Understanding sperm-egg interactions using fish and insect models

James Jonathan Bemrose

Thesis submitted for the degree of Doctor of Philosophy School of Biological Sciences, University of East Anglia

September 2021

© This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that use of any information derived there from must be in accordance with current UK Copyright Law. In addition, any quotation or extract must include full attribution.

Thesis abstract

Sperm-egg interactions operate at the end of the struggle to reproduce and therefore have a significant influence over reproductive fitness and gene flow. In this thesis I utilise the externally fertilising Atlantic salmon and internally fertilising *T. castaneum* as complementary model species to understand sperm-egg interactions. Atlantic salmon's (*Salmo salar*) external fertilisation system allows control and paired in vitro fertilisation experiments to reveal drivers and mechanisms of sperm-egg interactions. *Tribolium castaneum* flour beetles also enable tight control and generous replication, plus a generation time that enables experimental evolution. These systems and approaches were used to investigate both applied and fundamental questions about reproduction, fertilisation and gametic interactions in both wild and farm salmon. In Atlantic salmon, aquaculture and conservation hatcheries employ an artificial fertilisation process to reproduce offspring, which can sometimes lead to suboptimal hatch rates. Across a series of fertilisation tests, where potential factors within standard hatchery and farm gamete handling methods were investigated, we found wild Atlantic salmon hatch rate to be maximised when eggs were fertilised 'dry' (gametes are mixed together before the addition of river water) on the day of gamete stripping. In farm Atlantic salmon, we found no particular gamete handling factor to affect hatch rates, with females and eggs also able to tolerate external storage for extended periods of time. Overall, in both farm and wild fish, hatch rates were maximised when fertilised 'dry' with minimal post-ovulatory storage duration, and we found no evidence that any of these artificial gamete handling factors increased the risk of abnormal ploidy levels among offspring. However, if such storage is

needed, eggs should be left within the coelomic cavity for up to 14 days, rather than exposing females to multiple checking and stripping events. Despite genetic and phenotypic divergence between wild and farm salmon through domestication, we found no barriers to hybridisation at the gamete level. Moreover, in our tests, farm Atlantic salmon sperm dominated paternity when in competition with sperm from wild males for eggs from wild females, suggesting that sperm quality has not been degraded through domestic selection for aquaculture. We also found no influence that ovarian fluid identity affected the outcomes of these *in vitro* fertilisation competitions. In *T. castaneum*, experimental evolution for 130 generations under high versus low opportunities for sexual selection and cryptic female choice did not reveal any evidence for divergence in female ability to choose the 'right' kind of sperm when inseminated with both conspecific and heterospecific sperm. A further experiment on these high and low sexual selection line females found no evidence that divergence in offspring production rates had evolved: across 100 days of oviposition, females from high and low sexual selection lines produced similar offspring number at similar rates when males were freely accessible (in contrast to previous findings that the lines differ in reproductive output under sperm limitation). In summary, these combined experiments add to our applied and basic knowledge of processed at the gamete level that can have important consequences for reproduction. Through looking at the gamete level in Atlantic salmon, I was able to determine measures to improve production and survival rates of the early life stages. In *T. castaneum* I focused on the influence of sexual selection intensity on female ability to assert cryptic female choice and manipulate offspring production rate, and I found no clear evidence for such postcopulatory choice, despite clear opportunities for its actions to have evolved.

Access Condition and Agreement

Each deposit in UEA Digital Repository is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the Data Collections is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form. You must obtain permission from the copyright holder, usually the author, for any other use. Exceptions only apply where a deposit may be explicitly provided under a stated licence, such as a Creative Commons licence or Open Government licence.

Electronic or print copies may not be offered, whether for sale or otherwise to anyone, unless explicitly stated under a Creative Commons or Open Government license. Unauthorised reproduction, editing or reformatting for resale purposes is explicitly prohibited (except where approved by the copyright holder themselves) and UEA reserves the right to take immediate 'take down' action on behalf of the copyright and/or rights holder if this Access condition of the UEA Digital Repository is breached. Any material in this database has been supplied on the understanding that it is copyright material and that no quotation from the material may be published without proper acknowledgement.

Table of contents

Table of figures

Table of tables

Acknowledgements

Firstly, thank you to my supervisor, Matt Gage for this opportunity. Matt has pushed me to become a better scientist and has been an exemplary model of how to do this. I have grown as a person and a scientist and without Matt this would have never been possible. Thanks, as well to David and Ram, for their support and mentorship throughout the process.

Thanks to the Zekes: Ellen, Ellie, Jen, Tom, and Becky for being such a supportive and funny office. A particular thanks to Siobhan and Tom; it has been great to go through this journey together - thank you for being there for me in all aspects of my PhD.

Mum and Dad none of this would of happened without you, this journey begins and ends with your support, love and care. In my toughest times you have been there for me, in the best times you have celebrated my successes. This PhD journey begins with the love you instilled in me of the natural world, it starts with rock pools, walks through the woods and trips to Colchester Zoo. This journey to a PhD ends with you by my side, with unfailing support, copious proof reading and I hope lots of pride.

Finally, Charlotte you have been everything for me during this time from a proof-reader to a friend - no words could adequately describe what your support has meant to me.

Chapter 1

General introduction

1.1 Thesis aims

In this thesis I explore sperm-egg interactions in externally fertilising Atlantic salmon and internally fertilising *T. castaneum*. Atlantic salmon's external fertilisation allows us to control for confounding variables that are introduced in internal fertilisation. Whilst, *T. castaneum* provide the opportunity to look at experimental evolution with rapid generations time which would not be possible in Atlantic salmon. These two model species provide excellent systems to explore pre and post-copulatory mechanisms of sexual selection. In this thesis I look to explore male and female control of fertilisation and reproductive success. The relevance of these model species is also due to their influence over food production. Atlantic salmon provide an important and rapidly growing source of animal protein, whilst *T. castaneum* are a major pest species in stored grains products. In Chapter 3, I investigate the influence of artificial fertilisation on the hatch and abnormal ploidy rate of farm and wild Atlantic salmon. This study looks to determine the potential cause of low hatch rate in hatcheries and the most effective fertilisation methods to maximise success. In Chapter 4, I test the effect of post-ovulatory oocyte ageing on successful fertilisation and abnormal ploidy in farm Atlantic salmon. I look to investigate whether post-ovulatory oocyte ageing may result in reduced egg quality and therefore lower hatch rates in the farm environment. This study looks to identify whether post-ovulatory oocyte ageing may be a causal mechanism for low hatch rates. In chapter 5, I explore the result of competition between farm and wild males for farm and wild females at the gamete level and the threat this poses. I look to determine whether the divergence observed between farm and wild Atlantic salmon has resulted in the formation of a barrier to hybridisation at the gamete level. Due to previous research finding ovarian fluid to a potential mechanism we look to further

identify ovarian fluids potential role in sperm selection. In Chapter 6, I look to understand whether long term experimental evolution of high and low sexual selection results in differential ability to assert CFC. This study will test whether sexual selection intensity alters females ability to utilise CFC to avoid hybridisation with a sister species. Finally, in chapter 7 I explore whether these sexual selection line females ability to manage sperm has diverged. This study builds upon previous research identifying differential offspring production under sperm limitation between high and low sexual selection females. I look to determine whether differential offspring production is the result of an intrinsically different offspring production rate with females from high or low sexual selection backgrounds.

1.2 Sexual selection

1.2.1 Gamete Production and Sexual Reproduction

Sexual reproduction is the dominant route of offspring production across multicellular taxa, ultimately occurring when recombined haploid gametes fuse to form a diploid zygote (Maynard Smith, 1977). Gametes vary significantly in shape, size and structure across taxa, but the process of karyogamy, the fusion of gamete (haploid) nuclei, is conserved across animal kingdoms (Fincham, 2001). Although this process of haplotype fusion follows a conserved process, it is becoming increasingly recognised that a number of mechanisms involving competition and choice have evolved along the route from gamete release to haplotype fusion and fertilisation, and that these can have important influences upon individual reproductive success.

During sexual reproduction both males and females come under sexual selection as defined by Darwin (1859, 1871), as traits or behaviours that increase the chance of successful competition between rivals and improved opportunity for mate acquisition. Historically, it

was believed that sexual selection ceased with acquisition of a mate through male competition or female choice (Darwin, 1871, Andersson, 1994). However, research over the last half-century has identified that sexual selection can also occur through post-copulatory processes of sperm competition and cryptic female choice, suggesting that these post-copulatory mechanisms also influence reproductive success and gene flow, possibly to the same extent as male-male competition and female mate choice (Parker, 1970; Smith, 1984).

1.2.2 Sperm competition

Sperm competition occurs when two or more males compete for a single female's ova, being first identified in Parker's seminal work (Parker, 1970). Sperm competition has been observed to occur in a wide range of organisms, being widely recognised in both internal and external fertilisation systems (Parker, 1970; Birkhead, 1987; Gage *et al.*, 2004; Simmons, 2001; Birkhead & Hosken 2008). Sperm competition provides a strong force of sexual selection, affecting both males and females at a number of levels from morphological adaptions to behavioural changes (Birkhead & Møller 1998). At the gamete level, sperm competition is widely recognised to influence the evolution and allocation of sperm when fertilisation is won through the 'raffle principle' (Parker, 1990; Birkhead & Hosken 2008). The 'raffle principle' would suggest that when in competition males should produce large numbers of 'tiny' sperm to maximise fertilisation success (Parker, 1982; Gage and Morrow, 2003). But investment in sperm number may come at the cost of sperm quality meaning sexual selection through sperm competition can result in the production of traits for both sperm number and/or sperm quality. Two opposing hypotheses exist for the relationship between the development of sperm number and sperm quality. Gómez Montoto *et al.*, (2011) define the two hypotheses as a) sperm quantity and quality co-evolve under sperm

competition, b) energetic constraints force the trade-off between sperm number and sperm quality. In Gómez Montoto *et al.*, (2011) study of 18 rodent species they identified sperm quality and sperm number to improve under sperm competition in a complementary fashion with overall sperm quality a combination of both factors. Sperm quality has been identified as a key factor in winning sperm competition with traits of velocity (Gage *et al.*, 2004) length (García-González and Simmons, 2007), sperm morphology (Lüpold *et al.*, 2009) and longevity (Alavioon *et al.*, 2019) providing sperm with a competitive advantage. In this thesis we investigate sperm competition in both the *T. castaneum* and Atlantic salmon model species. Through controlled split ejaculate split clutch experiments, we explore sperm competition between wild and farm Atlantic salmon to determine the risk at the gamete level of genetic introgression. In *T. castaneum* sperm competition is assessed between *T. castaneum* and their sister species *T. freemani* under sequential and constant mating experiments for females under high or low sexual selection pressure.

1.2.3 Cryptic female choice

Cryptic female choice (CFC) occurs when females are not passive participants in post-copulatory sexual selection. Cryptic female choice is defined as “female-mediated morphological, behavioural or physiological mechanisms that bias fertilisation towards the sperm of specific males ” (Thornhill, 1983). Non-random mating patterns produced through CFC provide genetic benefits through increased survival of genetically viable and diverse offspring (Ivy & Sakaluk 2005; Simmons, 2005; Evans et al., 2012; Rosengrave et al., 2016). This fitness benefit is a result of selection for “good” or compatible genes, resulting in CFC influencing gene flow and therefore population genetic structure (Ivy & Sakaluk 2005;

Stapper et al.,2015). CFC specifically being also observed as a mechanism to prevent inbreeding (Gasparini and Pilastro, 2011; Lovlie *et al.*, 2013) and hybridisation (Yeates *et al.*, 2013). Over 20 potential mechanisms of cryptic female choice were defined by Eberhard (1997), including disruption of copulation, sperm ejection, sperm activation and differential fertilisation. The precision of these mechanisms increases with proximity to fertilisation due to fewer sperm being present (Firman *et al.*, 2017). The processes of cryptic female choice and sperm competition are key components of sexual selection. They provide selective pressure to produce competitive gametes, evolve mechanisms for sperm-egg interactions and gain fitness benefits. There is growing evidence that these gamete level processes can have population-level effects (Andersson and Simmons, 2006).

The importance of CFC as a mechanism of post-copulatory sexual selection remains highly debated as exemplified by (Ward, 2000) and (Simmons, Parker and Stockley, 1999) studies on the golden dung fly explaining the same result through opposing female controlled CFC (Ward, 2000) and male controlled sperm competition (Simmons, Parker and Stockley, 1999). The difficulty in providing clear empirical evidence of CFC is that male and female influences over fertilisation success are constantly interacting making it hard to decipher each sex's individual role. Furthermore, it is a cryptic process meaning it is not readily observed making it difficult to determine the mechanisms and their role. The influence and importance of CFC is explored throughout this thesis. In Atlantic salmon we look to explore ovarian fluids potential role as a mechanism of sperm selection both within ejaculate and between competing males through studies of fertilisation method and inter-strain gamete compatibility. In *T. castaneum* we explore females ability to assert CFC under high or low sexual selection

1.3 Model species

It is becoming increasingly recognised that a number of mechanisms have evolved along the route from gamete release to haplotype fusion that can have important influences upon fertilisation and individual reproductive success. In this thesis, I aim to advance our understanding of the mechanisms operating at the gamete level, and how they influence fertilisation outcomes. I have used the externally fertilising Atlantic salmon and internally fertilising *Tribolium spp.* with focus on *Tribolium castaneum*. I will look at how mechanisms operating at the gamete level influence fertilisation success and male-female compatibility. We focus, on Atlantic salmon and *T. castaneum* as they carry traits that make them great models for advancing our understanding of how reproduction works at the gamete level. Additionally, Atlantic salmon as a source of animal protein and *Tribolium castaneum* as a pest species are also important due to their impacts upon food production. Atlantic salmon is a rapidly growing source of animal protein being farmed within aquaculture with the prediction that its importance as a source of food is going to grow even further. *Tribolium spp.* are important to study for the opposing reason as they are a major pest species of stored products. We therefore look to develop theoretical understanding that can be applied to the improvement of reproduction efficiency in Atlantic salmon and further understanding of the influence of gamete level interaction on the population dynamics of a major pest species.

1.3.1 Atlantic salmon

The Atlantic salmon (*Salmo salar*) is a member of the Salmonidae Family, with one of the most diverse and complex life histories observed within the teleosts (Crête-Lafrenière, Weir and Bernatchez, 2012). Atlantic salmon have an anadromous life history: eggs hatch in

freshwater where fry grow to parr before migration to sea as smolts, and then returning to their natal river after oceanic feeding to breed. The movement between riverine and oceanic eco-systems forges an ecological link between oceanic and terrestrial eco-systems, with Atlantic salmon acting as a keystone species for the passage of important nutrients from the sea into oligotrophic upland systems (Willson & Halupka 1995; Limburg and Waldman, 2009). As a wild species, Atlantic salmon are a species of historical and cultural significance (Wigan, 2013), and of conservation relevance due to the current decline in wild population numbers (WWF, 2001).

Wild Atlantic salmon populations are distributed throughout the temperate and sub-arctic waters of the north Atlantic ocean (Aas *et al.*, 2011). The majority of Atlantic salmon populations are anadromous, with a few resident freshwater Atlantic salmon populations also occurring in both lentic (Pepper, Oliver and Blundon, 1985) and lotic freshwater systems (Sandlund *et al.*, 2014). Atlantic salmon spawn in natal rivers releasing sperm and eggs into gravel nests, where zygotes develop to eyed eggs, eventually hatching into juvenile alevins with a reliance on yolk deposits from the mother (Aas *et al.*, 2011). Once yolk deposits have been exhausted, the fry leave the gravel nest to independently search for food, developing over time into parr. Parr are the predominant freshwater juvenile life stage, occupying territories and developing cryptic colouration to suit their bottom-feeding life style (McCormick *et al.*, 1998). The length of the parr stage can vary from 1-3 years, depending on the acquisition of resources, photoperiod and temperature, before reaching the critical length of 7-12cm for smolting (Wedemeyer, Saunders and Clarke, 1980; Skilbrei, 1988)

Once an individual has reached a critical mass and environmental conditions are optimal, parr transition to the next life stage through the process of smoltification, in which

behavioural and morphological adaptions are developed that facilitate seaward migration, out to oceanic feeding grounds (McCormick *et al.*, 1998). These oceanic feeding grounds provide the resources needed to undergo rapid growth and the energetically costly process of maturation (Jones, 1959).

1.3.2 Atlantic salmon reproduction

The Atlantic salmon breeding pattern is a complex and energetically-costly mating system. Both genders expend 59% of their total energy reserves through migration and gamete production (Jonsson, Hansen and Jonsson, 1991). These high energetic demands lead to low survival rates of adults through and after a breeding season of 11% (Fleming, 1996).

Pre-spawning

Reproductively maturing Atlantic salmon return to natal rivers between spring and autumn, depending on river location, type and age class (Stabell, 1984; Aas *et al.,* 2011). Despite the scale and distance of their seaward migration, previous studies have found that salmon successfully return to their own natal freshwater system 97-99% of the time, rarely straying into different systems (Stabell, 1984).

During their time at sea, salmon remain on their ancestral feeding grounds, feeding opportunistically on more than 40 different species of fish and invertebrate (Dixon *et al.*, 2012). The timing of the return migration is dependent on age, with multi-sea winter individuals returning to natal freshwater systems significantly earlier through the season than one-sea-winter grilse (Jonsson, Jonsson and Hansen, 1990). The exact timing of migration is also dependant on river characteristics, such as water velocity and temperature (Webb and Hawkins, 1989; Jonsson et al., 2007).

During return migration, individuals begin to invest in gonadal development, with females investing approximately 25% of their body weight into gonadogenesis, and males investing comparatively less, at only 4-6% (Fleming, 1996). From the onset of the migration until spawning, the mean gonadosomatic index (GSI = [gonad weight / total tissue weight] × 100), a commonly-used score of reproductive development, increases from 0.4 to 3.2 in males and 2.8 to 28.6 for females (Jonsson, Jonsson and Hansen, 1997). As salmon migrate towards natal rivers, individuals begin to develop secondary sexual characteristics (SSCs). The most obvious SSC is the development of the male 'kype', an extension of the lower jaw which is used during intra-sexual competition as a physical determinant of male dominance (Darwin 1910; Järvi, 1990). SSCs also develop in females (Quinn & Foote 1994; Johnson *et al.,* 2006).

Once females have successfully returned to natal spawning grounds, they locate suitable spawning habitats and begin to construct nests and form redds (Fleming, 1996). Redds are defined as the continuous area of disturbed gravel in which eggs are deposited (Fleming, 1998). The number of nests produced on the redd relates to female size, with smaller grilse digging three to five nests, whilst larger multi-sea-winter females creating up to 14 (Fleming, Lamberg and Jonsson, 1997). To identify optimal spawning sites, females undertake test digging (Fleming, 1996). If the site is unsuitable, females will abandon the nest, even in the latter stages of construction (Fleming, 1996; Esteve, 2005).

While females are constructing redds, sexually mature males undertake short-range migrations within the natal river to locate mature and ovipositing females (Healey and Prince, 1998). The aptitude of a male to acquire a mate is determined by their competitive ability during male-male behavioural interactions (Fleming, 1996). Male Atlantic salmon will determine a hierarchy through a combination of aggression and visual cues (Fleming, 1996; Johnson, Carlson and Quinn, 2006). The high level of male-male competition is driven by an

operational sex ratio (OSR) that is often male biased: female sexual activity typically lasts 7-10 days whereas males can remain sexually active for more than a month (Webb and Hawkins, 1989). Due to this male biased OSR, females are regularly attended by multiple anadromous males taking up secondary (satellite) positions, and sexually mature but non-anadromous precocious parr (Järvi, 1990).

Mate selection is primarily focused on female size, with previous studies observing that large individuals have a higher fecundity (Reid and Chaput, 2012), so that intrasexual competition for the largest and most fecund individuals is expected to be higher (Sargent, Gross and Van Den Berghe, 1986). Females, although constrained by the outcome of male competition, are not passive in mate selection. Females choose mates on the basis of multiple anatomical and behavioural variables including male SSCs (Järvi, 1990). Females will attack males deemed sub-optimal during spawning to reduce their fertilisation success (Petersson, 1997; Blanchfield and Ridgway, 1999; Berejikian *et al.,* 2000).

Salmonids exhibit high phenotypic variation, displaying variance within species and genders in behavioural, physiology, morphology and life history traits. One alternative life-history phenotype is the precocious parr reproductive tactic where males mature before undertaking oceanic migration (Vladic and Jarvi, 2001). Due to high competition, sneaker-males attempt to avoid competition by investing no energy into the development of SSCs, choosing instead to use subterfuge to fertilise eggs deposited by females (Gage, Stockley and Parker, 1995; Fleming, 1996). The lack of SSC development puts these sneaker-males at a competitive disadvantage with older, larger mature males. However, sneaker-males overcome this by investing large amounts of energy into both gonadal development and gamete production (Lehnert *et al.*, 2017). Previous studies have found that sneaker-males invest 36% more energy into gonadal development (calculated as a percentage of total body mass) and

produce 1.8x more spermatozoa per unit of body mass compared to anadromous males; in addition, these spermatozoa are more motile and successful in sperm competitions (Gage, Stockley and Parker, 1995; Fleming *et al.*, 1996; Vladic and Jarvi, 2001).

Spawning

Atlantic salmon regularly engage in polyandrous mating as a result of the male-biased OSR and asynchrony in spawning times between the sexes (Webb and Hawkins, 1989). Multiple paternity is most pronounced in areas of high redd density, and hypothesised to be a result of increased male density including the presence of mature male parr, preventing a single male achieving mate-monopolisation of a female (Weir *et al.*, 2010). Weir *et al* (2010) parentage analysis in redds of wild Atlantic salmon identified polyandry to be the norm, with an average of 8 different males, and up to 16, fertilising the eggs in a single female's nest. These naturally high levels of polyandry are supported by Taggart *et al* (2001) study finding that over 50% of anadromous male spawners would contribute to reproduction across more than one redd.

Males will remain in close proximity to females, with dominant males quickly swimming towards the redd once the female begins ovulating. Pre-copulatory mate monopolisation by dominant males facilitates increased fertilisation success at ovulation (Caldeira *et al.*, 2018). Previous studies have shown that, during Atlantic salmon spawning, secondary or satellite males will attempt to gain access to the female 13%-63% of the time (Fleming, Lamberg and Jonsson, 1997). Garant et al (2001) found that male salmon gain access to females based on hierarchical position or, during mass spawning events, multiple males will release spermatozoa simultaneously.

When released into freshwater, salmon sperm undergo a number of structural and biochemical changes during activation. Sperm motility in salmonids is initiated when the decrease in potassium ion (K+) concentration within storage in the male reproductive tract occurs on release into freshwater at ejaculation (Alavi and Cosson 2006). Efflux of K+ results in the hyperpolarization of the flagellar membrane, which allows external Ca2+ influx through calcium channels (Cosson, Billard and Letellier, 1989; Alavi and Cosson, 2006). Ca2+ influx in combination with hyperpolarization of the flagellar membrane is hypothesised to activate cAMP cyclic adenosine monophosphate (Dumorné *et al.*, 2017).

1.3.3 Sperm competition and Cryptic female choice

The pattern of polyandrous external fertilisation observed in Atlantic salmon is widespread across the teleosts (Stockley *et al.*, 1997; Rakitin *et al.,* 1999; Immler & Taborsky 2009; Alonzo *et al.,* 2016), providing ample opportunity for post-copulatory sexual selection within sperm competition and cryptic female choice (Loo *et al.*, 2018). Sperm competition drives the production of morphological (Järvi, 1990), behavioural (Mjølnerød *et al.*, 1998) and physiological adaptions (Stockley *et al.*, 1997) to reproduction in males, while CFC will encourage mechanisms to evolve that allow females to be most likely to be fertilised by the 'right' sperm.

Polyandrous external fertilisation subjects males to a dual selective pressure for fertilisation. Firstly, sperm must be adapted to abiotic factors within the aquatic environment, including selection from osmotic pressure, temperature and turbulence (Morisawa *et al.*, 1983; Pennington, 1985; Billard *et al.*, 1986). Secondly, biotic factors created through sperm

competition and CFC will drive the evolution of competitive sperm traits, including sperm quantity and cell quality (Gage *et al.,* 2004; Liao *et al.,* 2018).

Sperm competition in salmon

Intensity of sperm competition in teleosts has been observed to modulate sperm production, increasing ejaculate investment with greater risk from sperm competition (Shapiro *et al.,* 1994; Marconato *et al.,* 1995; Marconato & Shapiro., 1996; Fuller, 1998). Investment in greater sperm production has been identified as a mechanism to facilitate increased fertilisation success (Gjerde, 1984; Mjølnerød *et al.,* 1998; Stoltz and Neff, 2006). Sperm number has been found to influence competitive success, with externally fertilising species generally maximising fertilisation through production of many small sperm, due to intense sperm competition (Parker, 1982; Gage & Morrow., 2003). There are exceptions to this rule, with greater sperm length observed to facilitate competitive success in externally fertilising cichlids and gastropod molluscs (Balshine, 2001; Oppliger *et al.,* 2003; Fitzpatrick *et al.,* 2009). In Atlantic salmon, flagellum length was found to have no influence on competitive success (Gage *et al.*, 2004).

Research has also identified sperm form and function to be a determining factor in fertilisation success. A number of sperm traits play a role within sperm competition, such as sperm motility (Gage *et al.*, 2004), length (Oppliger *et al.*, 2003), longevity (Levitan, 2000), and ATP production (Vladić, Afzelius and Bronnikov, 2002).

Gage *et al* (2002) study of sperm morphometry identified longer sperm and/or longer sperm flagella had no influence on velocity, instead sperm length had a negative relationship with longevity. Previous research identifying sperm length to reduce with sperm competition

intensity also indicated that greater length does not correlate with sperm competition risk across species (Stockley *et al.*, 1997). Despite these findings greater sperm midpiece length was identified by Vladic & Jarvi, (2001) to promote fertilisation success with greater midpiece size facilitating increased ATP production which in turn enhanced motility.

Motility has been identified as a key sperm trait for achieving competitive success. Sperm velocity is observed across a number of species to enhance external fertilisation success, including in Atlantic cod (Skjaeraasen *et al.*, 2009), sea urchins (Levitan, 2000) and lake trout (Butts *et al.*, 2012). This relationship between velocity and fertilisation success also applies in Atlantic salmon, with *in vitro* fertilisation competitive experiments determining relative contribution of sperm number and motility traits. Gage *et al.,* (2004), examined the influence of Atlantic salmon sperm concentration, velocity, longevity, and sperm length upon competitive fertilisation success, and found a significant positive relationship between relative sperm velocity and fertilisation success. The work therefore indicated that the relative speed at which an individual male's sperm swim can be a crucial determinant of sperm competition success.

Cryptic female choice in salmon

While sperm competition operates, cryptic female choice may also play a role in fertilisation success (Eberhard, 1997; Firman *et al.*, 2017). Recent work has identified the value of examining mechanisms of cryptic female choice within externally-fertilising aquatic environments, where experimental control and isolation of adult effects is possible. Studies of the broadcast spawner *Mytilus galloprovincialis* identified that eggs employ chemoattractants to attract sperm from genetically compatible male partners, selecting

between sperm of female conspecifics. These egg chemoattractants therefore determine mate choice by biasing sperm migration patterns (Evans *et al.*, 2012).

Mytilus galloprovincialis ova have also been observed to release soluble eggs factors, which remotely modulate the physiology and swimming behaviour of sperm through interactions with sperm surface glycans, which in turn influence Ca2+ influx, a key determinant of fertilisation success (Yoshida, Kawano and Yoshida, 2008). Sperm glycans can change the strength of the acrosome reaction, and have been identified to influence specific male-female interactions and thereby selection for specific (compatible) males as a mechanism of cryptic female choice (Kekäläinen and Evans, 2017).

In studies of cryptic female choice within Atlantic salmon, a possible mechanism of interest is ovarian fluid. The role of ovarian fluid as a post-copulatory sperm selection mechanism has been observed across salmonid species (Olsén *et al.*, 2001; Lahnsteiner, 2002; Turner and Montgomerie, 2002; Rosengrave *et al.*, 2008a). The biochemical and/or biophysical interactions between the constituents of ovarian fluid and sperm are hypothesised to act as a mechanism of sperm choice (Alonzo, Stiver and Marsh-Rollo, 2016; Rosengrave, Montgomerie and Gemmell, 2016). Ovarian fluid can influence swimming linearity, direction and longevity of sperm (Yeates *et al.*, 2013; Lehnert *et al.*, 2017), and through this allow conspecific sperm precedence to avoid hybridisation between *Salmo trutta* and *S. salar* (Yeates *et al.*, 2013). I propose to investigate whether equivalent mechanisms influencing CSP between these two species also act between populations but within Atlantic salmon. Research on Chinook salmon has identified that ovarian fluid influences sperm swimming behaviour at the species level, allowing influences on sperm velocity that translate into fertilisation success, and therefore acting as a mechanism of cryptic female choice based on male compatibility (Rosengrave *et al.,* 2008, Rosengrave *et al.,* 2016). Lehnert et al., (2017)

identified in Chinook salmon that ovarian fluid selected for sperm of males that were more distantly related. Conversely, Butts *et al.*, (2012) found sperm in ovarian fluid of a related female had significantly higher velocity this suggests a recognition system for specific sperm genotypes.

The role of ovarian fluid within a species has also been observed to interact with sperm swimming speed and embryo survival (Rosengrave, Montgomerie and Gemmell, 2016).

In order to demonstrate CFC, there must be a clearly hypothesised type of male or sperm that will benefit female fitness. Gene complexes with profound influence on fitness have been explored, with variation at the vertebrate major histocompatibility complex (MHC) identified as a possible region of importance in Atlantic salmon, and some evidence using a paired and balanced in vitro sperm competition design to reveal female sperm selection of males with a genetically similar MHC genotype (Yeates *et al.*, 2009). Contradictory evidence also exists suggesting no effect of non-random mating based on MHC class in Atlantic salmon (Promerová *et al.*, 2017). This finding is supported by Lehnert, (2017) who also found no relationship between MHC II diversity and paternity success. Further research is therefore needed to determine which genes could play an important role in gamete compatibility, as well as developing further insight into the possible role of MHC as a sperm-egg signalling system.

Once sperm have been released into the turbulent water column, they attempt to locate and access the surface of the egg and the single egg micropyle, a narrow canal leading through the chorion (Yanagimachi *et al.*, 2013). The micropyle is the sole point of access to fertilisation in salmonids, wide enough to allow a single sperm to enter the ova at a time (Yanagimachi *et al.*, 1992). Once a sperm cell makes penetrates the micropyle, the resulting activation initiates the block to polyspermy and closure of the canal, preventing additional sperm from entering

the egg (Kobayashi and Yamamoto, 1981). Post-fertilisation salmonid eggs will undergo hardening of the chorion, creating a mechanism to prevent mechanical damage during burial within the redd (Zotin, 1958).

1.3.4 Aquaculture

Atlantic salmon dominate across world aquaculture, with production growing by 43% from 2009-2014 the most significant increase in European aquaculture (FAO, 2015). During the 1970s, efforts were made to develop Atlantic salmon for aquaculture, with mass-production beginning in the late 1980s. Atlantic salmon aquaculture has rapidly developed into a global industry in a relatively short time-frame with production growing from 58,979 tonnes in 1986 to 2,423,000 tonnes in 2018 (Tveteras, Nystoyl and Jory, 2019).

Global large-scale commercial production of Atlantic salmon has developed in Australia, the United Kingdom, Atlantic Canada, Chile, Russia, Pacific US, New Zealand and Ireland. The largest aquaculture producer of Atlantic salmon is Norway, with its extensive coastline and riverine system facilitating development of the Atlantic salmon industry with 1,303,564 tonnes produced in 2014 worth 44,439 NOK million, 56% of global production (http://www.ssb.no/en/jord-skog- jakt-og fiskeri/statistikker/fiskeoppdrett/aar).

Domestication of Atlantic salmon was initiated in Norway as part of a government-led scheme in response to a large decline in the wild salmon farming industry in the 1960s, threatening rural Norwegian communities with economic depression (Sønvisen, 2003). Since its initiation, this programme has successfully domesticated Atlantic salmon over 10 generations of line breeding, promoted by phenotypic and genotypic variance across strains, moderate generation times, and high fecundity (Solberg *et al.,* 2013). Development of commercial traits has focused on improved growth rates (Solberg *et al.,* 2013), carcass

quality (Quinton, McMillan and Glebe, 2005) and reduced premature maturation (Rowe and Thorpe, 1990). Breeding of disease resilience is also under development, with success in producing resistance to infectious pancreatic necrosis (Kjøglum *et al.,* 2008; Houston *et al.,* 2010).

In many measures related to food production of a popular and nutritious product, Atlantic salmon farming has been a great success. However, the rapid development and increasing scale of the industry has resulted in environmental concerns about sustainability, chiefly arising from major issues of sourcing feed, disease spread, and genetic introgression of escaped farm fish into locally adapted wild populations. One focus of this thesis is exploring how we can improve production efficiency in aquaculture under the increasing pressure and demand experienced by the Atlantic salmon industry. In this thesis we explore a range of factors (fertilisation method, external oocyte ageing, mechanical damage, temperature) that may influence low hatching rates in the Atlantic salmon industry to improve overall production with increased early fertilisation and survival rates. Juvenile Atlantic salmon can have poor hatch and offspring survival rates in hatcheries (Johnson, 2003; Sutela *et al.*, 2007; Kocik and Sheehan, 2008; Thayer and Hamlin, 2016). Hatch rates as low as 50% have been observed across Atlantic salmon producing regions (Johnson, 2003; Sutela *et al.*, 2007; Kocik and Sheehan, 2008; Thayer and Hamlin, 2016). Craik and Harvey, (1986) assessed farm hatcheries observing mortality between stripping and the eyed egg stage to be as high as 40%, and up to 11% between eyed egg and hatch. Sutela (2007). Atlantic salmon eggs survival to the eyed stage to be as low as 48%, with 25-50% mortality rates of alevins previously being observed (Bergstrøm.E, 1973; Rye, Lillevik and Gjerde, 1990).

Furthermore, we look to explore the impact the aquaculture industry can have on wild population through assessment of genetic introgression risk at the gamete level. Through

controlled split-clutch and slit-ejaculate experiments we look at the proportional paternity of farm males when competing with wild males for both wild and farm females. This allows the assessment of whether any barriers to genetic introgression have formed at the gamete level after divergence of farm salmon from their wild counterparts through artificial selection over 50 years in the aquaculture industry.

Atlantic salmon are a mobile species kept within farms at unnaturally high densities, and sea cage security produces a containment issue for the industry. Despite improved operational management of farms, such as Norway's implementation of technical standards for sea cages reducing farmed escapees by 400,000 individuals per annum (Jensen *et al.*, 2010), expansion of the industry has kept escapee numbers high (www.fiskeridir.no). Escapees from Norwegian farms alone range from under 200,000 to over 900,000 individuals per annum between 2000 and 2015 (www.fiskeridir.no). These are the known and reported incidences, with actual figures for escapee numbers estimated to be two- to four-fold higher (Skillbrei, Heino & Svasand 2015; Glover et al.,2017). There is a major disparity in numbers of individuals, between Atlantic salmon within aquaculture and the wild resulting in homecoming Norwegian Atlantic salmon outnumbering farmed standing stock 250-700x fold (Gross 1998; Johansen et al.,2011). Therefore, even a low percentage of farmed Atlantic salmon escaping will make up a relatively large proportion of the wild Atlantic salmon population size.

These farmed escapees have become genetically distinct from their wild counterparts due to lack of predation, medical treatment and regular feeding resulting in the relaxation and loss of wild, locally-adapted traits (Bourret *et al.*, 2011). The issue of farmed escapes is that individuals are domesticated enough to lose local adaption (Fleming, 1997), but are still viable within the wild, resulting in farmed salmon spawning with wild individuals (Roberge

et al., 2008). The resultant hybridisation between farmed and wild individuals leads to genetic introgression of farmed genes, being observed to occur across the Atlantic salmon's natural range (Crozier, 2000; Bourret *et al.*, 2011; Glover *et al.*, 2013). The disparity in farmed and wild population size has even resulted in farmed individuals outnumbering wild conspecifics on some spawning grounds (McGinnity et al.,2003; Jonsson & Jonsson 2006). Life history adaptations make genetic introgression potentially more harmful to wild Atlantic salmon populations. Return to natal rivers to spawn produces distinct areas of local adaptation, causing genetic structure across populations, and possible early reproductive isolation (Taylor, 1991; McConnell *et al.*, 1995; Moffett and Crozier, 1996). Farm escape introgression has caused changes in genetic structures towards those allelic frequencies shown by farmed individuals, potentially disrupting and diluting local adaptions and causing ecological destabilisation (McGinnity *et al.*, 2003). These local adaptions are essential to population survival and breeding success, with changes to life history, morphology and physiology adapted to the ambient environmental conditions of the natal river (Brian et al.,1981; Järvi 1990; Pakkasmaa & Piironen 2001; Witten & Hall 2003; Kinnison et al.,2003; Vincent et al.,2013; Gradil et al.,2016; Jensen et al.,2017). Genetic introgression will result in homogenisation and erosion of wild population genetic structures, and loss of local adaption causes outbreeding depression which can reduce the fitness of 'hybrid' individuals and their populations (José Mork 1991; McGinnity et al.,2003; Roberge et al.,2008; Schindler et al.,2010; Glover et al.,2013;). This fitness impact results in vulnerable wild Atlantic salmon populations coming under risk of extirpation and wild Atlantic salmon extinction (McGinnity *et al.*, 2003). Populations at greatest risk of cumulative fitness depression could be subject to an extinction vortex, with those showing high immigration rates from farmed individuals into accessible natal rivers (McGinnity *et al.*, 2003; Glover *et al.*, 2012, 2017; Karlsson *et al.*,

2016). This extinction pressure is further accentuated when the genetic distance between farmed and wild individuals is increased, further increasing the potential for fitness depression (Einum and Fleming, 1997).

Chapter 2

The effect of artificial fertilisation methods on hatch rates and chromosomal abnormality in farm and wild Atlantic salmon

Abstract

Artificial fertilisation and rearing methods are crucial to the production of adult Atlantic salmon in both aquaculture and conservation hatcheries. Despite the widespread reliance on artificial fertilisation, low hatch and survival rates are regularly observed in both aquaculture and conservation hatcheries. This study uses a series of fertilisation trials exploring how common factors within the hatchery process could result in reduced hatch rates. We examined how variation in gamete storage duration, temperature, mechanical damage and whether gametes were mixed before or after the addition of fresh water ('dry' versus 'wet' fertilisation) affected hatch. I also explore how these factors might cause abnormal ploidy, and therefore low survival rates, in both wild and farm Atlantic salmon. Overall, this experiment examined the hatch rates across a total of 5489 wild salmon eggs and 6103 farm eggs. Hatch rates averaged 61.7% farm and 59.2% wild, so there are evidently some important factors reducing potential fertility following hatchery fertilisation protocols. Following our trials, we found wild Atlantic salmon to be less resilient to pre-

fertilisation gamete storage duration and ‘dry’ versus ‘wet’ fertilisation method than farm Atlantic salmon. Fertilising ‘dry’ (before the addition of river water) improved hatch rates by 24.6% compared to ‘wet’ fertilisation (sperm and eggs activated by river water and then mixed) in wild strain fish. Pre-fertilisation gamete storage duration also caused a decline in hatch rate in farm Atlantic salmon of 17.9%. Mechanical shaking and storage temperature did not affect subsequent hatch rates. We also did not observe any significant effect of any fertilisation factor on abnormal ploidy rates among either farm or wild offspring, finding only three cases of triploidy among one thousand one hundred and fourteen offspring screened. Overall, our results suggest that conservation hatcheries must ensure fertilisation takes place ‘dry’, on the day of strip, to ensure high offspring production rates. Similarly, despite being non-significant, farm Atlantic salmon offspring production was highest under these conditions. In further investigation of abnormal ploidy, genotyping of the eyed-egg stage may provide a clearer picture of whether triploidisation is causing early mortality with this studies focus on hatchlings.

2.1 Introduction

Atlantic salmon aquaculture has undergone significant growth in the last 50 years, with worldwide production increasing more than 40-fold from 58,979 tonnes in 1986 to 2,423,000 tonnes in 2018 (Tveteras, Nystoyl and Jory, 2019). Growth is expected to continue further, and forecasted to reach 2,740,000 tonnes per annum by 2021 (Tveteras, Nystoyl and Jory, 2019). In the longer term, the World Bank forecasts production to almost double

to 4,015,000 tonnes per annum by 2030, in line with growing demand for animal protein by an expanding human population (World Bank, 2013).

Although there has been rapid growth and expansion of salmon aquaculture, there are some important barriers that hinder production and sustainability, which must be overcome to meet growing human demand and reach acceptable environmental standards (Olafsen *et al.*, 2012). Over the last 50 years where salmon aquaculture has shown intensive growth, there have been drastic declines in wild Atlantic salmon populations, and salmon farming is one of a number of factors that have been blamed for this major decline (McGinnity *et al.*, 2003; Ford and Myers, 2008; Roberge *et al.*, 2008; Limburg and Waldman, 2009; Schindler *et al.*, 2010). Wild Atlantic salmon peak harvest has declined from over 8 million in 1983 to 3.4 million in 2016 (NASCO, 2019), with Limburg and Waldman, (2009) observing 46 population extirpations to have occurred across Europe and North America. Gross (1998), stated that over 94% of adult Atlantic salmon on Earth were farm fish, and that this percentage is only be expected to grow more with the expansion of salmon farming and declines in wild populations.

In this chapter, I explore routes to improve hatchery and farm production of both wild and farm salmon in order to simultaneously improve conservation hatchery production as well as farming efficiency. There are a range of increasing environmental pressures on Atlantic salmon across their complex anadromous life histories. The quality and extent of freshwater habitat is essential to the survival and overall production rate of natal rivers. Atlantic salmon's freshwater habitat quality is threatened by a range of issues such as pollution (Magee *et al.*, 2003; Hesthagen, Larsen and Fiske, 2011; Forseth *et al.*, 2017a)and man-made structures (Lundqvist *et al.*, 2008; Thorstad *et al.*, 2008, 2017; Nyqvist *et al.*, 2017). In freshwater and at sea, Atlantic salmon face further issues of over-fishing (Hard *et al.*, 2008) ,

parasitic infection including parasites from aquaculture (Johansen *et al.*, 2011; Krkosek *et al.*, 2013) and global warming (Walsh and Kilsby, 2007; Jonsson and Jonsson, 2009).

Farm salmon and the salmon aquaculture industry provide further pressure on wild Atlantic salmon populations through issues including genetic introgression (Crozier, 2000; Fleming *et al.*, 2000; McGinnity *et al.*, 2003; Naylor *et al.*, 2005; Leaniz *et al.*, 2007; Roberge *et al.*, 2008; Jensen *et al.*, 2010; Glover *et al.*, 2012, 2013; Vincent, Kent and Bernatchez, 2013) and farm to wild transferral of parasites and disease (Jones, 2009; Johansen *et al.*, 2011). A parasite of concern is the salmon louse (*L.salmonis)* with serious infestations causing mortality of adult salmon (Jones,2009). Escapee farm salmon can survive and hybridise with wild Atlantic salmon with hybridisation and therefore introgression observed across Atlantic salmons natural range (Glover *et al.*, 2009, 2017, 2020; Karlsson *et al.*, 2016). In brief, farm salmon have become genetically distinct to their wild counterparts losing local adaptations (Bourret *et al.*, 2011). Local adaptations are crucial to the survival and fitness of wild salmon (Brian, Dell and Leggett, 1981; Järvi, 1990; Pakkasmaa and Piironen, 2001; Kinnison, Unwin and Quinn, 2003; Witten and Hall, 2003; Vincent, Kent and Bernatchez, 2013; Gradil *et al.*, 2016; Jensen *et al.*, 2017). Genetic introgression can lead to local adaptions being disrupted or lost with the introduction of farm genes leading to reduced offspring survival and recruitment in future generations.

For Atlantic salmon populations to maintain high populations numbers, survival and production needs to occur in both marine and freshwater environments. To combat the decline in wild Atlantic salmon populations, conservation methods have been put in place including habitat improvement, tighter wild salmon fisheries policies for both commercial

and leisure anglers, and mitigation of farm salmonid impacts (Gausen, 1993; Vøllestad, Skurdal and L'Abée-Lund, 2014; Poirier *et al.*, 2017). Whilst the aquaculture industry has looked to control disease management through pathogen detection, chemical use and introduction of pathogen consuming companion species such as lumpfish, in order to minimise parasite and disease load in farms and onward transfer and impact on wild populations (Jones *et al.*, 2014; Føre *et al.*, 2017). The mitigation of farm salmonids can be further achieved by the continued improvement of sea cage security to prevent mass escapee events that have occurred regularly since the inception of aquaculture (Jensen *et al.*, 2010; Glover *et al.*, 2012).

Where wild populations are at risk due to low fish numbers, leading to the potential for extirpation or extinction, one management solution is the use of conservation hatcheries which can be used to release hatchery-reared offspring to supplement wild populations (Gausen, 1993; Araki and Schmid, 2010). Conservation hatcheries protect juvenile developmental stages from mortality and the environmental/ anthropogenic impacts mentioned above. The creation of juveniles occurs through the use of stripping of adults of ripe gametes and applying artificial fertilisation methods (Gausen, 1993; Araki and Schmid, 2010). The basic principles behind artificial fertilisation in salmonids is the stripping of mature gametes from mature adult fish in the autumn/winter, mixing of stripped sperm and eggs with river water to enable fertilisation, followed by transfer of the fertilised eggs to incubation trays where they are stored in slow-flowing water until hatch the following early spring. Similar *in vitro* fertilisation methods are employed by farms as an integral part of the food production cycle. In both of these contexts, hatchery and farm managers aim to maximise the fertility and hatch success of juveniles through *in vitro* fertilisation and incubation methods.

These *in vitro* fertilisation methods are key to farm Atlantic salmon aquaculture production and supplying the growing demand for this animal protein source. *In vitro* fertilisation is a key method in farms and hatcheries to enable offspring production, because captive fish will not spawn naturally in farm or hatchery broodstock tanks. Research into hatcheries has identified that juvenile Atlantic salmon can have poor hatch and offspring survival rates (Johnson, 2003; Sutela *et al.*, 2007; Kocik and Sheehan, 2008; Thayer and Hamlin, 2016). In the salmon aquaculture industry, egg survival and hatch rates have declined over the last decade. Hatch rates as low as 50% are occurring across Atlantic salmon producing regions (Johnson, 2003; Sutela *et al.*, 2007; Kocik and Sheehan, 2008; Thayer and Hamlin, 2016). Craik and Harvey, (1986) assessed farm hatcheries observed mortality between stripping and the eyed egg stage (when embryos are at an advanced stage) to be as high as 40%, and up to 11% between eyed egg and hatch. Furthermore, Sutela et al.,(2007) measured survival of Atlantic salmon eggs to the eyed stage to be as low as 48%, depending on water conditions. Later in development, 25-50% mortality rates of alevins have been observed (Bergstrøm.E, 1973; Rye, Lillevik and Gjerde, 1990). In an assessment of Scotland's Atlantic salmon farms, survival of ova laid down for hatching was 66%; it is likely that this mortality rate is an underestimate, as only a proportion of ova that had undergone artificial fertilisation will be laid down to hatch. Johnson (2003) further observed that Peboscot strain Atlantic salmon had 46% fry survival rates. These studies demonstrate that, across different Atlantic salmon producing regions, there exists the potential for high mortality at the early development stages from eyed-egg to fry, with a hypothetical combined mortality of over 90% being realistic from fertilisation to the fry stage as shown by the above research.

In natural spawnings, which usually take place in autumn and early winter as temperatures drop and river flow rises, the female digs a nest among the gravel on the spawning redd, and releases eggs into the nest at spawning (Crisp and Carling, 1989; Fleming, 1996; Esteve, 2005; Louhi, Mäki-Petäys and Erkinaro, 2008). Typically, multiple males engage in spawning with each female (Garant, Dodson and Bernatchez, 2001; Weir *et al.*, 2010), releasing clouds of milt as the female releases her eggs and ovarian fluid, with both sperm and ova therefore being activated by water before uniting for fertilisation (Alavi and Cosson, 2006; Fleming et al., 1997). After fertilisation, the female buries her nest by covering over the area with gravel which she moves downstream with the current (Fleming, 1996). Within artificial fertilisation, adult male and female salmon in full breeding condition are 'stripped' of their gametes into dry containers, mixing sperm and ova (or milt and eggs) prior to addition of water to activate the gametes and enable fertilisation (Taylor *et al.*, 2011). Stripping is done by applying abdominal pressure to the fish to express gametes, which readily pass out of the salmon's vent when in full breeding condition (Craik and Harvey, 1984). Care must be taken to avoid any contamination or 'pre-activation' of gametes through accidental contact with water, mucus or urine. Gametes may sometimes have to be stored or transported before fertilisation, for example if the hatchery is situated away from where the adult broodstock are held, or if gametes are distributed out from breeding sites to growing-on farms (Poon and Johnson, 1970; Wagner, Arndt and Roubidoux, 2006). After fertilisation, eggs are poured onto incubation trays, where through-flow keeps the developing eggs oxygenated until they hatch the following spring.

Despite the existence of artificial fertilisation in fish for many decades (Ghittino, 1980; Bellard, 1988), hatcheries and farms can struggle to achieve good fertility and hatch rates (Craik and Harvey, 1986; Thayer and Hamlin, 2016), and we know little about any genetic

consequences of non-natural breeding. Throughout the *in vitro* fertilisation process, a number of factors involved with artificial fertilisation in hatcheries and farms could compromise fertility, hatch and offspring genetics: 1) whether fertilisation takes place 'dry' (gametes mixed before activation by water) or 'wet' (gametes activated by water and then mixed together), 2) duration of gamete storage after stripping but before fertilisation, 3) gamete storage temperature, and 4) post-fertilisation mechanical stress. Using a multi-factorial breeding design, I explore whether these factors within artificial fertilisation in hatcheries and farms might compromise fertility and hatch, and whether there might be consequences for offspring in the form of abnormal chromosome inheritance through the production of partial or fully triploidised offspring with chromosomal abnormalities. Comprehensive experimental studies assessing the influence of artificial fertilisation on low hatchability and later survival of hatchery salmonids are limited; consequently, so is our understanding of 'best-practice' to achieve maximum egg fertility and fry production rates. Artificial fertilisation in Atlantic salmon allows for powerful experimental designs to test fertility issues using split-clutch and split-ejaculate designs to minimise confounding variables between individual males and females and their compatibility. Post-strip *in vitro* fertilisation, together with different experimental treatments, allows replicated and paired comparisons to be made between different storage and fertilisation methods. In this chapter, I therefore describe the results of experiments assessing different combinations of factors on fertilisation and hatch success, including *in vitro* conditions, post-stripping oocyte ageing, storage temperature and mechanical stress.

The Atlantic salmon aquaculture industry predominantly uses the 'dry' *in vitro* fertilisation method, where undiluted milt is directly placed onto eggs and ovarian fluid in the absence of water, and gametes are then mixed together. The popularity of dry fertilisation is

founded upon traditional knowledge that it increases fertilisation rate because the micropyle remains open longer before water activation, increasing the time in which sperm can be added and fertilisation can take place (Leitritz and Lewis, 1976). (Leitritz and Lewis, 1976), hypothesise that the micropyle remains open longer in 'dry' fertilisation as the hardening process is delayed when water is not present (Leitritz and Lewis, 1976). Because fertilisation takes place in a matter of seconds in salmon (Yeates *et al.*, 2007), dry fertilisation is favoured to avoid any delays in combining spermatozoa and ova once activation by water has begun.

Despite the traditional popularity of 'dry' fertilisation for artificial breeding, important aspects of natural spawning will be reduced or removed, including the activation of both gametes by water before they come together for fusion, and the possibility of sperm-egg compatibility (Yeates *et al.*, 2013). In natural fertilisation, a significant amount of ovarian fluid, which comprises 10 to 30% of the volume of the spawned egg mass, is also released into the nest at spawning (Rosengrave *et al.*, 2009). Dry or wet fertilisation could theoretically alter the 'natural' concentration and volume of ovarian fluid surrounding the ova, potentially disrupting or removing any impact on fertilisation, sperm selection, and/or post-fertilisation embryonic development and the shedding of the egg's second polar body (Lahnsteiner, 2002; Hatef, Niksirat and Alavi, 2009; Immler *et al.*, 2014; Alavioon *et al.*, 2017; Promerová *et al.*, 2017). *In vitro* fertilisation further deviates from natural spawning, removing the opportunity for pre-spawning behaviours through hierarchical competition and polyandry (Mylonas, Fostier and Zanuy, 2010; Weir *et al.*, 2010). These are obviously overridden or randomised though hatchery production, though a large-scale experiment investigating polyandry benefits found no improvement in egg fertility or offspring fitness

when eggs were fertilised by single-male versus multi-male sperm batches (Lumley *et al.*, 2016).

Post stripping gamete storage is a further issue that can occur due to artificial fertilisation, with the potential for delays between stripping of broodfish and fertilisation of the ova (Glover *et al.*, 2015). Delays to fertilisation can be caused by several factors, such as variation in the timing of selected broodfish coming into breeding condition, or transportation of unfertilised gametes between hatchery or farm facilities. Although delays to fertilisation can be relatively short through a matter of hours,, they are a major departure from natural spawning where gametes meet within seconds of being shed into freshwater (Withler and Morley, 1968; Poon and Johnson, 1970; Piper et al., 1986; Rizzo, Godinho and Sato, 2003; Allan and Vidal, 2004; Weingartner and Zaniboni, 2006; Samarin *et al.*, 2011). The impacts of gamete storage after stripping and pre-fertilisation have been examined across a range of teleosts, with declines in egg fertilisation and survival seen after just 30 to 60 minutes of storage in freshwater species such as the freshwater characin *Prochilodus marggravii* (Allan and Vidal, 2004; Weingartner and Zaniboni, 2006; Rizzo et al., 2003). In Salmonids, gamete storage can have varying impacts, with pink salmon eggs being viable for only six hours after stripping at 6°C (Poon and Johnson, 1970), whereas sockeye salmon fertility remains stable even after gametes have been stored at 9.9°C for 48 hours (Withler and Morley, 1968).

