Mechanisms and consequences of hybridisation between Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*)

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This thesis is dedicated to my parents.
Better ones a girl could not wish for; never have I wanted for a single thing.

Without you, this would not have been possible. Thank you.

Abstract

Relatively little research has been done to investigate the way postcopulatory, prezygotic mechanisms act to isolate species at the level of the gamete. This thesis uses the naturally-hybridising, externally-fertilising system of Atlantic salmon, *Salmo salar*, and brown trout, *S. trutta*, to investigate mechanisms of hybridisation through sperm-egg interactions, much of which is poorly understood.

Salmon and trout experience conspecific sperm precedence during *in vitro* sperm competition experiments, when sperm volumes and release times are equalised. This thesis firstly aimed to explore the dynamics of gametic interactions underlying this reproductive isolation. Manipulating the sperm entry time in interspecific sperm competitions significantly influenced the observed conspecific sperm precedence. A 2 second delay to the entry of conspecific sperm did not give hybridising males first-male sperm precedence, but neither did they gain precedence with paternity being shared between males; suggesting a mechanism of selection for conspecific sperm. Selection mechanisms were investigated through *in vitro* sperm competitions where egg ovarian fluid type was manipulated. Results showed that conspecific ovarian fluid allowed conspecific sperm significantly higher fertilisation success when competing against heterospecific sperm, regardless of which species eggs were under competition. This is the first evidence for cryptic female choice via a reproductive fluid in an external fertiliser.

The second objective of my thesis was to investigate the potential consequences of salmon-trout hybridisation for wild populations. This was achieved through comparing the early life and reproductive fitness of hybrids and pure species. Both reciprocal hybrid crosses had comparable early life fitness to pure species. Importantly however, neither reciprocal cross exceeded pure juveniles for any fitness measures. This suggests the replacement of parental species by hybrids is unlikely. Both hybrid crosses were capable of producing viable sperm and able to fertilise over 50% of both salmon and trout eggs. Neither cross gained paternity success when competing for trout eggs with conspecific males, while very low paternity was gained under sperm competition with Atlantic salmon for salmon eggs. The main threat posed by hybridisation to vulnerable salmon populations appears to come from wasted reproductive effort, through the production of reproductively unfit hybrids. The implications of this are discussed.

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Chapter 1

Introduction

This thesis uses a naturally hybridising, externally fertilising system to examine (1) how hybridisation is avoided at the gamete level, and (2) the consequences of hybridisation for offspring fitness. Coyne and Orr (2004) argue that reproductive isolation, reproductive barriers that prevent gene flow between populations, is the key to how closely related species remain isolated, even in sympatry. Barriers that act to isolate species can operate before fertilisation, through prezygotic mechanisms, or after fertilisation through postzygotic mechanisms. Further to this, prezygotic isolation can function on two levels. Species can be prezygotically isolated through precopulatory mechanisms that occur prior to mating to inhibit copulation, as well as through postcopulatory mechanisms, which operate after interspecific mating at the gamete level, but before fertilisation occurs (Coyne & Orr 2004). When reproductive isolation is based around postcopulatory mechanisms, important insights can be gained into the gamete level mechanisms of sperm-egg compatibility, which are relatively poorly understood. It is believed that divergent evolution, the build-up of genetic differences in allopatry, is likely to have resulted in reproductive isolation between many closely related species (Palumbi 1994).

Atlantic salmon and brown trout are sympatric sister species that coexist in rivers across much of their distribution, and are known to hybridise (Garcia-Vazquez et al. 2002; Garcia de Leaniz & Verspoor 1989; Hórreo et al. 2011; Verspoor & Hammar 1991). Salmon and trout are external fertilisers, where gametes are spawned and then fertilised in the external environment, where males and females are known to mate with multiple partners (Fleming 1996; Martinez et al. 2000; Weir et al. 2010). This established risk of hybridisation, and a multi-male spawning pattern, create clear criteria for the evolution of mechanisms that allow postcopulatory control of fertilisation. With incomplete reproductive isolation and external fertilisation, salmon and trout provide an excellent system in which to study the mechanisms of postcopulatory reproductive isolation, and the sperm-egg interactions that underlie them. Under external fertilisation, the potential for female control of the fertilisation process is reduced compared with internal fertilisation, allowing more targeted investigations of mechanisms influencing fertilisation compatibility. Using an externally fertilising system allows tighter experimental control, where manipulation of sperm and egg traits can be achieved *in vitro* and, importantly, it is possible to measure sperm form and function traits in the micro-environment to which the gametes are naturally adapted. I therefore use the

salmon-trout hybridisation system in this thesis as an informative system to investigate reproductive isolation in sympatric species, where precopulatory reproductive isolation barriers can be overridden.

Having examined postcopulatory mechanisms of reproductive isolation between salmon and trout, I then go on to look at the potential consequences on various aspects of offspring fitness when hybridisation does occur. Atlantic salmon receive high conservation priority, as populations have been declining around the world due to exploitation and habitat change by humans (Verspoor et al. 2007). Moreover, salmon have considerable commercial importance from their global fisheries status, and through sport fishing (Verspoor et al. 2007). Because of this, salmon are vulnerable to negative impacts on population growth, and hybridisation is one of a variety of factors to have an adverse effect on salmon populations, especially in the context of environmental change (Hindar & Balstad 1994). Therefore, it is important to understand the impact that hybridisation has on threatened salmon populations. The other part of this thesis will examine the fitness consequences of hybridisation in order to generate applied information on the potential for hybridisation and introgression, and the ecological impact of hybrids.

1. 1. Hybridisation

Hybridisation is widely defined as: “the interbreeding of individuals from what are believed to be genetically distinct populations, regardless of taxonomic status…” (Rhymer & Simberloff 1996)**.** With increasing refinements in molecular techniques, it has become clear that hybridisation between what were thought to be well established species occurs more widely than previously believed, occasionally resulting in hybrids that have sufficient fitness to allow introgression to occur (Arnold 1997). The argument that introgression via hybridisation (see 6.1.2) can play an adaptive evolutionary role in species divergence has long been accepted in plants (Rieseberg et al. 2003), and is increasingly gaining support in animals (Arnold 1997; Dowling & Secor 1997; Grant & Grant 1992; Seehausen 2004); with numerous cases providing evidence for the hybrid origin of some animal species (Abbott et al. 2011; Gross & Rieseberg 2005; Hermansen et al. 2011; Jacobsen & Omland 2011; Kunte

et al. 2011; Mallet 2007; Schwarz et al. 2005). Yet, the persistence of distinct species implies hybridisation is selected against, and many hybrid zones show evidence that hybrids are unfit relative to parental genotypes (Barton & Hewitt 1985; Hewitt 1988). Hybrids are expected to be less fit than parental types as the recombinant genotypes within hybrid individuals have been 'untested' by natural selection, and on average should be less well adapted (Barton 2001; Burke & Arnold 2001). As well as this, hybridisation can lead to breakdowns in local adaption and co-adapted gene complexes, resulting in hybrids with reduced fitness or viability (Barton & Hewitt 1989). Hybrid zones are maintained through selection against unfit hybrids and dispersal (Barton & Hewitt 1985; Barton & Hewitt 1989). Indeed, if hybrids are fit then hybrid zones become hybrid swarms where populations are made up of parent types, hybrids and backcrossed individuals. The fact that many species do hybridise successfully, with subsequent gene flow, means that the original way species were defined, the absence of gene flow (Mayr 1963), is too restraining to effectively classify species (Coyne & Orr 2004). More recently scientists have defined species by substantial, but not necessarily complete, reproductive isolation (Coyne & Orr 2004).

1.2. Reproductive Isolation

The way in which reproductive isolation arises between diverging populations is poorly understood (Eady 2001). Mechanisms of isolation fall into broad categories: those that occur before mating (precopulatory), those that occur after mating but before fertilisation (postcopulatory, prezygotic), and those that occur after fertilisation (postzygotic). In allopatry, populations develop reproductive isolation through barriers (of all types) that form as a result of genetic drift, mutation or as by-products of local adaption due to natural selection (Coyne & Orr 2004; Dobzhansky 1951; Mayr 1963). But how is reproductive isolation maintained in sympatry? Where inferior hybrid genotypes occur, individuals that mate with heterospecific partners will produce offspring with lower fitness than those individuals that mate with conspecifics, resulting in selection against hybridisation (Dobzhansky 1937; Mayr 1942; Mayr 1963). Selection against hybrids has been suggested to result in reinforcement. This process occurs when the production of maladaptive hybrids lowers the reproductive fitness in a species, resulting in the strengthening of pre-existing

reproductive isolation through natural or sexual selection (Marshall et al. 2002). The idea of reinforcement first pioneered by Dobzhansky (1940), and later developed by Blair (1955), gives natural selection an unambiguous role in isolating species (Marshall et al. 2002). Reinforcement has fallen in and out of favour with evolutionary biologists since its inception. Criticisms mainly stemmed from the lack of empirical and experimental evidence (Butlin 1987; Rice & Hostert 1993), as well as the restrictive conditions under which reinforcement is likely to occur, leading to suggestions it is unlikely to be important in nature (Marshall et al. 2002; Spencer et al. 1986). However, recent theoretical models have suggested reinforcement is plausible, and that selection against hybridisation can effectively drive the evolution of prezygotic reproductively isolating barriers (Dieckmann & Doebeli 1999; Kirkpatrick & Ravigne 2002; Liou & Price 1995; Turelli et al. 2001). While documented cases of reinforcement have been published (Higgie et al. 2000; Jiggins et al. 2001; Noor 1995; Saetre et al. 1997), empirical examples are still rare, possibly because of the difficulties in actually being able to identify reinforcement (Marshall et al. 2002).

1.2.1. Precopulatory reproductive isolation

The most obvious mechanisms that reproductively isolate species are those that prevent interspecific mating from occurring. Such barriers are termed precopulatory, prezygotic reproductive isolation as they act before copulation and thus zygote formation. Such mating barriers are often closely linked to ecological differences that arise in both allopatry and sympatry. They include behavioural, mechanical, temporal and habitat-based mechanisms of isolation. In reality, all barriers to reproduction could be the result of environmentally imposed selection, not just precopulatory, prezygotic isolating barriers. This is perhaps with the exception of mechanical isolation, where sexual selection and coevolution of male traits and female preference are likely to be involved in the evolution of divergent genitalia between species, resulting in incompatibilities and reproductive isolation (Eberhard 1985).

Behavioural isolation

Behavioural isolation occurs when differences in mating behaviours or preferences act to reduce attraction, and thus mating between different species. Behavioural isolation can take

the form of attraction to conspecific rather than heterospecific visual cues, such as bright plumage, ornaments, mating calls and pheromones. However, it can be hard to identify the traits that act to behaviourally isolate species, and the best studies are those that can manipulate traits such as mating calls and plumage (Coyne & Orr 2004). In a cryptic assemblage of 5 green lace wing insects in Europe, individuals across all 5 species preferred conspecific mating calls to any other heterospecific species (Noh & Henry 2010). A low level of genetic differentiation coupled with the strong premating behavioural isolation, is indicative that the species group has recently diverged (Noh & Henry 2010). The assumption beneath this form of isolation is that females have evolved to prefer the traits exhibited by conspecific males, and are thus more attracted to mate with them. This is clearly demonstrated in the sympatric butterflies *Pieris occidentalis* and *P. protodice*. Production of natural hybrids between these two species is not seen in the wild, despite no obvious hybrid sterility or inviability in lab crosses (Wiernasz & Kingsolver 1992). In field trials female *P. occidentalis* were found to reject mating from heterospecific *P. protodice* males, who have lighter wing colouration than conspecific *P. occidentalis* males. When *P. protodice* males had their wing colouration experimentally darkened to resemble that of conspecific males, their mating with *P. occidentalis* females significantly increased (Wiernasz & Kingsolver 1992). For this behavioural isolation to evolve, traits that attract mates (usually evolved by males) and preferences (usually evolved by females) for those traits must coevolve (Coyne & Orr 2004).

Variation in such traits is thought to evolve through sexual selection (Andersson 1994). While there is a wealth of literature on how behavioural cues and visual signals influence mate choice within species, less work has gone into testing how behavioural and visual cues act between species to isolate them (Williams & Mendelson 2010), and how these cues may evolve (Coyne & Orr 2004). Sexual selection within a species results from biases in fertilisation selecting for traits that specifically maximise lifetime reproductive success. This could result in the coevolution of male traits and female preferences within an isolated population and could instil behavioural changes that would reproductively isolate species (reviewed by Coyne & Orr 2004). But not all varying sexually selected traits result in behavioural isolation (Ryan 1998). Auklet sea birds vary in the crests they develop during the breeding season, but there was no evidence that females preferred crests from

conspecific males over that of heterospecifics (Jones & Hunter 1998). This could be due to decoupling of male trait and female preference evolution, and male traits may have diverged due to ecological reasons (Ryan 1998). While the debate continues as to how behavioural isolation may evolve (Coyne & Orr 2004), the importance of behavioural cues in isolating sympatric taxa is evident in species that cannot coexist without it. Female cichlids in Lake Victoria are known to strongly favour conspecific males in lab crosses when they are able to distinguish their colour (Seehausen et al. 1997), resulting in reproductive isolation. However, under poor light conditions where females are unable to distinguish male colour, assortative mating breaks down. In areas of Lake Victoria that are subject to high turbidity caused by eutrophication, reduced light levels lead to increased hybridisation as a result of the breakdown in assortative mating (Seehausen et al. 1997). There are fewer species of cichlid present in turbid areas of Lake Victoria than clear, implying species fuse when they are unable to distinguish colour (Coyne & Orr 2004; Seehausen et al. 1997). In this case at least it seems behavioural isolation is the main barrier maintaining distinct species status, and the system provides an excellent example of how fast environmental change (eutrophication) can break down evolved isolating systems.

Mechanical isolation

Mechanical mechanisms of reproductive isolation separate species through incompatibilities in the morphology of reproductive structures, resulting in impeded gene flow. Pollinator isolation involving structural incompatibilities is common in plants, occurring when pollinators cannot cross-fertilise flowers of differing shapes. For example, sister species *Mimulus lewisii* and *M. cardinalis*, show almost complete isolation in sympatry, though hybrids can be produced in laboratory experiments (Ramsey et al. 2003). *M. cardinalis* has long red tubular flowers pollinated almost entirely by humming birds, and *M. lewisii*, with broad low pink flowers, is pollinated almost exclusively by bees (Ramsey et al. 2003). Bees are unable to access the nectar and thus pollen of *M. cardinalis* flowers, and humming birds do not gain enough nectar from *M. lewisii* flowers, resulting in neither pollinator crossing pollen between species. Pollinator isolation is produced mainly by differences in flower colour and nectar loads given up by the flowers themselves (Schemske & Bradshaw 1999), and is most likely to evolve in allo or parapatry (Coyne & Orr 2004).

There are less examples of mechanical isolation in animals. However, genital morphology shows remarkable variation across animal species, particularly in insects. It is suggested such variation has evolved due to postcopulatory sexual selection (Arnqvist & Danielsson 1999a). Theory has postulated that mismatched genitals between species would reduce the efficiency of fertilisation and lead to reduced fertilisation success (Sota & Kubota 1998); known as the lock and key hypothesis. Yet, this theory has largely come under criticism due to lack of empirical evidence showing differences in genital morphology actually leading to reduced fertilisation success between closely related species (Eberhard 1985; Goulson 1993; Porter & Shapiro 1990). A recent example however has found that divergent body size in the lizard, *Plestiodon skiltonianus* species complex is a significant barrier to reproduction. Differences in the size of female and male genitals constrain alignment for penetration, inhibiting copulation (Richmond et al. 2011). One of the best examples of mechanical isolation in animals is to be found in carabid beetles. In two closely related species, *Carabus (Ohomopterus) maiyasanus* and *C. (O.) iwawakianus*, experiments show that males will mate indiscriminately with either conspecific or heterospecific females, but fertilisation with heterospecific females is low, and heterospecific females often suffer high mortality (Sota & Kubota 1998). This mortality is due to the fact that males have a penile appendage of a corresponding size to a pouch within the conspecific female's vagina. When females are subject to heterospecific mating, the appendage is the wrong size and ruptures the vaginal wall, sometimes resulting in death (Sota & Kubota 1998). Even if death does not follow a heterospecific mating, females can often be found with pieces of broken appendages within their vaginal pouch preventing any further reproduction from occurring (Sota & Kubota 1998).

Mechanical isolation could also arise through antagonistic sexual selection driving the coevolution of genital diversity through sexual conflict; the conflict that arises between the sexes due to differences in optimal fitness strategies. Sexual conflict can lead to adaptation and counter adaptation by the sexes in a bid to control the outcome of sexual encounters (Arnqvist and Rowe 1995). Through this conflict, evolution of elaborate genital morphology and secondary sexual structures could ensue (Eberhard 1985). If sexual conflict occurred in isolated populations, the resulting changes in genital morphology could lead to incompatibilities in mating with closely related species, inhibiting gene flow and leading to

successful reproductive isolation. Debate therefore continues as to whether genital morphology in animals is driven by reinforcement against production of maladaptive hybrids, or via sexual selection (Coyne & Orr 2004; Eberhard 1985; Hosken & Stockley 2004; Mutanen et al. 2006).

Temporal isolation

Temporal barriers to reproduction between species occur when gene flow is inhibited through differences in breeding seasons, breeding duration, flowering time and pollen shedding. Sessile, free spawning marine invertebrates live sympatrically in close proximity to each other, making it very likely that gametes of different species would come into contact if released at the same time. The brittle stars *Acrocnida brachiata* and *A. spatulispina*, live sympatrically in the intertidal and subtidal regions off the west coast of France. Investigations into isolating mechanisms found that spawning asynchrony is a strong barrier to hybridisation, with peak spawning times separated on average by 15 days (Muths et al. 2010). In some species of coral it has been observed that a temporal separation of mere hours can effectively isolate sympatric species (Knowlton et al. 1997). Closely related *Montastraea* species inhabit coral reefs in the tropics of the Western Atlantic, and have peak spawning times that differ by 1.5-3 hours. Despite the low temporal separation between gamete release, sperm from the first species to spawn has become too dilute within the water column to effectively fertilise eggs from the later spawning species, producing effective isolation (Knowlton et al. 1997). However, some sympatric broadcast spawning species release gametes synchronously, leading to gamete mixing. In these cases reproductive isolation comes from gametic incompatibilities (Geyer & Palumbi 2005), as discussed below. Temporal barriers to hybridisation can be become disrupted through habitat disturbance; potentially leading to species fusing in to hybrid swarms (Behm et al. 2010; Heath et al. 2010; Lamont et al. 2003). This has been seen in the plant genus *Banksia* in Australia. Here, hybrids are not naturally found in undisturbed habitats, but threaten to extinguish parent species in disturbed areas. It is thought increases in flower number in disturbed habitats have led to overlapping flowering times and cross pollination, resulting in hybridisation (Lamont et al. 2003).

Temporal isolation could potentially evolve through reinforcement of postzygotic mating barriers. Evidence for reinforcement as the evolutionary basis of temporal barriers has been observed in frogs from the genus *Rana* in Texas. When in allopatry the breeding seasons of these species overlap considerably, yet when they are in sympatric ranges, breeding times are displaced making them reproductively isolated (Hillis 1981). Hybrids of *Rana* species show reduced viability (Hillis 1988), highlighting a present but incomplete postzygotic reproductive barrier. It is possible that displaced breeding times in sympatric assemblages evolved to isolate species through selection against the maladaptive hybrids produced by interspecific mating (Coyne & Orr 2004). However, displaced breeding times in sympatry may also have evolved as a way to reduce interference between mating song (Noor 1999).

Habitat isolation

Habitat isolation is based on the failure of a species to successfully utilise another species' habitat. Genetic based differences in habitat preferences or tolerance may evolve in allopatry, as populations are naturally selected to adapt to their different environments. These differences can then go on to isolate species if they come back into secondary contact, through limiting or completely eliminating encounters and thus interspecific mating (Coyne & Orr 2004). An obvious example of habitat isolation can be seen in host-specific parasites whose hosts are allopatric. The beetle parasites in the genus *Ophraella* are adapted to a single, or few host plants that are allopatric. *Ophraella* species are unable to survive or lay eggs on other species host plants, completely preventing gene flow if secondary contact was to occur (Futuyma et al. 1995). Adaptations to abiotic environmental factors can also lead to reproductive isolation. Recently diverged species of *Gammarus* amphipods show marked differences in their ability to tolerate different salinities, preventing them from hybridising (Kolding 1985). Sympatric species also show reproductive isolation through utilisation of different niches within a habitat. Some coral species in the genus *Montastraea* are isolated by growing at different depths on a reef, with different species adapted to different light levels, resulting in gametes never coming into contact in the water column (Knowlton & Jackson 1994).

1.2.2. Post copulatory, prezygotic reproductive isolation

Post copulatory, prezygotic barriers act at the gametic level to isolate species after copulation has occurred, but before zygotes are formed, and for this reason sometimes come under the term gametic isolation. These barriers to reproduction act late within the life cycle of a species and can be hard to measure (Coyne & Orr 2004), therefore receiving relatively little attention compared to precopulatory reproductive isolation (Eady 2001). It has been understood for a long time that gametic interactions can influence the reproductive fitness of males and females within a species, and therefore maybe a major factor in isolating species (Coyne & Orr 2004; Eady 2001). Postcopulatory, prezygotic isolating mechanism can be divided into either non-competitive or competitive mechanisms.

Non-competitive postcopulatory, prezygotic isolation

Non-competitive isolation barriers are those that reduce or block heterospecific fertilisations in monogamous heterospecific matings, i.e. in the absence of any competition for fertilisation with conspecific sperm or pollen (Coyne & Orr 2004). In animals, failed sperm transfer and loss or expulsion of sperm from the reproductive tract in interspecific matings, are both examples of non-competitive mechanisms that bias fertilisations to conspecific males. Work with *Drosophila* has provided solid evidence for postcopulatory, prezygotic isolation (Chang 2004; Price 1997; Price et al. 2001; Price et al. 2000). When female *Drosophila simulans* were mated monogamously to males of a closely related species, *D. sechellia*, few sperm were transferred to the female's reproductive tract, even when copulation times were prolonged (Price et al. 2001). In contrast, when *D. simulans* females were mated to males of a different species in the same clade, *D. mauritiana*, large amounts of sperm are transferred to the female but very few go on to be stored within her reproductive tract, and fewer eggs are laid compared to conspecific matings (Price et al. 2001). In the reciprocal mating, *D. simulans* males are able to transfer large volumes of sperm to *D. mauritiana* females, which are retained, but these sperm are subsequently rapidly lost from the reproductive tract and thus fail to be utilised (Price et al. 2001).

Other non-competitive mechanisms of prezygotic isolation involve the inviability or reduced motility of gametes within a heterospecific reproductive tract or stigma (Gregory & Howard

1994; Niklas 1997; Patterson 1946). As well as this, heterospecific sperm or pollen can fail to be attracted to heterospecific gametes (Miller 1997; Williams & Rouse 1988; Yost & Kay 2009). In sympatric plant species *Costus pulverulentus* and *C. scaber*, pollen of *C. scaber* effectively adheres to and germinates on styles of *C. pulverulentus*, but pollen tube growth is insufficient to reach ovules resulting in no fertilisation (Yost & Kay 2009). Perhaps the most studied postcopulatory, prezygotic barrier arises via intrinsic gamete incompatibility. Here, despite encountering eggs, heterospecific sperm (or pollen) fail to successfully fertilise. Lack of fertilisation is most likely due to incompatibilities in molecular recognition mechanisms (Kresge et al. 2001; Palumbi 2009; Vacquier 1998). Probably the best studied intrinsic gamete incompatibly is in free spawning marine invertebrate sea urchin and abalone species. Sperm in these species fertilise eggs via a sperm-egg binding process (Swanson & Vacquier 1997) described in detail in section 4.4.1. Studies using *in vitro* fertilisation have highlighted that these sperm-egg binding reactions are highly species-specific (Swanson & Vacquier 1997; Vacquier & Lee 1993). This gamete recognition mechanism seems a highly important stage in that allows abalone and sea urchins to avoid hybridisation, which is confirmed further when combined with the fact that fertilisation of eggs is biased toward conspecific sperm when exposed to both heterospecific and conspecific sperm (Geyer & Palumbi 2005; Swanson & Vacquier 1998) as discussed below.

Competitive postcopulatory, prezygotic isolation

This mechanism is also generically termed conspecific gamete precedence (CGP), and is defined as the preferential utilisation of sperm or pollen from a conspecific male when a female's ova are exposed to both heterospecific and conspecific male gametes (Howard 1999). This barrier therefore only operates when females are exposed to both heterospecific and conspecific gametes during mating, so that hybridisation is not prevented when ova are exposed only to heterospecific sperm or pollen (Coyne & Orr 2004). In animals the mechanism is referred to as conspecific sperm precedence (CSP) and in plants as conspecific pollen precedence (CPP). Potential for fertilisation of females by multiple males is a common mating system in both animals and plants, and evidence strongly suggests that CGP can play an important role in promoting the reproductive isolation of more closely related species (reviewed by Howard 1999). In order to detect CGP, sperm or pollen competition experiments are needed, as heterospecific mating in the absence of competition can usually

produce hybrids that do not appear under interspecific competition conditions (Geyer & Palumbi 2005; Harper & Hart 2005; Howard 1999). *Drosophila* again provides a good example of CSP in animals. In conspecific mating in the majority of *Drosophila* there is a strong effect of mating order, where the last male to mate in a sequence fertilises the majority of the female's eggs (Price 1997). This last-male sperm competition success appears to be linked to the last male's seminal fluid (Harshman & Prout 1994; Price et al. 2000; Prout & Clark 2000). Yet, when *D. mauritiana* males are mated with *D. simulans* females in competition with conspecific males, they suffer severely reduced paternity success regardless of which order they are mated (Price 1997), providing clear evidence of CSP. In a later study, Price et al. (2000) found that when *D. mauritiana* males were mated second with *D. simulans* females, the conspecific *D. simulans* sperm already present in *D. simulans* female's reproductive tract outcompeted *D. mauritiana* sperm, preventing heterospecific fertilisation. When *D. mauritiana* males were first to mate with *D. simulans* females, ejaculates from the second-mating conspecific males displaced the stored *D. mauritiana* sperm, again ensuring the majority of the females eggs were fertilised by conspecific sperm (Price et al. 2000). In plants, CPP can successfully isolate species through conspecific pollen fertilising the majority of ovules. In the Louisiana irises *Iris fulva* and *I. hexagona*, heterospecific pollen tubes were found to grow more slowly than conspecific pollen tubes, allowing conspecific pollen to outcompete heterospecific pollen through faster tube growth (Carney et al. 1996).

Fertilisation in most animals follows a relatively uniform series of stages, providing distinct steps where both competitive and non-competitive mechanisms of CSP can act. Firstly, sperm are released, either into the female's reproductive tract or the external environment. At this point sperm can be attracted to eggs via chemicals released from the surface of the egg (Al-Anzi & Chandler 1998; Cherr et al. 2008; Eisenbach & Giojalas 2006; Inamdar et al. 2007; Miller 1997; Zatylny et al. 2002). A chemoattraction protein has been recognised in the toad *Xenopus laevis* (Al-Anzi & Chandler 1998) termed 'alluring' (Xiang et al. 2001). Present in the outer jelly layer, this protein diffuses from the jelly to attract sperm and guide them towards the egg (Al-Anzi & Chandler 1998). Evidence in some species suggests that these sperm chemoattractants can be species-specific. Across a range of holothurian and ophiuroid starfish species, ovarian extracts have been found to induce sperm motility and act

as chemoattractants, guiding sperm toward the egg (Miller 1997). Many of these chemotactic reactions have been found to be specific at the family level, and in one case at the species level in the genus *Bohadschia* (Miller 1997), showing that differential chemoattraction of sperm could play a role in reproductive isolation (Coyne & Orr 2004).

Once sperm have been attracted to the ovum, the next step in the fertilisation process is the attachment of sperm to the egg envelope. Sperm-egg contact and penetration are fundamental stages in all sexually reproducing systems, though the mechanisms are not homologous (Geyer & Palumbi 2005). Sperm bind to the egg envelope as a result of the interaction between proteins on the surface of the sperm and glycoproteins associated with the egg (Evans 2012; Wassarman 1999). In many mammalian species, sperm fusion to the egg envelope at the zona pellucida is species specific (Roldan & Yanagimachi 1989; Snell & White 1996; Wassarman 1999; Wassarman et al. 2001; Yanagimachi 1994), however, the molecular mechanisms behind species specificity in many systems are not well understood (Swanson & Vacquier 1998). As described above, one of the best studied systems of sperm-egg attachment is that found in sea urchins. In sea urchins, as in mammals, sperm undergo an acrosome reaction and fuse to the egg envelope (Vacquier & Moy 1977). Interspecific reproduction experiments have shown that heterospecific sperm have significantly reduced attachment to the vitelline envelope of sea urchin eggs (Glabe & Vacquier 1977; Metz et al. 1994; Palumbi & Metz 1991) allowing CSP to apply in some species (Geyer & Palumbi 2005). The sea urchin *Echinometra oblonga*, and as yet unnamed *Echinometra* species, have high levels of interspecific fertilisation under no mate choice lab experiments, but no natural hybrids have been described in the wild (Geyer & Palumbi 2005). Competitive *in vitro* fertilisations found that eggs of both species showed high preference for conspecific sperm when provided with mixed sperm from both species, providing evidence that interactions at the level of gamete provide an opportunity for complex mating-system dynamics (Geyer & Palumbi 2005). Both abalone and sea urchin sperm proteins show high amino acid divergence across species; it is this divergence that is thought to result in incompatibility between heterospecific sperm and egg membrane receptors (Palumbi 1999; Palumbi 2009; Swanson & Vacquier 1998; Swanson & Vacquier 2002b). Amino acid divergence is hypothesised to arise in one of two ways: (i) directional selection from coevolution of egg and sperm proteins to increase fertilisation efficiency, or (ii) from cyclic selection, possibly

encouraged by sexual conflict, where sperm evolve to increase fertilisation efficiency and egg penetration rate, leading to counter-evolution by eggs to slow sperm entry to avoid polyspermy (Palumbi 1999). These mechanisms of evolution could be accelerated by reinforcement as a result of hybridisation avoidance (Palumbi 1999), eventually resulting in CSP (Geyer & Palumbi 2005).

After attachment to the ovum, sperm have to penetrate the egg envelope in order to allow fusion with the egg membrane and subsequent fertilisation by fusion with the female pronucleus. Enzymatic proteins have been cited as facilitating this process in mice and ascidians (Matsumoto et al. 2002; Vacquier 1998). The bindin protein in sea urchin sperm has been implicated in mediating fusion between sperm and egg, in addition to the attachment of sperm to the vitelline envelope (Ulrich et al. 1998). An 18-kDa protein that coats the plasma membrane of abalone sperm following the acrosome reaction has also been linked to the fusion of sperm and egg (Swanson & Vacquier 1995). While many potential proteins mediating sperm egg fusion in mammals have been identified, it is likely that a combination of proteins play a role in facilitating sperm-egg fusion events (Kaji & Kudo 2004; Ying et al. 2010), with their coevolution driving reproductive isolation.

In species with internal fertilisation, the female reproductive tract presents added complexity and can play an important role in the fertilisation process, where sperm can be ejected, fail to navigate the tract successfully, or be attacked by the female's immune system (Howard 1999). Within species, it has been hypothesised that females may have the ability to bias the paternity of their offspring via cryptic mechanisms operating in the reproductive tract, in a process known as cryptic female choice (CFC) (Birkhead 1998b; Birkhead & Pizzari 2002; Eberhard 1996). Mechanisms of CFC are harder to define than those that operate in sperm competition (Birkhead 1998b; Eberhard 1996), because it has been a particular challenge to isolate female-controlled effects, if they exist, from the recognised male-controlled effects within differential fertilisation (Birkhead 2000; Pilastro et al. 2004; Pitnick & Brown 2000). Despite this, there has been an increasing body of evidence for female differential control of fertilisation at the level of the gamete (Reviews by: Birkhead 1998b; Eberhard 1996; Holman & Snook 2006). A number of potential mechanisms exist for a female's ability to bias individual male fertilisation success, including biasing the retention of sperm within the

tract to favour males with preferred phenotypes (Pizzari & Birkhead 2000), differential sperm storage (Eberhard 1996; Fedina 2007; Hellriegel & Bernasconi 2000) using internal muscular activity (Hellriegel & Bernasconi 2000), differential sperm transfer of higher quality sperm through bursa muscular contractions (Fedina 2007). These mechanisms, together with sperm-egg recognition or chemotaxis, could be used by females exposed to conspecific and heterospecific ejaculates to bias the paternity of her offspring to conspecific males in order to avoid hybridisation. Theory predicts that it will be female reproductive adaptations that primarily allow CSP (Price 1997), mainly due to the fact that hybridisation is invariably more costly to the reproductive fitness of females than males due to higher female investment (Parker & Partridge 1998).

1.2.3 Postzygotic reproductive isolation

Postzygotic reproductive isolation mechanisms act to prevent hybrid development or continuation after individuals from two separate species have mated and a zygote is successfully created. There are two distinct forms of postzygotic reproductive isolation, extrinsic and intrinsic. Extrinsic forms of postzygotic isolation occur when hybrids with phenotypes intermediate to those of their parent species have low fitness due to being maladapted to the habitat they are born into. Intrinsic postzygotic isolation occurs when hybrids have inherent developmental or functional defects that lead to partial or complete inviability or sterility. Both extrinsic and intrinsic mechanisms of isolation result in hybrids being unable to reproduce, either through pre-reproductive mortality, or via sterility, both of which lead to no gene flow back to parental populations or between hybrid individuals.

Extrinsic postzygotic isolation

Extrinsic isolation mechanisms can occur when there is an ecological disparity between hybrids and the habitat they are born into (Matsubayashi et al. 2010). This concept of extrinsic postzygotic isolation can be readily visualised with host dependent species. If F_1 hybrids of two species that live and breed on different host plants had adaptive characteristics intermediate to that of the parental species, they would have reduced fitness on each host compared to that of either parental species (Matsubayashi et al. 2010). In host races of *Eurosta solidaginis*, a fly that used different species of host plant to reproduce and

feed its larvae, hybrid larvae had lower fitness than both parental species on both host plants (Craig et al. 2007). Evidence suggests there was a poor correspondence between hybrid performance and parental optimum habitat, providing evidence that host races are extrinsically reproductively isolated (Craig et al. 2007). It is problematic however, to distinguish between reduced fitness due to mismatches between phenotype and environment, with reduced fitness that arises from inherent defects (Coyne & Orr 2004; Matsubayashi et al. 2010). Creating backcrosses and assessing these in each parental environment should reveal higher fitness in the environment of the backcrossed individual's pure parent, as they will have the majority of genes in common, and intrinsic reductions in fitness should not be linked to habitat (Coyne & Orr 2004; Egan & Funk 2009).

Extrinsic viability could be partly explained if intermediate hybrids also show intermediate behavioural phenotypes (Coyne & Orr 2004). Migration routes in passerine birds are thought to be heritable (Helbig 1991). Hybrids of two populations of blackcap birds with different migration routes to separate wintering grounds had intermediate migration direction (Helbig 1991). Any change in migration path would be deleterious to hybrids in the wild, as hybrids would likely reach unsuitable breeding grounds. Bensch et al. (1999) suggested an intermediate migration route taken by hybrids of two willow warbler subspecies, *Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula,* was the reason for the low recruitment of hybrids seen in populations. An intermediate route would lead them over the Sahara desert, resulting in severe food and water shortages, and certain death (Bensch et al. 1999).

Intermediate behaviour in hybrids can also lead to another form of extrinsic postzygotic isolation called behavioural sterility (Coyne & Orr 2004). Here, reduced fitness occurs due to hybrids being behaviourally or phenotypically intermediate to that of parents, leading to mate rejection or non-attraction. The green tree frog *Hyla cinerea* is sympatric with the barking tree frog *Hyla gratiosa,* and the two show high hybrid viability (Mecham 1960). *H. cinerea* males show greater variation in the mate calls females respond to when they are sympatric with *H. gratiosa* than when they are in allopatry (Höbel & Gerhardt 2003). Interspecific hybrid males produce mating calls that are different from parent males and unattractive to females of both parental species, leading them to reject hybrid males (Höbel

& Gerhardt 2003). Extrinsic isolation is not, however, always a fixed barrier to reproduction, and isolation mechanisms can be removed through changing environmental conditions. This is demonstrated extremely well by Darwin's finches on the Galapagos Islands. Hybrids were rare and did not reproduce before the El Niño climatic event due to reduced feeding efficiency from intermediate beak morphologies (Grant & Grant 1993; Grant & Grant 1996b). Yet, after this climatic shift, some hybrid and backcrossed individuals were demonstrating equivalent, and in some cases, higher fitness than parental species in terms of recruitment, reproduction and survival (Grant & Grant 1993). Higher fitness was due to hybrid beaks being better equipped to access new seed types made available as a result of the environmental change (Grant & Grant 1996b).

Intrinsic postzygotic isolation

Intrinsic postzygotic isolation occurs when hybrids have reduced fitness due to inherent developmental abnormalities that lead to inviable or sterile hybrids (Coyne & Orr 2004). Intrinsic isolation is much more widely studied due to the relative ease of studying such mechanisms in the laboratory (Ramsey et al. 2003). Intrinsic isolation can be divided into hybrid inviability, where hybrids fail as embryos or die before reproducing, and hybrid sterility, where hybrids are incapable of producing functional gametes. There are many examples in the literature of hybrid inviability. The model species complex *Drosophila* again provides a well-known example of hybrid inviability between *D. melanogaster* and *D. simulans*, and is reviewed by Sawamura (2000). Hybrid sterility can occur for physiological reasons, where hybrids are incapable of producing functional gametes (Coyne & Orr 1989; Coyne & Orr 2004). It is often only the heterogametic sex that suffers sterility, a phenomenon first noted by Haldane (1922) and dubbed Haldane's rule. Animals can also be behaviourally sterile, where neurological or pheromonal defects mean hybrids are incapable of reproduction. This differs from extrinsic behavioural sterility as the hybrid inability to mate arises from genetically disrupted behaviour, rather than behaviour that is intermediate of parent phenotypes (Coyne & Orr 2004). Dobzhansky (1936) and Muller (1942) postulated that sterility and non-viability in hybrids arises due to pleiotropic side-effects of genetic interactions formed when species are in allopatry. The Dobzhansky-Muller model draws together the ideas of Dobzhansky (1936) and Muller (1942) to propose that genetic substitutions built up by a species when in allopatry, while small scale enough not to reduce

the fitness of that species will, when brought together with genes from a divergent species, result in inviability or sterility in the F_1 hybrid or backcross generations (Coyne & Orr 1998; Russell 2003). Alleles brought together from divergent species have never been 'tested' together and may well result in hybrids with reduced fitness (Coyne & Orr 1998). The Dobzhanzky-Muller model underpins almost all modern work on the genetics of postzygotic isolation (Coyne & Orr 1998), and there is now strong evidence that hybrid sterility and inviability arise through locus incompatibilities (reviewed by Orr 1997).

1.3. Atlantic salmon and brown trout

Atlantic salmon, *Salmo salar*, and brown trout, *Salmo trutta*, are two closely related teleost fish in the Salmonidae family. Both species spawn in freshwater and show a large variation in life histories. Individuals in some populations move from natal freshwater streams to the sea and return to breed, while others remain resident in freshwater for the whole of their life cycle (Elliot 1994; Fleming 1996). Atlantic salmon are native to the temperate and subarctic regions of the North Atlantic Ocean, and typically adopt an anadromous life history returning to their natal rivers to spawn between September and February. Brown trout, once native only to Europe, are now found worldwide after repeated introductions (Elliot 1994), and populations all over the world show vast variation in life history. In some populations all individuals spend their entire lives in natal streams, growing slowly to become small, mature brown trout. In other populations, adults migrate from the stream to the nearest lake, while others adopt the anadromous life history and migrate to sea. Further to this, some populations express more than one of these life histories simultaneously (Elliot 1994). This, along with the wide variety of colours brown trout exhibit, has led to many subdivisions being classified as different species in the past. Brown trout are now most often classified as the *Salmo trutta*, but are understood to be polytypic (Elliot 1994; Hindar et al. 1991). However, some scientists still distinguish between sea-going trout, *Salmo trutta trutta* and non-sea-going trout, *Salmon trutta fario*. This distinction is probably not relevant in populations where female sea-going trout return to rivers where they are likely to have eggs fertilised by smaller resident males, with evidence showing that the two types are fully inter-fertile with little genetic distinction (Elliot 1994). The brown trout used in this thesis are all

offspring of wild fish from a population that contains both resident and anadromous individuals that spawn together.

In anadromous Atlantic salmon populations males display two reproductive phenotypes. Firstly, males can return from the ocean to spawn as large anadromous males with developed sexual traits; alternatively, males can mature as precocious parr while still in their natal stream and before seaward migration. Anadromy is a costly life history trait in terms of survival due to the huge energy cost of migrating (Fleming 1996). Mature parr avoid this cost by not migrating to the ocean and are more likely to go on to breed again, either by maturing as parr again the next year, or smolting and migrating to sea to return as anadromous males (Fleming 1996). Mature parr spawn by sneaking into a female's nest to fertilise eggs, while anadromous males fight for access to females (Fleming 1996). Both fighting anadromous males and sneaker males generate intense sperm competition, where sexual selection can operate at the level of the gamete. Mature salmon parr tend to have superior ejaculate quality, with higher levels of motile sperm with increased ATP content compared to anadromous males, thought to be the result of sexual selection generated from sperm competition (Vladić & Jävri 2001). Parr also father the majority of paternity under *in vitro* sperm competitions against anadromous males, with the higher levels of sperm ATP (i.e. higher energy reserves) being linked to fertilisation success (Vladić et al. 2010). Brown trout populations can have similar dimorphism between males during spawning, as large anadromous sea trout return and spawn with females in rivers with resident males that are much smaller (Elliot 1994). Sperm competition can be less intense in brown trout as large anadromous sea trout are often more effective at guarding females and chasing off smaller rivals (Jones & Ball 1954). In this thesis only large, mature anadromous Atlantic salmon and brown trout are used in fertilisation experiments and investigations into the gametic dynamics of hybridisation.

1.4. Hybridisation in Atlantic salmon and brown trout

Data from the literature shows that hybridisation is common within all major lineages of salmonids (Taylor 2004), and recognised in every genus (Heath et al. 2010). Like many

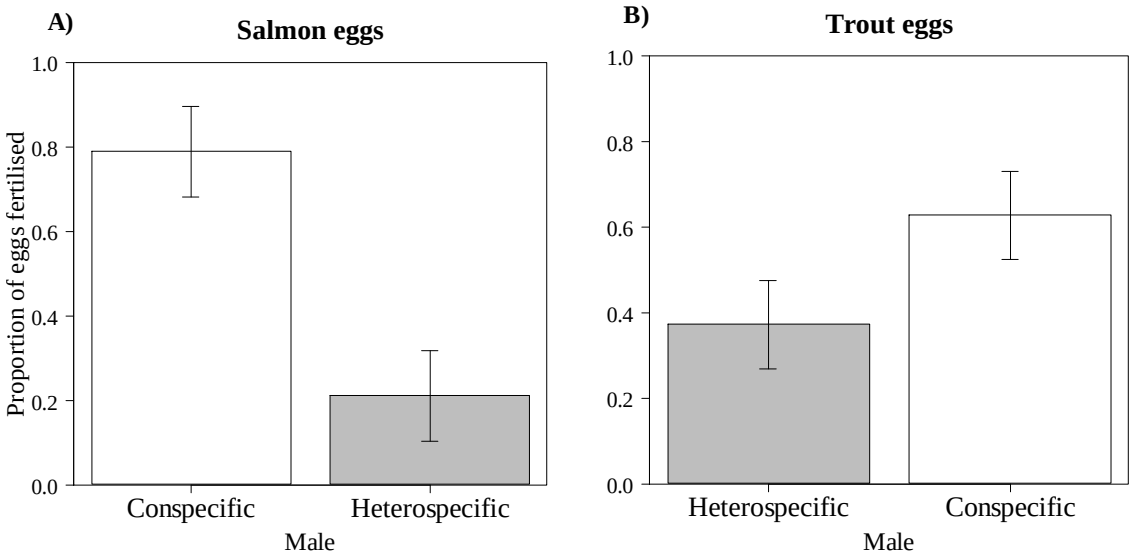
other teleost fish in sympatry, salmonids have weak isolating barriers to reproduction (Verspoor & Hammar 1991). Species are often only isolated by temporal and spatial mechanisms (Docker et al. 2003; Heggberget et al. 1988; Taylor 2004), and behavioural isolations are poor, often appearing non-existent (Grant et al. 2002). As well as weak precopulatory reproductive barriers, many salmonid species also appear to have weak postcopulatory barriers (Chevassus 1979; Taylor 2004). However, this varies from species to species, with some salmonid hybrids showing heterosis over parental types (Seiler & Keeley 2007) and others being extrinsically selected against (Hagen & Taylor 2001) .

