Vgll3 knockout as a means to delay male maturity during large smolt production of Atlantic salmon (*Salmo salar L.*)
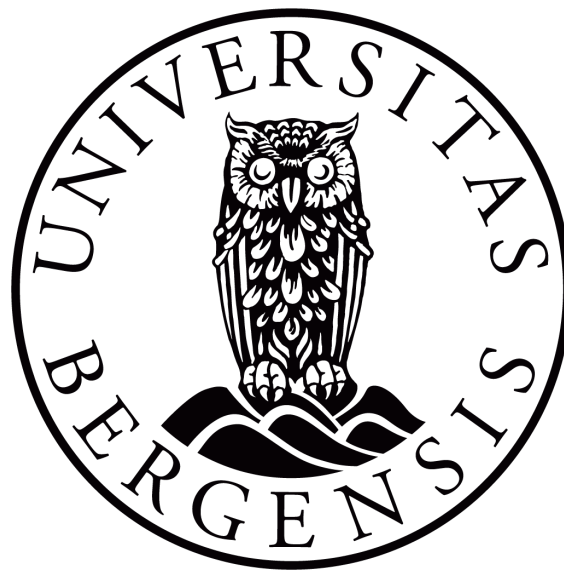
Master´s thesis in Aquaculture

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1st of December 2023

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Abstract

With a 100 billion NOK export value, Atlantic salmon (*Salmo salar L.*) has become one of the most important export products for Norway. A highly intensified production in recirculating aquaculture systems (RAS) has pushed the physical limits to achieve the fastest growing fish for the market, which has led to problems concerning early sexual maturation of males. New gene editing technology such as CRISPR/Cas9 could solve the maturation problems that the industry is facing, by creating frameshift (FS) mutations that lead to a lack of function. An interesting gene that has been linked to maturation in vertebrates is *vestigial-like protein 3 (vgll3)*. Researchers have studied this gene for nearly a decade and believe that *vgll3* plays a key part in the age of maturation for Atlantic salmon. The objective of the research presented in this thesis was to explore whether fish with two mutated copies of the *vgll3* allele (FS/FS, i.e. *vgll3* knockout) differ in their timing of maturation in modern production environments, compared to fish with two unedited copies of the *vgll3* wildtype allele (WT/WT), or heterozygous fish that have one mutated and one unedited *vgll3* allele (FS/WT). This was accomplished by having four families of gene edited male salmon with different *vgll3* genotypes (FS/FS, FS/WT and WT/WT) reared in a common garden experiment, following a typical intensive "large smolt" production environment of stable 13 °C water temperature with continuous light from first feeding, up until the fish reached 1.4 kg. Maturation was characterized by the combined use of ultrasound, testes histology, testes weight (the gonadosomatic index, GSI), and the plasma levels of the main androgen in salmon, 11-ketotestosterone (11-KT). The *vgll3* knockouts (FS/FS) had the lowest maturation levels (39%) of the three groups, followed by wildtype (73%) and heterozygous (96%). There were growth and performance differences between the groups when the fish were 30-40 g, knockouts being significantly smaller, and condition factor was significantly lower in knockouts around 0.8 kg, compared to heterozygous fish. The growth spurt observed in heterozygotes was as expected from a maturing salmon, unlike the knockouts which had the steady growth of an immature salmon. There were significant differences in SGR between the groups throughout the experiment. Data gathered indicates that *vgll3* knockout reduces the likelihood of male salmon entering sexual maturation during modern large smolt production and pushes the standard thresholds of GSI and 11-KT associated with immature salmon. Using *vgll3* knockouts on a commercial scale could be a part of the solution to solve the ongoing maturation problem that the aquaculture industry is experiencing with male Atlantic salmon.

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Acknowledgement

I first want to thank all the great people of Institute of Marine research (IMR) and Matre Research Station, who have helped and guided me along the way. I greatly appreciate the help from Roger Lille-Langøy and Anne Torsvik, which was essential for the completion of the tedious laboratory work. I must also praise Lise Dyrhovden for being a lovely lady during our many samplings together, where your wits and tough love was much needed on those long days.

To the Institute of Biology at University of Bergen, with Tom Ole Nilsen as my supervisor, thank you for giving me the opportunity to work with IMR in such a thorough and professional study. The collaboration with world leading scientists within my field was a unique experience that I probably would not have gotten any other place.

I must also thank my co-supervisors Thomas Fraser and Anna Wargelius. From a visual point of view, the thesis would never have looked so beautiful without the help of Thomas to create graphs and figures during gruesome Teams sessions. Your smirk and funny comments were a relief in-between my tantrums from terrible computer skills. I am also grateful for Anna that put this perfect team of scientists together, giving me the best people to work with that IMR has to offer.

Last, but not least, I must thank my main supervisor Erik Kjærner-Semb. This thesis could not have been done without your invaluable contribution and guidance. On my side, Erik and I hit it off quite early, and I knew from the very start that we could do great together. From our first days of work at Matre, to our weekly meetings more than a year later, you became a far greater mentor than I could ever hope for. And for that, you have my forever gratitude.

Note: This thesis uses the reference style of the seventh edition of the American Psychological Association's Publication Manual (APA7th).

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Abbreviations

Cas9 – CRISPR associated protein 9

Chr – Chromosome

CRISPR – Clustered regularly interspaced short palindrome repeats

DA – Dopamine

FSH – Follicle-stimulating hormone

FS – Frameshift

Gapdh – Glyceraldehyde-3-phosphate dehydrogenase

GSI – Gonadosomatic index

GnRH – Gonadotropin releasing hormone

HPG – Hypothalamic-pituitary-gonadal

IGF1 – Insulin-like growth factor 1

IF – In-frame

IMR – Institute of Marine Research

ISA – infectious salmon anemia

LH – Luteinizing hormone

PCR – Polymerase chain reaction

PIT – Passive integrated transponder

RAS – Recirculating aquaculture system

SGR – Specific growth rate

SdY – Sexually dimorphic on the Y-chromosome

SNP – Single nucleotide polymorphism

Vgll3 – Vestigial-like protein 3

WT – Wild-type

11-KT – 11-ketotestosterone

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1. Introduction

As the world's population steadily increases, the quest for highly nutritional food sources has never been more dire. Probably the most promising industry that can fulfill this role is the aquaculture industry. Being among the most sustainable ways of producing animal protein, aquaculture can play a key role in food security as the health benefits from consuming seafood is a result of the high content of proteins, vitamins, healthy long-chain fatty acids and minerals (Larsen et al., 2011). Unlike the fishing industry, aquaculture has a great potential to expand worldwide in both high-income and low-income countries, which is why there is no surprise that the Food and Agriculture Organization of the United Nations recommends further research in this field (Gonzalez Fischer & Garnett, 2016).

One of the more popular aquaculture species in the western world is Atlantic salmon (*Salmo salar L.*). This fish is favored for its vibrant red colored filet and impeccable taste. However, with the direction that the aquaculture industry is heading today, salmon production is facing several sustainability issues. Increased production intensity has been leading to problems such as early sexual maturation in male smolt. Early maturation is unfavored in production as it results in poor growth and osmoregulatory problems (Taranger et al., 2010). Individuals that mature will also be more susceptible to disease, which can have serious economic and welfare consequences. In addition to the negative effects on the individual, early maturation can increase the risk of genetic introgression between escaped farmed salmon and wild conspecifics, as mature escapees are more likely to enter rivers to spawn (Taranger et al., 2010), which is an environmental concern in Norway.

With the help of CRISPR/Cas9 technology for precise gene editing, early maturation in salmon has the potential to be drastically reduced by aiming to change genes associated with the timing of maturation and/or the functionality of the reproductive system. One of the genes that looks promising is the *vestigial-like protein 3* (*vgll3*) gene. Studies show that a single locus in this gene has a significant impact on what age maturation occurs at sea (Ayllon et al., 2015), as well as regulating parr maturation in the rivers (Ahi et al., 2022). Managing to solve the riddle of early maturation with gene editing can make aquaculture more sustainable than what it is today.

Using this technique could also be the first step in the right direction to increase resistance to common illnesses during production.

1.1 Atlantic salmon

Atlantic salmon (*Salmo salar L.*) is an anadromous fish in the salmonid family that usually lives a great portion of its life in the ocean (Hoar, 1988). During the spawning period the salmon return to the rivers where they were born. Salmon is an endemic species for the North Atlantic and can be found from its southern range in Portugal and the USA, to its most northern ranges around Norway, Iceland, and Canada (OECD, 2017). In Norway the salmon has been hunted for a long time and as of 2021 the conservation status of salmon, according to Artsdatabanken (2021), has dropped from "viable" to "near threatened". For a long time, the fish has been an important local food source but with the increasing demand globally, salmon has become an important export product for Norway, resulting in an export value of more than 100 billion NOK in 2022 (Norges Sjømatråd, 2023).

The life cycle of salmon is quite extraordinary, and Robin Ade illustrated this beautifully in 1997 with a drawing of all its stages (Figure 1). Wild salmon spawn in the rivers during October and November, with some populations being later in December to January. The female fish bury big orange eggs under gravel and pebbles. This layer of gravel and pebbles functions as protection while allowing filtration of water to bring fresh oxygen. This is crucial in the early stages of life, as several studies have shown how badly a lack of oxygen can affect development of salmon later in the life cycle (Kelly et al., 2020; Krasnov et al., 2021). The embryos will hatch after winter and the alevins will stay hidden in the gravel until the yolk sac has been fully used. The time during early development in salmon is often described using day degrees (d°C), which is calculated by adding together the average daily temperatures. Given that temperature is so important for the speed of development, two eggs can, for instance, hatch at completely different dates due to temperature differences within or between rivers. In the case of Atlantic salmon, hatching happens between 340-450 d°C from fertilization (Solberg et al., 2014), lasting three to four days, with another 220-280 d°C to fully utilize the yolk sac (Fiedler et al., 2023; Gorodilov, 1996). At this stage the fry emerges from the gravel, barely 2-3 cm long. Parr is the freshwater juvenile, recognizable by its sand brown skin and dark spots along the lateral line, called parr marks. With an empty yolk sac, the parr must dwell in the river and hunt for food to

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sustain itself in this growth phase. This will continue for two to five years before reaching a critical size for a developmental stage known as smoltification. Smolt is the description of juvenile salmon migrating downstream, towards the ocean. Smoltification is the transformation that adapts and prepares the fish for seawater entry.

Figure 1: Life cycle of the Atlantic salmon. Source: © Robin Ade and Atlantic Salmon Trust

During smoltification, Atlantic salmon undergo significant physiological, morphological, and behavioral changes that prepare them for their migration to the ocean (McCormick, 2012). These changes include increased hormone production, which triggers the smolt window, a critical period during which the salmon must choose to migrate to the ocean or remain in the river (McCormick, 2012). As the season progresses, the salmon experiences increased linear growth, skin and scale color changes, and the development of a distinct dark line at the ends of its fin. These changes are accompanied by high energy demand, leading to a decrease in the salmon's condition factor (McCormick, 2012). Behavioral changes also occur, such as increased negative rheotaxis, schooling behavior, and imprinting, which allows the smolt to memorize the chemical structure of the water in its natal river (Hasler et al., 1978; McCormick, 2012).

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These transformations enable the salmon to adapt to the saline environment and migrate downstream to the ocean.

After one or more winters in the sea the salmon will return to the rivers for breeding. The females usually stay longer in the sea, while some of the males choose a more different approach and return sooner (Mobley et al., 2021). During the spring and early summer, the salmon have increased growth and build up their fat deposits. This is to prepare them for the long travel during late summer when feeding and growth cease. At this point there are a variety of changes that have happened to the fish. Their gonads have had a rapid growth over the summer and a lot of the qualities appreciated by the aquaculture industry have reduced, such as skin color and filet properties. Once returned to the rivers the salmon have fulfilled their life cycle. While most of the salmon will perish, up to five percent will survive to repeat the cycle (Persson et al., 2023).

1.2 Production systems used in aquaculture

There is a wide array of systems that can be used to cultivate fish on a bigger scale. The main types of systems that are used today are ponds, open water cages, flow-through and recirculating aquaculture systems. Aquaculture can be traced back to ancient human societies, dating back thousands of years. Their method was primitive, but it closely resembles pond systems that are still widely used today. Pond systems are either natural or artificial basins, which vary in size depending on what species that is being farmed and is the least intensive production method (Baluyut, 1989). The method involves making a pond to create an environment where production of aquatic organisms is possible. Compared to pond systems, cages or net pens were a new method of cultivating fish. This method allows for a much greater intensity than that of pond systems and is ideal for producing large quantities of fish. The basics of this system is building a floating net enclosure, making a confined space for the fish to grow in larger bodies of water. One of the upsides of using cages and net pens is the wide range of places it can be placed either it be coastal waters, lakes or fjords (Baluyut, 1989). Another great benefit is that once the enclosure has been placed it is cheap to operate as there is no heating of water. In open water, the temperature will be influenced by the seasonal variations and continuously be replaced by natural flows which maintains a high quality for the fish. However, the main benefit from using net pens, the open exchange with the environment, is also what is

challenging for production. The surrounding environment can present a challenge since the lack of control of the environment compared to other methods can represent suboptimal conditions for production (van de Vis et al., 2020). Problems such as algae bloom or salmon louse outbreaks are among those conditions that a farmer has no ability to control.

Flow-through systems, also known as raceway, are tanks or ponds where water flows through the system. Flow-through as a rearing system is a great way to constantly change the water in the tanks and keep the water at a high quality. The principle for this technology is that water is acquired from a source nearby, either freshwater from a river or seawater in the ocean, adding water continuously to ensure good water quality in the tanks where the cultivated species is kept. In general, there are two types of flow-through systems, conventional and intensive. Systems using the conventional method require an abundant flow of water to ensure enough oxygen for the fish in the tanks, and depending on the water temperature, the intensity of the flow must be regulated (van de Vis et al., 2020). Systems using the intensive flow-through method places an aerator or pure oxygen to supply additional oxygen to the water. In some instances, equipment to regulate the raw water temperature can be installed to the flow-through systems, which allows for more control of the environment in the tanks. Regulating water temperature is expensive, since the water is not being reused, which is a negative aspect of this technology that holds it back from being accessible for everybody. On the other hand, flow-through systems are easy to operate and have high operational safety. Together with low investment cost, this makes flow-through systems ideal in a hatchery or during start feed early in the production cycle.

The latest and most fashionable technology in aquaculture now is production in recirculating aquaculture systems, known as RAS. This method allows for a production in complete separation from the elements. The basic premise for this method is that 95-99% of the raw water that is taken in will be recycled and reused (European Commission, Directorate General for Maritime Affairs and Fisheries, 2020). Raw water is transported from a source nearby and goes through an extensive water treatment process before it enters the system. The water treatment consists of five steps, usually in a specific order depending on the site, that being mechanical filter, biofilter, degasser, oxygen enrichment and UV disinfection (European Commission, Directorate General for Maritime Affairs and Fisheries, 2020). Water that comes from the tanks

to be recycled will go through the same treatment before it is reused. RAS facilities demand a high quality and maintenance in the equipment for it to be efficient and safe to use during production. This method is more complex and requires more surveillance due to higher infectious risk. Although it is expensive to install a RAS facility, the cost of operations are generally low because of the reuse of already heated water.

1.3 Maturation in male Atlantic Salmon

The external cues of photoperiod and temperature are two of the most important factors for maturation in salmon, and both can be adjusted during production. Photoperiod is important for maturation because it regulates the timing of both the parr to smolt transformation and sexual maturation (Oldham et al., 2023). In commercial facilities photoperiod can be used to manipulate and modify the timing of maturation salmon during production (Pino Martinez et al., 2021), tricking the salmon into believing it missed the maturation window. Photoperiod is usually referred to as light (L) or dark (D) and commercially the photoperiod follows a regime to accelerate growth. From first feeding, salmon are normally raised in constant light (LD 24:0). After a period of this regime an artificial winter signal is introduced (LD 12:12), lasting up to eight weeks, before returning to constant light (Pino Martinez et al., 2021). This technique is used in smolt production on land in both flow-through systems and RAS. The other factor, water temperature, also affects the timing of maturation in salmon, and generally high-water temperatures are associated with early maturation (Mobley et al., 2021). If a production is run on water temperatures of up to 15 °C, it is not uncommon that most male salmon mature (Åsheim et al., 2023). Temperature also plays a key role in affecting growth performance of salmon during production (Crouse et al., 2022), and farmers use high temperature to push growth and maintain the biofilter efficiency. Together, temperature and photoperiod can advance the onset of maturation, especially in RAS production and flow-through systems with water heating capabilities, since the push for a fast-growing fish demands much light and temperatures that are essential for a high growth rate.

Sexual maturation in salmon is a play between external and internal cues that trigger the maturation process. External cues are things such as the important environmental factors photoperiod and temperature, as mentioned earlier, as well as diet. Internal cues are things such as genetics, size, energy status, growth factors and biological clock. Together these cues send

signals that the fish is ready for maturation to the brain, which puts the hypothalamic-pituitary-gonadal (HPG) axis to work. The hypothalamus will release gonadotropin releasing hormone (GnRH) and dopamine (DA) as it sees fit, which function as stimulatory factors and inhibitory factors respectively (Klein, 2003). GnRH is a neuropeptide hormone which is a key regulator for reproduction while DA is an amine that has an inhibitory effect on reproduction, among other functions. The hormones GnRH and DA are taken up by the pituitary, which produces several types of peptide hormones. The gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are produced by two different types of gonadotroph cells in the pituitary (Crespo et al., 2022) and released into the bloodstream to regulate gonadal hormone and germ cell production (Crespo et al., 2022), directly affecting the gonads (testis for males and ovaries for females). During spermatogenesis there is a cellular development of spermatogonial stem cells which give rise to many developmental stages of germ-cells, which are spermatogonia, spermatocytes, spermatids, and spermatozoa (Gilbert, 2000). Also, during spermiogenesis the spermatids begin to form a tail, which is called spermiation. This describes the process of germ-cell supporting Sertoli cells that release spermatozoa into the testis's lumen.

When maturing, the male gonads will increase greatly in size, taking up most of the abdominal cavity. Testis is divided into two main compartments: the interstitial compartment and the tubular compartment. The interstitial compartment contains a steroidogenic cell type called Leydig cells, which produce androgens such as 11-ketotestosterone (11-KT) and testosterone 'in fish (Schulz et al., 2023). The tubular compartment contains germ cells and Sertoli cells. The Sertoli cells are supporting cells that produce growth factors, signaling molecules such as insulin-like growth factor 1 (IGF1), which work to support developing germ cells (Schulz et al., 2023). Sertoli cells are the only phagocytotic active cell type in the spermatogenic tubules, and they remove apoptotic germ cells and cellular remnants discarded by developing germ cells. The testes are also used in the calculation of gonadosomatic index (GSI), which along with 11-KT are used as indicators to determine if a male salmon is maturing or not. GSI is calculated by weighing the gonads and dividing that number on the total body weight and multiplying by 100. Generally, the GSI value for an immature male is between 0.06 and 0.11, with higher values found in maturing males (Oldham et al., 2023; Pino Martinez et al., 2023b). To measure plasma concentrations of 11-KT, blood samples are analyzed for quantifying the presence of

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11-KT in the plasma, and generally the 11-KT threshold for identifying a maturing salmon is >1-2 ng/ml (Fraser et al., 2023b).