During storage and transportation, gametes may also be exposed to unnatural temperatures (Wagner, Arndt and Roubidoux, 2006). High pre-fertilisation temperatures can impact upon gamete quality, and therefore fertilisation success and embryonic development (Pankhurst *et al.*, 1996; Aegerter and Jalabert, 2004). In Atlantic salmon, sub-optimal temperatures during vitellogenesis can also negatively affect egg quality (King *et al.*,

2003). Stripping and fertilisation of Atlantic salmon gametes requires handling and occasional transport of gametes, so they may be stored outside the fishes' bodies for hours or more (Krise, 2001), when temperature variation has the potential to impact upon gamete quality. Increasing external storage and whole organism rearing temperature has been shown to exacerbate the impacts of post-strip oocyte ageing, reducing egg quality (Withler and Morley, 1968; Poon and Johnson, 1970; Piper et al., 1986; Rizzo, Godinho and Sato, 2003; Allan and Vidal, 2004; Weingartner and Zaniboni, 2006; Samarin *et al.*, 2011). Standard aquaculture *in vitro* fertilisation practices may also result in mechanical shock to fertilised eggs. After fertilisation, developing eggs are placed into incubation trays within the hatchery system, and there is the potential to introduce mechanical stress to both gametes and subsequent zygotes as they are moved from fertilisation bowls onto incubation trays. Mechanical shock of salmonid embryos during development increases mortality rates, with Crisp, (1990) observing a 14-17% egg mortality of brown trout shocked with 7,700-8,390 ergs (**unit** of energy in the centimetre-gram-second system) immediately after fertilisation, and 50-60% mortality when shocked with 8,000 ergs at 10-20% complete development (eyed-egg stage). Mechanical shock can also cause major non-lethal physiological changes to embryos, so our experiment also measures rates of Unintentional Spontaneous Triploidisiation (UST) (Aegerter and Jalabert, 2004; Flajšhans, Kohlmann and Ráb, 2007; Glover *et al.*, 2015).

I examine how different fertilisation treatments might act on chromosomal abnormality by assessing the incidence of nintentional offspring triploidy. Triploidy can be purposefully induced to create sterile offspring through heat or hydrostatic pressure shock, with 97-100% success using these methods in laboratory conditions (Johnstone 1985; Mcgeachy et al.,1997; Devlin et al.,2010). Implementation of shock occurs immediately after fertilisation,

when the second polar body has yet to be extruded (Benfey, 2001). Failure of extrusion in the zygote post-fertilisation results in the maternal egg component remaining diploid, with a paternal haploid contribution resulting in triploidisation (Benfey, 2016). Controlled application of shock prevents the polar body extrusion process, resulting in triploid salmon being maternally driven (Nomura et al.,2013; Glover et al.,2015; Benfey 2016). Triploidy can also occur spontaneously, which I define here as Unintentional Spontaneous Triploidisation (UST). UST has been observed to occur in the aquaculture environment, at an average rate of 2% across Norwegian farms, and up to 17 - 28% in individual rearing cages (Glover *et al.*, 2015). These figures are likely to be underestimates of UST at egg development and hatch, because triploid salmon have lower survival rates than diploids (Taylor *et al.*, 2011). UST has not been observed to any significant extent in wild river systems (Jørgensen *et al.*, 2018). Triploid Atlantic salmon without special rearing experience have high numbers of abnormalities with 30% of triploid adults having lower jaw deformities and 60% lacking primary gill filaments (Sadler, Pankhurst and King, 2001; Amoroso *et al.*, 2016). Additionally, triploids have reduced flesh quality compared to diploid individuals reducing the quality of the product for the aquaculture industry (Bjørnevik *et al.*, 2004). The causal mechanisms of UST are currently unknown, but triploid individuals, if not reared under special conditions, are both an economic and ethical issue for the aquaculture industry, as well as a potential cause of low hatch rates of artificially bred salmonids if UST contributes to embryo death (Bjørnevik *et al.*, 2004; Ozerov *et al.*, 2010; Fraser *et al.*, 2013).

In this study, I therefore examine the effects of some potential factors that can be common within typical artificial fertilisation processes for farming and breeding salmon. I experimentally examine their impact on the hatch rates of both wild and farm origin Atlantic salmon. I examine: 1) fertilisation methods (‘dry’ versus ‘wet’), 2) post-stripping oocyte

storage duration and ageing, 3) storage temperature, and 4) mechanical shock after fertilisation. I measure and compare the effects of these artificial factors through a controlled and replicated split-clutch and split-ejaculate experimental design within a matrix that controls for between-individual fertilisation variance. As well as hatch rate, I measure how the above factors influence the rate of Unintentional Spontaneous Triploidisation in artificially-bred Atlantic salmon of both farm and wild origin, with an overall aim to improve fertilisation and hatch rates in both the hatchery and farm.

2.2 Materials and methods

2.2.1 Study area and fish groups

The main fertilisation experiment was conducted in Norway at the Institute of Marine Research (IMR) hatchery facility in Matre (84 km north of Bergen) in autumn and winter, 2018. Two strains of Atlantic salmon were used: 1) wild background Atlantic salmon taken from the River Etne (south-west Norway), and 2) one-sea-winter farmed fish taken from Marine Harvest's MOWI strain (12th generation of farm breeding). Farm adults for stripping were provided and maintained by MOWI Ltd, and gametes were fertilised, embryos reared, and all analysed at IMR, Matre. Etne fish were provided from hatchery facilities in which wild caught salmon from the river Etne are reared. All salmon in the experiment were stripped on the day before the first round of fertilisation, with gametes stored on wet ice at 1°C during transport to the Matre hatchery facility. Gametes from ten females and ten males were used in this experiment from both farm MOWI and wild Etne salmon strains (= total 40 adult fish).

Mowi and Etne salmon were utilised as there exists no ancestral link between the farm and wild salmon. The ancestral wild Mowi and Etne salmon strains are from geographically isolated regions of Norway

2.2.2 The fertilisation 'matrix' experiment

In vitro fertilisations using split clutches and ejaculates from individual males and females were conducted so that gamete combinations could be exposed to multiple experimental treatments while equalising adult identity (Figure 1). Due to logistical limitations all experiments were completed within 24 hours of gamete stripping in both farm and wild salmon. In previous studies of internal oocyte ageing and the ageing of milt no impact of 24 hour ageing was observed (Aegerter and Jalabert, 2004).To apply all these different treatments to gamete combinations, eggs from each female were separated into 8 separate batches, each containing an average of 36 eggs (Etne: mean N = 35 (±6.25 SD), MOWI: mean N = 38 (±6.23 SD), overall range = 20-56). Egg batches were photographed before each experimental treatment so that precise number could be counted for each fertilisation trial. For each male, eight 100 µl aliquots of undiluted milt were used to fertilise the eggs of a randomly selected female across all the eight different treatments per male x female combination (Figure 1).

The different treatments were:

1) Variation in post-strip and pre-fertilisation gamete storage temperature at either 1 °**C or** 10 °C**.** Eggs and milt were kept in sealed plastic bags in polystyrene boxes and maintained at either 1°C on wet ice, or at 10°C in a temperature-controlled room for 24 or 72 hours depending on gamete storage treatment.

2) Variation in post-strip and pre-fertilisation gamete storage duration at either 24 hours or 72 hours from strip. Gametes were held as described in 1) above, before being fertilised 'dry' versus 'wet', followed by 'shaken' or 'not shaken' mechanical stress (see below).

3) Variation in fertilisation mode through either 'dry' or 'wet' fertilisation. In dry fertilisation, gametes were mixed (60 seconds) and fertilised before the addition of activating water. In wet fertilisation, gametes were mixed and fertilised after the addition of activating water**.** Eggs were fertilised in 500 ml flat-bottomed plastic bowls (Desertcart.co.uk) by a randomly-assigned sperm sample from a male of the same strain. *In vitro* fertilisations used 100 µl of undiluted milt and 200ml of Matre river water, applying general principles described in Yeates et al., (2014). Dry fertilisation (Johnson *et al.*, 2004) took place by pipetting the milt directly onto the egg batch and mixing gametes together, before adding activating water, while wet fertilisation took place by placing the 100 µl of milt to the side of the egg batch in the fertilisation bowl, and then adding activating water to mix gametes together. Eggs and sperm were left to stand for 120 seconds after mixing of gametes (dry) or once activated with water (wet), to ensure full fertilisation opportunities, before placing into either the mechanical stress treatment. Each of the female's egg batch was fertilised by a different male, generating a total of 10 Etne and 10 MOWI male x female crosses, and 80 + 80 fertilisation trials with each male-female pair producing eight fertilised egg batches.

4) Variation in mechanical stress through fertilised eggs being exposed to a 'shaken' or 'not shaken' treatment (Figure 1). Fertilised eggs within the 'shaken' treatment were exposed to mechanical stress post-fertilisation by placing the bowl containing the fertilised egg batch in 200ml of river water onto an orbital shaker running at 161 rpm for 2 minutes, before being placed into the Matre hatchery.

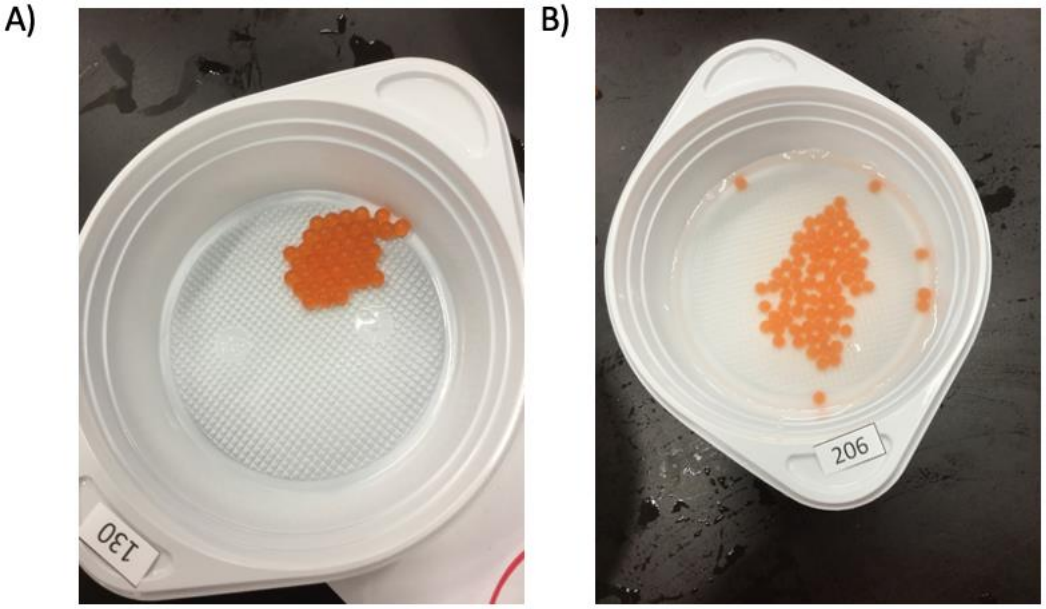

Figure 1: Image showing A) Egg batch pre-fertilisation in fertilisation tray (dry fertilisation method), B) Egg batch post-fertilisation in fertilisation tray with addition of river water (dry fertilisation method).

After experimental treatments, fertilised egg batches were moved to flow-through hatchery trays, with each egg batch being placed into hatchery trays of 45cm^2 with individual egg batches in chambers of 15cm^2 and labelled with a unique code. Water temperatures within hatchery systems were maintained throughout development at ~6°C, with a flow of approximately 10-litres of water per minute per trough containing 7 trays. Egg batches were treated daily with Pyceze^{TM} until the eyed egg stage to prevent fungus. Dead eggs or hatchlings were removed from the trays throughout development. Hatchlings were allowed to develop until the alevin stage prior to first feeding (approximately 20 days post hatch) at which point they were terminated and counted and stored for genetic analysis. Across this fertilisation experiment applying a matrix of treatments, hatching success relative to starting egg number was recorded for a total of 160 Etne individual trials and 160 Mowi trials, with a total of 5489 Etne eggs being fertilised to yield 3268 hatchlings, and a total of 6103 MOWI eggs fertilised to yield 3779 hatchlings.

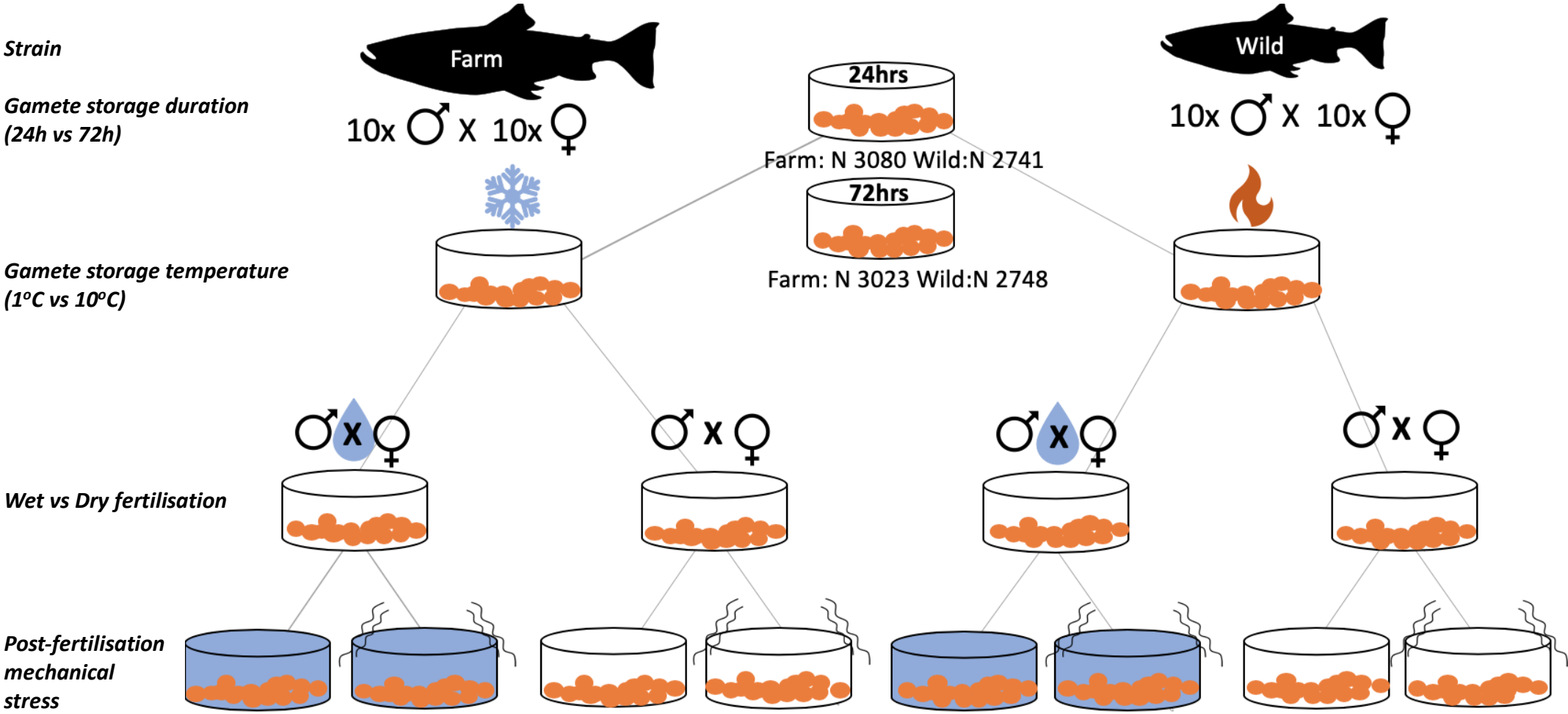

Figure 2: Fertilisation matrix design using four combined treatments of: 1) storage temperature, 2) storage time, 3) fertilisation method, and 4) mechanical stress. Gametes stripped from 10 males and 10 females of either salmon strain were divided to enable a split-clutch and split-ejaculate factorial design. Each female produced 16 egg batches with an average of 36 eggs per batch (Wild 35± 6.25, Farm 38 ± 6.23, range 20-56). Each egg batch was fertilised with 100µl of sperm and was exposed to a combination of the four fertilisation treatments. Eggs were reared to hatch, recording % hatch and assessing ploidy status among offspring. Replication involved 10 MOWI (farm) and 10 Etne (wild) male x female crosses, split into multiple treatments as above. A total of 11,592 ova were used in the experiment, and almost 2000 hatchlings from the different treatments assessed for triploidy.

2.2.3 Hatching success

Throughout development, egg batches were monitored, and dead or infected eggs and early hatchlings removed. At 20 days after hatch, surviving hatchlings in each treatment batch were photographed to count and score hatching success relative to the starting egg number for each of the treatment combinations from Etne and MOWI backgrounds.

2.2.4 Assessment of Unintentional Spontaneous Triploidy (UST)

Ploidy status of individual hatchlings was determined using a panel of established microsatellite loci that have been shown to be informative and accurate in previous studies of salmonids (Glover et al., 2015; Grimholt et al., 2002; Norris et al., 1999; Sánchez et al., 1996; Slettan et al., 1995; Stet et al., 2002; Genbank no. AY372820)**.** DNA was extracted from a single eye of each hatchling using the hotshot genomic DNA preparation method, described in Truett et al.,(2000). In brief, tissue was added to a 96-well plate along with 75µl of alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA). The plate was then incubated for 25 minutes at 95°C, after which 75**µ**l of neutralizing reagent (40 mM Tris-HCl at a pH of 5) was added to halt denaturation post-incubation.

Polymerase chain reaction (PCR) was carried out in a 10 µl volume reaction consisting of 2 µl of DNA (unspecified concentration) and 8 µl of PCR Mastermix composed of the following multiplex primers: forward/reverse primers MHC 1 (Grimholt *et al.*, 2002), MHC 2 (Stet *et al.*, 2002), SSsp3016 (Genbank no. AY372820), SsOsl85-a (Slettan, Olsaker and Lie, 1995), Ssa197-a (Norris et al.,1999) and SsaF43-a (Sánchez *et al.*, 1996). The PCR program took place with denaturation at 94°C for four minutes followed by 30 cycles of denaturation at 94°C for fifty seconds, annealing at 55°C for fifty seconds, extension at 72°C for eighty seconds, and extension at 72°C for 10 minutes. The overall program ran

for two hours and thirty minutes. PCR products were run on an ABI3730 automated sequencer at the Institute of Marine Research (Havforskningsinstituttet) Bergen, Norway. Triploidy was confirmed using microsatellite DNA genotyping and the identification of three clearly identifiable alleles per locus. The number of identifiable alleles per locus was determined using the genotyping software GeneMapper v5.0 (Applied Biosystems). An individual fish was confirmed as triploid if it displayed three clear alleles at two or more genotyped loci, as this has been found to highly accurate in a range of fish and insect species, including successful identification in Atlantic salmon (Liebert *et al.*, 2004; Garner *et al.*, 2008; Hernández-Urcera *et al.*, 2012; Glover *et al.*, 2015).

2.2.5 Statistical analysis

All data analyses were carried out in R-studio using R-version 3.4.1 (2009-2019) (RStudio Team, 2020*)*. Generalised mixed models (GLMMs) were conducted using the 'glmmTMB' package (Bolker,2018). To model percentage hatch rates (hatch rate is defined as the proportion of eggs hatching and surviving 100-120 days post-fertilisation, independent of fertilisation rate in the egg batch) across the multiple treatment combinations, a GLMM was fitted with a beta-binomial error distribution with the number of offspring hatched coded as the response variable. A binomial distribution was used in the model but had a large degree of over-dispersion leading to the use of a beta-binomial model to account for the over dispersion. The main effects model included all explanatory variables of storage duration, storage temperature, fertilisation method and mechanical stress as binary factors. The identification of each male x female pair was fitted as a random effect. Two separate main effects models were constructed for MOWI farm and Etne wild salmon background to identify strain-dependent effects across the combined fertilisation matrix experiment. Separate models were constructed to assess interactions between fixed effects.

Models were constructed as in the main effects model, to assess specific interactions a single interaction between two of the main effects (Temperature x Mechanical stress) was inserted, a separate model to assess interactions was produced for each possible interaction between the four matrix factors.

All GLMM's were assessed for overdispersion using the glmmtmb internal overdispersion parameter for betabinomial family. The "glmmtmb package calculates dispersion by returning the value of ϕ, where the conditional variance is $\mu(1 - \mu)/(1 + \phi)$ (i.e., increasing ϕ decreases the variance.) This parameterization follows Ferrari and Cribari-Neto (2004)

Overdispersion was observed in both the wild strain main effects model (4.87) and the farm strain main effects model (5.89) using the dispersion calculation described above. Removal of the random effect (= pair identification) reduced overdispersion in both the wild (1.49) and farm (2.73) models, while significant main effects remained, so when overdispersion was accounted for, output from the no-random-effect models are shown in Supplementary Table 1. All P-values reported were calculated through the wald t-test within the glmmtmb and lme4 packages.

2.3 Results

2.3.1 Hatch rates across different combined treatments in the fertilisation matrix experiment

Overall hatch rate

Egg hatch rate across all treatments was 59.2% for wild (Etne) egg batches and 61.7% for farm (Mowi) salmon egg batches.

Hatch rate following fertilisation through either 'dry' or 'wet' conditions.

Egg hatch rate in wild (Etne) salmon egg batches was significantly higher when fertilised dry (71.6 %± 3.01) (mean ± S.D.) in comparison to wet (47.0% ± 3.14) across all treatment combinations (Table 2 (a), Figure 3). Egg hatch rate was unaffected by fertilisation method in farm salmon dry fertilisation (62.8% ± 2.94) versus wet fertilisation (60.7% ± 2.98) (Table 2 (b), Figure 3).

Hatch rate effects from post-strip and pre-fertilisation gamete storage duration at either 24 hours or 72 hours from strip.

Similarly, post-strip oocyte ageing resulted in a significant decline in wild strain salmon, with egg hatch rate significantly lower following 72 hours of post-strip sotrage (50.3% ± 4.21) versus 24 hours (68.2% ± 3.23) (Table 2 (a), fig,4), but egg hatch rates of farm salmon were again unaffected by post-strip ageing after 24hrs (64.8% ± 2.80) or 72hrs (58.7% ± 3.10) (Table 2 (b), fig.4).

Hatch rate effects following different post-strip and pre-fertilisation gamete storage temperatures.

Pre-fertilisation storage temperature (1°C: 62.7% ± 3.08, 10°C: 60.8 %± 2.84) had no significant effect on proportional hatch rates of wild egg batches (Table 2 (a)) and farm egg batches (1°C: 57.4% ± 3.94, 10°C: 61.5% ± 3.80) (Table 2 (b)).

Hatch rate effects of post-fertilisation mechanical stress.

The application of mechanical stress to just-fertilised eggs and zygotes had no significant effect on proportional hatch rates in wild egg batches (no shake: 59.6% ± 2.85, shake: 63.9% ± 2.76) or farm egg batches (no shake: 57.8% ± 3.13, shake: 62.7 %± 3.23).

An assessment of interactions between factors in the fertilisation matrix found no significant interaction was observed between any treatment in farm or wild salmon egg batches (Table 2).

2.3.2 Ploidy status

The frequency of unintentional spontaneous triploidisation (UST) did not increase with any of the artificial fertilisation factors, with only a 0.27% triploidy rate found throughout, after genotyping 1,114 individuals (Table 1).

Table 1: Individual farm and wild salmon offspring genotyped from a selection of predicted low impact and high impact artificial fertilisation treatments.

Fry genotyped n=	**Temperature (°C)**	**Shake**	**Ageing**	**Fertilisation**	**Triploid individual n=**
202	1 °C	No	Day 1	Wet	0
245	1 °C	No	Day 1	Wet	0
161	1 °C	Yes	Day 3	Wet	0
167	10 °C	Yes	Day 3	Wet	0
155	1 °C	Yes	Day 3	Dry	1
184	10 °C	Yes	Day 3	Dry	2

Table 2. (A) A summary of the GLMM fixed effects analysis for hatching rates of wild strain individuals under the fertilisation matrix with number of hatched offspring as the response variable and random effect of mated pair (Var (Variance) =1.945). (B) A summary of the GLMM fixed effects for hatching rates of farm strain individuals under the egg fertilisation matrix with number of hatched offspring as the response variable and random effects of mated pair (Var=0.781). The baseline for both GLMM's was set as fertilisation wet, ageing 24hrs, 1°C temperature and no shake

Fixed Effects	**Estimate**	**Standard Error**	**z score**	**Pr(>|t|)**
(A) Wild Etne salmon				
Fertilisation - Dry	1.34	0.436	3.080	0.02 **
Storage ageing - 72hrs	-0.83	0.422	-1.983	0.047*
Temperature - 10 °C	0.38	0.412	0.94	0.350
Shake	0.61	0.411	0.15	0.887
(Fertilisation Dry* Ageing 72hrs)	-0.01	0.597	-0.021	0.983
(Fertilisation Dry* Temperature 10 °C)	0.28	0.626	0.449	0.653
(Fertilisation Dry* Shake)	-0.15	0.607	-0.250	0.802
(Ageing 72hrs * Temperature 10 °C)	-0.33	0.585	-0.573	0.566
(Ageing 72hrs * Shake)	0.02	0.578	0.04	0.966

(Temperature 10 °C * Shake)	0.06	0.582	0.10	0.919

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
(B) Farm MOWI salmon				
Fertilisation - Dry	0.26	0.405	0.652	0.515
Storage ageing - 72hrs	-0.11	0.396	-0.282	0.778
Temperature - 10 °C	-0.01	0.397	-0.03	0.971
Shake	0.61	0.411	1.489	0.136
(Fertilisation Dry* Ageing 72hrs)	0.11	0.542	0.22	0.828
(Fertilisation Dry* Temperature 10°C)	0.04	0.546	0.08	0.934
(Fertilisation Dry* Shake)	-0.02	0.57	-0.04	0.970
(Ageing 72hrs * Temperature 10 °C)	0.249	0.53	0.47	0.640
(Ageing 72hrs * Shake)	-0.43	0.55	-0.79	0.429
(Temperature 10 °C * Shake)	-0.17	0.55	-0.31	0.759

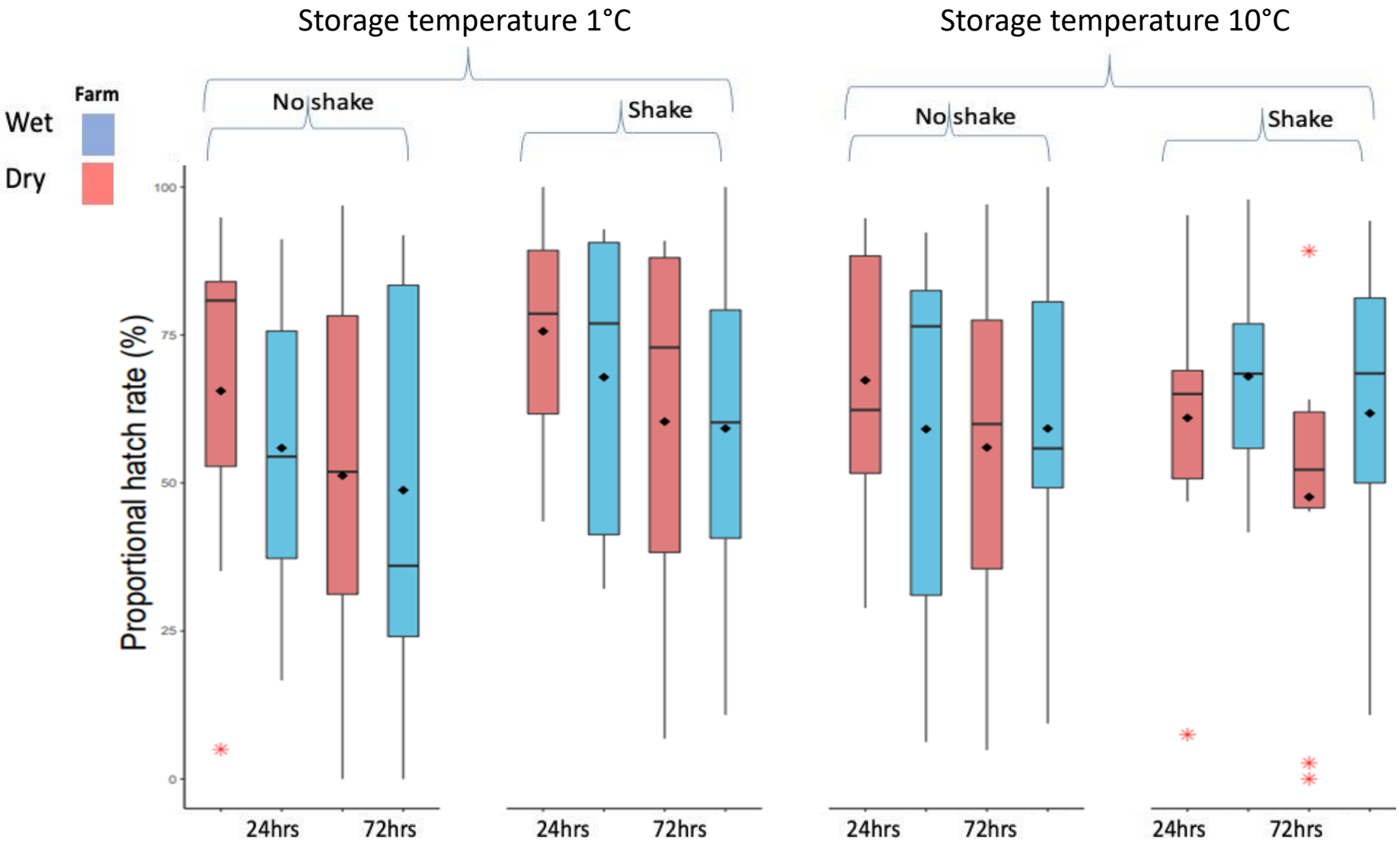

Figure 2: Hatch rates of MOWI farm Atlantic salmon egg batches fertilised under a combination of artificial fertilisation treatments. No significant difference is observed across any single treatment. Fertilisation method: dry (62.8% ± 2.94) (mean ± S.D.), wet 60.7% ± 2.98; post-strip ageing: 24hrs (64.8% ± 2.80), 72hrs (58.7% ± 3.10); storage temperature 1°C (57.4% ± 3.94) or 10°C (61.5% ± 3.80); mechanical stress shake: (63.9 %± 2.76), no shake (59.6% ± 2.85). In total 160 crosses were carried out to produce 3779 hatchlings from 6103 eggs. Boxplots are derived from 10 egg batches produced by 10 separate male x female pairings for each treatment. Mean is represented by a black diamond, median by a black horizontal bar and outliers in a red star.

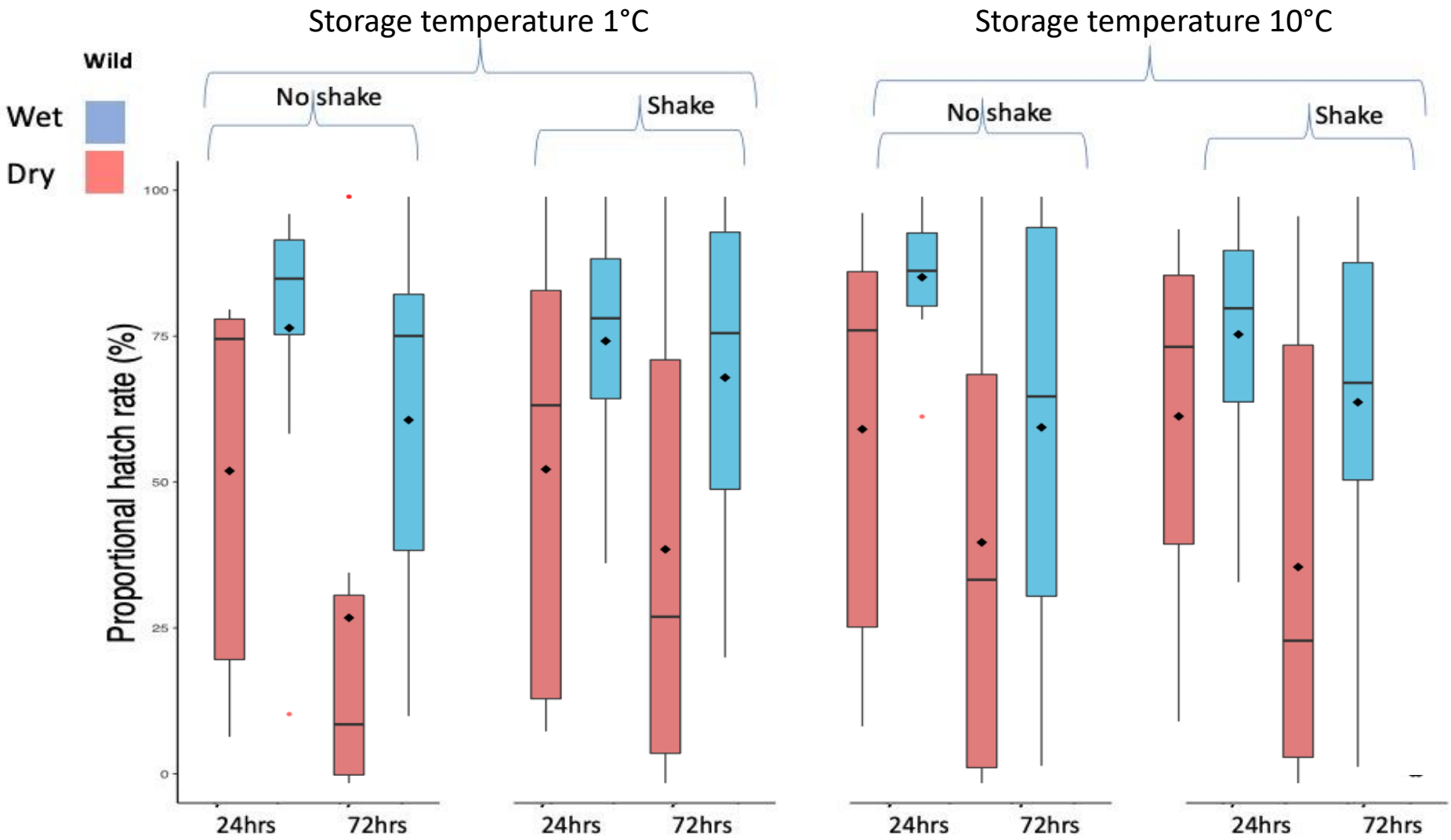

Figure 3: Hatch rates of Etne wild Atlantic salmon egg batches fertilised under a combination of artificial fertilisation treatments. Hatch rate was significantly higher when fertilised dry (71.6 %± 3.01) (mean ± S.D.) then wet (47.0% ± 3.14), and when fertilised 24 hours post-strip (68.2% ± 3.23) when compared to 72 hours post-strip oocyte ageing (50.3% ± 4.21). No significant difference was observed between temperature treatments 1°C (62.7% ± 3.08) or 10°C (60.8 %± 2.84) or mechanical stress shake (63.9 %± 2.85), no shake treatments (57.9% ± 3.24). In total, 160 crosses were carried out to produce 3268 hatchlings from 5489 eggs. Boxplots are derived from 10 egg batches produced by 10 separate male x female pairings for each treatment. Mean is represented by a black diamond, median by a black horizontal bar and outliers in a red filled circle.

2.4 Discussion

In this study we investigated the impact of artificial fertilisation on the fertilisation and early survival rates of farm and wild Atlantic salmon offspring. Using a series of multifactorial trials, we explored experimentally whether possible factors within artificial fertilisation used by Atlantic salmon hatcheries and farms could contribute to reduction in offspring production or chromosomal abnormality. We identified wild salmon to be more sensitive to some factors within artificial fertilisation processes using our fertilisation matrix experiment (varying oocyte-ageing, storage temperature, mechanical damage and fertilisation method) (Fig.1)

In MOWI farm strain salmon, we identify no significant effect of any individual treatment factor on hatch rate (Fertilisation method (difference=2.1%), Post-strip oocyte ageing (6.1%), Storage temperature (4.1%), mechanical stress (4.9%)). By contrast, the hatchery treatments, fertilisation method and post-strip oocyte ageing were observed to significantly affect wild salmon hatch rates. Fertilisation 'dry' of wild salmon produced 24.6% higher hatch rates when compared to 'wet' fertilisation of eggs, and fertilisation on the day of stripping produced 17.7% greater proportional hatch rates than those eggs aged post-strip for a further 48 hours. Maximal hatch rates were achieved for both wild and farm salmonid egg batches when fertilised 'dry' on the day of stripping. No treatment induced a significant increase in UST among yolk-sac dependent fry after evaluation of 1123 individuals.

2.4.1 Farm versus wild strain hatch rates

Under identical hatchery conditions, farm salmon achieved superior hatch rates across all treatments when compared to wild conspecifics, with farm gametes and subsequent embryos demonstrating greater resilience to the artificial spawning and rearing process (Fig. 2 versus 3).

Sperm traits have been observed to diverge under domestication in farm Atlantic salmon, with wild Atlantic salmon having significantly longer flagella (Camarillo-Sepulveda *et al.*, 2016). Domestication can act on gamete biology as well as wider whole animal effects. Our result of farm salmon adapting to the domesticated environment is consistent with research finding farm salmonid's increased resilience to captivity based stressors. Solberg *et al.*,(2013) found domestication to have resulted in the increased resilience of farm salmonids to heightened stress during the captive rearing process. Additionally, Harvey *et al.*, (2016) observed significantly higher survival rates of farm salmonids in hatchery conditions when compared to wild salmonids under a variety of dietary regimes. Through 12 generations of artificial fertilisation and rearing, farm salmonids have experienced both intentional and unintentional selective pressures across a range of traits, including breeding biology. Domestication-mediated selection has resulted in large scale changes to gene expression of farm salmonids (Roberge *et al.*, 2008; Tymchuk, Sakhrani and Devlin, 2009), resulting in rapid divergence of farm genotype and phenotype from wild conspecifics (Gjedrem, Gjøen and Gjerde, 1991; Fleming *et al.*, 1996; Jonsson and Jonsson, 2006; Solberg, Skaala, *et al.*, 2013). Bicskei *et al.*, (2014, 2016) demonstrates that divergence occurs early in development, observing transcriptional differences between farm and wild salmonids in pathways related to organogenesis. The genetic divergence of crucial developmental processes in farm Atlantic salmon is potentially an adaptive response to unnatural or

different stressors introduced through artificial fertilisation. Early developmental genetic divergence of captive salmonids provides a potential explanation for our finding of increased resilience of farm salmonid eggs and embryos to aquaculture fertilisation and husbandry practices.

2.4.2 Fertilisation mode under 'dry' versus 'wet' conditions

We observed no significant difference between fertilisation method on the hatch rate of farm salmonid egg batches. In both wild and farm salmonids, however, the greatest hatch rate was achieved using 'dry' fertilisation, and in wild salmonids the fertilisation method was significant and a major determinant of hatch rate success.

The aquaculture industry primarily uses 'dry' fertilisation, and we confirm this to be superior for offspring production through our experiment. 'Wet' and 'dry' fertilisation methods vary through the timing of river water addition to sperm and eggs, being pre-fertilisation in 'wet' and post fertilisation in 'dry'. Our finding and the view of the aquaculture industry, that 'wet' fertilisation is an inferior fertilisation method, may possibly result from river water pre-fertilisation displacing and diluting ovarian fluid, disrupting or diluting its important role in fertilisation.

In natural spawning, Atlantic salmon undergo synchronous gamete release (Fleming, 1996; De Gaudemar and Beall, 1999), where sperm and eggs are activated in water prior to possible fusion. During female gamete release, ova are accompanied by the expulsion of a large volume of ovarian fluid (Rosengrave *et al.*, 2009), resulting in dominant male gametes being ejaculated into a fertilisation micro-environment with a high concentration of ovarian fluid (Lahnsteiner, 2002; Rosengrave, Montgomerie and Gemmell, 2016). Concentration of ovarian fluid is hypothesised to be at its highest in teleosts surrounding the micropyle, and

on the egg's surface due to its high viscosity and jelly coat (Litvak and Trippel, 1998; Rosengrave et al., 2008; Turner and Montgomerie, 2002). The high concentration of ovarian fluid surrounding eggs is potentially lost in 'wet' fertilisation, but retained in 'dry' fertilisation, as ovarian fluid is not added to egg batches in the wet condition, with the only ovarian fluid present being that which was introduced within the small egg batch.

Ovarian fluid plays an important role in fertilisation, acting as a potential buffer to environmental conditions (Hatef, Niksirat and Alavi, 2009), preventing disease (Jensen *et al.*, 2009) and importantly acting as a mechanism of post-copulatory sexual selection (Satake *et al.*, 2006; Gasparini and Pilastro, 2011; Medina *et al.*, 2012; Alonzo, Stiver and Marsh-Rollo, 2016; Devigili *et al.*, 2018, Yeates et al). The potential loss of these important functions may provide an explanation for the observed difference in hatch rates of wild salmonids under 'wet' versus 'dry' fertilisation conditions.

The influence of ovarian fluid as a mechanism of sexual selection has been observed across a range of fish species including salmonids: Brown trout (Hatef, Niksirat and Alavi, 2009) , Arctic charr (Turner and Montgomerie, 2002) , chinook (Rosengrave, Montgomerie and Gemmell, 2016) as well as Zebrafish (Wilson-Leedy, Kanuga and Ingermann, 2009) and guppies (Gasparini and Pilastro, 2011). Ovarian fluid has physical and biochemical constituents that can modify sperm velocity , longevity and swimming direction (Satake *et al.*, 2006; Gasparini and Pilastro, 2011; Medina *et al.*, 2012; Alonzo, Stiver and Marsh-Rollo, 2016; Devigili *et al.*, 2018). Dilution and displacement of ovarian fluid is likely to impact upon pre-fertilisation and potential post-fertilisation biological function. Lahnsteiner, (2002), observed ovarian fluid's role in stabilising gametes was lost once diluted at a ratio of 1:8 ovarian fluid to water. In the current study 200ml of river water was added with ~1ml of

ovarian fluid, resulting in a far higher (~1:200) dilution ratio than has been identified to be sufficient to neutralise any role of ovarian fluid.

Lehnert et al (2017), observed that fertilisation can take place without ovarian fluid in Atlantic salmon using 'wet' fertilisation. In salmonids though, ovarian-fluid mediated sperm selection has been identified to occur both between species (Yeates *et al.*, 2013) and between males of the same species (Alonzo et al., 2016; Butts et al., 2012; Lehnert et al., 2017; Rosengrave et al., 2016, 2008). Rosengrave (2016), identified ovarian fluid's role in inter-ejaculate sperm selection to be adaptive, with cryptic female choice enhancing fitness of embryos and increasing offspring survival early in development. In assessment of ovarian fluid increasing embryonic survival, Hatef (2009) observed survival to the eyed embryo stage to be higher after activation of sperm in ovarian fluid than freshwater in brown trout.
A growing area of interest in post-copulatory sperm selection is the influence of within-ejaculate selection on offspring fitness. Previous research has identified intra-ejaculate selection to have a significant effect on the life history of offspring (Immler *et al.*, 2014; Alavioon *et al.*, 2017, 2019). Ovarian fluid is a promising mechanism of sperm selection within-ejaculates, facilitating selection for sperm that will confer enhanced survival on subsequent offspring. Within-ejaculates, large variation is observed between the phenotype of sperm cells (Joly *et al.*, 1989; Immler *et al.*, 2014; Bennison *et al.*, 2015; Hemmings, Bennison and Birkhead, 2016). Research into intra-ejaculate selection has identified phenotype selection to result in significant changes to some offspring life history traits. The influence of intra-ejaculate selection on offspring is further observed in Atlantic salmon, with Immler *et al.,*(2014) observing selection for sperm with intermediate longevity producing offspring with rapid development. One potential explanation for wild salmon's

increased fertilisation and survival rates in ‘dry’ fertilisation is ovarian fluid’s role in selecting the optimal sperm within an ejaculate; in ‘wet’ fertilisation this selection mechanism would be lost.

Alternatively, or in addition to sperm selection, ovarian fluid may act as a protective mechanism to gametes both from osmotic stress and disease. Osmotic stress activates gametes, but thereafter has the potential to cause damage (Kholodnyy *et al.*, 2020). Ovarian fluid’s high ionic concentration (Hatef, Niksirat and Alavi, 2009) could act as a buffer to rapid changes in osmotic pressure, reducing stressors during synchronous gamete release. Ovarian fluid’s stabilising role in salmonids has been previously observed, with eggs losing fertility after 1 minute in water (Billard *et al.*, 1986), but remaining fertile for more than 10 minutes in ovarian fluid (Lahnsteiner, 2002). Jensen *et al.,*(2009) identified a further potential role in Atlantic cod, with ovarian fluid containing immune transcripts that are hypothesised to prevent bacterial colonization of eggs post-fertilization. In ‘wet’ fertilisation, therefore, the protective role of ovarian fluid would be lost, resulting in the potential for egg quality to diminish, thereby reducing reproductive success.

We observed no significant difference between wet versus dry fertilisation methods on fertilisation and survival rates in farm salmonids. The key difference between these methods is the timing of river water’s addition to gametes, and its potential impact upon ovarian fluid location and concentration. In farm salmonids, the lack of difference between wet and dry indicates that the potential loss of ovarian fluid’s post-copulatory role has a reduced effect on farm fish in comparison to wild Atlantic salmon. Similarly to this study, Beirão et al.,(2014) identified that low concentrations of ovarian fluid in wild Atlantic cod confered increased sperm swimming linearity and velocity, while ovarian fluid in farm cod resulted in a marked decrease to sperm linearity and velocity, suggesting that ovarian fluid

quality or sperm sensitivity had decreased under captive rearing and resulting in reproductive dysfunction. Artificial fertilisation in Atlantic salmon aquaculture over 12/13 generations is predicted to have resulted in relaxed sexual selection, and this may have resulted in the degradation of ovarian fluid as the importance of its role has been diminished in the artificial fertilisation process, and therefore so has the potential for its effect on farm salmon's reproductive success.

2.4.3 Post-strip and pre-fertilisation gamete storage duration

In our assessment of post-strip ova storage effects, we observed a significant negative incluence on the hatch rates of embryos in Etne wild fish under longer storage duration. The impact of post-strip storage duration and/or temperature varies across different salmonids. Piper et al.,(1986) observed chinook salmon eggs stored at 1°C for 48 hours to have 47% egg mortality compared, with eggs stored at 10°C being viable for only two hours (Barnes, Sayler and Cordes, 1999). In contrast, Withler and Morley (1968) found no decline in fertility of sockeye salmon stored at 9.9°C after 48 hours, and only after 70 hours did fertility decline when stored at 9.9°C. Poon and Johnson (1970), observed pink salmon eggs to remain fertile for only six hours at 6°C , whilst Withler and Morley (1968) showed pink salmon ova to remain fertile for over 46 hours when stored at 3.2°C. Samarin (2011) showed pikeperch (*Sander lucioperca*) to have increased embryonic failure with increased post-strip gamete storage time; this failure was far greater when stored under higher temperatures. Interestingly, in our experiment a negative effect of post-strip ovum ageing was observed at both 1°C and 10°C, indicating that the ageing process is causing the negative decline in hatch rate, and not the relationship between ageing and temperature, with temperature having a non-significant effect on farm and wild salmonids in this experiment.

Ageing through storage of ova from farm strain females did not result in a significant reduction to hatch rates, indicating resistance to the impacts of post-strip storage that can occur during artificial fertilisation. Delays between killing of broodfish and fertilisation are expected to occur during peak production in aquaculture (Glover *et al.*, 2015), producing a potential selective pressure on farm embryos to resist unnatural post-strip ageing over 50 years of farm production.

2.4.4 Post-strip and pre-fertilisation gamete storage temperature

No significant differences in the hatch rates of wild or farm strain salmon were observed when pre-fertilisation storage temperatures differed between 1°C and 10°C. Similarly, Bonnet, Jalabert and Bobe (2003) observed *in vitro* storage of unfertilised rainbow trout eggs for 48hrs at 12°C to have no significant impact on developmental success of embryos once fertilised compared with eggs stored at 1°C. Norwegian farm and wild unfertilised ova resisted our high temperature treatment (10°C) indicating a natural adaptation. Adaptation to the higher storage temperature treatment may result from internal storage temperatures within the coelomic cavity ranging from 10-12°C in nature (Aegerter and Jalabert, 2004). Whilst at these temperatures, hatch rates can remain at 73% for 14 days post-ovulation before being manually stripped, as shown in our assessment of post-ovulatory oocyte ageing (Chapter 3: The effect of post-ovulatory oocyte ageing on hatch rate and unintentional triploidy in farm Atlantic salmon). During and immediately after spawning, unfertilised ova can also experience post-strip temperature fluctuations in river water temperature. The Norwegian river monitoring programme observed river temperatures during October (start of peak spawning) to reach 9.1 - 9.8°C in the Vosso, Vikedalselva,

Bjerkreimselva, Otra, and Drammenselva rivers. In the Glomma, Alna, Skienselva, Storelva, and Orrelva rivers, October water temperatures can be 10.4 - 12.1°C at least. (The Norwegian river monitoring programme- water quality status and trends (2017)).Our high temperature treatment is therefore within a range that is occasionally encountered in nature, with future work exploring how temperatures above 12^{o}C might impact fertilisation and development in Atlantic salmon.

2.4.5 Mechanical stress

Despite anecdotal evidence that hatcheries and the aquaculture industry are careful to avoid mechanical stress for eggs after fertilisation, we observed no impact of post-fertilisation shaking on the hatch success of farm and wild salmon fertilised eggs. Our findings are supported by previous research on hatchery practice identifying salmonids eggs to withstand mechanical stress for the first 48 hours after fertilisation (Piper et al., 1986). Crisp (1990) also observed minimal impacts of mechanical stress surrounding the period of fertilisation. The timing of any mechanical shock is important, with Crisp (1990) identifying that shock sensitivity peaks on reaching the 20% embryo development stage in Atlantic salmon. In Pacific salmonids, sensitivity has been observed to increase steadily post-fertilisation, with a log linear increase through development (Jensen and Alderdice, 1989). The resistance of gametes to mechanical shocks is likely an adaptive response to natural mechanical stressors introduced during the release of eggs into the nest, and the burial of gametes in the redd with gravel soon after spawning. We therefore find that just-fertilised Atlantic salmon eggs are resistant to any damage from mechanical stress induced by artificial fertilisation, when eggs are poured into incubation trays.

2.4.6 Conservation hatcheries versus the aquaculture industry and management recommendations

The results of this study show that unnatural fertilisation factors that might affect gametes, such as handling, storage and fertilisation mode, have a generally non-significant impact on the proportional hatch rates of MOWI farm strain salmon. However, a longer post-stripping egg storage duration, and the application of 'wet' fertilisation methods, both reduced the hatch rates of wild Etne salmon. In re-stocking programmes, conservation hatcheries attempt to produce 'living gene banks', both to enhance and re-establish vulnerable populations (Gausen, 1993). Research into hatchery stocking has identified that the artificial rearing environment can produce rapid changes in phenotype and genotype of salmonids, reducing survival rates and fitness of re-introduced individuals (Araki, Cooper and Blouin, 2007; Araki and Schmid, 2010). The combination of vulnerable wild populations and sub-optimal rearing programmes increases the importance of maximising production through successful artificial fertilisation. To maximise hatch rates, our experiment allows some practical recommendations. Firstly, we suggest that fertilisation should take place 'dry', with eggs bathed in some ovarian fluid. Additionally, all eggs should be fertilised on the day of stripping to reduce possible ageing or storage impacts on the unfertilised ova. Storage temperatures do not seem to matter between 1^{o}C and 10^{o}C, and some mechanical damage can be tolerated through the first few minutes following fertilisation.

Despite Farm Atlantic salmon hatch rates showing no significant difference between our experimental treatments, hatch rates were maximised when egg batches were fertilised 'wet' (a 2% increase), with minimal post-strip egg storage duration: a 6% increase. In addition, we observed in Chapter 3 that Atlantic salmon ova were viable and showed no

deleterious hatch rates when eggs were retained for up to 14 days within the female after ovulation (Chapter 2: internal ovum ageing). Our findings therefore show that eggs of MOWI farm strain females remain viable for far longer and at higher percentages when retained in the coelomic cavity (14 days), then when stored externally post-strip, after the female broodfish has been killed (48hrs). Therefore, farms and hatcheries, if faced by mismatched timing that could risk prolonged storage of eggs after stripping, could maximise production rates by allowing retention of ova within the female until fertilisation can be achieved on the same day.

Overall, therefore, our findings suggest that farm strain salmon gametes have become more resilient to artificial handling practices than their wild strain conspecifics. Despite greater resilience to the current experimental treatments, however, there still exists a significant 20-40% failure-to-hatch rate in both farm and wild egg batches. One possibility that we hypothesised before the experiments, was that some embryo mortality could have resulted if embryos had experienced unintentional triploidisation through artificial fertilisation practices. Mechanical shaking, varying temperatures and storage durations, as well as 'dry' versus 'wet' fertilisation methods, might have created conditions that slowed the shedding of the egg's second polar body, leading to chromosomal abnormalities for embryogenesis. However, our study found no evidence that triploidy had occurred when screened at the hatched fry stage (Table 1). Although it is conceivable that UST was not induced by artificial fertilisation, one possibility is that UST does occur, but kills offspring during embryogenesis, explaining the 20 to 40% failed hatch rates, especially under 'wet' fertilisation conditions. This possibility is now being explored by new experiments conducted by Marco Graziano, and there is indeed evidence for the existence of UST in the embryo stage, which is currently under further analysis and checking.

2.5 Supplementary material

Table 4 (A). Summary of the GLMM fixed effects analysis for hatching rates of Etne wild strain gametes through the full fertilisation matrix experiment, with number of hatched offspring as the response variable. (B) Summary of the GLMM fixed effects analysis for hatch rates of MOWI farm strain gametes through the full fertilisation matrix experiment, with number of hatched offspring as the response variable. The baseline for both GLMMs was set as fertilisation mode wet, post-strip ageing 24hrs, 1°C temperature, and not shaken. No random effect was coded in this model due to the random effect causing overdispersion, removal of the random effect allows assessment of normally distributed data.