Atlantic salmon and brown trout are frequently sympatric in rivers across much of their endemic European range, as well as in North America. Reproductive isolation between the two appears to be mainly in the form of differential peak spawning time, with brown trout spawning on average 15 days earlier than salmon (Heggberget et al. 1988). Overlaps in spawning time together with poor habitat segregation results in only partial reproductive isolation between the two species, which is vulnerable to environmental disturbance and change (Heggberget et al. 1988). Hybridisation between these species was recognised artificially as early as 1887 (Day 1887), but was first confirmed in the wild using biochemical markers in the 1970's (Payne et al. 1972). Since then, reports of hybridisation in Europe and North America have been widespread (Garcia de Leaniz & Verspoor 1989; Gephard et al. 2000; Hartley 1996; Hindar & Balstad 1994; Hurrell & Price 1991; Jansson et al. 1991; Jansson & Ost 1997; McGowan & Davidson 1992b; Payne et al. 1972; Verspoor 1988). In some cases relatively high rates of hybridisation have been recorded. In rivers in Northern Spain average hybridisation was documented at 2.3%, cited as higher than previous levels recorded in Europe (0.1%) and North America (0.8%) (Garcia de Leaniz & Verspoor 1989). A later study in a Swedish river found 13% of juveniles sampled were of hybrid origin (Jansson et al. 1991). This was exceeded in an English river where 18.18% of individuals sampled were hybrids (Hartley 1996). Some sites in Northern Europe have seen rates of salmon and trout hybridisation increase (Hindar & Balstad 1994; Jansson & Ost 1997), and anthropogenic causes are thought to be behind the observed rises. Reduced spawning grounds, stocking and aquaculture escapes have all been linked to cases of salmon-trout hybridisation (Garcia de Leaniz & Verspoor 1989; Hindar & Balstad 1994; Jansson & Ost 1997). In a restored river section in Sweden, where salmon and trout were re-

introduced to reduced spawning grounds, hybridisation was observed to reach as high as 41% (Jansson & Ost 1997).

Salmon and trout have been shown to have incomplete reproductive isolation (Heggberget et al. 1988) and are obviously inter-fertile. Any salmon and trout males hybridising with heterospecific females however are likely to experience sperm competition from conspecific males. Prior to this thesis preliminary work carried out sperm competitions, *in vitro* between salmon and trout males for eggs from either salmon or trout females (work done by S. Yeates). In these sperm competitions equal volumes of salmon and trout sperm were added to dry beakers containing salmon or trout eggs, with care taken not to allow contact between eggs and sperm within the beakers. Water was then rapidly added to heterogeneously mix the gametes, replicating the natural gametic environment (Gage et al. 2004), and simulating simultaneous gamete release by both males. Eggs were subsequently incubated until offspring hatched, when DNA samples were collected for microsatellite paternity analysis. DNA from the offspring, mother and two potential fathers in a cross was scored at 3 microsatellite loci that amplify in both salmon and trout (Cairney et al. 2000). This allowed unambiguous assignment of offspring to either competing male in the cross, letting the proportion of paternity salmon and trout males gained to be determined when they competed for conspecific or heterospecific eggs. Results from this work showed that there is CSP between salmon and trout (Figure 1.4.1); with conspecific males achieving significantly more paternity when competing for conspecific eggs (S. Yeates unpublished data). Despite the presence of CSP however, heterospecific males are still able to achieve a relatively large proportion of paternity, 37% on average across the two species (S. Yeates unpublished data). The CSP seen in Atlantic salmon-brown trout sperm competition suggests selection could be acting at the gamete level to isolate these species further to prevent hybridisation. One of the key aims in this thesis is to investigate the mechanisms that mediate this conspecific sperm precedence.

Figure 1.4.1: A) Mean ± 1 S.E.M fertilisation success of Atlantic salmon males (conspecific, white bars) in competition with brown trout males (heterospecific, grey bars) for Atlantic salmon eggs (n = 15 crosses). B) Mean ± 1 S.E.M fertilisation success of brown trout males (conspecific, white bars) in competition with Atlantic salmon males (heterospecific, grey bars) for brown trout eggs (n = 15 crosses). S. Yeates unpublished data.



1.5. External fertilisation as a model system

Postcopulatory sexual selection can be a powerful force shaping the reproductive physiology and behaviour of males and females (Birkhead & Møller 1998; Birkhead 1998b; Birkhead & Parker 1997). Gametic interactions can influence the reproductive fitness of males and females within a species, and with different populations under divergent selection, sperm-egg interactions may be a major factor in reproductively isolating species from each other (Coyne & Orr 2004; Eady 2001). However, many aspects of gamete competition and choice both within and between species remain unexplored and poorly understood. Under internal fertilisation direct observation of gametes is challenging, and experimental control of male and female effects within a reproductive tract clearly creates a major challenge (Howard 1999); as does recreating the exact natural fertilisation conditions. Female reproductive tracts are often complex and hostile environments that can, particularly in the case of mammals, bring about physiological changes in sperm (Eady 2001; Howard 1999 and references therein). Further to this, internal reproductive environments can conceal cryptic

mechanisms of female choice or sperm competition making it hard to observe and manipulate them (Eberhard 1996; Engqvist & Sauer 2003). Much of the literature has focused on gamete interactions in internally fertilising species, where sperm adaptation to the female's reproductive tract may produce confounding influences (for example: Birkhead & Moller 1992; Briske 1996; Fedina & Lewis 2004; Hellriegel & Bernasconi 2000; Pizzari & Birkhead 2000). In addition to this, there is potential for direct uncontrolled selection arising from CFC mechanisms on sperm from different males within sperm competition experiments (Eberhard 1996). This could further complicate studies of gamete function and interaction. Because of this, teasing apart the role of sperm from the role of eggs or the reproductive tract can be difficult for species with internal fertilisation (Engqvist & Sauer 2003; Evans et al. 2003; Pilastro et al. 2004; Pizzari & Birkhead 2000; Ward 2000).

This thesis aims to further the understanding of gamete interactions that lead to reproductive isolation under interspecific hybridisation, by using externally spawning and naturally hybridising Atlantic salmon and brown trout as a model system. Under external fertilisation, the gametic environment is more simple and under less female (or male) control. This situation allows tighter experimental control of sperm and egg traits under *in vitro* fertilisation, and allows controlled manipulation and analysis of gametes in the microenvironment to which they are naturally adapted (Gage et al. 2004). Male and female salmonids also mate with multiple mates (Fleming 1996; Martinez et al. 2000; Weir et al. 2010) and are at risk of hybridisation, creating clear criteria for the evolution of mechanisms that allow postcopulatory control of fertilisation.

1.6. Threats of hybridisation

Atlantic salmon are viewed with high conservation importance and are known to be declining in the majority of their distribution (Parrish et al. 1998; WWF 2001). Declines are often due to exploitation and habitat change by humans (Verspoor et al. 2007), thus salmon are vulnerable to negative impacts on population growth. Hybridisation is one of a variety of factors to have a negative effect on salmon populations, and has been seen to be increasing (Hindar & Balstad 1994; Jansson & Ost 1997). In Norway, salmon-trout hybridisation was

found to significantly rise between 1986 to 1992 (Hindar & Balstad 1994), being positively linked to high numbers of Atlantic salmon escaping from aquaculture nets at the time (Hindar & Balstad 1994). Hatchery reared and domesticated strains of fish, like those that escape from aquaculture nets, show lower reproductive fitness compared to wild fish through altered breeding behaviour as a result of both deliberate and unintentional selection during domestication (Fleming 1996; Levin et al. 2001). Further increases in hybridisation could be of greatest concern to threatened or vulnerable populations of Atlantic salmon, which have been shown to be more susceptible to hybridisation (Hindar & Balstad 1994).

An obvious threat of hybridisation to declining Atlantic salmon populations is that of introgression. Introgressive hybridisation results in non-native genes entering a population through interbreeding with closely related species, and can lead to a collapse of multispecies assemblages into a hybrid swarm (Seehausen et al. 2008). In a study of westslope cutthroat trout hybridising with rainbow trout, only a 20% admixture of rainbow trout genes was enough to cause a 50% reduction in the reproductive success of cutthroat trout, causing a population decline (Muhlfeld et al. 2009). Evidence of introgressive hybridisation between Atlantic salmon and brown trout has recently been observed in streams where brown trout were stocked, however the threat of introgression receded as hybridisation declined with the cessation of stocking (Castillo et al. 2008). Another threat to salmon to arise from hybridisation is a reduction in effective population size through outbreeding depression. Atlantic salmon females produce larger eggs per unit of body weight compared to other species (Armstrong et al. 2003), with each egg a high energy reproductive investment. Production of unfit hybrids would be highly detrimental to the reproductive fitness of females, as sterile or unviable hybrids would result in the removal of reproductive resources (i.e. reproducing adults) from the system (McGinnity et al. 2003).The threatened salmonid species, *Salvenlinus confluentus* hybridises with the introduced brook trout, *S. fontinalis*, with little evidence of hybrids beyond the F1 generation. This has resulted in wasted reproductive effort reducing the effective population size of *Salvenlinus confluentus*, with severe negative effects on population survival (Allendorf et al. 2001; Leary et al. 1993). Production of hybrids that are reproductively unfit can have further negative impacts if those hybrids are ecologically fit at all or some life stages. Ecologically fit hybrids have the potential to out-compete one or both of the parental species, resulting in reductions in pure

species fitness. A clear example of this has been seen in the pecos pupfish, *Cyprinodon pecosensis* which is threatened with replacement by the hybrids it produces with a closely related species, the introduced sheepshead minnow, *C. variegates*. Hybrids of these species have elevated swimming performance and faster growth; both of which increase food acquisition, reduce the threat of predation and allow hybrids to gain and hold breeding territories, meaning they can effectively outcompeting pecos pupfish (Rosenfield et al. 2004).

To understand the impact that hybridisation with brown trout has on threatened Atlantic salmon populations, knowledge on the fitness of hybrids is needed. With this thesis I therefore also aim to investigate the fitness of hybrids at early life stages, to assess whether they have the capacity to dominate or outcompete salmon or trout individuals for system resources. As well as this, I try to explore whether salmon-trout hybrids have the ability to proliferate and be an avenue of introgression, or if they are an evolutionary dead end which removes reproductive resources from the pure systems. By carrying out assessments of fitness, as detailed in this thesis, I hope to be able to contribute further knowledge on the fitness of hybrids at early life stages and hopefully infer any applied impacts hybrids could have on wild populations of Atlantic salmon and brown trout.

1.7. Thesis overview

This thesis is separated into 2 main objectives divided over 4 experimental data chapters. My first objective is to use externally fertilising Atlantic salmon and brown trout to experimentally investigate the mechanisms of postcopulatory, prezygotic reproductive isolation at the gamete level. Atlantic salmon and brown trout show CSP when males compete for conspecific eggs (Figure 1.4.1), with the conspecific male achieving the majority of paternity. The salmon-trout *in vitro* fertilisation system used in this thesis (Chapters 3 and 4) presents an excellent opportunity to establish whether CSP between these two species is mediated by eggs or by sperm, or both. I use reciprocally balanced sperm competition experiments, where individual males are analysed in both the conspecific and heterospecific ‘role’, and determine sperm competition success via parentage assignment. In

order to gain further insight into the mechanism of CSP within this hybridising system, and assess any variation in temporal dynamics under hybridisation at the gamete level, I use a 2 second experimental delay for one of the male's sperm to enter the fertilisation set (Chapter 3). The general hypothesis in this chapter is that eggs will favour conspecific sperm in fertilisation, even if heterospecific sperm are given a 2 second timing advantage in the fertilisation competition. The extent of any differences in CSP under sperm delay versus simultaneous release will provide insight into the dynamics of sperm competition, cryptic female choice, and differential fertilisation success. Following assessment of the effect of relative delay in the sperm competition, I investigate whether a female's ovarian fluid mediates CSP within salmon-trout hybridisation. Ovarian fluid is released with the eggs at spawning, but its specific role is so far poorly understood. Using sperm competition and fertilisation experiments that vary the presence of either species' ovarian fluid, I measure whether this fluid controls CSP, and investigate whether it differentially influences conspecific sperm motility and / or spermatozoa chemoattraction (Chapter 4). The hypothesis in this chapter is that ovarian fluid will favour conspecific sperm in fertilisation independently of eggs are being fertilised.

The second objective of my thesis looks at the potential consequences for offspring fitness when hybridisation does occur. Atlantic salmon are viewed with high conservation importance as populations have been declining around the world (Verspoor et al. 2007). Further to this, threatened or vulnerable populations of Atlantic salmon have been shown to be more susceptible to hybridisation (Hindar & Balstad 1994). Negative effects on population growth can occur through competition with hybrids, reductions in populations through wasted reproductive effort in the production on unfit hybrids and loss of adaptation through introgression. To understand the impact that hybridisation can have on threatened salmon populations, I developed the information on relative hybrid fertility, to gain a better understanding of the fitness of hybrid offspring (chapters 5 and 6). I firstly assessed the relative fitness of reciprocal hybrids compared to pure species through measuring different fitness traits at early life stages in a controlled and, for the first time to my knowledge, a semi-natural environment (Chapter 5). Measuring the relative fitness of hybrids in natural environments is fundamental to being able to anticipate the ecological and evolutionary impact hybrids may have on parental populations (Parris 2001). My aim was to provide a

better understanding of salmon-trout hybrid fitness at early life stages in an attempt to infer any impacts hybrids could have on wild populations of Atlantic salmon and brown trout. Secondly I assessed the reproductive capability of male F_1 hybrid parr, including when competing against adult male salmon and trout for salmon and trout eggs via *in vitro* sperm competitions (Chapter 6). To gain an idea of the ability of hybrid males to fertilise salmon and trout eggs in general, *in vitro* fertilisation trials with F_1 hybrid males fertilising salmon and trout eggs in the absence of competition were carried out. Sperm motility traits of hybrids were also assessed, in conjunction with sperm competitions, to compare sperm function of F1 hybrid males to that of adult anadromous salmon and trout males. Any hybrid males in a population that go on to spawn could be subject to the postcopulatory selection generated by sperm competition, and their success or failure will determine whether introgression is a real threat to Atlantic salmon populations.

Chapter 2

Experimental methods

2.1. Introduction

The 4 data chapters that comprise this thesis examine the fertilisation dynamics and compatibility between two closely related salmonid species, the Atlantic salmon (*Salmo salar*) and the anadromous brown trout (*Salmo trutta*). This general methods chapter aims to identify the core methods that form the main basis for the majority of the experiments described in this thesis. By collating the methods into a single chapter, repetition of techniques used throughout the following chapters can be avoided. The main methods of gamete collection, *in vitro* fertilisation techniques and recording of sperm traits are described here in detail. In the data chapters themselves there will be descriptions on how these methods were employed to achieve each experiment, along with specifics of experimental design. Methods that are particular to a single experiment are detailed within individual data chapters. With the exception of chapter 5, 'Quantitative fitness measures of salmon-trout hybrids at early life stages', the general experimental design was to carry out *in vitro* fertilisation and sperm competition assays with concurrent sperm trait analyses. These experiments were performed under a variety of conditions to better understand fertilisation dynamics and compatibility in the salmon-trout hybridisation system.

2.2. Study site and gamete collection

All field work for this thesis was carried out at the Norwegian Institute of Nature Research (NINA) research station in Ims, southwestern Norway, near the city of Stavanger (58º59'N, 5º 58'E), during the spawning seasons of 2008, 2009 and 2011, and the summer of 2010 (chapter 5). The research station is located at the mouth of the River Imsa, a small 1km long river with a catchment of 128 km^2 that empties into the Boknafjord (Einum & Fleming 1997; Fleming et al. 1994). The hatchery is supplied with water directly from the River Imsa. The broodstock Atlantic salmon and brown trout used for all but one of the experiments in this thesis were first generation hatchery fish derived from sympatric wild populations of these species in the Figgjo River, close to the NINA hatchery. Eggs and sperm were collected from wild fish and fertilised in the hatchery, where offspring were grown to adulthood to be

used as broodstock. The brown trout in the Figgjo River are a mix of anadromous and resident individuals. Figgjo fish at NINA were housed in holding tanks of 7000 litres.

In order for *in vitro* fertilisation and sperm competition experiments to be carried out, gametes from ripe Atlantic salmon and brown trout males and females had to be collected. Fish were checked almost daily by hatchery staff from October until eggs and milt were free flowing. Fish were then taken from the holding tanks and lightly anaesthetised with chlorobutanol (2ml per 10l of water). When fish reached a suitable state of anaesthesia, they were removed from the water and stripped of their gametes and a small fin clip taken and placed in 90% ethanol for later genotype analysis. Stripping consists of applying gentle pressure to the abdomen in a downward motion from head to vent, to expel gametes. Gametes were collected into polythene bags that were filled with oxygen and kept on ice until needed (for a maximum of 6 days). Einum and Fleming (2000) found minimal changes in eggs stored in this way up to 10 days after collection. Throughout the stripping process each fish had to be kept free of water, urine and mucus around the vent. Urine has been shown to activate sperm within the seminal fluid of freshwater fish (Billard et al. 1995; Dreanno et al. 1998; Linhart et al. 1995; Linhart et al. 1999; Poupard et al. 1998; Rurangwa et al. 2004) leading to immotile sperm and resulting in an unusable sample. Therefore great care was taken to avoid any moisture or water coming into contact with milt or eggs to avoid activation of gametes prior to experiments.

2.3. Measurement of Sperm traits and analysis

Sperm traits were recorded alongside *in vitro* fertilisation experiments. Sperm trait recording was carried out as close to fertilisation as possible (maximum 1 hour) in order to capture an accurate representation of sperm behaviour. By recording male motility traits in conjunction with fertilisation experiments, behavioural traits of a male's sperm at the time it entered the *in vitro* fertilisations were captured. This eliminates the confounding effects that sperm storage time could potentially have on sperm behaviour were sperm motility traits to be recorded hours or days after the fertilisations took place. Sperm from both Atlantic salmon and brown trout males was diluted with a trout extender (80 MM NaCl, 40 mM KCl, 1mM

$CaCl_2$ and 20 mM Tris, adjusted to pH 9 (Billard 1992)) prior to entering the fertilisation and measuring of sperm traits.

Salmonid milt is more viscous than water. If milt is used undiluted during *in vitro* fertilisations sperm may not activate evenly across the sample, as it would in the turbulent conditions of the redd. Billard and Cosson (1992) showed that, without dilution, activation of sperm for motility analysis results in a heterogeneous mixture of motile and immotile sperm swimming at different velocities and trajectories, with some sperm become progressively more activated after the initial activation. To obtain synchronous motility of sperm for accurate motility measures sperm needs to be diluted by at least 100 fold (Billard 1992). All *in vitro* fertilisation in this thesis used a 2 step activation procedure (Billard 1992) to ensure simultaneous activation of sperm within fertilisations as well as accurate motility measures. The 2 step process involves an initial dilution in the trout extender. Trout extender is of a similar osmolarity to trout seminal fluid, allowing sperm to remain immotile for several hours (Billard 1992), providing dilution without fear of sperm activating prior to use in experiments. The next step is activation of sperm in the activating medium (usually river water) which is done in the fertilisation experiment or during sperm trait recording. Yeates (2005) found no adverse effect of trout extender on the motility of Atlantic salmon sperm within 5-6 hours after stripping. In addition to this, Atlantic salmon sperm in trout extender behaves similarly to undiluted sperm, allowing extender to be used as the dilution medium for both salmon and trout in experiments without confounding effects on sperm function (Yeates 2005).

2.3.1. Recording of sperm motility

Spermatozoa activity was recorded on a Sony Hi8 tape deck connected to JVC video camera (TK-1280E) which was fixed to an Olympus CK40 inverted stage microscope at x400 under dark field phase illumination. Sub samples of a male's milt in extender were activated with river water or ovarian fluid depending on the experiment. After activation 0.7µl was immediately transferred to a well of a 12 well multitest glass slide (ICN Basingstoke, UK, depth~0.0116 mm) and a cover slip was carefully but rapidly put in place. Just prior to

activation the videotape was started and, using the videos on screen counter, the exact time of activation was noted. Noting the exact time of activation allowed motility throughout the lifetime of spermatozoa to be recorded, as well as comparisons of lifespans to be made between males under varying conditions. The time from activation, placement on the slide and image resolution was minimised as much as possible in order to capture as much of the sperm movement after activation as possible. Any samples that took longer than 10 seconds to achieve a recordable image after activation were abandoned and repeated. At the start of each video tape a 1000 µl graticule slide was recorded for around 1 minute. This allowed calibration by the automated sperm tracker (see below) to record actual distance during video analysis.

When recording sperm activity, sperm drifting across the field of vision due to too much fluid on the slide should be avoided as this movement can be interpreted as motility by automated sperm trackers, even if drifting sperm are actually immobile (Kime et al. 2001). The 0.7 µl volume of activated sperm placed on to the microscope slide was found to be optimal volume to avoid drift (Yeates 2005). To obtain an image with a manageable number of spermatozoa (50-100) at x400 magnification and ensure even activation of spermatozoa within a sub sample, milt in extender and activation medium volumes had to be adjusted for each male. If there were too many or too few sperm in the image, or the image was unfocused, it was abandoned and the process repeated.

Temperature has an effect on sperm motility in Atlantic salmon and brown trout, with decreased motility at higher temperatures (Vladić & Järvi 1997). For this reason the temperature of the river water used during *in vitro* fertilisations and activation of sperm for recording motility was measured. This ensured that there was no significant deviation in temperatures which may have influenced sperm behaviour. The randomised design of experiments ensured that there would be no directional bias in results caused by fluctuations in water temperature, with the average temperature of the water 10.2 ºC ± 0.11 (1 S.E.M). To further ensure river water remained at a constant low temperature that sperm in natural spawning would be exposed to, motility recordings were carried out in a cold room with an average air temperature of 6.5 º C ± 0.35 (1 S.E.M).

2.3.2. Motility analysis

Sperm motility traits were measured through analysis of the Hi8 video tapes by a computer assisted sperm analysis (CASA) system, the Hobson Sperm Tracker (Hobson Vision Ltd, Baslow, UK) at the Zoological Society of London (Permission W.V. Holt). CASA is a widely used method of obtaining accurate measurements of semen motility parameters not measurable or observable manually (Verstegen et al. 2002). The Hobson Sperm Tracker can track up to 200 individual sperm simultaneously in real time and generate 14 parameters of movement. Importantly the Hobson Sperm Tracker can be standardised for fish sperm which have a much shorter life span than mammalian sperm and have rapid velocity (Kime et al. 2001).

Motility parameters calculated by the sperm tracker include: curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), mean angular displacement (MAD), straightness (STR) and percentage motile sperm (%MOT). The parameters suggested most useful for studying sperm motility in fish are VCL, VSL, LIN and %MOT (Kime et al. 2001; Rurangwa et al. 2004). Both salmon and trout sperm swim with curved trajectories (Dziewulska et al. 2011; Kime et al. 2001) and VCL (measured in $\mu m\ s^{-1}$) provides the velocity of spermatozoa along the path trajectory (Rurangwa et al. 2004). VSL (also measured in $\mu m\ s^{-1}$) provides the velocity along the straight line path of the track (the distance between the start and end point); if the trajectory of the sperm is straight then VSL will equal VCL. The LIN is a useful measure of sperm trajectory (Rurangwa et al. 2004) and is simply VSL/VCL, the closer to 1 the straighter spermatozoa swim. The percentage of motile sperm in a males ejaculate is a good indicator of the number of sperm available to fertilise eggs and a good measure of a male's fertility. Throughout the experiments within this thesis, longevity (spermatozoa lifespan) of sperm was also deemed to be an important parameter to measure that could account for interspecific male differences. The other parameters automatically calculated by CASA have been found to be of little use when studying fish sperm function (Rurangwa et al. 2004). For this reason VCL, LIN, motility and sperm longevity motility traits were the main focus of sperm motility analysis throughout this thesis. Table 2.1.1 describes how each of these parameters is calculated.

Table 2.1.1: Motility parameters measured by the Hobson Sperm Tracker and by hand with details on how each of these parameters is calculated.

Motility Parameter	**Calculation**
VCL- Curvilinear velocity ($\mu m\ s^{-1}$)	The sum of the incremental distances moved in each frame along the sampled path divided by total time of the track.
LIN- linearity (%)	The straight line distance between start and end points divided by the sum of incremental distances along the actual path or VSL/VCL*100.
% Mot- percentage motility (%)	This is the number of motile sperm within the field of analysis divided by the sum of all sperm in the field multiplied by 100. Motility was manually calculated by freezing the video image as soon as it stabilised. Vibrating sperm with no progressive motility were considered to be immobile.
Longevity	Whilst the sperm tracker is capable of recording longevity, the videos analysed in this thesis were not tracked until sperm movement ended. Longevity was subsequently calculated manually. The video was stopped when sperm showed no more progressive forward motion. Time from activation until this cessation of movement was used as the lifespan of the spermatozoa. Vibrating sperm were considered to have ceased their progressive forward motion. When the majority of sperm reached this point the longevity measure ceased.

The other parameters were investigated in each experiment conducted, however as expected no significant relationships emerged, thus results were omitted from this thesis. The

parameter settings for the Hobson Sperm Tracker were already saved within the tracker for salmon and trout from previous work. The "trail draw" facility, which tracks the trail of the sperm across the screen, was set to track sperm trails for 4 seconds for observation on tracking, allowing necessary adjustments to the parameters (Yeates, 2005). The tracker was set to operate at a frame of 50 Hz and the "minimum track point" setting was 50 frames. The "search radius" used was 8.13 µm-10.56 µm and the "threshold" set to +30/100 with objective at x40 (Yeates 2005). Salmonid sperm is short lived in water, c 30-60 seconds (Billard et al. 1986) and shows a marked decrease in fertilisation success after 10 seconds (Hoysak & Liley 2001), suggesting most of the activity occurs in the early stages. This is why it is very important to get a stable image as soon as possible after activation. Tracking was started 10 seconds after activation and tracking periods were set to 15 seconds (the shortest time the sperm tracker can calculate percentage of motile sperm). The most useful data is from the first tracking period, 5-20s after activation (Kime et al. 2001).

2.3.3. Sperm counts

Sperm were counted within 24 hours of stripping as sperm left sitting for several days begin to aggregate, inhibiting even spread within samples and accurate counts (S. Yeates personal communication). The cell density of a male's sperm sample was calculated using an improved Neubauer haemocytometer according to previously established protocols (Gage et al. 1998). Samples of sperm in extender were diluted in water and 15µl were transferred to the haemocytometer under the cover slip. After letting the sperm settle, cells in 4 areas were counted to give a mean. This mean was then multiplied by the dilution factor and volume of the sperm sample to give a male's sperm density. This allowed for comparisons of sperm numbers entering the competition from the two different males.

2.4. *In vitro* fertilisations and egg rearing

2.4.1. In vitro *fertilisations*

When studying the dynamics of fertilisation there is a need to recreate the typical environment gametes experience during reproduction, whilst still maintaining experimental control. Using external fertilisers, such as salmonids, provides a major advantage as one can often easily retrieve large amounts of gametes and have control over sperm concentrations, egg numbers, the time gametes enter the fertilisation and their duration within the fertilisation, thus allowing complete experimental control. External fertilising systems also eliminate any confounds the internal reproductive environment may have on fertilisation or sperm competition. The use of the salmonid system also provides the additional benefit of being able to accurately recreate the gametic micro-environment (Gage et al. 2004), which can be a particular challenge in experimental fertilisations of internally fertilising species. The methods of *in vitro* fertilisation used throughout this thesis allow for rapid simultaneous mixing of gametes, simulating the release of sperm over egg batches within salmon redds.

The following method is the basic set up for all *in vitro* fertilisations (IVF) experiments in this thesis, with manipulations to this general method detailed within individual chapters. IVF's were carried out in 500ml dry plastic beakers. Care was taken to ensure each beaker was free of moisture to avoid activating the eggs before addition of sperm and water. For means number and range of eggs used in each experiment are detailed in individual chapters.

2.4.2. Sperm competitions

Many of the experiments within this thesis have placed sperm from two males of different species in competition for a female's eggs, allowing the examination of paternity patterns under different conditions. Sperm competition experiments were carried out in the same way as above (see section 2.4.1), except that sperm from two males are in the IVF. To ensure milt from each male has equal chance of fertilising the eggs the two sperm samples are mixed together by gently pipetting up and down or introduced into the stream of river water being

poured over the eggs. Known volumes of sperm are used to allow parallel calculation of the number of sperm from each male entering the competition (see section 2.3.3) which can then be controlled for in statistical analysis.

2.4.3 Egg rearing

After fertilisation trials and sperm competitions, eggs were placed into individually numbered egg trays and placed in incubation troughs. The channels provide a constant supply of slow flowing oxygenated water at an average rate of 10 l/ min, in line with standard hatchery protocols. Eggs were reared between November and April of each spawning season. Temperatures ranged from 2.7-12.8 º C in 2008-09, with an average water temperature of 4.92 º C ± 0.18 (1 S.E.M), and between 9.9-2.5 in 2009-10, with an average water temperature of 4.9 º C ± 0.13 (1 S.E.M).

Eggs that are infertile or die during incubation can turn white, making them easy to distinguish from live eggs within the incubators. It is important that these eggs are removed regularly throughout the incubation period to prevent fungal infection which can be transmitted to live eggs and potentially confound results. Hatchery staff at NINA regularly treated all eggs in the hatchery with anti-fungal chemicals to combat fungal infections.

2.4.4. Egg scoring

Fertilisation success of males in monogamous fertilisation trials (in the absence of sperm competition) can be determined within a week of fertilisation by soaking eggs in a 5% acetic acid solution (Hoysak & Liley 2001). Temperature determines how quickly embryos can be detected using this method and eggs in these experiments were left to develop for 10 days before scoring. The eggs were placed in the acetic acid solution for approximately 15 minutes until the embryos within turn white and are easily differentiated from empty, unfertilised eggs. For each fertilisation cross the number of fertilised and unfertilised eggs

was counted. The number of successfully fertilised eggs was divided over the sum of fertilised and unfertilised eggs to give a proportion of a male's fertilisation success.

For sperm competitions DNA paternity analysis had to be carried out in order to detect the fertilisation success of each male within the competition. Sperm competition eggs were therefore left until hatching, or just before, and then preserved in 95% ethanol for subsequent DNA extraction and genotyping of offspring.

2.5. Paternity analysis

Microsatellites have become a common tool in assessing paternity and are now widely used tool to study populations as well as identify individuals. Microsatellite DNA analysis was used to assign paternity of offspring derived from sperm competitions to either of the two males in the competition. For each sperm competition, up to 27 offspring had their DNA extracted and were genotyped at 3 loci. All mothers and potential fathers were also genotyped by extracting DNA from tissue samples collected at stripping, allowing paternity to be unambiguously assigned to each offspring.

2.5.1. DNA extraction

DNA was extracted from adult fish using fin tissue collected at the time of stripping gametes, and from fin tissue of developing or hatched out offspring. A modified salt extraction technique (Aljanabi & Martinez 1997) was used to efficiently extract the DNA. Due to the large number of individuals to genotype, extractions were done in 96 well plates (ABgene, surrey, UK and STARLAB (UK) Ltd, Milton Keynes, UK). A small amount of tissue (*c.* 5mm) from each individual was placed in a well along with 50 µl of TEN buffer (400mM NaCl, 10mM Tris-HCL [pH 8], 2mM EDTA [pH 8] and 2%SDS [9:1]) and 2.5 µl of proteinase K (20 mg/µl). Plates were then incubated overnight at 55-60 ºC. After incubation 15 µl of 6mM NaCl solution was added to each well and centrifuged at 3000 rpm for 25 minutes; after which 14 µl of supernatant was transferred to a new plate with 30 µl of

100% cold ethanol. Plates were then left at -20 ºC for at least 1 hour then spun at 3000 rpm for 30 minutes. The supernatant was discarded and DNA pellets washed with 70% cold ethanol. Pellets were then dried at 50 ºC and 100 µl of dH20 was subsequently added to each well and plates left at 37 ºC to allow for re-suspension of the DNA pellet. Plates where then stored at -20 ºC until needed.

2.5.2. PCR

Paternity was assigned to offspring using 3 pure microsatellite loci, Ssa408, ssa410 and Ssa417 (Cairney et al. 2000). The primers used were chosen as they amplify in both Atlantic salmon and brown trout with polymorphism (Cairney et al. 2000). PCR was carried out in 10 µl volume reaction multiplexes containing; 1 µl of DNA (unspecified concentration), 5 µl of 2 x PCR Mastermix with 1.5mM $MgCl_2$ (ABgene), 0.95 µl of forward labelled primers (0.2 µl Ssa408, 0.3 µl Ssa417 and 0.45 µl Ssa410) and 095 µl reverse primers (same volumes). Primers were labelled with NED (Ssa408), FAM (Ssa410) and HEX (Ssa417) (Applied Biosystems). The PCR ran at an initial 3 minute denaturation at 94 ºC, preceding 29 denaturing (94 ºC for 15 s), annealing (61ºC for 15 s) and extension (72ºC for 15 s) cycles. Samples were finally incubated at 72 ºC for 30 minutes.

2.5.3. Genotyping

PCR products were run on an ABI3730 automated sequencer at the NERC Biomolecular Analysis Facility at the University of Sheffield. Samples were run with Genescan-500 ROX labelled size standard (Applied Biosystems). Fragment lengths of PCR products were determined using the genotyping software GeneMapper v4.0 (Applied Biosystems). Often only a single locus was needed to unambiguously assign paternity in each 2 male competition. More loci were needed when assigning paternity to F_2 individuals produced in Chapter 6. Parentage was assigned by comparing alleles of the mother and the two potential fathers to those of the offspring. Parentage was unambiguously assigned in all offspring.

Chapter 3

How is conspecific sperm precedence influenced when the introduction of hybridising sperm to *in vitro* fertilisation competitions is delayed?

3.1. Introduction

3.1.1. Sperm competition and sperm precedence

The reproductive success of individual males in most mating systems is influenced by both precopulatory and postcopulatory mechanisms of sexual selection (Andersson & Iwasa 1996; Andersson 1994; Birkhead & Møller 1998). Precopulatory adaptations such as courtship displays, dominant aggressive displays and mate guarding are known to play important roles in male mating success. However, we now recognise that this mating success does not necessarily translate into fertilisation success, due to the influence of the postcopulatory sperm competition and cryptic female choice mechanisms (Eberhard 1996; Parker 1970). These postcopulatory processes can have profound effects upon fertilisation success (Birkhead & Møller 1998; Birkhead & Pizzari 2002; Eberhard 1996), meaning males will be under direct selection to produce ejaculates that are favoured by the different mechanisms of sperm competition, and cryptic choice evolved by females. A recognised mechanism within sperm competition is the raffle principle (Parker 1982; Parker 1990; Parker 1998), where numerical superiority of an individual male's spermatozoa can provide him with the highest probability of fertilisation success. This raffle principle is recognised in passerine birds (Birkhead 1998a; Immler et al. 2011b), insects (Gage & Morrow 2003) and fish (Stoltz & Neff 2006), and explains why sperm are so numerous and tiny, and why anisogamy is maintained (Parker 1982). Although the raffle principle is fundamental to many mechanisms of sperm competition, relative sperm number does not always fully explain fertilisation success (Birkhead & Møller 1998; Simmons et al. 2003), and there are a number of other processes by which an individual male can achieve sperm precedence (Snook 2005).

Sperm precedence is usually measured experimentally as the proportion of offspring sired by the second male to mate, known as P2 (Boorman & Parker 1976). A large proportion of the literature on sperm precedence describes second (or last) male precedence, particularly in insects (Birkhead & Parker 1997; Gwynne 1984; Simmons 2001) and birds (Birkhead 1998a; Birkhead & Moller 1992; Briske 1996). Last male sperm precedence is also

described in other taxa, including marine invertebrates and mammals (Diesel 1990; Kraaijeveld-Smit et al. 2002) but research is less well established. However, first male sperm precedence is also expressed across both vertebrate and invertebrate taxa, including mammals, amphibians, butterflies and arachnids (Agoze et al. 1995; Jones et al. 2002; Lacey et al. 1997; Watson 1991). P2 has become a convenient measure of sperm competition, and therefore the selective forces acting on males and females to maximise their reproductive success; allowing the underlying mechanism of sperm competition to be researched. Parker (1970) argued that sperm competition will cause simultaneous selection on males to remove rivals sperm and to prevent their own sperm from being displaced. P2 can be viewed as a balance between these two selective forces (Birkhead & Parker 1997; Parker 1970). For example, a very high P2 where the last male to mate fertilises most of the a females eggs, as is found in many odonates (Córdoba-Aguilar et al. 2003; Waage 1984), will lead to selection on males to invest in postcopulatory guarding to protect his mating investment from being wiped out by a subsequent male (Waage 1984). By contrast, a low P2, as is found in salmon (Mjølenrød et al. 1998; Yeates et al. 2007), will lead to selection on males to be first to spawn when a female releases eggs, and on sperm to be effective at rapidly locating and fusing with the egg.

The structure and function of the fertilisation environment has a major influence upon sperm precedence, with females evolving sperm storage organs, or fertilisation mechanisms, that can spatially and temporally influence when and where fertilisation takes place relative to insemination (Pitnick et al. 2009). In addition to female-derived effects, sperm precedence in internal fertilisers can result from transference of proteins in the seminal fluid of an ejaculate. In *Drosophila*, accessory gland proteins can increase oviposition in a female and decrease her receptivity to subsequent mating, acting as a chemical mate guard (Wolfner 1997; Wolfner 2002). Seminal proteins can also disable and displace the previous male's sperm to result in a very high P2 (Clark et al. 1995). Copulatory plugs are a physical mate guarding mechanism employed by males to inhibit rivals, and are described in species of snake (Devine 1975; Shine et al. 2000), primate (Dixson 1998; Dixson & Anderson 2002) marine invertebrate (Barker 1994) and insect (Baer et al. 2001; Matsumoto & Suzuki 1992). A solid plug of material is deposited by the male to block the opening to the female reproductive tract, preventing further insemination by other males as well as preventing

sperm leakage. Copulatory plugs may have evolved through sexual selection to prevent females re-mating, thereby eliminating the cost associated with mate guarding and freeing males to re-mate more quickly with additional mates, increasing fitness (Shine et al. 2000). At the other end of the scale, males that encounter previously mated females have evolved physical mechanisms to replace rival males sperm with their own. Mechanisms of displacement include physical removal of sperm left by the previous male, as seen in insects (Boorman & Parker 1976; Córdoba-Aguilar et al. 2003; Xu & Wang 2010), and sperm repositioning; where males forces sperm of previous males into the far reaches of females storage organs far from the fertilisation site (Córdoba-Aguilar et al. 2003), in some cases sealing it off (Diesel 1990). It is clear from the complex structures of male and female genitalia that a range of mechanisms could exist to influence sperm precedence at this key stage in reproduction at the cryptic level of the gamete. Added complexity arises from evidence that females can influence paternity of their offspring through manipulation and selection of sperm (Eberhard 1996).

3.1.2. Sperm precedence in salmonids

In the past, evidence for sperm competition in the literature has largely focused on internally fertilising species, with work on external fertilisers restricted mostly to game theory (Ball & Parker 1996; Parker 1982; Parker et al. 1996). However, since evidence for mating effects and sperm competition in free spawning animals was seen, a shift to empirical studies of sperm competition in external spawners occurred (Balshine et al. 2001; Bishop et al. 2000; Boschetto et al. 2011; Byrne 2004; Byrne et al. 2003; Gage et al. 2004; Hoysak & Liley 2001; Hoysak et al. 2004; Marshall et al. 2004; Mjølenrød et al. 1998; Stoltz & Neff 2006; Taborsky 1998; Yeates et al. 2007; Yeates et al. 2009). The advantage of studying sperm precedence in external fertilisers comes from the fact that experiments can be more focused on understanding the specific roles of sperm and egg within fertilisation dynamics, without confounds from the whole animal. Compared to internal fertilising species, external fertilisers have fundamental differences in reproduction that lead to different forces of selection. Notably there is no female reproductive tract to influence the sperm, meaning that there is no potential for direct female control of sperm, although selection from eggs and

reproductive fluids released with them can still occur (Rosengrave et al. 2008; Simmons et al. 2009). In external fertilisation, male reproductive success is much more heavily dependent upon the competitive quality of a male's gametes (Benzie & Dixon 1994; Casselman et al. 2006; Williams & Bentley 2002). This is particularly so in salmonids where ageing ejaculates lead to reduced fertilisation success (Mjølenrød et al. 1998), and faster more competitive sperm increase a male's paternity (Gage et al. 2004; Liljedal et al. 2008). Higher sperm ATP content has even been linked to increased fertilisation success (Vladić et al. 2010). These features mean that external fertilisers can present useful models for understanding the specific roles of gamete form and function in the control of fertilisation precedence without confounds of internal biology.

Salmonids present an excellent model system for studying what factors drive sperm precedence. Mating patterns generally involve multiple males for a single female's eggs resulting in strong sperm competition, with one recent paternity study of natural spawning in Atlantic salmon revealing an average of 8, and up to 16, different males involved in fertilising a female's egg batches (Weir et al. 2010). In addition to intense postcopulatory sexual selection, the ability to recover gametes and perform *in vitro* fertilisation experiments under conditions that mimic the natural gametic microenvironment provide useful practical aspects to this model system (Gage et al. 2004). Previous work on mechanisms of sperm precedence in salmon has demonstrated that, when sperm are experimentally released simultaneously, it is a male's average sperm velocity that explains significant variation in sperm competition success (Gage et al. 2004). This result suggested an important mechanism of sperm competition involves a race by sperm to locate the ovum and then swim down the single micropyle (Kobayashi & Yamamoto 1981; Yanagimachi et al. 1992), thereby selecting for fast-swimming sperm. Similar results have been found in other fish species (Casselman et al. 2006; Gasparini et al. 2010; Liljedal et al. 2008). A logical prediction from these initial findings was that if there was a short delay in the introduction of a male's sperm into any competition, it should experience a disadvantage in fertilisation success. Delays of 2 seconds in both sockeye and Atlantic salmon indeed revealed a significant decrease in the delayed male's fertilisation success (Hoysak et al. 2004; Yeates et al. 2007). The findings from *in vitro* sperm competition experiments suggest that the spatial positioning and timing of sperm release relative to female spawning is of great importance (Hoysak & Liley 2001;

Mjølenrød et al. 1998). Similar observations of fertilisation dynamics in medaka show that micropyles are occupied within the first 6 seconds of sperm release (Iwamatsu et al. 1991), again suggesting the importance of a race to locate and penetrate the egg micropyle.

There is therefore established evidence for first-male sperm precedence in salmon, with a disadvantage for delayed males in fertilisation success. In this chapter, I examine how a timing delay impacts on a conspecific sperm competition advantage recorded within salmon-trout hybridisation. In Chapter 1, I presented preliminary data on fertilisation and sperm competition dynamics between salmon and trout (figure 1.4.1, Yeates unpublished data), which identified a clear conspecific sperm competition advantage when sperm from both species were introduced simultaneously to eggs. Conspecific sperm precedence (CSP) is the non-random utilisation of sperm from conspecific males when both conspecific and heterospecific males mate with a female (Howard 1999), regardless of male order (discussed in 1.1.2). CSP could result from conspecific sperm out-competing heterospecific sperm because heterospecific sperm are less compatible in terms of physiological or chemical adaptations and fail to fertilise eggs (Howard 1999). CSP represent a form of cryptic female choice in salmonids: both heterospecific and conspecific sperm are capable of fertilising eggs in the absence of sperm competition, but when females or eggs are provided with a 'choice' of sperm, the conspecific sperm are favoured in fertilisation success. Here, the salmon-trout *in vitro* fertilisation system presents an opportunity to establish whether CSP is mediated by eggs or by sperm, because one can run reciprocally balanced sperm competition experiments where individual males are analysed in both the conspecific and heterospecific 'role', and determine sperm competition success via parentage assignment. Having already established that conspecific sperm have a ~70% sperm competition advantage when sperm number and release are equalised (figure 1.4.1), I use a 2 second experimental delay for one of the male's sperm to the fertilisation set, in order to gain further insight into the mechanism of CSP within this hybridising system. In the absence of hybridisation, two-male competitions in salmon where one male is given a 2 second delay results in a disadvantage in fertilisation success to about 20% of the egg batch (Yeates et al. 2007). The general hypothesis under test in this chapter therefore is that a 2 second timing advantage will not allow heterospecific sperm the majority of paternity, due to the presence of CSP. The extent of any differences in CSP under sperm delay versus simultaneous release may provide

insight into the dynamics of sperm competition, cryptic female choice, and differential fertilisation success.