As the fish starts to mature several physiological changes are happening. Most notably are the anabolic and catabolic effects happening to the body. The anabolic effects result in increased feed consumption, growth, lipid and pigment levels. This growth spurt is in direct contrast to immature fish that will grow at a steady rate. The catabolic effects are the transfer of nutrients and pigments, reduced digestion and reduced osmoregulation. To acquire enough energy to sustain the gonad growth the nutrients and pigments are transferred from the muscles and other tissue, which results in poor filet quality, since this is where most of the lipids are stored (Horn et al., 2018). The pigments are transferred to the skin, coloring the skin brown for the return to the rivers. The reduced digestion is a result of degenerated intestinal and hepatic tissue, which ultimately will lead the fish to its demise. Reduced osmoregulation are adaptations in the gill, kidney and intestine to make it ready for the transition from saltwater to freshwater, which makes them acclimated to freshwater while still being in saltwater (Hvas et al., 2018). In general, the fish will also have increased skin thickness and mucus production, which along with all the other physiological changes, are all unfavorable in production.

There are a variety of reproductive strategies that a male salmon can choose from. Maturing in certain stages during the life cycle is a trade-off between safety and risk. While some individuals prefer low risk, others want to push the limit hoping for a better outcome. The general trade-off for early maturation is that the fish gets access to the mating pool with very little energy investment, at the expense of being smaller and having a hard time competing with the larger males. The earliest reproductive strategy is seen in males which mature in freshwater, called precocious parr, before any sea migration. The precocious parr has developed an interesting strategy by being small and to still have the similar features of a regular immature parr, allowing them to sneak up on larger spawning salmon and fertilize the eggs without having to fight with larger males or use energy on courtship displays with females (Fleming, 1996). Precocious parr represent a genetic and ecological resource as occurrence of these males can be high in some rivers. When spawning is complete the precocious parr has two choices, to stay in the river or proceed to carry out smoltification the following spring and migrate to the oceans. The river has a limited supply of nutrition for the parr that has reached roughly 15 cm and staying there

is not sustainable in the long term. The most reasonable alternative is to migrate to the ocean however, they can stay in the rivers and mature again the coming autumn (Fleming, 1996). Alternatively, the salmon can mature shortly after it has reached seawater, a different strategy referred to as post smolt maturation. These are the individuals that complete their smoltification and migrate to the ocean but mature after less than one winter in seawater (Mobley et al., 2021). This strategy is rarely observed in nature (Klemetsen et al., 2003). Smaller male post smolts will have similar features of a smolt, staying silvery and looking immature, and potentially using female mimicry as a mating strategy, as this method has been observed in other species of salmon (Kano et al., 2006). The larger post smolts will look more like the next strategy, grilse, darkened in the color and fight for females against the bigger males.

The main difference between the last few strategies is how long the salmon risk staying at sea. The general trade-off for later maturation is that by staying longer in the sea the salmon can grow larger and increase its fighting potential against other males, which will provide greater access to females, at the expense of risking its survival by not returning to the rivers before their demise. If the salmon decide to mature the second autumn in seawater it is called a grilse. This fish will have a more similar look to the normal fish that mature after two or three winters in seawater, but they will not have fully developed features. While males have all these alternatives, females usually mature later than their male counterparts. Females can mature during or after smoltification, but typically do not obtain reproductive capacity until after a year or more at sea (Mobley et al., 2021), which is why early female maturation is not considered a problem during production. A fully mature male salmon that has returned to the rivers after three or more winters in the ocean will have a drastically different appearance than what it had when it first left the rivers. The jaws can elongate up to fifty percent during maturation, creating a hook of connective tissue from the lower jaw that fits into a hollow in the upper jaw, and the fish develop an intense red and green coloration on their entire body (Fleming, 1996).

1.4 Atlantic salmon in aquaculture

Although farmed salmon are the same species as the wild variation, their features and life cycles are completely different. Through extensive breeding programs farmed salmon have acquired traits or enhanced their existing traits to that which is desired by the customers. While wild salmon hunt and have a varied diet, farmed salmon are given a processed, high-fat, high-protein

diet with the goal of maximum growth in the least amount of time. This feeding regime is necessary, because compared to the 450 thousand salmon that return from the ocean to the rivers in Norway (Forseth et al., 2023), up to millions of fish from each production location must be fed. From a commercial point of view salmon must be produced all year round to feed the growing consumer base.

As of 2023 there are only a small handful of land-based facilities that have the entire production of salmon on land in Norway. Most of the industry have an option for combining producing hatchery fish on land, with either flow-through system or RAS, and once the fish has reached the smolt stage and a satisfying weight, it will be transferred to sea cages. This way of farming has been maintained largely to the fact that the economic risks concerning full on-land based production have been too high. Trials of salmon production on land in RAS facilities have shown that when salmon have reached the harvest size of four to five kg, up to eighty percent of the males have undergone precocious maturation (Good & Davidson, 2016). This is a large difference compared to natural production systems like sea cages, where maturation can be as low as six percent (Hansen et al., 2017). In a production where both males and females are present, these mature male salmon account for up to forty percent of the volume of fish. Due to competitive disadvantages, big companies in Norway usually do not make their production numbers public. However, a study from New Brunswick Canada found that the largest economic problems for the salmon aquaculture industry at that time was early maturation, and with an estimated gross revenue of 250 million dollars, losses due to grilsing could expect to be 11-24 million dollars (McClure et al., 2007). An economic loss of revenue between five and ten percent is something that most companies either cannot sustain or accept.

Maturation problems during production is a result of a paradigm shift that has happened to the industry over the last decades. High temperatures in RAS production are the cause for early maturation, but the reason is that juveniles are kept longer in RAS facilities to make them grow bigger before release, thus spending less time in the sea. Less time in the sea means less likelihood of sea lice infestation, a choice between risking mature smolt, instead of risking revoking production permit because of too many lice on the fish. Previously, juveniles were commonly kept on land until a size of 50-150 g, before undergoing a smoltification regime and released into a net pen. Now, the new normal is producing "large smolt". This describes

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producing fish which is seawater ready at 250-350 g, with some facilities even keeping the fish until 1 kg has been reached (Dempsey et al., 2023). Despite the fish being called large smolt, they are not traditional smolts, rather these are fish that appear to develop some seawater traits, but not every characteristic of a true smolt (Pino Martinez et al., 2023a). The reason why maturation has not been a problem in the past is that the effect temperature has on maturation first kicks in after the fish has reached 100 g (Fraser et al., 2023b). In other words, it is hard to make a male juvenile mature with the use of elevated temperatures if the weight is less than 100 g. Some argue that early maturation is a result of accumulation of steroids in the water reused in RAS facilities (Good & Davidson, 2016; Summerfelt, 2014), but the same maturation problems are still being observed in flow-through systems using elevated temperatures.

1.5 *Vestigial-like protein 3 (vgll3)*

When it comes to maturation for salmon, environmental factors such as light and temperature play a substantial role in the decision-making process of when to initiate the maturation process. However, genetics also plays a major role in the life history of salmon, as it already has been well-established that age of puberty is a heritable trait in salmon (Nævdal et al., 1978). Previous studies have found that a region located on chromosome (Chr) 25 is strongly linked to age maturity in Atlantic salmon (Ayllon et al., 2015; Barson et al., 2015). This region includes the *vestigial-like protein 3* (*vgll3*) gene, shown to be the gene with the strongest link to maturation (Ayllon et al., 2015), and a single nucleotide polymorphism (SNP) near the human *vgll3* gene has been linked to age for puberty in humans as well (Cousminer et al., 2013).

Some models have explored the effects of knocking out *vgll3* in mice (Figeac et al., 2019; Horii et al., 2023), however nobody has explored if *vgll3* is essential for the initiation of maturation. Nevertheless, in Atlantic salmon, the *vgll3* locus has two alleles, for early (E) and late (L) maturation. Exploration carried out in the studies done by Ayllon and colleagues (2019) and Barson and colleagues (2015) suggest that nearly forty percent of the variation in the age of maturation in Atlantic salmon could be explained by SNPs in the *vgll3* locus. Both studies showed a significantly higher odds ratio for delaying maturation in homozygous LL individuals, and respectively for the EE individuals there was a significantly lower odds ratio for delaying maturation. For heterozygous EL individuals Ayllon and colleagues (2019) observed an intermediate probability to delay maturation.

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Salmon have been observed with several polygenic heritability's, and the *vgll3* locus seems to be affecting some of them. Effects from *vgll3* can be seen in body condition as well as maturation. Higher body condition in both sex of salmon that are *vgll3* EE imply a positive covariance between body condition and maturation (Debes et al., 2021; House et al., 2023). It is assumed that individuals that uphold a higher body condition during periods when they are more receptive to environmental triggers of puberty, have a higher reserve of energy and are therefore more likely to initiate maturation earlier (Debes et al., 2021). Interestingly it has been discovered that body condition varies with the seasons in both sexes and individuals that have both the L alleles have a lower body condition in the spring, but towards autumn and breeding the body condition will elevate, a direct opposite to that of the individuals with both E alleles (House et al., 2023).

1.6 Gene editing technique

In 2012 a new gene editing technique was presented by Emmanuelle Charpentier and Jennifer A. Doudna (Jinek et al., 2012) for which they were awarded the 2020 Nobel Prize in chemistry, recognizing the impact of this method in modern genetic work. They named the method clustered regularly interspaced short palindromic repeats, or CRISPR, with the goal to make gene editing cheaper and easier to use. The method was inspired by how a bacteria's defense system works against viruses. The gene editing technique has two components, using the CRISPR associated protein 9 (Cas9) to make double-stranded cuts in a genome and a short RNA sequence, called guide RNA, to guide Cas9 to precisely where the editing should take place (Jinek et al., 2012). This technique was initially a great tool for biomedicine, however ever since its discovery it has seen a growth in use as a powerful tool to develop a more efficient agribusiness, and among these is aquaculture.

Before using the CRISPR/Cas9 technique an individual will have its original, wild-type (WT) genetics intact. Gene editing is all about adding or removing small pieces of DNA, called insertions and deletions, or indels (Sanders & Mason 2016). When an indel mutation with a size divisible by 3 occurs within a coding region it is called an in-frame (IF) mutation. While a frameshift (FS) mutation is the result of indels with sizes not divisible by 3, which shifts the reading frame of the coding sequence so that subsequent codons have been altered (Sanders &

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Mason, 2016). The latter of the mutations, FS indels, frequently lead to premature stop codons which are more likely to have greater impact on the individual than IF indels. Another effect of the FS indels is that all the amino acids between the location corresponding to the CRISPR cut site and a novel stop codon will be replaced with different amino acids. When the DNA has been altered in such a way that a gene is no longer functional, like premature stop codons in a FS mutation, it is called a knockout.

The first to use CRISPR/Cas9 technology to study salmon was the Institute of Marine Research (IMR) (Edvardsen et al., 2014). This study proved that the F0 generation of fish could be used in a functional study, laying the foundation for a variety of studies from scientific communities in aquaculture around the world. Other studies suggest that CRISPR/Cas9 and other gene editing tools are going to be an important tool to increase resistance to common illnesses such as infectious salmon anemia (ISA) and parasites such as salmon louse (*Lepeophtheirus salmonis*) (Elaswad & Dunham, 2018; Robinson et al., 2023), which will be vital to minimize loss of salmon in an intensifying production and prevent harming nearby wild salmon populations. However, to date, gene edited fish are not permitted for food production in Europe (NOU 2023: 18).

1.7 Research aim and purpose

The purpose of this experiment is to investigate whether it is possible to obtain immature male large smolts by using CRISPR/Cas9 technology to knock out the *vgll3* gene. To do so, several indicators of maturity prevalence were recorded in four families of salmon produced from F0 *vgll3* knockouts, which had various levels of *vgll3* genotypes including *vgll3* loss-of-function mutations, over time in environmental conditions relevant to modern large smolt production, which suffers from high incidences of pre-harvest maturation.

This thesis will discuss the results acquired from the experiment during the period between September 2022 and July 2023. The research aim is to study the effects on parameters such as growth, physiology, and gonad development over time in male large smolts with different *vgll3* genotypes including *vgll3* loss-of-function mutations.

The hypothesis for this thesis is two-parted. i) *Vgll3* is essential for initiating maturation, and ii) Knocking out *vgll3* does not have an impact on growth performance of the fish.

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2. Material & method

2.1 Experimental fish and husbandry

2.1.1 Production of *vgll3* knockout line

The experimental fish used in this study were created in November 2021. This F1 generation originated from a *vgll3* knockout F0 generation that was produced from Aquagen eggs and sperm. The fertilization itself took place on the 16th of November 2021, resulting in four families (E-22-1, E-22-2, E-22-3 and E-22-4) from two male knockouts and two female knockouts from F0 mutants (Figure 2). The parent fish were mosaic and had frameshift (FS) rates from 81- to 86%, In-frame (IF) from 12- to 19%, and wild-type (WT) rates from 0- to 7% in the *vgll3* gene. These four families, with a total of 1032 offspring, were reared separately until September 2022, but once they were PIT tagged, they were mixed and reared together. The parents (F0 generation) were produced in a 2017 study that has not yet been published. The F0 generation were considered mosaic, having many mutations that carry on to the next generation, which could result in different genotypes, including FS or IF mutations as well as WT alleles.
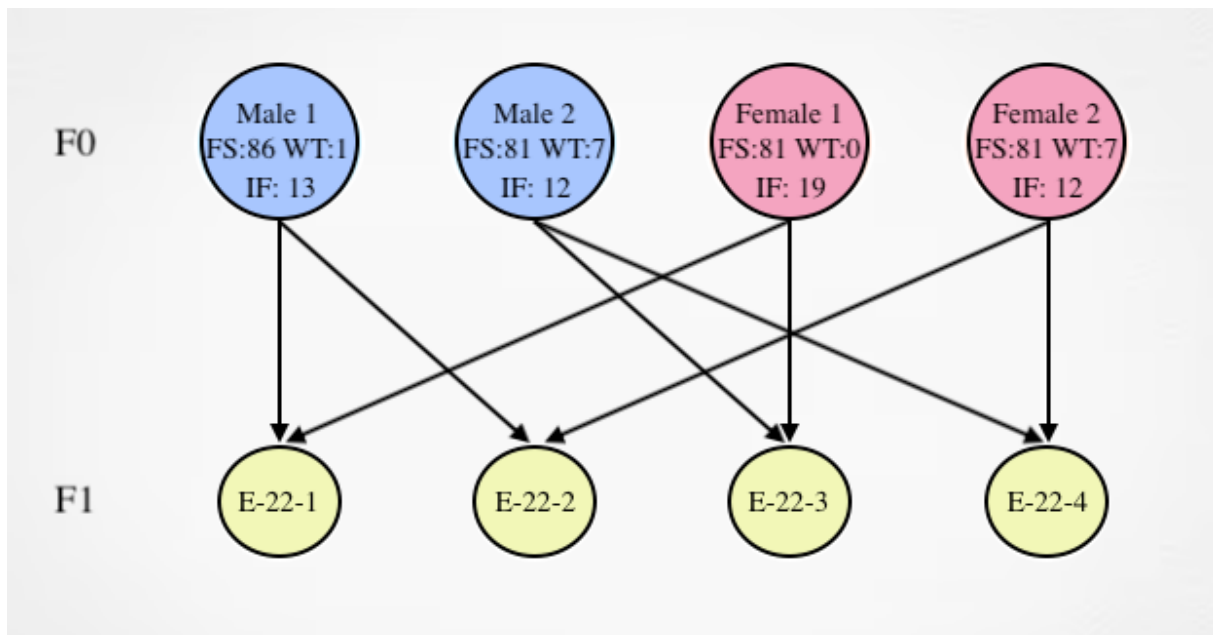

Figure 2: Four families (F1) created from two male knockouts and two female knockouts (F0). Mutation rates (percentage) for F0 males and females are in the blue and pink circles respectively.

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2.1.2 Husbandry

For the entire period of the experiment the fish have been located at the Matre Research Station in Masfjorden, Norway. Following the fertilization, the embryos were placed in a hatchery for about four and a half months at 6 °C until they reached start feeding at around 600 d°C, upon which they were moved to 1x1x1m start feeding tanks. At start feeding on the 4th of April 2022, the water temperature in the tanks was increased to 13 °C, with continuous light. A few days after start feed all the larvae with obvious deformities were removed from the hatchery. Throughout the experiment the fish have been fed with pellets from Skretting. The pellet size was chosen to fit the size of the fish. In the period from the 2nd of September 2022 to the 13th of June 2023 the fish were moved between tanks to fit their current size and number, usually in combination with fish samplings (see *2.3 Fish sampling*). On the 1st of July 2022, the fish were moved from start-feeding tanks to tanks holding roughly 400 L (1m width x 1m length x 0.9m height).

On the first sampling of this experiment on the 2nd of September 2022, the fish were moved to one tank that held 6000 L (3 m diameter x 1.2 m height). On the 17th of November 2022, the fish were manually inspected for deformations such as jaw, spinal or tail deformities, before being separated into healthy and deformed individuals (see Figure 5 in section 3.1.1 Deformities for examples of deformations). The deformed fish were transferred to a separate tank, leaving 500 non-deformed fish to remain in the main tank. On the 25th of November, a decision was made to euthanize the deformed fish and they were removed from the experiment entirely. To fully grasp the scope of deformities the fish were also studied with radiography to help quantify the severity of the malformations. The fish were placed on plastic trays in groups of 3 to 5 individuals, each tray having a number formed in lead that differed from each other.

On the 2nd of February 2023, the males and females were separated based on genetic screening for the sex marker gene *sdY* (see *2.2.4 Sex determination*). The females were saved for future experiments, while the males were moved to two 2500 L tanks (2 meter width x 2 meter length x 0.8 m height). For the next three samplings males would rotate between tanks of similar size, with a decreasing number of individuals per sampling. Matre Research Station is not equipped with RAS facilities which is why all the tanks that the fish alternated between during this

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experiment used a type of flow-through technology, with the possibility to control the water temperature and photoperiod, which closely simulates a RAS production environment.