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
(A) Wild Etne salmon				
Fertilisation - Dry	0.94	0.203	4.656	<0.001***
Storage ageing - 72hrs	-0.58	0.201	-2.885	0.004*
Temperature - 10°C	0.06	0.199	0.282	0.777
Shaken	0.09	0.199	0.499	0.618
(Fertilisation Dry * Ageing 72hrs)	0.61	0.399	1.522	0.128
(Fertilisation Dry * Temperature 10°C)	-0.21	0.398	-0.508	0.611
(Fertilisation Dry * Shaken)	-0.01	0.398	-0.022	0.982

(Ageing 72hrs * Temperature 10°C)	-0.31	0.398	-0.785	0.432
(Ageing 72hrs * Shaken)	0.23	0.398	0.595	0.552
(Temperature 10°C * Shaken)	-0.32	0398	-0.827	0.408

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
(B) Farm MOWI salmon				
Fertilisation Dry	0.11	0.168	0.660	0.510
Ageing 72hrs	-0.165	0.169	-0.979	0.327
Temperature 10°C	-0.161	0.168	-0.958	0.338
Shaken	0.159	0.169	0.934	0.350
(Fertilisation Dry * Ageing 72hrs)	-0.12	0.337	-0.358	0.720
(Fertilisation Dry * Temperature 10°C)	-0.461	0.337	-1.368	0.172
(Fertilisation Dry * Shaken)	-0.21	0.34	-0.614	0.539
(Ageing 72hrs * Temperature 10°C)	-0.03	0.33	-0.09	0.926

(Ageing 72hrs * Shaken)	-0.24	0.33	-0.79	0.427
(Temperature 10°C * Shaken)	-0.17	0.55	-0.31	0.759

Chapter 3

The effect of post-ovulatory oocyte ageing on hatch rate and unintentional triploidy in farm Atlantic salmon

Abstract

In the majority of fish species, reproduction is achieved within conservation hatcheries or aquaculture using in vitro fertilisation following manual stripping of gametes from mature adults. This manual stripping means that there may be temporal mismatches between when eggs are released from the female coelomic cavity versus when natural spawning might take place. Checking and stripping causes fish stress, so their frequency is balanced against that need to monitor the maturity timing correctly and maximise fertility and hatch. There is therefore ample opportunity through artificial breeding in salmon for eggs to have been ovulated by the female, but 'aged' in the coelomic cavity before stripping, and this might lead to reduced fertility which is a general problem for salmon farming. If, for example, checking and stripping takes place weekly through the breeding season, eggs could age for seven days in the female after ovulation. In this study, we examine the consequences of post-ovulatory oocyte ageing in farm salmon for offspring hatch rate and ploidy disruption. In the artificial aquaculture environment female Atlantic salmon retain eggs within the coelomic cavity as they are unable to spawn within the aquaculture environment. The inability to spawn means farm salmon are manually stripped. The manual stripping process can cause distress and damage to individuals, to prevent post-ovulatory oocyte ageing the stripping process may have to occur multiple times. Post-ovulatory oocyte ageing can occur when there is mismatch in timing between ovulation and manual stripping causing impacts to egg viability. We also look to identify the influence of post-ovulatory oocyte ageing on rates of abnormal ploidy in offspring. The issue of post-ovulatory oocyte ageing is accentuated by the continued intensification of the Atlantic salmon aquaculture industry. In

this study we partially stripped farm Atlantic females and males 1, 14 and 21-days after female ovulation. At each stage fertilisation of egg batches took place, we then incubated egg batches to the hatchling stage allowing us to assess hatch rates and abnormal ploidy of offspring. We found the hatch rate of Atlantic salmon to not significantly decline up to 14 following ovulation, but there was a significant decline by the time eggs had aged for 21 days within the female coelomic cavity following ovulation. Atlantic salmon ova in this study are therefore able to resist any deleterious effects of post-ovulatory oocyte ageing far longer than most teleosts previously studied. Triploidy was not observed to occur at any significant rate between day 1 and day 21 internal aged oocytes at the fry stage. This indicates that the farm aquaculture industry would maximise production rates by leaving ova within the coelomic cavity. This is opposed to the common held belief in the industry that eggs will deteriorate rapidly with repeated checks and stripping events for females being potentially more detrimental.

3.1 Introduction

Atlantic salmon is an essential future source of animal protein with growing demand from the human population. The World Bank predicts that demand and growth will continue to rise to a production of 4,015,000 tonnes per annum by 2030 (World Bank, 2013).

This rapid development and growth of the industry has encountered some barriers to production, which need to be overcome to meet growing human demand (Olafsen *et al.*, 2012). Fertility and gamete quality pose a major obstacle to future growth and expansion (Bromage,1992; Bobe & Labbé 2010). Intense domestic selection, an unnatural aquaculture rearing environment, and artificial methods of reproduction have combined to create

reductions in gamete quality so that maximising the production of offspring is not always achieved. Craik & Harvey's (1986) assessment of farm hatcheries observed embryonic mortality between stripping and the eyed egg stage to be as high as 40%, with a further loss of up to 11% between eyed egg and hatch. Later in development, mortality rates of alevins have been observed to be as high as 25-50% (Bergstrøm.E, 1973; Rye, Lillevik and Gjerde, 1990). It is likely that much higher mortality occurs at these early stages within Atlantic salmon aquaculture, but these data are not readily available for these developmental stages. This is a persistent and systemic issue for Atlantic salmon aquaculture in both Europe and the United States, with fertility, egg hatch and survival rates declining over the last decade. Hatch rates as low as 50% have been observed in both regions, contributing to falling production, potential loss of important selection lines, and increasing expenditure (Johnson, 2003; Sutela *et al.*, 2007; Kocik and Sheehan, 2008; Thayer and Hamlin, 2016). Sutela et al., (2007) observed survival of farm Atlantic salmon in a Finnish hatchery to the eyed-egg stage (138 days since fertilisation) to range between 48%- 69% depending on water quality and hand picking of dead eggs. Johnson, (2003) further observed that Peboscot strain Atlantic salmon had 46% fry survival rates. There are therefore fertility issues within salmon aquaculture and hatcheries which need to be addressed, this chapter explores the impact of post-ovulatory egg ageing on breeding success, when females in the hatchery are not stripped for artificial fertilisation at a time when they would naturally choose to spawn.

A major factor affecting survival of early developmental stages in salmonids is gamete quality, particularly that of the ova. Egg quality in the aquaculture industry is defined as those exhibiting low mortality at fertilisation, the eyed-egg stage, and during the hatch and first feed stages (Bromage *et al.*, 1992; Brooks, Tyler and Sumpter, 1997). Egg quality can

vary widely across different aquaculture systems (Bromage *et al.*, 1992; Brooks, Tyler and Sumpter, 1997). In the captive rearing environment, artificial husbandry practices can have a significant impact on egg quality. Pre-ovulation, brood stocks environmental conditions (diet, temperatures , photoperiod), spawning induction method and acute stress have all been identified to impact egg quality (Mylonas et al., 2010; Sprague et al., 2016; Sutela et al., 2007). Post-ovulation, internal and external oocyte ageing, gamete handling and water quality of hatchery systems can result in lower survival rates of both eggs and the early developmental stages (Brooks,1997; Bobe and Labbé, 2010).

A basic factor that can impact ova quality within aquaculture is post-ovulatory oocyte ageing (Samarin and Miroslav Blecha, Dmytro Bytyutskyy, 2015). In Atlantic salmon aquaculture, post-ovulatory oocyte ageing occurs when ovulated oocytes are retained in the coelomic cavity for longer periods than would be natural, due to an inability to spawn within the aquaculture rearing environment (Zohar and Mylonas, 2001). Farm salmon are manually stripped in the aquaculture production cycle, when eggs and milt are manually extruded from ripe adults for *in vitro* fertilisation. As stressful handling of adult fish is kept to a minimum, there can be a mismatch between the natural timing when a ripe female would naturally spawn, and when she is manually stripped, resulting in the potential for post-ovulatory oocyte ageing. This potential for over-maturation is likely to increase with continued intensification of the Atlantic salmon aquaculture industry. The current consensus in Atlantic salmon aquaculture is that Atlantic salmon hens should be stripped as soon as possible after ‘ripening’, when eggs are released into the coelomic cavity and are easily released from the vent with light pressure (Jobling, 2002).

Post-ovulatory oocyte ageing is known to negatively impact upon the viability of eggs and embryos. The impacts of post-ovulatory oocyte ageing have been observed in a range of fish

species including freshwater species: pike perch (Samarin and Miroslav Blecha, Dmytro Bytyutskyy, 2015), common carp (Boulekbache *et al.*, 1989) Capsian kutum (Samarin *et al.*, 2011) ; anadromous rainbow trout (Bonnet, Jalabert and Bobe, 2003; Aegerter and Jalabert, 2004) and a catadromous anguillid, Japanese eel (Nomura et al., 2013). During post-ovulatory oocyte ageing, there is a period of degeneration in which a number of physical and chemical changes take place, impacting upon fertilisation and offspring survival rates (Samarin et al., 2015). Post-ovulatory oocyte ageing causes a sharp decline in fertilisation and survival rates after hours in pike perch, common carp and Japanese eels (Boulekbache *et al.*, 1989; Nomura *et al.*, 2013b; Samarin and Miroslav Blecha, Dmytro Bytyutskyy, 2015), whilst declines in rainbow trout have been observed after 7-days (Aegerter and Jalabert, 2004). During post-ovulatory oocyte ageing, further developmental issues are observed with increased larval malformation (Aegerter and Jalabert, 2004) and ploidy anomalies (Aegerter and Jalabert, 2004; Flajšhans et al., 1993; Nomura et al., 2013; Samarin et al., 2016). Incidences of triploidy have been observed to increase with oocyte ageing in rainbow trout held at 12°C having 0% triploids at 0 days post ovulation (DPO), whilst at 21 DPO the incidence of triploidy was 30-32% (Aegerter and Jalabert, 2004). In the Japanese eel, normal diploid individuals made up 94.2% of offspring when eggs were fertilised immediately, whilst eggs that were retained in the body cavity for 4hrs had a normal diploid rate of 56.7% (Nomura et al., 2013). Given that abnormal offspring ploidy can occur in Atlantic salmon, this study explores whether oocyte ageing could disrupt genetic development in the zygote. The issue of ploidy anomalies has been identified as a new gamete quality and hatch rate issue that could be associated with post-ovulatory oocyte ageing (Glover *et al.*, 2015; Samarin and Miroslav Blecha, Dmytro Bytyutskyy, 2015). In farm Atlantic salmon, triploidy can be purposefully induced, but it has also been observed to occur spontaneously, referred

to as unintentional spontaneous triplodisation (UST). UST has been observed to occur in the salmon aquaculture environment, occurring at an average rate of 2% across Norwegian farms, and up to 17-28% in specific rearing cages (Glover *et al.*, 2015). Current estimations likely undervalue rates of triploidy through the early developmental stages, since Atlantic salmon triploids show elevated mortality under conventional aquaculture conditions (Fraser *et al.*, 2013; Hansen *et al.*, 2015). UST has not been observed to any significant extent in wild river systems (Jørgensen *et al.*, 2018). The causes behind UST in Atlantic salmon are currently unknown, but they present both economic and ethical issues for the industry, and a potential cause of low hatch rates and/or mortality in artificially-reared salmonids (Bjørnevik *et al.*, 2004; Ozerov *et al.*, 2010; Fraser *et al.*, 2013).

Comprehensive experimental studies assessing the influence of husbandry practices on low hatchability of hatchery salmonids are limited, so our understanding of 'best-practice' to achieve maximum production rates is also curtailed. Here, I report on an experiment to assess the impact of post-ovulatory oocyte ageing on hatch rates in farm Atlantic salmon. External fertilisation allows for robust experiments using split-clutch and split-ejaculate paired designs. The paired design involved all females being fertilised by every male separately at each oocyte ageing period. This will minimise confounding variables such as between-individual variation in male and female fertility, and reproductive compatibility between mating pairs. In this study we therefore examine the effect of post-ovulatory oocyte ageing on the survival and hatch rate of Atlantic salmon eggs and embryos. We part-stripped individual females at three separate times across a 21-day period following ovulation. In parallel to measures of hatch rate, we also assess whether post-ovulatory oocyte ageing results in unintentional spontaneous triploidisation. The study will assess the

importance of achieving accurate measures of ovulation timing, and speed of subsequent stripping, to maximise fertility and hatch rates within Atlantic salmon aquaculture.

3.2 Materials and methods

3.2.1 Study area and fish groups

The experiment was conducted in Norway at the Institute of Marine Research in Matre (84km north of Bergen) and a MOWI hatchery centre (south of Bergen), south-west Norway. Females for stripping were provided and maintained by MOWI Ltd, and gametes were fertilised, reared and analysed at IMR, Matre.

MOWI farm strain Atlantic salmon (12th generation) were used, with individuals coming from MOWI brood stock. To assess post-ovulatory oocyte ageing, ten females were partially stripped three times (on the 11th, 21st and 31st of January 2018), timed to align with 1, 14 and 21 days after ovulation. At each stripping event, milt was collected from five MOWI males, and the same individual males were used throughout the experiment. To preserve the same female x male crosses throughout, all individuals were fin and pit tagged.

3.2.2 Ageing fertilisation protocol

Once stripped, gametes were transported to the Institute of Marine Research hatchery facility in Matre, north of Bergen. Gametes were transported at each occasion on wet ice in

polystyrene boxes at 1°C. Prior to fertilisation, photographs were taken of each egg batch to count egg number.

Fertilisation experiments took place at Matre within 2-4 hours of stripping. Fertilisation was achieved by pipetting 1ml of undiluted milt from a single male (n=5) onto egg batches in 500 ml bowls containing 62 to 149 eggs (mean= 99) from each female (n=10) with gamete mixing for 60 seconds (fig.1), and then adding 200 ml of river water for another 60 seconds. A total of 139 crosses were completed in this experiment, after controlling for fertilisation failure. Mowi male 9002 was removed from the analysis, with male 9007 removed from the day 1 treatment, due to failure to produce any offspring. Additionally, the cross between females 5214 and male 9004 at day 21 was removed for failure to produce offspring. This resulted in 40 crosses at day 1, 50 at day 14 and 49 at day 21.

After fertilisation, egg batches were moved to flow-through hatchery trays with individual rearing chambers, each labelled with a unique code. Hatchery conditions and treatments are as described in Chapter 1 (Hatchery matrix). Water temperatures within hatchery systems were maintained throughout at ~6°C with the flow-through system passing approximately 10-litres of water per minute. *Egg batches were treated with Pyceze™ (anti-microbial) daily from fertilisation to the eyed egg stage to prevent fungal infection.* Embryos were allowed to develop for 100-120 days, with termination of the hatched egg batch and an alevin count occurring just prior to first feeding. Throughout development, each egg batch was assessed visually weekly for egg mortality and, in the later stages of development, fry mortality. Post-termination photographs were taken of alevins to determine proportional hatch rate (in relation to the count of the initial egg batch), with individuals stored in ethanol to allow for scoring of ploidy status.

3.2.3 Confirmation of triploidy using microsatellites

To determine ploidy status using DNA, we used a panel of established polymorphic microsatellites (Glover *et al.*, 2015). DNA was extracted from a single eye of each hatched alevin using the hotshot genomic DNA preparation method as described in Truett et al.,(2000). In brief, tissue was added to a 96-well plate along with 75µl of alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA). The plate was then incubated for 25 minutes at 95°C, afterwards 75**µ**l neutralizing reagent (40 mM Tris-HCl at a pH of 5) was added to halt denaturation post-incubation.

Polymerase chain reaction (PCR) was carried out in a 10 µl volume reaction consisting of 2 µl of DNA (unspecified concentration) and 8 µl of PCR Mastermix composed of multiplex primers (forward/reverse primers MHC 1 (Grimholt et al., 2002), MHC 2 (Stet et al., 2002), SSsp3016 (Genbank no. AY372820), SsOsl85-a (Slettan, Olsaker and Lie, 1995), Ssa197-a (Norris et al.,1999) and SsaF43-a (Sánchez *et al.*, 1996). The PCR program took place with denaturation at 94°*C for four minutes followed by 30 cycles of denaturation at* 94°*C for fifty seconds, annealing at 55°C for fifty seconds, and extension at 72°C for eighty seconds, and an extension at 72°C for 10 minutes; the overall program ran for two hours and thirty minutes. PCR products were run on an* ABI3730 automated sequencer at the Institute of Marine Research (Havforskningsinstituttet) Bergen, Norway.

Triploid individual salmon were confirmed using microsatellite DNA genotyping and the identification of three clearly identifiable alleles at individual loci. The number of identifiable alleles per locus was determined using the genotyping software GeneMapper v5.0 (Applied Biosystems). To confirm triploidy, an individual fish was reported as triploid if it displayed three clear alleles at two or more of the genotyped polymorphic microsatellite loci (Glover *et al.*, 2015). All individuals were screened at the fry stage 100-120 days post-fertilisation with 935 individuals analysed.

3.2.4 Statistical analysis

To analyse variation in hatch rate (hatch rate is defined as the proportion of eggs hatching and surviving 100-120 days post-fertilisation, independent of fertilisation rate in the egg batch which is unknown) within individual male-female pairings at three different times post ovulation, Generalised Linear Mixed Models (GLMM) were applied using the 'lme4' package in R (Bates *et al.*, 2015). To model proportional hatch rates at each stripping event, a GLMM was fitted with a binomial error distribution, model estimates were determined using laplace approximation, with the proportional response variable of hatch: non-hatch assessed with the explanatory variable of days post-ovulation. Both female and male ID were coded as random variables to control for individual variation. Due to the independent variable 'days since ovulation' having three levels, two models were produced: one set the intercept as day 1, and the second with the intercept coded as day 14 to enable pairwise comparisons between all levels. Over-dispersion was assessed using the dispersion_glmer function within the blmeco package (Korner-Nievergelt *et al.*, 2015). The dispersion_glmer function gave an output of 4.472 for both day 1 and day 14 intercept models indicating a high level of over-dispersion. Due to overdispersion an observation level random effect (OLRE) (Harrison, 2014) was coded as a random factor, with the output of the dispersion_glmer after addition of the OLRE reducing to 1.013.

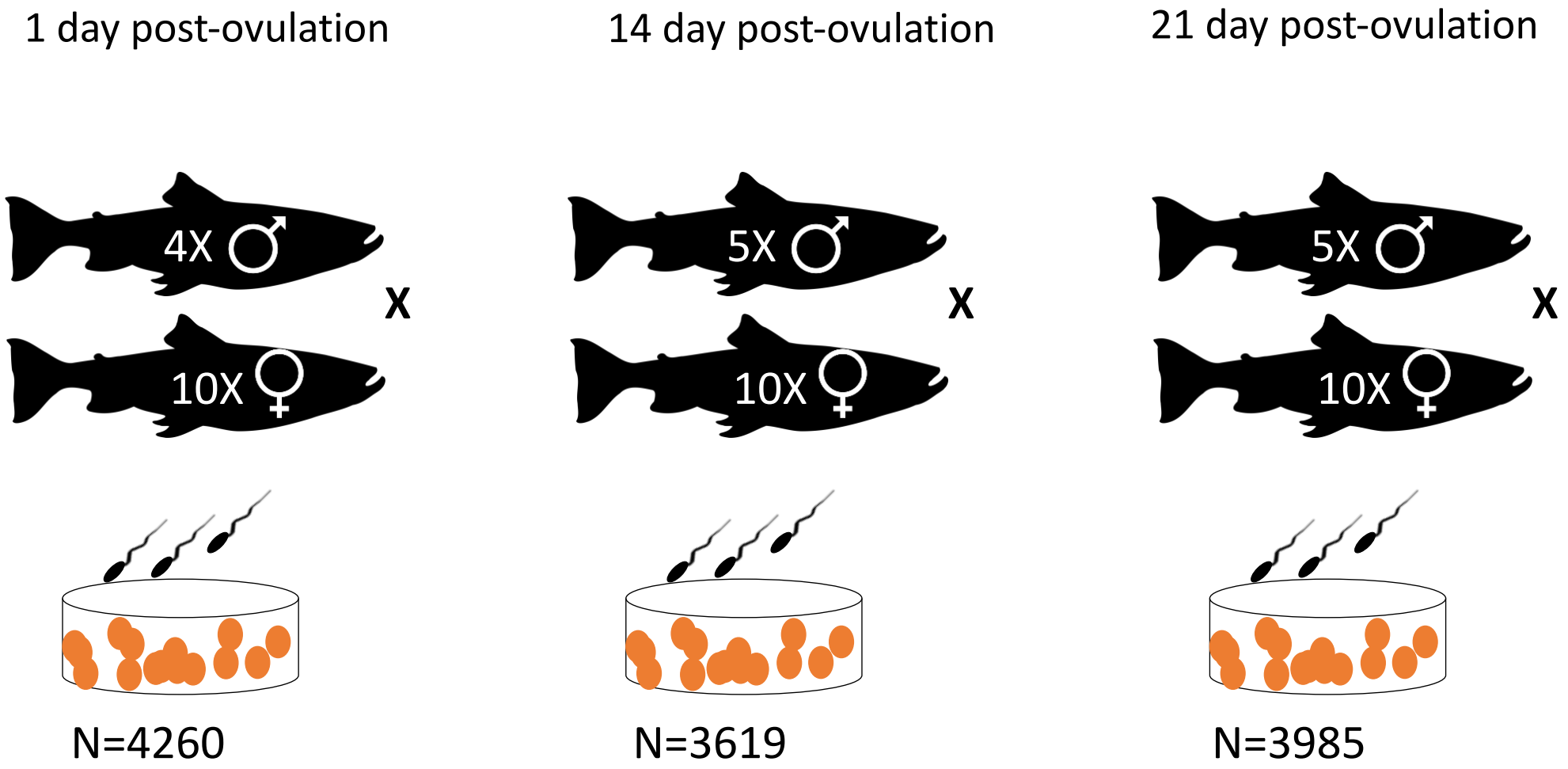

Figure 1: Post-ovulatory ageing experimental design. Gametes were stripped from 10 females and five males with a split-clutch and split-ejaculate design in which all males fertilised all females reducing any individual-based effects. Each female produced five egg batches at three time points 1,14 and 21-days post-ovulation with an average of 99 eggs per batch (±1.61). Each egg batch was fertilised with 1ml of undiluted milt, post-fertilisation offspring were reared to the fry stage and assessed for ploidy status and egg hatch rates. After controlling for failure to produce offspring ten females produced 14,909 ova split across 139 egg batches fertilised by four males.

3.3 Results

3.3.1 Hatch rate

Egg hatch rate was unaffected by a 14 day delay in female stripping: there was no difference in hatch rates between egg batches fertilised on either day 1 or day 14 following female ovulation (Day 1 = 67% (SE± 5.65) hatch success; Day 14 = 73% (± 3.97) hatch success, Figure 2). However, by day 21 following ovulation, hatch rate had reduced to 38.9% (± 4.37), showing significant differences in comparisons between both Day 1 and Day 21, and Day 14 and Day 21 (Table 1).

Table 1. A summary of the GLMM fixed effects for egg hatch rates from MOWI salmon at three separate post-ovulatory ageing time points. Proportional hatch rate was the response variable, and male and female identity in the paired crossing design were coded as random effects. The intercept for the GLMM was set as either Day 1 post-ovulation (A), or Day 14 (B) to allow comparison between Day 14 and Day 21 hatch rates. (A) Var (Variance): Female = 1.864, Male = 0.420 (B) Var: Female = 1.488, Male = 0.373.

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
A)				
Intercept (Day 1)	1.235	0.581	2.127	0.057
Day 14	-0.121	0.3020	-0.402	0.687
Day 21	-2.261	0.3070	-7.367	<0.001***

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
B)				
Intercept (Day 14)	1.1142	0.579	1.921	0.057
Day 1	0.121	0.3020	0.402	0.687
Day 21	-2.1402	0.3042	-7.035	<0.001 ***

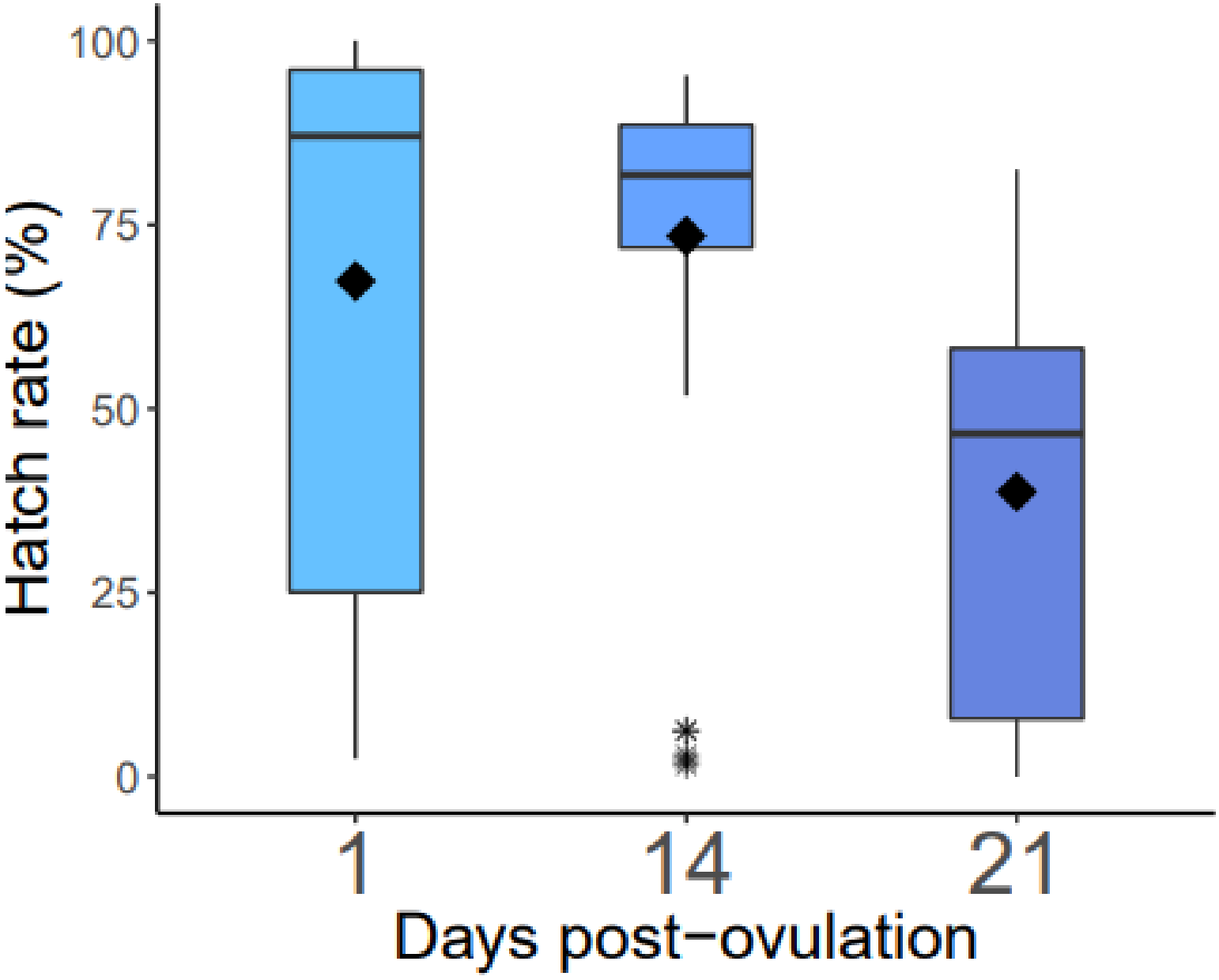

Figure 2: Hatch rates of farm Atlantic salmon egg batches fertilised at 1, 14 and 21 days post ovulation. No significant difference is observed between day 1 (67% (SE± 5.65) and day 14 (73% (± 3.97)) post ovulation, but a significant decline in hatch rate was observed between day 1 and day 21 (38.9% (± 4.37)), and between day 14 and day 21. Boxplots are derived from 4 x 10 crosses at day 1 = 4260 eggs , 5 x 10 crosses at day 14= 3619 eggs , 5 x 10 crosses at day 21= 3985 eggs producing a 139 total crosses.Means are represented by a black diamond, medians by black horizontal bars, and outliers by a black star.

3.3.2 *Ploidy status*

The frequency of unintentional spontaneous triploidy (UST) did not increase with post-ovulatory oocyte ageing, with a 0% triploidy rate observed across treatment timings on day 1 versus 21, despite the genotyping of 936 offspring (Table 1).

Table 2: Individual MOWI salmon offspring genotyped from groups fertilised on day 1 versus day 21 post ovulation showed no evidence for any triploidy.

Fry genotyped	**Ageing**	**Triploid individual No**
371	Day 1	0
565	Day 21	0

3.4 Discussion

This study investigated whether the ageing of eggs within ovulated females of farm Atlantic salmon caused a reduction in hatch rate (as defined in the methodology) or an increase in unintentional spontaneous triploidy. Eggs were aged within individual females across a 21-day post-ovulatory oocyte treatment, and part-stripped and fertilised at 1, 14 and 21 days post-ovulation. After controlling for some male infertility, a total of 139 male x female crosses were available for analysis. We found no significant change in the hatch rates (6% average change) of MOWI farm salmon eggs between day 1 and day 14 post ovulation. However, after 21 days post-ovulation, hatch rates had declined significantly compared with the day 14 treatment, with a 34.1% decline in hatch rate from day 14 to 21. A detailed evaluation of UST rates in 936 fry originating from eggs that had aged by 1 versus 21 days post ovulation found that no triploid individuals existed in either treatment. Our finding that in-vivo ageing results in degradation to egg quality, in particular declining fertilisation rate has been observed in both mammals and fish species. In humans in-vivo oocyte ageing has been observed to result in reduced fertilisation rates and embryo development (Miao *et al.*, 2009). In-vivo ageing of eggs has further been found to reduce fertilisation rates across a range of mammals including; cows (Rizos *et al.*, 2002) and pigs (Demond *et al.*, 2016).

Egg hatch was unaffected over delays of up to 14 days before female stripping, with no difference in hatch rates between egg batches fertilised on either day 1 or day 14 following female ovulation (Day 1 = 67% (SE± 5.65) hatch success; Day 14 = 73% (± 3.97) hatch success). However, by day 21 following ovulation, hatch rate had reduced to 38.9% (± 4.37), showing significant differences in comparisons between both Day 1 and Day 21, and Day 14 and Day 21 (Table 1).

Across fish species, a consistent deleterious effect on ova and embryo viability has been observed as a result of post-ovulatory oocyte ageing (Aegerter and Jalabert, 2004; Bonnet et al., 2003; Craik and Harvey, 1986; Nomura et al., 2013; Samarin et al., 2016). In assessment of the Japanese eel, 4 hours storage post-ovulation resulted in a decrease to 54.6% fertility, and 27.8% hatch, compared to 85.3% and 71.4% respectively in eggs immediately fertilised (Nomura et al., 2013a). In assessment of the effect of post-ovulatory oocyte ageing on common carp, Samarin et al.,(2015) identified a linear decline over time in the eyeing and hatching rate, with a 69% (80% 1 hour post-ovulation 11% 12hpo) drop in hatching and a 66.5% (69% 1hpo- 2.5% 12hpo) reduction in eyeing rate after 12-14 hours post ovulation, compared to immediate fertilisation.

We therefore find that farm Atlantic salmon can tolerate pre-strip, post-ovulatory oocyte ageing for up to 14 days, whereas other fish species show impacts on fertility and hatch rate after minutes / hours (Bonnet et al., 2003; Craik and Harvey, 1986; Nomura et al., 2013; Samarin et al., 2016). Our finding of increased resistance to oocyte-ageing in salmonids is consistent with previous work in farm rainbow trout showing resistance to oocyte ageing for up to 7 days after ovulation when held at 12°C, after which hatch rates declined (Aegerter and Jalabert, 2004), suggesting that salmonids may be more resistant to this egg ageing process than other fish species.

Although not significantly different, the highest hatching rates were observed in the day 14 treatment. The trend of greatest viability after the period of initial ovulation has been observed to occur in teleosts. (Samarin et al., 2015); in common carp, the highest eyeing and hatching rates were observed 2-4 hours post ovulation (hpo) with an increase of 7.5% compared with hatching rates at 0-2 hpo. Additionally, Samarin and Miroslav Blecha, Dmytro Bytyutskyy (2015) found that pike perch eggs fertilised 3-9 hours after ovulation had the highest viability, and lowest embryo mortality and larval malformation and ploidy anomalies. Furthermore, Aegerter and Jalabert (2004) identified the greatest egg viability rates of rainbow trout to be after 7 days post-ovulatory oocyte ageing.

The natural spawning behaviour of Atlantic salmon provides a possible selective pressure to explain some resistance to egg damage from post-ovulatory oocyte ageing. Mature female Atlantic salmon can go through multiple spawning bouts, releasing eggs on up to 7 redds in a single mating season (Fleming, 1996). These redds can vary from a few metres to up to 5 km apart for the same female (Taggart *et al.*, 2001). Fleming (unpubl. data) observed the average time between successive spawnings by individual females to be 24 hours, but ranging between 4 hours and 9 days, with the time between nest initiation and spawning taking up to 48 hours (Webb and Hawkins, 1989). During these extended mating periods, there is ample opportunity for time delays between ova released in the first and last redd, with up to 11 redds in total for individual females. This time delay will exert selective pressure on females and ova to avoid deleterious egg ageing effects, and maximise fitness through successive spawnings. Only when we extend post-ovulatory oocyte ageing to 21 days do we see a significant decline in egg hatch rate.

In addition to unexpected findings of no decline in hatch rate across 14 days, we found no evidence for triploidisation of fry, even when assaying eggs which had been exposed to 21

days of post-ovulatory ageing. We identified no incidences of triploidisation at all among fry fertilised as eggs at day 1 versus day 21 after ovulation across 936 offspring screened. Post-ovulatory oocyte ageing has previously been observed as a causal factor for spontaneous triploidisation in pike (Samarin *et al.*, 2016), Japanese eel (Nomura *et al.*, 2013a), tench (Flajšhans, Kvasnička and Ráb, 1993) and salmonids (Aegerter and Jalabert, 2004). Samarin *et al.,*(2016), found the incidences of triploidy in northern pike larvae to increase from 0% at day 2 to 14% at day 6. In tench, post-ovulatory oocyte ageing at 24°C resulted in a 5% triploidy rate after 5 hours (Flajšhans, Kohlmann and Ráb, 2007). Nomura *et al.*, (2013) observed increasing abnormal ploidy of embryos as a result of oocyte ageing the majority of which were triploid (86.5%). Aegerter and Jalabert (2004) found triploidisation in rainbow trout to be associated with post-ovulatory oocyte ageing when under high temperature treatments for 7 days post-ovulation. Aegerter and Jalabert (2004) also identified that adenosine triphosphate (ATP) levels in eggs reduced throughout post-ovulatory oocyte ageing, with this reduction hypothesised to result in the disruption of cytoskeletal organisation, preventing release of the second polar body and therefore resulting in triploidisation.

Aegerter and Jalabert (2004) hypothesise that ATP-mediated oocyte ageing may provide a potential explanation for our finding that salmonid Atlantic salmon have extended resistance to post-ovulatory oocyte ageing. The underlying effects of fish oocyte ageing are currently unknown, but there is a growing body of evidence that oxidative stress contributes significantly to the effects of oocyte ageing (Tarín., 2000 ; Takahashi *et al.*, 2003). Elevated levels of reactive oxygen species (ROS) cause ROS-induced mitochondrial dysfunction, which results in the eventual depletion of ATP in the oocyte, impacting on essential functions (Takahashi *et al.*, 2003; Nazmara, Salehnia and HosseinKhani, 2014; Samarin, Samarin and

Policar, 2018). Operational mitochondria are the primary source of ATP production in oocytes and embryos (Dumollard, Duchen and Carroll, 2007). Optimal energy production is essential for oocyte and embryo development, with mitochondrial dysfunction having major impacts (Babayev and Seli, 2015). We hypothesise that a plausible explanation for Atlantic salmon's ability to resist post-ovulatory oocyte ageing results from their relatively high oocyte ATP concentrations. The disparity in ATP concentration between salmonids of other teleosts have previously been observed. Salmonid species rainbow trout and chinook salmon, have relatively high ATP concentrations compared to other teleosts (Wendling *et al.*, 2000; Aegerter and Jalabert, 2004), with rainbow trout ATP concentrations in eggs being 100x higher than that of common carp (Boulekbache *et al.*, 1989; Aegerter and Jalabert, 2004). Wendling (2004), further observed steelhead trout unfertilised ova total ATP content to be twice that of unfertilised carp and loach eggs (Boulekbache *et al.*, 1989), but 75% the ATP content of the unfertilised chinook salmon eggs (Wendling *et al.*, 2000). The greater ATP concentrations present in salmonid ova could allow optimal energy production and therefore cell maintenance over a longer period of time, allowing salmonids to maintain normal oocyte and embryonic development over longer spawning periods, commensurate with natural reproduction operating over a number of days post ovulation (Dumollard, Duchen and Carroll, 2007).

As hypothesised by Wendling (2000) the greater intracellular ATP content of salmonids eggs may facilitate successful fertilisation after post-ovualtory oocyte ageing or storage, preventing the relatively quick decline observed in other species. This hypothesis is supported by Srivastava and Brown (1991), who identified calorific content of Atlantic salmon eggs to be a predictor of egg quality. In carp, a species with 50% the ATP content of

salmonid species, decline in embryo viability and ATP concentrations are observed after 9 hours, as opposed to over 14 days in our study (Boulekbache *et al.*, 1989).

The change in hatch rate observed in this experiment could also be driven entirely or in part by the ageing of males. As with females, males aged over the course of the experiment providing opportunity for both whole organism and gamete level ageing effects. The age of male Atlantic salmon has previously been found not to affect the quality of fresh sperm regardless of life history (Erraud *et al.*, 2021). In further support (Camarillo-Sepulveda *et al.*, 2016) found no impact of energy availability on sperm between 3 and 5 years. In contrast many studies have found sperm quality to decline with age over the course of years as a result of oxidative stress (Judycka *et al.*, 2020). In Rainbow trout males that are 2-3 years old have been found to have better sperm quality then 4-year-old males (İnanan and Yılmaz, 2018; Risopatrón *et al.*, 2018). Ageing effects can have a clear impact on male sperm quality, but in this experiment all males aged 21 days. The short period of time in which males aged suggests that sperm quality is unlikely to have been majorly impacted. Therefore, the decline in this experiment is far more likely to be the result of internal oocyte ageing then age related declines in sperm quality.

Alternatively, the lack of triploid individuals identified within our study may result because any triploid individuals had perished at the developmental or early post-hatch stages, so they no longer existed when we assessed ploidy at the fry-stage 100-120 days since fertilisation. Triploid individuals can suffer higher mortality, especially under stressful conditions (Fraser *et al.*, 2013; Sambraus *et al.*, 2017), so the decline in offspring production among the eggs fertilised at 21 days since ovulation could be explained if a greater number of these late-fertilisation individuals were triploid. Additional experiments have been conducted by Marco Graziano and colleagues (Pers. Comm.) where embryos and alevins are

tested at earlier developmental stages, which should reveal whether UST can explain reduced hatch rates and offspring production in Atlantic salmon.

In conclusion we have observed farm Atlantic salmon to resist post-ovulatory oocyte ageing over a 14-day period, which is far longer than the majority of fish species previously studied. Our finding suggest that the salmon aquaculture industry is able to hold hens for 14 days before stripping without loss of hatch rates in offspring. The ability to hold hens for 14 days should reduce the need for regular ovulation checks, reducing stress and possibly increasing egg quality. Additionally, our study suggests that the greatest fertilisation rates are achieved some days after initial ovulation, meaning immediate stripping may reduce potential production rates. An important next step is to replicate this experiment on wild Atlantic salmon that have not undergone the domestic selection process, in order to test whether they experience similar responses to post-ovulatory oocyte ageing. Further study is needed to elucidate the mechanisms behind Atlantic salmon's ability to resist oocyte ageing. It is important to measure both ATP levels through the use of a luminometer, and mitochondrial function which can be measured using electrode probes to assess oxygen consumption (Lanza and Nair, 2010). Oocytes will be measured at 1,14 and 21 days to provide clarity over ATP and mitochondrial functions potential influence in oocyte ageing and salmonids resistance. Finally, an important area for future research is to assess the impact of the partial stripping process on mechanical damage to oocyte. It is important to determine partial strippings effect to ensure it is not producing the results observed.

Chapter 4

Inter-strain gamete compatibility under sperm competition between unrelated farm and wild Atlantic salmon strains.

Abstract

Large numbers of farm Atlantic salmon escape from the aquaculture into wild Atlantic salmon populations. Farm escapees have been observed to enter natal spawning grounds and hybridise with wild populations. Hybridisation leads to the degradation of wild Atlantic salmon's genetic structure and localised adaptations to the natal river. The loss of these localised adaptations can put wild populations at risk of extirpation. In this study, I explore potentials for hybridisation between farm and wild individuals at the gamete level. We fertilised farm and wild egg batches with sperm from wild and farm males under sperm competition. Males competed four times, twice for eggs from a farm female in either farm ovarian fluid or wild ovarian fluid, and twice again for eggs from a wild female in farm and wild ovarian fluid. Through this study we look to identify whether any barriers exist to prevent hybridisation following divergence of farm and wild individuals, and what influence ovarian fluid has on competitive fertilisation outcome. Egg batches were assessed for paternity using microsatellite markers, allowing us to determine parentage success between sperm from farm and wild males. We found farm Atlantic salmon sperm to win greater than equal paternity when

competing for eggs from wild females, and an equal share of paternity when competing for eggs from farm females. We found no significant effect of ovarian fluid on paternity outcome within eggs from both wild and farm females. We therefore observe no barrier to hybridisation at the gamete level, and even fertilisation dominance by farm sperm, indicating that the aquaculture industry must take steps to reduce escapee numbers because of clear risks of genetic introgression into wild populations.

4.1 Introduction

Farm and wild salmon are currently experiencing opposing population trajectories. Between 1986 and 2018 there has been an increase of over 4000% in farm Atlantic salmon production (Tveteras, Nystoyl and Jory, 2019), whilst the catch of wild Atlantic salmon are estimated to have declined 57.5% from 1983-2016 (NASCO, 2019). This disparity has resulted in a major imbalance as demonstrated by Norway's standing stock of farm salmon being approximately 400 million individuals compared to the approximate 0.5 million adult wild salmon returning to breed in Norway's coastal waters (Forseth *et al.*, 2017).

The decline of wild Atlantic salmon is associated with the intensive growth of aquaculture (McGinnity *et al.*, 2003; Ford and Myers, 2008; Roberge *et al.*, 2008; Limburg and Waldman, 2009; Schindler *et al.*, 2010), environmental pressures such as climate change (Walsh and Kilsby, 2007; Jonsson and Jonsson, 2009; Sundt-Hansen *et al.*, 2018) and pollution (Magee *et al.*, 2003; Limburg and Waldman, 2009; Hesthagen, Larsen and Fiske, 2011; Skaala *et al.*, 2014), as well as human

impacts through fishing and habitat exploitation (Lundqvist *et al.*, 2008; Fjeldstad *et al.*, 2012; Vøllestad, Skurdal and L'Abée-Lund, 2014; Nyqvist *et al.*, 2017).

A major threat to wild Atlantic salmon populations posed by the aquaculture industry is the escape of farm salmon into the wild. Farm salmon are kept in unnaturally high densities, with poor sea cage security producing containment issues for the industry. Despite improved operational management of farms, such as for example Norway's implementation of technical standards for sea cages is reducing farmed escapees by 400,000 individuals each year (2007-2009) (Jensen *et al.*, 2010), expansion of the industry has kept escapee numbers high (www.fiskeridir.no). Escapees from Norwegian farms alone range from under 200,000 to over 900,000 individuals per annum between 2000 and 2015 (www.fiskeridir.no). These are the known and reported incidences, representing the minimum estimate, with actual figures for escapee numbers estimated to be 2-4x higher resulting in farm escapees outnumbering wild conspecifics (Glover *et al.*, 2017; Skillbrei.Ove, Mikkio Heino, 2017).

Farm salmon escapees have been observed in the natal rivers of wild Atlantic salmon populations across their natural range (Clifford, McGinnity and Ferguson, 1998; Fleming *et al.*, 2000; Fiske, Lund and Hansen, 2006; Glover *et al.*, 2012). These farm escapees are also present on spawning grounds (Crozier, 2000; Bourret *et al.*, 2011; Glover *et al.*, 2013) and have been observed to outnumber wild individuals (McGinnity *et al.*, 2003; Jonsson and Jonsson, 2006).

The farm escapees present on spawning grounds are able to hybridize with wild individuals causing genetic introgression (Crozier, 2000; Fleming *et al.*, 2000; McGinnity *et al.*, 2003; Naylor *et al.*, 2005; Leaniz *et al.*, 2007; Roberge *et al.*, 2008; Jensen *et al.*, 2010; Bourret *et al.*, 2011; Glover *et al.*, 2012, 2013)**.** Genetic introgression has been identified to be widespread in wild Atlantic salmon supporting rivers. In assessment of 109 salmon supporting rivers mean farm salmon genetic introgression levels were 6.4% ranging from 0-42.2% (Karlsson *et al.*, 2016). In 51 of these rivers significant genetic introgression had occurred when compared to historical samples (Karlsson *et al.*, 2016). In a separate assessment of 21 Norwegian salmon populations, four had significant temporal genetic

changes, one of these the river Opo had 100% of fish were excluded from historical samples (Glover *et al.*, 2020), 15 rivers in the experiment showed no significant temporal change as a result of genetic introgression with density of native populations thought to be a key predictor of susceptibility. Glover *et al.*, (2020)**,** further identified that rivers in 10 of the 13 aquaculture production zones covering most of Norway have a moderate to high risk of future farm introgression.

Genetic introgression threatens the genetic profile of wild populations as farm individuals through domestication have genetically diverged losing adaptive traits present in wild salmon (Fleming, 1997). Over 50+ years of artificial selection farm salmon's morphology, physiology and behaviour have diverged from their wild ancestors. The divergence of farm salmon is a result of the artificial aquaculture environment which provides different developmental forces such as the removal of predation whilst providing food and medical treatment (Bourret *et al.*, 2011).Farm Atlantic salmon escaping at the marine stage and entering freshwater breeding grounds are far larger in mass being 2-4x bigger than wild salmon under aquaculture conditions (Glover *et al.*, 2009; Solberg, Zhang, *et al.*, 2013; Harvey *et al.*, 2016). Farmed Atlantic salmon are not only larger but also display a divergent external morphology with changes to fin size and proportions (Fleming and Gross, 1994) and internal morphology with changes to heart morphology (Fraser *et al.*, 2013). Behaviourally farm salmon have been observed to display bold feeding behaviours with little predator awareness (Einum and Fleming, 1997), furthermore inappropriate spawning behaviour of farm individuals has been observed such as spawning at different times and different reaches in the river (Webb *et al.*, 1991; Fleming, 1996). Physiologically farm Atlantic salmon parr have different hormone profiles, maturity age, fin healing and swimming performance compared to wild parr (Jonsson and Jonsson, 2006).

The importance of life history adaptations to wild Atlantic salmon makes genetic introgression of maladaptive farm traits a major threat. Atlantic salmon returning to natal rivers to spawn produces distinct areas of adaptation, causing localised genetic structure across populations, and early

reproductive isolation (Taylor, 1991; McConnell *et al.*, 1995; Moffett and Crozier, 1996). Local adaptions are essential to population survival and breeding success, with changes to life history, morphology and physiology adapted to the ambient environmental conditions of the natal river (Brian, Dell and Leggett, 1981; Järvi, 1990; Pakkasmaa and Piironen, 2001; Kinnison, Unwin and Quinn, 2003; Witten and Hall, 2003; Vincent, Kent and Bernatchez, 2013; Gradil *et al.*, 2016; Jensen *et al.*, 2017).

Genetic introgression of maladaptive traits from farm individuals can lead to the erosion of wild population genetic structures, and loss of essential local adaptions causing outbreeding depression which can reduce the fitness of 'hybrid' individuals and their populations (José Mork, 1991; McGinnity *et al.*, 2003; Roberge *et al.*, 2008; Schindler *et al.*, 2010; Glover *et al.*, 2013). Genetic introgression leads to the erosion of local adaptations in wild salmon genetic structure (McGinnity *et al.*, 2003; Bourret *et al.*, 2011). The loss of these adaptations creates a risk of cumulative fitness depression putting vulnerable wild Atlantic salmon populations under risk of extirpation and ultimately extinction (McGinnity *et al.*, 2003; Fraser *et al.*, 2008). The risk of extinction events in wild salmon populations is accentuated by wild population density (Glover *et al.*, 2020), repeated annual farm immigration numbers into natal rivers (McGinnity *et al.*, 2003; Jonsson and Jonsson, 2006; Karlsson *et al.*, 2016; Glover *et al.*, 2017, 2020) and genetic distance between farm escapees and wild individuals (Einum and Fleming, 1997).

Despite growing evidence for the potential impact of genetic introgression, it is difficult to demonstrate unequivocally that ecological destabilisation is the direct result of farmed introgression potentially, until it is too late and the wild population has become extinct. Environmental fluctuations and anthropogenic impacts from fishery pressure, disease, pollution, water abstraction and other impacts on ecological health will also confound the relationship between farm salmon introgression and ecological disruption. However, a major meta-analysis clearly identified a negative impact of salmon farms on wild individuals. Ford and Myers, (2008) compared wild populations with

and without pressure from neighbouring farms and found a significant increase in mortality rates of those with neighbouring farms.

Our study looks to identify the potential for hybridisation of farm and wild individuals and therefore the subsequent potential for genetic introgression at the gamete level. We are investigating whether any reproductive barriers exist at the gamete level when farm males compete against wild males in sperm competition. We focus on wild and farm individuals which are distinct with no ancestral relation. Farm males success under sperm competition is an important factor due to polyandry being the norm, Weir *et al.*, (2010) identified an average of 8 different males, and up to 16, fertilising the eggs in a single female's nest.

In this study we examine proportional paternity between pairs of farm and wild males competing for farm and wild females to understand what influence genetic divergence may play in influencing sperm competition and or cryptic female choice. External fertilisation allows for a split-clutch and split ejaculate crossed design allowing us to minimise variability in individual performance and reproductive compatibility. This study also looks at the influence of ovarian fluid on proportional paternity with male pairs competing for females in self-ovarian fluid and non-self-ovarian fluid. Our focus on ovarian fluid is due to previous research identifying it as an important post-copulatory sexual selection mechanism (Lahnsteiner, 2002; Hatef, Niksirat and Alavi, 2009; Immler *et al.*, 2014; Alavioon *et al.*, 2017; Promerová *et al.*, 2017). In natural fertilisation, ovarian fluid is ~10 - 30% the volume of the spawned egg mass (Rosengrave *et al.*, 2009). Ovarian fluid has been identified to influence sperm by modifying velocity, longevity, and linearity of movement (Rosengrave *et al.*, 2008b; Butts *et al.*, 2012; Lehnert, Heath and Pitcher, 2012; Alonzo, Stiver and Marsh-Rollo, 2016). Ovarian fluid mediated sperm selection has been identified in salmonids between-species (Yeates *et al.*, 2013) and between males of the same species (Rosengrave *et al.*, 2008b; Butts *et al.*, 2012; Alonzo, Stiver and Marsh-Rollo, 2016; Rosengrave, Montgomerie and Gemmell, 2016; Lehnert *et al.*, 2017). Our study looks to identify the influence of ovarian fluid on sperm selection between farm and wild males when competing for self and non-self-females in self or non-self-ovarian fluid. By

removing whole organism affects we are able to identify whether a barrier to hybridisation and genetic introgression has developed at the gamete level between two distinct strains of Atlantic salmon through repeated in-vitro fertilisation.

4.2 Materials and methods

4.2.1 Study area and fish groups

The fertilisation experiment was conducted in Norway at the Institute of Marine Research (IMR) hatchery facility in Matre (84km north of Bergen) in autumn and winter 2018. Two strains of Atlantic salmon were used: 1) wild background Atlantic salmon taken from the River Etne (south-west Norway), and 2) one-sea-winter farmed fish taken from Marine Harvest's MOWI strain (12th generation of farm breeding). Farm adults for stripping were provided and maintained by MOWI Ltd. Etne fish were provided from hatchery facilities in which wild caught salmon from the river Etne are reared. Gametes were transported on each occasion on wet ice in polystyrene boxes. MOWI and wild Etne strains (known onwards as farm and wild) were fertilised, reared and analysed at IMR, Matre. All salmon were stripped the day before the *in-vitro* fertilisations took place with gametes stored on wet ice in polystyrene boxes. Gametes from ten females and ten males were used in this experiment from both farm MOWI and wild Etne salmon strains.

4.2.2 *Sperm competition experiment*

In vitro fertilisations consisted of paired sperm competitions with reciprocal testing of males and females through the use of split clutch and split ejaculates. This experimental design allowed for control of individual variation and male / female driven effects.

Sperm competition design (figure 1):

1. **Farm male 1** v **Wild male 1** competing for **Farm female 1** in **Farm female ovarian fluid.**
2. **Farm male 1** v **Wild male 1** competing for **Farm female 1** in **Wild female ovarian fluid.**
3. **Farm male 1** v **Wild male 1** competing for **Wild female 1** in **Wild female ovarian fluid.**
4. **Farm male 1** v **Wild male 1** competing for **Wild female 1** in **Wild female ovarian fluid.**

Sperm from each male pair competed four times, twice for eggs from the same farm female and again for eggs from the wild female's egg batches (average: 72, range: 52-94 eggs). Each female produced two egg batches, one fertilised within their own ovarian fluid (1ml) and another in the alternative strain's non-self-ovarian fluid (1ml). A total of 40 crosses took place with 10 farm and 10 wild females.

Fertilisations took place 'wet', in which gametes are mixed and fertilised after the addition of activating water. Eggs were fertilised in 500ml flat-bottomed plastic bowls, (Desertcart.co.uk) by two randomly assigned farm and wild males using 100 µl of both male's sperm. Both male's sperm were pipetted equidistantly from the egg batch with the addition of 200ml of river Matre water to enable in-vitro fertilisation, applying general principles described in Yeates et al., (2014).

Once Matre river water had been added, gametes were left to stand for 120 seconds for fertilisation to take place. A picture of the fertilisation trays is shown in the methodology of chapter 2 (Artificial hatchery fertilisation methods effect on hatching rates in farm and wild

Atlantic salmon, page 51). After experimental treatment, fertilised egg batches were moved to flow-through hatchery trays, with each egg batch being placed into hatchery trays of 45cm^2 with individual egg batches in chambers of 15cm^2 labelled with a unique code. Water temperatures within hatchery systems were maintained throughout at ~6°C with a flow of approximately 10-litres of water per minute per trough containing 7 trays. Egg batches were treated daily with Pyceze^{TM} until the eyed egg stage to prevent fungal growth. Embryos were left to develop until the alevin stage with termination beginning prior to first feeding (approximately 20 days post hatch). Photographs were taken pre-fertilisation to gain accurate egg number counts and post-termination to gain accurate hatchling numbers. Hatching success relative to starting egg number was recorded with a total of 1389 eggs producing 1075 hatchlings for wild females and 1483 eggs producing 803 hatchlings, 854 hatchlings from both the farm and wild origin underwent paternity analysis.

