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Faculty of Biosciences, Fisheries and Economics, Department of Artic and Marine Biology

Effects of diet and the *vgll3* locus on male maturation and sperm traits of Atlantic salmon

Regine Amanda Larsen

Master's thesis in aqua medicine BIO-3955, (60 hp) June 2024

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Abstract

A challenge in Norwegian Atlantic salmon (*Salmo salar*) aquaculture is unwanted early maturation in male fish. Environmental factors such as diet and genetic factors such as the *vgll3* locus, are key mediators of male maturation and reproduction. In this study, different feeding treatments, alongside the *vgll3* locus, was analyzed to assess their influence on maturation and sperm traits on four-year old Atlantic salmon raised in a common-garden. This study utilized first-generation hatchery broodstocks from two Finnish populations to produce fish with different *vgll3* genotypes. Fish were fed either a high fat feed or low fat feed representing a ~22% reduction in dietary energy. Sperm traits were assessed using Computer-Assisted Sperm Analysis (CASA) system. Significant differences in body length were observed between populations, with Oulu River males being smaller than Neva River males. Maturation showed no significant differences in *vgll3* genotypes, diet, or population. No significant *vgll3* effects were found on sperm traits, but Neva River fish had significantly higher linearity and straightness, and low fat diet fish had higher average pathway velocity and curvilinear velocity estimates. Sperm traits generally performed better with a low fat diet suggesting that a low fat diet might be more beneficial for sperm quality in Atlantic salmon.

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Regine Amanda Larsen

Table of contents

List of tables

List of figures

List of Abbreviations

ALH	Amplitude of Lateral Head displacement (μm)
BCF	Beat-Cross Frequency (Hz)
CASA	Computer-Assisted Sperm Analysis
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FCR	Feed Conversion Ratio
GLMM	General Linear Mixed Model
GnRH	Gonadotropin-Releasing Hormone
GWAS	Genome-Wide Association Study
HF	High fat feed
LC-PUFA	Long-chain polyunsaturated fatty acid
LIN	Linearity (%)
LBS	Lammi Biological Station
LF	Low fat feed
LMM	Linear Mixed model
PCR	Polymerase Chain Reaction
PIT	Passive Integrated Transponders
PUFAs	Polyunsaturated fatty acids
RAS	Recirculating Aquaculture System
SD	Standard Deviation
SE	Standard Error
SNP	Single Nucleotide Polymorphism
STR	Straightness (%)
TL	Total length
VAP	Average Path Velocity (μm/s)
VCL	Curvilinear Velocity (μm/s)
Vgll3	Vestigial-like protein 3 gene
VSL	Straight Line Velocity (μm/s)
WOB	Wobble (%)

x

1 Introduction

1.1 Introduction to Atlantic salmon

The Atlantic salmon (*Salmo salar*) is a keystone species in aquatic ecosystems and serves as a cornerstone of global fisheries and aquaculture industries (Friedland et al., 2009). It holds a significant ecological, economic, and cultural importance worldwide, captivating the attention of scientists, conservationists, and stakeholders alike (Jonsson & Jonsson, 2011). Known for its remarkable migration journey, ecological role in freshwater and marine ecosystems, the wild Atlantic salmon stands as an iconic symbol of biodiversity, sustainability and human-environment interaction (Einum & Fleming, 1997).

In contrast to its wild counterpart, farmed Atlantic salmon has become a major industry, with global production reaching millions of tons annually (FAO, 2020). Major producing countries include Norway, Chile, Scotland, and Canada, which together dominate the market (Naylor et al., 2000). Farmed Atlantic salmon is a vital resource in the global food supply, providing a significant and sustainable source of protein and essential omega-3 fatty acids to meet the world's increasing demand for nutritious food (Mozaffarian & Rimm, 2006).

Farmed salmon is considered one of the most efficient and sustainable sources of protein compared to other animal production for several compelling reasons. Salmon have impressive feed conversion ratio (FCR), requiring only about 1.2 kg of feed to produce 1 kg of body mass, which is significantly lower than terrestrial animals (Naylor et al., 2009). FCR is an essential metric for assessing the efficiency of feed utilization in aquaculture, measuring the amount of feed required to produce one kg of fish biomass (Nordgarden et al., 2003). This efficiency stems from their ectothermic or cold-blooded nature and lower energy requirements for body maintenance compared to endothermic or warm-blooded animals like chicken, pigs and cattle (Boyd & McNevin, 2015; Jonsson et al., 2013). The carbon footprint of farmed salmon is relatively low due to their efficient feed conversion and the reduced need for land-based feed production (Naylor et al., 2009). Additionally, farmed salmon generally use less freshwater compared to livestock, further contributing to their sustainability (Verdegem et al., 2006). Advances in aquaculture technology, such as improved breeding practices, disease management, and recirculation aquaculture systems (RAS), also contribute to the sustainability of salmon farming (Good & Davidson, 2016). These technologies help

optimize resource use and minimize environmental impact, thus promoting sustainability in salmon farming. Farmed salmon have also become synonymous with non-sustainability and negative human-environment interactions. Concerns have been raised about issues such as pollution, disease transmission to wild populations, and the considerable ecological footprint of fish farming practices for quite some time (Naylor et al., 2000; Naylor et al., 2021). Therefore, addressing these challenges is essential to ensure that the benefits of farmed salmon can be realized without compromising environmental integrity and biodiversity.

1.2 Maturation in Atlantic salmon

The life history of Atlantic salmon is characterized by its complex maturation process. Maturation, the transition to sexual maturity, is a critical life event in Atlantic salmon, significantly impacting populations dynamics, reproductive success, and commercial value (Good & Davidson, 2016; Mobley et al., 2021). This complex process is regulated by a multitude of genetic, environmental, and physiological factors to maximize the number of offspring, otherwise known as reproductive success (Garcia de Leaniz et al., 2007; Mobley et al., 2021). The considerable variation in age and size at maturation within this species is believed to be an evolutionary adaption to diverse river and ocean environments, aimed to maximize reproductive success (Good & Davidson, 2016; Mobley et al., 2021). Maturation is energetically demanding and thus involves trade-offs with other aspects of fitness such as growth and survival (Buoro et al., 2010; Mobley et al., 2021). This process necessitates the redirection of stored resources that were previously allocated for growth or maintenance towards gonad growth and development, gamete production, the development of secondary sexual structures, and sexual behaviors (Bernardo, 1993; Mobley et al., 2021).

1.3 Maturation in farming

In the context of farming, sexual maturation in Atlantic salmon, particularly male fish, poses significant challenges for aquaculture operations. Early maturation, characterized by individuals reaching sexual maturity at a younger age or smaller size than optimal for commercial production, can lead to reduced growth rates, reduced fish welfare, increased mortality, diminished flesh quality, and decreased market value (Fjelldal et al., 2011). Additionally, early maturation may disrupt production schedules and increase management complexities, as mature individuals may require separate handling and different nutritional

regimes to ensure optimal health and welfare (Leclercq et al., 2010). Effective management of maturation processes is crucial in Atlantic salmon farming to maintain productivity and economic viability. Various strategies have been explored to mitigate early maturation, including selective breeding programs aimed to enhancing desirable traits, the use of photoperiod manipulation to influence hormonal cycles, implementation of specific dietary adjustments to delay sexual maturity and production of sterile triploid salmon (Fjelldal & Hansen, 2010; Peruzzi et al., 2018; Taranger et al., 2010; Taranger et al., 1999). Moreover, genetic and environmental factors influencing maturation rates must be comprehensively understood and managed. Research has shown that both genetic predisposition and environmental conditions such as water temperature and light exposure significantly impact maturation timing (Aykanat et al., 2019; Barson et al., 2015; Good & Davidson, 2016; Jonsson & Jonsson, 2011).

The use of land-based recirculating aquaculture system (RAS) has been associated with an increased incidence of early maturation, particularly in males (Good & Davidson, 2016). Factors such as water temperature fluctuations, photoperiod manipulation, stocking densities, and feed composition in RAS can influence the hormonal balance and physiological development of the fish, potentially leading to early maturation (Fjelldal et al., 2011; Melo et al., 2014). Therefore, a multifaceted approach combining genetic selection, environmental control, and advanced husbandry techniques is essential to address the challenges posed by early maturation in Atlantic salmon effectively. By addressing these issues, the aquaculture industry can improve growth performance, enhance product quality, and achieve more predictable and efficient production cycles, ultimately contributing to the sustainable and profitability of Atlantic salmon farming operations.

1.4 Genetic regulation of maturation

The gene Vestigial-like protein 3, or *vgll3,* is involved in a range of biological functions, such as reproductive processes, maturation timing, adipocyte differentiation, and lipid metabolism (Ahi et al., 2024; Ayllon et al., 2015; Barson et al., 2015; Halperin, 2013; Mobley et al., 2021). The *vgll3* gene has emerged as a significant regulator of maturation timing and dynamics in Atlantic salmon populations (Barson et al., 2015). The *vgll3* gene is part of a large effect locus that explains up to 40% of variation in sea age at maturity, or the years spent at sea before maturing and returning to freshwater rivers to spawn (Barson et al., 2015).

The *vgll3* locus encompasses alleles for early (E) and late (L) maturation, with the E allele being dominant in males (Barson et al., 2015). Genetic variations within the *vgll3* locus have been associated with differences in maturation phenotypes, including age at maturity and reproductive success (Mobley et al., 2019; Mobley et al., 2020). The *vgll3* gene is believed to influence maturation through its involvement in hormonal signaling pathways and reproductive physiology, highlighting the genetic basics of maturation variability in Atlantic salmon (Kurko et al., 2020).

Variation in *vgll3* is associated with gene transcript structure in immature male gonad tissues, suggesting *vgll3* controls testis differentiation in Atlantic salmon (Verta et al., 2020). Additionally, *vgll3* is associated with the regulation of adipogenesis in mouse, highlighting its crucial role in lipid storage and metabolism in other vertebrates (Halperin, 2013). Several Genome-Wide Association studies (GWAS) have revealed a significant association between the *vgll3* region and maturation in Atlantic salmon (Ayllon et al., 2015; Ayllon et al., 2019; Barson et al., 2015). Although a genetic basis for age at maturity has been found (Barson et *al.*, 2015), these strategies are not solely determined by genetics, but also exhibit phenotypic plasticity, as genotype-environment interactions and growth conditions play a role in the sexual maturation process (Debes et al., 2021).

1.4 Environmental and dietary influences on maturation

Environmental factors, including dietary components, temperature, and photoperiod, have been shown to influence maturation processes in both wild and farmed Atlantic salmon (McCormick et al., 2013; Mobley et al., 2021; Taranger et al., 2010). Diet plays a crucial role in the maturation process of Atlantic salmon due to its influence on energy allocation and physiological development (Kadri et al., 1996). Maturation is an energetically demanding process, requiring substantial resources for gonad growth, gamete production, and the development of secondary sexual structures (Bernardo, 1993; Mobley et al., 2021). Therefore, the availability and quality of food resources directly impact the energy reserves and nutritional status of the fish, which in turn affect their ability to undergo maturation. Studies have demonstrated that variation within the *vgll3* locus and dietary components play a significant role in shaping maturation dynamics in Atlantic salmon (Aykanat et al., 2019; Debes et al., 2021; Mobley et al., 2021; Åsheim et al., 2023). High-quality proteins and essential fatty acids are crucial for the synthesis of hormones like gonadotropin-releasing

hormone (GnRH) and gonadotropins, which are key regulators of gonad development (van der Kraak, 2009). Adequate levels of these nutrients support the hormonal pathways necessary for maturation. Furthermore, studies have revealed that dietary compositions rich in specific nutrient can influence maturation rates in salmonid fishes, also underscoring the significance of dietary factors in reproductive physiology (Farkas et al., 2021; Heath et al., 1997).

