Effects of vgll3 on maturation and its interaction with salinity in Atlantic salmon (*Salmo salar, L*)

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Abstract

New emerging strategies in the production of Atlantic salmon (*Salmo salar*), for instance post-smolt and big smolt in land-based facilities experience maturation. High production rates, such as 24 hours photoperiods and high temperatures combined with full rations of feed, promote post-smolt maturation ("jacking"). The gene vgll3 regulate the weight at which maturation happens. Fish with the allele E (early) enter puberty earlier than fish with the allele L (late). As there are two alleles in each gene, we get the three genotypes EE, EL and LL. Eggs from three heterozygote mothers have been fertilised by a heterozygote male, producing three families all with theoretical 25% EE, 50% EL and 25% LL. The study finds that the allele E promotes maturation at a smaller size than the L allele, meaning EE fish mature first, then EL and lastly LL. Findings in this study reinforce the idea that EE fish mature earlier than EL, and EL fish mature earlier than LL. As salinity and maturation have been linked previously, this study finds that salinity does affect testis growth but does not affect jacking.

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Abbreviation list (alphabetic order)

SHORT	LONG
UN	The United Nations
AIC	Akaike Information Criterion
AKAP11	A-kinase anchor protein 11
BPG AXIS (HPG AXIS)	Brain-pituitary-gonad axis (hypothalamus-pituitary-gonad axis)
CF	Condition factor
FTS	Flowthrough system
FSH	Follicle-stimulating hormone
GLM	Generalised linear model
GOLT	Gill-oxygen-limitation theory
GSI	Gonadosomatic index
GNRH	Gonadotropin-releasing hormones
GH	Growth hormone
LME	Linear mixed-effects models
LM	Linear models
LH	Luteinising hormone
PIT	Passive integrated transponder
PCR	Polymerase chain reaction
RAS	Recirculating aquaculture systems
SIX6	Sine oculis-related homeobox 6
SNP	Single nucleotide polymorphism
NAOH	Sodium hydroxide
SGR	Specific growth rate
SDGS	UNs Sustainable Development Goals
VGLL3	Vestigial-like protein 3

1 Introduction

1.1 Aquaculture and the global demand for fish

The United Nations (UN) projects the human population to reach 9.7 billion by 2050. More people in the world means more food consumption. Seafood is considered to represent a largely untapped potential to feed the world. Our oceans cover 70% of the earth's surface, yet seafood only accounts for 17% of animal proteins consumed by humans in 2017 (FAO, 2020).

Wild capture is the traditional way of acquiring seafood. However, with modern technology and high demand for fish, fish stocks are on the brink of collapse in large parts of the world due to overfishing (Jackson et al., 2001). To combat this, governments worldwide have introduced quotas for fishing, meaning there is a limit on how much wild capture is available each year. As such, the demand for many fish species has started to outweigh the supply from wild fisheries. Atlantic salmon (*Salmo salar*) is a historically significant fish for all of Europe; however, the wild population is declining, and it is becoming increasingly rare to find wild individuals. The wild capture of Atlantic salmon globally peaked in 1973 at 15,387 tonnes. Wild capture has stagnated since 1996 due to reduced wild stocks. In 2018 wild capture salmon is down to only 2278 tonnes (FAO, 2020).

Aquaculture is another way of acquiring seafood. Aquaculture is rearing aquatic animals or cultivating plants in water, including algae, shellfish, or fish. Compared to wild capture, aquaculture is a more controlled way of acquiring specific marine resources, as the population are bred for harvest. Its growth is fuelled by increasing demand for seafood worldwide, which wild fisheries cannot supply. Aquaculture has been growing steadily since 1970, and in 2018, 45% of global marine food production came from aquaculture (FAO, 2020). Aquaculture may be a way to reach several of the UNs Sustainable Development Goals (SDGs) by protecting our oceans. Increased food production decreases world hunger and increases economic growth globally. In 2018 global production of Atlantic salmon was 2.4 million tonnes, compared to the wild capture of Atlantic salmon, at only 2278 tonnes globally (FAO, 2020) (Figure 1).

1.2 The Norwegian aquaculture industry

Norwegian aquaculture was set in motion in the early 1970s, with Atlantic salmon as the main species of interest. Initially, the industry was mainly dominated by small-scale family-owned businesses due to low governmental involvement and local aquaculture licenses (Hovland and Møller, 2010). Due to geographical and environmental factors, Norway was a seemingly perfect place for salmon farming, and aquaculture in Norway grew fast. However, this led to overproduction and, in turn, bankruptcy for a large majority of small-scale businesses. The government acted by changing licensing laws, allowing growing companies such as Mowi ASA (previously known as Marine Harvest) to buy up small-scale companies, creating a paradigm shift.

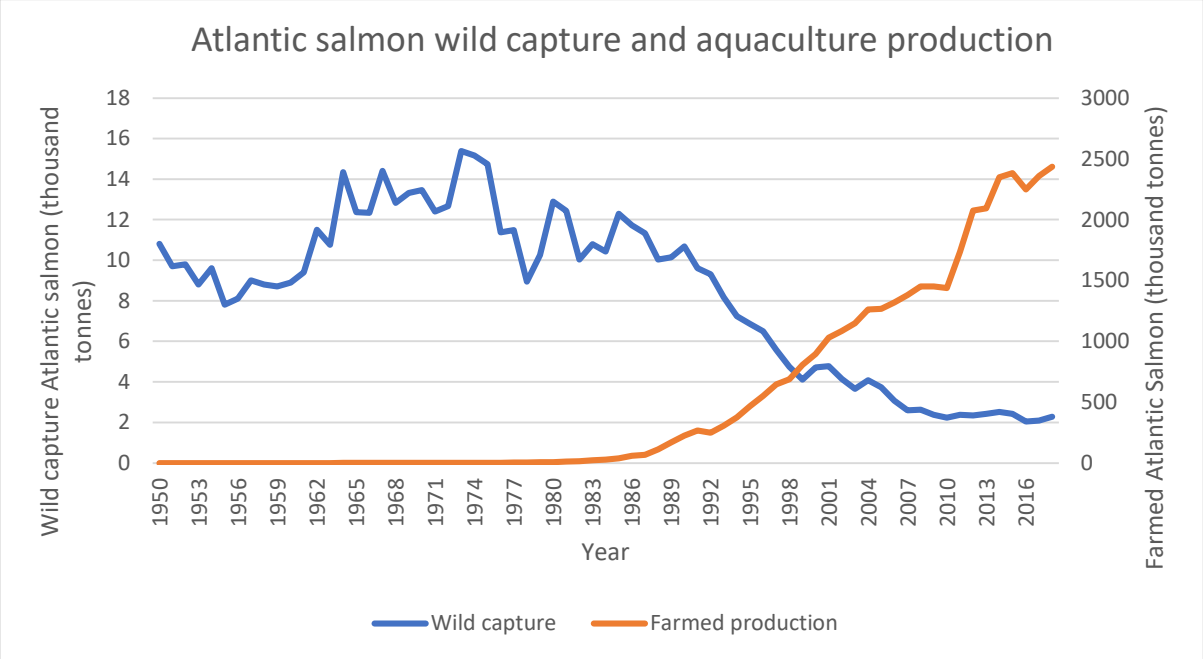

Figure 1: All reported Atlantic salmon wild capture (blue line) and aquaculture production (orange line) from 1950-2018 in thousand tonnes (raw data from FAO, 2020). Note that the scales are different.

Today, aquaculture is one of Norway's most lucrative industries, second only to oil and gas. Norway is the biggest producer of Atlantic salmon in the world. Norwegian aquaculture produced 1.3 million tonnes of fish in 2018 (FAO, 2020), with 1.1 million tonnes exported at a value of 67.8 billion NOK (Sjømatråd, 2018).

There are now over 1000 aquaculture farms in Norway. They are located from the southwest coast of Norway up to Russia in the northeast. Farms are spread evenly along the coast, with an exceptionally high density of farms in Western Norway (Vestland). Locations are mostly protected from open sea wind and waves behind islands or in fjords. Norway

produces more than 50% of all the Atlantic salmon globally, twice the amount of the second-biggest producer, Chile (FAO, 2020).

1.3 Life history of Atlantic salmon

Atlantic salmon are anadromous, which means they spend parts of their lives in rivers and freshwater systems, and parts in the ocean (OECD, 2017). The fish reproduce in the fall and lay their eggs in the gravel, where they remain well protected within the riverbed. Eventually, the fish will hatch into alevins, living off their yolk sac until they can consume feed. Alevins develop into fry and then eventually parr. Before migrating to the North Atlantic Ocean, the fish needs to undergo a significant transformation to become a smolt, a developmental process known as smoltification or the parr-smolt transformation (Stefansson et al., 2020). The parr must adapt to a different environment, especially the higher salinity environment in the ocean. Therefore, the gills must hyperosmoregulate, as the fish goes from being hyperosmotic in freshwater to hypoosmotic in seawater. Morphology changes occur as well. Going from brown to a silvery colour and streamlined shape. Behaviour changes from a territorial- to a schooling fish. After migrating to the sea, the fish grows rapidly in size over one or several years. If the fish is ready and spring comes, the fish undergoes sexual maturation then returns to its river of origin to reproduce (Figure 2).

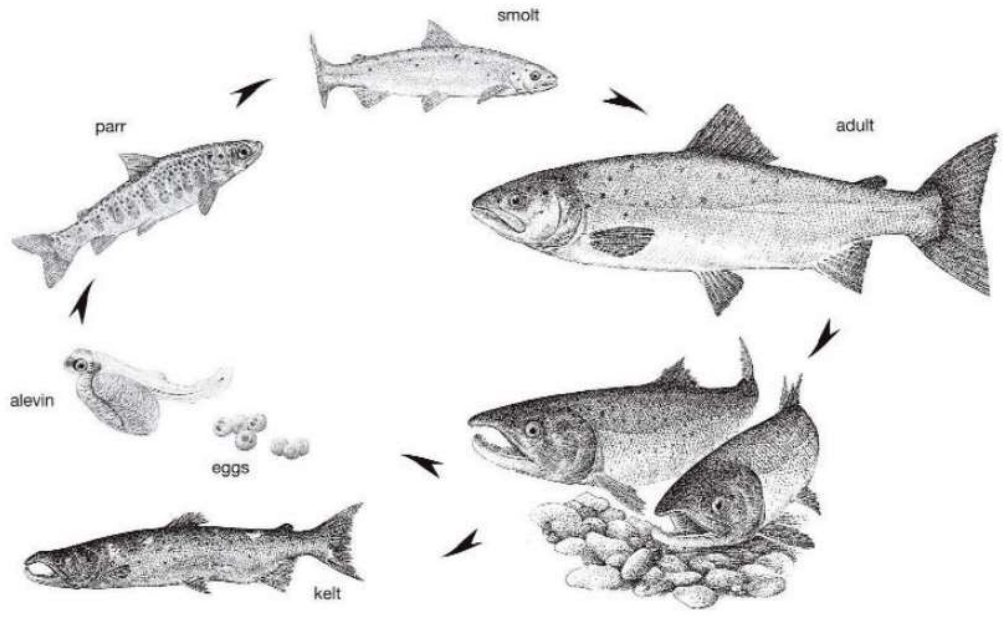

Figure 2: Schematic overview of the Atlantic salmon (Salmo salar) life cycle, from eggs to sexually mature adults. Modified after Sarah Wroot |(Hendry and Cragg-Hine, 2003).

1.4 Atlantic salmon farming

The farmed salmon goes through a similar, but shorter life cycle than the wild salmon, as the growth rate of farmed salmon is more than twice that of the wild salmon (Glover et al., 2009). Farmed salmon start as eggs in a hatchery and is then transferred to a land based freshwater facility. In the land-based facility, farmers regulate the growth through the feed to prepare the fish for sea transfer; the fish is usually around 60-100 grams at this stage. To prepare the fish for the ocean, the farmer needs to make sure the fish can survive the transfer to a more saline environment. Through artificial photoperiods, or with natural lighting, the fish is induced into smoltification. Photoperiods simulate seasons changing, preparing the salmon for the future in the ocean. After the fish has become smolt, it is ready to be transferred to the sea. The fish is in the sea for 10-18 months before harvested prior to sexual maturation. It is crucial to harvest the fish before sexual maturation as it reduces the quality of the harvested filet and leads to immunosuppression for the fish (Aksnes et al., 1986). Therefore, mature fish can be considered to experience lower wellbeing and may also be more susceptible to disease, which could infect other fish in the same cage or beyond and lead to reduced growth or death (Føre et al., 2016).