Due to the paired nature of this experiment when an in-vitro fertilisation resulted in no offspring both that egg batch and the second egg batch from the female were removed from the analysis. Under this control three females were removed from the experiment with Dam E1, M9, M10 egg batches controlled for leaving 16 females fertilised by 20 males.

4.2.3 Hatching success

Throughout development, egg batches were monitored, and dead or infected eggs and early hatchlings removed. At 20 days after hatch, surviving hatchlings in each treatment batch were photographed for counting hatching success relative to the starting egg number for each of the treatment combinations from Etne and MOWI backgrounds.

4.2.4 Assessment of paternity

Parental assignment was determined through molecular-based parentage analysis through the family analysis program (FAP), this programme utilises exclusion-based parental assignment based on known parental genotypes (Taggart, 2007). To determine parental genotypes we undertook microsatellite DNA analysis with amplification of five highly polymorphic microsatellites (Glover,2012), MHC 1 (Grimholt *et al.*, 2002), MHC 2 (Stet *et al.*, 2002), SsOsl85-a (Slettan, Olsaker and Lie, 1995), Ssa197-a (Norris et al.,1999) and SsaF43-a (Sánchez *et al.*, 1996). The methodology for sample preparation to undertake DNA analysis is outlined in the assessment of Unintentional Spontaneous Triploidy in Chapter 1 (Artificial hatchery fertilisation methods effect on hatching rates in farm and wild Atlantic salmon).

4.2.5 Assessment of sperm number

Although the experiment employed a paired design to control for many between-individual variables, I checked for variation in relative sperm number between the male sperm samples in order to assess its influence on fertilisation outcome. To assess sperm number, images were taken of two milt samples per male, with all sperm samples for use in fertilisation trials standardised across males at 500ml of milt and 500ml of ovarian fluid. A 1ml sample was then analysed under 200x dark field phase contrast microscopy with images taken using a grasshopper®3 USB3 VISION™ camera attached to the microscope. Images were then analysed manually using ImageJ count to assess sperm number. The field of vision was standardised for all males to ensure comparability with each male's sperm number assessed in two separate samples.

4.2.6 Statistical analysis

All data analysis was carried out in R-studio using R-version 3.4.1 (2009-2019) (RStudio Team, 2020).

Farm male paternity

To determine sperm competition success the farm (Mowi) males were analysed using Wilcoxon tests, comparing successful farm male's fertilisation against the null expectation of equal paternity between farm vs wild males. Wilcoxon tests were carried out to assess farm male's paternity for the experiment overall, and also separately assessing the response of farm and wild females.

To model whether farm male's proportional paternity was different when competing for farm or wild females we constructed GLMM models using the 'glmmTMB' package in r (Bolker,2018). The models were fitted with a binomial error distribution, the response variable being proportional paternity (farm or wild) with the explanatory variable of female strain (farm or wild). Male competing pair ID and female ID were fitted as a random effect. To ensure the model was not over-dispersed we utilised the blmeco package with the function dispersion_glmer showing no over dispersion (1.16) in the model.

Ovarian fluid

To model ovarian fluid origin's (farm v wild) effect on proportional paternity of farm and wild males we constructed GLMM models. These models were fitted with a binomial error distribution, model estimates were determined using laplace approximation, the response variable was proportional paternity (farm or wild) with the explanatory variable of ovarian fluid origin (farm or wild). Male (farm & wild) competing pair ID was fitted as a random effect. Three separate models were constructed for both farm and wild females as well as farm and wild females separately to identify strain-dependent effects. The disperson_glmer function showed no overdispersion in either the farm female (0.04) or wild female (1.03) models.

To assess sperm number a glmm was carried out using “glmmtmb” with sperm number as the response variable against the dependent variable of male strain and random effect of male pair. All P-values reported were calculated through the wald t-test within the glmmtmb and lme4 packages.

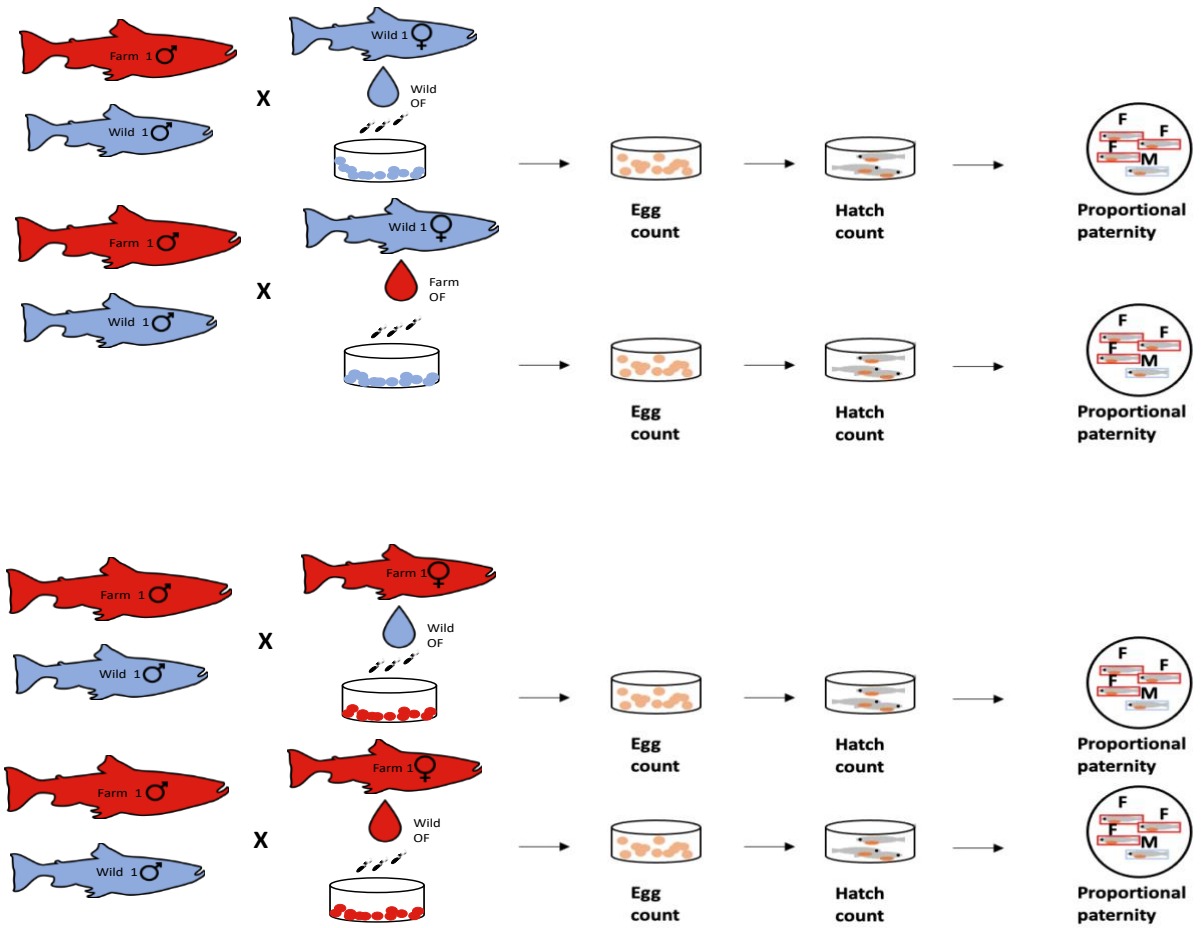

Figure 1: Sperm competition design: Farm / Wild male pairing compete four times 1) **Farm male 1** v **Wild male 1** competing for **Farm female 1** in **Farm female ovarian fluid 2) Farm male 1** v **Wild male 1** competing for **Farm female 1** in **Wild female ovarian fluid** 3)**Farm male 1** v **Wild male 1** competing for **Wild female 1** in **Wild female ovarian fluid 4) Farm male 1** v **Wild male 1** competing for **Wild female 1** in **Wild female ovarian fluid.** To enable reciprocal testing of pairs we used a split-clutch, split -ejaculate design. Each female produced two egg batches with an average of 72, range: 52-94 eggs. Each egg batch was fertilised with 100µl of sperm from both the farm and wild male. Eggs were reared to hatch, recording % hatch and assessing paternity. Replication involved 40 crosses with 10 farm and 10 wild females and males. A total of 2772 ova were used in the experiment producing 1878 hatchlings from different

4.3 Results

4.3.1 Sperm competition

Farm males were no less competitive than wild males. In fact, when competing for wild females, farm males gained greater than equal paternity, with an average 85% share (Mann-Whitney z= 3.290, P= <0.001, n=18). When competing for farm females, farm males were also highly competitive, gaining 64% paternity share (Mann-Whitney z= 1.062, P= 0.2882, n= 16).

Farm males were identified to have significantly higher proportional paternity then wild males when competing for wild females (85%) as opposed to farm females (64%) (Table.1).

Table 1: A summary of the GLMM fixed effect for proportional paternity of Mowi males when in competition with Etne males for Mowi (Farm) or Etne (Wild) females. Proportional paternity was the response variable with the fixed effect of female strain (Wild or Farm) and random effect coded as male pair ID and female ID. The intercept for the GLMM was set as Etne (wild) female, random effects variance male pair ID (Var=13.75) and Female ID (Var=0.007).

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
(A) Overall				
Mowi female	-1.029	0.305	-3.367	<0.001

4.3.2 Ovarian fluid

Proportional paternity of farm salmonid males showed no significant difference when fertilised in farm or wild ovarian fluid (OF) (Farm OF: 75.2%, Wild OF: 76.2%). Farm males proportional paternity was unaffected by OF origin when competing for wild females (Farm OF: 84.9% , Wild OF: 85.6%). Similarly, no significant difference in proportional paternity was observed as a result of OF origin when competing for farm females (Farm OF: 60.3%, Wild OF: 66.8%). All P-values reported were calculated through the wald t-test within the glmmtmb and lme4 packages.

Table 2: A summary of the GLMM fixed effect for proportional paternity of Mowi males in competition for Mowi (Farm) or Etne (Wild) females, (A) all females (B) Etne Females (C) Mowi females. Proportional paternity of Mowi males was the response variable with the fixed effect of ovarian fluid origin (wild vs farm) and random effect of male pair ID. The intercept for the GLMM was set as Etne ovarian fluid, random effects variance male pair ID A) Var = 14.69, B) Var=20.54, C Var=5.771)

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
(A) Overall				
Mowi ovarian fluid	-0.398	0.268	-1.485	0.138
(B) Etne females				
Mowi ovarian fluid	-0.471	0.361	-1.305	0.192
(C) Mowi females				
Mowi ovarian fluid	0.648	0.485	1.338	0.181

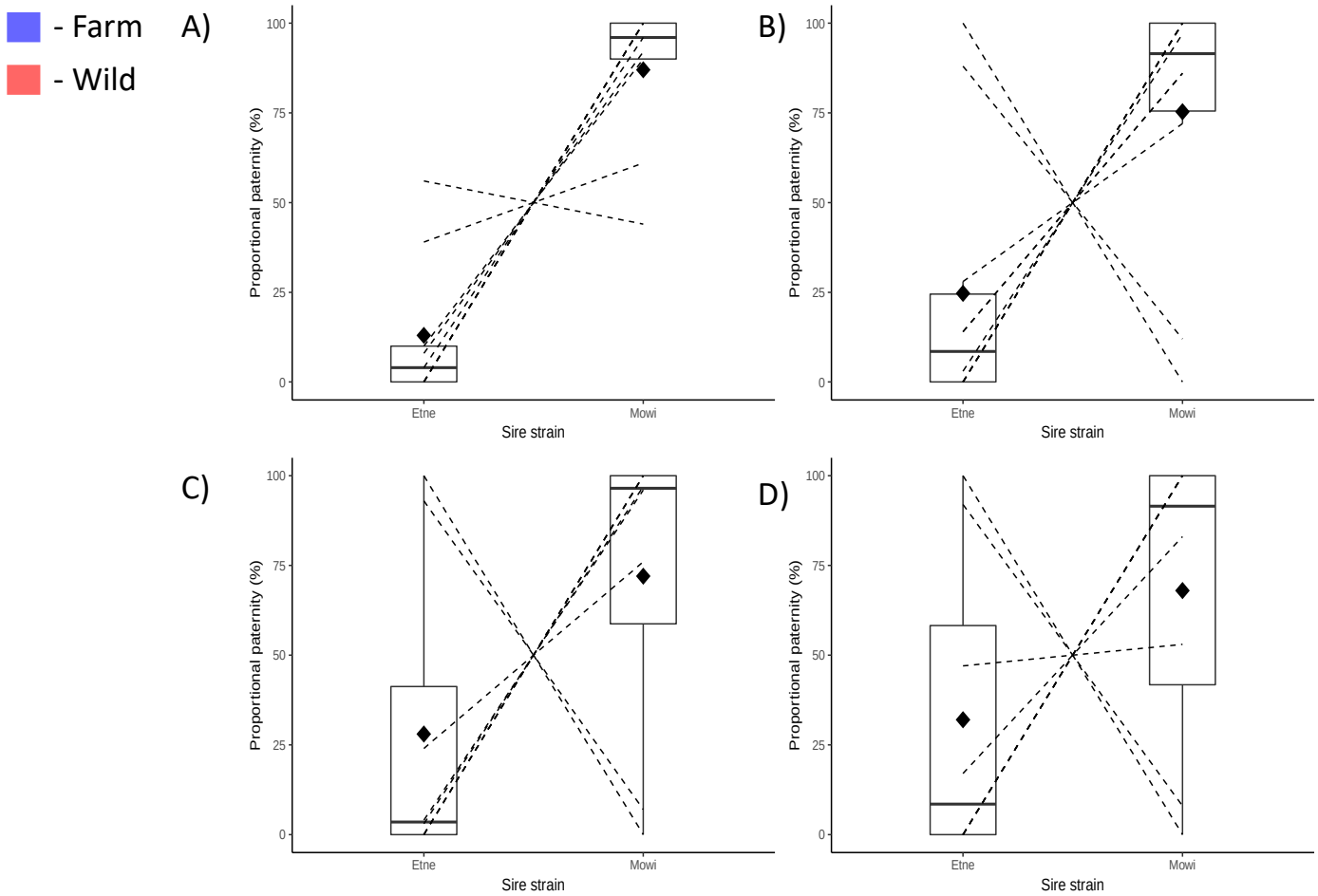

Figure 2: Proportional paternity of Farm (blue) and Wild (light red) males competing under sperm competition for A) Wild female in wild ovarian fluid, B) Wild female in farm ovarian fluid, C) Farm female in wild ovarian fluid, D) Farm female in farm ovarian fluid. Farm Atlantic salmon gained significantly greater share than equal paternity when competing for wild females (85%) and an equal share of paternity when competing for farm salmon (65%). No significant difference in the proportional paternity of farm male salmon was observed between wild and farm ovarian fluid overall (Farm OF: 75.2%, Wild OF: 76.2%)., in wild females (Farm OF: 84.9%, Wild OF: 85.6%) or farm females (Farm OF: 60.3%, Wild OF: 66.8%). Each dot represents a males proportional paternity with lines connecting the competing males and black diamond representing means. Boxplots are derived

4.3.3 Sperm number

In assessment of sperm number no significant difference was observed between Etne (980, individual sperm number) and Mowi males (1280) (P = 1.00).

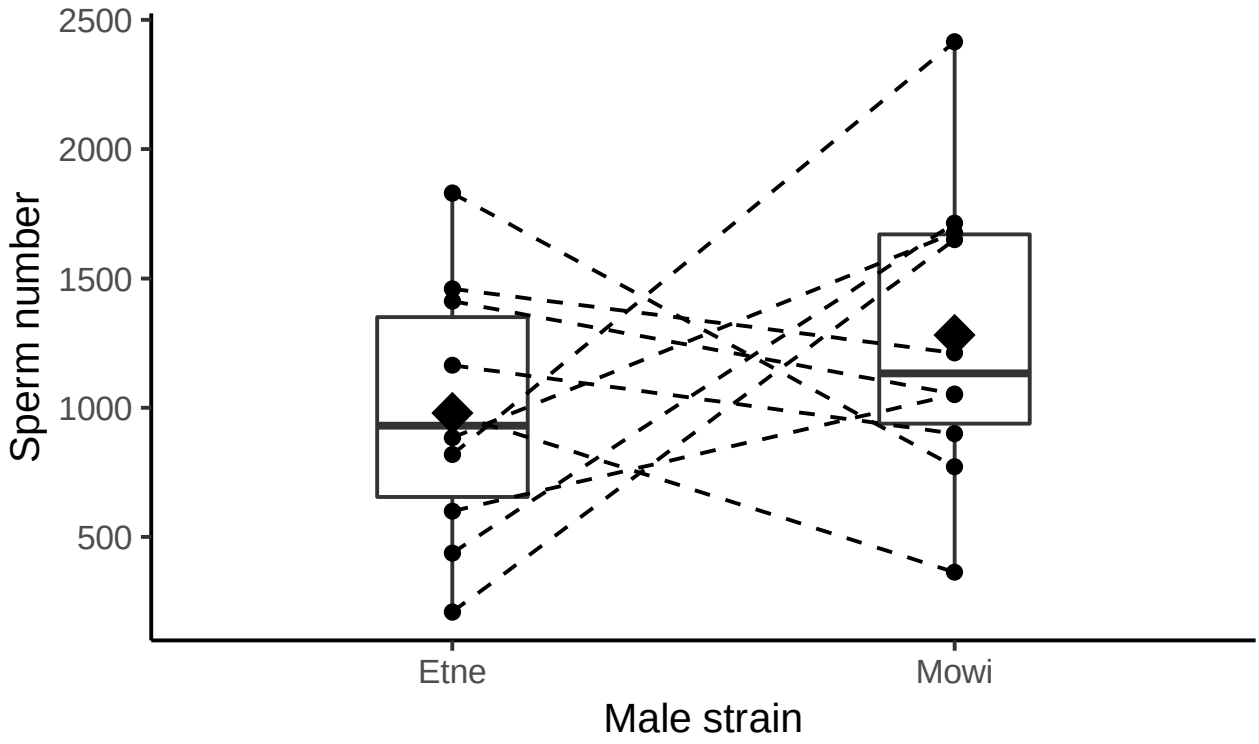

Figure 3: Sperm number of individual males averaged across stills taken from videos under microscopy in both Etne and Mowi males. The dashed line connects the competing males with the mean as a large black diamond, median as a horizontal black line and individuals males as a black circle. No significant difference was observed between Etne (980) and Mowi (1280) male sperm number.

Table 3: Average sperm number per males across video sperm counts, each column represents the Etne and Mowi male in competition.

Etne male	Sperm number	Mowi male	Sperm number
E13	978	M11	364
E14	884	M12	1677
E15	1412	M13	1053
E16	1164	M14	900
E17	819	M15	2415
E18	600	M16	1051
E19	1830	M17	772
E20	438	M18	1714
E21	210	M19	1650
E22	1460	M20	1212

4.4 Discussion

This study investigated whether a barrier to hybridisation exists at the gamete level between genetically unrelated farm and wild Atlantic salmon. Through 38 controlled fertilisations carried out under a powerful reciprocal mating, split clutch and split ejaculate design (Fig.1), we have identified no barrier to reproduction at the gamete level between farm Mowi and wild Etne Atlantic salmon whilst under sperm competition. We identify sperm from male farm salmon to gain a significantly greater than equal paternity share when competing for eggs from female wild salmon. When competing for farm salmon females, farm male sperm still gained a paternity majority, but this was not significantly greater than equal paternity. Ovarian fluid was manipulated in the experiment, with each female's egg batches split into treatments containing their own self-ovarian fluid and the other bathed in non-self-ovarian fluid from a female of the opposite farm or wild strain. Ovarian fluid had no significant effect on paternity, and this was true for competitive fertilisations for eggs from both farm and wild strain salmon.

4.4.1 Farm vs wild sperm competition

Farm salmon sperm achieved a greater proportion of paternity when competing with wild male sperm for eggs from farm and wild females. Our finding that farm salmon gametes are viable and have not undergone degradation or a reduction in competitiveness through aquaculture is supported by previous research, with Yeates et al., (2014) finding farm gametes have equivalence in function , fertility , competitiveness and compatibility. Here, we test compatibility between unrelated Mowi farm and Etne wild strains, whereas Yeates et al.,(2014) compared the Aquagen farm strain with one of its most important wild ancestors from the river Namsen. Camarillo-Sepulveda et al., (2016), observed no difference

in biochemical variables and no difference in metabolic enzyme activity in genetically unrelated farmed and wild Atlantic salmon sperm. In Chinook salmon, Lehnert et al., (2012) observed that farm males produced sperm with greater longevity and density compared to wild males, also suggesting that farming practices may lead to increased sperm performance. The equivalence of farm and wild gametes in Atlantic salmon suggests that domestication has not been sufficiently long or misdirected to result in selection up or down for sperm function (Yeates *et al.*, 2014). Additionally, Atlantic salmon's natural mating system is founded on a highly accurate ability to locate and spawn in natal rivers, observed to be as high as 97-99% effective (Stabell, 1984). This efficient pre-copulatory sexual selection mechanism of homing to natal rivers may result in female Atlantic salmon having a reduced ability to select between sperm based on strain / origin, and instead select for sperm based on performance only, such as motility (Gage *et al.*, 2002). Atlantic salmon females are far more likely to have mechanisms to prevent inbreeding than outbreeding depression, because of philopatry to spawn in natal streams. The chance of a male arriving at an inappropriate natal spawning site and breeding with a more unrelated individual female would be rare in natural mating systems, so there would be little selective pressure for females to guard against this.

Our finding that sperm from farm males under male-male sperm competition are dominant has been previously hypothesised to result from selective competition incited in the aquaculture fertilisation process (Lehnert, Heath and Pitcher, 2012). Gamete performance of males has the potential to vary widely across farm salmon strains, and within strains across farms. Aquaculture practice during both fertilisation (use of mixed milt) and rearing (inappropriate operational sex ratios) is likely to vary across farms. The potential for divergence in farm practice could result in variation to the intensity of sexual selection

pressures. Sexual selection and its effect on gamete quality is rarely controlled in the aquaculture production process, but may result in farm males showing both improved and degraded sperm quality depending on the farm's fertilisation and rearing processes.

The dominance of farm males in this experiment indicates that the risk from genetic introgression into wild populations following farm escape is high, as no reproductive barrier or degradation of sperm quality has occurred in the domestication process. The widespread and severe genetic introgression in some rivers is further (Glover *et al.*, 2020) supported by our finding that farm gametes are more than capable of fertilisation and competition with wild gametes. Genetic introgression is occurring across Atlantic salmon's natural range (Crozier, 2000; Bourret *et al.*, 2011; Glover *et al.*, 2013) and, at its most extreme, has removed the natural genetic structure of populations resulting in genetic extirpation (Glover *et al.*, 2020). In assessment of farm salmon's reproductive ability, whole organism studies have observed that farm salmon are disadvantaged due to inappropriate spawning behaviour. Farmed males are less aggressive (Gross, 1998) and have been observed to fail to enter female nests to spawn when oviposition is occurring (Fleming *et al.*, 1996). Furthermore, farmed individuals in wild systems have also been observed to spawn at different reaches in the river, and at different times to wild fish, reducing their ability to interbreed (Webb *et al.*, 1991). Importantly, these studies focus on mature Atlantic salmon escaping from farms; as suggested by Yeates et al., (2014) more research is needed to understand the implications of farm salmon early in development escaping and having a more natural development that could potentially result in adaptation to the natural environment and result in mitigation of inappropriate behaviour. If farm salmon after a period of time, whether as juveniles or adults, are able to develop natural behaviours, our

finding that gamete performance is equal or greater is a key indicator of the threat posed by genetic introgression.

The extent of genetic introgression is variable across Atlantic salmon populations, the vulnerability of wild populations to introgression is thought to be linked to wild population density (Glover *et al.*, 2020), repeated annual farm immigration numbers into natal rivers (McGinnity *et al.*, 2003; Jonsson and Jonsson, 2006; Karlsson *et al.*, 2016; Glover *et al.*, 2017, 2020) and genetic distance between farm escapees and wild individuals (Einum and Fleming, 1997). One potential further factor could be the intensity of unintentional sexual selection in surrounding farms and therefore the potential for improved sperm quality / competitiveness. Wild populations surrounded by farm males with high sperm quality may come under greater pressure from introgression.

4.4.2 Ovarian fluid

Ovarian fluid origin had no effect on the outcome of in vitro sperm competitions between farm and wild salmon sperm, when competing for farm or wild egg batches of two genetically distinct strains. Our finding that ovarian fluid does not influence post-copulatory sperm selection is in contradiction to the majority of literature on ovarian fluid. In Lehnert et al, (2017) study of Chinook salmon, ovarian fluid was identified to influence the outcome of sperm competition, selecting for males that were less related to the female. Conversely, Butts *et al.,*(2012) found sperm activated in ovarian fluid of a related female had significantly higher velocity than when activated in ovarian fluid of a non-related female, suggesting a recognition system for specific sperm genotypes. The role of ovarian fluid as a potential post-copulatory sperm selection mechanism has been observed across salmonids (Olsén *et al.*, 2001; Lahnsteiner, 2002; Turner and Montgomerie, 2002; Rosengrave *et al.*, 2008a), and there is ample opportunity for further discovery in this interesting male-female

relationship close to the point of fertilisation. In many experiments examining the influence of ovarian fluid, fertilisation took place with sperm being directly pipetted onto egg batches (dry) (Butts *et al.*, 2012; Alonzo, Stiver and Marsh-Rollo, 2016) or with sperm being pre-activated in ovarian fluid and passed into a flow of water towards the egg batch (Rosengrave *et al.*, 2008a; Lehnert *et al.*, 2017). In our experiment. egg batches were not pre-activated and fertilisations took place in a 500ml fertilisation tray (fig.2) with water added directly onto eggs and milt to allow fertilisation to take place. One potential explanation for our finding that ovarian fluid has no effect on proportional paternity is that, when water is added to the fertilisation tray, this dilutes and displaces ovarian fluid from the egg batch, neutralising ovarian fluid's potential post-copulatory sexual selection role. Thus, our in-vitro fertilisation trial conditions may not create the ovarian fluid rich micro-environment gametes would encounter in a natural mating system.

The potential for the role of ovarian fluid to have been neutralised means this experiment is focusing to a greater extent on the outcome of male-male sperm competition. Our results suggest that, independent of ovarian fluid, farm salmon are producing more competitive sperm than wild fish, whether that be velocity (Gage *et al.*, 2004), longevity (Alavioon *et al.*, 2019) or swimming linearity (Stoltz and Neff, 2006).

4.4.3 Farm v Wild experimental procedure effects

In all sperm competition experiments where two strains of Atlantic salmon are utilised, it is important to investigate the potential impact of divergent rearing and stripping processes. In our experiment, milt was collected from Mowi and Etne males at separate locations, despite consistent stripping methodology, minor differences may be introduced due to different teams carrying out the process, potentially affecting sperm quality and ultimate

fertilisation outcome. Additionally, sperm samples were transported in this experiment from different locations to the hatchery in Matre, introducing potential transport effects such as storage duration or temperature (Wagner, Arndt and Roubidoux, 2006). Our study was carried out with an experienced team to minimise the potential impact of different transport and stripping methodologies, but we cannot rule out practical effects to have confounded gamete performance.

In this experiment, we used Mowi Atlantic salmon, one of the most widely used strains of farm salmon in the world, and Etne strain salmon, which importantly were genetically wild but reared under similar environmental conditions. Etne salmon are reared and maintained in a conservation hatchery, and artificial rearing environments can be detrimental to wild fish fitness (Araki and Schmid, 2010). In brown trout and steelhead, the hatchery environment can cause lower reproductive fitness of individuals (Reisenbichler and McIntyre, 1977; Hansen, 2002). In Atlantic salmon, hatchery released individuals have lower survival and genetic diversity than their fully-wild counterparts (McGinnity *et al.*, 2003; Blanchet *et al.*, 2008). An important factor when comparing these two strains in direct sperm competition is therefore the potential effect that an artificial hatchery environment may have had on comparisons between farm and wild strain Atlantic salmon. Farm Atlantic salmon, despite being under artificial conditions, are likely to have adapted to a greater extent to the artificial fertilisation and rearing environment, as observed in Chapter 1 of this thesis where farm Atlantic salmon were more resilient to some artificial fertilisation factors. Importantly, for all sperm samples, density has been assessed as a metric of sperm quality, with no significant difference between wild and farm individuals (Figure 3).

4.4.4 Threat of genetic introgression at the gamete level

This study finds that when sperm from farm and wild and genetically unrelated salmon compete that farm gametes show superior performance, and that ovarian fluid identity did not influence relative paternity outcome. The significant advantage in proportional paternity for farm salmon sperm indicates that no barrier to reproduction exists after mating and through farm-wild hybridisation, and therefore that introgression by farm genes into wild populations is a major threat to wild salmon conservation. Despite the significant findings of the study only a modest sample size has been achieved and analysed meaning that any findings need to be assessed with caution

This finding suggests that the aquaculture industry should maintain and increase efforts to mitigate the threat posed by farm Atlantic salmon male escapes. Evidence has shown that triploidy can be a highly effective sterilisation technique (Murray et al., 2015), but it is currently not being utilised due to triploid individuals providing ethical and economic concerns to the industry (O'Keefe and Benfey, 1997; Bjørnevik *et al.*, 2004; Ozerov *et al.*, 2010; Fraser *et al.*, 2015). The gamete equivalence and even dominance in this experiment of farm strain males suggests that further research should go into developing aquaculture systems that can support the divergent needs of triploid or sterile individuals, such as the need for improved nutrition and housing conditions (Fjelldal *et al.*, 2016; Smedley *et al.*, 2016). Future research could also investigate sperm competition between farm and wild males from different farm and wild strains to understand whether our finding is consistent across multiple genetic backgrounds, or whether this dependent on the strains competing. Future research could also look to utilise wild salmon that have not experienced an artificial rearing environment, to determine both environmental and genetic impacts on gamete performance in tandem.

Chapter 5

Does experimental evolution under heightened sexual selection enable cryptic female choice in the flour beetle *Tribolium castaneum*?

Abstract

Cryptic female choice is defined as “female-mediated morphological, behavioural or physiological mechanisms that bias fertilisation towards the sperm of specific males ” (Thornhill, 1983). Cryptic female choice (CFC) is a difficult mechanism to observe and represent experimentally, due to the difficulty in isolating female-driven effects due to the intimate nature of male-female interactions. Through the use of long term experimental (130 generations) high sexual selection and low sexual selection *Tribolium castaneum* lines we look to identify whether females ability to assert CFC varies. High sexual selection line females are maintained in operational sex ratios of 90 males to 10 females whilst low sexual selection line females are maintained in operational sex ratios of 10 males to 90 females as sexually mature adults. We hypothesise that an evolutionary history of heightened opportunity for CFC under strong sexual selection will enable mechanisms of sperm selection to evolve. We test this hypothesis by mating each female to two males in sequence, who either inseminated the ‘right’ versus ‘wrong’ type of sperm, and assess whether paternity bias towards fertilisation by the ‘right’ kind of sperm occurs more frequently in females from lines that have experienced greater evolutionary opportunity for CFC. The ‘right’ kind of sperm was provided by a conspecific marker male, while the ‘wrong’ kind of sperm was from congeneric T. freemani males, where fertilisations will produce infertile hybrid offspring. We observed no difference between lines with stronger versus

weaker opportunities for CFC. High sexual selection and low sexual selection lines across any of the mating scenarios. Across 317 females from 3 independent lines per SS regime, we found that, on average, the 'right' kind of conspecific sperm gained 62% fertilisation precedence, indicating a general fertilisation advantage for the 'right' kind of conspecific sperm. However, we found no evidence that the strength of CFC could evolve, with no difference in conspecific fertilisation precedence for females that had experienced a greater evolutionary opportunity for sperm choice.

5.1 Introduction

Historically, it was believed that sexual selection ceased with acquisition of a mate through male competition or female choice (Andersson, 1994). Parker's seminal work (Parker, 1970) provided additional understanding that a number of mechanisms have evolved across taxa which operate along the route from gamete release to haplotype fusion and fertilisation, and which we now know can have profound influences upon individual reproductive success (Simmons and Garcia-Gonzalez, 2021). Research over the last half century has identified that sexual selection can occur through post-copulatory processes of sperm competition (Parker, 1970) and cryptic female choice (Thornhill, 1983; Eberhard, 1997). Post-copulatory mechanisms also influence reproductive success and gene flow, possibly to the same extent as male-male competition and female mate choice (Parker 1970; Smith 1984). There is growing evidence that these gamete level processes can have population-level effects (Simmons., 2001; Andersson and W. Simmons, 2006; Palumbi, 2009).

Sperm competition occurs when two or more males compete for a single female's ova (Parker 1970). The important role of sperm competition has become well established, being observed to occur in a wide range of organisms in both internal and external fertilisation systems (Birkhead, 1987; Birkhead and Moller, 1998; Gage et al., 2004; Parker, 1970; Simmons., 2001; Smith, 1984; Birkhead and Hosken, 2008; Godwin et al.,2017). Sperm competition provides a strong force of sexual selection, affecting both males and females from morphological adaptations to behavioural changes (Birkhead & Møller 1998; Godwin et al.,2017). At the gamete level, sperm competition influences the evolution and allocation of sperm numbers (Parker 1990; Birkhead & Hosken 2008), as well as traits of sperm velocity (Gage *et al.*, 2004), and sperm morphology (Oppliger *et al.*, 2003; García-González and Simmons, 2007; Lüpold *et al.*, 2009; Godwin *et al.*, 2017). Sperm competition drives the evolution of more competitive ejaculates with Godwin et al.,(2017), for example, revealing that ejaculates from male flour beetles following 77 generations of selection under high levels of male-male competition gained 20% greater paternity than ejaculates from similar populations under weak selection from male-male competition. Additionally, sperm competition can provide females with direct fitness benefits (Firman, 2011) through an increased chance of fertilisation (Sheldon and Burke, 1994) and lower probability of infanticide (Hrdy, 1977).

The post-copulatory process of cryptic female choice (CFC) has received less attention (Eberhard, 1997), potentially due to a greater focus on sperm competition and the difficulty in proving when CFC is taking place in isolation from effects driven by sperm competition, and so its importance in controlling reproductive fitness has been debated (Birkhead, 1998; Fedina, 2007). Cryptic female choice is defined as "female-mediated morphological, behavioural or physiological mechanisms that biased fertilisation towards the sperm of

specific males ” (Thornhill, 1983). Over 20 potential mechanisms of cryptic female choice were defined by Eberhard (1997), including disruption of copulation, sperm ejection, sperm activation and differential fertilisation. The precision of these mechanisms increases with proximity to the ova due to fewer sperm being present (Firman *et al.*, 2017). Previous research on cryptic female choice (CFC) suggests that females are not passive participants in post copulatory sexual selection (Eberhard, 1997; Yeates *et al.*, 2009; Gasparini and Pilastro, 2011; Firman *et al.*, 2017), and it is hypothesised to occur widely, including across fish (Rosengrave *et al.*, 2008a; Rosengrave, Montgomerie and Gemmell, 2016; Butts *et al.*, 2017), insects (Fricke and Arnqvist, 2004; Briceño and Eberhard, 2009), mammals (Wissenschafts-Verlag, Keil and Sachser, 1998; Fitzpatrick *et al.*, 2020) and broadcast invertebrate spawners (Evans *et al.*, 2012). CFC specifically has been observed as a mechanism to prevent inbreeding (Gasparini and Pilastro, 2011; Lovlie *et al.*, 2013) and avoid hybridisation (Yeates *et al.*, 2013), both of which will be more costly to females than males. Non-random mating patterns produced through CFC could provide genetic benefits through increased survival of genetically viable and diverse offspring (Ivy & Sakaluk 2005; Simmons 2005; Evans et al.,2012; Rosengrave et al.,2016). This fitness benefit is a result of selection for “good” or compatible genes, resulting in an influence of CFC on gene flow and therefore population genetic structure (Ivy & Sakaluk 2005; Stapper et al.,2015).

Despite a large body of experimental and theoretical work suggesting that cryptic female choice is a common and powerful force of post-copulatory sexual selection, however, direct evidence for its impact has been challenging to reveal (Eberhard, 1997). This scarcity of direct evidence for CFC may be in part due to the difficulty in experimentally isolating this process from factors including sperm competition, differential fertilisation rates and differential offspring survival rates (Gilchrist and Partridge, 1997).

In this chapter, we use experimental evolution to explore whether mechanisms of CFC can evolve under divergent intensities of sexual selection, and test whether cryptic female choice can evolve to discourage fertilisation by the 'wrong' kind of sperm and/or encourage fertilisation by the 'right' kind of sperm. In this experiment, the 'wrong' kind of sperm comes from a close genetic relative *T. freemani* that can hybridise with *T. castaneum*, and which will generate non-fertile hybrid offspring (Robinson, Johnson and Wade, 1994; Wade and Johnson, 1994). By contrast, the 'right' kind of sperm comes from a conspecific male carrying a genetic marker to reveal paternity.

T. castaneum is an ideal model (Lumley *et al.*, 2015; Godwin *et al.*, 2017; Lewis *et al.*, 2020; Pointer, Gage and Spurgin, 2021) for exploring CFC, being long-lived and easily maintained (Pointer, Gage and Spurgin, 2021) that is promiscuous and stores sperm from multiple males. Virgin females can to mate with 12 males an hour (Pai et al., 2007; Pai & Yan, 2003). *T.castaneum* males, once sexually mature, are able to copulate with a female every 2-3 minutes (Lewis, 2004). In studies of sperm competition in *T.castaneum,* variation in male fertilisation success has been shown to be high, with males regularly failing to fertilise a female (Bloch Qazi, Herbeck and Lewis, 1996). Research on CFC in *T. castaneum* has identified females to have multiple mechanisms that might influence fertilisation success of males pre-copulation (Edvardsson and Arnqvist, 2000), during copulation (Fedina, 2007) and post-copulation (Fricke and Arnqvist, 2004). Edvardsson and Arnqvist (2000), observed that a female's perception of copulatory courtship behaviour governs subsequent fertilisation success of sperm post-copulation.

T. castaneum females also readily accept matings with males of the close relative *T. freeman,* and together they hybridise to produce sterile offspring (Hinton, 1948; Juan et al., 1993; Wade & Johnson, 1994; Wade et al., 1994). When studying cryptic female choice,

little to no premating reproductive isolation has been observed between these species (Wade and Johnson, 1994). However, the production of hybrid offspring that are effectively reproductive dead ends should be under evolutionary selection to avoid, and CFC is an obvious mechanism to enable the avoidance of such hybrid fertilisations. Fricke and Arnqvist (2004), observed that *T. castaneum* females were able to utilise conspecific sperm precedence to select against heterospecific sperm. Heterospecific males were closely related suggesting conspecific sperm precedence has developed rapidly. Conspecific sperm precedence has been observed between *T. castaneum and T. freemani* the focal species of this study (Wade and Johnson, 1994). We therefore explore whether such mechanism of CFC become strengthened under conditions where there is great opportunity for female choice, and/or weakened when such choice has been restricted.

In this study, we used *T. castaneum females* from the sexual selection lines that were established in 2004 at the University of East Anglia (UEA) (Lumley et al 2015) to test between individuals from replicate populations evolving under experimentally high and low sexual selection regimes. Preliminary assessments of the female reproductive tracts from these regimes reveal that females evolving under histories of high sexual selection show greater complexities in their sperm storage organs and potentially their ability to implement CFC (unpubl.data). Using this, we look to identify whether a history of evolving under high or low opportunities for sexual selection and cryptic female choice enable changes in the ability of CFC to select against heterospecific sperm and favour conspecific sperm.

5.2 Materials and methods

5.2.1 Stocks

The hybridising flour beetles, *Tribolium castaneum* and *Tribolium freeman,* were used in this experiment as models for studying CFC. T. *freemani* adults are 1.4 times larger than *T. castaneum* adults in body size (Nakakita, 1983). All beetles used in the experiment were maintained under standard conditions within a controlled environment facility (CEF) at the University of East Anglia (UEA) at 30 ± 1 °C, 60 ± 5% RH and 16L: 8D photoperiod. Their fodder mix consisted of 10% organic brewer's yeast, 90% organic strong white bread flour, topped with organic jumbo oats for traction (Doves Farm, UK). This experiment used the long-term *T.castaneum* sexual selection lines, created in 2006/7 from the widely used *Georgia* lab strain. At the time of the experiment 130 generations of experimental evolution had elapsed over 14 years of line selection and management.

The sexual selection lines are maintained as non-overlapping generations exposed to two contrasting adult sexual selection regimes: male biased and female biased both of which have three independent replicate lines (Fig.1). Male biased & female biased treatments are designed to assert high sexual selection pressure (male biased) and low sexual selection pressure (female biased) on females. Both male biased and female biased treatments are given 7-days to mate and oviposit within each generation and, during this period, strong versus weak sexual selection pressure and opportunities are created by controlling the adult sex ratios, while equalising the effective population sizes. Strong sexual selection is applied by biasing the adult operational sex ratio where there are 90 males and 10 females, while contrastingly weaker sexual selection is applied at each adult generation by housing only 10 males with 90 females. Sex ratios are maintained each generation through pupal sexing, and 18-22 days after adults are removed, the resulting pupae are sexed using microscopy and collated for the next generation of male-biased and female-biased replicate lines. In Petri dishes (5cm Petri dish Thermo Fisher brand), isolated pupae are then left for 10 days to

eclose and sexually mature in single-sex groups. The correct sex ratios are then placed into 250ml colony jars with 150ml fodder for 7 days mating and oviposition, under divergent strengths of male-male competition and female choice.

To assess the outcome of any cryptic female choice, a female was mated to two experimental males, one of which was a *T. freemani* individual and the other a *T.castaneum* reindeer mutant (RDHD). *T.freemani* are a closely-related sister species of *T.castaneum* that can successfully mate and produce infertile hybrid offspring. *T.freemani* are maintained in non-overlapping generations: after 40 days, sexually-mature adults are introduced to fresh fodder for 7 days to mate and oviposit to found the next generation under standard conditions. The second male is a conspecific *T.castaneum* carrying a dominant homozygous mutation resulting in swollen clubbed antennae for identification of offspring. These reindeer mutant *Tribolium castaneum* are utilised to identify paternity, with all offspring carrying the clubbed reindeer antennal the phenotypic marker being sired by the conspecific male. *Tribolium castaneum* (RD mutant) individuals are also maintained in non-overlapping generations: after 35 days, sexually mature adults are introduced to fresh fodder for 7 days to mate and oviposit to found the next generation.

This experiment was performed on long-term sexual selection lines of *T. castaneum*, created in 2006/7 from Georgia 1 (GA1) strain individuals. The *T. freemani* beetles were acquired from the United States Department of Agriculture (USDA) as viable larvae in 2018, which were then reared and maintained at UEA under standard conditions. Prior to initiating this study, the sexual selection lines had been under selection for 130 generations (14 years). They have been maintained as non-overlapping generations with two independent treatments male biased (MB) and female biased (FB), both of which have three independent replicate lines (Fig.1). Both MB and FB treatments are paired for 7-days to mate and oviposit

in a generation; during this period, we produce high and low sexual selection pressure by artificially manipulating sex ratios, 90:10 male: female in the MB treatment and 10:90 male: female in the FB treatment. Sex ratios are maintained each generation through pupae sexing, which is carried out after 18-22 days post adult removal, and the sex of the pupae is determined by using a dissecting microscope with an external LED light source (GT vision Ltd). Isolated pupae are then left for a further 10 days to eclose into sexually mature adults: in single-sex groups of 15 pupae per Petri dish in standard fodder mix. Upon sexual maturity, the adults are placed into 250ml colony jars with 150ml fodder with oats in the correct sex ratios for an additional 7 days for mating and oviposition under standard CEF conditions.

5.2.2 Focal males

To investigate the actions of cryptic female choice, two focal males were used in the experiment: a *T. freemani* wild type heterospecific and a *T. castaneum* reindeer mutant (Rdhd- Reindeer honey dipper). *T. freemani* are a closely-related sister species (Angelini and Jockusch, 2008) of *T. castaneum* that can successfully mate and produce infertile hybrid offspring (Fricke and Arnqvist, 2004) which are approximately 1.5 times larger in body size than *T. castaneum* offspring (Unpublished work). The second male introduced is a conspecific *T. castaneum* with a dominant homozygous visible mutation, producing a phenotype of swollen clubbed antennae compared with the filiform wild type (Bernasconi *et al.*, 2006; Godwin *et al.*, 2017)(fig.2). All offspring sired by the reindeer mutant *T. castaneum* male will carry the visible antennal mutation (Fig. 1), enabling paternity identification.

Figure 1 Picture showing a Reindeer mutant *T.castaneum* on the left and a standard *T.castaneum* on the right. Image shows the major phenotypic difference which allows us to determine paternity. Reindeer *T.castaneum* have bulbous antennae as opposed the filiform antennae of the *T.castaneum* male credit: Nadine

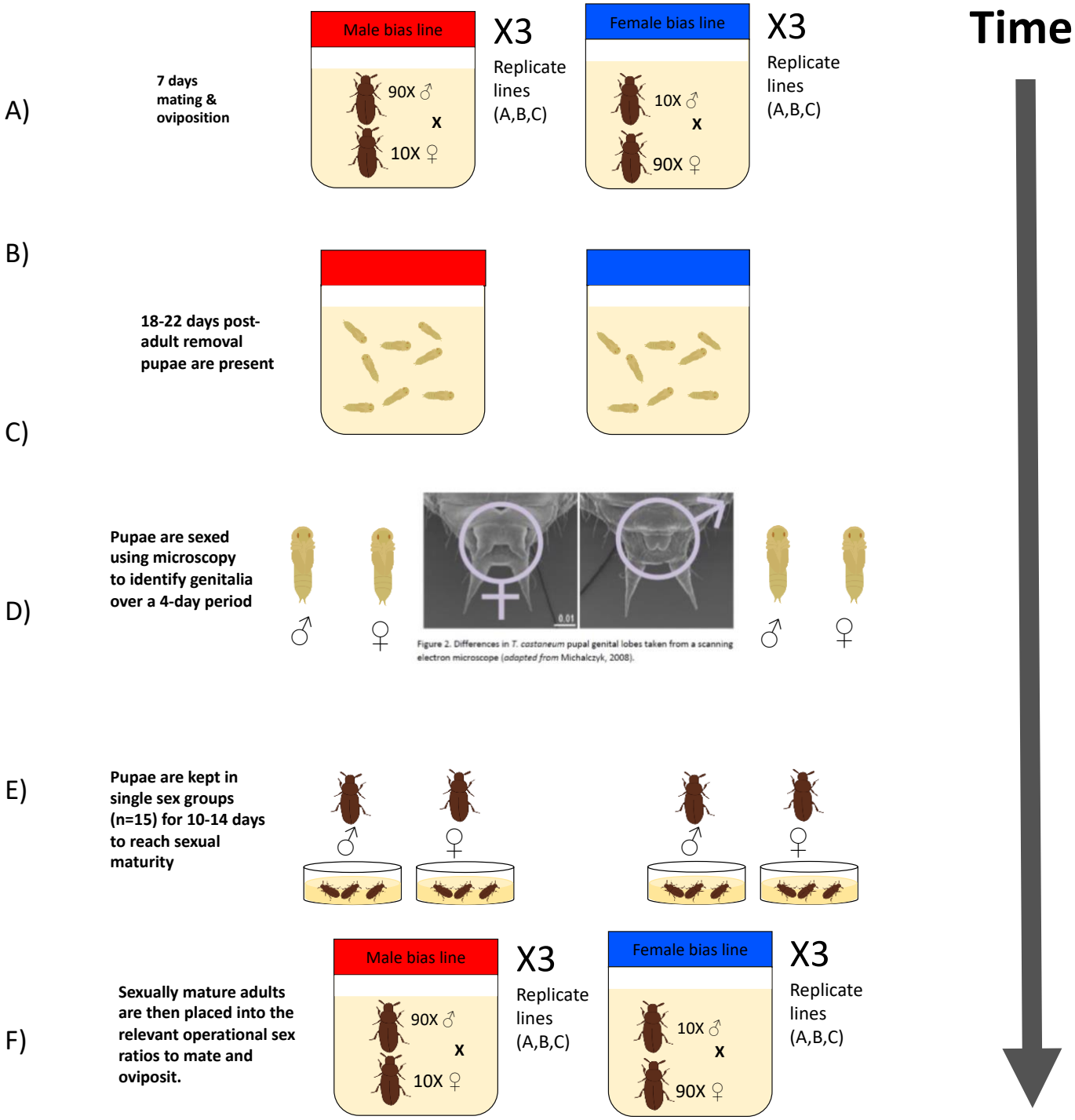
Time
A)
7 days mating & oviposition
Male bias line
90X ♂
X
10X ♀
X3
Replicate lines (A,B,C)
Female bias line
10X ♂
X
90X ♀
X3
Replicate lines (A,B,C)
B)
18-22 days post-adult removal pupae are present
C)
D)
Pupae are sexed using microscopy to identify genitalia over a 4-day period
♂
♀
0.01
♂
♀
Figure 2. Differences in T. castaneum pupal genital lobes taken from a scanning electron microscope (adapted from Michalczyk, 2008).
E)
Pupae are kept in single sex groups (n=15) for 10-14 days to reach sexual maturity
♂
♀
♂
♀
F)
Sexually mature adults are then placed into the relevant operational sex ratios to mate and oviposit.
Male bias line
90X ♂
X
10X ♀
X3
Replicate lines (A,B,C)
Female bias line
10X ♂
X
90X ♀
X3
Replicate lines (A,B,C)

Figure 2: Maintenance protocol for sexual selection line. A) Sexually mature adults are given 7-days to mate and oviposit in their respective 90:10 ratios, male biased 90 males 10 females and females biased 10 males and 90 females. B/C) Adults are removed, and offspring are given 18-22 days to mature to pupae at which point they are sexed under a microscope. D) Once sexed adults are kept in single-sex groups for 10 days to reach sexual maturity. E) Individuals are then assorted into the relevant sex ratio's and are given 7 days to mate and oviposit. Generation 130 of these sexual selection lines.

5.2.3 Experimental procedures

All pupal sexing was completed within 9 days of first pupation observed. Once sexed, all-female pupae were kept individually in ~0.75ml fodder and ~2-3 oats (individual number) within a 3ml Eppendorf (hole for ventilation) to ensure that any failure to correctly sex individuals did not result in mating prior to the experiment. Males of both species were kept in small groups of 15 in 5cm plastic Petri dishes with ~7.5ml of fodder and a layer of oats for traction. All pupae were given a minimum of 10-days to attain sexual maturity. During maturation, females were marked with a non-toxic, water-soluble paint-pen (uni Posca paint pen) on the thorax to allow future identification when mating was terminated.

Sequential mating protocol: two-male sperm competitions and CFC

In an attempt to focus on postcopulatory influences on paternity outcome, an experimental design was applied in which individual females were mated to the heterospecific and conspecific male in sequence, and the resulting paternity then tracked following up to 40 days of female oviposition (Fig. 3). In this design, the *T. castaneum* sexual selection line virgin female was paired first with a heterospecific *T. freemani* male for 48hrs in a 7ml mating vial containing ~1.5ml fodder (standard). The vial lids contained holes to allow for

ventilation. Females were then separated from the first males, and placed in a fresh vial on their own for a 48hr period of oviposition to ensure the first mating was successful and had transferred sperm (using subsequent offspring checks). After the completion of 48hrs of oviposition, the singly-mated females containing *freemani* sperm were then transferred to another vial and paired with the second, conspecific *T. castaneum* reindeer male mutant for 12hrs. Males were discarded once mating was terminated. The 48h and 12h mating periods were chosen in order to (1) maximise the chances of sperm transfer by both heterospecific and conspecific males in order to assess their relative fertilisation precedence in male-biased and female-biased females, while (2) aiming to reduce the possibility for variable mating frequency to have influenced the paternity outcomes.

All mating and oviposition vials were maintained in a controlled environment facility at standard conditions of 30 ± 1 °C, 60 ± 5% RH and 16L: 8D photoperiod. Here, vials were incubated to allow offspring development through to adulthood before being counted and scored for phenotype.

After both mating periods were completed, the female was transferred to a Petri-dish with ~7.5g fodder, and allowed to oviposit for 10 days, before being transferred to fresh Petri-dishes repeated for 4 x 10-day blocks. All Petri-dishes were subsequently incubated for 35-days after each female was moved onto the next 10-day oviposition block, under standard conditions. Paternity was scored among offspring by visually assessing for the presence or absence of the reindeer 'clubbed-antennae' phenotypic marker. Thus, after two mating opportunity periods for each female with a heterospecific and conspecific male, followed by oviposition for 40 days across four 10-day blocks and then all resulting offspring allowed to develop to adulthood, we could calculate the proportions of conspecific versus heterospecific offspring produced by for the females from the two contrasting sexual

selection backgrounds, and therefore whether either regime was more be able to encourage conspecific fertilisation precedence when given a choice between different males' sperm.

In total, n=79 matings took place, with n=43 by females from the male-biased regime and n=36 from the female-biased background. Each regime had three independent replicate lines, in which matings and sperm competitions were successfully executed (Male-biased line A n=14, Male-biased line B n==16, Male-biased line C n=13; Female-biased line A n=9, Females-biased line B n=11, Female-biased line C n=16).

In order to assess post-copulatory cryptic female choice, it was essential that both males had successfully inseminated each female, which we confirmed by checking their mating and oviposition vials for the presence of relevant offspring, and any females that had not produced offspring from both *T. freemani* and *T. castaneum* possible fathers throughout all oviposition periods were excluded. Females that produced <20 offspring in total were also censored from the analysis, ensuring that a representative average of n=156 offspring per female could be analysed for paternity biased. Following these controls, the final analysis sample size was n=67 overall in total, with n=38 for male-biased females and n=29 for female-biased females across replicate lines (Male-biased A=11 ,Male-biased B=15 , Male-biased C =12; Female-biased A=9 , Female-biased B=9 , Female-biased C=11). Results are presented both for trials in which both males achieved some paternity, and for trials in which the conspecific male achieved all paternity.

