using the statistical software R v3.4.3 and shape analyses were performed using package "geomorph" (R package version 3.0.7) by Adams *et al.* (2017). The critical *P* value was set to 0.05, and results are presented as mean ± standard error of mean (SEM). Shape data are presented as mean ± SD.

# 8 Results

## 8.1 Fish size and condition

There was a significant effect (two-way ANOVA) between diet groups where groups fed standard fishmeal-based diets (STD) were both longer (F (1, 491) = 49.727, $P$<0.001) and heavier (F (1, 491) = 32.536, $P$<0.001) than groups fed an experimental diet (EXP) containing hydrolyzed fish protein, but there was no significant effect of ploidy in either weight, length or condition factor (Figures 8.1-8.3). However, it was observed a significant interaction between ploidy and diet on weight (F (1, 491) = 5.643, $P$ = 0.018) and condition factor (F (1, 491) = 5.403, $P$ = 0.021).

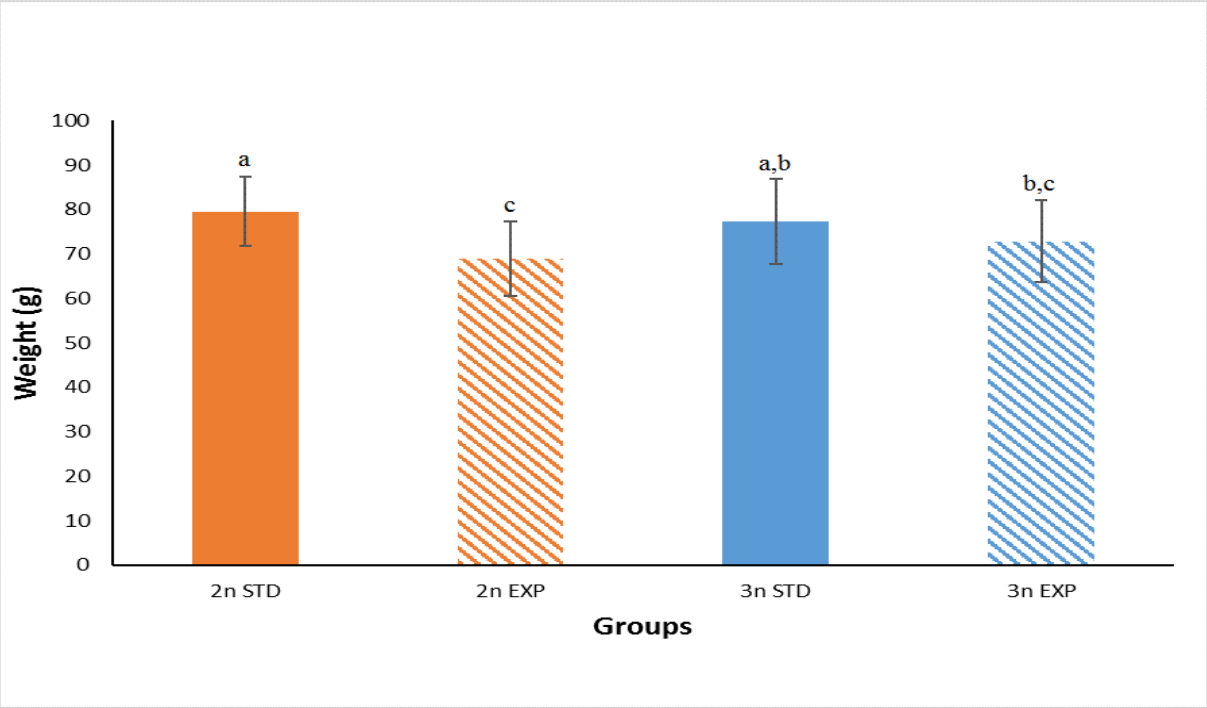


Figure 8.1. Body weight of diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD) and hydrolyzed fish protein diet (EXP). Different letters denote a significant difference (P<0.05) and data are presented as means ± SEM (n=3).

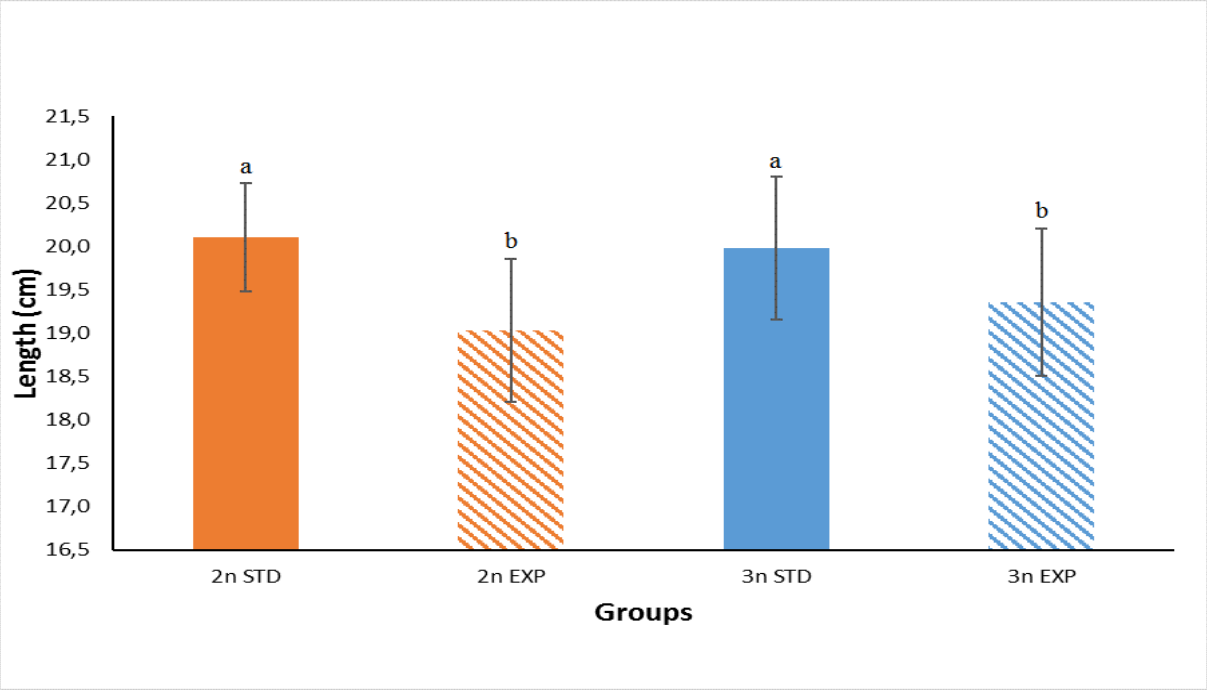


Figure 8.2. Body length (fork length measurements) of diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD) and hydrolyzed fish protein diet (EXP). Different letters denote a significant difference ($P<0.05$) and data are presented as means ± SEM (n=3).

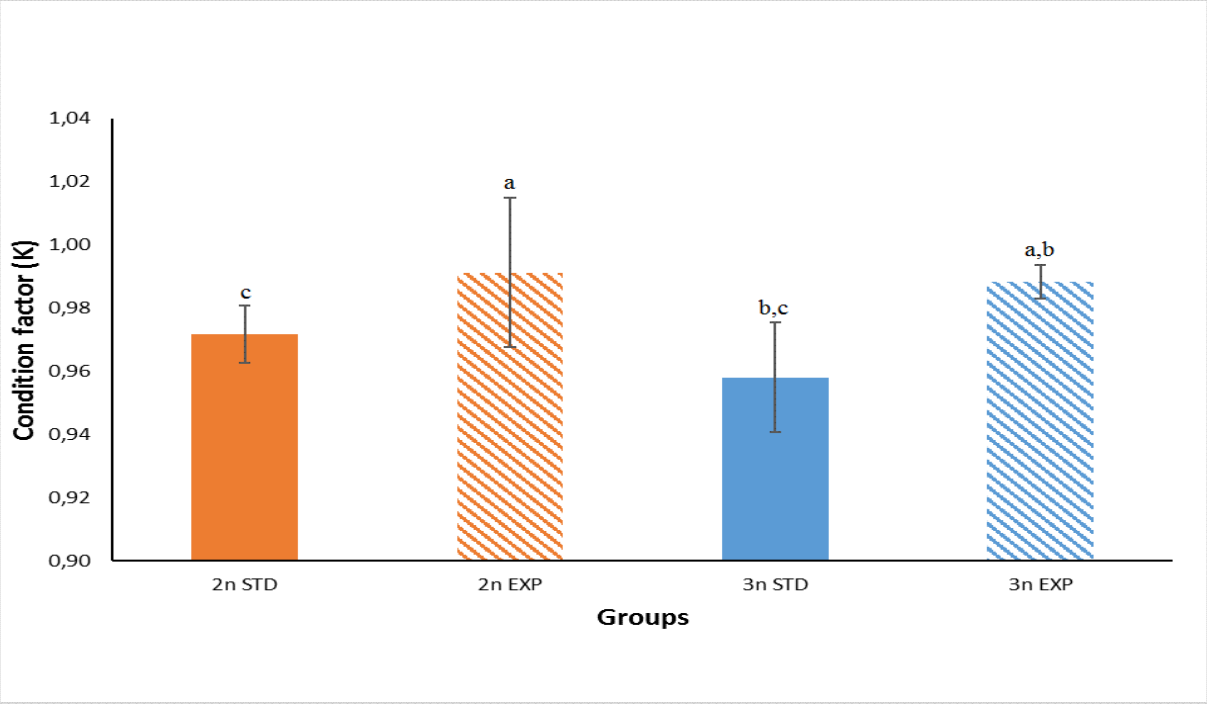


Figure 8.3. Condition factor of diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD) and hydrolyzed fish protein diet (EXP). Different letters denote a significant difference ($P<0.05$) and data are presented as means ± SEM (n=3).

## 8.2 Deformities – External inspection

There was a significant diet effect on shortening of the operculum (F (1, 491) = 4.320, *P* = 0.04) even with the frequency of such deformity being very low across groups (range: 0-3%; Figure 8.4). Approximately the same frequency was observed for external lower jaw deformities (LJD; Figure 8.5), and no significant effects of either ploidy, diet or their interaction was found (Appendix Table 11.1).

