Improving Atlantic Salmon *(Salmo salar)* conservation and farming sustainability, through the gamete level

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“Per aspera ad astra”

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A brief statement of Purpose

This Ph.D. project focused on reproductive biology in Atlantic salmon (*Salmo salar*) with the aim to address pure and applied questions about sperm form and function, fertilisation compatibility, inbreeding, gamete plasticity and epigenetic effects. Using gametes from wild and farmed salmon strains, I generated key data on 1) hatchery methods of gamete handling to optimise fertility, 2) gamete function, fertility and reproductive potential of farm salmon escapes 3) compatibility and fitness consequences of reproduction between genetic siblings 4) impacts of and responses to thermal variation at the gamete level and 5) effect of the physical properties of salmon ovarian fluid in sexual and natural selective mechanisms. On one side, conducted experiments aimed to delineate causes and effects of artificial coexistence between escaped farm salmon and native populations, generating reproductive potential data to inform management of wild fish facing farm introgression, and the sustainability of salmon aquaculture under rising global demand for animal protein. On the other one, gamete interactions and fertility across different levels advance our understanding of gamete biology, sperm-egg interaction, sexual selection, local adaptation, hybridisation, speciation, phenotypic plasticity, and cryptic female choice.

In the first chapter of my thesis, I review the scientific literature across two main, but related, research areas: 1) Salmon farming sustainability and wild Atlantic salmon conservation, and 2) gamete evolution and sperm and egg biology. Following, I delineate four experimental Work Packages and chapters that respectively examine: 1) dry *versus* wet fertilization techniques in the hatchery and their relevance to fertility, 2) inbreeding avoidance at the gamete level and offspring fitness consequences, 3) thermal plasticity in gametes and adaptive epigenetic inheritance, and 4) influence of the non-Newtonian viscoelastic properties of salmon ovarian fluid on natural and sexual selective mechanisms.

Summary of the main experimental findings

This body of research has allowed me to produce results that shine a light on sexual selection at the gamete level in an externally fertilising animal model, in a variety of contexts, and that could be applied to improve salmon conservation and sustainability in the aquaculture sector.

Specifically, in the first experimental Chapter (Chapter 3), we explored the effects of two *in vitro* fertilisation methods on gamete fertility, compatibility, embryo development and reproductive outcomes in farmed and wild strains of Atlantic salmon; with the aim to improve hatcheries' sustainability and wild salmon reintroduction efforts. We revealed that farmed salmon strains were far more resilient to different fertilisation techniques, their embryonic development was faster, and they manifested a higher degree of chromosomal abnormalities as compared to wild fish. When wild and farmed males competed over fertilisation of eggs from wild and farmed females, farmed male paternity rates ranged from 70 to 100% in both types of females, suggesting a clear threat to wild genetic pools following escapes. Our results stand in contrast with previous findings and present important insights that can help improving sustainability and reintroduction efforts in conservation aquaculture.

In Chapter 4, we tested the presence of inbreeding avoidance mechanisms at the gamete level that could arise from the philopatric return of salmon to their natal streams to spawn, in a context of polyandry. We hypothesised that in salmon, inbreeding avoidance would have to evolve at the gamete level and could be based on the interaction between sperm and the ovarian fluid released with eggs at oviposition, which may play a key role in determining fertilisation success. We compared sperm motility parameters in sibling and non-sibling ovarian fluid, and assessed fertilisation and hatching success, growth rate and paternity in sperm competition trials between sibling and non-sibling males. We found that sperm activated in ovarian fluid of sibling females showed lower values of motility-related parameters and led to an average of 36% reduction in fertilisation rates in the resulting crosses. Furthermore, offspring from sibling crosses were smaller before the onset of sexual maturation, but we found no difference in survival rates between sibling and non-sibling cross. When sperm from sibling and non-sibling males were competing simultaneously for the same egg batch, we found no influence of this on paternity, but surprisingly, sibling-sired offspring showed consistently higher degrees of multi-locus heterozygosity across the five

microsatellite loci analysed. Our findings indicate that post-mating inbreeding avoidance mechanisms have evolved at the gamete level in salmon, but that this does not necessarily affect the sperm competitiveness of sibling males. Our results have direct implications for conservation aquaculture and salmon farming sustainability due to the effect that inbreeding could have in these sectors.

We applied the acquired knowledge on sexual selection at the gamete level, gamete and embryo performance and survival rates to establish in Chapter 5 the presence of mechanisms of thermal plasticity of gametes and/or thermal selection which could affect egg and sperm function and competitiveness. We tested the hypothesis that gametes primed to a specific temperature positively influence hatching success and embryonic development in the same temperature environment and compared sperm and eggs. We incubated half of the eggs and sperm collected from wild Atlantic salmon at two temperatures (cold and warm) and performed *in vitro* fertilisations where we crossed warm and cold-incubated gametes for each of the mating pairs and reared one half of each clutch in cold temperature and the other in warm temperature. We monitored hatching success, hatching time, embryo survival before and after the eyed stage and presence of developmental abnormalities. We found that when the temperature for eggs, sperm and embryo development matched, embryos hatched earlier than in scenarios where gamete incubation and development temperature did not match Warm temperature exposure during embryo development generally caused increased rates of deaths after the eyed stage. Interestingly, we observed opposite effects of gamete incubation temperature on offspring fitness between eggs and sperm, where warm incubation was beneficial for eggs but detrimental for sperm which in turn negatively affected hatching success. Overall, we showed that gamete plasticity did not significantly improve offspring fitness, suggesting that these stages are particularly vulnerable to a changing environmental temperature.

In the last experimental section, Chapter 6, we gained insights on the physical structure of this fluid and potential impacts on reproduction; while its biochemical effects in relation to sperm energetics have been investigated, the influence of the physical environment in which sperm compete remains poorly explored. Using soft-matter physics approaches of steady-state and oscillatory viscosity measurements, we simulated the frequencies resembling those exerted by sperm swimming through the fluid near eggs. We demonstrated that this fluid, which in its relaxed state is a gel-like substance, displays a non-Newtonian viscoelastic and

shear-thinning profile, where the viscosity decreases with increasing shear rates. We concurrently found that the ovarian fluid can also display a shear-thickening phase at high frequencies , provided it is probed gently enough. We highlight the presence of a unique frequency-dependant structural network with important implications on sperm energetics and fertilisation and that could forunish the basis for a novel, bio-physically mediated, sexual selection mechanism.

We suggest how ovarian fluid physical properties deserve more attention when studying processes of sexual selection and that mechanisms enabled by non-Newtonian reproductive fluids within female internal genital tracts, like lubrication, facilitation and capacitation, should also be applied to the external fertilization environment. This opens new avenues into the study of cryptic female choice with important implications for understanding the evolution of sexual traits and exploring the underestimated role of physical properties of the fertilization environment that surrounds the gametes.

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2 General introduction

Statement of attributions: M. Graziano wrote the first complete draft of this Chapter, and received and applied review comments the Supervisors M. Gage, M. Taylor and S. Immler in all the following modifications that resulted in this final version.

2.1 Abstract

Wild Atlantic salmon (*Salmo salar*) populations have seen a drastic decline worldwide over the past century. During the same period, the commercial farming sector for this species has seen one of its biggest expansions ever witnessed. This has been identified as one of the main causes for reduction in wild salmon numbers, together with a series of other environmental risk factors associated with salmon aquaculture questioning its sustainability. Continuous and massive escapes of artificially selected salmon from aquaculture facilities have been found to quickly dilute locally adapted wild genotypes, contributing to their decline. A proposed mitigation strategy to recover salmon populations consists of the production of wild Atlantic salmon within conservation hatcheries through artificial fertilisation and rearing methods. However, low hatching and survival rates from this method are still observed in both aquaculture and conservation hatcheries, which require further development. We explored the effects of two *in vitro* fertilisation methods commonly used in hatcheries on gamete fertility, compatibility, embryo development and reproductive outcomes in farmed and wild strains of Atlantic salmon. We also examined how variation in fertilisation techniques might cause chromosomal abnormalities and embryonic deaths, explaining the lower numbers achieved in hatcheries; research which intends to improve hatcheries' sustainability and wild salmon reintroduction efforts. In a second set of experiments, using the same farmed and wild strains, we assessed reproductive performance at the gamete level in a simulated competitive

fertilisation environment. We found that farmed salmon strains were far more resilient to different fertilisation techniques, their embryonic development was faster, and they manifested a higher degree of chromosomal abnormalities as compared to wild fish. When wild and farmed males competed over fertilisation of eggs from wild and farmed females, farmed male paternity rates ranged from 70 to 100% in both types of females, suggesting a clear threat to wild genetic pools following escapes. Our results stand in contrast with previous findings and present important insights that can help improving sustainability and reintroduction efforts in conservation aquaculture.

2.2 Sustainability of Atlantic salmon farming and wild Atlantic Salmon conservation

2.2.1 Background and Rationale/Justification

Atlantic salmon (*Salmo salar* L.) aquaculture is one of the longest established fish farming methods, and represents one of the largest and most productive forms of the entire aquaculture industry (FAO, 2010). *S. salar* is one of the best-researched fishes, and its aquaculture plays a global role in the 'blue revolution'. First started in Norway in the early 1970s, salmon farming has now grown to become one of the country's largest economic exports. Since then, Atlantic salmon is the most significant species farmed in Norway and, in 2012, its production in the country was 1 232 095 tons, with a total of 1006 coastal marine farms licensed (Taranger et al., 2015)). To get a sense of its importance, *S. salar* is the eighth most cultured fish species in the world and with a yearly production of 2.07 million tons, it is the first most cultured both in marine-based farming systems and among all the marine/anadromous cultured fish species (FAO, 2013).

Aquaculture of salmon in Norway, as well as in the other countries where the species is farmed in significant numbers, starts with the production of eggs and juveniles in on-land freshwater facilities, followed by growth of fish in open marine cages, with gamete stage to slaughter for market cycles taking 2.5 to 3 years. Similar to a few other farmed fish species,

technical standards for the production of aquaculture infrastructures have evolved dramatically, especially during the last two decades. However, the rapid development and success of open sea cage production systems have not occurred without environmental challenges, in Norway or elsewhere. A compelling amount of evidence has identified various environmental impacts of Atlantic salmon aquaculture following its rapid expansion. This has generated concerns that management guidelines and targets to address potential negative effects might not have been developed fast enough and in concert with the process, throwing the sustainability of salmon farming into question from environmental perspectives (Taranger et al., 2015).

Some of the environmental challenges include effects of organic waste and inorganic nutrient load on benthic communities and on ecosystemic levels (Bannister et al., 2014; Buschmann et al., 2006; Kutti et al., 2007, 2008), transfer of parasites to wild populations (Krkošek et al., 2013, 2014; Skilbrei, 2012; Skilbrei et al., 2013; Torrissen et al., 2013) ecological interaction effects (Jonsson et al., 2006), "unnatural" transmission of disease (Glover, Solberg, et al., 2017; Glover, Sørvik, et al., 2013; Taranger et al., 2015) and reproduction with genetic disruption of wild populations (Clifford et al., 1998a, 1998b; Crozier, 2000; Glover et al., 2008; Glover, Pertoldi, et al., 2013; Glover, Solberg, et al., 2017; Skaala et al., 2004, 2012, 2019). The Norwegian government established in 2009 a series of environmental guidelines for sustainability in the "Strategy for an Environmentally Sustainable Norwegian Aquaculture Industry" (Norwegian Ministry of Fisheries and Coastal Affairs, 2009; Taranger et al., 2015)(Table 1, adapted from Taranger *et al.*, (2015). Likewise, the Norwegian Institute of Marine Research (NIMR), initiated a risk assessment of Norwegian salmon farming in 2010 that has been conducted yearly since.

According to severity, geographical extent, and duration and/or reversibility of the various impacts related to open sea cage salmon farms in coastal waters, the IMR has based Norwegian's risk assessment on the following hazards: (i) genetic introgression of escaped farmed salmon into wild populations, (ii) impact of salmon lice (*Lepeophtheirus salmonis*) on native populations, (iii) potential disease transfer from farmed to wild salmonid populations, and (iv) local and regional impacts of organic load and nutrients from marine salmon farms (Taranger et al., 2015). However, in this thesis focus will be given to the first of the above-mentioned aspects, as this is the area that more relates to my PhD research.

Table 1.1 *Identified hazards, the process of concern, and endpoint of concern for goals 1–3 for the future development of the Norwegian aquaculture industry as established by the Norwegian government in 2009 (adapted from Taranger et al., 2014).*

Hazard	Process of concern	Endpoint of concern
Genetic interaction (Goal 2)	Farmed escaped salmon successfully interbreed with wild salmon populations	Changes observed in the genetic characteristics of wild salmon populations
Salmon lice (Goal 1)	Salmon lice from fish farming affects wild fish	Salmon lice from fish farming significantly increase the mortality of wild salmonids
Viral diseases (Goal 1)	Disease transmission from fish farming affects wild fish	Viral transmission from fish farming significantly increase the mortality of wild salmonids
Discharges of organic material: (i) local effects (ii) regional effects (Goal 3)	Emissions of organic materials to the surrounding environment	(i) Unacceptable change in sediment chemistry and faunal communities in the production zone (ii) Significant change in bottom communities beyond the production zone—regional impact
Discharges of nutrients: (i) local effects (ii) regional effects (Goal 3)	Emissions of nutrients to the surrounding environment	(i) Nutrients from fish farms results in local eutrophication (ii) Nutrients from fish farms results in regional eutrophication

2.2.2 Salmon escapes and interaction with wild conspecifics

To counter the exploitation of wild living resources for food (Myers & Worm, 2003), domestication and captive production of some key species have come into play as feasible alternatives (Teletchea & Fontaine, 2014). To improve production in captivity, selective breeding and the use of non-local strains are often applied, which then create potential problems if these non-local, domestic strains escape into the wild, potentially affecting via gene flow and causing ecological disruption (Ellstrand et al., 1999; Randi, 2008). Such escapee-driven gene flow has been found to be common in fishes where, for human nutritional purposes, the usually uncontrolled harvest of wild populations is replaced by large-scale aquaculture production, as in salmonids. From the early pioneering days in 1970, a fast and almost continual growth has made Atlantic Salmon aquaculture the world's most economically important industry within fisheries and aquaculture. The first Norwegian breeding program, that gave life to the commercial strain now widely known as Aqua-Gen, from 1972 onwards focused on accelerating growth rates and food conversion (Gjedrem, 2000, 2010; Gjedrem et al., 1991). Afterward, the programme combined other commercially important traits such as age of sexual maturation (1980), furunculosis susceptibility (1989), fat content and fillet colour (1990) and resistance to infectious salmon anaemia (1992) (Gjedrem, 2000, 2010; Gjedrem & Gjøen, 1995; Glover, Solberg, et al., 2017). Inclusion of these traits in the breeding program occurred concomitantly to a series of genetic studies that demonstrated significant heritability estimates for some traits, such as body weight (Gjedrem & Aulstad, 1974; Gjerde & Gjedrem, 1984) chance of mortality associated with vibriosis infection (Gjedrem & Aulstad, 1974) and smoltification (Skaala et al., 2019). It is intuitive then, after five decades and several generations of artificial selection, to question how farmed salmon are now genetically different from their wilder counterparts, and a problem occurs in the fact that tens of thousands of farmed salmon frequently escape from open sea cages, posing a genetic risk to wild populations (160.000 farmed Atlantic salmon in one single episode in Chile (2017) and 56.000 in another single accident occurred during February 2018, data from Marine Harvest). Despite the strenuous development of more efficient production systems, technical and operational failures still occur, leading to a very high number of escapes (Bolstad et al., 2021; Jensen et al., 2010). Although the vast majority of escapees disappear post-escape because they may be unsuitable for wild survival (Hansen, 2006;

Skilbrei et al., 2010, 2013), every year relevant numbers of farmed salmon are still observed on wild spawning grounds, despite efforts to reduce escapes (Fiske et al., 2006; Zhang et al., 2013).

A minor portion of these escapees is formed by juvenile males from inland facilities that survive, mature precociously and can potentially spawn with wild fish. Juvenile escapees of both sexes that eventually survive can also migrate to sea and return in the future as adults and spawn with wild fish (Lacroix & Stokesbury, 2004). The major portion of wild escapes come from open sea cages where the post-smolts and adults are maintained (Crozier, 1993; Glover, 2010). Still, due to increasing global production, each year thousands or hundreds of thousands of farmed salmon escape into the natural environment in Norway (Figure 2.1). As suggested by Glover et al., (2017) these statistics underestimate the real entity of the problem, thus it can be reasonably assumed that the numbers of salmon escapes are in the order of millions, yearly. The estimated number of wild adult salmon recorded during their upstream migration to spawn was ~1 million in the mid-1980s and ~0.5 from 1983–2014 (Taranger et al., 2015). In Norway (50% of the global production), the estimate of salmon escaping annually from commercial fish farms has probably been in the order of millions in the period 2005–2011 (Skilbrei et al., 2015.). According to Glover et al., (2017), in Norway the number of farmed Atlantic salmon escapees probably exceeds the number of wild adult salmon returning to their spawning grounds in their upstream migration in most years. Triploid salmon, that are sterile, constituted ~54 000 of the 157 000 reported escaped salmon in Norway in 2015, although such statistics are not available for other years. Strikingly, a recent analysis estimated that the correct number of farmed salmon escaping from Norwegian farms in the period 2005–2011 was 2–4 times higher than the official statistics (Skilbrei et al., 2015.).

The potential for sexually mature farmed salmon to interact and reproduce with their wild counterparts is proven. Some of these fish reach the spawning grounds (Carr et al., 1997; Carr & Whoriskey, 2006; Morris et al., 2008) and participate in spawning with native populations (Carr et al., 1997; Lura et al., 1993; Lura & Sægrov, 1991; Webb et al., 1991, 1993), with the chance of gene flow from farmed to wild populations. The fact that large numbers of farmed escapees have been observed on the spawning grounds of some native populations has generated global concerns regarding the ecological and evolutionary consequences this may have for recipient populations. Spawning of adult escapees has been reported in rivers in

Scotland (Butler et al., 2005; Webb et al., 1991, 1993), Norway (Lura et al., 1993; Lura & Sægrov, 1991; Szegrov et al., 1997), Canada (Carr et al., 1997) and outside the species' native distribution range on the Pacific coast of North America (Volpe et al., 2000).

It has been shown that the relative spawning success of adult farmed salmon in the wild is significantly lower than their wild counterparts (Fleming et al., 1996, 2000; Weir et al., 2004). Based upon common-garden studies in seminatural spawning arenas, estimates of the spawning success of farmed escapees accounted for 11 to 19% the reproductive success of the wild equivalents (Fleming, 1996; Fleming et al., 1996). It has to be considered though, that their relative success is varied and might be multifactorial and case-specific (Fleming et al., 2000; Weir et al., 2004). The relative spawning success of adult farmed escapees has been suggested to change also in relation to the life stage at which the escape took place (Fleming et al., 1997; Weir et al., 2004). For instance, some experiments have shown how adult farmed males were able to reach 24% of spawning success in the spawning arenas at IMS field station (Fleming et al., 2000).

Comparative spawning studies between wild and farmed salmon have also been conducted in completely natural systems, leading to the conclusion that farmed identities are less competitive (Fleming et al., 2000). As reviewed by Glover *et al.*, (2017), two pivotal implications result from the studies on farmed *vs.* wild interaction in the wild. From 10% of adult farmed escapees that reach the spawning grounds and participate in in mating, the resulting potential genetic contribution is likely to be lower than 10%. And, differences in reproductive success between the males and females strongly suggest that the majority of the genetic contribution derives from farmed females spawning with wild males, thus producing hybrids.

Sperm quality has been shown to influence the reproductive success of farmed escapees in the wild. Several studies have illustrated the difference in sperm morphology (Gage et al., 1995, 1998, 2004) and fertilization success among individuals (Yeates et al., 2014). Nonetheless, when farmed and wild salmon have been reared under the same conditions (Yeates et al., 2014) or taken directly from the environment they are adapted to (farms and wild environment) (Camarillo-Sepulveda et al., 2016), differences have not been shown in either sperm and egg quality or *in vitro* fertilization success. Thus, leading to the conclusion that, despite their inferior competitiveness, if escaped farmed individuals manage to partake in wild spawning, their fertilization success will be similar to that of wild individuals.

Therefore, there is potential for genetic interaction between these escapees and the native populations. After decades of “unnatural” selection, where for unnatural we intend a selection that has followed routes that are not strictly the ones operated by the environment in which salmon live, wild Atlantic Salmon genotype has been negatively affected by the escape of farmed counterparts (Glover, Solberg, et al., 2017)) adding other technological concerns associated with its production (Lorenzen et al., 2012). This has been also documented by using comprehensive simulated release experiments and statistical modeling (Skilbrei et al., 2010, 2015). As a consequence for instance, Norwegian authorities have implemented DNA tracing tools to identify the farm of origin for escapees where these have not been reported (Glover, 2010; Glover et al., 2008; Z. Zhang et al., 2013). In this case, the risk assessment has been evaluated considering both the number of farmed salmon escaping into the wild (reported and unreported) and the actual physical mixing of farmed escapees observed on the spawning grounds of wild populations with the subsequent level of genetic introgression resulting from successful spawning. Finally, some studies have also reviewed the consequence of genetic introgression for both the short-term fitness consequences, and the longer-term evolutionary ones (Glover, Solberg, et al., 2017; Skaala et al., 2019)

So far, to improve the sustainability of this aquaculture sector, the containment strategy has been the most used approach. Conversely, the fitness of hatchery fish produced via supportive breeding for deliberate introduction into the wild has been improved to preserve wild stocks (Araki et al., 2008; Araki & Schmid, 2010b). There might also be substantial effects in wild populations caused by the interaction with native brood stocks that are non-locally adapted or domesticated.

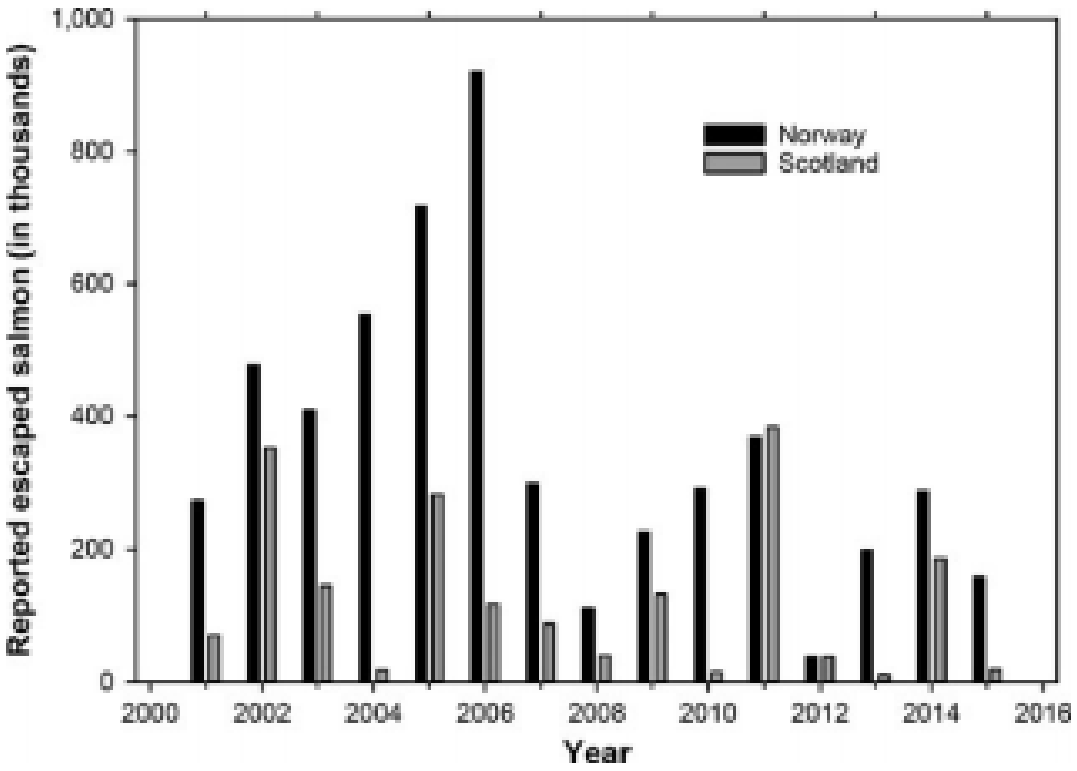

***Figure 2.1**. Reported numbers of farmed escaped Atlantic salmon in Scotland www.aquaculture.scotland.gov.uk and Norway www. fiskeridir.no in the period 2001 to 2015. Triploid salmon constituted ~54 000 of the 157 000 reported escaped salmon in Norway in 2015, although such statistics are not available for other years. A recent analysis estimated that the correct number of farmed salmon escaping from Norwegian farms in the period 2005–2011 was 2–4 times higher than the official statistics (Skilbrei, Heino et al., 2015). Adapted from Glover et al., 2017).*

2.2.3 Genetic impact of farmed salmon on wild conspecifics

Atlantic salmon display a clear population genetic structure throughout their native range (Ståhl, 1987, Verspoor et al., 2005). This structure is the ultimate result of several processes, such as recolonization patterns, genetic isolation by distance (Glover et al., 2012) and by landscape features which affect connectivity (Dillane et al., 2008)). In addition to differences in allele frequencies of molecular genetic markers, Atlantic salmon populations display different life-history traits and associated strategies. While much of this phenotypic variation is driven by the environment, some of these differences are influenced by underlying genetic variation that reflect adaptive responses to their native streams (Fraser et al., 2011; Garcia De

Leaniz et al., 2007; Taylor, 1991) according to the evolutionary phenomenon known as local adaptation (Taylor, 1991).

Throughout the years, salmon breeding programs have successfully selected for fish that grow multiple times faster under farming conditions (Glover et al., 2009; Solberg, Skaala, et al., 2013; Solberg, Zhang, et al., 2013). In addition to traits that have been specifically selected, genetic changes in non-targeted ones have also been observed, such as changes in predator awareness (Einum & Fleming, 1997), stress tolerance (Solberg, Skaala, et al., 2013), and gene transcription (Roberge et al., 2006). Moreover, decreased genetic variation, has been revealed by the use of molecular genetic markers (Norris et al., 1999; Skaala et al., 2004), and lower estimates of heritability for growth (Solberg, Skaala, et al., 2013) have been observed in wild populations. It is important to remember that Norwegian farmed Atlantic salmon, that dominate the global production, derive from over 40 Norwegian rivers, and have undergone ten or more generations of intense domestic selection (Gjedrem, 2010). The limited farmed effective population size has been correlated with reduced genetic variation using molecular genetic markers due to founder effects, while the reduction in heritability for growth is likely to be a result of successful directional selection for this trait over multiple generations.

Estimation of cumulative introgression of farmed salmon genes is statistically challenging, although several studies have observed farmed-derived changes in wild population in Canadian (Bourret et al., 2011) , Irish (Clifford et al., 1998a, 1998b; Crozier, 1993, 2000) and Norwegian waters (Glover et al., 2012; Skaala et al., 2006). However, so far, the most complete analysis to elucidate the introgression dynamics in the last 4 decades has taken place in Norway by merging Bayesian Computation, and genetic data for wild-historical, wild-contemporary and a varied range of farm samples that were genotyped for a set of informative single-nucleotide polymorphic markers (Glover, Pertoldi, et al., 2013; Karlsson et al., 2011, 2016).

Using a multifactorial approach, a Norwegian study that surveyed 20 rivers (Glover, Pertoldi, et al., 2013), has revealed less introgression of farmed Atlantic salmon in many Norwegian populations, as compared to the reported numbers of escapees in these populations, and estimations from introgression models (Glover, Pertoldi, et al., 2013; Glover, Wennevik, et al., 2020). Glover *et al.* (2013) concluded that the spawning success of farmed escaped salmon has been generally lower than expected by computational models, at least in most of

the Norwegian rivers. These results were consistent with earlier estimates of spawning success obtained in common garden experiments (Fleming et al., 1996, 2000). However, results from the Glover *et al.,* (2013) study, showed high levels of introgression in some of the native populations analyzed, and together with a previous study using microsatellites, reported decreased genetic differentiation over time among populations (Glover et al., 2012; Glover, Pertoldi, et al., 2013). These data, taken together, may suggest that the uncontrolled introgression of farmed salmon might lead to erosion of population genetic structure among native populations (Mork, 1991).

More challenging than estimating introgression itself is estimating the effects of this on life history traits, population fitness, and long-term evolutionary capacity of wild populations. Wild populations display large natural variation in marine survival, and at the same time are influenced by a wide range of anthropogenic factors (Parrish et al., 1998), which may potentially mask biological changes caused by introgression of farmed salmon. Nevertheless, comparative studies in Ireland and Norway have demonstrated additive genetic variation for fitness in the wild, with offspring of farmed salmon displaying lower survival than fish of native origin (Fleming et al., 2000; McGinnity et al., 1997; Skaala et al., 2012) as also shown for other salmonids (Araki et al., 2008; Araki & Schmid, 2010).

In summary, understanding the effects of coexistence and genetic interaction between farmed escaped and wild salmon on the spawning grounds of native populations is of pivotal importance for four main reasons: 1. Farm escaped salmon have undergone through a directional selection for commercially important traits within breeding programs; 2. domestication selection (non-targeted genetic changes associated with adaptative responses to the human-controlled environment and following reduction in natural selection pressure); 3. random genetic changes during domestication (initial founder followed by genetic drift across generations); 4. ancestry differences as farmed salmon may be of non-local or mixed-origin (Ferguson et al., 2007; Glover, Solberg, et al., 2017). These potential problems mean that understanding the potential for reproduction between farm and wild salmon and their hybrids is an important area of research.

The Atlantic salmon has now become a model system for understanding direct genetic interactions between domesticated and wild fish stocks (Bekkevold et al., 2006). Given its huge production and consumption, the risk for highly-valued wild populations, and the many years since salmon farming was initiated, further studies are needed for a comprehensive risk

assessment that would be beneficial both for wild populations and for commercial aquaculture. For instance, ‘hybrid’ fish generated through escapees’ interactions in the wild could show reduced reproductive performance, allaying concerns about introgression, or they may have similar or even superior reproductive performance. My Ph.D. will address this question at the gamete level. Given the variability in reproductive strategies and local adaptive phenomena exerted by *S. salar*, and taking into account the artificial selective mechanisms operated by breeding programs, more research is needed. This should focus for instance on farm vs. wild salmon interactions and the gametic and sexual selective mechanism (both pre- and post-copulatory), using both *in vitro* and *in vivo* assays of reproductive potential to produce scientific evidence that will contribute to making long term reliable provisional data for salmon escapes. For this reason, the following part of this literature review will focus on gamete evolution in Atlantic salmon.

2.3 Gametes and evolution

2.3.1 Background and Rationale/Justification

In the living world, organisms grow energetically, compete, communicate, migrate in order to transmit their genes to future generations. Sexual selection is the evolutionary selection process that favours the genes that can enhance the frequency that confer a reproductive advantage. This process, that Darwin thought to be exclusively precopulatory, has been more recently discovered to follow more complex rules. Darwin assumed female's monogamy whereas, starting 30 years ago, an increasing number of studies shows diffuse polyandry and promiscuity in a multitude of taxa (Birkhead & Moller, 1998; Lüpold et al., 2009). The implication of these discoveries lies in the fact that sexual selection persists after the mere event known as copulation. The postcopulatory sexual selection includes both male-male competition and cryptic female choice.

The male-male competition, namely "sperm competition" occurs between sperm cells of different males that compete in order to fertilise the same egg/set of eggs (Parker, 1970). Sperm must survive and perform outside of the male's body and compete against rival sperm when trying to fertilise the egg. Spermatozoa face numerous challenges during their journey to the egg (Birkhead et al., 1993) and, the vast majority of them will not achieve the scope of passing male's genes to future generations. Thus, sperm experiences intense selection trying to fertilise the egg with important implications for the evolution of its quality.

Fertilization in animals occurs both outside and inside the female reproductive tract, with external fertilization considered the ancestral state (Levitan & Petersen, 1995). It can be guessed how the above-mentioned kind of competition gains extreme importance in external fertilisers. In species with internal fertilization, sperm is released directly inside the female reproductive tract by apposite organs or taken up by females in moment antecedent the sperm-egg fusion inside the female's body. The latter is the selective environment that prevents most of the sperm from even reaching the egg (Birkhead et al., 1993). In contrast, in externally fertilizing species, gametes are typically released into an aquatic environment where they meet and fuse, forming the zygote. In these species, sperm face extrinsic environmental stressors (e.g., osmotic, ph, temperature, reactive oxygen species (ROS)) and

variable environmental conditions (e.g., water turbulence, siltation) that change the dynamics of fertilization success (Billard, 1986; Hirano et al., 1978; Pennington, 1985).

Being fertilization-competent is therefore not enough for most sperm to secure fertilization because sperm competition (Parker, 1970), cryptic female choice (female preference for sperm from certain males (Eberhard, 1996; Firman et al., 2017; Thornhill, 1983), and environmental selection, all shape the evolution of sperm traits by selecting through postcopulatory sexual mechanism the sperm cells that are best able to outcompete rivals, be selected by female preferences, and overcome environmental challenges.

2.3.2 Reproductive biology of Atlantic salmon: sperm behaviour as a meaningful evolutionary constraint

Salmonid fish are keystone species with a remarkably complex array of life history traits within one Family. An evolutionary history, characterized by a fast radiation (Phillips & RÁb, 2001), local adaptation phenomenon (Leániz et al., 2007; Primmer, 2011b), and variability in reproductive strategies, make salmon a model system to understand natural and sexual selection and their relation in defining processes such as speciation, gamete evolution and the role of hybridization in an evolutionary context. Among this family, Atlantic salmon (*Salmo salar*) show an extraordinary variability of life histories, phenotypes and reproductive adaptations which have derived from intense competition to maximize reproductive success (e.g., nest building, intense male hierarchy and competition, large female reproductive investment and vast differences in size- and age-at-maturity among individuals) (Fleming, 1996). This species is polyandrous (Jordan et al., 2007), females can be fertilised up to 16 males in one next (Weir et al., 2010), and show asynchronous gonadal maturation (Scott, 1987). These characteristics, together with the capacity by males to spawn rapidly and in multiple sessions, result in male-biased operational sex ratios that generate intense male competition for mates (Fleming, 1996; Fleming et al., 1997; Fleming & Einum, 1997; Webb et al., 2007; Webb & Hawkins, 1989). Thus, male secondary sexual characters associated with fighting and status signalling, alternative reproductive tactics to access the eggs (e.g., "sneaky" males,) (Fleming, 1996), and sperm competition are a pivotal phenomenon for individual reproductive success (Yeates, 2005).

Sperm competition occurs when sperm different individuals compete simultaneously to fertilise a set of eggs (Parker, 1970). This form of intra-specific competition is abundant in nature and it has been documented across a variety of taxa (reviewed in Birkhead & Moller, 1998; Birkhead & Pizzari, 2002). Usually, sperm competition occurs mainly in species where males have low access to females and in polyandrous species in order to maximize reproductive success (Taborsky, 1998, 2001). Consequently, distinct reproductive traits can be selected and result in the evolution of alternative reproductive tactics (size at maturity, behaviour, colour polymorphisms) (Taborsky et al., 2001). Teleost fish, because of their (generally) external fertilization, morphological variations, and parental care, show a relevant divergence in reproductive tactics and performances among the taxa (reviewed by Knapp & Kneff, 2008) that generate opportunities for sperm competition (Taborsky et al., 2008). In males, competition for mates is a common characteristic seen in the family Salmonidae (Blanchfield & Ridgway, 1997; de Gaudemar, 1998; Fleming' And & Gross, 1994; Quinn et al., 1996). In fact, while the evolution of female reproductive traits has been shaped by mechanisms of natural selection, males' reproductive ones have been differentially modulated by sexual selection (Fleming, 1996). As a consequence, sperm competition adds variation to the sexual selection resulting from a relative fertilizing efficiency of male gametes, generating post-copulatory, intra-sexual selection. Sperm characteristics (speed, motility, physic-chemical parameters, egg-sperm interactions) are essential for fertilization (Yeates, 2005); and significant natural variations in sperm performances are well established in this species (Gage et al., 2004; Vladić et al., 2010) as in other animal models, revising the model for which sperm cells are mere "DNA delivery-machines" (Birkhead & Moller, 1998; Birkhead, 1998; Karr et al., 2009). The regulation of these processes is made even more interesting by another major attribute. In salmonids, eggs quantity is more abundant relative to sperm and, in contrast to what happens in other animal models, except the majority of teleosteans, the fertilisation of the egg happens in a predetermined, confined, zone (micropyle) which is barely large enough to allow the passage of one spermatozoon (Hart, 1990; Kobayashi & Yamamoto, 1981; Yanagimachi et al., 1992)..

In males, the reproductive dynamics that influence fertilization are of particular interest because of the presence of alternative reproductive tactics (Taborsky, 1998). In another Salmonid, the sockeye salmon *Oncorhynchus nerka*, it has been hypothesized that sperm characteristics may vary among male phenotypes due to the different selective pressures to

which these biological entities are exposed (Hoysak & Liley, 2001). For instance, "sneakers" males, those males that steel paternity to large anadromous males, and so have a greater risk of sperm competition, invest extremely in gonadal development and sperm quality. Mature Atlantic salmon parr show higher gonado-somatic indices (GSI), relatively higher sperm numbers (Gage et al., 1995) and greater sperm motility than large anadromous males do (Daye & Glebe, 1984; Gage et al., 1995). The presence of a heritable basis in establishing male parr maturity ((Garant et al., 2003; Gjerde & Gjedrem, 1984; Nævdal, 1983; Nævdal et al., 1978)), alongside the effect of environmental factors and energy reserves (e.g., lipids) in determining the threshold between growth-rate and pubertal development (Berglund, 1992), evidence for reproductive traits mediated by frequency- and status- dependent selection exists (Fleming, 1996). In contrast to mature male parr, anadromous males are likely to invest heavily in behavioural activity during reproduction, fighting and somatic growth, causing them to have significantly lower survival rates than their female counterparts (Fleming, 1996; MacDonald, 1970; Purdom, 1993). In anadromous males, access to mating opportunities is positively correlated with body size and breeding success (Webb & Hawkins, 1986). However, we still don't know how sperm quality differences vary among populations, and their eventual relation to body size in anadromous males.

In order to correlate breeding success as a function of size and link it to the evolution of different reproductive strategies and phenotypes in this species, parent and off-spring genotyping experiments and sperm competition trials should, therefore, be conducted. Moreover, the body-size alone cannot justify the variability in reproductive techniques adopted by this species and their extraordinary different coloration and patterning among the spawning males. In coho salmon *Oncorhynchus kisutch* males with less intense red spawning coloration showed higher sperm velocities than males with darker red spawning coloration and no relationship was found between male body size and sperm traits (Fleming et al., 1997). In the *S. salar*, it has been shown that differential coloration patterns (female-like) adopted by some males to reduce intra-sexual competition (e.g., aggression) (Fleming et al., 1998). In anadromous individuals from both sexes, the gonadal investment is about 59% of their total energy reserves to mate, with larger individuals expending more than smaller ones (Jonsson et al., 1991, 1997), suggesting that also quality and not necessarily only the quantity of gametes is a meaningful evolutionary constraint for reproductive success in this species. On average, only 11% of the spawners, survive to breed on another year even if, in some populations, it may be as high as 43% (Ducharme, 1969). Jonsson et al., (1997) demonstrated

that in Atlantic salmon, the chance of repeat breeding decreases with increasing fish size, reflecting a general increase in energy expended during reproduction (Jonsson et al., 1997).

While the general knowledge on the reproductive biology in this species is well established, a lack of information is present regarding its evolutionary causes and consequences.
Reproductive success, as a focused evolutionary driving force for gametes (and the genes they contain), is crucial to understand natural and sexual selection (Gilbert & Endler, 1987), local adaptation (Williams, 1966), and stability of genetic, phenotypic, and behavioural traits within a population. Thus, the high reproductive investment showed by *S. salar* is by itself a plausible explanation for the peculiar reproductive adaptations mentioned above.

2.3.3 Sperm behaviour and intra-specific post-copulatory sexual selection

Sperm competition and cryptic female choice are considered to be the most relevant mechanisms of post-copulatory sexual selection (reviewed by Birkhead & Pizzari, 2002). In Atlantic salmon, nesting females do express mate choice in order to gain genetic benefits for the offspring (Fleming et al., 1997). In internal fertilisers, females can favour certain partners over others, before mating, and with the female reproductive tract during and after mating (Eberhard, 2004). However, in external fertilisers such as Salmonids, the lack of internal genital traits involved during mating, constrains mate choice (Eberhard, 1996, 2004). Despite pre-copulatory choices, external fertilisers are generally assumed to be unable to determine parenthood; the female cannot influence fertilization since this process occurs outside of her body. Importantly, it has recently been shown, that in these species, cryptic female choice can be mediated by the ovarian fluid (Evans et al., 2013; Evans & Sherman, 2013; Gasparini & Pilastro, 2011; Yeates et al., 2013) and by gamete-recognition proteins thus addressing the fertilization success to genetically compatible conspecifics (Evans et al., 2013; Evans & Sherman, 2013; Rosengrave et al., 2016).