The tanks were only run on freshwater and the temperature was holding roughly 13 °C and the lights were kept on constantly. Temperatures were recorded from the 2nd of September 2022 to the 13th of June 2023, as shown in Figure 3. From September 2022 to February 2023, the temperatures were measured automatically, while from February 2023 to June 2023 it was manually measured and written down regularly by personnel on site.
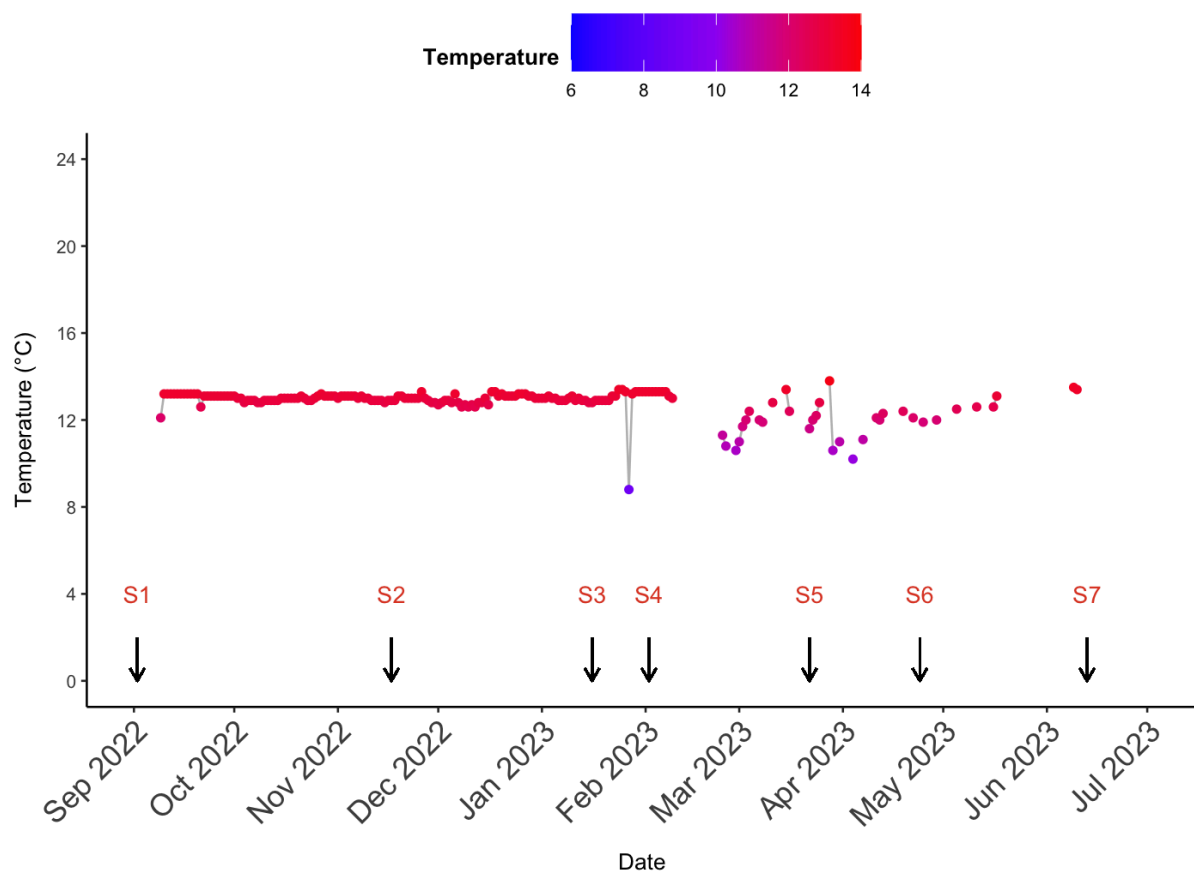

Figure 3: This graph shows water temperature that has been recorded over the course of the experiment, blue indicating colder and red indicating warmer temperature. Each dot was the average temperature that day. The arrows indicate time of sampling (S1-7).

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2.1.3 Ethics statement

Throughout the whole experiment there was an effort to remove ill, deformed or dead fish from the tanks at Matre Research Station. These fish were therefore euthanized humanely as soon as a problem was detected. This was a precaution to ensure good animal welfare for all the individuals, and not let any individual suffer needlessly. This experiment (FOTS permit number 29251) was approved by the Norwegian Animal Research Authority. The experiment followed strict guidelines in accordance with the Norwegian Animal Welfare Act of the 19th of June, in force from the 1st of January 2010. It was mandatory for all personnel involved to have undergone sufficient training, approved by the Norwegian Food Safety Authority, before they could partake in the experiment. Also, as a part of the experiment, working in the laboratory required a short course in environment, safety and health, which was provided by the staff on site.

2.2 Genetic work

2.2.1 PIT tag and fin clipping

By the end of summer 2022, the fish size allowed for implanting passive integrated transponder (PIT) tags into each individual. On the 2nd of September 2022, 1032 fish were tagged and fin clipped. To anesthetize the fish, Finquel vet. was mixed in a transport tub filled with water, approximately 0.1 g per liter water, with sodium bicarbonate as a buffer. When the fish were calming down, they were taken up one by one, to be inserted with the PIT tag. The tag was inserted using a scalpel, making a small insertion in the abdominal cavity, roughly half a centimeter caudally from the pectoral fin on the right side. A fin clip was also taken at the same time, a small sharp scissor was used to completely remove the fat fin, ensuring there would be sufficient genetic material to work with. The fin clips were stored in 100% ethanol on 96-well plates and stored in a freezer. Fin clips were collected for DNA extraction for subsequent analysis of genotypes and sex markers.

2.2.2 DNA extraction

With the fin clips secured, DNA extraction could proceed. All the fin clips were cut into smaller pieces, so that the material would be broken down for DNA extraction and to avoid tissue overload. The small tissue pieces were placed in a new 96-well plate and a lysis solution consisting of Lysis LBH (Beckman Coulter Inc) supplemented by Proteinase K (1,5 µg/µl) was

added to the wells. After the solution was added to the plate with DNA it was sealed and incubated at 55 °C on 125 rpm, for 18-20 hours. After the incubation, the tissue will be broken down and DNA ready for extraction. For the extraction of genomic DNA, the DNAdvance protocol from Beckman Coulter Inc (PN B66866AC) customized for Biomek i5 Automated Workstation was used. To conduct the extraction two methods are started from the "Biomek Method Launcher" program, being "Transfer of Lysates 1 to 5 plates - 200 µl" and "DNAdvance B5 1 to 5 plates - 200 µl".

2.2.3 Genotyping of mutations

Preparation of sequencing library

The first step of genotyping was the preparation of a sequencing library for amplicon sequencing. The sequencing library was prepared using two rounds of polymerase chain reaction (PCR). The protocol was originally based on Gagnon and colleagues (2014), but adjusted for salmon (Straume et al., 2020). For the first PCR (PCR1), forward and reverse primers specific to the CRISPR target site in *vgll3* on Chr 25 were used. The primer sequences were from a study that by the time of writing, has not yet been published. A mastermix was made for all the PCR1 reactions (n=1032) by mixing all the reagents in Table 1 (except for DNA).

Reaction mix was dispensed in wells on 96-well plates, and 1 µl DNA was added to each well. The plates were briefly centrifuged to collect the liquid at the bottom of the wells and run in the thermocycler with settings listed in Table 2. When the PCR had finished, PCR products were diluted 1:4 according to Gagnon and colleagues (2014) by adding 30 µl water to the PCR products. A subset of the samples was inspected by running gel electrophoresis to identify bands of expected size (Figure B1 in *Appendix B*). The second PCR (PCR2) was performed to attach index sequences which allows for multiplexed sequencing. Master mix containing reagents for PCR2 reactions was prepared for each plate as listed in Table 3 (except for DNA and i5 primer) and dispensed to wells on 96-well plates. Ninety-six different i5 indexing primers were used to indicate well positions on a plate, while 11 different i7 indexing primers were used to indicate plate number. One µl i5 primer corresponding to the well position was added to each well. One µl diluted PCR-product from PCR1 was used as input in PCR2 and added to individual wells. PCR2 was run in the thermocycler with settings specified in Table 2.

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Table 1 - Reaction mix for each PCR1 reaction.

<u>**Mix per 10 µl reaction:**</u>

2 µl Q5 Reaction Buffer

0.2 µl 10 mM dNTPs

0.1 µl Q5 HotStart Polymerase

1 µl 2.5 µM F/R primer mix

5.7 µl water

1 µl DNA

Table 2 - Thermocycler settings for PCR1 and PCR2. 30 cycles were used for PCR1, and 20 cycles was used for PCR2.

Start	Cycling	End
98 °C 30 s	98 °C 10s, 66 °C 20s, 72 °C 30s	72 °C 3 min, 4 °C inf

Table 3 - Reaction mix for each PCR2 reaction.

<u>**Mix per 10 µl reaction:**</u>

2 µl Q5 reaction buffer

0.2 µl 10 µM dNTPs

0.1 µl Q5 polymerase

1 µl 2.5 µM i7 primer (unique per plate)

1 µl 2.5 µM i5 primer (unique per well)

4.7 µl water

1 µl diluted DNA from PCR1

For each 96-well plate, 2 µl PCR2-products from each well were pooled into new tubes. From each of the tubes, the pooled PCR-products were loaded on a gel for gel extraction (see Figure B2 in *Appendix B*). Gel bands of expected size were excised, and DNA was extracted using QIAquick Gel Extraction Kit (Qiagen). DNA concentrations of each extracted pool was measured with Qubit (Thermo Fisher) and adjusted to 3.1 ng/µl by adding water, which corresponds to 10 nM for an amplicon of the expected size. All the 10 nM pools were then

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pooled into a final master pool, constituting the sequencing library, which was used for DNA sequencing. The samples were sent to Flødevigen Research Station for analysis where the IMR has the MiSeq sequencing machine (Illumina). The sequencing was done using Miseq Kit version 3 with 300 bp paired-end reads.

Sequence analysis

Samples were demultiplexed based on i5 and i7 index sequences, and all reads were required to match the target primer sequences in the 5' ends of both the forward and reverse reads. Sequence data was analyzed similarly to previous studies in salmon CRISPR mutants (Güralp et al., 2020; Straume et al., 2020) using an in-house built pipeline for amplicon-sequencing based mutation analysis. Each sequence read was aligned to the amplicon reference sequence from the Atlantic salmon reference genome. For each read, a custom script was used to determine the presence of insertions and deletions (indels) and the size of the indels. Reads were divided into categories depending on how the reading frame was affected, i.e., indels with a size divisible by 3 were counted as in-frame (IF) mutations and remaining indels were counted as frameshift (FS) mutations. Reads not containing any indels were counted as wild-type (WT) if they did not contain any substitutions. Percentages of IF, FS and WT were calculated for each individual. A subset of aligned sequence reads was inspected using Geneious 10 (Biomatters development), to ensure that the correct target was sequenced and that the correct mutation types were identified by the pipeline. Individuals were further divided into genotype groups based on identified mutations. If the reads from an individual contained more than 90% reads supporting a single mutation type (FS, IF or WT), that individual was considered homozygous (FS/FS, IF/IF or WT/WT). Individuals were defined as heterozygous if the reads from a given individual showed at least 40% support for two different mutation types (FS/WT, FS/IF or IF/WT). Individuals having FS/FS (knockouts), FS/WT (heterozygotes) and WT/WT (wildtypes) genotypes were used in the experiment, while individuals having genotypes IF/IF, FS/IF and IF/WT were removed from the experiment.

2.2.4 Sex determination

Sex determination is genetic in salmon, with males being XY and females being XX. To figure out the sex of each individual, a qPCR-based *sdY*-test was performed, based on the work done by Ayllon and colleagues (2020), with some corrections. The correct primer and probe sequences used can be found in *Appendix B,* see Table B1. The sex-determining gene in salmon,

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sexually dimorphic on the Y-chromosome (*sdY*), has 4 exons present in males. In very rare cases these exons can also be present in females. This way it is possible to differentiate between the two sexes using a qPCR assay. The housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) was used as a positive control to confirm the presence and amplification of DNA in the sample. For each of *sdY* exons 2 and 4 and *gapdh*, 40X assay mixes were made (Table 4), and were used in the qPCR reaction mix (Table 5). The qPCR was run using QuantStudio 5 (Thermo Fisher).

Table 4 - 40X Assay mixes for *sdY* exon 2 and 4 and *gapdh*.

40X Assay mixes for exon 2, exon 4 and gapdh
36 µl 100 µM forward primer
36 µl 100 µM reverse primer
8 µl 100 µM probe
20 µl water

Table 5 - Reaction mix for qPCR reactions.

PCR reaction mix (5 µl reactions)
2.5 µl 2X TaqMan Universal Master Mix
0.125 µl 40X *gapdh* assay mix
0.15 µl 40X *sdY* exon 2 assay mix
0.175 µl 40X *sdY* exon 4 assay mix
1.05 µl water
1 µl DNA

The final mixture was dispensed to wells on a 384-plate using a multi dispenser pipette adding 4 µl to the wells. After the 384-plate was filled it was covered and spun down, collecting the liquid in the bottom of the wells. Then 1 µl of template DNA was added to each well. Male and female control samples were also included. The 384-plate was covered with optical cover film and ran with the presence/absence program on the qPCR machine. The following program was used; a Pre-Read stage (60 °C for 30 seconds), a Hold Stage (95 °C for 10 minutes), a PCR stage (40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute) and Post-Read stage (60 °C

for 30 seconds). Amplification curves were manually inspected to determine the genotypic sex, see Figure B3 and B4 in *Appendix B*. Samples showing clear amplification of *sdY* exons 2 and 4 were counted as males, while samples lacking amplification of both exons were counted as females. Samples lacking amplification of one of the exons, and samples lacking amplification of *gapdh* were defined as undetermined.

2.3 Fish sampling

During the period from the 2nd of September 2022 to the 13th of June 2023 there were a total of 7 samplings (2nd of September, 17th of November, 17th of January, 2nd of February, 22nd of March, 24th of April and 13th of June). The goal was to have a sampling every one to two months, as this would provide evenly spread-out information and at the same time, give the least disturbance as the fish were growing. Some aspects of the sampling were only done once (22nd of March), such as blood sampling, but the things that were done each time was measuring length and weight. Gentle care of the fish was a key criterion for success as this would give the least stress to the fish and prevent unnecessary loss of important individuals. Until genotype and sex was determined through genetic work, the fish were kept in the same tanks, however, during the sampling in February, the fish were also separated into two groups, male and female.

From this point the males would be in focus whilst the females would go on to brackish water. The females would still be monitored and saved for future studies. The fish with the wrong genotypes and deformations were euthanized. To study gonad growth, a limited number of fish was selected from two genetic group for dissecting (24th of April). To avoid dissecting all the individuals, an ultrasound machine was used to detect gonads. This would allow for a bigger quantity of gonad detection and fewer deaths. All results from the ultrasound were recorded and analyzed by three different people. Blood samples and histology samples were taken to support the data already being gathered.

2.3.1 Growth and performance

Every one to two months there was sampling where the length and weight of all the fish that were alive would be measured. The fish were transported in a tub from their tank to the working station. The transport tub was mixed with water and Finquel vet, 0.1 g per liter of water with sodium bicarbonate as buffer, to anesthetize the fish before the next procedure. A measuring

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board that could measure up to 50 cm and a weight scale that could measure down to 1 g were used to measure the fish. To measure length, the fish was put on the table with the anterior end of its mouth at 0 cm. The fork length was measured from the tip of the mouth to the posterior end, to the middle of the caudal fin, see Figure 4.

Figure 4: An Atlantic salmon laying on a measuring board. The red stripes indicate the borders used in length measurements.

Length and weight would later be used to calculate condition factor and specific growth rate (SGR). All data retrieved during sampling was written in a separate Microsoft Excel sheet before it would be implemented in the master document. In the first samplings when the fish were small the size was measured down to one millimeter. At this stage every millimeter would have a big impact on condition factor and SGR, and miscounting would give the wrong impression of the fish. When the fish grew bigger the measuring would be measured to the closest half or whole centimeter as the difference in SGR and condition would be less or near insignificant. After length and weight was measured the fish would be transported to a clean new tank where they would stay until the next sampling. Condition factor was calculated as shown in Formula 1, while SGR was calculated as shown in Formula 2.

Formula 1: $Condition\ factor = \frac{100 * weight\ (g)}{length\ (cm)^3}$

Formula 2: $SGR = \frac{\ln Startweight\ (g) - \ln Endweight\ (g)}{Number\ of\ days} * 100$

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2.3.2 Dissection and ultrasound for detection of gonads

To avoid killing off all the male fish in one sampling, ultrasound was used as a non-invasive tool for the detection of gonads (Næve et al., 2019) on the 24th of April and 13th of June. This would allow for analysis on each individual and document maturation over time. Regular dissection was used to support results from ultrasound. To use the instrument the fish would still have to be anesthetized, but it was always done after measuring length and weight. The fish would be placed in a tray on its lateral side. The tray would be filled with water to calm the fish. To do the ultrasound the probe was placed behind the operculum and stride along the lateral line. This would give a clear view of most of the intestine, vertebrae and mature gonads. While doing the procedure it was important to keep the ultrasound machine away from water as the machine is not waterproof and could be ruined. The operator of the ultrasound reader was blinded to the fish genotype, to avoid bias in assignments of maturation status.

During the sampling on the 24th of April, a handful of individuals from two genetic group (FS/FS and FS/WT) was dissected to look for gonad growth, as this is an indicator for sexual maturation. Because of the low number of WT/WT individuals and observed difference with ultrasound in maturation rates between FS/FS and FS/WT, only fish with FS/FS and FS/WT genotypes were included in the sampling of gonads. The selected fish would be put in a tub with a strong mixture of Finquel vet. 1000 mg/g so they would be euthanized before dissection. Each fish was easily opened using a simple scalpel making an insertion from the anus, following a shallow cut towards the gills. With the abdomen opened, the intestines were removed so that the gonads could be observed. An immature male would have thin pink strings going alongside their swim bladder, while mature males would have huge white gonads almost resembling a second liver (see Figure 10 in *3.3 Maturatio*n for examples of immature and mature gonads). During the dissection, 30 of the gonads were photographed and collected for calculating gonadosomatic index (GSI) and cut into smaller pieces to study gonad tissue, see *2.3.3 Histology*. GSI was calculated as shown in Formula 3.

Formula 3: $GSI = \frac{Gonad\ weight\ (g)}{Fish\ weight\ (g)} * 100$

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2.3.3 Histology

To further study the maturation of the male large smolt, pieces of the gonads were dissected and cut to about the size of a small pea and studied with histology. A total of 30 males with FS/FS and FS/WT genotypes, 15 individuals each, were randomly chosen and dissected during the sampling on the 24th of April 2023. Because of the low number of WT/WT, this group was excluded from sampling of gonads as mentioned in *2.3.2 Dissection and ultrasound for detection of gonads*. The gonad tissue was placed in small plastic tissue cassettes and stored in a locked bottle with Bouin's fixative solution for 48 hours. This fixative preserves the tissue's morphology for histological analysis. All samples were rinsed in increasing concentrations of ethanol, then xylene, to remove the fixation solution before embedding in hot paraffin. Once cooled down, the block consisting of paraffin and tissue was sliced to a thin section. With the section ready, heat was used to remove the paraffin and replace it with water through several infiltration baths. The sections were stained with hematoxylin and eosin staining, coloring nuclei blue and cytoplasm red, respectively. Histological sections were scanned with a digital slide scanner (Hamamatsu NanoZoomer S60) using a 40× source lens (resolution 220 nm/pixel). Pictures of the thin section of gonad tissue were viewed in NDP.view2 (Hamamatsu Photonics K.K.) and classified as mature or immature based on the presence or absence, respectively, of spermatids and/or spermatozoa.

2.3.4 Plasma steroid hormone levels

As a supplement to visual changes and dissection, blood samples helped understand hormonal levels. On the 22nd of March 2023, blood sampling was done on all the 119 live males, during a regular sampling. While the fish was anesthetized for length and weight measuring, the fish could also have their blood extracted. A syringe with a needle would penetrate the skin, just behind the anal fin, and gently push towards the veins laying on the ventral side of the spine. There was a need to avoid using too much pressure, as that could result in hurting the spine, giving the fish permanent and unwanted injuries.