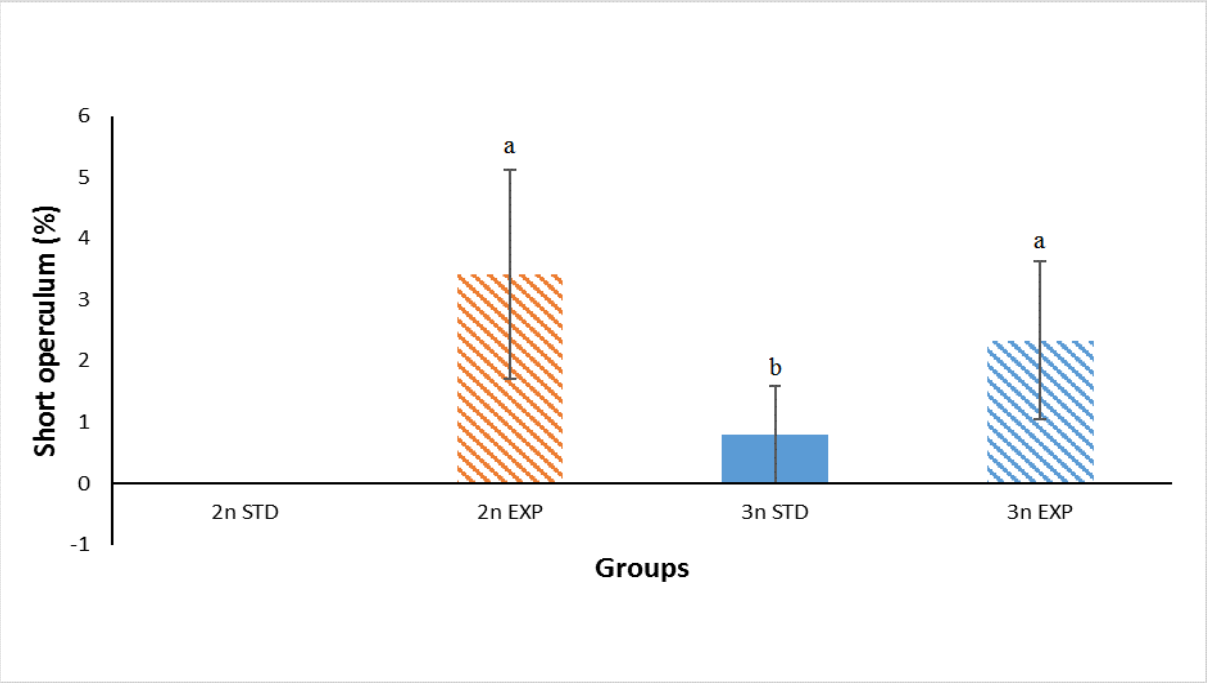


Figure 8.4. Percentage of operculum shortening in diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD) and hydrolyzed fish protein diet (EXP). Data presented as mean ± SEM (n=3).

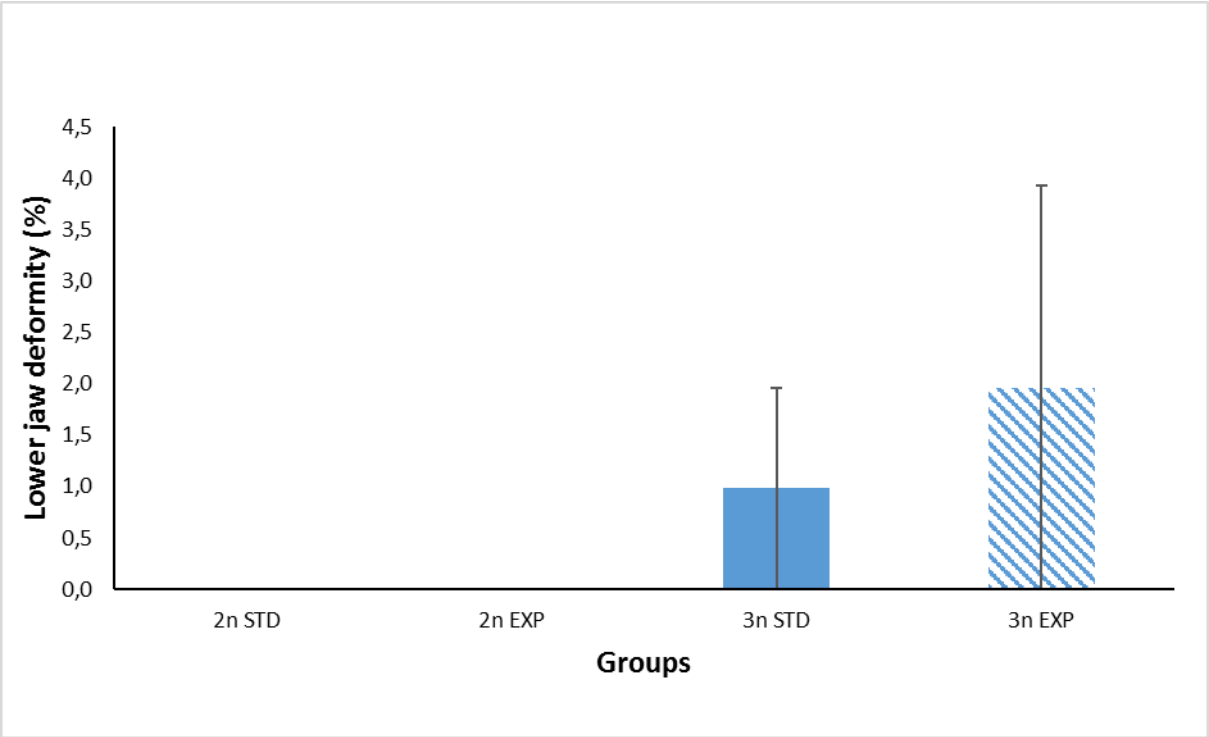


Figure 8.5. Percentage of external observations of lower jaw deformity in diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD) and hydrolyzed fish protein diet (EXP). Data presented as mean ± SEM (n=3).

## 8.3 Deformities – Radiological inspections

There was a significant ploidy effect on vertebral numbers and skeletal deformities (Figure 8.6 and Appendix Table 11.1). There were slightly but significantly fewer vertebrae in triploids (STD diet, 57.82 ± 0.45; EXP diet 57.78 ± 0.42) than in diploids (STD diet, 58.03 ± 0.43; EXP diet, 58.00 ± 0.48). Triploids had a significant ($F(1, 491) = 21.247$, $P<0.001$) higher incidence of skeletal deformities than diploids in both diets (Figure 8.6 and Appendix Table 11.1), and four types of skeletal anomalies could be observed (Figure 8.7): type 3 (two-sided compression with reduced intervertebral space), type 5 (one-sided compression), type 6 (compression and fusion (two-three vertebrae)) and type 8 (multiple side compression and fusions) following Witten *et al.* (2009). Overall, while all types of skeletal anomalies were equally represented among diploid fed a STD diet, a combination of vertebral compressions and fusions (type 6 and 8) seemed to predominate among fish fed EXP diet, independently from their ploidy. Irrespective of both ploidy and diet, there was a trend of higher incidents of skeletal deformities in the anterior part of the spinal column, corresponding to the cranial trunk region (R1; V1-8) and the caudal trunk region (R2; V9-30) (Figures 8.8-8.9).

In diploids fed both diets, the highest prevalence of deformities was in the R2 region (Figure 8.8). Similar observation was seen in triploids only with an overall higher occurrence in region R2 and high peaks in region R1 (Figure 8.9). Both diploid groups had a peak in the tail fin region (R4; V50-58). Similar could be seen in triploids fed EXP diet. In addition, triploids fed STD diet had a small peak in the posterior part of the tail region (R3; V31-49).

Kyphosis was observed in all experimental groups, but at low frequency (range: 1-3%). Similar frequency was observed in hyper-radiodense (range 2-4%). Data of kyphosis and hyper-radiodense are in the appendix (Appendix Table 11.1).

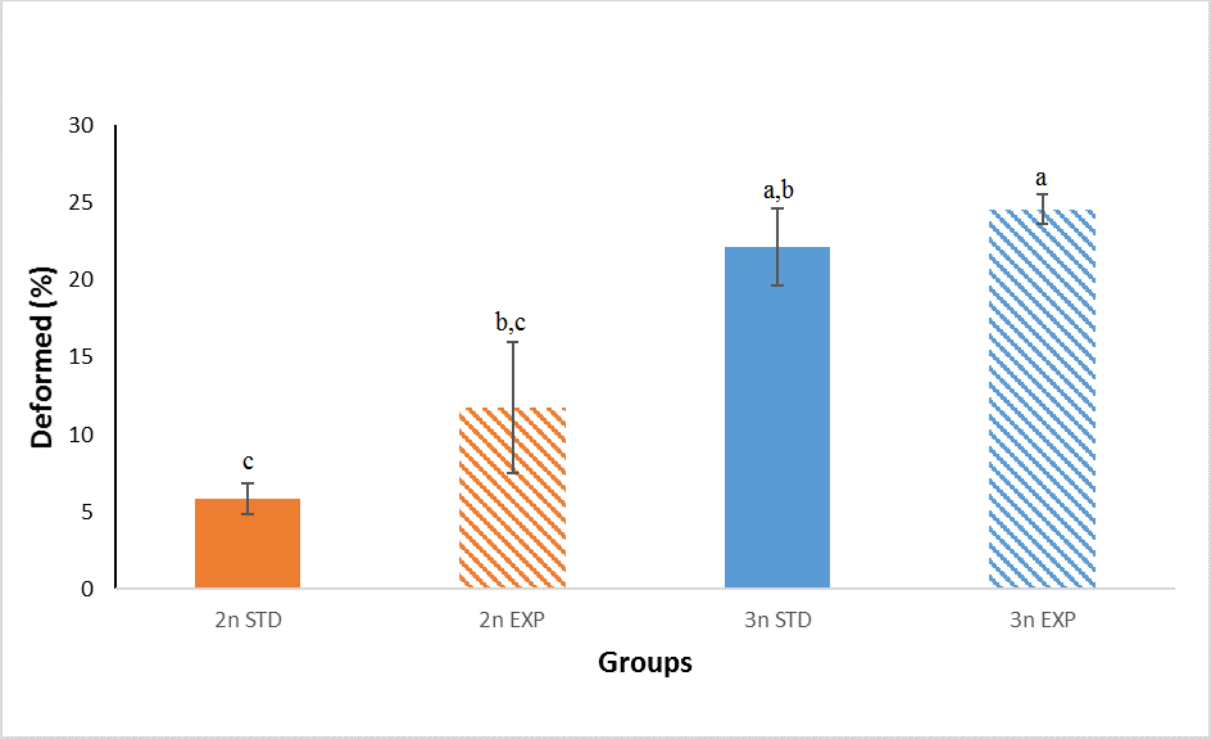


Figure 8.6. Percentage of spinal (vertebral) deformities in Atlantic salmon (*Salmo salar* L.) smolt among diploids (2n) and triploids (3n) observed by radiography. STD = Standard diet; EXP = Experimental diet. Different letters denote a significant difference ($P<0.05$) and data are presented as means ± SEM (n=3).