3.2. Methods

Sperm competition trials were run in 2008 at the Norwegian Institute of Nature Research (NINA) research station in Ims, Southwestern Norway using Atlantic salmon and brown trout originally sourced from the Figgjo River, and raised in the hatchery to spawning age (see 2.2). Adult salmon and trout were randomly assigned to experimental groups each containing 1 female and 1 male of both species. 15 such groups were constructed, giving a total of 15 females Atlantic salmon, 15 male Atlantic salmon, 15 female brown trout and 15 male brown trout (60 fish in total). When adults came into spawning condition (evidenced by free-running milt and eggs), they were stripped of their gametes using established hatchery methods (2.2). Because the fish within any one group did not necessarily ripen simultaneously, stripped gametes were then stored on ice in oxygenated bags until all four fish had ripened. In any one group, the maximum time required for storage of gametes until needed for fertilisation experiments was 5 days. Storage of gametes in this manner does not compromise quality or fertility (Einum & Fleming 2000), and the balanced paired experimental design meant that storage should not directionally confound the comparisons of fertilisation precedence. A fin clip was taken from each fish as it was stripped and placed in 95% ethanol for genotyping and parental assignment.

3.2.1. In vitro *sperm competition experiments*

Sperm competition trials were carried out in IVF beakers as detailed in section 2.4.1, except that this experiment introduced sperm into a flow of river water poured over the eggs with a 2 second delay in sperm ‘release’ between the two males (figure 3.2.1). The sperm competition experimental design is presented in table 3.2.1, with each group of four fish allowing the creation of competitions between salmon and trout sperm for either salmon or trout eggs, with a 2 second delay in the introduction of either male’s sperm. The experiment

is then repeated, with the order of sperm introduction reversed (Table 3.2.1). Thus, CSP for both salmon and trout can be compared within the same male according to whether his sperm is first or second to be introduced to the *in vitro* competition.

Figure 3.2.1: An illustration of the specific *in vitro* fertilisation conditions used for this experiment.

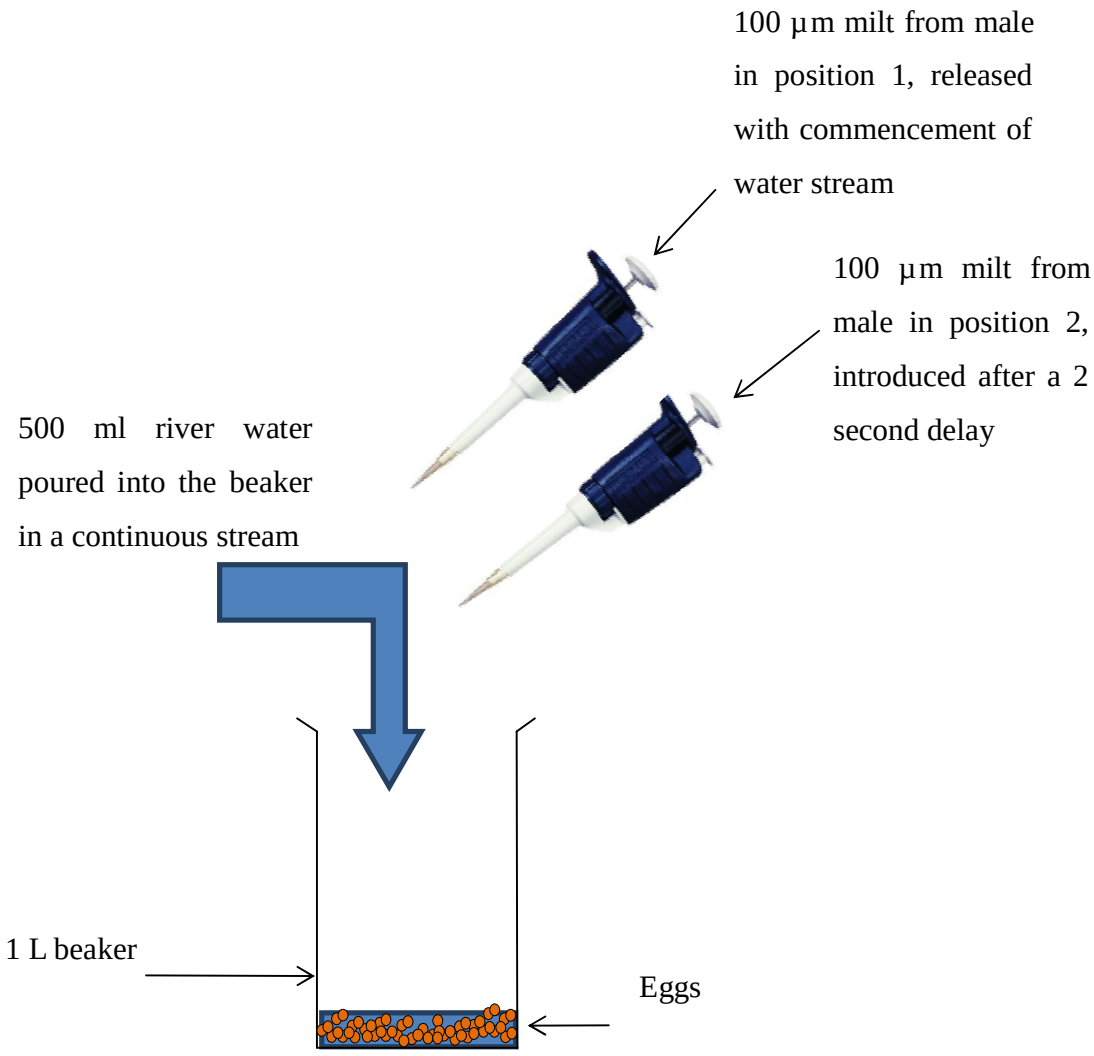


Table 3.2.1: Experimental design of crosses created in each group of fish. Either Atlantic salmon or brown trout eggs from the group's females were competed for by the Atlantic salmon and brown trout males in the group. Sperm from the male in position 1 was introduced to the stream of water poured over the eggs on stream commencement and milt from the male in position 2 was introduced to the stream 2 seconds later. Sperm from the same males was then introduced into the competition in reversed order to create a reciprocally balanced design. N is the number of replicates.

Eggs	**Male in Position 1**		**Male in Position 2**	**n**
Salmon	Salmon		Trout†	15
Salmon	Trout†	**2 second**	Salmon	15
Trout	Trout	**delay**	Salmon†	15
Trout	Salmon†		Trout	15

†heterospecific male in the cross

On average 71 ± 8 (S.D) (range 57-106) salmon or trout eggs were placed into a dry IVF beaker, after which 500 ml of river water at 10.23 ºC ± 0.4 (S.D) was gently poured over the eggs in a steady stream. At the commencement of the water stream, 100 µl of milt mixed in extender (see 2.3) from one male was added to the stream of water in a single plunge of a Gilson pipette, this male was in position 1 (P1); 2 seconds later, 100 µl of milt in extender from the second male was added in the same way, this male was in position P2 (P2). The sperm competition was then run again with each male now in the opposite position.

After the river water and milt from both males had been added, the eggs were left for at least 3 minutes for full fertilisation to take place. A photograph of the egg batch was then taken which allowed subsequent counting, and then the fertilised eggs were added to an individually-numbered incubator to develop (see 2.4.3). This process was then repeated

across all 15 groups. At egg hatch, approximately 120 days later, the emerging alevins were humanely killed in 95% ethanol to preserve tissue for later microsatellite paternity analysis.

3.2.2. Microsatellite paternity analysis

Between 16 and 27 offspring were genotyped per cross, with an average of 21 offspring per cross typed. Paternity analysis of offspring was determined as described in section 2.5. Paternity was assigned using 3 pure microsatellite loci, Ssa408, ssa410 and Ssa417 (Cairney et al. 2000) with calibrated PCR. PCR products were run on an ABI3730 automated sequencer at the NERC Biomolecular Analysis Facility at the University of Sheffield. Samples were run with Genescan-500 ROX labelled size standard (Applied Biosystems). Fragment lengths of PCR products were determined using the genotyping software GeneMapper v4.0 (Applied Biosystems). Once parental genotypes were known, a single locus was usually needed to unambiguously assign paternity in each 2-male hybridisation competition, and paternity was unambiguously assigned in all cases.

3.2.3. Sperm trait analysis

Spermatozoa motility traits were analysed in the same way as described in 2.3. Sperm motility traits were analysed on sperm subsamples within 30 minutes of their respective *in vitro* sperm competition trials. The parameters suggested most useful for studying sperm motility in fish are curvilinear velocity (VCL µm s^{-1}), straight line velocity VSL µm s^{-1}), sperm path linearity (LIN %), sperm motility (% MOT) (Kime et al. 2001; Rurangwa et al. 2004). As well as this, previous studies of sperm competition in salmonids have shown that a spermatozoa's curvilinear velocity is important in achieving fertilisation success (Gage et al. 2004; Liljedal et al. 2008). As this study was investigating sperm competition between two species of salmonids sperm VCL and MOT were deemed important to measure, along with sperm longevity (lifespan). For details on CASA and how traits were measured and calculated see 2.3.

3.2.4. Statistical analysis

Each male was used twice in a sperm competition trial, once in each timing position, creating a paired design. Paternity success, the proportion of eggs fertilised, of males was square root arcsine transformed in an attempt to achieve normality. However transformation did not produce a normally distributed data set with homogenous variance, so non-parametric statistics were used.

Average paternity success of individual males within a competition was compared when the hybridising male was P1 competing for salmon eggs and when the hybridising male was P2 competing for salmon eggs, using Wilcoxon rank sum tests. The same tests were used to compare paternity success between males when competing for trout eggs. The Wilcoxon rank sum test is the non-parametric equivalent of the independent samples t-test, using medians, and is based on the magnitude of difference between pairs of data points. The null hypothesis was that a delay of 2 seconds between sperm release does not affect fertilisation success of hybridising and conspecific males.

Spearman rank correlations were carried out to determine whether correlations existed between relative paternity success (proportion of eggs fertilised) and sperm motility traits (measured in 3.2.3) of all males, when the hybridising males were P1 in the sperm competitions. This was done to see if there was any association between increased sperm motility traits and increased fertilisation success. The same tests were carried out on the paternity success of all males and their sperm motility traits when conspecific males were P1 in sperm competitions. This created 3 multiple comparisons within a data set for each treatment (hybridising male P1, conspecific male P1) as paternity success was compared to three sperm traits in 3 individual spearman rank regressions. Fertilisation data used when the hybridising male was in P1 and the conspecific male was in P1 were from separate sperm competitions, and therefore were independent data for analysis (see table 3.2.1). To account for multiple comparisons of paternity success with multiple sperm traits, a Dunn-Sidak correction factor was used to adjust significance threshold to try and avoid type I errors.

$$\alpha_i = 1 - (1 - \alpha_e)^{1/n}$$

Where n is the number of comparisons and α_e is the 0.05 confidence level. Within each hybridising and pure males sperm traits there are 3 multiple comparisons, making the new significance threshold 0.016.

All statistical analyses were done using the R Project for Statistical Computing software version 2.13.1.

3.3. Results

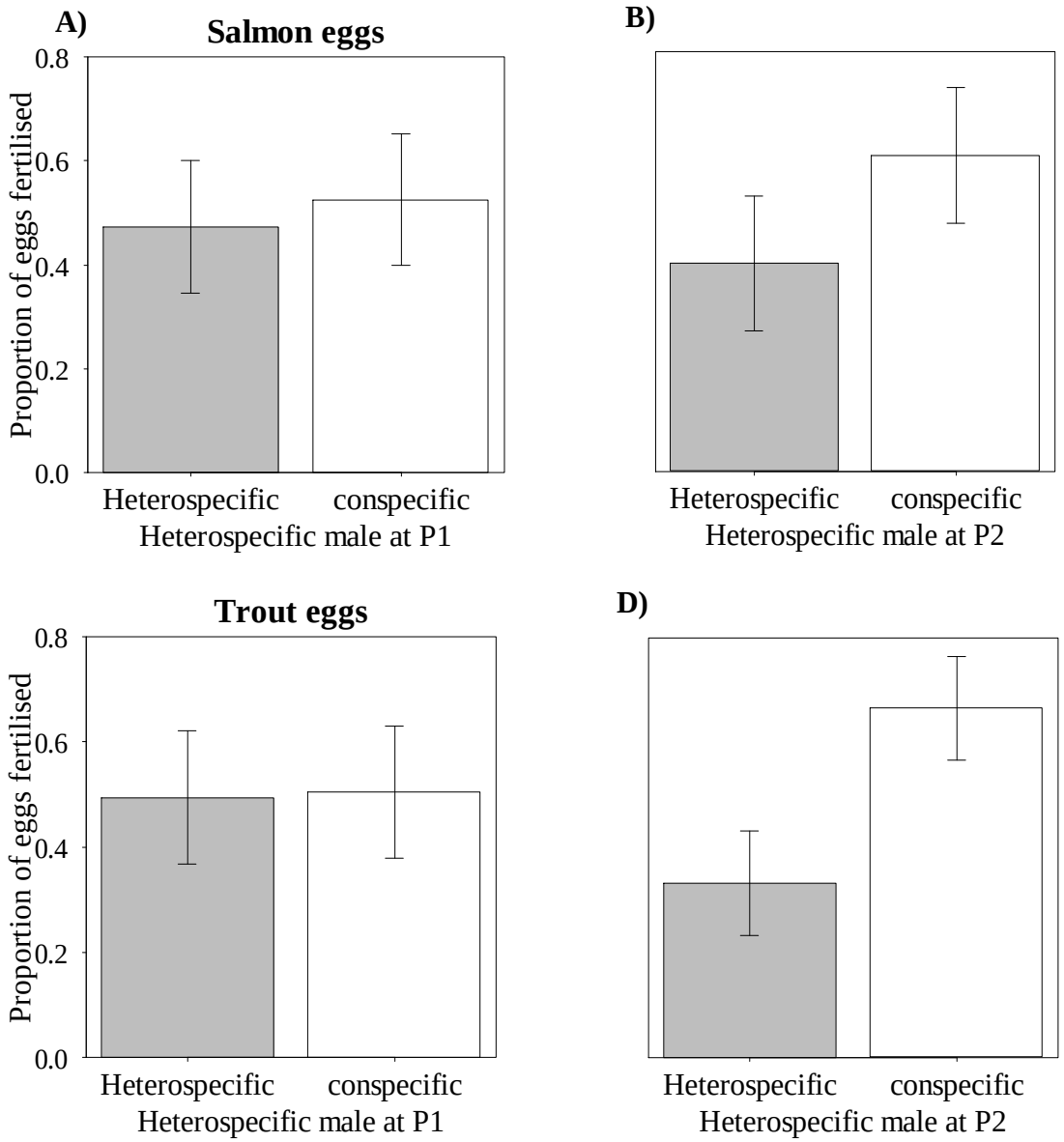
Overall results showed that a 2 second delay in the release of one of the competing males' sperm significantly influenced conspecific sperm precedence in salmon-trout hybridisations. A 2 second delay in the introduction of conspecific sperm to the competition did not give hybridising males the expected first-male sperm precedence advantage, but a 2 second delay in the introduction of heterospecific sperm gave hybridising males a significant fertilisation disadvantage.

3.3.1. In vitro *sperm competitions with a 2 second delay in one males sperm release*

When competing for Atlantic salmon eggs, hybridising males (brown trout) and conspecific males (Atlantic salmon) had no significant difference in their fertilisation success when the hybridising male was in P1, with a 2 second timing advantage (W= 90, P= 0.3613). Hybridising males gained an average of 48 ± 13 (1 S.E) % fertilisation success compared to 52 ± 12 (1 S.E) for conspecific males. The pattern of shared paternity was repeated when males competed for brown trout eggs with the hybridising male in P1 (figure 3.2.1c), with the hybridising males (Atlantic salmon) and conspecific males (brown trout) having no significant difference in paternity success (W=116, P=0.902). The hybridising males gained 49 ± 13 (1 SE) % paternity on average and conspecific males 51 ± 12 (1 SE) %.

However, when heterospecific males competed in P2, with a 2 second delay, their fertilisation success severely decreased as conspecific males' sperm took significant advantage of being first to enter the competition. When fertilising salmon eggs the heterospecific trout male gained significantly less paternity (W= 56, P=0.02), with 39 ± 13 (1 SE) % on average compared to a 61 ±13 (1 SE) % average for conspecific salmon males. Similarly, when fertilising trout eggs the heterospecific male salmon showed a significant fertilisation disadvantage in P2 (W= 21, P= <0.0001), fertilising only 34 ± 10 (1 SE) % of eggs on average compared to 66 ± 10 (1 SE) % achieved by conspecific trout.

Figure 3.3.1: Comparisons of fertilisation success between hybridising and conspecific males when A) hybridising males (open bars) are P1 in the competition with conspecific males (grey bars) for salmon eggs. B) Hybridising males are P2 in the competition with conspecific males for salmon eggs. C) Hybridising males are P1 in the competition with conspecific males for trout eggs. D) Hybridising males at P2 in competition with conspecific males for trout eggs.



3.3.2. Relative fertilisation success and sperm motility behaviour

Sperm motility traits are a good indicator of the quality of a male's sperm (Kime et al. 2001), and studies in fish, including Atlantic salmon, have shown that sperm velocity has an important bearing on a male's sperm competition success (Casselman et al. 2006; Gage et al. 2004; Gasparini et al. 2010; Liljedal et al. 2008). Spearman rank correlations between male fertilisation success (proportion of eggs fertilised) under sperm competition, and sperm motility traits described in 3.2.3 were used to see if there was any association between increased sperm motility traits and increased fertilisation success. Tests were carried out on fertilisation success when hybridising males were P1 in a sperm competition (Figure 3.3.2) and when conspecific males were P1 (Figure 3.3.3). Correlations of fertilisation success and sperm motility traits when hybridising males were in P1 showed no association between VCL, sperm longevity or percentage motile sperm ($S = 30306.32$, $P = 0.61$; $S = 26663.3$, $P = 0.17$; $S = 36487.41$, $P = 0.91$ respectively). Similarly, correlations showed no association with sperm traits and increasing fertilisation success when pure males were in the P1 position (VCL: $S = 32502.99$, $P = 0.99$; longevity: $S = 32152.29$, $P = 0.93$; percentage motile: $S = 34523.8$ $P = 0.76$).

Figure 3.3.2: Scatter plots of paternity success (proportion of eggs fertilised) for males in sperm competitions when the hybridising male (open circles) was P1 with a 2 second timing advantage over the conspecific males (closed squares), against measured sperm motility traits; A) curvilinear velocity, B) Sperm longevity. C) Percentage of motile sperm.

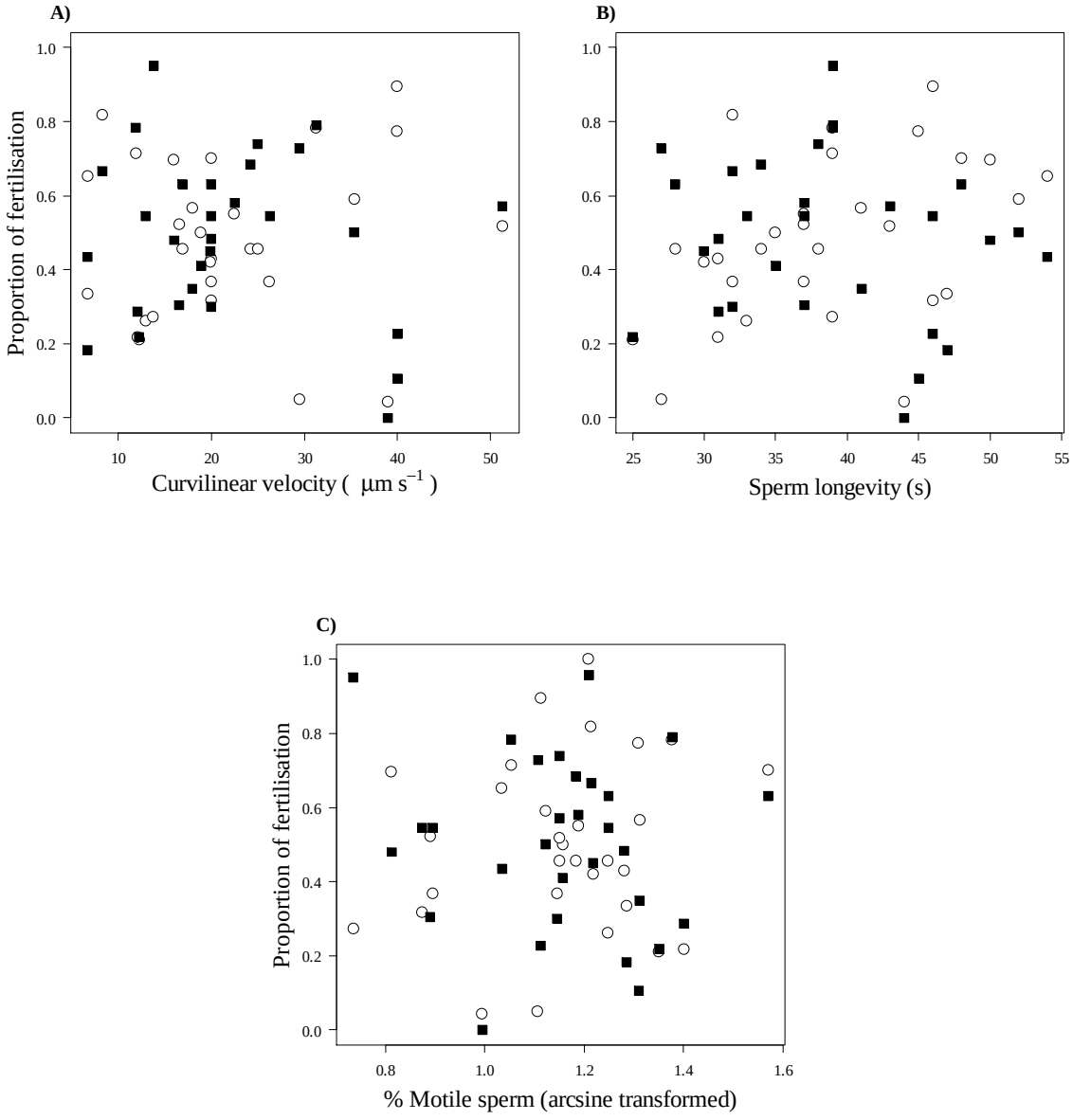
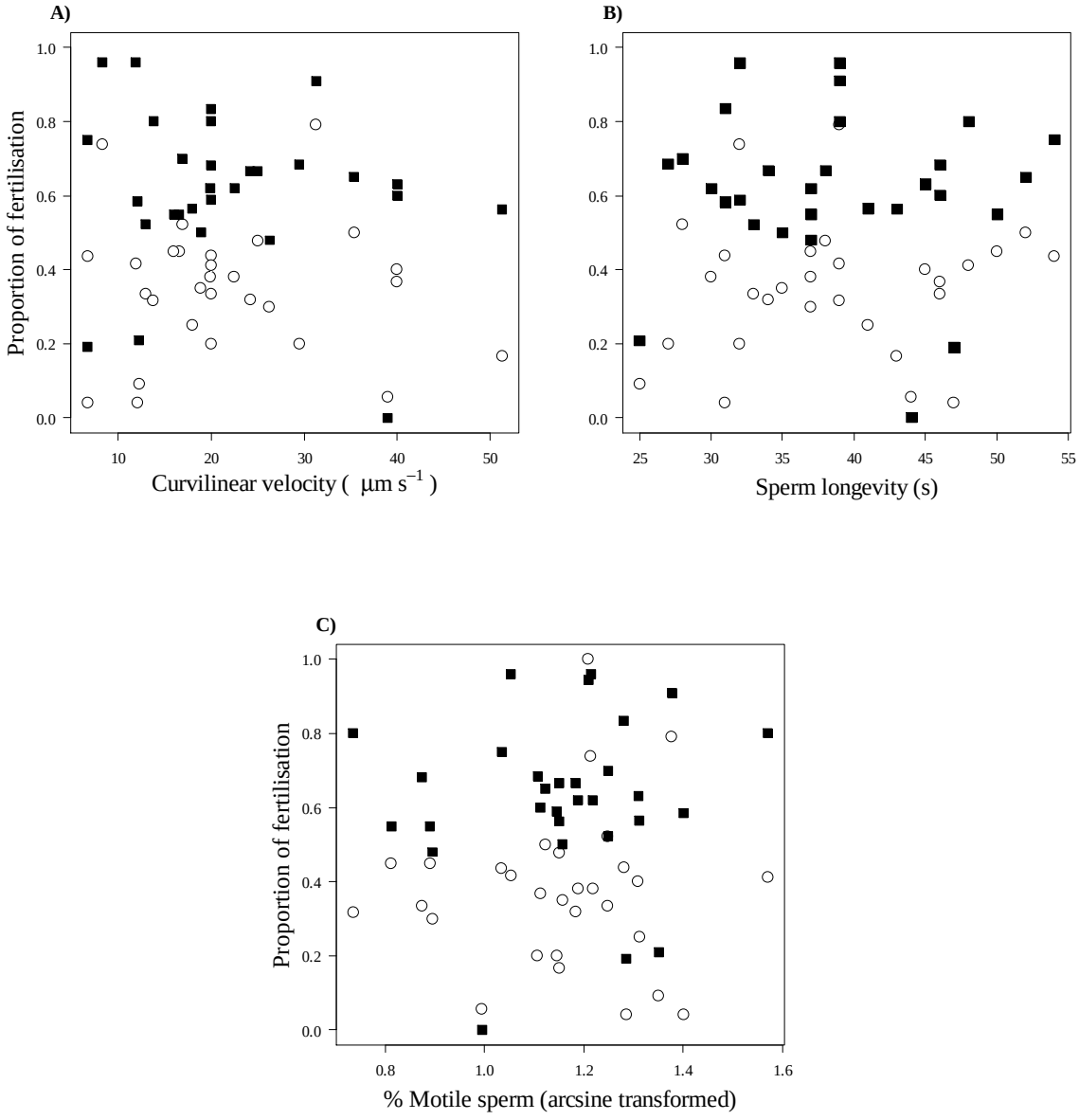


Figure 3.3.3: Scatter plots of paternity success (proportion of eggs fertilised) for males in sperm competitions when the conspecific male (blue circles) was P1 with a 2 second timing advantage over the hybridising male (red circles) against measured sperm motility traits; A) curvilinear velocity, B) Sperm longevity. C) Percentage of motile sperm.



3.4. Discussion

Previous work has shown that Atlantic salmon males with a 2 second advantage over competitors in a two male *in vitro* sperm competition father significantly more paternity of a female's offspring (Yeates et al. 2007). This finding highlighted how important the relative timing of sperm release is for the reproductive fitness of Atlantic salmon males. The aim of my study here was to explore whether this first-male sperm precedence is affected when sperm from one of the competing males is from a heterospecific male attempting to hybridise. Preliminary findings for this project (Chapter 1) showed that there is a conspecific sperm competition advantage in the salmon-trout hybridisation system, such that heterospecific sperm tend to fertilise about 37% of the eggs when competing against sperm from a conspecific male (S. Yeates unpublished data). This chapter examines the consequences for conspecific sperm precedence (CSP) when a timing delay is introduced. Results showed that the CSP advantage effectively removed any first-male sperm precedence for heterospecific sperm when in position one (P1) with a 2 second advantage, with both males in the competition achieving near equal 50% fertilisation. This situation is then significantly reversed when sperm from heterospecific males are in position 2 (P2), with a 2 second delay. Now sperm from conspecific males achieves significantly higher paternity and heterospecific males suffer a significant decrease in fertilisation success. Salmon sperm fertilised 34% of trout eggs when P2 in competition with trout sperm, and trout sperm 39% of salmon eggs when P2 on competition with salmon sperm. Because the patterns are similar from both salmon and trout perspectives, I will discuss the results for both species together as either heterospecific or conspecific males in fertilisation competitions.

One of the aims of this experiment was to determine whether the CSP advantage we see under simultaneous sperm release was altered in any particular manner by delayed sperm introduction. If eggs possess mechanisms that allow them to preferentially 'select' conspecific sperm through the fertilisation process, we might expect a timing delay to reveal this For example, we might suppose a 2 second delay to impart a very strong fertilisation disadvantage for heterospecific sperm if conspecific sperm are able to populate the micropyle first. Similarly, if eggs possess a filtering mechanism for conspecific sperm that

takes place over a longer time period than the rapid fertilisation dynamics we see in salmonids (Hoysak & Liley 2001; Liley et al. 2002), then we might expect conspecific sperm to achieve precedence, even though they are disadvantaged in the second-male P2 competitive position (Yeates et al. 2007). My results suggest that a passive choice mechanism may exist within salmon-trout hybridisation, and that a simple explanation can be offered. When heterospecific sperm are first to enter the fertilisation competition, they probably gain advantage during the first few seconds of the fertilisation process through sole access to the eggs; but when conspecific species' sperm enter the competition, any first-male advantage to heterospecific sperm is countered by the CSP advantage. By contrast, when heterospecific sperm enter the competition after conspecific sperm, the heterospecific sperm retain the lower second-male paternity seen in Atlantic salmon sperm precedence, while the conspecific sperm achieve the significant majority of fertilisations. Fertilisation in salmon is markedly different to other species in that there is no acrosome reaction, and thus no fusing of sperm to egg membrane (Hoysak & Liley 2001). In salmonid fertilisations, sperm enter the egg through a single small hole called the micropyle (Yanagimachi et al. 1992). The micropyle diameter is close to a single sperm head width, so that the order of sperm entry could be critical to fertilisation success (Yanagimachi et al. 1992), and my results support this model of salmonid fertilisation. Evidence in some fish show that the micropyle can be occupied in the first 6 seconds (Iwamatsu et al. 1991), making timing in fertilisation crucial to success, with a delay of mere seconds meaning the difference between paternity gained or lost for a male (Hoysak et al. 2004; Yeates et al. 2007).

When sperm precedence has been observed in intraspecific salmon mating previously, there has been variation across males, with some males fertilising the majority of eggs regardless of the timing position (Mjølenrød et al. 1998; Withler & Beacham 1994; Yeates et al. 2007). This suggests that sperm quality, as well as timing and position of a male play a large role in sperm competition success. Because of this, sperm traits were measured in conjunction with sperm competitions to examine whether sperm motility could explain any differences between species, or mating order in terms of relative fertilisation success. Sperm velocity has been shown to be an important trait for sperm competition success in Atlantic salmon when sperm from competing males are released simultaneously, and relative numbers between males show low variation (Gage et al. 2004). In nature, salmon males experience reduced

sperm precedence as the number of spawnings they have participated in increases due to the quality of their ejaculate diminishing (Mjølenrød et al. 1998). However, for the majority, especially at the beginning of the spawning season, salmon that gain access to eggs first enjoy paternity precedence, with dominant males achieving >80% of paternity (Mjølenrød et al. 1998; Yeates et al. 2007). I therefore examined the possibility that variance between sperm motility traits of speed, percentage motility, or longevity influenced fertilisation success of males in competition as hybridising, or pure males when P1. However, no clear relationships between sperm traits and fertilisation success were evident. It is possible that the experimental controls of mating order and heterospecific versus pure status, within relatively small spawning groups (N=15) had overpowered any specific effect of sperm motility on fertilisation success. In addition, it is possible that my methods of sperm analyses were not able to measure the critical initial first few seconds of activation when sperm are at their fastest velocity and when the majority of fertilisations take place (Hoysak & Liley 2001). In sockeye salmon >80% fertilisation occurs in the first 5 seconds (Hoysak & Liley 2001). The method of recording sperm motility used in this study meant the first 5 seconds of motility after activation was missed, potentially allowing for critical velocity measures to be lost.

In contrast to findings here, there are a number of example systems of CSP where heterospecific males achieve a significant disadvantage even if they gain primary access to the female. In the ground crickets *Allonemobius fasciatus* and *A. socius*, reproductive isolation only exists via CSP, and regardless of the order with which a female is mated the majority of offspring are sired by the conspecific male (Gregory & Howard 1994; Howard et al. 1998). This strong conspecific precedence persists even when a female is mated to a heterospecific male multiple times, and a conspecific only once (cited in Howard 1999). In sea urchins and abalone, externally fertilising marine invertebrates, there is strong species specificity between sperm and eggs, although hybridisation can take place (Metz et al. 1994; Shaw et al. 1994). In both organisms, the mechanism of CSP is well described and controlled by sperm proteins that bind with, and penetrate, the egg vitelline envelope (Metz et al. 1994; Shaw et al. 1994). In both groups, hybridising sperm can penetrate the egg, but do so with far less efficiency and speed, giving conspecific sperm a significant advantage. The rapid sperm-egg association in salmonids through a micropyle clearly provides females

with a lower discriminatory capacity against potentially hybridising sperm than these other systems. Despite the relatively low discrimination with other systems, my results and those of S. Yeates demonstrate clear evidence of some CSP at the gamete level, which I investigate further in the next chapter.

While hybridisation is a well-established phenomenon between salmon and trout in the wild (Hórreo et al. 2011), it is not clear whether hybridisation occurs in the presence of both conspecific and heterospecific males. Yet, if heterospecific males can increase their share of paternity when they have a first-male timing advantage it could have negative impacts on the fitness of the heterospecific male and female, as well as impacting on population genetic structure. My results showed that while not achieving the majority of paternity when P1, heterospecific males were able to gain on average an extra ~20% paternity over that gained in competition with simultaneous sperm release. Sneaking Atlantic salmon males and trout are a likely route of hybridisation between these species (Garcia-Vazquez et al. 2002; Gephard et al. 2000). Brown trout mostly spawn in single male female pairs due to the aggressive nature of brown trout males (Garcia-Vazquez et al. 2002), but evidence suggests that some hybridisation does naturally occur (Gephard et al. 2000; Hartley 1996; Jansson & Ost 1997; McGowan & Davidson 1992b). However, if Atlantic salmon mature parr were able to sneak into brown trout spawning, they could potentially decrease the paternity of brown trout males by 50% in achieving close proximity and thus first access to the eggs. Similarly, reproductively active male trout could engage in salmon spawning (Garcia-Vazquez et al. 2001). Mature salmon parr have been shown to have superior ejaculate quality, with higher levels of motile sperm with increased ATP content, compared to anadromous males (Vladić & Jävri 2001). Parr also win the majority of paternity under *in vitro* sperm competitions, with their higher levels of ATP (i.e. higher energy reserves) being linked to fertilisation success (Vladić et al. 2010). In cases where salmon parr are able to sneak in to brown trout spawnings, their superior spermatozoa could allow significant fertilisation success, despite losing out to CSP. Repeating these present experiments with mature Atlantic salmon parr and brown trout would answer this question.

Large size and aggression in salmon and trout has been shown to play an important role in breeding success of anadromous males (Fleming 1996; Jones & Ball 1954) by increasing

proximity to the female, and allowing primary access to the eggs. Brown trout, as mentioned, are highly aggressive in spawning with some fights between males lasting more than ten minutes (Jones & Ball 1954). If brown trout males were able to gain first access to Atlantic salmon females they could decrease the paternity, and thus fitness, of subordinate Atlantic salmon males. This could have significant implications in declining Atlantic salmon populations and be of particular relevance where the two species are forced together through reduced spawning grounds and stocking activities, and when population levels of either species become polarized (Castillo et al. 2008; Hindar & Balstad 1994; Jansson & Ost 1997). However, mating behaviour of both males and females will play a role in how successful hybrid males are. In natural spawning experiments Atlantic salmon males were shown to chase away brown trout males and hybridisation only occurred in the absence of conspecific males, females also altered their spawning behaviour by delaying gamete release and only mated with trout of intermediate size (Beall et al. 1997). These changes in spawning behaviour could mean that any advantage in paternity hybrid males may have had by spawning first with females would become irrelevant. Nevertheless, only a small number of spawning studies have been done and this present work would benefit from increased sample size and more *in vivo* spawning experiments under varying population density conditions to assess the risks of hybridisation.

Chapter 4

Ovarian fluid mediates conspecific sperm precedence in salmon-trout hybridisation

4.1. Introduction

With the emergence of sperm competition research (Parker 1970) came the realisation that postcopulatory sexual selection can be a powerful force shaping the evolution of reproductive physiology and behaviour (Birkhead 1998b; Birkhead & Parker 1997). Female promiscuity leads to sexual selection persisting after copulation, as gametes from multiple individuals compete for fertilisation success. The previous chapter discussed how male fertilisation success in competition can depend upon relative timing of gamete introduction, and in this chapter I specifically examine how females might control differential fertilisation success. Postcopulatory sexual selection does not only take the form of sperm competition, there is also potential for cryptic female choice (CFC) (Birkhead & Pizzari 2002). CFC was initially defined by Eberhard (1996) as a "female controlled process or structure that selectively biases paternity to conspecific males with a particular trait". This definition only covers CFC that occurs by active female choice, ignoring passive mechanisms such as genetic incompatibilities, and encompassing behavioural traits that are not essentially cryptic (Birkhead 2000). Pitnick and Brown (2000) later revised the definition to ''non-random paternity biases resulting from female morphology, physiology, or behaviour that occur after coupling". By removing the concept of control from the definition the authors removed the implication that females have absolute authority over a male's sperm, encompassing both passive and active control mechanisms to give a more objective definition of CFC (Birkhead 2000; Pitnick & Brown 2000). Under this definition, only female mediated processes that generate sexual selection need be demonstrated in order to qualify as CFC (Pitnick & Brown 2000). However, the mechanisms that produce CFC are less well defined than mechanisms of sperm competition (Birkhead 1998b; Eberhard 1996), mainly because it has been a particular challenge to isolate female-controlled effects, if they exist, from the recognised male-controlled effects within differential fertilisation (Birkhead 2000; Pilastro et al. 2004; Pitnick & Brown 2000). A confounding problem also arises because traits that allow males to win sperm competitions and fertilisations may co-vary with female preference. Finally, there may be theoretical challenges in setting criteria for when selection on CFC should exist (Birkhead 1998b). In this chapter, I use the hybridisation and external fertilisation system of salmon and trout to examine the potential for CFC.

4.1.1. Mechanisms of cryptic female choice

Despite the problems with isolating CFC there has been an increasing body of evidence for female differential control of fertilisation at the level of the gamete (Reviews by: Birkhead 1998b; Eberhard 1996; Holman & Snook 2006). A number of potential mechanisms exist for a female's ability to bias which individual male fertilises her eggs. One such mechanism is the physical manipulation of ejaculates (Matthias 2010; Pizzari & Birkhead 2000). In wild fowl, the majority of copulations are forced by males; yet females have been shown to consistently bias the retention of sperm within their reproductive tract to favour males with preferred dominant phenotypes (Pizzari & Birkhead 2000). By retaining a larger volume of sperm from a preferred male, a female increases the chance that male has of successfully fertilising her ova. Females achieve this active selection through manipulating behaviour of dominant males to reduce the occurrence of insemination by subordinates, or failing this, females will differentially eject sperm according to a male's status, retaining dominate male ejaculates (Pizzari & Birkhead 2000). Further physical female manipulation of male fertilisation success can occur in the form of differential sperm storage (Eberhard 1996; Fedina 2007; Hellriegel & Bernasconi 2000). Internal muscular activity of female yellow dung flies has been shown to effect sperm storage and separation of ejaculates (Hellriegel & Bernasconi 2000). Differential sperm transfer in females of the red flour beetle, *Tribolium castaneum*, biased toward males with higher quality sperm, was also suggested to be a function of female bursa muscular contractions (Fedina 2007). Other non-physical mechanisms of female control have also been postulated, including: varying oviposition timing, where a females delay in oviposition can lead to reduced fertilisation success of a male (Barbosa 2009), and manipulation of clutch size, where females can differentially bias the number of eggs they lay depending on which males they mate with (Arnqvist & Danielsson 1999b; Bretman et al. 2006; Thornhill 1983).

One situation where CFC could play an important role is under inbreeding avoidance. Females could use postcopulatory mechanisms to avoid the associated costs of inbreeding (Bretman et al. 2004; Tregenza & Wedell 2002), where there may be greater selection on females to avoid inbreeding (Pizzari et al. 2004). In plants that grow bisexual flowers, where male and female reproductive organs are in close proximity, pollen has a tendency to land on

the stigma of the same flower it originated from. If the plants own pollen subsequently germinates, the resulting fertilisation would lead to inbred progeny and reductions in the population's genetic variation. However, mechanisms have evolved to allow plants to avoid self-fertilisation (Kao & McCubbin 1996), and one such strategy is self-incompatibility. In plants that only have a single morphology for their flowers the most common incompatibility is termed gametophytic, where incompatibility is based on the genotype of the pollen. In the Solanaceae plant family a single polymorphic locus, S, that determines pollination has been identified (Kao & McCubbin 1996). For example, if a plant carries the alleles S_1 and S_2 then its pollen will either carry the S_1 or the S_2 allele. If pollen from this plant then land on its own stigma, germination is stopped as the pistil recognises either the S_1 or S_2 allele as originating from itself. If pollen originating from a plant with the genotype S_2S_3 lands on the style, then pollen with the haplotype S_2 will again be rejected as self, due to the shared nature of the allele; however, the S_3 allele will match neither from the original plant, and thus be allowed to fully germinate through the style to the ovary and complete fertilisation (Kao & McCubbin 1996).

In animals, similar mechanisms are thought to exist, but are not as well characterised as those in plants. Female sand lizards, *Lacerta agilis*, show no precopulatory mate choice (Olsson et al. 1996a), often leading to copulations with closely related males. Olsson et al. (1996b) found that closely related males sire a significantly lower proportion of offspring than distantly related ones, suggesting that females can actively select sperm of less related males, and thereby avoid the cost of inbreeding (Olsson et al. 1996a; Olsson et al. 1996b). A similar phenomenon has been identified in the field cricket, *Gryllus bimaculatus*, where evidence for postcopulatory female avoidance of inbreeding has been experimentally demonstrated (Bretman et al. 2004; Tregenza & Wedell 2002), and mediated by differential sperm storage (Bretman et al. 2004). Similar findings for differential sperm storage have been recognised in the related cricket, *Teleogryllus commodus*. In this species sperm storage in females was found to be correlated with the attractiveness of the male providing the sperm (Hall et al. 2010). A positive link between male attractiveness and differential fertilisation success has been empirically observed in the guppy, *Poecilia reticulata*. As in other guppy species females are attracted to mate with more colourful males (Godin & Dugatkin 1996; Houde 1987). To isolate the link between male colouration and sperm competitiveness,

Evans et al. (2003) controlled for ejaculate size, and through artificial insemination found postcopulatory selection for male phenotypic traits that reflected those preferred by females in precopulatory mate choice. This suggested colourful males produce competitively superior ejaculates, or that females encourage brighter males to fertilise their eggs (Evans et al. 2003). In later work, Pilastro et al. (2004) found that females can bias the number of sperm transferred toward more attractive males. The authors suggested that CFC may refine male fertilisation bias, seen under sperm competition, in favour of more colourful males (Pilastro et al. 2004).

There is, therefore, evidence for the existence of CFC. Yet, a large literature identifies the problems in experimentally isolating this phenomenon (Andersson & Simmons 2006; Birkhead 1998b; Kempenaers et al. 2000; Pitnick & Brown 2000; Telford & Jennions 1998). Under internal fertilisation, experimental control of male and female effects in this complex environment clearly creates a major challenge (Howard 1999). Female reproductive tracts are often complex environments that can, particularly in the case of mammals, bring about physiological changes in sperm (Eady 2001; Howard 1999 and references therein), and potentially conceal CFC mechanisms, making it hard to observe and manipulate them (Eberhard 1996; Engqvist & Sauer 2003). Because of this, teasing apart the role of sperm from the role of eggs or the reproductive tract in apparent cases of CFC can be difficult for species with internal fertilisation (Engqvist & Sauer 2003; Evans et al. 2003; Pilastro et al. 2004; Pizzari & Birkhead 2000; Ward 2000). Under external fertilisation, the gametic environment is simpler and under less female (or male) control. This allows tighter experimental control to be achieved, making CFC, if present, easier to identify. I therefore select the freshwater fertilisation environment of salmonids to test for the existence of female control of differential fertilisation at the postcopulatory level of the gamete.