1.5 Issues with salmon farming

Escapees, pollutants, animal welfare, drugs, salmon lice, and excessive mortality after sea transfer are just some of the challenges the industry is facing (Lekang et al., 2016).

One of the most prominent modern challenges in Norwegian aquaculture is the salmon lice (*Lepeophtheirus salmonis*), an ectoparasite that infects salmonids. Aquaculture farms are often located in fjords, where salmon migrate through to reach their river to spawn. As fish in a sea cage are exposed to the natural environments, the ectoparasite can easily access a vast number of hosts. Further, the lice will occur in an unnaturally high amount. The parasite poses issues regarding the wellbeing of wild salmon and the farmed salmon, which again causes economic problems for the farmers (Torrissen et al., 2013). Once a cage is infected, the salmon lice are hard to remove. The industry must treat the fish to keep the number of salmon lice to a minimum level. There are several ways to treat the salmon for lice, mechanical, thermal and chemical being the most commonly used methods, all of which reduce fish health and welfare. Treatment is harmful and may lead to mortality (Overton et al., 2019). In 2017 the economic cost of treatments against the ectoparasite was calculated to

exceed 5 billion NOK (Iversen et al., 2017). Preventative measures such as specialised feed and cleaner fish are also used to keep the number of salmon lice low (Lekang et al., 2016).

Excessive mortality in the sea is not unheard of in open-net pens. Environmental challenges like previous mentioned sea lice and disease as well as physiological processes and the change of osmoregulation may lead to mortality. The effects of this are that 20% of salmon die before reaching harvest size (Bleie and Skrudland, 2014). Most of the mortality occurs shortly after sea transfer (Bang Jensen et al., 2020, Nilsen et al., 2020, Aunsmo et al., 2008a).

1.6 Post- and big smolt production

A new set of strategies are emerging from the industry to combat salmon lice and disease. Traditionally, fish have been transferred to the sea at 60-100 grams. The new strategies entail increasing the time fish spend in a controlled land-based system and transferring the fish to the sea when they reach a bigger size. The idea behind this is that larger fish will have better immune systems and deflect lice attacks or disease infections better (Lekang et al., 2016).

The big-smolts strategy works by inducing smoltification when the fish is bigger compared to the classical weight. Bigger smolt requires freshwater; technology such as RAS (recirculating aquaculture systems) will solve this (Lekang et al., 2016).

The post-smolt strategy works by inducing smoltification at 60-100 grams and growing the fish bigger on land. Post-smolt requires the use of seawater in a land-based system (Lekang et al., 2016). As land-based facilities equal increased environmental control than in the sea, the farmer can regulate optimal growth parameters. These strategies will decrease the time fish is in the sea's exposed environments and are less likely to be epicentres for lice and disease outbreaks (Valdes-Donoso et al., 2013).

1.7 Reproduction phenotypes in salmon

Salmon are highly plastic; they have various reproductive phenotypes, both seen in wild and farmed salmon.

In wild salmon, phenotypes entail *mature parr*, known as sneaker males, which has a small body size and reach sexual maturity and can reproduce without leaving the river. *Grilse* are slightly larger and spend one winter at sea before returning to spawn in rivers after just one year. Lastly, *large adult salmon* is the most common for Atlantic salmon, and these fish spend 2-5 years in the sea before they return to their river of origin. The distribution between these phenotypes is most visible among the male fish (Thorpe, 1994). For male fish, the body size is not essential for reproductive success. Their incentive for growth is mainly for sexual selection and to combat other males. In comparison, female fish need a larger body size as it directly affects egg quality and fecundity (Heinimaa and Heinimaa, 2004).

These principles apply to farmed salmon as well, where male fish also have the most variation in phenotypes. In land-based on-growing facilities, farmers induce optimal growth conditions such as high temperatures and constant light, which leads to early male maturation (Good and Davidson, 2016)

Post-smolt maturation, known as *jacking,* is rarely seen in the wild for Atlantic salmon, however common in other salmonid species. Jacking is increasingly common in the salmon farming industry. Through the farmer's induction of smoltification, the salmon can enter puberty (Melo et al., 2013). The same environmental conditions induce both maturation and smoltification. Spring triggers smoltification and puberty presumed that the salmon is above a specific size or energy threshold. To simulate spring, farmers use a “square-wave light regime”, which entails reducing the amount of light the fish is exposed to and increasing it again in 4 weeks. The increase of light every day will trick the fish into believing that spring has arrived. This early maturation causes the salmon to mimic female phenotypes. Therefore, the jacks are challenging to identify as they look like female fish (Fjelldal et al., 2011).

1.8 The neuroendocrine and environmental control of salmon reproduction

The brain-pituitary-gonad-axis (BPG-axis, also known as the HPG-axis, Figure 3) is the endocrine pathway for maturation. In brief, neuropeptides in the brain regulate gonadotropin-releasing hormones (GnRH) in the midbrain, which signals to the gonadotrophs in the pituitary. The pituitary is an endocrine gland located at the base of the hypothalamus. Gonadotrophs then release follicle-stimulating hormone (FSH) and luteinising hormone (LH) into circulation to regulate gonad development (gametogenesis). The gonads themselves

produce and release steroid hormones such as 11-ketotestosterone, which stimulates spermatogonial proliferation (Taranger et al., 2010). Gametogenesis is the building of gonads, which the fish requires for reproduction. Throughout the entire axis, feedback loops from the gonads regulate GnRH and FSH/LH (Bone and Moore, 2008). Simultaneously, peripheral signals can act on the axis, providing information on the fish's physiology and energy status.

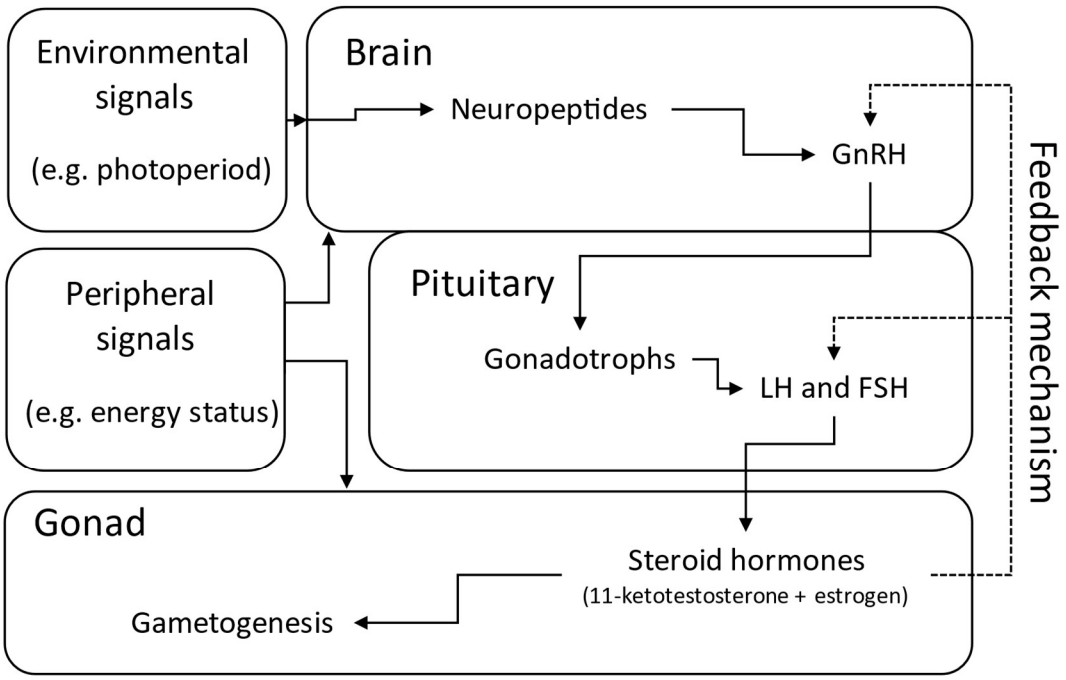

Figure 3: Schematic diagram of the brain-pituitary-gonad-axis (BPG-axis) in Atlantic salmon from environmental inputs through the brain and pituitary and finally gonads undergoing gametogenesis. Peripheral signals and feedback mechanisms have effects through the whole axis.

1.9 Body size, energy threshold and growth rate

Salmon does not enter puberty at a specific body size or a certain age (Morita and Masa-aki, 2006). Instead, life history predicts that maturation is attained once body size and growth thresholds are reached within a given period of the year. Therefore, maturation is commonly linked to increased growth and energy (Campbell et al., 2006), and - Atlantic salmon will only initiate maturation if the fish is in adequate condition (Kadri et al., 1996), meaning it is capable of surviving and reproducing on the energy stored in its body. During maturation, leptin is upregulated; the hormone frees up fat stores and turns them into energy

required for maturation (Frøiland et al., 2010). Salmon is a fat fish, meaning the lipid stores from salmon are in the muscles. When the lipids are used as energy during maturation, the filet will have a lower value for human consumption. Coho salmon (*Oncorhynchus kisutch*) that is genetically modified with increased growth hormone (GH) production has proven to enter puberty earlier than what is usual, indicating that GH has effects on maturation through higher growth factors (Devlin et al., 2004).

1.10 Environmental factors

1.10.1 Light

Generally, changes in day length regulates when fish enter puberty (Bayarri et al., 2009). This has been shown by numerous studies whereby the time of spawning during the year can be altered by artificially manipulating photoperiod (Björnsson et al., 1994, Adams and Thorpe, 1989).

This mechanism's evolutionary reasoning is that the fish only have a small window in their life to reproduce. Salmon are seasonal spawners. The fish need to synchronise their reproduction, and this is achieved by using seasonal change as the trigger, which is reliable every year. By synchronising maturation, the fish will enter puberty simultaneously; it increases the fish's chance to reproduce. Salmon begin maturation in the spring. The mechanisms used by salmon to sense light include the melatonin system and opsins. Melatonin is called the “time-keeping” hormone and is produced during the dark and broken down over time during light periods. Therefore, melatonin levels are elevated during the night and are reduced during the day. Opsins are light-sensing proteins usually found in the eye but are also found in the brain in salmon (Philp et al., 2000). Previous work has shown that melatonin inhibits maturation in salmon (Porter et al., 1999) as found in mammals (Ebling and Foster, 1989). Alternatively, opsins may also be involved in the mechanisms that control seasonal maturation (Porter et al., 1998), as suggested in birds (García-Fernández et al., 2015). However, the mechanism(s) by which melatonin and/or opsins regulate the BPG has not been determined.

1.10.2 Temperature

Salmon are ectotherm organisms, where metabolism is positively correlated with temperature. Given there is enough food for salmon, the high metabolism associated with higher temperatures increases growth. Increased growth will, as stated in part 1.9, increase chances to induce maturation, although the mechanism is unclear. One possibility is that temperature alters the way fish perceive light signals, as out-of-season reproduction is generally reported to occur when temperatures are unnaturally high (Fjelldal et al., 2011, Imsland et al., 2014). For example, melatonin signalling is known to be both photoperiod and temperature-sensitive (Falcón et al., 2010). In addition, opsins may also have a role in sensing temperature (Leung and Montell, 2017), although little is known about this in fish. Alternatively, the gill-oxygen-limitation theory (GOLT) predicts that fish will mature at smaller body sizes when reared on warmer temperatures. Here, GOLT theories that i) oxygen supply becomes limiting in larger fish due to a negative association between body size and gill size, ii) temperature is positively associated with oxygen demand, and iii) reproduction is itself metabolically demanding. As such, fish mature at a smaller body size at increasing temperature to not exceed their metabolic limits (Pauly, 2021, Meyer and Schill, 2021).