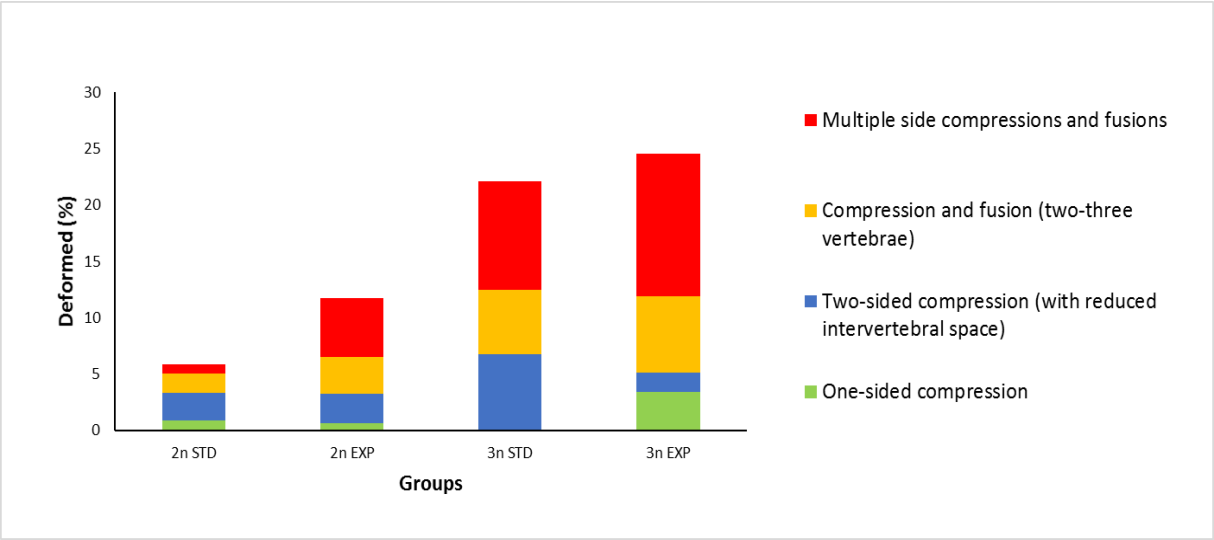


Figure 8.7. Types of vertebral deformities observed in diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt. Mean prevalence of vertebral deformity types in smolt fed a standard fishmeal-based diet (STD) and hydrolyzed fish protein diet (EXP). Classification of deformities according to Witten *et al.* (2009)

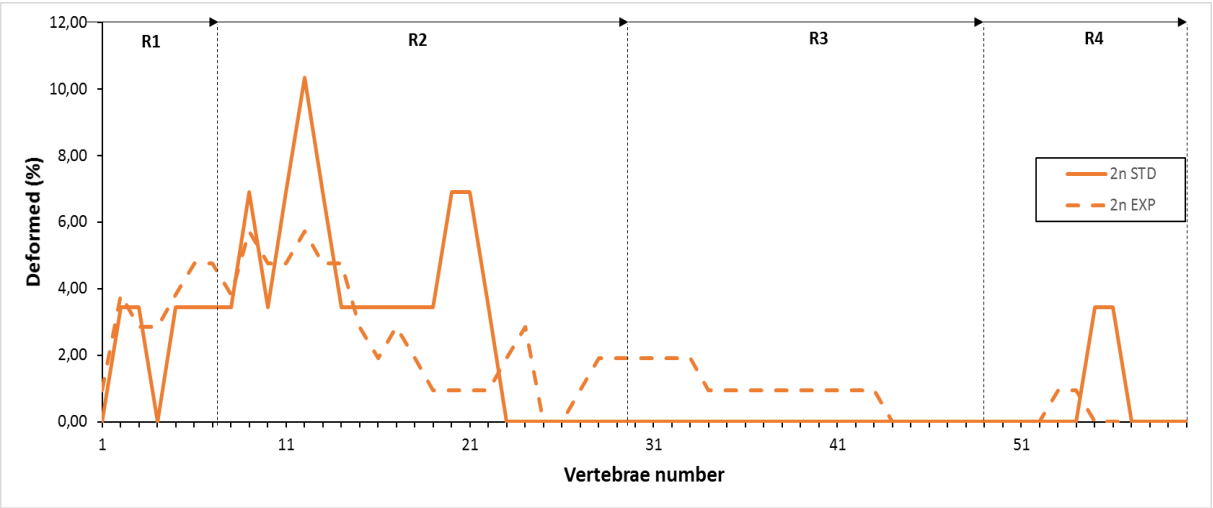


Figure 8.8. Distribution of deformed vertebrae in diploid (2n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD; solid line) and hydrolyzed fish protein diet (EXP; dashed line). Vertebral regions according to Kacem *et al.* (1998). R1 = cranial trunk; R2 = caudal trunk; R3 = tail region; R4 = tail fin.

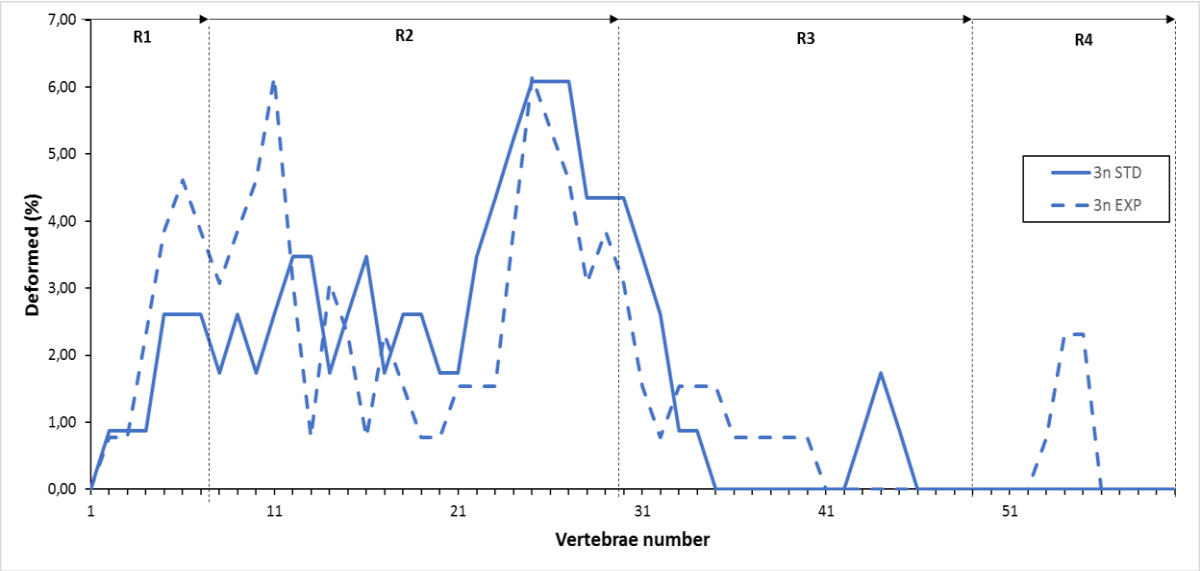


Figure 8.9. Distribution of deformed vertebrae in triploid (3n) Atlantic salmon (*Salmo salar* L.) smolt fed a standard fishmeal-based diet (STD; solid line) and hydrolyzed fish protein diet (EXP; dashed line). Vertebral regions according to Kacem *et al.* (1998). R1 = cranial trunk; R2 = caudal trunk; R3 = tail region; R4 = tail fin.

## 8.4 Shape analysis

The four groups differed significantly in their average body shapes (F (3, 487) = 10.656, $P = 0.001$), and there was a significant effect in shape between groups while accounting for the shape covarying with size ($R^2 = 0.011$, F (3, 487) = 1.873, $P = 0.016$). Pairwise test was carried out and found a significant shape difference between diploid fed different diets ($P = 0.019$), while accounting for size (Appendix Figure 11.1).

There was a significant ploidy (F (1, 491) = 23.003, $P = 0.001$) and diet (F (1, 491) = 4.865, $P = 0.002$) effect on average body shape as well as a significant interaction between ploidy and diet (F (1, 491) = 4.707, $P = 0.001$), and a pairwise test showed a significant difference ($P = 0.013$) in average body shape between diploids fed the two different diets.

Figures 8.10-8.12 are scatterplots with five plotted PC axes explaining a total of 73.3% of the variation observed between groups (PC1 = 37.2%; PC2 = 14.7%; PC3 = 8.4%; PC4 = 7.0%; PC5 = 6.0%). The first axis (PC1) can be interpreted as a degree of body curvature (body flexion or dorsoventral curving). The second axis (PC2) can be interpreted as body stretching and contracting along the antero-posterior axis and the dorsoventral axis, especially effecting the tail region (R3). The third axes (PC3) shows the degree of compression and stretching centered around the dorsal fin, and effects the middle part of the shape. The fourth axes (PC4) shows mainly a curving and stretching of the cranial and caudal trunk and a stretching of the

tail fin. PC4 seems to mostly effect the shape around the anal fin. The fifth axes (PC5) seems to show a whole stretch and contraction of the fish body in the antero-posterior axes. All body shape variations are shown in Figures 8.10-8.12 (and in Figure 11.3 in the appendix). Body shape variations from the control group (undeformed fish from 2n STD) are shown in Figure 8.13 by means of vector plots illustrating how each landmark moves in relation to the control groups position and Figure 8.14 are an extension from the vector plot to illustrate how the whole shape changes as each landmark move with help of a TPS (thin-plate spline) deformation grid and how it affects the shape of the fish. 2n EXP seems to have a dorsal curving, as depicted in Figure 8.13 showing as midpoint landmarks move upwards while the cranial and tail region move downwards. Triploids fed both diets display an elongation between landmark 1-2 and stretching in the whole middle part of the body (between landmark 4-5 and 10-11). This can be seen in Figure 8.13 showing that the snout landmark is moving down in both triploid groups, but 3n STD cranial part are more affected in a downward curving, while 3n EXP seems to have the dorsal cranial part stretched backward in the antero-posterior axes. The effect on shape from skeletal deformities are shown in Figures 8.15-8.18 with help of scatterplots and TPS grids. No significant differences were found between deformed and not deformed shapes among all groups.

