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

# A comparison of the ontogeny of the digestive system and growth of juvenile diploid and triploid Atlantic salmon (*Salmo salar* L.) fed a standard diet and an experimental diet containing high proportions of hydrolysed proteins

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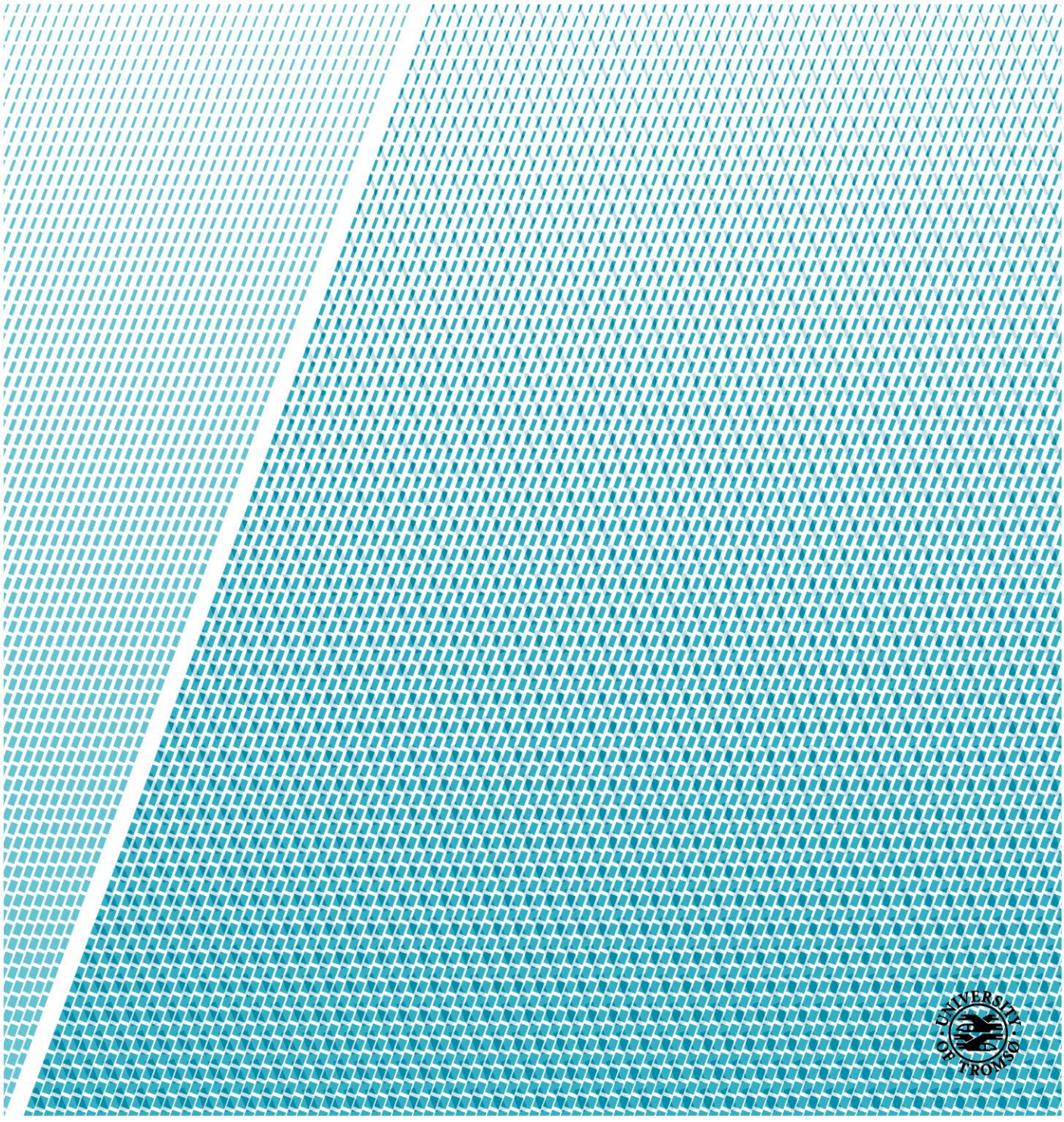

# Forord

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Lars Olav Ulleberg

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# Abstract

The purpose of the study was to evaluate and compare the effect of different diets on the gut structure of diploid and triploid juvenile Atlantic salmon (*Salmo salar*), and analyze the overall performance in terms of growth and survival from hatch until the end of smoltification.

At the beginning of the experiment, fish were divided into two groups, one diploid group and one triploid group. Four weeks after start feeding the fish were divided into four groups, two groups of different ploidy fed a standard diet, whereas the other two groups fed an experimental diet in which 45% of fishmeal (FM) fraction was replaced with hydrolysed proteins. The triploid fish fed standard diet (group 3) were the best performing after the initial phase and throughout the end of the experiment, both in terms of growth and survival. The diploid and triploid fish fed standard diet (group 1 and 3) were also superior in terms of survival compared to the fish fed experimental diets (group 2 and 4). Comparisons of the ontogeny of the digestive system between groups with respect to ploidy and diet revealed no obvious differences despite some variable trends. Under the present experimental conditions, the same conclusions can be drawn for the histomorphological traits measured in the digestive organs of these fish that were overall comparable.

Results in present work show that the use of experimental diets containing high levels of hydrolysed proteins overall did support but not enhanced growth in triploid or diploid salmon from start feeding until the end of the smoltification process.

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# 1 Introduction

## 1.1 Scientific background

The risk of interactions between farmed and wild stocks of Atlantic salmon (*Salmo salar*) increases as Norwegian aquaculture expands. Farmed escapees have both economic and environmental impact on the aquaculture industry (Glover *et al.*, 2017). Another problem is the onset of sexual maturation which results in poor flesh quality due to energy allocation towards the gonads rather than somatic growth. One approach to solve these problems has been the production of sterile (triploid) Atlantic salmon, and the performance of triploid salmon is currently evaluated on a commercial scale in Norway (Hansen & Fjelldal, 2015). Recent studies have revealed significant differences in gut morphology between diploid and triploid fish raised on the same diets and under similar conditions (Peruzzi *et al.*, 2013; Peruzzi *et al.*, 2015). For the triploid salmon, little is known about the nutritional requirements and optimal rearing conditions are not identified. Detailed information of the ontogeny of the digestive system is lacking for triploid salmon, as is it for triploids in other species, in particular histomorphology, key enzyme activity and gene expression. To optimize growth it may be necessary to use a specialized diet that could be in synergy with the gut morphology of the triploid salmon, and thus assess the functional ontogeny and physiology of the fish (Peruzzi *et al.*, 2015).

This master thesis is a part of a NFR financed project studying the functional ontogeny and physiology of the digestive system in juvenile diploid and triploid salmon (*Salmo salar*). Nofima manages the project and the partners are UiT The Arctic University of Norway, Salmobreed AS, Skretting AS, Nord University (Bodø) and Polytechnic University of Valencia (Spain). Representing UiT The Arctic University of Norway, this master thesis is more specifically aimed at comparing the performance of diploid and triploid Atlantic salmon (*Salmo salar*) fed a standard diet for diploid fish and an experimental diet containing high proportions of hydrolysed proteins for triploid fish.

By analyzing fish growth, survival and digestive tract morphology from hatching until smoltification, the goal is to highlight any differences in the ontogeny of the digestive system between the diploid and triploid salmon and decide if the triploids perform better on the diet containing more digestible proteins.

## 1.2 Atlantic salmon

### 1.2.1 Atlantic salmon: the species

Atlantic salmon (*Salmo salar*) is an anadromous carnivorous fish species belonging to the family Salmonidae (Luna & Kesner-Reyes, 2017). They are naturally distributed in the Atlantic Ocean, lakes and connected rivers, and spawn in rivers between October and November (Kottelat & Freyhof, 2007). After hatching in fresh water they will stay in this habitat for one to several years before becoming smolts migrating to the ocean (Thorstad *et al.*, 2010). After this feeding and growth period (1 to 4 years) at the sea they return to fresh water to spawn. The smoltification process is not just a physiological process which makes the salmon ready for seawater, but also includes the appearance of various morphological characteristics. The transition from a parr to a smolt lowers the condition factor as a more torpedo shaped body is attained, the parr marks disappear and the salmon becomes silvery along the sides. Dorsally they are bluish green and ventrally they are white (Luna & Kesner-Reyes, 2017).

### 1.2.2 Atlantic salmon: a cultured fish

In 2015 2.38 million tons of Atlantic salmon were produced worldwide (FAO, 2017). Almost 1.6 million tons are produced in Europe with Norway figuring as the largest producer with 1.3 million tons followed by the United Kingdom with 172,000 tons. The second largest continent is America with a total production of 750,000 tons with Chile standing for most of the production with 608,000 tons, and Canada with 120,000. Asia and Oceania are also producers of Atlantic salmon, but with relatively small quantities compared to the other continents (FAO, 2017). The Atlantic salmon is thus farmed within and outside its area of natural distribution where it is an exotic species (Eastern Atlantic – Northern America, Chile, Australia). The statistics is only based on Atlantic salmon, no other salmonids or other freshwater fishes are included.

## 1.3 Farmed fish escapees

### 1.3.1 Environmental consequences of farmed salmon escapees

As long as the fish farming takes place in the ocean, there is always a risk of fish escaping. In Norway where the fish is native, the risk of interaction between farmed Atlantic salmon and their wild counterparts is high, but in the other countries where the Atlantic salmon figures as

an exotic species this is not a problem when considering genetic interaction alone. But the escape of farmed fish can be negative in a number of ways; escaped fish, including triploids, can transmit diseases, compete for food and disrupt mating (triploid males). This is a problem in all places where Atlantic salmon is produced (Scotland, Ireland, Canada etc.). Disease epidemics are more likely to occur in dense populations, and farmed salmon is the largest reservoir of known pathogens. The farmed salmon figures as vectors for transmission of pathogens into new geographical areas (Johansen *et al.*, 2011). There will always be a risk of disease interaction between farmed and wild fish due to the open net pens; when considering salmon this can be even more unfavorable after the fish escapes due to direct interaction. The transmission of diseases between different species must also be considered. Fjelldal and co-workers (2014) proved that triploid male Atlantic salmon does interact with wild females, and they show the same spawning behavior as mature diploid wild males.

### 1.3.2 Genetic consequences of farmed salmon escapees in Norway

In Norway, approximately 1.3 million tons of Norwegian Atlantic salmon were sold in 2015 (FAO, 2017) and the production is expected to increase every year. Today there are normally 200.000 farmed Atlantic salmon in each sea cage and the Norwegian wild salmon population is estimated to be around 520.000 (Miljødirektoratet, 2015). There are concerns about the negative consequences of genetic interactions between wild and escaped farmed Atlantic salmon in its native range of distribution (Benfey, 2015). Due to a large number of farmed salmon, a large escape event can therefore be disastrous for the wild Atlantic salmon population in Norway when considering the genetic consequences.

The farmed salmon are different from the wild populations in terms of genetic differences, phenotypic differences and lower reproductive success (Weir & Grant, 2005), the farmed salmon are not adapted to living in the wild. In the 1970's salmon from more than 40 Norwegian rivers were collected and used to make a domesticated salmon (Gjedrem *et al.*, 1991). Today, the different domesticated strains used by companies are very different from the fish they were originally founded on. The breeding program has changed the genetic makeup of the farmed salmon, due to selection of economically important traits (Solberg *et al.*, 2013).

Not only are the farmed Atlantic salmon different form the wild Atlantic salmon, but the wild populations are also divided into different meta populations (Jensen *et al.*, 2010; Glover *et al.*,

2017), and strong evidence for local adaptions in salmonids has been shown (Garcia de Leaniz *et al.*, 2007; Primmer, 2011). There are indigenous populations in local rivers and significant genetic change due to farmed salmon is a risk (Cotter *et al.*, 2000), the concern is based on the adaptive differences existing between native and farmed salmon, and possible reduction in survival when losing their diversity (Taylor, 1991). Farmed salmon offspring show lower survival in the wild, in addition to lowered fitness, which can be harmful to vulnerable populations (McGinnity *et al.*, 2003).

Escaped farmed salmon can move over large areas, and a number of studies have shown that farmed salmon does interact with wild salmon (Gausen & Moen, 1991; Carr *et al.*, 1997; Skaala *et al.*, 2012; Fjelldal *et al.*, 2014; Karlsson *et al.*, 2016; Glover *et al.*, 2017). Skaala and co-workers (2012) also demonstrated that farmed salmon progeny overall showed a significantly lower survival than hybrids and wild progeny. Surviving hybrids can continue to interact with wild populations and thus affect the wild population gene pool (Glover *et al.*, 2017), affecting their local adaption. The farmed salmon can also interfere with the wild population through competition and thus indirectly affect the natural production of wild progenies.

Norway has over 200 rivers which contain native Atlantic salmon populations (Glover *et al.*, 2013), many of these populations have been exposed to a large number of farmed escapees (Fiske *et al.*, 2006), and Glover and co-workers (2013) strongly suggested that the genetic changes observed are due to farmed escapees. It is reported (Skaala *et al.*, 2012; Glover *et al.*, 2013; Karlsson *et al.*, 2016) that there is enough information to talk about the consequences farmed escapees have on the genetic structure of wild salmon, rather than questioning if they actually have been interacting. The same study by Karlsson and co-workers (2016) revealed that there are no regions in Norway without farmed introgression, and demonstrated that the level of genetic introgression in wild populations is closely associated with the proportion of escapees over the last 25 years. Escapees must be considered as a considerable problem in aquaculture due to the possible detrimental genetic and ecological effects on wild populations (Svåsand *et al.*, 2007; Jensen *et al.*, 2010).

There are a number of different reasons for the escape of farmed Atlantic salmon. Jensen and co-workers (2010) showed that in Norway, following a period from 2006 to 2009, escapees (in number of individuals) were mainly due to structural failures (68%), followed by

operational failures (8%), while external factors and other factors represented smaller percentages. In the period 2001 - 2015 the number of escapees of Atlantic salmon have varied a lot, from lowest with 38,000 in 2012 to highest 921,000 in 2006, and in 2015 170,000 salmon escaped from Norwegian farms (Fiskeridirektoratet, 2016a). In proportion to the production in tons the number of escapees have declined over the years, but due to the ever increasing production the number of escapees have remained relatively high (Glover *et al.*, 2017) (Fig 1.1).

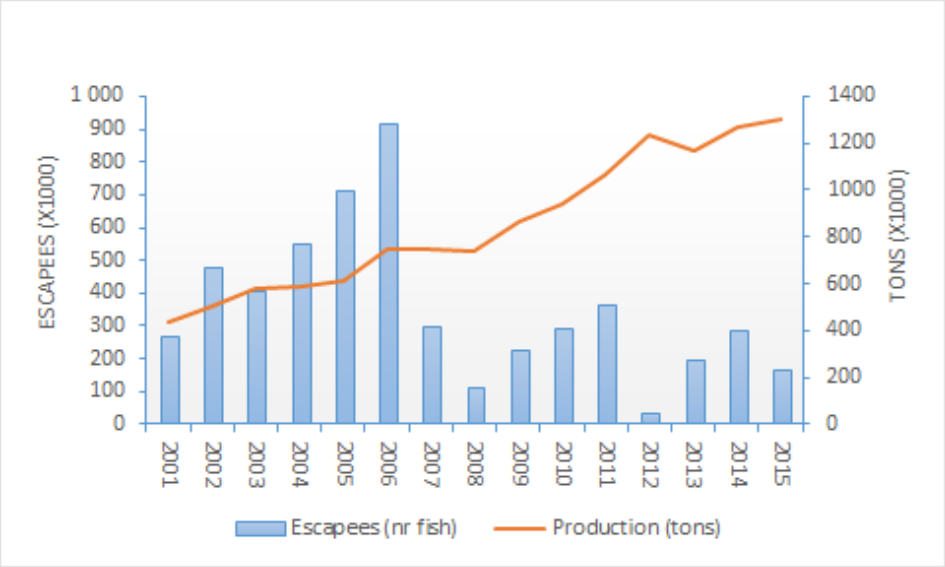


Figure 1.1. Overview of the number of escapes (x1000) and production in tons (x1000) of Atlantic salmon in Norway in the period 2001 – 2015. Adapted from Fiskeridirektoratet (2016a).

As the aquaculture industry expands, the sea cages can be hypothesized to be moved to more exposed areas, and thus be more vulnerable to storms and bad weather (Fiskeridirektoratet, 2016b). Using triploid farmed salmon will not solve or alleviate the problems related to disease transmission, competition for food and disrupting of interbreeding between farmed escapees and wild salmon, but triploids will stop genetic mixing, and thus preserve the integrity of the wild genetic gene pool, which is one of the main concerns in Norway.

## 1.4 What are triploids?

"Polyploids can be defined as organisms with one or more additional chromosome sets with respect to the number most frequently found in nature for a given species" (Piferrer *et al.*, 2009), in that respect triploids contain one extra set of homologous chromosomes, often received from the maternal part. Due to the extra set of homologous chromosomes, a larger cell nucleus is required and this often results in larger and fewer cells (Benfey, 2011). The

increased cell size seems to be compensated for; an example is the increased size of the erythrocytes which results in a decreased number of these cells. The hematocrit value will therefore remain the same (Tiwary *et al.*, 2004).

As early as in the 1940's manipulations of ploidy trials were carried out (Maxime, 2008), and the induction of triploidy is an alternative approach to produce sterility in animals, and is very useful for problems facing the aquaculture industry today. It's widely known that sexual maturation decreases body growth rates due to the fact that fish divert energy from somatic growth towards gonadal development (Piferrer *et al.*, 2009). The flesh quality is altered by sexual maturation (McClure *et al.*, 2007), in addition to the incidence of diseases also associated with sexual maturation (Piferrer *et al.*, 2009). The sterility observed in triploids is a result of an extra set of chromosomes which is retained in the egg nucleus interfering with meiotic division of the chromosomes early in gametogenesis (when cells enter meiosis). As a result of this, gonadal development is reduced and/or aneuploid gametes are produced, but these cannot produce viable offspring (Benfey, 1999; Tiwary *et al.*, 2004).

There is however differences between the sexes. Although neither males or females can produce functional gametes due to the odd number of chromosome sets, they differ both with respect to production of the gametes and development of secondary sexual characteristics (Benfey, 2011). Starting with the effects on the gamete production, most of the germ cells do not progress through the first meiotic prophase regardless of sex. Because of the triploid state, most of female germ cells will not progress through the first meiotic prophase whereas the male ones may undergo both meiotic divisions. As a result of this, the females display a small number of small primary oocytes scattered in a primordial gonad, and the males a reduced number of aneuploid primary spermatocytes (Benfey, 1999). Summarized, the ovarian growth in females is almost completely suppressed and they will not be able to produce eggs resulting in viable offspring, the few oocytes in females that undergo vitellogenesis being rarely ovulated (Benfey, 2011). The male testis however looks normal, but the milt is diluted and due to the aneuploid spermatozoa and resulting aneuploid embryos the offspring will not survive beyond hatching.

The difference observed between the sexes regarding development of secondary sexual characteristics is due to hormone production. The hormone producing cells in the gonads of male triploids are not affected by the triploid status, and consequently they will go through

the stages related to sexual maturation. The females however, are hormonally sterile and their gonads similar to that of immature fish. The female oocytes do not go through meiosis, and therefore will not reach the stage where the eggs are covered in hormone producing cells. Because of this they will not produce enough hormones to develop the sexual characteristics we see under normal sexual maturation (Cantas *et al.*, 2011). It is the combination of the lack of estrogen, gonadotropin and vitellogenin production that prevents female triploid salmon – as in the case of other cultured species - from becoming sexually mature (Benfey *et al.*, 1989; Cotter *et al.*, 2000).

## 1.5 Making triploids

One way to induce polyploidy in fish is to physically destroy the meiotic spindle to retain the second polar body within the egg. When fish eggs are released (oviparous fishes), they are blocked at metaphase stage of meiosis 2. The entry of spermatozoa leads to the resumption of meiosis 2. By applying a physical shock shortly after this phase, cell division can be suppressed and accordingly prevent the extrusion of the second polar body containing a second set of maternal chromosomes (Piferrer *et al.*, 2009). Done correctly and at the right moment this will result in production of triploid salmon eggs (Fig 1.2).

In fish, suppression of cell division is commonly achieved by either pressure or thermal treatments, the thermal shock being either a cold or a heat shock (Piferrer *et al.*, 2009). Pressure shocks have proven more successful, more effective and less harmful for the egg, and are most commonly used to induce triploidy in fish (Maxime, 2008; Piferrer *et al.*, 2009). When applying a pressure shock, the fertilized eggs are exposed to a short abrupt increase in hydrostatic pressure. The variables are timing, intensity and duration of the shock (Benfey, 1999; Piferrer *et al.*, 2009); the values for the different variables are temperature dependent and species-specific, and therefore a precise protocol is required for each fish species.

Different methods exist for determining ploidy level. Direct methods include genotyping of microsatellite DNA markers, measurement of DNA content and karyotyping figuring as perhaps the most accurate method, but very time consuming. Indirect methods include nuclear or cell size measurements as in flow cytometry (Piferrer *et al.*, 2009). As stated earlier, the nuclei in triploid cells contain 50% more DNA because of the extra set of maternal chromosomes, and the nuclei and cells are therefore larger than their diploid counterparts (Fraser *et al.*, 2012). Flow cytometry is a precise, rapid and direct method to measure DNA

content (Tiwary *et al.*, 2004; Piferrer *et al.*, 2009). Flow cytometry allows to estimate the DNA content of a large number of nuclei stained with specific fluorescent dyes, and thus determine if the nuclei derive from a triploid or diploid individual.

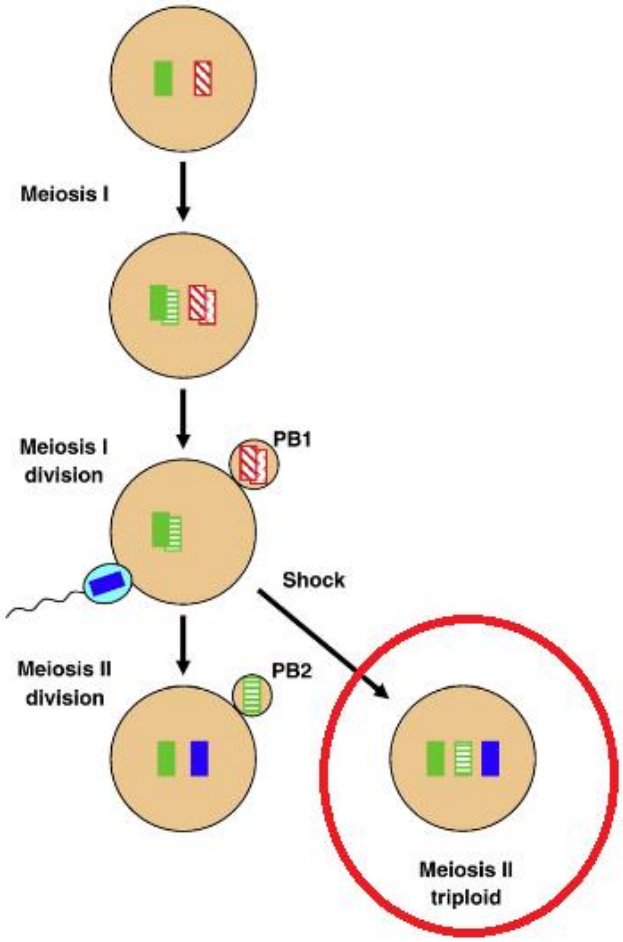


Figure 1.2. Methods of artificial induction of triploidy in fish by thermal or mechanical shocks via retention of the second polar body. Adapted from Piferrer *et al.* (2009).

## 1.6 The advantages of using triploids

The use of triploids has many potential useful applications in aquaculture, both with respect to performance improvement and genetic containment of farmed fish. The diversion of energy from gonadal to somatic growth and the expected higher growth potential observed in some cultured species is of high interest (Tiwary *et al.*, 2004; Piferrer *et al.*, 2009). Triploidy can also be used as a tool to prevent early sexual maturation in cultured stocks like in the case of farmed Atlantic salmon in Tasmania (Benfey, 2009), and consequently prevent the following detrimental effect on flesh quality.

Triploidization is practical, economical and figures as the most effective method for the production of sterile fish (Maxime, 2008). Because triploids are at least gametically sterile, the genetic impact on wild populations can be significantly overcome by using triploids in production. But of course, escaped triploids can always transmit diseases and compete for food and space. Male triploids can also compete with wild fish for mating. However, a study by (Cotter *et al.*, 2000) showed that triploids, in addition to their incapability to produce viable offspring, were not returning back to fresh water as often as diploids.

In addition to the genetic containment of farmed fish, triploids may also show good growth potential (Piferrer *et al.*, 2009), which is vital if the industry should adapt the production of triploids. In Atlantic salmon, triploid have proved to grow equally well as diploids, at least during the freshwater phase (Benfey, 2011; Fraser *et al.*, 2013). A study by Fjelldal & Hansen (2010) also showed that there were no ploidy effects on survival between first feeding and until the end of the freshwater period. A poorer survival observed during the egg-stage and until start-feeding may be due to the triploidization procedure (Piferrer *et al.*, 2009).

Triploids are not considered genetically modified organisms (GMOs) according to the European Union regulations (EU, Directive 90/220/CEE) and, as such, they are not subject to the tight rules applying to the use and containment of GMOs in farming (Piferrer *et al.*, 2009).

## 1.7 The disadvantages of using triploids

### 1.7.1 Skeletal deformities

Some triploids have proven to be more prone to skeletal deformities than their diploid counterparts (Sadler *et al.*, 2001). Although the etiology of this condition is not fully known, addition of extra phosphorus to their diet significantly reduced the occurrence of some deformities in Atlantic salmon (Fjelldal *et al.*, 2016). A study by Fjelldal & Hansen (2010) emphasized that the normal bone morphology observed in triploids suggests that the skeletal deformities observed are not entirely due to the triploid status, but there must be external factors involved. If the suboptimal environmental conditions trigger skeletal deformity, this may indicate different environmental requirements for triploids. Occurrence of skeletal deformity can thus be genetic, environmental or a nutritional problem, and maybe not directly related to ploidy. The high growth rates in the freshwater phase have also been hypothesized to lead to skeletal abnormalities. Jaw deformity and lower jaw deformity syndrome is also

more frequent in triploids and fish with this condition cannot be sold whole due to their appearance. In addition to the skeletal deformities eye cataract are widespread in triploid production, but seems to be a nutritional problem due to inadequate amount of the amino acid histidine (Taylor *et al.*, 2015).

### 1.7.2 Culture conditions and environmental tolerance

Suboptimal water conditions tends to impact triploid performances more severely than diploid ones (Sambraus *et al.*, 2017). Atkins & Benfey (2008) proved that triploids have lower thermal optima than diploids. This was also suggested by Sambraus and co-workers (2017), and using diploid temperature regimes in rearing could be the reason for the triploids poorer performance. Triploids seem to be especially prone to chronic stress under suboptimal rearing conditions (poor water quality) (Maxime, 2008). Lower survival under chronic stress is prominent in triploids, and hence the use of triploids has been limited due the inability to cope with chronic stress. The chronic stress is mostly because of handling procedures, although high water temperatures might affect performance and induce mortality during summer when water temperature peaks.

The already mentioned jaw deformities, which prevent the mouth from fully closing, can alter normal respiration when moving water across the gills; this can lead to reduced respiratory efficiency, fish welfare and growth. Graham and co-workers (1985) revealed in a study on blood oxygen carrying capacity in Atlantic salmon that triploids have lower hemoglobin concentrations than diploids. The same study concluded that triploids had a 1/3 reduction in blood oxygen carrying capacity, this may be the reason for the problems observed with regard to chronic stress under warm temperatures. Even so, the aerobic capacity seems to be the same in both diploids and triploids at least in the species investigated by Sezaki and co-workers (1991), possibly because of increased cardiac output.

Absence of primary gill filaments on the branchial arches was also strikingly prominent in triploids compared to diploids in the study by Sadler and co-workers (2001). The triploid Atlantic salmon have a lower gill surface area and different studies have confirmed missing filaments on the gill arches. The larger cells due to the ploidy situation reduce the surface to volume ratio, this can influence transport and diffusion distances as well as signaling pathways (Benfey, 1999).

## 1.8 Digestive system

The digestive system of fish consists of a long tube called the gastrointestinal tract (GIT) and stretches from mouth to anus; associated organs are the liver which secretes bile from the gallbladder and the pancreas which secretes digestive enzymes (Kryvi & Poppe, 2016). The types of secretive enzymes vary throughout the digestive tract. The whole system includes the mouth, pharynx, esophagus, stomach, pyloric caeca, intestine and anus. Different regions have different functions resulting in optimal nutrient acquisition. Between the different compartments, there are sphincters, which can influence the retention time of food. The main purpose of the GIT is to digest food, and make the nutrients in the food suitable for absorption (Ray & Ringø, 2014). But GIT also has a number of other functions, including osmoregulation and regulation of the immune system.

The Atlantic salmon is a carnivorous fish species and possesses both a stomach and multiple pyloric caeca, the latter located right after the stomach. The salmon also has a U-shaped stomach (Fig 1.3). The gastrointestinal tract for all fish species has a basic organization, but there are large variations depending on feeding habits, variation in ontogeny and phylogeny and diet, and their digestive system is optimized for efficient digestion and absorption of their natural diet.