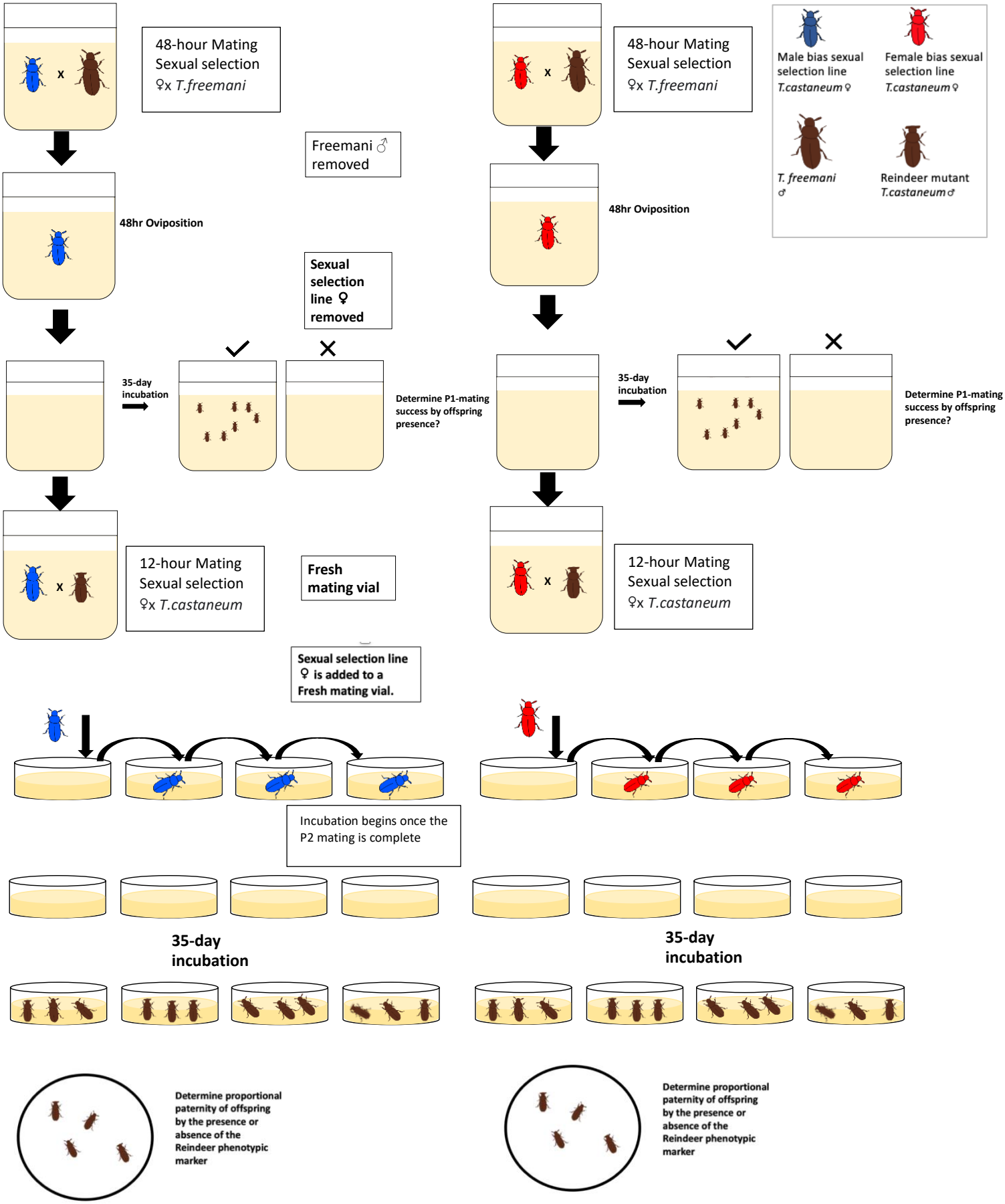

Figure3: Sexual selection (SS) background T.castaneum females were mated with a T.freemani male for 48hrs after which the T.freemani male was removed and the T.castaneum SS females then oviposited for 48hrs after which they were removed. The mating vial was then incubated under standard conditions (30 ± 1 °C, 60 ± 5% RH and 16L: 8D photoperiod) for 35 days after which the presence or absence of offspring was identified to determine P1 mating success. SS females were then transferred to a fresh vial to mate with a T.castaneum reindeer mutant male for 12hrs, after which both individuals were removed. SS females were then placed into a fresh vial every 10 days for a total of 50 days. Once the adult female was removed vials were incubated for 35 days under standard conditions. After 35-days paternity was assigned to offspring via visual identification of the reindeer clubbed antennae phenotypic marker.

Continuous trio mating protocol: pre- and post-copulatory effects when females are maintained with two competing males

In an attempt to focus on both precopulatory and postcopulatory influences on paternity outcome, an experimental design was applied in which individual females were housed continuously with the heterospecific and conspecific males, and the resulting paternity then tracked following up to 20 days of female oviposition within these trios (Fig. 4). The three adult beetles (Reindeer male *T.castaneum*, *T. freemani* male and a female from either the Male=biased or Female-biased Sexual Selection experimental evolution regimes) were simultaneously placed in a standard vial with *ad libitum* fodder to compete, choose, fertilise and oviposit for five days. To control for larval densities, the trios were rotated into new oviposition blocks (with new vials and fodder) every five days, with each vial containing ~5 pieces of oats and ~2.84ml fodder (standardized measuring spoon). All vials with eggs were incubated for 35-days under standard conditions upon removal of the adults. As in the previous experimental design for the sperm competition experiment, offspring paternity was determined after 35-days, by identifying the Rdhd phenotypic marker (Fig.2). In total, 273 mating trios were maintained across four 5-day blocks, with non-biological zeros due to premature deaths and escapees being excluded from final analysis. A final total of 248 matings took place across 62 trios (Male biased regime n= 31 , Female biased regime n=31), with replication in their respective lines of Male-biased A=12 , Male-biased B=10 , Male-biased C =10; Female biased A=6 , Female biased B=12 , Female biased C=12).

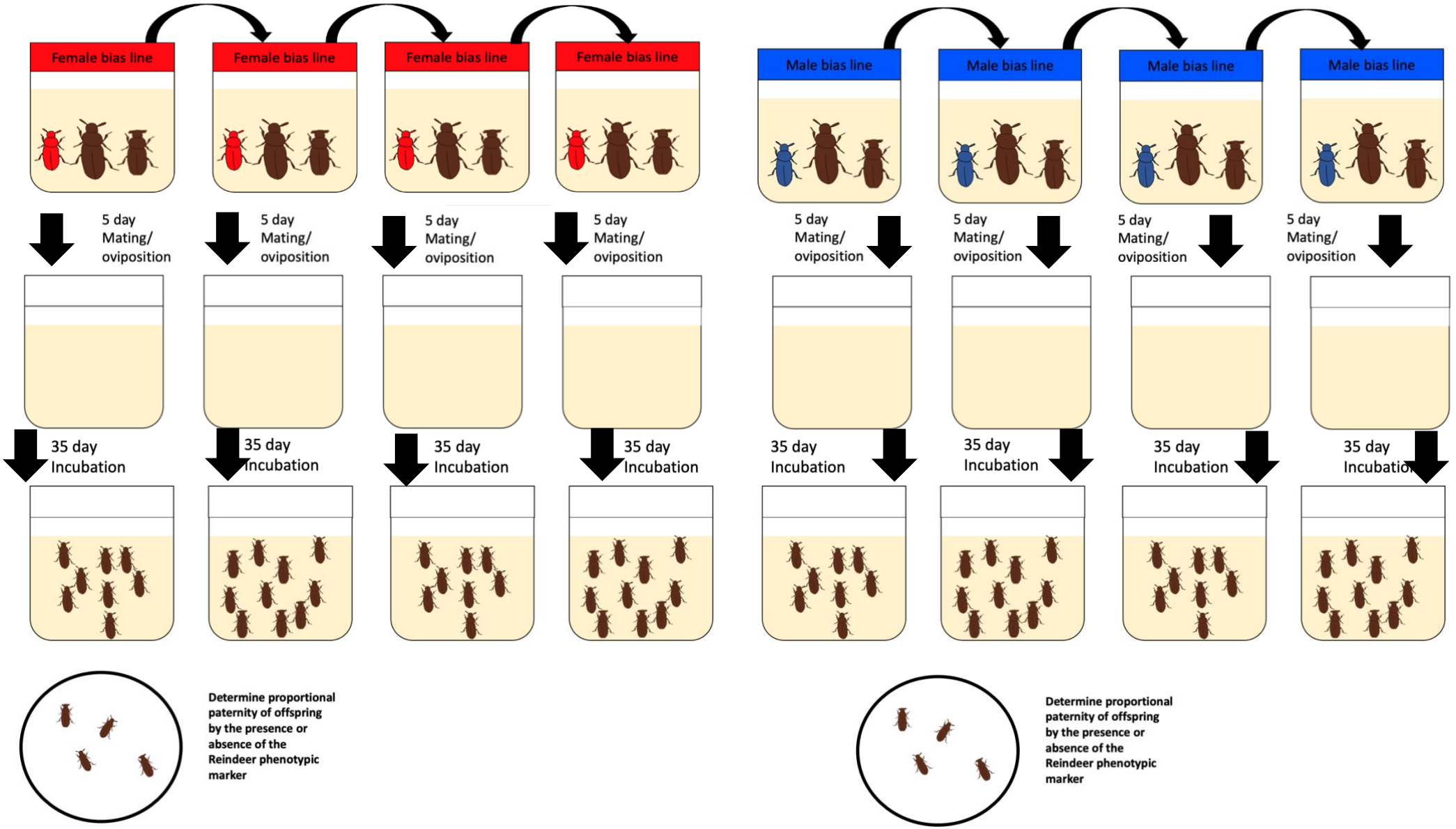

Figure 4: Sexual selection background T.castaneum females are kept in trio's with a T.freemani and T.castaneum reindeer male in mating vials for 5-day mating/oviposition before they are transferred to a fresh vial. In total the trio's were kept in mating vials for 4x5-day blocks with 35 days incubation until offspring where frozen and then counted to assess proportional paternity.

5.2.4 Statistical analysis

Data analysis was carried out in R-studio version 1.14.1106 using R-version 3.4.1 (R-Core team 2019) (RStudio Team, 2020). Generalised mixed models (GLMMs) were conducted using the 'glmmTMB' package (Bolker,2018). To compare between replicate lines of the same treatment, we used Wilcoxon-signed rank tests using the 'Wilcox.Test' function found in the 'stats' package in R. To analyse changes in proportional paternity and offspring number over time, we utilised both the lme4 (Bates *et al.*, 2015) and 'glmmTMB' package.

Sequential mating: two-male sperm competitions and CFC

Proportional paternity

To model proportional paternity (Reindeer *T. castaneum* vs. *T. freemani*) a GLMM (Goldstein, Bryk and Raudenbush, 1993) was fitted with a binomial error distribution, as the response variable 'paternity' is proportional. The fixed effect of sexual selection line origin (male biased vs. female biased) was coded with a random effect of replicate line ("A, B & C"), with each treatment having three replicates to account for variation.

To determine overdispersion of the model, I utilised the overdisp function (Bolker,2017) detecting overdispersion of count data, as proposed by Cameron and Trivedi (1990) based on the following equation, where H0 is the equidispersion given by Var(Y |X) = E(Y |X) as follows: Var(Y |X) = E(Y |X) + Φ[E(Y |X)]2. If the overdisp function identifies overdispersion the P value is <0.05.

In the proportional paternity model significant over dispersion was observed using the overdisp_ fun function chisq = 2357 , ratio = 36,828, P= <0.0001. To account for overdispersion, an observation level random effect was added to the model which

controlled for overdispersion shown through the overdisp_fun function, chisq=8.593 , ratio= 0.136 , P=1.000 (Harrison, 2014).

To model proportional paternity over time, I produced a glmm focusing on block 1 (1-10 days) and block 2 (11-20) this was due to the high number of 0's and low offspring production observed in block 3 (21-30) (0 offspring in 41/ 67 matings) and block 4 (0 offspring in 52/61 matings).

To model changes from block 1–2, I utilised the glmer function from the lme4 package to carry out a glmm with a binomial error model, with conspecific/heterospecific paternity producing a proportional response variable. The fixed effect was block two levels days 1-10 and days 11-20 with a random effect of individual replicate line. This was carried out separately for male biased and female biased sexual selection lines to identify changes over time.

Offspring number

To model differences in total offspring production of *T. castaneum* and *T. freemani* between male biased and female biased sexual selection lines, I utilised a GLMM (Goldstein, Bryk and Raudenbush, 1993). In both the analyses, offspring counts quantifying the number of reindeer vs. freemani offspring was overdispersed.

I used the "overdisp" function (Fávero *et al.*, 2020) to account for this and utilised a negative binomial error model "nbinom2" in the glmmtmb package which utilises a built-in measure to account for overdispersion. Following the use of a negative binomial error structure both the reindeer (0.937) and *freemani* (0.492) models were no longer overdispersed. Both models had sexual selection origin as a fixed effect with the replicate lines of each sexual selection line coded as the random effect.

To assess changes to offspring number through time a glmm was created using the 'glmmtmb' function with a negative binomial error structure. Block day 1-10 / day 11-20 was coded as the fixed effect with offspring number as the response variable and a random effect of replicate lines. Two separate models were produced to assess both *T. castaneum* offspring number and heterospecific *T. freemani* offspring number; this was done twice with separate models for male biased and female biased lines.

Continuous mating in the trio competitions

Proportional paternity

To model proportional paternity for constant data, we constructed a glmm model with a response variable of proportional paternity with the fixed effect of sexual selection line origin, model estimates were determined using laplace approximation. The random effect of replicate line was coded for with a binomial error model. Using "*overdisp"* we identified overdispersion , chisq = 14.55 , ratio = 0.105 , P=1.000 which was accounted for with an OLRE (Harrison, 2014)., chisq-10096 , ratio- 72.63, P=0.000.

To assess proportional paternity over time, a GLMM was produced using "glmmtmb" with a binomial error model and a 'cbind' proportional paternity as the fixed effect, model estimates were determined using laplace approximation. Block (as previously described above) was the response variable, and replicate line the random effect. The model was run twice for both female biased and male biased sexual selection regimes. Overdispersion was observed in both male biased and female biased models using the overdisp_fun function, so an OLRE (Harrison, 2014) was inserted into the model controlling for overdispersion.

Offspring number

As was done for the sequential mating analysis: to assess offspring number, GLMMs were constructed with sexual selection origin as the fixed effect, with the number of offspring as the response variable. The replicate line of each sexual selection regime was coded as a random effect with a negative binomial error structure.To assess offspring production over time, GLMM models were produced for both male and female biased sexual selection regimes, as described in the sequential mating section.

Wilcoxon signed rank tests

Wilcoxon rank tests were used to assess median values of replicate lines to ensure there were no significant differences between replicate lines of the same sexual selection treatment. Wilcoxon tests were carried out to assess differences for both proportional paternity and total offspring production of reindeer *castaneum* and *freemani* in sequential and constant mating. No significant differences between replicate lines of sexual selection treatments were observed in the experiment, allowing for analysis of replicate lines to be combined into sexual selection treatments male biased/ female biased. All P-values reported were calculated through the Wald t-test within the glmmtmb and lme4 packages.

5.3 Results

5.3.1 Differential fertilisation precedence: sequential matings

Proportion paternity

In the sperm competition trials, although the sperm of conspecific *T. castaneum* reindeer males achieved clear general precedence above 90%, there was no evidence that this level was different among the offspring batches in females from male-biased versus female-biased sexual selection lines. Conspecific *T.castaneum* reindeer sperm gained 96.4% (± 1.94) of competitive fertilisations in female-biased females, and 95.6% (±2.63) in male-biased females (Table 1, Figure 4). There was therefore no evidence that a selection history of greater opportunities for cryptic female choice within male-biased female lines enabled more effective CFC. When no controls were implemented, there remained no significant difference between male biased (81.5%) and female biased (79.8%) sexual selection lines (Figure 4). There were also no significant differences in % paternity between the replicate lines of the either sexual selection treatments (Table 2, Figure 4): Male biased, line A 96.2% ± 2.50, line B 95.7 ± 1.73, line C 95.8% ± 7.83); Female biased, line A 97.1% ± 10.15, line B 94.7% ± 2.86, line C 96.3% ± 2.26).

Table 1: A summary of the GLMM fixed effect analysis comparing relative paternity of *T. castaneum* (Reindeer) v *T. freemani* fertilisation precedence through sperm competition trials within female biased versus male biased sexual selection line females. Proportion paternity is the response variable, with a random effect of replicate line (Variance associated with random effect of block=1.417^{e-09}). The baseline for the GLMM is the female biased results.

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
Intercept (Female biased)	2.408	0.294	8.206	<0.001 ***
Male biased	-0.121	0.302	-1.018	0.308

Table 2: Results of Wilcoxon signed rank tests for pairwise comparisons of sexual selection replicate lines within-treatments (A) females from male biased high sexual selection background proportional paternity B) females from female low sexual selection background proportional paternity) proportional paternity (Reindeer: Freemani offspring).

	Comparison	W	P value
A)	**MA / MB**	**339**	**0.87**
	MA / MC	**269**	**0.91**
	MB/MC	**349**	**0.85**
B)	**FA / FB**	**162**	**1.00**
	FA / FC	**214**	**0.67**
	FB / FC	**214**	**0.55**

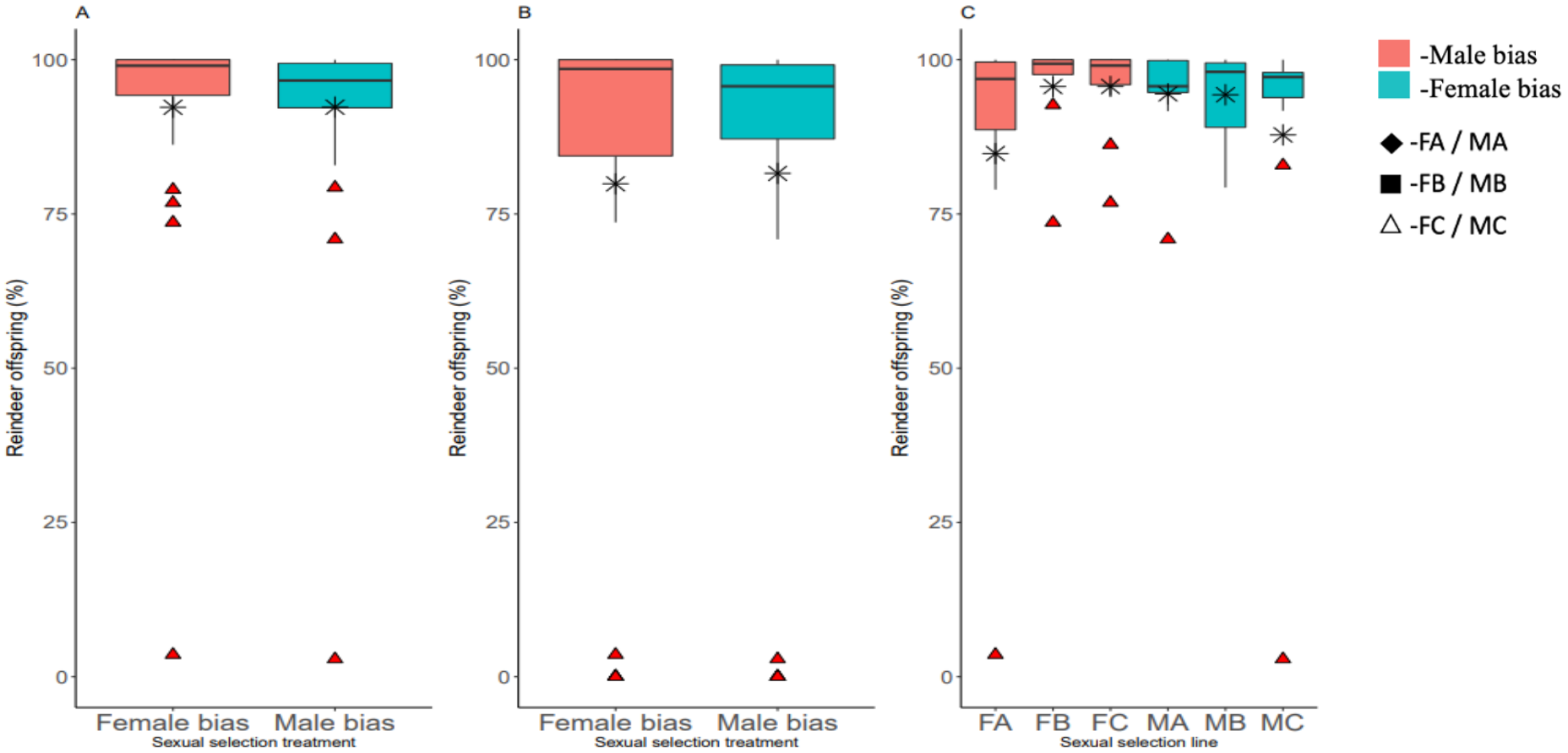

Figure 5: Relative paternity achieved by conspecific *T. castaneum* Reindeer sperm when competing within females from either female biased or male biased sexual selection line s. Each female is mated first to a *T. freemani* male (48hr opportunity) and then a *T. castaneum* reindeer mutant (12hr mating). A) no significant difference was observed between male biased (95.6%) and female biased (96.4%) sexual selection lines when successful mating can be confirmed for both males. B) No significant difference was observed between male biased (81.5%) and female biased (79.8%) sexual selection lines when we could not confirm both males had successfully transferred sperm. C) No significant difference in proportional paternity of conspecific *T. castaneum* offspring between replicate lines of the same sexual selection treatment with controls; male biased (line A 96.2%, line B 95.7, line C 95.8%); female biased (line A 97.1%, line B 94.7%, line C 96.3%). In total, 67 matings took place in which both males evidently transferred sperm, and 80 matings in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 4 x 10 day blocks of oviposition. Both sexual selection treatments (male biased & female biased) are represented by three independent replicate lines with individual lines (3 per regime) by diamonds, squares and triangles. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

Offspring number

In a similar manner to the relative paternities, there were also no significant differences in the absolute numbers of offspring between treatments and male types. Number of *T.castaneum* (reindeer) offspring produced was similar between female sexual selection background; Reindeer *T. casteneum* offspring number: Male biased = 6305 (± 17.1), Female biased = 5211 (± 17.9) (Figure 5, Table 3), and between replicate lines of the same treatment in male biased (line A 1960 ± 33.3 , line B 2546 ± 29, line C 1799 ± 28.0) and female biased lines (line A 1783 ± 41.8 , line B 1772 ± 28.2, line C 1656 ± 17.87) (Figure 5, Table 4). *T. freemani* offspring number production was not significantly different between females from male biased (*T.freemani* offspring number=287 ± 1.58) and female biased regimes (194 ± 1.93) (Figure 5, Table 3), and between replicate lines of the same treatment (male biased line A 76 ± 3.33, line B 111 ± 2.56, line C 100 ± 2.59; female biased line FA 80 ± 4.48, line B 52 ± 2.83, line C 62 ± 2.95) (Table 4, Figure 5).

Table 3: A summary of the GLMM fixed effect for the number of offspring sired by a A) *T. castaneum* (Rdhd) B) *T. freemani* male when competing for a female or male biased sexual selection line female under sequential mating with number of offspring (A) Reindeer or B) *freemani*) the response variable and sexual selection replicate line the random effect, A) var= 6.708e-10 B) var= 1.176e-09)

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
Reindeer A)				
Intercept (Female biased)	5.191	0.130	39.92	<0.001 ***
Male biased	-0.08	0.172	-0.46	0.644
Freemani B)				
Intercept (Female biased)	1.90	0.268	7.10	<0.001 ***
Male biased	0.121	0.355	0.34	0.733

TABLE 4**:** Wilcoxon signed rank tests for pairwise comparisons of sexual selection replicate lines within-treatments A) number of Reindeer sired offspring between 1) Male biased 2) Female biased sexual selection replicate lines, B) number of Freemani sired offspring 1) Male biased 2) Female biased, after sequential mating.

A) ***T. castaneum*** **(Reindeer)**	**Comparison**	**W**	**P value**
1)	**MA / MB**	**89**	**0.75**
	MA / MC	**74**	**0.64**
	MB/MC	**95**	**0.82**
2)	**FA / FB**	**42**	**0.92**
	FA / FC	**62**	**0.36**
	FB / FC	**68**	**0.17**
B) T. freemani	**Comparison**	**W**	**P value**
1)	**MA / MB**	**85**	**0.91**
	MA / MC	**56**	**0.55**
	MB/ MC	**64**	**0.21**
2)	**FA / FB**	**46.5**	**0.62**
	FA / FC	**61**	**0.39**
	FB / FC	**53.5**	**0.78**

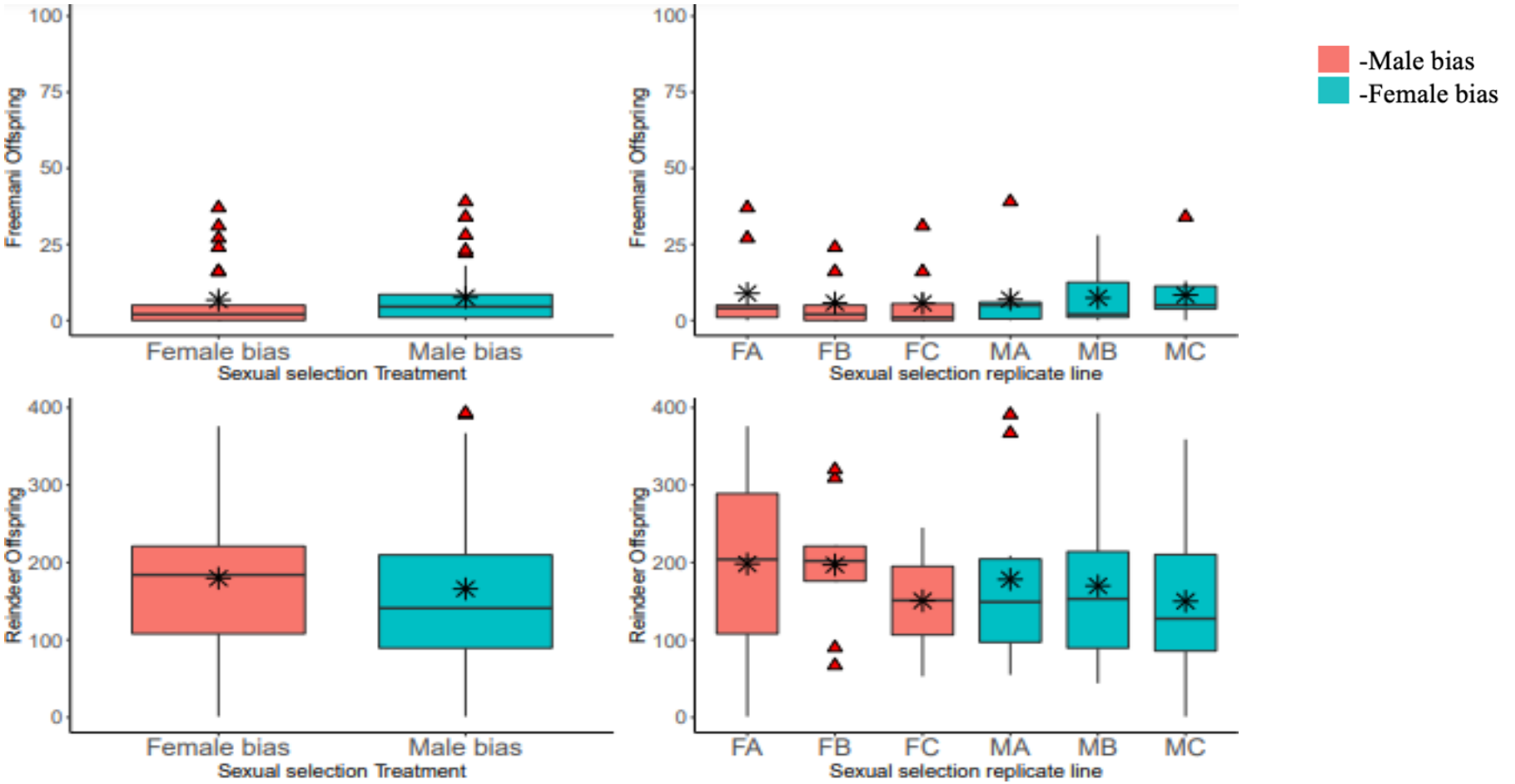

Figure 6: Number of offspring produced by (Graph C and D) conspecific *T. castaneum* Reindeer sperm and heterospecific *T.freemani* sperm (Graph A and B) when competing within females from either female biased or male biased sexual selection lines. Each female is mated first to a *T. freemani* male (48hr opportunity) and then a *T. castaneum* reindeer mutant (12hr mating). A) No significant difference was observed between male biased (287) and female biased (194) sexual selection lines in *T.freemani* offspring number. B) No significant difference was observed between replicate lines within Male biased (MA 76, MB 111, MC 100) and Female biased lines (FA 80, FB 52, FC 62). C) No significant difference was observed between male biased (6305) and female biased (5211) sexual selection lines in *T.castaneum* offspring number. D) No significant difference was observed between replicate lines within Male biased (MA 1960, MB 2546, MC 1799) and female biased (FA 1783, FB 1772, FC 1656). In total, 67 matings took place in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 4 x 10 day blocks of oviposition. Both sexual selection treatments (male biased & female biased) are represented by three independent replicate lines with individual lines (3 per regime) by diamonds, squares and triangles. The means are represented as stars, medians as black

Conspecific proportional paternity over time

Proportional paternity of conspecific *T*. *castaneum* (Reindeer) offspring was not significantly different between block 1 and block 2 in male biased (*T.castaneum* proportional paternity block 1: 95.5% ± 3.55, block 2: 93.6% ± 5.12) or female biased (block 1: 99.4% ± 5.3, block 2: 95.7% ± 6.5) sexual selection lines (Table 5/Figure 6).

Table 5: A summary of the GLMM fixed effect for proportional paternity of *T. castaneum* (Reindeer) v *T. freemani* males competing for a A) Female biased or B) Male biased sexual selection line female over block 1 (day 1-10) and block 2 (Day 11-20) with a fixed effect of proportional paternity and response variable of block with the random effect of replicate line A)var= 2.119e-16 , B) var= 2.443.

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
A) Male biased				
Intercept (Day 1-10)	2.92	0.726	4.019	<0.001 ***
Day 11-20	-0.14	1.028	-0.141	0.888
B) Female biased				
Intercept (Day 1-10)	3.99	2.916	1.350	<0.001 ***
Day 11-20	-0.73	1.090	0.674	0.500

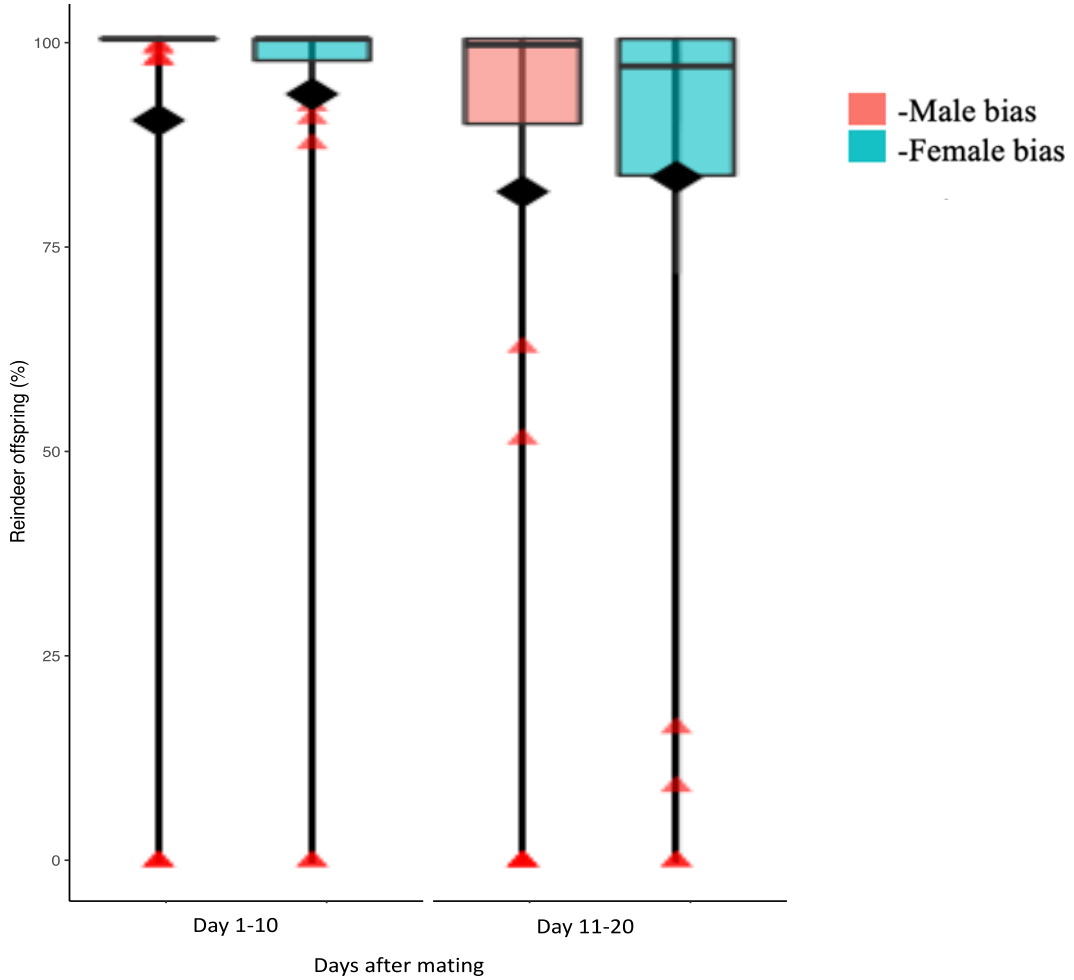

Figure 7: Relative paternity achieved by conspecific *T. castaneum* Reindeer sperm when competing within females from either female biased or male biased sexual selection lines. Each female is mated first to a *T. freemani* male (48hr opportunity) and then a *T. castaneum* reindeer mutant (12hr mating). No significant difference was observed in proportional paternity between sexual selection treatment block 1 and block 2 in male biased (block 1: 95.5% , block 2: 93.6%) and female biased lines (block 1: 99.4% , block 2:95.7%). Boxplots are derived from the total offspring produced over 2 x 10 day blocks of oviposition. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

Offspring production through time

No significant difference was observed in offspring production rates of *T.castaneum* (Reindeer) offspring between block 1 and 2 in male biased ((*T.castaneum* offspring number), block 1: 3245 ± 6.36 , block 2: 1961 ± 7.31) or female biased (block 1: 2698 ± 6.11 , block 2: 1781 ± 9.19) lines under sequential mating (Table 6, Figure 7). No significant difference was further observed in *T.freemani* offspring production number between block 1 and 2 in male biased (block 1: 151 ± 2.02, block 2: 133 ± 1.12) or female biased (block 1: 14 ± 0.19 , block 2: 80 ± 0.77) lines under sequential mating (Table 7, Figure 7).

Table 6: A summary of the GLMM fixed effect for offspring production of *T.castaneum* A) Male biased B) Female biased sexual selection lines under sequential mating and the response variable time- block 1 (Day 1-10) and block 2 (Day 11-20) with random effect of line A) var= 0.0865 B) var= 0.158.

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
A) Male biased				
Intercept (Day 1-10)	4.42	0.139	31.72	<0.001 ***
Day 11-20	-0.36	5.229	-1.79	0.07.
B) Female biased				
Intercept (Day 1-10)	4.50	2.756	4.39	<0.001 ***
Day 11-20	-0.19	0.259	-0.73	0.463

Table 7: A summary of the GLMM fixed effect for offspring production of *T.freemani* A) Male biased B) Female biased sexual selection lines under sequential mating and the response variable time- block 1 (Day 1-10) and block 2 (Day 11-20) with random effect of line A) var= 2.462e-09 B) var= 0.2949

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
A) Male biased				
Intercept (Day 1-10)	13.02	4.930	2.64	<0.001 ***
Day 11-20	-0.75	5.229	-0.144	0.885
B) Female biased				
Intercept (Day 1-10)	0.97	0.517	1.884	0.05.
Day 11-20	0.60	0.60	1.00	0.316

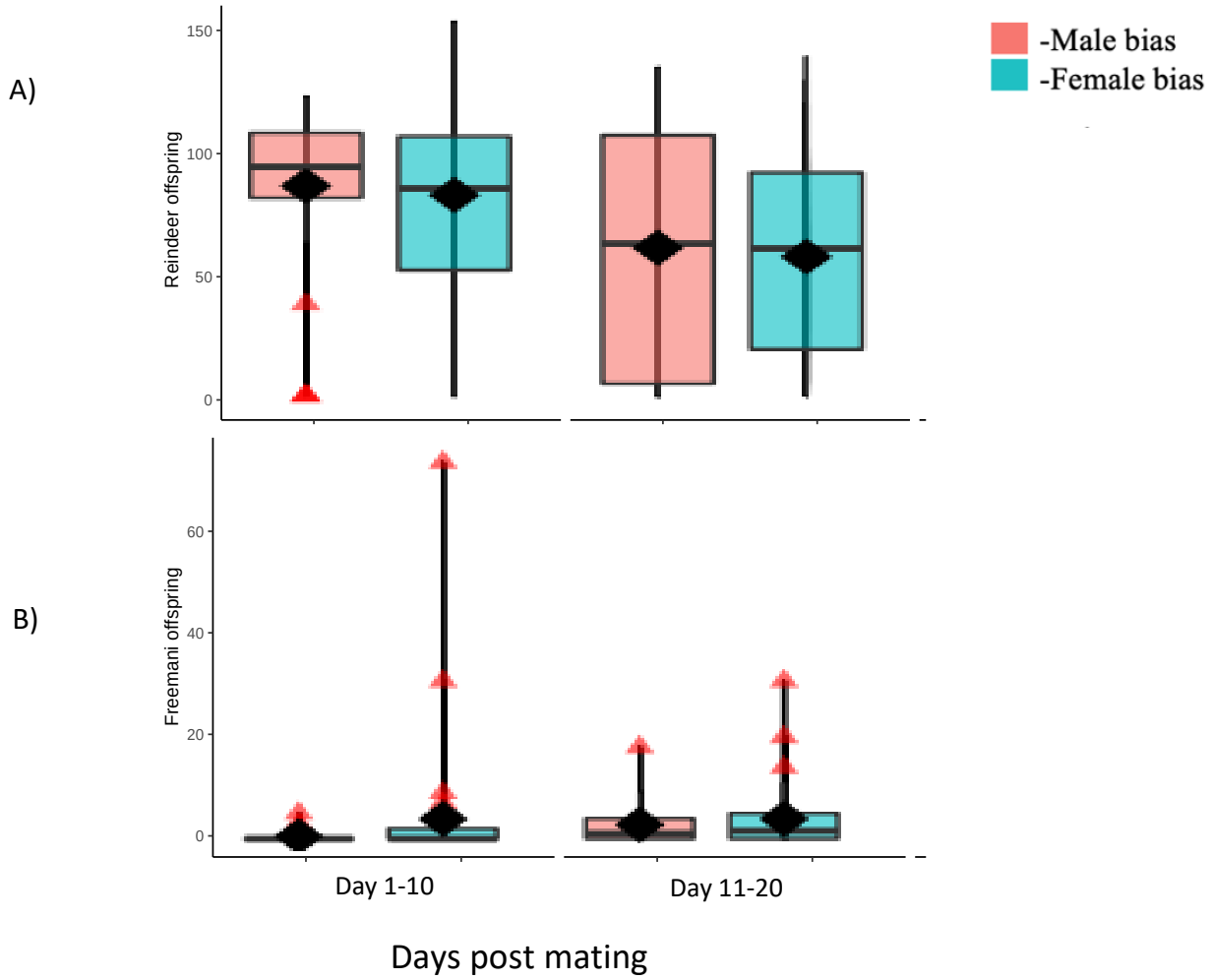

Figure 8: Number of offspring produced by conspecific *T. castaneum* Reindeer (Graph A), *T.freemani* sperm (Graph B) when competing within females from either female biased or male biased sexual selection lines. Each female is mated first to a *T. freemani* male (48hr opportunity) and then a *T. castaneum* reindeer mutant (12hr mating). A) No significant difference was observed in *T.castaneum* offspring number in block 1 and 2 male biased (block 1: 3245 , block 2: 1961) and female biased lines (block 1: 2698 , block 2: 1781). B) No significant difference in T.freemani *offspring* number was observed between block 1 and 2 in male biased (block 1: 151, block 2: 133) and female bias lines (block 1:14, block 2:80).In total, 67 matings took place in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 2 x 10 day blocks of oviposition. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

5.3.2 Continuous mating in the trio competitions

Proportional paternity

The proportion of *T.castaneum* (Reindeer) offspring sired by a female biased (71% ± 10.8) or male biased (79% ± 9.42) sexual selection line female was not significantly different (Table 8, Figure 8) when interspecific competition was present with a *T.castaneum* (Reindeer) and *T.freemani* male. In assessment of independent lines no significant difference was observed in the proportional paternity of reindeer males between lines of the same treatment, Male biased (MA 88.1% ± 19.22, MB 77% ± 17.85, MC 71.7% ± 18.14) Female biased (FA 71.4% ± 8.25, FB 69.5% ± 6.02, FC 73.6% ± 6.57) (Table 9,Figure 8).

Table 8: A summary of the GLMM fixed effect for proportional paternity of *T.castaneum* (Reindeer) v *T.freemani* males competing for a Female biased or Male biased sexual selection line female with proportional paternity the response variable and a random effect of Replicate line (Var=1.417^{e-09}).

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
Intercept (Female biased)	0.281	0.149	1.882	0.059
Male biased	0.304	0.212	1.436	0.151

Table: 9 Results of Wilcoxon signed rank tests for pairwise comparisons of sexual selection replicate lines within-treatments A) number of *T.castaneum* (Reindeer) sired offspring between 1) Male biased 2) Female biased sexual selection replicate lines, B) number of *T.freemani* sired offspring 1) Male biased 2) Female biased after constant mating

	Comparison	W	P value
A)	**MA / MB**	**882**	**0.80**
	MA / MC	**1065**	**0.53**
	MB/MC	**801**	**0.34**
B)	**FA / FB**	**962**	**0.11**
	FA / FC	**1055**	**0.51**
	FB / FC	**1548**	**0.36**

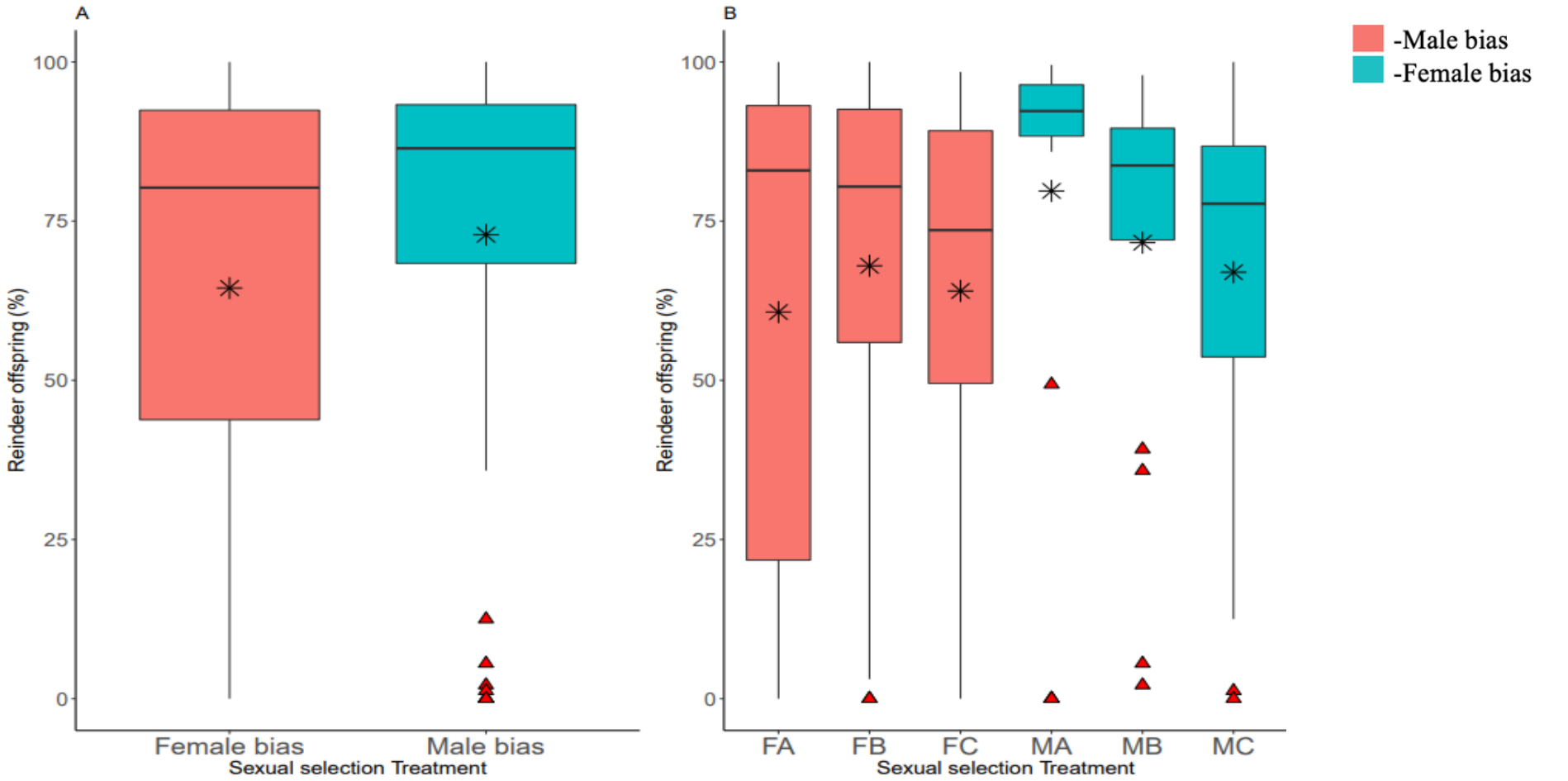

Figure 9: Relative paternity achieved by conspecific *T. castaneum* Reindeer sperm when competing within females from either female biased or male biased sexual selection lines. Each female is kept with a constantly present *T.castaneum* conspecific and *T.freemani* heterospecific male. A) No significant difference was observed between male biased (79%) and female biased (71%) sexual selection lines. B) No significant difference was observed between individual replicate lines of the same sexual selection treatment Male biased (MA 88.1%, MB 77%, MC 71.7%) Female biased (FA 71.4%, FB 69.5%, FC 73.6%).In total, 62 matings took place in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 4 x 5 day blocks of oviposition. Both sexual selection treatments (male biased & female biased) are represented by three independent replicate lines with individual lines (3 per regime) by diamonds, squares and triangles. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

Offspring number

The total number of *T.castaneum* (Reindeer) sired offspring produced by male biased females (10115 ± 5.07) was not significantly different from female biased females (9379 ± 5.56) (Table 10, Figure 9), with no significant difference observed between sexual selection replicate lines within the same treatments, Male biased (MA 4245 ± 7.08, MB 2454 ± 8.78, MC 3416 ± 9.22) Female biased (FA 2366 ± 8.24, FB 3753 ± 8.25, FC 3260 ± 8.11) (Table 11, Figure 9). The number of *T.freemani* sired offspring also showed no significant difference between Male biased (2649 ± 3.73) and Female biased (3757 ± 3.82) (Table 10, Figure 9) sexual selection lines females and replicate lines within the same treatment in male biased (MA 947 ± 6.58, MB 1641 ± 6.66, MC 1169 ± 6.09) and female biased (FA 571 ± 7.38 , FB 732 ± 11.65, FC 1346 ± 6.57) (Table 11, Figure 9).

Table 10: A summary of the GLMM fixed effect for the number of offspring sired by a A) *T.castaneum* (Reindeer) B) *T.freemani* male when competing for a female or male biased sexual selection line female under constant mating with number of offspring (reindeer or freemani) the response variable and sexual selection replicate line the random effect A) var= 2.418e-09 B) var=

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
A) Reindeer				
Intercept (Female biased)	4.82	0.126	38.29	<0.001 ***
Male biased	0.18	0.183	1.03	0.305
B) Freemani				
Intercept (Female biased)	3.90	0.155	25.14	<0.001 ***
Male biased	-0.27	0.223	-1.19	0.232

TABLE 11**:** Results of Wilcoxon signed rank tests for pairwise comparisons of sexual selection replicate lines within-treatments A) number of Reindeer sired offspring between 1) Male biased 2) Female biased sexual selection replicate lines, B) number of Freemani sired offspring 1) Male biased 2) Female biased after sequential mating.

Fixed effects	**Comparison**	**W**	**P value**
A) Reindeer			
1)	**MA / MB**	**306**	**0.06**
	MA / MC	**359**	**0.15**
	MB/MC	**199**	**0.49**
2)	**FA / FB**	**232**	**0.19**
	FA / FC	**235**	**0.30**
	FB / FC	**368**	**0.76**
B) Freemani			
1)	**MA / MB**	**85**	**0.91**
	MA / MC	**56**	**0.55**
	MB/MC	**64**	**0.21**
2)	**FA / FB**	**258**	**0.44**
	FA / FC	**289**	**0.96**
	FB / FC	**381**	**0.61**

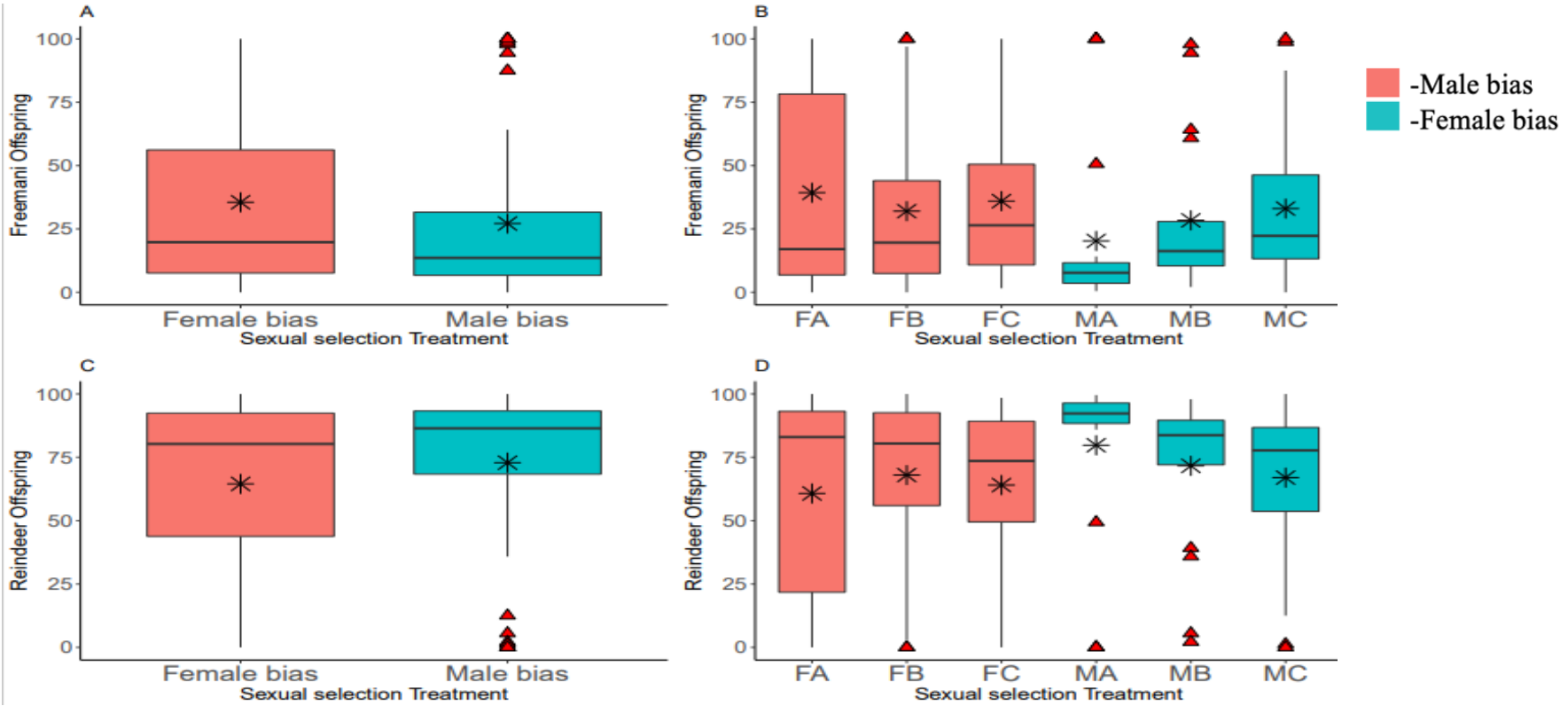

Figure 10: Number of offspring produced by (Graph C and D) conspecific *T. castaneum* Reindeer sperm and heterospecific *T.freemani* sperm (Graph A and B) when competing within females from either female biased or male biased sexual selection lines. Each female is kept with a constantly present *T.castaneum* conspecific and *T.freemani* heterospecific male. A) No significant difference was observed between male biased (2649) and female biased (3757) sexual selection lines in *T.freemani* offspring number. B) No significant difference was observed between replicate lines within male biased (MA 947, MB 1641, MC 1169) and female biased treatments (FA 571, FB 732, FC 1346. C) No significant difference was observed between male biased (10115) and female biased (9379) sexual selection lines in *T.castaneum* offspring number. D) No significant difference was observed between replicate lines within male biased (MA 4245, MB 2454, MC 3416) Female biased treatments (FA 2366, FB 3753, FC 3260). In total, 62 matings took place in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 4 x 5 day blocks of oviposition. Both sexual selection treatments (male biased & female biased) are represented by three independent replicate lines with individual lines (3 per regime) by diamonds, squares and triangles. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

Conspecific proportional paternity through time

No significant statistical differences were observed in proportional conspecific paternity between both blocks when interspecific competition was present across both females from male biased (block 1: 65.6% ± 8.26, block 2: 74.7% ± 9.38) or female biased (block 1: 53% ± 6.36, block 2: 49% ± 5.38) regimes (Table 12, Figure 10).