The blood was centrifuged down for 3 min with 14000 G at 4 °C, to be able to separate plasma from blood cells. This plasma was subsequently used to analyze 11-ketotestosterone (11-KT) hormone levels in each individual. The plasma samples were sent to Austevoll Research Station for analysis where the IMR has the required machines and trained personnel to do the tests. The

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analysis of sex steroids, specifically 11-KT, was conducted using ELISA (Butler & Oudit, 1994) and confirmed in accordance with previously established protocols (Andersson et al., 2013).

2.4 Statistical analysis

The analysis of data was done in RStudio version 4.2.2 as well as GraphPad Prism. RStudio was used to produce the graphs and figures with ggplot to illustrate the results. GraphPad Prism was used to do statistical analysis to compare the results between the different subject groups. The D'Agostino-Pearson omnibus test was used for testing if the values of the different groups were following a normal distribution. Several groups did not follow a normal distribution, therefore the non-parametric Kruskal-Wallis test was used with Dunn's multiple comparisons test. When only two groups were compared, which was only the case for GSI, the non-parametric Mann-Whitney test was used. In all cases, P-values below 0.05 were considered as significant and significance level is indicated with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

3. Results

3.1 Mutations

From the four families of 1032 PIT tagged fish, a total of 252 were males without deformities that had relevant genotypes (FS/FS, FS/WT and WT/WT) for studying. The three genotypes of interest FS/FS, FS/WT and WT/WT had 155, 76 and 21 male fish respectively, as shown in Table 7. The remaining fish were either females, had deformities or contained unwanted or unknown genotypes, and were therefore not the focus of this study. The male distribution of interesting genotypes within each family can be seen in Table 8, with family E-22-1 and E-22-3 containing most fish, 137 (FS/FS: 101, FS/WT: 26, WT/WT: 10) and 64 (FS/FS: 50, FS/WT: 10, WT/WT: 4) respectively. In the two other families, E-22-2 and E-22-4 had fewer individuals, with 27 (FS/FS: 1, FS/WT: 23, WT/WT: 3) and 24 (FS/FS: 3, FS/WT: 17, WT/WT: 4), respectively. For a complete overview over all genotypes of both males and females in all families, see Table A1 in *Appendix A*.

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Table 7: Mutation rates of all the fish. Bold text highlights the number of male fish with genotypes of interest.

Genotype	**Male**	Female	Unknown sex
FS/FS	**155**	157	17
FS/WT	**76**	57	11
WT/WT	**21**	18	0
FS/IF	89	86	15
IF/IF	61	56	18
IF/WT	8	18	0
Unknown	70	80	11
Total	480	472	80

Table 8: Distribution of genotypes of interest in males from all families.

Family	E-22-1	E-22-2	E-22-3	E-22-4
FS/FS	**101**	**1**	**50**	**3**
FS/WT	**26**	**23**	**10**	**17**
WT/WT	**10**	**3**	**4**	**4**
Total	137	27	64	24

3.1.1 Deformities

To comply with ethics guidelines and to avoid biased results, deformed fish were identified by manual inspection and using X-ray imaging and were removed from the experiment (Table 9). A total of 232 (22.5 %) fish had visible deformities, most notably was the lower jaw deformation, which was identified among 143 individuals. Other deformities that were found included upper jaw deformation (pug-head), tail deformity and spinal deformity. In the four

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families the males had deformation rates between 16- and 40% (Table 10), and the complete distribution of deformities in all families with all the genotypes of both sexes can be seen Table A1 in *Appendix A*. X-ray imaging of deformed fish confirmed the presence of jaw and spine malformations (Figure 5).

Table 9: Deformities in all fish. Number of fish with any type of deformity and number of fish with lower jaw deformities per genotype, independent of sex. Genotypes of interest are highlighted in bold. Deformities were characterized on the 25th of November.

Genotype	Total number of fish	Deformed fish	Lower jaw deformity	% Deformity	% Lower jaw deformity among deformed fish
FS/FS	**329**	**134**	**116**	**40.7**	**86.6**
FS/WT	**144**	**26**	**3**	**18.1**	**11.5**
WT/WT	**39**	**2**	**0**	**5.1**	**0.0**
FS/IF	190	20	4	10.5	20.0
IF/IF	135	11	1	8.2	9.1
IF/WT	26	3	0	11.5	0.0
Unknown	169	36	19	21.3	52.8
Total	1032	232	143	22.5	61.6

Table 10: Male deformed fish from genotypes of interest in each family.

Family	E-22-1	E-22-2	E-22-3	E-22-4
FS/FS	**43**	**1**	**21**	**1**
FS/WT	**3**	**10**	**1**	**2**
WT/WT	**1**	**0**	**0**	**1**
Total	47	11	22	4

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Among the genotypes of interest, it was observed the highest deformity rate among fish having the FS/FS genotype, with a deformity rate of 40.7%, among which upper jaw deformity accounted for 86.6% of the identified deformities. This deformation rate in FS/FS genotype was significantly higher than any other genotype, with P-value of < 0.0001 when compared to FS/WT and P-value of 0.0016 when compared to WT/WT. Among the two other genotypes of interest, FS/WT and WT/WT, there were observed deformity rates of 18.1% and 5.1%, respectively. Where most of the deformed fish in the FS/FS genotype group had lower jaw deformities, the FS/WT group had only 11.5%, while the WT/WT group had no lower jaw deformations (see Figure C1 in *Appendix C* for a visualization of the distribution of deformities between each genotype).
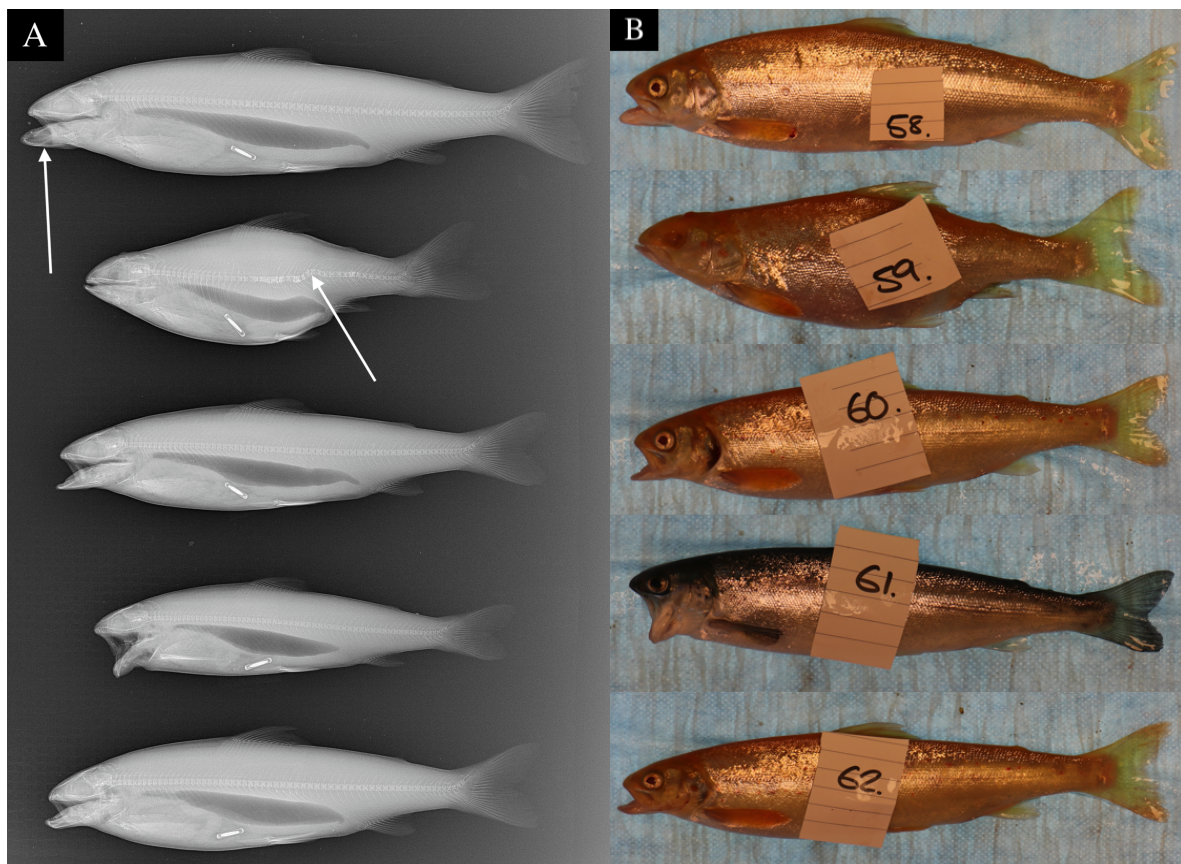

Figure 5: A) X-ray imaging of jaw and spine deformities. Five deformed individuals analyzed by X-ray imaging on the 25th of November 2022 were selected as representative for jaw and spine deformities. The bright cylinders inside the fish are the PIT-tags. The arrow on the first fish from the top is pointing to the enlarged lower jaw which was common among the deformed fish. The arrow on the second fish from the top is pointing to the area of which the spine has

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deformed. B) Pictures of the same deformed fish in the same order, captured by a regular camera.

3.2 Growth and performance

By the final sampling (13th of June) the fish had increased their length by 3.5 times and increased their weight by 45 times, relative to the initial sampling (2nd of September). At the first sampling the average length was 13.52 cm (±2.13), and weight was 33.77 g (±13.11). Nine months later, average length and weight had increased to 46.18 cm and 1459.40 g, respectively. Average lengths and weights of FS/FS, FS/WT and WT/WT males from all samplings are listed in Table 11, with distributions of length, weight, condition factor and SGR per time point and genotype shown in Figure 6, 7, 8 and 9, respectively. In *Appendix C*, the number of individuals during each sampling can be seen in Table C1. A complete table of each genotype's average length and weight can be found in Table C2 and C3, while significant differences and P-values for all results in *3.2 Growth and performance* can be found in Table C4, C5, C6, C7 and C8.

Table 11: Average length and weight of the males 2022-2023. Numbers in parenthesis are the standard deviation.

Date	2nd Sep	17th Nov	16th Jan	2nd Feb	22nd Mar	24th Apr	13th June
Mean length (cm)	13.52 (±2.13)	22.98 (±3.06)	32.13 (±3.06)	33.22 (±3.13)	39.09 (±3.92)	42.79 (±4.46)	46.18 (±4.26)
Mean weight (g)	33.77 (±13.11)	159.88 (±51.16)	443.67 (±132.63)	469.02 (±135.29)	819.85 (±264.32)	1104.18 (±379.18)	1459.40 (±412.43)

3.2.1 Length and weight

The general trend for length and weight was that the FS/FS genotype was smaller, and it was significantly smaller on the 2nd of September 2022 in comparison to the FS/WT and WT/WT genotype. However, at all other consecutive samplings there was no significant difference in length and weight between any of the three genotypes (Figure 6 and Figure 7). The FS/WT genotype generally had the largest fish, while the WT/WT genotype had sizes closer to the FS/FS genotype. Although the FS/WT genotype group contained the largest fish, the group also

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had the largest variation in length and weight throughout the whole experiment. The full list of significant differences and P-values are shown in Table C4 and C5 in *Appendix C*.
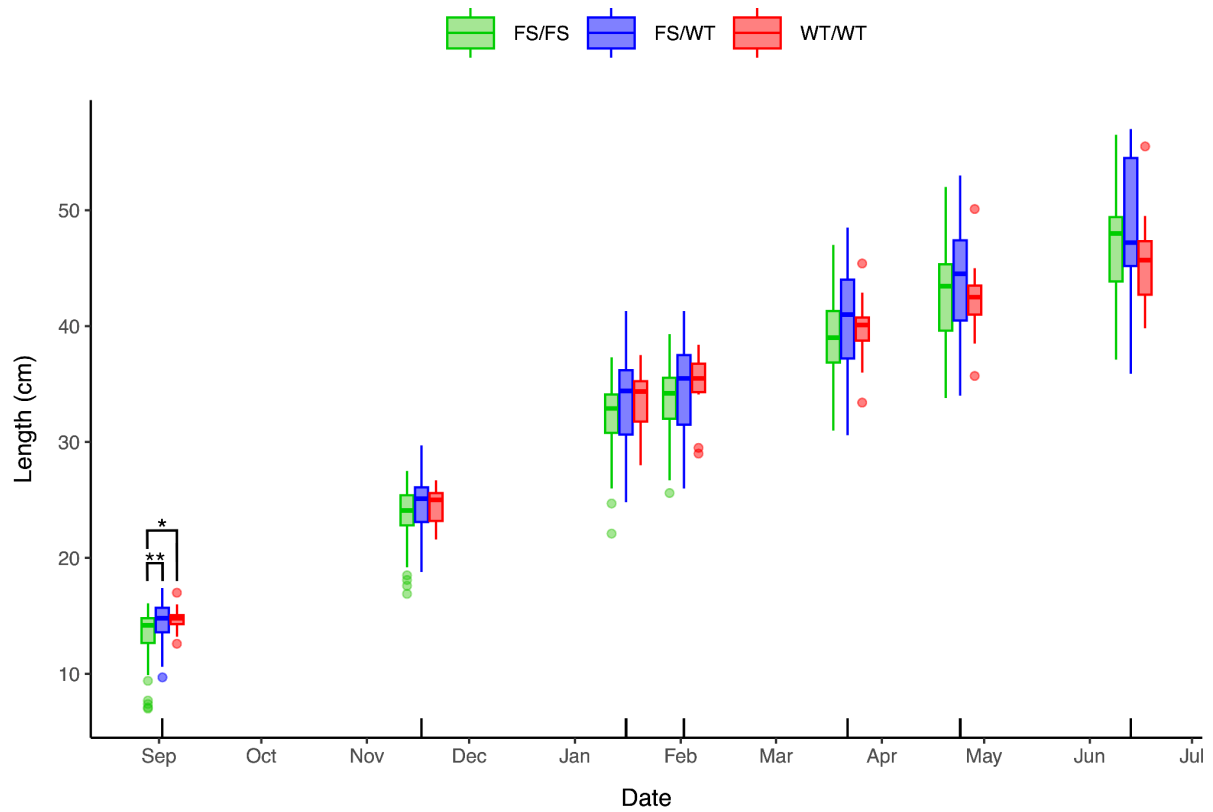

Figure 6: Boxplots showing length distributions among each genotype per sampling point. The vertical dashes along the x-axis indicate sampling time (S1-7) and the asterisk illustrate significant differences between the groups (* = P < 0.05, ** = P < 0.01). Boxplots show the medians (central line), 25th and 75th percentiles (box margins), and the largest and smallest values within ×1.5 the 75th and 25th interquartile ranges (arms). The points in the graph are all values outside the range of the arms.
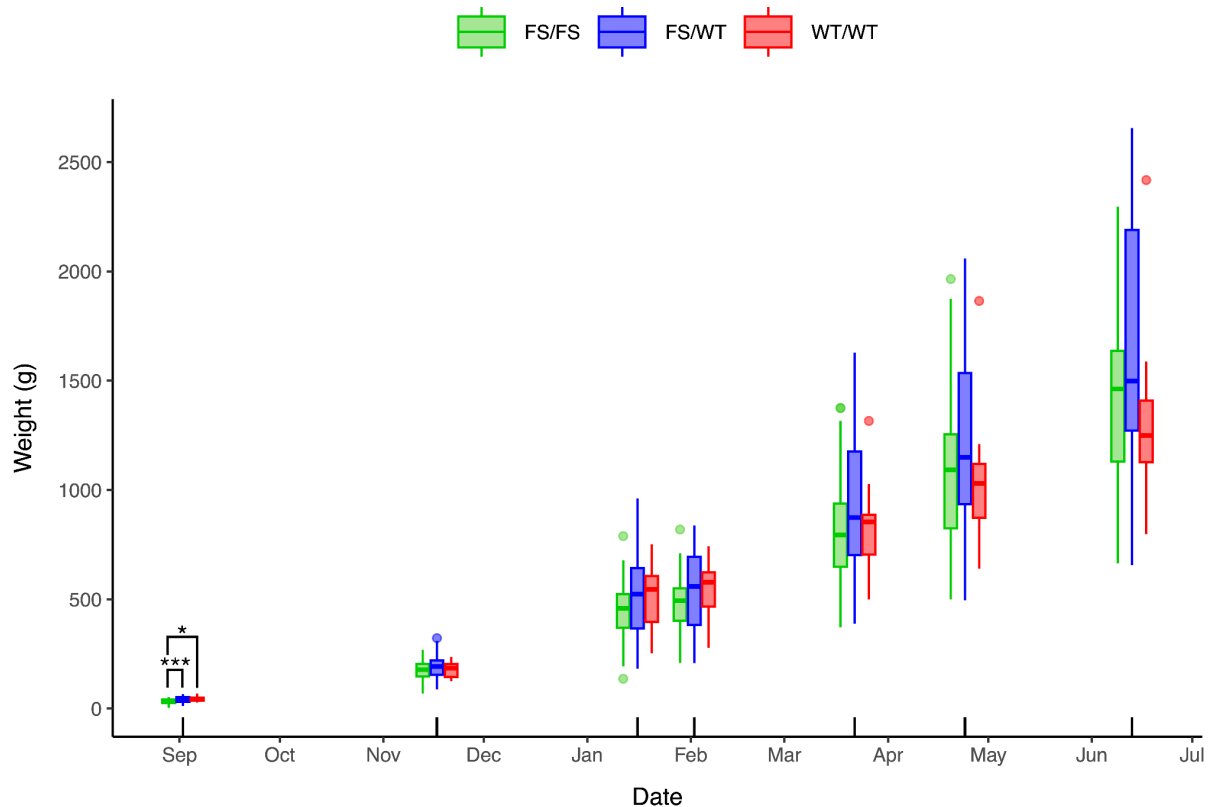
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Figure 7: Boxplots showing weight distributions among each genotype per sampling point. The vertical dashes along the x-axis indicate sampling time (S1-7) and the asterisk illustrate significant differences between the groups (* = P < 0.05, *** = P < 0.001). For details of boxplot, see Figure 6 legend.