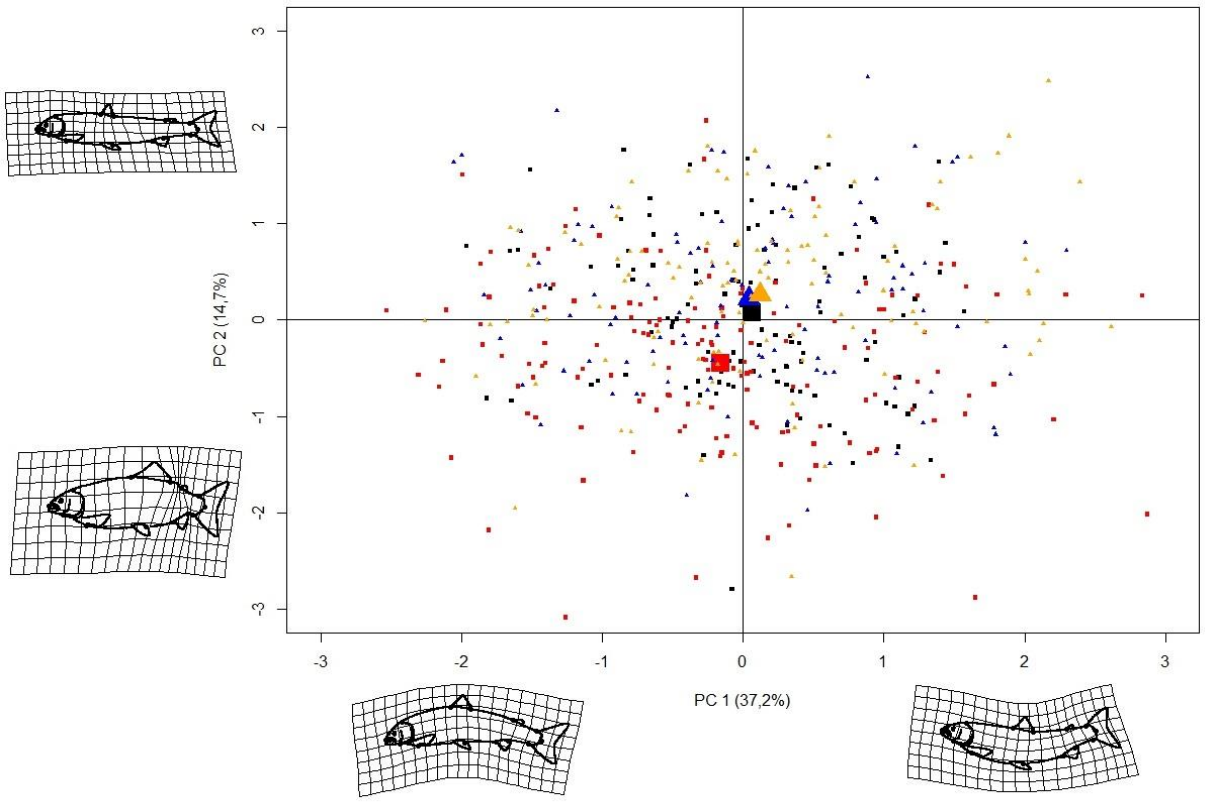


Figure 8.10. Scatterplot of ordination results depicting variation in geometric morphometry within and among groups, where 2n STD (black square), 2n EXP (red square), 3n STD (blue triangle) and 3n EXP (orange triangle). Shape variation along the ordination axes (PC1 and PC2) are illustrated by the shape configuration at ±1 standard deviation from the mean and larger figures represent the mean shape of the groups.

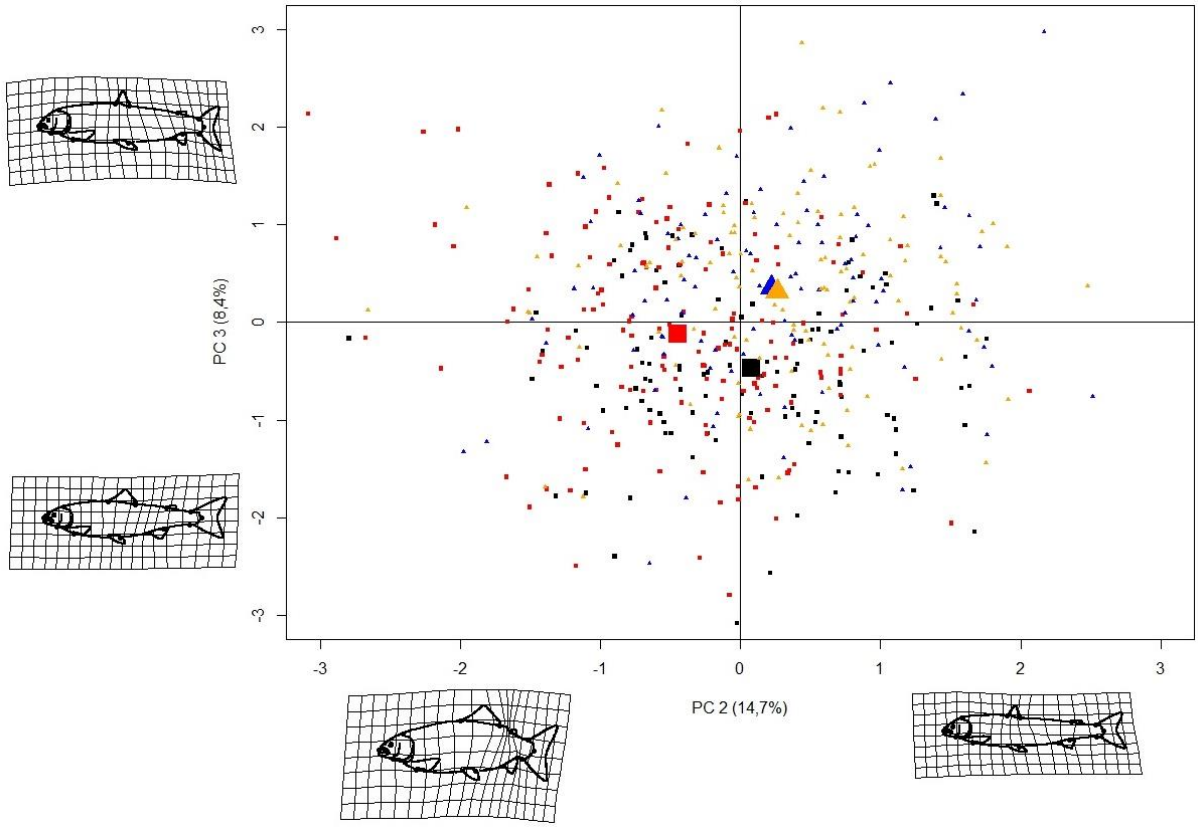


Figure 8.11. Scatterplot of ordination results depicting variation in geometric morphometry within and among groups, where 2n STD (black square), 2n EXP (red square), 3n STD (blue triangle) and 3n EXP (orange triangle). Shape variation along the ordination axes (PC2 and PC3) are illustrated by the shape configuration at ±1 standard deviation from the mean and larger figures represent the mean shape of the groups.

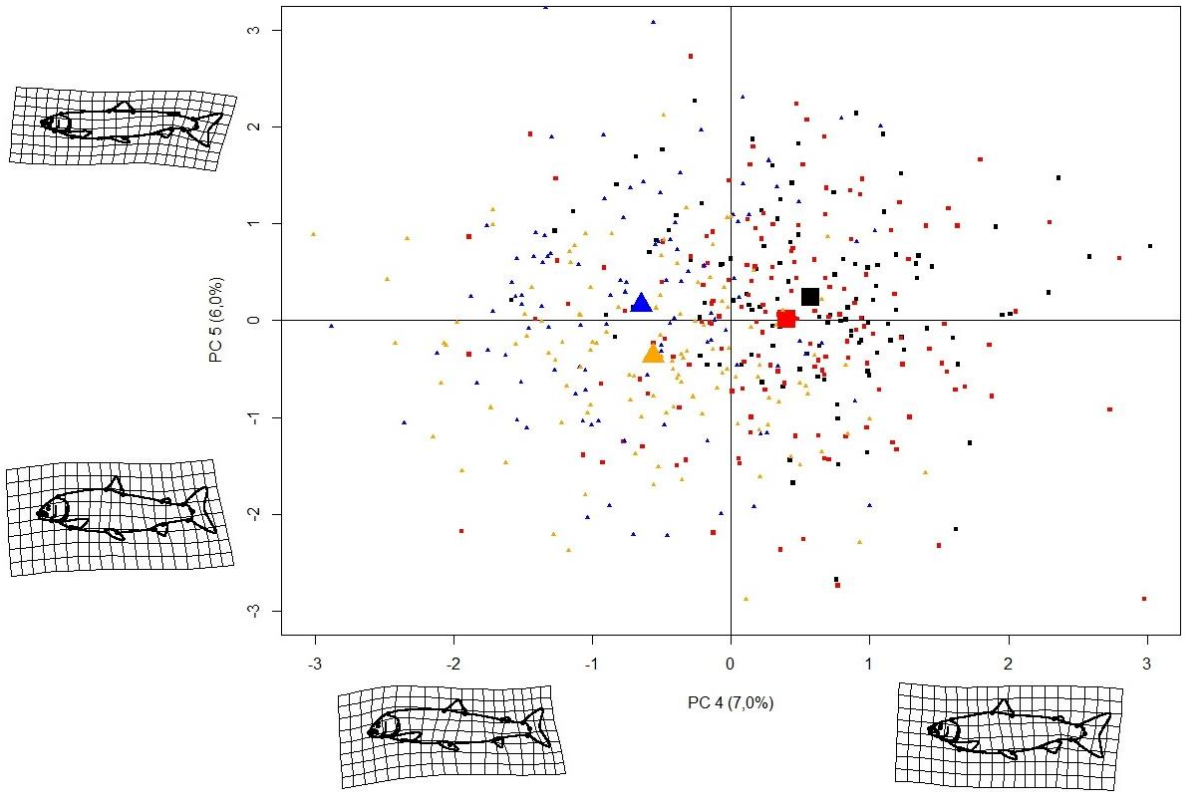


Figure 8.12. Scatterplot of ordination results depicting variation in geometric morphometry within and among groups, where 2n STD (black square), 2n EXP (red square), 3n STD (blue triangle) and 3n EXP (orange triangle). Shape variation along the ordination axes (PC4 and PC5) are illustrated by the shape configuration at ±1 standard deviation from the mean and larger figures represent the mean shape of the groups.

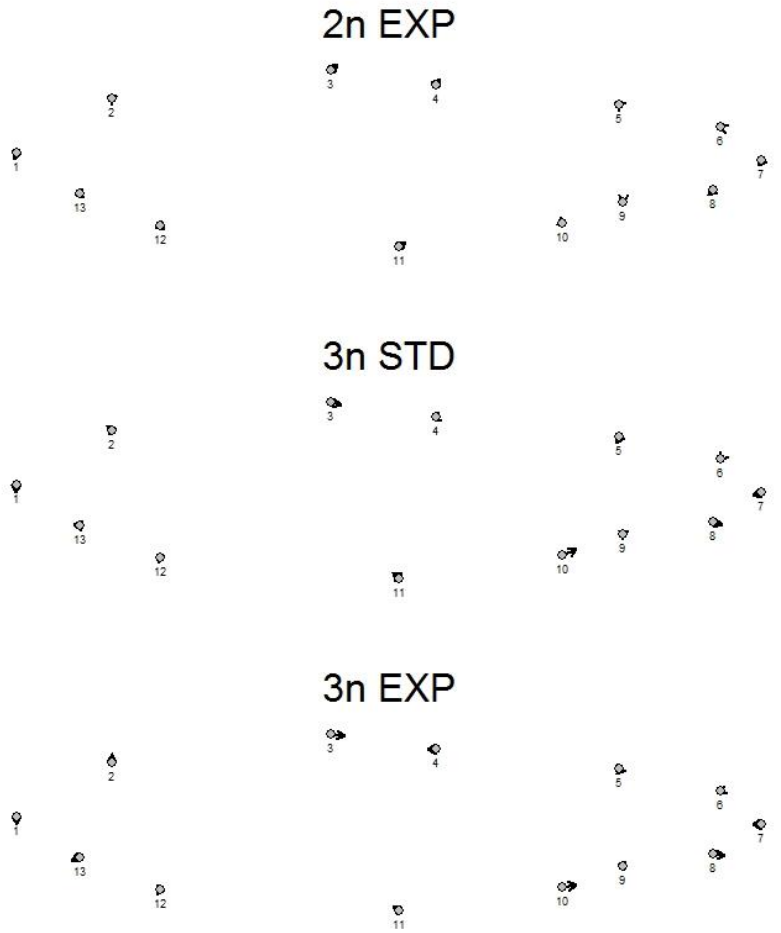


Figure 8.13. Landmark-based vector plots depicting magnitude (3x enhancement) and direction of mean shape deviation between diploid (undeformed fish from 2n STD) control and experimental groups (diploid fed experimental diet (2n EXP), triploid fed standard diet (3n STD) and triploid fed experimental diet (3n EXP).