A recent study by Åsheim et al. (2023) on male maturation in Atlantic salmon, using a common garden design, found that body condition and body mass was strongly associated with maturation probability in male Atlantic salmon. The study also revealed that the *vgll3* genotype, particularly the early maturation *vgll*3*E allele, was significantly associated with a higher maturation probability. However, the study by Maamela et al. (2023), also using the same fish, did not find any significant effect of the feed on female maturation. These studies suggest that the diet can affect maturation differently in males and females.

Diet and feeding practices are crucial considerations in aquaculture management, optimizing growth, maximizing reproductive performance, maintaining fish health, and ensuring economic profitability and environmental sustainability (Hardy, 2010). Recent advances in feeding technology include under-water feeding systems and integrated feed barges, improving feed efficient, reducing waste, and enhancing fish welfare by delivering feed directly to the fish with precise control (Moe Føre et al., 2022). Feeding *ad libitum* or allowing fish to consume food freely and in abundance, ensures that the fish have access to sufficient energy to support energetically demanding processes of maturation and spermatogenesis (Taranger et al., 2010). Competition for food in aquaculture settings can also lead to stress, aggression, and uneven growth among the populations (Jobling, 2001). Proper growth is essential for achieving reproductive competence and maximizing reproductive success (Einum & Fleming, 2000). The condition factor, weight and length of the fish are crucial parameters influencing maturation and sperm quality in Atlantic salmon, serving as indicators of overall health and nutritional status (Vasemägi et al., 2010). The condition factor is influenced by nutrient supply, and a higher nutritional status will allow higher energy consumption for further growth or processes that are necessary for maturation and investment in sperm cells (Ng'oma et al., 2017; Taranger et al., 2010).

It is well established that dietary fats play a crucial role in various physiological processes, including reproductive functions in various fish species, including Atlantic salmon (Bell & Sargent, 2003; Izquierdo et al., 2001; Lahnsteiner et al., 2009; Tocher, 2003). Fatty acids (FAs) are the primary components of the sperm membrane, governing the fluidity of the lipid layer (Lenzi et al., 1996). Polyunsaturated fatty acids (PUFAs), particularly omega-3 fatty acids like docosahexaenoic acid (DHA) and eicosapentaenoic acids (EPA), have been associated with improved sperm quality (Bell & Sargent, 2003; Tocher, 2003). However, fatty acids can have both positive and negative effects on sperm quality in fishes, depending on their type and concentration. An excess or imbalance of certain fatty acids such as highly unsaturated fatty acids, oleic acid, cholesterol and linoleic acid have been shown to influence sperm quality and fertility negatively in Atlantic salmon (Díaz et al., 2021), which also applies for mammals (Collodel et al., 2020; Crean & Senior, 2019; Esmaeili et al., 2015; Jensen et al., 2013). High levels of polyunsaturated fatty acids (PUFAs) can make sperm membranes more susceptible to lipid peroxidation, which can damage the sperm midpiece, axoneme, and DNA, leading to loss of sperm motility (Izquierdo et al., 2001; Lahnsteiner et al., 2009). Thus, while fatty acids are essential for sperm health in fishes, their imbalance or excess can potentially impair sperm quality and reproductive success.

1.6 Sustainable feed production

The steady decline in wild fish catches combined with the rising demand for aquaculture feed has led to a rapid reduction in the availability of fishmeal and fish oil, accompanied by a significant increase in their prices (FAO, 2020). Intensive global efforts are underway to develop alternative sources of protein and essential n-3 long-chain polyunsaturated fatty acids (LC-PUFA) for use in aquacultures driven by the depletion of wild fish populations and the environmental impact of soy production, including rainforest destruction (Albrektsen et al., 2022). Significant advances in feed ingredients for sustainable aquaculture have been made, focusing on low-trophic marine species like krill, copepods and algae, which offer sustainable nutrient sources and reduce reliance on traditional feeds (Albrektsen et al., 2022; Kousoulaki et al., 2022). Novel microbial ingredients from bacteria, yeast, and microalgae present promising alternatives converting non-food organic waste into high quality nutrients, supporting a circular economy. Insect-based feed, including black solider fly, yellow mealworm, and crickets, provide protein-rich, environmentally friendly options reared on organic waste (Albrektsen et al., 2022). Advanced processing technologies improve

nutritional quality and reduce anti-nutritional factors, ensure feed safety and efficacy (Albrektsen et al., 2022).

While these advancements are promising, there is a potential risk of compromising feed quality. Alternative feed ingredients may have different nutritional profiles compared to traditional sources like fishmeal and fish oil, requiring assurance that they provide adequate essential nutrients. Anti-nutritional factors in some alternative feed, particularly plant-based diets, may interfere with nutrient absorption or digestion, necessitating proper processing to mitigate these issues (Hardy, 2010). Additionally, unconventional feed sources such as insects or microbial biomass may carry contaminants if not produced under controlled conditions, impacting feed safety and the health of aquacultures species. The palatability and digestibility of these new ingredients may vary, affecting their acceptance and utilization in some cultured species (Merrifield et al., 2011). Compliance with regulatory standards and quality measures is essential to ensure feed safety and quality.

Alternative food resources in aquaculture, such as plant-based ingredients, insects, and microbial sources, face challenges in providing sufficient omega-3 fatty acids (EPA and DHA), essential for growth and health of salmonid species. To address this, researchers are exploring strategies like supplementing feed with microalgae-derived oils rich in DHA, selective breeding to enhance fish's capacity to biosynthesize EPA and DHA, formulating combination diets to balance essential nutrients, and using nutritional additives to improve omega-3 absorption (Leaver et al., 2008; Sprague et al., 2017). Ongoing research and innovation aim to overcome these challenges, ensuring the nutritional adequacy of alternative aquafeeds for salmon species and promoting sustainable feed practices.

A balanced diet with adequate intake of all macronutrients like protein, fats, and carbohydrates, aids in the production of essential hormones, energy metabolism, and the mitigation of oxidative stress (Izquierdo et al., 2001). Ensuring a balanced diet with appropriate levels of dietary fats, protein and carbohydrates is essential for maintaining optimal health and reproductive success.

1.5 Reproductive strategies

Reproductive strategies in Atlantic salmon are intricately shaped by a combination of environmental cues, genetic predispositions, and individual experiences (Mobley et al., 2021). One of the defining features of their reproductive behavior is anadromy, where adults migrate from marine habitats to freshwater rivers and streams for spawning (Fleming, 1998). However, there are also freshwater-resident forms of Atlantic salmon, known as landlocked salmon, which complete their entire life cycle in freshwater environments, adapting uniquely to these habitats (Cauwelier et al., 2018). Within the freshwater environments, Atlantic salmon exhibit a variety of spawning strategies, including synchronous spawning events, staggered spawning periods, and even prolonged spawning runs (Jonsson & Jonsson, 2009). These strategies are influences by factors such as water temperature, photoperiod, and the availability of suitable spawning habitats (Fleming, 1998). Additionally, the timing of migration and spawning can vary among populations. Reflecting adaptations to local environmental conditions and selective pressure (Quinn et al., 2005).

In numerous populations of Atlantic salmon, the proportion of males and females available for reproduction is heavily skewed towards males (Fleming, 1996, 1998; Fleming & Reynolds, 2004). This skewed sex ratio impacts the level of competition between the sexes and determines which sex compete more intensely for the other, thus influencing the strength of mate competition, leading to increased selection pressure on males to maximize their productive efforts (Kvarnemo & Ahnesjo, 1996).

1.6 Sperm traits

Sperm traits in Atlantic salmon encompass a diverse array of characteristics crucial for male reproductive success and fertilization dynamics (Gage et al., 2004; Morrow & Gage, 2001). Among these traits, parameters such as linearity (LIN), curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) are integral for understanding sperm swimming characters and efficiency (Pitcher et al., 2007). LIN describes the alignment of the sperm trajectory, with higher values indicating straighter paths and potentially more efficient swimming towards the eggs (Parker et al., 1996). VCL, VSL, and VAP represent different aspects of sperm velocity, with faster swimming speeds associated with improved chances of fertilizing eggs (Gage et al., 2004; Morrow & Gage, 2001). Moreover, the beat cross-

frequency (BCF) reflects the frequency of lateral head oscillations, influencing the propulsion and forward movement of sperm (Pitcher et al., 2007). Additionally, sperm elongation and straightness (STR) play significant roles in determining swimming efficiency and penetration capabilities. ALH, which measures the lateral movement of the sperm head, influences the agility and navigation ability of sperm within the aquatic environment. Additionally, wobble (WOB) reflects the irregularity of sperm movement, with lower wobble indicating smoother, more coordinated swimming motions that could contribute to increased fertilization success (Pitcher et al., 2007).

By considering how genetic factors and environmental conditions, including diet, shape sperm traits, we can gain deeper insight into the dynamics driving male fertility of Atlantic salmon. This understanding is crucial for informing conservation efforts, aquaculture practices, and strategies aimed at preserving the genetic diversity and reproductive health of Atlantic salmon populations.

1.7 Research objectives

Understanding the interplay between genetic and environmental factors is crucial for unraveling the complexities of maturation and sperm traits, and its implications for population dynamics, conservation efforts, and sustainable recourse management. In this study, I will investigate how the *vgll3* gene and dietary energy content affect maturation and sperm traits in Atlantic salmon. This study is a continuation of a common garden experiment that is described previously (Debes et al., 2021; House et al., 2021; Maamela et al., 2023; Åsheim et al., 2023). I will use the same experimental setup used in these experiments. This study is different because it analyzes maturation and sperm trait data from four-year old males. These data have not yet been published. Using data from the common garden experiment, I will investigate the following:

1) **Are there any differential *vgll3* genotypic effects or environment-dependent effects in terms of different feeding treatments on male maturation?**
I expect to find that different *vgll3* genotypes will affect maturation, specifically that the *vgll3**EE locus will be associated with a higher maturation rate, whereas the *vgll3**LL locus will correspond with lower maturation rate. I also expect that the high fat treatment will result in earlier maturation due to better nutrition support compared to the low fat treatment. These

expectations are grounded in the understanding of the *vgll3* genotypes' role in maturation timing and that nutrition, particularly fat content in the feed, influences maturation processes.

2) **Are there any differential *vgll3* genotypic effects or environment-dependent effects in terms of different feeding treatments on phenotypic sperm traits?**

The *vgll3* locus has been implicated in the regulation of reproductive timing and maturation in various species. Given the role of *vgll3* locus in controlling timing of sexual maturation, it is reasonable to hypothesize that the v*gll3* locus could also influence other reproductive traits, such as sperm quality. I also expect that high fat treatment may enhance sperm quality due to improved nutritional support, while low fat treatments might result in reduced sperm quality. These expectations are based on the understanding that nutrition, particularly fat content, plays a role in reproductive health and sperm development.