In salmonids, the ovarian fluid is a viscous substance that is released with the eggs and comprises 10-30% of the total volume of the spawned egg mass (Rosengrave, Montgomerie, et al., 2009; Rosengrave, Taylor, et al., 2009). At fertilization, spermatozoa face an increasing ovarian fluid concentration as they approach the egg in order to fertilise it. The highest ovarian fluid concentration has been found in proximity with the micropyle (Rosengrave et al., 2008). The positive relation between OF and sperm behaviour is now well documented,

salmonids included (Rosengrave, Montgomerie, et al., 2009; Turner & Montgomerie, 2002). The ovarian fluid contains various nutrient, metabolites, and hormones (Hirano et al., 1978; Ingermann et al., 2001; Lahnsteiner et al., 1995), that can play an important role in influencing sperm behavior. A recent study evidenced the effects of ovarian fluid pH on sperm behaviour and motility in the rainbow trout *Oncorhynchus mykiss* (Wojtczak et al., 2007). Additionally, in the chinook salmon *O. tshawytscha* it has been shown that sperm behaviour is differentially modulated by the ovarian fluid depending on male identities, suggesting that some males bring genetic information that is more attractive for the female and so for the offspring (Rosengrave et al., 2008). These findings address the importance of reproductive fluids in post-copulatory selective mechanism in fish. More importantly, these results become relevant especially in Salmonids, in which sperm cells are motionless until they come in contact with the water (if not considering the role of ovarian fluid) and only show motility (in water) for approximately 30s (Billard & Cosson, 1992; Billard, 1986), this reason brought Huxley (1930) to think that sperm cells are severely maladapted to fresh-water life. The short fertile window of gametes, together with sperm competition and sperm-egg/ovarian fluid interactions make the Salmonids a challenging model with great opportunity to understand how evolution acts on and shapes gamete biology.

3 Hatchery gamete handling and management: embryo development and hatching rates under different fertilisation techniques and reproductive competitiveness between farmed and wild salmon

Chapter's contributions:

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Statement of attributions: M. Gage and K. Glover provided fundings, operational facility and instrumentations. M. Solberg and K. Glover provided the fish and screened their genetic backgrounds prior to the experiments. M. Graziano and M. Gage conceptualised the work. M. Graziano, M. Gage and D. Murray conducted the fertilisation experiments. M. Graziano and L. Dryhovden monitored the fish until hatch and collected the samples. M. Graziano conducted the CASA experiments and the sperm competition trials. Data extraction, curation and analysis were performed by M. Graziano. Throughout the experiments hatchery technical support received was kindly ensured by L. Dryhovden and I. Helge. Sørvik and S. Kutnar trained M. Graziano to perform molecular analysis according to Lab's protocols and validated the diploid/triploid status of genotyped individuals and helped assign paternity. M. Graziano performed the molecular analyses. M. Graziano created all the tabs, figures and illustrations. M. Graziano wrote the first complete draft of this work and all the following versions after receiving the review comments and suggestions from the Supervisors M. Gage and S. Immler.

Keywords:
Conservation aquaculture, farmed salmon introgression, sperm competition, sperm quality, ovarian fluid, reproductive competitiveness

3.1 Abstract

Wild Atlantic salmon (*Salmo salar*) populations have seen a drastic decline worldwide over the past century. During the same period, the commercial farming sector for this species has seen one of its biggest expansions ever witnessed. This has been identified as one of the main causes for reduction in wild salmon numbers, together with a series of other environmental risk factors associated with salmon aquaculture questioning its sustainability. Continuous and massive escapes of artificially selected salmon from aquaculture facilities have been found to quickly dilute locally adapted wild genotypes, contributing to their decline. A proposed mitigation strategy to recover salmon populations consists of the production of wild Atlantic salmon within conservation hatcheries through artificial fertilisation and rearing methods. However, low hatching and survival rates from this method are still observed in both aquaculture and conservation hatcheries, which require further development. We explored the effects of two *in vitro* fertilisation methods commonly used in hatcheries on gamete fertility, compatibility, embryo development and reproductive outcomes in farmed and wild strains of Atlantic salmon. We also examined how variation in fertilisation techniques might cause chromosomal abnormalities and embryonic deaths, explaining the lower numbers achieved in hatcheries; research which intends to improve hatcheries' sustainability and wild salmon reintroduction efforts. In a second set of experiments, using the same farmed and wild strains, we assessed reproductive performance at the gamete level in a simulated competitive fertilisation environment. We found that farmed salmon strains were far more resilient to different fertilisation techniques, their embryonic development was faster, and they manifested a higher degree of chromosomal abnormalities as compared to wild fish. When wild and farmed males competed over fertilisation of eggs from wild and farmed females, farmed male paternity rates ranged from 70 to 100% in both types of females, suggesting a clear threat to wild genetic pools following escapes. Our results stand in contrast with previous findings and present important insights that can help improving sustainability and reintroduction efforts in conservation aquaculture.

3.2 Introduction

Atlantic salmon (*Salmo salar*) aquaculture is projected to surpass three-million tonnes per annum in 2022, making it one of the most dominant productions worldwide. It is certainly the one which has seen the biggest production increase in the last five decades and is forecasted for even greater expansion in the future (Tveteras et al., 2019). However, as expected the rapid growth of this sector has not come unaccompanied: a set of sustainability issues must be overcome in concert with the blue-economy revolution, along with unquestionable ethical standards and practices (Dadswell et al., 2021; Torrissen et al., 2011). This colossal production has been found responsible for several environmental impacts, and sadly also as one of the factors driving the decline of wild salmon populations worldwide (Limburg & Waldman, 2009; McGinnity et al., 2003; Schindler et al., 2010). As thermally sensitive, locally adapted, externally fertilising, and migratory fishes, Atlantic salmon are already profoundly affected by anthropogenic environmental impacts. Climatic changes (Jonsson & Jonsson, 2009; Thorstad et al., 2021; Walsh & Kilsby, 2007), pollution (Forseth et al., 2017; Magee et al., 2011), parasitic infections (Johansen et al., 2011; Krkošek et al., 2013, 2014), reduction in suitable spawning and marine habitats (Marschall et al., 2011; Thorstad et al., 2017, 2021) and overfishing (directly and indirectly through depletion of biomasses - salmon diets) (Czorlich et al., 2022; Hard et al., 2008), have all been proven to conspire in the decline of wild salmon. Put into context, while farmed salmon production was growing from 59,000 to 2,400,000 tonnes per year between 1983 and 2018, wild salmon populations experienced a drastic decline in their numbers with a concomitant harvest of the ‘wild’ stocks diminishing from 8 to 3.4 million fish per year within the same period (NASCO Annual Report, 2021). More than a decade ago, Gross (1998) calculated that roughly 94% of adult Atlantic salmon on the planet were farmed fish, and this estimate has likely grown recently.

Among the factors threatening the survival of wild salmon populations, the aquaculture industry has an indisputable detrimental role. This is due to the issues caused by the exorbitant number of farmed salmon escaping into the wild, leading to genetic introgression and shifts in life histories (Bolstad et al., 2017; Glover et al., 2012, 2018; Skaala et al., 2006), transfer of diseases and parasites (Johansen et al., 2011; Mordecai et al., 2021; Shea et al.,

2020), reduction in genetic variability, and the dilution of locally adapted phenotypes (Bourret et al., 2011; Glover, Pertoldi, et al., 2013; Glover, Wennevik, et al., 2020; Primmer, 2011; Vincent et al., 2013).

Atlantic salmon farm escapees have been shown to survive and hybridise with their wild counterparts with farm genotype introgression being observed across the entire natural range of the species (Glover et al., 2009; Glover, Solberg, et al., 2017; Glover, Wennevik, et al., 2020; Karlsson et al., 2016). Through decades and several generations of artificial selection within the hatcheries, farmed salmon have been selected for a multitude of commercially relevant traits, making them genetically different from wild fish (Gjedrem, 2000, 2010; Gjedrem & Gjøen, 1995; Glover, Solberg, et al., 2017). In addition to the volitional selection of traits for commercial purposes, these genetic changes have affected salmon fitness in the wild due to domestication, and several studies have reported such changes in non-targeted traits like predator avoidance, aggression and feeding behaviour (Einum & Fleming, 1997; Houde et al., 2011).

Sexually mature farmed salmon can reach spawning grounds following escapes (Carr et al., 1997; Carr & Whoriskey, 2006; Morris et al., 2008) and partake in reproductive events within wild populations (Carr et al., 1997; Lura et al., 1993; Lura and Sægrov, 1991; Webb et al., 1993, 1991). Concerningly high numbers of farmed escapees have been detected in the spawning grounds of some native populations, thus generating global concerns regarding the ecological and evolutionary consequences for recipient populations and the whole environment (Butler et al., 2005; Webb et al., 1991, 1993); Norway: (Lura et al., 1993; Lura & Sægrov, 1991; Szegrov et al., 1997), Canada: (Carr et al., 1997). Although the relative spawning success of adult farmed salmon in nature seems to be lower than their wild counterparts (Fleming, 1996d; Weir et al., 2004, 2010b), the continuous and substantial afflux of escaped farmed fish keeps sustaining introgression rates in the wild, that can be as high as 47 % (Glover, Pertoldi, et al., 2013). In seminatural spawning arenas, estimates of the spawning success of farmed fish accounted for 11 to 19 % of the reproductive success in comparison to the wild fish (Fleming et al., 1996). Relative spawning success of adult farmed escapees has been suggested to change also in relation to sex and the life stage at which the escape took place (Einum & Fleming, 1997; Fleming et al., 1996; Weir et al., 2004), with adult farmed males reaching a quarter of the total spawning success in semi-artificial spawning arenas (Fleming et al., 2000). In completely natural systems, comparative studies

between wild and farmed salmon at spawning have drawn the conclusion that farmed fish are generally less competitive (Fleming et al., 2000). Also, genetic studies suggest differences in reproductive success between farmed males and females, with the latter likely driving most of the genetic contributions to introgression (Glover, Solberg, et al., 2017). However, we still do not know whether these different performances are due to different fertilisation rates rather than reproductive potential; or due to different embryonic, juvenile, and adult survival. Thus, testing competitiveness at the gamete level between farm and wild fish strains could prove an important tool in mitigating farmed introgression in the wild, and in evidencing its causes from a reproductive point of view.

Sperm quality has been shown to control the reproductive success of farmed escapees in the wild. Several works from our lab have illustrated differences in sperm morphology and fertilisation success among farmed and wild individuals (Gage et al., 1995, 1998). Even so, no differences have been demonstrated in either sperm/egg quality or *in vitro* fertilisation success (Yeates, 2005; Yeates et al., 2014; Yeates & Gage, 2003), leading to the conclusion that, although less competitive, escaped farmed individuals could have similar reproductive potential to wild fish. Therefore, there is a great need to assess fertilisation dynamics and reproductive output on farm strains in comparison to wild fish, partially to explain the number of genetic interactions recorded in nature between these escapees and the native populations. Dynamics of sexual selection and reproductive success may be regulating genetic introgression, driving the loss of locally adapted traits through the introduction of farm genes, and leading to the lower offspring fitness and survival observed in nature (Bourret, O'reilly, et al., 2011; Fraser et al., 2008, 2011; Leániz et al., 2007; Primmer, 2011; Vincent et al., 2013).

To counteract the drastic decline in wild Atlantic salmon populations, several conservation strategies have been put in place, including more rigid policies for escapes management and withdrawal of resources, habitat improvement, and reintroduction of wild fish at different life stages, as well as the mitigation of salmon farm impacts (Bradbury et al., 2020; Bui et al., 2020; Hare et al., 2019; Mahlum et al., 2021; Powell et al., 2018; van Leeuwen et al., 2020; Yatabe et al., 2020). The mitigation strategies have focused on reducing the mass escapes from sea cages which still constitute the main source of the problem (Glover et al., 2012; Jensen et al., 2010). In this regard, some countries use wild-heritage local salmon strains, which are adopted for commercial production purposes because of the lower impact they

have on wild populations during escapes and hybridisation in the wild (Gjedrem, 2012; 2000).

Refurbishment of native salmon populations includes production and successful rearing of wild salmon strains, both in inland freshwater facility and offshore, and use these fish for reintroduction programs in the wild to compensate for the fitness loss (Araki et al., 2007; Araki & Schmid, 2010). In this context, the use of conservation hatcheries to complement mitigation strategies and supplement wild populations is therefore pivotal (Araki & Schmid, 2010; Gausen, 1993). Within these infrastructures gametes from adult fish are collected during spawning season in the wild, and hatchery methods common to commercial aquaculture are applied to fertilise and rear the offspring, that is until it reaches a stage which is less prone to predation and anthropogenic stressors. Because of the inability of Atlantic salmon to spawn within common hatchery tanks, *in vitro* fertilisation is indispensable to enable and optimise offspring production.

Within hatcheries, offspring survival and hatching rates are still very low and, in some cases, only half of the fertilised eggs eventually result in hatched offspring (Craik & Harvey, 1984; Hare et al., 2019; Johnson, 2011; Sutela et al., 2007; Thayer & Hamlin, 2016). High mortality has been recorded preponderantly between fertilisation and the moment in which embryos reach the eyed stage, later in embryonic development (40% vs 11% before and after the eyed stage respectively). After this, also fry survival within commercial aquaculture has the potential to be halved, further affecting productions (Sutela et al., 2007). There are several reasons to believe that common *in vitro* fertilisation protocols, despite having been used for decades, might still be sub-optimal due to the unnatural conditions in which both gametes and offspring are exposed. This is reflected by the lack of substantial improvement on fertility and embryo survival in salmon farms over the last 40 years (Craik & Harvey, 1984; Thayer & Hamlin, 2016). Several factors could in fact influence the reproductive outcome within the hatcheries, and there is a lack of studies which have tried to identify the genetic consequences of artificial fertilisation protocols in this species.

Artificial fertilisation in salmon aquaculture has usually adopted two main methods: ‘dry’ fertilisation, when gametes are in contact (and likely fertilisation takes place) before water is added; or ‘wet’ fertilisation when gametes are activated by water and mixed. *In vitro* fertilisation with the ‘dry’ method, where sperm and eggs within their ovarian fluid come into

contact in the absence of water, is the most popular technique commercial salmon hatcheries. The reason for this is commonly believed to be the result of delayed chorion formation/hardening and the micropyle remaining open for longer under ‘dry’ conditions, thus increasing the time for sperm to be added and for fertilisation to take place (Leitritz & Lewis, 1976). However, more recent studies have shown that fertilisation in this species truly occurs in a matter of seconds (Yeates et al., 2007), so dry fertilisation could favour immediate contact, activation within the ovarian fluid, and gamete fusion to avoid any delays.

In vitro fertilisation protocols may change important characteristics typical of natural spawning conditions such as a gradient of ovarian fluid, an optimal pH and viscosity. *In vitro* fertilisations could also remove or diminish the beneficial effects of the ovarian fluid associated with intra- and inter-ejaculate sperm selection, affecting both pre- and post-mating choice and consequently embryonic fitness and survival (Alavioon et al., 2017, 2019; Immler et al., 2014; Promerová et al., 2017). Previous experiments conducted by our lab have shown that dry fertilisation methods led to a 20 % higher hatching success compared to wet fersilisation (Bemrose et al., 2021). However, they found no evidence for any effect of IVF method on genetic aspects in the offspring such as Unintentional Spontaneous Triploidisation (UST). It has been postulated that UST could be involved in embryonic death, and this could partially explain the low hatching success in artificially bred salmonids within the aquaculture industry (Bjørnevik et al., 2004; Fraser et al., 2013; Ozerov et al., 2010; Salimian et al., 2016) and suggest that the impairment caused by trisomic or triploid assets should be investigated at early developmental stages, before these the individuals bearing these chromosomal assets are purged.

In this study, we examined two main aspects that can help improving the status of salmon populations in the wild: (I) refinement of commercial and conservation hatchery procedures to resolve sustainability issues and ameliorate offspring production, and (II) testing the reproductive competitiveness of farm and wild fish. To do this, we first examined the effects of dry and wet artificial fertilisation methods in farm and wild fish, to determine whether differences in hatching rate are correlated with differences in fertilisation rates, or whether occurrence of chromosomal abnormalities and UST before hatching, and embryo survival post fertilisation may explain the differences between wet and dry fertilisation outcomes.

Additionally, we tested for differences in shared paternity and reproductive competitiveness between farm and wild males, trying to understand if sperm behaviour was affected by different ovarian fluid concentrations in the two strains as a consequence of artificial selection in the aquaculture.

3.3 Materials and methods

3.3.1 Fish origin and handling procedures

The experiments were conducted at the hatchery facilities in Matre (Norway), owned by the Norwegian Institute of Marine Research (NIMR). Two strains of Atlantic salmon (*Salmo salar)* were used for our reproductive competitiveness trials and to test the effects of different fertilisation methods on the reproductive outcome: 'wild' genetic background Atlantic salmon caught from the river Etne (south-west Norway), and one-sea-winter 'farmed' fish made available by the commercial headquarters of Marine Harvest's (MOWI strain, 13th generation of farm breeding, MOWI Ltd). One day before the experiment started, ten adults per sex from each strain were stripped for their gametes after assessing the status of gonadal maturation. During transportation to the Matre hatchery facility, all eggs (including the ovarian fluid) and sperm batches were kept in sealed containers on ice at ± 1°C until use.

3.3.2 Fertilisation experiments under dry vs. wet fertilisation mode

Split clutches and ejaculates from individual males and females were created to benefit from a full-factorial design, so that the two in vitro fertilisation techniques could be applied to control for adult identity (See Figure 3.10 supplementary material). To this end, eggs from each female were divided in four batches containing roughly ~100 eggs each, and we created ten farmed crosses under dry fertilisation *versus* ten farmed crosses under wet fertilisation, and the same procedures were applied to the wild fish. We generated two experimental replicates for each strain: one replicate was terminated 15 days after fertilisation (d.p.f.) to monitor fertilisation rates and determine differences in embryonic development, and the other replicate was used to monitor hatching success and presence of chromosomal abnormalities and USTs in the developing embryos (Figure 3.2). We generated a total of ten crosses per strain subjected to two treatments, 20 + 20, each treatment consisting of ~ 400 eggs per female and 40 eggs rearing units in total. Each egg batch was photographed before the experimental treatments to later retrieve the exact number of eggs used in each fertilisation trial. For each male, four 100 µl aliquots of undiluted milt were used to fertilise the eggs of a randomly selected female with two different treatments (dry and wet) and two types of females (farmed and wild) for each. The eggs were fertilised in 500 mL flat-bottomed bowls by a randomly assigned sperm sample from a male of the same strain.

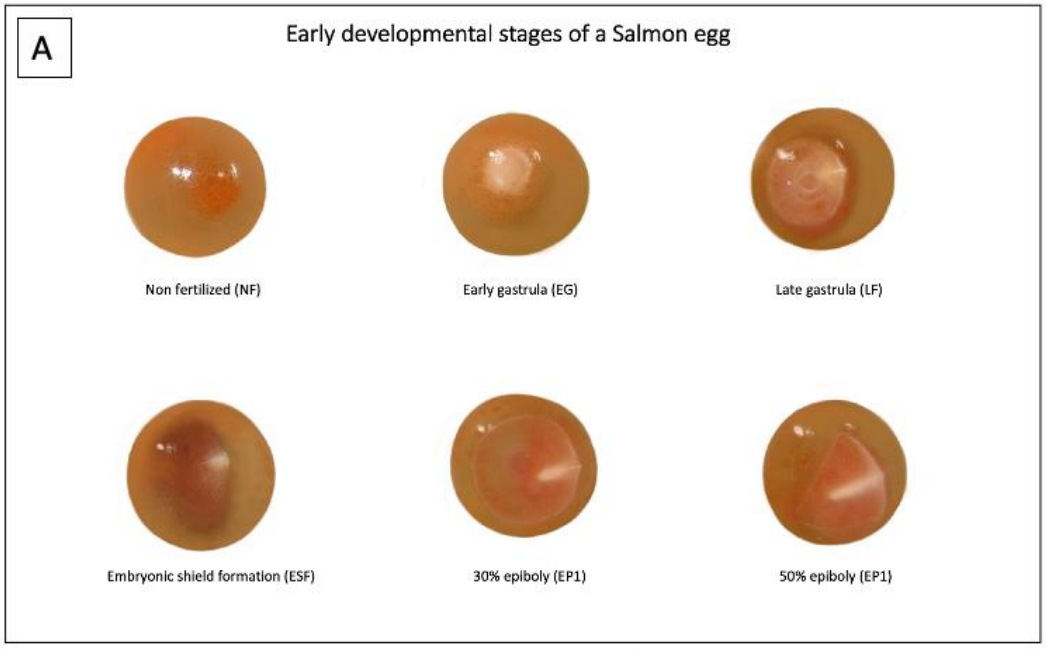
In the dry fertilisation treatment (Johnson, 2004), gametes were in direct contact before 1000 mL of river water was added. Fertilisations took place applying general principles as described in (Yeates et al., 2014) by pipetting the milt directly onto the egg batch and gently mixing gametes together, before adding activating water in dry fertilisation. In the wet fertilisation treatment, the 100 µl of milt were pipetted to the side of the egg batch in the fertilisation bowl, and subsequently mixed by adding the activating water. Gametes were left to stand for 120 seconds after mixing (dry) or once activated with water (wet), to maximize the fertilisation potential.

Fertilised egg batches were moved into a recirculating aquaculture system (RAS) hosting hatchery trays of 45 cm^2, with inner individual PVC units of 15 cm^2 which were labelled with a unique identifier. Fertilised eggs were incubated in full darkness at 6°C ± 0.44 and oxygen kept at saturation in the system.

3.3.3 Developmental speed in farmed and wild strains under different fertilisation modes

At 15 dpf, we assessed fertilisation success by putting one set of eggs (~100 eggs) into a solution of 6% acetic acid to dissolve the chorion and have a clearer view of the inner developing embryo. One macro picture of the inner PVC was taken and used to assess differences in developmental speed. The different stages of embryonic development were assigned according to Gorodilov (1996) and Nagasawa et al., (2013); (Figure 3.1A, B).

Figure 3.1 ***(A)*** *Stages of embryonic development in Atlantic salmon (Salmo salar: Non-fertilised egg (NF), Early gastrula (EG), Late gastrula (LG), Embryonic shield formation (ESF), 30 % of epiboly completed (EP1) and 50 % of epiboly completed (EP2) (from left to right corner respectively);* ***(B)*** *Representative macro picture of different developmental stages within an egg-batch at the time the picture was taken.*



3.3.4 Hatching success, embryonic deaths and chromosomal abnormalities

The second set of egg batches was left to develop to track embryonic abnormalities and USTs in dying embryos over time. Egg batches were assessed for fertilisation success within few minutes after fertilisation by gently mixing and counting the eggs that had turned white (some mechanical damage can be tolerated especially within the first few minutes following fertilisation, Bemrose et al., 2021 in progress). After this first screening, eggs were monitored every three days for embryo mortality until the last fry had hatched. At every count, until the first embryo had reached the eyed stage, eggs in every batch were gently mixed to find and remove the dead whitened eggs. No mixing was conducted from the eyed stage onward to avoid any embryonic damage. The dead eggs and were collected and stored according to treatment and developmental stage (dry/wet, pre-eyed/post-eyed) in 70% EtoH for future genotyping analyses of relative abundance of triploids and occurrence of chromosomal abnormalities. When all embryos had hatched, total offspring numbers for each cross and treatment were counted by two different operators to control for systematic biases.

3.3.5 Assessment of chromosomal abnormalities and Unintentional Spontaneous Triploidy (UST)

Ploidy status of embryos which died during development was assessed using a panel of 13 established microsatellite loci that were already proven to be useful for similar purposes in previous studies on salmonids (Glover et al., 2015; Grimholt et al., 2002; Norris et al., 1999; Sanchez et al., 1996; Slettan et al., 1995a, 1995c; Stet et al., 2002a)

DNA extraction procedures varied between embryos before and after reaching the eyed stage. For the non-eyed embryos, we used the Chelex method (Walsh et al., 1991). Briefly, each fertilised egg was put in 200 µl 5 % Chelex (Biorad Laboratories) solution (5% Chelex, 10 mM Tris, 1mM EDTA and 1 % SDS) and after this 6 µl of Proteinase K at a concentration of 25 µg/ml were added. This mixture was then placed at 55 °C over-night followed by a 15 min incubation at 95 °C.

For eyed embryos, we extracted the eyes and used the HotSHOT genomic DNA preparation method, as described in Truett et al., (2000a). Here, the eyes from each embryo were added into a 96-well plate along with 75 µl of alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA). Plates were then incubated for 25 minutes at 95 °C, after which 75 µl of neutralizing reagent (40 mM Tris-HCl, pH 5) was added to block the post-incubation denaturation. 2 µl of this DNA for each sample were used to perform polymerase chain reactions (PCRs) in a 10 µl total volume reaction containing 8 µl of PCR Mastermix composed of the following multiplex forward/reverse primers: Ssa202, SsaD144, SsaD157, Sp1605, Sp2216, Ssa14, Ssa171, Ssa289, MHC 1 (Grimholt et al., 2002), MHC 2 (Stet et al., 2002b)**,** SsOsl85-a (Slettan et al., 1995a), Ssa197-a (Norris et al., 1999) and SsaF43-a (Protein and Microsatellite Single Locus Variability in Salmo Salar L. (Atlantic Salmon), 1996). All the PCR programs were set to gradually reach a denaturation temperature *of 94*°C (4 min) followed by 30 cycles of denaturation at *94*°C (50 s), annealing at 55°C (50 s), gradual extension at 72°C for (80 s), and extension at 72°C (10 min). PCR products were transferred and run on an *ABI3730* automated sequencer at the Institute of Marine Research (Havforskningsinstituttet) Bergen, Norway.

DNA Microsatellite genotyping data were analysed to investigate the presence of chromosomal aberrations. These included: triploid, trisomic and uni-parental disomic individuals. The genotyping software GeneMapper (GeneMapper, version 5.0, Applied Biosystems) was used to determine the number of clearly identifiable alleles for each locus. An individual was confirmed as a triploid if it displayed three clear alleles at two or more

loci, as this has been found to be a reliable method to identify triploids in Atlantic salmon (Garner et al., 2008; Glover et al., 2015; Hernández-Urcera et al., 2012; Liebert et al., 2004) (three clearly identifiable alleles per locus/multiple loci/all-markers); and uniparental-disomic individuals if the individual was missing at least one allele, either from the mother or from the father side (unipaternal or unimaternal disomic).

3.3.6 Sperm competition assays

For each egg batch, eggs and ovarian fluid were gently separated by using a sieve, and the ovarian fluid and the eggs were briefly kept in different clean beakers. From these drained egg batches, 200 eggs were randomly selected, split in two 100-egg batches and transferred to two new clean containers. 5 mL of either self-strain ovarian fluid from the same female or non-self-strain ovarian fluid from another female, were added to these egg batches and gently mixed with the eggs. Five males per each strain were randomly selected and used to form a total of 20 unique sperm competition trios where each male was compared to a farm or wild female, in presence of self-strain or non-self-strain ovarian fluid. The 100 eggs in each run across the five male blocks were fertilised using a total of 200 µl of sperm from the two males (100 µl from a MOWI male and 100 µl from a ETNE male). Briefly, individual sperm samples from each male were gently homogenised with a pipettor, and the samples from a farm and a wild male were placed on the opposite sides of a dry 1L plastic beaker having an inner concave portion containing the eggs and the ovarian fluid to avoid premature sperm activation. Sperm and eggs were simultaneously activated and mixed through the addition of 200 ml of natural river water at say exact temperature again here. As described above, the fertilised and hardened eggs were transferred in the RAS hatchery system and monitored until hatching. Successfully hatched alevins were then counted and 40 individuals per cross were randomly sampled, euthanised, and placed in 70 % EtoH for subsequent paternity analyses.

3.3.7 Paternity assignment after sperm competition experiments

To assign parents to the offspring resulting from the sperm competition trials in the successfully hatched embryos, individuals were genotyped using a panel of five microsatellite markers. The five strategic microsatellite loci were amplified for each fish using a panel of markers from Glover et al., (2016): SsaOsl85 (Slettan et al., 1995b), MHC I (Grimholt et al.,

2002), MHC II (Stet et al., 2002), Ssa197 (O'Reilly et al., 1996) and SsaF43 (Sánchez et al., 1996). This particular set of microsatellites has been used extensively (Glover et al., 2016) and has proven useful for a series of population genetic studies (Harvey, Tang, et al., 2017), in reconstructing pedigree history (Jørgensen, Solberg, et al., 2018; Solberg, Zhang, et al., 2013), to permit identification of conjoined twins (Fjelldal et al., 2016), to track the source of farmed salmon escapes at the farm level and to identify trisomic, triploid and haploid individuals (Glover et al., 2016; Glover, Harvey, et al., 2020; Harvey, Fjelldal, et al., 2017; Jørgensen et al., 2018). Thus, highly experienced laboratory personnel have been trained to run and interpret genotypes of these markers for a variety of purposes. On each DNA extraction plate, two blank cells were added as negative controls.

PCRs were run for the five selected microsatellites and their products were analysed by an ABI 3730 Genetic Analyser (Applied Biosystems, Foster City, CA) at the Genetics Laboratory of the Institute of Marine Research in Bergen, Norway and sized by a 500 LIZ™ size-standard. Alleles binned automatically in the program Genemapper™ were independently checked by two researchers prior to exporting the data for statistical analysis. Offspring paternity assignment was conducted adopting the user-friendly exclusion-based family assignment program FAP (Taggart, 2006), allowing to link offspring to their familiar origins for known parental genotypes and crosses. Paternity was therefore assigned to each offspring according to allele sharing between the two putative sires (MOWI and ETNE), the mother used in the sperm competition trial and their offspring. The use of exclusive male + male x female in each of the crosses enabled an unequivocal paternity assignment because each individual alevin could only had been sired by one of the two males that were competing in each trio.

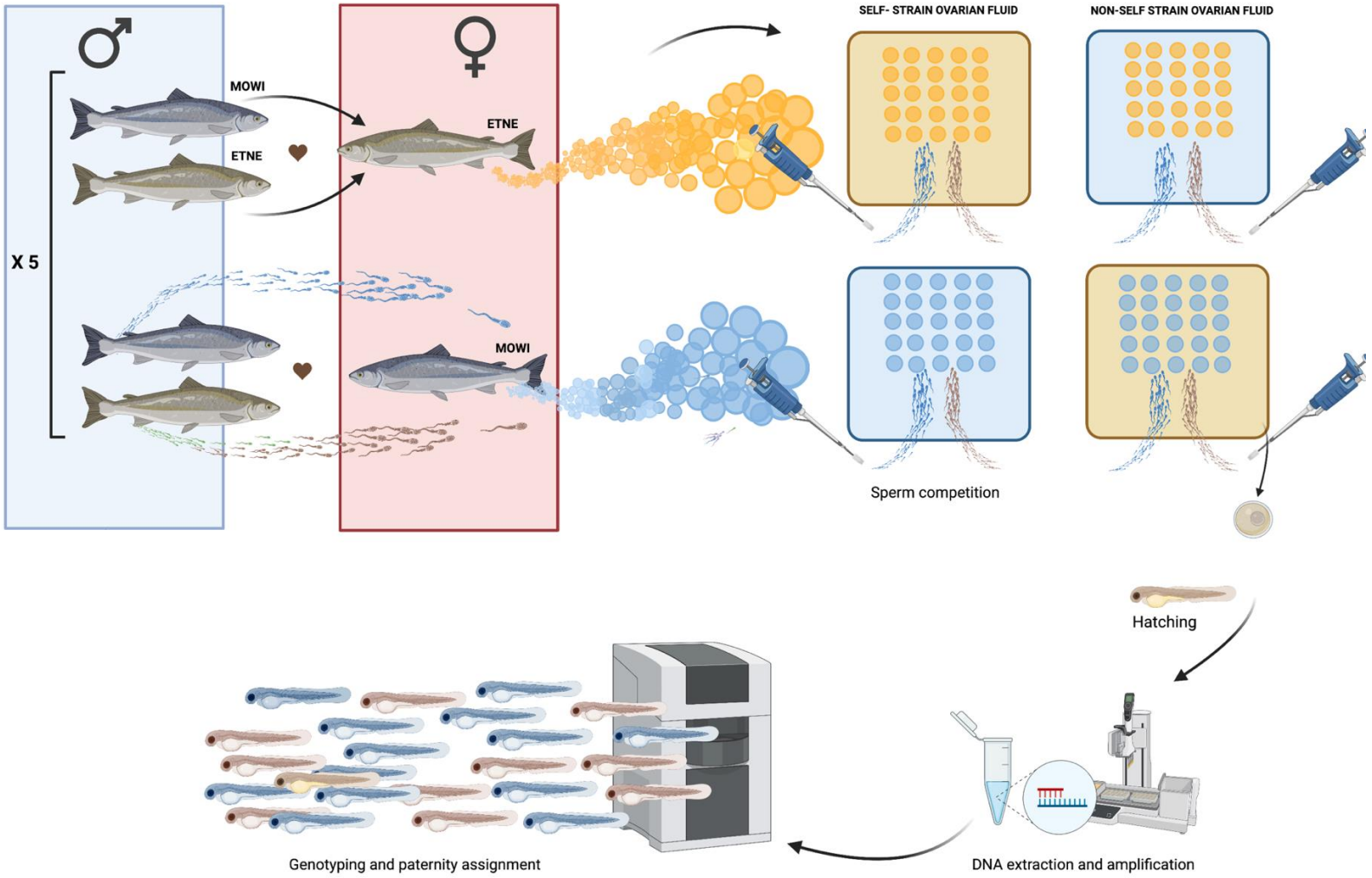


Figure 2.2 *Experimental design of the sperm competition experiments*

3.3.8 Sperm swimming behaviour assessment

For each of the ten experimental males used per strain, an aliquot of 1.5 ml of ejaculate was collected from the main flask after gentle mixing to homogenise the sample, transferred into an Eppendorf vial, and placed on ice. From each vial, 0.8 ul of undiluted ejaculate was directly pipetted and activated in a 4 µl solution of either pure river water, or 25, 50, 75 or 100 % ovarian fluid under a microscope (UOP, Tokyo, Japan, equipped with a 20× negative-phase contrast objective) using double-chambered Micro tool™ Cytonix sperm slides (Cytonix, Beltsville, MD 20705, USA). These slides are specifically designed to ensure quick and homogenous mixing, reduce wall effects, and stop the baseline flow caused by pipetting within the shortest amount of time, to produce trustable sperm activity recordings at five seconds post activation (spa). Each milt aliquot was placed at the entrance of the chamber and subsequently activated by flushing the ovarian fluid trough the entrance to fill the chamber. Video recording started at five seconds post activation to track sperm activity through a camera (Grasshopper2 digital camera, FLIR systems®, British Columbia, Canada) mounted onto the microscope. For each male, sperm activity was recorded in water and in proportionally increasing concentrations of ovarian fluid belonging to a wild Etne female in three experimental replicates per sample, continuously from 5 to 60 spa. Subsequently, videos were exported and analysed through the Computer Assisted Sperm Analyzer (CASA) automated plug-in available for the FIJI ImageJ software following the methodology introduced by Purchase and Earle. (Purchase & Earle, 2012a)

Briefly, recorded videos were converted to binary b/w images, labelled with an ID containing information about male and female IDs, experimental replicate, and strain and organised in a folder. Preliminary trials on a smaller sample of videos were performed to establish the optimal input parameters to feed to the CASA software (details on parameters can be found in the Supplementary material). The software opened the videos organised in the folder one by one and acquired tracking information at a rate of 30 frames s^{-1}. The percent of motile cells (MOT), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN = VSL/VCL), beat-cross frequency (BCF), wobble (WOB) and progression (PROG) were collected for individual cells and the average for every second calculated. All sperm motility, fertilisation and sperm competition trials were performed at a temperature of 6–7 °C, at a similar air temperature.

3.3.9 Statistical analysis

All data analyses were carried out in R Studio (RStudio (2020), Integrated Development for R. RStudio, PBC, Boston, MA) (v 1.3.1093) equipped with *car* (Fox and Weisberg, 2011), *glmmTMB* (Brooks et al., 2017), *(readxl), (lme4), (lmerTest), (DHARMa), (lsmeans), (merTools), (dplyr), (tidyverse), (rstatix), (ggpubr),(arsenal), (knitr), (survival),* and *lmerTest* (Kuznetsova et al., 2017) packages to perform exploratory analysis, run the main models, perform post-hoc tests and create output tabs. Graphical figures were plotted using *ggplot2* (Wickham, 2011), *(ggpubr), (sjPlot), (sjmisc)* and *(qqplotr).* Data were analysed using Linear Mixed Effect Models (LMMs) and Generalised Linear Mixed Effect models (GLMMs) in *lme4* (Bates et al., 2015) and *glmmTMB* (Brooks et al., 2017). To determine the error distributions, the relationship between the variance and the mean of the response variable and the assumptions for data distribution were checked (Crawley, 2012). Models were fitted using Restricted Maximum Likelihood (REML) methods to enable refinement and validation (Thomas et al., 2013). Residuals from linear models were checked for violations of normality and homoscedasticity. Significance of fixed effects in LMMs were obtained using *t*-tests with Satterthwaite's approximation for degrees of freedom implemented in *lmerTest* (Kuznetsova et al., 2017). Main effects, contrast analyses and interactions, when present, where extrapolated trough the *emmeans* and *emtrends* functions. For all the variables analysed, a selection of the model structure able to better explain our results was conducted for each of the analysed parameters by comparing residual dispersion, predicted values, AICs and BICs for each of the computed models through the 'summary' function output and through *DHARMa* residual diagnostic. Additionally, improved performances between the different models tested were compared by using the '*anova'* function.

3.3.9.1 Proportion of fertilised eggs, dying embryos and hatching success

The variation in number of fertilised eggs, embryos dying after reaching the eyed stage and successfully hatched embryos and calculated as proportions in relation to the starting number of eggs for each egg batch (function *cbind* in R), were all modelled in Generalised Linear

Mixed Effect Models (GLMMs) in "*glmer" in lme4* (Bates et al., 2015) with binomial error structure (logit link). Main effects included all explanatory variables of fertilisation mode (dry vs. wet), strain origin (farmed vs. wild), and all their possible interactions to explore strain-dependent effects across the combined fertilisation matrix experiment. Male and female IDs were both initially fitted as a random effect; however, after running this structure, model failed to converge, therefore, we included only female ID as random factor in our final models.

3.3.9.2 Sperm swimming behaviour in wild ovarian fluid

Sperm activity parameters MOT%, VCL, VAP, LIN, BCF, WOB and PROG were analysed in GLMMs (*glmmTMB*, Brooks et al., 2017) with the responses set on Gaussian distribution due to better residual diagnostics of these over the LMMs. Variations in VCL were analysed using LMMs. All the models included the strain origin (MOWI vs. ETNE), ovarian fluid percent (0, 25, 50, 75 and 100 %), the time from activation (SPA, 5 to 60s) and their interaction computed as fixed factors, and the different female ID (1 to 10) and male ID (1 to 20) with the three experimental sperm samples tracked per male nested by male ID. Random slopes were also included for these experimental males to account for the factorial design.

3.3.9.3 Sperm competition trials

Microsatellite genotyping data were used to determine the percentage of offspring sired by each farm or wild male in the sperm competition experiments. Paternity rates confirmed in GeneMapper were transferred to RStudio and analysed. The variation in paternity percentage between strains was modelled in a generalized linear mixed model (*glmmTMB* in R) with binomial error structure (logit link) including strain origin, strain of the female and strain of the ovarian fluid (two levels each, farmed vs. wild. Models included male and female IDs as random factors and included random slopes.

3.4 Results

3.4.1 Fertilisation, development, and hatching success

Fish origin influenced the developmental speed, with farmed embryos showing more advanced developmental stages compared to their wild counterparts when sampled at 15 dpf. The fertilisation treatments did not affect developmental speed in either strain but in wild fish, the number of embryos at earlier developmental stages were significantly with the wet technique (Fig 3.3, Table 3.1, 3.2). Similar fertilisation rates were observed in farm and wild fish as a consequence of the different fertilisation techniques (Fig 3.4A, Table 3.3). However, the proportion of dead embryos before reaching the eyed stage was significantly higher in wild crosses when these were fertilised with the dry technique (0.74 ± 0.09, z ratio= 8.29, $P<0.001$), showing higher early embryonic mortality in the wet wild groups. The proportion of dead embryos at the eyed stage was significantly lower for wild than for farm, but only under wet fertilisation conditions (-1.71 ± 0.71, z ratio= -2.4, P=0.0165), whereas post-hoc tests revealed no influence of fertilisation mode in farm fish (0.10 ± 0.06, z ratio= 1.53, $P> 0.05$). This resulted in significantly lower hatching success for wild fish fertilised through the wet technique (Fig 3.4B, C, Table 3.3, 3.4, 3.5, 3.6) when compared to farmed (-1.78 ± 0.812, z ratio= -2.196, P= 0.0281). A generally positive effect of dry fertilisation on hatching success rate was detected in both strains, but with lower estimates in farm fish (1.026 ± 0.14 and z ratio= 7.076 *vs* 0.59 ± 0.10 and z ratio= 5.65, $P<0.001$, for wild and farm fish respectively).