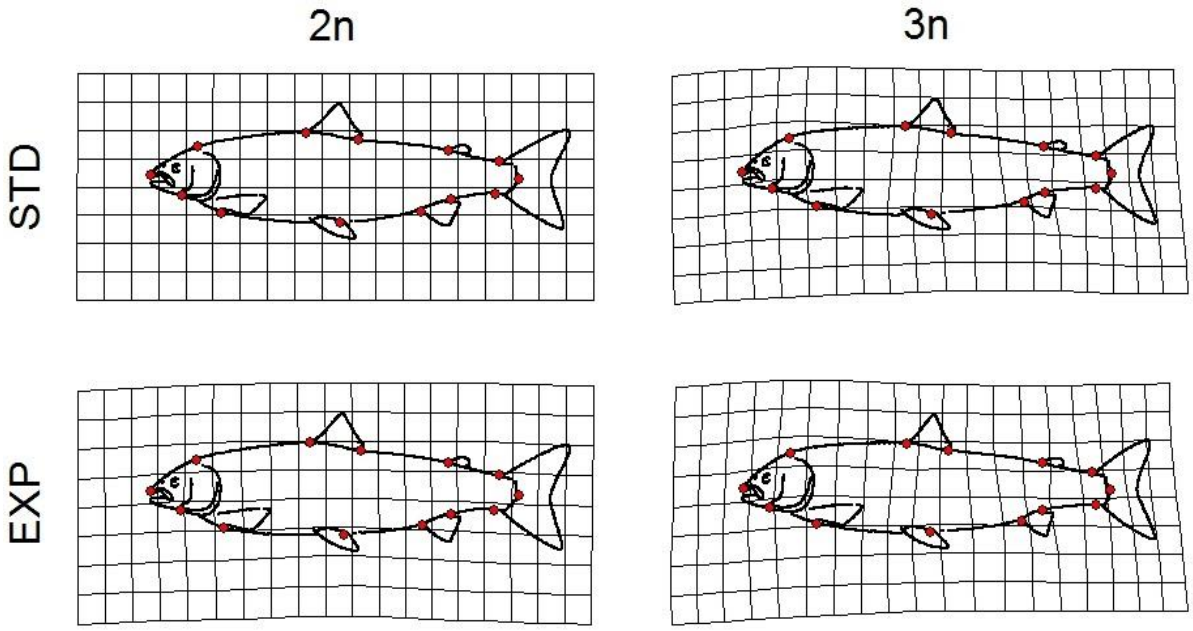


Figure 8.14. Thin-plate spline (TPS) deformation grids visualizing changes in mean shape between diploid (undeformed fish from 2n STD) control and experimental groups (diploid fed experimental diet (2n EXP), triploid fed standard diet (3n STD) and triploid fed experimental diet (3n EXP). Shape changes are illustrated as stretches, compressions and shearing of the entire landmark configuration relative to the control group and 3x enhancement are used to better visualize changes in shape among groups.

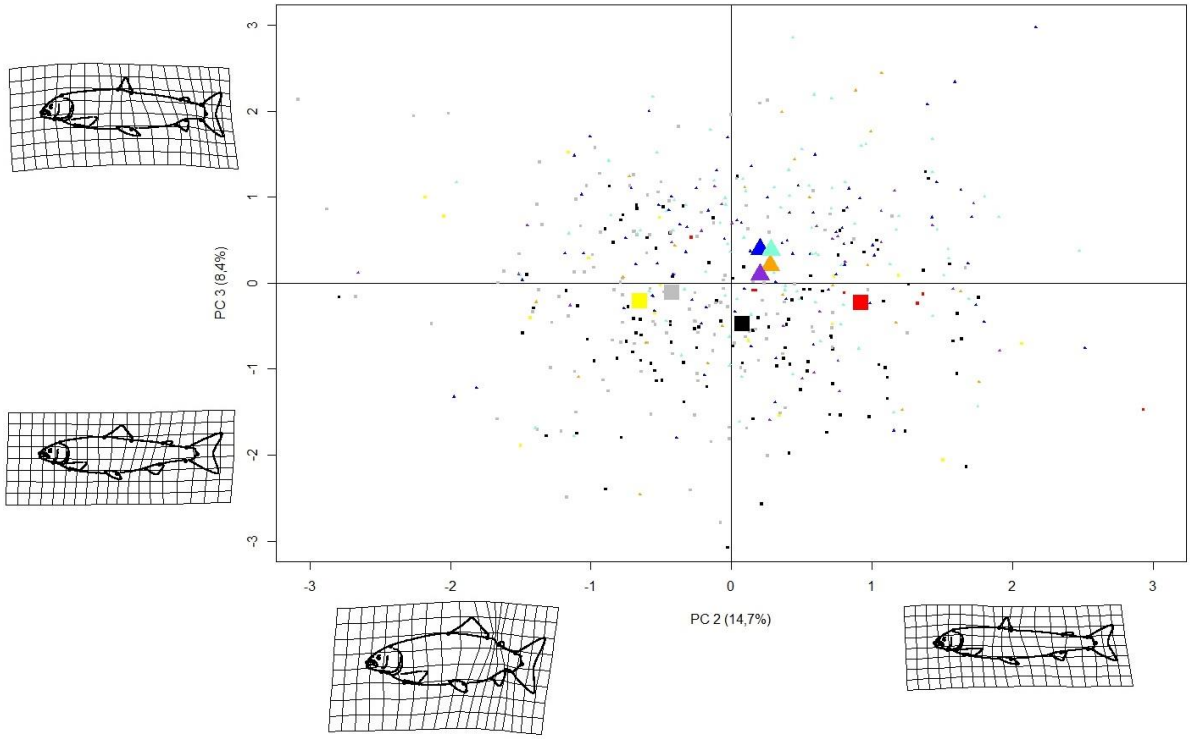


Figure 8.15. Scatterplot of ordination results depicting variation in geometric morphometry within and among groups, where 2n STD without deformities (black square), 2n STD with deformities (red square), 2n EXP without deformities (gray square) and 2n EXP with deformities (yellow square), 3n STD without deformities (blue triangle), 3n STD with deformities (orange triangle), 3n EXP without deformities (turquoise triangle) and 3n EXP with deformities (purple triangle). Shape variation along the ordination axes (PC2 and PC3) are illustrated by the shape configuration at ±1 standard deviation from the mean and larger figures represent the mean shape of the groups.

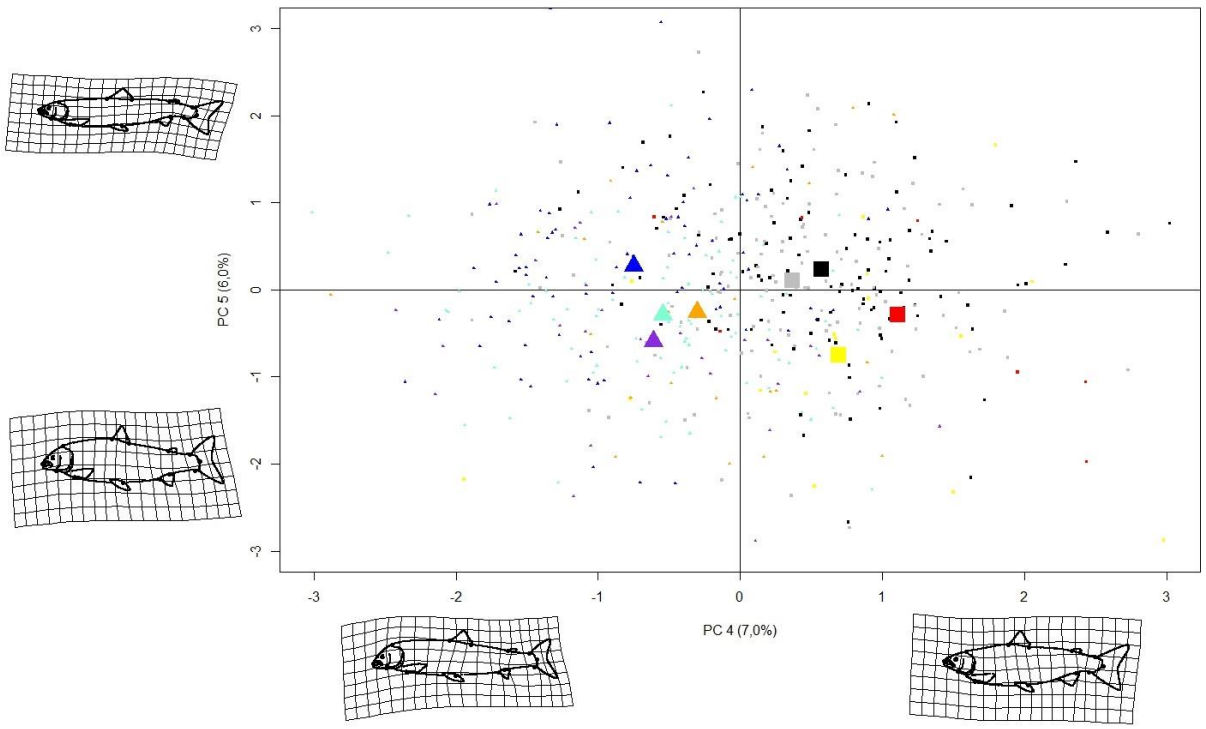


Figure 8.16. Scatterplot of ordination results depicting variation in geometric morphometry within and among groups, where 2n STD without deformities (black square), 2n STD with deformities (red square), 2n EXP without deformities (gray square) and 2n EXP with deformities (yellow square), 3n STD without deformities (blue triangle), 3n STD with deformities (orange triangle), 3n EXP without deformities (turquoise triangle) and 3n EXP with deformities (purple triangle). Shape variation along the ordination axes (PC4 and PC5) are illustrated by the shape configuration at ±1 standard deviation from the mean and larger figures represent the mean shape of the groups.