2. Method

2.1 Experimental setup

2.1.1 Parental stocks and crosses

Details on the population of origin, breeding, and rearing protocols of the experimental fish are provided by Åsheim et al. (2023), Maamela et al. (2023), Debes et al., (2021) and House et al., (2021). Briefly, the experiment involved first-generation hatchery Atlantic salmon broodstocks from two different populations: the Oulu River (Oulujoki) in Finland and the Neva River (Nevajoki) near St. Petersburg in Russia. The parental individuals were homozygous for *vgll3* genotypes and grouped into 2x2 crosses, whereby one individual of each sex had a *vgll3**EE genotype, and one of each sex had a *vgll3**LL genotype, resulting in families with *vgll3**EE, *vgll3**EL, *vgll3**LE and *vgll3**LL genotypes (the *vgll3* allele originating from the mother is listed first). In total, there were 14 2x2 crosses from the Oulu River, and 13 2x2 crosses from the Neva River, resulting in 50 and 42 families respectively, with 1470 offspring (Figure 1).
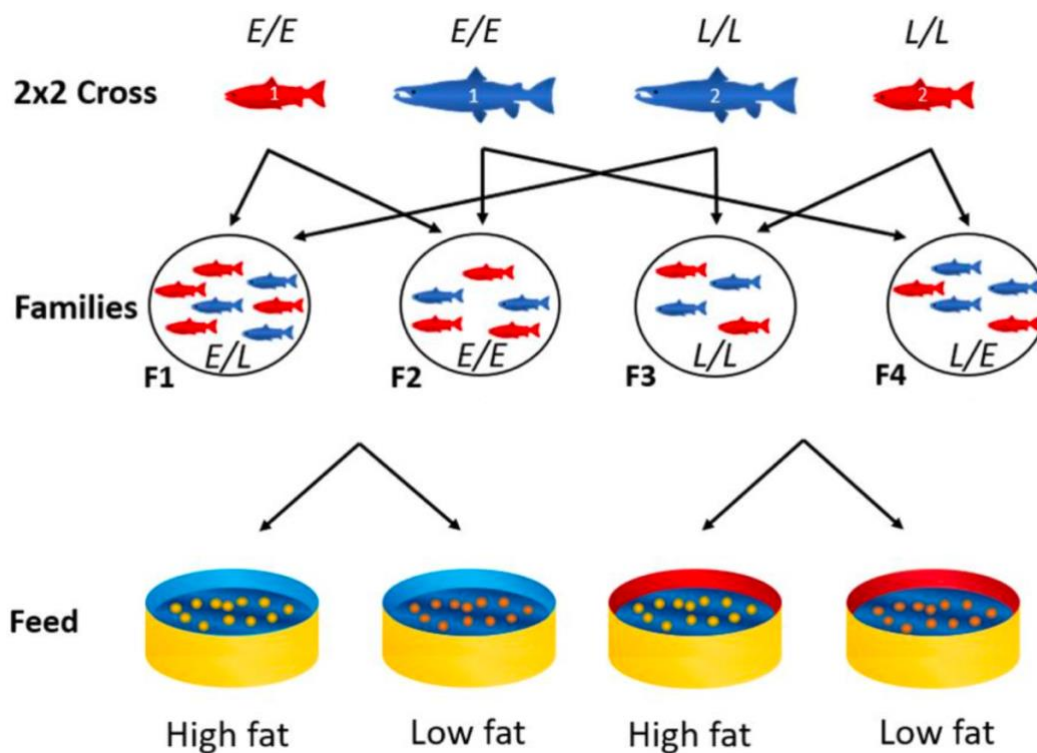
Figure 1. Experimental setup. Parental individuals (homozygous for the *vgll3* locus) from two populations were crossed and grouped into 2x2 crosses. The families were split across tanks with either high fat feed or low fat feed. The colors of the fish represent sex, red is females, and blue is males (Jagusch, 2020).

2.1.2 Rearing conditions

The offspring of the crosses were hatched in late 2017 in incubators at the University of Helsinki Viikki Campus and were transported after first feeding to Lammi Biological Station (LBS) in March 2018. At LBS the fish were split among twelve circular tanks with a flow-through system of water supplied from a local lake. The water was treated with UV light to reduce pathogen load before entering the tanks. The tanks had a circular flow created by a horizontal spray bar, and the water temperature followed seasonal lake temperature curve. The water was heated ~1°C from ambient lake water temperature which corresponds to the warm treatment in Maamela et al., (2023), Åsheim et al., (2023), Debes et al., (2021) and House et al., (2021) papers. The mean temperature of the water was 8.6°C. Lighting was automated to follow local sunrise/sunset times.

2.1.3 Initial feeding

Initially, all the fish were given a common Hercules Baltic blend (Raisioaqua, Raisio, Finland) fish feed of appropriate pellet size, allowing them to feed *ad libitum* based on their mass. To ensure that the fish were fed *ad libitum*, the feeding rate parameter was adjusted based on weekly appetite checks, and if increased feeding activity was noted, the feeding rate parameter of the automatic feeding system was increased to ensure the fish were fed in excess. The feed was administered by using an automatic feed system (Arvo-Tec Oy, Huutokoski, Finland).

2.1.4 Experimental feeding

Starting from July 10th 2019, the six tanks used in this experiment were divided into two treatment combinations, resulting in three tanks of each feed treatment. After March 2021, the fish were evenly distributed in to an additional six tanks to compensate for their growth. Tanks of different treatment were evenly distributed throughout the research facility to minimize treatment-location correlations. The pellet size was adjusted based on the fish size (Åsheim et al., 2023). The fish in the high fat treatment received regular Raisioaqua Baltic Blend aquaculture-grade feed (17-26% fat, 18.10-20.40 KJ/g depending on pellet size), and the fish in the low fat treatment received feed that was replaced with a custom-made fat-reduced feed of the same brand. This resulted in pellets of similar size and shape to the high fat feed but with lower fat content (12-13% fat, 17.25 KJ/g) (Table 1). This low fat content results in a 20-25% decrease in energy content compared to the high fat feed, as verified by independent analysis conducted by Synlab Oy, Karkkila, Finland (Table 1).

The relative contrast in fat content between the two feed treatments varied according to pellet size, with the distinction becoming more pronounced as the pellet size increased with the age of the fish (Åsheim et al., 2023). The high fat feed had higher fat and overall energy content, whereas the low fat feed contained a relatively higher proportion of protein (Åsheim et al., 2023). This feeding strategy was selected over food reduction to minimize the potential behavioral effects associated with reduced food quantities, such as heightened territorial behavior among more aggressive individuals (Orlov et al., 2006). The decreased energy content in the low fat treatment has the potential to affect the condition of the individuals and adipose tissue synthesis (Todorþeviü, 2009). Therefore, comparing fish raised on low and high fat feed allows for the investigation of the impact of nutritional status on maturity rates and sperm traits.

Table 1. Nutritional overview of different feed types and pellet sizes. The table provides a nutritional overview of the different feeding treatments, including the intended fish size range for each pellet size (Åsheim et al., 2023).

Size (mm)	Fish size (g)	Type	% Moisture	% Ash	% Fat	% Protein	% Carbs	Energy (kj/g)
0,8	0.8-4	High fat	4,8	8,4	16,9	57,6	12,3	18,1
1,2	4-15	High fat	5,4	7,2	17,7	53	16,7	18,4
1,2	4-15	Low fat	6,3	6,8	13,4	53,3	20,2	17,5
1,7	15-30	High fat	6,3	7,3	21,6	48,9	15,9	19,0
1,7	15-30	Low fat	8,4	7,9	13,7	51,1	18,9	17,0
2,5	25-70	High fat	5,5	6,6	22,3	47	18,7	19,4
2,5	25-70	Low fat	6,0	7,3	12,8	52,2	21,7	17,3
3,5	60-125	High fat	4,9	5,8	26,2	45,3	17,8	20,4
3,5	60-125	Low fat	5,6	6,9	11,7	53,6	22,2	17,2
5	110-500	High fat	7,7	4,9	29,7	38,4	19,6	20,8
5	110-500	Low fat	8,3	6,5	9,7	50,3	25,8	16,5
7	450-2300	High fat	5,9	4,3	33,7	35,8	20,0	22,0
7	450-2300	Low fat	6,4	6,1	9,0	48,8	29,3	16,6

2.1.5 Genotyping and sequencing

Before the initiation of the experiment, both parents and experimental progenies underwent genotyping using multiple-PCR for 177 single nucleotide polymorphisms (SNPs) (Aykanat et al., 2016). Sequencing was conducted on an Illumina platform (MiSeq or Next-Seq). Additionally, 984 brood stock individuals, from which the parents were selected, were sequenced using Ion Torrent. Parentage was assigned to offspring using a maximum likelihood-based method (Jones & Wang, 2010). Here, 131 unlinked SNPs and information about the crossing scheme of the parents was used to reconstruct grandparents of the parental

generation. The assignment of experimental individuals to their parents was accomplished through a likelihood approach using 141 usable SNPs, with only immediate parental information utilized in the form of the unique identity of sires and dams (Anderson, 2010). The SNP panel also encompassed the locus of interest, *vgll3*[TopSNP] (Barson et al., 2015) and the sex determining locus, *sdY* (Yano et al., 2013) used for sex typing.

2.2 Sampling

At the first measurement in April 2019, each fish was identifiable through Passive Integrated Transponders (PIT) tags implanted in the abdominal cavity. A small fin clip was taken from the caudal fin of each fish for genotyping, sex determination, and parental assignment, following the methods outlined in Debes et al., (2021). This procedure enabled individual identification from that point forward. From November/December 2021 all fish at LBS were examined for total length (TL, ± 1mm) and assessed for maturity. Fork length (length from snout to fork of tail) was measured to the nearest mm using digital fish-measurement board. Maturation status was checked by carefully stroking each individual's abdomen towards the vent, with the fish releasing milt categorized as mature. Before any measurements or procedures were performed, the fish were anesthetized with Tricaine methanesulfonate (MS-222,120mgL^{-1}) buffered with an equal amount of sodium bicarbonate. Mature males were then stripped by hand using lateroventral compression, and the sperm was collected in a Petri dish. While stripping, measures were taken to avoid contamination of the extracted ejaculate as water, mucus, blood, urine, and feces may lead to premature activation and subsequent expiration of the sperm. Therefore, only milt that was visibly free from contamination was transferred into 1.5mL Eppendorf-tubes and kept on ice for no more than 5 hours before the sperm traits were analyzed.

2.3 Sperm trait analysis

Sperm traits were analyzed using the Sperm Class Analyzer (SCA®, Microptic S.L., Barcelona, Spain), a computer assisted sperm analysis (CASA) system with a mobility and concentration module. A Basler aceA1300-200uc camera was mounted to capture sperm movement at 50fps for 0.5s on a Nikon Eclipse E200 LED phase contrast microscope, under negative phase contrast at 10x objective magnification (Figure 2). Since salmon sperm expires quickly after contact with an aqueous activation medium, a short measuring time was necessary. 2.5μL of the ejaculate were diluted in 997.5 μL MilliQ H_2O (1:400 dilution), simultaneously a stopwatch was started. 2.5μL of the dilution was thereafter injected into a

20μm two-chamber CytoGold slide (Microptic S.L., Barcelona, Spain). Prior to injection, a PE120 Peltier cooling stage system controlled by a T97 Controller was mounted onto the microscope and cooled the counting slide to $6 \pm 0.1°C$. Screen captures were taken for each ejaculate at 20, 30, 45 and 60s post-activation (Figure 2). The proportion of duplicated technical replicates was 33%.