Table 12: A summary of the GLMM fixed effect for proportional paternity of offspring sired by a A) Male biased B) Female biased sexual selection lines under constant mating and the response variable time- block 1 (Day 1-10) and block 2 (Day 11-20) with random effect of line A) var= 6615 B) 2137.

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
A) Male biased				
Intercept (Day 1-10)	13.02	4.930	2.64	<0.001 ***
Day 11-20	-0.75	5.229	-0.144	0.885
B) Female biased				
Intercept (Day 1-10)	12.11	2.756	4.39	<0.001 ***
Day 11-20	-0.54	3.175	-0.17	0.864

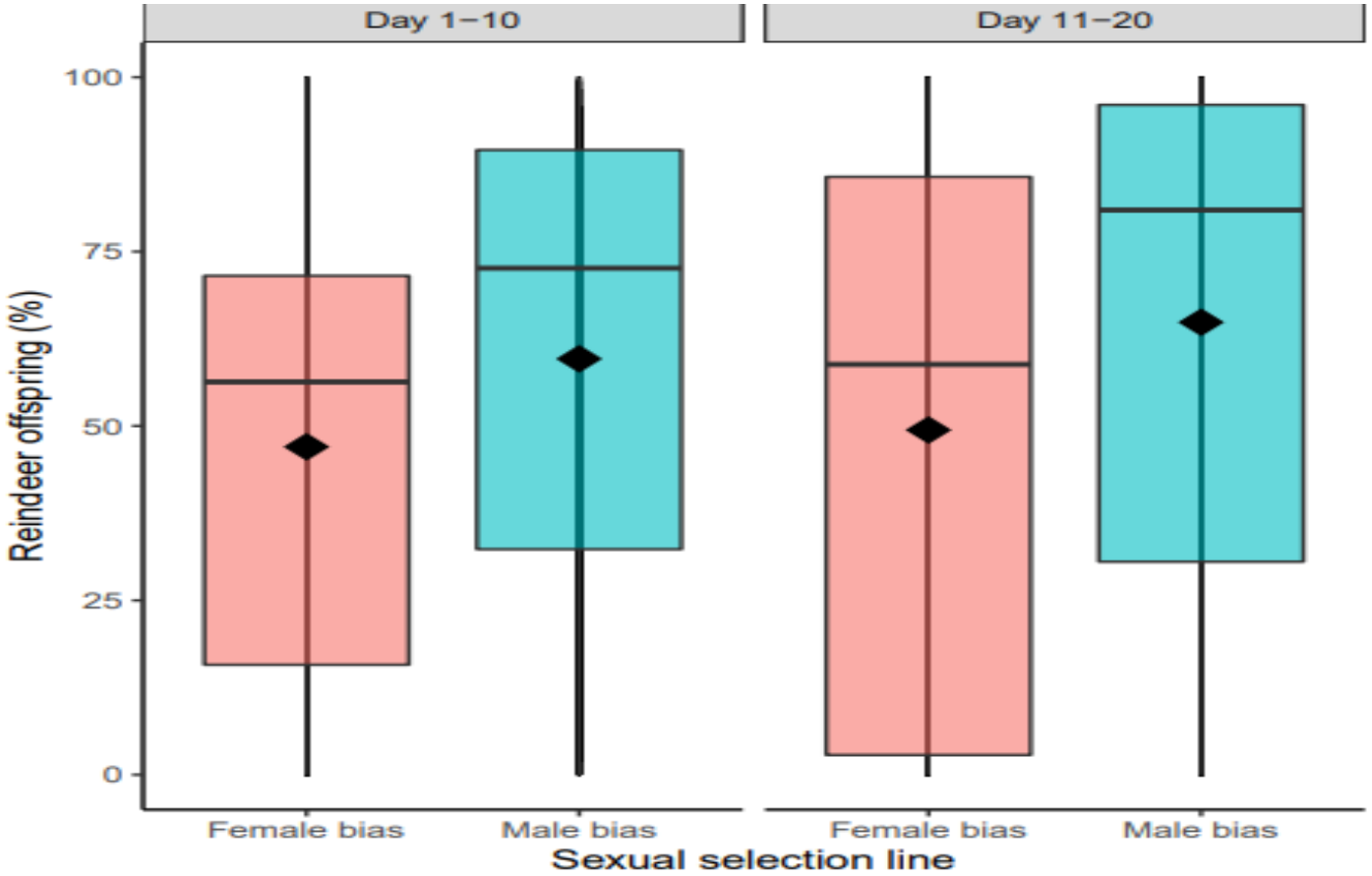

Figure 11: Relative paternity achieved by conspecific *T. castaneum* Reindeer sperm when competing within females from either female biased or male biased sexual selection lines. Each female is kept with a constantly present *T.castaneum* conspecific and *T.freemani* heterospecific male. No significant difference was observed between both *block 1 (day 1-10) and block 2 (day 11-20)* in male biased (block 1: 65.6%, block 2: 74.7%) and female biased t(block 1: 53%, block 2: 49%) regimes. In total, 62 matings took place in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 4 x 5 day blocks of oviposition. Both sexual selection treatments (male biased & female biased) are represented by three independent replicate lines with individual lines (3 per regime) by diamonds, squares and triangles. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

Offspring production through time

No significant difference was observed in total offspring production of *T.castaneum* (Reindeer) offspring between block 1 and 2 in male biased (block 1: 2022 ± 5.16, block 2: 1411 ± 4.71) or female biased (block 1: 1535 ± 4.26, block 2: 1331 ± 5.36) lines under constant mating (Table 13, Figure 11). No significant difference was further observed in *T.freemani* total offspring production between block 1 and 2 in male biased sexual selection lines (block 1: 1313 ± 6.06, block 2: 1380 ± 7.97) but a statically significant decrease in *T.freemani* offspring was observed in the female biased lines under constant mating (block 1: 1059 ± 5.42 , block 2: 476 ± 6.25) (Table 14, Figure 11).

Table 13: A summary of the GLMM fixed effect for offspring production of *T.castaneum* A) Male biased B) Female biased sexual selection lines under constant mating and the response variable time- block 1 (Day 1-10) and block 2 (Day 11-20) with random effect of line A) var= 2.261 B) var= 3.759.

Fixed Effects	Estimate	Standard Error	z value	Pr(>|t|)
A) Male biased				
Intercept (Day 1-10)	3.45	0.311	11.10	<0.001 ***
Day 11-20	-0.02	0.180	-0.124	0.901
B) Female biased				
Intercept (Day 1-10)	2.866	0.343	8.33	<0.001 ***

Day 11-20	-0.23	0.145	-1.63	0.101

Table 14: A summary of the GLMM fixed effect for offspring production of *T.freemani* A) Male biased B) Female biased sexual selection lines under constant mating and the response variable time- block 1 (Day 1-10) and block 2 (Day 11-20) with random effect of line A) var= 1.264 B) var= 0.1035

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
A) Male biased				
Intercept (Day 1-10)	3.06	0.212	14.32	<0.001 ***
Day 11-20	-0.65	0.135	-4.83	<0.001***
B) Female biased				
Intercept (Day 1-10)	3.59	0.19	18.80	<0.001 ***
Day 11-20	-0.03	0.262	-0.14	0.883

A)

B)

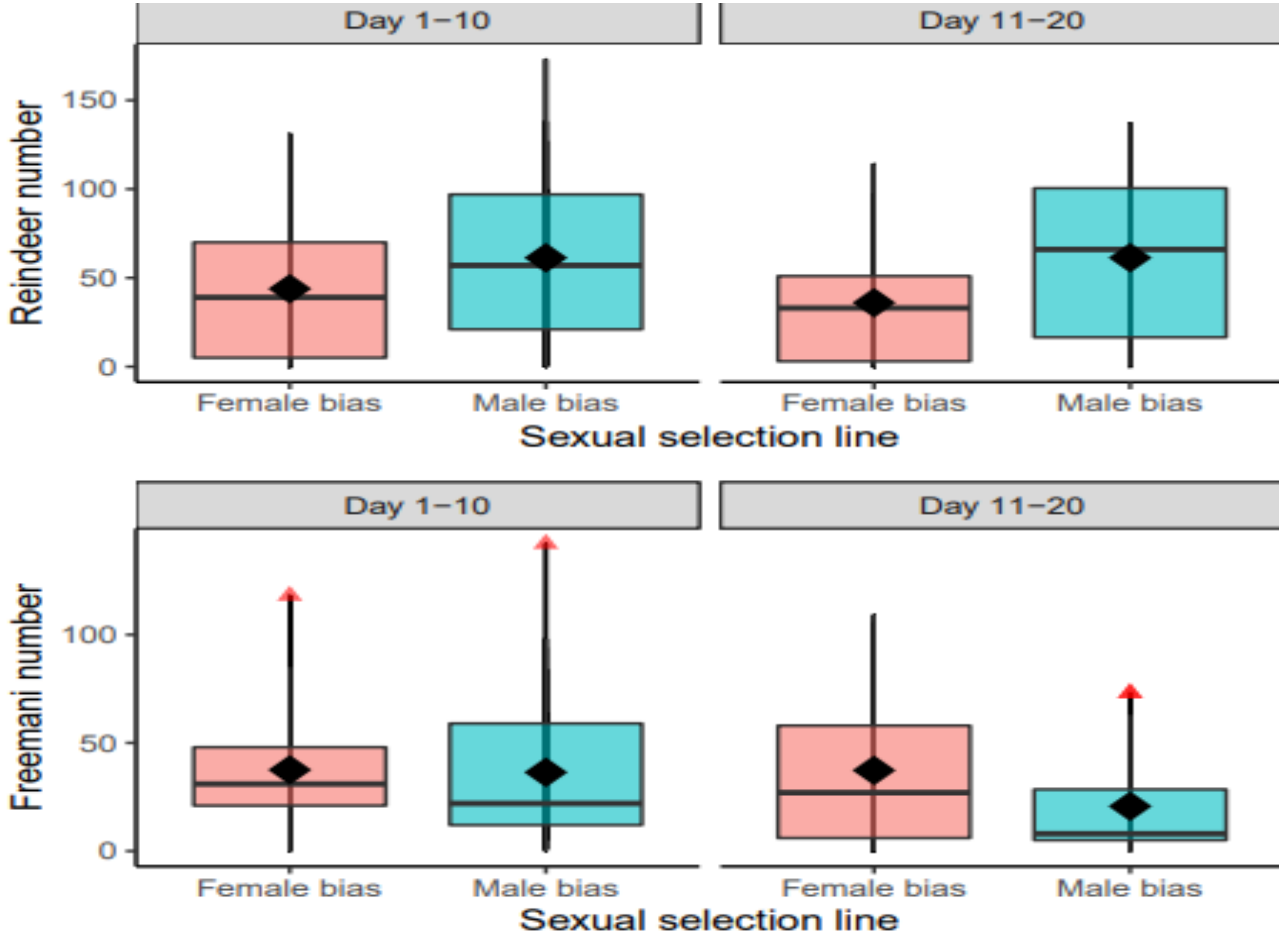

Figure 12: Number of offspring produced by conspecific *T. castaneum* Reindeer (Graph A), *T.freemani* sperm (Graph B) when competing within females from either female biased or male biased sexual selection lines. Each female is kept with a constantly present *T.castaneum* conspecific and *T.freemani* heterospecific male. A)No significant difference in *T.castaneum offspring* number was observed between block 1 and 2 in male biased (block 1: 2022, block 2: 1411) or female biased (block 1: 1535 , block 2: 1331). B) No significant difference in *T.freemani offspring* number was observed between block 1 and 2 in male biased (block 1:1059 , block 2: 476) but in female biased a statistically significant decrease occurred between blocks decrease in *T.freemani* offspring was observed in the female biased lines under constant mating (block 1:1059 , block 2: 476). In total, 67 matings took place in which mating opportunities were provided. Boxplots are derived from the total offspring produced over 2 x 10 day blocks of oviposition. The means are represented as stars, medians as black horizontal bars, and outliers by red filled triangles.

5.4 Discussion

Overall, our experiment found no evidence that ability to exert cryptic female choice (CFC) had evolved following very divergent opportunities for sexually-selected competition and choice. When females were inseminated with the 'right' and 'wrong' sperm from a conspecific and heterospecific male in quick sequence, and when females were continuously housed in trios with two such males, there was no evidence that females from a male-biased sexual selection background were able to bias fertilisation and/or mating towards conspecific sperm or males.

In general, conspecific males gained the majority of fertilisations in both sequential sperm competition and continuous trio conditions, typically around or above 90%, but this was no different between females from the male biased versus female biased selection backgrounds. Conspecific precedence result from both sexes, and the difficulty is determining the extent of mechanisms from either sex in driving conspecific paternity success, as they will be interacting constantly from mating and up to the fertilisation process. Our study demonstrates the complex challenges in demonstrating between male-driven and female-driven fertilisation biases but, interestingly, the use of both sequential sperm competition experiments that aimed to identify post-copulatory effects versus continuous mating trios where fertilisation outcomes will be driven by a combination of pre-copulatory and post-copulatory mechanisms, yield similar findings, and certainly little evidence that mate discrimination is greater than sperm discrimination.

5.4.1 Conspecific paternity precedence

We set out to test the hypothesis that high sexual selection (SS) origin females would have a greater ability to select against heterospecific males and their sperm than low sexual selection origin females, and therefore generate a higher conspecific paternity precedence when choosing between the two different male types and their sperm. In sequential matings designed to focus on post-copulatory sperm competition and CFC effects, we identified no statistically significant difference between conspecific proportional paternity berween high sexual selection (95.6%) and low sexual selection lines (96.4%), with both achieving high last male and conspecific sperm precedence. High conspecific paternity has been observed previously between the sister species *T.freemani* and *T.castaneum* used in this experiment. Robinson et al.,(1994), observed that, when *T.castaneum* females mated sequentially with a *T.freemani* and a *T.castaneum* male, the conspecific male won 91-100% of the subsequent paternity depending on mating order. Furthermore, Fricke and Arnqvist (2004), identified that *T.castaneum* males gained a significant proportion of paternity when in competition with *T.freemani* males. Fricke and Arnqvist, (2004) state that the high conspecific proportional paternity observed between the sister species is a result of conspecific sperm precedence.

In constant mating trios designed to reflect outcomes of both precopulatory female choice and post copulatory CFC, *T.castaneum* males gained the majority of fertilisations, but there was no statistically significant difference in conspecific paternity between high SS (79%) or low SS lines (71%). Interestingly, when both pre-copulatory mate choice and post-copulatory sperm choice are allowed to act together, the level of conspecific paternity precedence from the trios is reduced (~75%), compared with findings from the sequential experiments reflecting more of a focus on the outcome of just post-copulatory CFC effects; the opposite finding might have been hypothesised. Robinson, Johnson and Wade, (1994) found conspecific males gained 90%+ of copulations when given simultaneous access to *T.castaneum* and *T.freemani* males, which is 19% and 11% higher than that observed in the high and low sexual selection lines respectively here, but the same conspecific dominance is observed. The discrepancy between the extent of conspecific proportional paternity could be a result of final mating order, with random chance that a certain number of heterospecific males gained the last matings and therefore sperm precedence, reducing conspecific paternity over the oviposition period (Lewis and Jutkiewicz, 1998).

5.4.2 Sequential mating

Through the sequential mating trials, we can reduce pre-copulatory effects and allow focus on post-copulatory mechanisms for paternity outcome. The difficulty here, as previously stated, is to what extent our finding is influenced by male or female sexual selection mechanisms? Furthermore, sequential matings can help to control larval densities, reducing cannibalistic behaviour during development. The cost of hybridisation is far greater in females then males, as females will face higher individual fitness consequences from heterospecific mating mistakes and producing offspring that become reproductive dead

ends (Servedio, 2007; Hudson and Price, 2014). Therefore, females should be under selection to play a major role in our observed result of high conspecific proportional paternity in sequential mating.

T.castaneum female ability to use cryptic female choice (CFC) to avoid hybridisation has been observed in a range of vertebrate and invertebrate taxa (Welke and Schneider, 2009; Veen *et al.*, 2011; Yeates *et al.*, 2013). A proposed mechanism for *T.castaneum* females to prevent hybridisation post-copulation is through sperm selection in favour of conspecific gametes by biasing fertilitisation towards sperm of desirable, and against undesirable, sperm, as has been observed to occur in *T.castaneum* (Bloch Qazi, Aprille and Lewis, 1998; Fricke and Arnqvist, 2004; Fedina, 2007). The sperm selection mechanism has previously been observed to be refined enough to detect and select between two conspecific males (Fricke and Arnqvist, 2004), therefore *T.castaneum* females should be able to select between the more divergent sperm cells of *T.freemani* and *T.castaneum* males. Therefore, one potential explanation for the ~90% conspecific paternity levels found throughout this experiment is through females using CFC selection mechanisms such as female control of spermatophore transfer (Fedina, 2007), or differential sperm storage (Bloch Qazi, Aprille and Lewis, 1998; Lewis and Jutkiewicz, 1998) to minimise heterospecific sperm and maximise conspecific paternity. Fedina (2007) observed, by manipulating phenotypic quality through starvation of males, that females were able to control sperm quantity transfer, identified that fed males transferred significantly more sperm per spermatophore but only when mating with live females, begging the question whether starved males simply had less competitive sperm.

Mating sequence and last-male sperm precedence provide additional explanations for our high conspecific sperm findings. The *T.castaneum* conspecific males were second and last to

mate in the two-male sequence, and this was designed in order to encourage matings by the potentially less attractive heterospecific *freemani* males by giving them first mating. Last-male sperm precedence is a common outcome across insect sperm competitions (Birkhead, 1987). In *T.castaneum,* last male sperm precedence (often referred to as P_2) has been observed to result in a short term but very high competitive advantage of up to 90% during the first days after the competitive mating trial (Arnaud, Gage and Haubruge, 2001; Fricke and Arnqvist, 2004). In our experiment, 48hrs elapsed between the P_1 and P_2 matings, a greater time difference between matings has previously been observed to further accentuate the effects of last male sperm precedence as a result of sperm depletion / utilisation of the P1 male (Arnaud, Gage and Haubruge, 2001). Last male sperm precedence therefore provides a partial explanation for the dominance of conspecific fertilisation in this experiment, but the key outcome is that no differences in this overall level of P2 is seen between females of the male-biased versus female-biased sexual selection backgrounds. Previous research into last male sperm precedence in *T.castaneum* identified sperm precedence to relax after 1- 2 weeks of oviposition, perhaps because of sperm mixing within the female tract (Lewis and Jutkiewicz, 1998). We found continuously high last-male sperm precedence over the 40 day oviposition period, perhaps suggesting more complex CFC mechanisms are at play , such as cryptic male choice (Engqvist and Sauer, 2001; Reinhold, Kurtz and Engqvist, 2002), hybrid incompatibility (Drury *et al.*, 2013), sperm removal (Arnaud, Haubruge and Gage, 2001), or female reproductive tract traits described above which accentuate the dominance of conspecific males dominance of paternity for an extended period of time.

Sequential mating experiment limitations

One explanation for our results is that a mix of male and female post-copulatory mechanisms have resulted in the greater fertilisation success of conspecific males during sequential mating. One factor which may drive paternity outcome is female mate choice. T. castaneum femnales may be less willing to mate with a heterospecific male, generating a paternity bias. Three lines of reasoning do not suggest this to be the case. First, we gave the potentially less attractive *freemani* males the first mating in the experiment when females were virgins and more willing to mate. Secondly, we ran analyses in which it was clear that both males had successfully mated and similar precedence was found. Third, it is possible that females simply mated more often with the conspecific male, but we tried to reduce this impact by providing *freemani* males with 48 hours of mating opportunity, and only 12 hours for the *castaneum* male. And fourth, when we look at conspecific sperm precedence in the continuous mating trios, where mate choice is constantly available throughout the trial, the overall conspecific paternity level goes down compared with the sequential trials, from ~80 to ~95% (Figure 5) to ~75% (Figure 9).

A further potential limitation to this experiment is outlined by Gilchrist & Partridge (1997), who identified that differential juvenile survival of conspecific versus hybrid offspring may occur, under-representing the rates of heterospecific fertilisations. In this experiment where adult phenotype is utilised to determine reproductive success, a bias in observed paternity could occur as a result of reduced hybrid fitness and survival, and not mechanisms biasing sperm towards conspecific males. We observed the majority of females to have successfully mated with both males (83%) in our trials, with only a single female in the experiment to have failed to produce any offspring, but it is possible that the overall high conspecific fertilisation precedence we see may be partly driven by reduced hybrid offspring fitness.

5.4.3 Continuous mating in trios

In the trios where two males continuously competed for each female, females had the opportunity to remate multiple times with either or both males across the 20 days of each trial (as opposed to 48hr and 12hrs in the sequential mating experiment). The majority of females in the continuous trio mating treatments had mixed paternity from both males, showing that the majority of females mated with both conspecific and heterospecific males. The ability to remate with males provided females with both mechanisms of pre-copulatory mate choice and post-copulatory CFC. Fedina and Lewis (2015) state that *T.castaneum* females are more willing to remate with additional males and accept a spermatophore when a mated female is presented with a more attractive male, such as in this experiment where the conspecific male is constantly present. This could result in females willingly mating with the conspecific male second, but being far less receptive to mate with the less desirable heterospecific males. Nilsson et al., (2003) identified that females are more willing to remate rapidly with males of the same genotype. These studies suggest that females can bias fertilisation opportunities towards conspecific through pre-copulatory mechanisms, providing a potential explanation for their greater than equal share of paternity. However, as explained above, we would expect this greater mate choice opportunity in the trios to have resulted in even higher conspecific paternity shares compared with the sequential experiments, but this was not seen and, in fact, overall conspecific precedence was lower in the trios.

Evidence suggests that *T.castaneum* males exert little to any mate choice between females, with homosexual behaviour regularly observed in males as a result of inaccurate mate choice potentially due to the low ejaculate and mating cost to males (Sales *et al.*, 2018). The indiscriminate nature of male mate choice and lack of male-male competition indicates that

it is unlikely that male pre-copulatory competition plays a major role in the observed dominance of conspecific males.

Post-copulation, the same mechanisms as described in sequential mating are likely to have an effect, or even be accentuated over time, in the continuous mating trios. Both CFC (Fedina and Lewis, 2015) and last male sperm precedence (Lewis and Jutkiewicz, 1998) will influence reproductive success, as will male ability to reduce or remove a competing males spermatophore (Arnaud, Haubruge and Gage, 2001). *T.castaneum* females show an increased willingness to mate with *T.castaneum* males in combination with male ability to cause the ejection of competing spermatophores, also providing a strong mechanism which selects against *T.freemani* male reproductive success.

Constant mating trio limitations

As for the sequential mating experiments, the issue of differential offspring survival between 'pure' and hybrid zygotes, embryos and offspring may bias proportional paternity towards conspecific males, despite potentially equal fertilisation rates. A further limitation is that continuous trios will enable a stronger impact of variation in mating frequency by females with particular males, thereby eroding CFC-specific effects through less controlled impacts of pre-copulatory mechanisms.

5.4.4 Conspecific proportional paternity in sequential vs constant mating

We observed conspecific males gained a greater proportion of paternity in the sequential, as opposed to the constant mating experiment. There are multiple potential causal factors resulting in reduced paternity of conspecifics under constant mating. In constant mating the

major difference is the introduction of an extended opportunity to utilise pre-copulatory mechanisms. One hypothesis for increased success is that *T.freemani* males have greater body size; this facilitates greater opportunity to mate (Fedina and Lewis,2008). Due to the design of both experiments heterospecific males had the opportunity to gain last male sperm precedence in constant mating which was not possible in sequential mating. In 62 mating trios it is likely that a proportion *T.freemani* males will have gained last male sperm precedence in the final block which may have facilitated the greater heterospecific paternity observed.

5.4.5 Reproductive output: offspring number and changes through time in both offspring number.

Our hypothesis, would suggest that females from a female bias (low sexual selection) background should be more conservative with sperm usage due to the scarcity of males. Male bias background females should have access to an excess of males making the need for conservative sperm usage unnecessary. Despite this we did not identify any difference in the number of *T.castaneum* and *T.freemani* offspring produced by females from a high or low sexual selection background.

Previous research at UEA identified that when mated with a single male, offspring production is greater in female bias then male bias females 30-40 days post-copulation with no difference in the preceding days (Unpub.data). In our sequential mating experiment the majority of offspring were produced in the first two blocks (day 1-20) with 73% on average and a range from 60%-98% of offspring produced. As only 27% of offspring across the experiment were produced in the last two blocks (day 20-40) there is evidence for sperm

limitation to be occurring equally in both female bias and male bias females under sequential mating. The low offspring production and high number of 0's in blocks 3 and 4 occur in both male bias and female bias individuals; this is likely a result of potential sperm limitation, making determining any differences between females bias and males bias lines difficult. Therefore, our results suggest that high and low sexual selection lines are managing sperm under sequential mating to a similar extent across the 40-day oviposition period.

I identified, through research in this thesis that when constantly mated with a standard Georgia male, high and low sexual selection line females treatments utilised in this experiment showed no significant difference in offspring production rates over 100 days. Therefore, the similar offspring production rates between male bias and female bias females (over 20 days with two constantly present males) supports our finding that male bias and female bias sexual selection lines have intrinsically identical offspring production rates when males are available and sperm is unlimited.

5.4.6 Male and Female bias sexual selection lines

Across these experiments we observe no statistically significant difference between male bias and female bias sexual selection lines despite being highly divergent and long founded treatments. The lack of divergence as mentioned previously makes any inference on the influence of female vs male mechanisms difficult. We found that *T.castaneum* females produce offspring that are highly biased towards conspecific as has been found in previous experiments. Despite this, like many other studies we have found evidence for CFC to be inconclusive with difficulty determining the role of males and females and the extent of the individual sexes pre/peri and post-copulatory influence.

The lack of divergence between sexual selection treatments can be explained by two opposing theories, that CFC is a highly powerful mechanism independent of sexual selection pressure, or opposingly that females assert little to no CFC instead relying on polyandry promoting sperm competition to facilitate production of offspring with high fitness. Previous research at UEA into these sexual selection lines indicates that divergence has occurred with differences in sperm management. Divergence therefore seems to be occurring but potentially not in mechanisms of cryptic female choice and instead in sperm management to maximise polyandry and sperm competition. More research is needed to understand what effect this has on individual productivity and population dynamics.

In future study of female and male post-copulatory mechanisms, assessing both sequential mating orders having both a P_1 and then a P_2 conspecific experiment will further clarify male/females role in conspecific sperm selection. During these experiments it would be ideal to observe single mating's from the conspecific and heterospecific males to minimise the impact of mating success and isolate post-copulatory mechanisms.

It would be beneficial to identify the relative survival rates at each life stage of pure *T.castaneum* and hybrid offspring to determine the potential impact of genetic incompatibilities on the result. The controls mentioned above would isolate the individual roles of males and females to a greater extent allowing a more precise assessment of male and females role in copulatory success.

Chapter 6

Does experimental evolution under heightened sexual selection cause changes to offspring production rate in the flour beetle *Tribolium castaneum*?

Abstract

Ongoing research using the *T. castaneum* sexual selection lines at UEA has shown that females from the female-biased regime are better at maintaining their fertility under sperm limitation. This finding suggests that females can evolve to be more efficient at sperm management following an evolutionary history of male and sperm limitation. Here, I add to this finding by testing whether there are intrinsic differences in offspring production rates between females from the male-biased versus female-biased sexual selection regimes. Females from the sexual selection lines were therefore kept without sperm limitation and a control male to assess offspring production over a 100-day trial period, divided into 10-day blocks. Despite being under highly divergent sexual selection regimes, there was no difference in offspring production rates over the experiment between females from the male-biased versus female-biased evolutionary background. These results suggest that the improved offspring production rate under sperm limitation by female-biased regime females is the result of the experimental evolution of more efficient sperm usage mechanisms, and options to explore this in more detail are discussed.

6.1 Introduction

In this study, we look to build upon ongoing research at UEA by R. Vasudeva and colleagues in which *T. castaneum* females from male-biased versus female-biased sexual selection histories showed differing ability to store and manage sperm to maintain full fertility under sperm limitation. In applying different intensities of sexual selection through variation in the operation sex ratio (OSR), we have also created different risks of sperm limitation for adult females. High sexual selection line females are maintained in OSRs of 90 males to 10 females, whilst low sexual selection line females are maintained in OSRs of 10 males to 90 females as sexually mature adults. These divergent OSRs also give rise to divergent selection from risks of male limitation, with female-biased OSRs where only 10 males are available to fertilise 90 females at each adult generation creating some risk of sperm limitation (R. Vasudeva Pers. Comm.) Using trials where females were given a single mating, the low sexual selection background females that had evolved through higher risks of sperm and male limitation produced a significantly greater number of offspring than those females from a high sexual selection background (Figure 4). To show that this was also not due to female ageing or other factor driven independent of sperm availability, remating of the experimental females after most had ceased to produce any offspring restored their fertility and reproductive output, indicating that sperm limitation was behind the decline in reproductive output (Figure 4a) production. We can therefore hypothesise that female-biased females under male and sperm limitation has forced the evolution of more efficient sperm usage to maximise offspring production when males are rare. By ontrast, the male-

biased females, coming from an evolutionary history of ample sperm and male availability, have not experienced such strong selection on sperm storage and management.

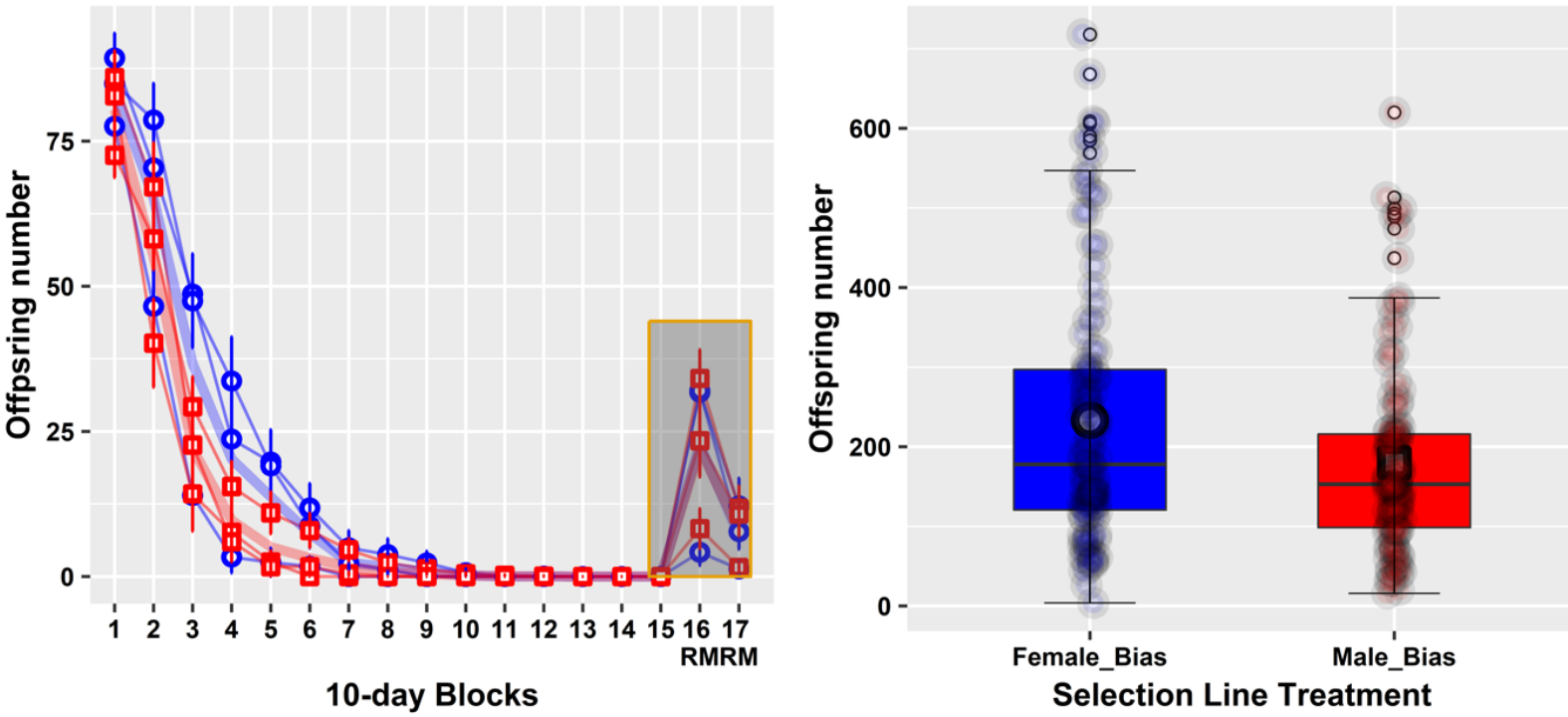

Figure 1: Figures left shows offspring production number over 170 days after a single mating, with a second mating occurring at block 16. Figure right shows the total offspring number produced over this period of time. Blue- female bias, red- male bias lines, figures produced by Ram Vasudeva.

Sperm storage by females is a widely recognised adaptation, allowing females to decouple fertilisation from the presence of males, and observed across huge numbers of vertebrate and invertebrate species (Birkhead and MØller, 1993, 1992; Bloch Qazi et al., 1998; Gasparini and Evans, 2013; Holt and Lloyd, 2010; Mendonca et al., 2019; Sever and Hamlett, 2002). The ability to store sperm provides multiple benefits to females, most obviously from being able to access male gametes but then reproduce independent of the physical

presence of male mates, but also providing the opportunity for greater female control of fertilisation via post-copulatory selection mechanisms (Bloch Qazi, Aprille and Lewis, 1998; Firman *et al.*, 2017), and allowing females to synchronise sperm levels and ovulation to maximise fertilisation rates (Sasanami *et al.*, 2013).

In insects, almost all of which employ sperm storage for reproduction, the bursa and spermatheca are responsible for receiving, maintaining and releasing sperm to fertilise eggs (Pascini and Martins, 2017). The spermatheca facilitates long term sperm storage by providing an optimal environment (Pascini and Martins, 2017), while also allowing ongoing sexual selection and CFC to operate (Eberhard, 1997). In this study, we explore whether sperm management in the female sperm storage organs of the polyandrous Tenebrionid flour beetle *Tribolium castaneum* can evolve. The sperm storage system of *T. castaneum* has been identified and characterised for a long period of time (Sinha, 1953). The male transfers a spermatophore during mating into the female's bursa copulatrix (Bloch Qazi, Herbeck and Lewis, 1996; Lewis *et al.*, 2005), acting as a short-term sperm storage system for initial deposition (Fedina and Lewis, 2008). Sperm become mobile in the bursa, with 4% moving into the spermatheca for longer term sperm storage (Bloch Qazi, Aprille and Lewis, 1998; Fedina and Lewis, 2008). After a single mating, *T. castaneum* females can produce offspring for up to 140 days post-copulation, showing a long term ability for sperm storage of about 60 days on average (Bloch Qazi, Aprille and Lewis, 1998). Recent research indicates that females may vary in their ability to manage and store sperm, so we investigate this further.

An alternative explanation for the increased fertility shown by females from the female-baised sexual selection lines is that, simply, these females intrinsically produce offspring at great rates, independent of sperm supply. To test this possibility, I therefore assess offspring

production rates in male- and female-biased females when they are not subject to sperm limitation. Females can maximise their fitness by having the optimal balance of survival, mating success and fecundity (Pincheira-Donoso and Hunt, 2017). These three factors interact with one another antagonistically under fecundity selection theory, the interaction between survival, mating success and fecundity are likely to vary based on sexual selection intensity (Pincheira-Donoso and Hunt, 2017). Females from high sexual selection intensity origins have adapted to resist the possible costs of polyandry (Michalczyk *et al.*, 2011), possibly trading this against fecundity. The variation in sexual selection pressure experienced by females therefore has the potential to result in variation to female reproductive patterns and investment into offspring production. We therefore determine whether the differences in offspring number between selection line females under sperm limitation observed by R. Vasudeva could also result from intrinsic differences in offspring production rates.

6.2 Materials and methods

6.2.1 Stocks

All beetles used in the experiment were maintained under standard conditions (30 ± 1 °C, 60 ± 5% RH and 16L: 8D photoperiod) in fodder (10% brewer's yeast , 90% organic strong white bread flour), topped with jumbo oats for traction.

Sexual selection lines

Long-term sexual selection line females were created in 2006/7 from Georgia strain individuals and, at the time of the experiment, 130 generations of selection had been applied. Sexual selection line females are maintained under two treatments: male-biased (90 males: 10 females) and female-biased (10 males: 90 females). The sexual selection lines are maintained as non-overlapping generations of 100 individuals, with sex ratios maintained through each generation using sexed pupae, completed 18-22 days after sexually mature adults from the previous generation are removed. Isolated pupae are then left for 10 days to eclose and sexual maturation in single-sex groups of 15 in 5cm petri dishes. The correct adult sex ratios are then placed into 250ml colony jars with 150ml fodder for 7 days mating and oviposition after which they are removed and frozen (see details in Lumley et al 2015). Standard Georgia control stock strain males were used for the matings.

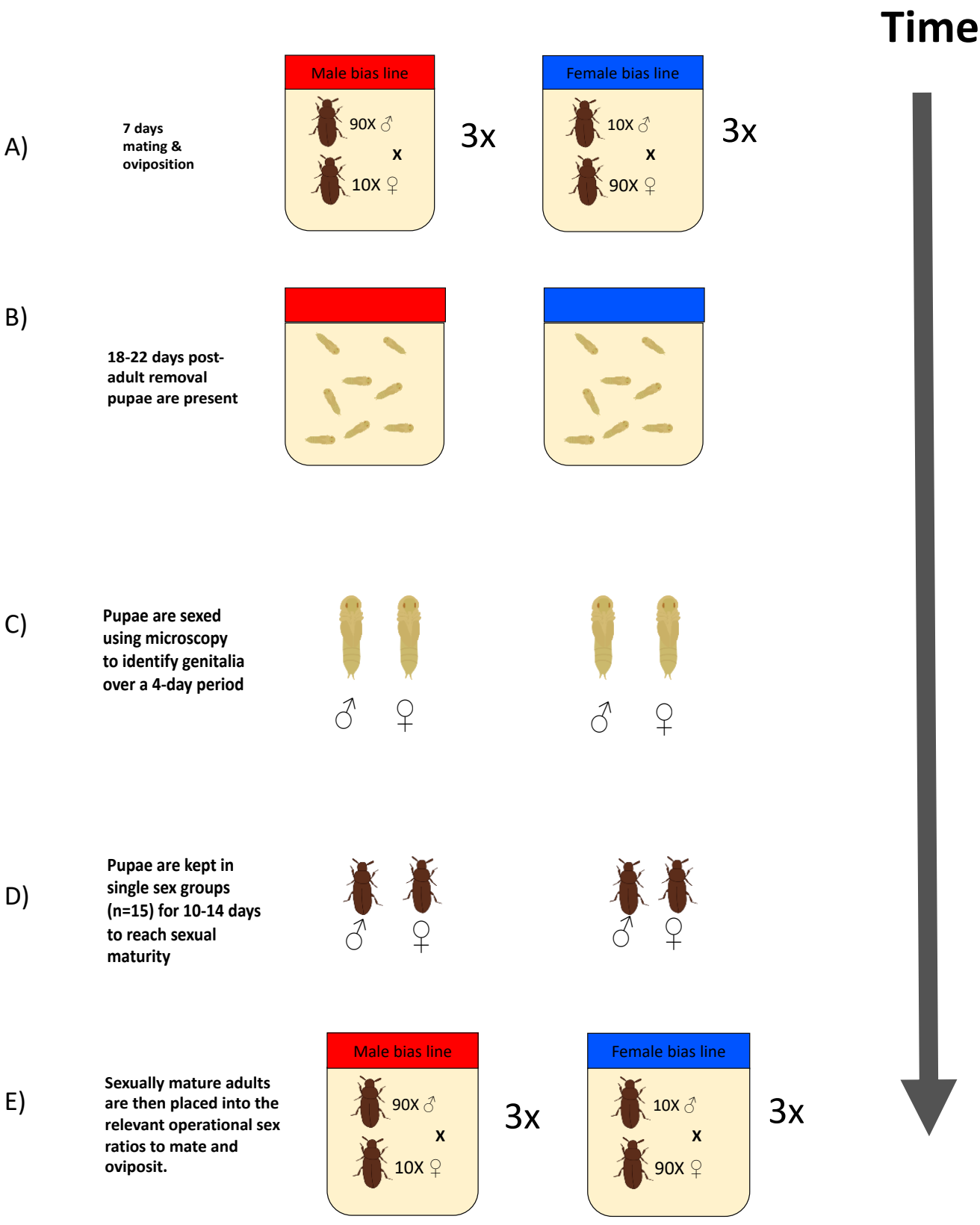

Figure 2: Maintenance protocol for sexual selection line. A) Sexually mature adults are given 7-days to mate and oviposit in their respective 90:10 ratios, male bias 90 males 10 females and females bias 10 males and 90 females. B/C) Adults are removed, and offspring are given 18-22 days to mature to pupae at which point they are sexed using a microscope. D) Once sexed adults are kept in single-sex groups for 10 days to reach sexual maturity. E) Individuals are then assorted into the relevant sex ratio's and are given 7 days to mate and oviposit

6.2.3 Offspring production rates of sexual selection line females

Pupal sexing was completed for all individuals used in the experiment within an 8 day time window. Males were sexed and kept in groups of 15, whilst females were maintained in groups of 20 of the same sexual selection line origin. All individuals were given at least 10 days to undergo eclosion and sexual maturation in 5cm plastic petri dishes with ~7.8g of fodder and a layer of oats (~3.8g). Once males and females were sexually mature, each female was randomly assigned a single Georgia strain control male, and placed into a mating Petri dish containing standard volume of fodder (~7.8g). Before adding couples to the mating Petri dishes, males were marked using a paint pen (uni-poscaTM) on the thorax to allow identification. Male female pairings were kept in mating Petri dishes for 10 days, and then transferred to new Petri dishes with fresh fodder and oats; this was carried out ten times over 100 days. The transfer of the pairing every 10 days allowed analysis of changes in offspring production rates over time, and prevented offspring density becoming extreme. After couples were removed from the mating Petri dishes, the resulting eggs and offspring were incubated under standard conditions as described above for 35 days, with reproductive output and relative fertility scored as the number of adult offspring produced in each 10-day block.

6.2.4 Statistical analysis

All data analysis was carried out in R-studio using R-version 3.4.1 (2009-2019) (RStudio Team, 2020).

To determine offspring production over the whole experiment a GLMM was constructed using the glmmTMB package in r (Bolker, 2018). The model was fitted with a negative

binomial error model with the response variable offspring count, explanatory variable of treatment (male bias vs female bias female origin) and random effect of block (1-10). GLM's were also constructed for each block (10 days) to determine any differences across the 100 day experiment. Offspring count was coded as the response variable with treatment as the explanatory variable (male bias vs female bias female origin) using a negative binomial error model.

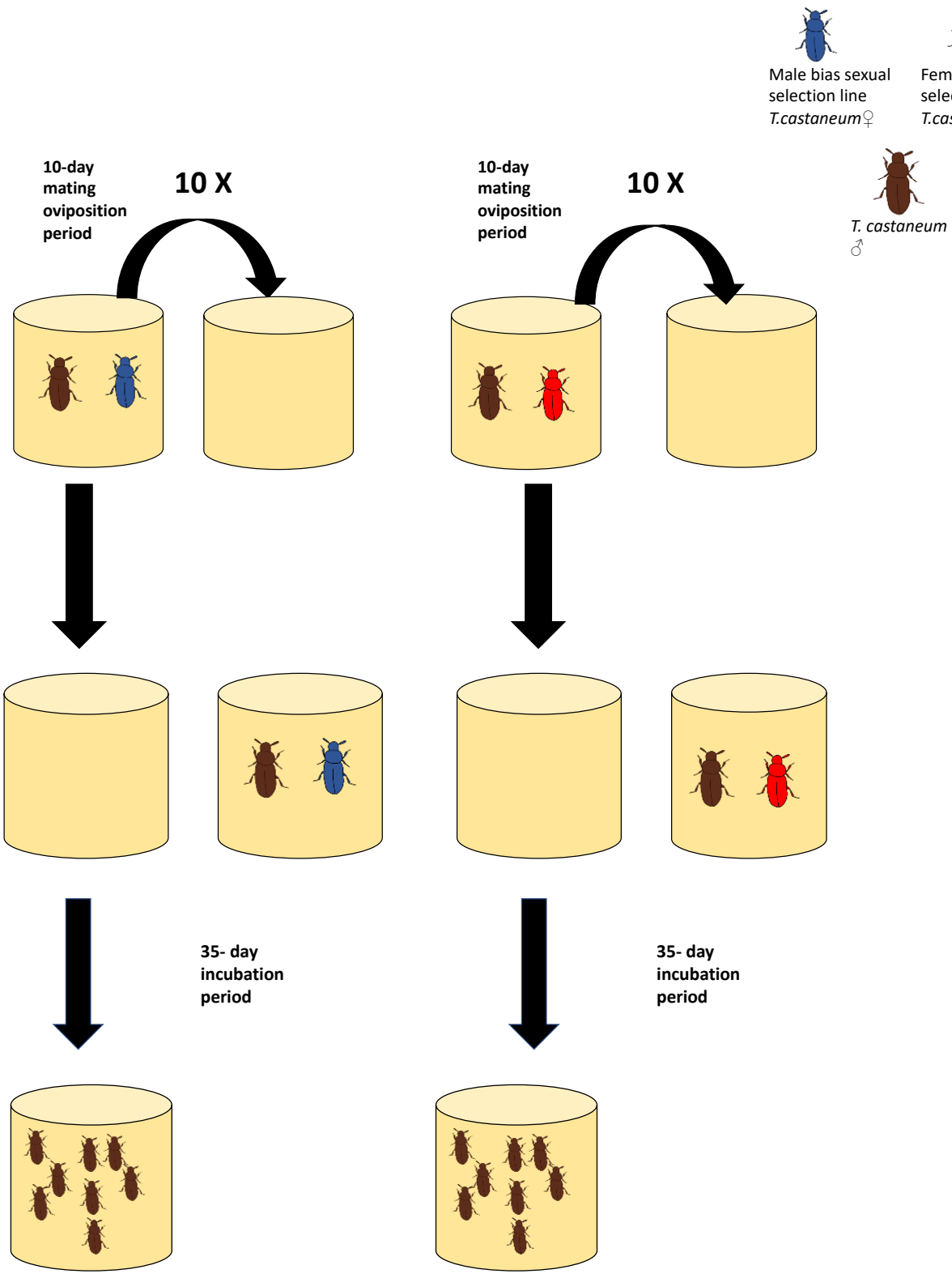

Figure 3: Sexual selection line females were mated with a standard Georgia strain T. castaneum male. Each pair was kept together for 100 days, with couples transferred to a fresh vial every 10 days. Once pairs had been removed, fodder was kept in standard conditions for 35 days to allow offspring to develop to mature to adults allowing us to count adult offspring number to score reproductive output.

6.3 Results

6.3.1 Offspring production rates of females from different sexual selection line backgrounds

Total offspring production over the whole experiment (100 days) did not differ significantly between male biased (total offspring = 14507, average= 56.2) and female biased females (total offspring=15104, average 62.5) (Table 2). Within each block (10 day period), no significant difference were observed between male biased and female biased female reproductive output (Table 1, Table 3). All P-values reported were calculated through the wald t-test within the glmmtmb and lme4 packages.

Table 1: Total and average offspring produced by females from either male biased or female biased sexual selection lines across each 10-day oviposition block, average per female.

Block	Average offspring number
1	Female bias=102.8
	Male bias=97.8
2	Female bias=86.8
	Male bias=89.7
3	Female bias=65.8
	Male bias=68.5
4	Female bias=65.5
	Male bias=60.9
5	Female bias=51.4
	Male bias= 48.6
6	Female bias=49.3
	Male bias= 36.5
7	Female bias=28.7
	Male bias=23.9
8	Female bias=30.1
	Male bias=18.1
9	Female bias=26.6
	Male bias=14.3
10	Female bias=24.0
	Male bias=11.8

Table 2: GLMM assessing overall offspring production number of female bias and male bias sexual selection lines over 100 days. The response variable of offspring number was coded with the fixed effect of sexual selection line origin with a random effect of block (Var- 0.308) to account for differences in offspring production rates

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
Intercept (Female bias)	3.8584	0.198	19.478	<0.001 ***
Male bias	-0.146	0.123	-1.194	0.232

Table 3: The results of GLM's assessing differences in offspring production number of males bias and female bias sexual selection lines at each block 1- 10. The response variable of offspring production number, fixed effect of female sexual selection line origin.

Fixed Effects	**Estimate**	**Standard Error**	**z value**	**Pr(>|t|)**
Block 1 (Day 1-10				
Intercept (Female bias)	4.63	0.082	56.07	<0.001 ***
Male bias	-0.05	0.119	-0.44	0.656
Block 2 (Day 11-20)				
Intercept (Female bias)	4.46	0.098	45.51	<0.001 ***
Male bias	0.03	0.139	0.23	0.817
Block 3 (Day 21-30)				
Intercept (Female bias)	4.19	0.122	34.14	<0.001 ***
Male bias	0.04	0.173	0.23	0.816
Block 4 (Day 31-40)				
Intercept (Female bias)	4.18	0.138	30.21	<0.001 ***
Male bias	-0.072	0.189	-0.38	0.705
Block 5 (Day 41-50)				
Intercept (Female bias)	3.940	0.155	25.28	<0.001 ***
Male bias	-0.05	0.216	-0.263	0.793

Block 6 (Day 51-60)				
Intercept (Female bias)	3.898	0.211	18.42	<0.001 ***
Male bias	-0.300	0.274	-1.093	0.28
Block 7 (Day 61-70)				
Intercept (Female bias)	3.360	0.250	13.43	<0.001 ***
Male bias	-0.186	0.349	-0.53	0.598
Block 8 (Day 71-80)				
Intercept (Female bias)	3.405	0.300	1.33	<0.001 ***
Male bias	-0.509	0.408	-1.24	0.222
Block 9 (Day 81-90)				
Intercept (Female bias)	3.281	0.348	9.40	<0.001 ***
Male bias	-0.618	0.480	-1.29	0.209
Block 10(Day 91-100)				
Intercept (Female bias)	3.181	0.383	8.289	<0.001 ***
Male bias	-0.708	0.528	-1.342	0.192

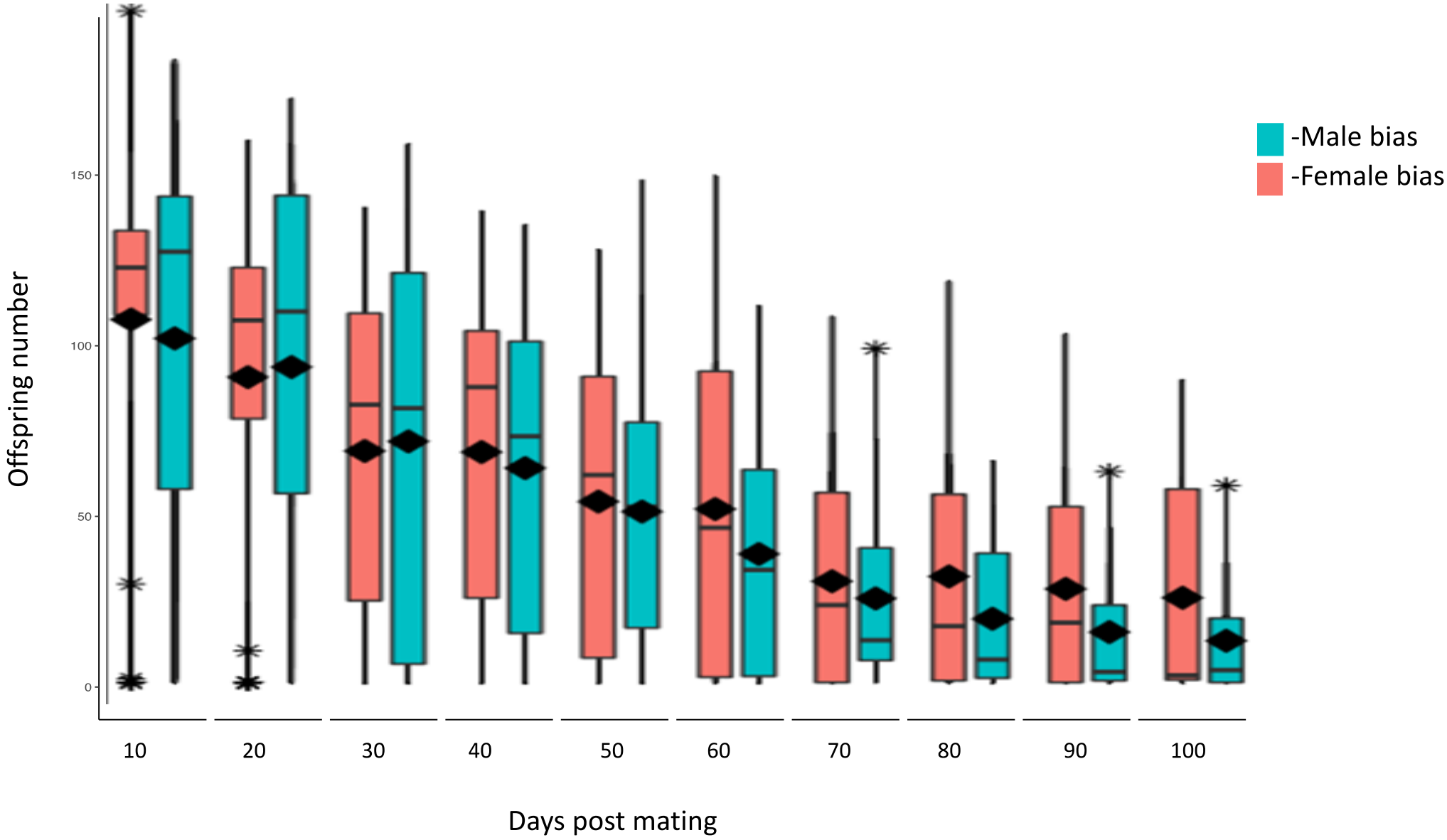

Figure 4: Offspring number produced by male biased (n=259 pairs) versus female biased (n=239 pairs) sexual selection line females within each 10-day oviposition block over a 100 day trial when provided with continuous male mates. Means are represented by a black diamond, medians by black horizontal lines, and jitters represent output from individual pairs.