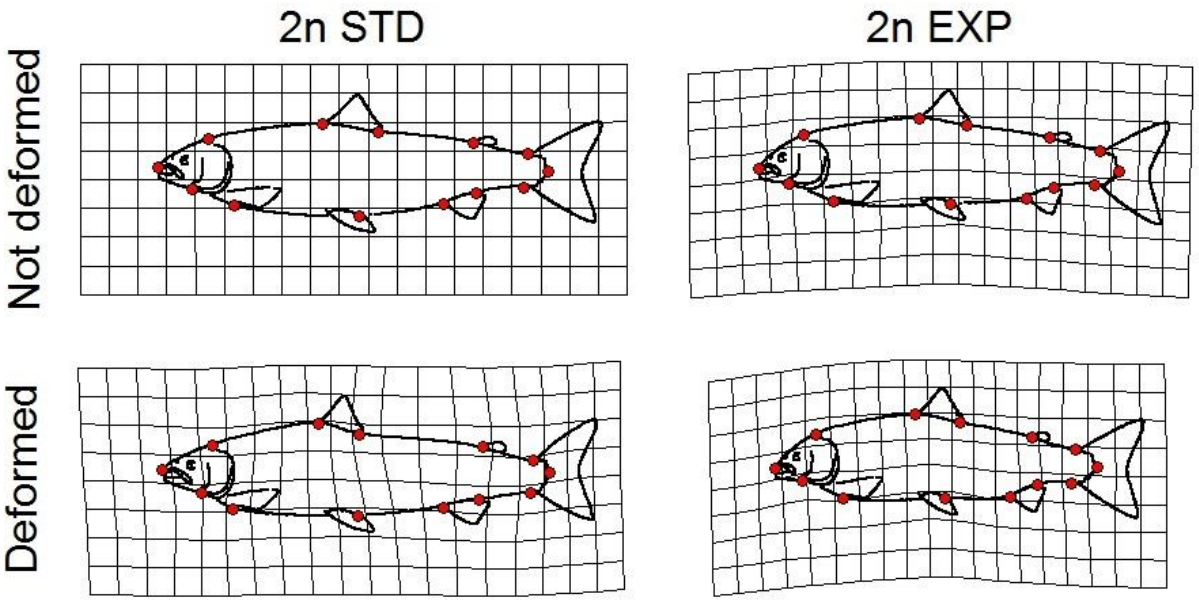


Figure 8.17. Thin-plate spline (TPS) deformation grids visualizing changes in mean shape between diploid (undeformed fish from 2n STD) control and diploid fed standard diet with deformities, diploid fed experimental diet without deformities and diploid fed experimental diet with deformities. Shape changes are illustrated as stretches, compressions and shearing of the entire landmark configuration relative to the control group and 5x enhancement are used to better visualize changes in shape among groups.

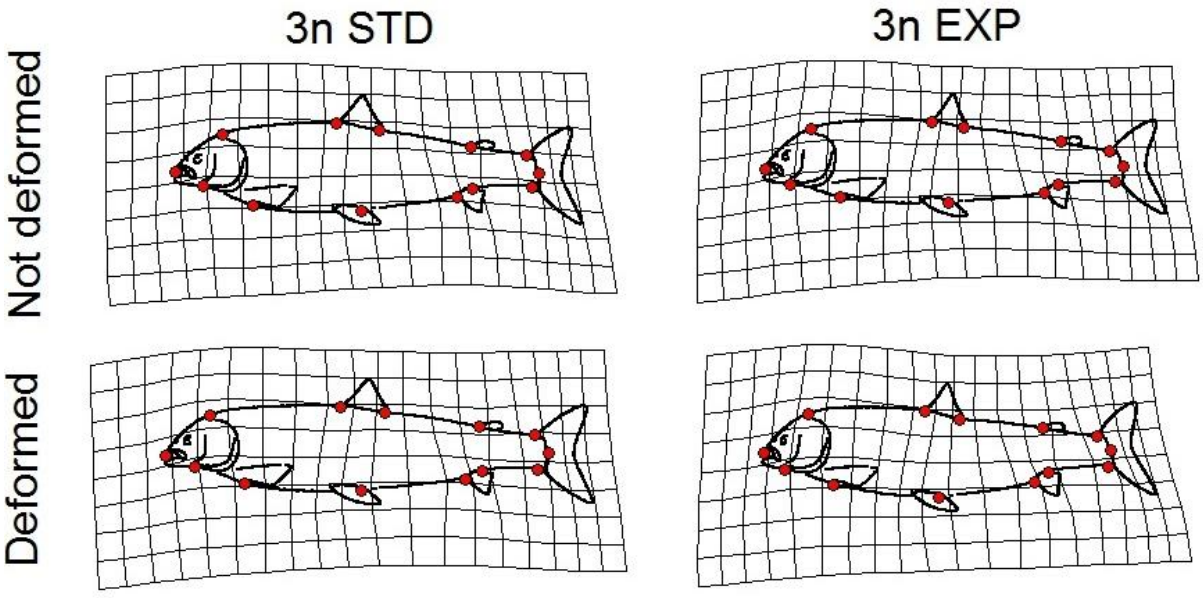


Figure 8.18. Thin-plate spline (TPS) deformation grids visualizing changes in mean shape between diploid (undeformed fish from 2n STD) control and triploid fed both diets with deformities and triploid fed both diet without deformities. Shape changes are illustrated as stretches, compressions and shearing of the entire landmark configuration relative to the control group and 5x enhancement are used to better visualize changes in shape among groups.

# 9 Discussion

The main goal of this thesis was to investigate whether diets containing high levels of phosphorus and hydrolyzed fish proteins may affect the prevalence and type of skeletal anomalies in diploid and triploid Atlantic salmon and how this may translate in measurable body shape differences. The final goal was to determine the degree of influence that ploidy, diet and/or skeletal deformities could have over such morpho-anatomical features in Atlantic salmon smolts using geometric morphometrics tools.

## 9.1 Fish size and condition

The fish used in this thesis originated from material collected within a research project focusing on the functional ontogeny and physiology of the digestive system in juvenile diploid and triploid Atlantic salmon (FUNGUT project) for which full data on fish performance including survival and growth were reported by Peruzzi *et al.* (2018). Fish used in Peruzzi *et al.* (2018) were smaller (range: 53-66g) and shorter (range: 16-18cm) compared to the fish used in the thesis and they had a higher condition factor (range: 1.22-1.27). This is probably because of critical selection of individuals under radiological inspection. Most of the small fish (under 30-35g) where thrown out if they were too difficult to analyze.

## 9.2 Skeletal anomalies

Spinal deformities were the most common skeletal anomaly found in this study. Both opercular shortening and LJD was low in all groups if any was observed. Interestingly, despite the low numbers of fish showing such anomalies, there was a significant diet effect on opercular shortening with fish fed the EXP diet being, although only marginally ($P = 0.04$) more affected than fish fed the STD diet, independently from their ploidy status. Peruzzi et al. (2018) reported low occurrence of opercular shortening in salmon parr (range 5-9%) and none at smolt stage suggesting that the fish had recovered from such a condition. If such, in the present work, it appears that the fish might had better recovery on standard diet then the experimental diet. One additional study has reported that Atlantic salmon have the ability to recover from opercular shortening (MacLean 1999), but the literature on this topic is scarce and further work might be required.

Lower jaw deformities (LJD) were only observed among triploids, as well as the incidents were low (≤2%). The low rearing temperatures combined with high available phosphorus might have had an effect on the development, especially the amount of available

phosphorus has been shown to affect the development of LJD in both ploidies. This agrees with earlier studies done on triploids (Benfey 2001, Sadler *et al.* 2001, Leclercq *et al.* 2011, Fraser *et al.* 2015, Amoroso *et al.* 2016a, Fjelldal *et al.* 2016).

A low percentage of individuals showed what they appeared to be hyper-radiodense vertebrae but because of the low numbers and degree of uncertainty regarding these observations the data were not statistically analyzed (Appendix Table 11.1). The reason behind the uncertainty being in the quality (contrast and light) of the x-ray pictures. Hyper-radiodense vertebrae are caused by ectopic cartilage that replaces the adipose tissue inside the trabecular network and has been observed in earlier studies on Atlantic salmon in freshwater phase (Helland *et al.* 2006).

There were no incidents of kyphosis in the control group (2n STD), as well as the frequency in the experimental groups were low (range: 1-3%). All observations of kyphosis were of a mild degree and were classified as a weak unnatural bend in the spinal column under x-ray inspection that could not be observed from an external view. Lordosis, kyphosis and scoliosis have been observed in earlier studies, but at a degree of severity that they could be observed externally. In farmed European sea bass (*Dicentrarchus labrax* L.), the spinal anomalies are usually of a lethal degree and may have a major impact on the survival in the early stages (Koumoundouros *et al.* 2002). Similar was observed by Sadler *et al.* (2001) in Atlantic salmon before first feeding, and especially in triploids that are of greater predisposition to develop spinal deformities and could be an explanation why there are different mortality rates between ploidy. But visible spinal deformities that develop after first feeding have been reported to be of a non-lethal degree and may be displayed throughout the individual's development (Sadler *et al.* 2001, Amoroso *et al.* 2016a). This agrees with findings in present work.

Radiological examination of vertebral deformities showed that triploids had significant less vertebrae number than diploids as reported by Peruzzi et al. (2018) and in agreement with other studies on triploid Atlantic salmon (Fraser *et al.* 2015, Amoroso *et al.* 2016a, Smedley *et al.* 2016). Recently, Baeverfjord *et al.* (2018) suggested that a lower number of vertebrae may influence the incidence of skeletal deformities in salmonids. Triploids had significant ploidy effect and higher incidents of spinal deformities than diploids but with no dietary effect. These findings agree with earlier studies on diploid and triploid Atlantic salmon during the freshwater phase (Fjelldal & Hansen 2010, Fraser *et al.* 2013, Fraser *et al.* 2015, Amoroso *et al.* 2016a, Benfey 2016, Fjelldal *et al.* 2016). Interestingly, Peruzzi *et al.* (2018) reported a significant

ploidy effect similar to this thesis, but also reported a ploidy diet interaction, as well as a large difference in the number of deformities reported in the experimental groups that were fed hydrolyzed fish protein. This did not agree with the findings in present work. Possible reasons for the different results in the occurrence of spinal deformities while working on fish from the same experiment could be that the number of fishes were reduced from 10 to 5 per plate in the x-ray screening, as well as a brand-new digital plate was used for the thesis. This may have provided a better resolution of the spinal column under image analyzes. Other factors could be that the computer screens used for image analyzes also had different resolution that gave better quality (contrast and light) of the x-ray image. Human error could cause major differences in results, with a less experienced person looking at skeletal deformities that over or under classifies certain deformities. The fish used in the thesis were general larger in all groups compared to Peruzzi *et al.* (2018) and that might have had an effect on the results. Larger fish (>50 grams) in general have more or fully calcified bone structure, and even with poor quality pictures, there was still possible to analyze the spinal column (Baeverfjord *et al.* 2018). There were also two to three times more individuals per group in the thesis, which could have provided a better insight into the incidents of skeletal deformities. Each factor mentioned could contribute in some degree to a different result and may be the reason why there was a significant interaction between diet and ploidy in Peruzzi *et al.* (2018) and not in the thesis. Other differences reported under the current work that did not fully agree with earlier studies. For example, detailed spinal column examination showed that most of the deformities occurred in the most anterior part (regions R1 and R2) of the fish while other studies reported more deformed vertebrae in the posterior part (R3 and R4) of the spinal column (Fraser *et al.* 2015, Amoroso *et al.* 2016a). Amoroso *et al.* (2016a) suggested that higher incidence of spinal deformities in the caudal region in the Atlantic salmon could be linked to dietary phosphorus deficiency. This could provide an explanation why different studies find deformities in different regions of the spinal column (Fraser *et al.* 2015, Amoroso *et al.* 2016a, Fjelldal *et al.* 2016, Peruzzi *et al.* 2018). Peruzzi *et al.* (2018) examined the dietary digestibility in the feed used under this study and found that all diets contained high concentrations of phosphorus and had high bioavailability. Fjelldal *et al.* (2016) studied the prevalence of spinal deformities in diploid and triploid Atlantic salmon fed three different experimental feeds with low (7.1g $kg^{-1}$), medium (9.4g $kg^{-1}$) and high (16.3g $kg^{-1}$) concentration of phosphorus. They found that most spinal deformities were located in the caudal region when fed a diet low in phosphorus, but when fed medium or high phosphorus diets, most of the deformities were found to the anterior/cranial part (V1-30) of the

spinal column. Their triploids fed with medium phosphorus concentration showed more deformed vertebrae in the trunk region than diploids, but they reported that there were no typical trends where the deformities developed in their high phosphorus group. This agrees with findings in present work, but the proportion of classified deformities that were found, only type 8 deformity (multiple side compression and fusion) agrees with their findings. Fjelldal *et al.* (2016) reported in general more of all other types of deformities.