The following sperm parameters were measured (Figure 3): elongation %, percent of the area of the sperm head calculated from the length (L) and width (W) of the sperm head [(L-W)/(L+W)]; straight line (rectilinear) velocity (VSL, μm/s), time-averaged velocity of a sperm head along the straight line between its first detected position and its last; curvilinear velocity (VCL, μm/s), time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope; average pathway velocity (VAP, μm/s), time-averaged velocity of a sperm head along its average path. This path is computed by smoothing the curvilinear trajectory according to algorithms in the CASA system); straightness (STR, %) linearity of the average path calculated as VSL/VAP; linearity of movement (LIN, %), The linearity of a curvilinear path, calculated as VSL/VCL; wobble (WOB, %), A measure of oscillation of the actual path about the average path, calculated as VAP/VCL; amplitude of the lateral displacement of the head (ALH, μm), magnitude of lateral displacement of a sperm head about its average path; and beat cross frequency (BCF, Hz), the average rate at which the curvilinear path crosses the average path. The sperm cells were categorized into different classes based on speed and progression: rapid-progressive, progressive, mobile, and immobile, which are the default settings for the CASA system.
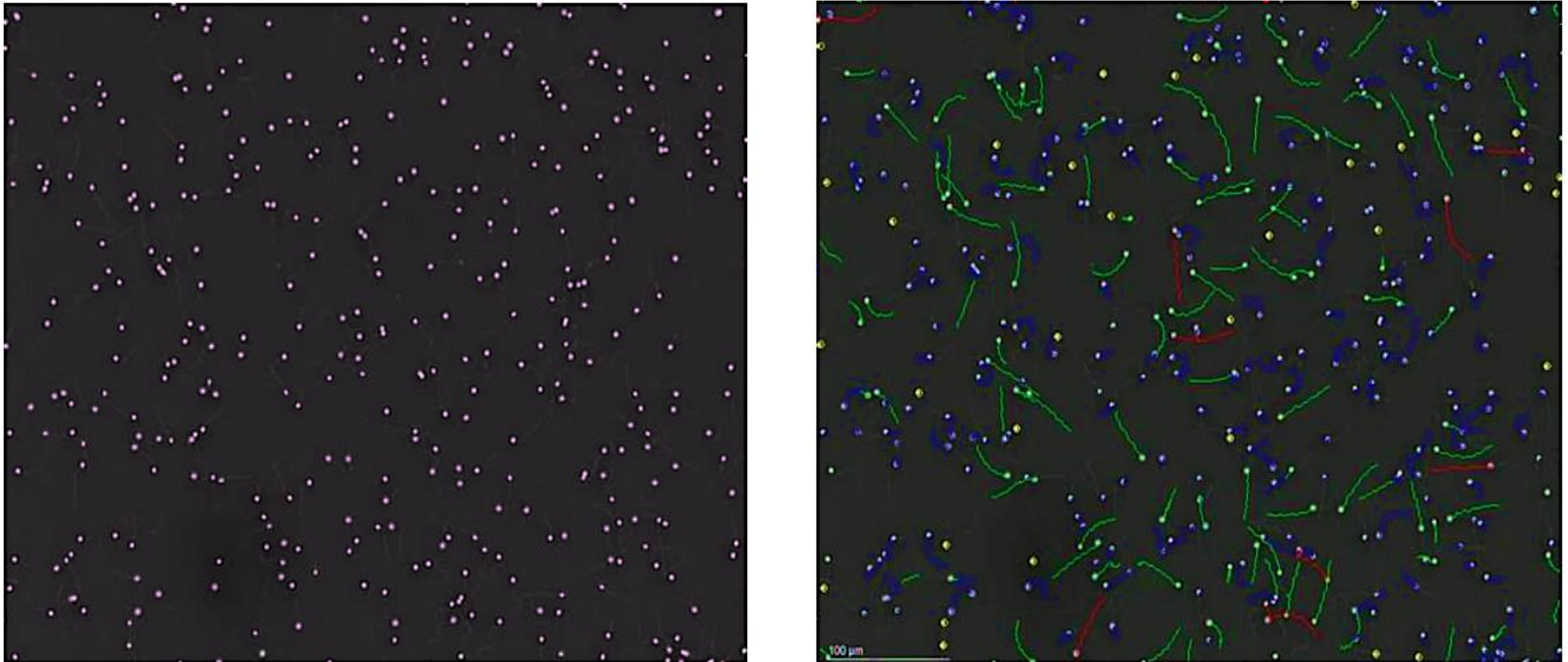
Figure 2. Sperm traits measured with CASA. On the left side, a raw snapshot captured under 10x objective magnification using negative phase-contrast. The white spots visible in the snapshots on the left panel are sperm cells. On the right side, a processed snapshot that shows the tracks of each sperm cell over a 0.5s time frame. The colors used to present the tracks are as follows: red for rapid progressive, green for progressive, blue for mobile, and yellow for immobile (Jagusch, 2020).
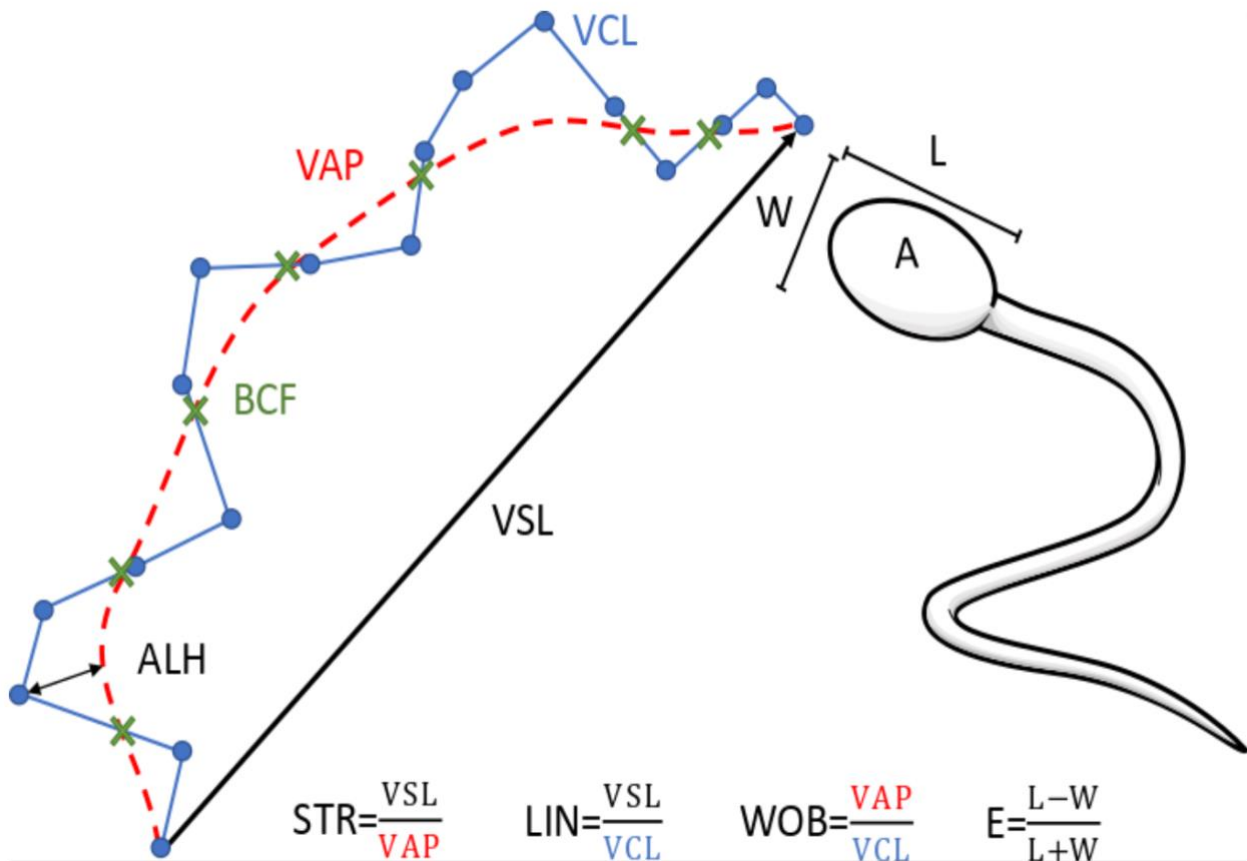
Figure 3. Sperm traits measured with CASA. By using the actual track (blue), the total distance bridge in the time frame (black), and the average track (red), we can calculate the rest of the traits shown in the figure. Elongation %, percent of the sperm head calculated from the length and width (L-W)/(L+W). Description of the abbreviations are found in the text (Jagusch, 2020)

2.4 Statistical analyses

I used a general linear mixed model (GLMM) approach to analyze the maturation data and Linear Mixed Models (LMM's) approach to analyze sperm trait data using the lme4 package (Bates et al., 2015) for the *R* environment (Zuur, 2009). Mixed effects modelling provides an effective method to incorporate random variables of interest, estimate their contributions to the residual variance, and generally yield more precise estimated of fixed effects (McNeish & Kelley, 2019). All statistical models were performed in R (R Core Team, 2022). Interactions were first tested and removed if non-significant ($\alpha = 0.05$). Model fits were assessed by inspecting residual plots (i.e., normal Q-Q plots, standardized vs fitted residuals, and histograms) using the DHARMa version 0.4.5 package (Hartig, 2022). All means are reported ± one standard error of the mean.

1.6 Body size

To test whether body size (total length) was different between males based on diet, *vgll3* genotype and the two different populations of origin, I conducted a LMM on body size (total length, cm) with diet and population as fixed factors and tank ID, maternal ID and paternal ID as random factors to address the nonindependence among the samples. The basic model structure for length:

$$\text{Length} \sim \text{feed treatment} + \textit{vgll}3 \text{ genotype} +$$
$$\text{ID\_Population} + (1|\text{ID\_Ma}) + (1|\text{ID\_Pa}) + (1|\text{Tank})$$

1.7.1 Maturation

I conducted a GLMM on male maturation with a binomial logit link (0 = not mature, 1 = mature). The fixed effects were the *vgll3* genotypes, feeding treatment and population. The random-effects Tank ID, maternal ID and paternal ID were included as random intercepts to address the nonindependence among the samples. The basic model structure for maturation:

$$\text{Mature males} \sim \text{feed treatment} + \textit{vgll}3 \text{ genotype} +$$
$$\text{ID\_Population} + (1|\text{ID\_Ma}) + (1|\text{ID\_Pa}) + (1|\text{Tank})$$

2.4.2 Sperm traits

For the analysis of sperm traits, a LMM was used for each of the nine sperm traits, with diet, *vgll3* genotype and population as fixed factors. Tank ID, maternal ID, and paternal ID were treated as random factors to address non-independence among samples. The model structure for each sperm trait:

$$\text{Sperm trait} \sim \text{feed treatment} + \textit{vgll}3 \text{ genotype} +$$
$$\text{ID\_Population} + (1|\text{ID\_Ma}) + (1|\text{ID\_Pa}) + (1|\text{Tank})$$

2.5 Ethical issues

The study was conducted under the licenses affiliated with the MATURATION project (ERC grant no. 742312), and all procedures and handlings were conducted in accordance with the European Union Directive 2010/63/EU under license ESAVI-2778-2018 by the Animal Experiment Board in Finland.

3 Results

In the analysis of maturation and body size there were 1439 males after removing individuals with error and NA values. Of those males there were 640 mature males, 397 mature males from the Oulu River and 243 from the Neva River. The following models presents the results of the LMM and GLMM for total length and maturation, respectively (Table 3, Table 4). Additionally, bar plots illustrating total length and maturation are included for visual reference (Figure 4, Figure 5).