1.10.3 Salinity

How salinity may affect maturation is an ongoing research topic. Pubertal males that are exposed to seawater show an increase in the speed of testis development (Melo et al., 2013), but to date, it is unknown whether salinity exposure can trigger puberty. Duston (1994) accounted that salinity exposure resulted in an increase in the number of mature parr with 5, 18, 23, 27% of males maturing following exposure to 0, 10, 20, and 31 ppt, respectively, suggesting it does. In contrast, de Fonseka et al. (in prep) observed no effect of salinity on the number of pubertal post-smolts (approx. 300g) reared at 12°C with 19, 14, 14, and 13% of males maturing at 0, 11, 23, and 35 ppt. Similarly, Ytrestøyl et al. (2020) also observed no effect of salinity (12, 22, 32 ppt) on maturity in post-smolts (450g) maintained in a RAS at 12°C, but the maturity was generally very low in this study (<1% in all groups). In the latter two studies, these fish had i) surpassed the size threshold for parr maturation but not reached the size threshold for jacking, and ii) been given photoperiod signals to induce smoltification, the same signal that induces jacking. Therefore, it would be interesting to determine whether salinity exposure alone can induce puberty in fish that have reached the size threshold for jacking and are not exposed to changes in photoperiod to induce

smoltification. Here, it is noted that salmon can tolerate seawater transfer without smoltification with increasing body size (McCormick et al., 1987). For example, fish greater than 14 cm in length is known to be capable of surviving and growing in seawater (McCormick & Saunders 1987; Duston 1994).

1.11 Genetics control of salmon reproduction

1.11.1 Family effects

There are prominent differences in early maturation depending on the family and strain of Atlantic salmon, revealing that early maturation is heritable. Atlantic salmon has gone through artificial selection over the last 50 years to increase the growth and robustness needed in aquaculture. Domesticated salmon strains dates to 1969, initially caught in rivers in Western Norway (Gjedrem et al., 1991).

Vestigial-like protein 3 (vgll3) explains some of the variation in maturation for Atlantic Salmon. Located in the genome area known as chr25, vgll3 is responsible for more than 35% of the variation of maturation. Vgll3 has alleles for early (E) and late (L) maturation, where E is dominant in males. The gene seems to have little effect on early maturation in females (Barson et al., 2015, Ayllon et al., 2015)

1.11.2 The gene Vgll3

The difference in potency of the gene is apparent in different settings. In wild salmon, the effects of vgll3 are more potent compared to farmed salmon (Ayllon et al., 2015). Vgll3 effects are more potent when the fish is getting a reduced amount of feed compared to fish on full feed rations to promote growth. There may be some indication that lipid stores, energy state and size is related to vgll3. Homozygous EE fish might mature with a lower energy status than LL fish (Kjærner-Semb et al., 2018). Other research has found that in mice, vgll3 is related to adipose tissue and lipid reserves (Halperin et al., 2013). Vgll3 is expressed in the gonads, heart and gills (Kjærner-Semb et al., 2018).

Post-smolt that have matured, known as jacks, are rare in the wild but standard in aquaculture. In land-based facilities, environmental factors such as constant light (24L0D) and high temperatures are expected, as it promotes growth. Together with enough feed, growth conditions are optimal. These factors also promote maturation, meaning one would

expect more jacks in aquaculture (Fjelldal et al., 2011). In this study, it is looked at how different vgll3 genotypes respond to modern farming strategies.

1.12 Aims of the thesis

Vgll3 controls processes of when the fish will enter puberty. By looking at different genotypes of vgll3, one can decide the effects on when the fish will enter puberty. Weight is especially important, as size together with length makes out condition factor, and indicates amount of energy stored.

Lacking research in vgll3s interactions with salinity is an interesting subject as little research exists on the subject. As vgll3 is expressed in the gills, which is the most important osmoregulatory organ in the fish, it will be of interest to identify if there is interaction with a change in salinity.

1.13 Study objective (Hypothesis)

i) EE Atlantic salmon males will start to mature/enter puberty at a lower weight than the LL male.

ii) Salinity exposure triggers post-smolt maturation (jacking).

2 Material and method

This thesis is part of a Research council of Norway project for the Institute of Marine Research in Norway called “Towards the sustainable production of male Atlantic salmon: the balance between genetic and environmental control for age at maturity” (RCN, #295100)”. I joined the project in September of 2020 and therefore did not partake in this experiment's earliest parts.

2.1 The fish and breeding

On the 19th Nov 2019, three half-sibling families were produced using defrosted (previously cryo-preserved) milt of a vgll3 heterozygous YY male (tag C457) crossed with fresh eggs from one of three Mowi strain females (tags AC22, 9056, A0BD).

In this experiment, only male fish were needed; therefore, an all-male population was the best option, as female fish would be discarded. The all-male population was possible through an extensive process. The first step is sex reversal of a male yolk sac larvae treated with estrogen; this causes the individual to become a neo female (XY-female). By breeding a neo female with a male, one will get 25% of the offspring as supermales (YY-male). By breeding a supermale with a female, 100% males (all XY) are expected (Figure 4), although some females have been observed in ”all-male” lines (Fjelldal et al., 2020). The exact reason for the occurrence of phenotypic and genetic females in such lines are currently unknown (Fjelldal et al., 2020).

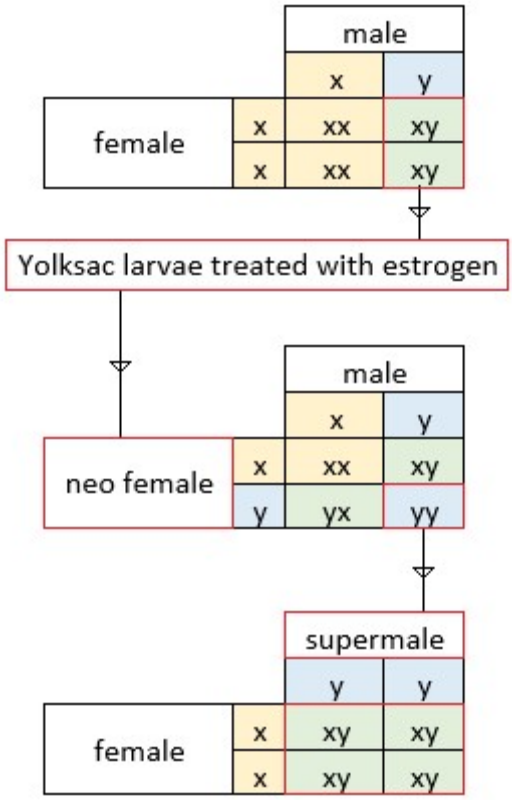

Figure 4: Schematic explanation of production of all-male population of salmon. Red squares indicate the most relevant part of each stage. Larvae treated with estrogen will turn in to neo-females, able to produce xy eggs. xy eggs will produce yy supermales 25% of the time. A yy supermale will have 100% male offspring.

The final all-male population was produced using genotyped individuals who were all VGLL-3 heterozygote EL. According to Mendelian genetics, the population's expected outcome will be 25% EE, 25% LL and 50% EL. The population have three different mothers but the same father. The three families are named T-20-1, T-20-6 and T-20-8.

2.2 Experimental design

All fertilised embryos were incubated at 8 °C, with each family in its own incubation tray. The first feeding (day 0) started on 28th Feb 2020. Approximately 2500 individuals from each family were moved to three tanks (n= 1tank per family). On day 53 (21st Apr 2020), the fish were divided into six tanks (n = 2 tanks/ family with 1250 fish per tank). On day 117 (24th Jun 2020), the fish were reduced and split between twelve tanks (n = 4 tanks per family with 400 fish per tank). On day 130 (7th Jul 2020), the fish were divided amongst eighteen tanks (6 tanks per family with 250 fish per tank). On day 187 (2nd Sep 2020), the fish were moved to three tanks (n= 1 per family). On day 199 (14th Sep 2020), 2550 random fish (n= 850 per family) were implanted with a passive integrated transponder (PIT tag, 2x12mm, RFID solutions, Stavanger). The same day, fin clips were removed and stored in 95% ethanol for genotyping, and the fish were divided into three tanks (n=1 tank per family, at a density of 21-26 kg per tank). On day 234 (19th Oct 2020), the fish had all been genotyped, the population was reduced and equally distributed into six tanks (n=250 fish per tank with 21-34 fish/genotype/family/tank). Following a 96-hour seawater trial starting on day 273 in which 0/10 fish died. Osmolality levels in the fish were measured through blood extraction from the caudal vein, at an average of 346.9 milliosmoles per kilo, whereas the control group in freshwater had an average of 318.2 milliosmoles per kilo. The water in three tanks was gradually switched from freshwater to seawater (35ppt) over a 12-day period (15, 25 and 35 ppt on day 279, 282 and 291, 3rd, 6th and 15th December 2020, respectively). From first feeding (day 0 until the end of the experiment (day 354, 16th Feb 2021), the temperature was maintained at approximately 13 °C and continuous daylight (24/0 hr light/dark). Throughout, the fish were fed an appropriate pellet size of a standard commercial diet (Skretting, Stavanger with 20% excess based on body weights and predicted growth rates).

2.3 Sampling

Sampling consisted of 7 sampling sessions (Table 1), and some minor adjustments to the population, and seawater challenges. These sessions consisted of checking the PIT tag, measuring length (centimetres, cm, 0.1) and weight (g ± 5 g) to the nearest 0.1 cm and g respectively, and extracting the gonads for weight measurement (mg) on a fraction of the fish, rounded to nearest 1 mg. The fish were netted out of tanks then put in a bath with a non-lethal dose of buffered 100 mg/L MS222 (Finquel) to anaesthetise so that handling the fish was less

stressful for both the fish and personnel. The gonad extraction required the fish to be euthanised. First, these fish were randomly preselected. Euthanising the fish happened in a bath with a lethal dose of buffered 500 mg/L MS222 (Finquel).

A small number (105) of female fish occurred, the ovaries of these fish were not measured. After October, all the remaining fish had been genotyped, and the genotypes were balanced, meaning fewer fish with genotype EL were sampled to reduce the number of sampled fish similar to genotype EE and LL.

Table 1: Amount of fish per genotype and amounts of gonads weighed at different sample points (days after first feeding), including average weights and notes explaining what specifics.

Days after first feeding	# of fish measured			# of gonads measured (count)	Size (g)	Notes
	Genotype					
	EE	EL	LL			
130	117	219	111	125	33	Fin clips were stored for 450 fish
166	111	220	116	181	90	Fin clips were stored for 450 fish
200	741	1497	741	450*	164	Pit tagging. Fin clips were stored for 3000 fish.
229	6	8	6	20	-	Seawater challenge
234	639	1276	611	484	283	-
272	489	546	455	431	484	-
273	7	7	6	20	-	Seawater challenge
274	7	9	4	20	-	Excess fish
312	324	379	301	454	706	-
354	175	196	165	490	922	Last sample

2.4 DNA extraction and qPCR analysis

Polymerase chain reaction (PCR) can artificially duplicate DNA several times, achieving higher concentrated mixtures of DNA. The process starts with NaOH mix and a tissue sample. A high temperature of 95 °C is induced to the mix, which will break the hydrogen bonds in the double-stranded DNA, resulting in two single-strands (denaturing). The single strands will act as a template and complement themselves back into a double-stranded DNA at 50-65 °C. This process will have duplicated the DNA. This can be done in several cycles to ensure a sufficient amount of DNA is produced. By adding a specific single nucleotide polymorphism (SNP) assay, identifying the genotype of the gene in question is possible (Fairchild et al., 2006).

The DNA is stored as fin clippings in 95% ethanol from 7th Jul, 12th Aug and 14th Sep 2020. The fin clips have been matched with pit tag identifications to match our data with the genotype. The DNA was extracted from the fin clips using HotSHOT DNA. Wells in a 96-well plate were filled with part of a fin clip and 15 µl of 50 mM NaOH, then heated to 95 °C for 20 minutes and cooled to 4°C for stability. After cooling, the samples were neutralised with 5 µl of 5 mM Tris-HCL and stored at -80 °C. The stored samples were thawed and transferred to a 384 well plate. 1.0µl of DNA sample were added 2.5 µl genotyping mix (TaqMan™ Universal PCR Master Mix, Applied Biosystems) and 0.125 µl single nucleotide polymorphism (SNP) assay (Table 2), then diluted with 1.375 µl RNA free water. The diluted samples were then transferred to a new 384-well plate before being put into a quantitative PCR (qPCR) machine (QuantStudio™ 5 Real-Time PCR System, 384-well, Applied Biosystems™). In total, 3896 individuals were genotyped.

Table 2: Single nucleotide polymorphism (SNP) assays used for qPCR in this thesis.