4.1.2. Conspecific Sperm Precedence

As discussed above, there is a clearly potential for CFC under postcopulatory inbreeding avoidance. At the other end of the genetic relatedness spectrum there will also be selection for hybridisation avoidance, potentially at the level of the gamete if other barriers to fertilisation have been overcome. Evidence strongly suggests that conspecific gamete

precedence can play an important role in isolating closely related species, and occurs in both plants and animals (reviewed by Howard 1999). In animals conspecific sperm precedence (CSP) is defined as the non-random utilisation of sperm from conspecific males by a female when she mates with both conspecific and heterospecific males (Howard 1999). CSP can represent a form of CFC, as in some cases females are potentially using fertilisation mechanisms to avoid fertilisation by heterospecific sperm, or promote fertilisation by conspecific sperm, when such a choice exists (Geyer & Palumbi 2005; Rugman-Jones & Eady 2007). Work prior to this thesis has already established that CSP exists in salmon-trout hybridisation when sperm volume and release time are equalised (figure 1.4.1), with both salmon and trout males achieving significantly more fertilisations when competing for conspecific eggs. In the previous chapter I showed that CSP is maintained when conspecific males have a 2 second advantage in sperm release. Further to this, heterospecific males are unable to gain significant paternity when they themselves have the 2 second advantage, suggesting some mechanism of differential selection comes into operation when conspecific sperm enter the sperm competition. In this study, I aim to investigate the underlying mechanism of the CSP observed in this system, and explore whether this is driven by female control.

There are many examples of CSP in nature, with examples primarily from insects (Fricke & Arnqvist 2004; Hewitt et al. 1989; Howard et al. 1998; Price 1997; Robinson et al. 1994), but also in free-spawning invertebrates (Geyer & Palumbi 2005; Harper & Hart 2005; Kresge et al. 2000; Metz et al. 1994; Vacquier et al. 1990) and fish (Immler et al. 2011a; Mendelson et al. 2007). An early example of CSP showed females of 2 karyotypically distinct populations of Alpine grasshopper, *Podisma pedestris,* had a highly significant propensity to be fertilised by males with the same karyotype as them, a trend which was still significant despite the fact that the first male to mate usually had sperm precedence (Hewitt et al. 1989). In many cases of interspecific hybridisation no choice fertilisation experiments show high interspecies fertilisation success, but low or no fertilisation success under sperm competition (Geyer & Palumbi 2005; Harper & Hart 2005; Rugman-Jones & Eady 2007; Wade et al. 1994). In such cases as these CSP is often only evident when eggs are exposed to both heterospecific and conspecific sperm, but the mechanism for CSP in most instances is not clear. Evidence from *Drosophila* suggest that a conspecific male's seminal fluid plays a

role in incapacitating and displacing a heterospecific male's sperm to achieve the majority of fertilisations, regardless of mating order, but that females mediate sperm competition (Price 1997). However, in free spawning invertebrates the mechanism of CSP is quite well established. In both sea urchins and abalone, sperm attach to the vitelline envelope surrounding the egg with sperm proteins called bindin and lysin, respectively (Glabe & Vacquier 1977; Metz et al. 1994; Palumbi 1992; Palumbi & Metz 1991; Vacquier et al. 1990; Vacquier & Lee 1993). Urchin and abalone eggs show high species specificity that is mediated by sperm bindin and lysin and egg receptors, leading to reproductive isolation between species (Metz et al. 1994; Swanson & Vacquier 1998; Vacquier et al. 1990). The evidence for CSP implies that it could be a significant factor in establishing or maintaining reproductive isolation, perhaps even playing a pivotal role in species formation in some cases (reviewed by Eady 2001; Lorch & Servedio 2007). While CSP could play an important part in reproductive isolation, it may simply be a by-product of male adaptations to sperm competition (Price et al. 2000); yet, on the other hand may be an important case of CFC. Theory predicts that female reproductive adaptations will primarily result in CSP (Price 1997), mainly owing to the fact that hybridisation can be much more costly to the reproductive fitness of females than males due to higher female investment (Parker & Partridge 1998), and would therefore be selected against.

4.1.3. External fertilisers as a model system

Pitnick and Brown (2000) recognised that by using external fertilisers to look for evidence of CFC it is possible to control male effects such as sperm number, velocity, motility and longevity. Salmonid fish are external fertilisers, therefore allowing experimental control of sperm and egg traits under *in vitro* fertilisation. Female salmonids also mate with multiple males (Fleming 1996; Martinez et al. 2000; Weir et al. 2010) and are under risk of hybridisation, creating clear criteria for the evolution of mechanisms that allow postcopulatory control of fertilisation. Recent work in salmonids has provided some evidence for CFC. Using a paired, within-female design, Yeates et al. (2009) showed that Atlantic salmon eggs are preferentially fertilised by sperm from males that are more similar to them at the major histocompatibility complex (MHC). There are two fundamental

biological traits that could play CFC roles in salmonid fertilisations: the ova and the ovarian fluid (OF) that surrounds the eggs and is released with them at spawning. The ovum in salmonids has a single micropyle which the sperm must access and enter to locate the female pronucleus. Mature salmon eggs (oocytes) are released from the ovaries into the body cavity where they are stored until they exit through the genital pore (Nagahama 1983). The eggs, and thus the mycropyle, are bathed in a considerable amount of fluid, between 10-30% of the eggs mass, which is thought to be secreted by the ovaries (Lahnsteiner et al. 1995a). OF has been shown to enhance motility traits of salmonid sperm by increasing velocity and the duration of progressive movement, or longevity (Dietrich et al. 2008; Lahnsteiner 2002; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007). OF can also enhance sperm traits in other species including cod, *Gadus morhua*, (Litvak & Trippel 1998) and the three spined stickleback, *Gasterosteus aculeatus* (Elofsson et al. 2003).

Explanations for the enhanced motility OF exerts on sperm include the inorganic composition of the fluid, as similar motility results were seen when brown trout sperm were activated in artificial saline solution (Lahnsteiner 2002). The pH of OF was also suggested to be the primary determinant of motility enhancement, with percentage motility, velocity and longevity of sperm being positivity correlated to pH, though it did not explain the variation found in the effect different OF had on sperm motility enhancement (Wojtczak et al. 2007). Lahnsteiner et al. (1995b) found intraspecific variation in the composition of OF from females of four salmonid species and postulated that the variation in the chemical make-up of OF between females could affect sperm traits of different males in different ways, potentially acting as a mechanism of CFC. Evidence for this has been seen in chinook salmon, *Oncorhynchus tshawytscha*, and Arctic char, *Salvelinus alpinus*, where the influence OF exerted upon a male was dependent upon which females OF its sperm are swimming in (Rosengrave et al. 2008; Urbach et al. 2005). In some cases within chinook salmon, an individual male's sperm velocity could be doubled in certain OF (Rosengrave et al. 2008). Because OF can be drained and rinsed from salmonid eggs before *in vitro* fertilisation, salmon-trout hybridisation presents an excellent opportunity to test the prediction that female salmonids use OF as a mechanism of CFC to promote the CSP observed in this system.

With OF potentially a mechanism for CFC within salmonids, it is also possible that OF could mediate CSP to reproductively isolate sympatric species. This could arise as a result of sperm being selected to be optimal for the chemical composition of their own species OF. In holothurian and ophiuroid starfish, ovarian extracts have been found to induce sperm motility and act as chemoattractants, guiding sperm toward the egg (Miller 1997). Many of these chemotactic reactions have been found to be species specific at the family level and in one case, in the genus *Bohadschia*, at the species level (Miller 1997), suggesting chemotaxis of sperm could play a role in reproductive isolation (Coyne & Orr 2004). Here I use hybridisation between the sympatric salmonids, Atlantic salmon and brown trout, two externally spawning teleost fish that undergo sperm competition, to investigate whether OF mediates CSP via its influence on sperm motility. Balanced fertilisation and sperm competition experiments were carried out to look for evidence of CSP under differential OF, and sperm traits were measured in a male's own and non-own species OF using computed assisted sperm analysis (CASA) to look for evidence that sperm are adapted to conspecific OF.

4.2. Methods

To investigate whether ovarian fluid (OF) could be a mechanism mediating reproductive isolation in salmon trout hybridisation, a series of *in vitro* experiments were set up to manipulate fertilisation conditions. Salmon and trout males and females were stripped of their gametes and stored until needed (see 2.2). In order to manipulate the egg and OF combinations in the experiments, salmon and trout eggs had to be separated from their OF. Eggs were poured into a fine mesh sieve over a dry plastic cup and letting the OF drain into the cup and each stored for later use. *In vitro* fertilisations were carried out using the same groups of fish used in Chapter 3 (n = 15 per cross), as described in section 2.4. The Transwell assays (see 4.2.4) used to investigate sperm attraction to OF were carried out in the spawning season of November 2011 and used hatchery reared salmon, derived from wild fish from the Figgjo River, and hatchery reared trout derived from wild fish from the Neva River, in the same catchment area. The particular conditions for each experiment are described in the sections below.

4.2.1. Fertilisation Trials

To explore whether OF had an effect on a male's ability to fertilise eggs in the absence of competition, male salmon and trout were crossed with salmon and trout females in either their own species (conspecific) or non-own species (heterospecific) OF (table 4.2.1).

Table 4.2.1: Fertilisation crosses of male salmon and trout with salmon and trout eggs in either conspecific or heterospecific (denoted in bold) ovarian fluid and n is the number of replicates.

Female	**Male**	**Ovarian Fluid**	**n**
Salmon	Salmon	Salmon	15
Salmon	Trout	**Salmon**	15
Salmon	Salmon	**Trout**	**15**
Salmon	Trout	Trout	15
Trout	Salmon	Salmon	15
Trout	Trout	**Salmon**	**15**
Trout	Salmon	**Trout**	**15**
Trout	Trout	Trout	15

For each cross an average of 67 ± 9 S.D (range 44-89) eggs were placed in a small mesh sieve and washed in an isotonic solution to rinse away any OF remaining on the surface of the eggs. The isotonic solution (90g NaCl in 10 l of water) allowed the eggs to be rinsed clean of OF whilst preventing egg activation prior to fertilisation. The sieve was then patted dry and the eggs added to a dry IVF beaker and 1ml of OF was pipetted directly onto the eggs. On the opposite side of the IVF beaker 15 µl of sperm was added with a pipette. To

carry out fertilisations, 100ml of river water was added to the beaker rapidly to ensure sufficient mixing of eggs and sperm and recreate the river bed fertilisation micro-environment (Gage et al. 2004; Yeates et al. 2009). After approximately 3 minutes, to allow for the eggs fertilisable period, eggs were photographed to allow the number to be subsequently counted, and added to an individually numbered incubator to develop. The process was repeated for all crosses. Once eggs had reached 10 days they were placed in 5% acetic acid to score developing embryos and give an individual male's fertilisation success (2.4.4).

4.2.2 Fertilisation rate

This experiment aimed to establish how quickly a male can fertilise heterospecific female eggs compared to conspecific eggs, and whether the fertilisation rate is influenced by OF. The basic protocol was to expose eggs to active sperm for limited periods, and explore the capacity of sperm to fertilise within that period. Eggs from each salmon female covered in her own OF, or trout OF were exposed to salmon or trout sperm for 2, 5 and 10 seconds (Table 4.2.2).

Table 4.2.2: Fertilisation crosses of male salmon and trout with salmon eggs in either the male's conspecific or heterospecific (denoted in bold) ovarian fluid. Each cross was repeated for egg exposure times of 2, 5 and 10 seconds. N is the replicates per exposure time.

Female	**Male**	**Ovarian fluid**	**Egg exposure time (s)**	**n per exposure time**
Salmon	Salmon	Salmon	2, 5 and 10	15
Salmon	Trout	**Salmon**	2, 5 and 10	15
Salmon	Salmon	**Trout**	2, 5 and 10	15
Salmon	Trout	Trout	2, 5 and 10	15

For each cross, at each time, an average of 63 ± 7 SD (range 46-104) eggs were washed in an isotonic solution to rinse away any OF remaining on the surface of the eggs (see above). Into a dry IVF beaker, 50µl of sperm was added with a pipette. In a separate beaker 1ml of salmon OF was added to 100ml of river water. The water containing the OF was then added to the sperm in the IVF beaker rapidly to ensure even mixing. Less than 1 second after sperm activation the eggs in the mesh sieve were dipped in to the river water for either 2, 5 or 10 seconds. After removal from the water the eggs were rinsed in 3 washes of river water to wash away any sperm on the surface of the eggs that could have fertilised eggs after the time constraints set by the experiment. Eggs were then photographed for counting and placed in individually numbered incubators to develop. The process was repeated for each cross. Fertility of each male in each cross was assessed using acetic acid (see above).

4.2.3. Sperm competition trials

In vitro sperm competition experiments were designed to test for any evidence of OF as a mechanism of conspecific sperm precedence (CSP). Eggs from salmon and trout were exposed to equal volumes of salmon and trout sperm simultaneously in the presence of either conspecific or heterospecific OF (table 4.2.3).

On average 77 ± 4 S.D (range 44-108) eggs were washed in isotonic solution to rinse away OF (4.2.1). The sieve was then patted dry and the eggs added to a dry IVF beaker with1ml of OF added to the eggs directly using a pipette. To the opposite side of the beaker 20 µl of salmon sperm and 20 µl trout sperm was added and mixed together with a pipette. To carry out fertilisations 100ml of river water was added to the beaker rapidly to ensure sufficient mixing of eggs and sperm and recreate the gametic fertilisation micro-environment. A photograph of the eggs was taken for counting. After approximately 3 minutes, eggs were added to an individually numbered incubator. This process was repeated for all crosses. Eggs from each cross were then left to develop until they hatched, when they were humanely killed and placed in 95% ethanol to preserve tissue for later microsatellite analysis.

Table 4.2.3: Competition crosses of male salmon and trout with either salmon or trout eggs in either the female's conspecific or heterospecific (denoted in bold) ovarian fluid and n is the number of replicates.

Female	**Males**	**Ovarian fluid**	**n**
Salmon	Salmon trout	Salmon	15
Salmon	Salmon trout	**Trout**	**15**
Trout	Salmon Trout	**Salmon**	**15**
Trout	Salmon trout	Trout	15

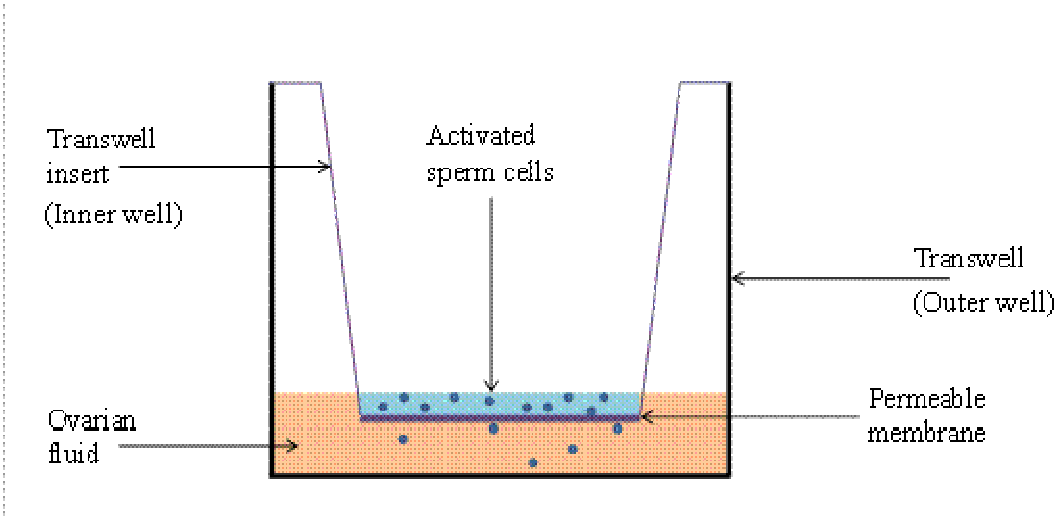
4.2.4. Sperm attraction to ovarian fluid

Modified Transwell migration assays (figure 4.2.1) were used to test if OF would act as a chemoattractant, with salmon and trout sperm migrating into conspecific species OF more than heterospecific species OF. Transwell assays are used to measure cell migration. The Transwells used in this experiment (Corning Life Sciences) consisted of an outer well within which sits a smaller insert. The insert contains a 10 µm thick permeable membrane at its base, with pores 8 µm in diameter at a density of 1 $x10^5$ cm^2. The chemoattractant, in this case OF, is placed in the bottom of the outer well and the test cells, in this case sperm, are placed in the insert where they can migrate through the membrane. Milt and eggs were stripped from the fish and stored as described in section 2.2., and OF was drained from the eggs as described in 4.2 of this chapter. All Transwell assays were carried out on the same day, avoiding confounding effects of gamete storage time.

For this experiment 200 µl of OF was placed in the outer well and 50 µl of river water in the outer well. 20 µl of sperm cells diluted in extender (see 2.3) was then activated in the inner well with river water, and the inner well then immediately placed into the outer well so that

the porous membrane came into contact with the ovarian fluid (figure 4.2.1). After 2 minutes the inner well was removed and the residual fluid on the base was washed off with a further 500 µl of water. The fluid in the outer well, now containing OF, water and any migrated sperm cells, was then pipetted into micro-centrifuge tubes for later analysis. Each individual male's sperm was tested twice with conspecific and twice with heterospecific species OF, plus a river water control. Numbers of sperm that had migrated into the lower well were counted using improved Neubauer haemocytometers as in section 2.3.3.

Figure 4.2.1: Diagram of the Transwell assay used to test and compare the migration of salmon and trout sperm to either conspecific or heterospecific species ovarian fluid.



4.2.5. Sperm trait analysis

To determine the effect of OF on sperm motility, computer assisted sperm analysis (CASA) was performed on sperm activated in its conspecific OF compared with water and heterospecific OF fluid. Traits shown to be important in assessing the quality of fish sperm recorded include curvilinear velocity (VCL), path linearity (LIN) and percentage motility (Kime et al. 2001; Rurangwa et al. 2004). Previous studies on the influence of OF on sperm motility behaviour have found that these traits are significantly enhanced by OF (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007). As well as this, the lifespan of spermatozoa was found to be increased by OF in salmonids (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie

2002; Urbach et al. 2005; Wojtczak et al. 2007) and other teleost fish (Elofsson et al. 2003; Litvak & Trippel 1998). For this reason the four traits (VCL, LIN, motility and longevity) were chosen to be the main focus of the sperm trait analysis. Other motility parameters were analysed for significant differences, but none were found and thus these analyses have been omitted from this thesis. Sperm motility for this experiment was recorded using the method described in section 2.3.

4.2.6. Microsatellite and paternity analysis

Between 13 and 26 offspring were genotyped per cross, with an average of 21 offspring per cross typed. Offspring were genotyped using the method described in section 2.5. PCR products were run on an ABI3730 automated sequencer at the NERC Biomolecular Analysis Facility at the University of Sheffield. Samples were run with Genescan-500 ROX labelled size standard (Applied Biosystems). Fragment lengths of PCR products were determined using the genotyping software GeneMapper v4.0 (Applied Biosystems). Once parental genotypes were known, often only a single locus was needed to unambiguously assign paternity in each 2 male competition involving Atlantic salmon and brown trout. All offspring were unambiguously assigned.

4.2.7. Statistical analysis

All statistical models were done using the R Project for Statistical Computing software version 2.13.1. For fertilisation trials, fertilisation rate and sperm competition experiments the proportion eggs fertilised by males was arcsine square root transformed to achieve normality (Shapiro-Wilks test) and homogenous distribution of variance (Bartlett's test).

For fertilisation trials, the fertilisation success of salmon males was compared when fertilising salmon eggs in conspecific and heterospecific OF using a paired t-test. The same test was used to compare salmon male's fertilisation success with trout eggs in conspecific and heterospecific OF. This was repeated for trout males. For fertilisation rates, the fertilisation success of salmon and trout males was compared across time in conspecific and

heterospecific OF using a 3 way ANOVA. Fixed factors were male species, egg exposure time and OF type. Non-significant interactions and factors were removed from the model in a stepwise fashion. For sperm competition trials, paternity success of the focal male in a competition was compared across females and OF type using an ANOVA, avoiding pseudoreplicated comparisons of the same male pairs. Egg type (female) and OF were fixed factors. Non-significant interactions and factors were removed from the model in a stepwise fashion.

Sperm counts from the Transwell assays were compared within salmon and trout using repeated measures ANOVAs to test for differences in sperm migrating through the membrane between conspecific and heterospecific ovarian fluid, as well as for differences between the two repeat measurements. Sperm count data was normally distributed with homogenous variance, after log transformation. Paired tests, comparing migration within salmon and trout males, was not used in favour of the repeated measures ANOVA in an attempt to capture any variation between repeats. Paired t-tests were used to see if there was a difference in the numbers of sperm moving into conspecific OF and water control, and heterospecific and water control, within a species.

Motility behaviour of sperm from salmon and trout males when activated in water was compared, within a species, to motility behaviour when activated in pure conspecific OF for four traits (VCL, LIN, longevity and motility). VCL and LIN were normality distributed with homogenous variances, as was proportion of motile sperm after arcsine square root transformation. Sperm longevity was normally distributed in all but salmon males concentrated OF, where even after transformation data were still not normal. Sperm traits were compared within males when sperm was activated in water and conspecific OF, and when they were activated in conspecific and heterospecific OF, using paired t-tests or Wilcoxon paired ranked sum test (the non-parametric equivalent of a paired t-test), when appropriate. Sperm count data were normally distributed and a 2 sampled t-test was carried out to analyse for differences between sperm numbers of salmon and trout males.

4.3. Results

Results of this study showed that salmon males had no significant difference in their ability to fertilise salmon or trout eggs when in conspecific compared to heterospecific ovarian fluid (OF). Trout males on the other hand, while having no significant difference in fertilisation success with salmon eggs in conspecific compared to heterospecific OF, had a significant fertilisation advantage with trout eggs in conspecific (trout female) OF. Under sperm competition, conspecific OF allowed a male significant paternity gains over the competing male, regardless of which species' eggs the males were competing for. OF was found to enhance sperm longevity and linearity above that of water, but there was no significant difference between any traits when a male's sperm was activated in conspecific compared to heterospecific OF.

4.3.1. Fertilisation success of Atlantic salmon and brown trout in conspecific and heterospecific ovarian fluid

Paired t-tests showed no difference in the fertilisation success of salmon males when fertilising conspecific salmon eggs in conspecific or heterospecific OF ($t = 1.13$, $df = 14$, $P = 0.27$). There was also no difference when fertilising heterospecific trout eggs ($t = 0.06$, $df = 14$, $P = 0.952$). For trout males, there was no difference in their fertilisation success in conspecific or heterospecific OF with heterospecific salmon eggs ($t = 1.62$, $df = 14$, $P = 0.125$), but there was a significance difference with conspecific trout eggs ($t = -3.37$, $df = 14$, $P = 0.004$), with trout males having a significantly higher fertilisation success with trout eggs in conspecific OF (figure 4.3.2).

Table 4.3.1: Mean percentage ± 1 S.E.M fertilisation success of salmon and trout males in single fertilisation crosses, with both salmon and trout eggs, in either conspecific or heterospecific ovarian fluid (OF).

Male	**Female**	**Mean ± S.E.M fertilisation success in conspecific OF**	**Mean ± S.E.M Fertilisation success in heterospecific OF**
Salmon	Salmon	82.9 ± 9	76.2 ± 10.9
Salmon	Trout	67.9 ± 12	62.9 ± 12.5
Trout	Salmon	79.9 ± 10.3	72.4 ± 11.5
Trout	Trout	77.7 ± 10.7	58.4 ± 12.7

Figure 4.3.1: Mean ± 1 S.E.M fertilisation success of Atlantic salmon males with salmon and trout eggs (n = 15) in either conspecific (open bars) or heterospecific (grey bars) ovarian fluid (OF).

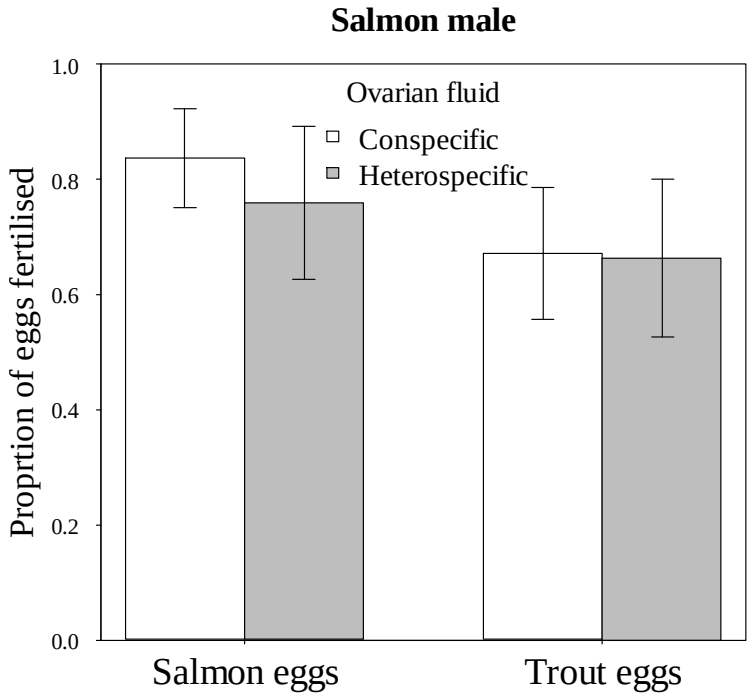
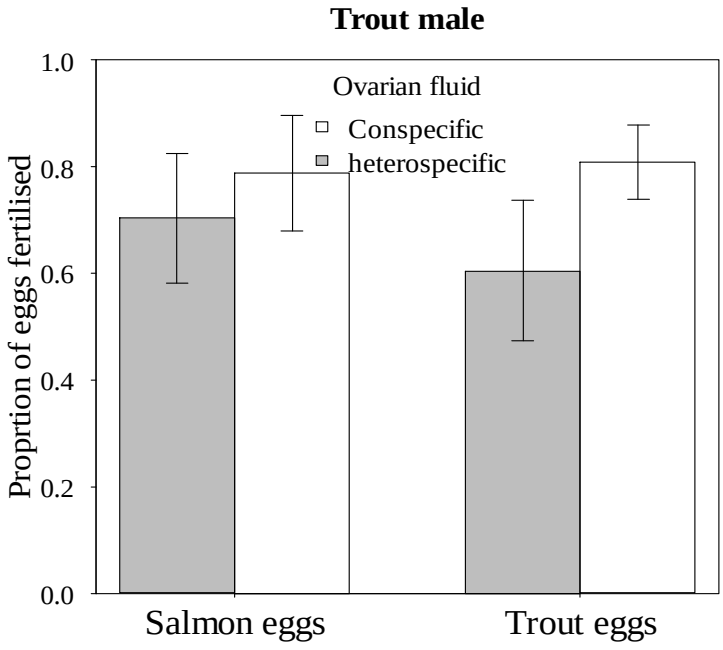


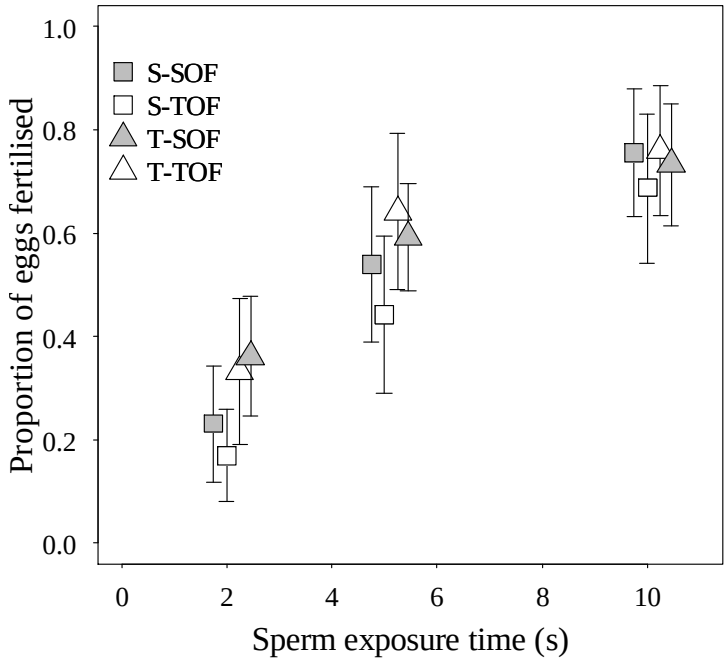
Figure 4.3.2: Mean ± 1 S.E.M fertilisation success of brown trout males with salmon or trout eggs (n = 15) in either conspecific (open bars) or heterospecific (grey bars) ovarian fluid.



4.3.2. Fertilisation rate of Atlantic salmon and brown trout males in conspecific and heterospecific ovarian fluid

Atlantic salmon eggs were exposed to salmon and trout sperm for different lengths of time in conspecific and heterospecific OF to see if OF influences the fertilisation rate of either species. ANOVA showed egg exposure time to sperm had a significant effect on the proportion of eggs a male fertilised, with fertilisation success increasing with increasing sperm exposure time ($F_{1,\ 177}$ = 102.96 P = <0.0001). Overall there was a significant difference in male fertilisation success ($F_{1,\ 177}$ = 9.09, P = 0.003), but there was no effect of OF on the ability of either salmon or trout sperm to fertilise salmon eggs at any time exposure (figure 4.3.3).

Figure 4.3.3: Mean fertilisation success ± 95% C.I of salmon and trout males with Atlantic salmon eggs (n=15) in conspecific or heterospecific ovarian fluid (OF) at 2, 5 or 10 seconds egg to sperm exposure. Eggs were fertilised by: salmon males with salmon OF (S-SOF, grey squares), salmon males with trout OF (S-TOF, open squares), trout males with salmon OF (T-SOF, grey triangles) and trout males with trout OF (T-TOF, open triangles).



4.3.3. Sperm competition success of Atlantic salmon and brown trout males in conspecific and heterospecific ovarian fluid

For each two male competition, the proportion of eggs fertilised (arcsine square root transformed) by the salmon male was compared in conspecific and heterospecific OF with conspecific (salmon) and heterospecific (trout) eggs. Salmon males, in sperm competition with trout males, had significantly higher fertilisation success when competing in conspecific OF ($F_{1,\ 56}$ = 4.85, P = 0.033) independent of which females egg they were fertilising ($F_{1,\ 56}$ = 0.96, P = 0.33, figure 4.3.4). When trout males are placed as the focal male in the analysis the results are the same. Trout males in competition with salmon males had significantly higher fertilisation in conspecific OF ($F_{1,\ 56}$ = 4.47, P = 0.038) regardless of the species eggs they were competing for ($F_{1,\ 56}$ = 0.82, P = 0.36).

Figure 4.3.4: Mean ± 1 S.E.M fertilisation success of Atlantic salmon males with salmon eggs and brown trout eggs (n = 15) in conspecific (open bars) and heterospecific (grey bars) ovarian fluid.

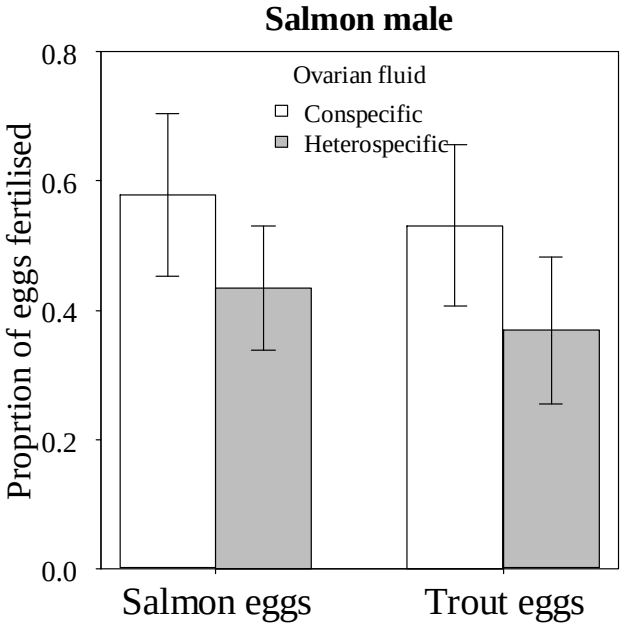
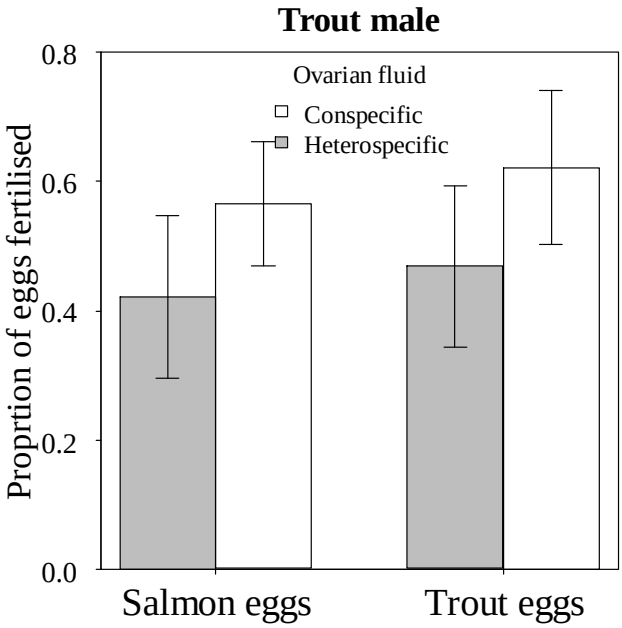


Figure 4.3.5: Mean ± 1 S.E.M fertilisation success of brown trout males with salmon and trout eggs (n=15) in conspecific (open bars) and heterospecific (grey bars) ovarian fluid.



4.3.4. Sperm attraction to ovarian fluid

Transwell cell migration assays were used to measure salmon and trout sperm migration through an 8 micron porous membrane into conspecific or heterospecific OF, and a water control. Repeated measures ANOVA showed that salmon males had significantly higher numbers of sperm migrating through the permeable membrane into conspecific OF compared to heterospecific OF ($F_{1, 17}$ = 48.26, P = <0.001). Trout males showed the same pattern, with significantly more sperm attracted to conspecific than heterospecific OF ($F_{1, 16}$ = 46.6, P = <0.001). Neither salmon nor trout males had significant variation between repeated sperm counts for each treatment. Paired t-tests were used to see if there was a difference in the numbers of sperm moving into conspecific OF and water control, and heterospecific and water control, within a species. Both salmon and trout had more sperm migrating into conspecific OF than into water, after correction for multiple comparisons (t = 3.1499, df = 17, P = 0.0058; t = 3.3166, df = 16, P = 0.0043 respectively). However, both males had no significant difference in the sperm migrating through the Transwell membrane into heterospecific OF and water (t = -1.923, df = 17, P = 0.071; t = -0.7649, df = 16, P = 0.45, salmon and trout males respectively).

Figure 4.3.6: Mean sperm ± 1 S.E.M of Atlantic salmon males in conspecific and heterospecific ovarian fluid (n = 18).

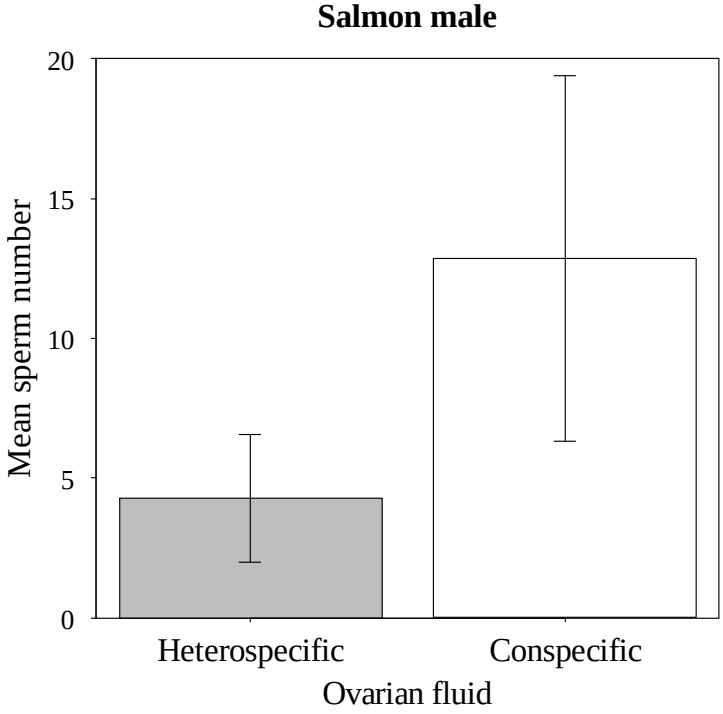
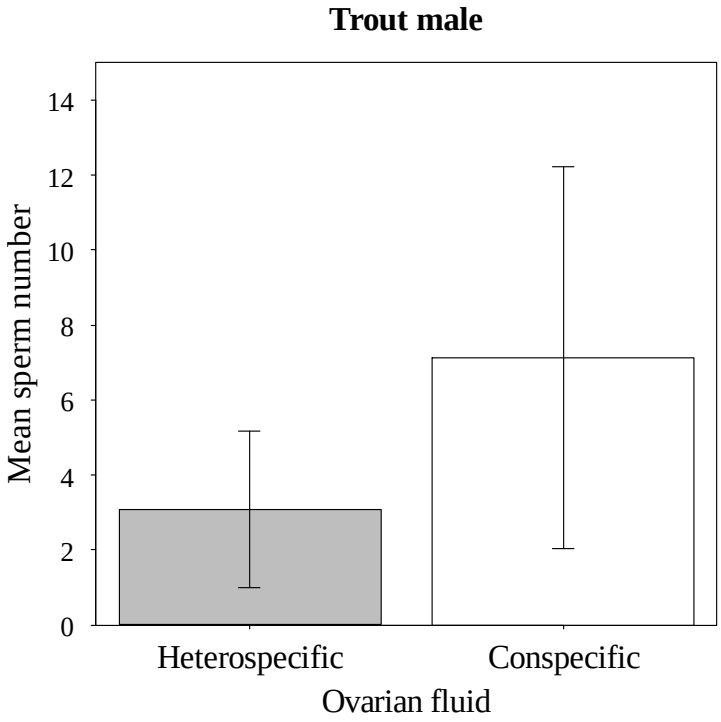


Figure 4.3.7: Mean sperm number ± S.E.M of brown trout males in Conspecific and heterospecific ovarian fluid (n=17).



4.3.5. Sperm motility traits and OF

Sperm from salmon and trout males were activated under a microscope in dilute and pure conspecific and heterospecific OF, as well as river water, and the behaviour of sperm recorded using CASA to measure motility traits. Sperm traits measured using both the Hobson Sperm Tracker and manual observation (see 2.3 for data collection and traits value calculations) were curvilinear velocity (VCL), linearity (LIN), sperm longevity and sperm motility. All sperm traits were checked for normality and compared within salmon and within trout males using either paired t-tests or Wilcoxon paired rank sum tests where appropriate. When comparing sperm motility traits activated in pure undiluted conspecific OF and activation in river water, sperm from both salmon and trout males showed a significant increase in sperm path linearity (paired t-test: $t = 4.8571$, $df = 15$, $P = 0.0002$, salmon; $t = 3.314$, $df = 14$, $P = 0.005$, trout). Both salmon and trout males also showed a significant increase in sperm longevity when activated with pure OF (Wilcoxon: $V = 136$, $df = 15$, $P = 0.0004$; $V = 120$, $df = 14$, $P = 0.0007$, respectively).

When sperm traits of salmon and trout males were compared between heterospecific and conspecific OF within males, there was no significant difference in any of the sperm traits (figure 4.3.9). Mean sperm number per µl for salmon and trout males were compared using an independent samples t test. There was no significant difference between males ($t = -1.2179$, $df = 29$, $P = 0.2335$).

Figure 4.3.8: Comparison of salmon (n = 15) and trout (n = 14) male sperm traits activated in conspecific ovarian fluid (open bars) and river water (grey bars). A) Mean VCL ± 1 S.E.M (µm s^{-1}). B) Mean LIN ± 1S.E.M. (%) C) Mean sperm longevity ± 1 S.E.M (s). D) Mean sperm motility ± 1S.E.M (%).

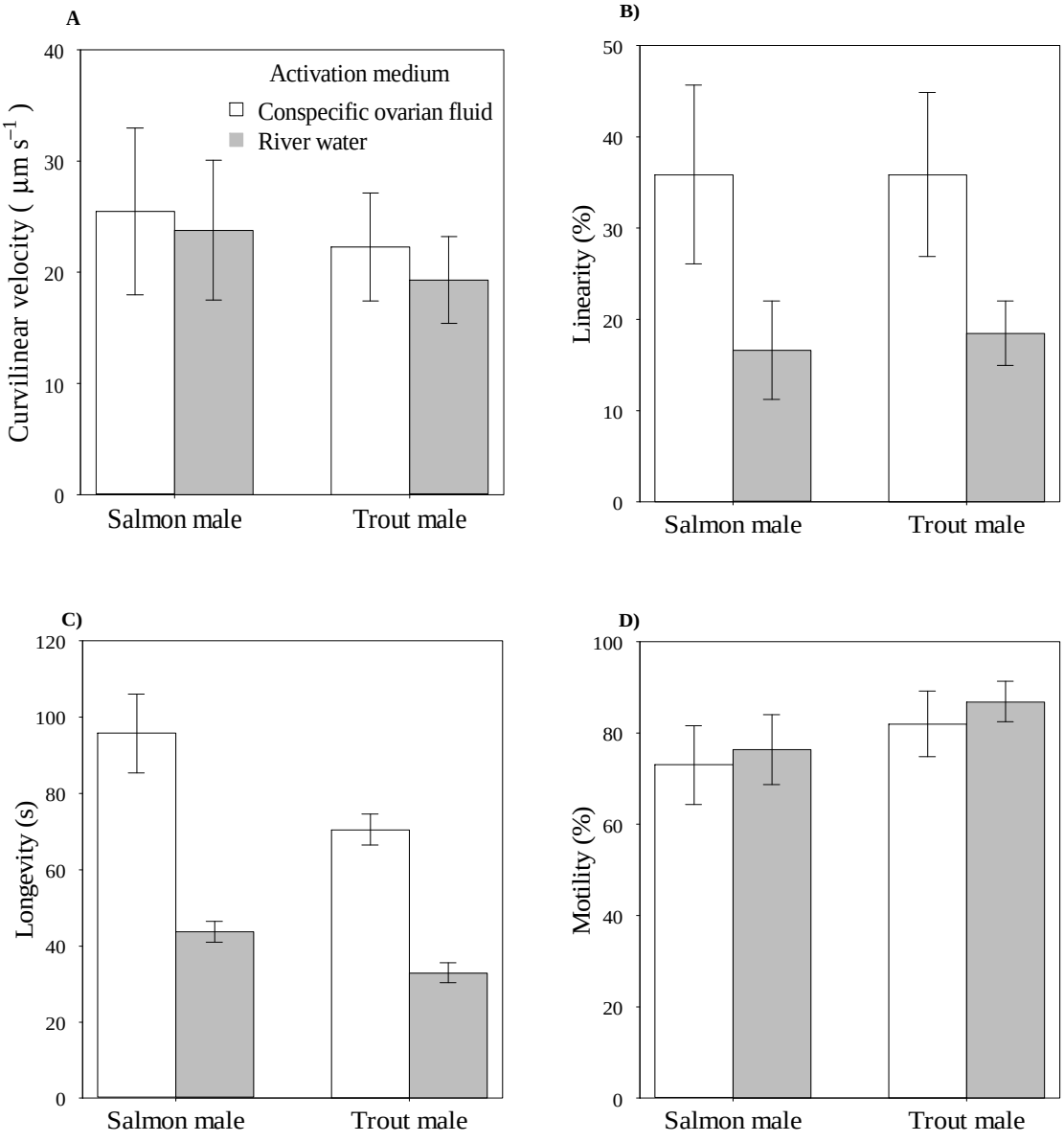
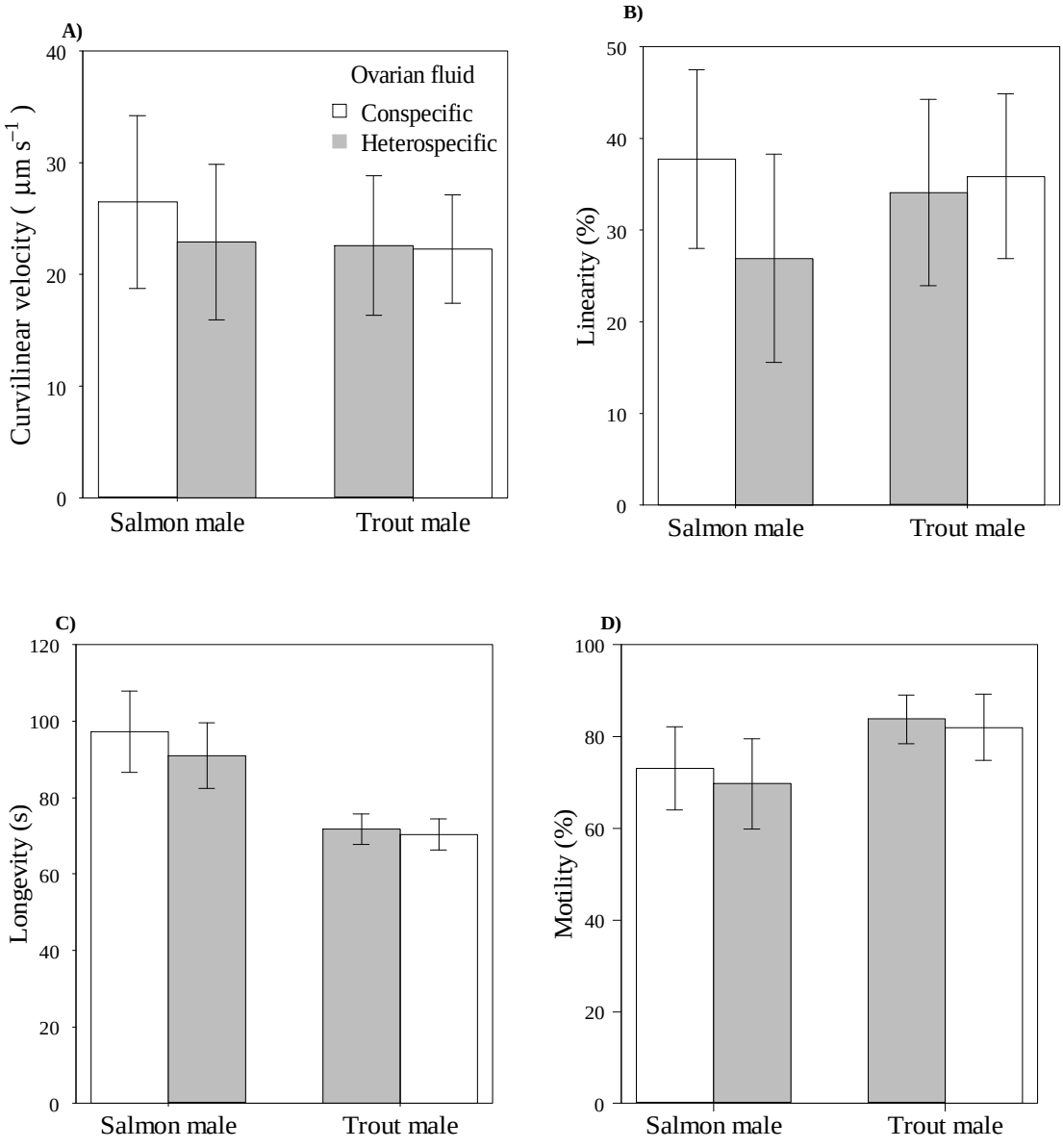


Figure 4.3.9: Comparison of salmon and trout sperm traits activated in conspecific (open bars) and heterospecific (grey bars) ovarian fluid. A) Mean VCL ± 1 S.E.M (µm s^{-1}). B) Mean LIN ± 1 S.E.M (%). C) Mean sperm longevity ± 1 S.E.M (s). D) Mean sperm motility ± 1 S.E.M (%).