3.1 Body size

Significant differences in total length were found between the two populations, with mature males from the Oulu River being significantly smaller than those from the Neva River (Table 3, Figure 4). No significant differences in total length due to the *vgll3* genotypes or feeding treatments, and no interactions were found. The random effects indicate that genetic factors (sire and dam) contribute significantly to the variability in total length, while tank effects are minimal.

Table 2. Descriptive analysis of dependent and explanatory variables. The results of the LMM's for length showing the fixed effect of feed, *vgll3* genotype, and population of origin, and the random effects of tank, sire and dam. Reference feed is high fat, reference *vgll3* genotype is *vgll3**EE, reference population is Neva. Asterix (***) by P values indicate the significance of the statistics at the α level of 0.05. Standard error (±SE) values indicate the precision of the estimated coefficients for the fixed effects. Standard deviation (SD) is a measure of the spread of values around the mean.

Total length

| Fixed effects	Estimate	±SE	t-value	P
Intercept	522.893	10.435	50.108	<0.001***
Feed (LF)	-7.455	5.076	-1.469	0.174
vgll3*EL	6.377	9.528	0.669	0.508
vgll3*LE	-3.638	10.382	-0.350	0.727
vgll3*LL	19.092	12.434	1.536	0.129
Pop (Oulu)	-101.018	11.599	-8.709	<0.001***
Random effects	**Variance**		**SD**	
Tank	15.23		3.903	
Sire	585.53		24.198	
Dam	602.82		24.552	
Residual	3370.24		58.054	

3.2 Maturation

No fixed effect reaches statistical significance, implying that neither feed treatment, *vgll3* genotype nor population significantly affects maturation (Table 4). Although the effect may not have reached statistical significance, it is notable that the p-values for *vgll3*LL and population (Oulu) are approaching 0.05 (Table 4), suggesting a trend for higher maturation in the *vgll3*LL locus compared to *vgll3*EE locus, as well a potential lower maturation rate in the Oulu River compared to the Neva River. It is also notable that the Neva River shows more variability in the proportion of mature males between genotypes and feed treatments, while Oulu has more uniform maturation among *vgll3* genotypes (Figure 5). The random effects show that genetic factors (sire and dam) contribute more to the variability in maturation than the environmental factor (tank) (Table 4). In this binomial GLMM we did not get any residual values. This may be because the model deals with binary outcomes where the variance in inherently tied to the mean through the binomial distribution.

Table 4. Descriptive analysis of dependent and explanatory variables. The results of the GLMM for the proportion of male maturation showing the fixed effect of feed, *vgll3* genotype, and population of origin, and the random effects of tank, sire and dam. Reference feed is high fat, reference *vgll3* genotype is *vgll3*EE, reference population is Neva. Asterix (***) by P values indicate the significance of the statistics at the α level of 0.05. Standard error (±SE) values indicate the precision of the estimated coefficients for the fixed effects. Standard deviation (SD) is a measure of the spread of values around the mean. Residual values are marked as "*na*", meaning they are not available for this binomial GLMM.

Maturation

Fixed effects	Estimate	±SE	t-value	P
Intercept	4.056	0.639	6.345	<0.001***
Feed (LF)	0.144	0.333	0.433	0.665
*vgll3*EL	-0.025	0.555	-0.045	0.964
*vgll3*LE	-0.769	0.563	-1.367	0.172
*vgll3*LL	-1.078	0.579	-1.862	0.063
Pop (Oulu)	-0.971	0.523	-1.858	0.063
Random effects	**Variance**		**SD**	
Tank	0.030		0.172	
Sire	0.266		0.515	
Dam	0.592		0.769	
Residuals	*na*		*na*	
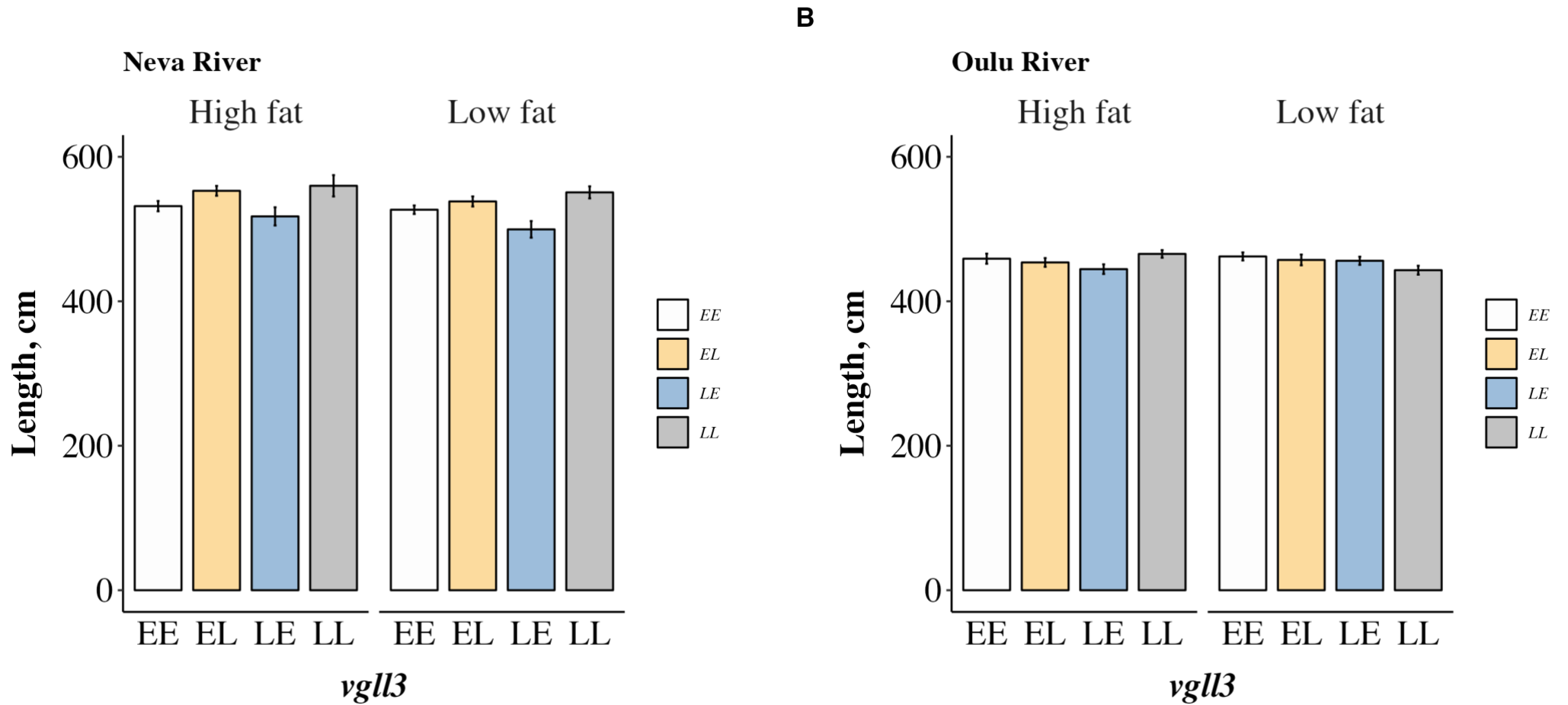
Figure 4. Length differences between the populations. The mean length of individuals of Atlantic salmon of the Neva River (A) and Oulu River (B) for different *vgll3* genotypes. High fat treatment on the left and low fat treatment on the right. Colors represent *vgll3* genotypes and standard error bars represent means ± SE.
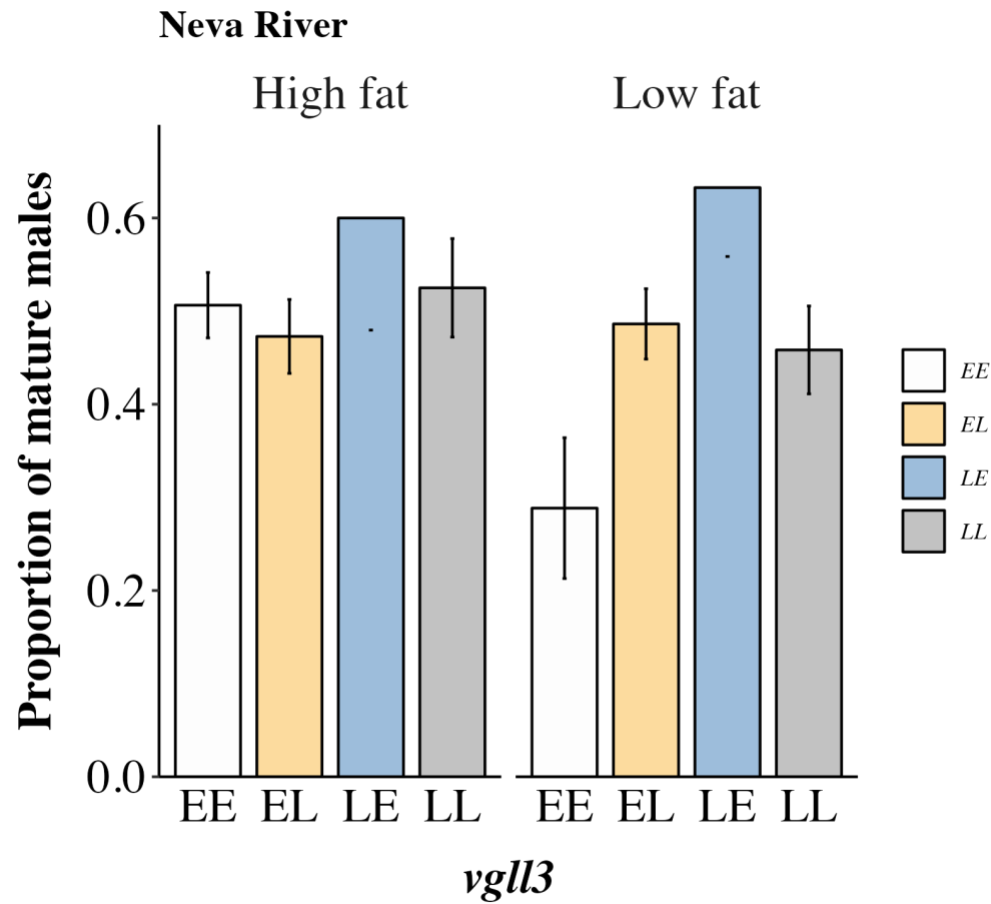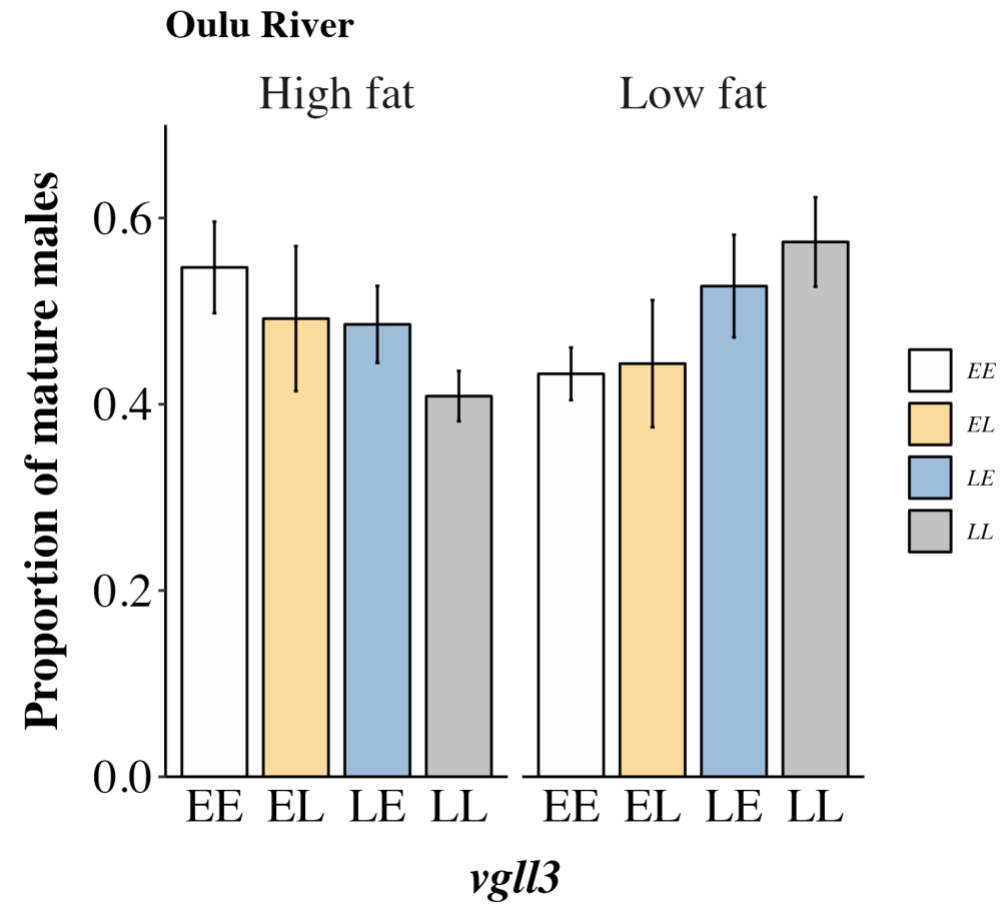
Figure 5. Proportion of mature males. The mean proportion of mature males for the *vgll3* genotypes for the Neva River (A) and Oulu River (B). High fat treatment on the left and low fat treatment on the right. Colors represent *vgll3* genotypes and error bars represent means ± one standard error of the mean.