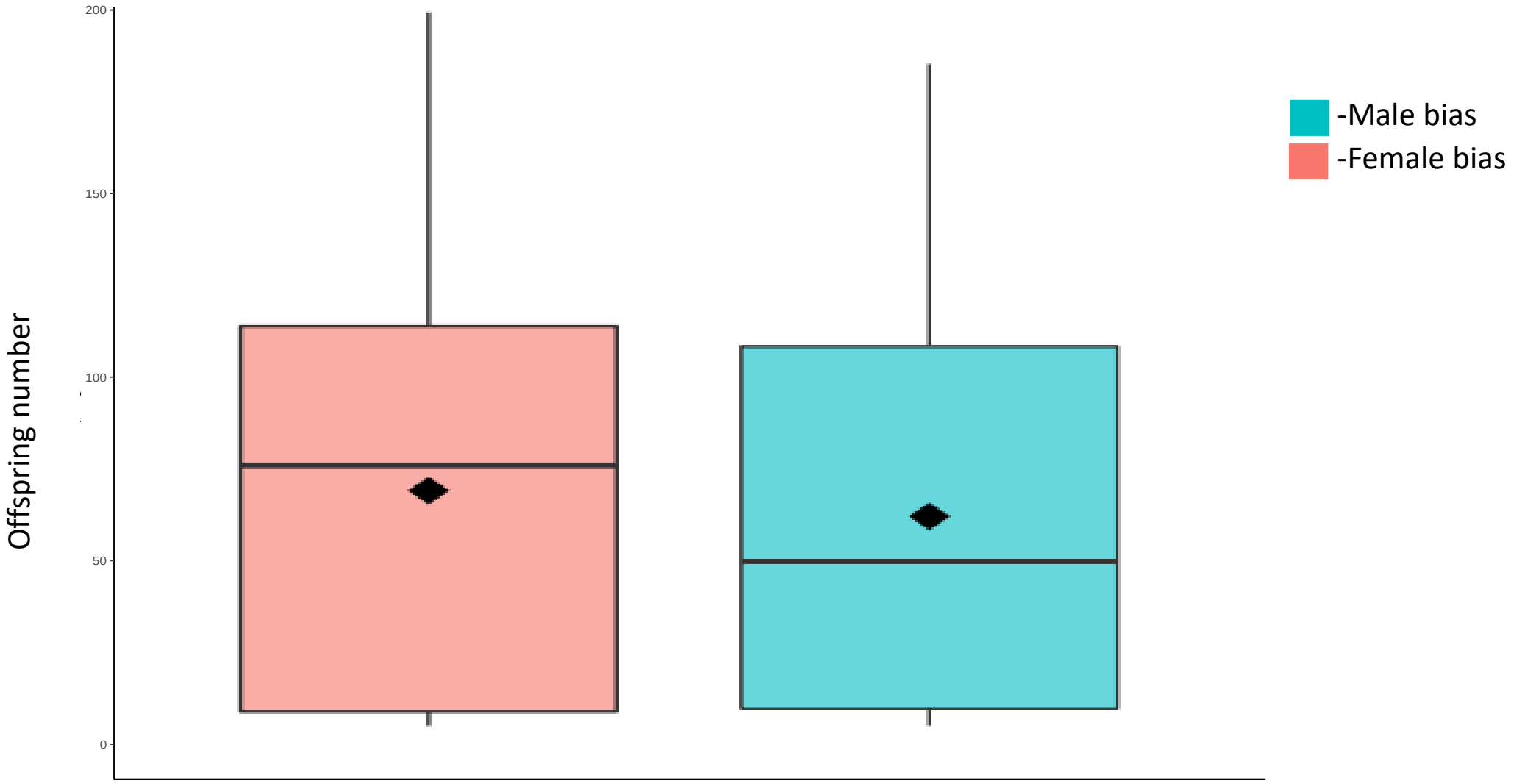

Figure 5: Offspring number of male biased (n=259) and female biased (n=239) sexual selection line females. Total output over a 100-day period when provided continuously with a standard georgia strain T. castaneum male. The mean is represented by a black diamond and the median a black horizontal line, with jitters representing the total output of individual pairs.

Discussion

Despite divergent evolutionary backgrounds in reproductive environment, we found that females from the male-biased and female-biased selection backgrounds produced almost identical offspring output profiles when male mates were provided continuously through oviposition and there was no sperm limitation. No significant differences were observed across the 100-day experiment trial, with male biased females producing on average 14,507 offspring, and female biased females producing 15,104. This finding indicates that the difference in offspring output under sperm limitation can be explained by differences in sperm storage and management, and not any intrinsic differences in reproductive output by male-biased versus female-biased background females.

This study builds upon previous research at UEA identifying differential offspring production rates of high and low sexual selection females under sperm limitation. This work has revealed that females from low sexual selection and female-biased regimes were able to produce a greater number of offspring under sperm limitation, especially over days 30 and 50 after their single matings. Showing that offspring production is equal between the lines when males are provided continuously indicates that differences in reproductive output under sperm and male limitation is due to evolved differences in sperm storage, usage and management efficiency, and not intrinsically different offspring production rates.

We did not observe the offspring production rate to vary between sexual selection lines suggesting that both are maximising offspring production when a male is constantly present. Due to *T. castaneum's* willingness and rapid re-mating system (Pai *et al.,* 2007; Pai & Yan, 2003), females should mate regularly under pair-living conditions, providing a high

level of sperm to the female. The limiting factor in offspring production rate when sperm are theoretically unlimited seems to be the female's ability to store and use sperm efficiently, which declines following mating, especially after 30 days. Despite being provided with a constantly present male here, offspring production still declined over the 100-day period (Figure 4), suggesting that female ageing is associated with reproductive output. Moreover, comparison of reproductive output decline under sperm limitation (Figure 1) reveals a more rapid and steep decline. And, importantly, there was no difference in the rate of decline between the male-biased and female-biased selection backgrounds in our experiment without sperm limitation (Figure 4).

Female ageing

The effects of female ageing or senescence on fecundity have been well studied across a range of species (Millery *et al.*, 2014; de Boer, Eens and Müller, 2018; Lemaître, Ronget and Gaillard, 2020; Žák and Reichard, 2021). The timing and influence of reproductive senescence shows wide variation across organisms, with mating pattern a key determinant of reproductive senescence's effect (Millery *et al.*, 2014; de Boer, Eens and Müller, 2018; Lemaître, Ronget and Gaillard, 2020; Žák and Reichard, 2021). In previous research on reproductive senescence at the University of East Anglia on *T. castaneum*, female reproductive output had decreased by 50% within 1 month of adult eclosion, compared to young females (Godwin, 2017 PhD Thesis). Here, we also find declining offspring output in both high sexual selection and low sexual selection females from block 1 (day 1-10) (average offspring number high= 103, low= 98) to block 4 (day 31-40) (high=66, low=61), and beyond (Figure 4). Therefore, one potential explanation for our finding of overall decline in offspring

output by individual females the experimental period is simply reproductive senescence of females.

Sexual conflict

In our experiment, constant access to males may create further impacts on female reproductive fitness through sexual conflict. Costs to females include energy involved in mating as well as physical (Le Galliard *et al.*, 2005) and chemical damage (Wigby and Chapman, 2004) caused by males onto females. There is a large body of evidence that sexual harassment can cause major impacts upon fecundity and survival (McLain and Pratt, 1999; Rossi, Nonacs and Pitts-Singer, 2010; Rankin, Dieckmann and Kokko, 2011; Maklakov *et al.*, 2013). In *T. castaneum,* sexual conflict has been shown to impact upon female fecundity, which is hypothesised to occur as a result of direct reduction in egg production or damage to females (Michalczyk *et al.*, 2011). In our experiment the reduction in fecundity may result from sexual conflict imposed by males. *T. castaneum* are a highly promiscuous and polyandrous species which would indicate that a single male may not impose a high cost of sexual selection. However, despite only a single male being present for each female, pairs were held together for 100 days in a closed environment, meaning the female would be limited in their ability to escape male harassment. During this 100-day period, the cost of sexual harassment of females could build, resulting in reduced fecundity during the latter period of oviposition. However, if this factor does explain a decline in female reproductive fitness, we might expect females from male-biased, high sexual selection backgrounds to be better able to resist such costly effects, as has been previously revealed (Michalczyk *et al.*, 2011).

Male spermatophore transfer

Male *T. castaneum* could influence female reproductive rates if there is variation in spermatophore transfer and sperm. Male insects are known to exert cryptic choice by biasing the quantity or quality of sperm they transfer to females (Aumont and Shuker, 2018). It has been observed that *T. castaneum* males preferentially mate with virgin females over already-mated females (Lewis and Iannini, 1995), so there may be some evidence for males investing in female mating status and 'quality' in this system and and use cryptic choice. In this experiment, females in block 1 are young, virgin females, whilst in subsequent blocks all females are mated and progressively older. Males may therefore provide less mating investment or low sperm density spermatophores when females become older. This pattern of reduced mating investment could, in combination with previous factors, result in the reduced offspring production among older females. In contrast with female output, Previous research at UEA (Godwin, 2017 PhD thesis) identified that male *T. castaneum* reproductive fitness showed no decline over 12 months, indicating that male quality is unlikely to be a factor in the reduction of offspring production over time.

High and low sexual selection intensity

In our experiment, and despite females coming from highly divergent sexual selection backgrounds, no difference in offspring production rate was observed. This suggests that sexual selection intensity does not determine female ability to use sperm when it is constantly available. Female biased females have evolved within a low male density environment, meaning that when males are present, females should be efficient in mating

and then storing an using sperm to produce the maximum number of offspring. Male biased females, by contrast, are reared in high male density environments with polyandry being common, so superfluous numbers of sperm will be the typical reproductive environment, and therefore of relaxed importance for reproducing females. Females in this experiment were kept at all times with another male in an enclosed environment, so that regular matings and awareness of the male's presence may be a key determinant of female offspring production rate. If there are differences in female ability to find or attract males, our experiment and results do not suggest such a phenomenon.

Previous research and future study

Our findings strengthen previous research that differences in offspring production between our sexual lines under sperm limitation could be due to evolved differences in the efficiency of sperm management, which would be a novel finding. Our study shows that this result is not due to intrinsically different offspring production rates between sexual selection regimes, but likely due to differences in sperm management. Further research is now being conducted to explore direct mechanisms here, including the direct imaging of fluorescent sperm within the bursa and spermathecae through storage time in different selection line backgrounds. This will allow us to determine whether differential offspring production under sperm limitation is the result of sperm management by allowing us to visualise sperm content moving within females over time. We can use this approach to test the hypothesis that females from low sexual selection environments with sperm limitation have developed more efficient sperm management mechanisms to maximise reproductive fitness when males are limited. We also aim to analyse the spermathecal and bursal structure of females

in different selection backgrounds, providing insight into how insect females store and manage sperm.

Chapter 7 General discussion

7.1 Thesis overview

The objective of this thesis is to advance our understanding of sperm-egg interactions and discover what factors can influence successful fertilisation. I addressed this in both the externally fertilising Atlantic salmon, and the internally fertilising *Tribolium castaneum.* First, I assessed the influence of the artificial fertilisation process on successful production of Atlantic salmon under hatchery conditions. In chapter 2, I focused on a matrix of factors surrounding the artificial fertilisation process and their influence on hatch rate and abnormal ploidy in wild and farm salmon. In chapter 3, I focused on the issue of post-ovulatory oocyte ageing in farm salmon. Chapter 2 and 3 allow us to explore factors influencing successful fertilisation at the gamete level, from both pure and applied scientific perspectives. Chapter 4 explores gamete interactions between farm and wild Atlantic salmon strains. This study allowed us to explore whether the selective divergence between farm and wild strains under domestication has resulted in the formation of a barrier to hybridisation, and the influence of genetic relatedness and ovarian fluid in determining successful sperm competition and fertilisation. The work also allows us to explore the risk of hybridisation by farm salmon escapes from an applied perspective, predicting genetic introgression.

By switching to *T. castaneum*, I was able to use experimental evolution to explore the influence of sexual selection intensity on reproductive evolution. In chapter 5, I explored female ability to assert cryptic female choice between conspecific and heterospecific males and their sperm, but found no evidence that CFC had evolved (desite changes in the ability of females to manage sperm efficiently under limitation). Finally, in chapter 6 and utilising the same sexual selection lines, I explored whether high and low sexual selection

backgrounds had changed sperm storage and utilisation patterns, or whether there were simple intrinsic differences in female reproductive output due to evolutionary background. This general discussion combines places experimental results of the thesis alongside current findings and implications for future study.

7.2 Main findings

Artificial fertilisation methods can constrain Atlantic salmon hatch rates, especially within wild strain fish. There was no evidence that unintentional spontaneous triploidy among hatched offspring could be driven by artificial hatchery practices.

Wild Atlantic salmon hatch rates declined significantly when fertilised under 'wet' fertilisation and when ova are fertilised following 72 hours storage after stripping. No significant difference was observed between fertilisation methods and external oocyte storage in farm Atlantic salmon. Our findings suggest that farm Atlantic salmon have begun to adapt to artificial fertilisation methods, with previous research finding farm Atlantic salmon to have gamete and whole organism adaptations to the artificial aquaculture environment (Solberg, Zhang, *et al.*, 2013; Camarillo-Sepulveda *et al.*, 2016; Harvey *et al.*, 2016). One potential hypothesis for the declines in hatch rate under 'wet' fertilisation is the dilution and dispersion of ovarian fluid during artificial fertilisation. Ovarian fluid is found in high concentrations in the natural gamete micro-environment, with highest concentrations surrounding ova in Salmonids (Litvak and Trippel, 1998; Olsén *et al.*, 2001; Lahnsteiner, 2002). The addition of river water pre-fertilisation could create mechanical forces that

disrupts this micro-environment, removing or reducing ovarian fluid's potential role in CFC, gamete protection and disease prevention (Hatef, Niksirat and Alavi, 2009; Jensen *et al.*, 2009; Yeates *et al.*, 2013). The impact of external oocyte ageing has been observed in both teleosts and salmonids specifically with reduction to ova viability (Piper et al., 1986; Barnes, Sayler and Cordes, 1999; Samarin *et al.*, 2011). Our finding, that farm salmon are not significantly impacted by these factors, suggest that adaptations to the aquaculture environment have occurred, or are occurring. Over 50 years of aquaculture, artificial fertilisation is likely to have weakened the importance of ovarian fluid as a post-copulatory mechanism of sperm choice, as well as providing a selective force for ova to resist external oocyte ageing. Despite not being significant in farm salmon, both wild and farm salmon were found to have their highest hatch rates under 'dry' fertilisation on the day of stripping, meaning both aquaculture and conservation hatcheries should ensure that this is achieved. There exists 20-40% failure to hatch rate in both strains that remains unexplained. One potential area of future study is to look at abnormal ploidy earlier in the development than the current study. This study focused on hatchling Atlantic salmon, with future study at UEA focusing on triploidy at the eyed-egg stage and already finding triploidy to be more common.

Farm Atlantic salmon are able to resist the impacts of post-ovulatory oocyte ageing for an extended period of time compared to most teleosts.

Farm Atlantic salmon had no significant decline in hatch rate after 14 days of post-ovulatory oocyte ageing, with a significant decline observed only after 21 days. In assessment of other

teleosts, the impacts of post-ovulatory oocyte ageing on egg viability occur 2-4 hours in carp (Samarin *et al.*, 2015), 3-9 hours in pikeperch (Samarin and Miroslav Blecha, Dmytro Bytyutskyy, 2015) and 7 days in rainbow trout (Aegerter and Jalabert, 2004). Our finding, of extended resistance to post-ovulatory oocyte ageing may result from Atlantic salmon's naturally extended spawning season, with repeated mating and egg batch production by females among multiple nests. We also found no evidence of unintentional spontaneous triploidisation as a result of post-ovulatory oocyte ageing. In previous research of post-ovulatory oocyte ageing, increased rates of triploidisation have been observed in Pike (Samarin *et al.*, 2016), Japanese eel (Nomura *et al.*, 2013a), Tench (Flajšhans, Kvasnička and Ráb, 1993) and salmonids (Aegerter and Jalabert, 2004).

The ability to resist post-ovulatory oocyte ageing and UST may result from ATP levels within eggs, ATP-mediated oocyte ageing is hypothesised by Aegerter and Jalabert, (2004). The salmonid rainbow trout has ATP concentrations 100 times higher than that of carp, providing a potential explanation for the large time difference before the impacts of post-ovulatory oocyte ageing. Atlantic salmon's resistance may therefore result from high ATP stores within eggs as an adaptive response to the natural extended mating system of Atlantic salmon.

Our findings suggest, from an applied perspective, that the aquaculture industry should leave eggs within the coelomic cavity as opposed to repeated checking and stripping events which can be stressful. Future study should look to identify ATP concentrations within ova throughout post-ovulatory oocyte ageing.

There is no evidence for a barrier to hybridisation between farm and wild Atlantic salmon at the gamete level, and clear evidence that farm salmon sperm can be highly competitive.

Farm male Atlantic salmon gained a significantly greater share of paternity than wild males when competing for wild females through in vitro fertilisation competitions. When in competition for farm female eggs, farm sperm gained equal paternity. Farm sperm paternity success indicates that the risk of genetic introgression is high at the gamete level, with no evidence for degradation of sperm quality or selection against farm sperm. The dominance of Atlantic salmon may result from selective competition incited in the aquaculture fertilisation process as hypothesised by Lehnert, Heath and Pitcher, (2012). Ovarian fluid was manipulated to identify its potential role as a mechanism of sperm selection. No significant effect was observed when manipulating farm and wild ovarian fluid on farm or wild egg batches. One potential reasons for this is the use of the 'wet' fertilisation methods that may have diluted and disrupted ovarian fluid's post-copulatory role (Rosengrave *et al.*, 2008a; Butts *et al.*, 2012; Alonzo, Stiver and Marsh-Rollo, 2016). Our findings provide further support for previous research that genetic introgression provides a major threat to the survival of wild Atlantic salmon population genetic structure. Divergence has not resulted in a barrier to hybridisation, with farm salmon sperm being highly competitive. Further research could look to build upon this by increasing replication, and studying different farm and wild strains under competition.

Experimental evolution of female *T. castaneum* under high versus low sexual selection intensity did not result in a strengthening of cryptic female choice.

T. castaneum females, after 130 generations of selection under strong versus weak opportunities for reproductive competition and choice, were sequentially or constantly mated with a conspecific and heterospecific males. We found no evidence that strong histories of sexual selection enabled females to improve their ability in exerting CFC when given a choice between conspecific or heterospecific sperm or males. Both proportional paternity and offspring number produced of conspecifc offspring were not statistically significant between high and low sexual selection background females. We observed conspecific males and their sperm to gain the majority of fertilisations in both sequential and constant matings, but no evidence that high sexual selection background females were able to increase conspecific sperm precedence. Two alternative hypotheses may explain this finding: 1) Cryptic female choice is a complete and powerful mechanism that does nopt change under relaxation or strengthening of sexual selection; 2) females have little influence over post-copulatory sperm choice, and instead facilitate sperm competition to achieve high offspring fitness through male-male interactions. Further research is needed to elucidate the female and male roles in this process. This could include assessing sequential mating with both a P_1 and then a P_2 conspecific experiment, with observation of a single mating taking place to minimise the potentially confounding effect of male mating success. Determining the survival rates of hybrid and pure *T. castaneum* offspring would further allow the ability to isolate CFC from offspring fitness.

Experimental evolution of sexual selection through male-biased versus female-biased operational sex ratios did not result in changes to offspring production by *T. castaneum* females.

T. castaneum females, when constantly mated with standard Georgia strain males, produced the same total and rate of offspring irrespective of whether they had evolved under high sexual selection and male-biased regimes, or low sexual selection and female-biased regimes. Through this 100 day experiment, similar declines in offspring production rates were observed in females from both sexual selection backgrounds. Our finding, suggests that differences observed in previous research by R. Rasudeva after applying sperm limitation, are the not the result of differences in intrinsic offspring production rates between sexual selection lines, but evolution of sperm management mechanisms. Future research could focus on measuring female reproductive morphology and visualising sperm within the reproductive tract to determine in vivo how changes in sperm management and fertilisation efficiency have changed under different levels of selection from sperm limitation.

References

Aas, O. *et al.,*(2011) *Atlantic Salmon Ecology*, *Atlantic Salmon Ecology*. doi: 10.1002/9781444327755.

Aegerter, S. and Jalabert, B. (2004) 'Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, Oncorhynchus mykiss', *Aquaculture*, 231(1–4), pp. 59–71. doi: 10.1016/j.aquaculture.2003.08.019.

Alavi, S. M. H. and Cosson, J. (2006) 'Sperm motility in fishes. (II) Effects of ions and osmolality: A review', *Cell Biology International*, pp. 1–14. doi: 10.1016/j.cellbi.2005.06.004.

Alavioon, G. *et al.,*(2017) 'Haploid selection within a single ejaculate increases offspring fitness', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1705601114.

Alavioon, G. *et al.,*(2019) 'Selection for longer lived sperm within ejaculate reduces reproductive ageing in offspring', *Evolution Letters*. doi: 10.1002/evl3.101.

Allan, R. and Vidal, E. (2004) 'TAXA DE FERTILIZAÇÃO DE OVÓCITOS DE CURIMBATÁ ARMAZENADOS POR DIFERENTES PERÍODOS DE TEMPO A TEMPERATURA AMBIENTE Bruno Estevão de Souza, Eduardo Antônio **Sanches, Diego Mendes Baggio**, Elizabeth Romagosa, Robie Allan Bombardelli e Eléxio Vidal'.

Alonzo, S. H., Stiver, K. A. and Marsh-Rollo, S. E. (2016) 'Ovarian fluid allows directional cryptic female choice despite external fertilization', *Nature Communications*, 7, pp. 1–8. doi: 10.1038/ncomms12452.

Amoroso, G. *et al.,*(2016) 'Concurrence of lower jaw skeletal anomalies in triploid Atlantic salmon (Salmo salar L.) and the effect on growth in freshwater', *Journal of Fish Diseases*, 39(12), pp. 1509–1521. doi: 10.1111/jfd.12492.

Andersson, W. Simmons, L. (2006) 'Sexual selection and mate choice', *Trends in Ecology and Evolution*, 21. Available at: https://ac.els-cdn.com/S0169534706001029/1-s2.0-S0169534706001029-main.pdf?_tid=edd23d02-e130-433f-8d76-110ed6174a83&acdnat=1520256696_727b803f52230f7f4093b8c8b0472c3e.

Andersson, M. (1994) *Sexual Selection*, *Sexual Selection*.

Angelini, D. R. and Jockusch, E. L. (2008) 'Relationships among pest flour beetles of the genus Tribolium (Tenebrionidae) inferred from multiple molecular markers', *Molecular Phylogenetics and Evolution*, 46(1). doi:

10.1016/j.ympev.2007.08.017.

Araki, H., Cooper, B. and Blouin, M. S. (2007) 'Genetic Effects of Captive Breeding Decline in the Wild', *Science*, 318(October), pp. 100–103. doi: 10.1126/science.1145621.

Araki, H. and Schmid, C. (2010) 'Is hatchery stocking a help or harm?. Evidence, limitations and future directions in ecological and genetic surveys', *Aquaculture*, 308(SUPPL.1), pp. S2–S11. doi: 10.1016/j.aquaculture.2010.05.036.

Arnaud, L., Gage, M. J. G. and Haubruge, E. (2001) *The dynamics of second-and third-male fertilization precedence in Tribolium castaneum*, *Entomologia Experimentalis et Applicata*.

Arnaud, L., Haubruge, E. and Gage, M. J. G. (2001) *Morphology of Tribolium castaneum male genitalia and its possible role in sperm competition and cryptic female choice*, *Belgian Journal of Zoology 131*. Available at: http://biblio.naturalsciences.be/associated_publications/bjz/131-2/volume-131-2-pp-111-115.pdf (Accessed: 2 June 2021).

Aumont, C. and Shuker, D. M. (2018) 'Cryptic male choice', *Current Biology*, 28(20). doi: 10.1016/j.cub.2018.07.071.

Babayev, E. and Seli, E. (2015) 'Oocyte mitochondrial function and reproduction', *Current Opinion in Obstetrics and Gynecology*. doi: 10.1097/GCO.0000000000000164.

Balshine, S. (2001) 'Sperm size of African cichlids in relation to sperm competition', *Behavioral Ecology*, 12(6), pp. 726–731. doi: 10.1093/beheco/12.6.726.

Barnes, M. E., Sayler, W. A. and Cordes, R. J. (1999) 'Transportation Influences on Inland Fall Chinook Salmon Egg Survival', *North American Journal of Aquaculture*, 61(1). doi: 10.1577/1548-8454(1999)061<0027:tioifc>2.0.co;2.

Bates, D. *et al.,*(2015) 'Fitting linear mixed-effects models using lme4', *Journal of Statistical Software*. doi: 10.18637/jss.v067.i01.

Beirão, J. *et al.,*(2014) 'Wild Atlantic cod sperm motility is negatively affected by ovarian fluid of farmed females', *Aquaculture Environment Interactions*. doi: 10.3354/aei00095.

Bellard, R. (1988) 'Artificial insemination and gamete management in fish', *Marine Behaviour and Physiology*. doi: 10.1080/10236248809378690.

Benfey, T. J. (2001) 'Use of sterile triploid Atlantic salmon (Salmo salar L.) for aquaculture in New Brunswick, Canada', *Ices Journal of Marine Science*, 58(2), pp. 525–529. doi: 10.1006/jmsc.2000.1019.

Benfey, T. J. (2016) 'Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (Salmo salar) as a case study', *Reviews in Aquaculture*, pp. 264–282. doi: 10.1111/raq.12092.

Bennison, C. *et al.,*(2015) 'Long sperm fertilize more eggs in a bird', *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2014.1897.

Berejikian, B. A., Tezak, E. P. and LaRae, A. L. (2000) 'Female mate choice and spawning behaviour of chinook salmon under experimental conditions', *Journal of Fish Biology*, 57(3), pp. 647–661. doi: 10.1006/jfbi.2000.1340.

Bergstrøm.E (1973) 'The role of nutrition in growth and survival of young hatchery reared Atlantic salmon', *Int. Atlantic Salmon Symp. 1972, 4, The International Atlantic Salmon Foundation*, pp. 265–282.

Bernasconi, G. *et al.,*(2006) 'Do spermathecal morphology and inter-mating interval influence paternity in the polyandrous beetle Tribolium castaneum?', *Behaviour*, 143(5). doi: 10.1163/156853906776759538.

Bicskei, B. *et al.,*(2014) 'A comparison of gene transcription profiles of domesticated and wild Atlantic salmon (Salmo salar L.) at early life stages, reared under controlled conditions', *BMC Genomics*. doi: 10.1186/1471-2164-15-884.

Bicskei, B. *et al.,*(2016) 'Comparing the transcriptomes of embryos from domesticated and wild Atlantic salmon (Salmo salar L.) stocks and examining factors that influence heritability of gene expression', *Genetics Selection Evolution*, 48(1). doi: 10.1186/s12711-016-0200-6.

Billard, R. *et al.,*(1986) 'Biology of the gametes of some teleost species', *Fish Physiology and Biochemistry*, 2(1–4), pp. 115–120. doi: 10.1007/BF02264079.

Birkhead, T. (1987) 'Sperm competition in birds', *Trends in Ecology and Evolution*, 2(9), pp. 268–272. doi: 10.1016/0169-5347(87)90033-4.

Birkhead, T. R. (1998) 'Cryptic female choice: Criteria for establishing female sperm choice', *Evolution*, 52(4), pp. 1212–1218. doi: 10.1111/j.1558-5646.1998.tb01848.x.

Birkhead, T. R. and Moller, A. P. (1998) *Sperm competition and sexual selection*, *Sperm competition and sexual selection*.

Birkhead, T. R. and MØLLER, A. P. (1992) 'Numbers and size of sperm storage tubules and the duration of sperm storage in birds: a comparative study', *Biological Journal of the Linnean Society*, 45(4). doi: 10.1111/j.1095-8312.1992.tb00649.x.

Birkhead, T. R. and MØLLER, A. P. (1993) 'Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals', *Biological Journal of the Linnean Society*, 50(4). doi: 10.1111/j.1095-8312.1993.tb00933.x.

Bjarne Gjerde (1984) 'Variation in semen production salmon and rainbow trout of farmed atlantic', *Atlantic*, 40, pp. 109–114.

Bjørnevik, M. *et al.,*(2004) 'Temporal variation in muscle fibre area, gaping texture, colour and collagen in triploid and diploid Atlantic salmon (Salmo salar L)', *Journal of the Science of Food and Agriculture*, 84(6), pp. 530–540. doi: 10.1002/jsfa.1656.

Blanchet, S. *et al.,*(2008) 'An integrated comparison of captive-bred and wild Atlantic salmon (Salmo salar): Implications for supportive breeding programs', *Biological Conservation*, 141(8), pp. 1989–1999. doi: 10.1016/j.biocon.2008.05.014.

Blanchfield, P. J. and Ridgway, M. S. (1999) 'The cost of peripheral males in a brook trout mating system', *Animal Behaviour*, 57(3), pp. 537–544. doi: 10.1006/anbe.1998.1014.

Bloch Qazi, M. C., Aprille, J. R. and Lewis, S. M. (1998) 'Female role in sperm storage in the red flour beetle, Tribolium castaneum', *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 120(4), pp. 641–647. doi: 10.1016/S1095-6433(98)10081-8.

Bloch Qazi, M. C., Herbeck, J. T. and Lewis, S. M. (1996) 'Mechanisms of sperm transfer and storage in the red flour beetle (Coleoptera: Tenebrionidae)', *Annals of the Entomological Society of America*, 89(6). doi: 10.1093/aesa/89.6.892.

Bobe, J. and Labbé, C. (2010) 'Egg and sperm quality in fish', *General and Comparative Endocrinology*, 165(3), pp. 535–548. doi: 10.1016/j.ygcen.2009.02.011.

de Boer, R. A., Eens, M. and Müller, W. (2018) 'Sex-specific effects of inbreeding on reproductive senescence', *Proceedings of the Royal Society B: Biological Sciences*, 285(1879). doi: 10.1098/rspb.2018.0231.

Bonnet, E., Fostier, A. and Bobe, J. (2007) 'Characterization of rainbow trout egg quality: A case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations', *Theriogenology*, 67(4), pp. 786–794. doi: 10.1016/j.theriogenology.2006.10.008.

Bonnet, E., Jalabert, B. and Bobe, J. (2003) 'A 3-day in vitro storage of rainbow trout (Oncorhynchus mykiss) unfertilised eggs in coelomic fluid at 12°C does not affect developmental success', *Cybium*, 27(1), pp. 47–51.

Boulekbache, H. *et al.,*(1989) 'Ageing of fish oocytes: Effects on adenylic nucleotides content, energy charge

and viability of carp embryo', *Comparative Biochemistry and Physiology -- Part B: Biochemistry and*. doi: 10.1016/0305-0491(89)90109-0.

Bourret, V. *et al.,*(2011) 'Temporal change in genetic integrity suggests loss of local adaptation in a wild Atlantic salmon (Salmo salar) population following introgression by farmed escapees', pp. 500–510. doi: 10.1038/hdy.2010.165.

Brian, E., Dell, R. I. D. and Leggett, C. (1981) 'Evidence of an Adaptive Basis for Geographic Variation in Body Morphology and Time of Downstream Migration of Juvenile Atlantic Salmon (Salmo sahr)'', (56049).

Briceño, R. D. and Eberhard, W. G. (2009) 'Experimental demonstration of possible cryptic female choice on male tsetse fly genitalia', *Journal of Insect Physiology*, 55(11), pp. 989–996. doi: 10.1016/j.jinsphys.2009.07.001.

Bromage, N. *et al.,*(1992) 'Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (Oncorhynchus mykiss)', *Aquaculture*, 100(1–3), pp. 141–166. doi: 10.1016/0044-8486(92)90355-O.

Brooks, S., Tyler, C. R. and Sumpter, J. P. (1997) 'Egg quality in ®sh: what makes a good egg?', *Reviews in Fish Biology and Fisheries*, 7, pp. 387–416. doi: 10.1023/A:1018400130692.

Butts, I. A. E. *et al.,*(2012) 'Ovarian fluid enhances sperm velocity based on relatedness in lake trout, Salvelinus namaycush', *Theriogenology*, 78(9), pp. 2105–2109. doi: 10.1016/j.theriogenology.2012.06.031.

Butts, I. A. E. *et al.,*(2017) 'Ovarian fluid impacts flagellar beating and biomechanical metrics of sperm between alternative reproductive tactics', *The Journal of Experimental Biology*, 220(12), pp. 2210–2217. doi: 10.1242/jeb.154195.

Caldeira, C. *et al.,*(2018) 'Comparison of sperm motility subpopulation structure among wild anadromous and farmed male Atlantic salmon (Salmo salar) parr using a CASA system', *Reproduction Fertility and Development*.

Camarillo-Sepulveda, N. *et al.,*(2016) 'Sperm traits in farmed and wild Atlantic salmon Salmo salar', *Journal of Fish Biology*, 88(2), pp. 709–717. doi: 10.1111/jfb.12801.

Clifford, S. L., McGinnity, P. and Ferguson, A. (1998) 'Genetic changes in an Atlantic salmon population resulting from escaped juvenile farm salmon', *Journal of Fish Biology*. doi: 10.1006/jfbi.1997.0566.

Cosson, M. P., Billard, R. and Letellier, L. (1989) 'Rise of internal Ca2+ accompanies the initiation of trout sperm motility', *Cell Motility and the Cytoskeleton*, 14(3), pp. 424–434. doi: 10.1002/cm.970140312.

Craik, J. C. A. and Harvey, S. M. (1984) 'Egg quality in rainbow trout: The relation between egg viability,

selected aspects of egg composition, and time of stripping', *Aquaculture*. doi: 10.1016/0044-8486(84)90350-8.

Craik, J. C. A. and Harvey, S. M. (1986) 'Egg quality in the Atlantic salmon', *Egg quality in mariculture*.

Crête-Lafrenière, A., Weir, L. K. and Bernatchez, L. (2012) 'Framing the Salmonidae Family Phylogenetic Portrait: A More Complete Picture from Increased Taxon Sampling', *PLoS ONE*, 7(10). doi: 10.1371/journal.pone.0046662.

Crisp, D. T. (1990) 'Some effects of application of mechanical shock at varying stages of development upon the survival and hatching time of British salmonid eggs', *Hydrobiologia*. doi: 10.1007/BF00012112.

Crisp, D. T. and Carling, P. A. (1989) 'Observations on siting,dimensions and structure of salmonid redds', *J.Fish.Biol.*, 34, pp. 119–134.

Crozier, W. W. (2000) 'Escaped farmed salmon , Salmo salar L., in the Glenarm River , Northern Ireland : genetic status of the wild population 7 years on', pp. 437–446.

Darwin, C. (1910) 'Charles Darwin and the origin of species', *The Eugenics Review*, 2(1), pp. 86–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22126884.

Demond, H. *et al.,*(2016) 'Preovulatory aging in vivo and in vitro affects maturation rates, abundance of selected proteins, histone methylation pattern and spindle integrity in murine Oocytes', *PLoS ONE*. doi: 10.1371/journal.pone.0162722.

Devigili, A. *et al.,*(2018) 'Possible glimpses into early speciation: the effect of ovarian fluid on sperm velocity accords with post-copulatory isolation between two guppy populations', *Journal of Evolutionary Biology*, 31(1), pp. 66–74. doi: 10.1111/jeb.13194.

Devlin, R. H. *et al.,*(2010) 'Occurrence of incomplete paternal-chromosome retention in GH-transgenic coho salmon being assessed for reproductive containment by pressure-shock-induced triploidy', *Aquaculture*, 304(1–4), pp. 66–78. doi: 10.1016/j.aquaculture.2010.03.023.

Dixon, H. J. *et al.,*(2012) 'Characterizing the trophic position and shift in Atlantic salmon (Salmo salar) from freshwater to marine life-cycle phases using stable isotopes', *ICES Journal of Marine Science*, 69(9), pp. 1646–1655. doi: 10.1093/icesjms/fss122.

Drury, D. W. *et al.,*(2013) 'Developmental trajectories and breakdown in F1 interpopulation hybrids of Tribolium castaneum', *Ecology and Evolution*, 3(7). doi: 10.1002/ece3.536.

Dumollard, R., Duchen, M. and Carroll, J. (2007) 'The Role of Mitochondrial Function in the Oocyte and Embryo', *Current Topics in Developmental Biology*. doi: 10.1016/S0070-2153(06)77002-8.

Dumorné, K. *et al.,*(2017) 'Protein phosphorylation and ions effects on salmonid sperm motility activation', *Reviews in Aquaculture*, pp. 1–11. doi: 10.1111/raq.12198.

Eberhard, W. G. (1997) 'Female Control: Sexual Selection by Cryptic Female Choice', *American Scientist*, 85(5), pp. 485–486. doi: Doi 10.1038/382772a0.

Edvardsson, M. and Arnqvist, G. (2000) 'Copulatory courtship and cryptic female choice in red flour beetles Tribolium castaneum', *Proceedings of the Royal Society B: Biological Sciences*, 267(1443), pp. 559–563. doi: 10.1098/rspb.2000.1037.

Einum, S. and Fleming, I. A. (1997) 'Genetic divergence and interactions in the wild among native, farmed and hybrid Atlantic salmon', *Journal of Fish Biology*, 50(3), pp. 634–651. doi: 10.1111/j.1095-8649.1997.tb01955.x.

Engqvist, L. and Sauer, K. P. (2001) 'Strategic male mating effort and cryptic male choice in a scorpionfly', *Proceedings of the Royal Society B: Biological Sciences*, 268(1468). doi: 10.1098/rspb.2000.1423.

Erraud, A. *et al.,*(2021) 'How age, captivity and cryopreservation affect sperm quality and reproductive efficiency in precocious Atlantic salmon (Salmo salar L. 1758)', *Aquaculture*, 544. doi: 10.1016/j.aquaculture.2021.737047.

Esteve, M. (2005) 'Observations of spawning behaviour in Salmoninae: Salmo, Oncorhynchus and Salvelinus', *Reviews in Fish Biology and Fisheries*, 15(1–2), pp. 1–21. doi: 10.1007/s11160-005-7434-7.

Evans, J. P. *et al.,*(2012) 'Assessing the potential for egg chemoattractants to mediate sexual selection in a broadcast spawning marine invertebrate.', *Proceedings. Biological sciences*, 279(1739), pp. 2855–61. doi: 10.1098/rspb.2012.0181.

FAO (2015) *Regional Review on Status and Trends in Aquaculture Development in Europe – 2015*, *Food and Agriculture Organization of the United Nations (Ed.)*. Available at: http://www.fao.org/3/a-i6865e.pdf.

Fávero, L. P. *et al.,*(2020) 'Overdisp: A stata (and mata) package for direct detection of overdispersion in poisson and negative binomial regression models', *Statistics, Optimization and Information Computing*, 8(3). doi: 10.19139/SOIC-2310-5070-557.

Fedina, T. Y. (2007) 'Cryptic female choice during spermatophore transfer in Tribolium castaneum (Coleoptera: Tenebrionidae)', *Journal of Insect Physiology*, 53(1), pp. 93–98. doi: 10.1016/j.jinsphys.2006.10.011.

Fedina, T. Y. and Lewis, S. M. (2008) 'An integrative view of sexual selection in Tribolium flour beetles', *Biological Reviews*. doi: 10.1111/j.1469-185X.2008.00037.x.

Fedina, T. Y. and Lewis, S. M. (2015) 'Evaluating cryptic female choice in highly promiscuous tribolium beetles',

in *Cryptic Female Choice in Arthropods: Patterns, Mechanisms and Prospects*. doi: 10.1007/978-3-319-17894-3_16.

Fincham, J. R. S. (2001) 'Gametes', *Encyclopedia of Genetics*, pp. 748–749. doi: 10.1006/rwgn.2001.0492.

Firman, R. C. (2011) 'Polyandrous females benefit by producing sons that achieve high reproductive success in a competitive environment', *Proceedings of the Royal Society B: Biological Sciences*, 278(1719). doi: 10.1098/rspb.2010.2791.

Firman, R. C. *et al.,*(2017) 'Postmating Female Control: 20 Years of Cryptic Female Choice', *Trends in Ecology and Evolution*, 32(5), pp. 368–382. doi: 10.1016/j.tree.2017.02.010.

Fiske, P., Lund, R. A. and Hansen, L. P. (2006) 'Relationships between the frequency of farmed Atlantic salmon, Salmo salar L., in wild salmon populations and fish farming activity in Norway, 1989-2004', *ICES Journal of Marine Science*. doi: 10.1016/j.icesjms.2006.04.006.

Fitzpatrick, J. L. *et al.,*(2009) 'Female promiscuity promotes the evolution of faster sperm in cichlid fishes', *Proceedings of the National Academy of Sciences*, 106(4), pp. 1128–1132. doi: 10.1073/pnas.0809990106.

Fitzpatrick, J. L. *et al.,*(2020) 'Chemical signals from eggs facilitate cryptic female choice in humans', *Proceedings of the Royal Society B: Biological Sciences*, 287(1928), p. 20200805. doi: 10.1098/rspb.2020.0805.

Fjeldstad, H. P. *et al.,*(2012) 'Removal of weirs and the influence on physical habitat for salmonids in a norwegian river', *River Research and Applications*. doi: 10.1002/rra.1529.

Fjelldal, P. G. *et al.,*(2016) 'Increased dietary phosphorous prevents vertebral deformities in triploid Atlantic salmon (Salmo salar L.)', *Aquaculture Nutrition*, 22(1), pp. 72–90. doi: 10.1111/anu.12238.

Flajšhans, M., Kohlmann, K. and Ráb, P. (2007) 'Autotriploid tench Tinca tinca (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing in vitro and in vivo', *Journal of Fish Biology*, 71(3), pp. 868–876. doi: 10.1111/j.1095-8649.2007.01557.x.

Flajšhans, M., Kvasnička, P. and Ráb, P. (1993) 'Genetic studies in tench (*Tinca tinca* L.): high incidence of spontaneous triploidy', *Aquaculture*, 110(3–4), pp. 243–248. doi: 10.1016/0044-8486(93)90372-6.

Fleming, I. (1997) 'Experimental tests of genetic divergence of farmed from wild Atlantic salmon due to domestication', *ICES Journal of Marine Science*, 54(6), pp. 1051–1063. doi: 10.1016/S1054-3139(97)80009-4.

Fleming, I. A. (1996) 'Reproductive strategies of Atlantic salmon: ecology and evolution', *Fisheries, Reviews in Fish Biology and Fisheries*, 6, pp. 349–416. doi: 10.1007/BF00164323.

Fleming, I. A. *et al.,*(1996) 'An Experimental Study of the Reproductive Behaviour and Success of Farmed and

Wild Atlantic Salmon (Salmo salar) Author (s): I. A. Fleming , B. Jonsson , M. R. Gross and A. Lamberg Published by : British Ecological Society Stable URL : http://ww', 33(4), pp. 893–905.

Fleming, I. A. (1998) 'Pattern and variability in the breeding system of Atlantic salmon (*Salmo salar*), with comparisons to other salmonids', *Canadian Journal of Fisheries and Aquatic Sciences*, 55(S1), pp. 59–76. doi: 10.1139/d98-009.

Fleming, I. A. *et al.,*(2000) 'Lifetime success and interactions of farm salmon invading a native population', *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2000.1173.

Fleming, I. A. and Gross, M. R. (1994) 'Breeding Competition in a Pacific Salmon (Coho: Oncorhynchus kisutch): Measures of Natural and Sexual Selection', *Evolution*, 48(3), p. 637. doi: 10.2307/2410475.

Fleming, I. A., Lamberg, A. and Jonsson, B. (1997) 'Effects of early experience on the reproductive performance of Atlantic salmon', *Behavioral Ecology [BEHAV. ECOL.]*., 8(5), pp. 470–480. doi: 10.1093/beheco/8.5.470.

Ford, J. S. and Myers, R. A. (2008) 'A global assessment of salmon aquaculture impacts on wild salmonids', *PLoS Biology*, 6(2), pp. 0411–0417. doi: 10.1371/journal.pbio.0060033.

Føre, M. *et al.,*(2017) 'Precision fish farming: A new framework to improve production in aquaculture', *Biosystems Engineering*, pp. 1–18. doi: 10.1016/j.biosystemseng.2017.10.014.

Forseth, T. *et al.,*(2017a) 'The major threats to Atlantic salmon in Norway', *ICES Journal of Marine Science*, 74(6), pp. 1496–1513. doi: 10.1093/icesjms/fsx020.

Forseth, T. *et al.,*(2017b) 'The major threats to Atlantic salmon in Norway', *ICES Journal of Marine Science*. doi: 10.1093/icesjms/fsx020.

Fraser, D. J. *et al.,*(2008) 'Mixed evidence for reduced local adaptation in wild salmon resulting from interbreeding with escaped farmed salmon: complexities in hybrid fitness', *Evolutionary Applications*, 1(3), pp. 501–512. doi: 10.1111/j.1752-4571.2008.00037.x.

Fraser, T. W. K. *et al.,*(2013) 'The effect of triploidy on the culture performance, deformity prevalence, and heart morphology in Atlantic salmon', *Aquaculture*, 416–417, pp. 255–264. doi: 10.1016/j.aquaculture.2013.09.034.

Fraser, T. W. K. *et al.,*(2015) 'The prevalence of vertebral deformities is increased with higher egg incubation temperatures and triploidy in Atlantic salmon Salmo salar L', *Journal of Fish Diseases*, 38(1), pp. 75–89. doi: 10.1111/jfd.12206.

Fricke, C. and Arnqvist, G. (2004) 'Conspecific sperm precedence in flour beetles', *Animal Behaviour*, 67(4), pp.

729–732. doi: 10.1016/j.anbehav.2003.08.014.

Fuller, R. C. (1998) 'Sperm competition affects male behaviour and sperm output in the rainbow darter', *Proceedings of the Royal Society B: Biological Sciences*, 265(1413), pp. 2365–2371. doi: 10.1098/rspb.1998.0585.

Gage, M. J. G. *et al.,*(2002) 'Relationships between sperm morphometry and sperm motility in the Atlantic salmon', *Journal of Fish Biology*, 61(6), pp. 1528–1539. doi: 10.1111/j.1095-8649.2002.tb02495.x.

Gage, M. J. G. *et al.,*(2004) 'Spermatozoal Traits and Sperm Competition in Atlantic Salmon', *Current Biology*, 14(1), pp. 44–47. doi: 10.1016/j.cub.2003.12.028.

Gage, M. J. G. and Morrow, E. H. (2003) 'Experimental evidence for the evolution of numerous, tiny sperm via sperm competition', *Current Biology*, 13(9), pp. 754–757. doi: 10.1016/S0960-9822(03)00282-3.

Gage, M. J. G., Stockley, P. and Parker, G. E. (1995) 'Effects of alternative male mating strategies on characteristics of sperm production in the Atlantic Salmon (*Salmo salar*): Theoretical and empirical investigations', 350(1334), pp. 391–399.

Le Galliard, J. F. *et al.,*(2005) 'Sex ratio bias, male aggression, and population collapse in lizards', *Proceedings of the National Academy of Sciences of the United States of America*, 102(50). doi: 10.1073/pnas.0505172102.

Garant, D., Dodson, J. J. and Bernatchez, L. (2001) 'A genetic evaluation of mating system and determinants of individual reproductive success in Atlantic salmon (Salmo salar L.).', *The Journal of Heredity*, 92(2), pp. 137–45. doi: 10.1093/jhered/92.2.137.

García-González, F. and Simmons, L. W. (2007) 'Shorter sperm confer higher competitive fertilization success', *Evolution*, 61(4), pp. 816–824. doi: 10.1111/j.1558-5646.2007.00084.x.

Garner, S. R. *et al.,*(2008) 'Juvenile growth and aggression in diploid and triploid Chinook salmon Oncorhynchus tshawytscha (Walbaum)', *Journal of Fish Biology*. doi: 10.1111/j.1095-8649.2008.01923.x.

Gasparini, C. and Evans, J. P. (2013) 'Ovarian Fluid Mediates the Temporal Decline in Sperm Viability in a Fish with Sperm Storage', *PLoS ONE*, 8(5). doi: 10.1371/journal.pone.0064431.

Gasparini, C. and Pilastro, A. (2011) 'Cryptic female preference for genetically unrelated males is mediated by ovarian fluid in the guppy', *Proceedings of the Royal Society B: Biological Sciences*, 278(1717), pp. 2495–2501. doi: 10.1098/rspb.2010.2369.

De Gaudemar, B. and Beall, E. (1999) 'Reproductive behavioural sequences of single pairs of Atlantic salmon in an experimental stream', *Animal Behaviour*. doi: 10.1006/anbe.1999.1104.

Gausen, D. (1993) 'The Norwegian gene bank program for Atlantic salmon (Salmo salar)', in *Genetic Conservation of Salmonid fishes*. Boston: Springer,Boston,MA, pp. 181–187.

Ghittino, P. (1980) 'The present and the future for artificial fertilization in fish-farming', *Bolletino di zoologia*, 47(3–4), pp. 391–396. doi: 10.1080/11250008009438695.

Gjedrem, T., Gjøen, H. M. and Gjerde, B. (1991) 'Genetic origin of Norwegian farmed Atlantic salmon', *Aquaculture*. doi: 10.1016/0044-8486(91)90369-I.

Glover, K. A. *et al.,*(2009) 'A comparison of farmed, wild and hybrid Atlantic salmon (Salmo salar L.) reared under farming conditions', *Aquaculture*, 286(3–4), pp. 203–210. doi: 10.1016/j.aquaculture.2008.09.023.

Glover, K. A. *et al.,*(2012) 'Three decades of farmed escapees in the wild: A spatio-temporal analysis of atlantic salmon population genetic structure throughout norway', *PLoS ONE*, 7(8). doi: 10.1371/journal.pone.0043129.

Glover, K. A. *et al.,*(2013) 'Atlantic salmon populations invaded by farmed escapees : quantifying genetic introgression with a Bayesian approach and SNPs'.

Glover, K. A. *et al.,*(2015) 'The frequency of spontaneous triploidy in farmed Atlantic salmon produced in Norway during the period 2007-2014.', *BMC genetics*, 16, p. 37. doi: 10.1186/s12863-015-0193-0.

Glover, K. A. *et al.,*(2017) 'Half a century of genetic interaction between farmed and wild Atlantic salmon: Status of knowledge and unanswered questions', *Fish and Fisheries*, 18(5), pp. 890–927. doi: 10.1111/faf.12214.

Glover, K. A. *et al.,*(2020) 'The future looks like the past: Introgression of domesticated Atlantic salmon escapees in a risk assessment framework', *Fish and Fisheries*. doi: 10.1111/faf.12478.

Godwin, J. L. *et al.,*(2017) 'Experimental evolution reveals that sperm competition intensity selects for longer, more costly sperm', *Evolution Letters*. doi: 10.1002/evl3.13.

Goldstein, H., Bryk, A. S. and Raudenbush, S. W. (1993) 'Hierarchical Linear Models: Applications and Data Analysis Methods.', *Journal of the American Statistical Association*, 88(421). doi: 10.2307/2290750.

Gómez Montoto, L. *et al.,*(2011) 'Sperm competition, sperm numbers and sperm quality in muroid rodents', *PLoS ONE*, 6(3). doi: 10.1371/journal.pone.0018173.

Gradil, K. J. *et al.,*(2016) 'Relationship between cardiac performance and environment across populations of Atlantic salmon (Salmo salar): a common garden experiment implicates local adaptation', *Evolutionary Ecology*, 30(5), pp. 877–886. doi: 10.1007/s10682-016-9847-2.

Grimholt, U. *et al.,*(2002) 'The major histocompatibility class I locus in Atlantic salmon (Salmo salar L.):

Polymorphism, linkage analysis and protein modelling', *Immunogenetics*, 54(8), pp. 570–581. doi: 10.1007/s00251-002-0499-8.

Gross, M. R. (1998) 'One species with two biologies: Atlantic salmon (*Salmo salar*) in the wild and in aquaculture', *Canadian Journal of Fisheries and Aquatic Sciences*, 55(S1), pp. 131–144. doi: 10.1139/d98-024.

Hansen, M. M. (2002) 'Estimating the long-term effects of stocking domesticated trout into wild brown trout (Salmo trutta) populations: An approach using microsatellite DNA analysis of historical and contemporary samples', *Molecular Ecology*, 11(6). doi: 10.1046/j.1365-294X.2002.01495.x.

Hansen, T. J. *et al.,*(2015) 'Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature', *Aquaculture*, 435, pp. 354–360. doi: 10.1016/j.aquaculture.2014.10.017.

Hard, J. J. *et al.,*(2008) 'Evolutionary consequences of fishing and their implications for salmon', *Evolutionary Applications*, 1(2). doi: 10.1111/j.1752-4571.2008.00020.x.

Harrison, X. A. (2014) 'Using observation-level randomeffects to model overdispersion in count data in ecology and evolution', *PeerJ*. doi: 10.7717/peerj.616.

Harvey, A. C. *et al.,*(2016) 'Plasticity in growth of farmed and wild Atlantic salmon: is the increased growth rate of farmed salmon caused by evolutionary adaptations to the commercial diet?', *BMC Evolutionary Biology*. doi: 10.1186/s12862-016-0841-7.

Hatef, A., Niksirat, H. and Alavi, S. M. H. (2009) 'Composition of ovarian fluid in endangered Caspian brown trout, Salmo trutta caspius, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium', *Fish Physiology and Biochemistry*. doi: 10.1007/s10695-008-9302-6.

Healey, M. C. and Prince, A. (1998) 'Alternative tactics in the breeding behaviour of male coho salmon', *Behaviour*, 135, pp. 1099–1124.

Hemmings, N., Bennison, C. and Birkhead, T. R. (2016) 'Intra-ejaculate sperm selection in female zebra finches', *Biology Letters*. doi: 10.1098/rsbl.2016.0220.

Hernández-Urcera, J. *et al.,*(2012) 'Development and validation of a molecular tool for assessing triploidy in turbot (Scophthalmus maximus)', *Aquaculture*. doi: 10.1016/j.aquaculture.2011.11.039.

Hesthagen, T., Larsen, B. M. and Fiske, P. (2011) 'Liming restores Atlantic salmon (Salmo salar) populations in acidified Norwegian rivers', *Canadian Journal of Fisheries and Aquatic Sciences*, 68(2). doi: 10.1139/F10-133.