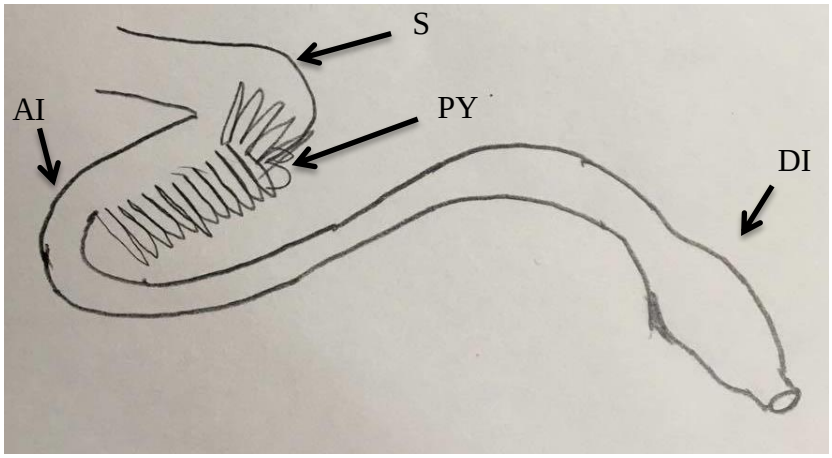


Figure 1.3. Schematic drawing gastrointestinal tract Atlantic salmon. S, stomach; PY, Pyloric caeca; AI, Anterior intestine; DI, Distal intestine. Adapted from Løkka *et al.* (2013).

The gastrointestinal tract of carnivorous fish is actually variable, even if they show the same feeding habits (Buddington *et al.*, 1996). Carnivorous fish also seem to have shorter guts than omnivorous and herbivorous species. The amount of plant material in the feed has been hypothesized to be related to the length of the intestine (Kramer & Bryant, 1995).

The feed starts to be digested in the stomach, which stores food and is expandable. It also mechanically digests the food with the help of contraction of smooth muscle, and chemically by secreting hydrochloric acid (HCl) and the digestive enzymes pepsinogen and lipase (Ray & Ringø, 2014). HCl denatures proteins, but also transform pepsinogen to pepsin making it readily available for enzymatic breakdown of food (Bakke *et al.*, 2010). After mixing and breakdown in the stomach, the food is now known as chyme (Young *et al.*, 2013) and will enter the pyloric caeca and then the intestine for absorption. Pyloric caeca are blind extensions of the intestine which both secrete digestive enzymes and absorb nutrients (Buddington *et al.*, 1996), as well as increasing the absorptive surface area (Buddington & Diamond, 1987). The lowered pH due to HCl in the stomach rises in the intestine, mainly due to secretion of bicarbonate from the pancreas, and thus makes the pH optimal for the digestive enzymes. The feed is now somewhat digested and is exposed for enzymatic breakdown in the intestine. A number of enzymes like lipase, phospholipase, amylase, and the proteolytic enzymes (trypsin, chymotrypsin) among others are produced in the pancreas and secreted into the anterior intestine (National Research Council, 2011). The proteolytic enzymes have to be activated in the intestine, but some can work directly after secretion such as amylase. The proteins and carbohydrates are broken down by these enzymes and the smaller fragments are hydrolyzed further in the brush border membrane (Buddington *et al.*, 1996). The nutrients are absorbed and travel across the membrane into the cells, and to the blood with the help of different transporters. Especially in carnivores, the rates of transporters for amino acids are high, and demonstrate the requirement for a protein rich diet. Overall, the ability to digest carbohydrates is low in fish.

Lipids have a more complex digestion. The anterior part of the intestine seems to be more involved in the absorption of lipids, and bile secreted in the anterior part of the intestine is highly involved in the digestion and absorption of lipids. During lipid digestion the fat globules breaks up to smaller droplets, called emulsification. This process increases the surface area for efficient digestive breakdown by the enzyme lipase. Bile produced by the liver prevents the droplets to re-associate (National Research Council, 2011). The triglycerides and phospholipids are hydrolyzed to mono- and diglycerides, which are passively absorbed across the brush border membrane.

Macromolecules can to some extent also be absorbed directly in the distal intestine (Buddington *et al.*, 1996), and also reabsorption of digestive components seems to be important in this area of the intestine (Nordrum *et al.*, 2000).

### 1.8.1 **Liver**

The liver is a metabolic organ and made of hepatocytes (liver cells) which are large polyhedral cells with round nuclei. Hepatocytes take up nutrients and other substances from the sinusoids (capillaries surrounded by liver cells). Furthermore, hepatocytes synthesize and secretes bile into a number of these small ducts (the bile capillaries) (Widmaier *et al.*, 2006). These ducts branch throughout the liver and end in the bile duct, this duct has a cylindrical epithelium surrounded by loose connective tissue and smooth muscle (Kryvi & Poppe, 2016). Depending on the amount of glycogen and fat, the appearance of the liver differs; apart from that it is a very homogenous tissue.

### 1.8.2 **Pancreas**

The pancreatic tissue is spread throughout the abdomen (Amin *et al.*, 1991), especially around the intestine, pyloric caeca, liver and mesenteric tissue. Exocrine cells that produce digestive enzymes and endocrine cells that produce hormones make up the pancreas. The pancreatic cells are characteristic with large nuclei located centrally, and the part of the cell facing the lumen of the gland duct (the apical part) is filled with vesicles called zymogen granules (Kryvi & Poppe, 2016). These vesicles contain the precursor pancreatic enzymes which will be activated in the intestine. The basal part of the pancreatic cells has a lot of endoplasmic reticulum (Kryvi & Poppe, 2016).

## 1.9 Ontogeny and histology of the digestive system of salmon

In terms of tissue, the whole digestive tract consists of the same basic structure, but the different layers of tissue of GIT vary in size throughout the system. The layers (from outer to inner) are: serosa, longitudinal muscularis, circular muscularis, submucosa and mucosa (Young *et al.*, 2013). The mucosa consists of three components; the muscularis mucosae (a thin layer of smooth muscle), lamina propria (a layer of loose connective tissue) and the epithelium which faces towards the lumen (Young *et al.*, 2013). The submucosa (a layer of connective tissue) separates the muscularis mucosae from the thick muscularis (Fig 1.4).

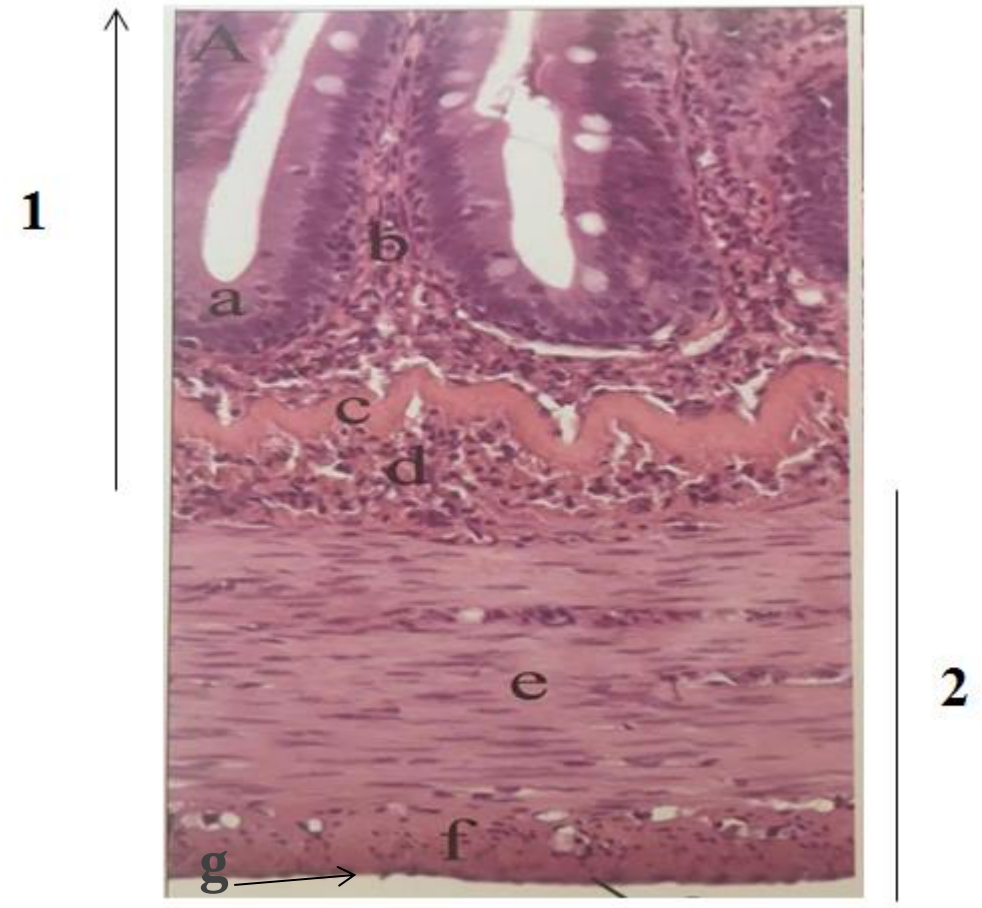


Figure 1.4. Section of the intestinal wall of Atlantic salmon illustrating the general organization of the intestinal wall. (1): The mucosa consisting of; a, simple columnar epithelium; b, lamina propria; c, submucosa with connective tissue/muscularis mucosae; d, granular cells in loose connective tissue. (2): The muscularis consisting of: e, smooth inner circular muscle; f, smooth outer longitudinal muscle; g, serosa. Adapted from Kryvi & Poppe (2016).

The mucosa is folded in order to increase the surface area of the intestine, and the amount of folding also differs throughout GIT. In each fold there is a number of cells with different functions. These cells are called epithelial cells and consists of enterocytes (absorptive cells), goblet cells (mucous cells) which secrete mucin and aids in digestion and protection of the mucosal lining (Bakke *et al.*, 2010), neuroendocrine cells (Buddington *et al.*, 1996) and intraepithelial leucocytes (Løkka *et al.*, 2013; Young *et al.*, 2013). The epithelium is mostly made up of the columnar absorbing enterocytes, and lining the epithelium are microvilli facing towards the lumen, commonly called the brush border membrane due to its appearance.

The submucosa supports the mucosa, and consists of the compact stratum compactum and mostly loose connective tissue. The stratum compactum is easily visible and is the transition between the submucosa and the inner circular muscularis, followed by the outer longitudinal

muscularis. The serosa consists of a thin layer of connective tissue and a layer of squamous epithelium (Young *et al.*, 2013).

The stomach is lined with columnar epithelium, and have gastric crypts which leads to the tubular/alveolar glands (Ray & Ringø, 2014). These cells produce a thick coating of mucous, figuring as a protective wall against gastric acid. The stomach is usually divided into two parts, the cardiac stomach figuring as the proximal, and the pyloric stomach as the distal part. Unlike mammals which have two different types of cells, the oxynticopeptic (parietal) cells release both acid and pepsinogen in fish, and are mainly found in the cardiac part of the stomach (Olsson, 2011). Following the stomach is the pyloric caeca, these open into the lumen of the intestine. They vary in number between species and with ploidy; for instance triploids are confirmed to have 20% less pyloric caeca than diploid salmon (Peruzzi *et al.*, 2015).

The distal intestine in Atlantic salmon is both morphologically and functionally different from the anterior intestine (Van den Ingh *et al.*, 1991). The distal intestine is composed of large complex folds, with smaller simple folds between them. The goblet cells located in the intestinal epithelium have often been described in the literature to be located at a larger concentration in the distal part of the intestine (Al-Hussaini, 1949).

The following is based on literature from Sahlman and co-workers (2015) and will figure as a short introduction and an overview when later comparing the selected parts of the digestive tract in triploid and diploid Atlantic salmon during the feeding experiment of this study. Atlantic salmon is highly developed at hatch; a week after hatch the mouth is open and the buccopharyngeal cavity contain mucous cells and taste buds in the epithelium. The esophagus leads to an incipient stomach, which is straight without gastric glands. The intestine is separated from the stomach with a pyloric sphincter. The intestine is straight with some mucosal folding and no goblet cells are present. The liver and pancreas seem to be functional a week after hatch; small glycogen vacuoles are present in the cytoplasm of the hepatocytes of the liver. The pancreas is diffusely arranged around the intestine.

From start-feeding and onwards mucous cells and taste buds increase in the buccopharyngal cavity and pharynx. In the esophagus taste buds appear and the epithelium folds longitudinally. Mucous cells become more numerous and the muscle layers increase in

thickness. The stomach attains a U-shape with mucous cells along the whole mucosa and is now divided in two distinct parts; gastric glands are also present. The pyloric caeca were developed at start-feeding and goblet cells appear in the epithelium of the intestine. The intestine can be divided into different parts, the anterior part posterior to the pyloric caeca and the distal part with mucosal folds being longer than those of the anterior part (Sahlmann *et al.*, 2015). Often there are no obvious marked difference between these parts of the intestine depending on species (Al-Hussaini, 1949). The intestine eventually forms a loop and the size of the folds and muscle layers increase onwards.

### 1.9.1 Triploid digestive capacity

This section discusses not only Atlantic salmon, but also different salmonid species due to limited information for Atlantic salmon only. Results show that triploids perform worse than diploids (Chiasson *et al.*, 2009), as good as (McGeachy *et al.*, 1995; McCarthy *et al.*, 1996), or better than diploids (Galbreath *et al.*, 1994; Oppedal *et al.*, 2003) with respect to growth rate and overall performance. A number of studies about feed conversion efficiency, energy utilization and digestibility of feed have been performed, and this can be used to assess why the triploids often under-perform when there actually is a growth potential present.

What we do know for certain is that triploids need a larger amount of different micronutrients in the diet to prevent eye cataract and skeletal deformities. For example, triploids require higher amounts of histidine (Taylor *et al.*, 2015), and a number of other studies show that triploid Atlantic salmon requires more dietary phosphorus than diploids in fresh water (Fjelldal *et al.*, 2016). In a study by Oliva-Teles & Kaushik (1987) triploids used less protein for energy. This type of differences suggests a different nutrient requirement in triploids compared to diploids.

But a number of studies investigating feed conversion efficiency, digestibility and nutrient retention in triploids show inconclusive results (Benfey, 2011), and often no difference between ploidies have been proved. A study by Burke and co-workers (2010) showed no difference in feed conversion ratio between ploidies in juvenile Atlantic salmon, but triploids showed a higher growth rate. It was hypothesized in the same study that the observed reduced cellular surface to volume ratio due to the presence of fewer and larger cells could reduce the efficiency of nutrient absorption, metabolism and retention. The same study did not reveal differences between ploidies when considering feed conversion and growth. The same authors

concluded that differences in growth could not be due to inefficiency in nutrient absorption through digestion, because there were no differences between ploidies with respect to feed digestibility. Within the same species, a study by Tibbetts and co-workers (2013) also showed that feed conversion efficiency, digestibility and nutrient retention efficiencies were equal between triploids and diploids.

In brook charr (*Salvelinus fontinalis*), a study performed by Sacobie and co-workers (2015) investigated if the differences in performance observed between ploidies were due to alterations in dietary energy utilization, and found that the triploid state did not affect digestibility rather suggesting a difference in metabolism and energy utilization. This was tested (Oliva-Teles & Kaushik, 1990) much earlier when they evaluated the growth and metabolic utilization of the diet of triploid rainbow trout (*Oncorhynchus mykiss*) juveniles. They found that protein efficiency ratios (feed:gain) was somehow superior in the diploid juveniles, however this difference was not significant. However, they did find that dry matter digestibility was significantly higher in triploid juveniles. They concluded that no difference existed between the ploidies with respect to metabolic and digestive utilization of the diet. However, triploids was as efficient as diploids at converting feed into growth in a recent study by Nuez-Ortín and co-workers (2017). The triploid state could affect metabolic tissues as liver and muscles, which is very important for growth and nutrient metabolism, in this study no differences were found with regard to ploidy when considering growth and nutrient utilization.

Triploids are different from diploids both in terms of general physiology (Benfey, 2011) and gut morphology (Peruzzi *et al.,* 2015). By having 20% fever pyloric caeca and 15% shorter guts than their diploid counterparts, they potentially have a different digestive capacity. Accordingly, this can play a role in determining the absorption of nutrients and the following growth of triploids (Peruzzi *et al.*, 2015).

The microbiota also plays an important role concerning host physiology. The microbiota in the GIT tract depends on gut structure, the mucosal immune system, and nutrients' absorption among others (Cantas *et al.,* 2011). The triploid state has an effect on these factors, and thus can have an effect on the gut microbiota. In the study by Cantas and co-workers (2011), they compared the culturable microbiota in diploid and triploid juvenile Atlantic salmon, and found 7.3% more bacteria within the whole gut in triploids. Several species of bacteria present

in the gut were significantly higher in the triploid gut. The study concluded that the triploid state may have an influence on the gut microbiota of the fish, as the different physiology of triploids provides a different environment in the gut.

In summary, triploid Atlantic salmon do show poorer growth, higher mortalities (Galbreath *et al.*, 1994; O'Flynn *et al.*, 1997), eye cataracts (Taylor *et al.*, 2015) and high prevalence of skeletal deformities (Fjelldal *et al.*, 2016), especially under sub-optimal rearing conditions. Sambraus and co-workers (2017) together with other studies suggest triploids have a lower temperature optima. This compared with findings by Handeland and co-workers (2008), that temperature is very important when considering growth, feed conversion rate and gut evacuation rates (in diploids), could mean that triploids display differences in feed efficiency when reared at temperatures not suitable for triploids. Sambraus and co-workers (2017) showed that with lower temperatures, triploids performed equal or better than diploids. This compared with findings by (Peruzzi *et al.*, 2015; Taylor *et al.*, 2015; Fjelldal *et al.*, 2016) implies that triploids are quite different, and that they require a diet more suitable for triploids and that they should be hypothesized to have some differences in the absorption of nutrients.

### 1.9.2 **Use of more digestible proteins**

The incorporation of protein hydrolysate in feed have proved to be successful in terms of growth in a number of fish species (Berge & Storebakken, 1996; Refstie *et al.*, 2004; Kotzamanis *et al.*, 2007). The protein hydrolysates discussed here are made of by-products of scrap fish and fish body parts, and are basically just enzymatically hydrolyzed fish protein. The reason for the positive effects observed when using protein hydrolysates could be due to the enzymatic degradation of proteins which increases their digestibility (Berge & Storebakken, 1996). The already mentioned study by Berge & Storebakken (1996) investigated the effects of substituting a minor portion of dietary fish meal with fish protein hydrolysate in terms of growth improvement. The results of the study indicated a positive effect on growth when including a small amount of protein hydrolysate, but it is not clear whether the effect was due to the hypothesized improved digestion and thus improved protein utilization, higher feed intake or other factors.

Pre-digested fish protein (protein hydrolysate) will be absorbed faster in Atlantic salmon than intact fish protein (Espe *et al.*, 1993). The study by Refstie and co-workers (2004) showed that inclusion of up to 15% protein hydrolysate stimulated growth in Atlantic salmon. The

observed growth improvement is probably due to the more efficient protein utilization. Increased protein efficiency and nitrogen digestibility were also confirmed to some extent in a study by Espe and co-workers (1999) when Atlantic salmon were fed diets containing variable levels (0-40%) of solubilized protein. To summarize, inclusion of hydrolyzed protein in the diet seems to be promising in terms of growth and overall performance.

## 1.10 The main objectives of the study

By analyzing fish growth, survival and digestive tract morphology of selected intestinal regions from hatching until the end of smoltification, the goal of this project is to highlight any differences in the ontogeny of the digestive system between the diploid and triploid salmon and decide if the triploids perform better on a diet containing more digestible (hydrolysed) proteins.

Little is known about the nutritional requirements for triploid Atlantic salmon, and the effects of diets on the gut structure. The hypothesis is that the experimental diet containing hydrolysed proteins is more suitable for the triploid Atlantic salmon.

The following objectives were set:

1. Analyze the general performance of the diploid and triploid juvenile salmon groups in relation to growth and survival until the end of smoltification.
2. Compare general digestive tract morphology of early juvenile diploid and triploid salmon.
3. Compare morphometrically intestinal structure of juvenile salmon fed standard and experimental diet until the end of smoltification.

# 2 Materials and methods

## 2.1 Location and experimental period

The feeding experiment with diploid and triploid Atlantic salmon was carried out at the Aquaculture Research Station (Kårvika, Tromsø, Norway) while the histological analyses were done at the University of Tromsø (UiT) the Arctic University of Norway. The experiment started 5.11.2015 and ended 12.12.2016. Due to an electric power failure and water stop occurred at the station on 2.12.2016 the December samples were excluded; the last samples included in the analysis was from 15.11.2016.

## 2.2 Experimental fish and design

The fish used for this project were Atlantic salmon originating from Stofnfiskur's breeding program (Stofnfiskur HF, Hafnarfjörður, Iceland). They were composed of n=20 families (half-sibs and full-sibs) originating from four- and five-year old males and females, which were stripped on 14.8.15 and 20.8.15. After fertilization the ova were handled according to commercial procedures, then split into two equal halves, where one half was exposed to a hydrostatic pressure shock of 9500 psi applied for 5 min, 300° degree-minutes (DM) post-fertilization at 5.2°C (Johnstone & Stet, 1995). The remaining un-shocked ova served as diploid controls. At eyed-egg stage (ca. 390 day-degrees, dd), the ova were shipped by air carrier to the Aquaculture Research Station in Kårvika (Tromsø, Norway). Newly hatched fry (n=20/family) were verified for their ploidy status by flow cytometry (Ploidy Analyzer, Sysmex Partec GmbH, Germany) using in-house methods (Peruzzi *et al.*, 2013). Of the n=20 diploid and triploid families received, only 17 families were actually used in the project. Three putative triploid families contained small percentages (2-5%) of diploids and were thus discarded along with their diploid control counterparts.

The ova were incubated in 34 hatching trays (n=17 trays / ploidy) in darkness at 5°C following standard in-house procedures. Hatching was completed around 11.12.2015 (570 dd) and one day prior to start-feeding (920 dd) the alevins were transferred to n=12 200L tanks with a density of approximately 3500 alevins/tank; three tanks per ploidy and feed type were used (see table 2.1 for details on experimental set up). The experimental tanks were located in two rooms and to minimize tank effect they were randomly distributed (n=6 tanks/room). The number of alevins per tank was determined by weighing (BW, g) two samples of n=100

alevins from each family and the average weight used to calculate the weight of alevins/family needed to achieve an equal contribution in terms of alevin number per each diploid and triploid family. Fish performance and ontogeny of the digestive system were investigated at approximately one-month intervals, starting from hatching (early ontogeny only) and until the end of smoltification following the sampling interval shown in Table 2.2.

Table 2.1. Experimental design: 2 of the groups (Group 1 and 3) were fed a standard diet and the other 2 groups (Group 2 and 4) an experimental diet. For each experimental group there were 3 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 |

Table 2.2. Sampling interval and nomenclature of the different stages from hatching to the end of the experimentation period adopted in the study given in weeks and corresponding age in day-degrees (dd) post-hatch.

| Weeks | Day degrees |
|---|---|
| Hatching | 570 dd |
| Start-feeding | 920 dd |
| 4 weeks after start-feeding | 1150 dd |
| 9 weeks after start-feeding | 1500 dd |
| 13 weeks after start-feeding | 1800 dd |
| 17 weeks after start-feeding | 2100 dd |
| 21 weeks after start-feeding | 2380 dd |
| 27 weeks after start-feeding | 2810 dd |
| 30 weeks after start-feeding | 3020 dd |
| 35 weeks after start-feeding | 3380 dd |
| 38 weeks after start-feeding | 3670 dd |

## 2.3 Rearing conditions and fish growth

After start-feeding (920 dd) the temperature was gradually raised (one degree every fifth day) until reaching 10°C using controlled conditions (heated water). This water temperature was

maintained until summer when the water supply was changed to ambient temperature for a period of approximately 2 months before returning to controlled water conditions (see Figure 2.1). The fish were daily fed a standard or experimental diet (see section 2.4) following the rearing procedures for Atlantic salmon in use at the Aquaculture Research Station. Fish biomass was monitored and re-adjusted when needed in order not to exceed 45 kg/m$^3$ following in-house rearing protocols. This resulted in four biomass re-adjustment periods (Biomass 1-4) during which the initial and final total biomass/tank recorded and the number of fish/tank estimated from the mean weight of n=50 fish/tank. Following the first three periods, the total biomass of fish was then set to the same level in each tank (data not shown in this thesis). On 20.5.2016 (1800 dd) the fish were moved from 100 L to 500 L tanks. Fish were kept under a continuous light regime (24 H, LL) throughout the experiment with the exception of the period required to simulate the onset of winter where the photoperiod was gradually reduced at 12L:12D. Fish mortality was recorded throughout the experiment and calculated taking into account the sampled fish and the biomass re-adjustments. An overview of the rearing conditions and the main operations is provided in Figure 2.1. Fish growth was estimated from start-feeding (920 day-degrees, dd) following the schedule shown in Table 2.2. At each sampling point, the body weight and length (BW, BL) of n=75 fish/group was measured to the nearest 0.01 point. All measurements were performed on randomly sampled fish that were euthanized using benzocaine following in-house procedures and national legislation (2015-06-18 no. 761, Regulation on the use of animals in experiments).

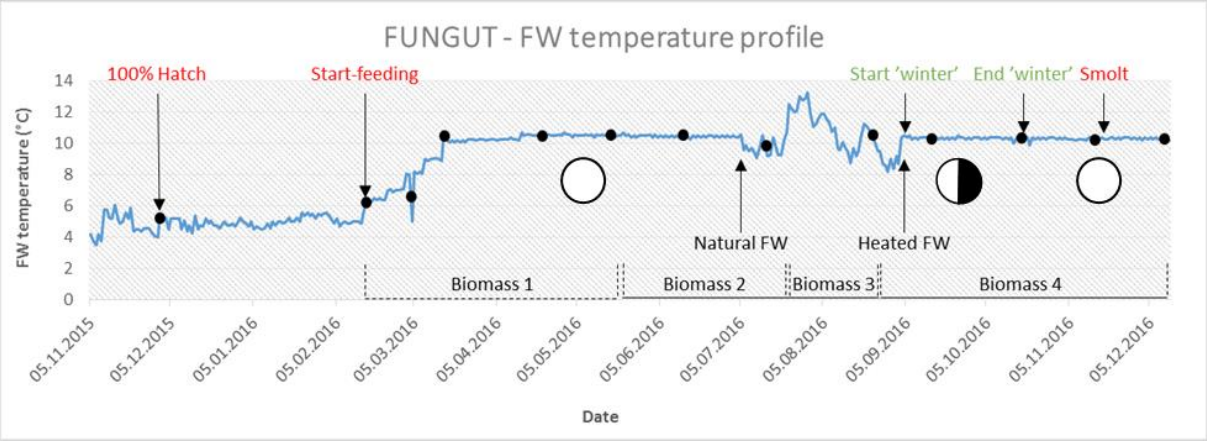


Figure 2.1. Summary of rearing conditions (temperature profile and main operations). Biomass re-adjustments– 4 periods (Biomass 1-4). Black dots represent sampling points, other relevant events are listed in the figure with arrows.

## 2.4 Diets

All diets were provided by Skretting AS (Stavanger, Norway). During start-feeding the size of the extruded pellets was 0.5 mm and the pellet size increased according to the fish growth, the maximum pellet size was 3.0 mm. In experimental diets, 45% of fishmeal (FM) fraction was replaced with hydrolysed proteins (CPSP Special G – hydrolysed FM) while lowering the fish oil levels. Regardless, the protein concentration (C PROT) was somewhat higher in the experimental diet. Additionally the experimental diets comprised a larger amount of a premix consisting of different micronutrients. The complete formulation of standard commercial and experimental diets used in this study is given in Tables 2.3-2.4. An inert marker (Yttrium) was added in the 3.0 mm diet for measuring feed digestibility (results not included here).

Table 2.3 Diet composition standard diets. SPC = Soy Protein Concentrate. *Marker Yttrium used in 3.0 mm diets only. SPC, Soy Protein Concentrate; TOT VOL, total volume; V MOIST, water stability %; C PROT, protein concentration; C FAT, fat concentration (G.Reisen, Skretting AS).

| | **Only fishmeal** | | |
|---|---|---|---|
| Diet Name | A, B, C | D | I, J, K |
| Pellet (mm) | 0.5/0.7/1.0 | 1.2 | 1.5/2.0/3.0 |
| Wheat | 7,2 | 6,1 | 6,9 |
| Wheatgluten | 10 | 10 | 10 |
| SPC | 14,4 | 16,7 | 17,9 |
| NA Fishmeal | 55 | 55 | 50 |
| CPSP Special G – hydrolysed FM | 0 | 0 | 0 |
| Fishoil Nordic | 11 | 10,8 | 11,6 |
| Water/Moisture change | 0 | 0 | 0,4 |
| Yttrium premix* | 0 | 0 | 0,1 |
| Premix (min, Vit, AA) | 2,4 | 1,4 | 3 |
| | | | |
| TOT VOL | 100 | 100 | 100 |
| V MOIST | 7,9 | 7,5 | 7,2 |
| C PROT | 55,9 | 56,8 | 56,0 |
| C FAT | 17,7 | 18,3 | 19,1 |

Table 2.4 Diet composition standard diets. SPC = Soy Protein Concentrate. *Marker Yttrium used in 3.0 mm diets only. SPC, Soy Protein Concentrate; TOT VOL, total volume; V MOIST, water stability %; C PROT, protein concentration; C FAT, fat concentration (G.Reisen, Skretting AS).

| | **With CPSP** | | |
|---|---|---|---|
| Diet Name | E, F, G | H | L, M, N |
| Pellet (mm) | 0.5/0.7/1.0 | 1.2 | 1.5/2.0/3.0 |
| Wheat | 5,4 | 5,5 | 6,9 |
| Wheatgluten | 10 | 10 | 10 |
| SPC | 14 | 16,2 | 16,7 |
| NA Fishmeal | 30 | 30 | 27,5 |
| CPSP Special G - hydrolysed FM | 25 | 25 | 22,5 |
| Fishoil Nordic | 9,4 | 9,2 | 10,2 |
| Water/Moisture change | 1,6 | 0,9 | 1,5 |
| Yttrium premix* | 0 | 0 | 0,1 |
| Premix (min, Vit, AA) | 4,7 | 3,3 | 4,6 |
| | | | |
| TOT VOL | 100 | 100 | 100 |
| V MOIST | 7,9 | 7,5 | 7,1 |
| C PROT | 60,3 | 59,0 | 56,9 |
| C FAT | 17,3 | 18,8 | 19,6 |

## 2.5 Histology samples

Randomly sampled fish (see section 2.3 for procedures) were fixed in 10% buffered formalin (Sigma-Aldrich, St.Loius, USA) for at least 48 hours before being transferred to 70% ethanol for storage until being prepared for histological studies. At hatch (570 dd) and at start-feeding (920 dd) n=20 diploid and n=20 triploid salmon were sampled as whole fish. From March (1150 dd) and until May (1800 dd) n=5 fish from each triplicate were fixed as whole fish, while from June (2100 dd) and until November (3670 dd) only the whole guts from n=5 individuals were dissected and fixed. The sampled fish were not fasted prior to sampling.