3.3   Sperm traits

After removing the mature males that were missing *vgll3* genotype information and replicates, we had 550 mature males that were included in the sperm trait analysis. The number of mature males was 266 in high fat treatment and 284 in low fat treatment in the different *vgll3* genotypes (Table 5). The following models presents the results of the LMM's for the different sperm traits (Table 6, Table 7, Table 8). Additionally, scatterplots illustrating all the sperm traits are included for visual reference (Figure 6, Figure 7, Figure 8).

There were no significant effects of *vgll3* genotypes on any of the measured sperm traits. However, we found significant higher estimates in LIN ($P < 0.008$) and STR ($P < 0.002$) for the Oulu River compared to the Neva River (Table 8). We also found significantly higher estimates in VCL ($P < 0.023$) and VAP ($P < 0.0037$) in the low fat treatment compared to the high fat treatment (Table 6).

Across all sperm traits, there was a general trend indication of better performance in the sperm traits with the low fat treatment compared to the high fat treatment, with the exception of elongation and wobble (Table 5). Even though wobble results with higher values, it does not indicate better performance. Thus, a high wobble is associated with lower sperm quality. Significant residual variance is observed in all traits, indicating other unaccounted factors may contribute to the variability in sperm traits.

Table 4. Table of sperm traits of mature males. The table summarizes the means±SE of the different sperm traits for the different *vgll3* genotype in high fat and low fat treatment. All measurements presented is at 20 seconds post-activation time.

Sperm traits	High fat				Low fat			
	*vgll3*EE	*vgll3*EL	*vgll3*LE	*vgll3*LL	*vgll3*EE	*vgll3*EL	*vgll3*LE	*vgll3*LL
VCL (µm/s)	82.45 ± 1.7 4	80.64 ± 1.47	83.96 ± 2.22	82.10 ± 2.22	90.01 ± 2.03	87.51 ± 2.17	91.52 ± 2.50	86.47 ± 2.20
VSL (µm/s)	52.54 ± 1.89	51.85 ± 1.57	50.10 ± 2.38	54.09 ± 2.15	60.98 ± 2.28	58.89 ± 2.10	60.74 ± 2.71	59.83 ± 2.09
VAP (µm/s)	73.75 ± 1.81	72.23 ± 1.53	74.72 ± 2.16	73.78 ± 2.25	81.55 ± 2.09	78.92 ± 2.20	82.93 ± 2.52	78.02 ± 2.17
STR (%)	77.67 ± 2.25	78.66 ± 1.02	73.91 ± 2.12	79.13 ± 1.54	80.80 ± 1.45	79.90 ± 1.46	79.30 ± 1.56	81.29 ± 1.31
LIN (%)	65.17 ± 1.53	65.81 ± 1.27	62.12 ± 2.35	67.84 ± 1.96	70.37 ± 1.83	68.44 ± 1.77	69.60 ± 2.08	70.02 ± 1.64
WOB (%)	88.83 ± 0.69	89.17 ± 0.48	88.97 ± 0.85	89.82 ± 0.99	90.70 ± 0.91	89.73 ± 0.99	91.12 ± 1.11	90.02 ± 0.78
ALH (µm)	1.21 ± 0.02	1.20 ± 0.01	1.21 ± 0.02	1.22 ± 0.02	1.24 ± 0.02	1.23 ± 0.02	1.24 ± 0.04	1.21 ± 0.02
BCF (Hz)	14.36 ± 0.32	14.44 ± 0.27	14.71 ± 0.54	14.56 ± 0.44	15.18 ± 0.41	14.81 ± 0.44	14.92 ± 0.46	14.71 ± 0.43
Elongation (%)	90.59 ± 0.08	90.56 ± 0.09	90.73 ± 0.11	90.50 ± 0.14	90.46 ± 0.11	90.42 ± 0.12	90.50 ± 0.11	90.55 ± 0.11
n	n = 92	n = 82	n = 43	n = 49	n = 75	n = 78	n = 64	n = 67

Table 5. Descriptive analysis of dependent and explanatory variables. Results of the Linear Mixed Models (LMM's) for VCL (µm/s), VSL (µm/s), and VAP (µm/s), at 20 seconds post-activation time. The table shows the fixed effects of feed, *vgll3* genotype, and population of origin, and the random effects of tank, sire ID and dam ID. The reference for feed is high fat feed (HF), *vgll3* genotype is *vgll3**EE, and population Neva River. Asterix (***) by P values indicate the significance of the statistics at the α level of 0.05. Standard error (±SE) values indicate the precision of the estimated coefficients for the fixed effects. Standard deviation (SD) is a measure of the spread of values around the mean.

Fixed effects	VCL (µm/s)				VSL (µm/s)				VAP (µm/s)			
	Estimate	±SE	t-value	P	Estimate	±SE	t-value	P	Estimate	±SE	t-value	P
Intercept	81.169	1.867	43.464	<0.001***	52.797	3.170	16.656	<0.001***	72.272	1.965	36.787	<0.001***
Feed (LF)	5.574	1.460	3.816	0.023*	6.681	3.924	1.703	0.163	5.505	1.721	3.199	0.037*
vgll3*EL	-2.021	1.889	-1.070	0.285	-1.917	2.013	-0.952	0.347	-1.793	1.919	-0.934	0.351
vgll3*LE	1.634	2.289	0.714	0.477	-1.157	2.246	-0.515	0.608	1.749	2.289	0.764	0.447
vgll3*LL	-1.701	2.267	-0.750	0.455	-2.083	2.259	-0.922	0.360	-1.733	2.262	-0.766	0.446
Pop (Oulu)	-0.460	1.773	-0.259	0.797	2.103	1.735	1.212	0.234	0.007	1.754	0.004	0.997
Random effects	**Variance**		**SD**		**Variance**		**SD**		**Variance**		**SD**	
Tank	0.005		0.071		19.728		4.442		1.139		1.067	
Sire	0.000		0.000		2.832		1.683		0.000		0.000	
Dam	6.611		2.571		1.507		1.228		5.149		2.269	
Residual	285.384		16.893		298.509		17.277		294.689		17.167	

Table 6. Descriptive analysis of dependent and explanatory variables. Results of the LMM's for WOB (%), ALH (μm), BCF (Hz), at 20 seconds post-activation time. The table shows the fixed effects of feed, *vgll3* genotype, and population of origin, and the random effects of tank, sire ID and dam ID. The reference for feed is high fat feed (HF), *vgll3* genotype is *vgll3**EE, and population Neva River. Asterix (***) by P values indicate the significance of the statistics at the α level of 0.05. Standard error (±SE) values indicate the precision of the estimated coefficients for the fixed effects. Standard deviation (SD) is a measure of the spread of values around the mean.

|	WOB (%)				ALH (μm)				BCF (Hz)			
Fixed effects	**Estimate**	**±SE**	**t-value**	**P**	**Estimate**	**±SE**	**t-value**	**P**	**Estimate**	**±SE**	**t-value**	**P**
Intercept	87.539	0.992	88.271	<0.001***	1.206	0.026	47.814	<0.001***	14.342	0.419	34.263	<0.001***
Feed (LF)	0.370	1.141	0.324	0.762	0.016	0.028	0.587	0.588	0.231	0.412	0.560	0.605
vgll3*EL	-0.152	0.780	-0.195	0.856	-0.014	0.020	-0.693	0.489	-0.229	0.371	-0.618	0.537
vgll3*LE	0.213	0.880	0.242	0.809	0.008	0.023	0.362	0.718	-0.144	0.445	-0.324	0.746
vgll3*LL	-0.143	0.863	-0.166	0.869	-0.022	0.023	-0.936	0.352	-0.201	0.440	-0.456	0.649
Pop (Oulu)	1.156	0.648	1.785	0.075	-0.003	0.018	-0.175	0.862	-0.292	0.342	-0.854	0.398
Random effects	**Variance**		**SD**		**Variance**		**SD**		**Variance**		**SD**	
Tank	1.405		1.185		0.000		0.003		0.132		0.364	
Sire	0.000		0.000		0.001		0.000		0.000		0.000	
Dam	0.000		0.000		0.000		0.023		0.218		0.467	
Residual	49.023		7.002		0.031		0.176		10.931		3.306	

Table 7. Descriptive analysis of dependent and explanatory variables. The table shows the results of the LMM's for elongation (%), LIN (%) and STR (%), at 20 seconds post-activation time. The table shows the fixed effects of feed, *vgll3* genotype, and population of origin, and the random effects of tank, sire ID and dam ID. The reference for feed is high fat feed (HF), *vgll3* genotype is *vgll3**EE, and population Neva River. Asterix (***) by P values indicate the significance of the statistics at the α level of 0.05. Standard error (±SE) values indicate the precision of the estimated coefficients for the fixed effects. Standard deviation (SD) is a measure of the spread of values around the mean.