Name **Vgll3 Exon3**	**5'-Sequence-3'**	**Application**
Chr25_28658151_**AD_F** Foreward primer	AGCCCAGGGATACACAGTGA	Allelic discrimination
Chr25_28658151_**AD_R** Reverse primer	GTGGGCCAGGCTGAGG	Allelic discrimination
Chr25_28658151_**AD_V/M** Primer early/late probe	CCACCTCTGT(G/C)TTCACA	Allelic discrimination

The program for qPCR was as depicted by Figure 5 A; samples were heated to 60 °C within 30 seconds, then heated to 95 °C for 10 minutes. The PCR cycle starts with 15 seconds at 95 °C before being cooled back to 60 °C for 1 minute. The PCR cycle was repeated 40 times for each sample.

To determine the genotype of subjects in the experiment, the qPCR machine was able to detect the primers that were duplicated. At 30 to 40 cycles, the machine detects an increase in the sequence we want to look at. Two probes are used, one for each allele, the difference in expression of these is how we can distinguish which genotype is present. Notice the graphs in Figure 5 B, C and D, where one can see an increase in the amplification of sequences, and these figures show the genotypes EE, EL and LL, respectively.

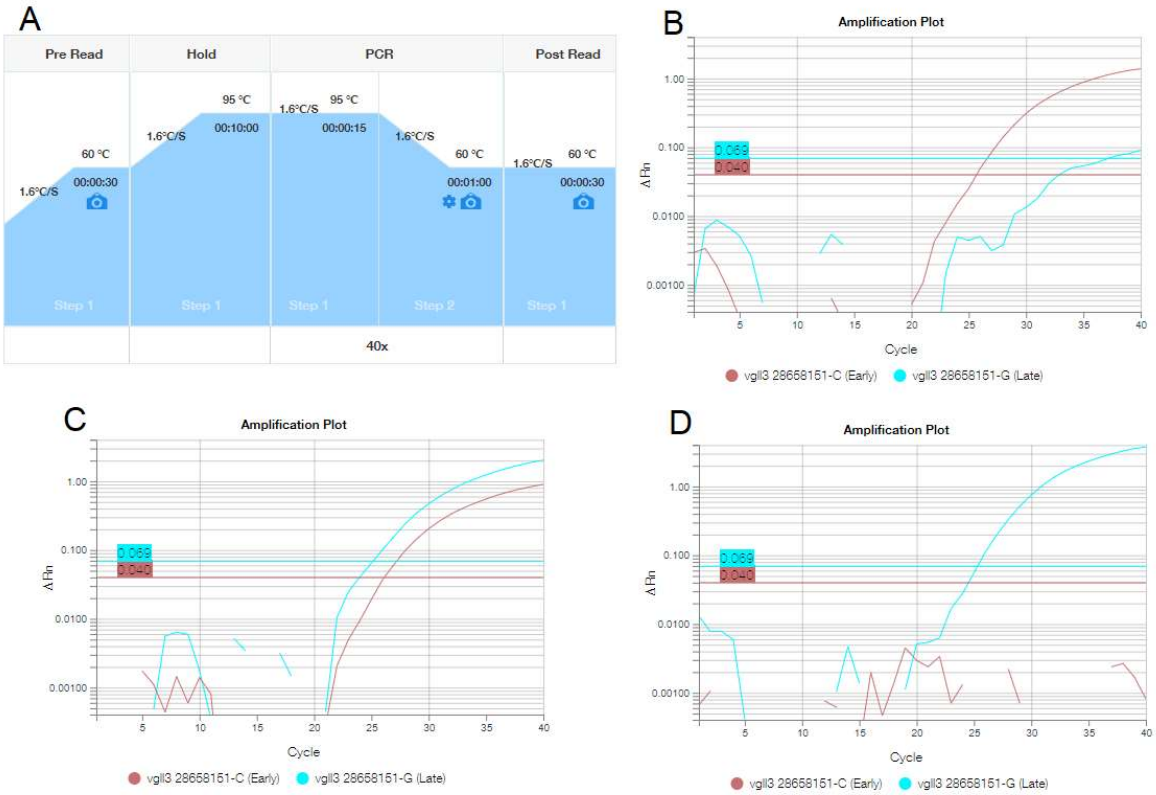

Figure 5: Screenshot of the qPCR program cycle (A), quality check of an EE subject (B), quality check of an EL subject (C) and quality check of LL subject (D) (Thermo Fisher Scientific, USA).

2.5 Statistics and Mathematics

To determine the length and the weight of the fish, the fish were weighed and measured on metric scales. The measurements were recorded in the dataset.

Condition factor (CF), or k-factor, describes the ratio of the weight compared to the length of the fish. A low condition factor means the fish is elongated, whereas a high condition factor implies the fish is fatter and shorter.

$$\text{Condition factor } = \frac{Weight\ (g)}{Length\ (cm)\ \text{^}3} * 100$$

Gonadosomatic index (GSI) is a calculation of the gonadal weight relative to body weight. The GSI is used to determine whether the fish is mature or not, and it is also an indication of how far the fish is in puberty development.

$$\text{Gonadosomatic Index} = \frac{Gonad\ weight\ (g)}{Body\ weight\ (g)} * 100$$

Specific growth rate (SGR) is a term used to identify growth in fish. It describes how much growth is happening each day over a period of time.

$$Specific\ growth\ rate = \frac{\left(\ln\left(Final\ Weight(g)\right) - \ln\left(Initial\ Weight(g)\right)\right)}{Time\ (Days)} * 100$$

Different statistical methods were tested to determine interactions between various varying factors. By starting with complex models and making the most parsimonious by backwards model building using the lowest **Akaike Information Criterion** (AIC) score, it was possible to get valid models reflecting the population. Weight, length, condition factors and maturity were tested.

To determine the response of the variables weight, length and condition factor for fish that were immature, variants of linear models were used. It was not preferable to use pubertal fish in these responses as rapid growth increases before maturation and is reduced during maturation, thus changing the models and can lead to misinterpretation of the data. **Linear models (lm)** describe a response variable defined by one or several predictor variables in a continuous manner. Whereas **linear mixed-effects models (lme)** are similar to linear models, but with the introduction of random effects. Linear models were used for day 130 as well as day 200. For days 166, 234, 272, 312 and 354, linear mixed-effects models were used. Predictor variables were family and genotype, as well as salinity for day 312 and 354, as the

fish were not introduced to different salinities before this timepoint. The random effect in the lmes introduced was “tank”, meaning random effects from the tanks. For the timepoints day 130 and 200, there were insufficient data from each family and genotype in each tank to do linear mixed-effects models.

A generalised linear model (glm) is a more flexible version of linear models; it is similar to linear models but allows for response variables to have error distribution models other than a normal distribution. Glms were used to determine the interactions between predictor variables family, genotype, and salinity for the binomial response variable pubertal fish. Glm was used for the timepoints day 312 and 354 as the earlier timepoints lacked representation of pubertal fish for each family and genotype.

Residual plots are used to determine whether a model meets specific criteria. A residual is the difference between the observed value and the predicted value. If the residual plot has a random pattern, the model is linear. If the residual plot is not random, either a U-shape or a fan-shape, the residual plot is not random, and the model is not linear.

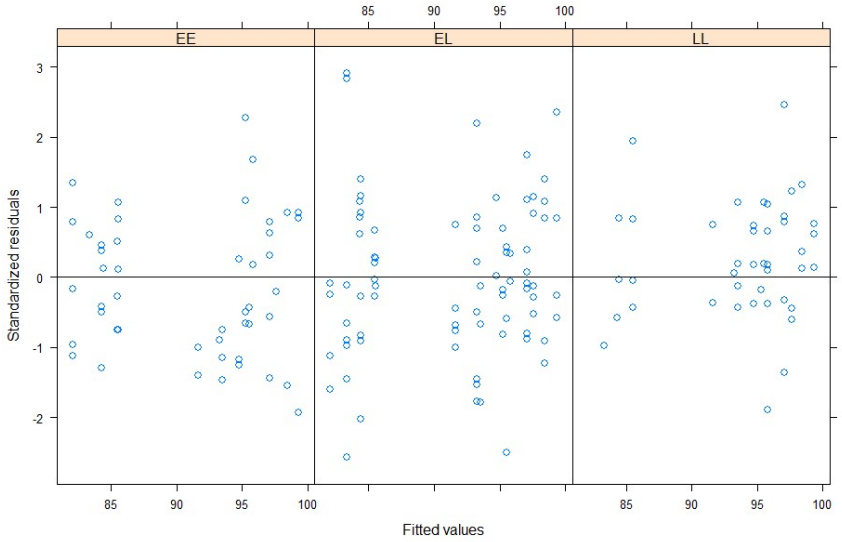

Figure 6: Three different residual plots by genotype for weight on day 166. Indicating a linear relationship.

A **Normal Q-Q plot** is a scatterplot that tells you if the residuals are normally distributed or not. If the points are in a straight line, your residuals are normally distributed and which is required for LM and LME models.

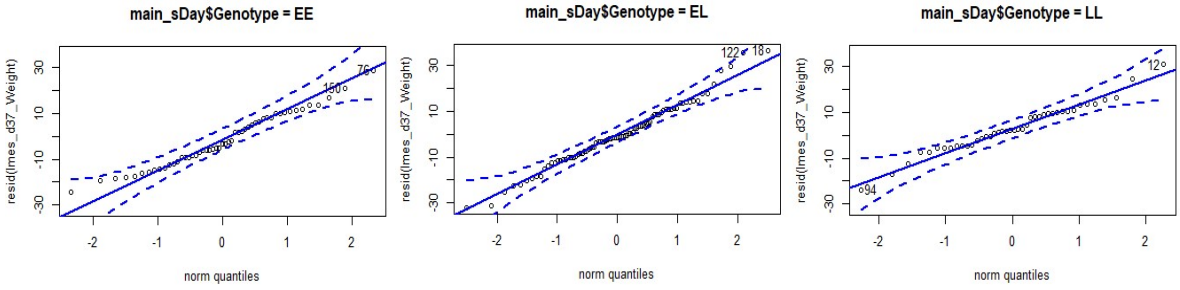

Figure 7: Three different normal Q-Q plots by genotype for weight on day 166. Indicating a normal distribution.

For data that is non-parametric, or not normally distributed, **Kruskal-Wallis** tests are used for statistical analyses. A Kruskal-Wallis, or "one-way ANOVA on ranks" can be used to determine statistically significant difference on two or more groups of independent or continuous variables.

A **Tukey HSD test** is a post-hoc test, the test will tell you differences within different groups. Tukey tests are used for GLM models to see significant difference between pairs of groups.

R was used for data analysis and statistics (R Core Team, 2019).

2.6 Determining maturity through GSI

In this experiment, maturity is decided by the value of the GSI for each fish. This value has been set at a GSI of 0.1. The reasoning behind this is that the gonads are very small (GSI <0.1) before the fish start maturing and the fish uses a lot of energy to develop mature gonads when the fish decides to enter puberty, meaning the gonads will grow rapidly compared to the weight of the fish, surpassing 0.1 GSI. Previous studies (Kjærner-Semb et al., 2018) have used a GSI of 0.1 as well, reinforcing the reasoning for using this value. Figure 8 and Figure 9 show a histogram of the distribution of GSI, showing a clear difference from low and high GSI.