3.2.2 Condition factor and specific growth rate

The average condition factor for all three genotypes stayed between 1.2 and 1.4 for the duration of the experiment (Figure 8), and there were two instances of significant difference between the groups. Statistics show that in September and March there was a significant difference in condition factor between FS/FS and FS/WT genotype. A decline in SGR for all genotypes was observed (Figure 9), starting at around an SGR of 2.2 for FS/FS and FS/WT genotype and 1.8 for WT/WT genotype. At the last sampling all genotypes had an SGR around 0.5. Statistics show there were quite a few instances where there was a significant difference in SGR between the group. In February there was a significant difference between FS/FS and FS/WT genotype, and a significant difference between FS/FS and WT/WT genotype in November, January and April. Between FS/WT and WT/WT genotype there was a significant difference in SGR in

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February and March. The full list of significant differences and P-values are shown in Table C6 and C7 in *Appendix C*.
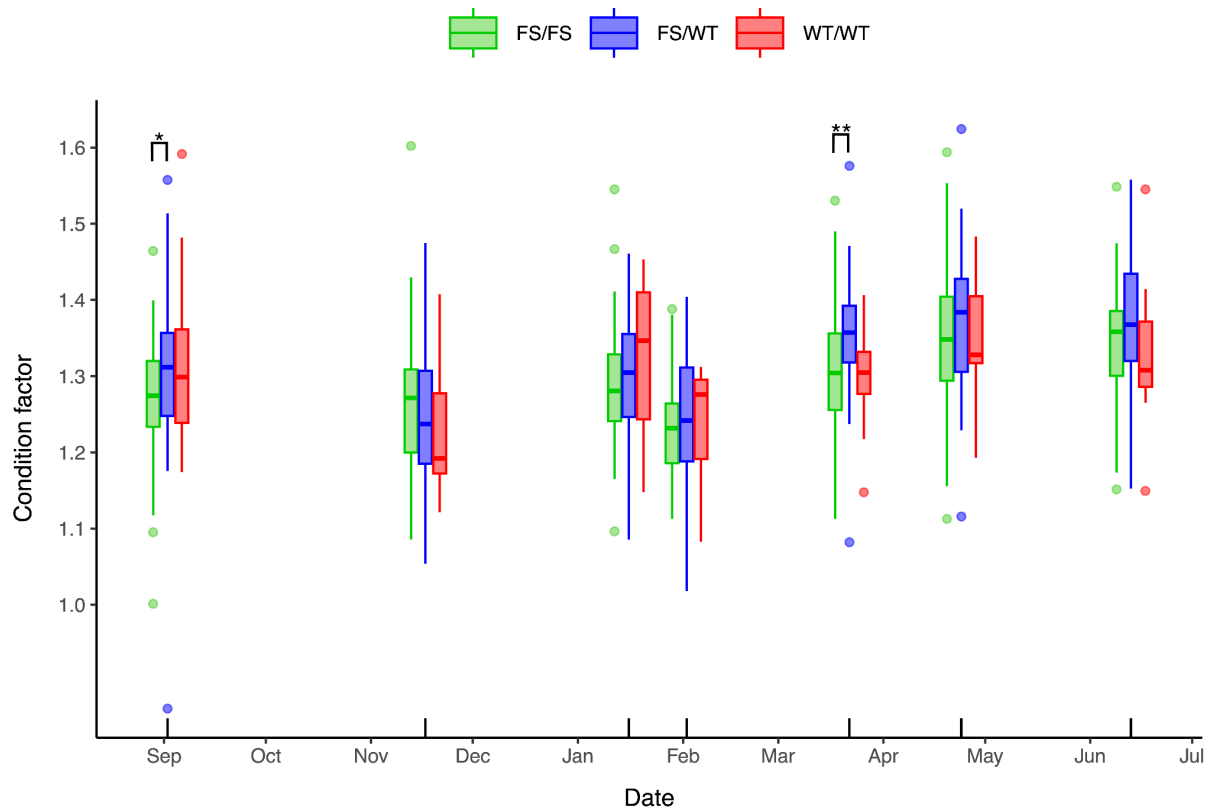

Figure 8: Boxplots showing condition factor distributions among each genotype per sampling point. The vertical dashes along the x-axis indicate sampling time (S1-7) and the asterisk illustrate significant differences between the groups (* = P < 0.05, ** = P < 0.01). For details of boxplot, see Figure 6 legend.

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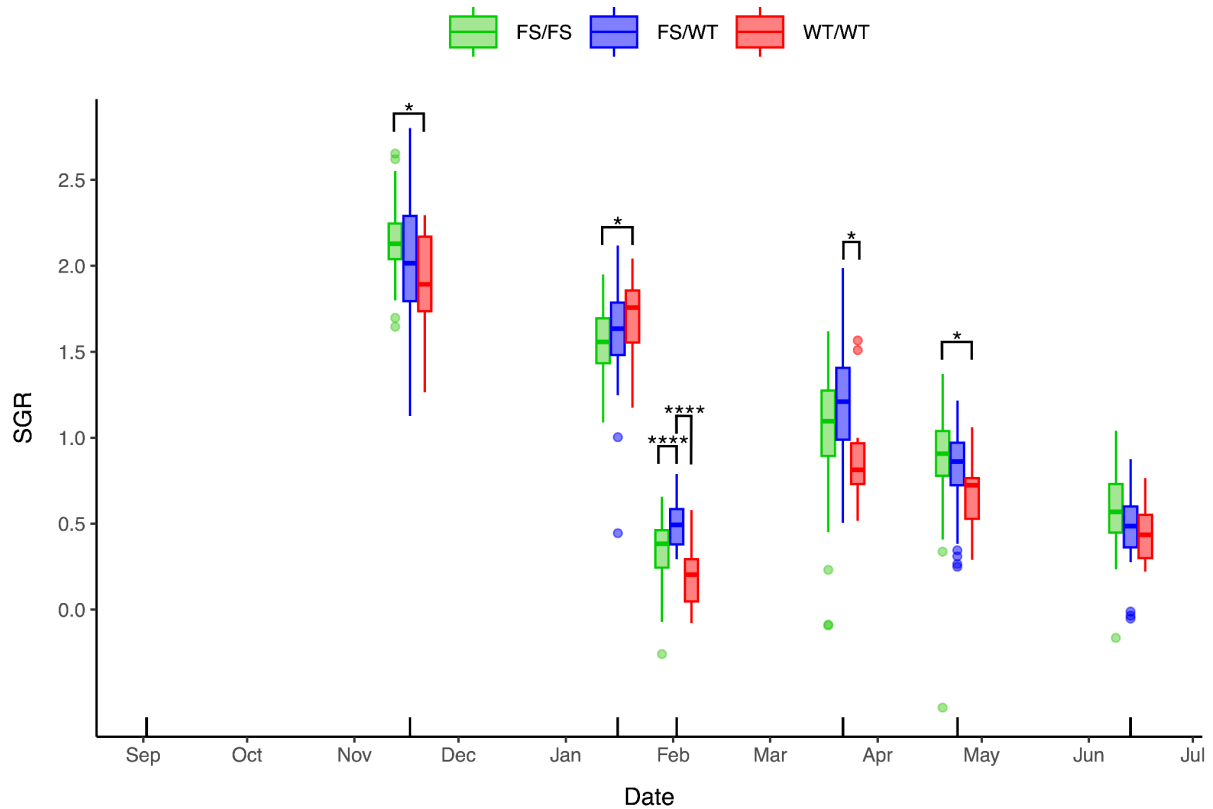
Figure 9: Boxplots showing SGR distributions among each genotype per sampling point. The vertical dashes along the x-axis indicate sampling time (S1-7) and the asterisk illustrate significant differences between the groups (* = P < 0.05, **** = P < 0.0001). For details of boxplot, see Figure 6 legend.

3.3 Maturation

Maturation status for each individual male was determined based on detection of gonads by ultrasound and supported by 11-KT levels in plasma. To confirm the accuracy of the method, 15 FS/FS and 15 FS/WT males were randomly selected for dissection of gonads. The combined accuracy of GSI, 11-KT and histology of the 30 individuals supported the assigned sex previously based on ultrasound, as seen in Figure 10.

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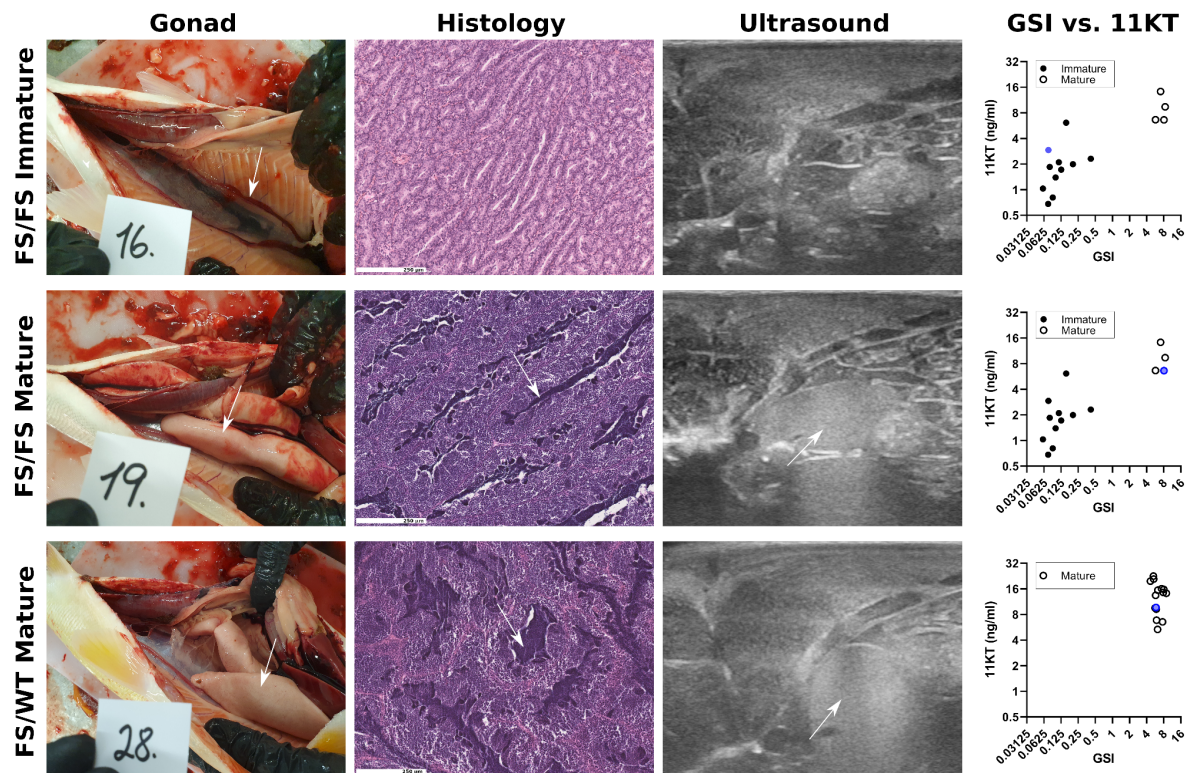*Figure 10: Characterization of male maturation. The panel shows an overview over the different methods used to characterize maturation in male salmon, with each row showing one individual representing either immature FS/FS, mature FS/FS or mature FS/WT. The white arrows in the left column indicate the gonads in each individual upon dissection. The white arrows in the second column indicate spermatozoa in mature males observed in gonad histology. The third column shows ultrasound readings, with white arrows indicating the presence of mature gonads. The fourth column shows GSI at sampling time plotted against 11-KT levels one month prior to sampling for all the individuals of the respective genotype. Based on the ultrasound, immature and mature males are indicated with filled and open circles, respectively, and the representative individual highlighted in blue.*

3.3.1 Ultrasound

Results from ultrasound on 24th of April (Figure 11) showed that of the FS/FS genotype there were 44 immature and 22 mature individuals, resulting in the lowest maturation among the three groups (33.3%). For the FS/WT genotype there were 3 immature and 38 mature individuals, which is a maturation of 92.6%, having the highest maturation among the three groups. The last genotype, WT/WT, had 3 immature and 8 mature individuals, resulting in 72.7% maturation. Results from ultrasound on 13th of June (Figure 11) showed that the FS/FS genotype had 31 immature and 20 mature individuals. During the sampling this group again

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showed the lowest maturation at 39.2%. In the FS/WT genotype there was only 1 immature individual while the remaining 24 were mature. This group was still maturing at the highest rate with 96% maturation. The WT/WT genotype showed no difference from previous sampling, still having 3 immature and 8 mature individuals, a 72.7% maturation rate (Figure 11).

On 24th of April the FS/WT genotype matured at a rate of 2.8 times higher than FS/FS genotype, being a significant difference ($P < 0.0001$) between the two of them. Seven weeks later, on 13th of June, a similar difference (2.4 times) in the proportion of mature males was observed between FS/WT genotype and FS/FS genotype, still being a significant difference ($P = 0.0015$). The difference in maturation between WT/WT genotype and FS/FS genotype was 2.2 times higher and 1.9 times higher respectively to the same dates, showing a similar difference between the two sampling dates. This is a significant difference ($P = 0.0458$), but only a significant difference on the first sampling. The number of individuals was not the same on both sampling because of individuals being dissected.
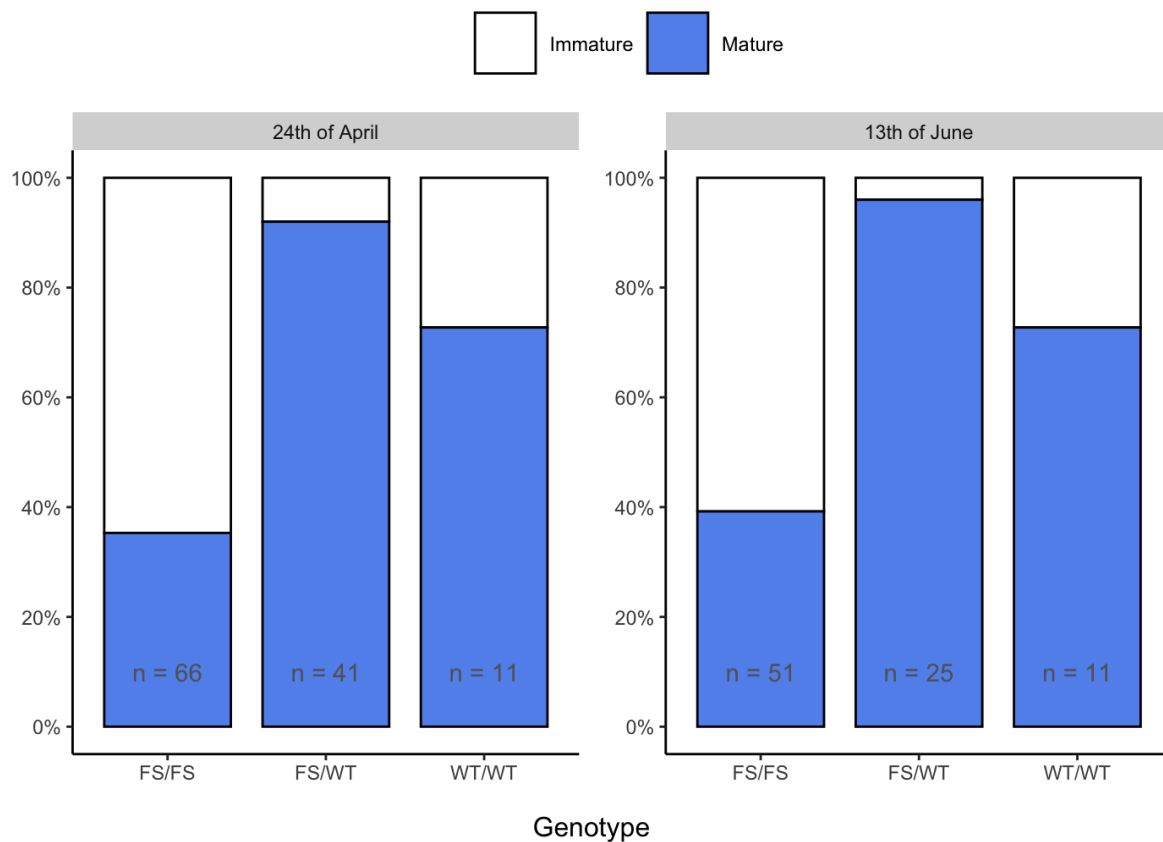

Figure 11: Percentage of males showing mature gonads detected by ultrasound for the genotypes FS/FS, FS/WT and WT/WT on 24th of April (left) and 13th of June (right). White and

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blue color indicate immature and mature individuals, respectively. Total number (n) of individuals analyzed by ultrasound per genotype is indicated within each bar.

3.3.2 Steroid hormone levels

The groups had widespread plasma steroid hormone levels on 22nd of March. Figure 12 shows that males from the FS/FS group had the overall lowest 11-KT values. The same figure shows WT/WT genotype had the highest overall 11-KT values, just passing FS/WT genotype at almost the same values. Statistics show that there is a significant difference in 11-KT between FS/FS and FS/WT genotype when looking at all individuals. When only comparing mature individuals in the same groups, there is no significant difference in 11-KT. There is no significant difference between WT/WT and the two other groups.

The mean value of 11-KT in the FS/FS genotype was 5.48 ng/ml, with 0.01 ng/ml and 32.22 ng/ml being the lowest and highest respectively for that group. All individuals with over 6.12 ng/ml were identified as mature by ultrasound one month later, except for two fish which were mature at 3.82 ng/ml and 5.62 ng/ml. While most of the individuals with 11-KT more than 3.5 ng/ml were identified as mature using ultrasound three months later, one individual which had 11-KT of 1.69 ng/ml three months prior had also matured. In the FS/WT genotype the group had a mean of 11.64 ng/ml, with 0.85 ng/ml being the lowest and 23.27 ng/ml being the highest. In this group all individuals having over 1.53 ng/ml 11-KT were identified as mature by ultrasound one month later, and three months later only one individual was not mature. The mean value for WT/WT genotype was 11.50 ng/ml, where the individual with the lowest 11-KT value was 0.01 ng/ml and the highest 11-KT value was 36.32 ng/ml. WT/WT genotype also had to surpass a 11-KT level of 6.19 ng/ml for all males to be identified as mature by ultrasound the following month and three months later, however there were only three individuals below this level, whereas two were immature and one was mature.
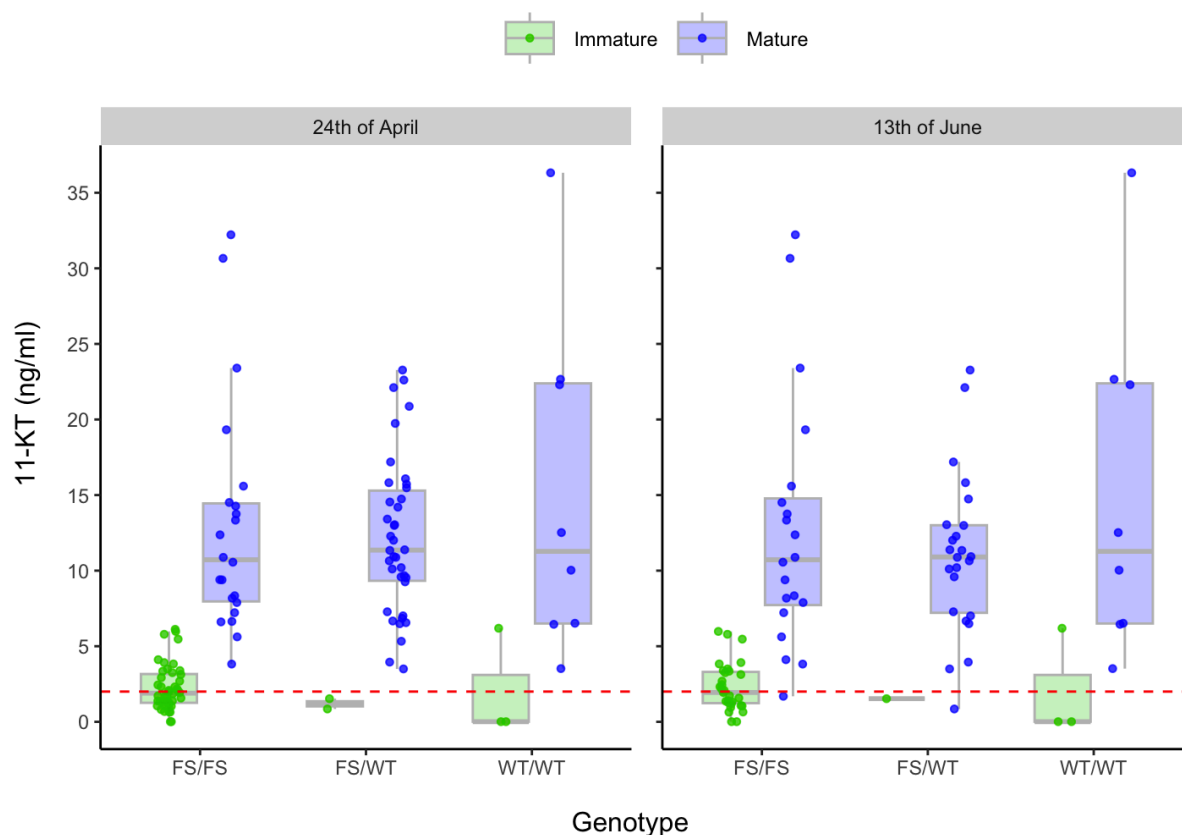
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Figure 12: These two boxplots illustrate the 11-KT values in the three genotypes taken on March 22nd. In each plot the FS/FS genotype is to the left, FS/WT genotype is in the middle and WT/WT genotype is on the right. Based on ultrasound results the blue dots indicate fish that would be mature one (left plot 24th of April) and three (right plot 13th of June) months later, while the solid green dots indicate fish that would stay immature one and three months later. The dashed red line indicated a previously reported 11-KT threshold of a maturing salmon at 2 ng/ml (Fraser et al., 2023b). Number of individuals per group is shown in Figure 11.