4.4. Discussion

Under non-competitive fertilisation conditions involving the sperm and eggs of single males and females, conspecific and heterospecific ovarian fluid (OF) has no influence on the ability of salmon sperm to fertilise either salmon or trout eggs. However, trout males had significantly higher success fertilising trout eggs bathed in conspecific OF, compared with trout eggs bathed in heterospecific salmon OF. But trout sperm fertilisation of salmon eggs was not affected by ovarian fluid. The rate at which salmon and trout males could fertilise salmon eggs was not influenced by OF. Exposure time to sperm increased fertilisation success and trout males had high success initially, but fertilisation success in neither species was significantly increased by their own species' OF. Under sperm competition, both salmon and trout males had significantly higher paternity success when competing for eggs bathed their own conspecific OF, independent of which species' eggs they were competing for. Thus, it was not egg identity, but OF identity, that allowed conspecific sperm to father the majority of offspring. This non-random fertilisation in salmon-trout inter-specific reproduction is known as conspecific sperm precedence (CSP), and in this case is seen under conditions of sperm competition rather than monogamous mating. CSP results from postcopulatory sexual selection operating on gamete choice or sperm competition. When females are faced with both conspecific and heterospecific ejaculates cryptic choice of conspecific sperm can occur, or conspecific sperm can outcompete heterospecific sperm to achieve differential fertilisation bias to the conspecific male (Howard 1999). Because of the element of sperm selection demonstrated within some cases of CSP, it can be considered a mechanism of CFC in some cases (Geyer & Palumbi 2005; Price 1997). Females are potentially using mechanisms to enable differential fertilisation in the avoidance of hybridisation (Geyer & Palumbi 2005; Howard 1999; Price 1997), which potentially evolved under sexual conflict, the avoidance of polyspermy or reinforcement

4.4.1. Conspecific sperm precedence in salmon-trout hybridisation

As considered in the introduction, a number of mechanisms have been identified that account for a female's apparent ability to bias which individual male fertilises her eggs. These mechanisms can be physically functional in nature, including female ejection of

ejaculates (Matthias 2010; Pizzari & Birkhead 2000), differential sperm storage (Eberhard 1996; Fedina 2007; Hellriegel & Bernasconi 2000), varying oviposition timing (Barbosa 2009) and manipulation of clutch size (Arnqvist & Danielsson 1999b; Bretman et al. 2006; Thornhill 1983). In interspecies, as well as intraspecies fertilisation, there is strong evidence that biochemical interactions between sperm and egg, rather than physical mechanisms, can be responsible for observed patterns of differential fertilisation seen during sperm competition, as well as being a barrier to hybridisation (Dziminski et al. 2008; Evans & Marshall 2005; Glabe & Vacquier 1977; Marshall & Evans 2005; Metz et al. 1994; Palumbi & Metz 1991; Roldan & Yanagimachi 1989; Shaw et al. 1993; Snell & White 1996; Wassarman 1999; Wassarman et al. 2001; Wedekind et al. 1996; Yanagimachi 1994). Sperm-egg contact and penetration are fundamental in all sexually reproducing systems, though the mechanisms are not homologous (Geyer & Palumbi 2005). In mammals, sperm fuse with the egg's extracellular coat (zona pellucida) and undergo the acrosome reaction to penetrate the egg (Wassarman 1999). In many mammalian species, sperm fusion to the zona pellucida is species specific (Roldan & Yanagimachi 1989; Snell & White 1996; Wassarman 1999; Wassarman et al. 2001; Yanagimachi 1994), however, the molecular mechanisms behind species specificity in mammals are not well understood (Swanson & Vacquier 1998). Species specificity as a result of incompatibility between sperm-egg interactions are best understood in a very few species of externally fertilising marine invertebrates.

In sea urchins, as in mammals, sperm undergo an acrosome reaction and fuse with the egg membrane known as the vitelline envelope, facilitated by a protein on the acrosome called bindin (Vacquier & Moy 1977). This protein mediates the adherence of sperm to the vitelline envelope via carbohydrate receptors in the glycoprotein layer (Vacquier & Moy 1977). Interspecific reproduction experiments have shown that heterospecific sperm have significantly reduced attachment to the receptors in the vitelline envelope of sea urchin eggs (Glabe & Vacquier 1977; Metz et al. 1994; Palumbi & Metz 1991), with those that do attach failing to form continuity with the eggs plasma membrane resulting in failed fertilisation (Metz et al. 1994). In the bivalve mollusc abalone, the sperm acrosome protein is called lysin and again reacts with the egg's vitelline envelope, where it binds with the vitelline envelope receptor for lysin (VERL) on the surface of the egg (Swanson & Vacquier 1997). After attachment to the VERL receptor, lysin non-enzymatically creates a hole in the extracellular

matrix of the egg to allow the sperm to enter and fertilisation to take place (Swanson & Vacquier 1997). As with bindin, lysin shows strong species specificity in the ability of sperm to attach to the egg membrane receptor (Lee et al. 1995; Lee & Vacquier 1992; Shaw et al. 1993; Vacquier & Lee 1993). Both abalone and sea urchins have high amino acid divergence across species as a result of positive selection, even in sympatric populations. It is this divergence that is thought to result in incompatibility between heterospecific sperm and egg membrane receptors (Palumbi 1999; Palumbi 2009; Swanson & Vacquier 1998; Swanson & Vacquier 2002b). Amino acid divergence is hypothesised to arise in one of two ways; directional selection from coevolution of egg and sperm proteins to increase fertilisation efficiency, or from cyclic selection, where sperm evolves to increase fertilisation efficiency and egg penetration rate, leading to evolution of eggs to slow sperm entry to avoid polyspermy (Palumbi 1999). These mechanisms of evolution are theorised to potentially occur under sympatric conditions (Gavrilets & Waxman 2002; Van Doorn et al. 2001) and could be accelerated by reinforcement as a result of hybridisation avoidance (Palumbi 1999) and eventually result in CSP (Geyer & Palumbi 2005). Empirical evidence for reinforcement accelerating the rate of evolution in gamete recognition proteins comes from *Echinometra* sea urchins. Populations of *E. oblonga*, that are sympatric with an as yet unnamed closely related congener, show higher divergence in bindin alleles compared to allopatric populations and are under positive selection (Geyer & Palumbi 2003). This divergence and selection suggests reinforcement might be occurring to fix mutations in populations that prevents cross fertilisation (Palumbi 1999).

While it is clear for a variety of vertebrate and non-vertebrate species that the surface of the egg plays a vital role in fertilisation success through surface receptor ligands, (Hirohashi et al. 2008) as described above, evidence suggests that in some cases eggs take a further role in the reproductive process by releasing proteins that actively attract sperm for binding (Al-Anzi & Chandler 1998; Cherr et al. 2008; Eisenbach & Giojalas 2006; Inamdar et al. 2007; Zatylny et al. 2002). In the sea urchin *Arbacia punctulata*, the peptide resact diffuses from the egg stimulating sperm motility and attracting sperm (Inamdar et al. 2007; Kaupp et al. 2003). The chemoattraction protein alluring (Xiang et al. 2001) has also been recognised in the frog, *Xenopus laevis*, egg (Al-Anzi & Chandler 1998). This protein diffuses from the egg jelly to attract sperm, but unlike in the sea urchin it does not induce motility (Al-Anzi &

Chandler 1998). In my study, ANOVA of sperm competition results found that the species egg type, for which males competed, had no effect on the fertilisation success of either Atlantic salmon or brown trout sperm. This suggests that it is not sperm interacting with the egg that produced the differential fertilisation observed, but rather it was the type of OF around the eggs that influenced the fertilisation process in some way. Studies of mammals have found that oviduct and folicullar fluids influence the motility of sperm (Imam et al. 2008; Satake et al. 2006; Suarez & Pacey 2006) and appear to act as chemoattractants that correlate strongly with egg fertilisation (Ralt et al. 1991). This suggests reproductive fluids can play a role not only in attracting sperm to the egg but also in its fertilisation. OF in salmonids is secreted from cells lining the ovarian cavity and is released with mature oocytes into the peritoneal cavity, before passing out of the genital pore with the release of eggs (Lahnsteiner et al. 1995a). Both seminal fluid and OF in salmonids are known to play an important role in egg storage *in vivo* (Billard & Cosson 1992; Lahnsteiner et al. 1995a). Seminal fluid of freshwater teleost fish, including the Salmonidae, has high levels of potassium ions (K^+) which keep gametes inactive before release (Billard & Cosson 1992; Morisawa & Suzuki 1980). On release and contact with water, salmonid sperm experience decreased extra cellular K^+ resulting in activation (Morisawa & Suzuki 1980). The same occurs in salmonid eggs when they come into contact with water. Eggs immediately activate and undergo osmotic swelling that closes the micropyle preventing fertilisation (Billard & Cosson 1992). Both sets of gametes are viable for a very short space of time in freshwater, around 30-40s (Billard 1983; Billard 1986; Billard 1992). However, OF has been shown to increase the fertilisable period of salmonid eggs and the longevity of spermatozoa (Billard 1983; Lahnsteiner 2002), giving rise to the possibility that salmonid OF has a role to play in fertilisation itself, and may give rise to the CSP observed in salmon-trout hybridisation.

4.4.2. Effects of conspecific and heterospecific ovarian fluid on salmon and trout sperm

Salmonid OF not only increases the lifespan of sperm, but has also been shown to increase the proportion of motile sperm and sperm velocity (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007). Sperm

swimming speed in salmonids is thought to be a major factor influencing a male's paternity success in sperm competition, and has been shown to be the primary determinant of paternity in male Atlantic salmon and Artic charr (Gage et al. 2004; Liljedal et al. 2008). Interestingly, some studies have found that within a species, the ability of OF to influence sperm mobility differs between females, with some females' OF consistently increasing sperm velocity higher than others (Dietrich et al. 2008; Rosengrave et al. 2008; Urbach et al. 2005). Variation within males has also been shown, with some males having consistently higher sperm velocity in OF regardless of which female's OF they are swimming in (Dietrich et al. 2008; Rosengrave et al. 2008; Urbach et al. 2005). These findings suggest that some females may be enhancing their chances of fertilisation success by increasing the swimming ability of sperm; and some males may have advantages in sperm competition through higher sperm velocities in OF (Dietrich et al. 2008; Gage et al. 2004; Liljedal et al. 2008). The co-variation between individual males' sperm within OF and variation within OF itself, is even proposed to generate compatibility between individual males and females, with some male-female combinations recording higher sperm velocity than others (Dietrich et al. 2008; Rosengrave et al. 2008; Urbach et al. 2005). This finding might mean that females have the ability to discriminate between ejaculates of individual males, achieving CFC and biasing fertilisation in favour of certain males. However, so far there has been no link between this potential compatibility, and other genotypic or phenotypic compatibilities between reproducing male and female salmonids. Evidence for CFC, via certain female-male interactions proving more successful than others, has been seen in other externally spawning amphibian and marine invertebrate species, and in these cases fertilisation success was directly linked to CFC (Dziminski et al. 2008; Evans & Marshall 2005), unlike in fish where evidence has indirectly hinted at the presence of CFC (Dietrich et al. 2008; Rosengrave et al. 2008; Urbach et al. 2005; Wojtczak et al. 2007). In the externally fertilising frog, *Crinia georgiana* half-sib polyandrous *in vitro* fertilisation experiments found there was a significant effect of male-female haplotype interactions on fertilisation success and offspring fitness (Dziminski et al. 2008). The combinations of parental haplotypes that resulted in the highest fertilisation success produced offspring with higher viability and faster juvenile development than those with low fertilisation success, suggesting a potential CFC mechanism selecting for compatible sperm (Dziminski et al. 2008). In my study, I use crosses between males and females where clear expectations of incompatibility can be

identified, and find a strong CSP effect on the fertilisation success of salmon and trout males under sperm competition, mediated by OF. In this case OF appears to be able to discriminate between ejaculates to bias fertilisation to conspecific sperm, providing evidence that OF can be a mechanism of CFC in salmonids to avoid hybridisation.

With the results of sperm competition suggesting that OF identity, not egg identity, influences the CSP seen in salmon-trout hybridisations, the mechanism appears to lie in the influence OF has on sperm behaviour. In this study I found that Atlantic salmon and brown trout sperm migrated through a permeable membrane in significantly higher numbers into OF from conspecific females, compared with OF from heterospecific females or water. This result suggests there may be a chemotactic response of salmonid sperm towards OF that is heightened by conspecific OF. Chemotaxis is the directional change in movement of cells up a concentration gradient of chemoattractant, or down a concentration gradient of chemorepellent (Eisenbach 1994). Chemotaxis of sperm toward eggs has been described in many taxa from marine invertebrates and fish, to amphibians and mammals (Eisenbach 1999; Eisenbach & Giojalas 2006). The sperm used in the chemotaxis assay in my study were activated prior to entering the Transwell with river water. This makes it unlikely that the movement of sperm toward OF through the permeable membrane was due a chemokinesis response (motility activation) of sperm to the OF, although the OF could have exaggerated sperm swimming through increased lifespan. In non-mammalian species the role of chemotaxis appears to be involved in drawing as many sperm to the egg as possible (Eisenbach 1999), especially important in externally fertilising species like salmon and trout, whose sperm need to find eggs in a more diffuse fertilisation environment compared with internally fertilising systems (Eisenbach 1999). However, as motility characteristics of sperm are known to be enhanced in OF (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007), it could simply be that sperm had enhanced swimming ability in conspecific OF that allowed more of the sperm to migrate through the membrane than in heterospecific OF or water. Both chemotaxis and sperm enhancement mechanisms are supported by the fact significantly more sperm cells migrated into a male's conspecific species OF than into water or heterospecific OF, but there was no difference in migration between heterospecific OF and water.

4.4.3 Potential mechanisms of ovarian fluid as a mediator of conspecific sperm precedence

The fact that conspecific OF appears to attract or stimulate more sperm to migrate through a permeable membrane, suggests that OF is influencing the motility of conspecific sperm in a way that heterospecific OF and water do not, providing an explanation for the bias in fertilisation of a females eggs seen under sperm competition. In a bid to uncover any mechanisms of this differential behaviour, sperm traits of males were recorded and measured using CASA when activated in conspecific and heterospecific OF's and water. Conspecific OF clearly increased the sperm path linearity and longevity of sperm over that of water in both salmon and trout, as seen in previous studies (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007). However, sperm velocity and percentage of motile sperm did not increase in conspecific OF over water for either species, as described for other salmonids in preceding studies (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007). Yet crucially, when comparing salmon and trout sperm traits within conspecific and heterospecific OF, there were no differences in any of the traits measured between the two OF's for either male, failing to reveal a species specific mechanism that could explain the differential fertilisation and sperm migration induced by conspecific OF. It is possible that the methods I used to measure the sperm motility of males were not fine scale enough. Due to the way the sperm is activated and placed on a microscope slide for image recording (see 2.3) the first 10 seconds of sperm activity are inevitably not captured. Spermatozoa speed declines rapidly after activation (Kime et al. 2001; Turner & Montgomerie 2002) and the initial period of activation is thought to be crucial in sperm competition of some fish species (Burness et al. 2004). Fertilisation dynamics in medaka show that micropyles are occupied within the first 6 seconds of sperm release (Iwamatsu et al. 1991), and 80% of fertilisation is complete after 5s in salmonids (Hoysak & Liley 2001), suggesting the importance of the initial activation period in the race to locate and penetrate the egg micropyle. If conspecific OF was to exert influence on sperm in these important first few seconds I would have missed it. Another possibility as to why there was no difference in sperm behavioural traits activated in conspecific and heterospecific OF, could be that the sample size used in this study wasn't big enough. Sperm traits were measured in each OF once for each 15 males of the two

species. The CASA system can only track between 50-100 spermatozoa on the screen at one time. This means that the mean trait measures for each male in each OF were comprised from 50-100 spermatozoa. When one considers the number of sperm contained within a male's ejaculate number in the billions, the measures in my study were unavoidably a tiny representation of what a male enters into a competition. It is possible that if the effects produced by OF on spermatozoa are subtle, my studied did simply not measure enough sperm to register them; the relatively large variances (seen on figure 4.3.9) support a relatively low power here to detect differences in sperm behaviour within conspecific versus heterospecific ovarian fluid. It is also possible that sperm must be first activated in water (as would occur naturally) before encountering a high OF concentration around the egg to reveal behavioural changes.

When fish spermatozoa begin to swim in water, they have a curved trajectory (Kime et al. 2001) swimming in an elliptical pattern. As shown in this study and others (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007), sperm path linearity increases in OF compared to water, so the sperm begin to swim with straighter trajectories. Path linearity is a measure of a spermatozoa's trajectory through a solution (Kime et al. 2001); with a high linearity meaning a straight line path. It is possible that when sperm reach OF around the eggs in a redd they switch from elliptical to straight line swimming up the chemical gradient, enabling them to reach the egg and micropyle faster. If conspecific OF were to more effectively make the transition in sperm from elliptical to straight line swimming, this may provide conspecific sperm with an advantage in reaching the egg first. In salmonids, it is the first egg to reach the micropyle that tends to successfully fertilise it, suggesting sperm competition involves a race by sperm to locate the ovum and then swim down the single micropyle (Kobayashi & Yamamoto 1981; Yanagimachi et al. 1992). Anything that would help one male's sperm locate eggs faster than another would influence the relative fertilisation success of both males in competition. A faster increase to a linear swimming trajectory in conspecific sperm could be a potential mechanism of chemotaxis in salmonid OF. This proposed mechanisms indirectly implied by the results of the Transwell assay and the CASA comparisons of sperm in conspecific OF versus water. In sea urchins, chemotaxis is due to ion induced changes in sperm flagella movement (Böhmer et al. 2005; Kaupp et al. 2003; Strunker et al. 2006). The

chemoattractant peptide resact binds with a receptor on the sperm and activates rapid production of cyclic guanosine monophosphate (Kaupp et al. 2003).This phosphate production in turn opens K+ channels, resulting in hyperpolarization of the cell membrane and an increased membrane potential, subsequently resulting in entry of Ca^{2+} into the cell (Strunker et al. 2006). Increases of Ca^{2+} cause the flagella of the sperm to beat in an asymmetrical fashion and produce a high curvature trajectory, while decreases in Ca^{2+} produce more symmetrical movements and straighter line swimming (Böhmer et al. 2005). Resact appears to induce spikes of Ca^{2+} in the spermatozoa, with spikes producing a curved trajectory and turning the sperm toward the source of attractant in units of response (Böhmer et al. 2005). It is possible that a protein in OF of salmon and trout could provide a similar mechanism. It is known that salmonid sperm is activated via changes in ionic concentrations (Morisawa & Suzuki 1980), and it is therefore also possible that changes in ionic concentration could lead to changes in sperm movement, with a component of OF mediating this through altering the ion balance within the sperm.

Results of my study provide evidence that CSP is mediated by OF, and not the egg, in salmon-trout hybridisation. These results present a clear example of CFC employed in the avoidance of hybridisation, with direct evidence that a female derived reproductive fluid biases fertilisation to conspecific males. However, isolation through CSP is not complete, with heterospecific sperm still achieving 35-40% paternity when competing for heterospecific eggs in heterospecific OF. It has been proposed that CSP is a precursor to complete gamete incompatibility in the evolution of postcopulatory, prezygotic reproductive isolation (Geyer & Palumbi 2005; Howard 1999), which could see salmon and trout become completely isolated in this respect further down the evolutionary timeline. Despite a clear effect of conspecific OF mediating sperm competition success, and some correlated sperm swimming behaviours that might explain how CSP is achieved, we have yet to isolate the specific mechanism. Further work is needed to determine what differences exist between salmon and trout OF, and to then isolate the effects of any different components within CSP and sperm behaviour.

Chapter 5

Quantitative fitness measures of salmon-trout hybrids at early life stages

5.1. Introduction

5.1.1. Hybridisation

Hybridisation, the interbreeding between individuals from genetically distinct populations, occurs in many plant and animal groups (Rhymer & Simberloff 1996). The persistence of distinct species despite hybridisation implies that hybrids are disadvantageous, and many hybrid zones show evidence that hybrids are unfit relative to parental genotypes (Barton & Hewitt 1985; Hewitt 1988). Maintenance of separate species with hybridising populations is due to selection against inferior hybrid genotypes and dispersal of pure genotypes into the hybrid zone (Barton & Hewitt 1985). Where inferior hybrid genotypes occur, individuals that mate with heterospecifics will produce offspring with lower fitness than those individuals that mate with conspecifics, resulting in selection against hybridisation (Dobzhansky 1937; Mayr 1942; Mayr 1963). Selection against hybrids has been suggested to result in reinforcement, a process where natural and sexual selection against unfit hybrids causes the strengthening of prezygotic isolation between sympatric individuals (Marshall et al. 2002). Although the evidence indicates that most inter-specific hybrids show reduced fitness relative to pure species individuals, there are examples where hybridisation has resulted in adaptive shifts within a species, for example in the face of rapid environmental change. One well documented case is that of Darwin's finches (*Geospiza* spp.) on the Galapagos Islands. Hybrids were rare and did not reproduce before the El Niño climatic event (Grant & Grant 1993). Yet, after this climatic shift some hybrid and backcrossed individuals demonstrated equivalent, and in some cases higher fitness than parental species in terms of recruitment, reproduction and survival (Grant & Grant 1992). High survival and fitness in hybrids was explained through their novel beak morphologies. These intermediate hybrid beak types were better equipped to access the new seed types that had become available as a result of the environmental change, allowing hybrids increased foraging efficiency, and thus fitness over parental species (Grant & Grant 1996b).

5.1.2. Environmental change and breakdowns in reproductively isolating barriers

Natural climatic shifts such as El Niño are rare however, and rapid environmental change is more commonly seen as a result of anthropogenic influences (Chapin et al. 1997; Lepers et al. 2005). Hybridisation is more likely to take place between species where environmental change has occurred, as habitat disturbance can lead to breakdowns in spatial and temporal reproductive isolation that previously isolated species (Anderson 1948; Arnold 1997; Coyne & Orr 2004; Hubbs 1955; Rhymer & Simberloff 1996). Under disturbed habitat conditions there are likely to be a wider range of novel habitat niches to exploit than in undisturbed habitats (Anderson 1948). As well as this, disturbed habitats provide increased opportunities for parent species to meet, escalating the likelihood of hybridisation (Arnold 1997). Disturbed habitats usually refer to environments that have either been disrupted directly or undergone indirect disturbance as a result of human activity nearby. Numerous studies have described changes in hybridisation patterns that have occurred in conjunction with anthropogenic environmental change (Behm et al. 2010; Lamont et al. 2003; Mecham 1960; Mercader et al. 2009; Schlefer et al. 1986; Seehausen et al. 1997; Taylor et al. 2006).

One of the main ways humans contribute to habitat disturbance is through land management, both on a local and regional scale (Foley et al. 2005). Modification on a local scale can increase hybridisation between sympatric species which are isolated by habitat niches (Rhymer & Simberloff 1996). Construction of artificial ponds and clearing of vegetation in the southeastern United States has led to an increase in hybridisation between the green tree frogs *Hyla cinerea* and *H. gratiosa* (Lamb & Avise 1986; Mecham 1960; Schlefer et al. 1986). In undisturbed habitats *H. cinerea* males call for mates whilst elevated on emergent vegetation near pond banks, in contrast to *H. gratiosa* males who call while submerged within the ponds themselves. In disturbed habitats the emergent vegetation has been cleared and is no longer available. This results in *H. cineara* calling to mates on the bank or on vegetation over hanging the ponds, and hybridisation consequently occurs through *H. cinerea* males intercepting *H. gratiosa* females (Lamb & Avise 1986; Schlefer et al. 1986). On a regional scale, habitat modification can lead to expansion ranges of a species into the geographical ranges of closely related species (Rhymer & Simberloff 1996). In the eastern

United States there was widespread habitat change from forests to agricultural grassland since colonisation until the 1970's (Drummond & Loveland 2010; Williams 1989). This led to large scale expansions of the grassland adapted mallard duck, *Anas platyrhynchos*, in areas occupied by the forest dwelling black duck, *Anas rubripes*, resulting in high incidences of hybridisation (Johnsgard 1967; Rhymer & Simberloff 1996). By providing permanent corridors in this way, habitat modification can link previously allopatric species that have not evolved reproductive isolation.

5.1.3. Hybridisation in freshwater fish

Hybridisation in freshwater fish is widespread (Hubbs 1955), and is more frequently observed than in any other vertebrate group (Campton 1987; Scribner et al. 2001). Several factors associated with freshwater fish mean they lend themselves easily to hybridisation; these factors include: external fertilisation, weak behavioural reproductive isolation, uneven numbers of one parental species compared to another, competition for restricted spawning grounds, reduced habitat complexity, and susceptibility of recently diverged forms to secondary contact (Campton 1987; Hubbs 1955; Scribner et al. 2001). Interbreeding between closely related species of fish can often be linked to natural ecological settings, or geological events (Scribner et al. 2001). Glaciations influenced the characteristics and the degree of connectivity of aquatic habitats (Hewitt 1996), and glacial effects are thought to have made important contributions to the adaptive radiation of fish in postglacial novel habitats (Schluter 1996). Fish species trapped in postglacial lakes after the retreat of ice were likely to have mixed, resulting in high hybridisation in Northern latitudes (Hubbs 1955).

Yet, as in many other taxa, human activities can play a large role in promoting hybridisation between fish above natural levels (Campton 1987; Scribner et al. 2001; Verspoor & Hammar 1991), with reproductive isolation between closely related sympatric species sensitive to habitat disturbances (Hubbs 1955). Heavy water management has led to reduced and modified aquatic habitats (Dowling & Childs 1992). These changes can result in reduced spawning grounds which constrain the reproductive activities of species and increases the likelihood of contact, which can lead to increased hybridisation (Rhymer & Simberloff

1996). Cichlid species in Lake Victoria were seen to undergo increased hybridisation in areas of the lake that were subject to high turbidity caused by eutrophication; this reduced light levels, impairing the assortative mating between species based on male colour (Seehausen et al. 1997; Verschuren et al. 2002).

As well as habitat modification, widespread introduction of fish species outside their endemic range resulting from aquaculture escapes, restocking and sport fishing is increasing (Gozlan 2008). This makes fish some of the most introduced aquatic species in the world, with some 624 recorded cases (Gozlan 2008). These introductions have often resulted in increased incidences of interspecific hybridisation (Scribner et al. 2001), and can lead to loss of genetic integrity (Allendorf et al. 2004; Muhlfeld et al. 2009; Utter 2000). While introductions account for only 17% of hybridisation (Scribner et al. 2001), it could be of high importance on a local scale (Allendorf et al. 2004; D'Amato et al. 2007). In some instances, local adaptation could be lost through introgression, threatening the persistence of the native species (Allendorf et al. 2004).

5.1.4. Salmonid hybridisation

One group of freshwater fish with high hybridisation rates are the salmonids. Data from the literature shows that hybridisation is common within all major lineages of salmonids (Taylor 2004), and has been seen in every genus (Heath et al. 2010). Like many other teleost fish in sympatry, salmonids have weak isolating barriers to reproduction (Verspoor & Hammar 1991). Species are often only isolated by temporal and spatial mechanisms (Docker et al. 2003; Heggberget et al. 1988; Taylor 2004), and behavioural isolations are poor often appearing non-existent (Grant et al. 2002). As well as weak precopulatory reproductive barriers many salmonid species also appear to have weak postcopulatory barriers as well; displaying little in the way of hybrids with reduced fitness (Chevassus 1979; Taylor 2004). However, this varies from species to species, with some salmonid hybrids showing heterosis over parental types (Seiler & Keeley 2007) and others being exogenously selected against (Hagen & Taylor 2001) .

The weak isolating barriers in salmonids make them susceptible to aquatic environmental change, which can lead to hybridisation (Castillo et al. 2008; Docker et al. 2003; Heath et al. 2010; Hindar & Balstad 1994; Jansson & Ost 1997). Reproductive isolation was found to breakdown between sympatric populations of cutthroat trout, *Oncorhynchus clarki*, and rainbow trout, *Oncorhynchus mykiss*, that had been subject to stocking of hatchery reared rainbow trout (Docker et al. 2003). Rainbow trout and cutthroat trout are subtly isolated by spawning time and spawning habitat, with rainbow trout spawning earlier and lower in river drainages (Docker et al. 2003). It is therefore unsurprising that these barriers are disrupted when large numbers of hatchery fish, which have not been selected to follow these spawning patterns, enter the system. In the same species pair, long term logging was also found to positively correlate with hybridisation. It was suggested the removal of trees increased erosion and sediment loads entering streams, possibly resulting in reduced areas available for spawning leading to increased contact and likelihood of hybridisation (Heath et al. 2010). The same study found anthropogenic disturbance in general was strongly correlated with high levels of hybridisation between the two trout (Heath et al. 2010).

5.1.5. Hybridisation in Atlantic salmon and brown trout

One of the best studied cases of hybridisation within the salmonids is that of Atlantic salmon with brown trout. These two species are sympatric in rivers across much of their endemic European range as well as in North America, where the brown trout was introduced for sport fishing. Isolation between these two species seems to come mainly from peak spawning time, as brown trout spawn on average 15 days earlier than salmon (Heggberget et al. 1988) Overlaps in spawning time, as well as poor habitat segregation, mean reproductive isolation between the two species is not complete (Heggberget et al. 1988). Hybridisation in the wild between Atlantic salmon and brown trout was first confirmed using biochemical markers in the 1970's (Payne et al. 1972). Since then reports of hybridisation in Europe and North America have been widespread (Garcia de Leaniz & Verspoor 1989; Gephard et al. 2000; Hartley 1996; Hindar & Balstad 1994; Hurrell & Price 1991; Jansson et al. 1991; Jansson & Ost 1997; McGowan & Davidson 1992b; Payne et al. 1972; Verspoor 1988), and in some

cases relatively high (Garcia de Leaniz & Verspoor 1989; Hartley 1996; Jansson & Ost 1997).

Some sites in Northern Europe have seen rates of salmon and trout hybridisation increase (Hindar & Balstad 1994; Jansson & Ost 1997). Anthropogenic causes are thought to be behind the observed rises in hybridisation with reduced spawning grounds, stocking and aquaculture escapes all being linked to cases of salmon-trout hybridisation (Garcia de Leaniz & Verspoor 1989; Hindar & Balstad 1994; Jansson & Ost 1997). In a restored river section in Sweden, salmon and trout were re-introduced to spawning grounds much reduced from their original size, after which hybridisation was seen to reach as high as 41% (Jansson & Ost 1997). In Norway, salmon-trout hybridisation was found to significantly rise between 1986 to 1992 (Hindar & Balstad 1994). This rise was positively linked to high numbers of Atlantic salmon escaping aquaculture nets at the time (Hindar & Balstad 1994). Hatchery reared and domesticated strains of fish, like those that escape aquaculture nets, show lower reproductive fitness compared to wild fish due to altered breeding behaviour as a result of deliberate and unintentional selection during domestication (Fleming 1996; Levin et al. 2001). It has also been suggested that farmed salmon are not adapted to the local spawning conditions they find themselves in after escaping nets, making them likely to be less discriminate about partners (Hindar & Balstad 1994). A study of hybrids in a sympatric population of salmon and trout in Scotland, found that all hybrids present were a result of farmed female salmon breeding with male trout (Youngson et al. 1993). The authors suggested this was due to farmed salmon females spawning at the wrong time, coinciding with trout spawning (Youngson et al. 1993). Large number of farmed fish escaping from nets can mean Atlantic salmon become the dominant species in some rivers, leading to brown trout being the outnumbered, rarer species, a factor thought to contribute to hybridisation (Hubbs 1955). Indeed, in many cases of extensive hybridisation between these species it is often adult salmon numbers that are low (Ayllon et al. 2004; Jansson & Ost 1997). Hybrids in Europe are commonly as a result of female Atlantic salmon crossing with brown trout males (Garcia-Vazquez et al. 2001), particularly so in salmon populations that are declining (Castillo et al. 2010). All of the cited examples provide good evidence anthropogenic actions can result in, and increase, hybridisation between salmon and trout. With demand for food and natural resources increasing, aquaculture and environment

modification are likely to continue; potentially leading to further increases in hybridisation. This could be of greatest concern to threatened or vulnerable populations of Atlantic salmon, which have been shown to be more susceptible to hybridisation (Hindar & Balstad 1994).

Atlantic salmon are viewed with high conservation importance, as populations around the world are in decline as a result of exploitation and anthropogenic habitat change (Verspoor et al. 2007). Thus, salmon are vulnerable to negative impacts on population growth, and hybridisation is one of a variety of factors to have a adverse effect on salmon populations. An obvious risk to declining Atlantic salmon populations is that of introgression via hybridisation. Introgressive hybridisation results in non-native genes entering a population through interbreeding with closely related species, and can lead to the collapse of multispecies assemblages into a hybrid swarm (Seehausen et al. 2008). A study of westslope cutthroat trout hybridising with rainbow trout, found that only 20% admixture of rainbow trout genes was enough to cause a 50% reduction in the reproductive success of cutthroat trout and a reduction in their population size (Muhlfeld et al. 2009). A study Atlantic salmon-brown trout hybridisation in Spanish rivers found evidence of introgressive hybridisation in areas where brown trout had been stocked (Castillo et al. 2008). However, rates of hybridisation were seen to decrease as stocking of trout was reduced, removing the threat of introgression (Castillo et al. 2008).

Another threat to salmon to arise from hybridisation is a reduction in effective population size through outbreeding depression. Atlantic salmon females produce a smaller number of larger eggs per kg of body weight compared to other freshwater fish (Armstrong et al. 2003). This means each egg is a high energy reproductive investment, and production of unviable hybrids would be highly costly to female Atlantic salmon fitness. Non-viable hybrids, arising in the F_1 generation or delayed until further backcrossing, often occur in species with mismatched chromosome numbers (Templeton 1986) like Atlantic salmon and brown trout. Producing reproductively unfit hybrids can result in the removal of reproductive resources (i.e. reproducing adults) from the system (McGinnity et al. 2003). A reduction in effective population size has been shown to have negative impact on the threatened bull trout, *Salvenlinus confluentus*, hybridising with the introduced brook trout, *S. fontinalis*. There is little evidence of hybrids beyond the F_1 generation between these two species, leading to

wasted reproductive effort having a detrimental effect on bull trout populations (Allendorf et al. 2001; Leary et al. 1993). Production of hybrids that are reproductively unfit can have further negative impacts if those hybrids are ecologically fit at all or some life stages. Ecologically fit hybrids have the potential to outcompete one or both of the parental species, thereby driving down pure species fitness via ecological loading. The Pecos pupfish, *Cyprinodon pecosensis*, is threatened with replacement by hybrids with a closely related species, the introduced sheepshead minnow, *C. variegates.* Hybrids have elevated swimming performance and faster growth, both of which increase food acquisition, reduce the threat of predation and gain and hold breeding territories (Rosenfield et al. 2004).

To understand the impact that hybridisation has on threatened salmon populations, knowledge on the fitness of hybrids is needed. To gain this knowledge, we need answers to a few key questions: what is the frequency of hybrids? Do hybrids have the potential to dominate and out-compete parental species for resources? Do hybrids have the ability to proliferate and disrupt local adaptation through introgression? Are hybrids infertile, with their production simply taking resources out of the system? With this study I aim to answer some of these questions and try and establish a clearer understanding of the fitness of salmon and trout hybrids. Previous studies investigating salmon and trout hybrids have created laboratory crosses and commented on the success of each reciprocal cross at hatch, but results from these studies have been conflicting. Early studies reported relatively high survival of both hybrid crosses (Chevassus 1979), however, the success of each reciprocal cross in subsequent studies varies (Chevassus 1979 and references therein; McGowan & Davidson 1992a and references therein; Refstie & Gjedrem 1975). More recent studies on salmon and trout hybridisation have reported very low survival from progeny derived from brown trout females and Atlantic salmon males (Álvarez & Garcia-Vazquez 2011; McGowan & Davidson 1992a). This cross does occur naturally in the wild, however, thought to be as a result of sneaking Atlantic salmon mature male parr (Garcia-Vazquez et al. 2002; Gephard et al. 2000; Hartley 1996; Jansson & Ost 1997). Reports on the fertility and backcrossing ability of salmon and trout hybrids are clearer with little discrepancy between studies. Maternal salmon hybrids have been shown to go on and mature and backcross with females of both species under culture (Johnson & Wright 1986; Wilkins et al. 1993) and natural spawning conditions (females only (Garcia-Vazquez et al. 2003)), and

introgression of brown trout genes into Atlantic salmon populations has been seen via maternal salmon hybrids (Castillo et al. 2008). Hybrids derived from brown trout females have been shown to produce backcrossed offspring with Atlantic salmon, though survival was low (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Nygren et al. 1975). Attempts to backcross either hybrid type to brown trout has been unsuccessful (Garcia-Vazquez et al. 2004).

In this chapter, reciprocal hybrids of Atlantic salmon and brown trout were measured for different fitness traits at early life stages in a controlled and, for the first time to my knowledge, hybrid fry were assessed for fitness in a semi-natural environment. More often than not, studies on the fitness of hybrids relative to their parents are carried out in the laboratory rather than natural setting. Consequently these studies only record reproductive isolation as a result of breakdown in genetic compatibilities, rather than through lack of adaptation to the environment (Hatfield & Schluter 1999). Measuring the relative fitness of hybrids in natural environments is fundamental in being able to anticipate the ecological and evolutionary impact hybrids may have on parental populations (Parris 2001) Furthermore, it gives insight into the forces behind the genetic components that form reproductive isolation (Hatfield & Schluter 1999). By carrying out assessments of fitness, as detailed in this study, I hope to be able to provide a more definitive answer on the fitness of hybrids at early life stages and hopefully infer any impacts they could have on wild populations of Atlantic salmon and brown trout.

5.2 Methods

5.2.1. Establishing pure and hybrid crosses

To assess differences in the development, growth and survival of Atlantic salmon and brown trout hybrids compared to their parental species, 11 families were constructed from randomly picked Atlantic salmon and brown trout populations, both of which were first-generation hatchery (2.2). Each family consisted of a single male and female Atlantic salmon and brown trout. These fish were then used to create pure salmon and trout crosses and both reciprocal hybrid crosses (table 5.2.1).

Table 5.2.1: Crosses created in each family in each of the 11 families created. Families consisted of one Atlantic salmon female and male, and one brown trout female and male. Each female and male were used twice, once to create a pure cross and once to create a hybrid cross. N is the number of replicates for each cross.

Female	**Male**	**cross created**
Atlantic salmon	Atlantic salmon	Pure salmon
Atlantic salmon	brown trout	Maternal salmon hybrid (F_1)
brown trout	Atlantic salmon	Maternal trout hybrid (F_1)
brown trout	brown trout	Pure trout

Gametes were collected and stored in the way described in section 2.2. To create each offspring cross a female's eggs were placed into a plastic bowl and the corresponding male's milt placed directly onto the eggs. The gametes were stirred and left for 3 minutes to allow fertilisation to occur. Eggs were then transferred to circular incubators in incubation channels (2.4.3). Each cross in each family had an individual incubator giving 44 incubators in total. An average of 679 ± 20 eggs were fertilised per cross.

5.2.2. Fitness measure: Embryo development

On the 20-01-2010, exactly 2 months after fertilisation and in the eyed stage, 10 eggs were removed from each cross in each family and preserved in 4% formalin. The process was repeated on the 02-03-2010, four days prior to the first offspring hatching. Preserved embryos were carefully dissected out of the egg by piecing the shell, peeling it away and gently lifting the embryo from the egg mass. Embryos were then placed on a microscope slide and orientated horizontally. Slides were placed under a Stereo Discovery V8 dissecting microscope (Carl Zeiss Ltd) along with graduated callipers set to a known distance of 1cm. Photos of embryos were taken with a PowerShot A650 IS digital camera (Cannon) attached

to the microscope. Photos were then transferred to a computer and embryo length was measured using the computer software ImageJ.

To compare embryo length across fish types a linear mixed effects model was used. One of the advantages of using a mixed effects model over a standard ANOVA is that they give improved estimates of within subject variance, i.e. the random effect, by pooling information across subjects (Fox 2002). In this case a linear mixed model gives a superior estimation of the random variation between female and male parents, to help reveal any differences in embryo length of offspring by taking this random variation into account. In the model embryo length was the response variable with fish type as the fixed factor and female and male identity of each offspring as random factors. Models were then fitted using the log-likelihood ratio test. This test expresses how likely the data are under one model than another. At the first time period, 20-01-2010, female identify of offspring significantly influenced the model and male did not, so male identity was removed from the model. When running a linear mixed model t stats are produced but not P values. To obtain P values Markov Chain Monte Carlo (MCMC) method was employed with 10,000 iterations. MCMC is a simulation method that creates a hypothetical population derived from the original data in the model, and using a sequence of random numbers, constructs a sample of the population (10,000 times in this case) against which P values are computed. The original model had pure salmon fish type as the intercept and each other fish type coefficient was compared to this intercept using MCMC. To gain P values for all pair-wise comparisons among the fish types for embryo length, the intercept of the model had to be changed and the model and MCMC re-ran with a different fish type as the intercept. This was done until all pair-wise comparisons had been made.