Several studies on triploid Atlantic salmon have reported a relative high proportion of skeletal deformities among their treatment groups, where the number of reported deformations in triploids and diploids exceed 40% and 20%, respectively (Fraser *et al.* 2013, Smedley *et al.* 2016). In this study, diploids and triploids fed a standard commercial (STD) diet showed relative low number of spinal deformities (Diploids, 5.83 ± 1.01 and triploids, 22.12 ± 2.47) compared to other studies on triploid Atlantic salmon. Higher rearing temperatures under critical stages like egg incubation, start-feeding and early growth in freshwater are usually the most important factor for increased skeletal deformities and many studies have seen a correlation between rapid growth and the occurrence of skeletal deformities (Amoroso *et al.* 2016a). This fact, in connection with genetic differences in the background of the fish, could provide an explanation why in this present study there are less skeletal deformities seen between the standard diet groups, irrespective of ploidy. Other dietary factors such as increased phosphorus availability can have great effects on skeletal deformities as reported in Fjelldal *et al.* (2016). These authors showed that the occurrence of skeletal deformities was much greater with low (diploids 35%, triploids 56%) or medium (diploids 17%, triploids 45%) phosphorus concentration contra high (diploids 8%, triploids 10%) concentration. As mentioned earlier, Peruzzi *et al.* (2018) examined the apparent digestibility (ADC) of diets used in the present study and found that the phosphorus concentration was around 18g $kg^{-1}$. Even with the high concentration of phosphorus and apparent high bioavailability in both ploidies in the current study, the amount of skeletal deformities found among triploids was still significantly higher than in diploids (Figure 8.6 and Appendix Table 11.1).

## 9.3 Shape analysis

This study revealed a significant difference in shape between ploidy and diet groups, as well as a correlation between size and shape amongst groups. The first axes of shape change is related to a degree of body curvature (Figure 8.10). Carpenter (1996) suggested that such shape change was probably caused by “arching effect” of fish bodies. He did a morphometric study

on lethrinid fishes and concluded that such shape differences might be a measurement artifact, resulting from problems in the preservation of the specimens, or be from a functional explanation. Valentin *et al.* (2008) did a study on redfishes (genus *Sebastes*) to investigate the arching effecting and whether specimens' posture, specimen preservation (freezing) or biological factors were responsible for the effect. Their results suggested it was not related to the preservation method (freezing) or a biological factor (size or species), but rather due to slight posture differences between fishes during landmark capture. Since this study was not initially designed for morphometric analysis, the right procedures for preparing specimens for such analysis were not followed (e.g. fins pinned down) and the variation of the first axes are probably from "arching effect" or posture differences, but it should not be excluded that some underlying functional explanation like spinal deformation are present in the variation. Especially can this be observed in the diploid EXP shape (Figures 8.14 and 8.17), and the reason might be that diploid EXP was the largest group (153 individuals) and would express most of the posture differences observed since it contained 37.2% of the variation. Posture differences can especially be observed for shape variation in the tail fin region (R4) that are explained by PC4 and PC5. The second axes can be interpreted as body stretching and contracting along the anteroposterior axis and the dorsoventral axis, especially in the tail region (R3). Some of the variation can be explained by the difference in condition factors between ploidies, but diploid fed EXP diet are the only group that shows a minor "short tail" tendencies (Figures 8.11 and 8.15). Short tail is characterized by homogeneously compressed vertebrae throughout the posterior part of the spine (Witten *et al.* 2005). In seawater, the incidents of caudal compressed vertebrae usually increase with increasing fish size, and the shape analysis may have revealed an early developmental stage of short tail. The cause of this condition is not yet established but are a well-known problem in Atlantic salmon farming and the co-occurrence with various other skeletal deformities makes it more difficult to find a direct cause of the short tail deformity (Baeverfjord *et al.* 2018). There were higher proportions of severe deformities (type 6 and 8) in the triploid groups and there might be a correlation between the number of observed deformities in the posterior caudal trunk region (Figure 8.10) and the compression around the dorsal fin (landmark 3 and 4), especially in the triploid EXP group (Figure 8.14 and Appendix Figure 8.5). Triploids fed both diets had a minor stretch between landmark 2 and 3 compared to the control and could be caused by the deformities observed in R2 region (Figure 8.10). Triploids fed EXP also seemed to have a minor compression between landmark 2 and 3 compared triploids fed STD (Figure 8.16). This might be caused by the anterior peak seen in

Figure 8.10 in R2 region from the triploid EXP group and not triploids fed STD but could also be affected by the compression of the dorsal fin. Both triploid groups have their cranial trunk bending slightly downward, like the shape shown for PC3 and PC4, while diploids were more drown to the opposite shape in the same axes. Similar observation has been seen in other experiments on farmed fish reared in tanks where the bend could be caused by fish feeding at the bottom of rearing tanks while under the most critical and intense growth stage in their life cycle (R. Primicerio, personal communication, October 10, 2018). The bending observed could also be caused by underlying deformities in the posterior caudal trunk region in triploids. In Figures 8.15-8.18, all individuals that were classified as deformed have been isolated from ploidy and diet groups to study the direct effect between deformed and not deformed shapes. The major issue with the design is that each individual with just one deformed vertebra was classified as deformed under the analysis. One or two deformed vertebrae will probably have a shape similar to the non-deformed grand shapes and thereby disrupt the true representation of shapes with moderate to severe deformities among groups. In Figure 8.7, where the severity of the deformed fishes is represented, less than half might reveal visible shape differences. Interestingly, the deformed diploids fed STD diet showed the most body shape difference compared to control, and this could be caused by the small sample size in that group (seven individuals). In such a small sample, a lot of variability will be visualized in the TPS grid.

There seems to be little diet differences caught by the components. By looking at PC5 there seems to be minor difference between diet groups that might be explained by a slimmer body shape among the groups fed STD diet (Figure 8.12). Groups fed STD were significantly larger than groups fed EXP and might have been further in the development of the slimmer and longer body shape of the smolt.

Lower jaw index showed no large differences between measured values, but there was a significant ploidy effect ($P<0.001$), as well as a significant diet ploidy interaction ($P = 0.021$). The differences are probably from phenotypic variation in the genome in the families used as well as there could be cranial shape difference between diploids and triploids (Figure 8.13).

Morpho-anatomical differences between diploids and triploids have been seen in other species like the common carp (*Cyprinus carpio* L.), bighead carp (*Hypophthalmichthys nobilis* Richardson), tench (*Tinca tinca* L.) and Indian catfish (*Heteropneustes fossilis* Block). In Atlantic salmon the LJD is reported as the most commonly observed abnormality in triploids (Benfey 2016), and other shape morpho-anatomical differences has not been reported. This agrees with the findings in this study were some LJD were found, but only in triploids, and

there was not enough to see any shape difference compared to the control. A problem with this design could be the different families used in the experiment. Phenotypic differences could contradict each other and hide the true differences in shape between triploid and diploids. But thus far, no studies have reported morphological differences in diploid and triploid Atlantic salmon before the onset of maturation or in the presence of a jaw deformity.

# 10 Conclusion

This study can conclude that a diet with 45% of the fishmeal replaced with hydrolyzed fish protein will potentially increase the amount of skeletal deformities in both triploid and diploid Atlantic salmon contra a standard diet containing high amount of phosphorus. It seems that a more important factor for reducing deformities would be lower rearing temperatures during the freshwater phase since there has been observed a correlation between rapid growth in triploids and the amount of deformities present. The increased amount of phosphorus has been shown to have an effect (Fjelldal *et al.* 2016), but the amount that can be absorbed are a limited factor. Thus, a combined effect with lower rearing temperature could be favorable to reduce deformities and would have less impact on effluent freshwaters. The use of feed with high phosphorus concentration will lead to an increased phosphorus concentration in the water. Phosphorus are a limited resource in primary production of salmon in freshwater, and high levels of phosphorus can lead to eutrophication (enrichment of water by nutrient salts that causes structural changes to the ecosystem) of freshwater lakes, rivers and streams (Peruzzi *et al.* 2018). If diets with high phosphorus are to be used in freshwater production of triploids, some sort of wastewater treatment should be required to not disturb the natural flora in effluent freshwater.

There were only slightly visual differences in shape between ploidy and diet groups. Most of the variation caught by the multivariate analysis were probably from placement differences ("arching effect") on the digital plate and the size differences seen between groups. Phenotypic variation within and amongst groups are also a variable that cannot be excluded as it might have had a major impact on the differences in shape. However, there was compression around the dorsal fin in both triploid groups, and triploid fed EXP had a compression between landmark 2 and 3. There might be a correlation between the number of severe deformities seen in the caudal trunk region in triploids and the compression observed in that area, especial around the peaks in Figure 8.10. Triploids also had more of a downward curving of the cranial trunk that might be caused by a bottom feeding behavior under development. It may also be caused by underlaying deformities in the cranial and caudal trunk (e.g. kyphosis). One of the extreme shapes in the second principal component axes found a severe compression in the tail region, where diploids fed EXP diet were the only group that favored that shape, but only minor shape differences could be observed. Overall, grand shapes of all deformed groups had a more compressed body shape than non-deformed groups (Figures 8.16-8.18). Further analyses are

needed to identify which factor (genetic, skeletal or ploidy) had an effect on shape differences observed between groups.

Triploids are seen as the only current feasible method to produce sterile fish and are a secondary security against introgression of genes in the wild population in the event of an accidental escape. If producers are to adopt the practice of producing triploids, the optimal rearing condition needs to be known and more commercial oriented research are required. When it comes to using hydrolyzed feed, earlier studies have shown that the amount used in feed can have a negative effect in performances compared to ordinary diets if not the ideal concentration are found for the specific species in question. More groups with different concentration of hydrolyzed protein are needed to find the optimal concentration if they are to be applied in aquaculture feed.