3.3.3 Histology

Of the 30 males sampled to confirm the correct scoring of maturation status, FS/FS contained 10 immature fish and 5 mature fish, while all 15 FS/WT fish were mature on 24th of April (Table 12). Individuals that had started to mature were considered as mature. All mature males were characterized by the presence of spermatids and/or spermatozoa, which was absent in immature fish. The histological difference between a mature gonad and an immature gonad can be observed in Figure 10. Of the 15 FS/FS fish, 10 of the individuals were immature while the remaining 5 individuals were mature, resulting in a maturation of 3.33% in this group. In the FS/WT genotype there were no immature individuals as all 15 fish were mature, resulting in a

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maturation of 100% in this group. The analysis of gonad histology confirmed the maturation status previously determined, all except for one, from the presence or absence of mature gonads identified by ultrasound.

Table 12: Histology 24th of April. 15 FS/FS and 15 FS/WT gonads analyzed by histology to confirm maturation status.

Genotypes	Immature	Mature	Maturation %
FS/FS	10	5	33.3
FS/WT	0	15	100.0

3.3.4 Gonadosomatic ndex

The GSI value for genotypes FS/FS and FS/WT varied greatly within each group and between each group, as shown in Figure 13. The results show that for the FS/FS genotype the mean value for GSI was 1.93, while the median was 0.12. Overall, the lowest GSI value was 0.06 and highest GSI value was 8.47. Results for the FS/WT genotype show that the mean value for GSI was 6.02 while the median was 5.91. Overall, the lowest GSI value was 4.67 and the highest GSI value was 8.89. Statistics show that there is a significant difference in GSI between FS/FS and FS/WT genotype (Table C8).
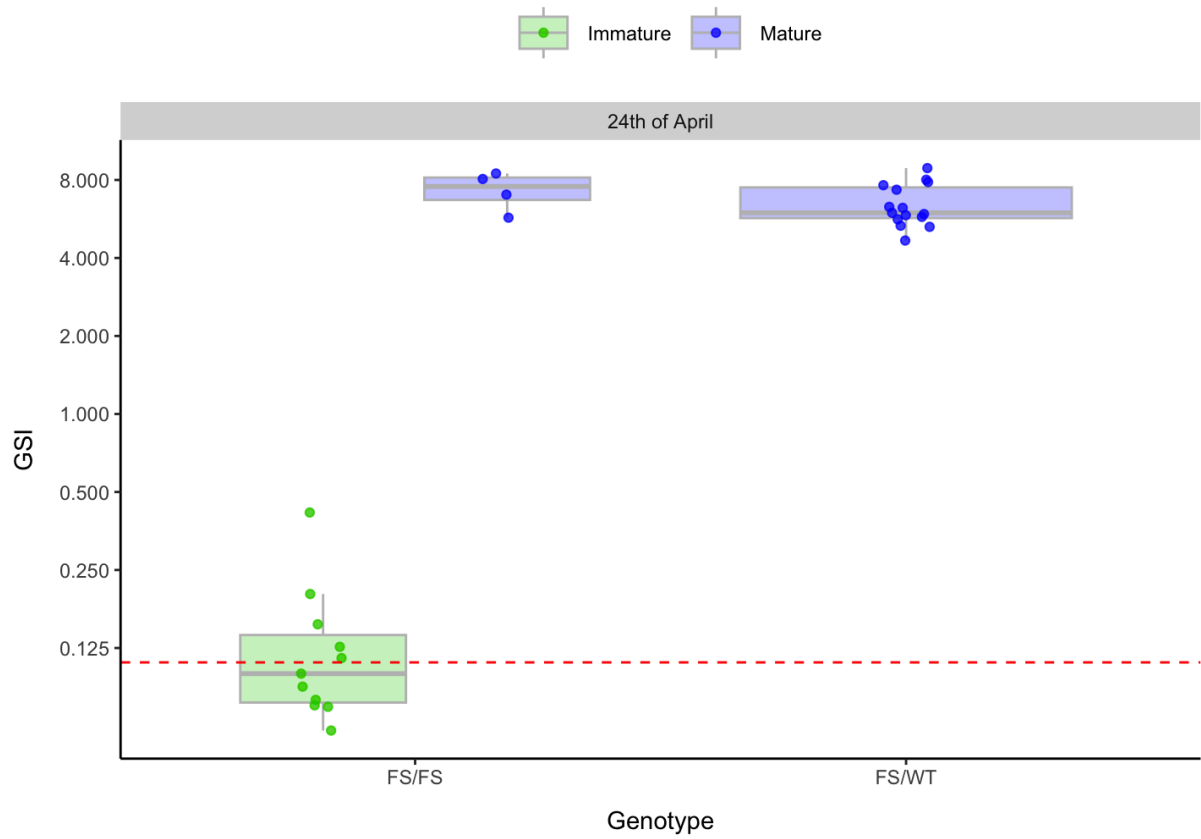
Figure 13: The graph shows a plot of the GSI in two genotypes, FS/FS (left) and FS/WT (right). Based on the ultrasound results the blue dots indicate mature fish while the whole green dots indicate immature fish within each genotype. The y-axis is log2 scaled. The dashed red line indicates a previously reported GSI threshold of a maturing salmon at 0.11 (Oldham et al., 2023). Both GSI and ultrasound were analyzed on 24th of April.

4. Discussion

The different *vgll3* mutations, especially *vgll3* knockout salmon, has helped to further our understanding of how this gene affects the different physiological parameters of the fish. Before discussing how *vgll3* affected development of large smolts, some clarifications must be made regarding the division of the three genetic groups that were studied in this experiment. Also, an unexpected event involving high deformation rates must be addressed as it had a big impact on one tenth of the fish. Although this event gave unwanted results, it still presented intriguing data that can be used in future research. The end of this segment will explore how gene edited salmon, such as *vgll3* knockouts, can be of benefit for the open market.

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4.1 Genotypes in the *vgll3* line

The experimental fish that were suitable for studying the *vgll3* gene´s impact on maturation consisted of 252 individuals, roughly one fourth of the fish initially included in the experiment. At the beginning of the experiment, the number of male fish in each genotype was 155 FS/FS, 76 FS/WT and 21 WT/WT. The skewed distribution of genotypes was a result of the F0 generation having a high prevalence of FS alleles (Figure 2), which they were able to transfer to the F1 generation. The number of knockouts and heterozygotes was sufficient in accordance with assessments of sample size in experiments (de Blas et al., 2020). Since the wildtype genotype was the control group it would have been beneficial for the group to be larger, however, this was a difficult task to resolve. Increasing the amount of wildtype fish would have meant introducing fish that were not related, which could introduce more bias into the experiment. The wildtype group gave sufficient data to compare with, much to the gratitude of ultrasound that allowed for saving the individuals from dissection throughout the experiment. Although it is generally not ideal to use a test group as a control group, the heterozygous fish showed much of the same phenotypes as the wildtype variant, and thus in the absence of a high quantity control group, were also used in comparing results against the knockouts.

Out of all the 1032 fish, 230 had unknown genotype or unknown sex. These individuals could have provided important information to the experiment, but due to lack of important information on these fish, these had to be removed from further analysis. Only 80 of these unknown fish were not assigned to a sex, so it was mainly the genotyping that had failed in this group of fish. There are several reasons that can apply in the case of genotyping errors such as low-quality DNA samples, poor sequencing quality, pipetting errors or contamination. Considering the amount of time and money that would have had to be spent on recovering these last specimens, it was decided that it was not worth running additional rounds of genotyping.

There was an uneven distribution of genotypes between the families, and individuals in general, which makes comparing results between them considerably harder. Two of the families stand out as the groups with the most individuals, with the E-22-1 family having almost half of the total number of individuals and the E-22-3 family almost one third. The two other families had a fraction of those numbers with both the E-22-2 and E-22-4 families having roughly one tenth of the total number of fish. The most optimal outcome would have been for the four groups to

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have an approximately even distribution of fish and genotypes of interest, which would allow studying if the results during this experiment were caused by *vgll3* knockout or family differences. For instance, in this case, the uneven distribution makes it wrong to compare knockout deformities in E-22-1 (79 out of 199) with E-22-2 (2 out of 3) if the goal is to explore whether deformities are the blame of *vgll3* knockout or family difference. It is less likely that the deformities are a family effect, especially lower jaw deformity, because then one would be able to see a pattern in all genotypes of a family. That is why, because of the low number of knockout fish in two of the families (Table 8) and the fact that E-22-1 and E-22-3 showed similar trends regarding deformations (Table 10), all fish were considered one family, and the results were interpreted accordingly. Alternatively, the two families with the lowest number of fish could be removed, giving a less biased view on effects of *vgll3* knockout in heterozygous fish.

4.1.1 Unexpected deformities

It is important to note that the euthanasian of deformed fish was mainly from an animal welfare perspective. The deformed fish would not serve a purpose in the study for several reasons, however most of them could probably live normal lives. A more precise description of the lower jaw deformation would be enlarged lower jaw, as most looked "normal" just with bigger jaws (Figure 5). It is unsure whether this would impact growth and welfare in later stages of life, but previous studies suggest that any deformation in the jaw has negative impacts on welfare (Amoroso et al., 2016; Noble et al., 2012). However, not all fish had this minor deformity and more crucial deformities such as spinal deformity would have held the fish back. These individuals have a hard time swimming efficiently, acquiring food is tougher and they generally come out on the losing side of life (Silverstone & Hammell, 2002). Based on the observations from Figure 5 the fish are exhibiting signs of distress or discomfort, and those reasons alone are sufficient to remove deformed fish from the experiment.

Looking at the deformation rates in each genotype group, one group stands out. The FS/FS genotype had considerably more deformations among their ranks than any other group, as observed in Table 9. There could be several possible explanations for this unwanted occurrence of deformities. One explanation could be that the *vgll3* gene is involved in development of the jaw or bone structure, and knocking out this gene completely would make it more likely that

the jaw would grow out of control. It has been observed in mice that *vgll3* plays a pivotal role in osteoblast differentiation by regulating the expression of osteogenic transcription factors and bone morphogenetic protein signaling (Yuan et al., 2022). Like salmon, mice are a vertebrate, and it could explain the high prevalence of jaw deformation in knockout fish while simultaneously explaining why the FS/WT genotype had half the number of deformities, especially the lower jaw deformation. Individuals with heterozygous genotype could lean on their WT allele for support (Jackson et al., 2018) and avoid deformation. Despite low numbers of fish with WT/WT genotype, there was no observed jaw deformation in this group, supporting previous statements that *vgll3* may play a pivotal role in development of bone structure.

Another possibility, which is probably more likely, is that during gene editing in the F0 generation, a region with a similar genetic code as the *vgll3* locus was affected. This event is called off-target genome editing and it refers to nonspecific and unintended genetic modifications that can arise using engineered nuclease technologies such as CRISPR/Cas9 (Höijer et al., 2022), which could be passed down to the next generation. With the premise of this theory it would be interesting to further study if there is off-target activity in the genome of *vgll3* mutants leading to jaw deformities, and observe if it can be used to reduce jaw deformities in salmon during production, as it is already established that jaw deformities lead to substantial economic losses and animal welfare implications (Amoroso et al., 2016; Noble et al., 2012). Both theories are purely speculation as there is no definite proof for any of the cases, however, when 80% of deformities in the knockouts are lower jaw deformities it is no longer a coincidence, it is a repeating event. It will be important for future studies to thoroughly examine if they acquire similar deformities when editing the *vgll3* gene in salmon, as that can give evidence to whether *vgll3* knockout is suitable to use for reducing maturation in salmon during large smolt production or not.

4.2 Growth and performance

During development there are some environmental factors that drive and dictate the speed of growth and timing of maturation in salmon, in particular temperature and light (Oldham et al., 2023; Pino Martinez et al., 2021; Pino Martinez et al., 2023b; Åsheim et al., 2023). With temperatures being either too warm or too cold the fish will lose appetite and growth stagnates (Stockwell et al., 2021). Light will help the fish to decide how active they should be as light

and different day lengths represent the seasonal variance. For this experiment the goal was to trigger maturation and to push the limits as quickly as possible, just as the industry does to the commercial salmon. Temperature and light regime were inspired by looking at studies that have shown that triggering maturation in salmon before and after smoltification can be quite easy with temperatures up to 16 °C combined with constant light (Fjelldal et al., 2011; Pino Martinez et al., 2021; Pino Martinez et al., 2023b). It is also reported that the high prevalence of mature smolt during production in RAS facilities is likely caused by temperature between 12 and 13 °C from first feeding and onwards (Fraser et al., 2023a; Pino Martinez et al., 2023b). Therefore, this experiment used temperatures around 13 °C and with continuous light. The average temperature throughout the whole experiment was 12.9 °C, staying true to the initial target.

Regarding the growth performance of the salmon, it was mostly as expected from an experiment simulating production in a RAS like setup with 13 °C and continuous light. All the genotypes had a relatively stable increasing growth from the start, with heterozygotes eventually having somewhat of a growth spurt, which is as expected from maturing individuals (Cousminer et al., 2013). It can be difficult to identify a growth spurt, as the fish are maturing randomly because of their inner clock that is unsynchronized by the environment they reside in (Pino Martinez et al., 2023a). It was observed that knockouts were significantly smaller than the other genotype in September 2022 which could suggest that *vgll3* plays a significant role in growth, at least during early development. Previously, Debes and colleagues (2021) found that the different *vgll3* genotypes can vary body condition, but not growth. Interestingly, although the wildtype genotype had high maturation it is not possible to observe a growth spurt, as wildtype are the group that ends up with the smallest individuals, which could simply be a result of too few individuals. A similar experimental setup was used by Crouse and colleagues (2022) to perform a study with similar environmental conditions in a RAS facility, observing growth in tanks holding 12 and 14 °C for an 8-month trial. At the end of the trial the fish were roughly 44 cm long and 1.3 kg (Crouse et al., 2022), which is approximately the same growth as in this experiment. The average length and weight after 9 months were 46 cm and over 1.4 kg. The fact that growth is approximately the same in this experiment, as a study using salmon with natural *vgll3* alleles, suggests that *vgll3* knockout does not affect overall growth, supporting claims already made by Debes and colleagues (2021). It should therefore not be any growth performing issues that will hold *vgll3* knockouts back during commercial production.

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Since the beginning of the experiment, fish with the FS/WT genotype had the overall biggest size of all the groups. Heterozygotes remained the largest during the whole experiment, as seen in Figure 6 and 7, while FS/FS was significantly smaller in September, and WT/WT an intermediate between the other two. During April there was a rapid increase in growth in the knockouts that allowed them to catch up with the wildtype fish, shifting balance and becoming the intermediate fish themselves. The trend could suggest that given enough time, knockouts would catch up with the heterozygotes or at least grow more efficiently. This idea is backed by looking at the condition factor (Figure 8) and SGR (Figure 9) after April, where knockouts catch up with or surpass their siblings of other genotypes. This was around the same time where many heterozygotes and wildtype individuals started to become mature. Although SGR is considered inappropriate for predicting growth in fish (Aunsmo et al., 2014), combined with the condition factor it can tell us something about how the salmon is growing. A fish's condition factor reflects the state of sexual maturity and degree of nourishment, and a high SGR means that the fish has grown at a faster rate over a specific period, in this case expressed as a percentage of body weight. This means that the knockout fish grew more during that period (in a percentage of weight) than the other fish. Assuming the fish continues to grow efficiently, this proposes the idea that knockouts can keep up their growth rate longer than the other groups, resulting in bigger and potentially immature fish.

There was also one incident in March when the condition factor was significantly lower in the FS/FS compared to the FS/WT genotype. Variation in condition of different *vgll3* genotypes have already been observed in previous studies (Debes et al., 2021; House et al., 2023), but these observations were seasonally dependent. It is explained that E allele individuals have a higher aerobic scope than L allele individuals, which allows them to accumulate more resources, and thus achieving an increased condition factor (House et al., 2023). It is thought that this mechanism is important during winter months, and the L allele individuals will lose condition faster than E allele individuals, resulting in lower and higher condition respectively to the two groups in the spring (House et al., 2023). It would also be beneficial to study and compare condition factors between only immature individuals, as maturation state dictates body condition and were not controlled for in this study. Our results suggest that knocking out *vgll3* has a transient impact on condition, but the mechanisms behind this are unclear.

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By the end of January 2023 there was an unexpected event where a malfunction in the water regulator system resulted in cold water entering the fish tanks. For a few hours the temperature in the water was below 5 °C with a sudden shift of probably just a few minutes. This is an important piece of information as other studies suggest that the lower limit for growth in Atlantic salmon is around 6 °C (Elliott & Hurley, 1997; Handeland et al., 2008). The temperature drop can be seen in Figure 3, the lowest daily average temperature for the entire experiment at just under 9 °C. As a precaution an extra sampling was performed on February 2nd to observe the impact of the temperature drop, despite having had a sampling about two and a half weeks earlier. Luckily, no fish were lost that day, showing that the gene-edited salmon still have a high resilience and adaptability to their surroundings. Interestingly, this temperature drop can possibly be observed in the SGR measured from 2nd February, showing all genotypes with an SGR between 0.25 and 0.5. The low SGR can also partly be explained by the fact there was a recent sampling, two weeks prior. Sampling is very stressful for the fish, and they will not return to regular feeding straight away. Longer time between sampling will reduce this effect. By the next sampling these levels had reverted to regular levels, on a declining trend. It is unsure how much and for how long this incident interfered with growth in the fish, but it is safe to say it had a notable impact, as studies show that lowering temperatures gradually can have a big impact on appetite, stress and mortality (Vadboncoeur et al., 2023). Further, because of the common garden design of the experiment, all fish in the experiment (including all genotypes) were equally affected by the temperature drop.