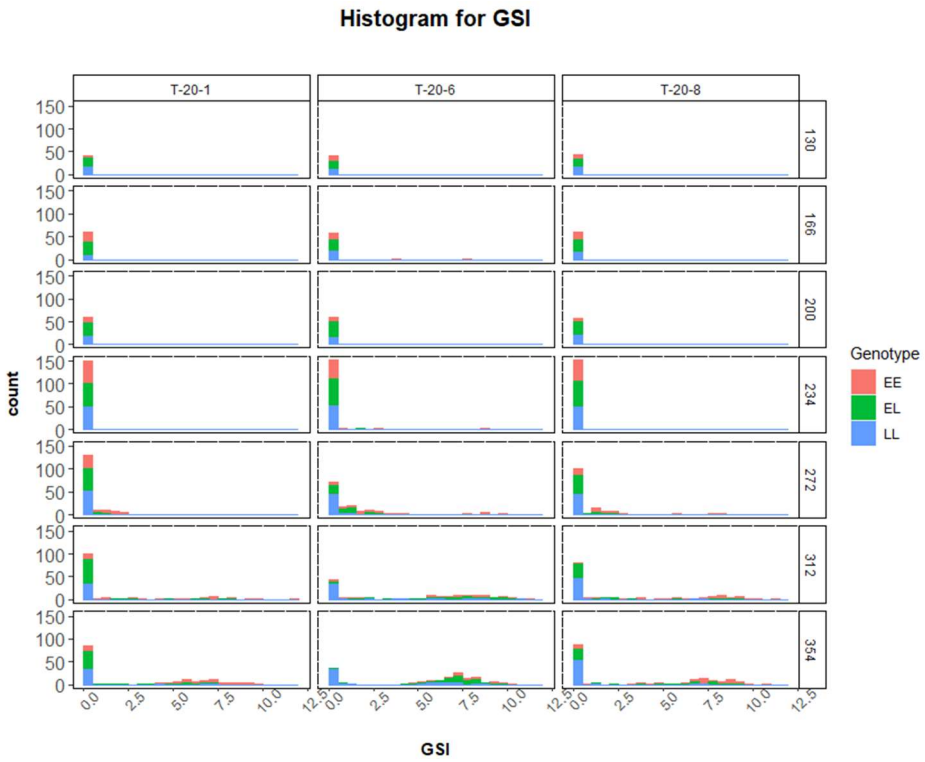

Figure 8:Histogram of count of GSI per value with a range of 0.5 by family, genotype and day.

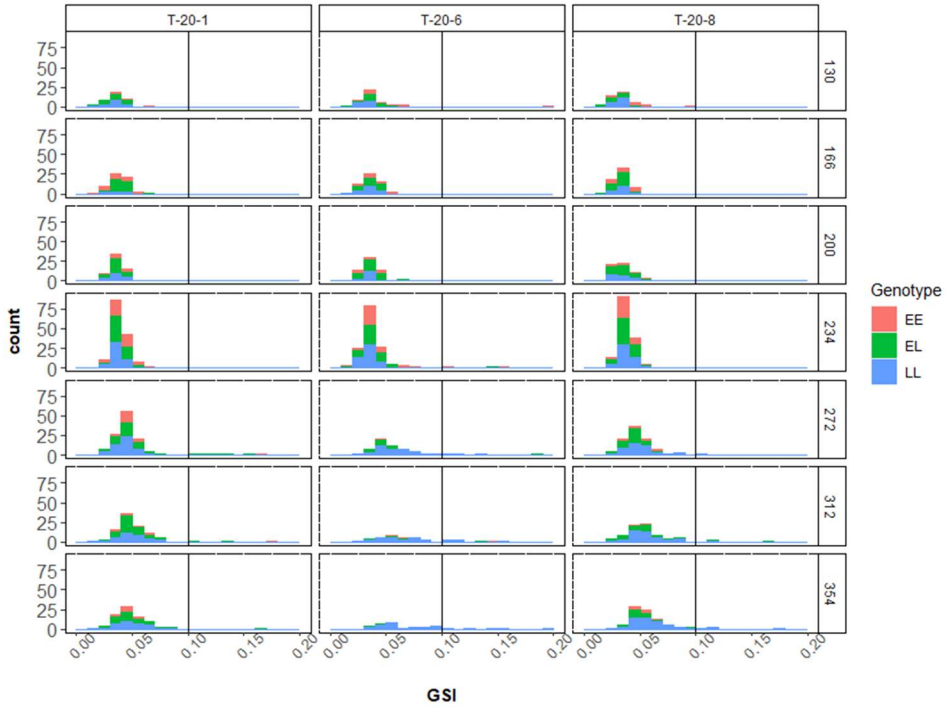

Figure 9: Histogram of count of GSI per value with a range of 0.02 by family, genotype and day, zoomed in to a GSI range of 0.00-0.20.

3 Result

3.1 Mortality

There was very little mortality of subjects in this experiment. In total, 15 (0.38%) fish died between sampling points. At day 272, half of the fish were moved to seawater without smoltification, which did not result in any deaths. Two fish died between day 272 and 312, and zero fish died between day 312 and 354. The two deaths were both in freshwater.

3.2 Maturation

3.2.1 Generalisation of puberty

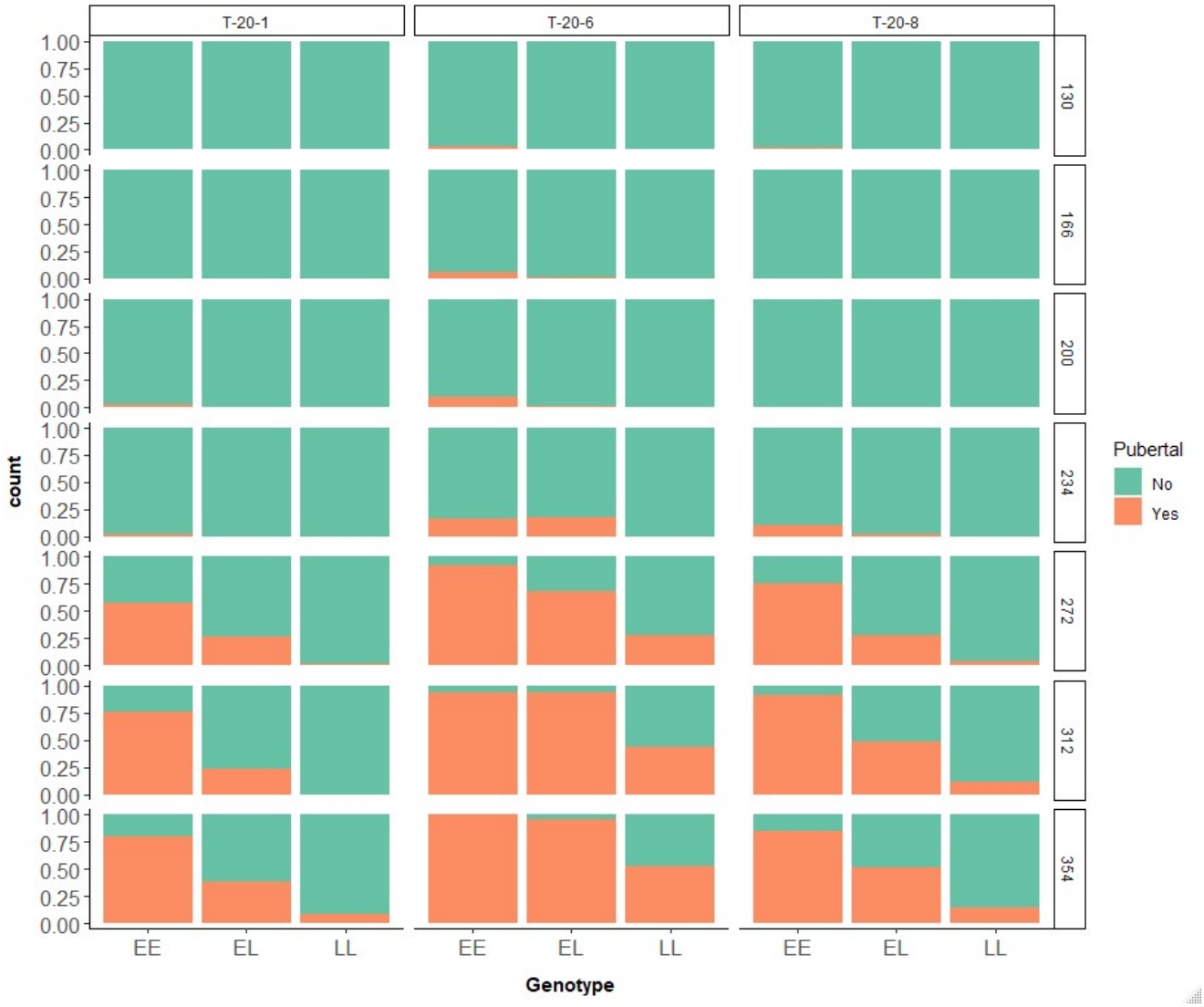

Figure 10: Bar plot for ratio of pubertal (orange) and nonpubertal (green) fish for each sample point by genotype, family.

Throughout the experiment, the general trend is that EE fish enter puberty (GSI >0.1) before EL fish and EL fish enter puberty before LL fish. Figure 10 depicts that the earliest pubertal EE fish occur at day 130 for family T-20-6 and T-20-8 and day 200 for family T-20-1. The earliest pubertal EL fish occurred at day 200 for family T-20-6, at day 234 for T-20-8, and at day 272 for T-20-1. The earliest pubertal LL fish occurred on day 272 for all families. In family T-20-1, the earliest pubertal EL fish and LL fish occur on the same day. However, there are notably more EL fish than LL fish that pubertal within the family.

3.2.2 Statistical analysis of pubertal fish

Where there was adequate information to do statistical analysis GLMs showed that genotype and family had a significant effect on initiation of puberty, but salinity did not (Table 3). No interactions were found between genotype, family, and salinity.

Table 3: Anova test of generalised linear models (GLM) on pubertal fish for timepoint 272, 312 and 354 on family, genotype and salinity.

Days from first feeding	LR	χ2	Degrees of freedom	p-value
272	Family	59.343	2	<0.001
	Genotype	158.035	2	<0.001
312	Family	102.315	2	<0.001
	Genotype	177.523	2	<0.001
	Salinity	1.866	1	0.1719
354	Family	101.846	2	<0.001
	Genotype	169.807	2	<0.001
	Salinity	2.609	1	0.1062

3.2.3 Statistical analysis of genotype and maturation

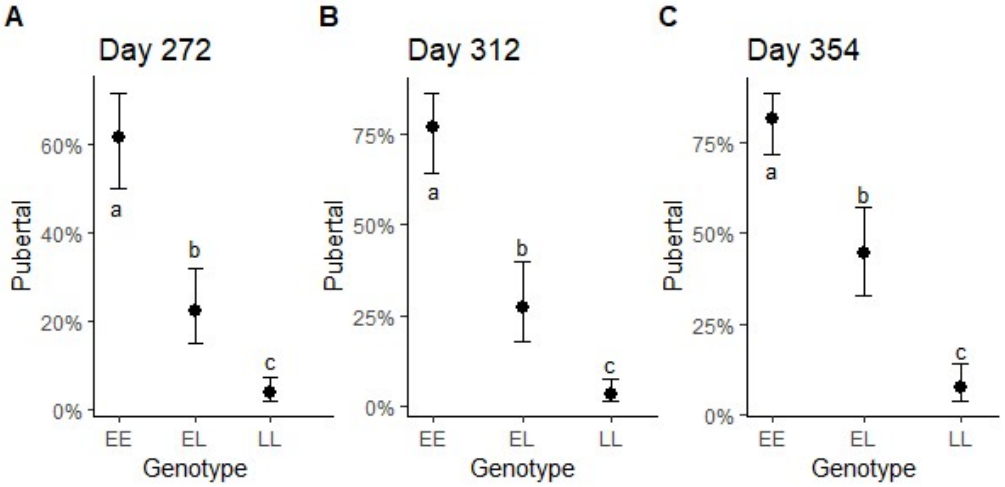

Figure 11: Generalised linear models of percentage of pubertal fish by genotype on days 272 (A), 312 (B) and 354 (C). Different lowercase letters indicate significant effects (maximum to minimum group means) within timepoint (post hoc, least square means ±95% CI, p <0.05)

On all days that were analysed statistically, there were significant differences between genotypes ($p < 0.0001$) (Figure 11). EE enter puberty earlier than EL, EL enter puberty earlier than LL.