3.4.2 Presence of chromosomal abnormalities and USTs

During embryonic development, a total of 155 dead embryos were collected, of which 120 could be assigned to their parents with certainty. The remaining 35 belonged almost entirely

to the wild strain and were from the groups annotated as ‘wet-pre-eyed stage’ (early embryonic deaths). At the end, we were left with only nine samples from the wild fish under wet fertilisation, because the extraction techniques we performed did not yield good results with early embryos consisting only of a few cells after first divisions. This lowered our statistical power and hampered the chance to model error distribution among paired individuals and crosses and explore inter strain differences under the two fertilisation treatments. We performed a Chi-Square test to compare the frequency of normal (fully diploid individuals) versus the ones presenting chromosomal aberrations. This revealed that, irrespective of the fertilisation method used, farm fish presented higher frequencies of chromosomal abnormalities (χ^2= 25.45, df= 1; P= 0.00125) (Table 3.7). In farmed fish, only 35 out of 77 embryos were fully diploid, 14 were trisomic for one or more markers, five were triploids, ten were uni-parental disomic individuals for one or more markers (seven from the mother and three from the father side). The remaining 13 embryos had different degrees of chromosomal mutations at on one or more markers, including the display of new alleles. In wild fish, four out of 44 embryos showed chromosomal abnormalities: one trisomic, one triploid, one was a uni-maternal disomic individual and one expressed a novel allele.

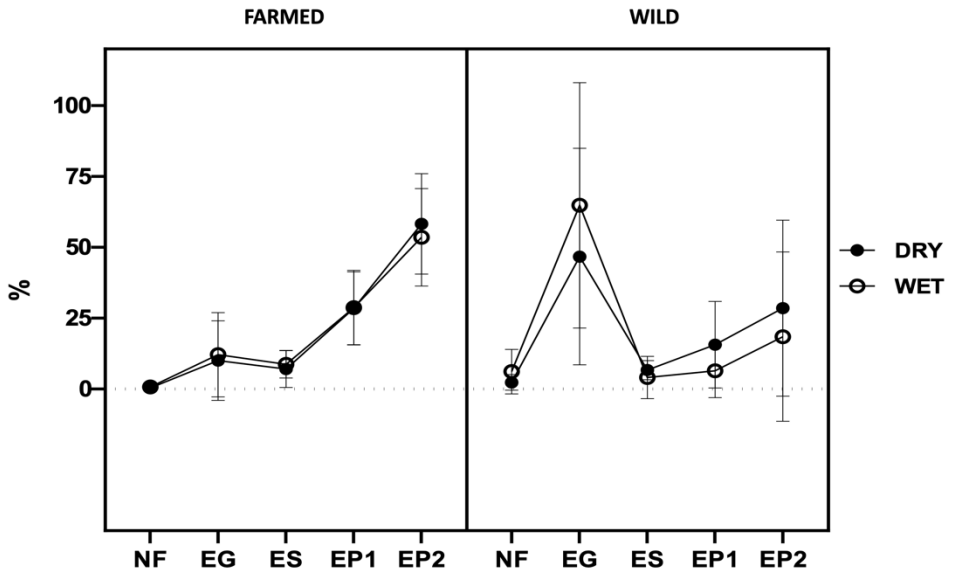


Figure 3.3 *Percentage at each of the different developmental stages: non-fertilised (NF), early gastrula (EG), embryonic shield formation (ES), 30% epiboly (EP1) and 50% epiboly (EP2) at 15 d.p.f. in salmon embryos (~ 100 eggs per female cross) obtained after dry and wet fertilisation in crosses of farmed (n= 10) and wild salmon (n= 10), Data are shown are as means ± SD.*

Table 3.2 *Analysis of Deviance Table (Type III Wald chisquare test) from a Generalized linear mixed model (glmmTMB in R)) for percentage at each developmental stage: early gastrula (EG), embryonic shield formation (ES), 30% epiboly (EP1) and 50% epiboly (EP2) at 15 d.p.f. in salmon embryos (~ 100 eggs per female) obtained after dry and wet fertilisation in farmed (n= 10) and wild (n= 10) crosses. Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

Response: STAGE OCCURRENCE (%)			
	Chisq.	***Df.***	***Pr(>Chisq)***
(Intercept)	*65.56*	*1*	***<0.001***
Strain	*19.18*	*1*	***<0.001***
Stage	*38.88*	*4*	***<0.001***
Fert mode	*4.91*	*1*	***0.027***
Strain:Stage	*33.19*	*4*	***<0.001***
Strain:Fert mode	*1.81*	*1*	*0.18*
Stage:Fert mode	*8.02*	*4*	*0.09*
Strain:Stage:Fert mode	*2.81*	*4*	*0.59*

Table 3.3 *Generalized linear mixed model (glmmTMB in R) for percentage at each developmental stage: early gastrula (EG), embryonic shield formation (ES), 30% epiboly (EP1) and 50% epiboly (EP2) at 15 d.p.f. in salmon embryos (~ 100 eggs per female cross) obtained after dry and wet fertilisation in crosses of farmed (n= 10) and wild (n= 10) crosses. Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					0.004	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	46.74	5.77	35.4, 58.06	1, 161	8.097	**<0.001**
Strain (farmed): Stage EG	49.55	12.05	2.59, 7.31	1, 161	4.11	**<0.001**
Strain (farmed): Stage ES	66.45	12.05	42.81, 90	1, 161	5.51	**<0.001**
Strain (farmed): Stage EP1	37.16	11.88	13.90, 61	1, 161	3.13	**<0.001**
Strain (farmed): Stage EP2	34.77	12.05	11.11, 58.40	1, 161	2.88	**0.0039**
Stage EG: Fert. Mode (wet)	-27.32	11.54	-49.98, -4.69	1, 161	-2.37	**0.017**
Stage ES: Fert. Mode (wet)	-28.13	11.54	-50.71, -5.49	1, 161	-2.43	**0.015**
Stage EP1: Fert. Mode (wet)	-20.71	11.54	-43.39, 1.92	1, 161	-1.74	0.07
Stage EP2: Fert. Mode (wet)	-14.26	11.54	-36.65, 8.36	1, 161	-1.23	0.21
Strain (farmed): Stage EG: Fert. Mode (wet)	25.48	17.05	-7.9, 58.90	1, 161	1.49	0.13
Strain (farmed): Stage ES: Fert. Mode (wet)	21.31	17.05	-12.21, 50.42	1, 161	1.25	0.21
Strain (farmed): Stage EP1: Fert. Mode (wet)	*20.24*	16.77	-12.65, 53.12	1, 161	1.206	0.22
Strain (farmed): Stage EP2: Fert. Mode (wet)	12.49	17.06	-20.92, 45.05	1, 161	0.73	**0.046**

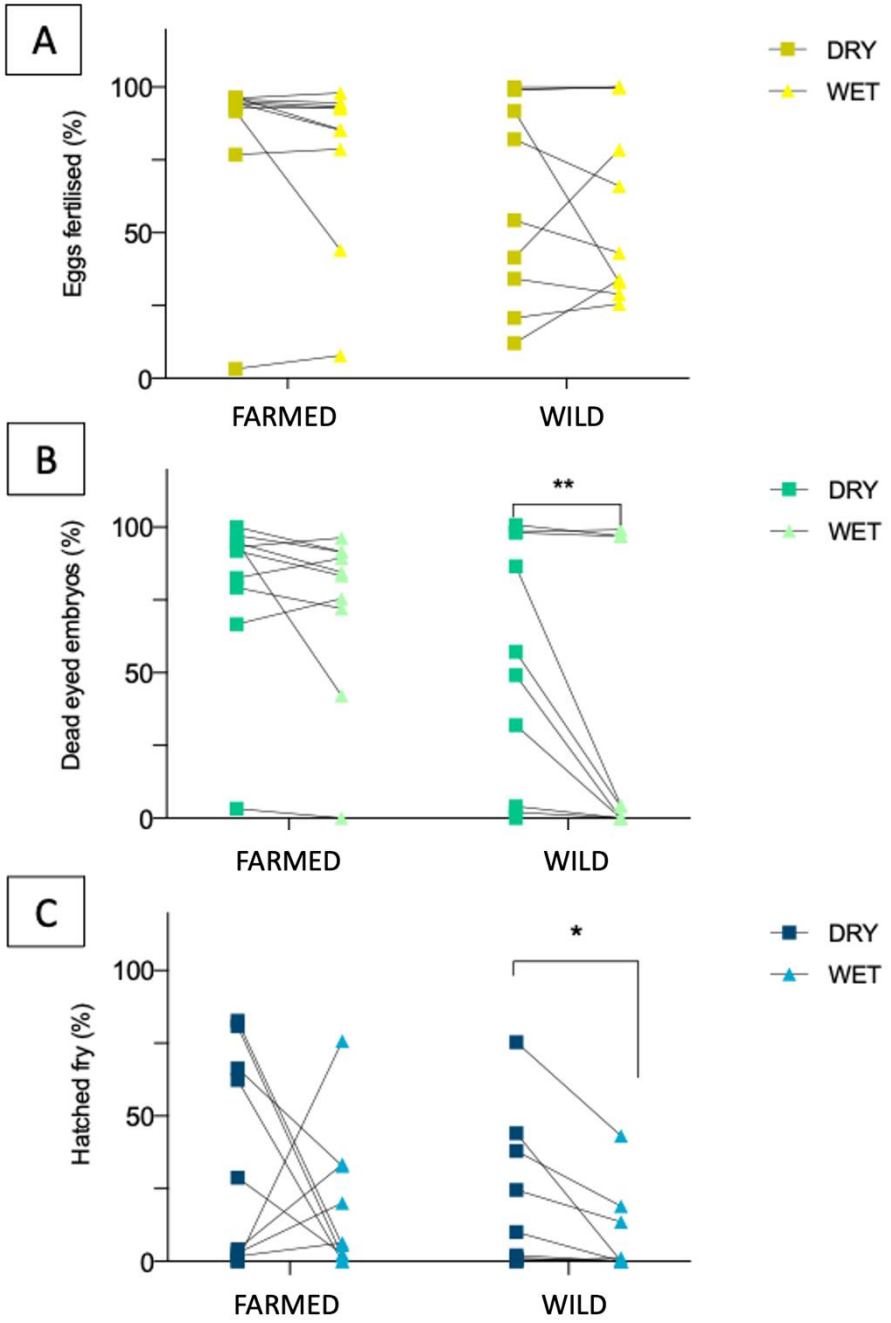


Figure 3.4 *Percentage of fertilised eggs (A), embryos that died after reaching the eyed stage (B) and total number of hatched fry (C) from dry and wet fertilisation in crosses of farmed (n= 10) and wild salmon (n= 10), data shown are individual values, *p< 0.05.*

Table 3.4 *Generalized linear mixed model (glmer in R) for the proportion of fertilised eggs from dry and wet fertilisation in farmed (n= 10) and wild (n= 10) crosses. Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					0.4024	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-0.58	0.20	0.46, 0.92	1, inf	-2.81	**0.0049**
Strain (farmed)	0.26	0.29	-0.34, 0.86	1, inf	0.89	0.37
Fert. Mode (wet)	-0.041	0.07	-0.18, 0.09	1, inf	-0.58	0.55
Strain (farmed)*:* Fert. Mode (wet)	-0.044	0.096	-0.23, 0.15	1, inf	-0.45	0.64

Table 3.5 *Generalized linear mixed model (glmer in R) for the proportion of embryos that died after reaching the eyed stage from dry and wet fertilisation in farmed (n= 10) and wild (n= 10) crosses. Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					2.44	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-1.57	0.51	-2.67, 2.31	1, inf	-3.097	**0.0019**
Strain (farmed)	1.071	0.72	-0.39, 2.58	1, inf	1.506	0.13
Fert. Mode (wet)	-0.74	0.09	-0.92, -0.57	1, inf	-8.22	**<0.001**
Strain (farmed)*:* Fert. Mode (wet)	0.63	0.11	0.41, 0.86	1, inf	5.67	**<0.001**

Table 3.6 *Generalized linear mixed model (glmer in R) for* the proportion of successfully hatched embryos *from dry and wet fertilisation* in farmed *(n= 10) and wild (n= 10)* crosses. *Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					1.73	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-3.03	0.58	-4.34, 2.62	1, inf	-5.201	**<0.001**
Strain (farmed)	1.35	0.80	-0.29, 3.09	1, inf	1.69	0.09
Fert. Mode (wet)	-1.026	0.14	-1.31, -0.74	1, inf	-7.076	**<0.001**
Strain (farmed): Fert. Mode (wet)	0.43	0.18	0.076, 0.78	1, inf	2.38	**0.017**

Table 3.7 *Percentage of fertilised eggs, embryos that died after reaching the eyed stage, and total number of hatched fry from dry and wet fertilisation in crosses of farmed (n= 10) and wild salmon (n= 10), (data shown are means ± SD).*

Variable								
	FARMED				**WILD**			
	DRY		**WET**		**DRY**		**WET**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Eggs Fertilised (%)	84.86	29.24	77.55	29.09	64.49	34.83	60.88	31.67
Reached the eyed stage (%)	79.91	28.61	72.62	29.82	52.53	41.54	30.26	46.63
Hatched fry (%)	32.97	35.98	17.18	24.11	19.56	25.69	7.68	14.20

Table 3.8 *Contingency table showing counts of dead embryos presenting chromosomal abnormalities and diploid fish in farmed and wild fish after from dry and we fertilisation protocols*

Variable				
	FARMED		**WILD**	
	DRY	**WET**	**DRY**	**WET**
	Count	**Count**	**Count**	**Count**
Chromosomal abnormalities (count)	24	18	2	2
Diploids (count)	21	14	33	7

3.4.3 Sperm swimming behaviour in farm and wild salmon sperm activated in wild female ovarian fluid

Strain origin affected sperm swimming traits when these were activated in water or increasing solutions of wild ovarian fluid. Farmed fish showed an overall 16.40 ± 6.27 higher motility rates when the effect of ovarian fluid was not accounted for. However, exposure of farmed sperm to increasing concentrations of wild ovarian fluid had negative effects at 25% that went extinguished with increasing concentrations of ovarian fluid of 50 and 75 %, until switching to a marginally significant (P= 0.051) positive effect in a 100 % concentration of wild ovarian fluid (See Fig. 3.5, Table 3.8 for details; see also Tables 3.13 and 3.14). Sperm curvilinear velocity (VCL) followed a similar pattern, being negatively affected in farm males by lower concentrations of wild ovarian fluid but showing a strong increase in pure ovarian fluid (13. 32 ± 1.87 μm/s). This was also consistent with a proportionally faster decline of this parameter with the seconds passed from activation when velocities where higher (See Fig. 3.6, Table 3.9; for details see also Tables 3.15 and 3.16). Average path velocity, straight line velocity and linearity all showed higher values in farm males but only in water and in concentrations of 25 to 50 % ovarian fluid. In higher ovarian fluid concentrations, the difference between farmed and wild was no longer significant; this was true both for the mean values across the entire sperm video recording time window as well as for the slopes showing velocity and linearity decline from 5 to 60 seconds post activation (see Fig. 3.7, 3.8, Table 3.10, 3.11 and Supplementary material Tables 3.17, 3.18 and 3.19, 3.20). In particular, farmed sperm linearity showed a reduction of 8.26 ± 1.27 % in pure ovarian as compared to their wild counterparts, but significantly higher values at lower concentrations, as did the parameters beat-cross frequency and sperm progression, both having increased under a gradient from pure water to pure ovarian fluid.

3.4.4 Sperm competition experiments in self and non-self ovarian fluid between farm and wild crosses

In the sperm competition experiments, farm males consistently achieved higher paternity shares across all trios analysed and independent of egg origin and origin of ovarian fluid used (65.83 ± 10 .25 % on average across all treatments, P< 0.001); (Figure 3.9; Tab 3.12).

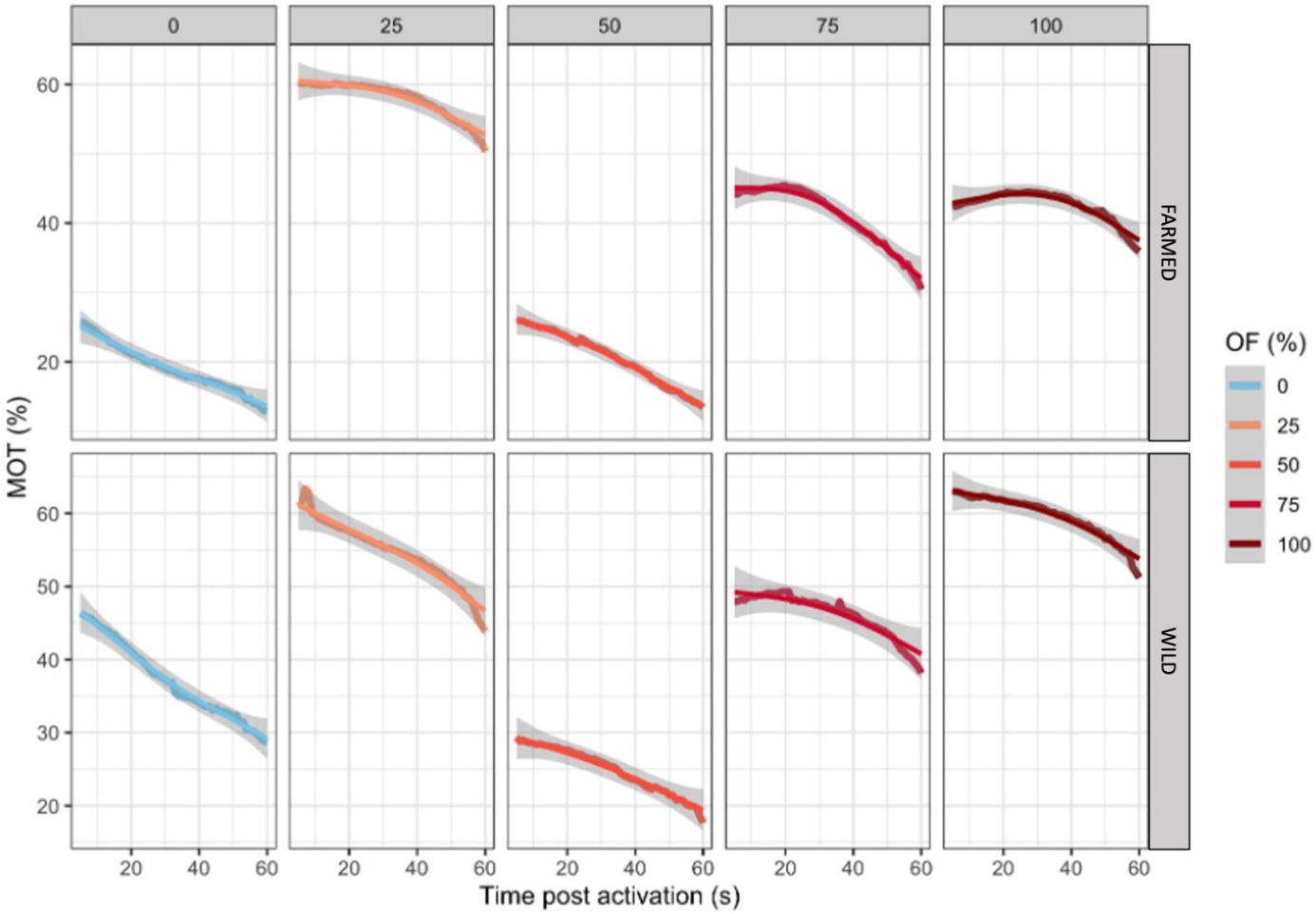


Figure 3.5 *Motility (%) of farmed (n= 10) and wild (n= 10) sperm following activation in 0 (pure river water), 25, 50, 75 and 100 % wild strain ovarian fluid (OF) and recorded each second from 5 to 60 seconds (s) post activation. Data shown represent the average between males (three replicates per male) and confidence intervals.*

Table 3.9 *Analysis of Deviance Table (Type III Wald chisquare test) from Generalised linear mixed effect model (glmmTMB in R) for variation in sperm motility (%) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

Response: MOT			
	Chisq.	***Df.***	***Pr(>Chisq)***
(Intercept)	*37.2042*	*1*	***<0.001***
OF	*857.7565*	*4*	***<0.001***
Strain	*6.8357*	*1*	***0.008***
spa	*51.4082*	*1*	***<0.001***
OF:Strain	*190.6364*	*4*	***<0.001***
OF:spa	*27.7089*	*4*	***<0.001***
Strain:spa	*7.2932*	*1*	***0.006***
OF:Strain:spa	*30.5822*	*4*	***<0.001***

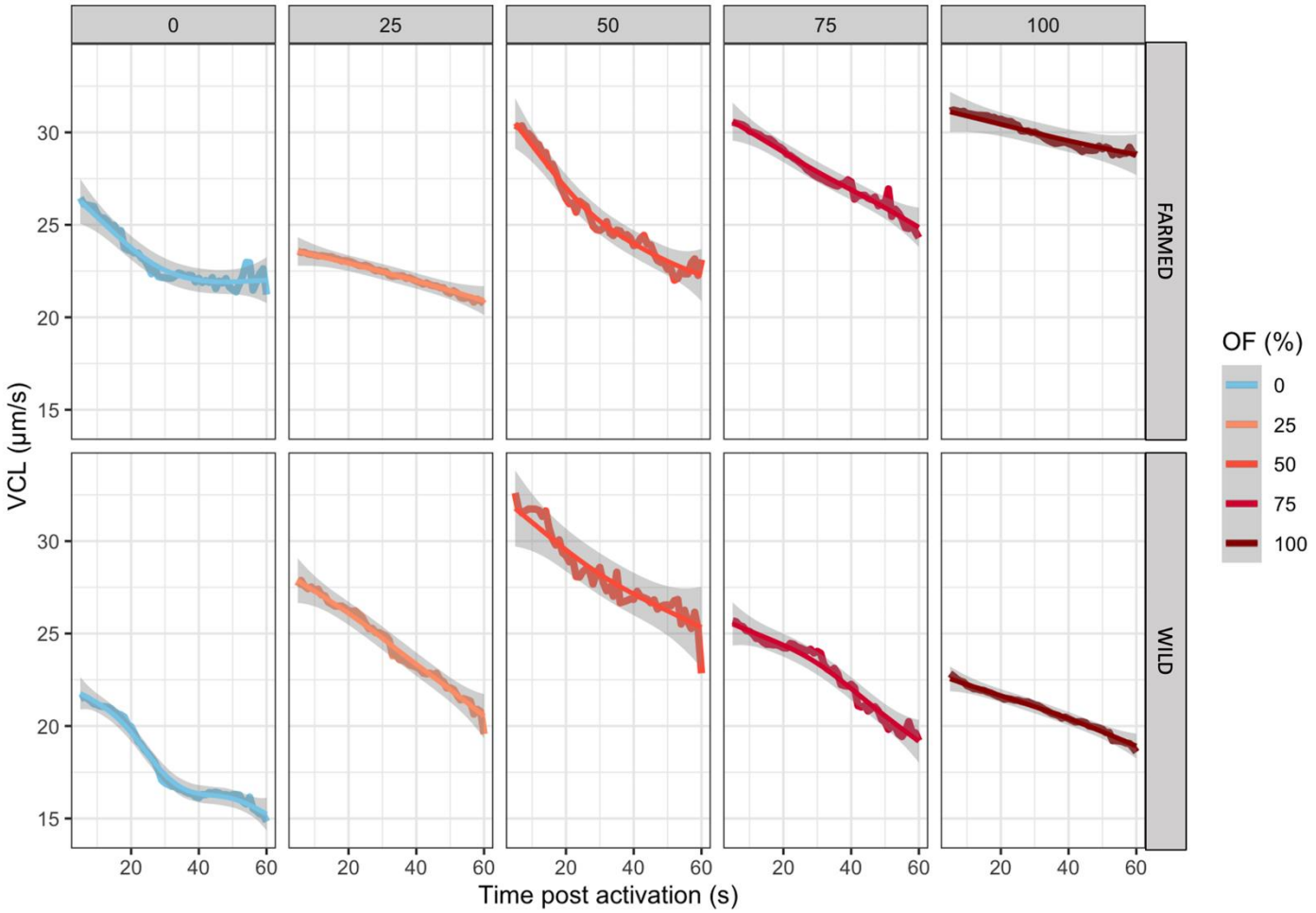


Figure 3.6 *Curvilinear velocity (µm/s) of sperm from farmed (n= 10) and wild (n= 10) males activated in 0 (pure river water), 25, 50, 75 and 100 % wild strain ovarian fluid (OF) and recorded each second from 5 to 60 seconds post activation. Data shown represent the average between males (three replicates per male) and confidence intervals.*

Table 3.10 *Analysis of Deviance Table (Type III Wald chisquare test) from a linear mixed effect model (lmer in R) for variations in curvilinear velocity (μm/s) from 5 to 60 s post activation, in farmed (n= 10) and wild (n= 10) salmon strains when activated in 0, 25, 50, 75 or 100% wild female ovarian fluid (OF). Estimates are provided with standard error (SE) and confidence intervals (CI).*

Response: VCL			
	Chisq.	***Df.***	***Pr(>Chisq)***
(Intercept)	*273.290*	*1*	***<0.001***
OF	*573.559*	*4*	***<0.001***
Strain	*2.615.*	*1*	***0.106***
spa	*2.0525*	*1*	*0.152*
OF:Strain	*170.267*	*4*	***<0.001***
OF:spa	*20.913*	*4*	***<0.001***
Strain:spa	*20.741*	*1*	***<0.001***
OF:Strain:spa	*30.077*	*4*	***<0.001***

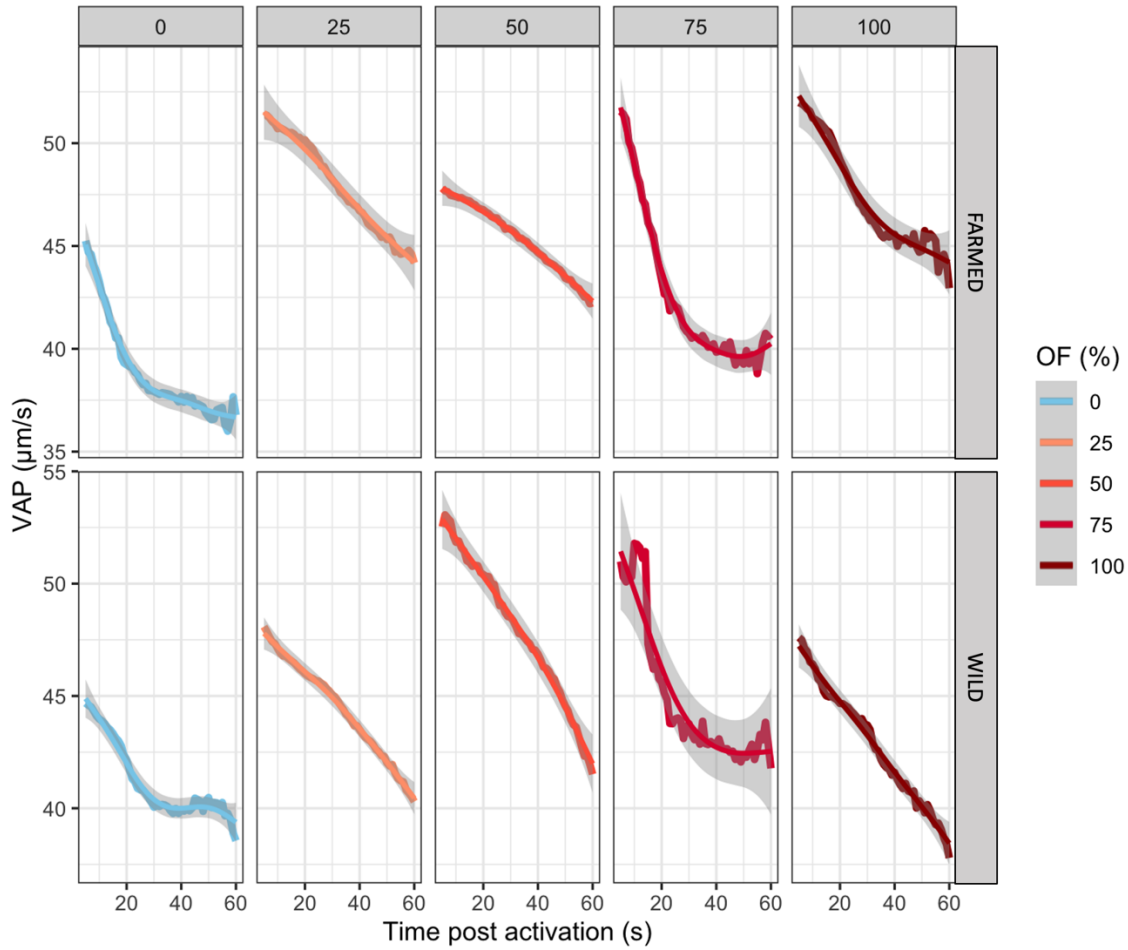


Figure 3.7 *Average path velocity (µm/s) of sperm from farmed (n= 10) and wild (n= 10) from Atlantic salmon activated in 0 (pure river water), 25, 50, 75 and 100 % wild strain ovarian fluid (OF) and recorded each second from 5 to 60 seconds post activation. Data shown represent the average between males (three replicates per male) and confidence intervals.*

Table 3.11 *Analysis of Deviance Table (Type III Wald chisquare test) from a Generalised linear mixed effect model (glmmTMB in R) for variation in average path velocity (μm/s) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

Response: VAP			
	Chisq.	***Df.***	***Pr(>Chisq)***
(Intercept)	*1429.138*	*1*	***<0.001***
OF	*294.889*	*4*	***<0.001***
Strain	*0.346*	*1*	*0.556*
spa	*92.345*	*1*	***<0.001***
OF:Strain	*190.131*	*4*	***<0.001***
OF:spa	*40.447*	*4*	***<0.001***
Strain:spa	*2.641*	*1*	*0.104*
OF:Strain:spa	*39.514*	*4*	***<0.001***

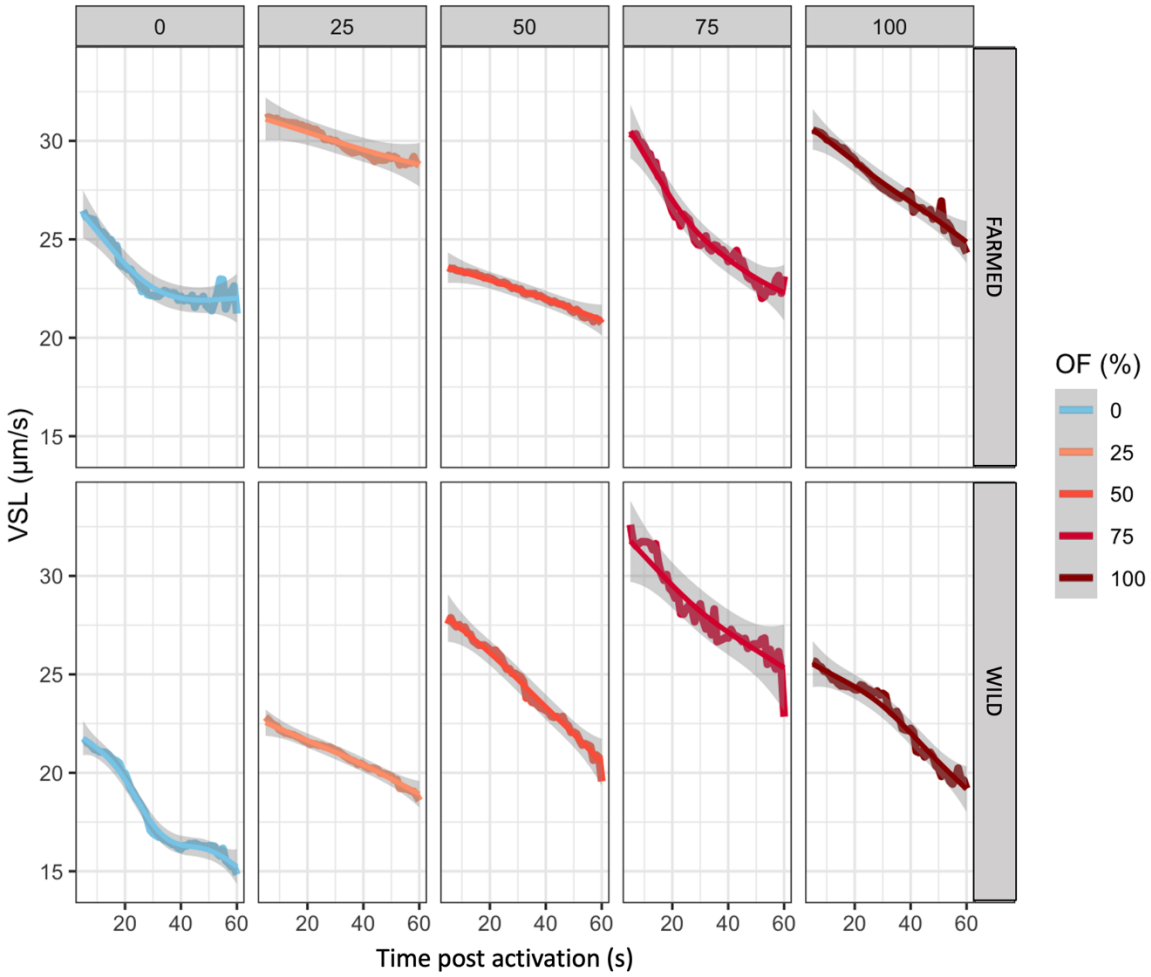


Figure 3.8 *Straight velocity (µm/s) of sperm from farmed (n= 10) and wild (n= 10) Atlantic salmon activated in 0 (pure river water), 25, 50, 75 and 100 % wild strain ovarian fluid (OF) and recorded each second from 5 to 60 seconds(s) post activation. Data shown represent the average between males (three replicates per male) and confidence intervals.*

Table 3.12 *Analysis of Deviance Table (Type III Wald chisquare test) from a Generalised linear mixed effect model (glmmTMB in R) for variation in straight velocity (μm/s) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

Response: VSL			
	Chisq.	***Df.***	***Pr(>Chisq)***
(Intercept)	*260.201*	*1*	***<0.001***
OF	*316.345*	*4*	***<0.001***
Strain	*0.7391*	*1*	*0.389*
spa	*30.838*	*1*	*0.152*
OF:Strain	*338.596*	*4*	***<0.001***
OF:spa	*58.748*	*4*	***<0.001***
Strain:spa	*9.779*	*1*	***0.002***
OF:Strain:spa	*32.103*	*4*	***<0.001***

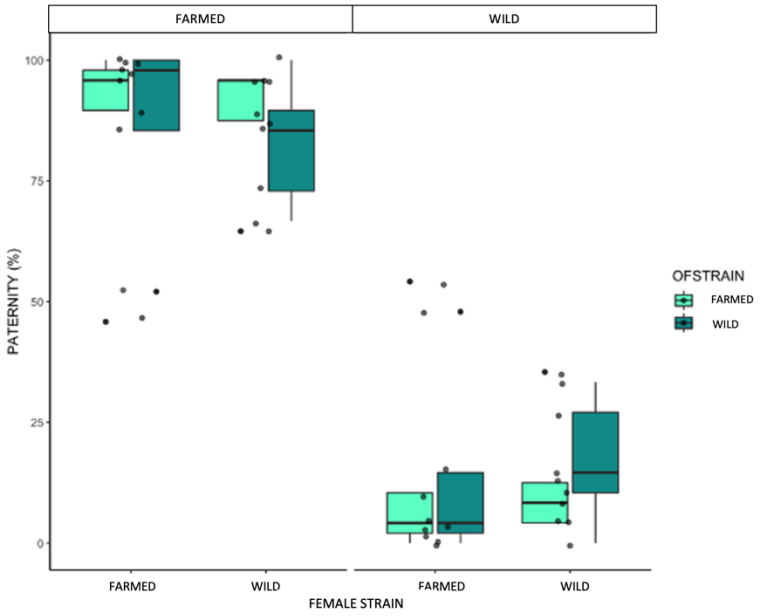


Figure 3.9 *Percentage of total offspring sired by each male within a specific trio between farmed and wild males and farmed and wild females and in presence of farmed (pale green) or wild (dark green) ovarian fluid. Data shown represent mean ± SD.*

Table 3.13 *Generalised linear mixed effect model (glmmTMB in R)) for paternity share (%) of farmed (n=10) and wild (n=10) males when these were competing to fertilise farmed (n=10) or wild (n=10) eggs in presence of self-strain or non-self-strain ovarian fluid. Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Male ID					18	
Female ID					0.0024	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	17.083	7.14	3.08, 3.11	1, 29	2.39	**0.017**
Strain (farmed)	65.83	10.10	46.02, 85.63	1, 29	6.51	**<0.001**
Female Strain (farmed)	-3.33	5.44	-14, 7.34	1, 29	-0.61	0.54
OF strain (farmed)	-4.16	5.44	-14.84, 6.51	1, 29	-0.76	0.44
Strain (farmed)*: Female Strain* (farmed)	7.50	7.70	-7.59, 24.23	1, 29	0.97	0.33
Strain (farmed)*:* OF strain (farmed)	9.16	7.70	-5.93, 24.26	1, 29	1.19	0.23
Female Strain (farmed)*:* OF strain (farmed)	4.58	7.70	-10.51, 19.68	1, 29	0.59	0.55
Strain (farmed)*: Female Strain* (farmed)*:* OF strain (farmed)	-10.83	10.89	-32.18, 10.51	1, 29	-0.99	0.32

3.5 Discussion

Our results confirm that the microenvironment experienced by gametes at fertilisation can have different effects in farmed and wild salmon strains and that this in turn affects the reproductive competitiveness between these two. Overall, we found that farmed fish were more resilient to different fertilisation techniques and their embryo development was faster, although they exhibited a higher degree of chromosomal abnormalities compared to wild fish. Only wild fish were sensitive to the two different fertilisation methods, with dry fertilisation leading to higher hatching success, thus highlighting the crucial role of the ovarian fluid in ameliorating the reproductive outcome. Our data suggest that after decades of artificial selection within the aquaculture sector, farmed salmon have a far greater reproductive potential when competing with wild fish *in vitro*, both when paired to wild and or farmed females, pointing out a clear threat to wild genetic pools following escapes. Altogether, our results highlight the importance of stopping farmed escapes into the wild.

We found that artificial fertilisation techniques did not affect fertilisation rates, but that the observed differences in hatching success could be explained by differential embryo mortality between farmed and wild fish under different fertilisation techniques. Specifically, wet fertilisation led to 49% lower hatching rates and a lower number of embryos reaching the eyed stage in wild fish. In contrast, fertilisation methods did not affect farmed fish in any of these traits. This suggests that wild fish were more sensitive to the microenvironment experienced by gametes during fertilisation. In their natural spawning grounds, Atlantic salmon fertilisation occurs after synchronous release of gametes in the water (de Gaudemar et al., 2000; de Gaudemar & Beall, 1999; Fleming, 1996; Hendry & Beall, 2004). Sperm are ejaculated directly into a fertilisation microenvironment consisting of substantial amounts of ovarian fluid (Rosengrave, Taylor, et al., 2009). Ovarian fluid can constitute up to 30 % of the total mass of the spawned eggs in this species (Lahnsteiner et al., 1995; Rosengrave et al., 2016), and the highest concentrations have been found in proximity with the micropyle (Litvak & Trippel, 1998; Rosengrave et al., 2008; Turner & Montgomerie, 2002). Dry fertilisation could therefore have preserved the function of ovarian fluid that is lost when large amounts of activating water were flushed onto the eggs to mix gametes. The 200 mL of

river water per ~1ml of ovarian fluid present within the eggs result in a very high dilution factor (~1:200) (Bemrose et al. 2021). Such a dilution is more than sufficient to neutralise any effect of the ovarian fluid, which is lost already at dilutions greater than 1:8 (Lahnsteiner, 2002). The presence of ovarian is also known to have a beneficial effect on egg viability in salmonids, as ovarian fluid maintains egg viability for up to ten minutes as opposed to only one minute in water (Billard et al., 1986). While our results clearly demonstrate the importance of a dilution effect of ovarian fluid for offspring viability and development in wild salmon, it is currently unclear, how exactly the dilution of the ovarian fluid contributes to embryo development and hatching rates. One possibility is the role of ovarian fluid in postcopulatory sexual selection in externally fertilising fish including salmonids (Alonzo et al., 2016; Devigili et al., 2018, 2021; Gasparini & Pilastro, 2011; Kholodnyy et al., 2022; Zadmajid, Myers, Sørensen, Butts, et al., 2019). The dilution of ovarian fluid following wet fertilisation could result in a reduction of its potential to exert ejaculate selection, which could explain the lower embryo fitness and hatching success observed in wild fish. It would be interesting to understand how in farmed fish, after decades of artificial selection and gamete manipulation within the aquaculture industry, several functions of the ovarian fluid might have been lost.

The ovarian fluid has been shown to modulate sperm velocity and guidance by modifying the biochemical environment in which sperm perform (Alonzo et al., 2016; Devigili et al., 2021; Firman et al., 2017). In salmonids, such selective effects have been demonstrated both within and across different ejaculates (Butts et al., 2012, 2017; Hatef et al., 2009; Rosengrave et al., 2016; Turner & Montgomerie, 2002b), from the same species (Butts et al., 2012; Rosengrave et al., 2008; Rosengrave, Taylor, et al., 2009a) or from different species (Yeates et al., 2013). Although fertilisation can certainly occur without ovarian fluid (Lehnert et al., 2017), its impact on the reproductive outcome in *in vitro* fertilisation protocols should not be neglected and call for a ‘naturalisation’ of these procedures. The role of the ovarian fluid in managing intra-ejaculate sperm selection has been found to be adaptive, with mechanisms of cryptic female choice from the females enhancing offspring fitness and survival during early development (Alonzo et al., 2016; Butts et al., 2012; Rosengrave et al., 2008, 2016). In brown trout (*Salmo trutta*) for example, survival to the eyed embryo stage was higher when sperm were activated in ovarian fluid as opposite to water (Hatef et al., 2009). Intra-ejaculate selection appears to optimise reproductive costs and improve offspring fitness (Alavioon et al., 2017; Immler et al., 2014; Zajitschek et al., 2014), and in Atlantic salmon, intra-ejaculate

selection for sperm with medium longevity produced faster developing offspring (Immler et al., 2014). All this evidence suggests that the use of ovarian fluid in artificial fertilisation protocols within the hatcheries should be a priority to facilitate sperm selection and exploit the natural selective potential put in place by evolutionary constrains to increase offspring fitness.