Fixed effects	Elongation (%)				LIN (%)				STR (%)			
	Estimate	±SE	t-value	P	Estimate	±SE	t-value	P	Estimate	±SE	t-value	P
Intercept	90.617	0.100	904.288	<0.001***	63.917	2.987	21.396	<0.001***	75.307	2.662	28.288	<0.001***
Feed (LF)	-0.136	0.098	-1.393	0.234	2.946	3.828	0.770	0.483	2.004	3.443	0.582	0.592
vgll3*EL	-0.111	0.098	-1.131	0.258	-0.569	1.662	-0.343	0.734	0.136	1.351	0.101	0.920
vgll3*LE	0.067	0.110	0.608	0.543	-2.226	1.819	-1.223	0.224	-2.58	1.505	-1.715	0.090
vgll3*LL	-0.035	0.108	-0.321	0.748	-0.463	1.852	-0.250	0.803	-0.115	1.554	-0.074	0.941
Pop (Oulu)	0.012	0.081	0.144	0.886	4.033	1.430	2.821	0.008**	4.109	1.229	3.343	0.002**
Random effects	Variance	SD			Variance	SD			Variance	SD		
Tank	0.006	0.076			19.788	4.448			16.402	4.050		
Sire	0.000	0.000			2.962	1.721			2.648	1.627		
Dam	0.000	0.000			0.744	0.863			1.903	1.380		
Residual	0.774	0.880			194.495	13.946			121.538	11.024		
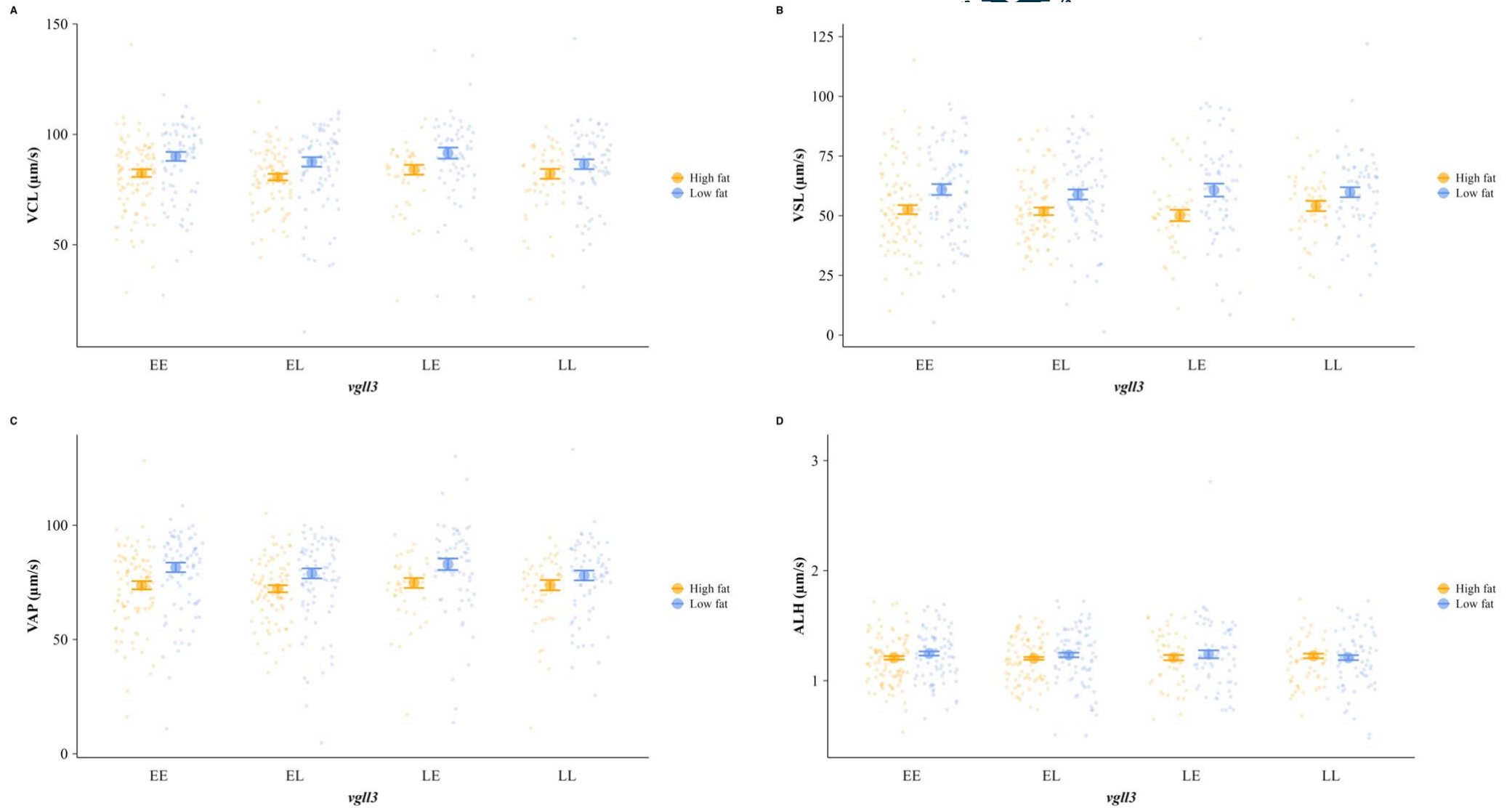
Figure 6. Mean ± SE of A) VCL (µm/s), B) VSL (µm/s), C) VAP (µm/s) and D) ALH (µm) for *vgll3* genotypes in high fat and low fat treatments. Large circles with error bars represent the mean ± *SE*, while small circles show individual data points. For clarity, individual points are jittered on the *x*- and *y*-axis.
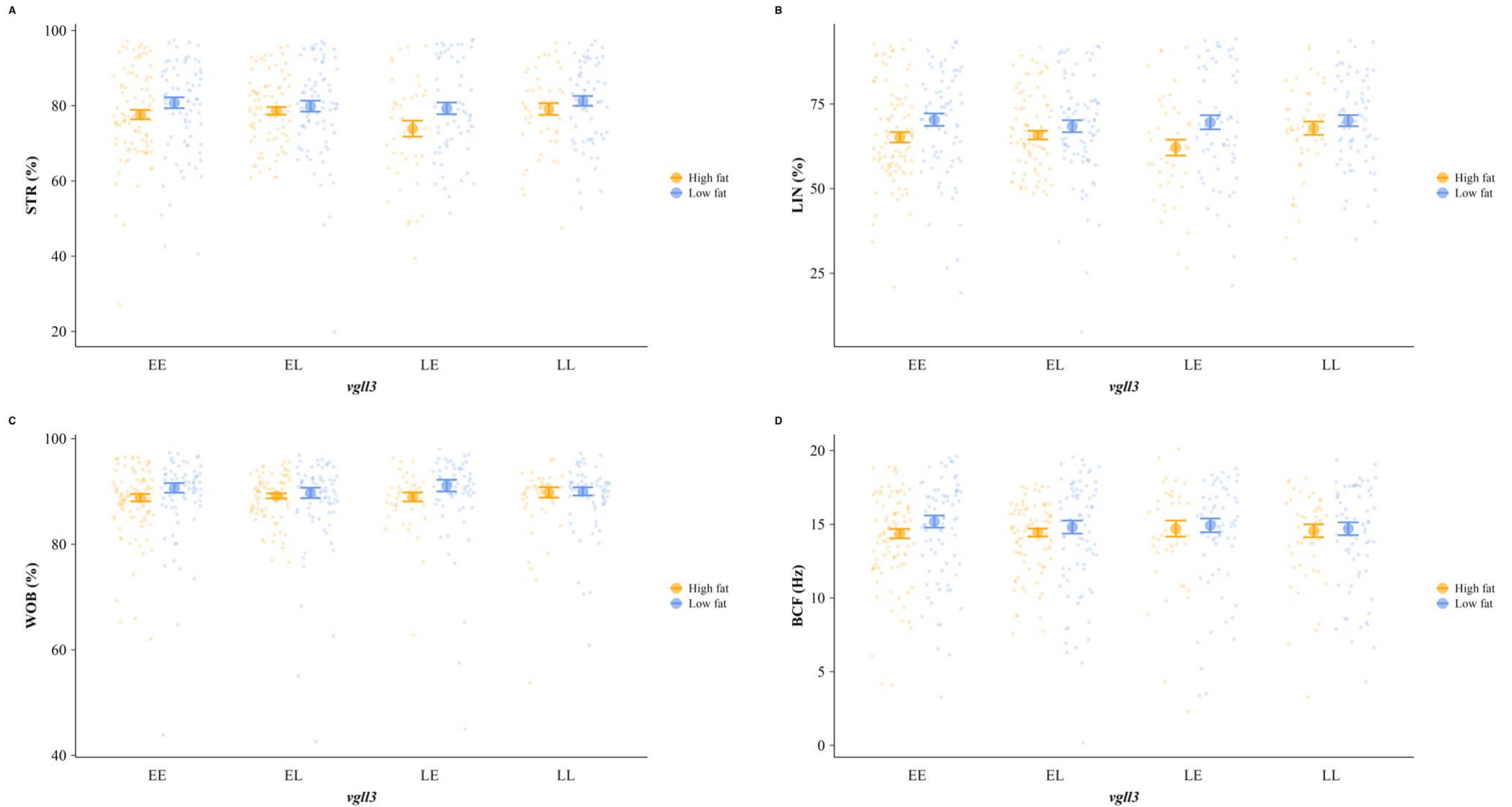
Figure 7. Mean ± SE of A) STR (%), B) LIN (%), C) WOB (%) and D) BCF (Hz) for *vgll3* genotypes in high fat and low fat treatments. Large circles with error bars represent the mean ± *SE*, while small circles show individual data points. For clarity, individual points are jittered on the *x*- and *y*-axis.
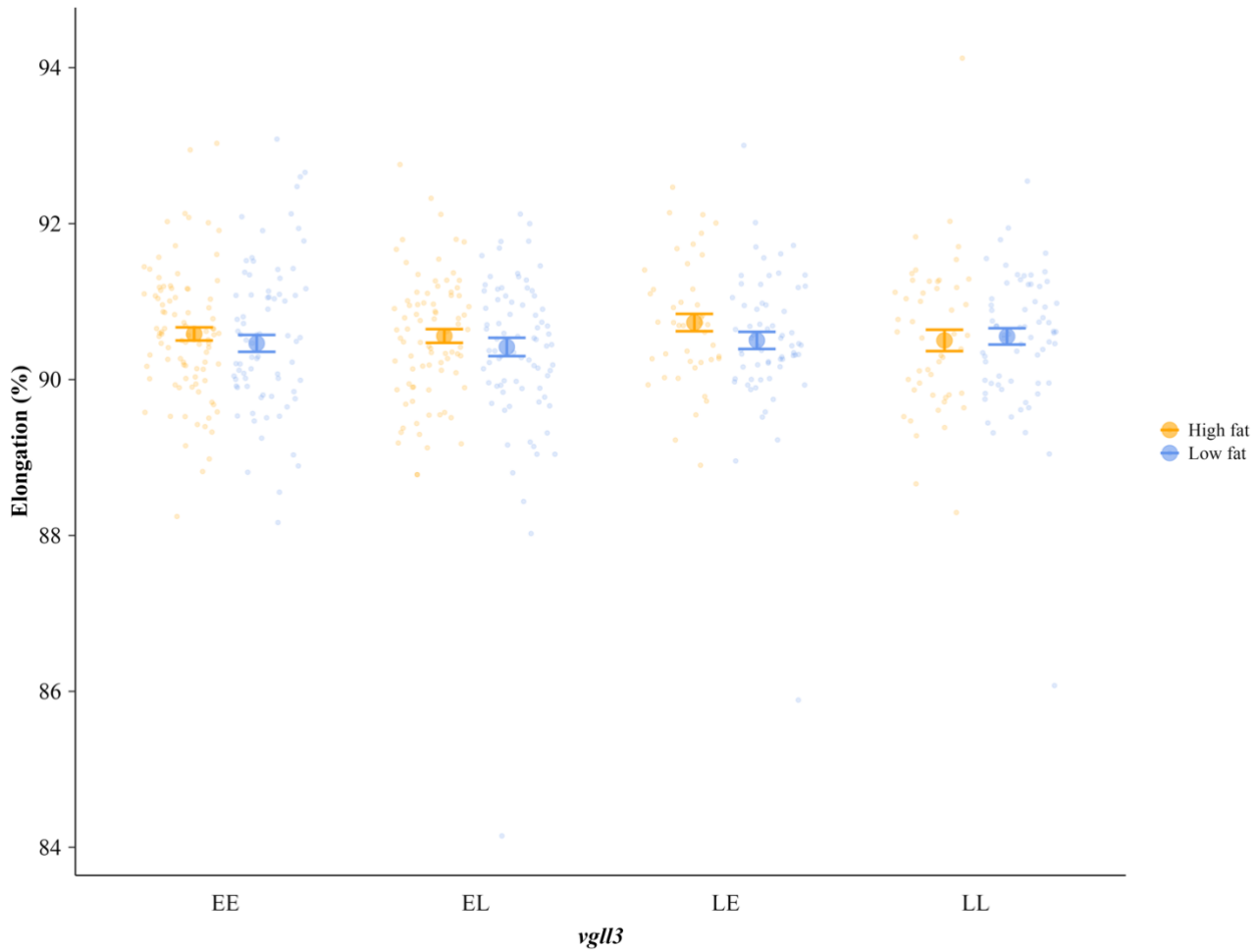
Figure 8. Mean ± SE of Elongation for *vgll3* genotypes in high fat and low fat treatment. Large circles with error bars represent the mean ± *SE*, while small circles show individual data points. For clarity, individual points are jittered on the *x*- and *y*-axis.