3.2.4 Statistical analysis of family and maturation

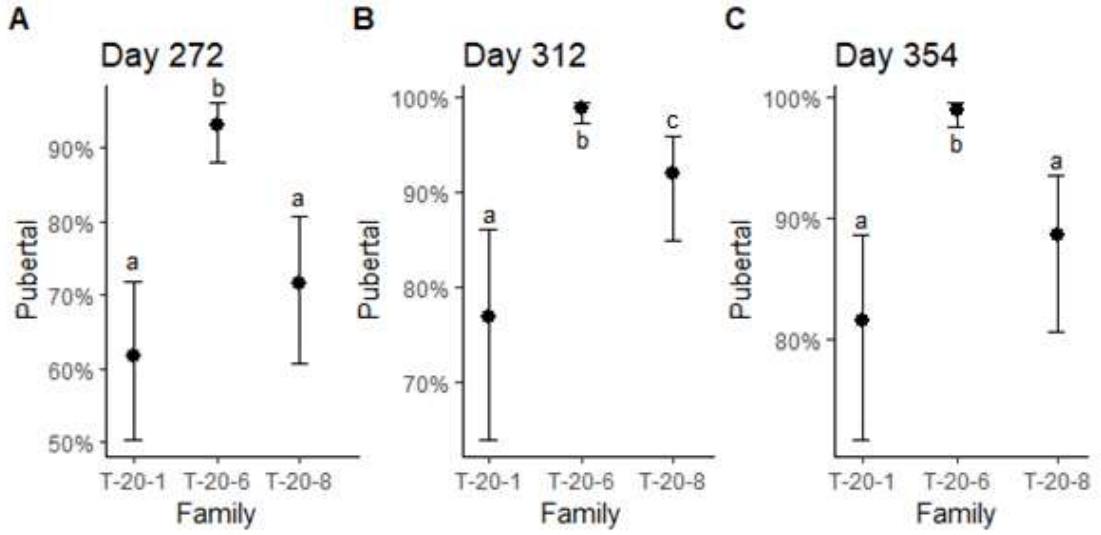

Figure 12: Generalised linear models of percentage of pubertal fish by family at days after feeding for day 272 (A), 312 (B) and 354 (C). Different lowercase letters indicate significant effects (maximum to minimum group means) within day after first feeding (post hoc, least square means ±95% CI, p <0.05).

On all days that were analysed statistically, there were significant differences between family T-20-1 and the others ($p<0.0001$). For T-20-1 and T-20-8 there were differences for day 272 ($p=0.2540$) and 354 ($p=0.11$), but not 312 ($p=0.0002$) (Figure 12).

3.2.5 Statistical analysis of salinity and maturation

On day 272, three out of the six tanks of fish were transferred to seawater (35 ppt). The fish in seawater tanks did not have significant differences in pubertal fish compared to the fish in freshwater on day 312 ($p=0.1719$) or day 354 ($p=0.1062$) (Figure 13, Table 3).

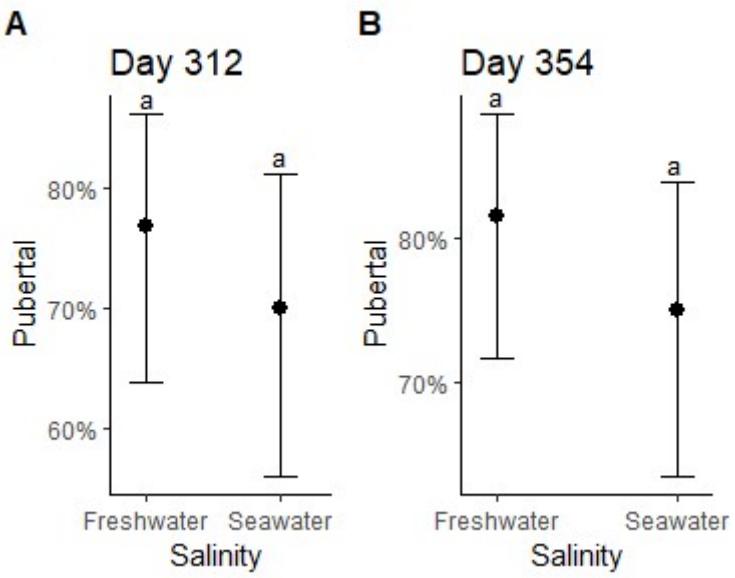

Figure 13: Generalised linear models of percentage of pubertal fish by salinity at days after feeding for day, 312 (A) and 354 (B). Different lowercase letters indicate significant effects (maximum to minimum group means) within days after first feeding (post hoc, least square means ±95% CI, $p<0.05$)

3.3 GSI

3.3.1 Description of GSI data

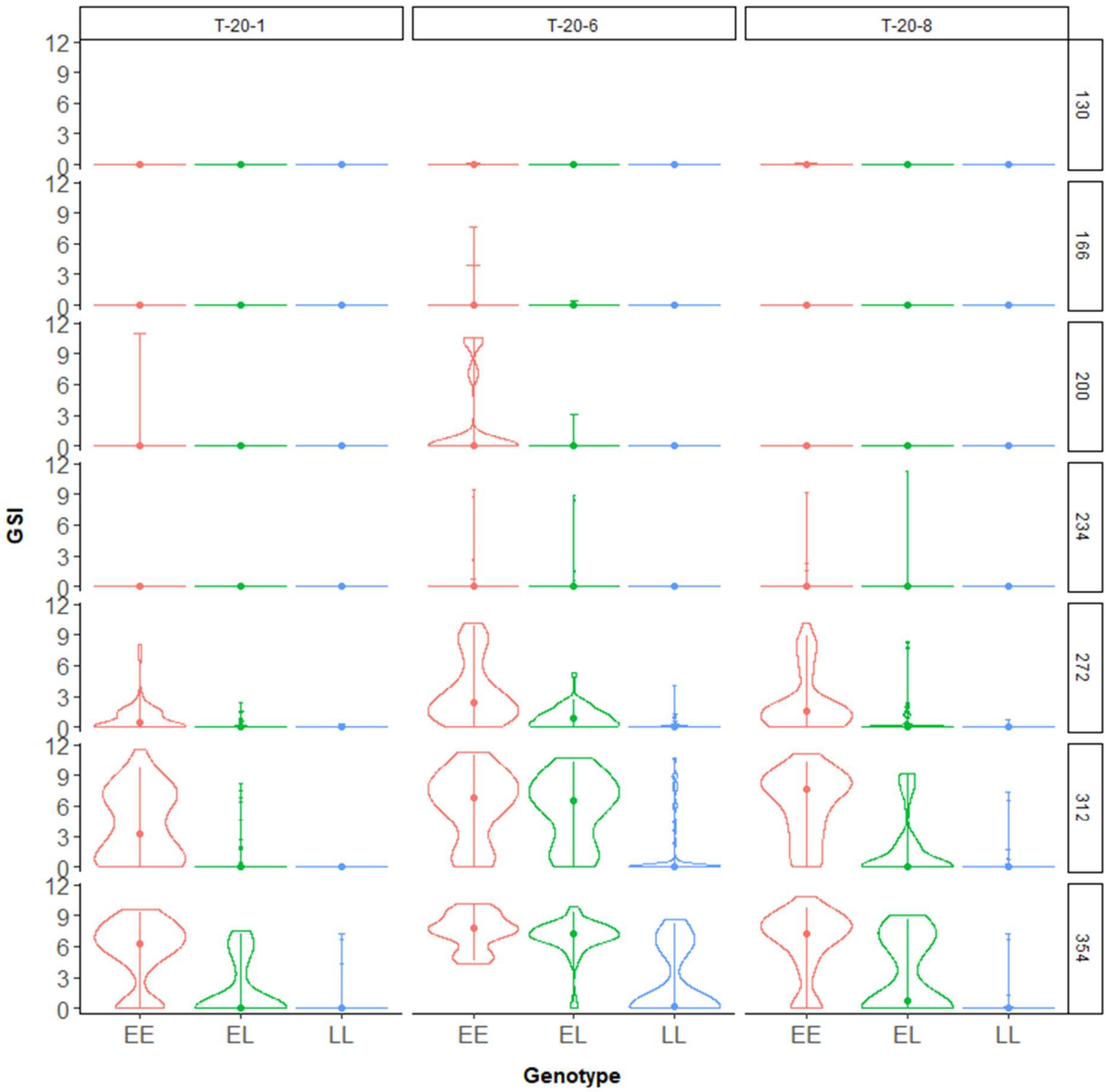

Figure 14: Violin plot illustrating amount of population within groups (family and genotype) at different values of GSI per day. Dots represent the median within the different groups.

There were notable trends for GSI depending on family and genotype. The fish with genotype EE develops a higher GSI earlier than EL and EL higher than LL in all families (Figure 14). Family T-20-6 develops higher GSI earlier compared to the family T-20-1 and T-20-8 (Figure 14).

Figure 15 depicts higher GSI value in freshwater fish compared to seawater. At 272 days after first feeding, the fish had a similar GSI violin plot, indicating very similar GSI profile for the groups. At 312 and 354 days after first feeding, freshwater fish had a higher GSI, indicated by the form and median in the violin plots.

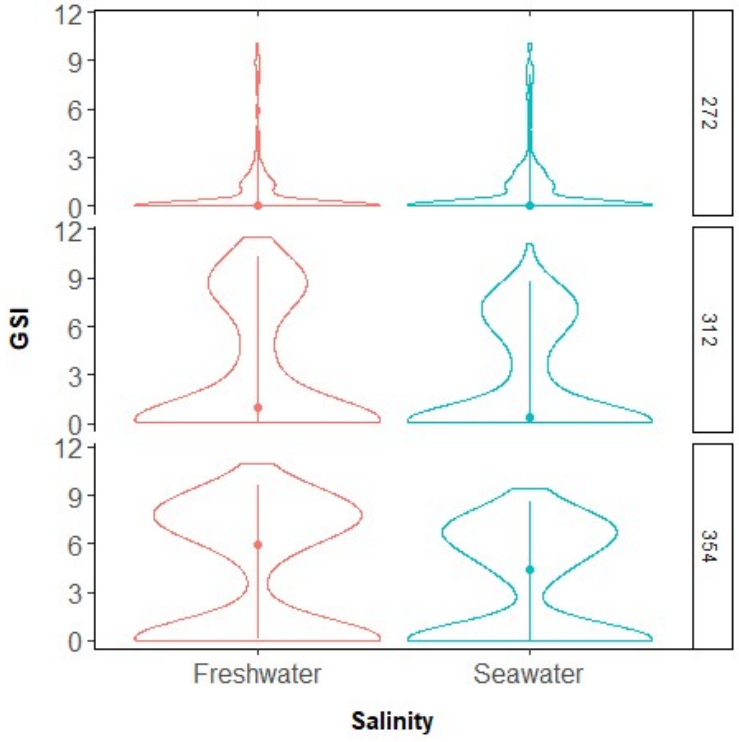

Figure 15: Violin plot illustrating amount of population at different values of GSI per day in freshwater (red) and seawater (blue). The dot represents the median of the groups.

3.3.2 Statistical analysis of GSI

It was found that family had a significant effect on GSI on days 272, 312, and 354 (Table 4) as family T-20-6 had significantly higher values than the other two families at all timepoints (Figure 16A-C). Fish kept in seawater also had a significantly lower GSI than those kept in freshwater (Table 4) on days 312 and 354 (Figure 16DE). Through statistical analysis for the day 272, 312 and 354, it was found that family had an effect on GSI for all the three days, salinity had an effect on day 312 and 354 (Table 4).

Figure 16 depicts a boxplot of the GSI for days 272, 312 and 354, showing higher GSI for family T-20-6 compared to the others. There is also notably higher GSI for fish in freshwater compared to fish in seawater

Table 4: A non-parametric Kruskal-Wallis tests of GSI depending on family or salinity by days from first feeding.