## 2.6 Tissue processing

All samples were transferred to standard plastic cassettes before tissue processing and embedding. The samples were dehydrated and embedded in paraffin wax (Histolab Products AB, Gothenburg, Sweden) using a Citadel 2000 Wax Bath (Thermo Scientific, Runcorn, United Kingdom). All chambers were filled with the appropriate substances, then the samples were placed in a proper metal basket, and using program A the samples went through a 15 hour bath cycle (see Appendix A). Samples were removed the following morning and brought to the lab for further processing.

### 2.6.1 Embedding

Each dehydrated sample were placed in a suitable metal embedding tray and placed on a pre heated tray on the Wax Dispenser WD -4 (Kunz, Valletuna, Sweden) and filled with paraffin wax holding approximately 60°C. Each metal tray with the samples and wax were stored in the fridge for minimum 24 hours.

### 2.6.2 Sectioning and staining

The histological sections of the digestive tract are based on both longitudinal samples of whole fish, cross sections of the anterior intestine and distal intestine from whole fish and cross sections of the respective dissected gut parts. The longitudinal sections of the smaller juveniles included the whole digestive tract except the head and caudal fin. In larger juveniles, a piece cut right after the end of the pyloric region represented the anterior intestine (AI), and a similar piece cut at the fore part of the distal intestine (DI) (darker color and thicker diameter) were made ready for cross sectioning (see (Sanden *et al.*, 2005)). The complete gastrointestinal tract (GIT) of Atlantic salmon including the different intestinal areas used for histological examination is shown in Figure 2.2.

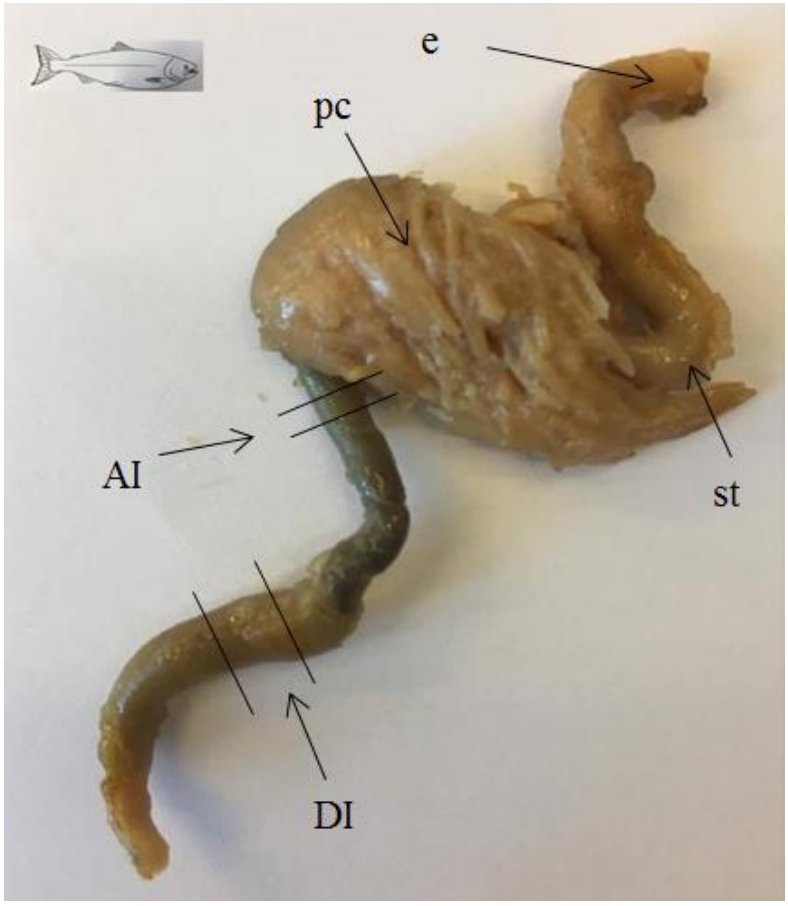


Figure 2.2. The complete gastrointestinal tract (GIT) of Atlantic salmon divided into different parts, AI, anterior intestine; DI, distal intestine; e, esophagus; pc, pyloric caeca; st, stomach.

Each paraffin block containing the desired tissue was sectioned using a Leica RM 2255 rotary microtome (Leica Biosystems, Nussloch, Germany) and cut using a Microtome Blade (MX35 Ultra 34°/80mm, Thermo Scientific). Some samples turned out to be dry and brittle and these were kept on ice and were rehydrated in a water bath to secure proper sectioning, pieces that were still inadequate after this treatment were not included in the morphometric analysis. The thickness of the sections was 5 µm.

Sections were heated at 37°C using a Slides Warmer XH – 2002 (Heco, Oslo, Norway) to remove excess water, and then kept at room temperature. Before staining the sections were placed in an incubator (Incubator Modell 100, Memmert GmbH + Co. KG, Schwabach, Germany) for minimum 6 hours to remove excessive wax. The sections were placed in Histo-Clear (Natural Diagnostics, Atlanta, USA) for at least 4 minutes, then a Linear Stainer (Leica ST4020, Leica Biosystems, Nussloch, Germany) were employed and the chambers filled according to the following setup with 90 seconds exposure in each chamber. Histo-Clear, Ethanol ≥ 98.8% (Sigma Aldrich, St.Loius, USA), Ethanol 96% (Sigma Aldrich, St.Loius,

USA), Ethanol 96%, Water, Instant Hematoxylin (Thermo Scientific, Kalamazoo, USA), Instant Hematoxylin, Water, Water, Instant Eosin (Thermo Scientific, Kalamazoo, USA), Ethanol 96%, Ethanol 96%, Ethanol ≥ 98.8%, Ethanol ≥ 98.8%, Histo-Clear. A cover glass was attached to each section using Eukitt quick-hardening mounting medium (Sigma-Aldrich, St.Loius, USA) for microscopy.

Each section was examined using a Leica DM 2000 LED light microscope (Leica Microsystems, Wetzlar, Germany) connected with a Leica DFC 295 Digital Color Camera (Leica Microsystems, Wetzlar, Germany). Photos of the sections were processed using Leica software application suite (LAS).

A comparison of the digestive tracts of diploid and triploid groups was performed for the earliest sample periods (hatch (570 dd) – 9 weeks after start-feeding (1500 dd)). And for the morphometric measurements the following evaluation of the intestinal morphology was conducted: width and length of cross sections of the intestinal mucosal folds, number of folds, thickness of the muscle layers as well as presence of goblet cells. Measurements of the anterior and distal intestinal fold heights (from tip of intestinal fold to basal cells) and widths (Fig 2.3 A) of 10 different folds per individual (section) were done for all sampling points (three individuals per tank, 9 individuals per diet group). 10 measurements per section were performed for the thickness of the intestinal wall excluding the folds (Fig 2.3 B). Two complete folds per section were used for counting goblet cells (Fig 2.3 C). The number of folds was determined from two cross sections of the intestine (Fig 2.3 D). The different sections were studied and measured in the microscope between 5x and 40x magnifications.

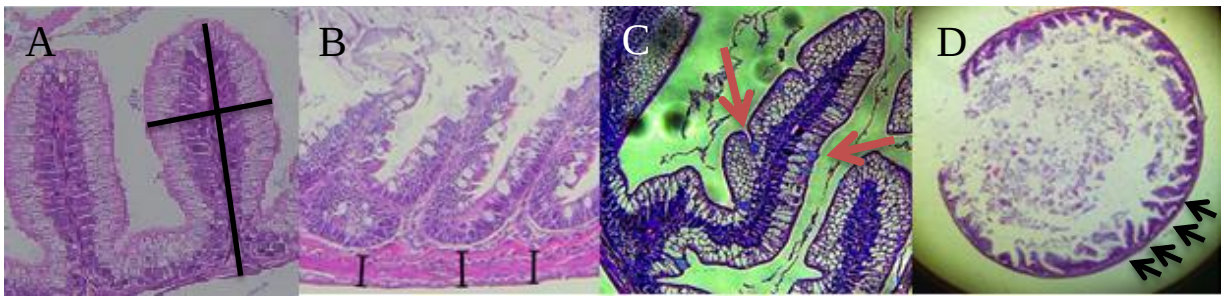


Figure 2.3. Examples of how the different intestinal tract measurements was performed. A: Height and width of intestinal folds. Height measured from tip of intestinal fold to basal cells, width measured across the middle of the fold (Black lines). B: Thickness of the underlying muscle tissue. Thickness of the intestinal wall measured from the serosa and up to the beginning of the folds (Black lines). C: Goblet cells. Visible goblet cells counted (Red arrows). D: Number of folds: Visible folds counted (Black arrows).

## 2.7 Statistical Analysis

Results on body weight and length at start-feeding (only two groups available, diploid and triploid) were analyzed by t-test. Percent mortality data were arcsin-transformed and analysed by general linear model procedure (GLM). Data on body weight and length, fold height, width, thickness, number of goblet cells and number of folds between groups at the anterior intestine and distal intestine respectively were analyzed by one-way ANOVA. Data normality and homogeneity of variances were checked using Levene's and Shapiro Wilk's test, respectively. When these parameter conditions were not met for linearity, the length measurements were logarithmically-transformed and the counting measurements arcsin-transformed, to improve the normality of their distributions. If there were no differences between the replicates within each group, the data were pooled and considered as a single group. When there were differences among replicates within one or several groups, the groups were compared by nested ANOVA using tanks nested in the dietary groups. In case of significant differences among groups, pairwise comparisons were made using Tukey's Post-Hoc test. All data have been recorded and processed in Microsoft Excel 2010 and the statistical analyses performed using SYSTAT v.13 (SYSTAT Software Inc., USA). The level of significance was $P<0.05$, and the results are presented as means ± 95% Confidence Intervals (CI) or Standard Deviation of means (STD).

# 3 Results

## 3.1 Mortality

Over the entire experimental period (cumulated data) mortality was higher ($P<0.001$) in groups fed the experimental diet (16-18%) than in those fed a commercial diet (8-10%) with no differences between ploidies (Fig 3.1). Most of the mortality (ca. 70% of total) occurred during the first rearing period (Period biomass 1) again with groups fed experimental diet (Group 2 and Group 4) showing higher mortality ($P<0.01$) than those fed a standard diet (Group 1 and Group 3) independent of their ploidy (Fig 3.2).

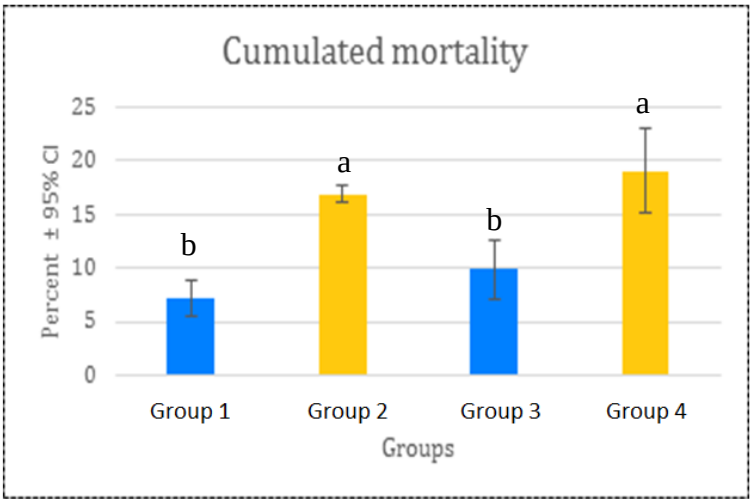


Figure 3.1. Percent mortality in the four experimental groups between 920 dd (start-feeding) and 3876 dd (December 5th). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

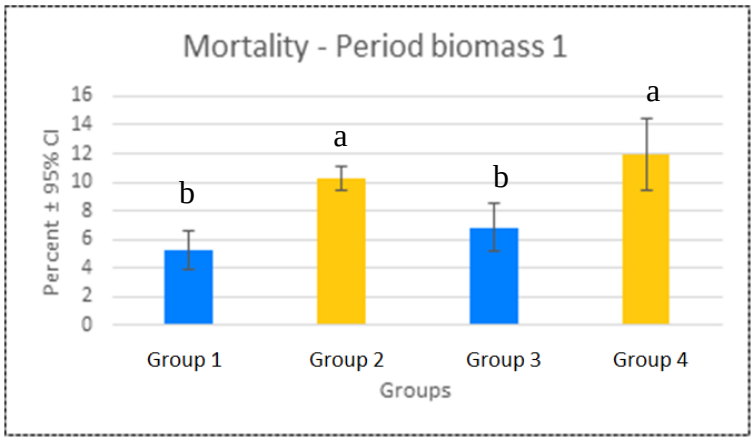


Figure 3.2. Percent mortality in the four experimental groups between 920 dd (start-feeding) and 1800 dd (end of period biomass 1). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

## 3.2 Hatch (570 dd)

### 3.2.1 **Early ontogeny**

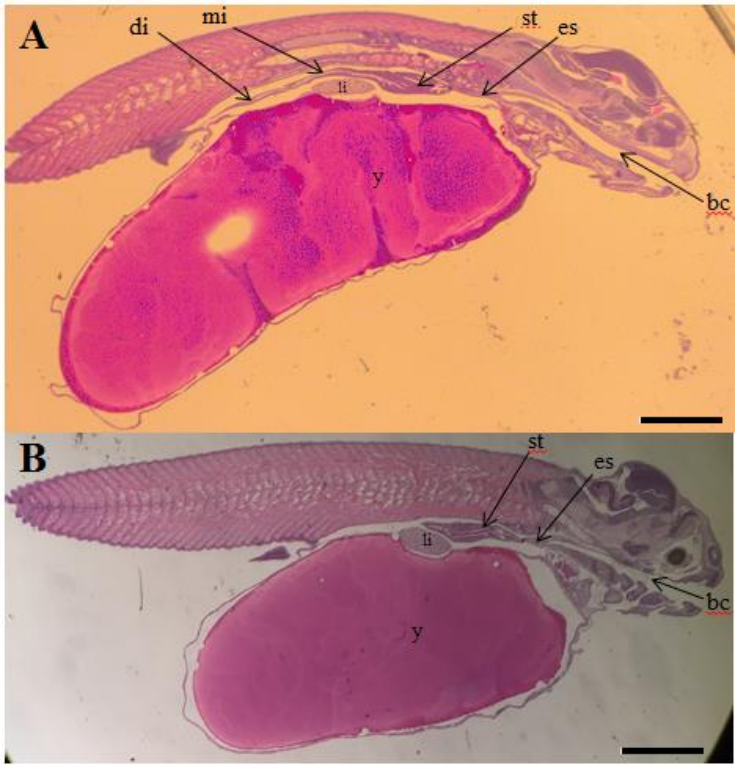


Figure 3.3. Longitudinal sections of whole individuals of diploid (2N) and triploid (3N) Atlantic salmon at hatch (570 dd). (A) Overview 2N individual. (B) Overview 3N individual. bc, buccopharyngeal cavity; di, distal intestine; es, esophagus; li, liver; mi, mid intestine; st, stomach; y, yolk sac. Scale. A-B, 1.75 mm.

At this stage four individuals per ploidy were used for comparisons of the digestive system, and no apparent general differences could be observed. The yolk sac was large at hatch (Fig 3.3). Stratified squamous epithelium lined the buccopharyngeal cavity and pharynx, mucous cells and taste buds were present (Fig 3.4 A-B). The esophagus was short, and scattered mucous cells were noted in the epithelium; no clear transitional zone between the esophagus and the stomach was noted (not shown). The stomach was present at hatch as a straight and sac-like structure with longitudinal mucosal folds (Fig 3.4 C). The folds were made of connective tissue (lamina propria) and columnar epithelium facing the lumen. Neither gastric glands or pyloric caeca were present at hatch. The intestine was a simple straight tube without coiling. In the anterior part of the intestine mucosal folding was initiated (Fig 3.4 D) while the distal part was lined with simple columnar epithelium without any sign of folding (Fig 3.4 E), and no mucous cells were present. The rectum was short with an open anus (not shown). The liver seemed to be functional at hatch with vacuolated hepatocytes. The pancreas was also

present with zymogen granules and endoplasmic reticulum in the exocrine part (Fig 3.4 G) (endocrine not shown).

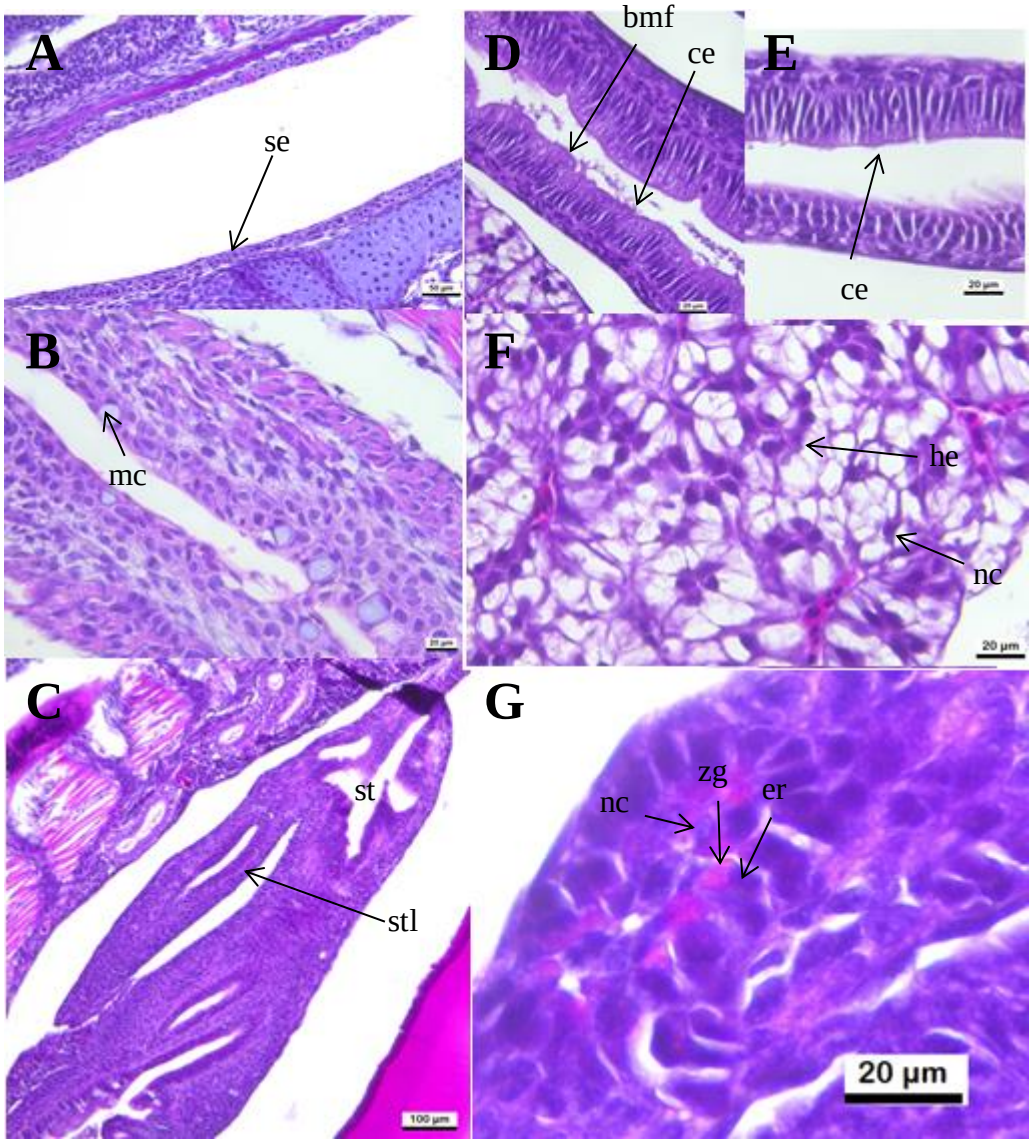


Figure 3.4. Longitudinal sections of several parts of the gastrointestinal tract and associated organs of diploid and triploid Atlantic salmon at hatch (570 dd). (A) Buccopharyngeal cavity 2N. (B) Esophagus 3N. (C) Stomach 3N. (D) Proximal intestine 2N. (E) Distal intestine 2N. (F) Liver 3N. (G) Pancreas 3N. bmf, beginning mucousal folding; ce, columnar epithelium; er, endoplasmic reticulum; he, hepatocytes; mc, mucous cells; nc, nucleus; se, squamous epithelium; st, stomach; stl, stomach longitudinal fold; zymogen granula. Scale. A-B, 50 µm. D-E, 20 µm.

## 3.3 Start-feeding (920 dd)

### 3.3.1 Growth

At start-feeding, only two groups of fish were present (diploids and triploids). These were analyzed for possible ploidy differences with regard to growth (weight) and length, but no significant differences were found (Fig 3.5). Diploids weighed 0.13 g ± 0.03, and triploids weighed 0.14 g ± 0.03. The length was 2.75 cm ± 0.13 and 2.79 cm ± 0.14 for diploids and triploids, respectively.

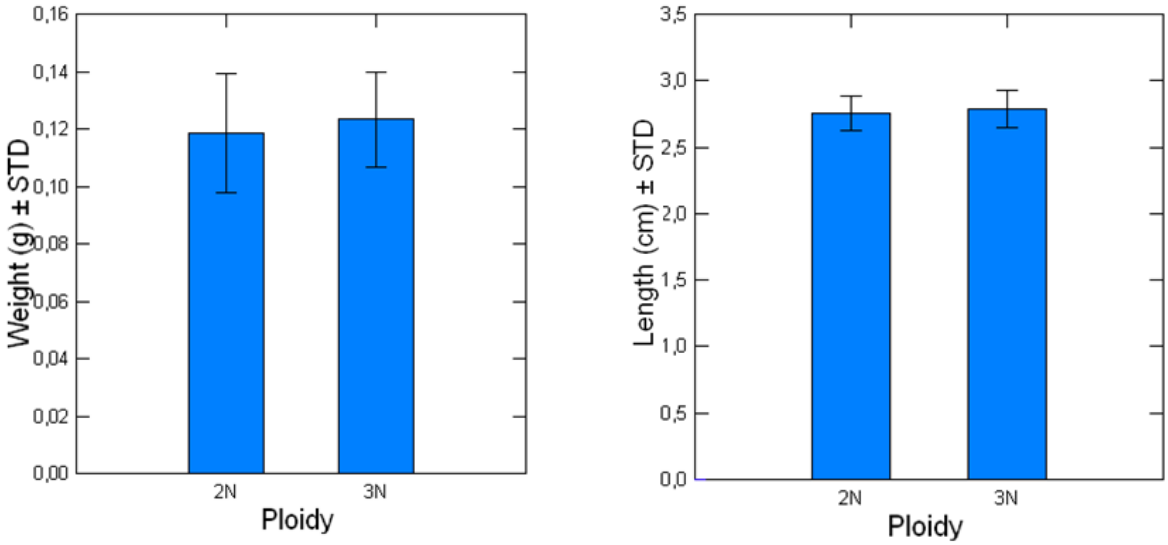


Figure 3.5. Body weight (g) (left) and body length (cm) (right) of the two groups at start-feeding (920 dd). Data with a common superscript are not significantly different.

### 3.3.2 **Ontogeny**

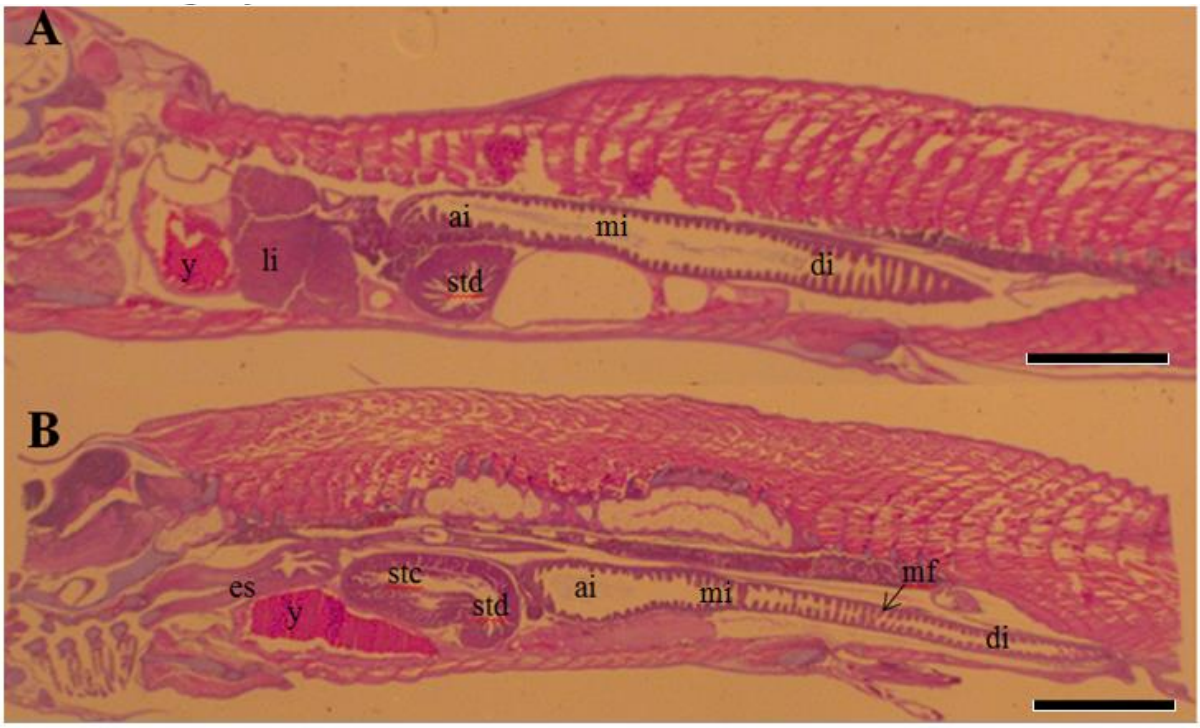


Figure 3.6. Longitudinal sections of the abdominal part of whole individuals of diploid and triploid Atlantic salmon at start-feeding (920 dd). (A) Overview whole individual (2N). (B) Overview whole individual (3N). ai, anterior intestine; di, distal intestine; es, esophagus; li, liver; mf, mucosal folds; mi, mid intestine; stc, stomach cardiac; std, stomach distal; y, yolk sac. Scale. A-B, 1.5 mm.

At this stage four individuals per ploidy was analyzed for the general histomorphology, and all individuals looked the same for both ploidies at this stage except some individual differences observed for the liver within each ploidy group.

**Yolk sac**. At start-feeding a small yolk rest was present in the abdominal cavity (Fig 3.6). **Pharynx.** The stratified squamous epithelium lined the buccopharyngeal cavity and pharynx, mucous cells and taste buds were present in large numbers (Fig 3.7 A-B). **Esophagus.** The epithelium had started to fold, and taste buds appeared together with increasing numbers of mucous cells in the epithelium (Fig 3.7 C-D). The esophagus was also made up of stratified squamous cells.