4.3 Understanding maturation in *vgll3* knockouts

Regarding the representation of genotypes in dissected fish in Figure 10, it is a shame not all the groups could be portrayed. For reasons already mentioned, the WT/WT genotype was left out of dissection to save the low number of individuals for the entire experiment. However, since all the dissected fish were randomly selected and nearly all fish were mature in the FS/WT genotype, no immature fish in this group were available for a more thorough examination. Being able to use histology to examine the gonads of immature fish of the two other genotypes could have given insightful information and be used for comparison if there was any difference to the knockout fish. It is most unfortunate that these groups are lacking from the analysis using histology and GSI, as they could have provided meaningful details to understanding maturation,

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especially regarding if there are tissue differences between the groups. Histology of mature testicles resembles analysis done by both Fjelldal and colleagues (2018) and Kjærner-Semb and colleagues (2018), so it is safe to say that mature knockouts and heterozygotes are unlikely to be sterile, which was a concern prior to the analysis. Having sterile fish would bring up a whole lot of other issues such as technical, ethical and commercial issues. Acquiring sterile fish was never the goal of this study, it was to delay maturation during large smolt production which, because of how the industry is developing, is where the increasing maturation problem is happening (Fraser et al., 2023a).

The results from the two rounds of ultrasound show a similar trend in that males of the FS/FS genotype have the lowest maturation percentage, and males with FS/WT genotype mature most frequently. Males with WT/WT genotype fall a place in between the two other genotypes, while still holding a high maturation percentage, but the percentage is a bit more uncertain because of the low number of fish in this group. Compared to the knockouts, both the wildtype and heterozygous fish were twice as likely to mature during the experiment, the latter almost three times. This exceptional difference between the knockouts and the two other groups supports the theory that *vgll3* controls age at maturation in Atlantic salmon (Ayllon et al., 2015), and can potentially help reduce the incidence of maturation during large smolt production.

The heterozygous fish on the other hand have one loss-of-function allele which in theory does not contribute to time of maturation and one allele for normal maturation. Wildtype fish will naturally have a wider array of varieties in their wildtype alleles. Here it would be beneficial to know whether the ratio of *vgll3* E and L alleles was similar between these two groups. The difference in maturation could be explained by higher frequency of E alleles in heterozygotes compared to the wildtypes. Therefore, in future studies it would be beneficial to determine the *vgll3* genotype of the heterozygotes and wildtype fish, to determine which functional allele they have, as this would benefit comparisons between maturation rates.

Prior to the sampling on 24th of April there was uncertainty as to whether the knockouts would mature at all. Many of the fish had started to show morphological changes that indicated maturation, but the fish could not be distinguished from each other by looks alone. After seven to eight months in a flow-through facility with water holding 13 °C and continuous light the

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results were as expected for the wildtype. With just over seventy percent maturation in the group the fish behaves just as predicted, supporting previous research (Crouse et al., 2022; Good & Davidson, 2016; Åsheim et al., 2023), that most of the male salmon will mature during a regime like this experiment has carried out. With the premise that heterozygous fish would act in accordance with that of the wildtypes, they too showed maturation numbers that were expected. Surprisingly, heterozygous fish had more than ninety percent maturation, almost thirty percent more maturation than wildtypes. Future studies will have to pay attention to what the effects of knocking out one single *vgll3* gene copy will have on heterozygous performance. The main experimental group from this experiment, *vgll3* knockouts, behaved as desired as they were not sterile, but showing lower maturation numbers, just over thirty percent. It was still a baffling dissimilarity to observe, and it was encouraging to see that the differences between the group continued to stay the same on 13th of June during the last sampling, although the *vgll3* knockouts had now reached nearly forty percent maturation, supporting previous finding that *vgll3* strongly impacts the time of maturation. In this study maturation probably started to occur around January or February, five to six months into the experiment. Data gathered from this experiment have provided interesting evidence, all of which support that further studies should be made to unravel the link between *vgll3* and maturation in salmon.

After two rounds of ultrasound the results are suggesting that a functional *vgll3 gene* is not required to initiate maturation for Atlantic salmon. This is proven by the near forty percent of the knockouts that have matured, despite complete lack of the gene entirely. This fact, however, does not change the stance that *vgll3* has a considerable influence over time of maturation in salmon (Ayllon et al., 2019), and this will have to be validated by future studies. With the stance that *vgll3* is not necessary for maturation, it is interesting to compare whether knockouts are more like salmon with natural EL or LL alleles. From the results, the knockouts behave differently from natural EE males. Having knocked out the *vgll3* gene entirely one could expect that the salmon will mature as an individual with natural EL genotype if both alleles push in opposite directions in regard to early or late maturation. However, studies show that individuals with LL alleles mature anywhere between fifteen and thirty percent, whilst EL individuals mature between sixty and one hundred percent (Fraser et al., 2023a; Åsheim et al., 2023). Comparing maturation with two experiments that has other environment factors (9 °C and 16 °C respectively) becomes purely speculation. However, the results in this experiment would

then place *vgll3* knockouts with its thirty to forty percent maturation somewhere between EL and LL alleles, but more closely resembling individuals having LL alleles. When observing maturation, it is important to remember that the amount of maturation within any given experiment is dynamic and highly influenced by the environment, genetics, and their interaction.

In this experiment, the maturation of males was primarily characterized using ultrasound as this technique has proven to be efficient in differentiating between mature and immature individuals in previous studies (Næve et al., 2019). It is important to note that ultrasound is not suggested to be the best method to discover maturation in salmon since it is less sensitive, having bigger issues spotting the first signs of maturation, however it allows for the least invasive repetitive examination to keep the fish alive. To further strengthen the claims done by ultrasound, maturation indicators from 15 FS/FS and 15 FS/WT individuals, such as 11-KT, GSI and dissection, were used to support the characterization. The results obtained from the 30 individuals show much overlap with that of the results from ultrasound, however, there were some discrepancies with elevated 11-KT and GSI values in some immature individuals compared to what was expected from the literature (Fraser et al., 2023b; Oldham et al., 2023). Based on previously reported 11-KT thresholds for maturing fish (2 ng/ml; Fraser et al., 2023b), the 11-KT values observed in the present experiment would suggest that 21 of the knockouts and one wildtype characterized by ultrasound as immature, should be mature, and they can be observed in Figure 12 as the green points above the red dashed line. The same can be said for GSI (Figure 13), where 5 of the knockouts passed the suggested GSI threshold for a maturing salmon (0.11; Oldham et al., 2023). If a lower threshold was used, for instance 0.06 as suggested by Pino Martinez and colleagues (2023b), all knockouts should be considered mature. For the knockouts it is quite possible that *vgll3* knockout affects maturation in salmon in a way of not preventing it, but instead pushes the limits or thresholds it needs for starting to mature. Observations from Fraser and colleagues (2023a) revealed a trend in which, individuals with natural *vgll3* alleles, the fish with L alleles had to become larger to achieve maturation. Parallels can potentially be pulled between these results, as knockouts were initially smaller than the other groups and would need to become bigger to mature. Regardless, evidence of the pushed threshold lies in Figure C2 in *Appendix C,* where in fact immature individuals have delayed maturation despite having high values of 11-KT and GSI. The fish are showing signs of early

maturation or puberty but lack spermatids and/or spermatozoa to be categorized as mature in this experiment. However, this is fascinating results, and it brings with it new questions, such as how far these thresholds are being pushed?

Although results from this experiment does not bring certain proof, a new limit for 11-KT and GSI in *vgll3* knockouts could be plausible (Figure C2 and C3). New threshold for 11-KT could be around 4.5 ng/ml, as that is the average of two immature males with higher 11-KT values than what is expected. While threshold for GSI could be around 0.2, as the highest GSI recorded for an immature individual was slightly over this. For this experiment there was only one blood sampling to keep handling of fish to a minimum. Taking blood with every sampling could cause unnecessary stress and harm to the fish, however the results have proven the need for a second blood sampling. It is therefore recommended for future studies to sample additional blood one or two months prior, in this case January or February, as more clustered data could help understand where the tipping point is for maturation in *vgll3* knockouts. To further support the claims, it would have been nice to have earlier ultrasounds as well. Unfortunately, during this experiment there was lack of sufficient experience in the personnel to accurately operate an ultrasound machine, which prevented use and acquisition of gonad imaging before 24th of April. That is why it is also recommended for future studies to acquire earlier ultrasound images. Earlier ultrasound will not harm the fish or inflict unnecessary stress in comparison to other methods, and quite frankly the ability to detect mature individuals with such precision will only benefit future studies.

Data from this experiment would suggest that *vgll3*, with its two natural alleles, offers a mechanism to the fish that acts like a catalyst in the presence of the gene. This statement is a further explanation of Barson and colleagues´ (2015) suggestion that *vgll3* has a key mechanism that regulates the coordination between fat reserves and maturation in salmon. This catalyst effect could be of interest to study, and to do so one would need to know heterozygous *vgll3* genotype, as mentioned earlier, and then compare maturation results between two groups. The first group would be one loss-of-function and one E allele (FS/E), while the second group would be one loss-of-function and one L allele (FS/L). This could prove if *vgll3* functions as a catalyst independently of the alleles or if the L allele has an inhibitory effect. Otherwise, if the L allele does not show an inhibitory effect, and *vgll3* knockouts show similar results as LL genotype

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individuals, then it would probably be easier for a fish farmer to breed for an entire stock of LL allele fish. That is why it is recommended for more *vgll3* studies, to get a clear understanding of the catalyst effects that the *vgll3* gene has on maturation in salmon, especially the catalyst effects of *vgll3* knockout in a RAS simulated environment.

A final aspect that can be useful for future studies is to divide the salmon into three separate maturation categories including immature, pubertal and mature. Being the intermediate stage between an immature and mature fish, the pubertal fish share many of the problems as a mature fish during production (Taranger et al., 2010), and dividing into one additional category could help understand and give a more accurate prediction of time of maturation in salmon. This experiment only differentiates between immature and mature salmon, which makes it hard to understand whether *vgll3* knockout prolongs the immature stage or the pubertal stage. The reason for the choice to only have two groups is that pubertal fish can be difficult to spot with ultrasound, since the gonads are not fully developed, and could only be categorized with GSI or 11-KT alone. However, since the ultrasound was so accurate in accordance with histology samples, which categorized maturation by the presence of spermatids and/or spermatozoa, the use of two categories in this experiment were justified.

4.4 Commercialization of gene edited salmon

The situation regarding production of salmon in Norway has been changing drastically the last few years. In the early 2000s smolts were transferred to sea with a size of 100 g, while now it is more common to keep the smolt on land until the fish has reached a size of 250 to 500 g (Afewerki et al., 2023). This has been a direct result of difficulties in obtaining new production licenses and the increase of costs in treating salmon lice. By keeping the fish on land for a longer period the company can improve the utilization of the already existing license and limit the impact of salmon lice (Afewerki et al., 2023). There is a high probability that this trend will continue, as regulations regarding licenses are getting stricter and the welfare of fish are put on the agenda more now than ever. However, keeping the fish on land for a longer period will intensify the current problem with maturation of smolt in RAS facilities (Fraser et al., 2023a), which will make it hard to produce immature smolt of bigger size. The data gathered during this experiment has illustrated that with the use of *vgll3* knockouts it is possible for a large smolt to reach a size of more than 1.4 kg without maturing. This could very much revolutionize the

industry by reducing time in sea to under a year, and effectively reduce treatment of salmon lice to a minimum, if any at all. It would not be a production problem using *vgll3* knockouts on a commercial scale as they have proven not to be sterile, however current regulations are limiting the feasibility of using such fish in production, as acquiring permission to use gene edited organisms to be used in food production is difficult (NOU 2023: 18). Luckily the future looks promising, with an extensive amount of work done by the Genetic Technology Committee appointed in November of 2020, the Norwegian government were in July of 2023 advised to soften the regulations in favor of gene technology, seeing the potential part that gene technology will play regarding sustainable food production. The potential use of CRISPR/Cas9 salmon, with modifications such as *vgll3* knockouts, can open new doors that can help improve production and animal welfare, as for instance increasing resistance to common illnesses.

5. Conclusion

In conclusion, this thesis has investigated and described the physiological effects of knocking out the *vgll3* gene in male Atlantic salmon. The experiment carried out on Matre Research Station has proved that it is possible to obtain immature male large smolts by using CRISPR/Cas9 technology to knock out the *vgll3* gene. During the period from September 2022 to June 2023 the fish grew to 1.4 kg, where nearly forty percent of the male *vgll3* knockouts were mature, less than half of what would be expected from normal male salmon in similar environments (13 °C and continuous light) during commercial production in RAS facilities. Producing *vgll3* knockouts on a commercial scale can help reduce early onset of sexual maturity in male large smolts, but prior to that regulations regarding the use of gene editing technology in food production in Norway must change.s

To answer the hypothesis presented, the results would suggest that i) *Vgll3* is essential for initiating maturation, is false. In this study there was nearly forty percent maturation in knockouts, proving that *vgll3* is not essential for initiating maturation in Atlantic salmon. ii) Knocking out *vgll3* does not have an impact on growth performance of the fish, is true. Observations from this experiment would suggest that *vgll3* knockouts overall do not have a difference in growth performance, supporting already existing literature, that *vgll3* does not affect growth in Atlantic salmon.

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The work presented in this thesis offers a unique opportunity for future studies, as this is the first experiment of its kind to knock out the *vgll3* gene to study male maturation in Atlantic salmon. This thesis explains the first documented results of maturation in *vgll3* knockout salmon, and much of the results will have to be validated. It is recommended that further research is carried out in studying the effects that the *vgll3* gene has on maturation in salmon, especially the effects of *vgll3* knockout. This thesis provides a clear starting point for designing future experiments.

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Appendix

Appendix A: Experimental fish and husbandry

Microsoft Excel sheet and R script

If there is a desire to have a look at the Microsoft Excel Master Document or RStudio Script used during the writing of this thesis, please contact "sebastianbraathen@hotmail.com".

Distribution of families and deformities

Table A1: Distribution of genotypes in all families. Bold text highlights the number of fish with genotypes of interest. Numbers include both males and females.

Family	E-22-1	E-22-2	E-22-3	E-22-4
FS/FS	**199**	**3**	**122**	**5**
FS/WT	**46**	**44**	**24**	**30**
WT/WT	**17**	**5**	**10**	**7**
FS/IF	86	15	61	28
IF/IF	64	19	44	8
IF/WT	10	4	8	4
Unknown	85	20	51	12
Total	507	110	320	94

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Table A2: Deformed fish in each family. Bold text highlights the number of fish with genotypes of interest. Number includes both sexes.

Family	E-22-1	E-22-2	E-22-3	E-22-4
FS/FS	**79**	**2**	**52**	**1**
FS/WT	**4**	**18**	**2**	**2**
WT/WT	**1**	**0**	**0**	**1**
FS/IF	6	6	2	6
IF/IF	5	2	3	1
IF/WT	1	1	1	0
Unknown	17	8	7	4
Total	113	37	67	15

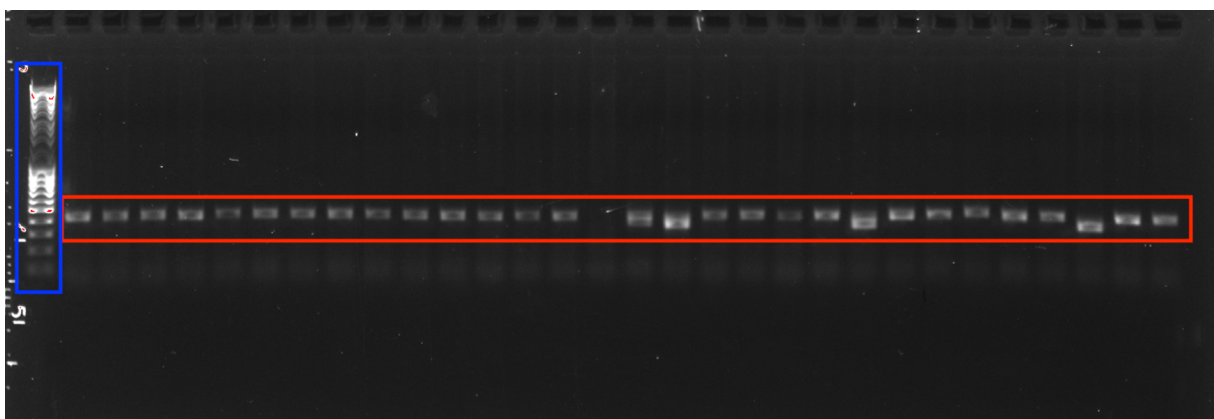## Appendix B: Genetic work

Miseq protocol

Figure B1: Figure illustrates subset of samples after PCR1. Red box indicates bands of expected size from a subset of samples. Blue box indicates a ladder showing bands of known sizes.
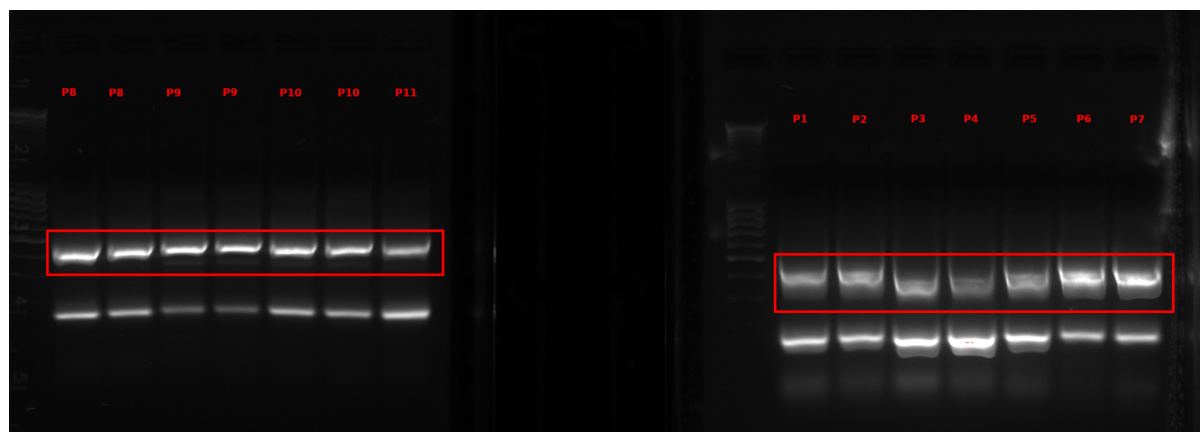
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Figure B2: Figure illustrates pools from each plate after PCR2. Plate number is indicated with letters above and the red boxes indicate excised gel bands.

### *sdY* protocol

Table B1: Correct primer and probe sequences.