5.2.3. Fitness measure: Hatching success

After fertilisation, eggs from each cross were photographed and the total number of eggs subsequently counted. All dead eggs and alevins were recorded up until 27 days after hatching had ceased for each of the 11 family groups created (5.2.1). This allowed the number of surviving fry on the day of hatch and 27 days post hatch to be counted to give a

percentage survival at hatching (n = 11) and percentage survival 27 days after (n = 11) for each cross in each family. This proportion was then square root arcsine transformed in an attempt to achieve normality, but the data was still not normality distributed; most likely due to the small sample size. Therefore a non-parametric Kruskal-Wallis test was used to compare survival across the groups at hatch and 27 days post hatch.

The day on which each cross in each family started to hatch was also recorded. Egg batches were then observed daily until hatching had ceased. This gave the number of days it took for each cross in each family to have every offspring hatch, termed hatch duration. The number of days to complete hatching was normally distributed with homogenous variance so was compared across the fish types using a one way ANOVA. Hatch duration was the dependent factor and fish type a fixed factor. Tukey Honest Significant Difference (Tukey HSD) post hoc test was used for pair wise comparisons between fish types.

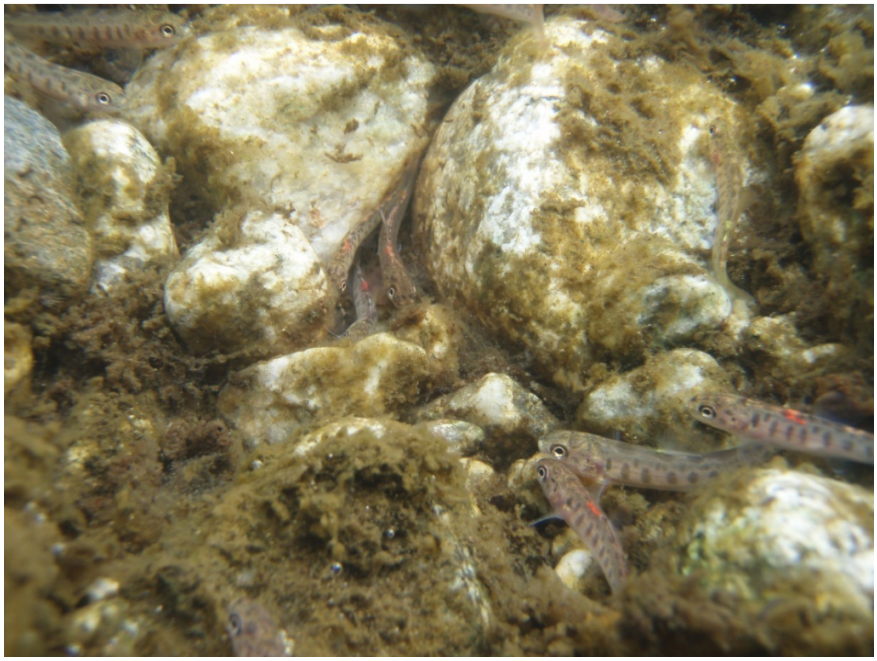
5.2.4 Fitness measure: Survival in a semi-natural stream

Two semi-natural streams, located near to the hatchery in Ims, constructed to be as similar as possible and each measuring 110 meters in length were used to assess growth and survival of hybrids compared to pure species in an environment close that which they would be exposed to in nature. Each stream is fed from the naturally occurring River Imsa that runs adjacent and in parallel to the streams. The flow rate of each stream can be manually controlled and each stream has a fish trap to monitor any fish migrating out of the stream. Within the streams, fish are exposed to competition, predation and disease that exist in the natural river. After hatching, crosses of the same type from different families were combined to give 4 $0.5m^3$ (500 l) tanks containing pure Atlantic salmon, brown trout and both reciprocal hybrid crosses. Densities of fish in all tanks were similar. Seven weeks after hatching, fry of all cross types were selected at random from their respective tanks and 50 of each type measured and weighed to give an average weight and length for each cross. A further 1800 fry (450 from each cross type) were then randomly selected and anesthetised for marking.
Fish were too small to tag individually with PIT tags or coded wires, therefore visual elastomer dye (VIE [Northwest Marine technology, Inc, USA]), was used to mark fish. Due

to the numbers of fish used in the experiment it was not possible to identify each fish individually. Fish were therefore identified as pure salmon, maternal salmon hybrids, pure trout, or maternal trout hybrids. Marks were made by injecting a VIE just under the skin of the fish. The VIE was prepared according to the manufacturer's instructions on the day of use and stored on ice. Two colours, yellow and red, were used with 2 different marking locations, giving 4 unique marks to identify each of the 4 crosses. A total of 450 fish of each cross were marked (n = 1800). Marked fish were then divided into two groups for high and low density treatments: 400 from each cross, 1600 in total, were combined into one portable tank, and 50 of each cross type, 200 in total, were placed into another. These were then left to recover from their anaesthetic for 1 hour.

Figure 5.2.1: The river park containing two experimental streams into which fish were released for 6 weeks. The high density stream was on the left the low density to the right. The streams were fed by the river Imsa beyond the fence in the left of the picture. Photo credit: Sian Diamond.

Figure 5.2.2: Fish marked with VIE just after release into one of the experimental streams. Photo credit: Sigurd Einum.



After the recovery period, fish were transported to the experimental streams (around 500m) on the 14th March 2010. In the first stream the 1600 fry (400 of each cross) were released at the top of the stream close to the flow outlet; this was the 'high density' stream. In the second stream, 200 fry (50 of each cross type) were released in approximately the same location as the first stream; this was the 'low density' stream.

Fish were then left unattended for 6 weeks. The trap was monitored for algae build up and any algae removed to prevent the trap from over flowing. During their time in the streams the fish were left to feed naturally from the river water flowing into the streams and were exposed to natural pathogens and predation. After 6 weeks, on the 24th and 25th of June 2010, the streams were electrofished three times each to maximise recapture of fish. The area beyond the trap was also electrofished to see if any fish had escaped the streams, but no tagged fish were found. After capture, fish were place in a high dose of anaesthetic to humanely kill them and then transported back to the research station at Ims, where they were individually identified via their mark. Most marks could be seen by the naked eye, but some required a UV light and orange UV filter glasses. Each individual recaptured fish was also measured on a fish board and weighed on electronic scales.

By sweeping the streams three times with electrofishing equipment it was hoped that most fish that still remained in the river would be recaptured to allow an accurate assessment of survival in high and low density streams across the fish types. As each stream was just one replicate of each density treatment it was not possible to have mean survival across fish type. Observed frequency of recaptured fish was the only survival data available. Because of this a G test goodness of fit analysis was used to compare the observed frequency of surviving individuals in each fish type to an expected frequency.

The observed frequency was the actual number of fish recovered for each fish type in each stream. The expected frequency for each fish type was calculated by dividing the starting number of fish (400 in the high density and 50 in the low density) by the total observed (recovered number of fish). The ratio of the observed and expected frequencies for each fish type was then calculated. The natural log of these ratios was then taken and multipled by the observed frequency. The sum of the natural log ratios was then taken and doubled to give the G value. This G value was then compared to the Chi Sq distribution with one fewer degrees of freedom than the number of categories. (5.3.3).

The null hypothesis of the G test is that observed frequency (recovered fish) in each category (fish type) is not significantly different to the expected frequency. The alternative hypothesis is that the observed frequencies are different from the expected.

5.2.5. Fitness measure: Length-weight relationships

In an attempt to assess the differences in weight and length gained (growth), if any, between the fish types from when they entered the experimental streams to when they were removed 6 weeks later, a sample of 50 fish from each group were randomly selected to be weighed and measured prior to entry of fish to the river to give an average weight and length for each population. Data were normally distributed (Shapiro-Wilks test), with homogenous variance (Bartlett's test), which were compared using one-way ANOVAs to explore any differences in length and weight from before the start of the experiment. When fish were recovered from the experimental stream river park, each was identified and weighed and measured as

mentioned previously. Length and weights were again compared using one way ANOVA's to see if any differences seen in fish prior to entry to the streams still existed.

Length-weight relationships in fish allow morphological and life history comparisons to be made between different species in the same or different environments (Petrakis & Stergiou 1995) and can be described by the equation 5.2.4

Equation 5.2.4:

$$W=aL^b$$

Where **W** is weight, L is length, **a** is the intercept which reflects the initial growth coefficient and **b** is the slope of the equation which describes the relative growth of the fish. By log transforming the weight and length data the relationship becomes linear (equation 5.2.5)

Equation 5.2.5:

$$\text{Log } W = \text{Log } a + \text{Log } L^b$$

The relative growth of fish can be estimated by using this linear regression on log transformed data. When the slope, b is equal to 3 fish are growing isometrically, where length and weight increase proportionally to each other. To see if the relative growth of the fish types in the two river treatments was significantly different to the isometric value of b (3) a t-test, H_0: b= 3, at the 95% confidence interval was applied equation 5.2.6 (Sokal and Rohlf, 1987).

Equation 5.2.6:

$$T_s = \frac{b - 3}{S_b}$$

Where $\mathbf{T_s}$ is the t test value to compare to the T distribution for 1 degree of freedom at the 95% confidence interval, **b** is the slope of the regression and $\mathbf{S_b}$ is the standard error of the slope. The comparison of T_s and the respective tabled critical values for 1 degree of freedom

allowed the statistical significance of b to be determined. If b has a value significantly lower than 3 the fish are in negative allometric growth, where weight is lower for a given length than predicted by isometry. A slope value significantly higher than 3 the fish are in positive allometric growth, where weight is higher for a given length than predicted by isometry. As it was not possible to mark fish in the streams individually it was not possible to obtain individual growth parameters for each fish and compare specific growth rates between groups and between treatments. In a bid to try assess whether any differences in growth between fish types existed in either stream, length-weight regression were compared between fish types using an ANCOVA analysis, with weight as the dependent variable, length as the explanatory variable and fish type as the covariate. If length and fish type show a significant interaction then the length-weight relationship is different between fish types.

All statistical analyses were carried out using the free open software R v 2.13.1.(2008)

5.3 Results

Overall results showed that maternal salmon hybrids (MSH) had equal, and in some cases, higher fitness compared to parental species in the measures used in this study. Maternal trout hybrids (MTH) on the other hand, while showing equal fitness to parental species juveniles for some measures, showed reduced fitness at others, most significantly for survival in a semi natural stream at high density.

5.3.1 Fitness measure: Embryo development

Embryo lengths were compared between pure crosses and reciprocal hybrid crosses as a proxy for fitness at two time periods. Eggs from Atlantic salmon and brown trout females had significantly different volumes ($t = 40.18$, $df = 1$ $P = <0.001$), with eggs produced by Atlantic salmon females being on average 34% bigger than trout eggs. There was a strong effect of egg size on embryo length at both time periods throughout the study, with larger eggs producing larger embryos ($R^2 = 0.13$, $P = <0.001$, $R^2 = 0.24$, $P = <0.001$, salmon and

trout eggs respectively). There was no difference in the volume of each egg type between the two time periods (2 way ANOVA, $F_1 = 2.475$, $P = 0.116$).

Linear mixed effect models (see 5.2.2) were used to compare embryo length across the fish types. For model structure see table 5.3.1. Of the embryos measured at the first time period (20-01-2010), salmon and MSH, hatched from Atlantic salmon eggs, were significantly longer than trout and MTH derived from brown trout eggs, (Figure 5.3.1a, all pair-wise comparisons $P = <0.01$). Within egg types, MTH and trout did not differ in embryo length ($P = 0.0881$). However, MSH were significantly longer on average than salmon embryos ($P = 0.0005$), making them the longest embryos on average of all the crosses. There was a significant influence of maternal identity on the model at the first time period. Paternal identity had no effect so was removed from the model (table 5.3.1). At the second time period (02-03-2010), four days prior to hatch, MSH embryos were still, on average, longer than all other embryos (Figure 5.3.1b, all pair-wise comparisons $P = <0.001$). Salmon and trout embryos no longer significantly differed in average length ($P = 0.9399$) and MTH were now significantly smaller than all other fish types, not just those from salmon eggs (Figure 5.3.1b, all pair-wise comparisons $P = <0.001$). Paternal identity of embryos now significantly influenced the model, as opposed to maternal identity (table 5.3.1).

Figure 5.3.1: Boxplots showing the median length of embryos from Atlantic salmon, brown trout (open boxes) and maternal salmon hybrids (MSH) and maternal trout hybrids (MTH, grey boxes), at two time periods throughout development. A) Embryo lengths of pure and hybrid fish types on the 20/01/2010. B) Embryo lengths of pure and hybrid fish types on the 02/03/2010. Boxplots show the median (lines inside the boxes), interquartile ranges (the boxes) and the maximum and minimum values (the whiskers). Dots are outlier values plotted individually.

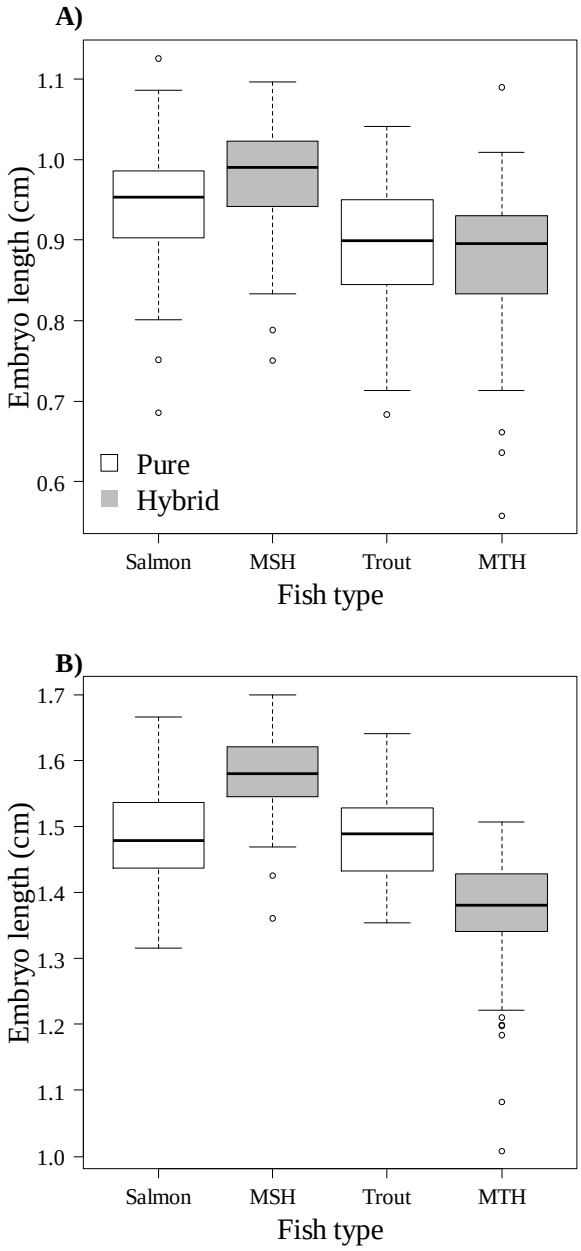


Table 5.3.1: Summary of model selection to compare embryo length across the fish types for the first and second time periods, with the global model listed first. Model structures with significantly lower log-likelihoods were poorer fits to the data. Of the models that did not significantly differ in their log-likelihoods, the model with the simplest structure was used. The final model is denoted in bold.

Model structure	**Log-likelihood §**
Time 1 (20/01/2010)	
embryo length ~fish type + egg volume + female.id + male.id + egg volume × fish type	410.33
embryo length ~fish type + egg volume + female.id + male.id	409.88
embryo length ~fish type + female.id + male.id	408.80
embryo length ~fish type + egg volume + male.id	396.06 *
embryo length ~fish type + egg volume + female.id	409.83
embryo length ~fish type + male.id	391.83 *
embryo length ~fish type + female.id	**408.73**
Time 2 (02/03/2010)	
embryo length ~fish type + egg volume + female.id + male.id + egg volume × fish type	649.72
embryo length ~fish type + egg volume + female.id + male.id	649.56
embryo length ~fish type + female.id + male.id	649.40
embryo length ~fish type + egg volume + male.id	648.77
embryo length ~fish type + egg volume + female.id	625.88 *
embryo length ~fish type + female.id	625.88 *
embryo length ~fish type + male.id	**648.55**

× Indicates interaction term

§Significantly lower logliklihoods are starred to show different significance levels. *0.05%, **0.01% and ***<0.001%

5.3.2 Fitness measure: Hatching success

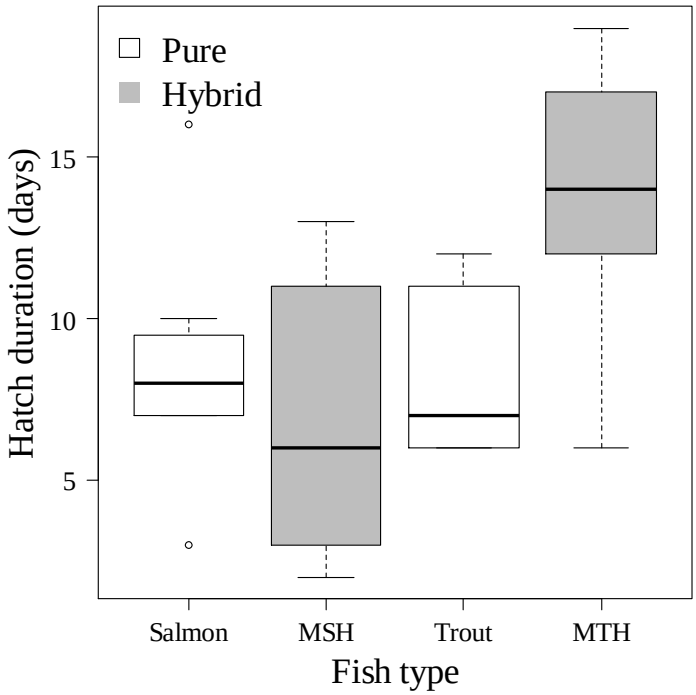
To investigate if and how the hatching success of hybrid offspring differs to that of parent species, the survival of offspring at hatch and 27 days post hatch between fish types was compared. There was no significant difference in survival between the different fish types at hatch ($X^2 = 5.163$, df = 3, P = 0.16) or 27 days post hatch completion ($X^2 = 6.85$, df = 3, P = 0.077). MTH had the lowest hatching success of all the four fish at hatch (table 5.3.2 and figure 5.3.2a) which decreased by 7.5% by 27 days post hatch, the biggest decline of all crosses (table 5.3.2). The P value at 27 days post hatch was close to significant (0.077), and maternal trout hybrid survival was over 10% lower than that of other fish types (table 5.3.2). Survival at this stage may have been significantly lower than the other crosses with a bigger sample size. Pure salmon had a large amount of variation in survival and both MSH and pure trout had extreme values, where the egg batch had suffered poor survival compared to the other replicates (figure 6.3.2b), which may also have contributed to the non-significance.

Table 5.3.2: Mean survival at hatch and 27 days post hatch of of Atlantic salmon, brown trout and maternal salmon hybrids (MSH) and maternal trout hybrids (MTH). Means are shown with standard deviation (S.D) and standard error of the mean (S.E.M).

Survival at hatch		**Mean survival**		
	Fish type	**(%)**	**S.D**	**S.E.M**
	Salmon	75.0	1.7	0.5
	MSH	77.8	1.7	0.5
	MTH	65.6	1.4	0.4
	Trout	72.4	1.1	0.3
Survival 27 days post hatch				
	Salmon	73.5	1.7	0.5
	MSH	74.4	1.7	0.5
	MTH	58.2	1.3	0.4
	Trout	71.2	1.1	0.3

The number of days each cross took to complete hatching, hatch duration (5.2.3) was also used as an evaluation of hybrid fitness. There was a significant difference between hatch duration of fish types ($F_{3,34}$ = 6.263, P = 0.001). MTH had a significantly longer hatch duration than any of the other fish types (Tukey HSD pair-wise comparisons <0.01), with the average number of days to complete hatching 13, compared to just 8 days and under for the other fish types (figure 5.3.2). No other pair-wise comparisons were significant. MSH egg batches, while not different from the pure crosses did have larger variation in the number of days it took them to complete hatching but had the shortest average at 7 days (figure 5.3.2).

Figure 5.3.2: Boxplot showing hatch duration of Atlantic salmon, brown trout (open boxes) and maternal salmon hybrids (MSH) and maternal trout hybrids (MTH, grey boxes). Boxplot shows the median (lines inside the boxes), the interquartile ranges (the boxes) and the maximum and minimum values (the whiskers) of arcsine transformed hatching success. Dots are outling values plotted individually.



To examine relationships between the length of embryos during development and the survival of cross types, Spearman rank correlations were carried out. There was a significant correlation between mean embryo length for each cross and its survival for the first time

period, 2 months post fertilisation (n = 40, r_s = 0.36, P = 0.023). This association had disappeared by the time the embryos had reached hatch, as there was no significant correlation between mean embryo length of a cross at the second time period, 4 days prior to the first eggs hatching, and its survival (n = 40, r_s = 0.24, P = 0.133).

5.3.3 Fitness measure: Survival in a semi-natural stream

Two month old pure species and reciprocal hybrid fry were placed in semi natural streams at high and low density (5.2.4) to examine hybrid fitness in a semi-natural environment alongside their parent species.

Table 5.3.3: Frequency table for the high and low density stream G tests, where O is the observed frequency (recovered number of fish), E is the expected ratio (the total number of fish that entered the stream), Ef is the expected frequencies calculated as E/ Σ (E)* Σ (O), R is the ratio calculated as O/Ef, and LnR is the natural log of the ratio calculated as O*ln(O*E).

	Fish type	O	E	Ef	R	LnR
High density stream	Salmon	205	400	195	1.0512	10.252
	MSH	237	400	195	1.2153	46.229
	MTH	92	400	195	0.4717	-69.11
	Trout	246	400	195	1.2615	57.153
Low density stream	Salmon	37	50	32	1.1562	5.3717
	MSH	32	50	32	1	0
	MTH	26	50	32	0.8125	-5.3986
	Trout	33	50	32	1.0312	1.0154

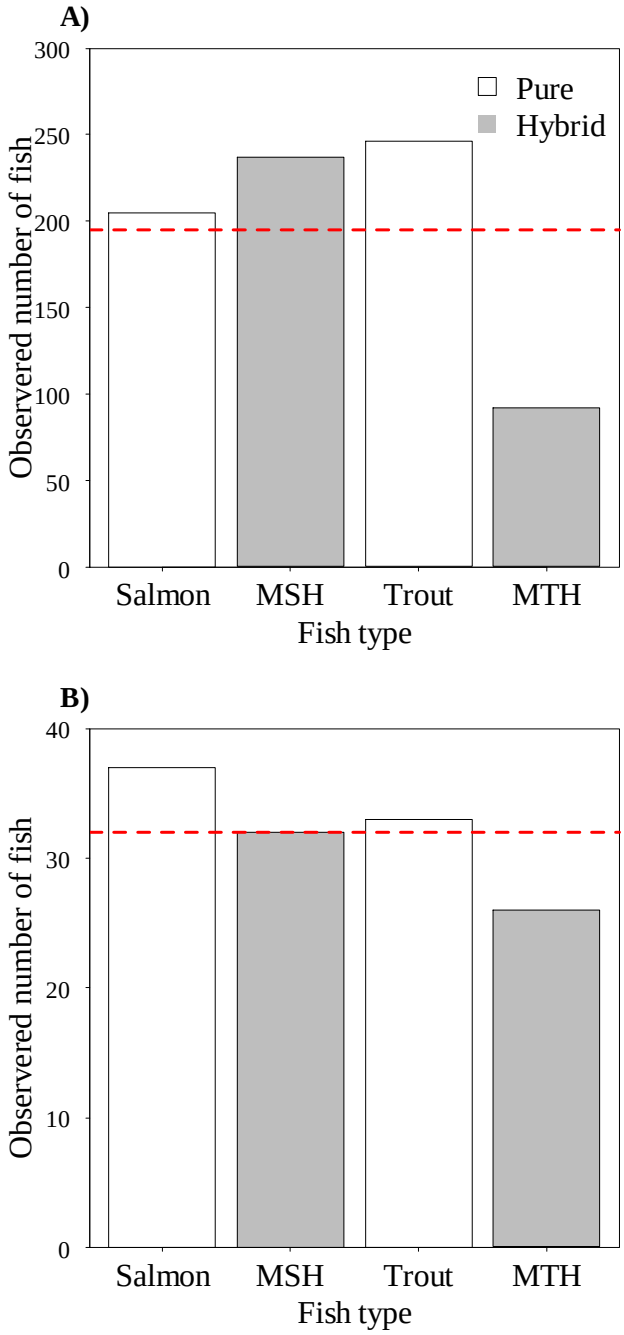
The G number = 2 * Σ (Observed*ln(Observed/Expected))

High density G number = 89.047

Low density G number = 1.9775

When the G number for the high density stream was compared to the Chi Sq distribution for 3 degrees of freedom there it was significant, indicating a significant difference between the observed frequency and expected frequency across the fish types (X^2_3 = 89.04, P = 0.001). Figure 5.3.4a shows the number of fish recovered from each fish type in the high density stream and the expected frequency as a red line. MTH frequency is much lower than the expected frequency, and likely to be the source of the significant difference. The G number if the low density stream was not significant when compared to the Chi Sq distribution for 3 degrees of freedom, indicating no difference in the observed frequency of fish and the expected across all fish types (X^2_3 =1.977, P = 0.577). In other words the observed numbers of fish in all groups did not statistically differ from the expected frequency (figure 5.3.4b).

Figure 5.3.4: Barplots showing the observed frequency of pure salmon and trout (open bars) and maternal salmon hybrids (MSH) and maternal trout hybrids (MTH, grey bars), after 6 weeks in a semi natural streams. The null expected frequency in each case is shown as a red dashed line. A) Observed frequency of each fish type in the high density semi-natural stream. B) Observed frequency of each fish type in the low density semi natural stream.



5.3.4. Fitness measure: Length-weight relationships

Before release into the experimental streams, a representative sample of 50 fish per cross was weighed and measured to give an average length and weight of each cross before the experiment started. ANOVA showed that MTH were significantly shorter (Tukey HSD pair-wise comparisons P = <0.0001) and lighter (Tukey HSD P = <0.0001) than the other fish types prior to entry into the experimental streams, no other fish types differed in length or weight.

When fish were recovered each fish type was significantly larger in the low density stream compared to the high density (T test, all P values <0.001). Within each stream, ANOVA again showed that MTH had the lowest length and weight across all the fish types (Tukey HSD <0.01). MSH in the high density stream were significantly longer than pure salmon on average (Tukey HSD P = 0.02), but significantly lighter than pure trout (Tukey HSD P = 0.03). In the low density stream MSH did not differ in average length compared to pure salmon and trout, but pure trout were heavier on average than any of the other fish types (Tukey HSD P = <0.03). Lengths and weights after 6 weeks in each stream for all fish types can be seen in table 5.3.3.

Table 5.3.3: Mean ± 1 S.E.M length (mm) and weight (g) of Atlantic salmon, brown trout and maternal salmon hybrids (MSH) and maternal trout hybrids (MTH) after 6 weeks in high and low density experimental streams.

Stream density	**Fish type**	**L (mm)†**	**S.E.M**	**Difference in L between streams (mm)**	**W (g)‡**	**S.E.M**	**Difference In W between streams (g)**
Low	Salmon	53.49	0.74		1.58	0.07	
High		47.09	0.38	6.39	1.07	0.03	0.51
Low	MSH	52.66	0.98		1.53	0.09	
High		45.44	0.43	7.21	0.97	0.03	0.56
Low	MTH	46.12	0.92		1.12	0.07	
High		38.52	0.46	7.59	0.53	0.02	0.58
Low	Trout	54.12	0.51		1.89	0.07	
High		46.15	0.39	7.97	1.08	0.03	0.80

† L = length; ‡ W = weight

Log length-weight regressions were carried out for each fish type. T-tests on the slope coefficients (5.2.5) of each regression revealed that none of the fish types in the low density river displayed deviations from isometric growth, as none of the slope coefficients significantly differed from 3 (table 5.3.4). In the high density river, MTH were the only fish type to deviate from isometric growth (table 5.3.4), with a slope coefficient of 2.714 ($t = 10.922$ $P = 0.029$). MTH in the high density river were exhibiting negative allometric growth, meaning for a given length these fish weighed less than a fish of the same length from one of the other crosses.

Table 5.3.4: The condition of Atlantic salmon, brown trout, maternal salmon hybrids (MSH) and maternal trout hybrids (MTH) juveniles, estimated by linear regression on log transformed data, in the high density and low density stream.

Stream	**Fish type**	**b‡**	**SE b**	**t value**	**P value**	
High density	Salmon	3.039	0.073	0.534	0.349	
	MSH	3.111	0.069	1.608	0.177	
	MTH	**2.714**	**0.22**	**10.922**	**0.029**	*****
	Trout	3.371	0.062	3.129	0.098	
Low density	Salmon	2.823	0.237	0.746	0.296	
	MSH	3.395	0.122	3.237	0.095	
	MTH	3.224	0.184	1.2174	0.219	
	Trout	3.371	0.176	2.108	0.141	

‡ b is the slope coefficient of the regression

In the high density river ANCOVA analysis produced a significant interaction between length and fish type, meaning the regression slopes are significantly different between the different fish types in the high density river (F_3 = 3.2593, P = 0.02). In other words, the relationship between weight and length varies between the fish types. From figure 5.3.5 it is clear to see that the MTH slope is different to the other cross types due to an outlier group that were underweight for their length. As well as this, all surviving MTH individuals were not above 45 mm long, where all other groups have large numbers of individuals surviving well above 45 mm, up to 60 mm (figure 5.3.5).

In the low density river, ANCOVA analysis showed no difference between the slopes of the different fish types as there was no significant interaction (F_3 = 2.0184, P = 0.115). MTH

relative growth was the same as the other fish types, with no very underweight individuals as found in the high density river (figure 5.3.6).

Figure 5.3.5: Scatter graph showing log weight and log length relationship for the four fish types in the high density river; salmon (green circles), maternal salmon hybrids (MSH, blue diamonds), trout (MTH, grey squares) and maternal trout hybrids (red triangles). Log weight-log length regression slopes for each fish type are overlaid on the points in corresponding colours.

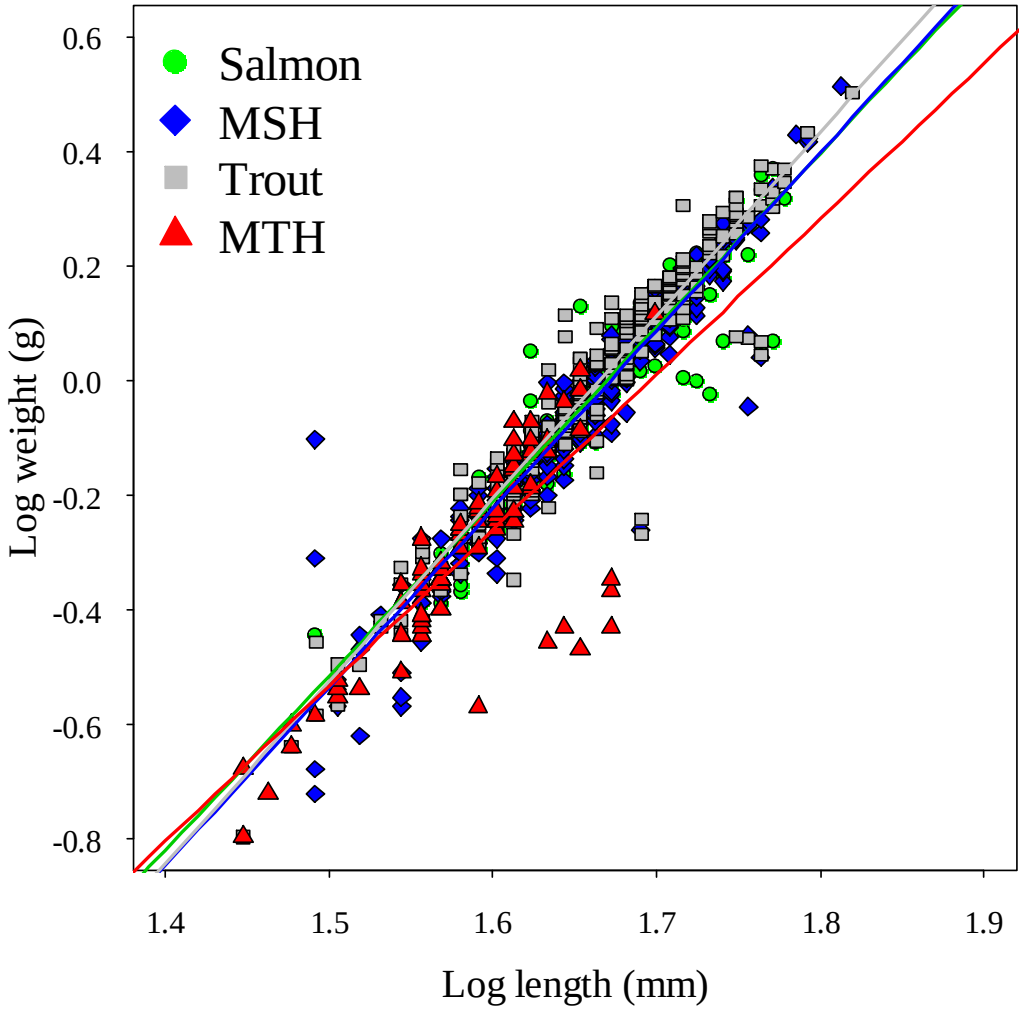
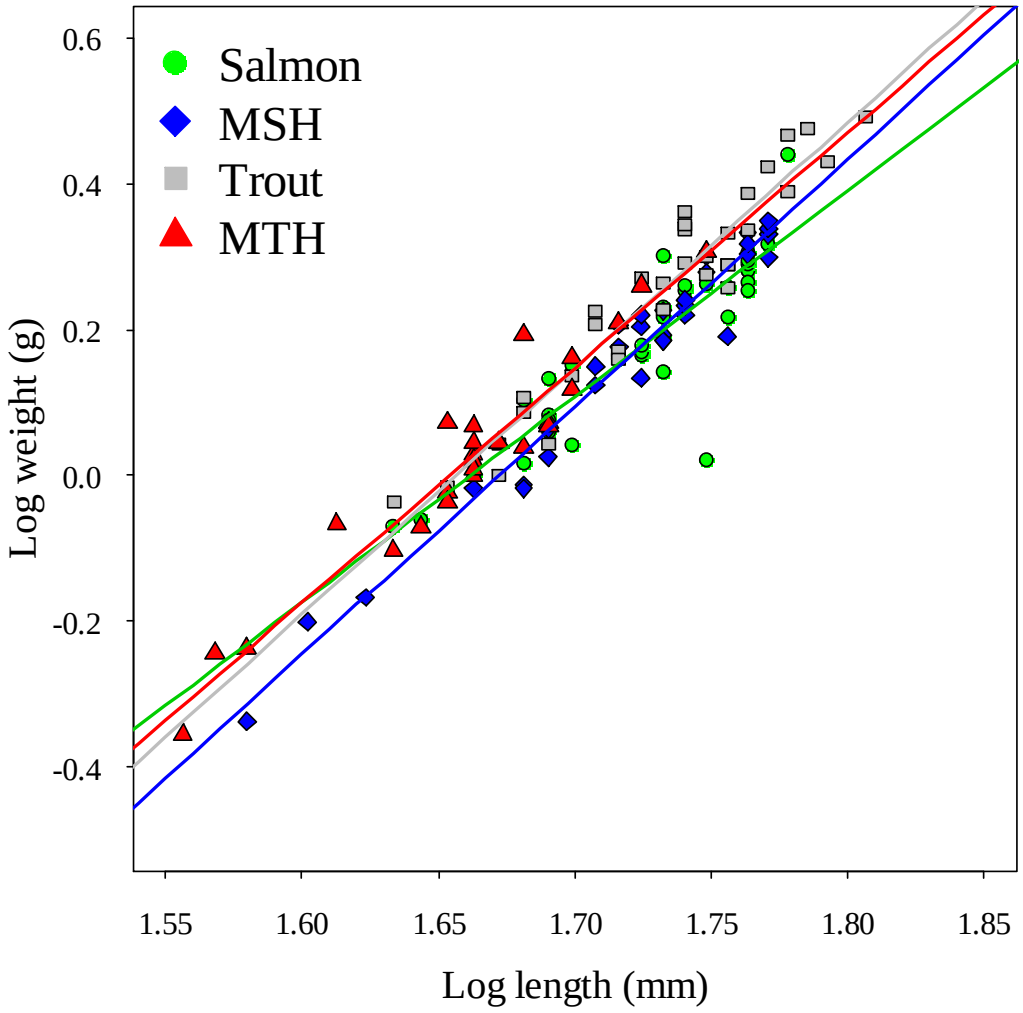


Figure 5.3.6: Scatter graph showing log weight and log length relationship for the four fish types in the low density river; salmon (green circles), maternal salmon hybrids (MSH, blue diamonds), trout (grey squares) and maternal trout hybrids (MTH, red triangles). Log weight-log length regression slopes for each fish type are overlaid on the points in corresponding colours.



5.4. Discussion

This study aimed to assess the fitness of salmon and trout reciprocal hybrids in relation to parental species at early life history stages. Results of my study suggest that F_1 maternal salmon hybrids (MSH) have no significant difference in fitness compared to pure salmon and trout at any of the life history stages examined, and may even have a fitness advantage at hatch. Conversely, maternal trout hybrids (MTH) suffered reduced fitness at some stages, yet had equal fitness to parent species at others, particularly under low density conditions. Importantly, neither hybrid cross exceeded fitness of parental juveniles at any life history stage measured in the study. These results are in contrast to those found in previous studies on F_1 salmon and trout reciprocal hybrid crosses. Chevassus (1979) reviewed the literature available at the time and concluded that MTH hybrids are superior, but that both perform as well or better than pure species. In later studies, results have shown that MTH are less viable with low hatch success and survival (Álvarez & Garcia-Vazquez 2011; Garcia-Vazquez et al. 2002).

5.4.1 Hatching success of hybrid crosses

Survival of hybrid crosses at hatch is an important measure of fitness. If the majority of hybrid offspring fail to hatch, or suffer high mortality soon after, they are unfit and would represent wasted reproductive effort. If hybridisation occurred on a large scale in this case, it could be damaging to whole populations (Allendorf et al. 2001; Leary et al. 1993). This study found no significant difference in survival at hatch, or 27 days post hatch across all the fish types. Previous studies have found that MTH suffered significantly higher mortality at hatch than parental species or MSH (Garcia-Vazquez et al. 2002; McGowan & Davidson 1992a). Natural spawning of wild trout females with wild male salmon in experimental streams produced MTH that suffered extremely low survival after emergence from redds, ranging from 0-1.95% (Garcia-Vazquez et al. 2002). In a study on artificial hybridisation between salmon and trout, McGowan and Davidson (1992a) noted that the majority MTH that suffered early mortality failed to absorb their yolk sacs. In this study, while survival of MTH was lower than that of the other crosses, it was not significantly so at hatch or 27 days

post hatch, by which time the egg sac has been absorbed. I found no evidence to suggest that MTH had problems with egg sac absorption or suffered fitness loss through severe mortality, as seen previously at these very early life stages (Garcia-Vazquez et al. 2002; McGowan & Davidson 1992a). The low survival of MTH seen by Garcia-Vazquez et al. (2002) may have been due to the fact wild fish spawned, and offspring developed and hatched, in natural stream channels rather than in a hatchery. The natural conditions experienced by eggs and offspring, opposed to the stable environment of a hatchery, may have exposed an inherent lack of fitness in MTH. Studies have found that wild salmonid fry have higher survival than domesticated hatchery fish in both wild and hatchery environments (Hyatt et al. 2005; McDermid et al. 2010; Miller et al. 2004). The fish used to create hybrid and pure offspring investigated in this study were wild broodstock fish, meaning they were hatched from the eggs of wild fish and raised in the hatchery until adulthood. Therefore the pure and hybrid crosses created in this study were 1st generation hatchery fish. In this population at least, I seem to find little evidence that salmon and trout reciprocal hybrids suffer inherent fitness losses at hatch, although it is possible that these results might be influenced by inherent hybrid fitness loss at hatch in wild redds.

5.4.2. Offspring size

In many species, including salmonids, juvenile body size positively correlates with fitness (Einum 2003; Einum & Fleming 1999; Fox & Czesak 2000; Marshall et al. 2006; Moran & Emlet 2001), especially in poorer conditions (Allen et al. 2008). Larger juvenile body size in fish is associated with amplified fitness traits such as higher survival (Cutts et al. 1999; Einum & Fleming 1999; Heath & Blouw 1998; Sogard 1997), faster growth (Cutts et al. 1999; Einum & Fleming 1999; Pitman 1979; Wallace & Aasjord 1984), increased swimming performance (Heath et al. 1999; Ojanguren et al. 1996) and predator avoidance (Segers & Taborsky 2011). In this study embryo length was used as a proxy for fitness to compare reciprocal hybrid crosses to parental species throughout development. MSH were found to be significantly larger than all other fish types for the duration of the study period; both at the eyed stage of development 2 months after fertilisation, and at 4 days prior to hatch. At the eyed stage, both trout and MTH were significantly smaller than salmon and MSH, but

not each other (figure 5.3.1a). This initial measurement period saw maternal identity of the embryo significantly influence the statistical model, suggesting the differences observed in embryo lengths between fish types were maternally derived at this stage, as previously seen in salmonids (Einum 2003; Einum & Fleming 1999; Heath et al. 1999), and other animals.

At the second, later time period just prior to hatch MTH were now significantly smaller on average than pure trout, making them the smallest of all the fish types (figure 5.3.1b). Pure trout were no longer significantly smaller than pure salmon, while MSH continued to be the largest embryos on average of all the fish types (figure 5.3.1b). The statistical model at this later time period showed that paternal identity now strongly influenced embryo size, opposed to maternal identity earlier in development. These results are in conjunction with those found by Heath et al. (1999) studying offspring size in chinook salmon, where maternal influences on early embryo development gave way to paternal effects in the latter stages. Previous studies have shown that egg size in salmonids varies considerably within females and between populations, accounted for in part by female age and size (Fleming & Gross 1990; Jonsson et al. 1996; L'Abee-Lund & Hindar 1990; Ojanguren et al. 1996; Quinn et al. 1995; Thorpe et al. 1984). Since egg size significantly influences the size of embryos in salmonids (Einum 2003; Einum & Fleming 1999; Heath et al. 1999), egg size has the potential to confound any differences seen between pure and hybrid fish. While Atlantic salmon eggs were larger than brown trout eggs, there was no significant difference in eggs size between females of the same species in this study. All fish in this study were of the same age and from the same populations (see 2.2), constraining the effect of potentially confounding egg size variation.

The fact that MTH were significantly smaller than their parent species at hatch has previously been noted (McGowan & Davidson 1992a), and underscores the potential for this hybrid type to suffer fitness losses post hatch. Studies of brook trout, brown trout and Atlantic salmon have all found that larger offspring benefit from higher survival and growth in early life than smaller ones, which can become exaggerated under certain environmental conditions (Einum & Fleming 1999; Hutchings 1991; Ojanguren et al. 1996). As well as higher survival and growth, larger individuals potentially have fewer predators due to gape restrictions, and the increased ability to escape predators through amplified swimming

performance (Heath et al. 1999; Ojanguren et al. 1996). MSH with their larger initial size could potentially have increased fitness over salmon and trout. Salmonid fry emerging from the nest quickly establish and defend feeding territories (Fausch 1984; Keenleyside & Yamamoto 1962; Titus 1990). The ability of salmon juveniles to acquire food in the short period of time after hatching, to enable fast growth, is a critical factor in the survival of young salmon and trout fry; with those unable to acquire suitable feeding ground either pushed out further downstream to less lucrative feeding positions, or to die (Elliot 1994; Keeley 2001; Seiler & Keeley 2007). Larger salmon fry have been seen to have a competitive advantage over smaller conspecifics when acquiring territories (Wańkowski & Thorpe 1979), resulting in increased feeding rates and faster growth (Cutts et al. 1999; Metcalfe & Thorpe 1992). However, body size is not the only determinant of fitness in emergent salmonid fry. Prior residence of juvenile fish within territories and high aggression, have both been shown to be important when establishing territories, independent of body size (Brännäs 1995; Cutts et al. 1999; Egglishaw & Shackley 1973; Harwood et al. 2003; Höjesjö et al. 2002; Metcalfe & Thorpe 1992). Dominant fish that are able to gain and maintain feeding territories can then go on to have increased body size, and the fitness benefits associated with it (Huntingford et al. 1990). A study on sympatric assemblages of Atlantic salmon and brown trout, when the two species were in direct competition, found aggression rather than body size was more likely to influence feeding success (Harwood et al. 2002). MTH could therefore have the potential to negate fitness loses suffered due to their smaller size through high aggression or prior residence.