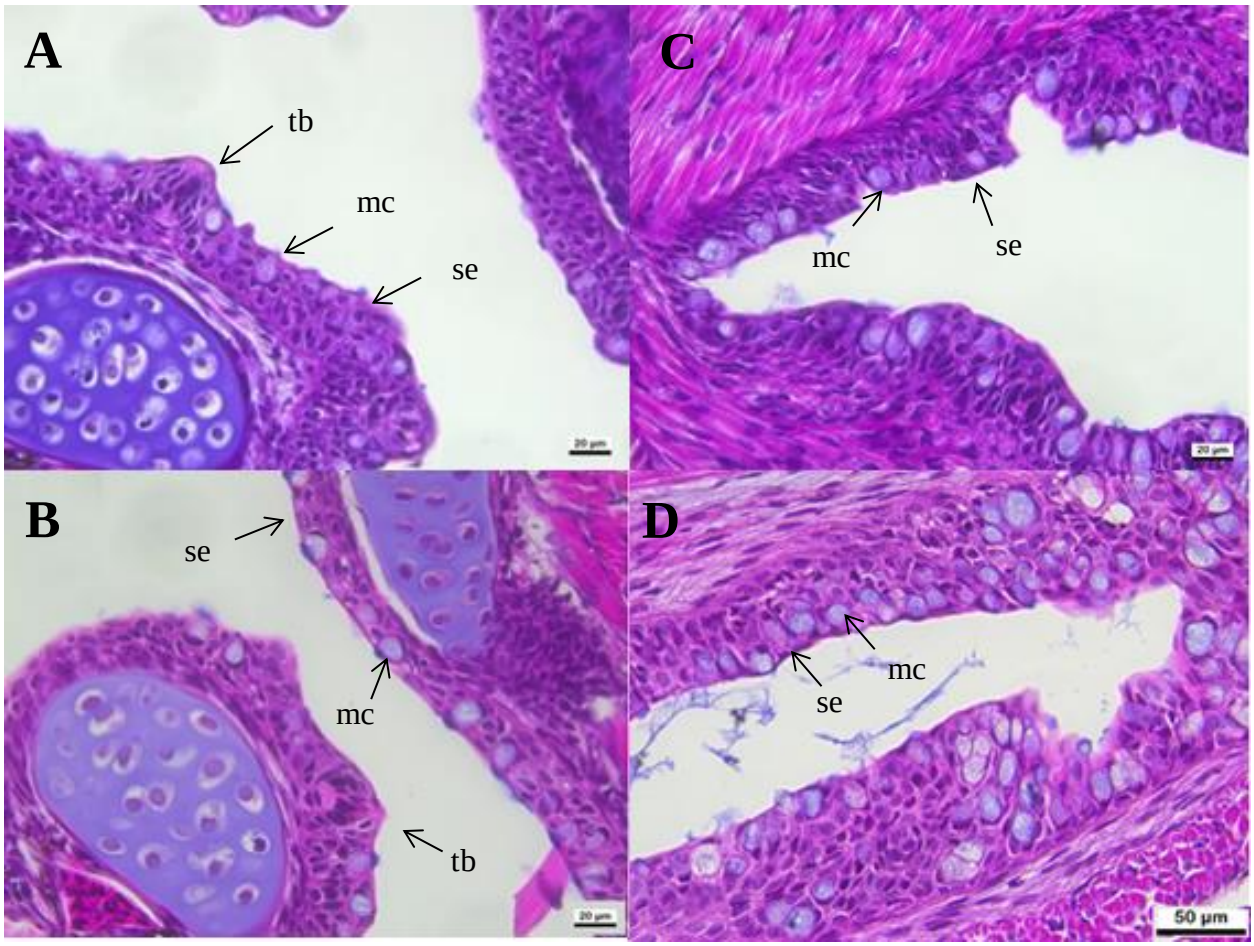


Figure 3.7. Longitudinal sections of the pharynx (A-B) and esophagus (C-D) of diploid and triploid Atlantic salmon at start-feeding (920 dd). (A) Diploid individual (2N). (B) Triploid individual (3N). (C) Diploid individual (2N). (D) Triploid individual (3N). mucous cells; se, squamous epithelium; tb, taste buds. Scale. A-C, 20 µm.

**Stomach.** At start-feeding the stomach was "U shaped" (Fig 3.8 A-B). The stomach was longer and divided in two parts with the mucosa of the cardiac part lined with simple secretory columnar cells with tubular gastric glands (Fig 3.8 C-D), and the distal part with a thicker muscle layer and more folded mucosa. Mucous cells were present in the epithelium along the full length of the stomach (not shown).

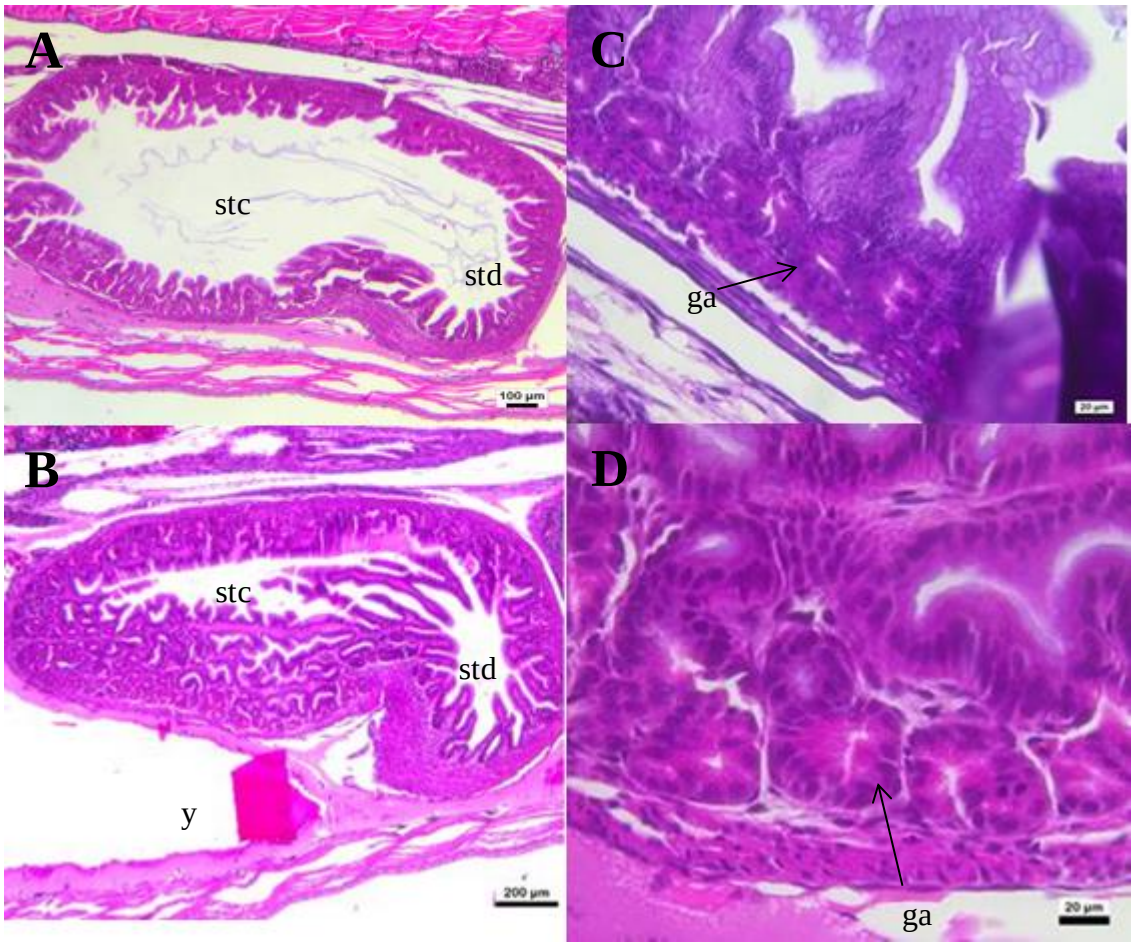


Figure 3.8. Longitudinal sections of the stomach (A-B) and gastric glands (C-D) of diploid and triploid Atlantic salmon at start-feeding (920 dd). (A) Diploid individual (2N). (B) Triploid individual (3N). (C) Gastric glands in the cardiac part of the stomach (2N). (D) Gastric glands in the cardiac part of the stomach (3N). ga, gastric pits; stc, stomach cardiac; std, stomach distal; y, yolk sac.

**Pyloric caeca.** The pyloric caeca buds were observed at start-feeding in both ploidies (Fig 3.9. A-B). Pancreatic tissue is located around the pyloric caeca buds.

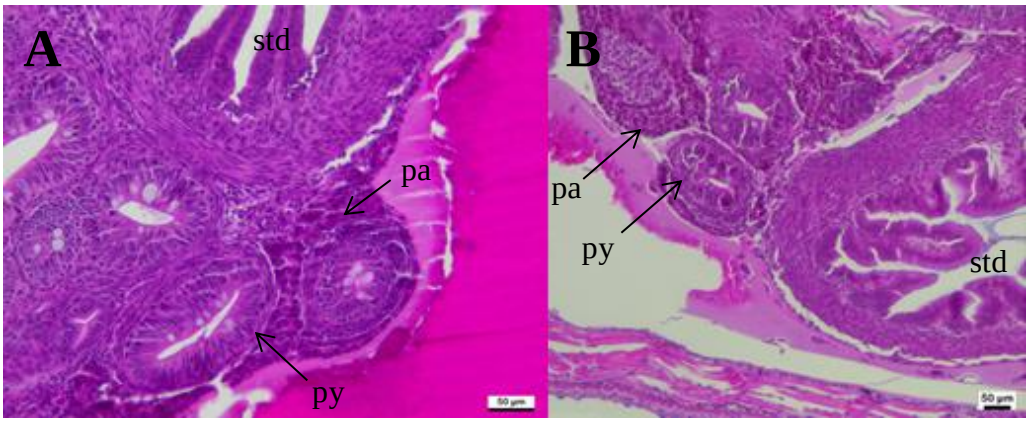


Figure 3.9. Longitudinal sections of early pyloric caeca and stomach of diploid and triploid Atlantic salmon at start-feeding (920 dd). (A) Early pyloric caeca (2N). (B) Early pyloric caeca (3N). pa, pancreatic tissue; py, pyloric caeca; stomach distal. Scale. A-B, 50 µm.

**Intestine.** At start-feeding the intestine was slightly coiled, and characterized by a simple folded mucosa (Fig 3.10 A-D). A few mucous cells could be observed in the folded epithelium. No supranuclear vacuoles present at this stage.

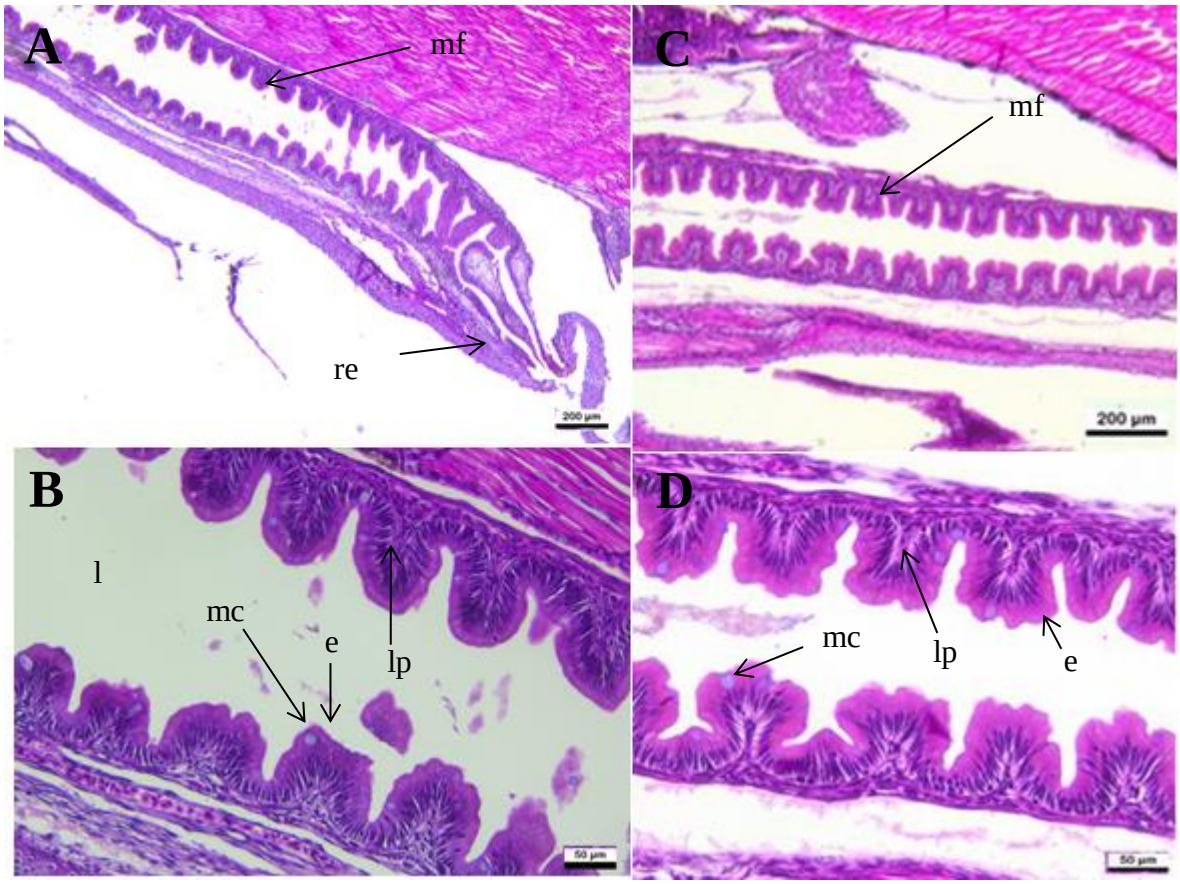


Figure 3.10. Longitudinal sections of the mid and distal intestine of diploid and triploid Atlantic salmon at start-feeding (920 dd). (A) Distal intestine (2N). (B) Distal intestine (2N). (C) Mid and distal intestine (3N). (D) Mid intestine (3N). e, enterocytes; l, lumen; lp, lamina propria; mc, mucous cells; mf, mucosal folds; re, rectum.

**Liver and pancreas**. At start-feeding the liver was larger than at hatch, and still had glycogen-vacuoles in the hepatocytes (Fig 3.11 A-B). As illustrated below, B contains more vacuoles than A, however this was not consistent for the triploid group as they showed variation between individuals, and one of the diploid individuals also showed the same amount as the triploid group. Pancreas tissue was present in the mesenteric tissue in the abdomen, especially around the pyloric caeca and anterior intestine at this stage. Zymogen granules were present (brighter part of the cell in the exocrine pancreas) at start-feeding (3.11 C-D).

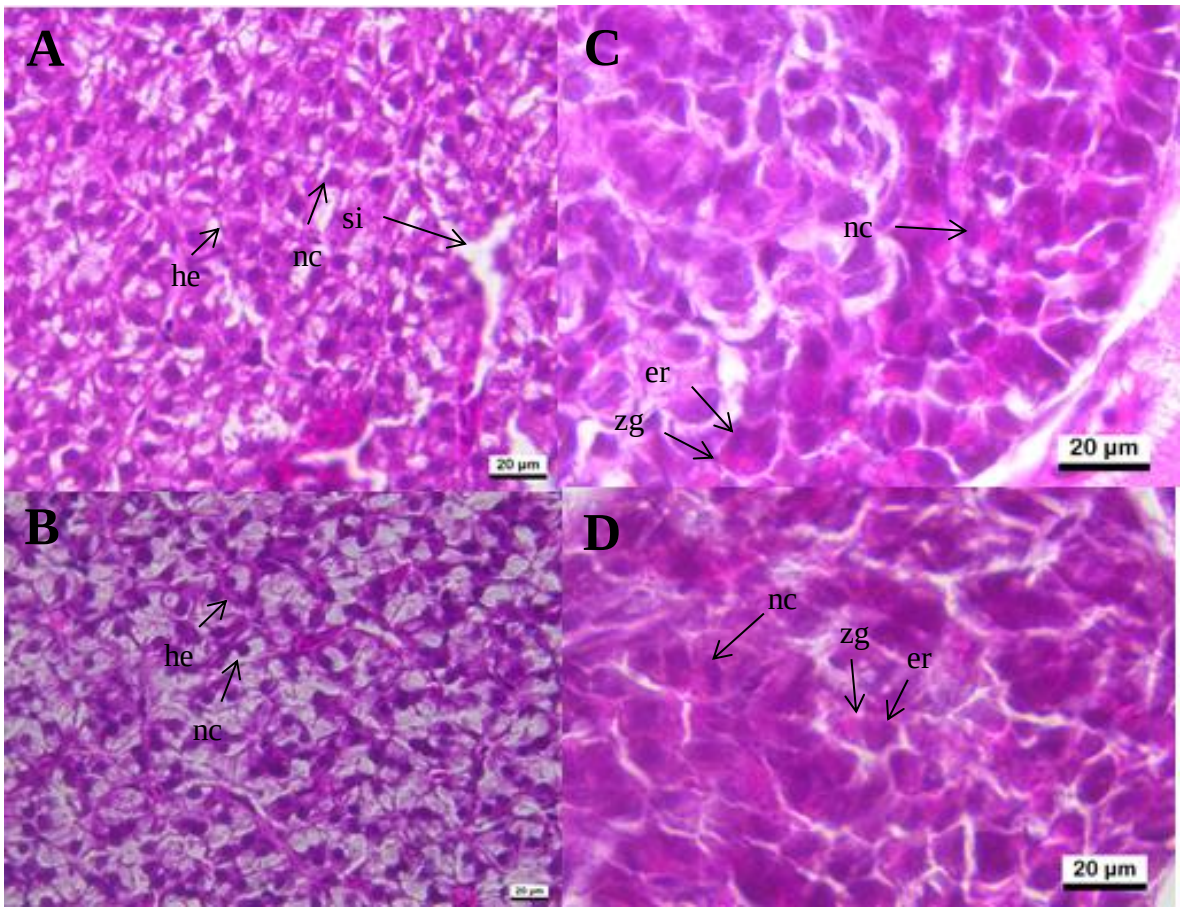


Figure 3.11. Longitudinal sections of liver (A-B) and pancreas (C-D) for diploid and triploid Atlantic salmon at start-feeding (920 dd). (A) Diploid (2N). (B) Triploid (3N). (C) Diploid (2N). (D) Triploid (3N). er, endoplasmic reticulum; he, hepatocytes; nc, nucleus; si, sinusoid; zg, zymogen granula. Scale. B, 20 µm.

### 3.3.3 **Morphometric measurements of the intestine**

Individual intestinal sections were analyzed for fold height and width, thickness of the intestinal wall and number of goblet cells. Sections from the proximal intestine at this sampling stage showed a very distended mucosa and were of a quality little suitable for measurements and therefore sections from the distal intestine were used for comparative analyses. The fold height and number of goblet cells did not vary between the two groups, but a difference was found in the width of the folds and the thickness of the intestinal wall. The fold width (75.5 µm ± 8.12) was significantly wider in the triploid group compared to the diploid group (66.26 µm ± 12.21) ($P$<0.001) (Fig 3.12). However, the intestinal wall proved to be significantly thicker in the diploid group, 13.92 µm ± 2.88, while that of the triploid group was 12.06 µm ± 2.34 ($P$<0.001) (Fig 3.13). Morphometric measurements where the groups do not differ significantly are listed in the appendix section B as tables and section C

as box plots; this is consistent for the rest of the morphometric measurements throughout the thesis.

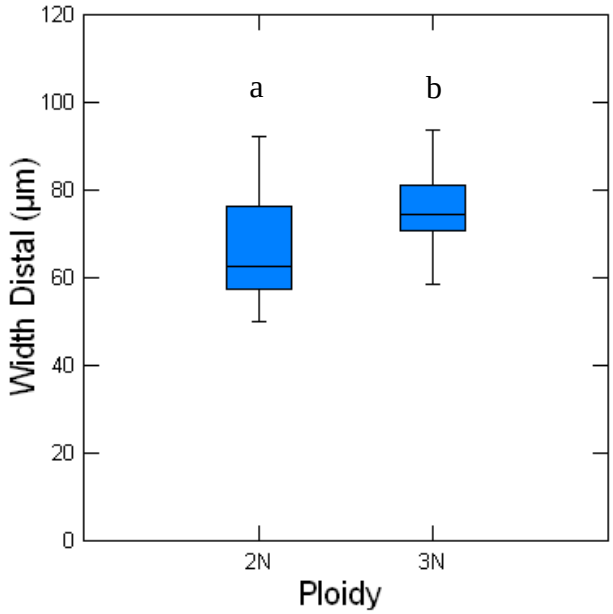


Figure 3.12. Fold width of the distal intestine (n=4 fish/ploidy) in each ploidy group at start-feeding (920 dd). Data with a common superscript are not significantly different. Box-plot to the left represents data from the diploid group and the box plot to the right represents data from the triploid group. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

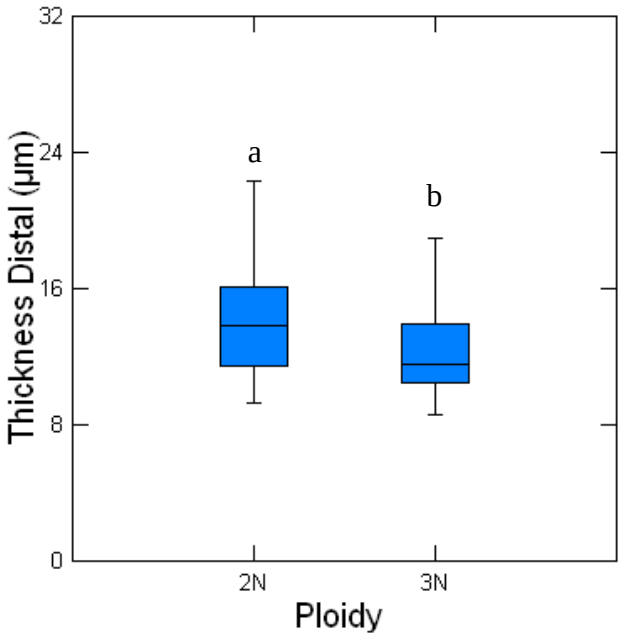


Figure 3.13. Thickness of the distal intestinal wall (n=4 fish/ploidy) in each ploidy group at start-feeding (920 dd). Data with a common superscript are not significantly different. Box-plot to the left represent data from the diploid group and the box plot on the right represent data from the triploid group. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.4 4 weeks after start-feeding (1150 dd)

### 3.4.1 **Growth**

At this stage fish could be analyzed for differences between both ploidy and diet. A difference in body weight between groups was observed. Group 1 weighed 0.25 g ± 0.06 and was significantly heavier than group 2 weighing 0.20 g ± 0.06 ($P$<0.05) and group 3 weighing 0.21 g ± 0.08 ($P$<0.05) (Fig 3.14). Group 4 weighed 0.22 g ± 0.07. Body length was not measured at this sampling day.

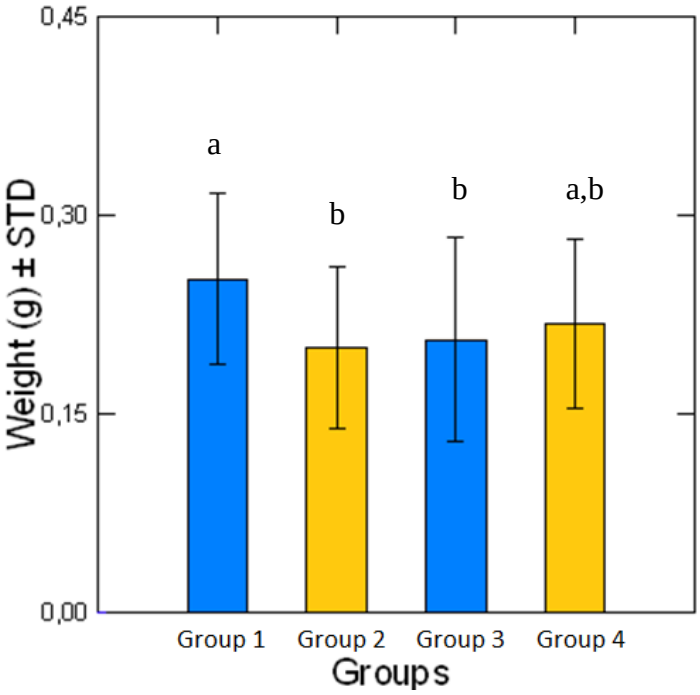


Figure 3.14. Effect of ploidy and diet on weight (g) of Atlantic salmon 4 weeks after start-feeding (1150 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.4.2 **Ontogeny**

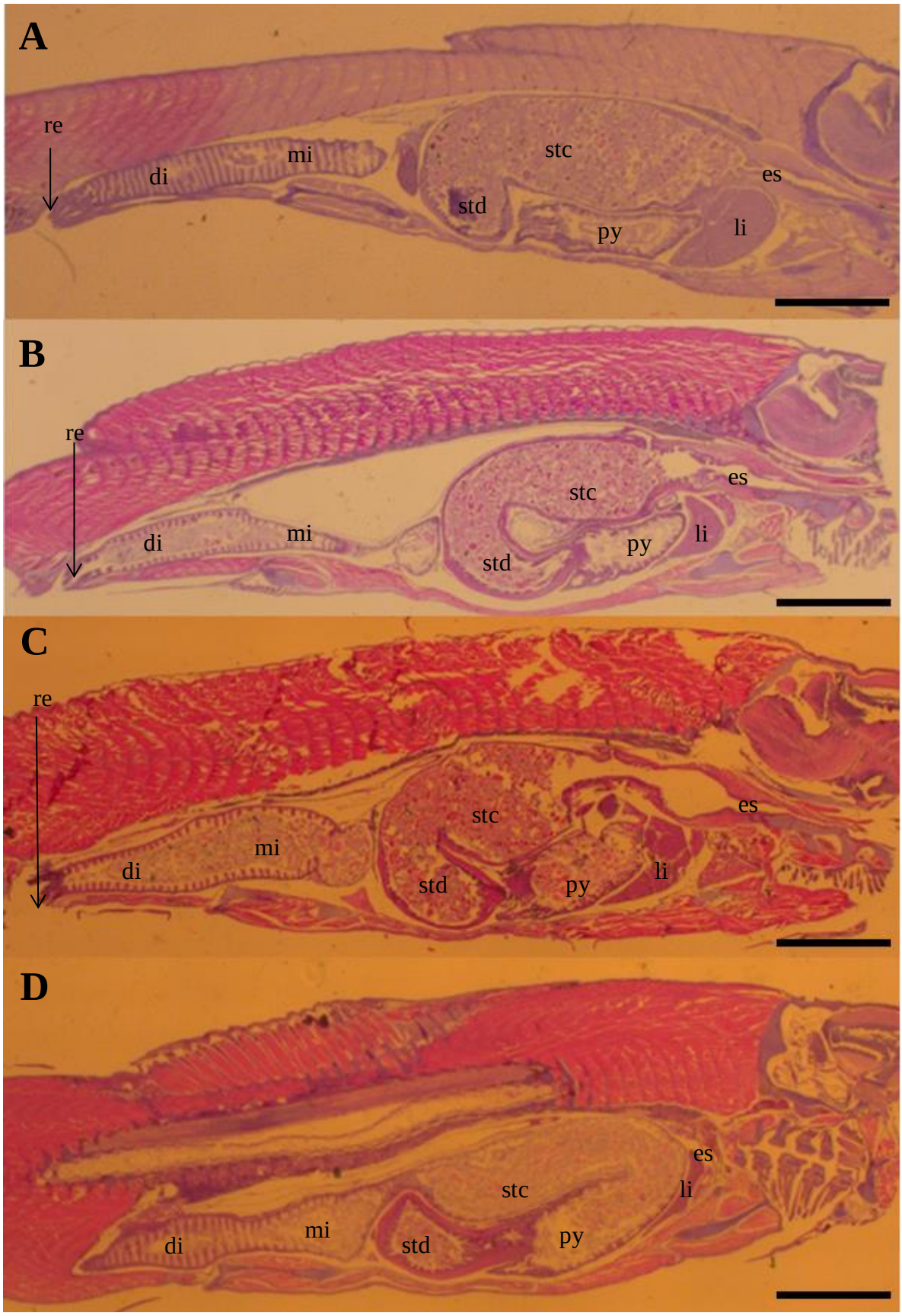

Figure 3.15. Longitudinal sections of the abdominal part of whole individuals of diploid and triploid Atlantic salmon fed different diets 4 weeks after start-feeding (1150 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4). di, distal intestine; es, esophagus; li, liver; mi, mid intestine; py, pyloric caeca; re, rectum; stc, stomach cardiac; std, stomach distal. Scale. A-D, 1.88 mm.

Nine individuals per group was compared at this stage but no obvious differences could be observed in the general histomorphology, except some individual differences for the livers within the same groups.

**Yolk sac.** 4 weeks after start-feeding the yolk sac was completely absorbed. **Pharynx.** A slight increase was observed in number and sizes of mucous cells, this was the only difference observed considering the morphology of the pharynx mucosa between start-feeding and 4 weeks after start-feeding (Fig 3.16 A-D). **Esophagus.** There were increased mucosal folding and taste buds, and mucous cells were also more numerous (Fig 3.16 E-H). A clear transitional zone between esophagus and stomach could be observed (Fig 3.15 A-B).