4 Discussion

4.1 Maturation

One of the hypotheses for this experiment was that the *vgll3* genotype and diet would affect sexual maturation on four-year-old male Atlantic salmon. However, the analyses revealed no significant differences in maturation rates between populations, *vgll3* genotypes, or feeding treatments. Previous research by Mobley et al. (2021) highlights the critical role of accumulating adequate energy reserves in maturation, guiding this investigation into the impact of mature males' length on these processes. The significant differences in length observed between the populations from Oulu and Neva highlight potential population-specific variations in growth patterns and genetics. Atlantic salmon populations often exhibit genetic diversity due to factors such as historical isolation, migrations patterns, and local adaptation (Debes et al., 2014; Åsheim et al., 2023). Genetic differences among populations can lead to variations in growth rates, body size, and ultimately at length at maturation. The result suggests that the population from Oulu may possess genetic traits that predispose individuals to reach maturation at smaller size. Similar results have been observed for both females and males in previous studies utilizing the same fish as in this experiment (Maamela et al., 2023; Åsheim et al., 2023), demonstrating significant differences in body size between the two populations. Despite the smaller body size of the fish from the Oulu River compared to the fish from the Neva River, we did not observe significant differences in maturation between the two populations. This may suggest that the populations have different threshold for body size and accumulated energy stores necessary to achieve the same level of maturation. These findings emphasize the complex interaction of genetic and environmental factors in the life history traits of Atlantic salmon.

The different feeding treatments did not reveal any significant differences in maturation ratio in this study. Interestingly, females from the same experiment also did not show differences in maturation based on feeding treatment (Maamela et al., 2023). This outcome contrasts with previous research demonstrating clear associations between diet and maturation (Jonsson et al., 2012; Jonsson et al., 2013). These unexpected results could be attributed to the feeding approach utilized in this experiment. Instead of decreasing food quantity, the fishes were fed *ad libitum.* When feed is available in abundance, dominant individuals are less likely to monopolize access to food, and subordinate fish are less prone to nutritional stress

malnutrition (Jobling, 2016). This promotes a stable environment within the group. This feeding regime may also enable the fish to accumulate sufficient lipid reserves for maturation regardless of the diet's energy content (Taranger et al., 2010). It is also possible that the reduction in feed energy content in this experiment was not substantial enough to elicit detectable dietary effects on maturation.

In Atlantic salmon, maturation is complex and moderated by a range of biological and environmental factors (Mobley et al., 2021). In controlled aquaculture environments, the strategic manipulation of light and temperature settings is recognized as a powerful tool that can significantly delay reproductive processes (Mobley et al., 2021; Taranger et al., 2010). This approach underscores the extent to which environmental controls can override the potential influences of dietary composition on the biological rhythms of cultures species, despite intensive feeding regimes. It is reasonable to hypothesize that environmental manipulations might interact synergistically or antagonistically with dietary factors, potentially modulating the physiological responses of fish in ways that are not yet fully understood. Additionally, the overall nutrition balance, encompassing proteins, vitamins, and minerals, along with fat contends, play a critical role in determining both growth and maturation in Atlantic salmon (Bendiksen et al., 2011). The diverse nutritional requirements suggest that a holistic approach to diet formulation could be key to optimizing physiological development and reproductive readiness. It is conceivable that the specific ration available of these nutrients might interact in complex ways to influence growth rates and the onset of maturation, potentially overriding the simpler effects of fat content alone.

While previous studies have reported significant associations between the *vgll3* locus and maturation (Ayllon et al., 2015; Ayllon et al., 2019; Barson et al., 2015), we could not find any significant effects of *vgll3* genotype on maturation in this study. There could be several reasons for this. Atlantic salmon are adapted to varying environmental conditions in the wild. Considering that *vgll3* affects sea age at maturity (Barson et al., 2015), and the experimental setup restricted the fish from going to sea, this could potentially disrupt the signaling pathway for maturation (Jonsson & Jonsson, 2011). Laboratory or controlled studies might not fully replicate these natural conditions. The lack of natural environment might impact the expression and interaction of genes related to maturation (Feil & Fraga, 2012), highlighting the importance of environmental context in genetic studies of maturation.

Genetic effects on complex traits like maturation are often influenced by multiple genes and environmental factors, making it challenging to detect individual gene effects in small-scale studies. The presence of a more complex genetic architecture, which includes multiple loci and gene environment interactions affecting maturation, could potentially obscure the impact of the *vgll3* locus in this particular study (Sinclair-Waters et al., 2020). Factors such as gene-environmental interaction and gene expression regulation may also contribute to the influence of the *vgll3* locus on biological processes (Virolainen et al., 2023).

Further research with larger sample sizes, diverse populations, and comprehensive genetic analyses may help elucidate the role of *vgll3* and other genes in regulation maturation in Atlantic salmon more effectively. It is also plausible that the observed results reflect a genuine absence of effect of diet, genetics and population differences, which aligns with other previous studies (Boulding et al., 2019; Mohamed et al., 2019). The results suggests that, at least within the scope of this study, variations in *vgll3* genotype or dietary composition did not exert a measurable effect on maturation or body length of the individuals.

4.2   Sperm traits

The other hypothesis for this experiment was that the *vgll3* genotype and diet will affect the sperm traits. In this experiment, I assessed a range of sperm traits to investigate the potential influence of diet and *vgll3* genotype on them. While we did not find any significant effects of the *vgll3* genotype on the sperm traits, we did find significant effects of feeding treatment and population for specific sperm traits.

Significantly higher effects were observed with the low fat feed in average pathway velocity (VAP, µm/s) and curvilinear velocity (VCL, µm/s). This unexpected finding that sperm traits from fish in the low fat treatment performed better raises intriguing questions about the interplay between diet and reproductive physiology in Atlantic salmon. It is known that dietary fats play a crucial role in various physiological processes, including reproductive functions (Lahnsteiner et al., 2009). High fat diets, particularly those rich in polyunsaturated fatty acids (PUFAs), have been shown to influence sperm quality and fertility in several species, including mammals (Collodel et al., 2020; Esmaeili et al., 2015). Lipids are the primary components of the sperm membrane, governing the fluidity of the lipid layer (Lenzi et al., 1996). However, an excessively high fat diet might disrupt metabolic processes or lead

to imbalances in nutrient intake. The low fat diet might have provided a more balances or optimal nutrition profile for the fish, leading to improved sperm quality. Adequate protein intake supports spermatogenesis, maintain sperm structure and function, regulates reproductive hormones, and helps mitigate oxidative stress (Félix et al., 2020). Considering the increased amount of protein due to increased pellet size in the low fat diet, this may have provided sufficient energy and essential nutrient without the potential negative effects associated with excessive fat consumption.

The significant differences observed in straightness (STR, %) and linearity (LIN, %), between the two populations could be attributed to genetic variation, environmental factors, evolutionary pressures, and reproductive strategies which all play roles shaping sperm traits across populations. Natural and sexual selection particularly influence the evolution of these traits in response to environmental challenges. Variation in reproductive studies may lead to development of traits that enhance competitive fertilization success, described in several studies (Gage et al., 2004; Morrow & Gage, 2001; Parker et al., 1996). However, the ultimate indicator of sperm quality is the fertilization rate. Therefore, further investigation should include examining these sperm traits in relation to fertilization rate to gain a comprehensive understanding of sperm quality and reproductive success.

The lack of observed effects of the *vgll3* genotype on sperm traits in this experiment could be attributed to several factors. Firstly, it's possible that the absence of effects of the genotype observed may be indicative of no strong link between maturation and sperm traits. The complexity of *vgll3*'s effects is further compounded by the interactions between genetic predispositions and environmental factors, such as temperature and photoperiod, which also play significant roles in shaping reproductive outcomes (Boulding et al., 2019). Environmental variability, such as differences in rearing conditions or habitat characteristics, could have masked any potential effects of the *vgll3* genotype on sperm traits. Environmental factors play a crucial role in shaping phenotypic variation and could confound the results. Additionally, genetic interactions with other loci could have influenced the observed effects (Sinclair-Waters et al., 2020). The genetic architecture underlying complex traits like sperm quality is often polygenic, meaning that multiple genes interact to determine the phenotype. Interactions between *vgll3* and other genes not considered in this analysis, may have obscure any direct effects of *vgll3* on the sperm traits. However, previous studies have suggested that *vgll3* may influence gametogenesis by regulating genes involved in cell fate commitment

(Kjærner-Semb et al., 2018; Kurko et al., 2020). Additionally, repression of *vgll3* expression in salmon testes has been linked to the induced onset of puberty, further underscoring its role in reproductive development (Kjærner-Semb et al., 2018; Verta et al., 2020). While the impact of *vgll3* on maturation is well-recognized, its specific effects on sperm characteristics remain an area ripe for further research.

5 Conclusion

This study investigated the influence of *vgll3* genotype and diet on sperm traits and maturation in Atlantic salmon. While *vgll3* genotype and diet did not show significant effects on maturation or length, population of origin and feeding treatment did exhibit significant effects. The absence of significant effects of *vgll3* on sperm traits suggests complex interactions involving other genetic and environmental factors. Population differences in sperm traits highlights the role of evolutionary pressures and reproductive strategies. The unexpected findings regarding sperm traits in different feeding treatments raise intriguing questions about interplay between diet and reproductive physiology, suggesting the need for further investigation into the specific dietary components influencing sperm quality. Overall, this study demonstrates the complexity of genetic and environmental influences on reproductive traits in Atlantic salmon.

By considering how genetic variations and environmental conditions influence maturation and sperm traits, we can gain deeper insight into the dynamics driving male fertility, maturation, and broader evolutionary landscape of Atlantic salmon. This holistic understanding is crucial for aquaculture practices and strategies, ensuring the preservation of genetic diversity, reproductive health, and appropriate maturation timing of Atlantic salmon populations. As the farming of Atlantic salmon intensifies, recognizing the impact of these factors becomes increasingly important for maintaining sustainable and healthy fish stocks in the face of changing environmental conditions and industry demands.

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