5.4.3. Hatch duration

Early emergence from the redd can enable higher dominance in the social hierarchy of salmonid juveniles (Metcalfe & Thorpe 1992), and has also been seen in other vertebrates, notably several bird species (Drummond 2006; Velando 2000). Early emergence in Atlantic salmon can also have repercussions on an individual's life history (Metcalfe & Thorpe 1992). Fry emerging from redds as little as one week ahead of conspecifics have been found to migrate to sea a year ahead of those emerging later (Metcalfe & Thorpe 1992). The time it takes to establish the prior dominance effect can therefore be very short. An experimental

stream study on Atlantic salmon showed that prior residency of just a day was enough to establish dominance over an area (Huntingford & Garcia de Leaniz 1997). When comparing the time it took for hatching to complete (see 5.2.3.) across the fish types in this study, MTH took significantly longer to complete hatching than any of the other crosses; taking on average 5 days longer for all alevins within an egg batch to hatch. MSH did not significantly differ in hatching time to either parent species. This corresponds to previous findings that have also shown hybrids issued from trout females taking longest to emerge (Álvarez & Garcia-Vazquez 2011; McGowan & Davidson 1992a). Results of previous studies, and this one here suggest that MTH would therefore emerge from redds almost a week after parental species. This is likely to put them at a real fitness disadvantage at the commencement of first feeding after emergence.

Late emergence, coupled with their significantly smaller size on hatching, suggests that MTH are less fit at first feeding and less likely to survive and persist in the river. Yet, as mentioned, aggression has been shown to influence which juveniles occupy superior feeding sites. Individuals with high aggression that arrive subsequent to establishment of territories can compete for and win feeding stations (Harwood et al. 2003). If MTH had elevated aggression they could negate their fitness loss of late emergence, much as aggression could combat their small size (see above). While aggression in juvenile salmonids can provide enhanced fitness, evidence from stream experiments indicates non-aggressive brown trout juveniles can be successful in heterogeneous habitat, growing just as fast as dominant juveniles (Höjesjö et al. 2002). This suggests that even if MTH are not aggressive, they still have the potential to survive and persist despite their small size and late emergence in some habitats. MSH on the other hand did not significantly differ in the time it took them to complete hatch compared to their parental species, meaning they should emerge from redds on average at the same time as pure salmon and trout fry. This, together with their larger size at hatch could potentially allow MSH individuals to compete for territories without facing prior residency effects in the way that late emerging juveniles would. However, there was a lot of variation in MSH hatch duration compared to that of pure salmon and trout. This variation could arise from the wide range of hybrid genotypes that are inevitability created when bringing together two species' genomes, leading to a wide range of hybrid fitness (Burke & Arnold 2001). F_1 hybrid genotypes can often exhibit heterosis as a result of

additive effects of alleles across loci, though this advantage often breaks down in subsequent generations (Burke & Arnold 2001). Results from this study suggest that MSH, while not exhibiting the vastly elevated fitness seen in heterosis, are certainly not suffering inherent fitness losses at these early life stages in the F_1 generation. This suggests that MSH have the ability to compete effectively with parental species. However, rearing in a hatchery environment leads to assessments of relative fitness of hybrids through measurements of genetic effects only, and doesn't give insight into how hybrid genotypes survive and compete under ecological selection in the wild (Hatfield & Schluter 1999). For this, fitness must be measured in semi-natural or natural settings.

5.4.4. Survival and growth in a semi-natural stream environment

While previous studies have looked at survival of salmon-trout F_1 hybrids in hatchery environments, this is the first study to my knowledge that has looked at early life stage hybrid fitness in the near wild. Other studies that have produced hybrids in natural spawning conditions, have then transferred the offspring to the hatchery environment (Garcia-Vazquez et al. 2002). Le Cren (1973) postulated that the likeliest factor influencing juvenile populations in salmonid species is density-dependent territorial behaviour. As discussed, both Atlantic salmon and brown trout juveniles fight to establish feeding territories very soon after emerging from the redd, with social hierarchies subsequently formed. Within these hierarchies the most dominant fish are in the most profitable feeding positions, where maximum food is gained for minimum energy expended (Fausch 1984). Competition for these feeding stations is density dependent, with high density creating high competition, and low density conditions allowing well-spaced territory to be held and reducing aggressive encounters (Kalleberg 1958). Mortality rates in streams have been to show to be related to, and be predicted by, salmon fry density (Bohlin et al. 2002; Gee et al. 1978).

In this study survival and growth was assessed among salmon and trout fry and their reciprocal hybrids in high and low density semi-natural streams. Pure trout fry had the highest survival in both high and low density streams (61 and 66% respectively). Brown trout have been shown to be competitively superior to Atlantic salmon in sympatric

assemblages, often due to increased levels of aggression (Harwood et al. 2002; Skoglund et al. 2011; Stradmeyer et al. 2008). The higher survival of trout across both stream treatments suggests that trout individuals were the most competitively able of all the fish types. Survival of all fish types was lower in the high density stream compared to that in the low density stream. It is assumed that the larger number of fish in the high density stream negatively affected survival. Previous findings have shown that fry stocked at lower densities have higher survival than when stocked at high densities (Bohlin et al. 2002; Gee et al. 1978; McMenemy 1995), as seen here in this study.

In the high density stream survival of MSH fry did not differ from the expected frequency computed by the G-test; neither did that of pure salmon and trout. This suggested survival of MSH was comparable to that of parental species, appearing intermediate between the two (figure 5.3.4a). This corroborates previous findings in this study that suggest MSH are capable of competing for space with pure species juveniles (see 5.4.2), and thus able to persist within a stream. On the other hand, MTH fry had significantly reduced survival in the high density stream, just 23% compared to 61 and 51% for pure trout and salmon fry respectively. This reduced survival suggests that MTH were unable to compete with the other fish types for feeding grounds at the head of the experimental stream (the release point), where feeding is more lucrative (Einum & Fleming 2000). MTH were significantly smaller and lighter than the other fish types when entering the streams (5.3.4). This smaller size could have led MTH to have a competitive disadvantage, leading them to be displaced downstream. In Atlantic salmon displacement downstream by dominant individuals results in less competitive juveniles only being able to access less profitable feeding grounds, as well as being at higher risk of predation (Einum & Fleming 2000). A study on rainbow trout, *Oncorhynchus mykiss* in semi-natural stream channels found, when emigration was barred, that smaller fish were less likely to occupy profitable areas of the stream, which was positively correlated to density (Keeley 2001). When emigration out of the stream was allowed, smaller fish in poor condition were more likely to leave; in both cases increased competition (density) increased mortality and led to decreased growth (Keeley 2001). The small size of MTH on entering the streams may have started them off at a disadvantage and not allowed them to compete for territories, leaving them to be displaced downstream. The low survival of MTH also suggests that they lack the high aggression seen in pure trout

(Harwood et al. 2002; Skoglund et al. 2011; Stradmeyer et al. 2008), that could see them compete for territories successfully independent of their body size, as postulated earlier.

In the low density stream the survival of all the fish types did not differ from the expected frequency. Survival of MTH fry was higher than in the high density treatment, yet it was still the lowest of all the groups. They were also longer and better conditioned than MTH in the high density stream, though again smaller than other fish types (Table 5.3.3). These results suggest that reduced competition in the low density treatment allowed MTH to feed in more profitable areas, resulting in higher survival and growth. This could have important implications if hybridisation occurs in low density populations in large rivers, as MTH may be able to survive and persist where they otherwise would be outcompeted. MSH were equal to parental species in terms of survival and growth, again suggesting that, in the F1 generation at least, these hybrids do not suffer fitness losses at early life history stages, and can persist to compete with parental species.

Length-weight relationships in the two streams were examined to assess fish growth, and whether this differed between fish types and between densities. When feeding conditions are poor, fish may lose weight and be lighter for their length than they would in good conditions (Jobling 2002). Length-weight relationships can also be a practical index on the condition of fish, which is useful for comparing life histories of populations in different regions (Petrakis & Stergiou 1995), and is usually assessed using regression analysis. The exponent b (the slope) of the regression reveals whether fish are undergoing isometric growth (Santos et al. 2002). In the high density stream MTH had a significantly different length-weight regression coefficient to the other fish types (5.3.4). Many of these fish were lighter for a given length than their fellow stream dwellers. These fish were also undergoing negative allometric growth (5.3.4), the only fish type in either stream to do so. MSH and parental species juveniles did not differ in their length-weight relationships. In the low density stream, all fish types were growing isometrically, and there was no difference in the way length varied with weight. The fact that the MTH length-weight relationship was the same as that of parental species in low density conditions, and was not in the high density stream, suggests negative allometric growth was due to environmental factors rather than endogenous to their growth. This again leads to the conclusion that both salmon and trout reciprocal crosses can survive

and compete with parental species, especially under less competitive environmental conditions.

5.4.5. Causes of variability between studies of salmon-trout hybrid fitness

Aside from the time it took to complete hatch (hatch duration), the results of the first half of my study are in conflict with more recent findings on F_1 MTH fitness. This study has clearly shown that MTH fry, while significantly smaller and taking longer to hatch have survival comparable to that of parental species and the reciprocal hybrid cross. This is in stark contrast to results of the most recent studies that have found survival at hatch and post hatch ranging from 0 to 1.95% in MTH (Álvarez & Garcia-Vazquez 2011; Garcia-Vazquez et al. 2002), and yet in conjunction with results of older studies that put survival of MTH fry at 68% (McGowan & Davidson 1992a) and in some cases higher (Chevassus 1979). So why is there so much variability when it comes to MTH results?

Chevassus (1979) put much of the variation in results seen down to lack of strict methods, poor control over environmental conditions and overripe gametes. Overripe gametes occur when ripe hatchery fish are not stripped of their gametes at the critical time window, leading to the gametes spoiling while still inside the fish. This may have explained the variation seen in results of earlier work, but our understanding of fish culture and the importance of experimental control has improved, enabling this problem to be avoided. Increased knowledge of gamete biology means sperm and eggs are stored on oxygen and ice, allowing storage without fertility impairment for up to ten days (Einum & Fleming 2000). Eggs that show over-ripeness are not used. This has allowed for superior and equivalent tests of hybrid fitness in more recent studies without confounds from unequal gamete ripening times, and it is therefore unlikely that these reasons explain why the results of my study differ to similar work carried out in the last ten years (Álvarez & Garcia-Vazquez 2011; Garcia-Vazquez et al. 2002). However, Chevassus (1979) also hypothesised that the variability in results could be due to the fact that studies often use fish originating from different populations.

The most recent works preceding this study (Álvarez & Garcia-Vazquez 2011; Garcia-Vazquez et al. 2002) were carried out using salmon and trout originating from Spanish populations, at the southern end of their European distribution. The fish used in this study originated from populations in Norway, the northern end of the species' European distribution. The study by McGowan and Davidson (1992a) was carried out on North American fish, a region where brown trout were introduced by humans around a century ago, making it a secondary contact zone. It has been noted many times that some hybrid genotypes are fitter than others within the same hybrid zone, and even within the same brood or cohort (Arnold et al. 1999; Arnold & Hodges 1995; Burke & Arnold 2001; Kruuk et al. 1999; Parris 2001), making it likely that some hybrid genotypes will be fitter in some populations compared to others. The genetic structure of Northern European Atlantic salmon has been found to be generally distinct from that of modern Southern European populations (Campos et al. 2008; King et al. 2001). The same has been found when comparing populations in North America and Europe (King et al. 2001; McConnell et al. 1995), providing evidence of genetic divergence across geographically isolated populations. It is therefore possible that different hybridising populations of salmon and trout have different levels of fitness expressed by their hybrids, due to divergence in parental genomes. There is often variation in hybrid fitness and introgression occurring across and between hybrid zones, making it important to compare them to determine the importance of intrinsic and extrinsic factors influencing their dynamics (Aboim et al. 2010). A comparison of two hybrid zones of pied and collared flycatchers showed that hybrids in a hybrid zone on the Baltic islands had apparently higher fitness than those hybrids in the Central European clinal zone. This was proposed to be the result of higher levels of introgression brought about by increased contact in the confined island habitat producing fitter hybrid genotypes (Sætre et al. 1999). As well as variation in hybrid fitness, genetic differences between populations has been postulated to result in differences in the direction of hybridisation seen between hybridising populations (Wirtz 1999). In Atlantic salmon and brown trout the direction of hybridisation is reported to be different in North America, where female brown trout hybridise with male salmon (Gephard et al. 2000), compared to Europe where the majority of hybrids are via female salmon and male trout (Garcia-Vazquez et al. 2002). Genetic differentiation between geographically distinct populations could therefore potentially be

responsible for hybrid fitness, as well as determining which cross is more prevalent, both of which could have implications for levels of introgression.

From the results presented here, I believe that it may not be possible to achieve an all-encompassing assessment of the relative fitness of salmon and trout hybrids at early life history stages; and that this can potentially only be done on an individual population basis. It is important in conservation terms to assess the threat posed by hybrids, especially to declining Atlantic salmon populations known to be at higher risk of hybridisation (Hindar & Balstad 1994). This will be especially true if the threat and impact of hybridisation varies with location, as the fitness and success of hybrids varies.

5.4.6. Conclusions

Results of my study suggest that salmon and trout F_1 hybrids have the ability to compete alongside parental crosses, particularly under low density, good growth conditions. Importantly, I also find that hybrids are not able to outcompete pure parental species during early life. However, it is possible that hybrids go on to perform poorly at later life stages. There has been relatively little experimental work looking at environmental fitness traits of salmon and trout hybrids when older than yearling parr. MSH created in natural spawning in experimental streams were noted to reach their third year under hatchery conditions (Garcia-Vazquez et al. 2002). A few studies have looked at salt water tolerance in salmon-trout hybrids. One study found that MSH had comparable survival and growth to that of pure Atlantic salmon when transferred to saltwater pens after 17 months freshwater rearing (Galbreath & Thorgaard 1997). Urke et al. (2010) found that 21% (250) of fish migrating downstream in the River Driva in Norway were MSH, most of which were adapted to enter full strength seawater, as were salmon but not trout. The fact that one fifth of the fish caught during migration to sea (Urke et al. 2010) is evidence that MSH are capable of surviving past early life history stages and of undergoing the smoltification process successfully. This, together with evidence of introgression of brown trout genes into a Atlantic salmon population (Castillo et al. 2008), shows that some hybrids are capable of returning to spawn after seaward migration. Production of post F_1 hybrid offspring was observed under natural

spawning conditions from 4 female F_1 MSH; 66.9% (83 of 124) of the resulting offspring survived until their first year under hatchery rearing conditions (Garcia-Vazquez et al. 2003).

My work is adding to the growing body of evidence that hybridisation between these two species may pose a threat to endangered populations in some circumstances. The survival of hybrids in natural conditions could also be an issue for pure salmon juveniles in declining populations. MSH juveniles appear particularly capable of competing for resources and could potentially displace pure salmon downstream, threatening their survival and increasing the risk of further population decline. Despite the fact both reciprocal hybrids appear ecologically fit in early life, evidence suggests they are reproductively unfit (Garcia-Vazquez et al. 2004) and could represent wasted reproductive effort for salmon and trout females. However, both hybrid crosses do seem capable of producing gametes and F_2 backcrossed offspring (Garcia-Vazquez et al. 2004). The next step of my thesis is therefore to investigate the reproductive fitness of salmon and trout reciprocal F_1 hybrid males when backcrossing to salmon and trout eggs, and whether paternity could be gained in competition with salmon and trout males for a female's eggs. This will allow insights into whether salmon-trout hybrids are capable of longer term, trans-generational fitness effects.

Chapter 6

Fertility and sperm competiveness of F_1 salmon-trout hybrid males

6.1. Introduction

Chapter 5 of this thesis investigated the fitness of salmon-trout hybrids at early life history stages, in relation to their pure parental species, in order to measure the ecological and evolutionary impacts hybrids pose. Both reciprocal hybrid crosses were found to have equal survival at hatch to parental species and could survive as well in semi-natural streams, particularly under low density conditions. In order to know if this equivalent hybrid fitness is trans-generational, or if salmon-trout hybrids are essentially an evolutionary dead end, information on the ability of these hybrids to produce viable sperm and backcross to parental species is needed. Many closely related species exist together in sympatry even when there is no F_1 hybrid inviability (Grant & Grant 1992). In many such cases, precopulatory and postcopulatory, prezygotic barriers maintain reproductive isolation (Coyne & Orr 1998). Precopulatory barriers are induced by differences in habitat use, mating behaviour, and reproductive timing (Coyne & Orr 1998), and are particularly well observed in birds (Grant & Grant 1996a). Postcopulatory, prezygotic barriers act to isolate species after mating has taken place but before a zygote is formed, as a result of conspecific sperm precedence and gamete incompatibility (Coyne & Orr 1998; Howard 1999). Postcopulatory, prezygotic barriers do exist between salmon and trout, as shown in chapters 3 and 4 of this thesis. However, as highlighted in these previous chapters, the prezygotic barriers to hybridisation are far from complete in this system, further evidenced by the discovery of natural hybrids in many wild populations in Europe and North America (Castillo et al. 2008; Castillo et al. 2010; Garcia de Leaniz & Verspoor 1989; Gephard et al. 2000; Hartley 1996; Hindar & Balstad 1994; Hurrell & Price 1991; Jansson et al. 1991; Jansson & Ost 1997; McGowan & Davidson 1992c; Youngson et al. 1993).

6.1.1. Postzygotic reproductive isolation

Postzygotic reproductive isolation can inhibit gene flow between species through hybrid inviability or sterility (Coyne & Orr 1989; Coyne & Orr 2004). Dobzhansky (1936) and Muller (1942) postulated that sterility and non-viability in hybrids is a pleiotropic side-effect of genetic interactions formed when species are in allopatry. The Dobzansky-Muller model

draws together the ideas of Dobzhansky (1936) and Muller (1942) to propose that genetic substitutions built up by a species when in allopatry, while small-scale enough not to reduce fitness in that species will, when brought together with genes from a divergent species, result in inviability or sterility in the F_1 hybrid or backcross generations (Coyne & Orr 1998; Russell 2003). Alleles brought together from divergent species have never been 'tested' together and the loss of co-adaptation may therefore result in hybrids with reduced fitness (Coyne & Orr 1998). The Dobzhanzky-Muller model underpins almost all modern work on the genetics of postzygotic isolation (Coyne & Orr 1998), and there is now strong evidence that hybrid sterility and inviability arise through locus incompatibilities (reviewed by Orr 1997). Situations can arise where hybrids are both viable and capable of reproducing, but are disadvantaged as they fail to mate with parent species, through mate discrimination or impaired mating behaviour (Coyne & Orr 1989). For example, male hybrids of limnetic and benthic sticklebacks had no disadvantage reproducing in the lab, but did in the wild with limnetic parents due to poor choice of intermediate habitats by hybrids, compared to parental males (Vamosi & Schulter 1999). This highlights the importance of conducting experiments within environments that present relevant selective processes in order to pick up habitat selection effects upon hybrids. But it is often combinations of isolation barriers to reproduction that are needed to completely isolate species from one another (Price & Bouvier 2002; Schluter 2001).

When hybrids are inviable or sterile they are effectively wasted reproductive effort for the parental individuals involved (Rhymer & Simberloff 1996), as they themselves are unable to reproduce. Total sterility in F_1 hybrids can result in replacement of one species by another; this is particularly the case when one of the hybridising species has been introduced and is a successful 'invader' (Largiadèr 2008). While F_1 hybrids can be fit, the F_2 and backcrossed generations can be inviable or sterile due to breakdowns in co-adapted genes or chromosomes (Barton & Hewitt 1989; Templeton 1986). Karyotypes regularly differ between different species, which can lead to chromosomal based hybrid sterility (Turelli et al. 2001). Different chromosome numbers in the parent species can sometimes result in recombination of chromosomes in F_1 hybrids that produce aneuploid gametes, which can die or cause zygotes to perish (Rieseberg 2001). Salmon and trout have a large difference in chromosome numbers, with trout having 80 compared to 58 (typically) for salmon

(Pegington & Rees 1967). The difference in number is thought to have evolved through redistribution and structural change as a result of fusion or fragmentation of chromosomes, rather than polyploidy (Rees 1964). While the numbers of chromosomes differ, the total amount of nuclear DNA is similar (Rees 1964), with trout having smaller but more numerous chromosomes (Pegington & Rees 1967). This difference in chromosome sizes is maintained within hybrid nuclei, leading to the suggestion that the change in karyotype through speciation arose through redistribution and structural change, rather than being genotypically determined (Pegington & Rees 1967). Because of this large karyotypic difference, salmon – trout hybrids were expected to be sterile; however, reports of fertile F_1's and production of F_2 and back-crossed offspring are established in the literature (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Nygren et al. 1975; Wilkins et al. 1993), suggesting postzygotic reproductive isolation may not be complete between these species. Having established the similar ecological fitness of salmon-trout hybrids in Chapter 5, this chapter will therefore investigate F1 hybrid fertility, and also measure this in the context of sperm competition.

6.1.2. The threat of introgression

When pre and postzygotic barriers to hybridisation are incomplete or break down, genes of one species can become introduced into the gene pool of another, via fertile recombinant hybrid and backcrossed genotypes. The resulting gene flow can lead to partial genetic mixing of the two species genomes in a process called introgression (Wright 1977). Introgression can play a positive role in the evolution of a species by proving sources of adaptive genetic variation (Grant et al. 2005) and enabling species to widen their ecological niche (Choler et al. 2004). However, the transfer of exotic genes into a population via introgression is more likely to create negative consequences, potentially resulting in genetic extinction of that species by swamping and replacing the endemic genome (Epifanio & Nielsen 2000; Hails & Morley 2005; Mallet 2005; Rhymer & Simberloff 1996), and leading to hybrid swarms (Seehausen 2004) and outbreeding depression (Rhymer & Simberloff 1996). Postzygotic barriers to hybridisation can be sex-biased due to the phenomenon known as Haldane's rule. Haldane (1922) noted that when one sex in the F_1 offspring of

interspecific crosses is missing, rare or sterile, that sex is more likely to be the heterogametic sex. Haldane's Rule does not appear to be a postzygotic isolation mechanism in salmon-trout hybridisation, where males are the heterogametic sex, due to reports of fertile male and female F_1 hybrids (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Nygren et al. 1975; Wilkins et al. 1993) and no described bias in abundance of one sex over another.

Introgression of genetic material between species can be asymmetrical, both in terms of which species undergoes introgression, and the introgressed genes themselves (Largiadèr 2008). Across natural hybrid zones, asymmetrical patterns of gene introgression have been observed due to particular genes being more 'readily' introgressed than others, perhaps due to a selective advantage conveyed to the backcrossed genotype (Avise 1994). Asymmetrical hybridisation and introgression can occur through several mechanisms. Size dimorphism between hybridising species can lead, for example, to the smaller species' females to be the only ones to hybridise successfully. This can be due to mechanical incompatibilities, for example where smaller males trying to mate with larger females are physically unable to copulate (Karl et al. 1995). Size dimorphism can also lead to asymmetrical hybridisation, when females of the smaller species accept mates from larger heterospecific males, but females of the larger species reject males of the smaller species due to sub-normal mating cues (Grant & Grant 1997). Poor mate discrimination, preference for heterospecific mates or gamete incompatibility in one hybridising species, can also result in asymmetry (Wirtz 1999).

It has long been proposed that the relative abundance of closely-related species can impact hybridisation dynamics and directionality of introgression (Hubbs 1955; Mayr 1963). If one of the hybridising species is less abundant than the other, females of the rarer species will be more likely to encounter heterospecific gametes through increased encounters with the more common species' males (Wirtz 1999). Thus, often the maternal species in a hybrid cross is from the rarer species, which has been observed in mammals, fish and birds (Avise & Saunders 1984; Hofmeyr et al. 1997; Lajbner et al. 2009; Väli et al. 2010), suggesting female mate choice can also play a role in asymmetrical introgression (Wirtz 1999). Males of a rare species can attempt to court females of a more common, closely related species, but

are rejected in favour of common conspecific males, resulting in no hybrids between rare species males and common species females; while on the other hand, females of the rare species initially reject common males, lack of conspecific encounters results in reduced mate discrimination in the rare species female and interspecific mating occurs, leading to uni-directional hybridisation and introgression (Wirtz 1999). In conjunction with this, F_1 hybrids are also more likely to mate with the more abundant species due to increased encounter rates, leading to backcrossed individuals from the more abundant species (Lepais et al. 2009). This could be damaging to the minority species, particularly if represented by a few individuals that produce a large proportion of hybrids, as they may become locally extinct due to gene swamping and dilution of the genome (Burgess et al. 2005; Lajbner et al. 2009; Lepais et al. 2009; Rhymer & Simberloff 1996; Rosenfield et al. 2000). This scenario could be of concern in the case of Atlantic salmon and brown trout. Atlantic salmon are known to be declining in the majority of their distribution due to anthropogenic influences (Parrish et al. 1998; WWF 2001). Trout, by contrast do not appear to be suffering to the same extent, showing no general declines in abundance (Jonsson & Jonsson 2011); however, declines in localised areas have been seen (Burkhardt-Holm 2008; Hansen et al. 2002; Jonsson & Jonsson 2011). Hybrids in Europe are commonly created as a result of female Atlantic salmon crossing with brown trout males (Garcia-Vazquez et al. 2001), particularly where salmon numbers are low (Castillo et al. 2010) and differences in relative abundance of the two species within a river can lead to extensive hybridisation in some cases (Ayllon et al. 2004; Jansson & Ost 1997). This is possibly as a result of lack of male salmon to spawn with. In a survey of southern European rivers, stocked brown trout inflated natural populations and hybridisation with Atlantic salmon was found to increase, with hybrids mostly from domesticated brown trout males and Atlantic salmon females (Castillo et al. 2008). Introgression of brown trout genes into Atlantic salmon populations resulted, though the effect was reversed after stocking ceased (Castillo et al. 2008).

6.1.3. Loss of local adaptation due to introgression

Both Atlantic salmon and brown trout show strong genetic differentiation among populations across river basins (Ferguson 1989; Hansen & Loeschcke 1996; Hindar et al.

1991; McConnell et al. 1995; McConnell et al. 1997; Nielsen et al. 1996) and even in continuous habitats (Heggenes et al. 2009). The large differentiation in genetic structure is brought about by strong natal fidelity and homing ability of anadromous salmonids (reviewed by Stabell 1984), with 94-98% of individuals returning (Jonsson et al. 2003), together with the low iteroparity of individuals resulting in constrained gene flow (Garcia de Leaniz et al. 2007). Low gene flow, together with large environmental differences across their distribution (3200 km in Europe [Garcia de Leaniz et al. 2007]) and wide genetic variation, provide Atlantic salmon populations with clear opportunities to become locally adapted to their environments. Local adaptation is present in populations when the average fitness of individuals originating in the population habitat exceeds the average fitness of conspecific immigrants entering the habitat (Lenormand 2002). The evidence for local adaptation in salmonids is mostly indirect, coming from ecological correlates with phenotypic traits (Garcia de Leaniz et al. 2007; Taylor 1991), with most phenotypic traits having heritable components (Carlson & Seamons 2008). Yet the evidence, both indirect and direct, makes a convincing case for local adaptation (Garcia de Leaniz et al. 2007). Several studies on Atlantic salmon have identified genetic differences between populations for traits that relate to fitness, such as body size, growth rate and survival (Friedland et al. 1996; Jonasson 1993; Jonasson et al. 1997; Jonsson et al. 2001). Introgression of non-native genes into a salmon population could potentially erode local adaptation by introducing genes that are non-adaptive, with evidence that population mixing can lead to outbreeding depression (Einum & Fleming 1997). This is of large concern for salmon and trout populations that are subject to domesticated fish of hatchery origin that enter populations through stocking and escapes. Hatchery reared fish have not been subject to river conditions and have been under relaxed natural selection pressures, with domesticated Atlantic salmon showing reduced survival and reproductive fitness in the wild (Fleming et al. 2000; McGinnity et al. 2003). However, escaped farmed fish are known to successfully reproduce with wild Atlantic salmon and introgression of genes from hatchery reared fish into wild populations is feared to have negative effects on the genetic integrity and fitness of wild populations (Bourret et al. 2011; Einum & Fleming 1997; Fleming et al. 2000; McGinnity et al. 2003; McGinnity et al. 1997). Tentative evidence of farmed salmon introgressing with a wild population of Atlantic salmon having an increasing negative impact over time has been seen in Canada. The Atlantic salmon population of the Magaguadavic River in The bay of Fundy has been in

severe decline for the last 20 years, which correlates with interbreeding with escaped farmed salmon (Bourret et al. 2011). A detailed genetic study on the population found strong, but not definitive, evidence for introgression of farmed genes within the wild population (reduced differentiation between farmed and wild salmon over time), that has resulted in significant alteration in genetic integrity with negative effects (Bourret et al. 2011). More controlled experiments have found evidence both for and against, that repeated breeding with farmed individuals reduces local adaptation (Frasier 2008).

Perhaps the best evidence for local adaptation is in pathogen resistance (Garcia de Leaniz et al. 2007). Salmon in the Baltic sea are resistant to the monogenean flatworm parasite *Gyrodactylus salaris*, after building up resistance through prolonged exposure when isolated in glacial lakes (Meinilä et al. 2004). Whereas Scottish and Norwegian stocks are susceptible or partly susceptible to infection due to a lack of exposure to the parasite (Bakke 1991; Bakke et al. 2002; Bakke et al. 1990; Dalgaard et al. 2003). Introduction of *G. salaris* to Norwegian rivers in the 1970's lead to population extinctions 5-7 years after the introduction event in infected rivers (Johnsen & Jensen 1986). Infection resistance is known to be heritable, with interspecific hybrids between Atlantic salmon and brown trout showing intermediate resistance to that of the parents (Bakke et al. 1999). Brown trout have innate resistance to *G. salaris* and hybrids fathered by brown trout (the most common cross in Europe) inherit this resistance. This could mean that trout and hybrids may be the survivors in infected rivers where the two species are sympatric, and may form a reservoir and dispersal mechanism of the parasite (Bakke et al. 1999). Introgression of salmon genes into brown trout also has the potential to disrupt trout innate ability to resist the parasite, threatening survival.

While there is little direct evidence to conclude that introgression disrupts local adaptation, the threat of introgression to Atlantic salmon is real and does occur between salmon and trout (Castillo et al. 2008). But how likely is introgression to occur under natural conditions in sympatric rivers? Interspecific mating could be of particular relevance where the two species are forced together through reduced spawning grounds and stocking activities, and when population levels of either species become polarized, where increased encounter rates may lead to increased interspecific mating and increased hybridisation (Castillo et al. 2008;

Hindar & Balstad 1994; Jansson & Ost 1997). However, mating behaviour of both sexes in both species will play a role in how successful hybrid males are. Natural spawning experiments showed Atlantic salmon males chased away brown trout males and hybridisation with female trout only occurred in the absence of conspecific males, and only with trout of intermediate size (Beall et al. 1997). These changes in spawning behaviour could mean hybridising males would not face sperm competition. However, only a single small scale spawning study has been done, which is unlikely to capture natural spawning conditions in a mixed species assemblage. Salmon are known to be polyandrous, with multiple males spawning for a female (Fleming 1996; Martinez et al. 2000), and sneak salmon male parr are thought to interfere with trout spawning to produce hybrids (Garcia-Vazquez et al. 2001; Gephard et al. 2000), meaning hybridising salmon and trout males will be likely to face sperm competition. Moreover, the incidence of hybrid males within natural spawnings may be difficult to determine in natural redds when precocious parr or smaller trout males are present. Therefore the primary goal of this study was to investigate the ability of hybrid sperm to compete against sperm from adult male salmon and trout in fertilisations of salmon and trout eggs. However, to gain an idea of the basic ability of hybrid sperm to fertilise salmon and trout eggs, *in vitro* fertilisation trials with F_1 hybrid males fertilising salmon and trout eggs in the absence of competition were also carried out. Due to financial constraints, I was unable to grow up F_1 hybrid offspring from these single fertilisation back crosses to assess hatching success and fitness through the juvenile stages. To achieve the principal aim of the study, sperm from F_1 hybrid males was competed against sperm from anadromous salmon and trout males for eggs from their conspecific females within *in vitro* sperm competitions. This design allowed the ability of hybrid sperm to gain paternity under competition with conspecific males to be assessed. Sperm motility traits of hybrids were also measured, in conjunction with sperm competitions, to compare sperm function to that of adult anadromous 'pure' salmon and trout males. Any hybrid males in a population that go on to spawn will conceivably be subject to the postcopulatory selection generated by sperm competition, and their success or failure will determine whether introgression is a real threat to Atlantic salmon populations or not.

6.2. Methods

To measure the fertility of hybrid F_1 males, *in vitro* fertilisation experiments were set up where sperm from male maternal salmon hybrids (MSH) and maternal trout hybrids (MTH) were used to fertilise salmon and trout eggs. Fertilisation success was compared to that of conspecific male controls. Further to this, the competitive ability of hybrid sperm in competition was also assessed though *in vitro* sperm competition experiments. Motility and behaviour of sperm from hybrid males and pure males were recorded in conjunction with *in vitro* sperm competitions in order to compare the behaviour of hybrid sperm to that from pure species males.

In 2007, S. Yeates created offspring batches of pure salmon and trout, and salmon-trout reciprocal hybrids, from adult 1st generation hatchery reared Atlantic salmon and brown trout originating from fish from the Figgjo River. After 2 years of development in the hatchery, these parr-stage fish were checked in November 2009 for sperm-producing males after anaesthetisation in chlorobutanol (0.5 ml per 10l of water). Spermiating male parr then had their milt collected into 10ml plastic vials by gently applying pressure to the abdomen in a downward motion from the pectoral fins to the vent, causing the milt to run into the vial. Care was taken to dry the vent and surrounding area of water, urine and faeces to prevent activation of the sperm (see 2.3). Samples were then stored on ice until needed in the same way as described in section 2.2. At the same time, adult anadromous Atlantic salmon and brown trout males and females were stripped of their gametes and also stored on ice until needed (see section 2.2). As all the fish were stripped, a sample of fin tissue was taken and placed in 95% ethanol for later DNA analysis that would allow offspring paternity analysis.

6.2.1. In vitro *fertilisation trials*

Each hybrid male type was back-crossed to batches of Atlantic salmon eggs (n= 20 crosses per hybrid male type) and brown trout eggs (n= 16 crosses per hybrid male type). To compare the fertility of the hybrid males to that of pure species males, conspecific control

crosses between Atlantic salmon males and the same females (n =20), and brown trout male and the same females (n=15) were performed. The six cross types are detailed in table 6.2.1

Table 6.2.1: Single fertilisation crosses carried out *in vitro* between maternal salmon hybrids (MSH), maternal trout hybrids (MTH), and Atlantic salmon and brown trout males with Atlantic salmon and brown trout females. N is the sample size for each cross.

Female	**Male**	**n**
Salmon	Salmon	20
Salmon	MSH	20
Salmon	MTH	20
Trout	Trout	15
Trout	MSH	16
Trout	MTH	16

The *in vitro* fertilisation trials were carried out as described in section 2.3.1. On average, 67 ± 12 eggs (range = 47-104) were placed on one side of a dry beaker, and 50 µl of sperm in extender (see 2.3) was added to the other side. 300 ml of river water, with an average temperature of 5.7 ± 0.15 ºC (range = 5.5-5.8 ºC) was added rapidly and the eggs then left to stand for a minimum of 3 minutes to allow complete fertilisation. Egg batches were then placed in individually numbered incubators and allowed to develop for ten days before fertilisation success was determined using the acetic acid method (see 2.3.4).

6.2.2 In vitro *sperm competition trials*

In vitro sperm competition trials were carried out using IVF beakers as detailed in section 2.4.2. Hybrid males were competed against salmon males for salmon eggs and against trout males for trout eggs, leading to 4 competitive treatments in each group (table 6.2.2).

Table 6.2.2: Sperm competition crosses carried out *in vitro*, between conspecific males and maternal salmon hybrid (MSH) and maternal trout hybrid (MTH) males. Males entered the competition simultaneously with equal volumes of sperm. N is the sample size for each cross.

Female	**Male 1**	**Male 2**	**n**
Salmon	Salmon	MSH	9
Salmon	Salmon	MTH	10
Trout	Trout	MSH	9
Trout	Trout	MTH	8

On average 68 ± 12 (SD) (range = 45-113) eggs were placed into a dry IVF beaker on one side. Equal volumes of sperm in extender from two males (40 µl per male) was mixed using a Gilson pipette and added to the opposite side of the dried beaker to the eggs. 300 ml of river water, with an average temperature of 5.7 ± 0.15 ºC (range = 5.5-5.8 ºC) rapidly poured over the eggs and sperm, after which the eggs were left to stand for at least 3 minutes. Egg batches were then placed into individually labelled incubators and offspring left to develop until hatch, when they were humanely killed and placed in 95% ethanol to preserve tissue for later microsatellite paternity analysis. Each egg batch was photographed before being placed into the incubator so the initial number of eggs that were subject to sperm competition could be counted. Eggs that died throughout development were picked and the numbers recorded, leaving only live embryos at hatch. Offspring still alive at hatch were counted and compared to the initial egg number, allowing the survival to hatch for each sperm competition to be calculated for each cross.

6.2.3. Sperm trait analysis

To measure differences in sperm traits between male types, sperm motility of each male was recorded after activation in river water, as described in 2.3.2. Traits recorded and analysed were curvilinear velocity (µm s^{-1}), sperm path linearity (%), percentage of motile sperm (%) and sperm longevity (s). These traits have been shown to be important when assessing the

viability and competitiveness of teleost sperm (Kime et al. 2001; Rurangwa et al. 2004), and thus were used to compare the quality of hybrid sperm to that of pure males. For detail on the sperm traits measured see 2.3.2. Spermatozoa were recorded in parallel with fertilisation and sperm competition experiments, allowing a representative record of the behaviour of each male's sperm entering the *in vitro* fertilisations.

6.2.4. Microsatellite and paternity analysis

Offspring of sperm competitions between hybrids and pure males were genotyped using the method described in 2.5. Between 7 and 22 offspring were genotyped per cross, with an average of 15 offspring per cross typed. Genotype peaks were read using GeneMapper v 4.0 (Applied Biosystems). Each offspring was unambiguously assigned to one of the males that took part in the sperm competition cross.

6.2.5. Statistical analysis

All statistical analysis was done using the R Project for Statistical Computing software version 2.13. For fertilisation crosses the proportion of eggs fertilised by each male was arcsine square root transformed to achieve normality and homogenous variance. To investigate differences between the fertilisation success of MSH and MTH males with salmon eggs compared to Atlantic salmon males, a one way ANOVA was performed. The same test was performed comparing MSH and MTH male success with trout eggs compared to trout males. In the case of a significant ANOVA result, Tukey's Honest Significant Difference (TukeyHSD) post hoc testing was used to see where the differences in fertilisation success occurred.

The sperm competition result data were not normally distributed, and could not be transformed to normality. To compare the paternity between males within a competition cross, the proportion of eggs won by each male in the competition was compared using a non-parametric Wilcoxon rank sum test. This test is the non-parametric equivalent of the

independent samples t-test, using medians, and is based on the magnitude of difference between pairs of data points. When a competition cross was unanimously won by a single male type, no statistics were necessary.

Curvilinear and longevity sperm trait data were normally distributed (Shapiro-Wilks test for normality) with homogenous variance (Bartlett test), and the percentage of motile sperm for each male was arcsine square root transformed to normality and homogenous variance. Linearity data (the percentage of net distance moved to total path distance) could not be arcsine transformed to normality. Differences in the individual traits between the male types was investigated using one way ANOVA's when the data were normally distributed with homogenous variance. When data were not normally distributed, the non-parametric version of ANOVA, the Kruskal-Wallis test was used.

6.3. Results

Of the 51 2nd year MSH checked, 22 were found to be with sperm, compared to 31 out of 51 MTHs. As the fish were not sexed it was not possible to compare the proportion of sperm producing males between hybrids, as differences may have been due to more females being randomly selected in one group than the other. Both reciprocal crosses were capable of fertilising both salmon and trout eggs in the absence of sperm competition, though they achieved approximately 30% lower fertilisation success than conspecific males. Under sperm competition for salmon eggs with conspecific males, both hybrid crosses had extremely low paternity success, siring less than 5% of the offspring typed. Neither reciprocal hybrid could backcross successfully with female trout eggs in competition with conspecific males.

6.3.1. Fertilisation trials

Both hybrid crosses were capable of fertilising eggs and producing live embryos ten days post fertilisation with both egg types. There was a significant difference in the fertilisation

success of salmon males and hybrid crosses for salmon eggs ($F_{2, 57} = 16.77$, P = <0.0001). Both MSH and MTH males has significantly lower fertilisation success with salmon eggs than salmon males (TukeyHSD pairwise comparisons: P = <0.0001). There was also a significant difference between the fertilisation success of hybrid crosses and male trout for trout eggs ($F_{2, 45} = 3.81.77$, P = 0.02). While both MSH and MTH fertilised significantly less eggs than trout males, the effect was not as strong as seen with salmon eggs (TukeyHSD: P = 0.04), and MTH were on the cusp of significance (P = 0.06). However, despite fertilising significantly less eggs than pure salmon males, MSH were still able to fertilise over 60% of salmon eggs and over 50% of trout eggs on average (64 ± 10.7 and 55.1 ± 11.1 (1 S.E.M) % respectively). MTH followed the same pattern as MSH with both salmon and trout eggs (61.5 ± 12 and 57.4 ± 11.4 (1 S.E.M) % on average respectively). There was no significant difference in fertilisation success of hybrid males when fertilising salmon or trout eggs (TukeyHSD pairwise comparisons: P = >0.05).

6.3.2. Sperm competition trials

To determine whether sperm from hybrid male parr were capable of fertilising eggs of salmon while in competition with pure males, *in vitro* sperm competition trials were run. Salmon hybrids and trout hybrids were only able to gain some paternity when competing with pure salmon males for salmon eggs. MSH achieved on average 4.8 ± 7.1 (1 S.E.M) % success with salmon eggs when competing against pure salmon males (figure 6.3.2), significantly lower than pure salmon males (Wilcoxon: W = 80, P= 0.0002). Trout hybrids had lower success than salmon hybrids when competing with salmon males for salmon eggs with 2.8 ± 5.2 (1 S.E.M) % paternity on average (figure 6.3.2), again significantly lower than salmon males (Wilcoxon: W = 80, P = 0.001). When hybrid crosses were competed with trout males for trout eggs, neither maternal salmon or trout hybrids were able to gain any paternity success (figure 6.3.2).

Figure 6.3.1: Mean ± 1 S.E.M proportion of eggs fertilised by hybrids and pure males. A) Maternal salmon hybrids (MSH [blue bars]), maternal trout hybrids (MTH [yellow bars]) and pure salmon males' (open bars) success fertilising salmon eggs. B) Maternal salmon hybrids (MSH), maternal trout hybrids (MTH) and pure trout males' success fertilising trout eggs.