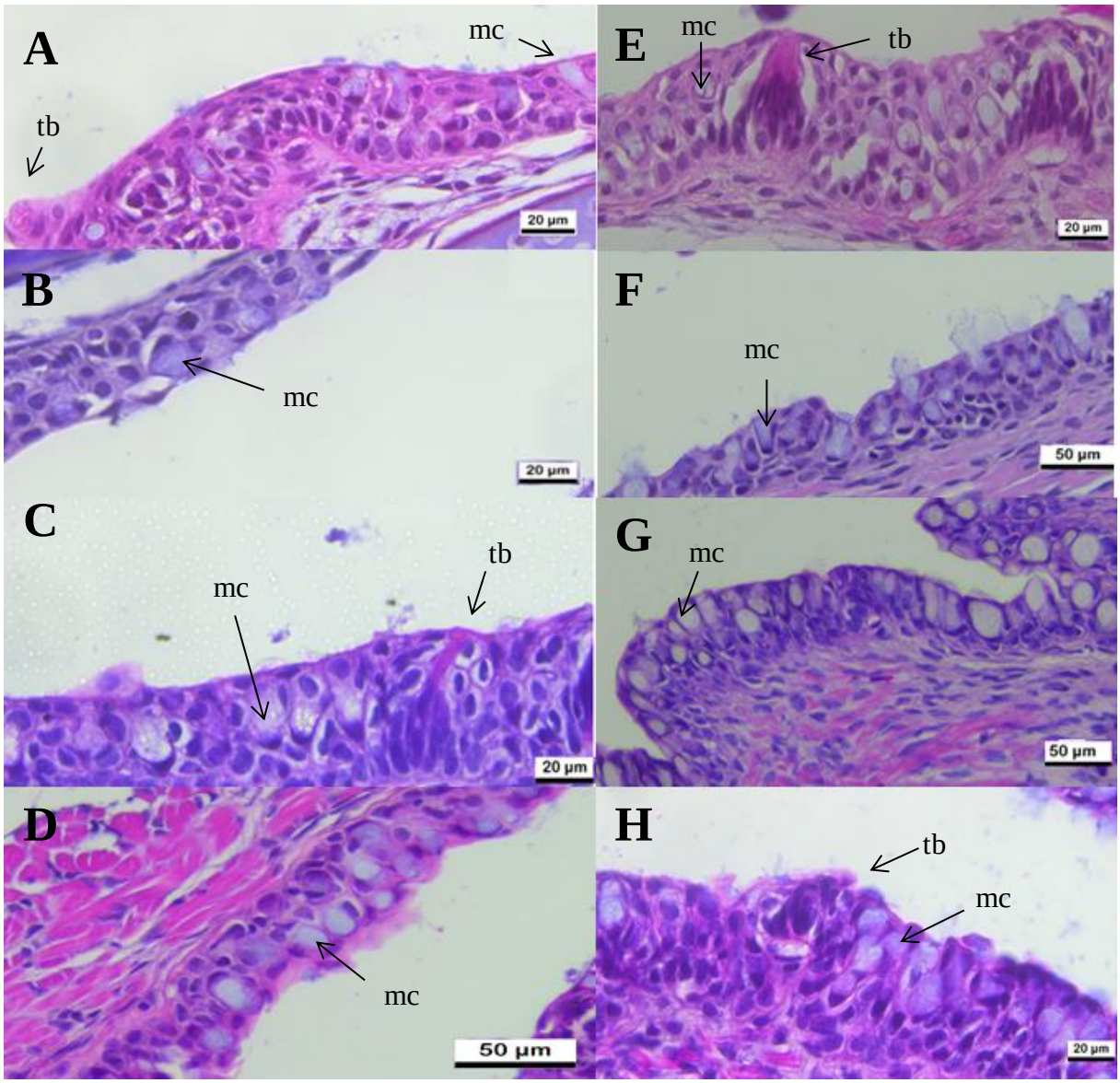


Figure 3.16. Longitudinal sections of the pharynx (A-D) and esophagus (E-H) of diploid and triploid Atlantic salmon fed different diets 4 weeks after start-feeding (1150 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4. E= Group 1. F = Group 2. G = Group 3. H= Group 4). Esophagus. mc, mucous cells; tb, taste buds.

**Stomach and pyloric caeca.** 4 weeks after start-feeding there was a clear difference between the cardiac and distal part of the stomach, as well as a general increase in stomach size. Stomach and pyloric stomach also received a more clear "U shape and loop look" (Fig 3.15 A-D). The muscle layer was thicker as well. Pyloric caeca larger and more numerous. There was an overall increase in mucosal fold height. **Intestine.** An increase in size of the intestine as well as fold height and width were noted. Small supranuclear vacuoles was also observed

in the enterocytes at this stage. Otherwise no major structural changes were obvious (Fig 3.17 A-D).

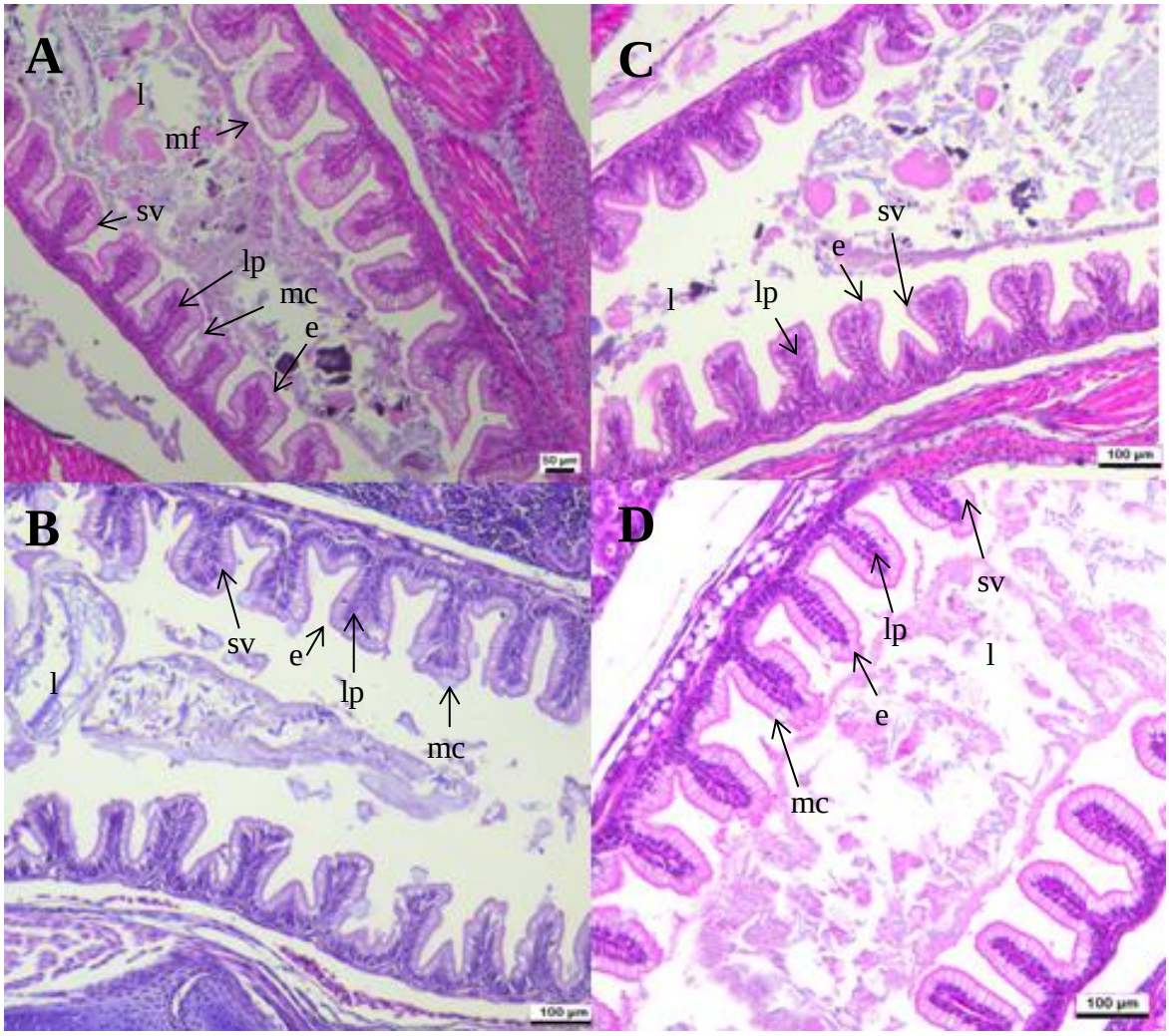


Figure 3.17. Longitudinal sections of the distal intestine of diploid and triploid Atlantic salmon 4 weeks after start-feeding (1150 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4). e, enterocytes; l, lumen; lp, lamina propria; mc, mucous cells; mf, mucosal folds; sv, supranuclear vacuoles.

**Liver and pancreas**. 4 weeks after start-feeding the hepatocytes had numerous glycogen granules and variable degree of vacuolization (Fig 3.18 A-D) and the total size of the organ was larger. A and C show less vacuolated liver, but again, this was not consistent for all individuals within the group, altogether there was a lot of variation for this observation. More pancreatic tissue was observed in the abdomen and mesenteric tissue (Fig 3.18 E-H).

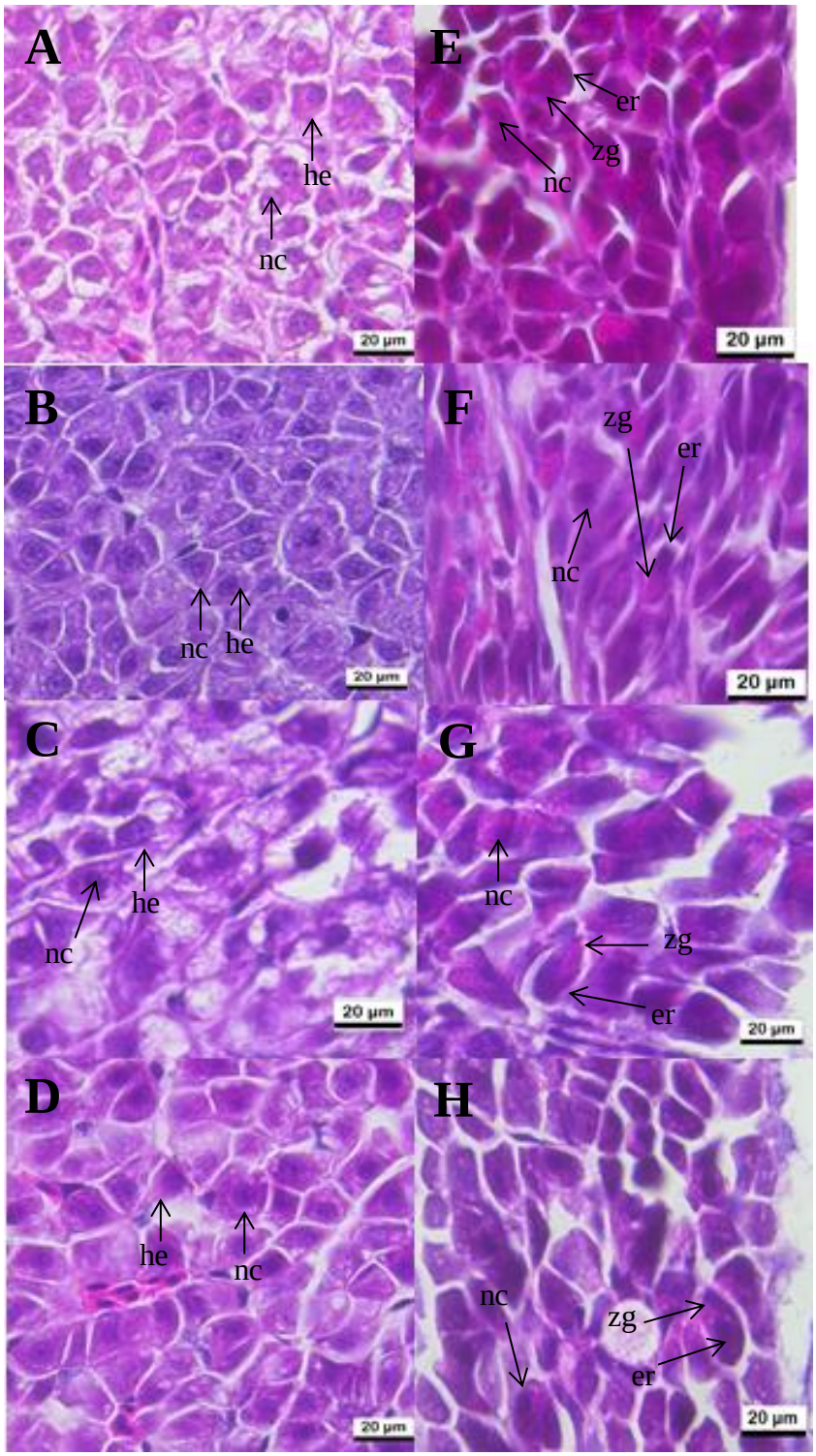


Figure 3.18. Longitudinal sections of liver (A-D) and pancreas (E-H) for diploid and triploid Atlantic salmon 4 weeks after start-feeding (1150 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4. E= Group 1. F = Group 2. G = Group 3. H= Group 4). er, endoplasmic reticulum; he, hepatocytes; nc, nucleus; zg, zymogen granula.

### 3.4.3 **Morphometric measurements of the intestine**

Sections of individuals from the four different groups were analyzed for fold height and width, thickness of the intestinal wall and number of goblet cells in the distal intestine, but also for thickness of the intestinal wall in the anterior intestine. No significant differences were found with respect to height or width of the folds or goblet cells in the distal intestine, neither were differences found in the intestinal wall thickness in the anterior intestine between the groups. However, the thickness of the intestinal wall in the distal intestine was significantly thicker in group 3 compared to all the other groups. Group 1 (12.95 µm ± 4.95) had a significantly thinner intestinal wall than group 3 (16.13 µm ± 7.23) ($P$<0.01). Group 2 (12.65 µm ± 4.47) also showed a significantly thinner intestinal wall compared to group 3 ($P$<0.01). Finally, group 3 had a significantly thicker wall than group 4 (14.66 µm ± 5.29) ($P$<0.05) (Fig 3.19).

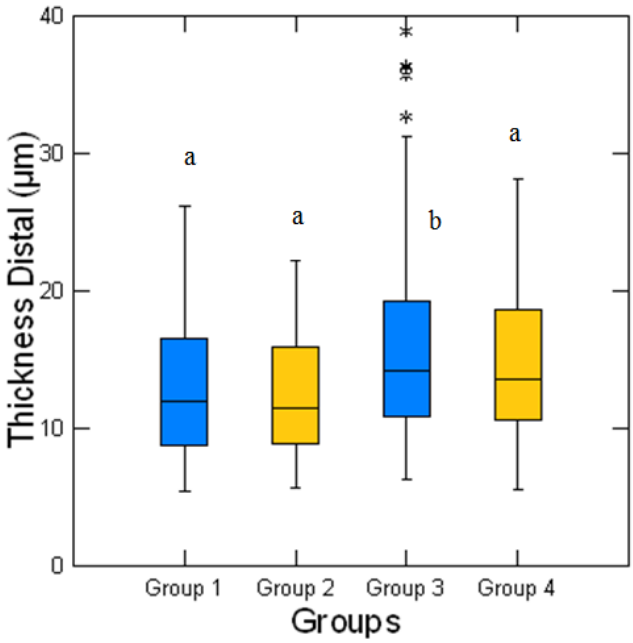


Figure 3.19. Thickness of the distal intestinal wall (n=9 fish/group) in the 4 experimental groups 4 weeks after start-feeding (1150 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Group 1 and 2 are diploid groups, and group 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.5 9 weeks after start-feeding (1500 dd)

### 3.5.1 Growth

Significant differences were observed between groups 9 weeks after start-feeding in terms of growth and body length. Group 3 weighing 0.74 g ± 0.06 was significantly heavier than group 4 weighing 0.62 g ± 0.07 ($P$<0.05) (Fig 3.20). Group 1 and group 2 weighed 0.69 ± 0.02 and 0.65 ± 0.06, respectively.

There was also a difference among groups in terms of body length. Group 3 (4.25 cm ± 0.1) was significantly longer than both group 2 (4.01 ± 0.12) ($P$<0.05) and group 4 (3.95 ± 0.14) ($P$<0.01) (Fig 3.20). Group 1 was 4.11 ± 0.06 cm in body length.

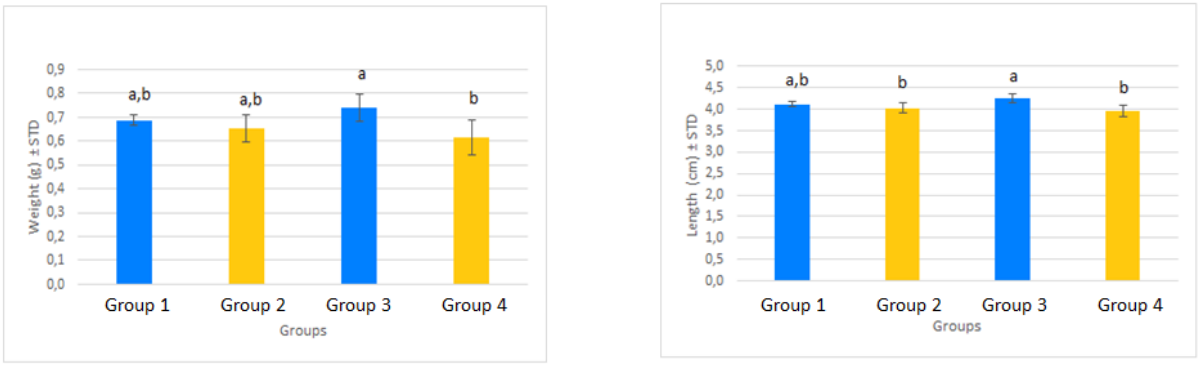


Figure 3.20. Effect of ploidy and diet on weight (g) (left) and length (cm) (right) of Atlantic salmon 9 weeks after start-feeding (1500 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.5.2 Ontogeny

**Pharynx.** A slight increase in number of mucous cells and their size were the only difference in the structure of the pharynx mucosa between 4 and 9 weeks after start-feeding (Fig 3.21).

**Esophagus.** No apparent major differences were noted in the esophagus compared to the sections from 4 weeks after start-feeding.

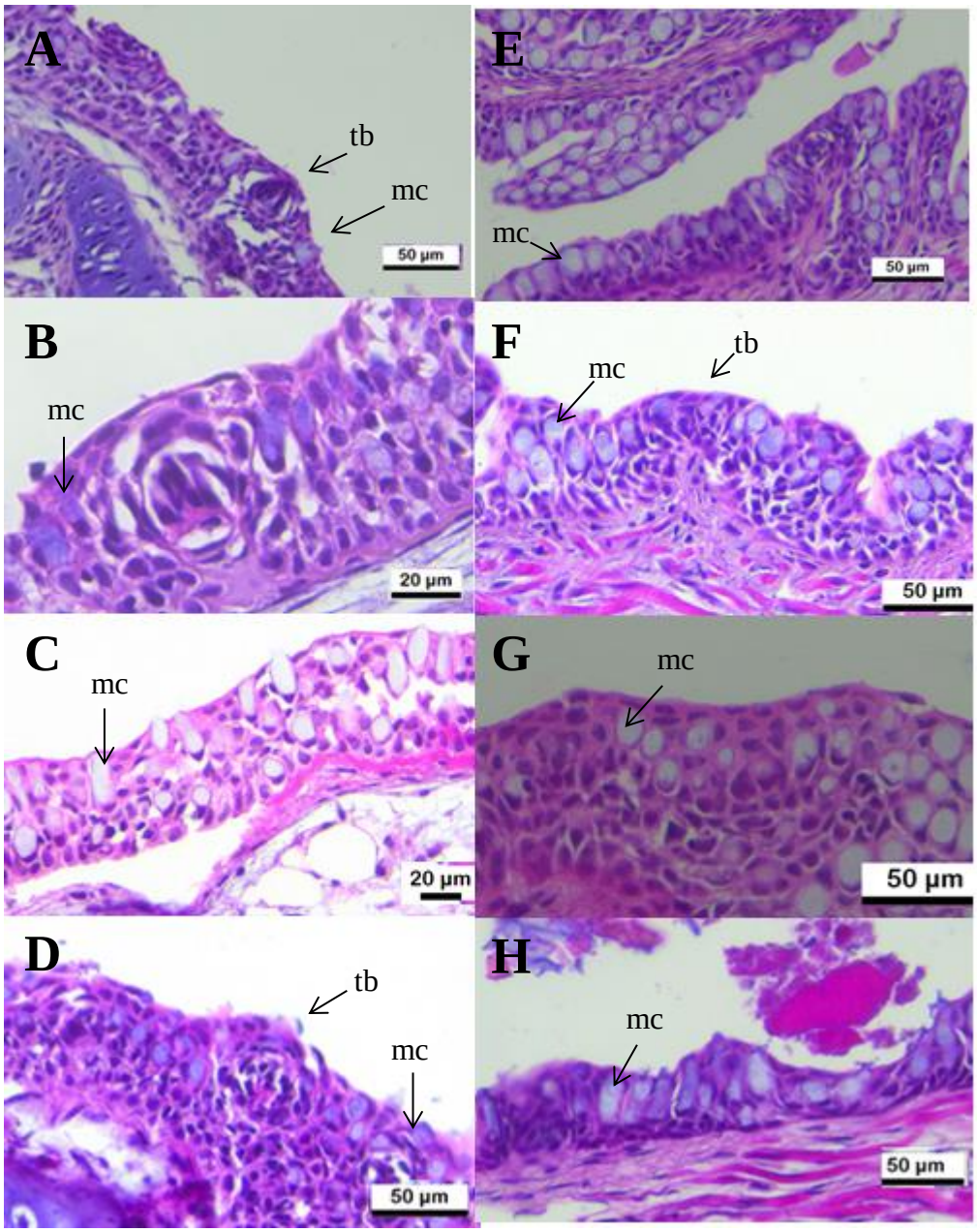


Figure 3.21. Longitudinal sections of the pharynx (A-D) and esophagus (E-H) of diploid and triploid Atlantic salmon fed different diets 9 weeks after start-feeding (1500 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4. E= Group 1. F = Group 2. G = Group 3. H= Group 4). mc, mucous cells; tb, taste buds.

**Stomach and pyloric caeca.** No major modifications were apparent in the stomach mucosa compared to the previous sampling point. A general increase in size as well as thickness of muscle layer were noted. There was a more distinct separation between the cardiac part and distal part of the stomach (not shown), and pyloric caeca were more numerous (not shown). **Intestine.** The intestinal fold heights and muscle layers had increased. Small supranuclear vacuoles was also present at this stage (Fig 3.22 A-D).

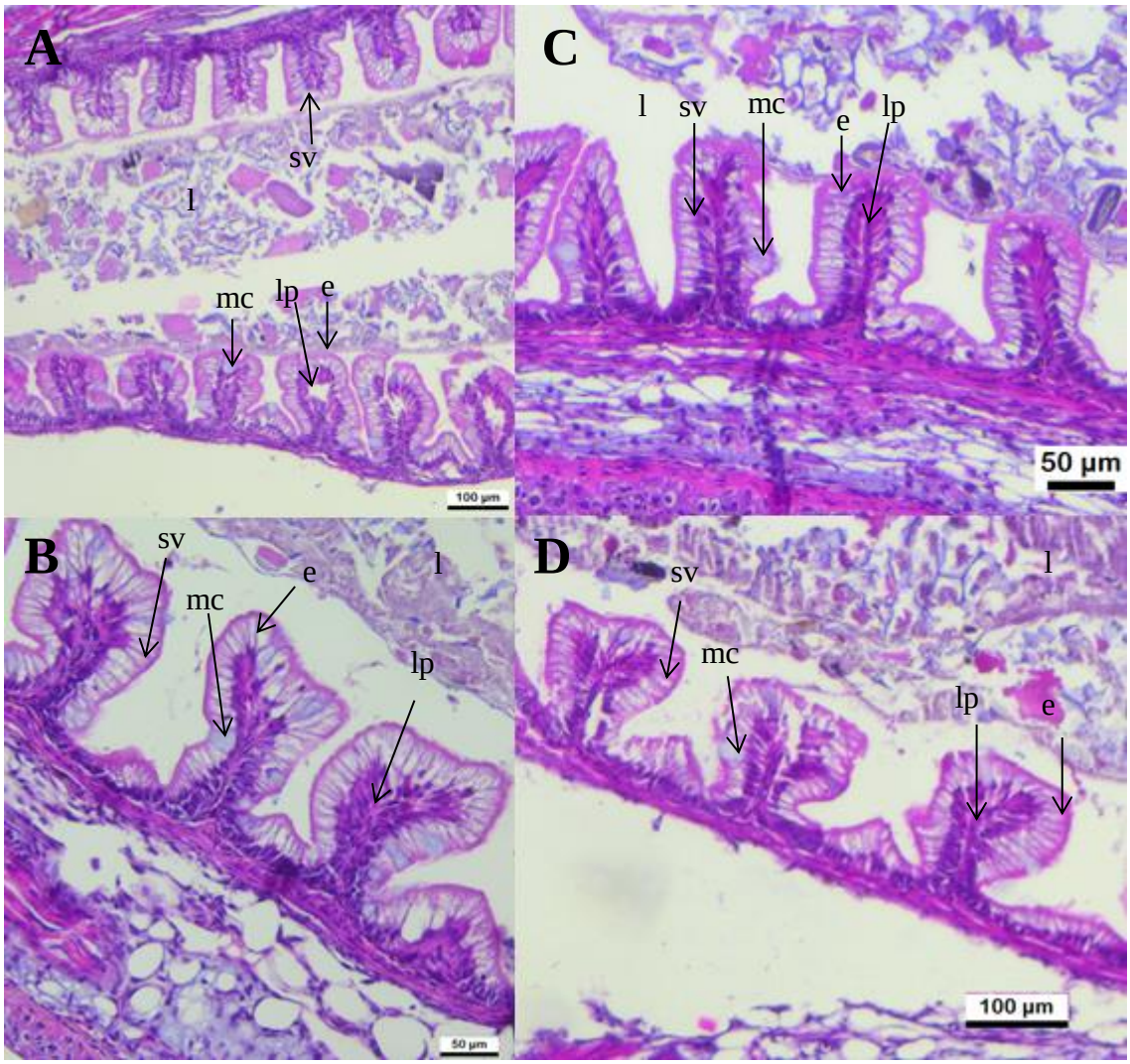


Figure 3.22. Longitudinal sections of the distal intestine for diploid and triploid Atlantic salmon 9 weeks after start-feeding (1500 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4). e, enterocytes; l, lumen; lp, lamina propria; mc, mucous cells; sv, supranuclear vacuoles.

**Liver and pancreas.** Still large and vacuolated liver (Fig 3.23 A-D). The pictures used for illustration shows less glycogen-vacuoles in C, but this was not consistent. All groups showed some degree of variation, but most of the individuals showed large glycogen deposits in the liver. No major modifications in the pancreas tissue (Fig 3.23 E-H).

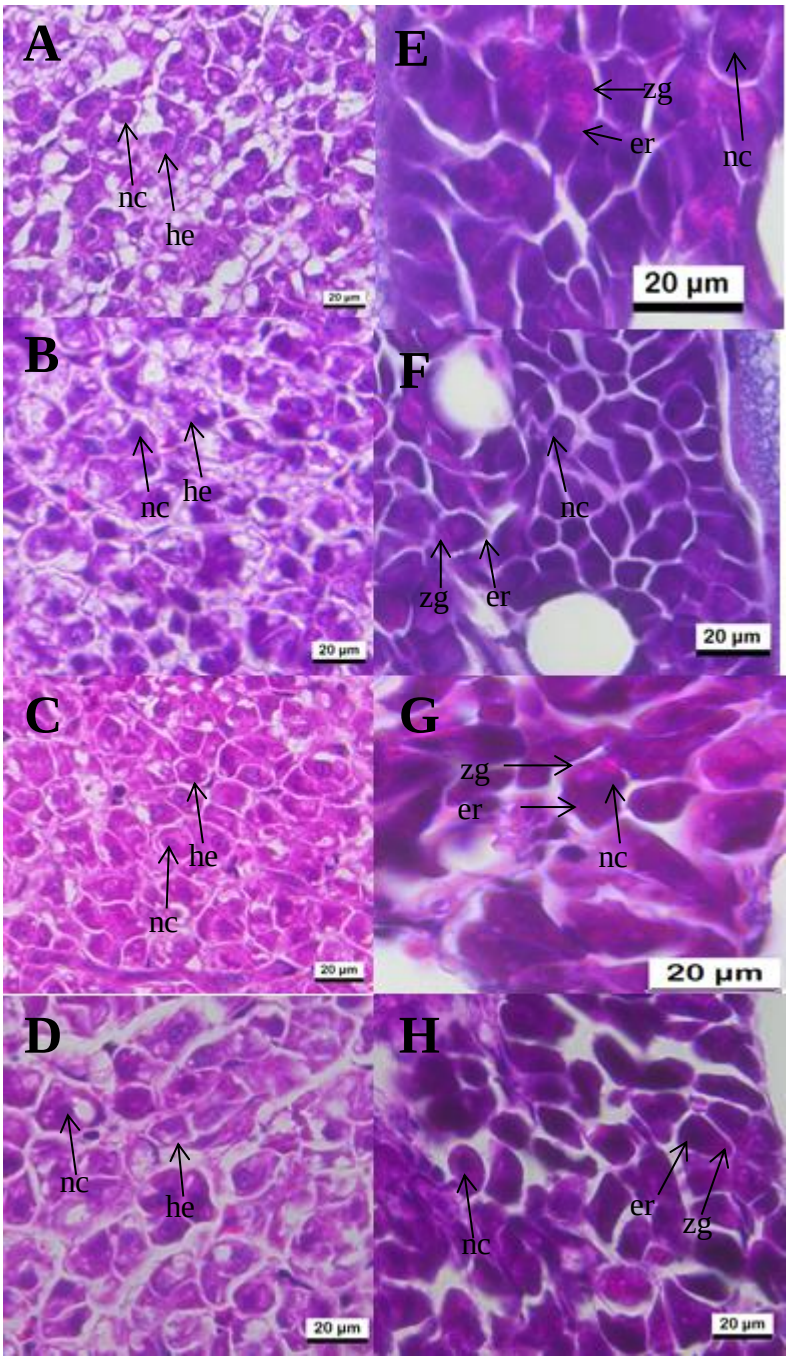


Figure 3.23. Longitudinal sections of liver (A-D) and pancreas (E-H) of diploid and triploid Atlantic salmon 9 weeks after start-feeding (1500 dd). (A= Group 1. B = Group 2. C = Group 3. D= Group 4. E= Group 1. F = Group 2. G = Group 3. H= Group 4). er, endoplasmic reticulum; he, hepatocytes; nc, nucleus; zg, zymogen granula.