Group	Figure	Days from first feeding	$\chi 2$	Degrees of freedom	p-value
Family	A	272	54.32	2	<0.001
	B	312	65.87	2	<0.001
	C	354	50.64	2	<0.001
Salinity	D	312	9.20	1	0.00242
	E	354	27.78	1	<0.001

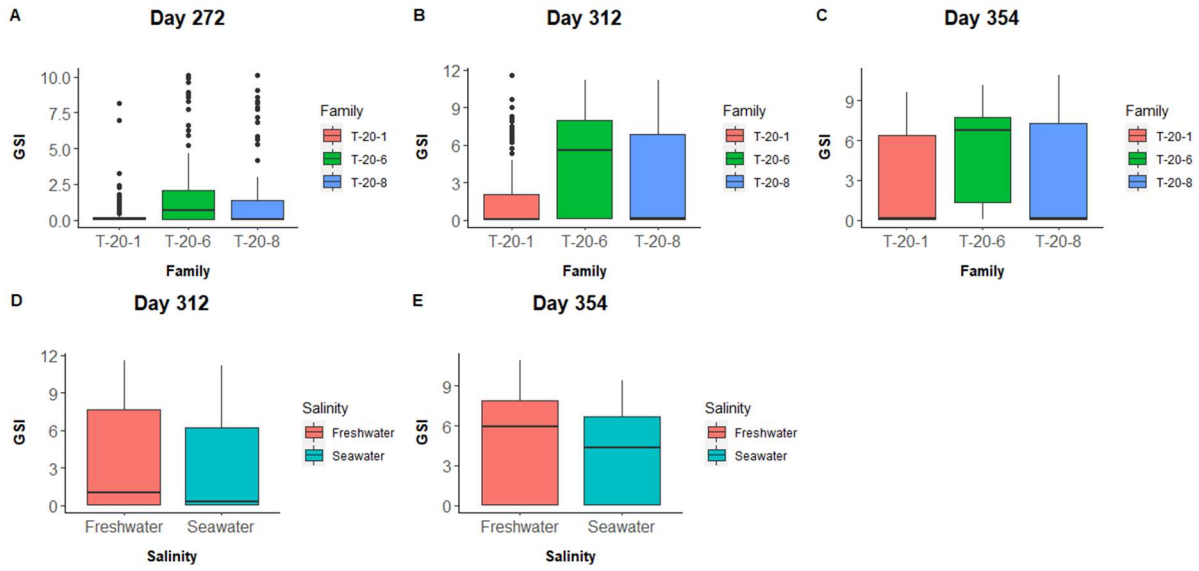

Figure 16:Box plot of GSI depending and family (A, B, C) or Salinity (D, E). The last three time points, Day 272 (A), 312 (B, D) and 354 (C, E).

3.4 Growth

3.4.1 Description of growth data

The fish grew from an average weight of 33 g on day 130 to 922 g on day 354. This is a total SGR of 1.31%. There are notable differences in growth for each genotype, each family and for different salinities, as seen in Table 5 and Figure 18. Family T-20-1 was smaller than the other families at the final sample point, although the SGR% was the same for all families at 1.31%, meaning that the increase in growth is the same. Fish with genotype EE grew less than EL and LL. EE had an SGR of 1.26%, whereas EL had 1.33%, and LL had 1.34%.

Table 5: Average weights (grams) for different families and genotypes per day, including total SGR%.

Day	Family			Genotype			Grand Total
	T-20-1	T-20-6	T-20-8	EE	EL	LL	
130	30	34	35	33	33	33	33
166	83	95	93	90	90	92	90
200	148	178	165	162	164	165	164
234	269	303	278	282	282	288	283
272	444	534	476	486	481	488	485
312	636	766	717	678	717	726	707
354	839	968	958	819	951	997	922
SGR%	**1.31%**	**1.31%**	**1.31%**	**1.26%**	**1.33%**	**1.34%**	**1.31%**

Puberty has a considerable effect on growth, consequently pubertal fish were excluded from the models to get a more accurate description of the growth. Figure 17 depicts growth by genotype (A, B and C) and family (D, E and F) for weight, length, and the condition factor. There seems to be little effect in growth from genotype, and more effects from family. T-20-6 is the family of fish that are biggest at the final timepoint, where T-20-8 is in the middle, and T-20-1 with the lowest weight. There is some difference in condition factor as well, where the heavy fish have higher condition factor.

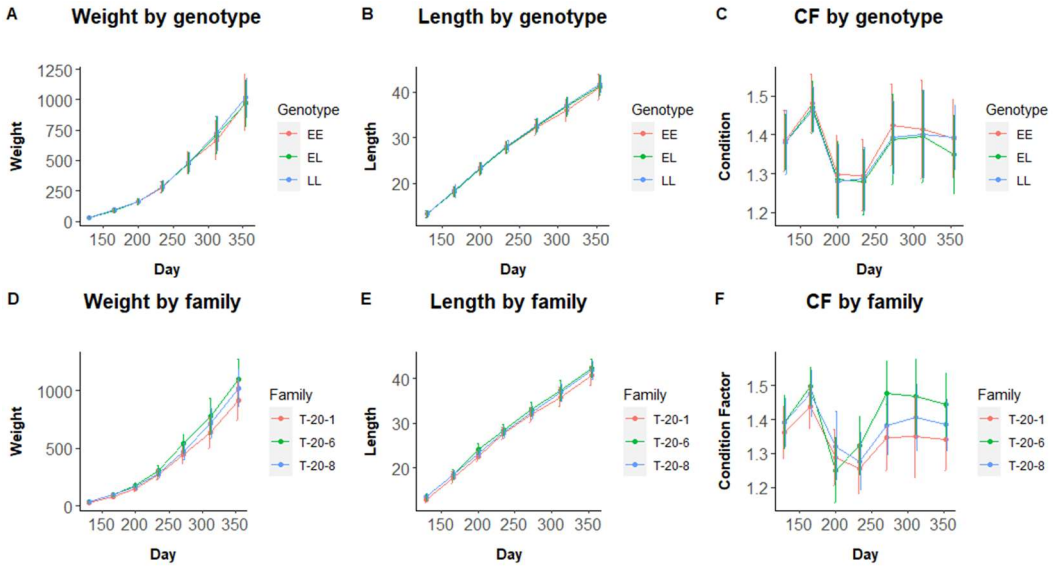

Figure 17: Collection of growth by weight (mean ±sd) (A,D), length (mean ±sd) (B,E), and condition factor (mean ±sd) (C,F) over time depending on genotype (A,B,C) and family (D,E,F).

3.4.2 Description of salinity data

Fish in freshwater grew faster from day 272-312, whereas, from day 312-354, the seawater group grew faster (Figure 18). From day 272-312, the freshwater fish had an SGR of 1.30%, whereas the seawater fish had an SGR of 0.81%. From day 312-354, freshwater fish had an SGR of 0.67%, whereas seawater subjects had an SGR of 0.99%.

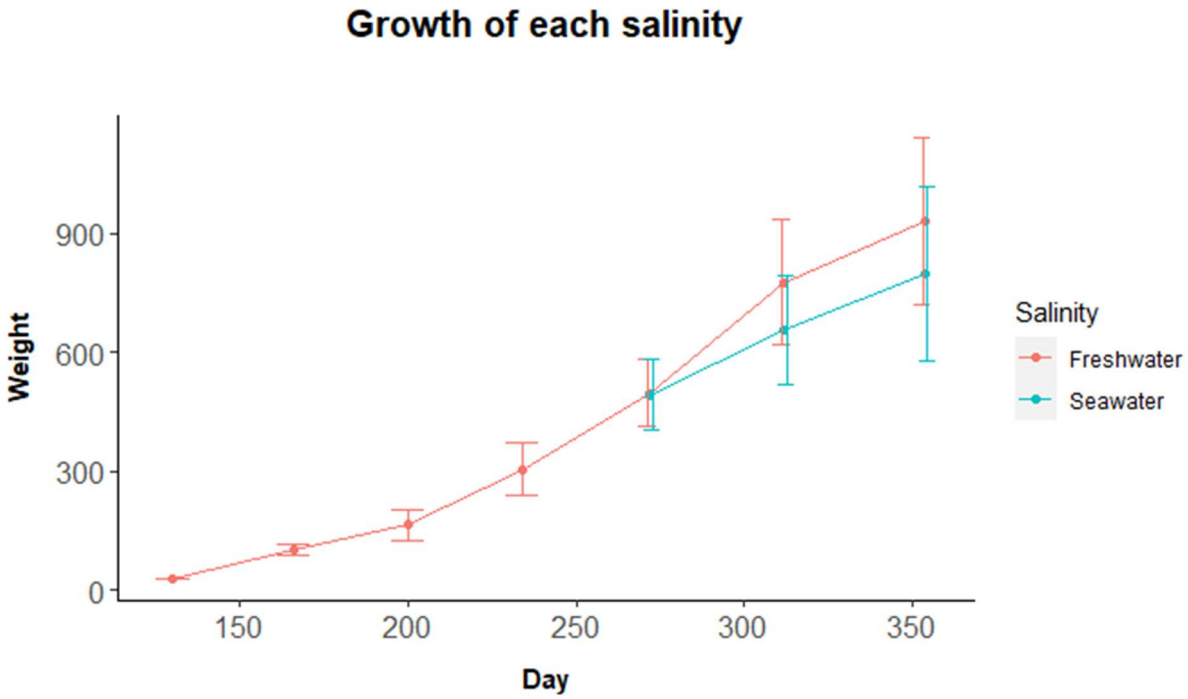

Figure 18: Weight (mean ±sd) of all fish, depending on which salinity the fish were put in at day 272.

3.4.3 Statistical analysis of growth

Through statistical analysis it was found that there is significance of effects of family, genotype and salinity for weight, length, and condition factor (Table 6). Family significantly influenced weight on all days (130, 166, 200, 234, 272, 312, 354). T-20-6 was the biggest family in weight, followed by T-20-8 and the smallest was T-20-1. Family influenced length for all days except 166 and 234, where the level of significance was lower, where family T-20-6 was longest, followed by T-20-8 and the shortest was T-20-1. Family significantly influenced the condition factor for day 166, 234, 312 and 354, little significance was found at day 200 and day 272, but not 130, where there was no influence. For all days with significance, family T-20-6 had highest CF, followed by T-20-8 and the lowest CF was family T-20-1. Genotype had some effect on weight for day 272, where EL had slightly lower weight than EE and LL. Genotype had some effect on CF for day 200, where EE had a slightly higher CF, and day 272, where EL had a slightly lower CF. Salinity had an effect for weight, length, and condition factor, but only for day 312, where growth was reduced in seawater. There was an interaction between family and salinity.

Table 6: Effects through linear models and linear mixed effects models of independent variables, family, genotype, and salinity, on response variables, weight, length, and condition factor, for days after first feeding. Only nonpubertal fish are used for models. Salinity was introduced after 272.

Day	Response variable	Statistic Model	Independent variable	$\chi 2$	Degrees of freedom	P value
130	Weight	LM	Family	393.38	2	<0.001
	Length	LM	Family	38.5.19	2	<0.001
	CF	LM	null	242.93	1	<0.001
166	Weight	LME	Family	22.68	2	<0.001
	Length	LME	Family	11.87	2	0.0026
	CF	LME	Family	16.56	2	<0.001
200	Weight	LM	Family	17819	2	<0.001
	Length	LM	Family	26.42	2	<0.001
	CF	LM	Family	0.05	2	0.020
			Genotype	0.04	2	0.038
234	Weight	LME	Family	37.81	2	<0.001
	Length	LME	Family	13.32	2	0.0013
	CF	LME	Family	46.18	2	<0.001
272	Weight	LME	Family	49.52	2	<0.001
			Genotype	10.15	2	0.0063
	Length	LME	Family	17.93	2	<0.001
	CF	LME	Family	70.09	2	<0.001
			Genotype	8.07	2	0.0177
312	Weight	LME	Family	72.57	2	<0.001
			Salinity	55.00	1	<0.001
			Family × Salinity	6.66	2	0.0359
	Length	LME	Family	43.67	2	<0.001
			Salinity	29.37	1	<0.001
			Family × Salinity	7.70	2	0.0213
	CF	LME	Family	64.63	2	<0.001
			Salinity	89.78	1	<0.001
354	Weight	LME	Family	32.59	2	<0.001
	Length	LME	Family	22.83	2	<0.001
	CF	LME	Family	31.25	2	<0.001

3.5 Prevalence of females

Females occurred in the supposedly all-male population. Family T-20-1 had 2.20% females, T-20-6 had 3.08% females, and T-20-8 had 2.23% females. There were no significant family effects (GLM: $\chi^2 = 3.4$, df = 2, p = 0.1803).

3.6 Distribution by family and genotype

The distribution of each genotype was as expected; 25.03% EE, 49.97% EL and 25.00% LL. The distribution of each family was 1/3 for each family.

4 Discussion

4.1 Generalisations

The focus of this study has been to identify the effects of different genotypes of the gene vgll3 in an all-male population of Atlantic salmon under the specific conditions 13 °C and light 24-hours per day. Through 354 days, the fish grew from parr to post-smolt and big smolts. The fish originated from three different mothers, making three families, all with a heterozygote genotype of the gene vgll3, EL. The outcome was thus 25% EE, 50% EL and 25% LL. Time of puberty is depended highly on fish genotype. EE fish matured first, then EL fish and lastly LL. Thus, it was fulfilling the hypothesis that the allele E promotes early maturation compared to the L allele. Results are similar to previous studies done on the gene vgll3 (Ayllon et al., 2019), where the allele E enters puberty earlier than L. As little is known about vgll3 and its interactions with salinity, 50% of the fish were moved to seawater to quantify the interactions with salinity. The study does not find an increased amount of pubertal fish in seawater compared to freshwater.