Holt, W. V. and Lloyd, R. E. (2010) 'Sperm storage in the vertebrate female reproductive tract: How does it

work so well?', *Theriogenology*. doi: 10.1016/j.theriogenology.2009.07.002.

Houston, R. D. *et al.,*(2010) 'The susceptibility of Atlantic salmon fry to freshwater infectious pancreatic necrosis is largely explained by a major QTL', *Heredity*, 105(3), pp. 318–327. doi: 10.1038/hdy.2009.171.

Hrdy, S. B. (1977) 'Infanticide as a primate reproductive strategy', *American Scientist*, 65(1), pp. 40–49.

Hudson, E. J. and Price, T. D. (2014) 'Pervasive reinforcement and the role of sexual selection in biological speciation', in *Journal of Heredity*. doi: 10.1093/jhered/esu041.

Immler, S. *et al.,*(2014) 'Sperm variation within a single ejaculate affects offspring development in Atlantic salmon'. doi: 10.1098/rsbl.2013.1040.

Immler, S. and Taborsky, M. (2009) 'Sequential polyandry affords post-mating sexual selection in the mouths of cichlid females', *Behavioral Ecology and Sociobiology*, 63(8), pp. 1219–1230. doi: 10.1007/s00265-009-0744-3.

İnanan, B. E. and Yılmaz, F. (2018) 'Seasonal and age-related changes in semen characteristics and composition of seminal plasma in sex-reverse female rainbow trout (Oncorhynchus mykiss) in comparison with normal males', *Animal Reproduction Science*, 196. doi: 10.1016/j.anireprosci.2018.07.009.

Ivy, T. M. and Sakaluk, S. K. (2005) 'Polyandry promotes enhanced offspring survival in decorated crickets', *Evolution*, 59(1), pp. 152–159. doi: 10.1111/j.0014-3820.2005.tb00902.x.

Järvi, T. (1990) 'The Effects of Male Dominance , Secondary Sexual Characteristics and Female Mate Choice on the Mating Success of Male Atlantic Salmon Safmo safaar', 132, pp. 123–132.

Jensen *et al.,*(2010) 'Escapes of fishes from Norwegian sea-cage aquaculture: Causes, consequences and prevention', *Aquaculture Environment Interactions*, 1(1), pp. 71–83. doi: 10.3354/aei00008.

Jensen, A. J. *et al.,*(2017) 'Rapid evolution of genetic and phenotypic divergence in Atlantic salmon following the colonisation of two new branches of a watercourse', *Genetics Selection Evolution*, pp. 1–12. doi: 10.1186/s12711-017-0298-1.

Jensen, I. *et al.,*(2009) 'Susceptibility of atlantic cod gadus morhua juveniles to different routes of experimental challenge with infectious pancreatic necrosis virus (IPNV)', *Diseases of Aquatic Organisms*, 85(2). doi: 10.3354/dao02066.

Jensen, J. O. T. and Alderdice, D. F. (1989) 'Comparison of mechanical shock sensitivity of eggs of five Pacific salmon (Oncorhynchus) species and steelhead trout (Salmo gairdneri)', *Aquaculture*. doi: 10.1016/0044-8486(89)90030-6.

Jobling, M. (2002) 'Handbook of Salmon Farming', *Aquaculture*. doi: 10.1016/s0044-8486(02)00347-2.

Johansen, L. H. *et al.,*(2011) 'Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway', *Aquaculture*, 315(3–4), pp. 167–186. doi: 10.1016/j.aquaculture.2011.02.014.

Johnson, J. H. (2003) 'Egg-to-fry survival of two strains of atlantic salmon (salamo salar) in stream Incubators under laboratory conditions', *Journal of Freshwater Ecology*. doi: 10.1080/02705060.2003.9663987.

Johnson, R. M. *et al.,*(2004) 'Family, induction methodology and interaction effects on the performance of diploid and triploid chinook salmon (Oncorhynchus tshawytscha)', *Aquaculture*, 234(1–4), pp. 123–142. doi: 10.1016/j.aquaculture.2004.01.024.

Johnson, S. P., Carlson, S. M. and Quinn, T. P. (2006) 'Tooth size and skin thickness in mature sockeye salmon: Evidence for habitat constraints and variable investment between the sexes', *Ecology of Freshwater Fish*, 15(3), pp. 331–338. doi: 10.1111/j.1600-0633.2006.00175.x.

Johnstone, R. (1985) 'Induction of triploidy in Atlantic salmon by heat shock', *Aquaculture*, 49(2), pp. 133–139. doi: 10.1016/0044-8486(85)90120-6.

Joly, D. *et al.,*(1989) 'Variation of sperm length and heteromorphism in drosophilid species', *Genetics Selection Evolution*. doi: 10.1186/1297-9686-21-3-283.

Jones, S. R. M. (2009) 'Controlling salmon lice on farmed salmon and implications for wild salmon', *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 4(October 2009), pp. 1–13. doi: 10.1079/PAVSNNR20094048.

Jones, S. R. M. *et al.,*(2014) 'Disease management mitigates risk of pathogen transmission from maricultured salmonids', *Aquaculture Environment Interactions*, 6(2), pp. 119–134. doi: 10.3354/aei00121.

Jonsson, B. and Jonsson, N. (2006) 'Cultured Atlantic salmon in nature: a review of their ecology and interaction with wild fish', *ICES Journal of Marine Science*, 63(7), pp. 1162–1181. doi: 10.1016/j.icesjms.2006.03.004.

Jonsson, B. and Jonsson, N. (2009) 'A review of the likely effects of climate change on anadromous Atlantic salmon Salmo salar and brown trout Salmo trutta, with particular reference to water temperature and flow', *Journal of Fish Biology*. doi: 10.1111/j.1095-8649.2009.02380.x.

Jonsson, B., Jonsson, N. and Hansen, L. P. (2007) 'Factors affecting river entry of adult Atlantic salmon in a small river', *Journal of Fish Biology*, 71(4), pp. 943–956. doi: 10.1111/j.1095-8649.2007.01555.x.

Jonsson, B. Y. N., Hansen, L. P. and Jonsson, B. (1991) 'Variation in Age , Size and Repeat Spawning of Adult Atlantic Salmon in Relation to River Discharge Author (s): Nina Jonsson , Lars P. Hansen and Bror Jonsson Source : Journal of Animal Ecology , Vol. 60 , No. 3 (Oct., 1991), pp. 937-947 Publishe', 60(3), pp. 937–947.

Jonsson, N., Jonsson, B. and Hansen, L. P. (1990) 'Partial segregation in the timing of migration of Atlantic salmon of different ages', *Animal Behaviour*, 40(2), pp. 313–321. doi: 10.1016/S0003-3472(05)80926-1.

Jonsson, N., Jonsson, B. and Hansen, L. P. (1997) 'Changes in Proximate Composition and Estimates of Energetic Costs During Upstream Migration and Spawning in Atlantic Salmon Salmo salar', *The Journal of Animal Ecology*, 66(3), p. 425. doi: 10.2307/5987.

Jørgensen, K. M. *et al.,*(2018) 'Investigating the frequency of triploid Atlantic salmon in wild Norwegian and Russian populations', *BMC Genetics*. doi: 10.1186/s12863-018-0676-x.

José Mork (1991) 'One-generation effects of farmed fish immigration on the genetic differentiation of wild Atlantic salmon in Norway', *Aquacutlure*, 98, pp. 267–276.

Judycka, S. *et al.,*(2020) 'Effects of glucose, methanol concentration, and time of equilibration on post-thaw sperm motility of rainbow trout semen', *Aquaculture*, 520. doi: 10.1016/j.aquaculture.2020.734996.

Karlsson, S. *et al.,*(2016) 'Widespread genetic introgression of escaped farmed Atlantic salmon in wild salmon populations', *ICES Journal of Marine Science: Journal du Conseil*, 73(10), pp. 2488–2498. doi: 10.1093/icesjms/fsw121.

Kekäläinen, J. and Evans, J. P. (2017) 'Female-induced remote regulation of sperm physiology may provide opportunities for gamete-level mate choice', *Evolution*, 71(2), pp. 238–248. doi: 10.1111/evo.13141.

Kholodnyy, V. *et al.,*(2020) 'How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish', *Reviews in Aquaculture*. doi: 10.1111/raq.12378.

King, H. R. *et al.,*(2003) 'Effect of elevated summer temperatures on gonadal steroid production, vitellogenesis and egg quality in female Atlantic salmon', *Journal of Fish Biology*. doi: 10.1046/j.1095-8649.2003.00137.x.

Kinnison, M. T., Unwin, M. J. and Quinn, T. P. (2003) 'Migratory costs and contemporary evolution of reproductive allocation in male chinook salmon', *Journal of Evolutionary Biology*, 16(6), pp. 1257–1269. doi: 10.1046/j.1420-9101.2003.00631.x.

Kjøglum, S. *et al.,*(2008) 'Selective breeding can increase resistance of Atlantic salmon to furunculosis, infectious salmon anaemia and infectious pancreatic necrosis', *Aquaculture Research*, 39(5), pp. 498–505. doi:

10.1111/j.1365-2109.2008.01904.x.

Kobayashi, W. and Yamamoto, T. S. (1981) 'Fine structure of the micropylar apparatus of the chum salmon egg, with a discussion of the mechanism for blocking polyspermy', *Journal of Experimental Zoology*, 217(2), pp. 265–275. doi: 10.1002/jez.1402170213.

Kocik, J. F. and Sheehan, T. F. (2008) 'Northeast Fisheries Science Center Publications , Reports , and Abstracts for Calendar Year 2007', *Northeast Fisheries Science Center Reference Document*, (November).

Korner-Nievergelt, F. *et al.,*(2015) *Bayesian Data Analysis in Ecology Using Linear Models with R, BUGS, and Stan*, *Bayesian Data Analysis in Ecology Using Linear Models with R, BUGS, and Stan*. doi: 10.1016/C2013-0-23227-X.

Krise, W. F. (2001) 'Sensitivity of Atlantic Salmon Eggs to Mechanical Shock during the First Six Hours after Fertilization', *North American Journal of Aquaculture*, 63(1). doi: 10.1577/1548-8454(2001)063<0034:soaset>2.0.co;2.

Krkosek, M. *et al.,*(2013) 'Impact of parasites on salmon recruitment in the Northeast Atlantic Ocean.', *Proceedings of the Royal Society: Biological Sciences*, 280(1750), p. 20122359. doi: 10.1098/rspb.2012.2359.

Lahnsteiner, F. (2002) 'The influence of ovarian fluid on the gamete physiology in the salmonidae', *Fish Physiology and Biochemistry*, 27(1–2), pp. 49–59. doi: 10.1023/B:FISH.0000021792.97913.2e.

Lanza, I. R. and Nair, K. S. (2010) 'Mitochondrial metabolic function assessed in vivo and in vitro', *Current Opinion in Clinical Nutrition and Metabolic Care*. doi: 10.1097/MCO.0b013e32833cc93d.

Leaniz, C. G. De *et al.,*(2007) 'A critical review of adaptive genetic variation in Atlantic salmon : implications for conservation', 82, pp. 173–211. doi: 10.1111/j.1469-185X.2006.00004.x.

Lehnert, S. J. *et al.,*(2017) 'Effects of ovarian fluid and genetic differences on sperm performance and fertilization success of alternative reproductive tactics in Chinook salmon', *Journal of Evolutionary Biology*, 30(6), pp. 1236–1245. doi: 10.1111/jeb.13088.

Lehnert, S. J., Heath, D. D. and Pitcher, T. E. (2012) 'Sperm trait differences between wild and farmed Chinook salmon (Oncorhynchus tshawytscha)', *Aquaculture*, 344–349, pp. 242–247. doi: 10.1016/j.aquaculture.2012.03.007.

Leitritz, E. and Lewis, R. C. (1976) 'Trout and salmon culture (hatchery methods)', *Fish. Bull. State Calif. , Fish Game Comm.*

Lemaître, J. F., Ronget, V. and Gaillard, J. M. (2020) 'Female reproductive senescence across mammals: A high

diversity of patterns modulated by life history and mating traits', *Mechanisms of Ageing and Development*, 192. doi: 10.1016/j.mad.2020.111377.

Levitan, D. R. (2000) 'Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin Lytechinus variegatus', *Proceedings of the Royal Society B: Biological Sciences*, 267(1443), pp. 531–534. doi: 10.1098/rspb.2000.1032.

Lewis, R. C. *et al.,*(2020) 'Polyandry provides reproductive and genetic benefits in colonising populations', *Ecology and Evolution*, 10(19). doi: 10.1002/ece3.6742.

Lewis, S. M. *et al.,*(2005) 'Sperm stratification and paternity success in red flour beetles', *Physiological Entomology*, 30(3). doi: 10.1111/j.1365-3032.2005.00450.x.

Lewis, S. M. and Iannini, J. (1995) 'Fitness consequences of differences in male mating behaviour in relation to female reproductive status in flour beetles', *Animal Behaviour*, 50(5). doi: 10.1016/0003-3472(95)80031-X.

Lewis, S. M. and Jutkiewicz, E. (1998) *Sperm precedence and sperm storage in multiply mated red ¯our beetles*.

Liao, W. B. *et al.,*(2018) 'Ejaculate evolution in external fertilizers: Influenced by sperm competition or sperm limitation?', *Evolution*, 72(1), pp. 4–17. doi: 10.1111/evo.13372.

Liebert, A. E. *et al.,*(2004) 'Triploid females and diploid males: Underreported phenomena in Polistes wasps?', *Insectes Sociaux*. doi: 10.1007/s00040-004-0754-0.

Lijalad, M. and Powell, M. D. (2009) 'Effects of lower jaw deformity on swimming performance and recovery from exhaustive exercise in triploid and diploid Atlantic salmon Salmo salar L.', *Aquaculture*, 290(1–2), pp. 145–154. doi: 10.1016/j.aquaculture.2009.01.039.

Limburg, K. E. and Waldman, J. R. (2009) 'Dramatic Declines in North Atlantic Diadromous Fishes', *BioScience*, 59(11), pp. 955–965. doi: 10.1525/bio.2009.59.11.7.

Litvak, M. K. and Trippel, E. A. (1998) 'Sperm motility patterns of Atlantic cod (Gadus morhua) in relation to salinity: Effects of ovarian fluid and egg presence', *Canadian Journal of Fisheries and Aquatic Sciences*, 55(8), pp. 187–1877. doi: 10.1139/f98-093.

Loo, J. *et al.,*(2018) 'High levels of polyandry, but limited evidence for multiple paternity, in wild populations of the western rock lobster (*Panulirus cygnus*)', *Ecology and Evolution*, (January), pp. 4525–4533. doi: 10.1002/ece3.3985.

Louhi, P., Mäki-Petäys, A. and Erkinaro, J. (2008) 'Spawning habitat of Atlantic salmon and brown trout: General criteria and intragravel factors', *River Research and Applications*, 24(3), pp. 330–339. doi:

10.1002/rra.1072.

Lovlie, H. *et al.,*(2013) 'Cryptic female choice favours sperm from major histocompatibility complex-dissimilar males', *Proceedings of the Royal Society B: Biological Sciences*, 280(1769), pp. 20131296–20131296. doi: 10.1098/rspb.2013.1296.

Lumley, A. J. *et al.,*(2015) 'Sexual selection protects against extinction', *Nature*, 522(7557), pp. 470–473. doi: 10.1038/nature14419.

Lumley, A. J. *et al.,*(2016) 'Post-copulatory opportunities for sperm competition and cryptic female choice provide no offspring fitness benefits in externally fertilizing salmon', *Royal Society Open Science*, 3(3), p. 150709. doi: 10.1098/rsos.150709.

Lundqvist, H. *et al.,*(2008) 'Upstream passage problems for wild Atlantic salmon (Salmo salar L.) in a regulated river and its effect on the population', *Hydrobiologia*. doi: 10.1007/s10750-008-9282-7.

Lüpold, S. *et al.,*(2009) 'Sperm morphology and sperm velocity in passerine birds', *Proceedings of the Royal Society B: Biological Sciences*, 276(1659). doi: 10.1098/rspb.2008.1645.

Magee, J. A. *et al.,*(2003) 'Effects of episodic acidification on Atlantic salmon (Salmo salar) smolts', *Canadian Journal of Fisheries and Aquatic Sciences*. doi: 10.1139/f03-015.

Maklakov, A. A. *et al.,*(2013) 'The effect of sexual harassment on lethal mutation rate in female Drosophila melanogaster', *Proceedings of the Royal Society B: Biological Sciences*, 280(1750). doi: 10.1098/rspb.2012.1874.

Marconato, A., And, V. T. and Marin, G. (1995) 'The mating system of Xyrichthys novacula: sperm economy and fertilization success', *Journal of Fish Biology*, 47(2), pp. 292–301. doi: 10.1111/j.1095-8649.1995.tb01896.x.

Marconato, A. and Shapiro, D. Y. (1996) 'Sperm allocation, sperm production and fertilization rates in the bucktooth parrotfish', *Animal Behaviour*, 52(5), pp. 971–980. doi: 10.1006/anbe.1996.0245.

Maynard Smith, J. (1977) 'The Evolution of Sex', (July).

McConnell, S. *et al.,*(1995) 'Isolation of salmonid microsatellite loci and their application to the population genetics of Canadian east coast stocks of Atlantic salmon', *Aquaculture*, 137(1–4), pp. 19–30. doi: 10.1016/0044-8486(95)01111-0.

McCormick, S. D. *et al.,*(1998) 'Movement, migration, and smolting of Atlantic salmon (*Salmo salar*)', *Canadian Journal of Fisheries and Aquatic Sciences*, 55(S1), pp. 77–92. doi: 10.1139/d98-011.

Mcgeachy, S. A. *et al.,*(1997) 'Comparisons of cultured triploid and diploid Atlantic salmonn', *Journal of Marine Science*.

McGinnity, P. *et al.,*(2003) 'Fitness reduction and potential extinction of wild populations of Atlantic salmon, Salmo salar, as a result of interactions with escaped farm salmon', *Proceedings of the Royal Society B: Biological Sciences*, 270(1532), pp. 2443–2450. doi: 10.1098/rspb.2003.2520.

McLain, D. K. and Pratt, A. E. (1999) 'The cost of sexual coercion and heterospecific sexual harassment on the fecundity of a host-specific, seed-eating insect (Neacoryphus bicrucis)', *Behavioral Ecology and Sociobiology*, 46(3). doi: 10.1007/s002650050606.

Medina, M. F. *et al.,*(2012) 'Effect of oviductal secretion components on the fertilizing capacity of amphibian sperm: Biological and ultrastructural studies', *Micron*. doi: 10.1016/j.micron.2011.08.001.

Mendonca, T., Cadby, A. J. and Hemmings, N. (2019) 'Sperm Gatekeeping: 3D Imaging Reveals a Constricted Entrance to Zebra Finch Sperm Storage Tubules', *Biophysical Journal*, 117(11). doi: 10.1016/j.bpj.2019.10.038.

Miao, Y. L. *et al.,*(2009) 'Oocyte aging: Cellular and molecular changes, developmental potential and reversal possibility', *Human Reproduction Update*. doi: 10.1093/humupd/dmp014.

Michalczyk, L. *et al.,*(2011) 'Experimental evolution exposes female and male responses to sexual selection and conflict in tribolium castaneum', *Evolution*, 65(3). doi: 10.1111/j.1558-5646.2010.01174.x.

Millery, P. B. *et al.,*(2014) 'The song of the old mother: Reproductive senescence in female drosophila', *Fly*. doi: 10.4161/19336934.2014.969144.

Mjølnerød, I. B. *et al.,*(1998) 'Mate and sperm competition during multiple-male spawnings of Atlantic salmon', *Canadian Journal of Zoology*, 76(1), pp. 70–75. doi: 10.1139/cjz-76-1-70.

Moffett, I. J. J. and Crozier, W. W. (1996) 'A study of temporal genetic variation in a natural population of Atlantic salmon in the river Bush, Northern Ireland', *Journal of Fish Biology*, 48(2), pp. 302–306. doi: 10.1111/j.1095-8649.1996.tb01123.x.

Morisawa, M. *et al.,*(1983) 'Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes.', *The Journal of experimental biology*, 107(1), pp. 95–103. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6421979.

Mylonas, C. C., Fostier, A. and Zanuy, S. (2010) 'Broodstock management and hormonal manipulations of fish reproduction', *General and Comparative Endocrinology*, 165(3), pp. 516–534. doi: 10.1016/j.ygcen.2009.03.007.

Nakakita, H. (1983) *Rediscovery of Triboliumfreemani Hinton: a Stored Product Insect Unexposed to Entomologists for the Past 100 Years*.

NASCO (2019) 'State of North Atlantic Salmon', *North Atlantic Salmon Conservation Organisation Report*, pp. 1–30. Available at: http://www.nasco.int/pdf/iys/SoS-final-online.pdf.

Naylor, R. *et al.,*(2005) 'Fugitive Salmon: Assessing the Risks of Escaped Fish from Net-Pen Aquaculture', *BioScience*, 55(5), pp. 427–437. doi: 10.1641/0006-3568(2005)055[0427:fsatro]2.0.co;2.

Nazmara, Z., Salehnia, M. and HosseinKhani, S. (2014) 'Mitochondrial distribution and ATP content of vitrified, In vitro matured mouse oocytes', *Avicenna Journal of Medical Biotechnology*.

Nilsson, T., Fricke, C. and Arnqvist, G. (2003) 'The effects of male and female genotype on variance in male fertilization success in the red flour beetle (Tribolium castaneum)', *Behavioral Ecology and Sociobiology*, 53(4). doi: 10.1007/s00265-002-0565-0.

Nomura, K. *et al.,*(2013a) 'Post-ovulatory oocyte aging induces spontaneous occurrence of polyploids and mosaics in artificial fertilization of Japanese eel, Anguilla japonica', *Aquaculture*, 404–405, pp. 15–21. doi: 10.1016/j.aquaculture.2013.04.016.

Nomura, K. *et al.,*(2013b) 'Post-ovulatory oocyte aging induces spontaneous occurrence of polyploids and mosaics in artificial fertilization of Japanese eel, Anguilla japonica', *Aquaculture*, 404–405, pp. 15–21. doi: 10.1016/j.aquaculture.2013.04.016.

Norris, A. T., Bradley, D. G. and Cunningham, E. P. (1999) 'Microsatellite genetic variation between and within farmed and wild Atlantic salmon (Salmo salar) populations', *Aquaculture 180: 247-264*, 180(3–4), pp. 247–264. Available at: http://www.sciencedirect.com/science/article/B6T4D-3XMGSTW-5/2/4f0c53c3cb4419c8e7d83a7e57721985.

Nyqvist, D. *et al.,*(2017) 'Migratory delay leads to reduced passage success of Atlantic salmon smolts at a hydroelectric dam', *Ecology of Freshwater Fish*, 26(4), pp. 707–718. doi: 10.1111/eff.12318.

O'Keefe, R. A. and Benfey, T. J. (1997) 'The feeding response of diploid and triploid Atlantic salmon and brook trout', *Journal of Fish Biology*, 51, pp. 989–997. doi: 10.1006/jfbi.1997.0505.

Olafsen, T. *et al.,*(2012) 'Value created from productive oceans in 2050. A report prepared by a working group appointed by the Royal Norwegian Society of Sciences and Letters (DKNVS) and the Norwegian Academy of Technological Sciences (NTVA).', pp. 1–83.

Olsén, K. H. *et al.,*(2001) 'Both ovarian fluid and female urine increase sex steroid hormone levels in mature

atlantic salmon (Salmo salar) male parr', *Journal of Chemical Ecology*, 27(11), pp. 2337–2349. doi: 10.1023/A:1012243323670.

Oppliger, A. *et al.,*(2003) 'Sperm length influences fertilization success during sperm competition in the snail Viviparus ater', *Molecular Ecology*, 12(2), pp. 485–492. doi: 10.1046/j.1365-294X.2003.01748.x.

Ozerov, M. Y. *et al.,*(2010) 'High Gyrodactylus salaris infection rate in triploid Atlantic salmon Salmo salar', *Diseases of Aquatic Organisms*, 91(2), pp. 129–136. doi: 10.3354/dao02242.

Pakkasmaa, S. and Piironen, J. (2001) 'Water velocity shapes juvenile salmonids', pp. 721–730.

Palumbi, S. R. (2009) 'Speciation and the evolution of gamete recognition genes: Pattern and process', *Heredity*. doi: 10.1038/hdy.2008.104.

Pankhurst, N. W. *et al.,*(1996) 'Effect of holding temperature on ovulation, egg fertility, plasma levels of reproductive hormones and in vitro ovarian steroidogenesis in the rainbow trout Oncorhynchus mykiss', *Aquaculture*. doi: 10.1016/S0044-8486(96)01374-9.

Parker, G. A. (1982) 'Why are there so many tiny sperm? sperm competition and the maintenance of two sexes', in *Sperm Competition in Humans: Classic and Contemporary Readings*, pp. 33–46. doi: 10.1007/978-0-387-28039-4_2.

Parker, G. A. (1990) 'Sperm Competition Games: Sneaks and Extra-Pair Copulations', *Proceedings of the Royal Society B: Biological Sciences*, 242(1304), pp. 127–133. doi: 10.1098/rspb.1990.0115.

Parker, G. A. (1970) 'SPERM COMPETITION AND ITS EVOLUTIONARY CONSEQUENCES IN THE INSECTS', *Biological Reviews*, 45(4), pp. 525–567. doi: 10.1111/j.1469-185X.1970.tb01176.x.

Pascini, T. V. and Martins, G. F. (2017) 'The insect spermatheca: an overview', *Zoology*. doi: 10.1016/j.zool.2016.12.001.

Pennington, J. T. (1985) 'The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adult aggregation, and synchronous spawning.', *Biological Bulletin*, 169(2), pp. 417–430. doi: 10.2307/1541492.

Pepper, V. A., Oliver, N. P. and Blundon, R. (1985) 'Juvenile Anadromous Atlantic Salmon of Three Lakes of Newfoundland', *Internationale Revue der gesamten Hydrobiologie und Hydrographie*, 70(5), pp. 733–753. doi: 10.1002/iroh.19850700512.

Petersson, E, J. T. (1997) 'Reproductive Behaviour of Sea Trout (Salmo trutta): The Consequences of Sea-Ranching Authors (s): Erik Petersson and Torbjörn Järvi Published by : Brill Stable URL :

http://www.jstor.org/stable/4535426 REFERENCES Linked references are available on JST', 134(1), pp. 1–22.

Pincheira-Donoso, D. and Hunt, J. (2017) 'Fecundity selection theory: concepts and evidence', *Biological Reviews*, 92(1). doi: 10.1111/brv.12232.

Piper et al.,(1986) 'Fish Hatchery Management', *Management*. doi: 10.1007/s13398-014-0173-7.2.

Pointer, M. D., Gage, M. J. G. and Spurgin, L. G. (2021) 'Tribolium beetles as a model system in evolution and ecology', *Heredity*. doi: 10.1038/s41437-021-00420-1.

Poirier, J. *et al.,*(2017) 'U. S. Fish and Wildlife Service Columbia River Fish and Wildlife Conservation Office Literature Review of Survival , Age at Return , Straying , Life History Diversity and Ecological Effects of Subyearling and Yearling Hatchery Release Strategies for Fal'.

Poon, D. C. and Johnson, A. K. (1970) 'The effect of delayed fertilization on transported salmon eggs', *Progressive Fish-Culturist*. doi: 10.1577/1548-8640(1970)32[81:TEODFO]2.0.CO;2.

Promerová, M. *et al.,*(2017) 'No evidence for MHC class II-based non-random mating at the gametic haplotype in Atlantic salmon', *Heredity*, 118(6), pp. 563–567. doi: 10.1038/hdy.2016.129.

Quinn, T. P. and Foote, C. J. (1994) 'The effects of body size and sexual dimorphism on the reproductive behaviour of sockeye salmon, Oncorhynchus nerka', *Animal Behaviour*, pp. 751–761. doi: 10.1006/anbe.1994.1300.

Quinton, C. D., McMillan, I. and Glebe, B. D. (2005) 'Development of an Atlantic salmon (Salmo salar) genetic improvement program: Genetic parameters of harvest body weight and carcass quality traits estimated with animal models', *Aquaculture*, 247(1–4), pp. 211–217. doi: 10.1016/j.aquaculture.2005.02.030.

Rakitin, A., Ferguson, M. M. and Trippel, E. a (1999) 'Sperm competition and fertilization success in Atlantic cod (Gadus morhua): effect of sire size and condition factor on gamete quality', *Canadian Journal of Fisheries and Aquatic Sciences*, 56(12), pp. 2315–2323. doi: 10.1139/f99-164.

Rankin, D. J., Dieckmann, U. and Kokko, H. (2011) 'Sexual conflict and the tragedy of the commons', *American Naturalist*, 177(6). doi: 10.1086/659947.

Reid, J. and Chaput, G. (2012) 'Spawning history influence on fecundity, egg size, and egg survival of Atlantic salmon (Salmo salar) from the Miramichi River, New Brunswick, Canada', *ICES Journal of Marine Science*, 71(May), pp. 1678–1685. doi: 10.1093/icesjms/fss153.

Reinhold, K., Kurtz, J. and Engqvist, L. (2002) 'Cryptic male choice: Sperm allocation strategies when female quality varies', *Journal of Evolutionary Biology*, 15(2). doi: 10.1046/j.1420-9101.2002.00390.x.

Reisenbichler, R. R. and McIntyre, J. D. (1977) ' Genetic Differences in Growth and Survival of Juvenile Hatchery and Wild Steelhead Trout, Salmo gairdneri ', *Journal of the Fisheries Research Board of Canada*, 34(1). doi: 10.1139/f77-015.

Risopatrón, J. *et al.,*(2018) 'Effect of the age of broodstock males on sperm function during cold storage in the trout (Oncorhynchus mykiss)', *Andrologia*, 50(2). doi: 10.1111/and.12857.

Rizos, D. *et al.,*(2002) 'Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality', *Molecular Reproduction and Development*. doi: 10.1002/mrd.1153.

Rizzo, E., Godinho, H. P. and Sato, Y. (2003) 'Short-term storage of oocytes from the neotropical teleost fish Prochilodus marggravii', *Theriogenology*. doi: 10.1016/S0093-691X(03)00108-0.

Roberge, C. *et al.,*(2008) 'Genetic consequences of interbreeding between farmed and wild Atlantic salmon: Insights from the transcriptome', *Molecular Ecology*, 17(1), pp. 314–324. doi: 10.1111/j.1365-294X.2007.03438.x.

Robinson, T., Johnson, N. A. and Wade, M. J. (1994) 'Postcopulatory, prezygotic isolation: Intraspecific and interspecific sperm precedence in Tribolium spp., flour beetles', *Heredity*, 73(2), pp. 155–159. doi: 10.1038/hdy.1994.114.

Rosengrave, P. *et al.,*(2008) 'A mechanism for cryptic female choice in chinook salmon', *Behavioral Ecology*, 19(6), pp. 1179–1185. doi: 10.1093/beheco/arn089.

Rosengrave, P. *et al.,*(2009) 'Chemical composition of seminal and ovarian fluids of chinook salmon (Oncorhynchus tshawytscha) and their effects on sperm motility traits', *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*. doi: 10.1016/j.cbpa.2008.09.009.

Rosengrave, P., Montgomerie, R. and Gemmell, N. (2016) 'Cryptic female choice enhances fertilization success and embryo survival in chinook salmon', *Proceedings of the Royal Society B: Biological Sciences*, 283(1827), p. 20160001. doi: 10.1098/rspb.2016.0001.

Rossi, B. H., Nonacs, P. and Pitts-Singer, T. L. (2010) 'Sexual harassment by males reduces female fecundity in the alfalfa leafcutting bee, Megachile rotundata', *Animal Behaviour*, 79(1). doi: 10.1016/j.anbehav.2009.10.023.

Rowe, D. K. and Thorpe, J. E. (1990) 'Suppression of maturation in male Atlantic salmon (Salmo salar L.) parr by reduction in feeding and growth during spring months', *Aquaculture*, 86(2–3), pp. 291–313. doi: 10.1016/0044-

8486(90)90121-3.

Rye, M., Lillevik, K. M. and Gjerde, B. (1990) 'Survival in early life of Atlantic salmon and rainbow trout: estimates of heritabilities and genetic correlations', *Aquaculture*. doi: 10.1016/0044-8486(90)90126-8.

Sadler, J., Pankhurst, P. M. and King, H. R. (2001) 'High prevalence of skeletal deformity and reduced gill surface area in triploid Atlantic salmon (Salmo salar L.)', *Aquaculture*, 198(3–4), pp. 369–386. doi: 10.1016/S0044-8486(01)00508-7.

Sales, K. *et al.,*(2018) 'Experimental evolution with an insect model reveals that male homosexual behaviour occurs due to inaccurate mate choice', *Animal Behaviour*, 139. doi: 10.1016/j.anbehav.2018.03.004.

Samarin, A. M. *et al.,*(2011) 'Effects of post-ovulatory oocyte ageing and temperature on egg quality in kutum rutilus frisii kutum', *World Applied Sciences Journal*, 15(1), pp. 14–18.

Samarin, A. M. *et al.,*(2015) 'Determination of the best post-ovulatory stripping time for the common carp (Cyprinus carpio Linnaeus, 1758)', *Journal of Applied Ichthyology*. doi: 10.1111/jai.12855.

Samarin, A. M. *et al.,*(2016) 'Post-ovulatory and post-stripping oocyte ageing in northern pike, Esox lucius (Linnaeus, 1758), and its effect on egg viability rates and the occurrence of larval malformations and ploidy anomalies', *Aquaculture*, 450, pp. 431–438. doi: 10.1016/j.aquaculture.2015.08.017.

Samarin, A. M. and Miroslav Blecha, Dmytro Bytyutskyy, T. P. (2015) 'Post-Ovulatory Oocyte Ageing in Pikeperch (Sander lucioperca L.) and its Effect on Egg Viability Rates and the Occurrence of Larval Malformations and Ploidy Anomalies', *Turkish Journal of Fisheries and Aquatic Sciences*, 15, pp. 711–722. doi: 10.4194/1303-2712-v15.

Samarin, A. M., Policar, T. and Lahnsteiner, F. (2015) 'Fish Oocyte Ageing and its Effect on Egg Quality', *Reviews in Fisheries Science and Aquaculture*, 23(3), pp. 302–314. doi: 10.1080/23308249.2015.1053560.

Samarin, Azin Mohagheghi, Samarin, Azadeh Mohagheghi and Policar, T. (2018) 'Cellular and molecular changes associated with fish oocyte ageing', *Reviews in Aquaculture*.

Sambraus, F. *et al.,*(2017) 'Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (Salmo salar L.) post-smolts', *Aquaculture*, 473, pp. 1–12. doi: 10.1016/j.aquaculture.2017.01.024.

Sánchez, J. A. *et al.,*(1996) 'Protein and microsatellite single locus variability in Salmo salar L. (Atlantic salmon)', *Heredity*, 77(4), pp. 423–432. doi: 10.1038/hdy.1996.162.

Sandlund, O. T. *et al.,*(2014) 'Spatial and temporal genetic structure of a river-resident Atlantic salmon (Salmo

salar) after millennia of isolation.', *Ecology and evolution*, 4(9), pp. 1538–54. doi: 10.1002/ece3.1040.

Sargent, R. C., Gross, M. R. and Van Den Berghe, E. P. (1986) 'Male mate choice in fishes', *Animal Behaviour*, 34(2), pp. 545–550. doi: 10.1016/S0003-3472(86)80123-3.

Sasanami, T. *et al.,*(2013) *Sperm Storage in the Female Reproductive Tract in Birds*.

Satake, N. *et al.,*(2006) 'Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct', *Journal of Experimental Biology*. doi: 10.1242/jeb.02136.

Schindler, D. E. *et al.,*(2010) 'Population diversity and the portfolio effect in an exploited species', *Nature*, 465(7298), pp. 609–612. doi: 10.1038/nature09060.

Servedio, M. R. (2007) 'Male versus female mate choice: Sexual selection and the evolution of species recognition via reinforcement', *Evolution*, 61(12). doi: 10.1111/j.1558-5646.2007.00247.x.

Sever, D. M. and Hamlett, W. C. (2002) 'Female sperm storage in reptiles', *Journal of Experimental Zoology*, 292(2). doi: 10.1002/jez.1154.

Shapiro, D. Y., Marconato, A. and Yoshikawa, T. (1994) 'Sperm economy in a coral reef fish, Thalassoma bifasciatum', *Ecology*, 75(5), pp. 1334–1344. doi: 10.2307/1937458.

Sheldon, B. C. and Burke, T. (1994) 'Copulation behavior and paternity in the chaffinch', *Behavioral Ecology and Sociobiology*, 34(2), pp. 149–156. doi: 10.1007/BF00164185.

Simmons, L. W. (2005) 'The Evolution of Polyandry: Sperm Competition, Sperm Selection, and Offspring Viability', *Annual Review of Ecology, Evolution, and Systematics*, 36(1), pp. 125–146. doi: 10.1146/annurev.ecolsys.36.102403.112501.

Simmons, L. W. and Garcia-Gonzalez, F. (2021) 'Can Sexual Selection Drive the Evolution of Sperm Cell Structure?', *Cells*, 10(5). doi: 10.3390/cells10051227.

Simmons, L. W., Parker, G. A. and Stockley, P. (1999) 'Sperm displacement in the yellow dung fly, Scatophaga stercoraria: An investigation of male and female processes', *American Naturalist*, 153(3). doi: 10.1086/303171.

Simmons L.W. (2001) *Sperm competition and its evolutionary consequences in the insects.* Princeton (NJ): Princeton University Press.

Sinha, R. N. (1953) 'The Spermatheca in the Flour Beetle (Tribolium castaneum Herbst)', *Journal of the New York Entomological Society*, 61, pp. 131–134.

Skaala, Ø. *et al.,*(2014) 'A conservation plan for Atlantic salmon (Salmo salar) and anadromous brown trout

(Salmo trutta) in a region with intensive industrial use of aquatic habitats, the Hardangerfjord, western Norway', *Marine Biology Research*, 10(3), pp. 308–322. doi: 10.1080/17451000.2013.810758.

Skilbrei, O. T. (1988) 'Growth pattern of pre-smolt Atlantic salmon (Salmo salar L.): The percentile increment method (PIM) as a new method to estimate length-dependent growth', *Aquaculture*, 69(1–2), pp. 129–143. doi: 10.1016/0044-8486(88)90192-5.

Skillbrei O, Mikkio Heino, T. S. (2017) 'Using simulated escape events to assess the annual numbers and destinies of escaped farmed Atlantic salmon of differnt life stages from farm sties in Norway', *ICES Journal of Marine Science*, 72(October), pp. 670–685.

Skjaeraasen, J. E. *et al.,*(2009) 'Sperm characteristics and competitive ability in farmed and wild cod', *Marine Ecology Progress Series*, 375, pp. 219–228. doi: 10.3354/meps07774.

Slettan, A., Olsaker, I. and Lie (1995) 'Atlantic salmon, Salmo salar, microsatellites at the SSOSL25, SSOSL85, SSOSL311, SSOSL417 loci', *Animal Genetics*, 26(4), pp. 281–282. doi: 10.1111/j.1365-2052.1995.tb03262.x.

Smedley, M. A. *et al.,*(2016) 'Dietary phosphorous and protein supplementation enhances seawater growth and reduces severity of vertebral malformation in triploid Atlantic salmon (Salmo salar L.)', *Aquaculture*, 451, pp. 357–368. doi: 10.1016/j.aquaculture.2015.10.001.

Smith, R. L. (1984) *Sperm Competition and the Evolution of Animal Mating systems*, *Academic Press, Inc*. doi: 10.1016/B978-0-12-100543-6.50023-4.

Solberg, M. F., Skaala, Ø., *et al.,*(2013) 'Does Domestication Cause Changes in Growth Reaction Norms? A Study of Farmed, Wild and Hybrid Atlantic Salmon Families Exposed to Environmental Stress', *PLoS ONE*, 8(1). doi: 10.1371/journal.pone.0054469.

Solberg, M. F., Zhang, Z., *et al.,*(2013) 'Growth reaction norms of domesticated, wild and hybrid Atlantic salmon families in response to differing social and physical environments', *BMC Evolutionary Biology*, 13(1). doi: 10.1186/1471-2148-13-234.

Sønvisen, S. A. (2003) 'Integrated Coastal Zone Management (ICZM). The allocation of space in Norwegian aquaculture - from local lottery to central planning?', (May). Available at: http://www.ub.uit.no/munin/handle/10037/324.

Sprague, M., Dick, J.R. and Tocher, D. R. (2016) 'Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015', *Scientific Reports*, 6(1), p. 21892. doi: 10.1038/srep21892.

Srivastava, R. K. and Brown, J. A. (1991) ' The biochemical characteristics and hatching performance of

cultured and wild Atlantic salmon (Salmo salar) eggs ', *Canadian Journal of Zoology*, 69(9). doi: 10.1139/z91-342.

Stabell, O. B. (1984) 'Homing and Olfaction in Salmonids: a Critical Review With Special Reference To the Atlantic Salmon', *Biological Reviews*, 59(3), pp. 333–388. doi: 10.1111/j.1469-185X.1984.tb00709.x.

Stapper, A. P., Beerli, P. and Levitan, D. R. (2015) 'Assortative mating drives linkage disequilibrium between sperm and egg recognition protein loci in the sea urchin strongylocentrotus purpuratus', *Molecular Biology and Evolution*, 32(4), pp. 859–870. doi: 10.1093/molbev/msv010.

Stet, R. J. *et al.,*(2002) 'Unique haplotypes of co-segregating major histocompatibility class II A and class II B alleles in Atlantic salmon (Salmo salar) give rise to diverse class II genotypes', *Immunogenetics*, 54(5), pp. 320–331. doi: 10.1007/s00251-002-0477-1.

Stockley, P. *et al.,*(1997) 'Sperm Competition in Fishes: The Evolution of Testis Size and Ejaculate Characteristics', *The American Naturalist*, 149(5), pp. 933–954. doi: 10.1086/286031.

Stoltz, J. A. and Neff, B. D. (2006) 'Sperm competition in a fish with external fertilization: The contribution of sperm number, speed and length', *Journal of Evolutionary Biology*, 19(6), pp. 1873–1881. doi: 10.1111/j.1420-9101.2006.01165.x.

Sundt-Hansen, L. E. *et al.,*(2018) 'Modelling climate change effects on Atlantic salmon: Implications for mitigation in regulated rivers', *Science of the Total Environment*, 631–632. doi: 10.1016/j.scitotenv.2018.03.058.

Sutela, T. *et al.,*(2007) 'Impacts of Water Quality and Hand-Picking of Dead Eggs on the Survival of Brown Trout and Atlantic Salmon Eggs', *North American Journal of Aquaculture*. doi: 10.1577/a06-028.1.

Taggart, J. B. *et al.,*(2001) 'Spawning success in Atlantic salmon (Salmo salar L.): A long-term DNA profiling-based study conducted in a natural stream', *Molecular Ecology*, 10(4), pp. 1047–1060. doi: 10.1046/j.1365-294X.2001.01254.x.

Taggart, J. B. (2007) 'FAP: An exclusion-based parental assignment program with enhanced predictive functions: Program note', *Molecular Ecology Notes*. doi: 10.1111/j.1471-8286.2006.01616.x.

Takahashi, T. *et al.,*(2003) 'Impact of oxidative stress on calcium oscillations at fertilization in aged mouse oocytes', *Fertility and Sterility*. doi: 10.1016/s0015-0282(03)01658-3.

Tarín., A. (2000) 'Consequences on offspring of abnormal function in ageing gametes', *Human Reproduction Update*.

Taylor, E. (1991) 'A review of local adaptation in Saimon. idx. with particular reference to Pacific and Atlahc'.

Taylor, J. F. *et al.,*(2011) 'Ploidy effects on hatchery survival, deformities, and performance in Atlantic salmon (Salmo salar)', *Aquaculture*. doi: 10.1016/j.aquaculture.2010.11.029.

Thayer, L. R. and Hamlin, H. J. (2016) 'Investigating factors contributing to reduced embryo survival in farm-raised Atlantic salmon, *Salmo salar* L.', *Aquaculture Research*, 47(12), pp. 3980–3989. doi: 10.1111/are.12848.

Thornhill, R. (1983) 'Cryptic Female Choice and Its Implications in the Scorpionfly Harpobittacus nigriceps', *The American Naturalist*, 122(6), pp. 765–788. doi: 10.1086/284170.

Thorstad, E. B. *et al.,*(2008) 'Factors affecting the within-river spawning migration of Atlantic salmon, with emphasis on human impacts', *Reviews in Fish Biology and Fisheries*. doi: 10.1007/s11160-007-9076-4.

Thorstad, E. B. *et al.,*(2017) 'Survival and behaviour of Atlantic salmon smolts passing a run-of-river hydropower facility with a movable bulb turbine', *Fisheries Management and Ecology*, 24(3), pp. 199–207. doi: 10.1111/fme.12216.

TR Birkhead, DJ Hosken, S. P. (2008) 'Sperm biology: an evolutionary perspective'.

Truett, G. E. *et al.,*(2000) 'Preparation of PCR-quality mouse genomic dna with hot sodium hydroxide and tris (HotSHOT)', *BioTechniques*, 29(1), pp. 52–54. doi: 10.1016/j.jacc.2014.05.073.

Turner, E. and Montgomerie, R. (2002) 'Ovarian fluid enhances sperm movement in Arctic charr', *Journal of Fish Biology*. doi: 10.1006/jfbi.2002.2018.

Tveteras, R., Nystoyl, R. and Jory, D. E. (2019) 'GOAL 2019: Global finfish production review and forecast', (December). Available at: https://www.aquaculturealliance.org.

Tymchuk, W., Sakhrani, D. and Devlin, R. (2009) 'Domestication causes large-scale effects on gene expression in rainbow trout: Analysis of muscle, liver and brain transcriptomes', *General and Comparative Endocrinology*. doi: 10.1016/j.ygcen.2009.05.015.

Veen, T. *et al.,*(2011) 'Premating reproductive barriers between hybridising cricket species differing in their degree of polyandry', *PLoS ONE*, 6(5). doi: 10.1371/journal.pone.0019531.

Vincent, B., Kent, M. P. and Bernatchez, L. (2013) 'environment interactions driving local adaptation', pp. 1–49. doi: 10.1111/evo.12139.This.

Vladić, T. V., Afzelius, B. A. and Bronnikov, G. E. (2002) 'Sperm Quality as Reflected Through Morphology in Salmon Alternative Life Histories1', *Biology of Reproduction*, 66(1), pp. 98–105. doi: 10.1095/biolreprod66.1.98.

Vladic, T. V. and Jarvi, T. (2001) 'Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism', *Proceedings of the Royal Society B: Biological Sciences*, 268(1483), pp. 2375–2381. doi: 10.1098/rspb.2001.1768.

Vøllestad, L. A., Skurdal, J. and L'Abée-Lund, J. H. (2014) 'Evaluation of a new management scheme for Norwegian Atlantic salmon Salmo salar', *Fisheries Management and Ecology*, 21(2), pp. 133–139. doi: 10.1111/fme.12057.

Wade, M. J. and Johnson, N. A. (1994) *Reproductive isolation between two species of flour beetles, Tribolium castaneum and T. freemani: variation within and among geographical populations of T castaneum MICHAEL*.

Wagner, E. J., Arndt, R. E. and Roubidoux, R. (2006) 'The Effect of Temperature Changes and Transport on Cutthroat Trout Eggs Soon after Fertilization', *North American Journal of Aquaculture*. doi: 10.1577/a05-065.1.

Walsh, C. L. and Kilsby, C. G. (2007) 'Implications of climate change on flow regime affecting Atlantic salmon', *Hydrology and Earth System Sciences*, 11(3). doi: 10.5194/hess-11-1127-2007.

Ward, P. I. (2000) 'Cryptic female choice in the yellow dung fly Scathophaga stercoraria (L.)', *Evolution*, 54(5). doi: 10.1111/j.0014-3820.2000.tb00712.x.

Webb, J. H. *et al.,*(1991) 'The spawning behaviour of escaped farmed and wild adult Atlantic salmon (Salmo salar L.) in a northern Scottish river', *Aquaculture*, 98(1–3), pp. 97–110. doi: 10.1016/0044-8486(91)90375-H.

Webb, J. and Hawkins, A. D. (1989) 'The movements and spawning behaviour of adult salmon in the Girnock Burn, a tributary of the Aberdeenshire Dee, 1986', *Scottish Fisheries Research*, 40(40), pp. 1–42.

Wedemeyer, G. A., Saunders, R. L. and Clarke, W. C. (1980) 'Environmental Factors Affecting Smoltification and Early Marine Survival of Anadromous Salmonids', *Marine Fisheries Review*, 42(6), pp. 1–14.

Weingartner, M. and Zaniboni, F. E. (2006) 'EFEITO DO CURTO PERÍODO DE ESTOCAGEM DOS OVÓCITOS E TEMPO DE ENXAGUE DA ÁGUA DE ATIVAÇÃO DOS GAMETAS NA REPRODUÇÃO DE DOURADO Salminus brasiliensis', *1 Congresso Brasileiro De Produção De Peixes Nativos De Água Doce*, (2000).

Weir, L. K. *et al.,*(2010) 'Multiple paternity and variance in male fertilization success within Atlantic salmon Salmo salar redds in a naturally spawning population', *Journal of Fish Biology*, 77(3), pp. 479–493. doi: 10.1111/j.1095-8649.2010.02690.x.

Welke, K. and Schneider, J. M. (2009) 'Inbreeding avoidance through cryptic female choice in the cannibalistic orb-web spider Argiope lobata'. doi: 10.1093/beheco/arp097.

Wendling, N.. *et al.,*(2000) 'Adenosine triphosphate levels of chinook salmon (Oncorhynchus tshawytscha)

eggs following in vitro maintenance and activation/fertilization', *Fish Physiology and Biochemistry*, (3), pp. 217–223.

Wigan, M. (2013) *The Salmon: The Extraordinary Story of the King of Fish*. William Collins.

Wigby, S. and Chapman, T. (2004) 'Female resistance to male harm evolves in response to manipulation of sexual conflict', *Evolution*, 58(5). doi: 10.1111/j.0014-3820.2004.tb00436.x.

Willson, M. F. and Halupka, K. C. (1995) 'Anadromous Fish as Keystone Species in Vertebrate Communities', *Conservation Biology*, 9(3), pp. 489–497. doi: 10.1046/j.1523-1739.1995.09030489.x.

Wilson-Leedy, J. G., Kanuga, M. K. and Ingermann, R. L. (2009) 'Influence of osmolality and ions on the activation and characteristics of zebrafish sperm motility', *Theriogenology*, 71(7). doi: 10.1016/j.theriogenology.2008.11.006.

Wissenschafts-Verlag, B., Keil, A. and Sachser, N. (1998) 'Reproductive Benefits from Female Promiscuous Mating in a Small Mammal', *Ethology*, 104, pp. 897–903.

Withler, F. C. and Morley, R. B. (1968) 'Effects of Chilled Storage on Viability of Stored Ova and Sperm of Sockeye and Pink Salmon', *Journal of the Fisheries Research Board of Canada*. doi: 10.1139/f68-240.

Witten, P. E. and Hall, B. K. (2003) 'Seasonal changes in the lower jaw skeleton in male Atlantic salmon (Salmo salar L.): remodelling and regression of the kype after spawning', pp. 435–450.

World Bank (2013) 'Fish to 2030: Prospects for fisheries and aquaculture', *Agriculture and environmental services discussion paper*, 3(83177), p. 102. doi: 83177-GLB.

Wwf (2001) 'The Status of Wild Atlantic Salmon: A River by River Assessment', *http://wwf.panda.org/about_our_earth/blue_planet/publications/?3729/TheStatus-of-Wild-Atlantic-Salmon-A-River-by-River-Assessment.*, p. 184 pp. Available at: http://wwf.panda.org/about_our_earth/blue_planet/publications/?3729/TheStatus-of-Wild-Atlantic-Salmon-A-River-by-River-Assessment.

Yanagimachi, R. *et al.,*(1992) 'Factors Controlling Sperm Entry into the Micropyles of Salmonid and Herring Eggs: fish/sperm/egg/micropyle/fertilization', *Development, Growth & Differentiation*, 34(4), pp. 447–461. doi: 10.1111/j.1440-169X.1992.00447.x.

Yanagimachi, R. *et al.,*(2013) 'Sperm Attractant in the Micropyle Region of Fish and Insect Eggs1', *Biology of Reproduction*, 88(2). doi: 10.1095/biolreprod.112.105072.

Yeates, S. *et al.,*(2007) 'A two-second delay confers first-male fertilization precedence within in vitro sperm

competition experiments in Atlantic salmon', *Journal of Fish Biology*, 70(1). doi: 10.1111/j.1095-8649.2006.01294.x.

Yeates, S. E. *et al.,*(2009) 'Atlantic salmon eggs favour sperm in competition that have similar major histocompatibility alleles', *Proceedings of the Royal Society B: Biological Sciences*, 276(1656), pp. 559–566. doi: 10.1098/rspb.2008.1257.

Yeates, S. E. *et al.,*(2013) 'Cryptic choice of conspecific sperm controlled by the impact of ovarian fluid on sperm swimming behavior', *Evolution*, 67(12), pp. 3523–3536. doi: 10.1111/evo.12208.

Yeates, S. E. *et al.,*(2014) 'Assessing risks of invasion through gamete performance: Farm Atlantic salmon sperm and eggs show equivalence in function, fertility, compatibility and competitiveness to wild Atlantic salmon', *Evolutionary Applications*, 7(4), pp. 493–505. doi: 10.1111/eva.12148.

Yoshida, M., Kawano, N. and Yoshida, K. (2008) 'Control of sperm motility and fertility: Diverse factors and common mechanisms', *Cellular and Molecular Life Sciences*, 65(21), pp. 3446–3457. doi: 10.1007/s00018-008-8230-z.

Žák, J. and **Reichard, M**. (2021) 'Reproductive senescence in a short-lived fish', *Journal of Animal Ecology*, 90(2). doi: 10.1111/1365-2656.13382.

Zohar, Y. and **Mylonas, C. C**. (2001) 'Endocrine manipulations of spawning in cultured fish: From hormones to genes', *Aquaculture*, pp. 99–136. doi: 10.1016/S0044-8486(01)00584-1.

Zotin, A. I. (1958) 'The Mechanism of Hardening of the Salmonid Egg Membrane after Fertilization or Spontaneous Activation'.