### 3.5.3 **Morphometric measurements of the intestine**

Sections were analyzed for fold height and width, thickness of the intestinal wall and number of goblet cells in the distal intestine, but also for thickness of the intestinal wall in the anterior intestine. No significant differences were observed with respect to height or width of the folds, thickness of the intestinal wall or number of goblet cells in the distal intestine. The only

parameter where a difference was observed between the groups was in the intestinal wall thickness in the anterior intestine. Group 1 (16.5 µm ± 4.3) had a significantly thicker intestinal wall than all groups; group 2 (11.44 µm ± 2.03) ($P$<0.001), group 3 (12.76 µm ± 2.51) ($P$<0.001) and group 4 (13.1 µm ± 2.93) ($P$<0.001). Group 4 also had a significantly thicker intestinal wall than group 2 ($P$<0.05) (Fig 3.24).

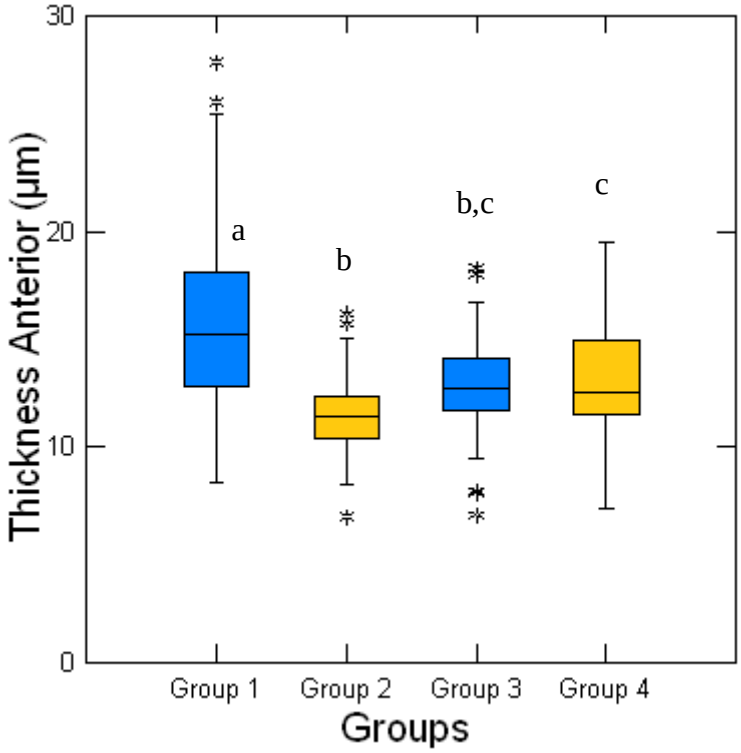


Figure 3.24. Thickness of the anterior intestinal wall (n=9 fish/group) in the 4 experimental groups 9 weeks after start-feeding (1500 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and group 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.6 13 weeks after start-feeding (1800 dd)

### 3.6.1 Growth

13 weeks after start-feeding (1800 dd) no difference in terms of length was observed, but the groups differed with regard to weight. Group 1 (1.68g ± 0.47) was significantly heavier than group 4 (1.39g ± 0.4) ($P$<0.05) (Fig 3.25). Group 2 and 3 weighed 1.55 g ± 0.43 and 1.60 g ± 0.53. The respective lengths from group 1 to 4 was: 5.53 cm ± 0.57, 5.44 cm ± 0.50, 5.47 cm ± 0.60, 5.27 cm ± 0.51.

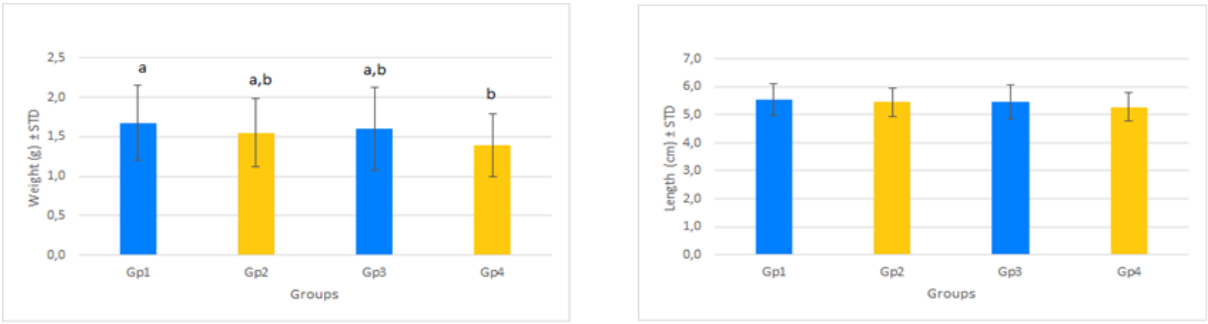


Figure 3.25. Effect of ploidy and diet on weight (g) and length (cm) of Atlantic salmon 13 weeks after start-feeding (dd 1800 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.6.2 **Morphometric measurements of the intestine**

Morphometric measurements were not performed at 13 weeks after start-feeding (1800 dd) due to bad quality sections.

## 3.7 17 weeks after start-feeding (2100 dd)

### 3.7.1 **Growth**

At this stage 17 weeks after start-feeding groups differed both in terms of growth and length, and the difference was observed in the same groups for both parameters. Group 3 weighing 4.11 g ± 1.22 was significantly heavier than group 2 weighing 3.28 g ± 0.83 ($P$<0.01) (Fig 3.26). Group 1 weighed 3.62 g ± 1.09 and group 4 weighed 3.72 g ± 1.56.

Group 3 was also significantly longer, measuring 7.28 cm ± 0.73 versus group 2 measuring 6.76 cm ± 0.62 in length ($P$<0.01). Group 1 and 4 measured 7.01 cm ± 0.71 and 7.02 cm ± 1.00, respectively.

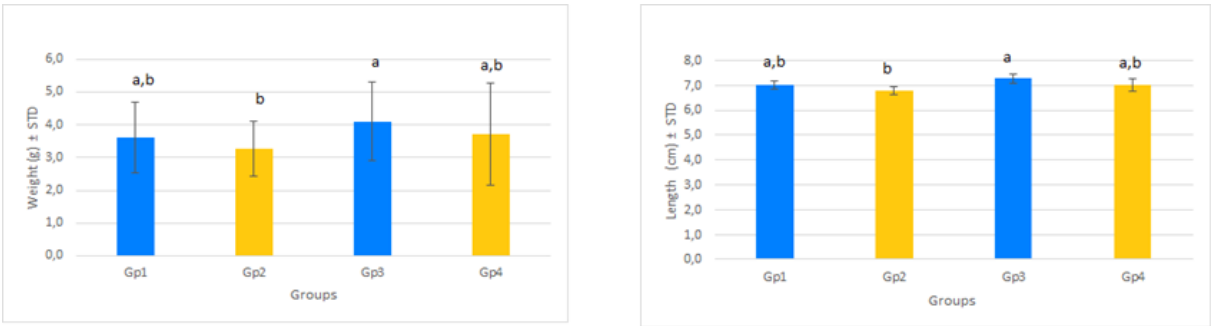


Figure 3.26. Effect of ploidy and diet on weight (g) and length (cm) of Atlantic salmon 17 weeks after start-feeding (2100 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.7.2 **Morphometric measurements of the intestine**

From 17 weeks after start-feeding and until the last sampling individuals were analyzed for all the different parameters; fold height and width, thickness of the intestinal wall, number of folds and number of goblet cells for both the anterior and the distal intestine. Differences were found with regard to fold height in the anterior intestine, thickness of the anterior intestinal wall and fold width in the distal intestine. Starting with the fold height in the anterior intestine, a significant difference was found between groups 1 and 2, with group 2 (140.38 µm ± 37.3) having significantly longer folds than group 1 (129.07 µm ± 22.33) ($P$<0.05) (Fig 3.27). Considering the thickness of the anterior intestinal wall, group 4 (21.95 µm ± 5.89) had a significantly thicker intestinal wall than group 2 (21.75 µm ± 5.89) ($P$<0.01) and group 1 (20.89 µm ± 6.34) ($P$<0.05). There was also a difference between groups 3 and 2, with group 3 (22.92 µm ± 4.8) being significantly thicker than group 2 ($P$<0.05) (Fig 3.28). The last parameter where a difference was observed was in fold width in the distal intestine. Here the folds in group 4 (117.98 µm ± 34.07) were significantly wider than in both group 2 (101.98 µm ± 24.32) ($P$<0.01) and group 1 (114.33 µm ± 32.92) ($P$<0.05) (Fig 3.29).

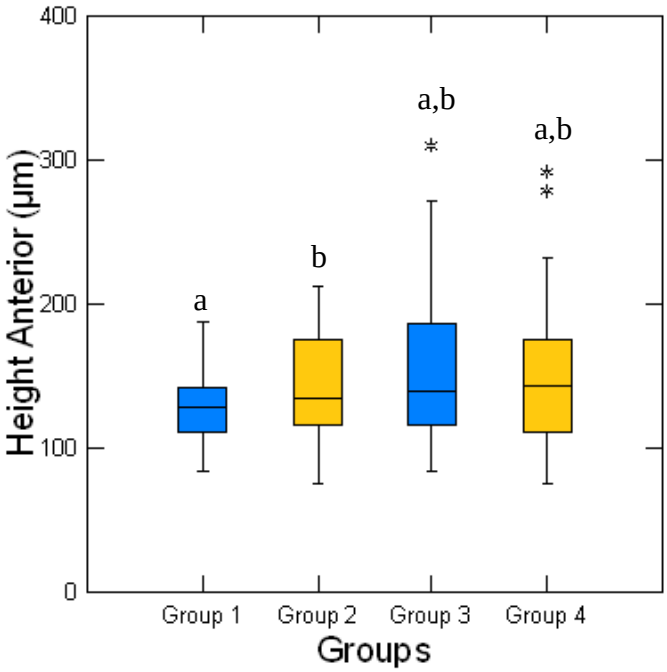


Figure 3.27. Fold height anterior intestine (n=9 fish/group) in the 4 experimental groups 17 weeks after start-feeding (2100 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

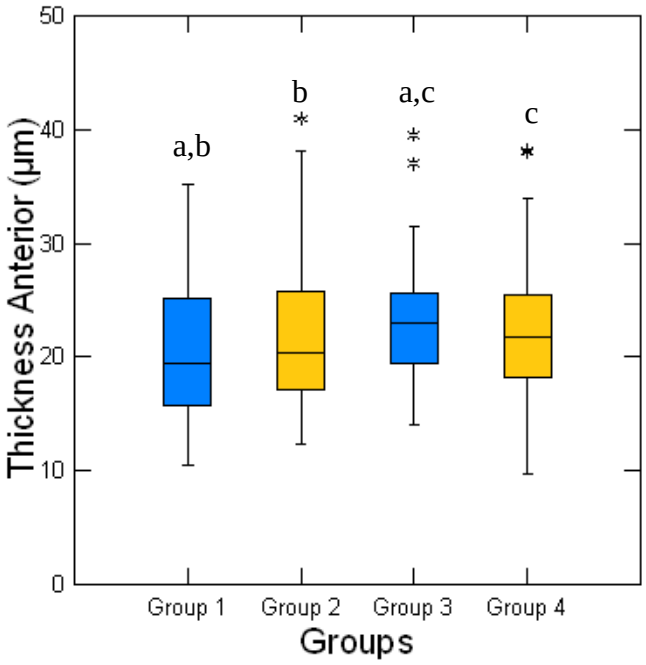


Figure 3.28. Intestinal wall thickness anterior intestine (n=9 fish/group) in the 4 experimental groups 17 weeks after start-feeding (2100 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

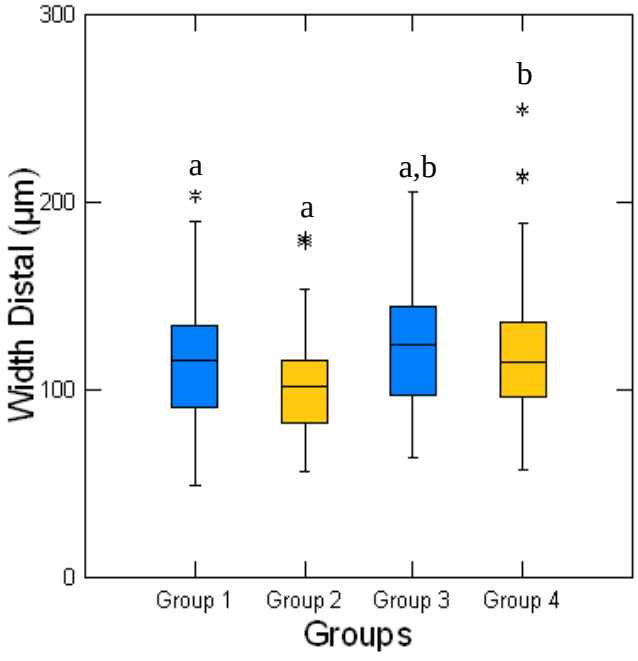


Figure 3.29. Fold width distal intestine (n=9 fish/group) in the 4 experimental groups 17 weeks after start-feeding (2100 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.8 21 weeks after start-feeding (2380 dd)

### 3.8.1 Growth

There were no differences observed in terms of growth 21 weeks after start-feeding (2380 dd), but group 3 was significantly different from the diploid groups in terms of length. Group 3 (9.23 cm ± 1.03) was significantly longer than group 1 (8.69 cm ± 0.95) ($P$<0.01) and group 2 (8.74 cm ± 1.01) ($P$<0.05) (Fig 3.30). Group 4 was 8.97 cm ± 1.14 in length. In terms of growth, the groups from 1 to 4 were 7.74 g ± 2.32, 7.70 g ± 2.56, 8.66 g ± 2.87, 7.76 g ± 2.79, respectively.

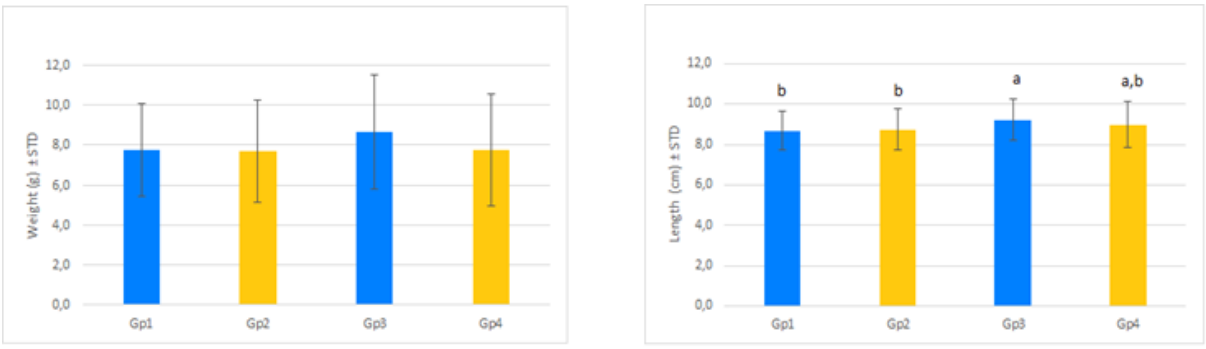


Figure 3.30. Effect of ploidy and diet on weight (g) and length (cm) of Atlantic salmon 21 weeks after start-feeding (2380 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.8.2 Morphometric measurements of the intestine

At 21 weeks after start-feeding differences were observed in anterior intestinal fold height, thickness of the distal intestinal wall and number of goblet cells in the distal intestine. Considering fold height of the anterior intestine, group 4 (179.98 µm ± 52.79) was significantly longer than both group 1 (162.48 µm ± 35.55) ($P$<0.05) and group 2 (151.35 µm ± 42.36) ($P$<0.05) (Fig 3.31). Number of goblet cells in the anterior intestine was different between groups 1 and 3, with group 1 (13.00 ± 1.61) having significantly more goblet cells than group 3 (10.00 ± 1.12) ($P$<0.05) (Fig 3.32). With respect to the thickness of intestinal wall of the distal intestine, group 2 (19.54 µm ± 7.32) had significantly thinner intestinal wall than group 3 (23.50 µm ± 7.02) ($P$<0.01), but thicker than group 4 (19.07 µm ± 6.19) ($P$<0.01). Group 3 was also significantly larger than group 4 ($P$<0.01) (Fig 3.33).

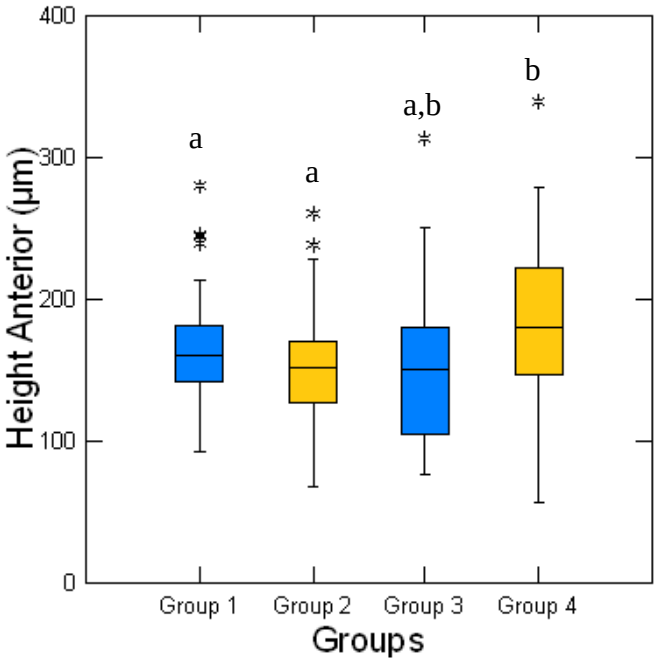


Figure 3.31. Fold height anterior intestine (n=9 fish/group) in the 4 experimental groups 21 weeks after start-feeding (2380 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

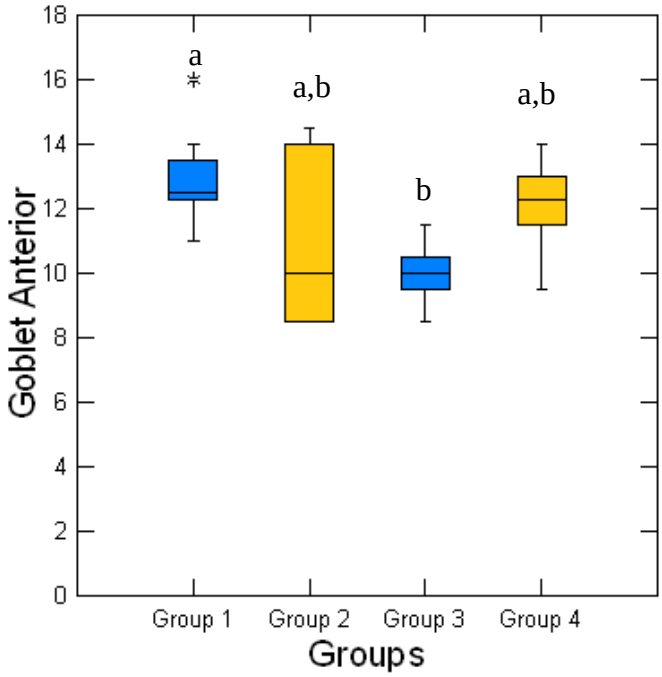


Figure 3.32. Number of goblet cells in anterior intestine (n=9 fish/group) in the 4 experimental groups 21 weeks after start-feeding (2380 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

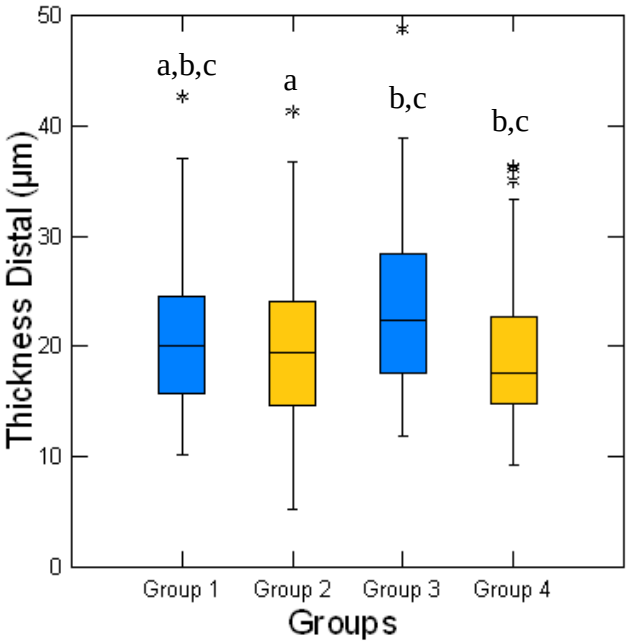


Figure 3.33. Intestinal wall thickness distal intestine (n=9 fish/group) in the 4 experimental groups 21 weeks after start-feeding (2380 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.9 27 weeks after start-feeding (2810 dd)

### 3.9.1 **Growth**

27 weeks after start-feeding there were no significant differences between the groups in terms of either growth or length of the fish. In terms of growth the groups (group 1 to 4) weighed 21.97 g ± 1.19, 21.22 g ± 0.95, 22.51 g ± 0.65, and 19.8 g ± 2.43 (Fig 3.34). From group 1 to 4 the groups measured 12.9 cm ± 1.9, 11.49 cm ± 0.14, 11.73 cm ± 0.03, and 11.27 cm ± 0.41 in length.

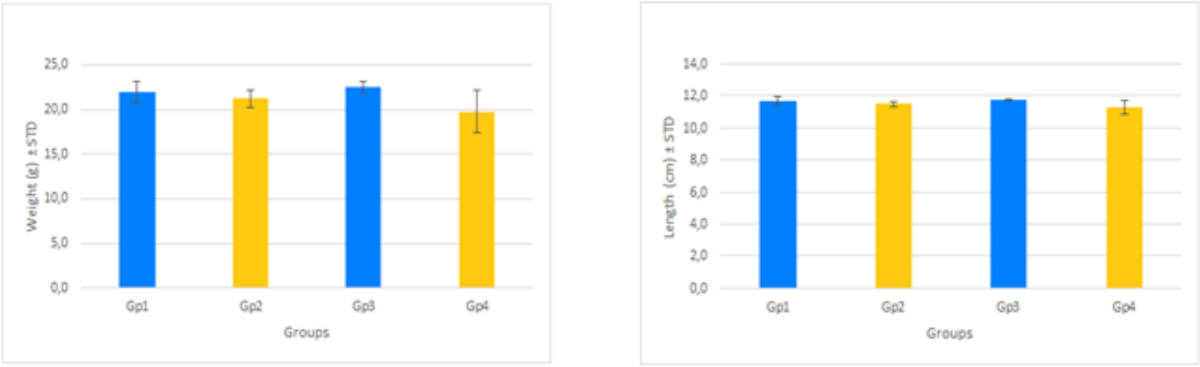


Figure 3.34. Effect of ploidy and diet on weight (g) (left) and length (cm) (right) of Atlantic salmon 27 weeks after start-feeding (2810 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.9.2 **Morphometric measurements of the intestine**

At this sampling stage significant differences between groups were found with regard to thickness of the anterior intestinal wall. Group 2 (33.30 µm ± 7.11) had a significantly thinner intestinal wall than both group 1 (41.23 µm ± 8.53) ($P$<0.001) and group 3 (42.29 µm ± 13.30) ($P$<0.001) (Fig 3.35).

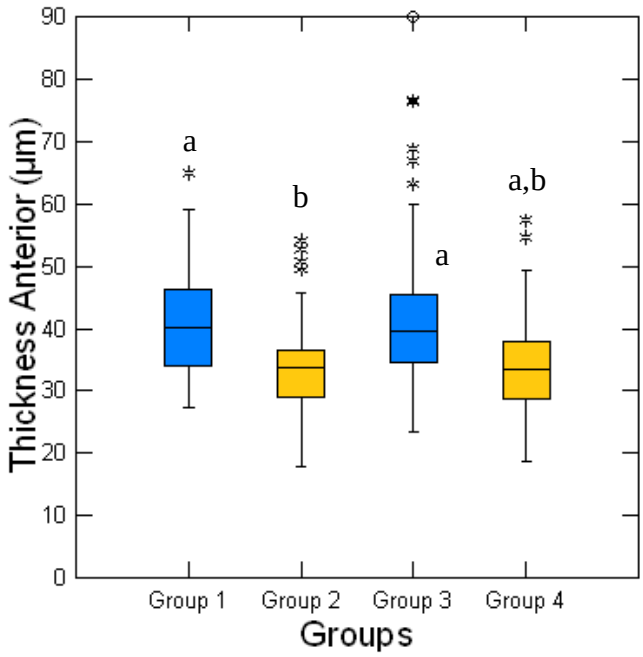


Figure 3.35. Intestinal wall thickness anterior intestine (n=9 fish/group) in the 4 experimental groups 27 weeks after start-feeding (2810 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.10 30 weeks after start-feeding (3020 dd)

### 3.10.1 Growth

At this stage 30 weeks after start-feeding there were no significant differences between the groups in terms of either growth or length of the fish. The groups (group 1 to 4) weighed 27.83 g ± 0.48, 27.57 g ± 1.05, 29.89 g ± 2.15, and 26.39 g ± 1.63, respectively (Fig 3.36). In terms of length, they (group 1 to 4) measured 12.86 cm ± 0.05, 12.83 cm ± 0.11, 13.18 cm ± 0.45, and 12.62 cm ± 0.34.

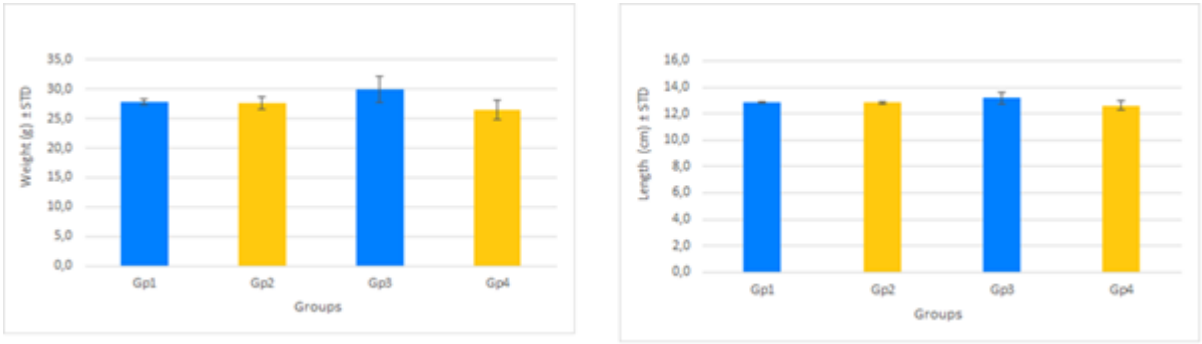


Figure 3.36. Effect of ploidy and diet on weight (g) (left) and length (cm) (right) of Atlantic salmon 30 weeks after start-feeding (3020 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.10.2 Morphometric measurements of the intestine

In the anterior part differences were found in terms of thickness of the intestinal wall; in the distal intestine differences were observed both with regard to fold height and thickness of the intestinal wall. The thickness of the anterior intestinal wall of group 1 (47.37 µm ± 12.44) was significantly thicker than that of group 2 (37.75 µm ± 7.53) ($P$<0.01), but thinner than group 3 (54.30 µm ± 13.97) ($P$<0.001). Group 3 was also significantly thicker than group 2 ($P$<0.001) and group 4 (38.44 µm ± 11.38) ($P$<0.001) (Fig 3.37). In terms of fold height in the distal intestine, the only difference between groups observed was significantly longer folds in group 2 (434.24 µm ± 153.9) versus group 4 (396.9 µm ± 132.89) ($P$<0.05) (Fig 3.38). The thickness of the distal intestinal wall of group 1 (42.67 µm ± 41.23) differed from all the other groups; while being 31.39 µm ± 9.86 for group 2 ($P$<0.001), 35.92 µm ± 9.83 for group 3 ($P$<0.001) and 31.3 µm ± 11.13 for group 4 ($P$<0.05), respectively (Fig 3.39).