4.2 Methodological considerations

At day 0-234 the families were kept in separate tanks, meaning family effects in this period are indistinguishable to random tank effects. Preferably, the families would have been mixed earlier to reduce random variability. To get a divided distribution, the fish were mixed after the genotyping was done to get an equal number of the different genotypes in each tank and equal family distribution in each tank.

The experiment was done in a flowthrough system (FTS), although it would be interesting to see effects in a recirculating aquaculture system (RAS) as a FTS does not allow a build-up of pheromones or other chemical factors which could potentially trigger puberty. However, early maturation should happen in both FTS and RAS, as the reason for triggering early maturation seems to be the intensification of the production (Imsland et al., 2014, Good and Davidson, 2016).

The fish experienced handling at sample points, which was unavoidable since handling was necessary to measure the fish. Fish experience stress during handling, but also from the anaesthesia they are subjected to prior the handling (Carey and McCormick, 1998). On day 200, the 3000 remaining fish were pit tagged; although this requires surgical

implantation, no deaths occurred as a cause of pit tag implantation. As the fish was at a mean weight of 164 g at this sample point, the fish were big enough to handle the procedure (Gries and Letcher, 2002). Individuals who were sampled at each sample point after day 200 were randomly preselected before sampling to get an equal number of fish per genotype, family, and tank.

On day 272, 50% of the fish were transferred to seawater; this was done by changing the tank's input, meaning no handling or transportation was needed, thus reducing potential stress. The fish did not go through a smoltification regime as to not change the constant conditions of the experiment. Ten fish were transferred to seawater on day 273, with ten fish in control (freshwater). The fish went through a 96-hour seawater challenge, where the salinity increased over time, up to 35 ppt. Out of the ten fish in seawater, all of them survived with a blood plasma osmolality of 346.9 milliosmoles. These values were well within the lethal limits of plasma levels (Conte and Wagner, 1965). Even though the fish were not smoltified before the transfer to seawater, previous studies have experienced few to no deaths on fish above a size of 14 cm (Duston, 1994, McCormick et al., 1989).

Throughout the whole experiment (day 0-354), the fish were kept in 13 °C and a 24-hour light regime. High temperature, such as 13 °C, is used to promote growth, but known to induce maturation, whereas 24-hour light regimes is known to inhibit maturation (Fjelldal et al., 2011, Imsland et al., 2014, Good and Davidson, 2016). As the conditions were constant, change in temperature and photoperiod did not occur, meaning the fish did not experience any change of seasons which, given the right conditions, is inducing puberty in wild Atlantic salmon. The fish received 20% excess feed based on body weight. Feed is the fish’s only source of energy and is an important factor of lipid storage.

GSI is a valid indication of identifying puberty and deciding how far along the fish is in sexual maturation. Therefore, a GSI of 0.1 was set as the difference between pubertal fish and nonpubertal fish, which is the same used by Kjærner-Semb et al. (2018). Through sampling of the gonads, there was a distinct difference between the gonads that had started to develop and the undeveloped testis, where the weight difference was immense.

Although the population was all-male in this experiment, there was a prevalence of females in the population. However, the experiment did not focus on females, and thus they were discarded. Sex chromosomes were not identified, meaning the study does not show if

the females are XX or XY. Previously, Fjelldal et al. (2020) found that of the females in their all-male population, 5% were XY, and 95% were XX.

4.3 Impact of vgll3 on the size of maturation

The pubertal fish in this study were indistinguishable from immature fish. These mature fish can be described as mature post-smolts or jacks. The phenotype is rarely seen in the wild for Atlantic salmon, however common in the Chinook salmon (*Oncorhynchus tshawytscha*) (Fjelldal et al., 2011).

Barson et al. (2015) found that vgll3 regulated early maturation in Atlantic salmon. Puberty is triggered by the brain-pituitary-gonadal axis (BPG axis) and is known to interplay with lipid reserves (Kaplowitz, 2008, Sam and Dhillo, 2010, Taranger et al., 2010). Studies in mammals have noted that vgll3 is linked to lipid reserves and sexual maturation (Halperin et al., 2013, Cousminer et al., 2013). The molecular link between the BPG axis and lipid reserves is unknown, but vgll3 seems to be a valid candidate. Energy storage, especially lipids, is essential to initiate pubertal gonad development as sexual maturation is extremely energy-consuming (Jonsson et al., 1997). Studies on Atlantic salmon that looked at vgll3 found that the allele E makes the fish mature earlier than L (Fjelldal et al., 2020, Ayllon et al., 2019). Fjelldal et al. (2011) found that fish with a higher condition factor (CF) were more likely to mature, as the CF in pubertal fish were higher than immature fish. CF is linked to lipid reserves (Herbinger and Friars, 1991), meaning that if the CF is high, there are more lipid reserves, which in turn affects the BPG axis and increases the chances for salmon to enter puberty. Family T-20-6 was both most likely to enter puberty early (Table 3, Figure 12). The CF was higher as well, suggesting a higher amount of lipid stores. This could be why T-20-6 is more likely to enter puberty earlier than T-20-8 and T-20-1.

The genotype group with the least pubertal fish was LL, followed by EL and final EE with most pubertal fish (Figure 10). Genotype was the most important factor in this experiment on whether the fish entered puberty or not. The data from testis weight revealed that EE fish developed a higher GSI earlier than EL and EL earlier than LL (Figure 14). This reinforces the hypothesis that EE fish enter puberty earlier than EL fish and EL fish enter puberty earlier than LL fish, and supports current knowledge of vgll3 (Kjærner-Semb et al., 2018, Ayllon et al., 2019, Fjelldal et al., 2020).

There is a clear link between family and growth, and in turn, family and puberty. The growth data analysis is of nonpubertal fish, as the fish grow differently during puberty. The family with the highest mean weight, T-20-6 (Table 5), is also the family with the most pubertal fish, and the family with the lowest mean weight, T-20-1, was the family with the least pubertal fish. An initial growth spurt is often linked to puberty, followed by a decline in growth as puberty progresses (Taranger et al., 2010, Imsland et al., 2014, Fjelldal et al., 2011). However, it is unknown whether the fish initiates puberty because of a growth spurt, or if the growth spurt happens because the fish initiates puberty. As the heaviest family in this study is more likely to initiate jacking, there is reason to believe that the growth spurt has happened before or during initiation in puberty. In contrast, Fraser et al. (2019) did not find the growth spurt in jacks; however, they did find that jacks were smaller at the final sample point. Because the statistical analysis was done for nonpubertal fish, we do not see the effects of puberty on growth spurt and thus may not determine whether the growth spurt might have happened.

4.4 Impact of salinity on maturation

As the Atlantic salmon migrates to the ocean, the body needs to change the regulation of salinity; the salmon needs ions in freshwater and needs water in seawater. The fish goes from being hyperosmotic in freshwater to being hypoosmotic in seawater. The transition to seawater, therefore, requires energy (Heggberget, 1992). Duston (1994) found an increase of mature parr with higher salinity, but de Fonseka et al. (in prep) and Ytrestøyl et al. (2020) did not. There was a lower mean of GSI in seawater than freshwater (Table 4, Figure 16DE). The Atlantic salmon needs to use the energy reserves to handle the change in osmolality instead of maturation, which could be why the GSI is lower in seawater. In addition, the fish is gaining less weight after the sea transfer, indicating that energy is needed for changes in osmolality instead of growth and gametogenesis, consistent with previous smolt studies (Berrill et al., 2006, Heggberget, 1992). Although there is significant difference in GSI between freshwater and salinity, there is not a significant difference in the percent of pubertal fish (Figure 13, Table 3). This effectively means that testis growth was greater in freshwater fish but did not affect initiation of puberty, which is consistent with Melo et al. (2013).

Figure 17 (C and F) shows that between day 166 and 272, there was a dip in the condition factor (CF). This happened before the fish was exposed to seawater. The reason

behind this is most likely because the fish reached a specific size and want a more streamlined morphology to handle the ocean, as a reduction in condition factor is associated with smoltification (Hoar, 1939, Wedemeyer et al., 1980). However, the CF returned up after the reduced period, increasing CF again. CF is a reasonable estimate of the lipid reserves in the fish (Herbinger and Friars, 1991), meaning that high energy reserves in the form of lipids may affect whether or not the fish enters puberty. It would be interesting to see what would have happened had the fish undergone seawater transfer earlier to match the change in morphology changes.

4.5 Context – how is this relevant to the industry?

As previous studies (Ayllon et al., 2019, Fjelldal et al., 2020, Verta et al., 2020) have found similar results on the effects of vgll3 as the current study, the industry can use artificial selection to reduce early puberty, especially in land-based on-growing facilities. Land-based facilities are increasingly popular (Bjørndal and Tusvik, 2019), big smolt and post-smolt production can produce Atlantic salmon at a higher temperature, which in turn reduces the time needed to produce fish ready for harvest. Post-smolt and big smolt are better prepared for seawater transfer, as they are bigger and have a greater chance of survival in the sea. By reducing exposure to open fjords and seawater from 18 to 10 months, it will lead to less disease and parasites such as salmon louse (Lekang et al., 2016), which may lead to an increase in the quantity and quality of Atlantic salmon production. In addition to health benefits, companies may increase production by freeing up legal biomass capacity, as the fish is in land-based facilities instead of the sea in a bigger part of the production cycle.

This study also has passive implications for wild salmon. Reduced pressure on disease and parasites because of post-smolt or big smolt production may lead to a safer environment along the Norwegian coast, increasing the probability of wild salmon surviving their journey and become able to reproduce in rivers. Artificial selection of certain genotypes will narrow the gene pool of the salmon, making escapees even more dangerous for wild populations. If the narrow gene pool of cultured Atlantic salmon mixes with the broad gene pool of the wild Atlantic salmon, there may be irreversible unpredicted consequences (Pulg et al., 2020).

Although all-female populations are also possible (Galbreath and Thorgaard, 1995), males grow faster (Rye and Refstie, 1995, Aunsmo et al., 2008b, Leclercq et al., 2010).

Through an artificial selection of vgll3 in an all-male population, the industry can further increase production rates.

4.6 Further research

As this study has differences in the three families (T-20-1, T-20-6 and T-20-8), there may be genetic differences with effects on maturation other than vgll3. There are other genes that may influence maturation similarly to vgll3. Notably, akap11 and six6 (Kurko et al., 2020), interactions between vgll3 and the former should be identified. The families may have different genotypes of six6 and akap11 and therefore should be genotyped in order to determine whether or not they are responsible for the difference in family effects.

As vgll3 was linked to size at maturity (EE fish mature at smaller sizes), it would be interesting to study how vgll3 affects oxygen delivery in salmon as this is predicted to regulate the size at maturation (Meyer and Schill, 2021). Interestingly, vgll3 is expressed not only in the gonads but in the heart and gills as well (Kjærner-Semb et al., 2018), two organs that play a central role in oxygen delivery. Gill-oxygen-limitation-theory (GOLT) predicts that smaller gills and bigger body size will reduce the oxygen available in the bloodstream and will reduce metabolism and thus might induce maturation, as it will be insufficient for the fish to become bigger in order to reproduce, as it is more oxygen demanding with a limited oxygen supply (Meyer and Schill, 2021, Pauly, 2021). Comparatively to GOLT, the allele E of vgll3 induces maturation much earlier than what is ideal in the wild.

4.7 Conclusion

Findings have shown that the vestigial-like protein 3 (vgll3) gene influences size of maturation in Atlantic salmon in constant light (24-hours a day) and 13 ºC. Fish with the genotype EE enter puberty first, thereafter the genotype EL and finally LL. This happens most likely because fish with the genotype EE has a lower energy (lipid) storage needed to initiate puberty. Seawater had no significant effect on the frequency of post-smolt maturation (jacking) of Atlantic salmon and is therefore unlikely a trigger for puberty in post-smolts. Artificial selection of the gene vgll3 can help the industry transition to more sustainable production through post-smolt or big smolt production.

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