Target	Primer/probe	Sequence 5'-3'
gapdh	Ss_gapdh_F	CCGCCACCCAGAAGACTGT
	Ss_gapdh_R	CTGGCGCCACGTCCAT
	Ss_gapdh_Pro	6FAM-TCCTTCTGGAAAGCTGTGGA-MGBNFQ
sdy exon 2	Ss_sdY_Exon2_F	CCTACAAGCCCTTCTCCCTGAT
	Ss_sdY_Exon2_R	GGGCTTTGGGAGAGAGATGAC
	Ss_sdY_Exon2_Pro	VIC-ATGGATGGGATCCC-MGBNFQ
sdy exon 4	Ss_sdY_Exon4_F	CCATGGGCTCAGCAGCTATT
	Ss_sdY_Exon4_R	GGAGGACTCAAGCCAGATCCT
	Ss_sdY_Exon4_Pro	NED-AAGCAAGCTCACGACTT-MGBNFQ

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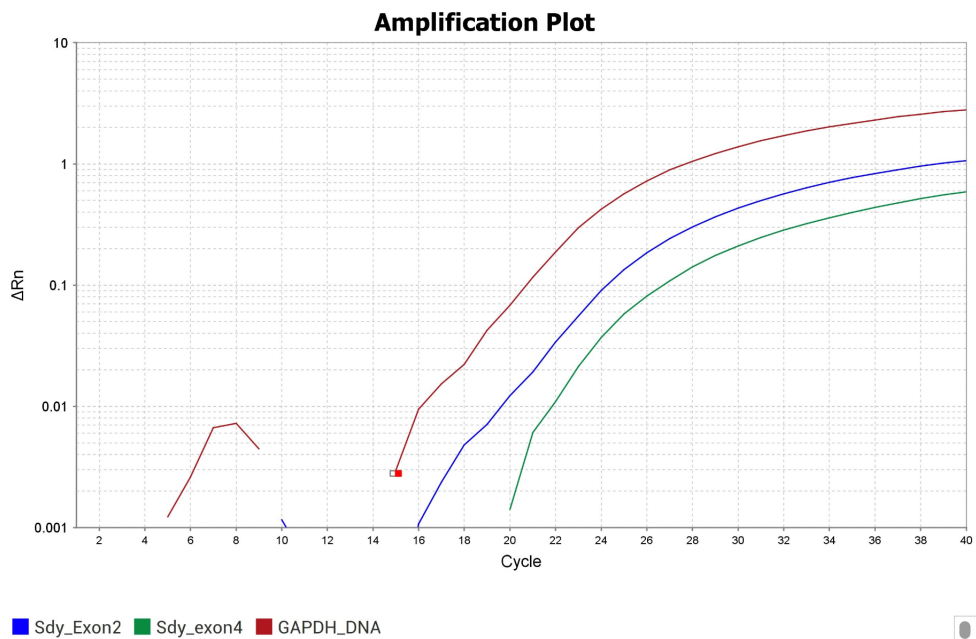
Figure B3: Amplification curve for male salmon. This plot shows a clear amplification of both sdY exons 2 (blue) and 4 (green). The red line confirms the amplification of the control gene gapdh.
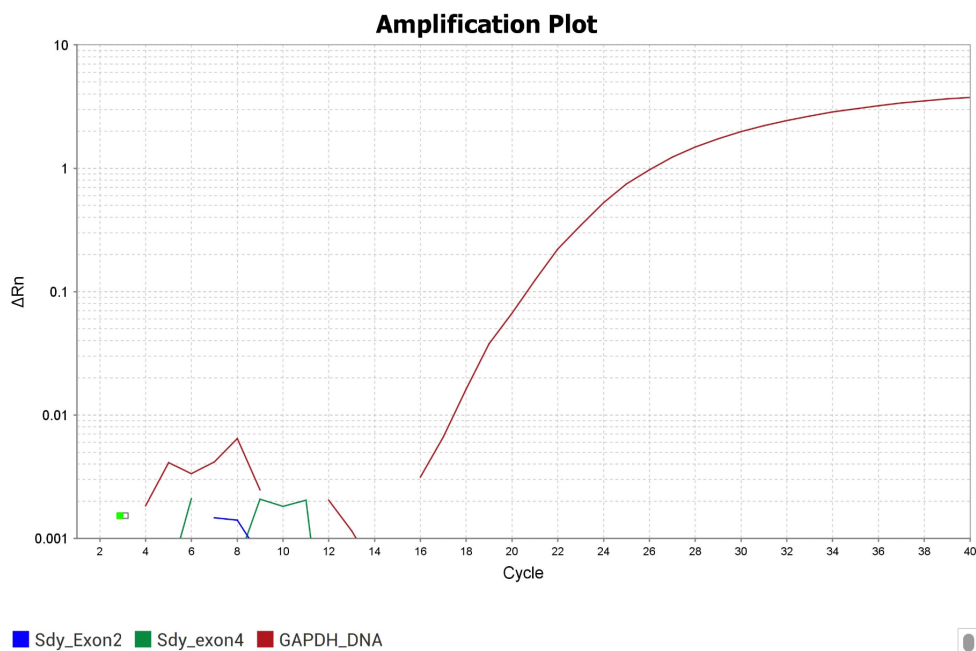
Figure B4: Amplification curve for a female salmon. This plot lacks both sdY exon 2 (blue) and sdY exon 4 (green). The red line confirms the amplification of the control gene gapdh.

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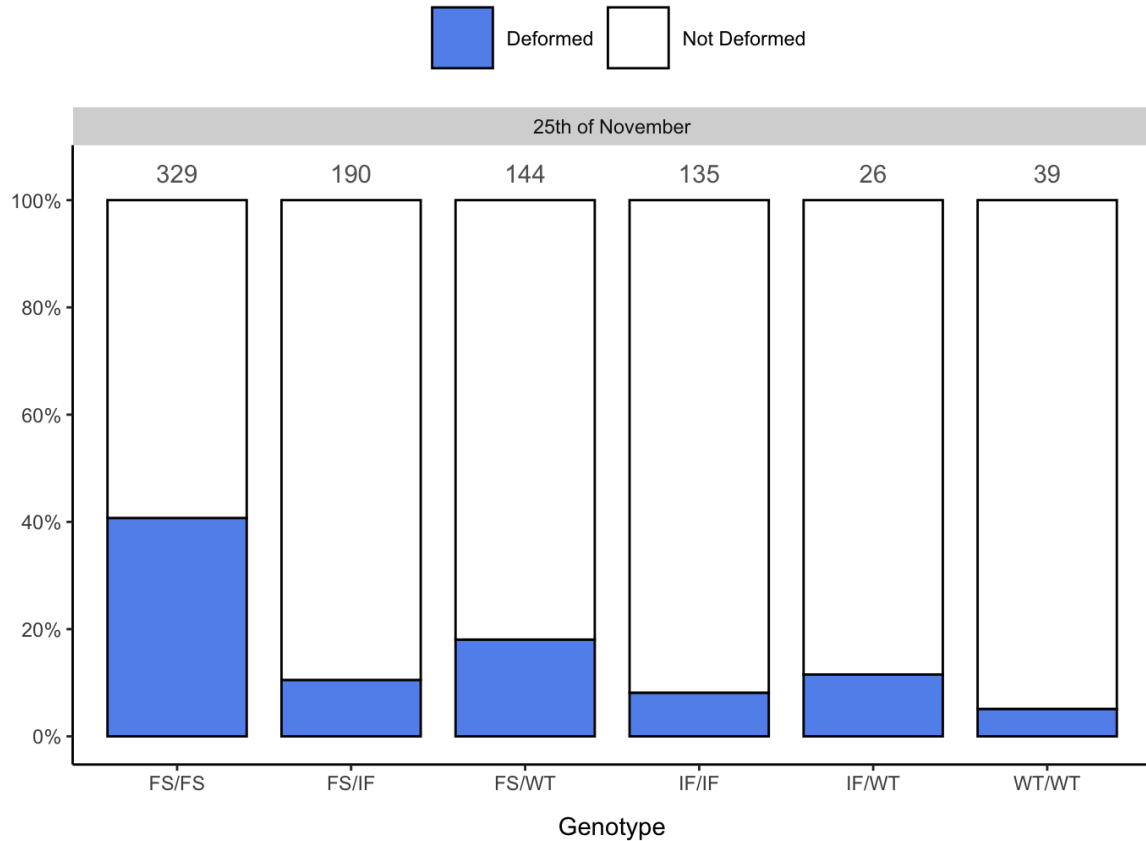## Appendix C: Fish sampling

Mutations

Figure C1: Figure showing proportions of deformed fish among each genotype. Blue bars indicate deformed fish, white bars indicate fish without any visible deformities.

Growth and performance

C1: Number of fish in each genotype per sampling.

Date	2nd Sep	17th Nov	16th Jan	2nd Feb	22nd Mar	24th Apr	13th June
FS/FS	89	79	77	67	67	66	51
FS/WT	60	57	55	42	41	41	25
WT/WT	19	17	16	11	11	11	11

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Table C2: Average length per sampling.

Date	2nd Sep	17th Nov	16th Jan	2nd Feb	22nd Mar	24th Apr	13th June
FS/FS	13.18 (±2.21)	23.02 (±3.09)	31.70 (±2.89)	32.92 (±3.01)	38.44 (±3.93)	42.33 (±4.33)	46.00 (±4.06)
FS/WT	14.30 (±1.75)	24.00 (±2.76)	32.81 (±3.31)	33.76 (±3.35)	40.10 (±4.46)	43.83 (±5.22)	47.25 (±4.45)
WT/WT	14.25 (±1.42)	23.97 (±2.10)	32.49 (±3.10)	33.50 (±3.48)	39.74 (±3.19)	42.36 (±3.70)	46.03 (±3.47)

Table C3: Average weight per sampling.

Date	2nd Sep	17th Nov	16th Jan	2nd Feb	22nd Mar	24th Apr	13th June
FS/FS	31.37 (±12.44)	160.66 (±49.43)	422.38 (±119.53)	452.10 (±125.72)	772.80 (±250.81)	1060.74 (±337.61)	1427.48 (±376.80)
FS/WT	40.58 (±12.46)	180.61 (±48.69)	475.14 (±148.52)	497.23 (±148.35)	911.90 (±310.06)	1216.67 (±445.12)	1587,31 (±439.61)
WT/WT	39.74 (±11.13)	172,92 (±48.76)	468.50 (±129.73)	488.64 (±138.09)	829.82 (±220.62)	1048.64 (±330.49)	1448.15 (±402.75)

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Table C4: Length significance and P-values. Significance is marked with an asterisk.

Length	FS/FS vs. FS/WT	FS/FS vs. WT/WT	FS/WT vs. WT/WT
September	0.011 **	0.03658 *	> 0.9999
November	0.1274	> 0.9999	> 0.9999
January	0.0788	0.4572	> 0.9999
February	0.5754	0.7139	> 0.9999
March	0.3514	> 0.9999	> 0.9999
April	0.6587	> 0.9999	0.7727
June	> 0.9999	0.7798	0.3061

Table C5: Weight significance and P-values. Significance is marked with an asterisk.

Weight	FS/FS vs. FS/WT	FS/FS vs. WT/WT	FS/WT vs. WT/WT
September	0.0005 ***	0.0184 *	> 0.9999
November	0.2299	> 0.9999	> 0.9999
January	0.0894	0.3314	> 0.9999
February	0.4686	0.7251	> 0.9999
March	0.1658	> 0.9999	> 0.9999
April	0.5172	> 0.9999	0.5590
June	> 0.9999	0.6427	0.2222

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Table C6: Condition factor significance and P-values. Significance is marked with an asterisk.

Condition factor	FS/FS vs. FS/WT	FS/FS vs. WT/WT	FS/WT vs. WT/WT
September	0.0404 *	0.6060	> 0.9999
November	0.4308	0.1763	> 0.9999
January	> 0.9999	0.2163	0.6777
February	> 0.9999	> 0.9999	> 0.9999
March	0.0045 **	> 0.9999	0.0638
April	0.2713	> 0.9999	0.6871
June	0.8711	0.4930	0.1353

Table C7: SGR significance and P-values. Significance is marked with an asterisk.

SGR	FS/FS vs. FS/WT	FS/FS vs. WT/WT	FS/WT vs. WT/WT
September	-	-	-
November	0.0815	0.0118 *	0.4839
January	0.2318	0.0268	0.4571
February	< 0.0001 ****	0.0679	< 0.0001 ****
March	0.1861	0.4550	0.0413
April	0.2584	0.0158	0.2838
June	0.1465	0.1446	> 0.9999

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Table C8: 11-KT and GSI significance and P-values. Significance is marked with an asterisk.

	FS/FS vs. FS/WT	FS/FS vs. WT/WT	FS/WT vs. WT/WT
11-KT	< 0.0001 ****	0.1958	0.6320
11- KT mature	> 0.9999	> 0.9999	> 0.9999
GSI	0.0027 **	-	-

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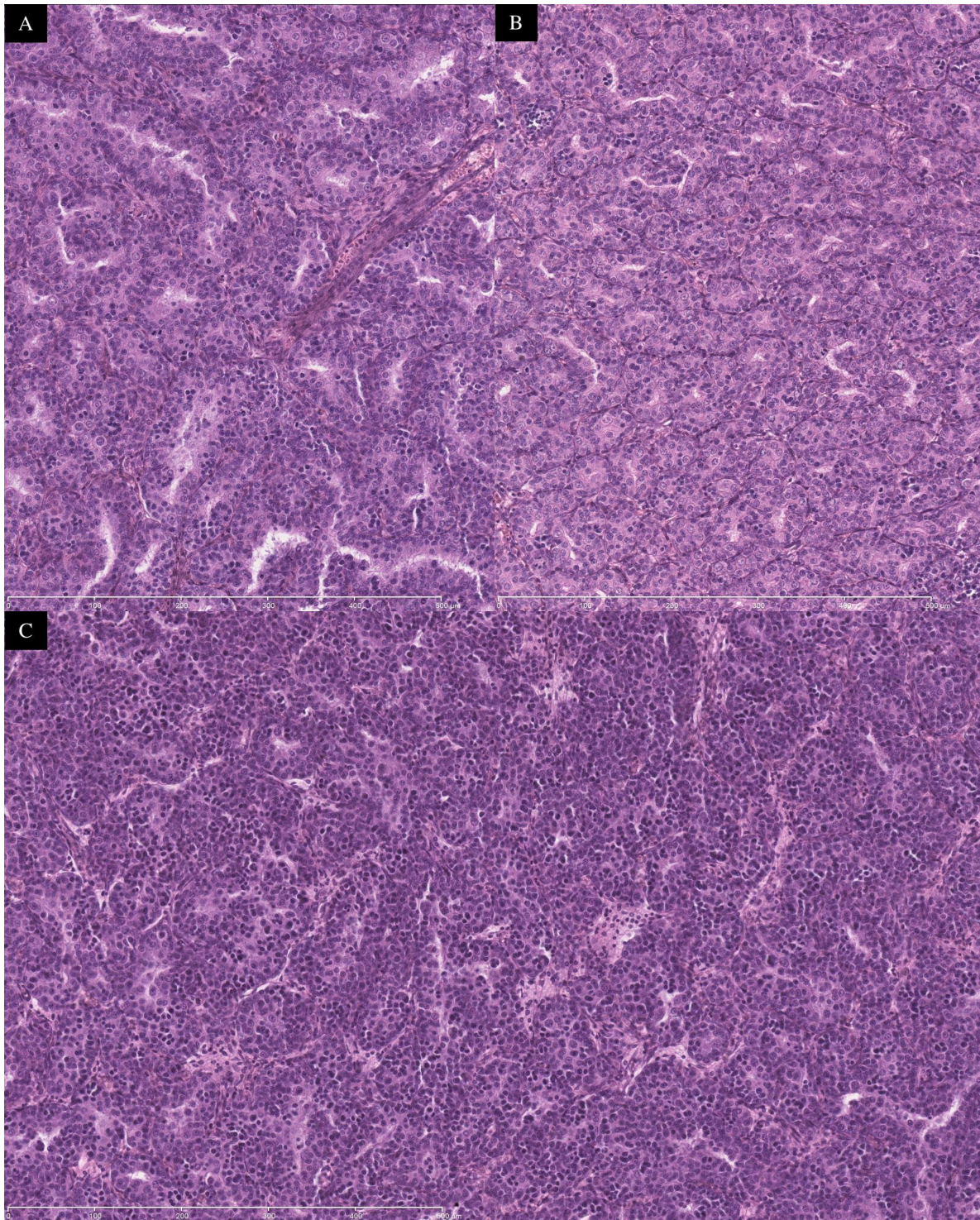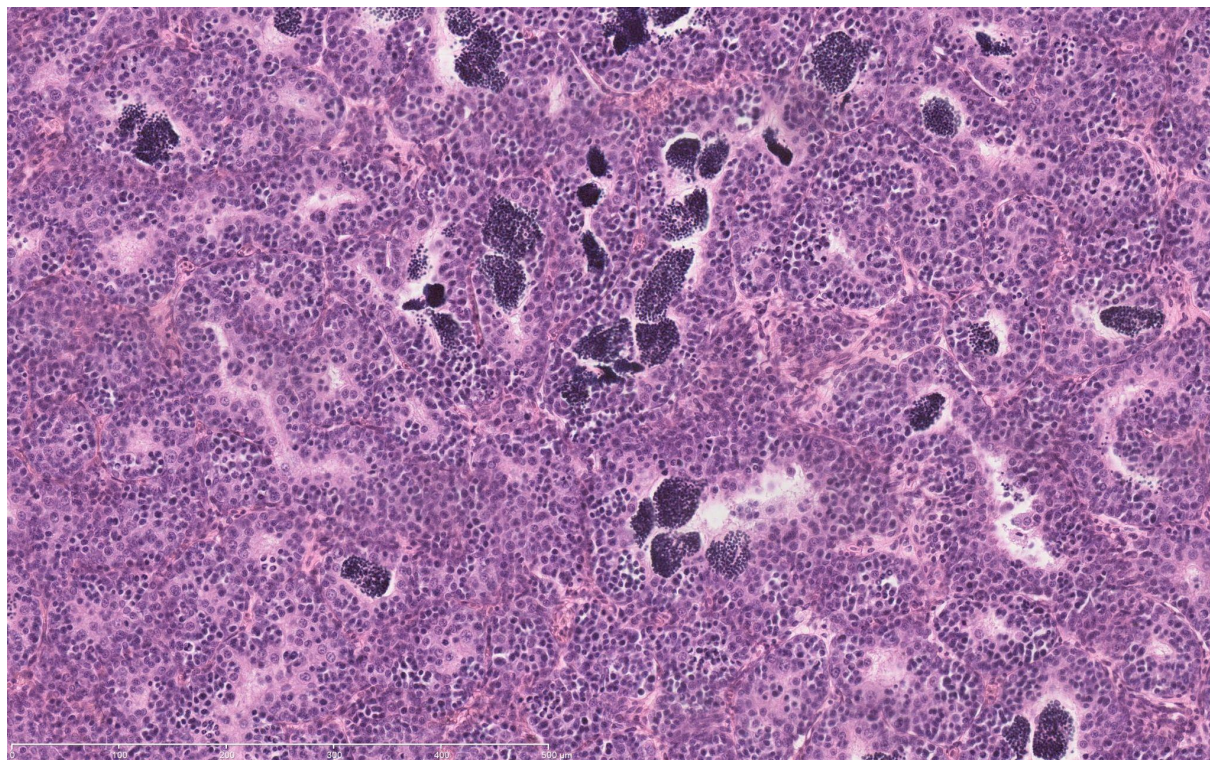
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Histology

Figure C2: A, B and C show three different immature vgll3 knockouts. A) has 11-KT of 1.72 ng/ml and GSI of 0.126. B) has 11-KT of 6.12 and GSI of 0.154. C) has 11-KT of 1.99 and GSI of 0.202.

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Figure C3: Presence of spermatids to categorize the fish as mature. This vgll3 knockout had a 11-KT of 2.31 and GSI of 0.417. The developmental stage of this fish behaves differently than what one would expect from other fish with the same GSI or 11-KT values, effectively showing the delay effect from knocking out vgll3. This individual was not categorized as mature with ultrasound.