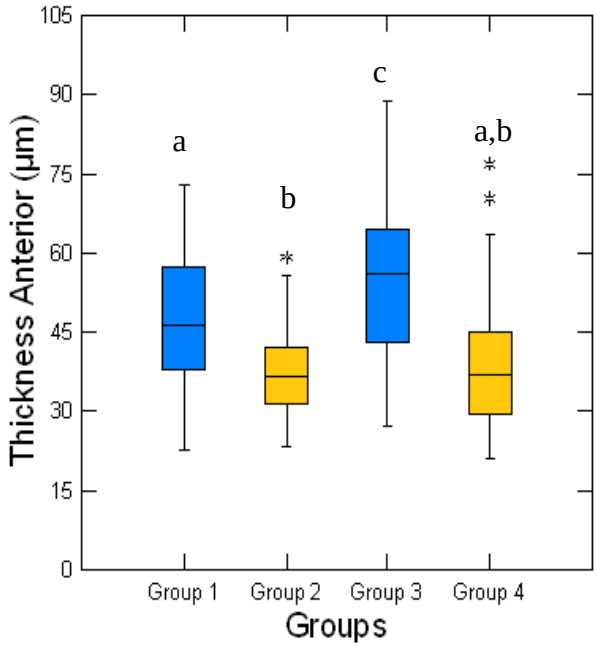


Figure 3.37. Intestinal wall thickness anterior intestine (n=9 fish/group) in the 4 experimental groups 30 weeks after start-feeding (3020 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

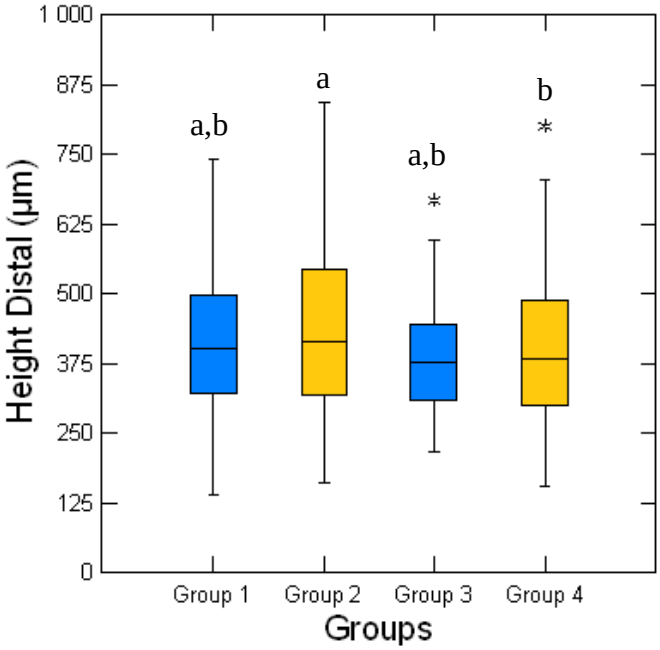


Figure 3.38. Fold height distal intestine (n=9 fish/group) in the 4 experimental groups 30 weeks after start-feeding (3020 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75$^{th}$ percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

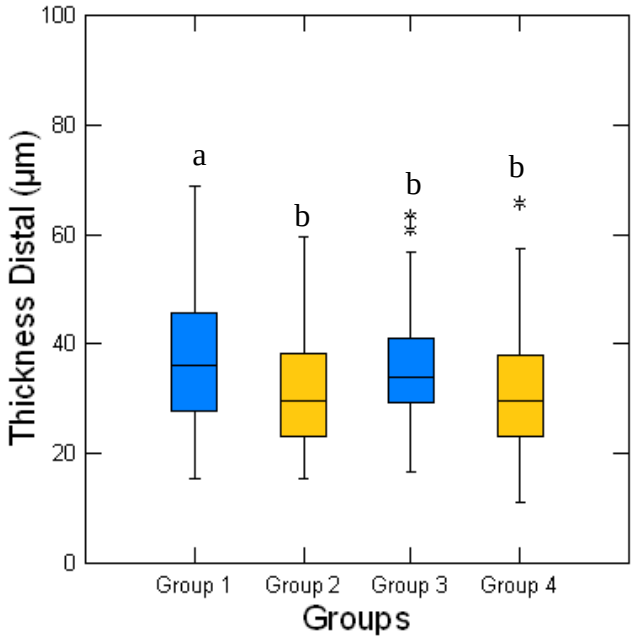


Figure 3.39. Intestinal wall thickness distal intestine (n=9 fish/group) in the 4 experimental groups 30 weeks after start-feeding (3020 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

## 3.11 35 weeks after start-feeding (3380 dd)

### 3.11.1 Growth

35 weeks after start-feeding there were no significant differences between the groups in terms of either growth or length of the fish. Weight from group 1 to 4 was 36.68 g ± 1.82, 35.81 g ± 1.35, 40.06 g 1.70, and 37.63 g ± 2.13 (Fig 3.40). Length from group 1 to 4 was: 14.01 cm ± 0.19, 13.78 cm ± 0.24, 14.36 cm ± 0.14, and 14.09 cm ± 0.25.

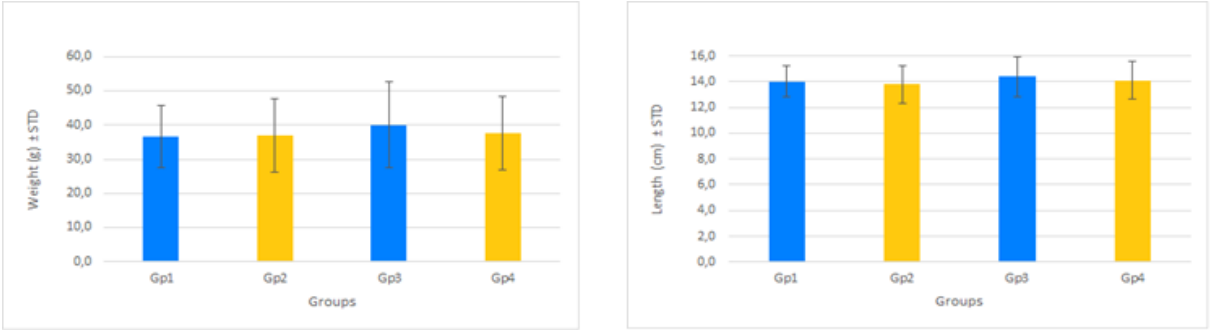


Figure 3.40. Effect of ploidy and diet on weight (g) (left) and length (cm) (right) of Atlantic salmon 35 weeks after start-feeding (3380 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.11.2 Morphometric measurements of the intestine

35 weeks after start-feeding no difference was found between groups for any parameter. The measurements are listed in tables in the appendix section B and presented as box plots in section C.

## 3.12 38 weeks after start-feeding (3670 dd)

### 3.12.1 Growth

38 weeks after start-feeding was the last sampling date and end of smoltification (3670 dd). At this stage group 1 had a mean weight of 56.44 g ± 1.58, group 2 weighed 53.15 g ± 1.57, group 3 66.56 g ± 1.26 and group 4 weighing 55.67 g ± 4.44. Group 3 was significantly larger than all the other groups (Fig 3.41); group 1 ($P$<0.001), group 2 ($P$<0.001), group 4 ($P$<0.001). Group 3 was also significantly longer than all other groups (Fig 3.41). The length was 17.53 cm ± 0.21, while that of group 1 was 16.38 cm ± 0.21, group 2 16.02 ± 0.22 and group 4 16.37 cm ± 0.27. Group 1 ($P$<0.001), group 2 ($P$<0.001), group 4 ($P$<0.001).

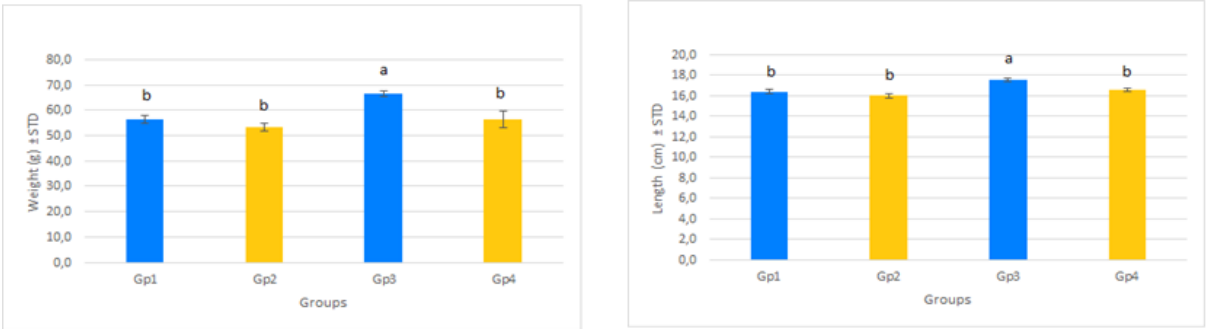


Figure 3.41. Effect of ploidy and diet on weight (g) (left) and length (cm) (right) of Atlantic salmon 38 weeks after start-feeding (3670 dd). Data with a common superscript are not significantly different. Blue bars express standard diet, and yellow bars express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups.

### 3.12.2 Morphometric measurements of the intestine

At the last sampling no differences were found for the parameters measured in the distal intestine, but for the anterior intestine fold height and thickness of the intestinal wall were significantly different between groups. Starting with the fold height group 4 had significantly longer folds (231.08 µm ± 68.88) than group 1 (195 µm ± 63.57) ($P$<0.001) (Fig 3.42). For the intestinal wall thickness group 3 (93.02 µm ± 37.59) was significantly thicker than all the other groups, group 1 (61.76 µm ± 18.97) ($P$<0.001), group 2 (60.2 µm ± 23.61) ($P$<0.001) and group 4 (56.36 µm ± 15.88) ($P$<0.001) (Fig 3.43).

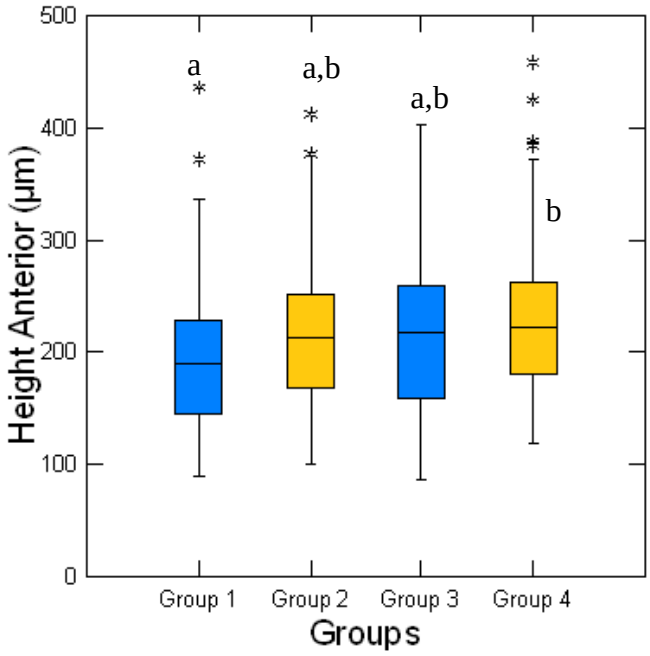


Figure 3.42. Fold height anterior intestine (n=9 fish/group) in the 4 experimental groups 38 weeks after start-feeding (3670 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

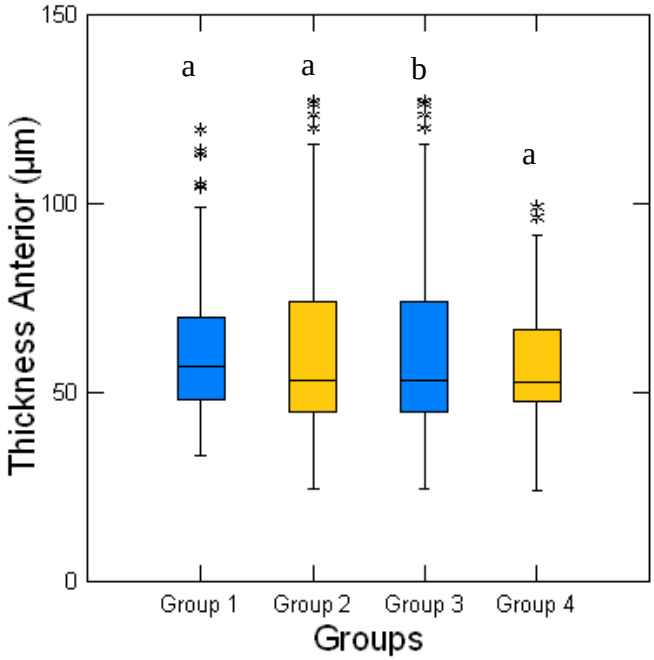


Figure 3.43. Intestinal wall thickness anterior intestine (n=9 fish/group) in the 4 experimental groups 38 weeks after start-feeding (3670 dd). Data with a common superscript are not significantly different. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

# 4 Discussion

The main goal of this project was to highlight any differences in the ontogeny of the digestive system between the diploid and triploid salmon and decide if the triploids performed better on a diet containing more digestible (hydrolysed) proteins.

## 4.1 Mortality

The groups fed the experimental diet showed an overall higher cumulated mortality than the other groups. The pellet size used in the beginning proved to have lower water stability (Table 2.3 and 2.4), and some of the feed probably dissolved before reaching the fish. This could be due to a reduced binding capacity of small pellets containing high proportions of hydrolyzed proteins affecting their water stability (Skretting AS, pers. comm.). This can be a plausible explanation for the increased mortality observed, as most of the mortality happened during the first rearing period with the smaller pellet sizes. The making of fish protein hydrolysates increases the number of polar groups and the solubility of the hydrolysate (Kristinsson & Rasco, 2000) which makes it more soluble in water. All diets possessed very high amounts of protein (>55%), and in the experimental diets a large portion of this amount was comprised of protein hydrolysate, and could thus be the reason for problems observed of leaching of nutrients and the pellet dissolving more rapidly than expected. The experimental diet pellets used in the beginning was also hypothesized to be less available to the fish by sinking faster than the standard diet pellets.

Triploids prove to show lower survival between fertilization and first feeding (O'Flynn *et al.*, 1997; Cotter *et al.*, 2002), but also after start-feeding in the fresh water rearing period according to some studies (Withler *et al.*, 1995). Triploids often show a poorer survival; it is not clear if this is due to the ploidy, diet or rearing conditions. In this experiment the diet would be the main cause for the mortality due to its effect on both ploidies. The feeding frequency was increased and prevented a further increase in mortality. The percent mortality observed in the experiment was, however, not higher than expected in triploids (Withler *et al.*, 1995; Cotter *et al.*, 2002).

## 4.2 Growth

A problem with growth studies is that differences which are believed to derive from treatment could be a result of different weights of the fish (Shearer, 1994). The fish used in this study were never size-sorted and were thus not of the same sizes, which could pose a problem when analyzing effects of the diets. There were relatively large size differences in the beginning which could be due to the use of different salmon families. A reason for not size grading is that this could hide treatment differences.

The growth results obtained in the present study showed that group 3 (triploid fish fed standard diet) was the best performing group at the end of the experimental period when the fish had reached smoltification, although not consistent for all samplings. Fraser *et al.* (2013) showed that triploids were both heavier and longer when transferred to seawater and this indicates that triploids do perform better in freshwater if reared under optimal conditions. In seawater this growth advantage is often lost, as shown in the same study. Taylor and co-workers (2013) also found that triploids grew 30% faster in freshwater and again slower in seawater (-7.5%). Overall, triploid salmonids generally perform better in freshwater compared to diploids (Sheehan *et al.*, 1999).

During the experiment growth varied between the groups. Group 1 was significantly larger than group 2 and 3, but not group 4, four weeks after start-feeding (1150). 9 weeks after start-feeding (1500 dd) fish from group 3 were the largest both in terms of weight and length, and those from group 4 were the smallest. 13 weeks after start-feeding (1800 dd) group 1 was again the largest both in terms of weight and length, but only significantly larger to group 4. 17 weeks after start-feeding (2100 dd) and onwards group 3 was the largest and longest, although never significant to all other groups. The exception was at 27 weeks after-start feeding (2810 dd) when group 1 was seemingly longer, but this was not significant. This group had a high standard deviation and a few large individuals can be the reason for this. From 30 weeks after start-feeding (3020) and onwards, group 3 was the largest and longest, although values were not significantly different before the last sampling point. At this point all other groups weighed and measured the same. Group 2 (diploids fed experimental diet) was the smallest and shortest group at the end of sampling (3670 dd), although not significantly smaller than the other groups (except group 3).

Interestingly, the trend was that groups 1 and 3 (fish fed standard diets) was almost always seemingly larger than group 2 and 4, although seldom significantly different. As group 1 (diploids fed standard diet) was larger than both groups fed experimental diet it turned out that the experimental diet containing more hydrolyzed proteins did not enhance growth. Salmonids need between 35 and 55% protein in dry feed to gain maximal growth (Einen, 2001), and as all diets contained >55% protein, the amount of protein was sufficient, but maybe the incorporation of hydrolysates was too high. In earlier studies a much smaller amount have been incorporated in the feed. Berge & Storebakken (1996) used diets with 5-8% hydrolysates and Refstie and co-workers (2004) used different diets ranging from 0 % to 15 %. The high amount included in the experimental diet of this study was maybe chosen to clearly indicate an effect of the diet. In freshwater triploids do show a growth advantage, why this was not the case for group 4 probably had to do with the formulation of the experimental diet.

The inconsistency of weight between groups at different sampling points could be due to the size differences between individual fish within each tank and group, although the number of fish sampled per group should overcome this problem. At the end of the experiment, the weight varied from 53 g to 66 g among the groups.

Results presented in the present study indicate that the diploids fed standard diet showed best growth in the initial phase (4 weeks after start-feeding), after this period all groups performed equally for a while before the triploid group fed the standard diet slowly took over and eventually showed overall best performance in terms of growth. However, both ploidy groups fed the standard diet was superior in terms of survival compared to the groups fed experimental diets in the period from start-feeding until smoltification. The observed growth advantage in fresh water for triploids (group 3) compared to the diploids (group 1) is in line with previous findings regarding the performance of triploids during the freshwater phase. This could be due to differences in the muscle fibers and their growth patterns as it seems that triploid salmonids show higher rates of muscle fiber requirement than their diploid counterparts (Suresh & Sheehan, 1998).

## 4.3 Ontogeny

Recent studies (Løkka *et al.*, 2013; Sahlmann *et al.*, 2015) have focused on the intestinal morphology and ontogeny of the digestive system of diploid Atlantic salmon. The

observations made in this study seem to match very much what is observed by Sahlmann and co-workers (2015) at the various stages. When comparing diploid and triploid digestive tract ontogeny in this study, no obvious major differences could be highlighted between the groups with relation to diet or ploidy.

**At hatch** diploid and triploid fish showed no apparent differences in histomorphology, and the observations made coincide with results from Sahlmann and co-workers (2015). Gross tract morphology appeared similar between groups. The liver and pancreas were well developed at this stage of development, and the hepatocytes were vacuolized (Fig 3.4 F). Ruyter and co-workers (2006) saw a tendency towards more fat in the liver early in the rearing period when the temperature was lower, and this is also in accordance with other studies from different species reared at low temperatures (Caballero *et al.*, 1999). This vacuolization continued and was also present at **start-feeding (920 dd)**, which means that fish used yolk sac nutrients to store lipids as well as glycogen in the liver. At this stage the liver cells differed slightly between individuals with regard to vacuolization in the same ploidy groups. The liver histology at this stage is only based on 4 individuals per ploidy group though, and conclusions should be taken with care.

Interestingly, both ploidies showed a quite well-developed stomach and gastric glands at this stage, which means they will be able to digest external feed. Only pyloric caeca buds were present, however, indicating that the digestive system was not fully functional. No differences in appearance of pyloric caeca and gastric glands were observed between ploidies. There were no supranuclear vacuoles present in the distal intestine at this stage, but the fish had not received any feed pellets before this sampling, and its known that supranuclear vacuoles decrease rapidly at fasting (Baeverfjord & Krogdahl, 1996). A large yolk sac was still present at this stage, which the fish could utilize while at the same time start the intake of external feed. Summarized the fish had not a fully developed digestive system at this stage, but both ploidies could probably digest and utilize external feed to some degree. No other major differences could be observed between ploidies at this stage.

**4 weeks after start-feeding (1150 dd)** small supranuclear vacuoles was observed in the enterocytes of all four groups, this indicates that the fish possess the ability of protein pinocytosis. Long pyloric caeca were present and looked fully functional, the stomach had also attained its final shape. This was the case in all four experimental groups, and no specific

differences in digestive tract morphology were noted among them. Vacuolization of the hepatocytes differed between individuals within each group also at this stage. Between 4 weeks and until **9 weeks after start-feeding (1500 dd)** there were no major histological changes, and no apparent differences between groups except these individual differences in hepatocyte vacuolization.

Most of the histomorphological changes in the digestive system occurred between start-feeding (920 dd) and 4 weeks after start-feeding (1150 dd) and there were no obvious differences in the development of the digestive system between either ploidy or diet groups.

## 4.4 Morphometric measurements of the intestine

Starting with the height of the anterior folds in the anterior intestine, the only significant difference observed was at 21 weeks after start-feeding (2380 dd) and at the final sampling with longer folds in group 4 compared to group 1. It is not meaningful to compare these two groups as they differ in both ploidy and diet, but group 4 had the longest folds at the end of project. Considering the width of mucosal folds in the anterior intestine, there was a trend with the folds in group 1 and 3 being bigger, although never significant at any sampling point.

Thickness of the intestinal wall in the anterior intestine varied considerably, but the trend was that the same groups, 1 and 3 possessed thicker intestinal walls than both group 2 and 4, with group 3 being much larger than all other groups. This extremely large values measured for the intestinal wall thickness in group 3 (see appendix B. Table B9) could be due to some of the individuals from group 3 being larger in terms of weight and length. This was investigated, and the individuals chosen for measurements were actually the largest ones in each tank (the 3 chosen of the 5 fish sampled per tank).

Number of folds in the anterior intestine was seemingly higher in group 3 at all sampling points, this was however never significant. This is in accordance with the growth data where group 3 overall showed to be the largest group, and a larger individual could have developed more mucosal folds.

Number of goblet cells could be expected to be more numerous in the triploid groups as this have been shown earlier in Atlantic cod (Peruzzi *et al.*, 2013). In the mucosal folds in the anterior intestine this was however not the case, and the only significant difference observed

was for group 1 (diploid) possessing significantly more goblet cells than group 3 (triploid group) in the sampling 21 weeks after start-feeding (2380 dd). The goblet cells counted were clearly visible when the measurements was performed, so the varying results observed for this type of measurements could not be due to the staining method used (H&E).

The only significant difference observed between groups with regard to mucosal fold height in the distal intestine was at 30 weeks after start-feeding (3020 dd) where group 2 fish (diploids fed experimental diet) had significantly higher folds than group 4 (triploids fed experimental diet). Otherwise, there were no trends observed for this type of measurement, other than that group 3 (triploids fed standard diet) actually seemingly had smaller folds at the last sampling compared to the other groups, although values were not significantly different. Considering the width of the mucosal folds in the distal intestine, the only significant difference observed was group 4 being significantly larger than group 1 and 2 at 17 weeks after start-feeding (2100 dd).

Regarding the thickness of the intestinal wall in the distal intestine, at 4 weeks after start-feeding (1150 dd) this was significantly higher in group 3 than all other groups. This was also the case at 21 weeks after start-feeding (2380 dd) when group 3 showed significantly higher values than all other groups except group 1. However, at 30 weeks after start-feeding (3020 dd) group 1 was significantly larger than all the other groups. To summarize, there were no consistent significant differences, but the trend was that groups fed standard diet (1 and 3) showed a thicker intestinal wall, with group 3 having the thickest one. This is consistent with findings of the anterior intestine, where group 1 and 3 overall showed the thickest intestinal walls. This also supports the growth data where the same groups showed highest weight.

No significant differences were observed when measuring the folds in the distal intestine. A trend towards more folds in the triploid groups (group 3 and 4) was noted in the beginning, but never significant and towards the end of the experiment no differences were registered.

When comparing number of goblet cells in the distal intestine, no significant differences were found, and it was hard to spot any trends between the groups. The distal intestine often shows more goblet cells compared to the anterior part (Al-Hussaini, 1949; Løkka *et al.*, 2013), but this was not observed in the present study.

Overall, the folds measured in the distal part were much larger than the ones in the anterior intestine. In addition, in accordance with the observations made by Løkka and co-workers (2013) and Korovina & Dorofeeva (1981), the intestinal wall in the distal area was thinner than the one in the anterior part. This is hypothesized to be due to more pronounced peristaltic movements occurring in the anterior part of the intestine compared to the other portions (Løkka et al., 2013).

It has to be noted that for several measurements the standard deviation was high (Appendix B). This large variation observed when measuring the different parameters was probably due to some methodological challenges (problems related to fixation, processing of the samples with all the different dehydration steps) resulting in histological preparations with variable quality and/or the method used for measuring the same preparations. Kryvi & Poppe (2016) also highlighted the problem with poor preservation of the epithelial tissue as a known problem. The fish were not starved prior to sampling and the intestines were not rinsed. If there was too little fixation fluid compared to tissue and the fixation process took too long, this can have resulted in autolysis of the mucosal folds and tissues (Williams & Nickol, 1989).

Variations in various morphometric measurements both within and between groups at the various samplings points were found. Nevertheless there were some observable trends, and the most notably was for groups fed the standard diet showing the thickest intestinal walls both in the anterior and distal part. Group 3 (triploid standard diet) overall had the thickest intestinal wall although seldom significantly different from the other groups.

# 5 Conclusion

In the present work the use of experimental diets containing high levels of hydrolysed proteins generally did support but not enhanced growth in triploid or diploid salmon until the end of the smoltification process. Within the same period and despite some variable trends and a degree of individual variability, the ontogeny of the digestive system was overall comparable between ploidies and diets. The same conclusions can also be drawn, at least under the present experimental conditions, regarding the histomorphological traits measured in the digestive organs of these fish.

Since farming of triploid salmon could prevent further genetic introgression of genes into the wild salmon population, it is highly important to evaluate their nutritional requirements and thereby achieve good performing triploids for production. There is still a need for scientific experimentation to figure out the optimal nutritional requirements for triploid Atlantic salmon. It would be interesting to carry out a feeding experiment with diets containing different amounts of hydrolysed proteins and try to find the optimal level of inclusion required for best growth and overall performance. The finding that triploid fish show differences in gut morphology from earlier studies imply that they should possess some compensatory mechanisms. Even if these were not observed in this study this deserves further attention.