We could not directly test for differences in chromosomal abnormalities and Unintentional Spontaneous Triploids (USTs) under different fertilisation treatments due to very low sample sizes in the wild fish. But we found evidence that numbers of chromosomal aberrations are mainly found in pre-hatch embryos. While a previous study found 3 cases of triploidy among 1114 hatched offspring (0.27%; Bemrose et al., 2021), we found 8 full triploids among 121 embryos (6.6 %), of which 6 belonged to farm offspring. In addition to the full triploids, unhatched embryos displayed a series of other chromosomal aberrations (trisomy, uniparental disomy) that could explain embryonic deaths. In fact, less than half of all fish that had died before hatch presented a normal diploid asset, highlighting the improbability of finding such abnormalities in adult fish. This could justify the low levels of USTs reported in adult fish previously reported in nature (0.017%), (Jørgensen et al., 2018), in farmed escapees (0.18 %) as well as in commercial aquaculture facilities (2%) (Glover et al., 2016). In wild systems, UST seems to be rare and/or virtually absent (Jørgensen et al., 2018), but this could also be due to the chance of finding triploid assets at early developmental stages to be very difficult. In Salmonids (Allendorf & Thorgaard, 1984; Schultz, 1979), as well as in other fish families (Ferris, 1984; Ferris & Whitt, 1978), polyploidy may have played a crucial role in shaping the speed and the rate of diversification, by adding the genetic variation necessary to respond to evolutionary changes (Otto & Whitton, 2000; Ramsey et al., 1998; Ohno, 1968, Ohno & Wolf, 1970). Thus, salmonids could provide an intriguing model to study phenotypic, genomic and ecological fitness traits of polyploid fish during development.

Triploidy can be easily induced to create sterile offspring either through heat or hydrostatic pressure shock, with extremely high success rates when these protocols are performed in controlled conditions (Benfey & Sutterlin, 1984; Chourrout, 1980, 1982; Devlin et al., 2010; van Eenennaam et al., 2020). These protocols cause the retention of the polar body in the egg, which turns the maternal genome diploid and the fusion with the paternal haploid results in triploidisation. It is known that heat and hydrostatic pressure can have severe structural

effects on microtubule and cytoskeletal structural integrity; although these effects may be reversible (Benfey & Sutterlin, 1984; Wilson et al., 2001). Cytoskeletal mechanisms are at the base of the extrusion of the second polar body (Duan & Sun, 2019; Maddox et al., 2012; Pickering et al., 1988), and crucial during the sensible pairing of chromosomes (Labella et al., 2011; Sato et al., 2009). Therefore, it can be hypothesised that osmotic stresses could play a role in disrupting microtubular and microfilament function leading to the decline of hatching rates in wet-fertilised wild groups (Hatef et al., 2009; Lahnsteiner et al., 1995; Rosengrave, Taylor, et al., 2009a). Osmotic stress, while involved in gamete activation, can also be damaging. The ionic composition of ovarian fluid (Hatef et al., 2009) could have a buffering role to withstand rapid changes in osmotic pressure and their detrimental effects on both gametes and on the tiny sperm in particular (Kholodnyy et al., 2020)020). Wet fertilisation and the resulting dilution of the ovarian fluid may cause the loss of this beneficial buffering effect (Hatef et al., 2009), and potentially drive the release of higher reactive oxygen species concentrations.

When testing in the second sets of experiments how the ovarian fluid from wild females could influence sperm swimming performances in farmed males, we found that despite higher motility of farmed sperm in water, ovarian fluid presence significantly increased farmed sperm motility at the highest concentration. In wild fish, which showed on average lower motility in water, increasing concentrations of ovarian fluid proportionally enhanced the number of actively motile cells. Curvilinear velocity was interestingly the only parameter showing higher values in farmed fish under the higher concentrations of ovarian fluid, whereas in wild strains, peak velocity was observed at a 25 % ovarian fluid concentration. Comparable to a study in cod (*Gadus morhua*) (Beirão et al., 2014), higher concentrations of ovarian fluid reduced average path velocity, straight velocity, linearity and beat cross frequency of farmed fish sperm, but had positive effects on wild sperm. This strain-dependent sperm swimming behaviour under different concentrations of wild ovarian fluid might be another sign of domestication and the long-standing interference in fertilisation processes.

Yeates et al., (2013) found no difference in reproductive competitiveness between farmed and wild salmon, both in terms of fertilisation potential, compatibility, hatch and during sperm competition trials. Our results mostly followed this pattern, but we found strikingly higher relative reproductive success of farmed males during sperm competition experiments with wild males. This was true for all the crosses, with paternity rates for farmed fish being

frequently higher than 90% of the total offspring. Importantly, greater paternity share for farmed fish occurred independently of the female strain and the ovarian fluid. This suggests that farmed escapees could pose a serious threat for introgression at the gamete level. But this would need to be tested also under natural spawning conditions. In fact, we used the wet fertilisation technique for our competition trials, and the patterns could look quite differently under dry fertilisation conditions.

Overall, we clearly show that farmed salmon could be even a greater threat to wild populations than previously believed. We performed detailed *in vitro* comparisons at the gamete level and across different reproductive stages from sperm behaviour in a complex and the natural microenvironment, to fertilisation, embryo development, survival and hatching success, using a robust paired design that minimised confounding variables between individuals and their compatibility consistently across the different experiments performed. Therefore, we believe our experiments are a good approximation for the real contexts of hatchery rearing method and reproductive competitiveness and sperm competition in the wild. It is speculative but not unrealistic to believe that the major reproductive competitiveness and resilience of farmed gametes could be representative of an increased selection for certain reproductive traits as a consequence of artificial selection in the aquaculture industry. The farm strain we used is likely one of the most intensively selected and commercially exploited salmon strains in the world. Therefore, the use of this commercial strain and the time passed between previous studies and ours could be consistent with and heightened selection for certain reproductive phenotypes. Sperm traits for instance have been reported to diverge because of domestication in Atlantic salmon (Camarillo-Sepulveda et al., 2016). In addition, our results show higher resilience to environmental stress in farmed salmon which is in line with studies finding increased resilience to stressors as a consequence of captivity (Solberg, Skaala, et al., 2013). Artificial selection in domesticated salmon has resulted in genetic divergence (Bolstad et al., 2017; Fleming, 1996d; Gjedrem, 2012). And resulting changes to gene expression patterns (Debes et al., 2012; Tymchuk et al., 2009). Such genetic divergence has the potential to be adaptive and driven by the response to artificial fertilisation techniques, further explaining our findings of differences between wild and farmed salmon.

Finally, the fertilisation trials could provide the conservation aquaculture sector with some protocols to reduce embryo mortality in wild salmon strain and alert policy makers and

farming companies to the strong reproductive prevalence of farmed genotypes in a scenario of escapes from the farms and hybridisation in nature. Specifically, the wet fertilisation methods should be avoided, and the presence of a more natural fertilisation environment in presence of ovarian fluid should be preferred for wild fish used in re-stocking purposes to maximise production and limit the detrimental effects of unnatural fertilisation protocols on already vulnerable wild populations. Concomitantly, the aquaculture sector should further limit the escapes of diploid farmed fish given their elevated reproductive potential. Finally, we suggest that policy makers and the public should push for tighter salmon farming policies such as making the use of local and regional strains mandatory. This means that when salmon escapes cannot be completely avoided, at least these can act as a supply of adapted and wild genotypes.

3.6 Supplementary material

TRAY	TREATMENT	STRAIN	MALE	FEMALE
99	DRY	MOWI	K150	2006
100	WET	MOWI	K150	2006
101	DRY	MOWI	O133	2008
102	WET	MOWI	O133	2008
103	DRY	MOWI	R131	2002
104	WET	MOWI	R131	2002
105	DRY	MOWI	G116	2003
106	WET	MOWI	G116	2003
107	DRY	MOWI	O116	2004
108	WET	MOWI	O116	2004
109	DRY	MOWI	B108	2014
110	WET	MOWI	B108	2014
111	DRY	MOWI	R109	2011
112	WET	MOWI	R109	2011
113	DRY	MOWI	O141	2005
114	WET	MOWI	O141	2005
115	DRY	MOWI	O118	2012
116	WET	MOWI	O118	2012
117	DRY	MOWI	K144	2013
118	WET	MOWI	K144	2013
149	DRY	ETNE	203	165
150	WET	ETNE	203	165
151	DRY	ETNE	201	168
152	WET	ETNE	201	168
153	DRY	ETNE	182	167
154	WET	ETNE	182	167
155	DRY	ETNE	193	170
156	WET	ETNE	193	170
157	DRY	ETNE	187	171
158	WET	ETNE	187	171
159	DRY	ETNE	174	188
160	WET	ETNE	174	188
161	DRY	ETNE	190	189
162	WET	ETNE	190	189
163	DRY	ETNE	186	192
164	WET	ETNE	186	192
165	DRY	ETNE	173	199
166	WET	ETNE	173	199
167	DRY	ETNE	178	200
168	WET	ETNE	178	200

Figure 3.10 *Crosses design used in the above-mentioned experiments*

Table 3.14 *Generalised mixed effect model (glmmTMB in R) for variation in sperm motility (%) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					5.68	
Male ID					129.3	
Replicate:Male ID					111.29	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	*27.59*	*4.53*	18.38, 36.85	1, 24.36	6.096	**<0.001**
OF25	*34.89*	1.42	32.11, 37.85	1, 1627	24.56	**<0.001**
OF50	*2.128*	1.46	-0.73, 4.99	1, 1629	1.45	0.145
OF75	*-22.071*	1.44	19.24, 24.89	1, 1.626	15.32	**<0.001**
OF100	*18.52*	1.47	15.63, 21.42	1, 1619	12.53	**<0.001**
Strain (farmed)	*16.40*	6.27	3.55, 29.17	1, 24.03	6.27	**0.0089**
spa	*0.202*	0.028	-0.26, -0.14	1, 1629	-7.17	**<0.001**
OF25: Strain (farmed)	*-17.27*	1.98	-21.15, -13.39	1, 1628	-8.73	**<0.001**
OF50: Strain (farmed)	*-14.88*	2.033	-18.87, -10.90	1, 1627	-7.32	**<0.001**
OF75: Strain (farmed)	*-14.29*	1.99	-18.21, -10.38	1, 1625	-7.15	**<0.001**
OF100: Strain (farmed)	*3.93*	2.022	-0.02, 7.89	1, 1627	1.94	0.051
OF25:spa	*0.057*	0.038	-0.017, 0.13	1, 1624	1.50	0.13
OF50:spa	*-0.067*	0.039	-0.14, 0.0096	1, 1624	-1.71	0.08
OF 75:spa	*-0.046*	0.039	-0.11, 0.32	1, 1624	-1.18	0.23
OF100:tpa	*0.106*	0.040	-0.079, 0.13	1, 1624	2.685	**0.0072**
Strain (farmed):spa	*-0.10*	0.038	-0.18, 0.02	1, 1624	-2.701	**0.0069**
OF25: Strain (farmed):spa	*-0.01*	0.053	-0.12, 0.09	1, 1624	-0.27	0.78
OF50: Strain (farmed):spa	*0.16*	0.055	0.05, 0.27	1, 1624	2.95	**0.0031**
OF75: Strain (farmed):spa	*0.21*	0.054	0.11, 0.32	1, 1624	4.07	**<0.001**
OF100: Strain (farmed):spa	*0.02*	0.055	-0.079, 0.13	1, 1624	0.50	0.61

Table 3.15 *Type III Analysis of Variance Table with Satterthwaite's method from a Generalized linear mixed effect model (glmmTMB in R) for variation in sperm motility (%)) from 5 to 60 s post activation in Mowi (n= 10) and Etne (n= 10) when activated in 0, 25, 50, 75 or 100% Etne ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

	Sum sq	***Mean Sq***	***Df***	***F value***	***Pr(>F)***
OF	450902	112726	*4*	364.6259	***<0.001***
Strain (farmed)	511	511	*1*	1.6534	*0.220*
spa	176995	176995	*1*	572.5150	***< 0.001***
OF: Strain (farmed)	58936	14734	*4*	47.6591	***< 0.001***
OF:spa	7463	1866	*4*	6.0349	***< 0.001***
Strain (farmed):spa	639	639	*1*	2.0682	*0.150*
OF: Strain (farmed):spa	9455	2364	*4*	*7.645*	***< 0.001***

Table 16 *Linear mixed effect model (lmer in R) for variation in curvilinear velocity (μm/s) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					119.68	
Male ID					59.41	
Replicate:Male ID					116.48	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	76.52	*4.62*	67.45, 85.59	1, 24.36	16.53	**<0.001**
OF 25	*23.39*	1.31	20.81, 25.97	1, 1627	17.75	**<0.001**
OF 50	*-2.69*	1.36	-5.36 -0.022	1, 1629	-1.97	**0.048**
OF 75	0.8031	1.33	-1.81, 3.43	1, 1.626	0.601	0.54
OF 100	0.63	1.36	-2.04, 3.31	1, 1619	0.46	0.64
Strain (farmed)	10.57	6.53	-2.24, 23.39	1, 24.03	1.61	0.105
spa	-0.037	0.02	0.09, 0.01	1, 1629	-1.43	0.15
OF 25: Strain (farmed)	-8.41	1.83	-12.0041, -4.82	1, 1628	-4.59	**<0.001**
OF 50: Strain (farmed)	-6.37	1.89	-10.091, -2.65	1, 1627	-3.35	**<0.001**
OF 75: Strain (farmed)	-1.02	1.85	-4.65, 2.59	1, 1625	-0.55	0.57
OF 100: Strain (farmed)	13.32	1.87	9.65, 16.99	1, 1627	7.12	**<0.001**
OF 25:spa	-0.12	0.035	-0.19, -0.05	1, 1624	-3.48	**<0.001**
OF 50:spa	-0.0089	0.037	-0.08, 0.06	1, 1624	-0.22	0.82
OF 75:spa	*-0.076*	0.037	-0.14, -0.005	1, 1624	-2.10	**0.035**
OF 100:spa	-0.11	0.038	-0.18, -0.043	1, 1624	-3.14	**0.0016**
Strain (farmed):spa	0.16	0.035	0.09, 0.23	1, 1624	4.55	**<0.001**
OF 25: Strain (farmed): spa	-0.16	0.049	-0.25, -0.06	1, 1624	-3.23	**0.0012**
OF 50: Strain (farmed): spa	-0.26	0.052	-0.36, -0.16	1, 1624	-5.070	**<0.001**
OF 75: Strain (farmed): spa	-0.21	0.050	-0.031, -0.011	1, 1624	-4.156	**<0.001**
OF 100: Strain (farmed) spa	-0.19	0.050	0.28, -0.09	1, 1624	-3.742	**<0.001**

Table 17 *Type III Analysis of Variance Table with Satterthwaite's method from a Linear mixed model (lmer in R) for variation in curvilinear velocity (μm/s) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

	Sum sq	***Mean Sq***	***Df***	***F value***	***Pr(>F)***
OF	*234401*	*58600*	*4*	*222.6991*	***<0.001***
Strain (farmed)	*646*	*646*	*1*	*2.4564*	*0.1311*
spa	*44043*	*44043*	*1*	*167.376*	***< 0.001***
OF: Strain (farmed)	*44812*	*11203*	*4*	*42.5748*	***< 0.001***
OF:spa	*25057*	*6264*	*4*	*23.8061*	***< 0.001***
Strain (farmed):spa	*2*	*2*	*1*	*0.0062*	*0.9371*
OF: Strain (farmed):spa	*7914*	*1979*	*4*	*7.5191*	***0.0047***

Table 18 *Generalized mixed effect model (glmmTMB in R) for variations in s Average path velocity (μm/s) from 5 to 60 s post activation, in farmed (n= 10) and wild (n= 10) salmon strains when activated in 0, 25, 50, 75 or 100% wild female ovarian fluid (OF). Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					0.74	
Male ID					0.006	
Replicate:Male ID					1.13	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	*42.41*	*1.11*	42, 46	1, 26.71	37.94	**<0.001**
OF 25	6.05	0.64	4.78, 7.31	1, 1595	9.40	**<0.001**
OF 50	6.21	0.66	4.91, 7.52	1, 1595	9.33	**<0.001**
OF 75	9.46	0.65	8.18, 1.07	1, 1595	14.47	**<0.001**
OF 100	10.27	0.66	8.95, 11.5	1, 1595	15.34	**<0.001**
Strain (farmed)	0.92	1.57	-2.15, 4.11	1, 1595	0.59	0.55
spa	-0.12	0.02	-0.14, -0.009	1, 1593	-9.61	**<0.001**
OF 25: Strain (farmed)	4.81	0.89	3.063, 6.57	1, 1593	5.38	**<0.001**
OF 50: Strain (farmed)	0.42	0.92	-0.14, 0.23	1, 1593	0.46	0.64
OF 75: Strain (farmed)	-5.16	0.91	-0.64, -0.39	1, 1593	-5.70	**<0.001**
OF 100: Strain (farmed)	-5.51	0.92	-0.73, -0.33	1, 1593	-6.03	**<0.001**
OF 25:spa	0.023	0.018	-0.012, 0.056	1, 1593	1.27	0.20
OF 50:spa	-0.082	0.019	-0.19, -0.0042	1, 1593	-4.60	**<0.001**
OF 75:spa	*-0.021*	0.018	-0.0064, 0.0005	1, 1593	-1.64	0.10
OF 100:spa	-0.012	0.024	-0.0051, 0.002	1, 1593	-0.89	0.37
Strain (farmed):spa	0.028	0.028	-0.003, 0.0019	1, 1624	1.63	0.10
OF 25: Strain (farmed): spa	-0.12	0.025	-0.017, 0.007	1, 15930	-5.04	**<0.001**
OF 50: Strain (farmed): spa	0.017	0.025	-0.003, 0.006	1, 15930	0.66	0.51
OF 75: Strain (farmed): spa	-0.031	0.035	-0.008, 0.0016	1, 15930	-1.27	0.20
OF 100: Strain (farmed): spa	-0.021	0.025	-0.007, 0.003	1, 15930	-0.85	0.39

Table 19 *Type III Analysis of Variance Table with Satterthwaite's method from a Generalised mixed effect model (glmmTMB in R) for variation in average path velocity (μm/s) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

	Sum sq	***Mean Sq***	***Df***	***F value***	***Pr(>F)***
OF	*27410*	*6853*	*4*	*108.579*	***<0.001***
Strain (farmed)	*1*	*1*	*1*	*0.012*	*0.914*
spa	*89022*	*89022*	*1*	*1410.543*	***< 0.001***
OF: Strain (farmed)	*12001*	*11203*	*4*	*47.5408*	***< 0.001***
OF:spa	*2366*	*592*	*4*	*9.3741*	***< 0.001***
Strain (farmed):spa	*10*	*10*	*1*	*0.1539*	*0.694*
OF: Strain (farmed):spa	*2495*	*624*	*4*	*9.8847*	***0.0047***

Table 20 *Generalised mixed effect model (glmmTMB in R) for variations in straight velocity (μm/s) from 5 to 60 s post activation, in farmed (n= 10) and wild (n= 10) salmon strains when activated in 0, 25, 50, 75 or 100% wild female ovarian fluid (OF). Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Female ID					16.35	
Male ID					0.029	
Replicate:Male ID					14.92	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	*23*	*1.47*	18.38, 36.85	1, 33.32	16.31	**<0.001**
OF 25	*-0.57*	0.63	32.11, 37.85	1, 1591	-0.903	0.36
OF 50	*5.56*	0.65	-0.73, 4.99	1, 1597	8.49	**<0.001**
OF 75	6.32	0.64	19.24, 24.89	1, 1597	9.83	**<0.001**
OF 100	8.32	0.66	15.63, 21.42	1, 1584	12.63	**<0.001**
Strain (farmed)	-1.78	2.11	3.55, 29.17	1, 31	-0.86	0.39
spa	-70	0.013	-0.26, -0.14	1, 1597	-5.53	**<0.001**
OF 25: Strain (farmed)	7.077	0.88	-21.15, -13.39	1, 1597	7.97	**<0.001**
OF 50: Strain (farmed)	3.84	0.92	-18.87, -10.90	1, 1597	4.202	**<0.001**
OF 75: Strain (farmed)	-2.08	0.81	-18.21, -10.38	1, 1597	-2.331	**0.019**
OF 100: Strain (farmed)	-7.93	0.93	-0.02, 7.89	1, 1598	-8.81	**<0.001**
OF 25:spa	0.19	0.017	-0.017, 0.13	1, 1596	1.11	0.26
OF 50:spa	*-0.092*	0.018	-0.14, 0.0096	1, 1596	-5.16	**<0.001**
OF 75:spa	*-0.035*	0.017	-0.11, 0.32	1, 1596	-1.99	**0.04**
OF 100:spa	0.21	0.018	-0.079, 0.13	1, 1596	1.18	0.23
Strain: Strain (farmed):spa	*-0.055*	0.023	-0.18, 0.02	1, 1596	-3.12	**<0.001**
OF 25: Strain (farmed): spa	*-0.028*	0.025	-0.12, 0.09	1, 1596	-1-17	0.24
OF 50: Strain (farmed): spa	0.103	0.024	0.05, 0.27	1, 1596	4.13	**<0.001**
OF 75: Strain (farmed): spa	0.038	0.024	0.11, 0.32	1, 1596	1.59	0.11
OF 100: Strain (farmed): spa	0.037	0.025	-0.079, 0.13	1, 1596	1.51	0.13

Table 21 *Type III Analysis of Variance Table with Satterthwaite's method from a Linear mixed model (lmer in R) for variation in average path velocity (μm/s) from 5 to 60 s post activation in farmed (n= 10) and wild (n= 10) when activated in 0, 25, 50, 75 or 100% wild ovarian fluid (OF). Chisquare test values (Chisq.), degrees of freedom (Df.) and adjusted Pvalues (Pr(>Chisq) are shown.*

	Sum sq	***Mean Sq***	***Df***	***F value***	***Pr(>F)***
OF	18417	4604	*4*	75.2627	***<0.001***
Strain (farmed)	40	40	*1*	0.6525	*0.426*
spa	41169	41169	*1*	672.9765	***< 0.001***
OF: Strain (farmed)	20716	*11203*	*4*	84.6591	***< 0.001***
OF:spa	*25057*	5179	*4*	1.3153	***< 0.001***
Strain (farmed):spa	581	581	*1*	9.4892	***0.002***
OF: Strain (farmed):spa	1964	491	*4*	8.0266	***< 0.001***

4 Limited inbreeding avoidance at the gamete level despite inbreeding depression in Atlantic salmon (Salmo salar)

Chapter's contributions:

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Statement of attributions: M. Gage and K. Glover provided fundings, operational facility and instrumentations. M. Solberg and K. Glover provided the fish and screened their genetic backgrounds prior to the experiments. M. Graziano and M. Gage conceptualised the work. M. Graziano, M. Gage and D. Murray conducted the fertilisation experiments. M. Graziano and L. Dryhovden monitored the fish until hatch and collected the samples. M. Graziano conducted the CASA experiments. M. Graziano and M. Solberg conducted the sperm competition trials between sibling and non-sibling crosses. Data extraction, curation and analysis were performed by M. Graziano. Throughout the experiments hatchery technical support received was kindly ensured by L. Dryhovden and I. Helge. A. Sørvik and S. Kutnar trained M.Graziano to perform molecular analysis according to Lab's protocol and curated with M.Graziano the microsatellite database in genemapper for paternity assignment. A. Sørvik supervised and validated the microsatellites-based paternity assignment process. M. Graziano performed all the molecular analyses. M. Taylor trained M. Graziano with genepop and p-link and helped with the interpretation of the population genetics measures. M. Graziano created all the tabs, figures and illustrations. M. Graziano wrote the first complete draft of this work and all the following versions after receiving the review comments and suggestions from the Supervisors M. Gage and S. Immler.

Keywords:
Fertilisation, cryptic female choice, ovarian fluid, kin recognition, inbreeding, sperm competition

4.1 Abstract

Inbreeding and the related increase in homozygosity and expression of deleterious alleles reduces fitness and adaptive responses known as inbreeding depression. To avoid inbreeding depression, a range of mechanisms to limit reproduction between close genetic relatives have evolved at all stages from pre-mating mate choice to post-mating gamete selection. In our study, we tested the level of inbreeding avoidance between sperm and egg in Atlantic salmon *Salmo salar* at the post-mating stages. Philopatric salmon return to their natal streams to spawn and the risk of breeding between close relatives is high. Female control over paternity is particularly difficult in external fertilisers and mechanisms of inbreeding avoidance would have to evolve at the gamete level and the interaction between sperm and ovarian fluid released with eggs at oviposition may play a key role in determining fertilisation success. In a paired breeding design, we compared sperm motility parameters in sibling and non-sibling ovarian fluid, and assessed fertilisation and hatching success, growth rate and paternity in sperm competition trials between sibling and non-sibling males. We found that sperm activated in ovarian fluid of sibling females exhibited an average 36% reduction in fertilisation rates in sibling crosses and offspring from sibling crosses were lighter and shorter before the onset of sexual maturation. However, we found no difference in survival rates between sibling and non-sibling cross offspring. Sperm competition trials between sibling and non-sibling males revealed no difference in relative paternity success when simultaneously competing over a female's eggs but surprisingly, sibling-sired offspring showed consistently higher degrees of multi-locus heterozygosity across the five microsatellite loci analysed. Our findings indicate that post-mating inbreeding avoidance mechanisms have evolved at the gamete level in salmon, but that this does not necessarily affect the sperm competitiveness of sibling males. Our results have direct implications for conservation aquaculture and salmon farming sustainability due to the high degree of inbreeding reported in these sectors.

4.2 Introduction

Inbreeding-induced loss of genetic diversity and increased homozygosity can reduce adaptive responses, thus increasing the risk for extinction (Caughley, 1994; Hedrick, 1999). By reducing the number of heterozygotes, inbreeding can deplete the genetic variability within a population, either by loss of alleles from random genetic drift or through inbreeding depression due to increased homozygosity in individuals (Wang et al., 2002b). High rates of inbreeding may affect and increase homozygosity leading to the heightened expression of deleterious alleles reducing reproductive fitness and survival which is known as inbreeding depression (Charlesworth & Charlesworth, 1987; Keller & Waller, 2002). Inbreeding and inbreeding depression are wide-spread and particularly affect the viability of small populations (Bijlsma et al., 2000; Keller & Waller, 2002) presenting a primary concern in conservation biology (Hedrick and Kalinowski, 2000).

Inbreeding avoidance mechanisms have evolved to control the rate of mating between close relatives anywhere before, during and after mating. At the premating stages, increased dispersion of one or both sexes and/or kin recognition strategies for instance are evolutionary routes to reduce the probability of inbreeding (Pusey & Wolf, 1996; Tregenza & Wedell, 2000). At the post-mating stage, cryptic female choice and a preference for sperm from non-related males as well as reduced parental investment in offspring sired between close relatives have been reported (Simmons, 2005; Zeh & Zeh, 1996, 1997). This implies that females have the ability to assess the genetic quality of potential mates and assess the match with their own genes to ensure heterozygosity (“good genes as heterozygosity” hypothesis; Landry et al., 2001; Qvarnström and Forsgren, 1998). Although the concept of “mate quality” itself is hard to define (Brown, 1997) and the incompatibility levels between mates may be the result of a number of different factors (Tregenza & Wedell, 2000; Zeh & Zeh, 1996, 2001), several studies support this hypothesis. Main supporting evidence of inbreeding avoidance mechanisms comes from studies on kin selection based on major histocompatibility complex (MHC) compatibility (Keane, 1990; Landry et al., 2001; Simmons, 1991; Yeates et al., 2013). However, the mechanistic cascade of events that enables any differential mate choice has not yet been identified, stimulating the proposal of different explanatory theories (Ziegler et al., 2005). Females are thought to be able to influence paternity at different stages of the reproductive process, before, during and after mating and before and after fertilisation (Birkhead, 1998; Birkhead et al., 1993; Gowaty,

1994; Zeh & Zeh, 1997). Several studies support female control of paternity before copulation (Gowaty, 1994). Female butterflies (*Bicyclus anynana,* Fischer et al., 2015) and female sticklebacks (*Gasterosteus aculeatusi*) for example show pre-mating preferences toward unrelated mates (Frommen & Bakker, 2006). On the other hand, post-copulatory inbreeding avoidance has been reported in red junglefowl (*Gallus gallus*) (Pizzari et al., 2004) and two cricket species, *Teleogryllus oceanicus* (Simmons et al., 2006) and *Gryllus bimaculatus* (Bretman et al., 2009). But pre- and post-mating mechanisms are not mutually exclusive and their co-occurrence have been shown across different species such as the Trinidadian guppies (*Poecilia reticulata;* (Daniel & Rodd, 2016; Gasparini & Pilastro, 2011; Glover et al., 2012) and house mice (*Mus domesticus;* Firman and Simmons, 2008; Penn & Potts, 1998; Potts et al., 1991). However, disentangling the role of the two sexes in determining reproductive success is a challenge (Birkhead, 1998). Externally fertilising organisms are particularly well suited to address these challenges because they offer an opportunity to study gamete interactions in a controlled fertilisation microenvironment outside the female's body; and in fact, it is here that some of the clearest demonstrations of sperm discrimination have been recorded (Alonzo et al., 2016; Yeates et al., 2009). To test whether similar mechanisms may form a mechanism of inbreeding avoidance among closely related individuals was the aim of this study.

Adult philopatric Atlantic salmon (*Salmo salar*) tend to return to their native streams to spawn (Garant et al., 2000; Jordan & Youngson, 1992). Furthermore, Atlantic salmon experience high rates of inbreeding rates due to farmed salmon escapes (Besnier et al., 2011; Glover et al., 2012) and as a result of inadequate breeding programs (i.e., low number of breeders, unnatural fertilisation regimes (Campton, 2004; Tave, 1986; Withler & Beacham, 1994). The risk of inbreeding among closely related individuals and the reduced control of females over paternity offers a perfect opportunity to study the evolution of post-mating pre-zygotic mechanisms of inbreeding avoidance based on a genetic kin recognition system at the gametic level (Jordan & Bruford 1998). However, non-random gamete fusion may not evolve because the costs may be high in external fertilisers, where quick association of gametes in the outer environment might reduce the chances for assortative mating (Wedekind et al., 2004). Nevertheless, in salmonids as well as other fish, there is evidence that cryptic female choice can be mediated by the ovarian fluid, a viscous substance released with eggs at oviposition (Rosengrave et al., 2016; Yeates et al., 2013). However, the presence of

inbreeding avoidance mechanisms between full siblings in externally fertilising fish is currently untested.

In the present study, we tested how genetic relatedness affects sperm behaviour and sperm-egg incompatibility and which mechanisms of inbreeding avoidance are found in farmed Atlantic salmon. We performed *in vitro* fertilisation assays to analyse sperm motility and velocity parameters to compare offspring fitness between sibling and non-sibling crosses and run sperm competition experiments to test relative reproductive success between sibling and non-sibling males. In addition, we assessed heterozygosity levels at five microsatellite loci including two MHC loci in the resulting offspring. These assays allowed us to explore whether a self- *vs.* non-self-recognition occurs strictly between gametes or also between sperm and the ovarian fluid.

4.3 Methods

4.3.1 Study species

All fish used in this experiment belonged to the commercial strain Mowi, which is one of the earliest strains used in salmon aquaculture and has been following a breeding regime for 13 generations. Since 1960, this strain has been artificially selected for growth, delayed gonadal maturation and fillet quality. This selection was exclusively targeting fish phenotypes until 1999, when pedigree techniques became widely available and a family selection program based on DNA fingerprinting was implemented (Fjelldal et al., 2009).

At the Norwegian Institute of Marine Research (NIMR) Research Station, multiple generations of domesticated Atlantic salmon populations have been produced (Glover et al., 2018; Harvey et al., 2016, 2018; Perry et al., 2019; Solberg et al., 2014; Solberg, Skaala, et al., 2013; Solberg, Zhang, et al., 2013). With the help of preventive genotyping and a productive collaboration between the NIMR and the private aquaculture sector, all the parental fish used in this study were from a well-known genetic background and known pedigrees (Taggart, 2006). Information about relatedness among the experimental fish was at the start of the experiment and was obtained with the help of microsatellite analyses in combination with exclusion-based pedigree determination using the program FAP (see genotyping and paternity assignment section for details). This genetic information was used to carefully select seven males and seven females to generate seven full sibling pairs, and seven non-sibling pairs with no relatedness between paired males and females (see Fig.1).

4.3.2 Gamete collection

Brood fish were stripped for gametes at the Mowi breeding facility in Askøy (western Norway), where they had been reared from feeding larval stage to adulthood in controlled conditions (natural photoperiod, 6 ± 3.3 °C). Males and females were gently stripped of gametes without anaesthesia due to its risk of impacting sperm function (Wagner et al., 2002). Stripping was conducted by using standard hatchery procedures as described in Gage et al., (2004). Briefly, gametes were collected from the urogenital pore by applying gentle abdominal pressure. Prior to stripping, urogenital pores were dried with a paper towel to avoid sperm activation before the start of the fertilisation and sperm competition experiments due to contamination with water, mucus, or urine. Once collected, milt samples from males were transferred into sterile flasks and kept on ice in sealed polystyrene boxes. Eggs including the ovarian fluid from the females were placed in sealed egg buckets and kept on ice. Gametes were then transported on ice and processed on the same day at the experimental aquaculture facilities and laboratories at the NIMR Research Station in Matre.

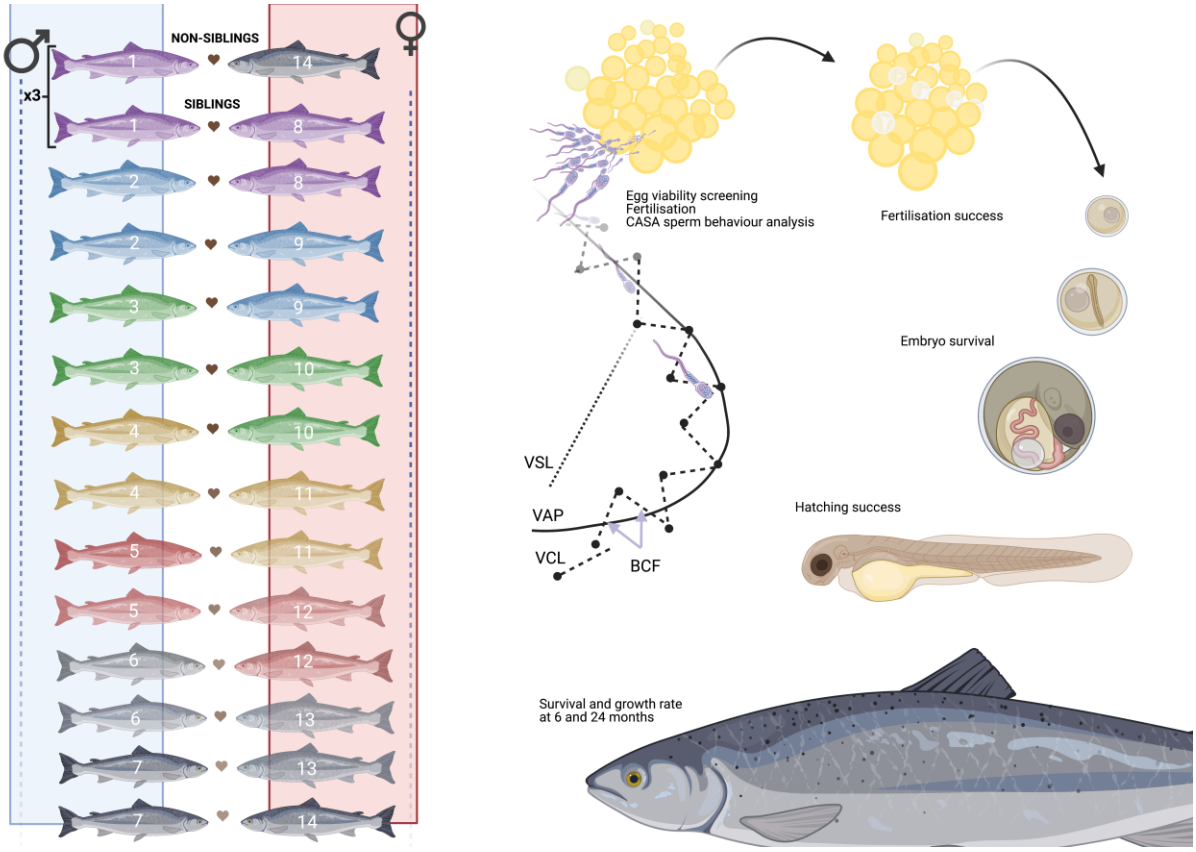

Figure 4.1 *Experimental design used in the homospermic experiment for sperm swimming behaviour, fertilisation-, hatching success and growth rates in non-siblings and sibling salmon crosses (N= 7 males x 7 females arranged in 28 unique crosses (3 replicates per cross). Individuals are siblings when they share the same colour.*

4.3.3 Sperm behaviour assessment

For each of the seven experimental males, an aliquot of 1.5ml of ejaculate was collected from the main flask after gentle mixing to homogenise the sample, transferred into an Eppendorf vial and placed on ice. From each vial, 0.8 ul of undiluted ejaculate was directly activated in a 4µl solution of 100% ovarian fluid under a microscope (UOP, Tokyo, Japan, equipped with a 20× negative-phase contrast objective) using double-chambered Micro tool™ Cytonix sperm slides (Cytonix, Beltsville, MD 20705, USA). These slides are specifically designed to ensure quick and homogenous mixing, reduce wall effects, and stop the baseline flow caused by pipetting within the shortest amount of time, to produce trustable sperm activity recordings at 5 seconds post activation (spa). The aliquot was placed at the entrance of the chamber and subsequently activated by flushing the ovarian fluid trough the entrance to fill the chamber. Video recording started at five seconds post activation to record sperm activity through a camera (Grasshopper2 digital camera, FLIR systems®, British Columbia, Canada) mounted to

the microscope. For every male, sperm activity in ovarian fluid belonging either to a non-sibling or a full-sibling female was recorded in three experimental replicates per sample, from 5 to 60 spa. The resulting videos were exported and analysed in a CASA automated plug-in available for the FIJI ImageJ software (Schindelin et al., 2012) as described in (Purchase & Earle, 2012). Briefly, recorded videos were converted to binary b/w images, labelled with information about male and female IDs, experimental replicate, and relatedness of the cross (sibling or non-sibling) and organised in a folder. Preliminary trials on a smaller sample of videos were performed to obtain the optimal input parameters to feed to the CASA software (details on parameters can be found in the Supplementary material, Fig 4.8). The software acquired sperm tracking information at a rate of 30 frames s^{-1}. The percent of motile cells (MOT), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN = VSL/VCL), beat-cross frequency (BCF), wobble (WOB) and progression (PROG) were collected for both individual cells and the averages for every second calculated. All sperm motility and fertilisation trials were performed at a water temperature of 6–7°C and similar air temperature.

4.3.4 Fertilisation and hatching success

For every experimental cross (sibling or non-sibling), two replicates of 100 eggs each were collected to conduct fertilisation trials and to monitor development to embryo stage. An additional 500 eggs per cross were monitored for post-hatching development. 100-egg batches were fertilised by using 100µl raw milt added per fertilisation and activated in 100ml natural river water at the temperature of 6 ± 0.62 °C. After 2 minutes (maximising fertilisation potential), fertilised egg batches were individually transferred into 7 x 7 cm hatching chambers created within a bigger 45 x 45cm perforated PVC tray. At this stage, eggs were gently mixed within the hatching chambers, counted, and individually screened for fertility (unfertilised eggs quickly turn white shade with patches of colour). Unfertilised eggs were counted and removed from the chamber. All the trays containing fertilised egg batches were kept in a controlled oxygen saturated Recirculating Aquaculture Systems (RAS) system at a temperature of 6 ± 1.7 until hatching. All the perforated PVC trays shared the same water during development and labelled groups were evenly distributed ensuring an equal number of sibling and non-sibling egg batches in each tray. At hatching, all embryos were counted and immediately placed in a 60% Etoh solution for future analyses.

The 500-egg batches were fertilised using 500 µl raw milt and placed in the same RAS system but in 15 x 15 hatching chambers. During development, each batch was checked daily to monitor embryonic development, to count embryonic deaths and to remove dead eggs/embryos from the hatching chamber. After hatching, alevins were randomly divided into three equally sized groups per replicate and transferred into six (three for non-sibling crosses and three for sibling crosses) larger RASs (1000 L) resulting in three RASs per experimental group. The alevins and juvenile fish were fed ad libitum from the start of the feeding phase. After six months, half the fish were collected, euthanised, and their bodyweight (BW) and fork lengths (FL) measured. The remaining half was split again into two groups and transferred into bigger RASs (5x5m each) and fed ad libitum for two years under natural photoperiod in natural sea water under the same conditions described above. After two years, the remaining fish were sampled for growth-related parameters.