# 11 Appendix

## 11.1 Skeletal anomalies

Table 11.1. Fish anomalies (Vertebrae numbers, operculum shortening, jaw deformity (external)), lower jaw index (LJI) and vertebral deformities (x-ray) of diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) fed fish meal (STD) diet and an experimental (EXP) diet containing high amount hydrolyzed fish protein. Numbers in bold and italic denote a significant difference (P<0.05) and data are presented as means ± SEM (n=3).

| | **Groups** | | | | **P-values** | | |
|---|---|---|---|---|---|---|---|
| | 2n STD | 2n EXP | 3n STD | 3n EXP | Ploidy | Diet | Interaction |
| **External inspection** | | | | | | | |
| **Operculum shortening (%)** | 0.00 ± 0.00 | 3.41 ± 1.71 | 0.79 ± 0.79 | 2.34 ± 1.29 | 0.972 | ***0.038*** | 0.487 |
| **Lower jaw deformity (LJD; %)** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.98 ± 0.98 | 1.96 ± 1.96 | 0.057 | 0.637 | 0.603 |
| **Radiological inspection** | | | | | | | |
| **Vertebrae (n)** | 58.03 ± 0.43 | 58.00 ± 0.48 | 57.82 ± 0.45 | 57.78 ± 0.42 | ***0.002*** | 0.638 | - |
| **Lower jaw index (LJI)** | 0.98 ± 0.00 | 0.98 ± 0.01 | 0.97 ± 0.01 | 0.98 ± 0.01 | ***<0.001*** | 0.408 | ***0.021*** |
| **Vertebral deformities (%)** | 5.83 ± 1.01 | 11.77 ± 4.22 | 22.12 ± 2.47 | 24.58 ± 0.99 | ***<0.001*** | 0.263 | 0.661 |
| **Hyper-radiodense (%)** | 1.71 ± 0.86 | 3.19 ± 1.10 | 3.57 ± 2.06 | 3.08 ± 1.94 | - | - | - |
| **Lordosis (%)** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | - | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Kyphosis (%)** | 0.00 ± 0.00 | 1.19 ± 0.60 | 3.15 ± 1.71 | 0.98 ± 0.98 | - | - | - |

## 11.2 Shape analysis

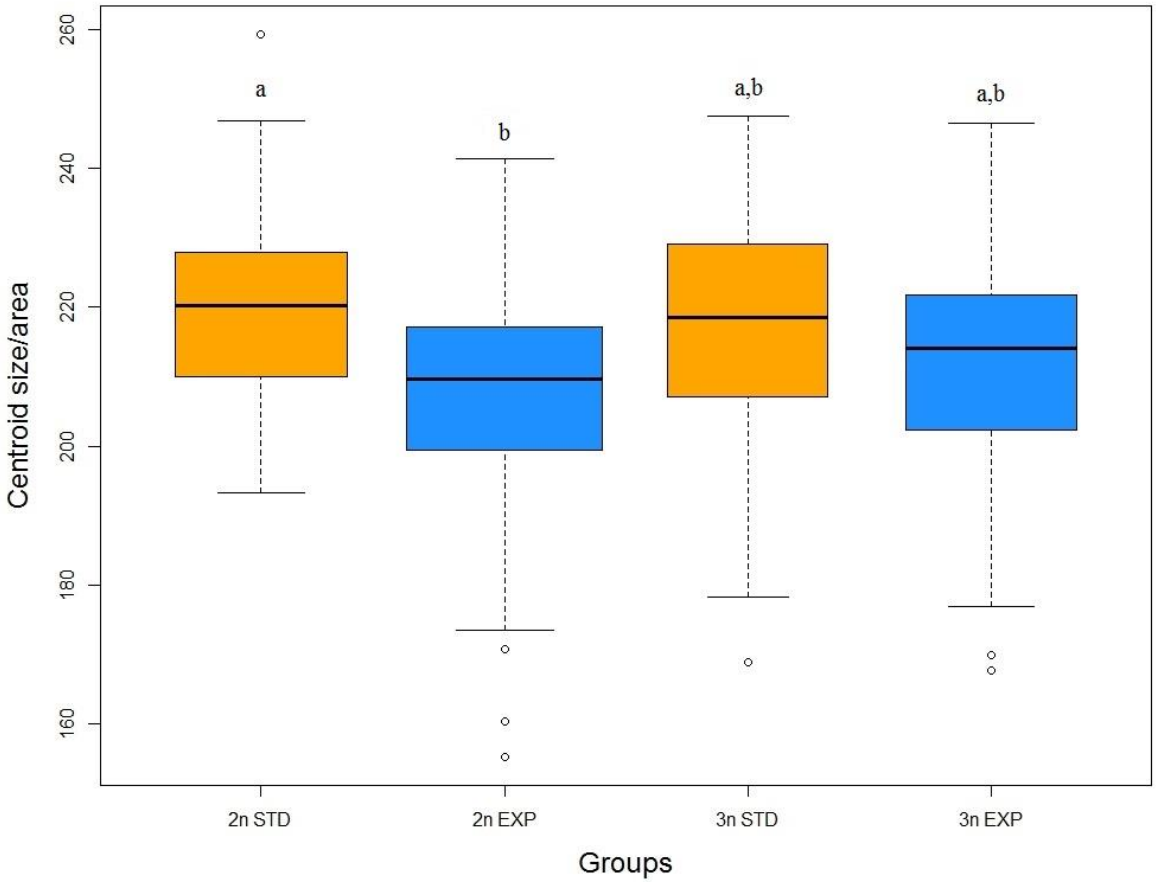


Figure 11.1. Body centroid size of diploid (2n) and triploid (3n) Atlantic salmon (*Salmo salar* L.) fed fish meal (STD) diet and an experimental (EXP) diet containing high amount hydrolyzed fish protein. Different letters denote a significant difference ($P<0.05$) and data are presented as means ± standard deviation.

Table 11.2. Procrustes distances between mean group shapes among ploidy and diet groups.

| **Procrustes distance in pixels** | **Groups** | | | |
|---|---|---|---|---|
| | 2n STD | 2n EXP | 3n STD | 3n EXP |
| 2n STD | 0.000 | 0.007 | 0.011 | 0.011 |
| 2n EXP | 0.007 | 0.000 | 0.011 | 0.012 |
| 3n STD | 0.011 | 0.011 | 0.000 | 0.005 |
| 3n EXP | 0.011 | 0.012 | 0.005 | 0.000 |

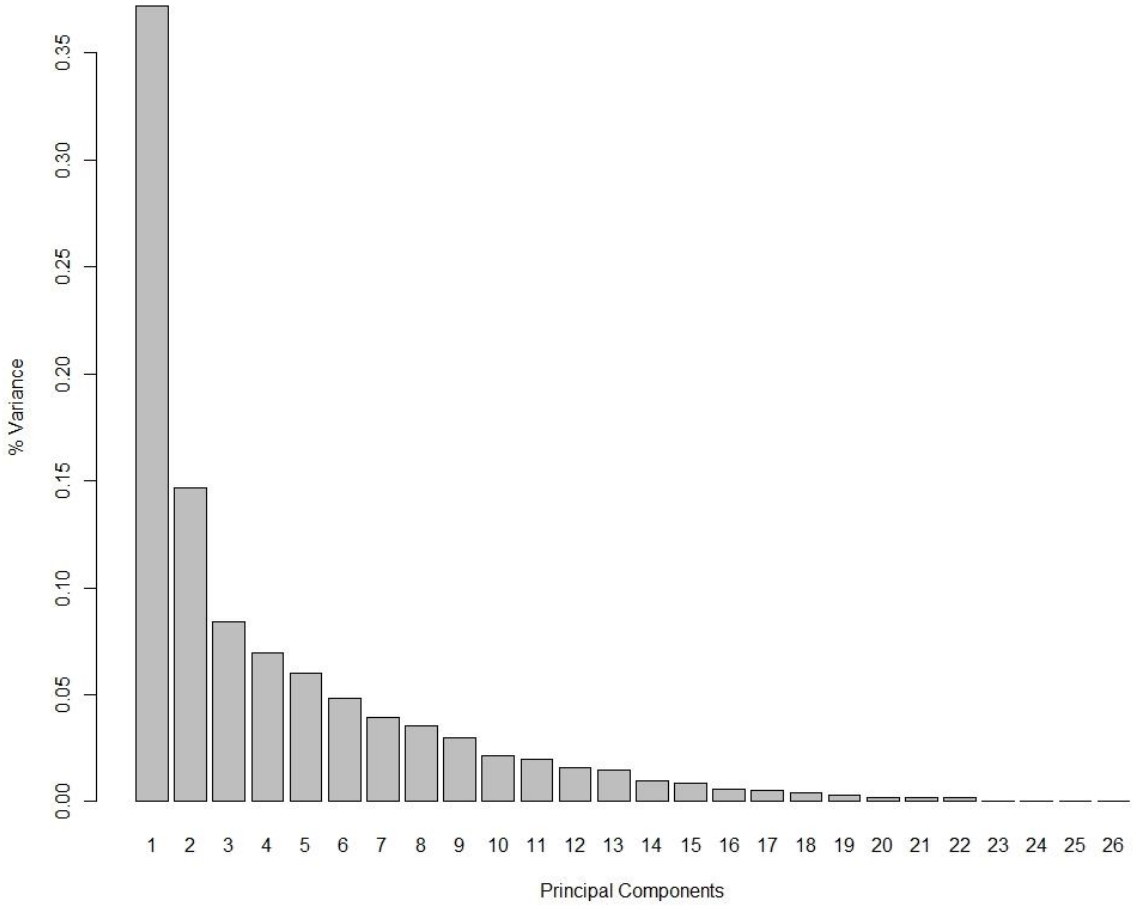


Figure 11.2. Proportion of variance explained by each principal component.

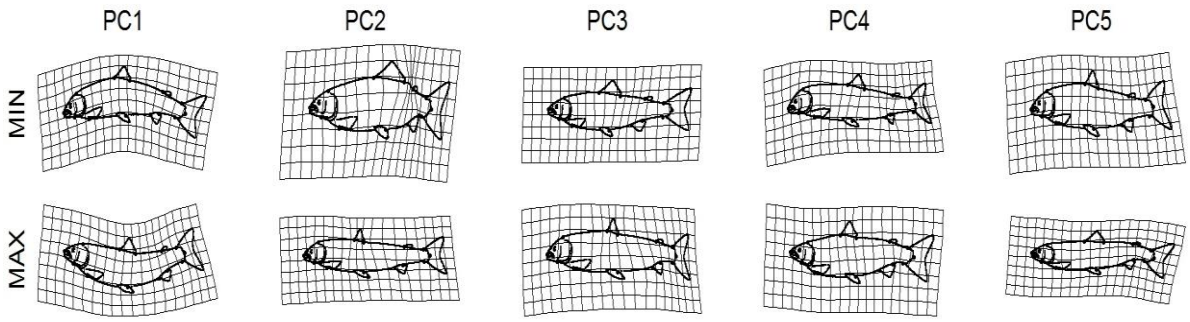


Figure 11.3. Shape differences in all principal components (PC) that explain at least 5% of the variance. MIN equals to -3 standard deviation from the mean and MAX equals to +3 standard deviation from the mean.

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