# 6 Appendix

## Appendix A. Embedding protocol

Table A.1. Citadel embedding protocol program A.

| Container | Contents |
|---|---|
| Container 1 | empty/pass |
| Container 2 | empty/pass |
| Container 3 | 96 % EtOH – 2 hours |
| Container 4 | 96 % EtOH – 2 hours |
| Container 5 | 100 % EtOH 2 hours |
| Container 6 | 100 % EtOH – 2 hours |
| Container 7 | 1:1 100 % EtOH and Histoclear – 1 hour |
| Container 8 | Histoclear – 1hour |
| Container 9 | Histoclear – 1 hour |
| Container 10 | Histoclear – 1 hour |
| Container 11 | 1:1 Histoclear and Paraffinwax – 1 hours |
| Container 12 | Paraffinwax – minimum 2 hours |

# Appendix B. Morphometric measurements

Table B.1. Morphometric measurements at start-feeding (920 dd). The values presented are average values ± standard deviation.

| | Distal intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Diploid | 87,91 ± 20,77 | 66,26 ± 12,21 | 13,92 ± 2,88 | | 3,7 ± 1,15 |
| Triploid | 92,00 ± 10,31 | 75,50 ± 8,12 | 12,06 ± 2,34 | | 3,50 ± 0,50 |

Table B.2. Morphometric measurements 4 weeks after start-feeding (1150 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | | | 11,14 ± 2,71 | | |
| Group 2 | | | 11,21 ± 1,55 | | |
| Group 3 | | | 11,73 ± 1,75 | | |
| Group 4 | | | 8,40 ± 2,43 | | |
| | Distal intestine | | | | |
| | Height Distal | Width Distal | Intestinal wall thickness | Folds Distal | Goblet Distal |
| Group 1 | 170,68 ± 45,07 | 88,31 ± 14,79 | 12,95 ± 4,95 | | 4,5 ± 1,18 |
| Group 2 | 159,33 ± 39,34 | 81,20 ± 12,90 | 12,65 ± 4,47 | | 5,30 ± 0,91 |
| Group 3 | 156,15 ± 42,98 | 76,49 ± 17,47 | 16,13 ± 7,23 | | 4,17 ± 0,52 |
| Group 4 | 185,98 ± 57,23 | 89,47 ± 17,71 | 14,66 ± 5,29 | | 3,67 ± 0,93 |

Table B.3. Morphometric measurements 9 weeks after start-feeding (1500 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | | | 16,15 ± 4,30 | | |
| Group 2 | | | 11,44 ± 2,03 | | |
| Group 3 | | | 12,76 ± 2,51 | | |
| Group 4 | | | 13,10 ± 2,93 | | |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 189,26 ± 56,01 | 99,52 ± 14,59 | 17,14 ± 7,08 | | 4,8 ± 1,04 |
| Group 2 | 209,10 ± 55,10 | 98,01 ± 16,00 | 15,69 ± 7,17 | | 7,00 ± 1,50 |
| Group 3 | 184,53 ± 38,91 | 94,11 ± 21,17 | 15,56 ± 5,53 | | 5,33 ± 0,29 |
| Group 4 | 169,05 ± 34,82 | 103,57 ± 21,29 | 14,72 ± 5,34 | | 5,33 ± 1,04 |

Table B.4. Morphometric measurements 17 weeks after start-feeding (2100 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 129,07 ± 22,33 | 85,05 ± 20,28 | 20,89 ± 6,34 | 27,88 ± 4,53 | 9,86 ± 1,44 |
| Group 2 | 140,38 ± 37,30 | 80,34 ± 23,97 | 21,75 ± 5,89 | 27,00 ± 4,23 | 12,36 ± 1,41 |
| Group 3 | 151,92 ± 46,76 | 94,73 ± 23,78 | 22,92 ± 4,80 | 28,71 ± 1,93 | 10,28 ± 1,15 |
| Group 4 | 147,95 ± 46,41 | 87,39 ± 31,10 | 21,95 ± 5,89 | 28,94 ± 4,07 | 10,17 ± 2,81 |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 240,81 ± 79,42 | 114,33 ± 32,92 | 18,39 ± 8,08 | 32,67 ± 3,98 | 9,94 ± 2,46 |
| Group 2 | 244,10 ± 86,99 | 101,98 ± 24,32 | 17,98 ± 5,20 | 33,78 ± 5,34 | 9,94 ± 1,89 |
| Group 3 | 258,38 ± 78,64 | 123,51 ± 32,58 | 18,92 ± 5,17 | 36,25 ± 6,77 | 10,00 ± 1,83 |
| Group 4 | 257,73±103,32 | 117,98 ± 34,07 | 15,51 ± 5,02 | 34,56 ± 4,69 | 9,94 ± 2,16 |

Table B.5. Morphometric measurements 21 weeks after start-feeding (2380 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 162,48 ± 35,55 | 97,78 ± 19,12 | 23,43 ± 6,52 | 33,31 ± 5,17 | 13,00 ± 1,61 |
| Group 2 | 151,35 ± 42,36 | 82,35 ± 19,50 | 25,56 ± 6,61 | 36,57 ± 6,18 | 10,92 ± 2,67 |
| Group 3 | 149,85 ± 47,39 | 89,53 ± 34,20 | 22,42 ± 7,50 | 31,88 ± 5,90 | 10,00 ± 1,12 |
| Group 4 | 179,98 ± 52,79 | 108,91 ± 27,15 | 25,74 ± 6,09 | 33,86 ± 4,84 | 12,08 ± 1,53 |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 286,38 ± 92,78 | 125,14 ± 34,41 | 20,85 ± 6,26 | 38,72 ± 6,83 | 10,8 ± 1,50 |
| Group 2 | 302,2 ± 100,25 | 113,94 ± 30,07 | 19,54 ± 7,32 | 38,72 ± 8,25 | 10,28 ± 3,80 |
| Group 3 | 319,8 ± 116,21 | 129,56 ± 39,01 | 23,50 ± 7,02 | 39,75 ± 3,69 | 10,81 ± 2,12 |
| Group 4 | 290,71 ± 99,68 | 132,45 ± 33,78 | 19,07 ± 6,19 | 37,56 ± 5,79 | 10,94 ± 3,03 |

Table B.6. Morphometric measurements 27 weeks after start-feeding (2810 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 192,64 ± 51,92 | 103,66 ± 28,77 | 41,23 ± 8,53 | 45,06 ± 5,35 | 12,22 ± 1,66 |
| Group 2 | 184,15 ± 49,04 | 102,52 ± 25,42 | 33,30 ± 7,11 | 44,83 ± 10,24 | 14,13 ± 2,39 |
| Group 3 | 202,92 ± 62,52 | 102,57 ± 21,47 | 42,29 ± 13,30 | 44,89 ± 5,62 | 12,06 ± 1,72 |
| Group 4 | 224,77 ± 58,82 | 113,77 ± 29,45 | 34,23 ± 7,72 | 40,89 ± 5,78 | 12,72 ± 2,05 |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 398,05±143,82 | 136,63 ± 50,27 | 31,15 ± 9,27 | 57,67 ± 7,99 | 12,50 ± 2,17 |
| Group 2 | 460,4 ± 170,01 | 142,08 ± 44,58 | 26,75 ± 7,65 | 58,61 ± 7,85 | 13,67 ±2,06 |
| Group 3 | 361,94±124,23 | 143,07 ± 42,93 | 30,21 ± 10,49 | 62,06 ± 12,61 | 12,94 ± 2,31 |
| Group 4 | 381,67±131,76 | 145,53 ± 45,86 | 28,16 ± 7,47 | 52,33 ± 6,30 | 13,39 ± 3,30 |

Table B.7. Morphometric measurements 30 weeks after start-feeding (3020 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 178,91 ± 51,36 | 93,17 ± 21,42 | 47,37 ± 12,44 | 44,33 ± 7,20 | 13,83 ± 3,22 |
| Group 2 | 183,14 ± 60,19 | 85,79 ± 25,91 | 37,75 ± 7,53 | 42,50 ± 8,22 | 10,58 ± 1,59 |
| Group 3 | 209,85 ± 67,34 | 97,32 ± 23,38 | 54,30 ± 13,97 | 47,83 ± 7,44 | 11,78 ± 2,11 |
| Group 4 | 191,19 ± 60,42 | 103,28 ± 27,14 | 38,44 ± 11,38 | 40,72 ± 6,74 | 12,25 ± 2,52 |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 415,44±133,24 | 129,27 ± 37,19 | 42,67 ± 41,23 | 56,00 ± 14,75 | 12,4 ± 3,13 |
| Group 2 | 434,24±153,90 | 136,97 ± 39,46 | 31,39 ± 9,86 | 51,31 ± 6,96 | 12,00 ± 1,60 |
| Group 3 | 390,29 ± 95,80 | 139,68 ± 40,77 | 35,92 ± 9,83 | 54,06 ± 7,70 | 11,83 ± 2,75 |
| Group 4 | 396,9 ± 132,89 | 151,51 ± 40,06 | 31,30 ± 11,13 | 47,67 ± 9,05 | 11,72 ± 2,15 |

Table B.8. Morphometric measurements 35 weeks after start-feeding (3380 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 186,67 ± 49,48 | 96,31 ± 27,36 | 59,62 ± 15,60 | 41,11 ± 9,41 | 13,4 ± 3,35 |
| Group 2 | 171,22 ± 53,63 | 97,31 ± 24,67 | 56,08 ± 22,57 | 41,31 ± 5,89 | 13,5 ± 3,36 |
| Group 3 | 177,81 ± 69,05 | 92,33 ± 20,97 | 60,64 ± 27,23 | 45,39 ± 3,90 | 11 ± 2,95 |
| Group 4 | 161,24 ± 38,48 | 98,94 ± 25,46 | 59,75 ± 28,55 | 44,11 ± 3,80 | 9,71 ± 2,83 |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 410,45±164,22 | 166,85 ± 49,96 | 34,88 ± 15,23 | 54,61 ± 13,81 | 12,4 ± 2,61 |
| Group 2 | 400,48±160,07 | 154,70 ± 42,33 | 35,04 ± 11,74 | 61,89 ± 13,70 | 13,11 ± 1,58 |
| Group 3 | 413,04±165,18 | 148,7 ± 44,52 | 31,71 ± 11,40 | 58,22 ± 9,28 | 11,72 ± 2,15 |
| Group 4 | 372,6 ±172,41 | 146,18 ± 46,49 | 33,32 ± 11,74 | 55,81 ± 10,26 | 11,93 ± 1,27 |

Table B.9. Morphometric measurements 38 weeks after start-feeding (3670 dd). The values presented are average values ± standard deviation.

| | Anterior intestine | | | | |
|---|---|---|---|---|---|
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 195,22 ± 63,57 | 102,04 ± 27,80 | 61,76 ± 18,97 | 47,94 ± 6,16 | 11,86 ± 2,06 |
| Group 2 | 216,58 ± 65,64 | 104,58 ± 29,97 | 60,2 ± 23,61 | 51,11 ± 8,64 | 11,86 ±3,68 |
| Group 3 | 219,24 ± 75,20 | 109,39 ± 29,50 | 93,02 ± 37,59 | 52,72 ± 8,09 | 12,06 ± 2,85 |
| Group 4 | 231,08 ± 68,88 | 107,64 ± 27,19 | 56,36 ± 15,88 | 48,28 ± 6,49 | 10,56 ± 1,33 |
| | Distal intestine | | | | |
| | Fold height | Fold width | Intestinal wall thickness | Fold number | Goblet cells |
| Group 1 | 386,72±161,89 | 139,75 ± 41,75 | 37,28 ± 11,22 | 60,17 ± 10,18 | 10,4 ± 1 75 |
| Group 2 | 387,44±167,24 | 135,12 ± 41,73 | 37,99 ± 10,75 | 66,28 ± 15,48 | 10,72 ± 2,98 |
| Group 3 | 329,06±163,53 | 136,59 ± 64,12 | 44,95 ± 37,59 | 55,25 ± 10,97 | 9,72 ± 2,05 |
| Group 4 | 391,87±161,69 | 148,44 ± 42,53 | 35,83 ± 14,21 | 53,11 ± 7,01 | 10,17 ± 2,30 |

# Appendix C. Morphometric measurements (Box Plots)

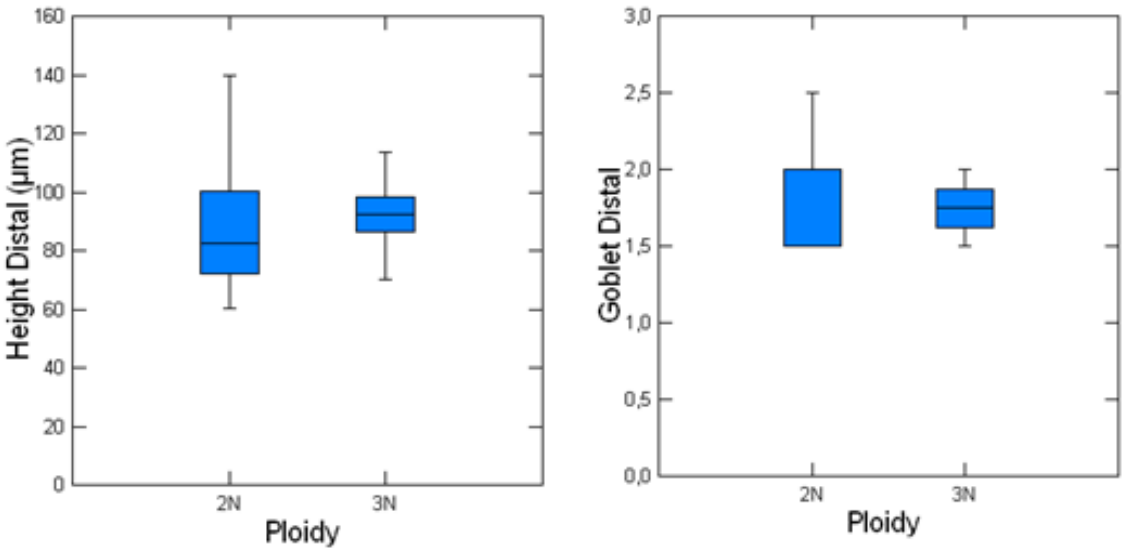


Figure C.1. Morphometric measurements (Box Plots) at start-feeding (920 dd). For the following box-plots no significant differences between groups were found. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

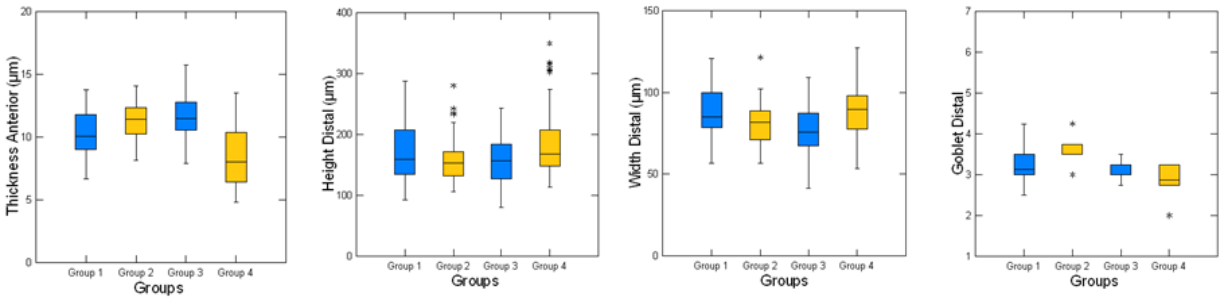


Figure C.2. Morphometric measurements (Box Plots) 4 weeks after start-feeding (1150 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

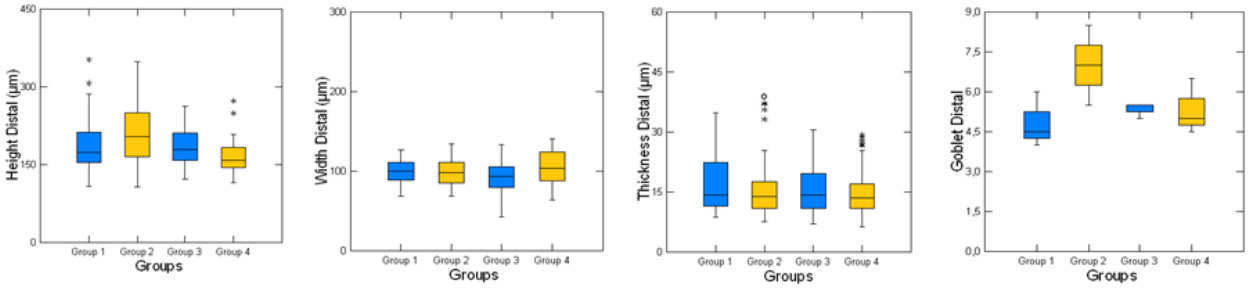


Figure C.3. Morphometric measurements (Box Plots) 9 weeks after start-feeding (1500 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

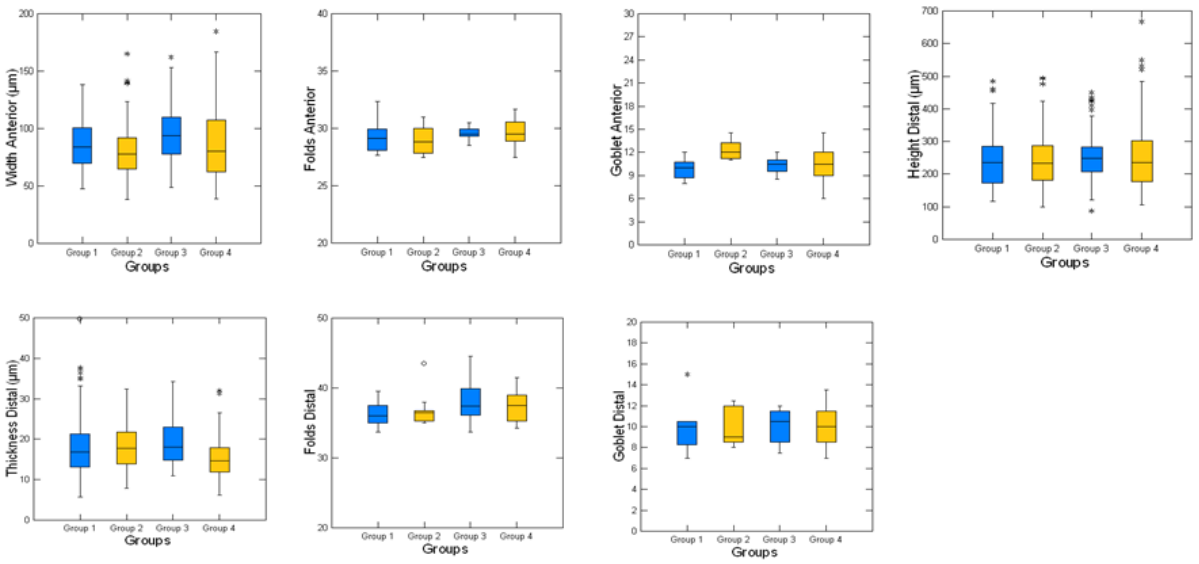


Figure C.4 Morphometric measurements (Box Plots) 17 weeks after start-feeding (2100 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

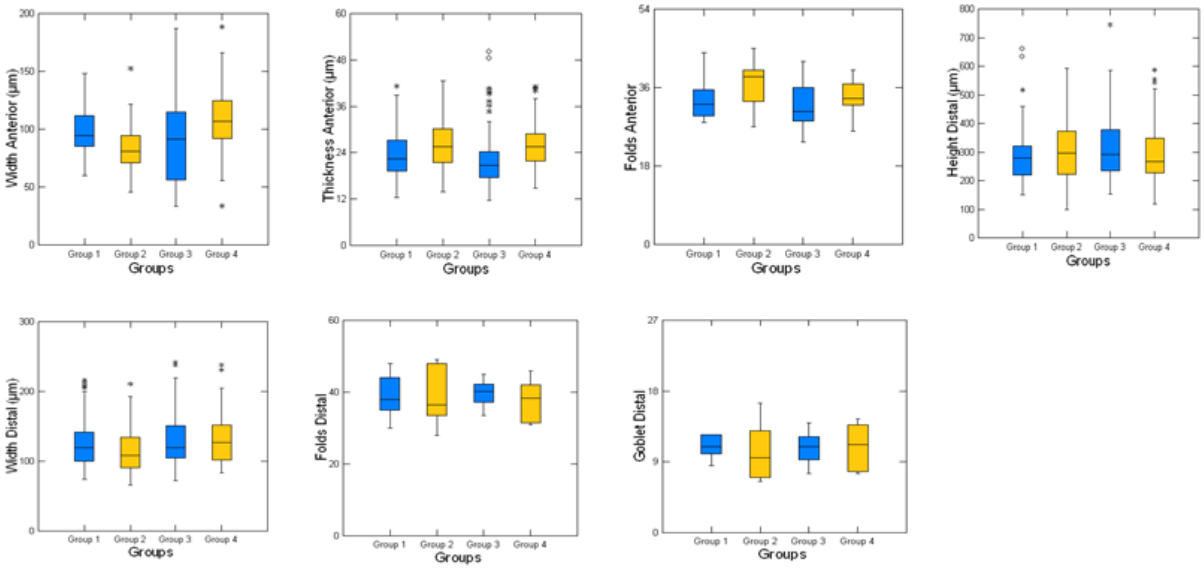


Figure C.5 Morphometric measurements (Box Plots) 21 weeks after start-feeding 2380 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

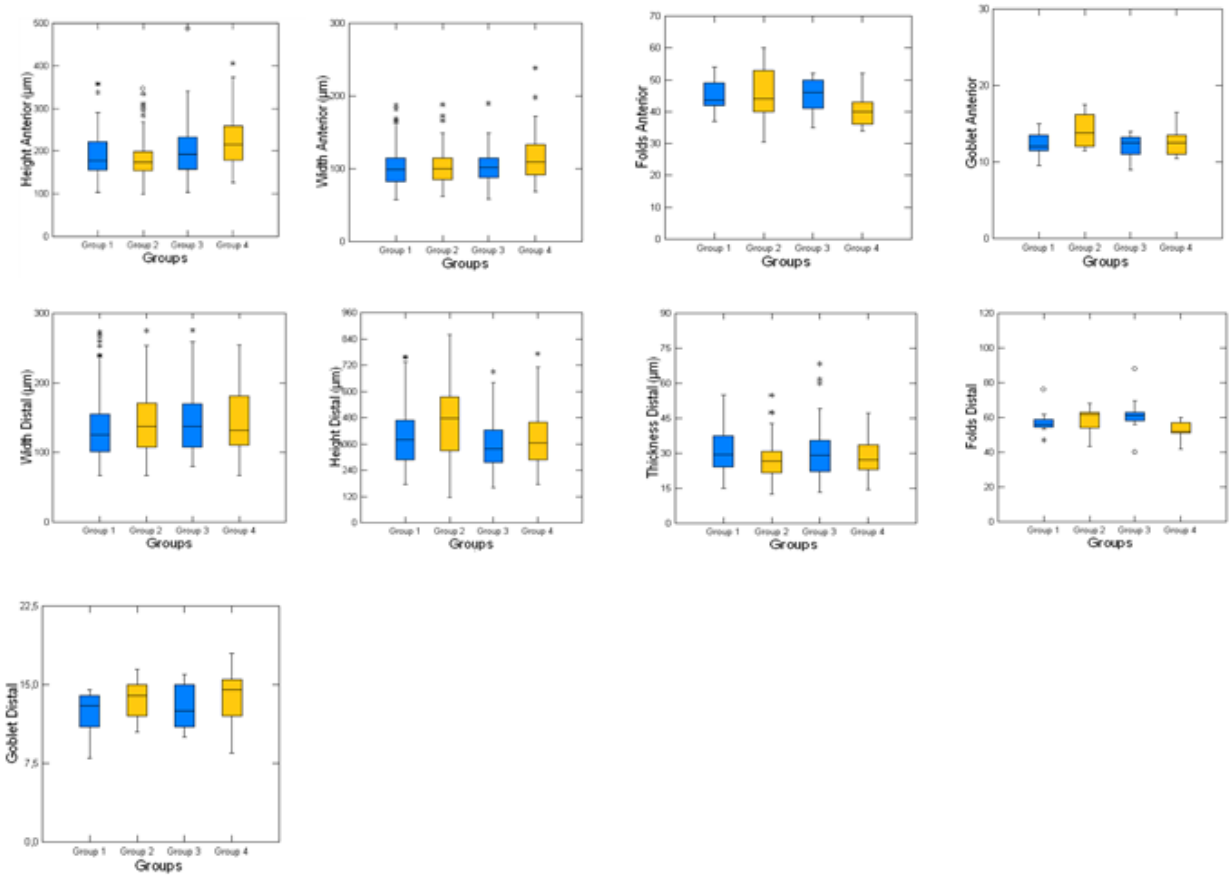


Figure C.6 Morphometric measurements (Box Plots) 27 weeks after start-feeding (2810 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

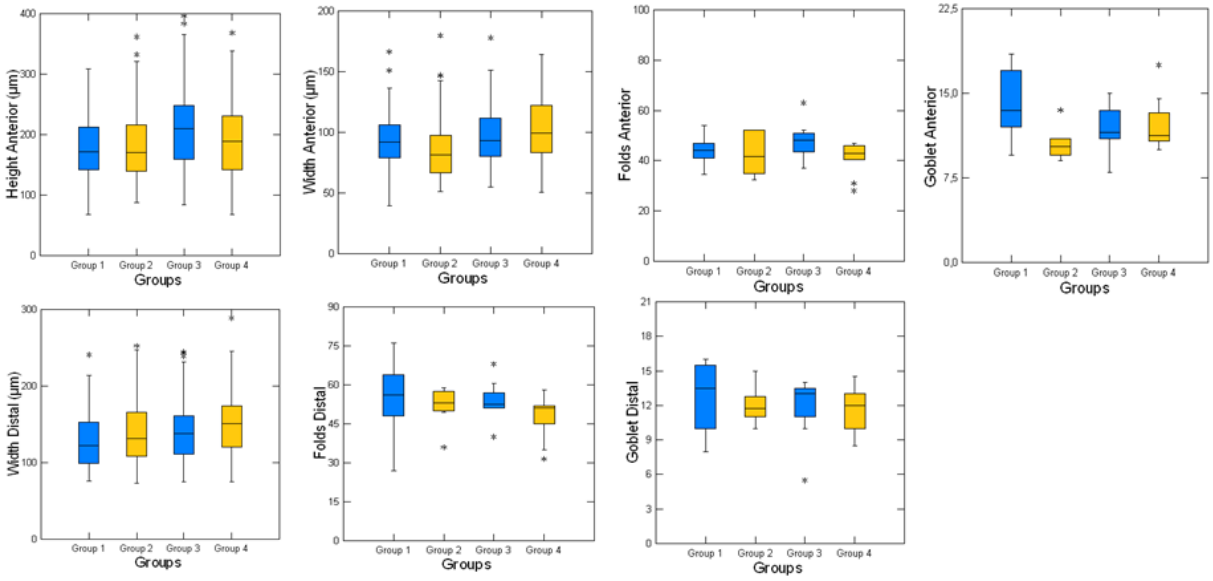


Figure C.7 Morphometric measurements (Box Plots) 30 weeks after start-feeding (3020 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

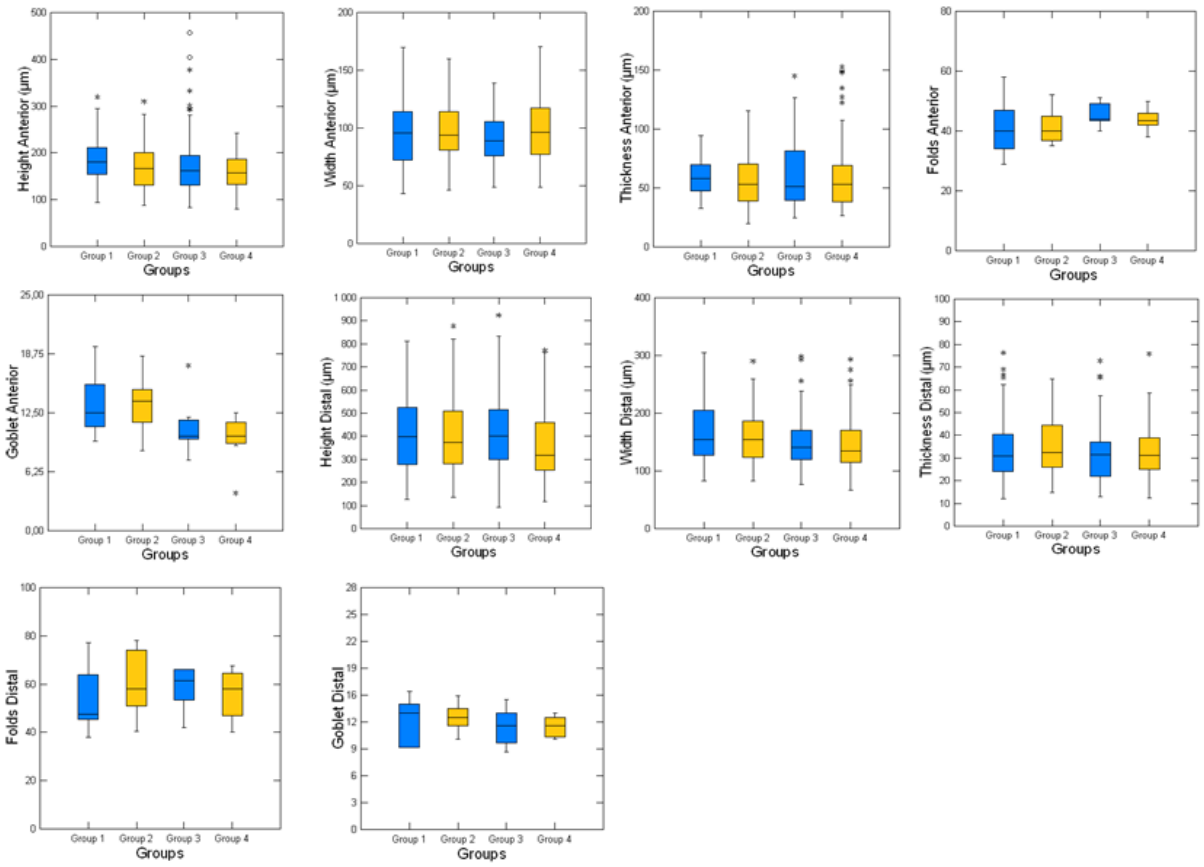


Figure C.8 Morphometric measurements (Box Plots) 35 weeks after start-feeding (3380 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

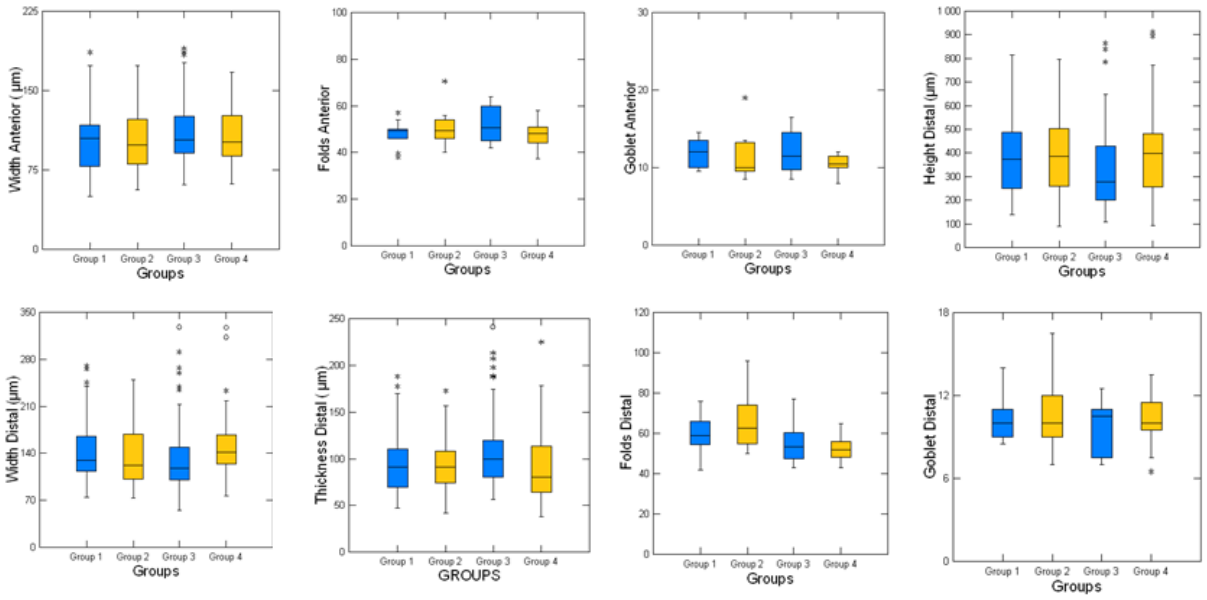


Figure C.9 Morphometric measurements (Box Plots) 38 weeks after start-feeding (3670 dd). For the following box-plots no significant differences between groups were found. Blue box-plots express standard diet, and yellow box-plots express experimental diet. Groups 1 and 2 are diploid groups, and groups 3 and 4 are triploid groups. Each box-plot has the following elements: median (solid bar in the box-plot); 25-75th percentile (rectangular box); 1.5 x interquartile range (non-outlier range of the box whiskers).

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