4.3.5 Sperm competition

Approximately 100 eggs from each female were fertilised using a total of 200µl of sperm from two males (100 µl from a full sibling male and 100 µl from a non-sibling male). Individuals were paired according to the same study design as for the fertilisation trials described above (Fig 2). The separate sperm samples from each male were gently homogenised with a pipette, and the samples from the sibling and non-sibling male placed on opposite sides of a dry 1L plastic beaker having an inner concave portion containing the eggs to avoid premature sperm activation. Sperm and eggs were simultaneously activated and mixed by the addition of 200ml natural river water at 6 ± 0.62 ℃. Immediately after the addition of water, pictures of the egg batches were taken for later counting of total number of eggs. As described above, the fertilised and hardened eggs were transferred into the RAS hatchery system and monitored until hatching. Hatched alevins were counted and 48 individuals per family were randomly sampled, euthanised and placed in 70% EtoH for subsequent paternity analyses.

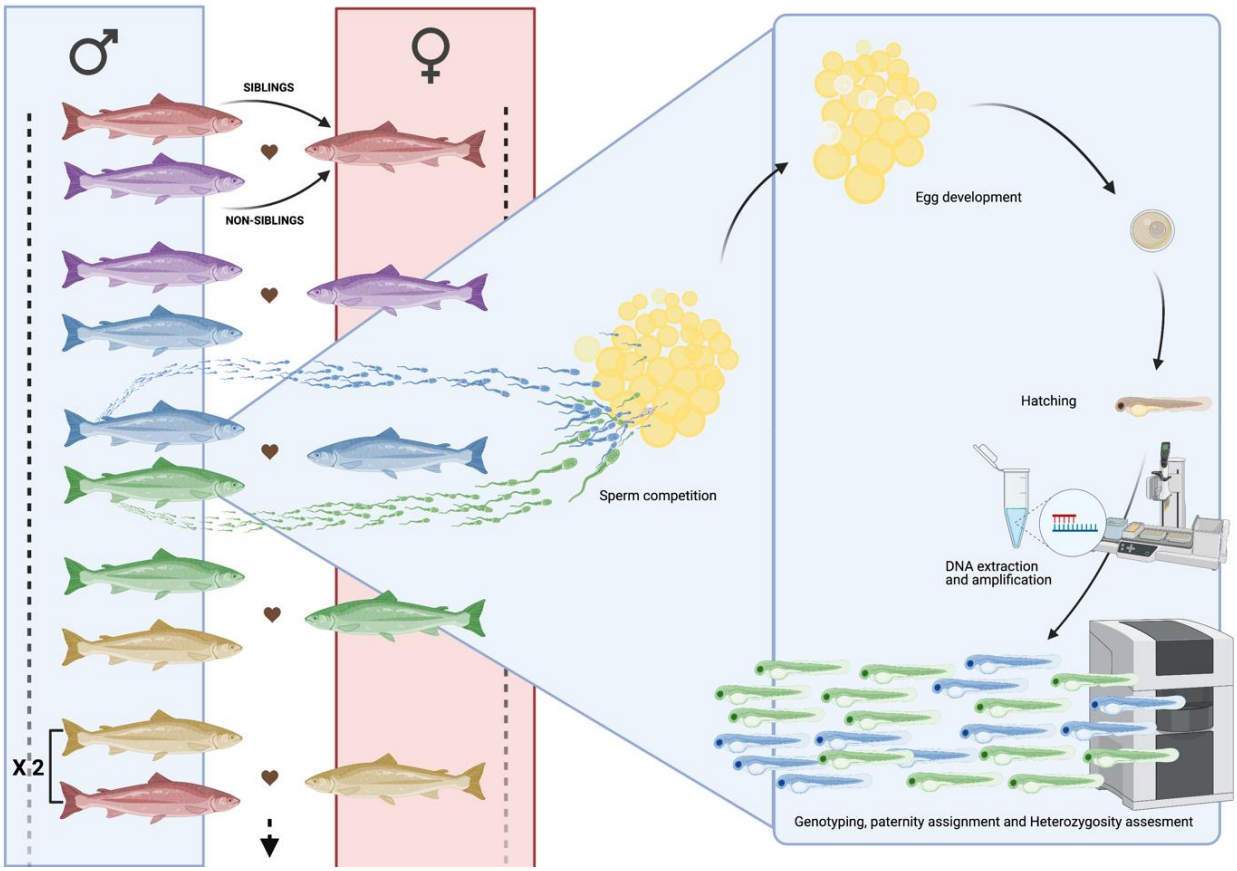

Figure 4.2 *Experimental design for the sperm competition (heterospermic) experiment; (N= 7 males x 7 females arranged in 7 unique trios (2 replicates per trio). Individuals are siblings when they share the same colour.*

4.3.6 Genotyping and paternity assignment

To assign parents to the offspring resulting from the sperm competition trials and examine the inheritance patterns in the hatched embryos, individuals were genotyped using a panel of five microsatellites: *SsaOsl85* (Slettan et al., 1995), *MHC1* (Grimholt et al., 2002), *MHC2* (Stet et al., 2002a), *Ssa197* (O'Reilly et al., 1996) and *SsaF43* (Sánchez et al., 1996). The selected set of microsatellites has been used for more than a decade on several thousands of Atlantic salmon in this laboratory (Glover, Solberg, et al., 2017) and has proven useful for population genetic studies (Harvey, Tang, et al., 2017), in reconstructing pedigree history (Jørgensen et al., 2018; Solberg, Zhang, et al., 2013), permit identification of conjoined twins (Fjelldal et al., 2016), to identify the source of farmed salmon escapes at the farm level and to identify trisomic, triploid and haploid individuals (Glover et al., 2015, 2016; Glover, Hansen, et al., 2017; Harvey, Fjelldal, et al., 2017; Jørgensen et al., 2018).

DNA was extracted from one eye of each alevin in 96-well format using the HotSHOT method as described by Truett et al., (2000). On each DNA extraction plate, two blank cells were added to serve as negative controls. PCRs were run for the five selected microsatellites and PCR products were analysed on an ABI 3730 Genetic Analyser (Applied Biosystems, Foster City, CA) at the Genetics Laboratory of the Institute of Marine Research in Bergen, Norway and sized by a 500LIZ™ size-standard. Alleles binned automatically in the program Genemapper™ were manually checked by two researchers prior to exporting the data for statistical analysis. Offspring paternity assignment was conducted adopting the exclusion-based family assignment program FAP (Taggart, 2006) permitting to link offspring to their familiar origins for known parental genotypes and crosses. The use of exclusive male + male x female in each of the crosses enabled an unequivocal paternity assignment because each individual alevin could only had been sired by one of the two males in each trio.

4.3.7 Statistical analyses

All analyses were performed using R Studio version 1.3.1093 (RStudio (2020), Integrated Development for R. RStudio, PBC, Boston, MA) equipped with *car* (Fox and Weisberg, 2011), *glmmTMB* (Brooks et al., 2017), *(readxl), (lme4), (lmerTest), (DHARMa), (lsmeans), (merTools), (dplyr), (tidyverse), (rstatix), (ggpubr),(arsenal), (knitr), (survival),* and *lmerTest* (Kuznetsova et al., 2017) packages to perform exploratory analysis, run the main models, perform post-hoc tests and create output tabs. Graphical figures were plotted using *ggplot2* (Wickham, 2011), *(ggpubr), (sjPlot), (sjmisc)* and *(qqplotr).* All the sperm motility data were analysed using Linear Mixed Effect Models (LMMs) and Generalised Linear Mixed Effect models (GLMMs) in *lme4* (Bates et al., 2015). To determine the error distributions, the relationship between the variance and the mean of the response variable and the assumptions for data distribution were checked (Crawley, 2012). Models were fitted using Restricted Maximum Likelihood (REML) methods to enable refinement and validation (Thomas et al., 2013). Residuals from linear models were checked for violations of the assumptions of normality and homoscedasticity. Significance of fixed effects in LMMs were obtained using *t*-tests with Satterthwaite's approximation for degrees of freedom implemented in *lmerTest* (Kuznetsova et al., 2017). Main effects, contrast analyses and interactions, when present, were extracted trough the *emmeans* and *emtrends* functions. For all the variables analysed, a selection of the best model structure to explain each variable was conducted by comparing residual dispersion, model predictions, AICs and BICs for each of the computed

models through the 'summary' function output and through *DHARMa* residual diagnostic. Additionally, performance between the different models was compared by using the '*anova'* function.

4.3.7.1 Sperm swimming behaviour in non-sibling and sibling ovarian fluid

Sperm motility parameters MOT%, VSL, LIN, BCF and PROG were analysed using LMMs, whereas VAP, VCL and WOB were analysed using GLMMs (*glmmTMB*, Brooks et al., 2017) due to better residual diagnostics of these over LMMs. All the models included the relatedness between mates (non-siblings or full siblings), the time from activation (SPA, 5 to 60s) and their interaction as fixed factors, and female and male ID as random factor, (ID 1 to 7), with the three experimental sperm samples tracked per each male nested by male ID. Random slopes were included for these experimental males to account for the fully factorial design.

4.3.7.2 Fertilisation and hatching rates in sibling and non-sibling crosses

The proportion of eggs successfully fertilised for each batch of eggs, as well as the final percentage of fertilised embryos that succeeded to hatch were modelled trough GLMMs. The final models included the relatedness between mates (non-siblings or full siblings) as fixed factor, and female and male ID as random factor (ID 1 to 7) with the two experimental replicates per male nested by male IDs.

4.3.7.3 Morphometrics

The comparison of fork length and the body weight between non-sibling and sibling offspring were modelled at 6- and 24-months post-hatching trough LMMs, following a similar approach as previous models. The final models included the relatedness between parents (non-siblings or full siblings) as fixed factor, and the tank ID as a random factor (3 tanks for sibling crosses and 3 tanks for non-sibling ones).

4.3.7.4 Sperm competition experiments and microsatellites analyses

Microsatellite genotyping data were used to determine the percentage of offspring sired by each male in the sperm competition experiments. Paternity rates and genotypic identities of all the individuals were transferred to R Studio and analysed for multilocus heterozygosity

(MLH) as well as for heterozygosity levels at the individual loci using the package *InBredR*. In Genepop (Genepop version 5.2.3.), parental pedigree and offspring genotypic data were used to compare Hardy-Weinberg equilibrium, allele frequencies (Genepop option 1.2) and inbreeding coefficients (Robertson & Hill 1984), (Genepop option 5.1) between sibling and non-sibling groups. The number of shared MHC1 and MHC2 alleles between parents were used as an independent variable in a *glmmTMB* mixed effect model testing for differences in paternity explained by the number of MHC alleles shared between mother and father (0, 1 or 2; our random variables) using a similar model selection approach as explained for previous variables. The observed (H_o) and expected (H_e) heterozygosity values for all the analysed markers were imported into RStudio, and the percentage of heterozygous individuals for each marker in sibling and non-siblings offspring using Levene Tests and mixed effect models. with Male and Female Id as random factors.

4.4 Results

Exposure of sperm to ovarian fluid from sibling females resulted in significantly reduced motility over time, lower velocity-related parameters and lower linearity and beat-cross frequencies compared to sperm exposed to ovarian fluid from non-sibling female (Figure 4.3, Tables 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6). This pattern was consistent among all males and true for all the sperm motility parameters analysed. Fertilisation rates were significantly lower (36 ± 8.22 %) in sibling crosses compared to non-sibling crosses (Fig 4.4A, Table 4.7). In contrast, hatching rates among fertilised eggs did not differ between the two groups (Fig 4.4B, Table 4.8) nor did mortality over the duration of the experiment. However, alevins in sibling crosses showed significantly lower body weight and standard length both at six- and 24-months post hatching (Fig 4.5, Table 4.9). In the sperm competition experiment, no effect of parent relatedness on paternity share was found (Fig 4.6, Table 4.10). Interestingly, multilocus heterozygosity was consistently higher in all sibling offspring that were genotyped, but the levels of single locus heterozygosity and inbreeding coefficients did not differ between sibling and non-sibling offspring (Fig 4.7, Table 4.11, 4.12).

Table 4.22 *Generalised liner mixed model (glmmTMB in R) model for percentage of motile sperm (%) exposed to sibling or non-sibling ovarian fluid from 5 to 60s post activation. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					152.28	
Male ID					185.47	
Replicate:Male ID					33.89	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	25.83	7.47	9.64, 41.7	1, 11.29	3.45	**0.0056**
Relatedness	-2.95	2.15	-7.76, -1.58	1, 17.50	-1.38	0.9034
Time post-activation (s)	-0.18	0.02	-0.21, -0.14	1, 2241.61	-9.38	**<0.001**
Relatedness * Time post-activation (s)	0.08	0.02	0.03, 0.13	1, 2241.88	3.14	**0.0017**

Table 4.23 *Linear mixed effect model (lmer in R) for sperm straight line velocity (μm/s) in sibling or non-sibling ovarian fluid from 5 to 60s post activation. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					3.66	
Male ID					0.001	
Replicate:Male ID					96.58	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	46.43	2.48	41.56, 51.30	1, 28.79	18.69	**<0.001**
Relatedness	-9.68	1.28	-12.19, -7.18	1, 13.90	-7.57	**<0.001**
Time post-activation (s)	-0.39	0.02	-0.45, -0.35	1, 2096.56	-16.52	**<0.001**
Relatedness * Time post-activation (s)	0.18	0.03	0.11, 0.24	1, 2097.63	5.545	**<0.001**

Table 4.24 *Linear mixed effect model (lmer in R) for sperm curvilinear velocity (μm/s) in sibling or non-sibling ovarian fluid from 5 to 60s post activation. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					139.44	
Male ID					261.83	
Replicate:Male ID					32.31	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	79.29	8.61	69.74, 90.90	1, 10.22	9.72	**<0.001**
Relatedness	-4.05	2.17	-0.02, 3.93	1, 13.91	-1.86	**<0.001**
Time post-activation (s)	-0.30	0.01	-0.33, -0.25	1, 2110	-17.90	**<0.001**
Relatedness * Time post-activation (s)	0.02	0.02	-0.04, 0.06	1, 2110	1.10	0.7424

Table 4.25 *Linear mixed effect model (lmer in R) for sperm average path velocity (μm/s) in sibling or non-sibling ovarian fluid from 5 to 60s post activation. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					20.70	
Male ID					0.0002	
Replicate:Male ID					84.60	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	62.59	2.86	56.98, 68.21	1, 22.01	21.84	**<0.001**
Relatedness	-4.52	1.50	-7.47, -3.62	1, 214.73	-3.014	**0.00258**
Time post-activation (s)	-0.41	0.02	-0.46, -0.36	1, 2092	-16.001	**<0.001**
Relatedness * Time post-activation (s)	0.12	0.03	-0.06, -0.20	1, 2093	3.61	**0.003**

Table 4.26 *Generalised liner mixed model (glmmTMB in R) for sperm linearity (%) in sibling or non-sibling ovarian fluid (Relatedness), from 5 to 60s post activation. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					24.53	
Male ID					62.51	
Replicate:Male ID					97.09	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	73.17	4.42	63.57, 83.14	1, 9.06	16.52	**<0.001**
Relatedness	-12.09	1.36	-14.79, -9.27	1, 43.24	-8.89	**0.001**
Time post-activation (s)	-0.21	0.02	-0.25, -0.17	1, 2092	-10.31	**<0.001**
Relatedness * Time post-activation (s)	0.17	0.03	0.12, 0.23	1, 2093	6.446	**<0.001**

Table 4.27 *Linear mixed effect model (lmer in R) for sperm beat cross frequency (Hz) in sibling or non-sibling ovarian fluid (Relatedness) from 5 to 60s post activation. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					0.30	
Male ID					2.36	
Replicate:Male ID					5.70	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	10.77	4.42	9.35, 12	1, 28	17.72	**<0.001**
Relatedness	-0.02	0.01	-0.58, -0.55	1, 320	-0.055	0.9560
Time post-activation (s)	-0.06	0.005	-0.05, -0.08	1, 2096	12.64	**<0.001**
Relatedness * Time post-activation (s)	-0.017	0.007	0.032, 0.034	1, 2097	-2.40	**0.0148**

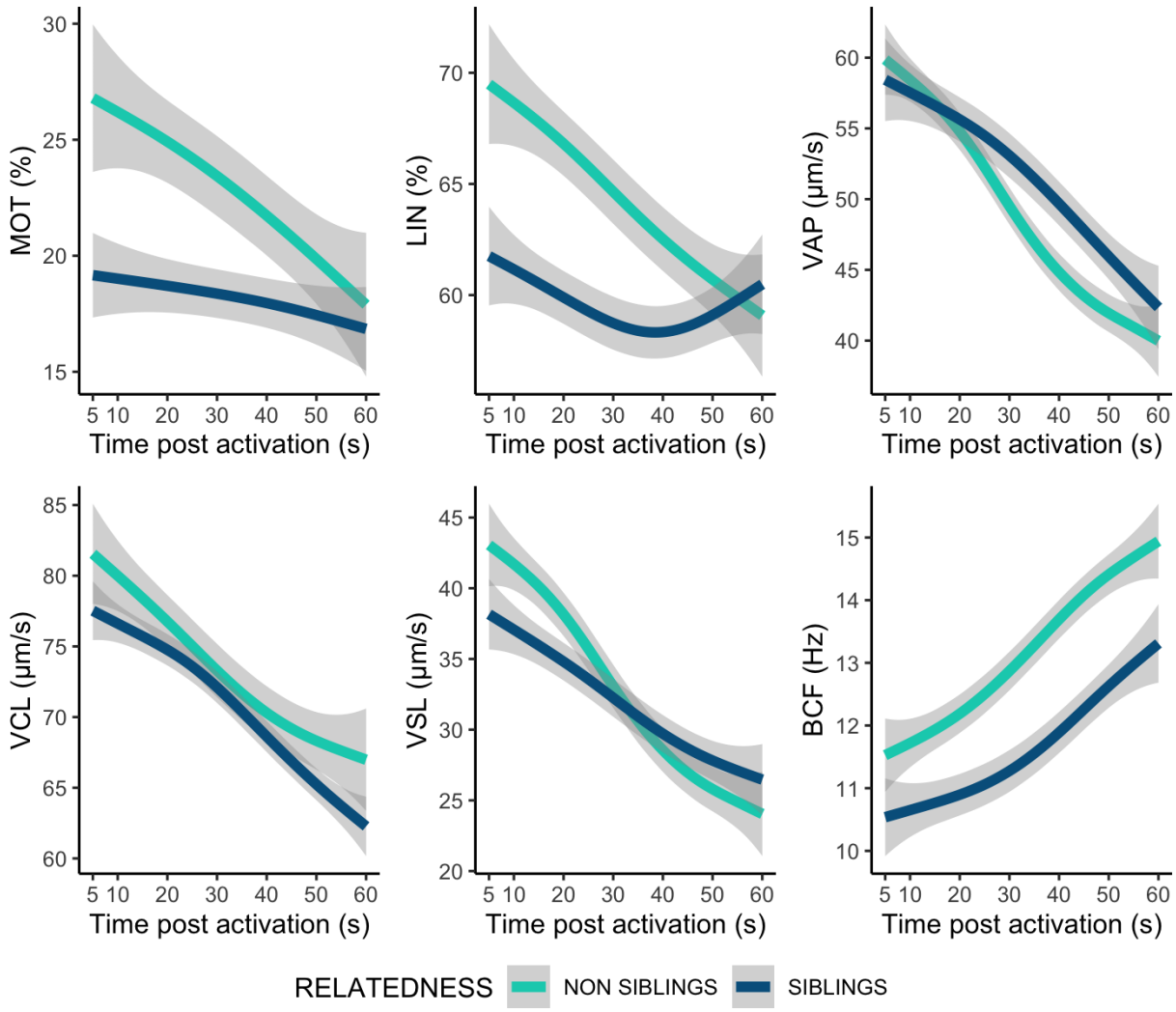

Figure 4.3 *Motility (%), linearity (%), average path velocity, curvilinear velocity, velocity on a straight axis and beat cross frequency (Hz) of sperm activated in non-sibling (green) or. sibling (blue) ovarian fluid recorded every second from 5 to 60 seconds post activation. Data shown represent the average between males (n=7) and the and confidence intervals (grey-shaded areas).*

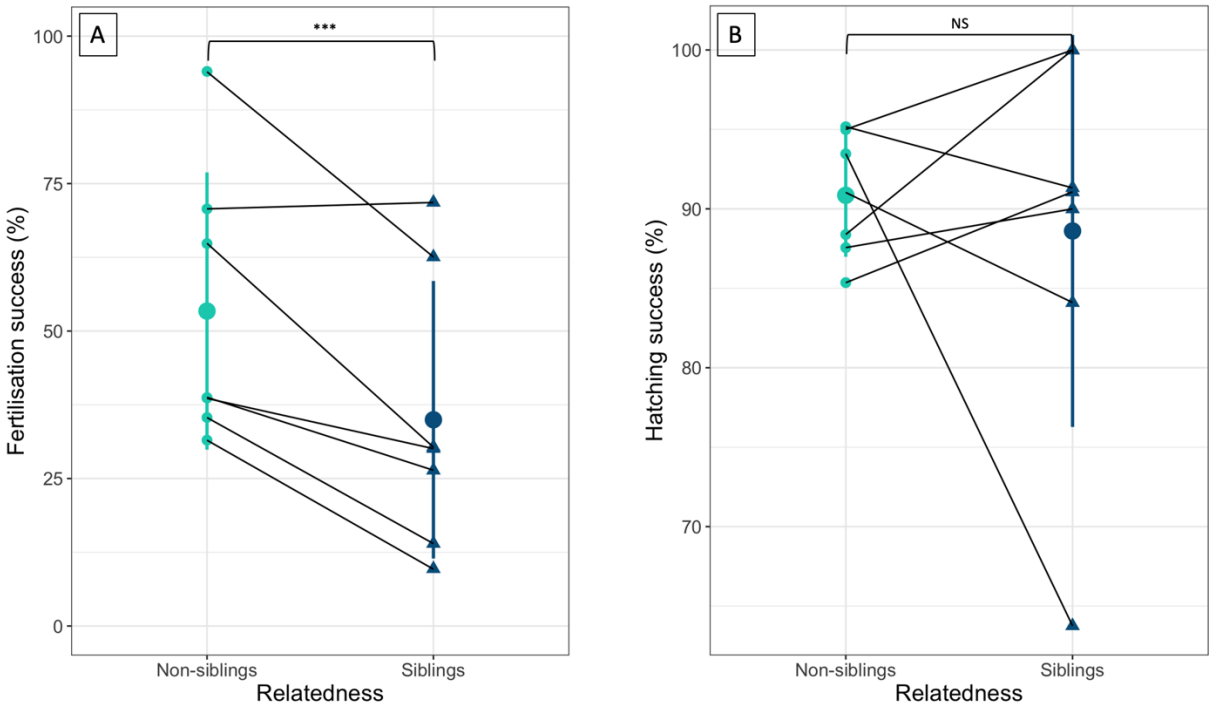

Figure 4.4 A) *Fertilisation and* ***(B)*** *hatching success (%) in non-sibling (N = 7) and sibling crosses (N= 7). Data are displayed as mean ± standard error (SE), dark lines connect each experimental female used in the experiment, (dots for non-siblings and triangles for siblings). Significance threshold: *** = p < 0.001.*

Table 4.28 *Generalised liner mixed model (glmer in R) for fertilisation success (%) in sibling or non-sibling crosses. The results are shown for a total of 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					0.195	
Replicate:Male ID					0.197	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-0.863	0.21	-1.32, -0.41	1, 8.70	-4.034	**<0.001**
Relatedness	-0.367	0.08	-0.53, -0.21	1, 9.77	-4.439	**<0.001**

Table 4.29 *Generalised liner mixed model (glmmTMB in R) for hatching success (%) in sibling or non-sibling crosses. The results are shown for a total og 14 split-design crosses with seven males and seven females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Female ID					12.33	
Replicate:Male ID					2.39	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	90.85	3.13	83.92, 97.78	1, 8.9	28.97	**<0.001**
Relatedness	-2.25	2.70	-8.05, 3.56	1, 13.05	-0.81	0.43

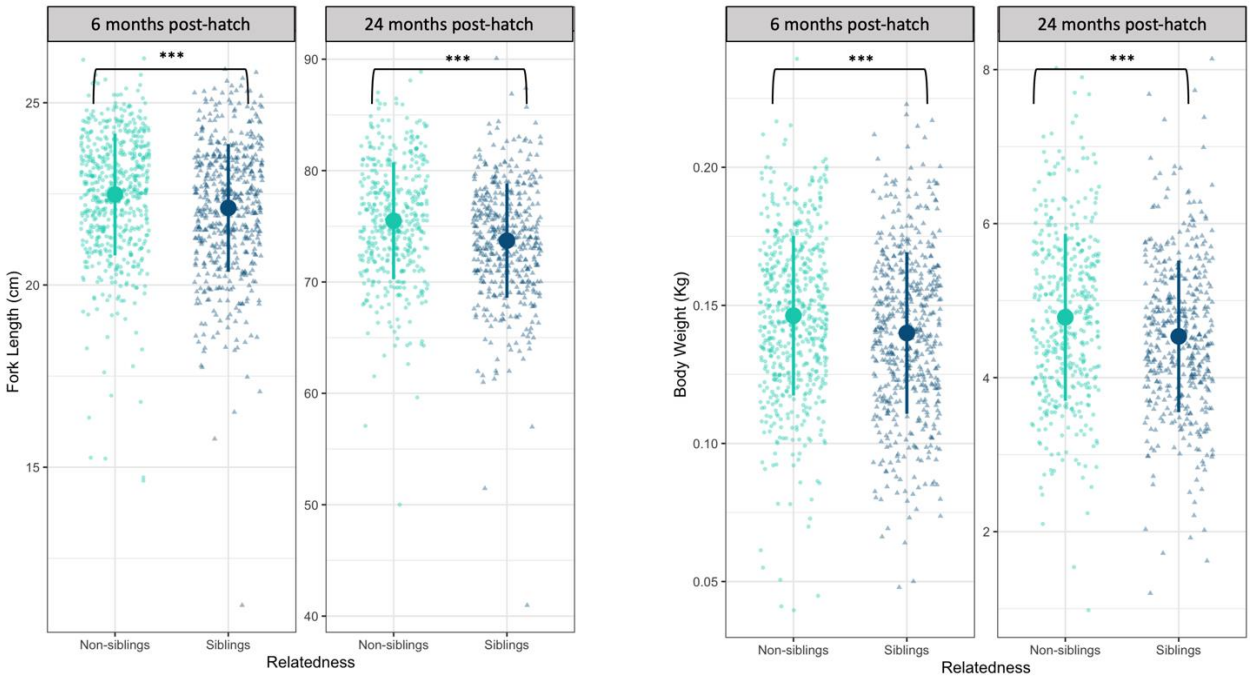

Figure 4.5 *Comparison of fork length (cm) and body weight (Kg) between sibling and non-sibling offspring measured at six- and 24-months post hatching. Samples sizes are 431 and 436 for sibling offspring and 576 and 544 for non-sibling at six- and 24-months respectively. The graphs display raw data (dots and triangles) and means (big dots) ± standard error (SE, vertical bars). Significance threshold: *** = p < 0.001.*

Table 4.30 *Linear mixed effect model (lmer in R) for changes in body weight (kg) and length (cm) in sibling or non-sibling (Relatedness) crosses at sampled at 6- and 24-months post-hatch. Models include the random factor for replicate experimental tank per relatedness status. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Body weight (Kg) 6 months post hatch **Variable**						
Random					**Variance**	
Tank					<0.001	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	0.14	0.001	0.143, 0.149	1, 1116	120.87	**<0.001**
Relatedness	-0.006	0.001	-0.010, -0.002	1, 1116	-3.63	**<0.001**
Fork Length (cm) 6 months post-hatch **Variable**						
Random					**Variance**	
Tank					<0.001	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	22.48	0.07	22.34, 22.66	1, 1116	316	**<0.001**
Relatedness	-0.36	0.01	-0.56, -0.16	1, 1116	-3.59	**<0.001**
Body weight (Kg) 2 years post-hatch **Variable**						
Random					**Variance**	
Tank					0.0168	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	4.79	0.09	4.61, 4.96	1, 878	53.34	**<0.001**
Relatedness	-0.26	0.07	-0.40, -0.13	1, 878	-3.78	**<0.001**
Fork Length (cm) 2 years post-hatch **Variable**						
Random					**Variance**	
Tank					0.45	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	75.52	0.46	74.62, 76.43	1, 878	163.65	**<0.001**
Relatedness	-1.85	0.34	-2.53, -1.17	1, 878	-5.33	**<0.001**

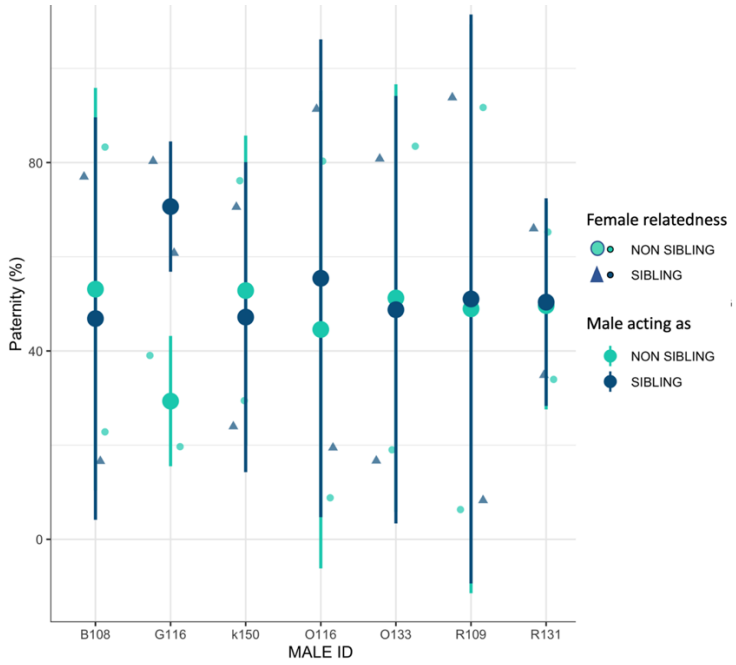

Figure 4.6 *Percentage of hatched offspring sired by non-sibling (green, n= 7) or sibling males (blue, n= 7) in paired sperm competition assays with females (n= 7). Data shown represent mean ± standard error (SE).*

Table 4.31 *Generalised mixed effect model (glmmTMB in R)) for paternity (log) by non-sibling (n= 7) and sibling fathers (n= 7) within each of the trios. Estimates are provided with standard error (SE), confidence intervals (CI) and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Male ID: acting as (NON-SIB or SIB)					0.0016	
Female ID					0.0019	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	47.09	8.35	3.12, 4.37	1, 14.82	11	**<0.001**
Acting as (NON.SIB or SIB)	-0.08	1.14	-2.32, 2.14	1, 13	1.14	0.940
MHC1share	-0.61	0.84	-2.27, 2.15	2, 20	-0.72	0.466
MHC2share	-0.07	0.55	-1.16, 1	2, 20	-0.14	0.886
Acting as SIB: MHC1share	0.90	1.18	-1.42, 3.23	2, 20	1.18	0.445
Acting as SIB: MHC2share	0.12	0.89	-1.63, 1.27	2, 20	0.89	0.887
MHC1share: MHC2share	0.26	1.03	-1.76, 2.29	2, 20	0.25	0.799
Acting as SIB: MHC1share: MH2share	-0.47	1.16	-2.75, 1.81	2, 20	-0.40	0.685

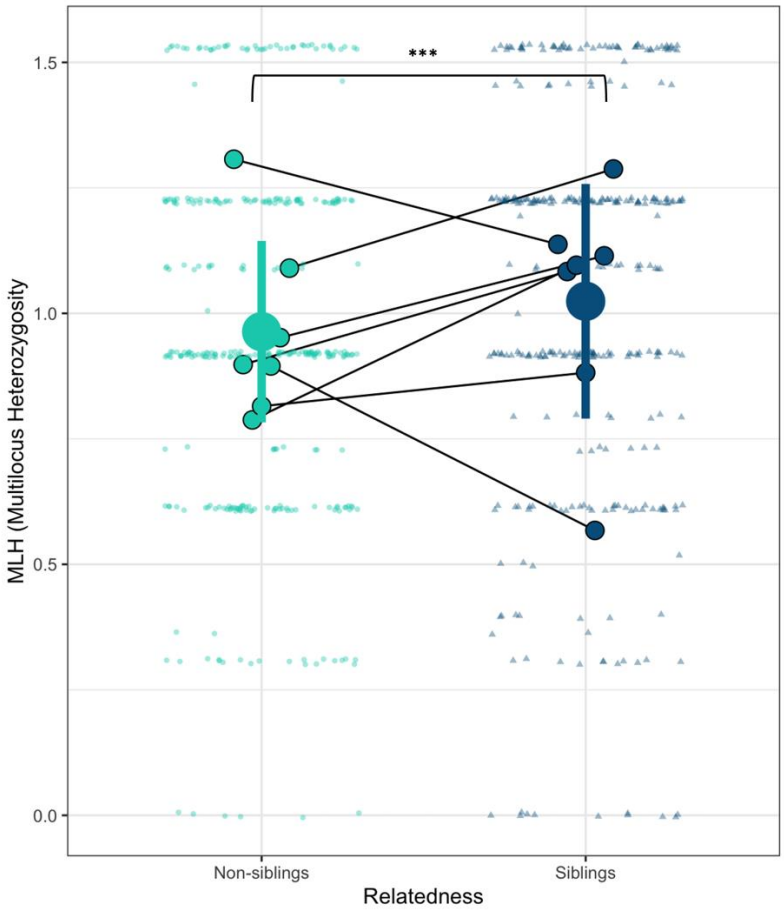

Figure 4.7 *Multilocus heterozigosity values in hatched non-sibling (n=326) and sibling (n=324) offspring from the sperm competition experiment. Dark lines connect the average values of offspring sired by either the non-sibling or the sibling male for each experimental female used in the trio (n= 14 trios total). Data shown represent mean ± standard error (SE).*

Table 4.32 *Linear mixed effect model (lmer in R) for multilocus heterozigosity in hatched and genotyped offspring hatched from the sperm competition experiment between a sibling (n=7) and a non-sibling father (n=7). Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Family ID					0.02	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	0.96	0.06	0.82, 1.10	1, 6.47	14.18	**<0.001**
Relatedness	0.06	0.02	0.01, 0.11	1, 642	2.36	**0.0185**

Table 4.33 *Expected (He) and observed (Ho) heterozygous individuals, and the inbreeding coefficients, Fis, according to Robertson and Hill (1984) (R&H) in 650 genotyped Atlantic salmon offspring at five microsatellite loci.*

Locus		He	H_o	R&H
MHC1	non-siblings	28.62	35	-0.1765
	Siblings	27.82	33	-0.1608
MHC2	non-siblings	20.23	20	0.1320
	Siblings	23.14	22.42	0.1470
SsOsl85	non-siblings	29.54	34.28	-0.1581
	Siblings	26.70	31.71	-0.1876
Ssa197	non-siblings	23.22	30	-0.1985
	Siblings	24.29	32.57	-0.2612
SsaF43	non-siblings	22.03	25.85	-0.1552
	Siblings	22.38	27.85	-0.2418

4.5 Discussion

Our results provide clear evidence for a differential effect of ovarian fluid from sibling and non-sibling females on sperm performance and fertilisation success. Sperm motility, velocity, and linearity as well as fertilisation success were significantly higher in the ovarian fluid of non-sibling females and the size and growth of alevins from sibling crosses was reduced. These findings suggest that while inbreeding depression is likely as indicated by the reduced size of juvenile and adult fish 6 and 24 months after hatching, inbreeding avoidance mechanisms have evolved at the gamete level mediated by the interaction between sperm and ovarian fluid. However, when sperm from sibling and non-sibling males were competing simultaneously for the same egg batch we found no bias in paternity. This opens the possibility that in presence of intense competition factors other than relatedness may play a role in determining the reproductive outcome. Our overall results therefore suggest that while the ovarian fluid may provide a pre-zygotic barrier to inbreeding, this barrier is relatively weak and may be overcome in situations of sperm competition.

4.5.1 Post-mating pre-zygotic mate choice

Our current knowledge on mechanisms of kin-recognition at the gametic level comes mainly from internally fertilising species and mammals in particular (de Boer et al., 2021). Most of these studies tested inbreeding avoidance across varying levels of genetic similarity (e.g., unrelated, half-sibling crosses, and inbred and outbred individuals; Pike et al., 2021). For instance, in houbara bustards (*Chlamydotis undulata*), unrelated males sired more offspring than cousins or half-siblings, but this effect was explained through differential embryo mortality among the crosses rather than by the presence of a pre-zygotic barrier to inbreeding (Vuarin et al., 2018). The presence of such mechanisms has been suggested in house mice, where a fertilisation bias against genetically related males was found and ascribed to an egg-driven sperm selection toward unrelated males (Ee et al., 2015). In the internally fertilising Trinidadian guppy (*Poecilia reticulata*), the ovarian fluid of unrelated females enhanced sperm swimming performance compared to ovarian fluid of related females (Gasparini and Pilastro, 2011). In this study, paternity in competition trials was clearly biased toward unrelated males which contrasts with our results. One possible explanation for these contrasting results are the fundamentally different reproductive strategies and life histories of

the two species: In externally fertilising species, females are expected to favour stronger pre-zygotic mechanisms, whereas live-bearers should theoretically invest more in post-zygotic mechanisms (Zeh & Zeh, 2000, 2006).

How the ovarian fluid differentially modulates the sperm according to male identity and kinship is still unknown. This process could involve signalling peptides dispersed within the ovarian fluid and receptors on the sperm, as found in mammals (Carlstedt et al., 1983; Paradisi et al., 2000; Spehr et al., 2003). Although MHC-peptides have been proposed as feasible candidates in such mechanisms in other external fertilisers, their identification in the ovarian fluid still has to be confirmed, and so does the MHC expression on sperm. However, our results suggest that these components if present in the ovarian fluid, are unlikely to be involved in this mechanism; at least for the farm strain of salmon used. Other reproductive proteins playing a role in the fertilisation process could be more important (Swanson & Vacquier, 2002). Future studies in fish should therefore focus on identifying peptides capable of modifying sperm behaviour, such the decapeptide 'speract' which affects sperm motility and is present in the reproductive fluid of sea urchins (Wood et al., 2007). In the zebrafish *Danio rerio*, variation in the ovarian fluid of different females was shown to differentially affect sperm behaviour across males, where the male x female interaction was particularly important in supporting its potential in cryptic female choice mechanisms (Poli et al., 2019), but the mechanisms underlying these observations are not known. Understanding the mechanistic series of events leading to a differential behaviour of sibling or non-sibling sperm in presence of ovarian fluid could therefore be a priority for future research. It would also be interesting to investigate whether the dynamics of mate selection in farmed salmon are also applicable to their wild counterparts, and what the consequences of this could be following salmon escapes in the natural environment.

4.5.2 MHC based mate choice and gamete fusion

We tested how fertilisation, hatching rates, and paternity under sperm competition were influenced by parental genetic background and by the number of alleles shared between partners, including at the *MHC1* and *MHC2* loci; two markers that have been found to be involved in mate choice in a variety of species (Jordan & Bruford, 1998; Neff et al., 2008; Penn & Potts, 1998; Pitcher & Neff, 2006; Potts et al., 1991; Reusch et al., 2001; Skarstein et

al., 2005; Wedekind et al., 1995, 1996). Their mechanistic role in mate choice and kin recognition is still highly debated (Ziegler et al., 2005), and we therefore tried to test the idea in the context of inbreeding avoidance mechanisms between sibling and non-sibling crosses in an externally fertilising animal model. Due to the solidity of our pairing structure, and the robustness of re-using the same males multiple times in a paired way, either in the role of sibling or non-sibling, we can confidently reject a possible role of any microsatellite loci used in this study in post-mating mate choice. We discuss our findings in view of previous experimental results.

Pre-zygotic mechanisms of kin-recognition in external fertilisers are still relatively poorly understood. In Atlantic salmon for example, sperm competition experiments between males and females with similar or dissimilar MHC1 alleles provided clear evidence of a fertilisation bias towards males with dissimilar MHC alleles (Yeates et al., 2009) In contrast, experimental testing for MHC*2*-based gamete fusion at the haploid level in Atlantic salmon provided no evidence for non-random fusion (Promerová et al., 2017). In the brown trout (*S. trutta*), females favoured mates with an intermediate level of MHC1 similarity but the exact mechanisms of mate choice and the relative importance of pre-and post-mating pre-zygotic mechanisms was not determined (Forsberg et al., 2007). We found no deviation from a Hardy-Weinberg equilibrium in sibling and non-sibling offspring for both MHC1 and MHC2 and we detected no significant difference in inbreeding coefficients between sibling and non-sibling offspring after sperm competition. Our contrasting results with the previous study on MHC1 (Yeates et al., 2009) could be due to the fact that we paired our mates according to their sibling non-sibling status, rather than according to their MHC*1* and MHC2 variability. This is similar to what Promerova et al., (2017) did and may explain why neither found any evidence for a role of MHC dissimilarity. Such a mechanism may only materialise in comparisons between mating partners at opposite ends of the MHC similarity spectrum and in fact the interaction between relatedness and MHC may make the detection of a non-random mating pattern hard. Another possible explanation for the discrepancy in the results could be the use of wild (Yeates et al., 2009) *versus* farmed (this study) populations. After decades of artificial selection due to aquaculture management (Campton, 2004; Glover, Solberg, et al., 2017), we cannot exclude that kin recognition mechanisms or MHC-based selection systems could have been weakened and diluted as described for a series of other inheritable traits such as kype size, growth, and reaction norms to different environments (Perry et al., 2019; Solberg, Skaala, et al., 2013; Teletchea & Fontaine, 2014). Finally, in a

whitefish (*Coregonus sp.*), there was also no evidence for MHC-based sexual selection (Wedekind et al., 2004) and the authors argued that MHC-based selection likely did not evolve in fish due to the higher costs of non-random gamete fusion in externally fertilising species. In such species, gametes are released into a hostile environment and quickly have to come into contact therefore reducing the chances for assortative mating. In addition, they suggested that selection for assortative mating should be weak because of the large amounts of eggs and the lower investment per egg compared to mammals, and because a generally lower risk of inbreeding might have not selected toward strategies to avoid its detrimental effects (Wedekind et al., 2004).

By controlling the starting number of eggs per female and precisely determining the number of fertilised eggs within minutes of contact between male and female gametes, we suggest that the difference in reproductive outcomes observed in the first set of experiments (homospermic fertilisation trials) could not be traced to differential early embryo mortality between non-sibling and sibling crosses. One of the motivations in support of highly variable *MHC* complexes is linked to the enhanced capacity of ensuring resistance to broader spectrum of fast-evolving parasites, bacteria and viruses (Agbali et al., 2010; Wedekind et al., 2004). Neither in the single-male experiment, nor after the sperm competition did we find differential hatching success, egg to adult, or embryonic survival in sibling and non-sibling crosses suggesting no MHC-mediated improved survival in our experiment. However, this is a cautious conclusion, since we did not specifically test for this, and because our eggs and embryos developed in very controlled RAS aquaculture settings.

4.5.3 Inbreeding depression and inbreeding avoidance mechanisms

Intriguingly, the presence of a pre-zygotic preference for unrelated sperm within the ovarian fluid, in combination with a small but significant level of inbreeding depression observed in sibling offspring would suggest that the barrier posed by the ovarian fluid is overall weak. This contrasts with the widely assumed concept that inbreeding depression should drive the evolution of inbreeding avoidance mechanisms, but new views, referred to as the ‘inbreeding paradox’(Reid et al., 2021), justify the coexistence of inbreeding depression without - or in the presence of a weak avoidance mechanism (Kokko & Ots, 2006; Szulkin et al., 2013). This is not a unique occurrence in nature and comparable results have been found in different

animal models like the sparrows *Passer domesticus* and *Melospiza melodia* (Billing et al., 2012; Olson et al., 2012; Reid et al., 2021). Whether this is the case only for the farm strain studied here or because we used full-sibling crosses rather than assessing the preference for unrelated males based on specific markers has to be explored further. Our results are instead in line with a study focusing on haploid selection and finding no haploid MHC2-based assortative gamete fusion in the same species (Promerova et al., 2017). Similarly, in brown bears (*Ursus arctos*), there is no evidence that females display mating preferences according to MHC similarity. The authors also found no association between the reproductive success of a male and the MHC alleles he carried, providing no support for any role of mate choice in shaping MHC polymorphism in this species (Kuduk et al., 2014).

Anadromous salmonids generally show high degrees of local adaptation, clearly distinguishable sub-populations and display homing behaviour, all suggesting that inbreeding should pose a concrete risk (Fleming, 1996; Leániz et al., 2007; Primmer, 2011; E. B. Taylor, 1991; Wang et al., 2002a). Importantly, local adaptation has been shown to be under strong selection in this species, therefore highlighting that outbreeding could in theory pose an even higher issue for offspring fitness than inbreeding (Bourret et al., 2011; Côte et al., 2014; Dionne et al., 2008). The proven rapid association of gametes in salmonids, after these are released into the external environment, might not allow a egg-sperm receptor-mediated kin recognition system in this model. Yeates et al., (2007) showed that a 2 second delay in sperm release resulted in a significant decrease in fertilisation success during sperm competition experiments (Yeates et al., 2007) and suggested that such a rapid association might not allow much time for kin recognition mechanisms at this stage (Yeates et al., 2009).

Nevertheless, the fact that we detected lower fertilisation rates and an overall number of hatched embryos in sibling crosses during the first set of experiments suggests that a mechanism allowing kin recognition and eventually control inbreeding avoidance could take place within the ovarian fluid, and that this seems not to be MHC-related. By focusing on the interaction between the ovarian fluid and sperm in the sperm motility assays, we controlled for confounding factors such as behavioural pre-mating preference and sperm activation time and separate them from kin-selection based mechanisms involving receptors on the eggs (Kobayashi & Yamamoto, 1981; Metz et al., 1994; Yanagimachi et al., 1992). Moreover, by comparing sperm from the same male in the role of either a sibling or non-sibling partner to a female, we controlled for inter-males difference in sperm velocity and characteristics that

could have otherwise biased our results. However, we cannot exclude that within the pairs and trios created here, the effect of relatedness could have been masked because of compatibility issues between mates, therefore confounding our results.

4.5.4 Conclusions

Our results show that the ovarian fluid from a farmed salmon strain can differentially regulate sperm swimming performance according to relatedness, but that this barrier does not seem to be strong enough to bias the paternity outcome and reduce inbreeding depression when unrelated and related males are simultaneously competing to fertilise a set of eggs. These results are in contrast with some other studies using wild salmonids and suggest that a strong inbreeding avoidance mechanism and/or an MHC recognition system could not evolve or was lost in farmed salmon strains, despite the decrease in fitness observed after inbreeding. We also suggest that a combination of pre- and post-zygotic mechanisms could mask the real strength of a bias in reproductive outcomes between mating partners of varying relatedness; and that further studies should look toward a genome-wide enhancement of heterozygosity or locally adapted traits, rather than focus on specific alleles. There is in fact evidence that mate preferences can operate not exclusively at the level of key individual loci and in a directional way, but that instead favourable allelic combinations could be assessed on a broader genomic scale (Mays & Hill, 2004; Neff & Pitcher, 2005; Tregenza & Wedell, 2000). For instance, a recent study identified 55 SNPs showing a signature of sexual selection and 611 SNPs involved in differential viability in the Gulf pipefish, *Syngnathus scovelli* (Flanagan et al., 2016). Similarly another genome-wide screening study in the plant *Mimulus guttatus* reported several hundreds of SNPs exhibiting a signature of viability selection (Monnahan et al. 2015), suggesting that although a few loci can show greater effects, mate choice might operate on a multitude of levels.

4.6 Supplementary Material

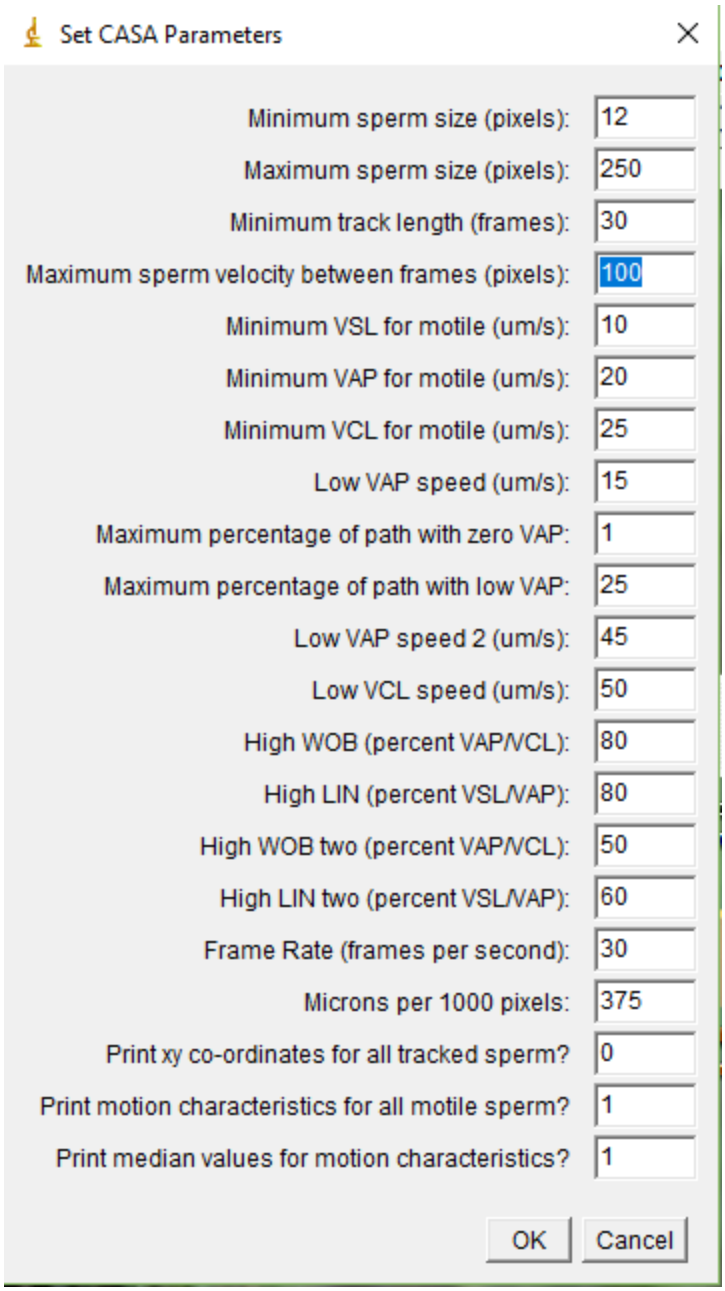

Figure 4.8 *Input parameters for the CASA automated sperm motility analyses used in this study*

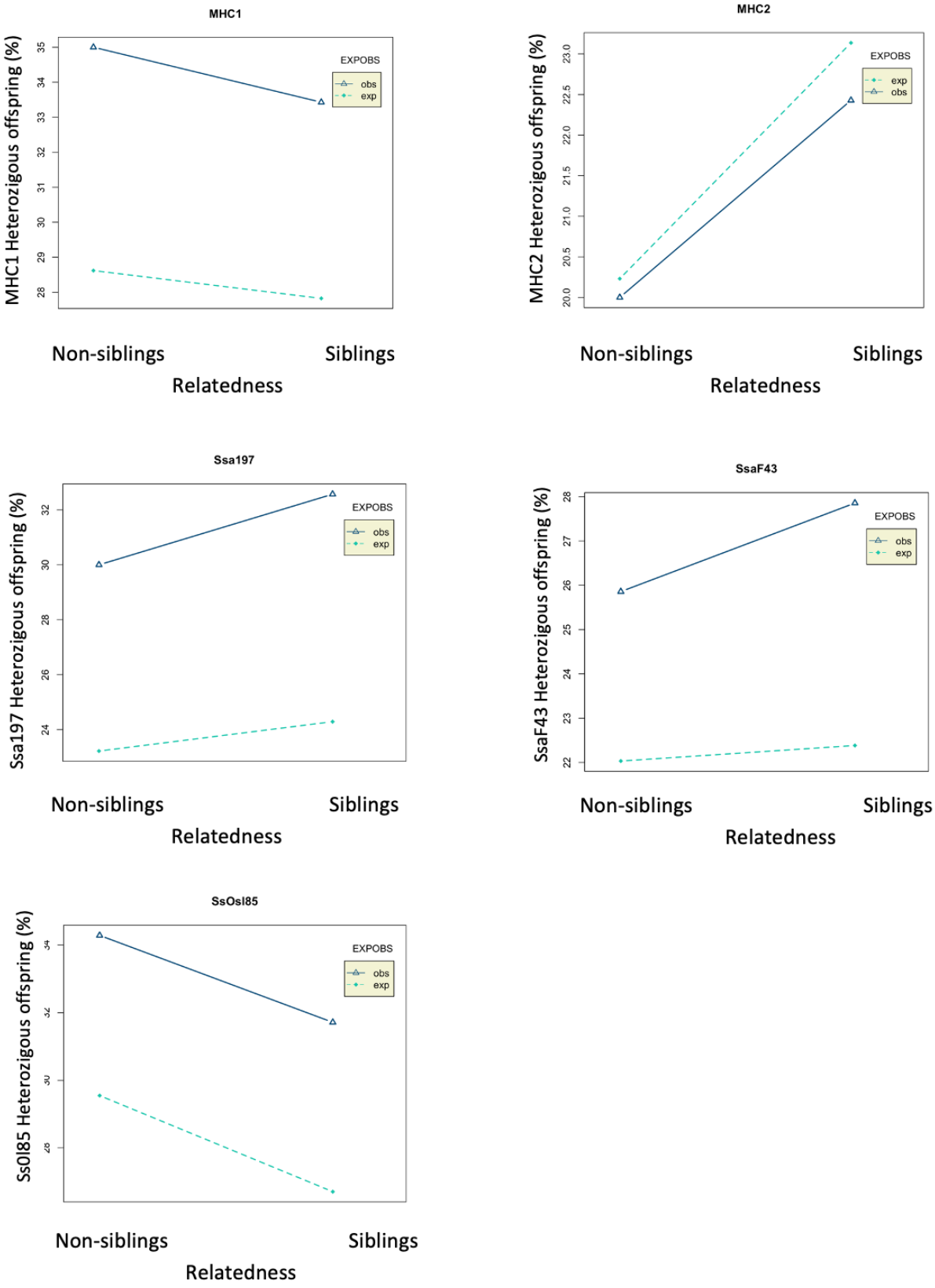

Figure 4.9 *Expected and observed heterozygosity of MHC1, MHC2, Ssa197, SsaF43 and SsOsl85, in non-siblings and sibling offspring (n=650) of Atlantic salmon (Salmo salar) after sperm competition.*

5 Pre-fertilisation gamete thermal environment influences reproductive success, unmasking sex-specific responses in Atlantic salmon (Salmo salar)

Chapter's contributions:

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Statement of contributions: M. Gage and K. Glover provided fundings, operational facility and instrumentations. M. Solberg and K. Glover provided the fish and the gametes. M. Graziano and M. Gage conceptualised the work. M. Graziano, M. Gage and D. Murray conducted the fertilisation experiments. M. Graziano and L. Dryhovden monitored the fish until hatch and collected the samples. M. Graziano extracted, curated and analysed the data. Hatchery technical support received was kindly ensured by L. Dryhovden and I. Helge. A. M. Graziano performed all the counts, measurements and screened for developmental abnormalities. M. Graziano created all the tabs, figures and illustrations. M. Graziano wrote the first complete draft of this work and all the following versions after receiving the review comments and suggestions from the Supervisors M. Gage and S. Immler.

5.1 Abstract

The environment that gametes perform in just before fertilisation is increasingly recognised to potentially affect offspring fitness. Recent findings have shown that both paternal and maternal pre-fertilisation environments can have profound consequences on fertilisation success, offspring size, growth, dispersal ability and survival. However, the relative contribution of male and female gametes, the adaptive significance and the underlying mechanisms are still largely unexplored. Here, we studied the mechanisms of thermal plasticity of gametes and/or thermal selection affecting egg and sperm function and competitiveness. We tested the hypothesis that gametes adapted to a specific temperature positively influence hatching success and the performance of the resulting embryos during development in the same temperature environment and compared sperm and eggs. We incubated half of the eggs and sperm collected from wild Atlantic salmon (*Salmo salar*) overnight at either 2° C (cold) or 8 °C (warm). We performed *in vitro* fertilisations where we crossed warm and cold-incubated gametes following a full-factorial design for each of the mating pairs and reared one half of each clutch in cold temperature and the other in warm temperature. We monitored hatching success, hatching time, embryo survival before and after the eyed stage and presence of developmental abnormalities. We found that when the temperature for eggs, sperm and embryo development were the same, embryos hatched relatively earlier than in scenarios where gamete incubation and development temperature were different. Warm temperature exposure during embryo development generally caused increased rates of deaths after the eyed stage. Interestingly, we observed opposite effects of gamete incubation temperature on offspring fitness between eggs and sperm, where warm incubation was beneficial for eggs but detrimental for sperm which in turn negatively affected hatching success. Overall, gamete plasticity did not significantly improve offspring fitness, suggesting that these stages are particularly vulnerable to a changing environmental temperature.

5.2 Introduction

Environmental changes are intrinsically related to key evolutionary concepts including adaptation, selection and speciation. Irrespective of the causes and strengths of environmental changes, they inevitably influence natural populations; the efficiency and degree of resistance of the latter depend on their ability to adjust through adaptive responses such as migration, phenotypic plasticity, and epigenetics (Gaitán-Espitia & Hobday, 2021; Hoffmann & Sgró, 2011). These mechanisms determine the long-term perspective of organisms to persist in the face of climate change (Bell & Ad Collins, 2008; Burrows et al., 2011; Pandolfi et al., 2011). The ability to evolve an adaptive response strongly depends on genetically heritable variation and on the demographic costs of such adaptation (Bell & Gonzalez, 2009). If environmental changes are too extreme or if they occur over periods that are evolutionarily irrelevant, high mortality and extinction will prevent an adaptive evolutionary response (Bell & Gonzalez, 2009; McGuigan et al., 2021). In such situations, phenotypic plasticity plays a key role, as it involves the differential expression of phenotypes according to local conditions and allows for an efficient strategy to cope with rapid environmental fluctuations (Chevin & Lande, 2010).

Phenotypic plasticity in the context of climate change is particularly important when it affects several generations, referred to as inter- or transgenerational plasticity, where the environment or condition of the parents affects the fitness of future generation(s) (Agrawal, 2001; Harmon et al., 2021). Such multi-generational responses can be adaptive when the environment experienced by the parent/s is a good predictor for the conditions to be encountered by the offspring (Burgess & Marshall, 2014; Chirgwin et al., 2018; Silva et al., 2021). In the seed beetle *Stator limbatus* for example, mothers raised under poor nutritional conditions pass on their environmental experience by increasing egg size to protect their offspring against starvation when these will develop on seeds with hard and thick coat causing high mortality rates (Fox et al., 1997). In the flour beetle *Tribolium castaneum*, males which experienced heat waves and sperm that were produced under such conditions produced offspring with reduced reproductive fitness and lifespan (Sales et al., 2018.; Vasudeva et al., 2019). In spiny chromis *Acanthocromis polyacanthus* and sheepshead minnows *Cyprinodon variegatus*, thermal tolerance in the offspring was enhanced by parental exposure to warm temperatures (Bernal et al., 2022; Salinas & Munch, 2012).

Among the abiotic environmental variables affecting organisms, temperature is one of the most studied (Harris et al., 2018; Radchuk et al., 2019); specifically in the last decade, with the changing climate manifesting high levels of short- and long-term fluctuations (Perkins et al., 2012; Raftery et al., 2017; Tomczyk & Bednorz, 2019). Across Europe for example, the Heat Wave Magnitude Index (HWMId, adimensional), expressing the duration and intensity of heatwaves, has increased steadily from an average of -2 to +2 between 1980 and 2015, and is projected to augment until 2100, even considering the most conservative of the predictions (Russo et al., 2015; R. Zhang et al., 2020). In riverine systems, global mean and high (95th percentile) water temperatures are projected to increase by 0.8-1.6 and 1.0-2.2 °C respectively according to the Special Report on Emissions Scenario (SRES) B1–A2 for 2071-2100 relative to 1971-2000 (van Vliet et al., 2013). Considering these catastrophic fluctuations, there is a need to assess how and if organisms are able to cope with such changes in their habitat, especially during sensitive life stages.

Organisms can respond in a multitude of ways to thermal variation, from changes in behaviour and phenology (Beever et al., 2017) to mitochondrial function (Hraoui et al., 2021; Menail et al., 2022) and osmoregulatory adaptations (Cooper, 2017; Gerber & Overgaard, 2018). Importantly, temperature affects a key aspect that arguably defines the survival of a species - its ability to reproduce. Experimental evidence from a wide array of species highlights how temperature affects fertility (B. S. Walsh et al., 2019b) and that the threshold temperature for fertility is much lower than for survival (Walsh et al., 2019). In fact, high temperatures have been shown to affect the germ line and the associated reproductive efficiency of both sexes (Pérez-Crespo et al., 2008; Vollmer et al., 2004), because thermal stress affects meiotic divisions much more that mitotic proliferation (Paupière et al., 2014; Sage et al., 2015). This is the reason why in mammals for example, testes descend during gonadal maturation for scrotal externalisation to ensure that spermatogenesis occurs at temperatures that are lower than body temperature (Moreno et al., 2012).

Male and female gametes may perform differently in varying environment and their optimal conditions may vary (Vasudeva et al., 2019). On their path to the egg, sperm try to achieve their mission in a ‘non-self and demanding environment’(Gage & Morrow, 2003; Parker, 1982; Reinhardt et al., 2015; Pitnick et al., 2009.). Sperm production and function are particularly sensitive to higher temperatures which result in lower sperm density (e.g., Paxton

et al., 2016, Cnidarian *Acropora digitifera*), lower motility (e.g., Porcelli et al., 2017, *Drosophila subobscura*; Batista et al., 2018, *D. tripunctata*), reduced sperm size (e.g. Vasudeva et al., 2014, cowpea weevil *Callosobruchus maculatus*), changes in biochemical composition (e.g., Dadras et al., 2017, several Teleosts) and increased rates of DNA damage (e.g. Pérez-Crespo et al., 2008, *Mus musculus*; Peña et al., 2019, *Sus sp.*). In the solitary tunicate *Styela plicata* for example, offspring sired by males raised in low-density populations had faster developments and higher hatching success than that of offspring deriving from high-density populations. Also, offspring had in general higher survival rates when their raising conditions matched that experienced by the parents, suggesting that the observed responses were adaptive (Crean et al., 2013). In the whitefish *Coregonus lavaretus*, the pre-fertilisation incubation of sperm to warmer temperatures resulted in smaller offspring with reduced swimming performance as compared to the colder treatment (Kekäläinen et al., 2018).

Similarly, eggs are highly sensitive to temperature experienced before fertilisation (Weber et al., 2011) where higher temperatures results in reduced production (e.g., Rukke et al., 2018, bed bug *Cimex lectularius)*, smaller size (e.g., Vasudeva et al., 2019, *T. castaneum*), reduced fertilisation capability (e.g., de Rensis et al., 2017, *Bos taurus*) and lower offspring fitness including lower survival rates (e.g. Sales et al., 2019, *T. castaneum*; Zheng et al., 2017, fruit moth *Grapholita molesta*, also reviewed by Hoffmann & Sgró, 2011; Walsh et al., 2019). External factors may influence gamete function and could therefore provide the basis for the selection of adaptive responses in the resulting offspring. Such a process would allow individuals to improve their reproductive fitness by priming their gametes to the environment during fertilisation and offspring development. Gamete thermal plasticity could evolve as an adaptive trait when the environment experienced by the parents matches that of the offspring (Burgess & Marshall, 2014). However, the mechanisms by which temperature affects gametic performance and how these influence offspring fitness remains poorly understood (Crean et al., 2013; Crean & Immler, 2021; Evans et al., 2019; Kekalainen & Evans, 2018; Läinen et al., 2018; Zajitschek et al., 2014).

Exploring the consequences of gametic thermal environments for offspring fitness is particularly impartant for stenothermal species like salmonids that are highly sensitive to thermal fluctuations (Pankhurst et al., 2011; Pankhurst & King, 2010; Thorstad et al., 2021). Minor changes in the thermal environment experienced by sperm for instance have been

found to have significant effects on its function in the common carp (*Cyprinus carpio)*, in the salmonids brown trout (*Salmo trutta)* and in the greyling (*Thymallus thymallus)* and in the turbot *Lota lota* (Dadras, Sampels, et al., 2017; Fenkes et al., 2016, 2017; Lahnsteiner & Mansour, 2012a). High temperatures can similarly yield to the increased production of reactive oxygen species (ROS) in human and mouse sperm that can lead to oxidative stress and disrupt the lipidic and proteic sperm and cause DNA damage (Chianese & Pierantoni, 2021; Lane et al., 2014; Menezo et al., 2016; Tunc & Tremellen, 2009).

We investigated the potential role of thermal plasticity and/or thermal selection in eggs or sperm and how this affects the resulting offspring and their adaptation to thermal variation. We tested the hypothesis that the priming of gametes to a warm or cold temperature before fertilisation should benefit embryo development and hatching success in the same temperature and vice versa. In addition, we aimed to disentangle sex-specific effects using a full-factorial design and study offspring fitness in crosses where pre-fertilisation temperatures of gametes were not matched. We used a full-factorial split-clutch design using eggs and sperm from wild Atlantic salmon (*Salmo salar*). We monitored offspring survival to hatching, hatching synchrony, embryonic age at hatching and hatching success. We found that hatching success and synchrony were influenced by the conditions experienced by gametes prior to fertilisation, with warm environments presenting a threat for reproduction. We also found that when both gametes match the developmental temperature, we observed a lowering of the embryonic age at hatch that might indicate an adaptive plastic response, but gamete plasticity did not enable significant improvement of offspring numbers in our experiments.

5.3 Methods

5.3.1 Fish origin and gamete handling conditions

All Atlantic salmon used in this study came from the commercial strain Mowi from the breeding facility in Askøy and were reared and maintained under the same environmental conditions as described in the previous chapters. Stripping of gametes was conducted using standard hatchery procedures as described previously (Gage et al., 2004; Yeates et al., 2014) without anaesthesia due to its influence on sperm function (Wagner et al., 2002). Briefly, gametes were collected from the urogenital pore by applying gentle abdominal pressure and prior to each stripping, urogenital pores were dried to avoid sperm activation before the start of the experiments due to contamination with water, mucus and/or urine. Using this procedure, eggs from ten females and sperm from ten males were collected. Sperm samples were transferred into sterile flasks kept on ice in sealed polystyrene boxes. Eggs in their ovarian fluid were placed into sealed egg buckets and kept on ice. Gametes were then immediately transported to Norwegian Institute of Marine Research (IMR) experimental aquaculture facilities and laboratories in Matre and processed within the same day.

5.3.2 Experimental design and gamete temperature priming

Gametes from ten males and females were split across four different pre-fertilisation thermal treatments. The full factorial design resulted in four different treatments for each pair: warm sperm X warm eggs, warm sperm X cold eggs, cold sperm X warm eggs, cold sperm X cold eggs. Following collection, eggs and sperm were incubated over night for 24 hrs at either 2°C (cold) or 8°C (warm). In vitro fertilisation assays were conducted by adding 100 µl raw milt to a batch of 200 eggs for each treatment and the mixture was subsequently activated with 200 ml of river water (5.7 ± 0.46°C). Each of the batches was split into two halves again after fertilisation and one half reared in cold or warm egg RAS units kept at 2 ± 0.72°C or 8 ± 0.97°C respectively. We monitored survival before and after the eyed stage, hatching success, hatching time and developmental abnormalities (such as spinal deformities, incomplete neurogenesis, or abnormalities of the yolk sack; ‘malformed embryos’) every 12 hrs for the duration of the experiment (75 days for warm incubated and 180 days for cold incubated embryos; Figure. 5.1).

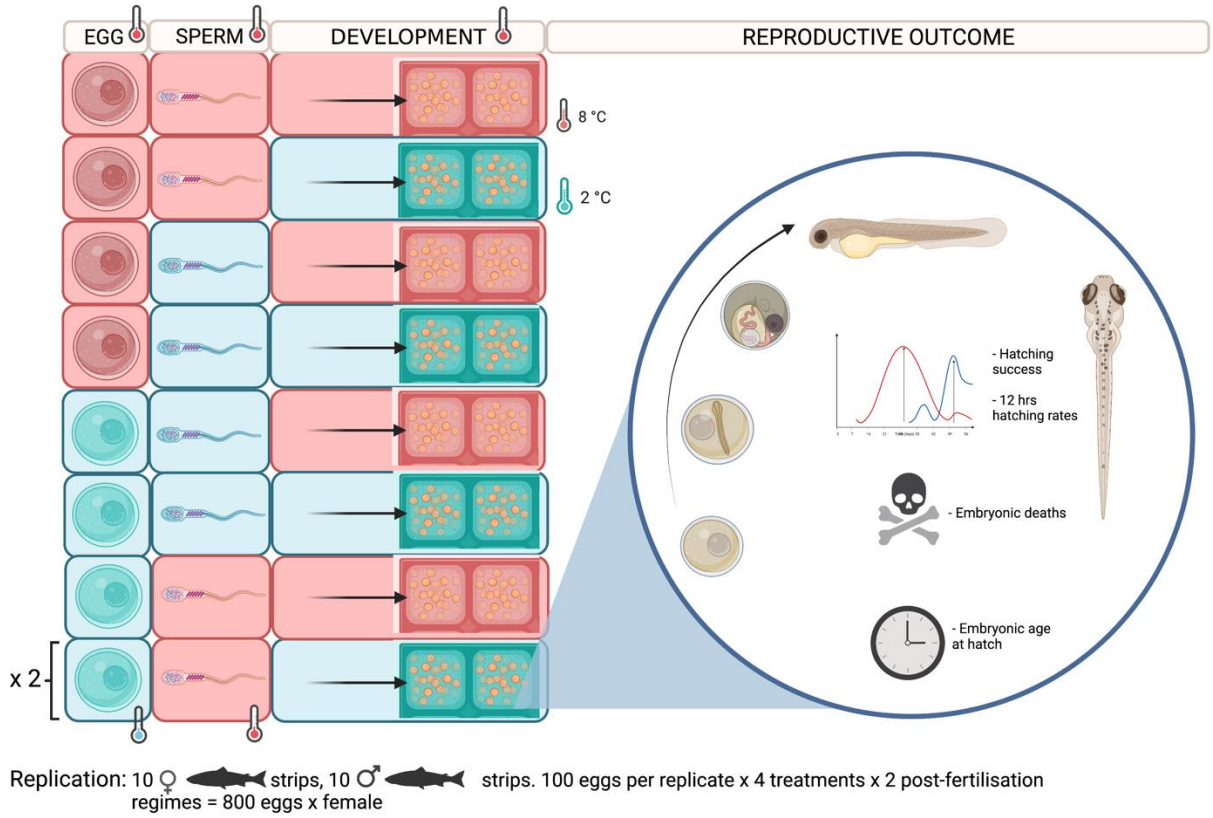

Fig 5.1 *Experimental design showing the treatments to which eggs, sperm, and embryos were exposed in each Family (n=10), two replicates of 200 eggs per cross per treatment), using a fully factorial design. On the right portion of the cartoon, the different measures of reproductive outcomes used to test the effects of post-release thermal environment on gametes function and offspring fitness are presented.*

5.3.3 Statistical analyses

All analyses were performed using R Studio (RStudio (2020), Integrated Development for R. RStudio, PBC, Boston, MA) (v 1.3.1093) equipped with *car* (Fox and Weisberg, 2011), *readxl*, *writexl*, *lme4*, *lmerTest* (Kuznetsova et al., 2017), *lmtest*, *DHARMa*, *dplyr*, *tidyverse*, Rmisc, *knitr and mass* (Venables and Ripley, 2002) packages to perform exploratory analysis, run the main models, perform post-hoc tests and create output tabs. Figures were generated using *ggplot2* (Wickham, 2011), *ggpubr*, *sjPlot*, *sjmisc*, *qqplotr*, *ggpub*, *magrittr*, *gridExtra*, *ggsignif*, *patchwork*, *RColorBrewer*. All data were analysed using Linear Mixed Effect Models (LMMs) and Generalised Linear Mixed Effect Models (GLMMs) in the package *lme4* (Bates et al., 2015). Error distributions were determined by checking the relationship between variance and mean of the response variables as well as the necessary

assumptions for data distribution (Crawley, 2012). All models were fitted using Restricted Maximum Likelihood (REML) methodology enabling model rectification and validation (Thomas et al., 2013). The residuals from linear models were also explored for normality and homoscedasticity. Significant main effects and interactions were extrapolated using *t*-tests with Satterthwaite's approximation controlling for degrees of freedom used as implemented in *lmerTest* (Kuznetsova et al., 2017) and by using the functions available through *emmeans* and *emmtrends*. Additionally, the runs from the models were also generated in the form of Analyses of Variance with output from Type III Wald Chi Square tests. Model performance was explored for each dependent variable of interest by comparing residual dispersions, model predictions, AICs and BICs for each of the computed models through the *lme4* 'summary' function output and through residual diagnostics implemented by the package *DHARMa*. Fitting improvements between different hierarchical model structures were tested for significance by using the '*anova'* function.

5.3.3.1 Embryonic deaths and abnormal development

The number of dead embryos whose development was arrested after reaching the eyed stage ('dead embryos at eyed stage'), the number of embryos that died while hatching ('dead embryos at hatching'), and the number of hatched embryos with evident abnormalities were all analysed as proportions on the number of starting eggs for each treatment with the *cbind* function using *glmer*. Models included pre-fertilisation temperatures for sperm and eggs respectively and the embryo development temperature as fixed effects and Family ID (1 to 10) as random effect. To account for our fully factorial design, we added random slopes for our replicate crosses.

5.3.3.2 Hatching rates

Effects of temperature on hatching was analysed using the *glmer* function with binomial error structure (logit link) from the package *lme4*. This model included the proportion of offspring hatched relative to the starting number of eggs (*cbind*) entered as response variable, and time post fertilisation, egg and sperm pre-fertilisation temperature and embryo development temperature and their interactions as fixed effects. Family ID was included as random factor with random slopes.

5.3.3.3 Hatching success

Differences in the total numbers of hatchlings following the thermal treatments, were modelled using *glmmTMB* with binomial error structure (logit link), for the better residual diagnostics that this model showed compared to other generalised linear models. We included the number of successfully hatched embryos on the total number of starting eggs (therefore accounting for the eggs that fail to develop) by using the *cbind* function and checked that the model was not over dispersed. Our fixed factors were egg and sperm pre-fertilisation temperature and embryo development temperature and their interactions. Family ID was included as random factor with randomised slopes.

5.3.3.4 Differences in embryonic age at hatch as a consequence of gamete priming and developmental temperature

Divergence in embryonic age at hatching among the thermal regimes was instead determined using *lmer* using a gaussian family structure. We included egg, sperm and developmental temperature (2 or 8 °C) as fixed factors, their interactions, and family ID as random factor.

5.4 Results

5.4.1 Effects on embryonic age at hatch

Age at hatching was not influenced by gamete pre-fertilisation temperature. Embryo development temperature had the strongest effect with warm temperature resulting in development to be faster by 146 days compared to the cold incubated embryos (mean ± SD warm: 60.5 ± 0.709 days; cold: 173.7 ± 0.710). However, there was a strong significant interaction between the gamete pre-fertilisation temperature and embryo development temperature: when egg pre-fertilisation temperature matched embryo development temperature, larvae started hatching on average 6.15 days earlier. We also found a significant three-way interaction between egg and sperm pre-fertilisation temperature and embryo development temperature; when these matched, age at hatching was a total of 6.60 days earlier (Fig. 5.2, 5.3; Table 5.1).

5.4.2 Proportion of hatchlings born every 12 hrs

Hatching rate was influenced by gamete pre-fertilisation temperature but showed opposite trends for egg and sperm. Hatching rates were on average 18 ± 5.1 % higher in eggs exposed to warm pre-fertilisation temperature but were significantly lower by 11 ± 5.5 % when sperm were exposed to warm temperature. Three-way interactions and post-hoc tests showed that when both eggs and sperm were exposed to warm pre-fertilisation temperatures, hatching rate was 28 ± 11 % lower. Developmental temperature showed again the greater impact with more than double the number of hatchlings recorded every 12 hrs recorded in warm groups as compared to cold groups and explaining a variation through time of 66 ± 5.2 % (Fig 5.4, Table 5.2).

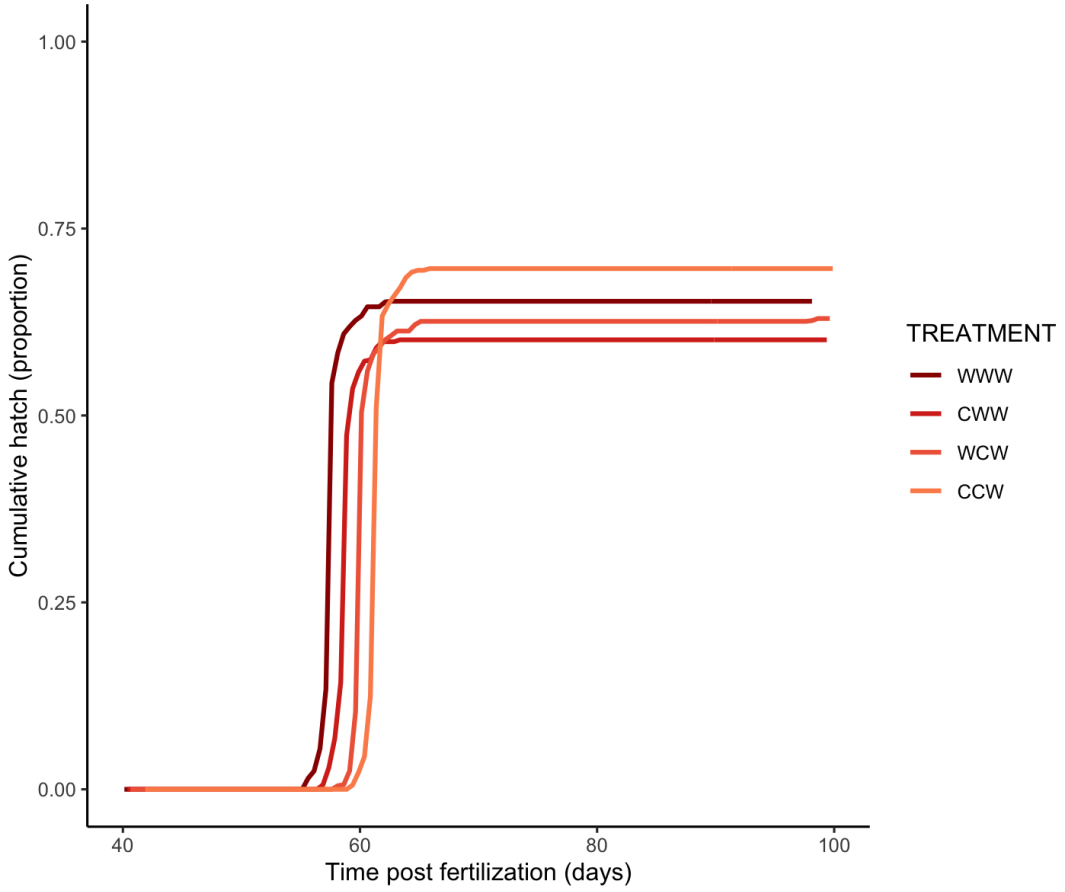

Figure 5.2 *Cumulative proportions of successfully hatched larvae following the different combinations of gamete incubation regimes (cold (C): 2°C; warm (W), 8°C) in batches developing in the warm embryo development treatment (8°C). Letters in the treatment acronyms represent the following order: egg temperature, sperm temperature, embryo development temperature. The results are shown as means ± SD for a total of 80 split-design crosses, with ten males and ten females crossed pairwise.*

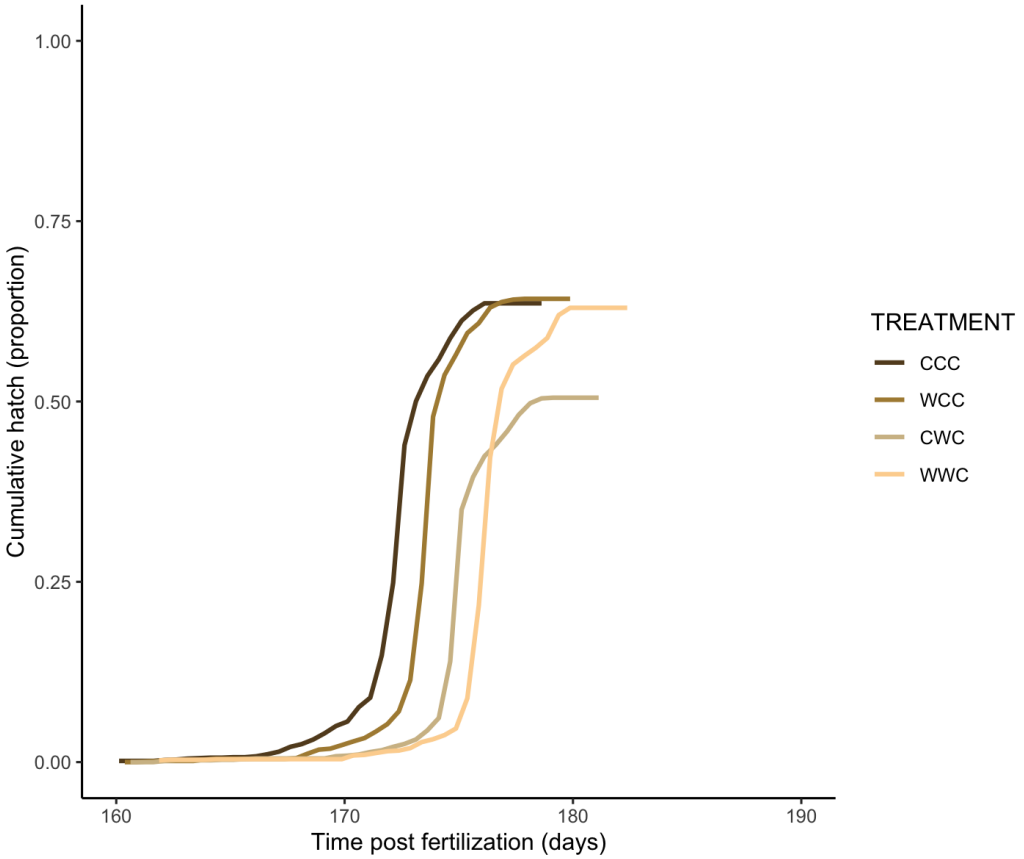

Figure 5.3 *Cumulative proportions of successfully hatched larvae in response to the different combinations of gamete incubation regimes (cold (C): 2°C; warm (W), 8°C) in batches developing in the cold embryo development treatment (2°C). Letters in the treatment acronyms represent the following order: egg temperature, sperm temperature, embryo development temperature. The results are shown as means ± SD for a total of 80 split-design crosses, with ten males and ten females crossed pairwise.*

Table 5.34 *Linear mixed effect model (lmer in R) for age at hatching in response to egg incubation temperature, sperm incubation temperature, and embryo development temperature. The results are shown for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Estimates are provided with standard error (SE) and confidence intervals (CI).*

Variable						
Random					**Variance**	
Family ID					4.89	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**t**	**p**
Intercept	137	0.73	172.20, 175.19	1, 9.78	235.83	**<0.001**
Egg	0.12	0.32	-0.50, 0.76	1, 6134	0.39	0.69
Sperm	-0.12	0.34	-0.80, 0.54	1, 6133	-0.37	0.71
Dev	-146	0.31	-115.24, -134	1, 6131	-360.92	**<0.001**
Egg*Sperm	-0.013	0.4	-0.92, 0.92	1, 6131	-0.003	0.99
Egg*Dev	-6.15	0.49	5.27, 7.03	1, 6133	13.69	**<0.001**
Sperm*Dev	-0.19	0.47	-1.11, 0.72	1, 6132	-0.41	0.67
Egg*Sperm*Dev	-6.60	0.65	-7.43, -4.88	1, 6131	-9.45	**<0.001**

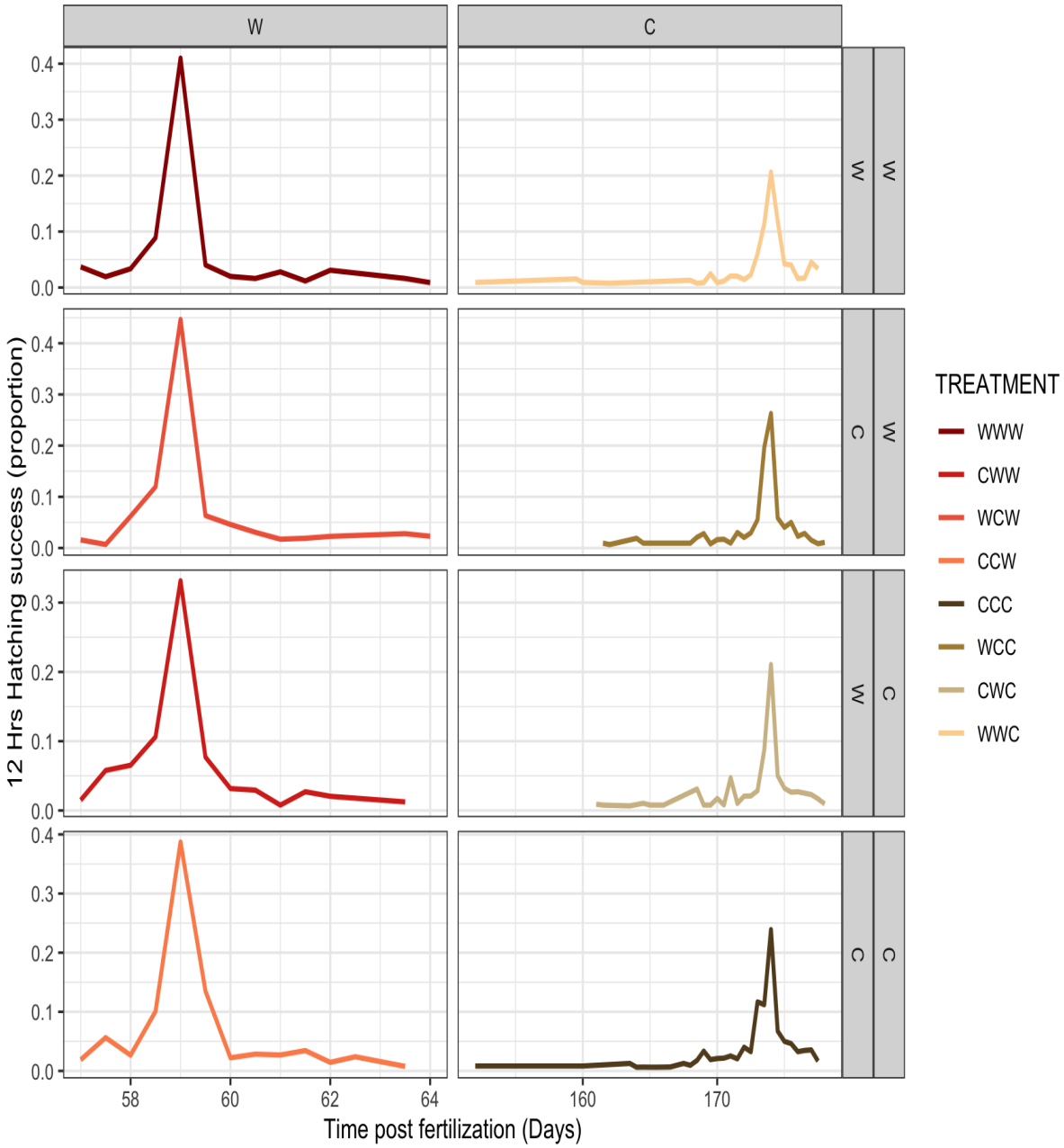

Figure 5.4 *Proportions of hatching rates assessed every 12 hrs under the different thermal regimes (cold (C): 2°C; warm (W): 8°C). Letters in the treatment acronyms are ordered as follows: egg temperature, sperm temperature, embryo development temperature. The results are shown as means ± SD for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Helpful to add symbols of eggs and sperm or more detailed descriptions in the captions.*

Table 5.35 *Generalised linear mixed effect model (glmer in R) for hatching rate in response to pre-fertilisation temperature for eggs, sperm, and embryo development temperatures (2 or 8 °C). The results are shown for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI), and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Family ID					0.08	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-2.96	0.09	-3.17, -2.76	1, 9.78	-30.28	**<0.001**
Egg	0.18	0.05	0.08, 0.29	1, 6134	3.62	**<0.001**
Sperm	-0.11	0.06	-0.23, -0.01	1, 6133	-2.13	**0.032**
Dev	0.67	0.05	0.56, 0.77	1, 6131	-12.67	**<0.001**
Egg*Sperm	0.04	0.07	-0.10, 0.19	1, 6131	0.55	0.58
Egg*Dev	-0.07	0.07	-0.21, 0.07	1, 6133	-0.91	0.36
Sperm*Dev	0.04	0.07	-0.11, 0.19	1, 6132	0.55	0.57
Egg*Sperm*Dev	-0.28	0.10	-0.49, -0.08	1, 6131	0.10	**0.007**

5.4.3 Hatching success

Total hatching success showed opposite trends in response to pre-fertilisation temperature for sperm and eggs, with warm-incubated sperm siring significantly lower numbers than warm-incubated eggs (a reduction in 20 ± 6.8 % and an increase in 14 ± 6.6 % respectively). We found no significant interaction between sperm and egg pre-fertilisation temperature, but we found a negative effect when egg pre-fertilisation temperature and embryo development temperature were 8°C (Fig. 5.5, Table 5.3).

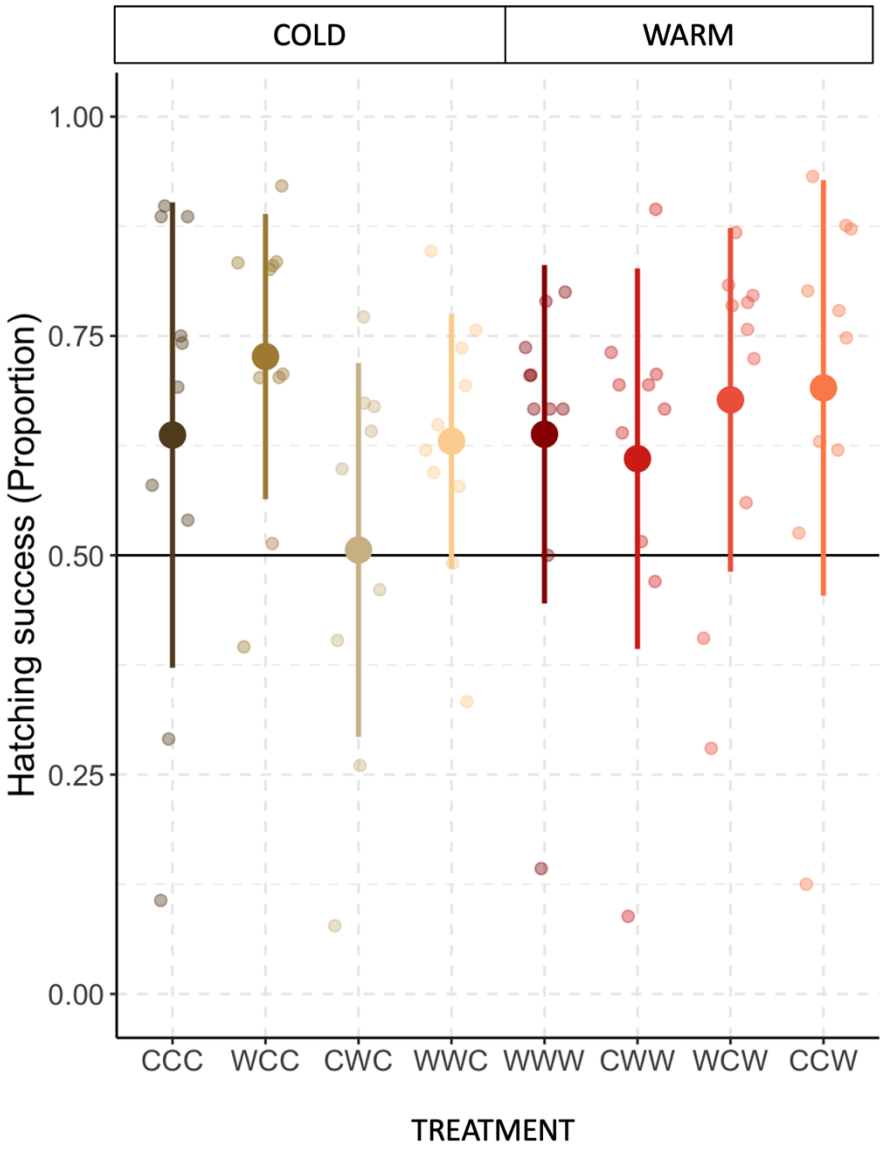

Figure 5.5 *Proportion of successfully hatched embryos in response to the different thermal regimes at the pre- and post-fertilisation stages (cold (C), 2°C or warm (W), 8°C). Letters in the treatment acronyms are ordered as follows: egg temperature, sperm temperature, embryo development temperature. The data are shown as mean ± SE for a total of 80 split-design crosses, with ten males and ten females crossed pairwise.*

Table 5.36 *Generalised linear model (glmer in R) for the proportion of successfully hatched embryos in response to pre-fertilisation temperature for eggs, sperm, and embryo development temperatures (2 or 8 °C). The results are shown for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI), and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Family ID					0.08	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-0.52	0.13	-0.79, -0.23	1, 9	-3.96	**<0.001**
Egg	0.14	0.06	0.01, 0.27	1, 70	2.11	**0.004**
Sperm	-0.20	0.06	-0.33, -0.07	1, 70	-2.94	**<0.001**
Dev	0.08	0.06	-0.04, 0.21	1, 70	1.29	0.15
Egg*Sperm	0.07	0.09	-0.12, 0.26	1, 70	0.74	0.32
Egg*Dev	-0.15	0.09	-0.34, 0.03	1, 70	-1.68	0.009
Sperm*Dev	0.08	0.09	-0.10, 0.27	1, 70	0.90	0.22
Egg*Sperm *Dev	-0.01	0.13	-0.27, 0.24	1, 70	0.07	0.94

5.4.4 Effect on embryonic deaths and developmental abnormalities

The proportion of embryos that died after reaching the eyed stage was significantly lower in the cold embryo development treatment than in the warm treatment. In the warm gamete pre-fertilisation temperature, the number of embryos dying after the eyed stage tended to be lower (non-significant). We found no effects of gamete pre-fertilisation temperature on embryo survival after the eyed stage (Fig 4.5, Tab 4.5) Embryos dying during the hatching process as well as embryos that hatched but showed clear signs of abnormal development were instead only affected by embryo development temperature and not by the pre-fertilisation gamete temperatures and only in cold-developed groups (Figs 4.6 and 4.7; Tabs 4.6 and 4.7).

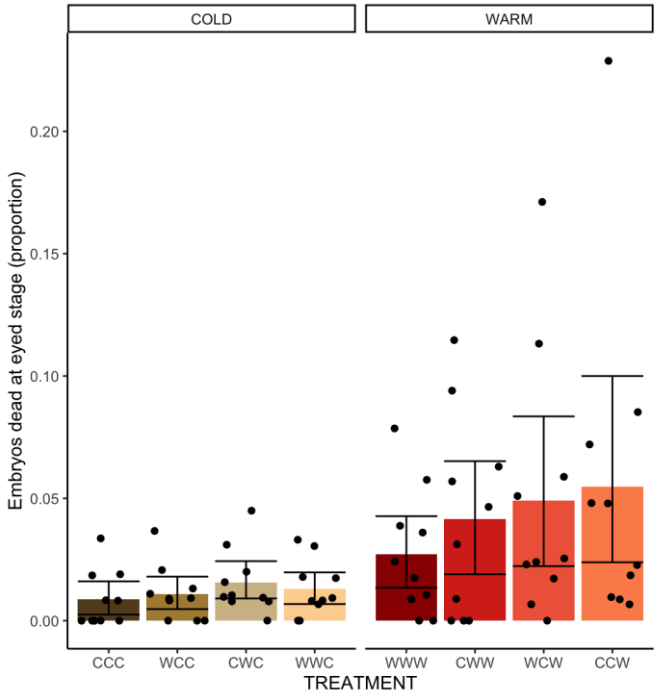

Figure 3 *Proportion of dead embryos after reaching the eyed stage in response to the different thermal regimes at the pre- and post-fertilisation stages (cold (C), 2°C or warm (W), 8°C). Letters in the treatment acronyms are shown in the following order: egg temperature, sperm temperature and embryo development temperature. The results are shown as means ± SD for a total of 80 split-design crosses, with ten males and ten females crossed pairwise.*

Table 5.37 *Generalised linear model (glmer in R)) for the proportion of embryos dead after reaching the eyed stage in response pre-fertilisation temperature for eggs and sperm, and embryo development temperatures (2 or 8°C). The results are shown for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI), and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Family ID					0.65	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	-5.01	0.40	-5.85, -4.23	1,49.57	-12.55	**<0.001**
Egg	0.19	0.41	-0.62, 1.02	1, 63	0.47	0.64
Sperm	0.53	0.38	-0.21, 1.31	1, 63	1.38	0.17
Dev	1.80	0.33	1.19, 2.50	1, 63	5.05	**<0.001**
Egg*Sperm	-0.29	0.53	-1.36, 0.76	1, 63	-0.55	0.58
Egg*Dev	-0.32	0.49	-1.23, 0.56	1, 63	-0.717	0.47
Sperm*Dev	-0.77	0.43	-1.63 0.065	1, 63	-1.793	0.073
Egg*Sperm*Dev	-0.0045	0.60	-1.20, 1.20	1, 63	0.61	0.99

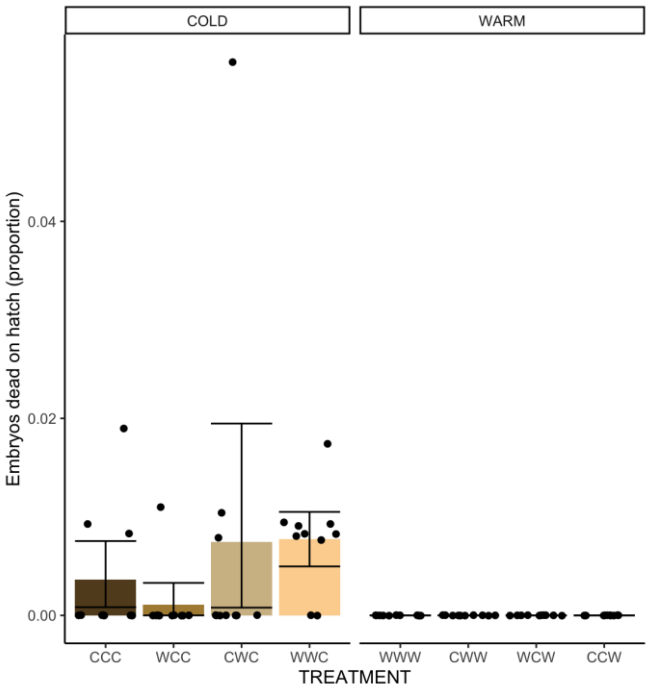

Figure 4 *Proportion of embryos dead during hatch in response the different thermal regimes (cold (C), 2°C or warm (W), 8°C Letters in the treatment acronyms are shown in the following order: egg temperature, sperm temperature and embryo development temperature. The results are shown as means ± SD for a total of 80 split-design crosses, with ten males and ten females crossed pairwise.*

Table 5.38 *Generalised linear mixed effect model (glmer in R) for the proportion of embryos dead during hatch in response pre-fertilisation temperature for eggs and sperm, and embryo development temperature (2 or 8 °C). The results are shown for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI), and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Family ID					0.00076	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	0.0036	0.0022	-0,00048, 0.0077	1, 71.75	1.66	0.078
Egg	-0.0026	0.0030	-0.00084, 0.0032	1, 63	-0.83	0.71
Sperm	0.0038	0.0031	-0.0020, 0.001	1, 63	1.23	0.095
Dev	-0.0037	0.0031	-0.0094, 0.0021	1, 63	-1.19	**0.0019**
Egg*Sperm	0.0029	0.0043	-0.0053, 0.011	1, 63	0.65	0.64
Egg*Dev	0.0026	0.0043	-0.0056, 0.011	1, 63	0.59	0.71
Sperm*Dev	-0.0038	0.0043	-0.012 0.0044	1, 63	-0.87	0.095
Egg*Sperm *Dev	-0.00029	0.0062	-0.012, 0.0090	1, 63	-0.46	0.65

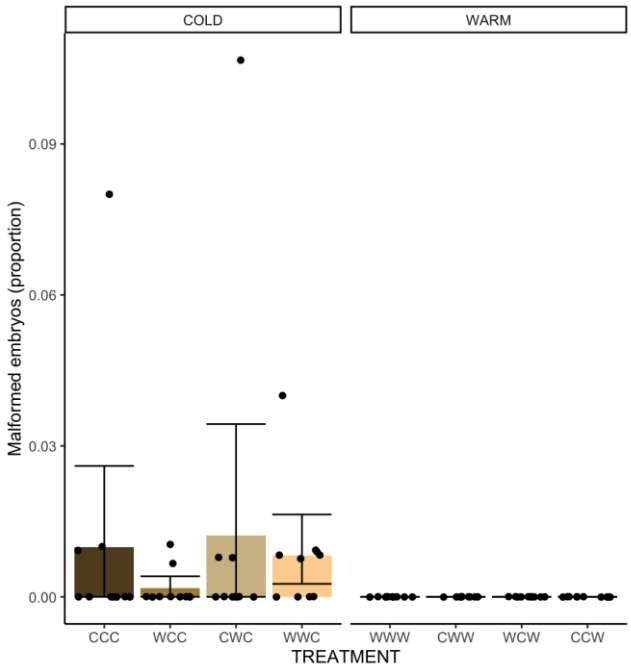

Figure 5 *Proportion of malformed embryos in response to the different thermal regimes at the pre- and post-fertilisation stages (cold (C), 2°C or warm (W), 8 °C). Letters in the treatment acronyms are shown in the following order: egg temperature, sperm temperature and embryo development temperature. The results are shown as means ± SD for a total of 80 split-design crosses, with ten males and ten females crossed pairwise.*

Table 5.39 *Generalised linear mixed effect model (glmer in R)) output for the proportion of malformed embryos hatched in response to pre-fertilisation temperature for eggs and sperm, and embryo development temperature (2 or 8 °C). The results are shown for a total of 80 split-design crosses, with ten males and ten females crossed pairwise. Estimates are provided with standard error (SE), confidence intervals (CI), and degrees of freedom (df1= k -1 and df2= n_{tot} – k, where k is the number of treatment levels and n_{tot} is the total number of observations).*

Variable						
Random					**Variance**	
Family ID					0.005	
Fixed	**Estimate**	**SE**	**CI**	**df1, df2**	**z**	**p**
Intercept	0.009	0.004	0.003, 0.013	1, 51.69	2.04	**0.047**
Egg	-0.008	0.005	-0.019, 0.0031	1, 63	-1.371	0.31
Sperm	0.002	0.006	-0.009, 0.0136	1, 63	0.384	0.46
Dev	-0.01	0.006	-0.021, 0.0014	1, 63	-1.66	**0.009**
Egg*Sperm	0.004	0.004	-0.011, 0.02	1, 63	0.50	0.72
Egg*Dev	0.008	0.008	-0.0078, 0.024	1, 63	0.97	0.31
Sperm*Dev	-0.002	0.008	-0.018 0.013	1, 63	0.27	0.46
Egg*Sperm*Dev	-0.004	0.011	-0.027, 0.018	1, 63	-0.35	0.72

5.5 Discussion

Our study provides clear evidence that in an externally fertilising species, the thermal environment experienced by gametes pre-fertilisation can influence reproductive fitness. Interestingly, the effect of the temperature experienced by gametes prior to fertilisation was opposite in sperm and eggs: sperm function was negatively affected by warm temperatures whereas eggs were negatively affected by cold temperature. In addition, we found that embryo development temperature was the main driver of differences in survival, age at hatching and developmental abnormalities, but it did not affect total number of offspring. Interestingly, the matching of gamete incubation temperature with embryo development temperature did not improve overall hatching success and again sperm and eggs showed opposite trends. Our findings contribute to the evidence that in ectotherms, where the environmental temperature directly determines body temperature, thermal fluctuations affect gamete function (Gasparini et al., 2018; Pinsky et al., 2019; Adriaenssens et al., 2012; Walsh et al., 2019). Such effects can occur before gametes are released into the environment e.g., during gonad development and gametogenesis or later e.g., during spawning and fertilisation (Crean & Immler, 2021). Externally fertilising and stenothermal organisms like salmon are recognised to be particularly vulnerable to environmental fluctuations because of their incapability to keep gametes at a roughly constant temperature and because of their narrow thermal tolerance range (Albright & Mason, 2013; Keshavmurthy et al., 2014). Environmentally induced modifications in gametes during gametogenesis have been intensively studied over the last decade, and mechanisms including thermally induced epigenetic changes or phenotypic selection within and among ejaculates have been documented (Crean & Immler, 2021; Lempradl, 2020; Yamada & Chong, 2017). However, the evolutionary consequences of gametes being exposed to variable environmental conditions including temperature are still largely unexplored (Crean & Immler, 2021). It is also unclear how the two sexes are affected by varying environments and how the sex-specific effects interact. We discuss our results and possible mechanisms that may explain the observed effects below.

5.5.1 Temperature effects on sperm and eggs

Sperm traits such as phenotype, swimming behaviour, longevity, and motility can be affected by environmental temperature (Dunn et al., 2012; Falkenberg et al., 2019; Fenkes et al., 2017; Läinen et al., 2018). In our study, sperm performance was negatively affected by warmer temperatures, and this translated into lower hatching success. These temperature effects on sperm can be explained by a series of non-mutually exclusive mechanisms. Sperm selection in response to varying environments could act in two ways: First, a specific gamete cohort could be selected that is particularly adapted to specific environmental conditions (Alavioon et al., 2017) and second, environmental conditions can hamper the biochemical and biomolecular pathways of gamete function with consequences for reproduction (also reviewed by Crean & Immler, 2021). The warmer temperature of 8°C could increase ROS production in sperm (Dadras et al., 2017), and their impact is known to reduce membrane fluidity, mitochondrial function, enzymatic activity and DNA integrity (Dadras et al., 2016; Menezo et al., 2016). Similar changes have been shown in inactivated sperm in the closely related brown trout *Salmo trutta* (Lahnsteiner & Mansour, 2012). Males in many taxa have been shown to vary sperm motility and phenotype in response to varying environmental conditions (Crean et al., 2013; Reinhardt et al., 2015; Zajitschek et al., 2014). Such changes can be induced by a variety of abiotic environmental factors such as pH, ionic content and temperature (Alavi & Cosson, 2005; 2006). Similar to sperm, egg quality is affected by environmental temperature and may cause variation in traits including size and biochemical composition of the yolk (Foo & Byrne, 2017; Vasudeva et al., 2019).

A particularly interesting finding was the opposite effect of temperature on sperm and eggs with eggs performing better at 8°C and sperm performing better at 4°C. This finding suggests that a global increase in temperature may be particularly important for male fertility, and the resulting offspring. Such a differences between males and females may be particularly important as salmon populations are characterised by a male biased sex-ratio with more males competing for a smaller pool of females (Fleming, 1996). Therefore, it is possible that warming-related effects could lower sperm competition dynamics and alter mate choice even more in an already threatened species. Post-release environmental temperature could also affect gamete function and phenotypic plasticity, as well as the interaction between male and

female haplotypes in response to environmental fluctuations. However, we found no interaction effects between gamete pre-fertilisation temperature and developmental environment that would support this idea (see also next section).

5.5.2 Gamete effects on offspring fitness

Pre-fertilisation temperature variation did not only affect gamete performance but also offspring fitness. Therefore, the pre-fertilisation thermal environment could have shaped offspring fitness via epigenetic factors triggered by such physiological changes (Danchin et al., 2011; Jenkins & Carrell, 2012). Several epigenetic markers such as DNA methylation, histone modifications, and modifications of cytoplasmic and nuclear proteins could be responsible for the variation in embryonic age at hatching and hatching rates through time (Castillo et al., 2014; Danchin, 2013). Interestingly, high ROS levels experienced by sperm have been linked to altered methylation patterns of the haploid DNA of sperm, as well as its lipidic and preotic content (Lane et al., 2014; Menezo et al., 2016). In mice, elevated ROS levels in sperm similarly impaired embryo development, without affecting affect sperm motility and fertilisation potential (Lane et al., 2014). Furthermore, temperature can affect several components of the seminal fluid including peptides, RNAs, enzymes and hormones which are known to influence offspring development (Chen et al., 2016; Crean et al., 2013, 2016; Crean & Bonduriansky, 2014). Additionally, changes in the composition of reproductive fluids can in turn affect gamete composition and the resulting offspring fitness (Immler, 2018). Such effects on sperm or seminal fluid components could have been altered in response to the thermal treatments performed in our experiment, leading to the observed lower hatching success observed among warm-exposed sperm groups. In the whitefish *Coregonus lavaretus*, offspring sired by sperm that had been exposed to 6.5°C pre-fertilisation exhibited poorer swimming performance and were smaller than their siblings sired by sperm kept at 3.5 °C (Läinen et al., 2018). In this study, no differences in sperm performance were observed between the two temperature regimes, nor were any effects on hatching success observed. One possible explanation for the differences between the Coregonus study and our own study on Atlantic salmon could be the smaller temperature range used in the former which may be less stressful for sperm than our treatments. In addition, these temperatures and their effects are likely to be highly species specific.

Interestingly, survival and normal development of embryos was mainly affected by embryo development temperature whereas pre-fertilisation gamete incubation temperature only showed an effect in interaction with the embryo development temperature. The number of dead embryos at the eyed stage was significantly higher in the warm-development temperature treatments and tended to be lower in groups where sperm had been exposed to warm pre-fertilisation temperature. Surprisingly, the number of hatched malformed embryos and that of offspring which died during hatching were observed exclusively in cold-developed groups, suggesting a different purging mechanism toward sub-optimal phenotypes operated by cold developmental temperatures. Within these treatments the number of abnormal embryos was generally higher when sperm were exposed to warm temperatures, although not significantly, thus arguably pointing out that the detrimental effects of warm pre-fertilisation incubation temperature on sperm might have exacerbated abnormal selection at fertilisation and/or developmental disruption during zygote formation. These observations suggest that the effects lowering hatching success likely occurred before or during fertilisation, or during early development stages rather than later in development. It is also possible that the strength of all these above-mentioned changes could have been stronger if we had used a larger number of mating pairs, thus augmenting the statistical power. The temperature we used in our experiment during fertilisation was intermediate and standardised across all treatments and we cannot exclude that the abrupt change in temperature at fertilisation may differentially affect some sperm cohorts leading to intra-ejaculate selection.

In crosses where egg and sperm pre-fertilisation temperatures and embryo development temperature were set to warm (8°C), offspring hatched on average almost a week earlier. This could be consistent with the idea of a gamete-driven adaptive response or with a physiological optimization (Angilletta et al., 2002). In salmon, the timing of hatching and larval emergence are tightly correlated (Beacham et al., 2012; Beacham & Murray, 2011; Berg & Moen, 1999). Early emerging alevins can acquire competitive advantage due to prior residency and show better growth and survival in the wild (Cutts et al., 1999). Consequently, the delayed hatching observed in the treatments were sperm and eggs did not match the embryo development temperature could pose a threat to offspring survival in warmer environments (e.g., sudden heatwave). This may be particularly important where rapid fluctuations in temperature occur such as those characteristic of man-induced climate changes over the last two decades (di Cecco & Gouhier, 2018). Thermally sensitive and cold-adapted

species like salmonids likely may not benefit from mechanisms that fully balance the overall loss in reproductive outcomes driven by higher temperatures, and therefore furnish an advantage to their offspring under environmental fluctuations. In the brook trout *Salvelinus fontinalis*, maternal and paternal temperatures modulate the methylation patterns in offspring which may drive their adaptation to predictable environments (Venney et al., 2022). Although in this study both females and males were exposed to cool and warm temperatures prior to fertilisation, and their offspring was split and developed in both environments, development temperature had no effect on methylation. This could be in line with our finding of no effect of embryo development temperature on hatching success. It appears that the conditions during gametogenesis play a key role. Heat shock proteins are continuously expressed during spermatogenesis and are known to play a pivotal role in sperm development. It has in fact been proposed that adaptations to different thermal environments could be adaptively modulated during gonadal development so that sperm function can better withstand future challenges and be effective at reproduction in a warmer environment (Dunn et al., 2012). The fact that we detected such changes when exposing gametes alone prior to their activation long after spermatogenesis has ended suggests that such processes may not be strictly parentally transmitted adaptations, but that that gametes alone could be involved.

5.5.3 Conclusion

In conclusion, our results highlight opposite effects to thermal regimes on eggs and sperm, where warm was overall beneficial for eggs but detrimental for sperm. These specific disadvantageous environments experienced by gametes hampered hatching success and the total number of offspring. The effects were predominantly influenced by the conditions experienced pre-fertilisation and priming of both gametes to future temperatures can benefit the offspring. Our findings revealed a switch in hatching time but not overall reproductive output as a consequence of gamete acclimation. This finding suggests that populations might not be able to successfully buffer the deleterious effects of unpredictable and significant temperature fluctuations on gametes. However, pre-fertilisation thermal regimes for gametes could be differentially applied to eggs and sperm in the aquaculture sector, to optimise fertilitisation rates, hatching success, timing and synchrony. Moreover, our results highlight that salmon males could be the most affected under climatic fluctuations. Future studies should focus on genetic and epigenetic changes in gametes and how these affect offspring fitness to better understand how populations could cope with climate changes

6 Frequency-dependent viscosity of salmon ovarian fluid has biophysical implications for sperm-egg interactions

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Statement of contributions: A. Yethiraj and C.Purchase provided fundings, operational facility and instrumentations. C.Purchase, M.Graziano, R. Carrow and S. Poulos collected the fish and gamets including the ovarian fluid. M. Graziano conceptualised the work. S. Palit trained M.Graziano for the rheology measurements. M. Graziano conducted all the rheology experiments. M. Graziano extracted, curated and analysed the data. Technical support in the soft matter lab was kindly given by T. Isawa and S. Palit M. Graziano created all the tabs, figures and illustrations. M. Graziano wrote the first complete draft of this work and all the following versions after receiving the review comments and suggestions from C. Purchase and A. Yethiraj and from the Supervisors M. Gage and S. Immler.

6.1 Abstract

Gamete-level sexual selection of externally fertilising species is usually achieved by modifying sperm behaviour with mechanisms thought to alter the chemical environment in which gametes perform. In fish this can be accomplished through the ovarian fluid, a substance released with the eggs at spawning. While its biochemical effects in relation to sperm energetics have been investigated, the influence of the physical environment in which sperm compete remains poorly explored. Our objective was therefore to gain insights on the physical structure of this fluid and potential impacts on reproduction. Using soft-matter physics approaches of steady-state and oscillatory viscosity measurements, we subjected salmon ovarian fluids to variable shear stresses and frequencies resembling those exerted by sperm swimming through the fluid near eggs. We show that this fluid, which in its relaxed state is a gel-like substance, displays a non-Newtonian viscoelastic and shear-thinning profile, where the viscosity decreases with increasing shear rates. We concurrently find that this fluid obeys the Cox-Merz rule below 7.6 Hz and infringes it above, thus indicating a shear-thickening phase where viscosity increases provided it is probed gently enough. This suggests the presence of a unique frequency-dependant structural network with relevant implications on sperm energetics and fertilisation dynamics.

6.2 Introduction

The micro-conditions of fertilization are poorly understood in the majority of animal species (Cosson, 2015; Eisenbach & Giojalas, 2006; Kholodnyy et al., 2019). Following ejaculation, sperm find and fertilise eggs, but this usually takes place in the presence of post-mating sexual selection arising from sperm competition with rival males (Birkhead & Pizzari, 2002; Parker, 2020), and cryptic female choice that biases paternity (Firman et al., 2017). We now know that polyandry (female mating with multiple males in a given breeding episode) is widespread and common in nature (Taylor et al., 2014), and that post-mating sexual selection

plays a crucial role in governing reproductive fitness (Simmons, 2005). It is likely to be responsible for the tremendous diversity in sperm morphology (Ramón et al., 2014; Pitnick, Hoksen and & Birkhead, 2008) and female reproductive tract morphological complexity (Kelly & Moore, 2016; Sloan & Simmons, 2019). Although many studies have revealed the importance of post-mating sexual selection for dictating variance in individual fertilization success (Gasparini & Pilastro, 2011; Kekalainen & Evans, 2018; Lüpold et al., 2012), we still understand little about the exact mechanisms that control the outcome of post-mating sexual selection and, ultimately, individual fertilisation success (Birkhead & Pizzari, 2002).

In terms of female control over paternity, internal fertilisation clearly offers greater direct opportunity to manage sperm and the fertilisation process, compared with external fertilisation. In internal fertilisers, sperm are deposited within the female reproductive tract, and then move from the insemination site either directly towards the egg for fertilisation, or indirectly via short- or long-term storage. Sperm can move under their own flagellar propulsion, or be moved by female tract mechanisms, but we rarely understand which sex is controlling sperm dispersal, and how, where and when this occurs through the whole process. Several female mechanisms could control sperm transfer, progress and activity; from mechanical contractions and hydrostatic pressures in the female tract, to sorting sperm from different males in designated organs, and through completely ejecting ejaculates or exerting spermicidal actions (Firman et al., 2017). Biochemical complexity in which these dynamics take place is also important, with evidence that the female tract can be either supportive or, at times, hostile to certain male gametes (Firman et al., 2017; Wolfner, 2011). Ostensibly, much remains to be discovered about this reproductive diversity, with recent *in vivo* research using GFP-tagged sperm revealing high levels of activity and interaction between sperm from different males and different areas of the female tract (Manier, Belote, et al., 2013; Manier, Lüpold, et al., 2013).

External fertilization, in which gametes fuse outside the body in an aqueous environment, appears to present far fewer opportunities for females to exert post-mating control over fertilisation. Interactions between gametes cannot benefit from a complex reproductive tract with opportunities for differential sperm uptake, storage, and management. However, despite its increased reproductive ‘simplicity’, studies have shown that external fertilization can indeed allow cryptic female choice via adaptations that encourage the ‘right’ sperm, or discourage the ‘wrong’ sperm, to fertilise (Firman et al., 2017). For example,

gamete recognition systems in or on the egg, and reproductive fluids that are released with the eggs, are known to influence sperm behaviour and fertilisation outcome (Evans et al., 2013; Yeates et al., 2013). It is the relative simplicity of these systems compared to internal fertilisers, and the tractability of external fertilisation for controlled *in vitro* fertilization experiments, that have enabled significant advances in understanding the outcomes and potential mechanisms that control sperm-egg interactions in the context of post-mating selection from sperm competition and cryptic female choice.

Some of our most fundamental knowledge about sperm-egg interactions comes from broadcast-spawning marine invertebrates. The associations between bindin molecules (Palumbi, 1999), and between lysin and its vitelline envelope receptor (VERL) (Swanson & Vacquier, 1997), have been described in detail in sea urchin and abalone (*Haliotis)* respectively, where biochemical mechanisms control against the risk of heterospecific sperm attachment or egg membrane penetration (Metz et al., 1994; Palumbi, 1999), influencing individual fertilisation success (Hussain et al., 2016). Similarly, more recent work has described the mechanisms by which female-derived chemoattractants within egg-associated reproductive fluids mediate post-mating mate choice, fertilization success and offspring fitness in mussels (Fitzpatrick et al., 2012; Oliver & Evans, 2014). In fish, females manufacture ovarian fluid, which is released into the coelomic cavity with maturing eggs (Hirano et al., 1978). It contains a complexity of nutrients, metabolites and hormones (Hirano et al., 1978; Ingermann et al., 2001; Lahnsteiner et al., n.d.), and once spawned shows the highest concentration in proximity to the micropyle entrance of eggs. Ovarian fluid identity of different females has been found to differentially impact sperm swimming behaviour and influence fertilisation outcome according to the genetic relatedness of males (Butts et al., 2012; Gasparini & Pilastro, 2011) and their spawning origin (Beirão et al., 2014). In salmonids, ovarian fluid comprises up to 30% of the spawned egg mass, and its influence on sperm is relatively well studied (Galvano et al., 2013; S. L. Johnson et al., 2020; Purchase & Rooke, 2020; Turner & Montgomerie, 2002a; Zadmajid, Myers, Sørensen, Butts, et al., 2019). There is increasing evidence that this reproductive fluid can act as a ‘fertilisation filter’ for or against sperm from different partners, enabling cryptic female choice. This facilitates sperm selection even in highly polyandrous externally fertilisers like Atlantic salmon (*Salmo salar*), where a single egg batch can be sired by up to 16 fathers (Weir et al., 2010a). Yeates et al. (Yeates et al., 2013) showed that ovarian fluid allowed females to apply conspecific sperm precedence when facing *in vitro* hybridization risks between Atlantic

salmon and brown trout (*Salmo trutta).* However, we do not yet know the exact mechanisms facilitating such choice.

Sperm swimming propulsion is created by the flagellum, whose function is influenced by chemical (Cosson, 2015; Kholodnyy et al., 2019) and physical (Cosson, 2015; Cosson & Prokopchuk, 2014; Holwill, 1977) conditions. The different responses of sperm behaviour reported in presence of ovarian fluid, and their resulting effects on fertilization (Alonzo et al., 2016; Galvano et al., 2013; Gasparini et al., 2012; Rosengrave, Taylor, et al., 2009a), have been associated to changes in pH (Wojtczak et al., 2007), ionic composition (Rosengrave, Taylor, et al., 2009b), and viscosity (Turner & Montgomerie, 2002a) that control flagellar beating (Kholodnyy et al., 2019). While the effects of chemistry (Rosengrave, Taylor, et al., 2009; Wojtczak et al., 2007) and temperature (Dadras, Dzyuba, et al., 2017; Dadras et al., 2016) have been more frequently investigated (Cosson, 2015; Dadras et al., 2016; Kholodnyy et al., 2019), the influence of changes in viscosity on swimming sperm remain poorly explored in external fertilisers (Kholodnyy et al., 2019; Lauga, 2007). There is evidence that fish ovarian fluid possesses structural properties that makes for a non-Newtonian viscous response (where viscosity changes depending on the force applied) that is very different to water (Rosengrave, Taylor, et al., 2009), and this peculiar viscous response could influence the biophysics of sperm swimming behaviour in external fertilisation environments. To describe such function, we conducted detailed measurements of its biophysical characteristics using a rheological approach commonly used in soft-matter physics. We sought to uncover the rheological nature of ovarian fluid when different forces are applied to it, thus exploring how its non-Newtonian behaviour could affect sperm activity, penetration, bioenergetics, and guidance to fertilisation in a context of sperm competition and cryptic female choice.

6.3 Materials and methods

6.3.1 Sample collection and preliminary measurements

Wild anadromous Atlantic salmon were collected in early September from a fish ladder at Grand Falls (48° 55' N, -55° 39' W) during their up-stream spawning migration on the Exploits River (Newfoundland, Canada). Following previous protocols (Rooke et al., 2019), fish were transferred to covered, outdoor tanks next to the river, and experienced ambient temperatures and light. Over two weeks in early November, females were assessed for ovulation using gentle abdominal pressure, fish were then anaesthetised using a solution of 2ml/L clove oil, measured for length and weight, and stripped of eggs after drying the urogenital pore. Each female's eggs (and associated ovarian fluid) were kept in sealed glass jars, enclosed with bubble wrap, and placed in a cooler of wet ice for transport to the laboratory. Each egg batch was separated from its ovarian fluid using a fine mesh net (Purchase & Rooke, 2020) within 10 hours of stripping. For each ovarian fluid we recorded volume and weight to deduce density, followed by pH and conductivity.

6.3.2 Rheological characterization of ovarian fluid

The mechanical properties of many soft biological materials are neither purely viscous (liquid-like) nor purely elastic (solid-like), and these rheological properties correlate strongly with their function (Storm et al., 2005). Structured fluids often do not flow until they reach a critical stress level, below which a material is considerable elastic, and above which the structure of the material breaks down and starts to flow. Two experiments were performed to define how the ovarian fluid's polymeric structure (and related physical properties that in turn would affect sperm swimming activity) can be modulated, depending on swimming sperm flagellar beat frequency. Specifically, we tested ovarian fluid 'behaviour', both under steady shear (i.e., "flow curves") and under small-amplitude oscillatory shear (SAOS). The former examines the viscoelastic response of the ovarian fluid by continuous deformation and breakup of internal networks, while the latter can probe

weaker internal structures (Ferry, 1980; Pearson, 1978). A preliminary rheological analysis (n= 5 fish) was conducted to assess different fluid preservation methods (see supplementary material). Each frozen sample was thawed at room temperature for 1hr prior to analysis, and measurements were made using 1.5 mL aliquots. All the analyses were performed in the Soft Matter Lab at Memorial University using an MCR 301 rheometer, equipped with a cone-plate (CP50-0.5, 50 mm diameter plate and cone angle, Anton Paar GmbH, St. Albans, UK) system. Ovarian fluid samples were individually filtered through a 200 μm sieve to remove any particulates (e.g., coagulated blood, ovarian tissue) that could influence the rheological measurements. Pipetted fluid was equilibrated for three minutes at the plate temperature of 6^{o}C, allowing for homogenous sample relaxation from any uncontrolled pre-shear imposed on the fluid during loading.

6.3.3 Steady-state shear properties

Samples were tested for their resistance to flow in order to measure their viscosity under a specific rate of deformation. To obtain a flow curve, the shear stress was measured for a range of shear rates ($\dot{\gamma}$), from 10 to 500 s^{-1} in 50 equally spaced steps. The resultant shear stresses of the ovarian fluid were measured to determine the *apparent* viscosity η_a, which was averaged across three aliquots per female (n= 11) and plotted as a function of the shear rate.

Among each of the three ovarian fluid aliquots per fish, a run with distilled water was performed as a control. For distilled water (pure Newtonian fluid), a theoretical positive relationship between shear stress and shear rate should be linear and the fit line should pass through zero. When the profiles of water runs were fitted, a positive intercept (typical for these kind of measurements) of 0.0133 Pa was concluded to be low-shear rate instrumental noise. It subtracted from all the water and ovarian fluid samples as standardization (shear stress - 0.0133 Pa)/(shear rate), creating a small change in values. A comparison of individual ovarian fluid viscosity profiles with distilled water for each of the instrumental replicates allowed us to assess variability among females.

The apparent viscosity of ovarian fluid decreased with increasing shear rates, in contrast with water whose apparent viscosity (η_a= 0.00151 ± 0.00003 Pa·s) was independent of shear rate. The apparent viscosity at $\dot{\gamma} = 10\ s^{-1}$ was roughly 10 times the viscosity of water

but returned comparable at 100 s^{-1} (see Results). For three females the ovarian fluid samples had apparent viscosities η_a in the order of 0.003 Pa·s at 10 s^{-1}, showing no meaningful differences with the rheological behaviour of water at the same shear rate. Likely, these samples were contaminated by urine and/or water during stripping of gametes and for these reasons were not included in the main results. The remaining 11 flow curves were globally fitted to the form $\eta_a = \frac{\sigma_{00}}{\dot{\gamma}} + \eta_\infty$, which is a simple equation incorporating an elastic component, the yield stress σ_{00} which must be overcome before there is flow, and a viscous component η_∞, which represents the viscosity at very high shear rates. This simple form was arrived at when fits to a more complicated formula, the Herschel-Bulkley equation $\eta = \frac{\sigma_{00}}{\dot{\gamma}} + \eta_\infty(\dot{\gamma})^{n-1}$ (Herschel, 1926) resulted in power laws *n* that were very close to unity.

6.3.4 Small Amplitude Oscillatory Sweeps

To preserve finer polymeric structures and obtain a dynamic profile that informs about the viscous and elastic components, we subjected the ovarian fluid to small-amplitude oscillatory shear. For these measurements, a sinusoidal deformation ($\gamma = \gamma_0 \sin \omega t$) was imposed on the sample at a fixed frequency ω and a maximum amplitude (γ_0) (Schoff & Kamarchik, 2005). Measurements were performed for a range of frequencies (ω), from 0.01 to 500 rad· s^{-1} in 24 equally spaced logarithmic increments. The storage modulus,

$$G'(\omega) = (\sigma_0 \,/\, \gamma_0) \cos \delta, \quad (1)$$

and the loss modulus,

$$G''(\omega) = (\sigma_0 \,/\, \gamma_0) \sin \delta, \quad (2)$$

were obtained as a function of frequency (ω). The modulus of the complex viscosity η* was obtained from the relation

$$|\eta^*| \equiv [(G')^2 + (G'')^2]^{1/2}/\omega, \quad (3)$$

while the damping factor (or loss factor) tan δ ≡ G”/ G′ represents the ratio between viscous and elastic contributions to the viscoelasticity.

6.3.5 Applicability of the Cox-Merz rule

The Cox-Merz rule, an empirical method to rationalize steady shear and oscillatory rheological data (Cox & Merz, 1958), was used to compare the two different rheological analyses adopted in our study. A strong correlation between two independent methodologies is a good consistency check. This rule states that the apparent viscosity ($\eta_a = \sigma/\dot{\gamma}$) at a specific shear rate ($\dot{\gamma}$) is equal to the complex viscosity ($|\eta^*(\omega)| = |G^*(\omega)|/\omega$) at a specific oscillatory frequency (ω), that is

$$\eta_a(\dot{\gamma}) = |\eta^*(\omega)| \quad (4)$$

When the rule is obeyed, rheological properties of a fluid can be described by either oscillatory or steady-state shear experiments (“Engineering Properties of Foods,” 2014).

6.3.6 Statistical analyses

All ovarian fluid measurements and fish morphological data (mean ± SD, 95% CI and Coefficient of Variation (CV%)) were summarised using the descriptive statistics function in GraphPad Prism, version 8.0.0, (GraphPad Software, San Diego, California USA). Rheometer reads were first standardized for instrumental error and the model fits were applied as described above. Subsequently, the average values of *G’* and *G”* (dependent variables) across all the sampled females were pair-wise compared trough t-tests at specific frequencies (independent variables) of interest within two shear stress ranges, 0.001 to 0.105 and 0.105 to 1 rad· s^{-1}, to double-check their uniformity within the plateau region and/or alternatively the prevalence of either the viscous or the elastic component of the ovarian fluid in this dimensional range. Normality of the residuals was ensured through D’Agostino-Pearson test followed by Shapiro-Wilk test (P= 0.2174 and 0.4697, respectively). Throughout the analyses, the statistical significance threshold used was α= 0.05.

6.4 Results

Ovarian fluid characteristics varied among individual females (Table 1). For context, coefficient of variation ((standard deviation / mean) *100) of fish length was 10% while body weight (which included eggs and ovarian fluid) was 34%. The amount of ovarian fluid produced for a given size of fish or mass of eggs was very inconsistent among females (CV ~50%). Conversely, fluid density, pH and conductivity were similar (<10%, and thus less variable than fish length). Apparent viscosity was highly variable among fish, but all exhibited clear non-Newtonian behaviour. The amount of variation declined with the shear rate applied, being CV=57% among females measured at 10 s^{-1} and CV=17% at 500 s^{-1} (Table 6.1, Figure 6.1).

Table 40 *Rows group wild Atlantic salmon female growth-related parameters (length (cm) and weight (Kg), ovarian fluid (OF) pH, conductivity (mS cm^{-1}), density (g/cm^3), volume (mL; per centimetre, per kilogram and per 10 grams of eggs) and apparent viscosity values at 10, 50, 100 and 500 rad• s^{-1} (Pa• s). In columns, left to right values are expressed as means ± standard deviations (SD), range (min-max), and coefficient of variation (CV %) among females (n=11).*

	Mean ± SD	Range (Min-Max)	CV (%)
Fish length (cm)	54.55 ± 5.714	40.90 - 74.20	10%
Fish weight (Kg)	1.556 ± 0.530	0.57 - 3.54	34%
OF volume (ml) per cm of fish	1.06 ± 0.54	0.23 - 2.20	51%
OF volume (ml) per Kg of fish	37.78 ± 19.05	7.97 - 64.98	50%
OF volume (ml) per 10 gr of eggs	2.38 ± 1.32	0.53 – 4.41	55%
OF pH	8.264 ± 0.117	8.010 - 8.57	1%
OF conductivity (mS • cm^{-1})	14.19 ± 0.674	11.77 - 15.19	5%
OF density (g • cm^{-3})	0.993 ± 0.091	0.8220- 1.530	9%
OF apparent viscosity (Pa • s) at 10 rad • s^{-1}	0.012 ± 0.006	0.006 - 0.029	57%
OF apparent viscosity (Pa • s) at 50 rad • s^{-1}	0.004 ± 0.001	0.002 - 0.008	40%
OF apparent viscosity (Pa • s) at 100 rad • s^{-1}	0.003 ± 0.001	0.002 - 0.005	32%
OF apparent viscosity (Pa • s) at 500 rad • s^{-1}	0.002 ± 0.000	0.001 - 0.003	17%

6.4.1 Ovarian fluid rheology in steady-state shear flows

To measure the viscosity under a linearly increasing rate of deformation, the ovarian fluid samples were tested for their resistance to flow for a range of shear rates (10 to 500 s^{-1}). The resulting shear stress responses from the deformed ovarian fluid were measured to determine the apparent viscosity of the material at each of the measuring points.

Atlantic salmon ovarian fluid showed non-Newtonian shear thinning behavior indicating successive loss of polymer entanglements with increasing shear rates (Figure 6.1). The Herschel-Bulkley equation fits (Figure 1) returned a mean value of yield stress σ_{00}= 0.09 (± 0.01 Pa) and a mean value of the high-shear viscosity η_{∞}= 2.3 (± 0.8 mPa · s) with the ovarian fluid showing an average 97% decline in viscosity as an increasing shear rate was applied through the rheometer's plate. Variability among all females and water control are also shown in Figure 6.1.

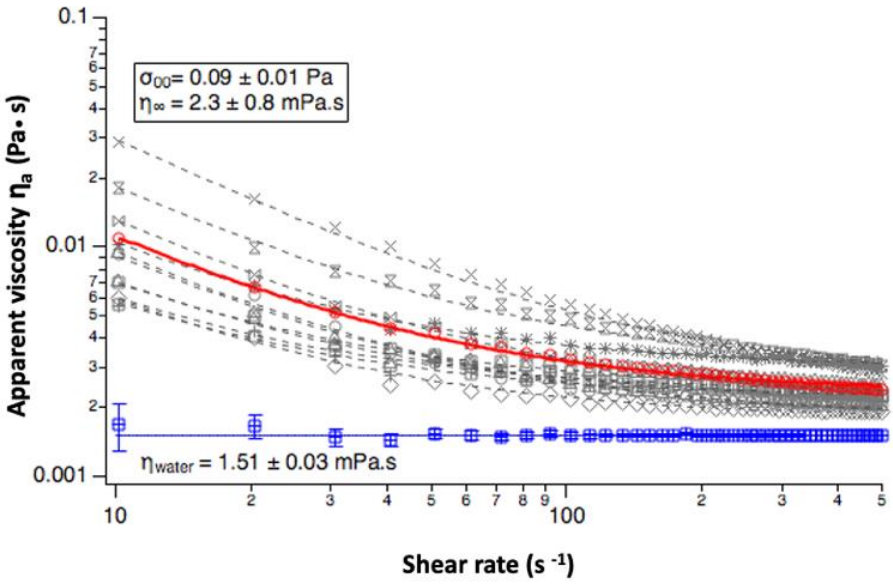

Figure 6.1 *Apparent viscosity obtained from the steady shear flow curves (η) of Atlantic salmon ovarian fluid samples (n= 11, in grey) and water controls in blue, plotted versus shear rate (s^{-1}) on a log-log scale. Grey symbols and dotted lines represent individual ovarian fluid means across 3 replicates per female and their fitted equations respectively, while the red and blue symbols and the continuous lines represent the mean across all ovarian fluid samples (red) and water controls (blue). The symbols* $\boldsymbol{\sigma_{00}}$ *and* $\boldsymbol{\eta_{\infty}}$ *are respectively the Yield stress and the apparent viscosity at high shear rates obtained from the fitting to the Herschel-Bulkley equation;* $\eta_{water\ instead}$ *represents the average apparent viscosity value of water within the analysed shear rates (mean ± SD).*

6.4.2 Small Amplitude Oscillatory Sweeps and dynamic shear properties of the ovarian fluid

The dynamic viscoelastic behaviour of the ovarian fluid dispersions was also determined by applying small amplitude oscillatory shear (SAOS) frequency sweep. The storage modulus *G'* and loss modulus *G''*, shown in Figure 2(A), were not different at low frequencies, with both having a value of approximately 0.1 Pa in the 5 measuring steps between 0.01 and 0.105 rad· s^{-1} (0.065 ± 0.011 Pa and 0.077 ± 0.001 Pa respectively (mean ± SD); P≥ 0.05, t= 2.77, df=4) and describe a pure viscoelastic fluid where the elastic and the viscous components of the fluid are comparable. Both *G'* and *G''* decreased slightly between 0.01 and 0.07 rad· s^{-1} (note the Log_{10}-Log_{10} axes) and thereafter maintained constant plateau values until the shear rate reached 1 rad s^{-1}. Note that this plateau value is numerically proximate, given the errors, to the value obtained for the yield stress in the steady shear measurements. Salmon ovarian fluid is therefore a gel-like structure at low frequencies and becomes more dominantly liquid-like at frequencies higher than 10 Hz. Interestingly, this structural shift occurs in a dimensional range that overlaps with the frequencies exerted by salmon sperm when swimming through the ovarian fluid to reach the egg (refer to dashed vertical lines in Figure 6.2A, B). This is confirmed also by the fact that at low frequencies, the gel-like structure is supported by a value of tan $\delta = G''/G'$ of 1 (crossover or gel point, See Fig 6.2 B), however between frequencies from 0.10 to 1 rad· s^{-1} (6 steps) the loss modulus *G''* (mean 0.081 ± 0.02) was marginally higher (P<0.001, t= 32.93, df= 5), than *G'* (0.052 ± 0.01). As observed through the study of their first and second derivatives, *G'* and *G''* trends start to slowly diverge, more intensely from 10 Pa (at 1.59 Hz) onward revealing a breakpoint in the polymer that exacerbates together with increasing shearing rates (Fig 6.4, 6.5 in supplementary material). Specifically, the storage modulus reached 0 Pa between 47.6 and 312 rad· s^{-1} (7.58 and 49.66 Hz), showing that the elastic response of the polymer under these frequencies is null (liquid-like); and viscous forces at their maximum in this frequency range instead prevailed. As a result, the absolute value of the complex viscosity (|η*|) decayed until reaching its a minimum of 0.005 Pa·s, at a frequency near 8 Hz (50 rad· s^{-1}), (see Fig 6.2 A). Interestingly, |η*| increased after this measuring point. Values of tan $\delta = G''/G'$, were similar at low frequencies and also showed a clear dependence in the same frequency range increasing to 34 ± 17 at the highest frequencies.

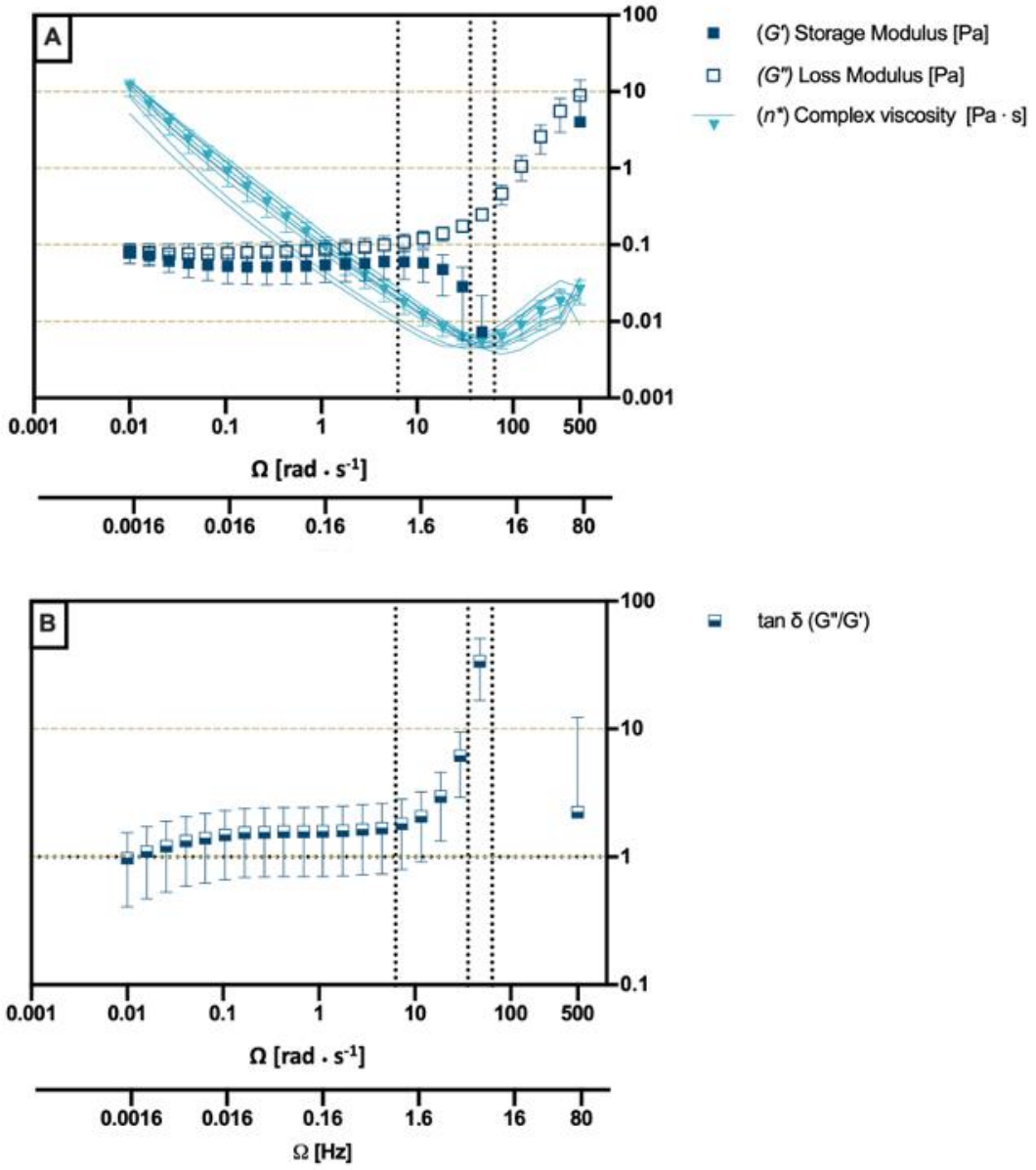

Figure 6. ***(A****) Storage modulus (G'), loss modulus (G") and complex viscosity (η*) of Atlantic salmon ovarian fluids (n= 11), to describe the relation between the viscous and elastic components of the fluid at increasing angular frequencies (0 <Ω< 500 rad· s^{-1}). Data are presented as means ± SD, continuous lines for η* represent individual ovarian fluid means across three replicates for each female).* ***B)*** *Loss factor (tan δ= G"/G') of Atlantic salmon ovarian fluids (mean ± SD) plotted versus frequency (rad· s^{-1}, Hz for reference), where tan δ= 100 for a liquid material with a pure viscous behaviour and tan δ= 0.01 for a solid material with an ideally elastic behaviour. Vertical dotted lines from left to right represent a reference baseline at 1 Hz and Atlantic salmon average sperm tail beat frequencies in Hz from Dzievulska et al., 2011a, b. Graph is plotted on log-log scale.*

6.4.3 Comparison of steady and oscillatory shear

The steady state properties of the ovarian fluid were compared with the dynamic states by applying the Cox-Merz rule. This rule, applied to polymers, enables the identification of secondary flow behaviours and/or breaking down of the fluid's polymeric network under a certain imposed stress. Apparent viscosities (η_a) obtained in the flow curves, and absolute values of complex viscosities ($|\eta^*|$) resulting from the small amplitude oscillatory sweep experiments, were plotted as a function of shear rate rad s^{-1}, fitted to the best trend and assessed for deviations between the curves' profiles (Figure 6.3). Ovarian fluid η and η^* followed the same trend with many remarkable similarities. When the oscillatory shear probed lower frequencies, the curves overlapped very closely between 10 and 50 rad· s^{-1}. From the steady shear results, we extracted a yield stress $\sigma_{00} = 0.09 \pm 0.01$ Pa, which is close to the *G'* plateau value of $\sigma_0 = 0.068 \pm 0.006$ Pa. However, above 50 rad· s^{-1} (8 Hz), there is an increase in $|\eta^*|$.

Beyond this frequency, the Cox-Merz rule was not obeyed, meaning that η_a and $|\eta^*|$ values obtained at a specific shear-rate are not equal when compared between the two different methodologies used. It should be noted that steady shear is much more disruptive to the gel structure than oscillatory shear. Thus, while we must be cautious with interpreting the rise in $|\eta^*|$ between 50 and 500 s^{-1}, it is nevertheless feasible that this rise is indicative of a rise in the SAOS viscosity.

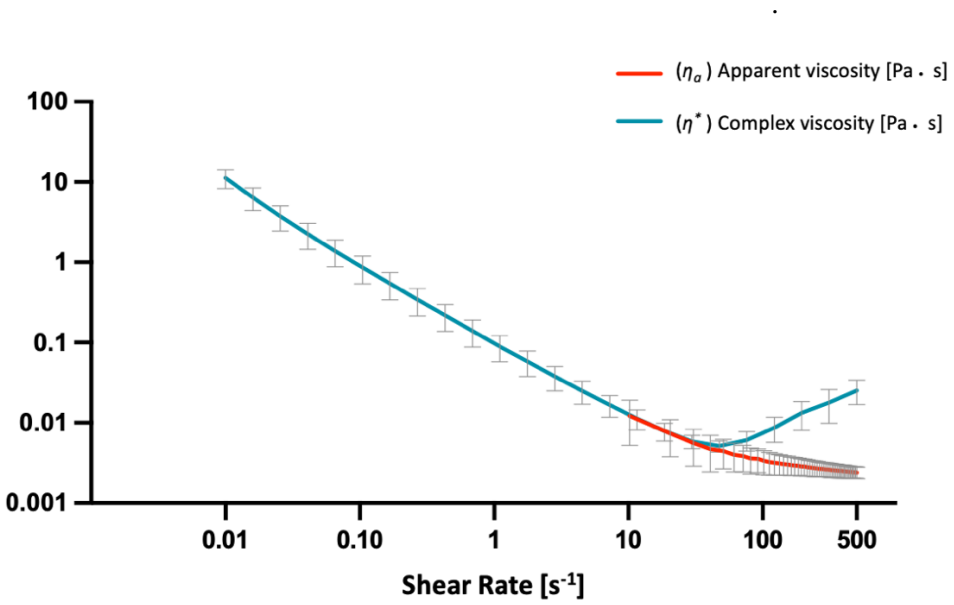

Figure 6.3 *Comparison between the steady state and the dynamic properties of Atlantic salmon ovarian fluid (OF). Apparent viscosity obtained from the steady shear flow curves (η_a) of Atlantic salmon ovarian fluid (OF) samples (N= 11, red) and complex viscosity (η^*) of Atlantic salmon ovarian fluids (n= 11, blue) plotted versus shear rate (s^{-1}). Values are presented as mean ± SD (grey vertical bars). Graph is plotted on log-log axes.*

6.5 Discussion

We describe the rheological characteristics of Atlantic salmon ovarian fluid to understand the possible involvement in sexual selection mechanisms. We subjected ovarian fluid from different females to both variable shear stresses (steady-state rheology) and angular frequencies (small-amplitude oscillatory sweeps), similar to those exerted by sperm swimming through ovarian fluid to fertilise eggs (Dziewulska, Rzemieniecki, & Domagała, 2011; Dziewulska, Rzemieniecki, Czerniawski, et al., 2011). This allowed for the identification of the main viscoelastic profile of the fluid, but also for inferring secondary flow behaviours and the eventual breaking down of macromolecular entanglements under a certain imposed stress. In particular, small-amplitude oscillatory measurements (SAOS) describe the viscous and elastic components within the ovarian fluid that could affect fertilisation dynamics. We found that the physical characteristics of salmon ovarian fluid clearly show a non-Newtonian viscoelastic nature, where shear-influenced changes in

viscosity and elasticity might have the potential to influence fertilization. Here, we discuss the structural characteristics of the ovarian fluid that could influence sperm and explore the potential of its non-Newtonian properties to be adaptive.

6.5.1 Shear-thinning behaviour in steady-state rheology and under small amplitude oscillatory sweeps

Our results indicate that ovarian fluid, which is a gel at its relaxed state (between solid- and liquid-like behaviour), is a shear thinning viscoelastic-liquid at low frequencies, and may exhibit a shear thickening phase at high frequencies. This shift from gel to a more passable medium, together with minimum viscosity values observed within the range of average beating frequencies exerted by salmon sperm, points out an interesting overlap that might be linked to 'bio-mechanical co-evolution' of female and male gametes. Specifically, Atlantic salmon ovarian fluid has a viscosity at its relaxed state that is on average 60 times that of water, being 0.09 Pa. A hypothetical beating frequency of 1Hz would yield to the absolute value of the complex viscosity $|\eta^*|$ of 0.017 Pa·s (five times lower than at its relaxed state), while a beating frequency of 12 Hz yields 0.006 Pa·s. Sperm movement occurs at low shearing rate (Brokaw, 1965, 1966) and the reported sperm beat cross frequency (BCF) values present in literature (~ 5-10 Hz) (Dziewulska, Rzemieniecki, & Domagała, 2011; Dziewulska, Rzemieniecki, Czerniawski, et al., 2011) are in a similar range of frequencies as used in our experiment. Fascinatingly, these frequencies correspond to either the shear thinning region or to the minimum values of apparent and complex viscosity reported, having a meaningful biological translation. Another intriguing possibility is that the departure from the Cox-Merz rule at high frequencies might actually signal an increase in viscosity when measured in a sensitive way (SAOS) that does not disrupt gel structure, but not when measured in a more disruptive manner (steady shear). There might therefore even exist an optimal beat frequency window below *and* above which the ovarian fluid is effectively more viscous.

Flagellar beating frequency varies considerably with temperature, pH, time, activation medium (e.g., water vs. ovarian fluid) and methodology used to detect it (Cosson, 2021; Zadmajid et al., 2019). Measures from other salmonids obtained at higher temperatures, in a diluted solution of ovarian fluid, and using stroboscopic techniques, show higher frequencies, such as ~50 Hz for *O. mykiss* (M. P. Cosson et al., 1985) and ~80 Hz for

O. tshawytscha (Butts et al., 2017). For this reason, the authors will more cautiously consider for this discussion a broader sperm beating frequency of salmon sperm in ovarian fluid going as up as 80 Hz.

Our viscosity measures are considerably higher than reported for other fish species, such as 0.0038 Pa.s (2.76 times that of water) for Arctic charr (*Salvelinus alpinus*) when measured at 0.5 Hz under a plate viscosimeter (Turner & Montgomerie, 2002). In (*O. Tshawytscha*) ovarian fluid viscosity decreased from 0.0042 to 0.0027 Pa·s as shear was increased from 7 to 72 Hz (Rosengrave, Taylor, et al., 2009a). These lower values reported in other species might be related to the higher starting frequency used as compared to ours. In fact, if paralleled to what we found in *S. salar,* the starting point of 7 Hz used for *O. tshawytscha* falls within the shear thinning phase of the fluid, implying that this was first probed already under a certain initial stress rather than at its relaxed state, thus masking a potentially higher relaxed state viscosity. In our case, by controlling for instrumental uncertainty and comparing two different rheological approaches, we had the advantage to precisely probe and extract realistic zero shear viscosities and low shear values. This is relevant because our results evidence not only that the gap in viscosity caused by increased shear is greater than previously thought, but so will be the biological implications resulting from different frequencies shearing the fluid.

6.5.2 Viscous and elastic components within the ovarian fluid that could affect fertilization dynamics

Viscous compounds were already known to influence the flagellum, resulting in a lower velocity (Brokaw, 1965). Brokaw (Brokaw, 1966, 1983) investigated sperm flagellar behaviour in response to increased viscosity in three marine Phyla (Anellida, Tunicata and Echinodermata), finding a decrease of both beat frequency and wavelength, similar to what was found in chinook salmon (Butts et al., 2017). These authors partially justified an observed increase in velocity and propulsive efficiency of sperm swimming in ovarian fluid through the non-Newtonian properties of this medium. These were firstly described in a study by Rosengrave and colleagues (Rosengrave, Taylor, et al., 2009b), who explored its response to shear rates under a constant rotational force (steady state properties). By including both steady state measurements and SAOS, we add crucial information on the specific elastic and

viscous components within the ovarian fluid that could justify the changes in sperm behaviour reported by other authors and further investigate its role during reproduction.

In view of our rheological results which show viscoelastic behaviour of salmon ovarian fluid, new considerations need to be made because the viscous (liquid-like) and elastic (solid-like) components of the fluid defining its changing complex viscosity cannot be neglected when analysing sperm energetics and outcome. In studies with internally fertilisers (mammals), viscoelastic reproductive fluids have been found to decrease spermatozoa velocities as viscosity increased while concurrently increasing their linearity (Suarez & Pacey, 2006). *Bos taurus* have higher thrust efficiencies of sperm when swimming in a non-Newtonian fluid rather than in a Newtonian one, which has been suggested might be due to a better energetic exploitation of the elastic responses of the fluid (Hyakutake et al., 2019). In our case, we observe a drop in absolute value of the complex viscosity as the frequency is increased up to 8 Hz (absolute viscosity minimum) when subjecting salmon ovarian fluids to SAOS, suggesting that until this point sperm find an increasingly thinner polymeric network that gets looser with frequency. This happens first in presence of a good elastic component that instead collapses in the 'armpit region', having the potential to positively influence sperm linearity and guidance. In this fluid, sperm with different tail beating frequencies, would in principle face substantially different polymeric structures within the shear-thinning phase. This shear-thinning flow behaviour could either facilitate sperm getting into the egg, or it could also enable cryptic female choice if a specific sperm, its morphological phenotype, swimming behaviour or another other trait, is favoured over the one of a rival male competing to fertilise the eggs. Moreover, if considering the reported within-male sperm variability observed in *S. salar* (Immler et al., 2014) , it is presumable that the physical properties of ovarian fluid might have a role also in within-male sperm selection.

Sperm traits are under strong selection (Fitzpatrick et al., 2020; Fitzpatrick & Lüpold, 2014), with recent studies evidencing a relation between some sperm traits and offspring fitness (Immler et al., 2014) and a correlation between sperm phenotype and genotype (Alavioon et al., 2017). Moreover, sperm within the same ejaculate can experience different stressors that negatively affect their swimming behaviour; the impairment of these 'abnormal' gametes is also reflected on a molecular level (e.g., DNA fragmentation) (Fernández et al., 2003), influencing the quality of the information transmitted to the zygote and accordingly its performance. Flagellar activity declines with time post-activation, while the osmotic- and ROS-derived damage experienced by the sperm cell increases (Kholodnyy et al., 2019). Therefore, the peculiar non-Newtonian properties of this fluid, shear-thinning at

low shear rates, followed possibly by shear-thickening, might help select the best performing sperm also within a single ejaculate with the objective to limit the chance of 'abnormal' sperm getting into the eggs. The frequency-dependent minimum in viscosity, raises therefore the intriguing possibility that the ovarian fluid selects for an optimal speed, providing a viscosity cost for both slow and fast beating sperm. Augmenting the swimming cost also for an ultra-fast fertilization could eventually allow selection based on further biochemical mechanisms, that are known to be pivotal for sperm egg interaction, can influence the reproductive outcome and have been advocated in reducing the hybridization risk with other species (Yanagimachi et al., 2017).

It is well accepted that the guidance within reproductive fluids occurs by means of chemical and biochemical cues that can differentially enhance the reproductive outcome from different males as demonstrated in a range of external fertilisers (J. Cosson, 2015; J. Cosson et al., 2008; Evans & Lymbery, 2020; Evans & Sherman, 2013; Kholodnyy et al., 2019; Yanagimachi et al., 1992; Zadmajid, Myers, Sørensen, Anthony, et al., 2019). We propose that more consideration should be given to the physical characteristics of the ovarian fluid that could affect sexual selection processes. Females might be able to facilitate the progression of the high quality and fast beating sperm, within and among ejaculates. Also, in view the variability observed across females; it could be suggested that these might have different capabilities to exert this selective potential, and is not to exclude how such potential could change with the hydration grade of the ovarian fluid as the reproductive season advances.

6.5.3 Shear-thickening behaviour

Under SAOS at the highest shear rates measured we observed a significant stiffening of the polymer. This did not occur in steady state measurements, where the fluid continued to thin up to 80 Hz. This difference could identify the presence of weak network associations that are broken in steady state flow measurements, where a continuous rotational force is applied on the fluid. In contrast, these interconnected networks are unaffected in the oscillatory shear tests. In this case, the ovarian fluids were subjected to sinusoidal shear stresses within a linear range small enough that the macromolecular entanglements are preserved. A shear thickening phase at very high frequencies, mostly in absence of any elastic component, would suggest that sperm swimming efficiency could be exclusively dependent

on its speed and on the fluid viscosity, without exploiting the positive effects on linearity that some elasticity would provide. The lack of elasticity on the other hand may also be promoting a more circular swimming pattern, which in a closely related species *(Oncorhynchus mykiss)* has been linked to augmenting the chances of fertilizing the egg (Wojtczak et al., 2007).

It could be further speculated that a shear thickening phase at high frequencies might also be linked to the 'necessity' for ovarian fluid to stay close to the eggs and not be washed away – an aspect of natural selection. Salmon spawn in rivers and an infinitely shear-thinning ovarian fluid would enhance its chances of being dispersed and diluted very quickly, thus depriving the eggs from its known beneficial effects on fertilization (Alonzo et al., 2016; Butts et al., 2012; Gasparini & Pilastro, 2011; Poli et al., 2019; Yeates et al., 2013). Other works have shown that a shear-thickening behaviour observed at high frequencies could be also derived from inertial forces. For example, a study of hagfish slime (Böni et al., 2016) presented similar behavior at low frequency with G"/G' of order one, indicating an ultra-soft material having weak elastic properties. However, in that study a rise in G'' (and drop in G') at higher frequencies was attributed to instrument inertia. We cannot exclude that this could also be the case here. Moreover, although the idea that the ovarian fluid may have a natural selective function at very high shear rates is indeed fascinating, this specific aspect lies outside the scope of this study and was not tested specifically. Future experiments should try to provide insights in this regard by testing the ovarian fluid dispersion capacity from eggs under different shear rates that could better simulate the riverbed waterflow. Additionally, it could be also tested whether the shear-thickening phase observed at the upper end of our analysed range is sincere and if this persists at very high frequencies with a beneficial effect on the eggs (e.g., higher diffusion, mechanical resistance, pathogen barrier) (Elofsson et al., 2003).

6.5.4 Conclusive remarks and future perspectives

Ovarian fluid physical properties deserve more attention and considerations when studying processes of sexual selection such as selection on sperm performance, sperm competition assays and fertilization trials, both *in vivo* and *in vitro*. The characteristic rheological behaviour of the ovarian fluid we report here underlines the importance of

including it as a preferred sperm activation medium over pure water to simulate a more natural fertilisation environment and benefit from its effects on sperm.

Our discovery yields a number of predictions to be tested in the future, including testing whether the physical properties of ovarian fluid act as a filter for specific sperm or, whether its structure only ameliorates sperm performance in general. Notably, our findings suggest that processes enabled by non-Newtonian reproductive fluids within female internal genital tracts, like lubrication, facilitation and capacitation, should also be applied to the external fertilization environment. This opens new avenues into the study of cryptic female choice with important implications for understanding the evolution of sexual traits and exploring the underestimated role of physical properties of the fertilization environment that surrounds the gametes both in nature and in artificial fertilization protocols.

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6.6 Supplementary material

6.6.1 Preliminary assessment of the ovarian fluid rheology and preservation method

After filtration and pH, volume, conductivity and density measurements were taken (see materials and methods section), each batch of ovarian fluid was divided in three separate falcon vials containing equal volumes that were then stored at - 80, -20, and 4 °C respectively. A preliminary rheological analysis, using a portion of these samples (N= 5) was conducted to assess the best preservation method. Being that the techniques used to assess the viscoelastic profiles of the samples were particularly time consuming (1 or 2 samples per day maximum), we wanted to avoid any bacterial degradation in the 4°C-stored samples that would eventually affect the fluid's structure. Nevertheless, we also wanted to ensure that the freezing thermal treatments would not affect the polymeric structure of the fluid. No observable hysteresis was found between the three thermal treatments when subjected to the experiments illustrated. Therefore, we opted for the samples stored at -20 °C for optimal processing through the duration of the experiments, and to these all the results are referred.

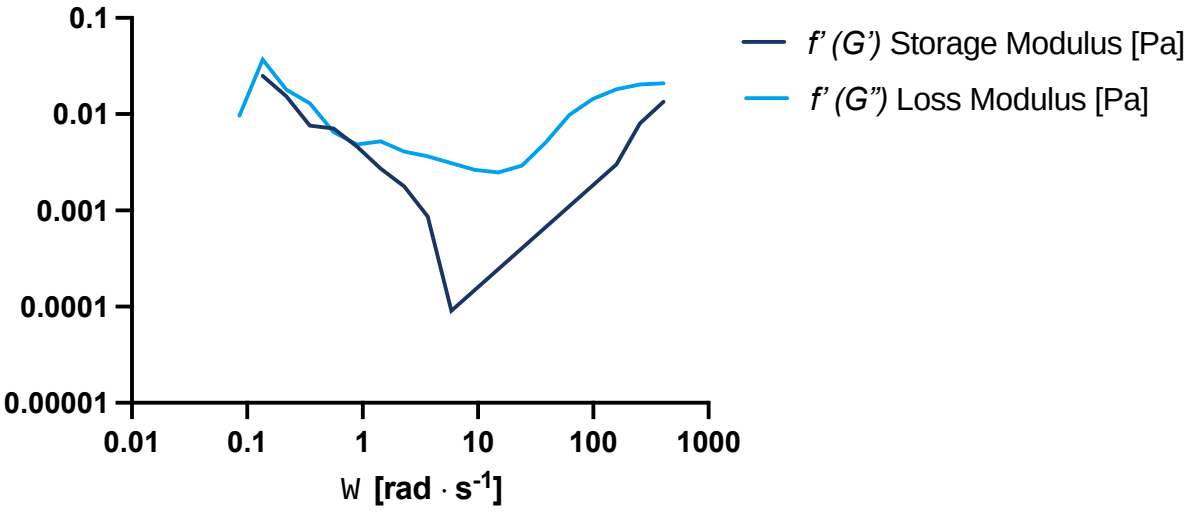

Figure 6.4. *First derivative (f') of the Storage modulus (G') and loss modulus (G") of Atlantic salmon ovarian fluids (n= 11), to describe the relation between the viscous and elastic components of the fluid at increasing angular frequencies (0 <Ω< 500 rad· s^{-1}). Data are presented as means.*

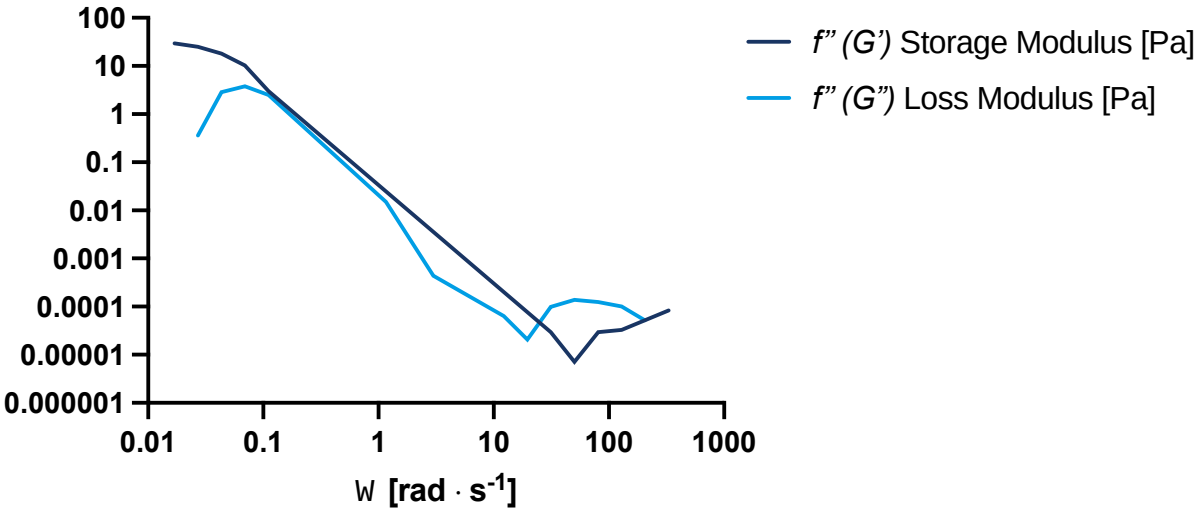

Figure 6.5 *Second derivative (f") of the Storage modulus (G') and loss modulus (G") of Atlantic salmon ovarian fluids (n= 11), to describe the relation between the viscous and elastic components of the fluid at increasing angular frequencies (0 <Ω< 500 rad· s^{-1}). Data are presented as means.*

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