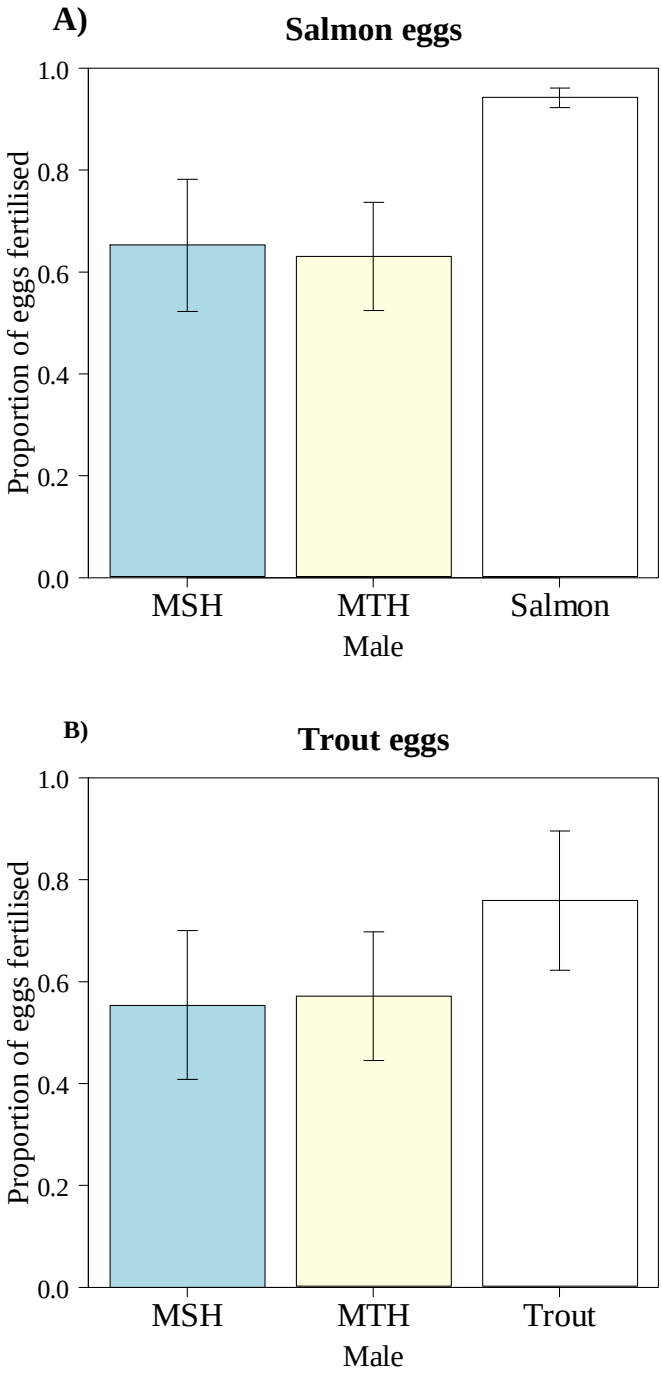
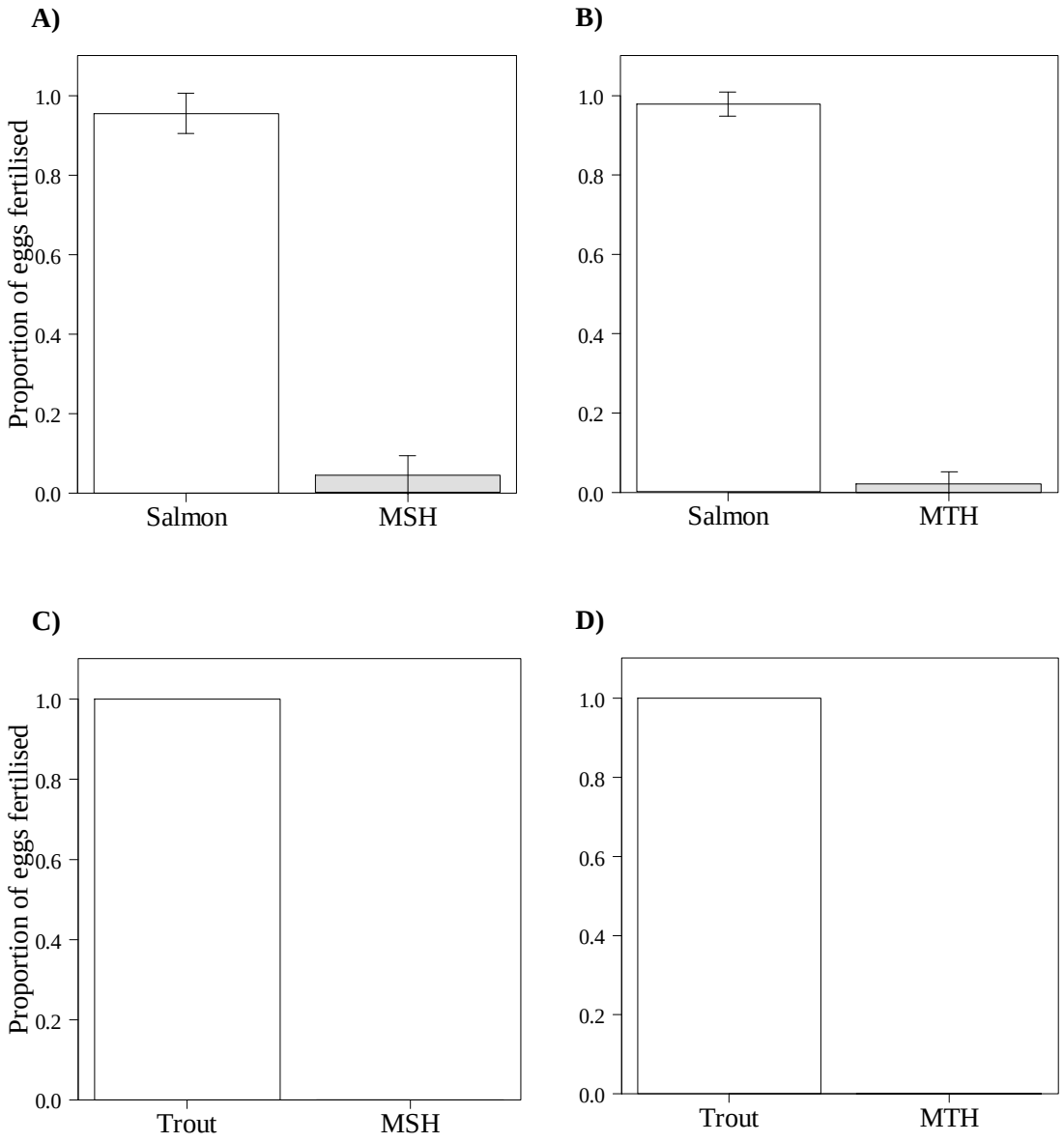


Figure 6.3.2: Mean proportion of eggs ± 1 S.E.M fertilised by hybrid and pure males under sperm competition. A) Paternity success of salmon males (open bar) in competition with maternal salmon hybrid (MSH) males (grey bar) for salmon eggs. B) Paternity success of salmon males (open bar) in competition with maternal trout hybrid (MTH) males (grey bar) for salmon eggs. C) Trout males (open bar) win all paternity when in competition MSH males for trout eggs. D) Trout males (open bar) win all paternity when in competition with MTH males for trout eggs.



Average survival to hatch ± 1 S.E.M of eggs fertilised in sperm competitions was calculated for each competition (6.2.3). Results are shown in table 6.3.1. All sperm competition crosses had lower average survival than the usual hatchery average for pure crosses of 95% (K. Bergesen personal communication).

Table 6.3.1: Mean ± 1 S.E.M percentage of eggs that survived to hatch for *in vitro* sperm competitions of maternal salmon hybrid (MSH) and maternal trout hybrid (MTH) male parr with adult anadromous salmon and trout males.

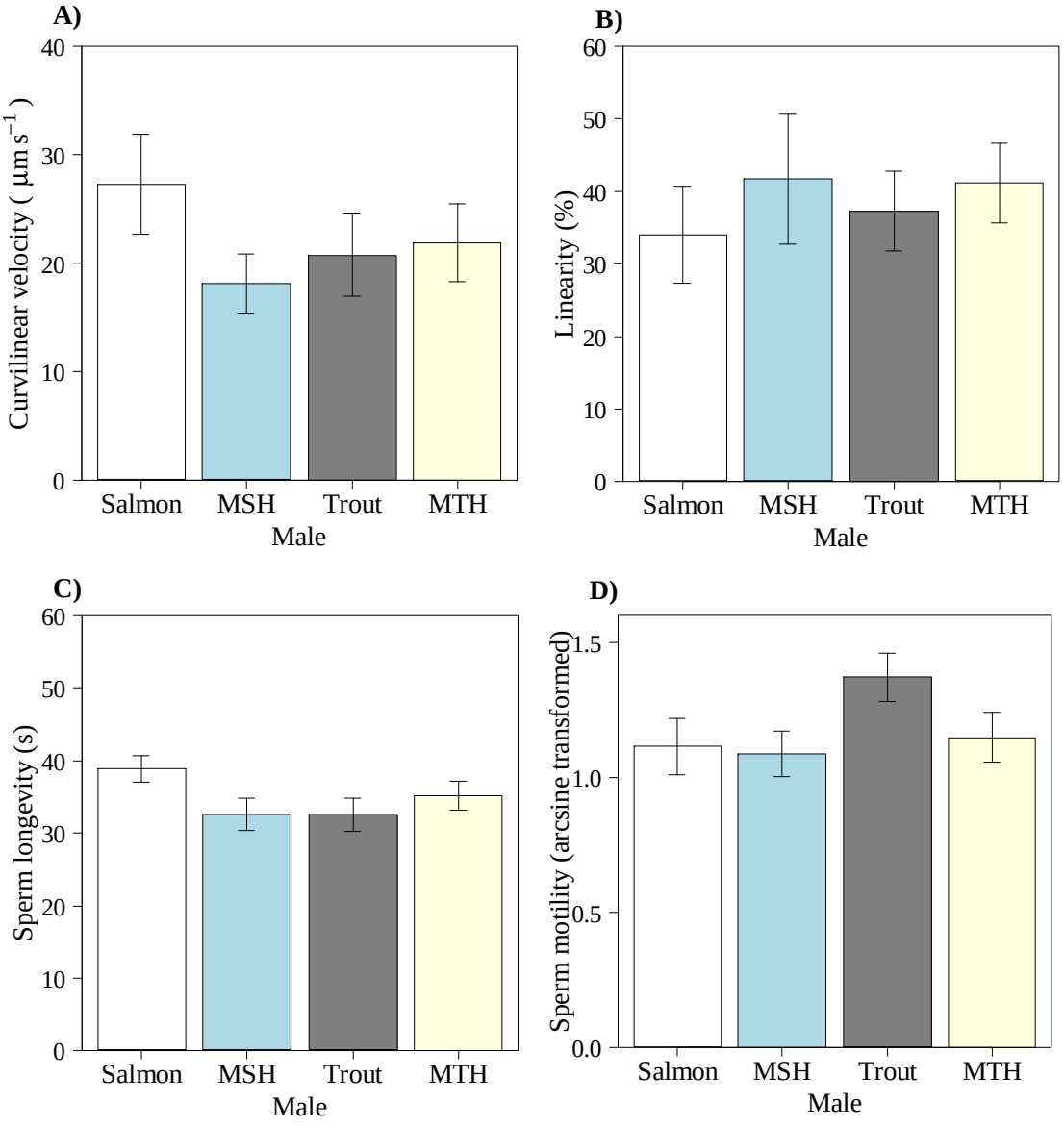
Eggs	Pure adult male	Hybrid parr male	% Embryos survived to hatch	SEM	Range %
Salmon	Salmon	MSH	74.2	3.1	37.7 - 96.5
Salmon	Salmon	MTH	75.5	2.7	56.5 - 92.6
Trout	Trout	MSH	58.2	6.3	16.6 - 89.6
Trout	Trout	MTH	54.9	6.7	0 – 90

6.3.3. Hybrid sperm motility traits

Sperm traits of hybrid males were measured in conjunction with sperm competitions, along with those of pure males, to explore for differences in motility and behaviour of spermatozoa originating from hybrids and pure males using computer assisted sperm analysis (see 2.3). No significant difference was found between hybrids and pure males in linearity ($F_{3, 72} = 1.07$, $P = 0.364$), a measure of sperm path trajectory. However, ANOVA did reveal a significant difference in curvilinear velocity between crosses ($F_{3, 72} = 4.23$, $P = 0.008$). Post hoc testing indicated that salmon males had significantly faster sperm MSH (TukeyHSD P= 0.004), but no other comparisons were significant. Sperm longevity was also significantly different ($F_{3, 72} = 7.92$, $P = 0.0002$), with post hoc testing showing salmon males to have significantly longer lived sperm than both hybrid crosses (TukeyHSD, P = <0.001 stats), no other comparisons were significant. Motility also differed between crosses ($F_{3, 72 =} 6.51$, $P = 0.0005$). Post hoc testing revealed trout males had significantly higher sperm motility than any other fish types (TukeyHSD, P = <0.01), no other comparisons were significant.

Previous work has also shown that salmon males have faster sperm than trout and that trout sperm are longer lived (personal observation) suggesting hybrid sperm is no less competitive than salmon or trout sperm.

Figure 6.3.3: Comparisons of sperm motility traits measured by CASA, between pure salmon (open bars) and trout (grey bars), maternal salmon hybrids, (MSH [blue bars]) and maternal trout hybrids, (MTH [yellow bars]). A) Mean ± 1 S.E.M sperm curvilinear velocity. B) Mean ± 1 S.E.M sperm path linearity. C) Mean ± 2 S.E.M sperm longevity in seconds. Mean ± 1 S.E.M sperm motility, arcsine square root transformed.



6.4. Discussion

This study aimed to assess the reproductive fitness of male F_1 salmon-trout reciprocal hybrids, both in terms of quantifying their ability to fertilise eggs of parental species and their competitive ability in sperm competition with conspecific males. Both maternal salmon hybrid (MSH) and maternal trout hybrid (MTH) parr were able to produce viable sperm that showed comparable motility traits to that of adult salmon and trout pure species males. When backcrossed to salmon eggs *in vitro*, hybrid males fertilised significantly less eggs than adult salmon males, but still gained over 60% fertilisation success (compared with 75 to 95% success for pure trout and salmon males respectively). When backcrossing to trout eggs *in vitro*, fertilisation success of hybrid crosses was over 50%, with MSH fertilising significantly less eggs than adult trout males. However, MTH had no significant difference in their ability to fertilise trout eggs, though this was on the cusp of significance. A bigger sample size may have revealed a reduced ability of MTH to fertilise trout eggs. Despite their clear potential to fertilise, both hybrid crosses were notably less successful in their reproductive ability under sperm competition. When competing with salmon males for salmon eggs, both reciprocal crosses were able to gain paternity, but average success was very low. MSH gained around 5% paternity and MTH 3%, obviously significantly lower than that of adult salmon males. When competing against trout males *in vitro*, neither hybrid cross was able to gain any paternity at all. This data suggests that hybrid males were perhaps unable to compete with sperm from conspecific males, or that hybrids were able to fertilise a large proportion of eggs in the sperm competition, but those eggs failed to develop and hatch.

6.4.1. F_1 hybrid backcrossing

The primary aim of this study was to investigate the ability of F_1 hybrid males to compete against adult salmon and trout, as sperm competition would be a likely scenario for spawning in natural populations. To determine the ability of hybrid males to fertilise salmon and trout eggs in the absence of sperm competition, single-pair *in vitro* fertilisation crosses were carried out. Earlier studies on the ability of hybrid offspring to backcross to salmon and

trout have shown that MSHs are capable of producing viable offspring (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Nygren et al. 1975; Wilkins et al. 1993). Survival of offspring by male MSH is very low with both salmon and trout females (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Nygren et al. 1975; Wilkins et al. 1993). Survival of offspring from female MSH were found to have relatively high survival (compared to that of male's offspring) when crossed with Atlantic salmon males (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004). Offspring from both reciprocal crosses have been shown to produce recombinant genotypes in post F_1 offspring when crossing back with Atlantic salmon, evidence that chromosome pairing at meiotic division is possible (Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Wilkins et al. 1993). MSH males are able to produce halploid gametes and are thus expected to be fertile; however, female hybrids of this cross have only been shown to produce diploid gametes (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004). The large eggs and triploid offspring produced by female MSH are indicative of unreduced gametes (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2003; Garcia-Vazquez et al. 2004), and when female hybrids were backcrossed to Atlantic salmon males, offspring had 2 sets of salmon chromosomes and 1 of brown trout, which must have originated from the hybrid mother (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2003; Garcia-Vazquez et al. 2004). Female MSH have been shown to spawn with Atlantic salmon males under natural spawning conditions, and offspring survived under hatchery conditions until the second year (Garcia-Vazquez et al. 2003). However, due to their triploid nature, offspring from this cross will likely be sterile (Garcia-Vazquez et al. 2003; Garcia-Vazquez et al. 2004). There have been no experiments examining spawning behaviour of MSH or MTH male spawning behaviour with adult salmon. As with male MSH, both sexes of MTH can produce haploid gametes (Garcia-Vazquez et al. 2004), resulting in the production of diploid offspring when backcrossed to Atlantic salmon, some of which survived, though survival was much lower in these crosses (Garcia-Vazquez et al. 2004). Due to the diploid nature of post F_1 offspring produced by MTH, there is no reason to suggest these offspring would not produce haploid gametes (Garcia-Vazquez et al. 2004) and thus be fertile and able to reproduce themselves.

Neither hybrid cross was able to gain paternity under sperm competition conditions when competing with brown trout males for brown trout eggs (figure 6.3.2 d-c). In previous

studies, all backcrosses to brown trout have failed during the embryo stage (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004). This raises the possibility of hybrid males being able to gain fertilisations when competing for brown trout eggs, but fail to achieve paternity due to their progeny failing to develop. This hypothesis is supported by the fact that both MSH and MTH parr are capable of fertilising over 50% of brown trout eggs in the absence of sperm competition, with live embryos at ten days. Further support comes from the fact that survival to hatch (6.2.3) of offspring from competitions for trout eggs against trout males was 54.9 ± 6.7% for MSH and 58.2 ± 6.3% for MTHs. This seems abnormally low as average survival to hatch in pure crosses at the Ims hatchery where all work for this thesis was carried out, is generally very high at 95% (K. Bergesen personal communication). This further substantiates the possibility that hybrid males were able to win fertilisations, but those embryos died during development. The sperm volumes throughout the sperm competitions were high enough to ensure 100% fertilisation of egg batches, so it is unlikely that eggs were sperm limited and did not get fertilised at all. In sperm competitions for Atlantic salmon eggs between hybrid males and salmon males, hybrids were able to gain small paternity success (less than 5%). Again both hybrid crosses were found to be capable of fertilising both salmon and trout eggs in the absence of competition, with over 60% success (figure 6.3.1). But, as discussed previously, studies have found very low survival of offspring from hybrid F_1 males backcrossed to Atlantic salmon females (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Nygren et al. 1975; Wilkins et al. 1993). This, combined with lower than average survival to hatch of backcrosses to Atlantic salmon eggs in competition with salmon males, with 74.2 ± 3.1% and 75 ± 3.1% for MSH and MTH respectively, again suggests that MSH and MTH were capable of gaining fertilisation success within the sperm competitions, but only a very small proportion survived to hatch. This hypothesis is supported in both salmon and trout backcrossing by analysis of hybrid sperm. CASA and analysis of the sperm motility trait data showed that both hybrid crosses have competitively equivalent sperm to parents in terms of motility characteristics (Gage et al. 2004). However, this of course is just conjecture. It is also possible that hybrid males have competitively inferior or less compatible sperm and were unable to gain fertilisations in competition with pure species males. Under both scenarios it seems that hybrids are postzygotically isolated from brown trout. To confirm the hypothesis that F_1 hybrid males are able to gain fertilisation under

sperm competition but they fail to hatch due to embryo death, detailed analysis of all embryos in a batch of fertilised eggs would be needed. Carrying out the same sperm competitions and then genotyping embryos as they die and all live embryos at hatch, would confirm the ability of hybrid males to gain fertilisation success under sperm competition.

The fact that all backcrosses of reciprocal hybrids to brown trout resulted in inviable offspring suggests genetic mismatching leads to fatal embryo developmental problems. Co-adapted gene complexes in parental species can be broken up in recombinant hybrid genotypes, where mosaic chromosomes are created composed of two divergent genomes (Renaut & Bernatchez 2010). As stated in the introduction, the Dobzhansky-Muller (B-D-M) model is used to explain hybrid breakdown and has been increasingly supported (Coyne & Orr 2004). A simple scenario in the B-D-M model proposes that a mutant allele **a** becomes fixed at locus **A** for one population, while in a second population separated from the first by allopatry, a mutant allele **b** gets fixed at the locus **B**. When the two populations come into secondary contact, F_1 hybrids would have copies of the wild type **A** and **B** alleles as well as mutant **a** and **b** alleles, leading to normal genetic interactions continuing with no negative effects on hybrid fitness. However, a proportion of the F_2 or backcross generation will be homozygous for both mutant alleles **a** and **b** which, having evolved in separate lineages where selection could not act positively on their interaction, will lead the alleles to interact poorly together, resulting in hybrid fitness breakdown (Burton et al. 2006; Coyne & Orr 2004). Genomic incompatibles, such as the ones described in the B-D-M model can lead to disruption of DNA transcriptions by RNA, impacting on the way genes are expressed and result in novel gene expression in hybrids, and is hypothesised as a pivotal factor in hybrid breakdown (Landry et al. 2007). Renault and Bernatchez (2010) found transcriptome-wide disruption was responsible for hybrid breakdown in the backcrossed progeny of hybrids from a species pair of dwarf and normal white fish (*Coregonus* spp. which reside within the Salmonidae family). F_1 hybrids backcrossed to normal type parents resulted in offspring with a genome comprising 25% dwarf and 75% normal type genes. Of the backcrossed offspring, 33% showed abnormalities that resulted in death. Renault and Bernatchez (2010) compared the transcriptome of parents and found it was virtually identical during embryonic development, yet all hybrids showed strong divergence in gene expression. When comparing transcriptome expression between healthy and deformed hybrids, the authors found over

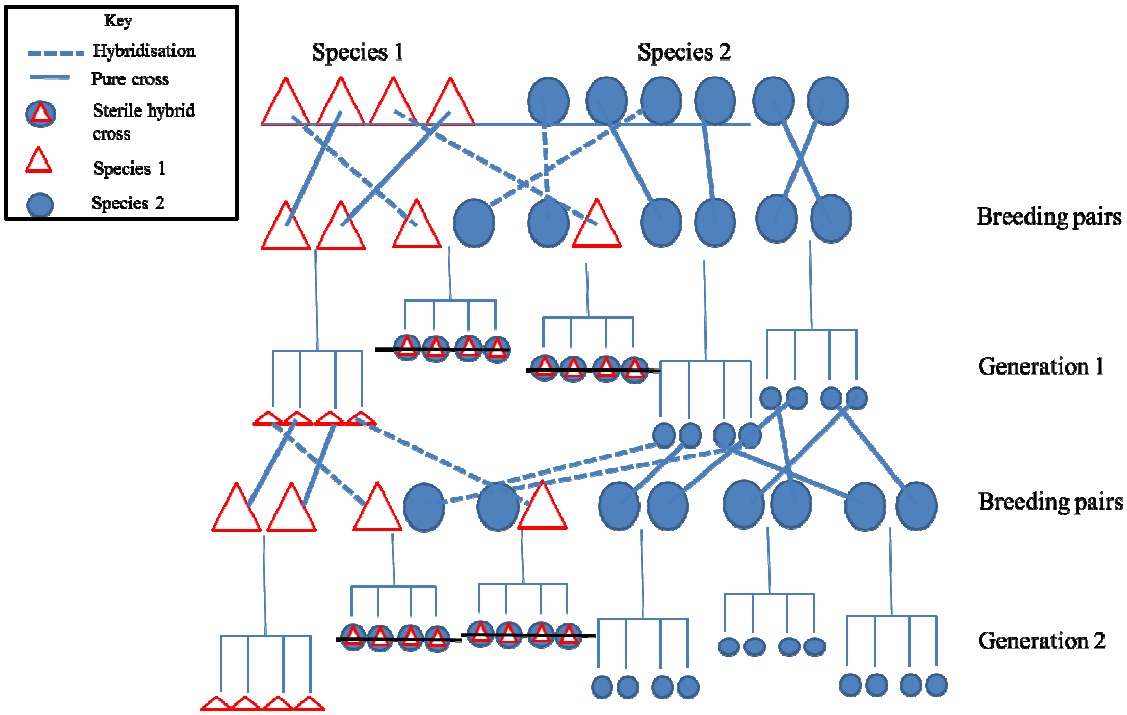
2000 genes were misregulated in abnormal hybrids, with a bias toward developmental genes, providing the mechanism for the observed hybrid breakdown (Renaut & Bernatchez 2010). This whitefish case is an example of regulatory incompatibles that can arise in hybrids and backcrosses through recombination of divergent genomes. A simple scenario that would give rise to regulatory incompatibilities would be the co-evolution of transcription factors and transcription binding sites within a genome (Landry et al. 2007). There has also been suggestion that hybridisation may lead to disruption in mitochondrial function and could be an underlying cause of hybrid breakdown (Burton et al. 2006). All work on salmon-trout hybrids has shown they suffer from a large degree of hybrid breakdown in backcross generations to Atlantic salmon, and total breakdown in backcrosses to brown trout; however the underlying causes have not been investigated. Due to the asymmetrical reproductive isolation between salmon and trout, resulting from failed backcrossing to brown trout, introgression into brown trout via hybrids is not possible it seems. However, viable diploid offspring from backcrossing to Atlantic salmon suggests introgression of trout genes into Atlantic salmon populations is possible (Garcia-Vazquez et al. 2004). Indeed, introgression of brown trout genes into an Atlantic salmon population has been seen in a Spanish river that had undergone sustained stocking with brown trout that led to increased hybridisation (Castillo et al. 2008). Introgression occurred at very low levels however, and the effect was seen to be reversed after stocking had ceased (Castillo et al. 2008).

6.4.2. Salmon-trout hybrids and wasted reproductive effort

Evidence from this study and previous work, seems to suggest that introgression is not a major threat to Atlantic salmon populations; it appears the larger threat posed by hybrids is that of wasted reproductive effort of pure salmon individuals put in to creating hybrids, leading to reductions in population size (figure 6.4.1). The consequences of reduced effective population size due to wasted reproductive effort can be seen in a few cases in the wild. The European mink has been in decline for over a hundred years (Maran & Henttonen 1995) with part of this decline blamed on the introduction of the larger, more competitive American mink. European mink are known to hybridise with male American mink, but the hybrid embryos abort in the womb resulting in wasted reproductive effort (Rozhnov 1993).

The wasted eggs that result from these non-productive couplings are thought to be accelerating the decline of European mink (Allendorf et al. 2001).

Figure 6.4.1: Hybridisation where the offspring are sterile or inviable can lead to declines in rare species (open triangles) while not impacting on the more abundant species (closed circles). In the scenario depicted below, the number of offspring have increased overall in 2 generations, but due to the sterility/inviability of the hybrid the rarer species declines in frequency (after Levin 2002 ; Rhymer 2008).



A similar situation occurs between the bull trout, *Salvelinus confluentus*, and the introduced brook trout, *S. fontinalis*. Hybridisation of the two species in sympatric assemblages results in displacement of the native bull trout (Leary et al. 1993). The hybrids produced are predominantly sterile (Leary et al. 1993) meaning any reproductive effort used in creating them is lost and reduces the effective population size contributing to the next generation. This has the largest effect on bull trout populations, as it is the females that predominantly produce the hybrids with male brook trout (Kanda et al. 2002). As well as this, bull trout do not sexually mature until 3-6 years of age, whereas brook trout can mature in 2 years, thus

any reduction in effective population size will have a larger negative impact on bull trout populations than brook trout (Leary et al. 1993). However, well documented cases where F_1 sterility and inviability cause loss of fitness in parent taxa are not common. If hybrids are inviable and die at early life stages, hybridisation may go unnoticed. which would explain the lack of empirical evidence for introgression (Largiadèr 2008). An additional explanation is simply that hybrid F_1 sterility and inviability have relativity little impact on the displacement of parental taxa (Huxel 1999), as long as sufficient parents continue to reproduce conspecific offspring in sufficient numbers (Largiadèr 2008).

The consequences of wasted reproductive effort for Atlantic salmon populations could be severe for declining populations. Individuals that spawn to produce functionally sterile salmon-trout hybrids are not contributing to the next generation, lowering the effective population size. The effective population size, simply stated, is all the individuals in a population that contribute to their genetic material to the next generation, i.e. excluding juvenile or sterile individuals. A population's effective population size is related to its viability, and can allow predictions about a population's potential extinction to be made (Newman & Pilson 1997). It is known that small populations are at risk of losing genetic variation through random genetic drift, regardless of their effective population size; but this can be compounded by a small effective population size, and populations can rapidly lose genetic variation (Nunney & Elam 1994). A small effective population size can lead to a reduction in heterozygosity which can have a negative impact on fitness (Allendorf & Leary 1986), and correlate with population extinction (Newman & Pilson 1997). If reproducing adults can survive an effective population size bottleneck and go on to breed again, then the genetic effect on the population will be much reduced (Nunney & Elam 1994). However, Atlantic salmon (particularly females) generally spawn once in their lifetime (Fleming 1996) meaning any reproductive effort that is wasted in a spawning season, lowers the effective population size of that generation permanently. Hybridisation between salmon and trout has been recorded at high levels (Jansson et al. 1991; Jansson & Ost 1997), and is at risk of increasing (Hindar & Balstad 1994). With Atlantic salmon are declining in much of their range (Parrish et al. 1998; WWF 2001), the impact of the production of functionally sterile hybrids to vulnerable populations could be high.

If genetic incompatibility leads to reduced viability of offspring, as seen in salmon-trout hybridisation, selection should favour pre or postcopulatory mechanisms to avoid it (Welke & Schneider 2009). It is hypothesised that polyandry has potentially evolved as a mechanism to avoid inbreeding (Michalczyk et al. 2011; Zeh & Zeh 1997), where genetic incompatibility is high, and numerous studies have provided good evidence that postcopulatory mechanisms play a role in inbreeding avoidance (Bretman et al. 2004; Jehle et al. 2007; Kraaijeveld-Smit et al. 2002; Mack et al. 2002; Olsson et al. 1996b; Thuman & Griffith 2005; Welke & Schneider 2009; Zeh & Zeh 1997). Although precopulatory mate discrimination (Roberts & Gosling 2003) and postcopulatory behavioural mechanisms such as sperm ejection (Pizzari et al. 2004) have been suggested as the mechanism behind the way females ensure fertilisation by a less related male, there is evidence for postcopulatory inbreeding avoidance. One such potential postcopulatory mechanism is cryptic female choice (CFC), where morphology, physiology, or behaviour of a female non-randomly biases the paternity of her offspring (Birkhead 1998b; Eberhard 1996; Pitnick & Brown 2000). CFC can only occur when a female's ova are exposed to two or more ejaculates, i.e. she is polyandrous. At the other end of the relatedness scale to inbreeding, CFC could also be used to avoid hybridisation via conspecific sperm precedence or heterogamy (Howard 1999), as seen in the salmon-trout hybridisation system in chapter 4, mediated by a female's ovarian fluid. Polyandry in my study system seems to reduce the risk of hybridisation, as a bias toward conspecific sperm will mean that the majority of eggs will not be wasted through hybrid fertilisations under sperm competition (Chapter 4). This is strongly reinforced when hybrid F_1 males attempt backcrossing under sperm competition. It is therefore possible that the high degree of polyandry seen in Atlantic salmon has evolved as a mechanism to allow CSP and avoid hybridisation.

Chapter 7

General discussion and conclusions

This thesis tackles both pure and applied questions surrounding the reproductive biology of a naturally-hybridising, externally-fertilising fish system. I present further evidence for the conspecific sperm precedence (CSP) seen in Atlantic salmon and brown trout hybridisation (Chapter 1, Chapter 3 and Chapter 4), and identify a mechanism that explains this CSP when sperm from both species are introduced simultaneously to eggs (Chapter3 and Chapter 4). A variety of *in vitro* fertilisation experiments were conducted in conjunction with gamete motility analysis, fertility scoring and microsatellite paternity screening, to explore the dynamics of gametic interaction within the incomplete reproductive isolation between these species. These findings are presented alongside a detailed examination of the ecological and reproductive fitness of F_1 salmon-trout hybrids, allowing a risk assessment of the impacts hybrids may have on wild populations. Reciprocal hybrid crosses were measured for different fitness traits at early life stages, under a combination of controlled and semi-natural environments (Chapter 5). In addition to this, *in vitro* fertilisation and sperm competition experiments were again used in combination with gamete motility and paternity analyses to compare sperm function of hybrids with adult salmon and trout (Chapter 6), and assess whether hybrids pose a real threat of gene introgression or represent wasted reproductive effort (Chapter 6).

7.1. Gametic reproductive isolation between Atlantic salmon and brown trout

Investigations into salmon-trout reproductive isolation in the past have shown the two species to be isolated mainly in the form of differences in peak spawning times, with brown trout spawning on average 15 days earlier than salmon (Heggberget et al. 1988). This segregation is far from complete, with widespread reports of hybridisation in Europe and North America (Garcia de Leaniz & Verspoor 1989; Gephard et al. 2000; Hartley 1996; Hindar & Balstad 1994; Hurrell & Price 1991; Jansson et al. 1991; Jansson & Ost 1997; McGowan & Davidson 1992b; Payne et al. 1972; Verspoor 1988), and in some cases relatively high (Garcia de Leaniz & Verspoor 1989; Hartley 1996; Jansson & Ost 1997). No studies have previously looked at the success of hybridising salmon or trout males under sperm competition, conditions which they are likely to face in the wild (Elliot 1994; Fleming

1996; Martinez et al. 2000; Weir et al. 2010). Work directly preceding this thesis carried out *in vitro* sperm competitions between salmon and trout males, and found that conspecific males won significantly more paternity than heterospecific males (figure 1.4.1). This provided clear evidence for the existence of a degree of reproductive isolation at the level of the gamete in the form of CSP, for the first time in this system (S. Yeates unpublished data). Because these findings exist under external fertilisation, the result suggests that salmonid eggs possess a mechanism that allows them to preferentially 'select' conspecific sperm through the fertilisation process, if such sperm are present. To determine the temporal dynamics of this CSP, I ran experiments which introduced a timing delay to sperm entering the competition. There is a first male sperm precedence in intraspecific sperm competitions in Atlantic salmon (Yeates et al. 2007), so I hypothesised that the mechanism of conspecific sperm choice might reveal CSP, even if the conspecific sperm are in the disadvantaged second-male position. My results indeed showed that when a 2 second advantage was provided to heterospecific males during *in vitro* sperm competition with conspecific males, first male precedence was not seen (Chapter 3). A 2 second advantage in sperm release did allow hybridising heterospecific males to boost their paternity over that gained under simultaneous release, but paternity on average was shared with conspecific males, with both achieving near equal 50% fertilisation success (figure 3.3.1a and c). These results further confirm that a mechanism of CSP is acting to confer an advantage to conspecific sperm when they enter the competition, even if they have been temporally disadvantaged. A simple scenario may be in operation in salmon-trout hybridisation to bring about the paternity patterns observed. When hybridising sperm are first to enter the sperm competition, they probably gain advantage in the first few seconds of fertilisation over that under simultaneous release, as they have sole access to the eggs. However, when conspecific sperm enter the competition, any first-male advantage to hybridising sperm is countered by the CSP advantage. This would result in the majority of the remaining fertilisations going to conspecific sperm. In reverse roles my results show that when heterospecific males enter sperm competitions as P2, with a 2 second delay to their sperm, they suffer a significant fertilisation disadvantage compared to conspecific males who won the significant majority of fertilisations. This produced results similar to those seen under simultaneous release (figure 1.4.1 and figure 3.3.1b and d). These paternity patterns suggest that, in the first 2 seconds when conspecific sperm have sole access to the eggs, mechanisms of CSP are not in effect.

However, when heterospecific sperm enter the competition, CSP mechanisms come into play, promoting fertilisation by conspecific sperm to produce the same paternity share as under simultaneous release. Evidence from some teleost fishes shows that the egg micropyle can be occupied within 6 seconds following gamete release (Iwamatsu et al. 1991), making timing in sperm release and crucial to fertilisation success (Hoysak et al. 2004; Yeates et al. 2007). The rapid sperm-egg association in salmonids through a micropyle possibly provides females with a lower discriminatory capacity against potentially hybridising sperm compared with other systems, particularly those with internal fertilisation where sperm must traverse selective barriers or are stored for long periods of time (Briske 1996; Hellriegel & Bernasconi 2000; Snow & Andrade 2005). Despite the very short time frame in which to discriminate against heterospecific sperm, my results demonstrate clear evidence of CSP at the gamete level.

7.1.1. Ovarian fluid as a mediator of conspecific sperm precedence

Having established CSP within sperm competitions where different males's sperm were released simultaneously or after a short delay, the next logical step was to investigate which mechanisms mediate the observed CSP within salmon-trout hybridisations (Chapter 4). CSP can represent a form of cryptic female choice (CFC), because females can be using reproductive selection mechanisms to avoid fertilisation by heterospecific sperm, or promote fertilisation by conspecific sperm, when a choice exists (Geyer & Palumbi 2005; Rugman-Jones & Eady 2007). Recent work has provided some evidence for CFC in salmonid species. Yeates et al (2009) showed that Atlantic salmon eggs are preferentially fertilised by sperm from males that are more similar to them at the MHC. Once female salmonids have released their eggs, there are two biological traits that could potentially play a role in CFC: the ova and/or the ovarian fluid (OF) that surrounds and coats the eggs and is released at spawning. The ovum in salmonids has a single micropyle which the sperm must access, enter and penetrate to locate the egg pronucleus. The eggs, and thus the mycropylar opening, are bathed in a considerable amount of fluid thought to be secreted by the ovaries (Lahnsteiner et al. 1995a). In salmonid species, OF has been shown to enhance motility traits of sperm by increasing velocity and the duration of progressive movement, or longevity (Dietrich et al.

2008; Lahnsteiner 2002; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007), making it a logical candidate for mediating CSP in salmon-trout hybridisation. Results from chapter 4, in which experiments controlled the presence of conspecific or heterospecific OF, showed that conspecific OF conveyed a significant paternity advantage to conspecific sperm, and that ovum identity played no role in the direction of CSP, i.e. salmon OF gave salmon sperm significantly higher fertilisation success when competing with trout sperm for either trout or salmon eggs (figure 4.3.4). The same pattern was observed from the trout male perspective (figure 4.3.4). These results strongly suggest that it is OF and not egg-sperm surface interactions that mediate CSP in salmon-trout hybridisation. My results also provide good evidence for CFC in an external fertiliser, where the potential for female postcopulatory control could be considered unlikely. Previous studies have suggested that OF could allow a mechanism of CFC in intraspecific salmonid fertilisation, as the influence OF has on sperm mobility differs between females and within males (Dietrich et al. 2008; Rosengrave et al. 2008; Urbach et al. 2005). These findings suggest that some females may have the ability to discriminate between ejaculates of individual males, achieving CFC and biasing fertilisation in favour of certain males, but no direct links to fertilisation success have been made. Here I directly link increases in fertilisation success to conspecific OF, providing strong evidence for OF operating as a mechanism of CFC to promote fertilisation by conspecific males and avoid hybridisation.

Following the isolation of OF as a factor driving CSP, I examined sperm function of salmon and trout in conspecific and heterospecific OF. Using a cell migration assay which quantifies the degree of cell movement through a permeable membrane into a chemoattractant, I found that activated sperm of both salmon and trout migrated through a permeable membrane into conspecific OF, compared with heterospecific OF (figure 4.3.6 and 4.3.7) or water (4.3.4). Direct examinations of salmon and trout sperm in their own OF compared with water, showed that OF caused significant changes in sperm swimming behaviour, specifically in terms of the straightness of the sperm swimming path, and increases in sperm swimming longevity (Chapter 4, figure 4.3.8). However, direct examinations of salmon and trout sperm activated in conspecific OF compared with heterospecific OF did not reveal differences in sperm motility traits (figure 4.3.9). It is possible that the methods used to compare sperm motility in conspecific versus heterospecific OF were not able to detect changes in sperm

motility because of the rapid nature of sperm activation and fertilisation. Fertilisation dynamics in salmonids show 80% of fertilisations are complete within 5 seconds of gamete activation (Hoysak & Liley 2001), suggesting that the initial activation period in the race to locate the micropyle is very important. The methods I used to measure sperm motility in this thesis may not have been fine enough scale to capture these crucial first seconds of fertilisation (discussed in Chapter 4). In addition, the relatively high variance in sperm motility traits within a small sample size of trials may have struggled to demonstrate any differences in sperm behaviour between conspecific versus heterospecific OF. It is possible that sperm must be first activated in water (as would occur naturally) before encountering a high OF concentration around the egg to reveal behavioural changes.

Whatever the effect of conspecific versus heterospecific OF, my motility results revealed a clear effect of OF on sperm behaviour compared with activation in water, with a straightening of the sperm swimming path and an increase in sperm motile lifespan. Previous work has demonstrated that when fish spermatozoa begin to swim in water they have a curved trajectory (Kime et al. 2001), following in an elliptical pattern. As shown in this study (figure 4.3.8) and others (Dietrich et al. 2008; Rosengrave et al. 2008; Turner & Montgomerie 2002; Urbach et al. 2005; Wojtczak et al. 2007), sperm path linearity increases in OF compared to water, so the sperm begin to swim in straighter trajectories in OF. Path linearity is a measure of a spermatozoa's trajectory through a solution (Kime et al. 2001), with a high linearity meaning a straight line path. A potential mechanism for the CSP induced by OF in salmon-trout hybridisation observed in this thesis could come from changes in sperm linearity caused by OF as a form of chemoattraction up an increasing OF concentration gradient. It is possible that when sperm reach OF around the eggs in a redd, they switch from elliptical to straight line swimming in order to continue up the chemical gradient, directing sperm to reach the egg and high concentration of OF in the micropyle faster. If conspecific OF generated a more specific signal to sperm that stimulated the transition from elliptical to straight-line swimming, this may provide conspecific sperm with a directional advantage in reaching the egg first. Anything that would help one male's sperm locate eggs faster than another's would influence relative fertilisation success in competition, as in salmonids it is the first egg to reach the micropyle, which is a single sperm head in diameter, that tends to successfully fertilise it (Yanagimachi et al. 1992). This proposed

mechanism is suggested by the results of the Transwell assay and the CASA comparisons of sperm in conspecific OF versus water (Chapter 4). In sea urchins, changes in sperm flagella movement occur when the egg peptide resact binds with a receptor on the sperm, activating rapid production of cyclic guanosine monophosphate (Kaupp et al. 2003). This rapid phosphate production opens K+ channels and increases the membrane potential, subsequently resulting in entry of Ca^{2+} into the cell (Strunker et al. 2006). This causes the flagellum of the sperm cell to beat in an asymmetrical fashion and produce a high curvature trajectory (Böhmer et al. 2005). Resact appears to induce spikes of Ca^{2+} in sea urchin spermatozoa, with spikes producing a curved trajectory and turning the sperm toward the source of resact in units of response (Böhmer et al. 2005). It is possible that a peptide in the protein-rich OF of salmonids could provide a similar mechanism (Rosengrave et al. 2008). Since ions regulate sperm activation in fish (Morisawa & Suzuki 1980), it is also possible that changes in OF ionic concentration could lead to changes in sperm movement. Whatever the specific regulator of sperm swimming behaviour in OF, my results show that it is relatively species-specific, which would be expected if divergence were promoted by reinforcement to allow CSP for hybridisation avoidance (Swanson & Vacquier 2002a).

7.1.2 Divergence in sperm–egg compatibility in closely related species

CSP between salmon and trout provide evidence of divergence in gametic compatibility between two very closely related species. But why should this occur? Strong gametic incompatibility is seen in externally fertilising marine invertebrate species of sea urchins and abalone, and can be considered as a form of mate recognition (Geyer & Palumbi 2003). In these invertebrates, sperm proteins bind with receptors on the egg envelope that allow penetration and fertilisation, and this binding has been shown to be species specific (Glabe & Vacquier 1977; Lee et al. 1995; Lee & Vacquier 1992; Metz et al. 1994; Palumbi & Metz 1991; Shaw et al. 1993; Swanson & Vacquier 1997; Vacquier & Lee 1993), and result in CSP (Geyer & Palumbi 2005). Divergence in sperm-egg recognition is thought to be due to divergence in sperm protein amino acid sequences, some of which have been shown to be under positive selection, resulting in incompatibility between heterospecific sperm and egg membrane receptors (Palumbi 1999; Palumbi 2009; Swanson & Vacquier 1998; Swanson &

Vacquier 2002b). Amino acid divergence is hypothesised to arise in one of two ways: directional selection from coevolution of egg and sperm proteins to increase fertilisation efficiency, or from cyclic selection, where sperm evolves to increase fertilisation efficiency and egg penetration rate, leading to evolution of eggs to slow sperm entry to avoid polyspermy (Palumbi 1999). While these mechanisms would most logically arise in allopatry to create incompatibility on secondary contact (Coyne & Orr 2004), evolutionary theory postulated that they have the potential to occur under sympatric conditions as well (Gavrilets & Waxman 2002; Van Doorn et al. 2001). Further to this, these mechanisms of selection could be accelerated in sympatry through reinforcement as a result of hybridisation avoidance (Palumbi 1999), resulting in reproductive character displacement in the form of CSP.

Reproductive character displacement (RCD) occurs when a trait crucial to reproduction differs between populations of a species that live in sympatry with closely related species, compared to populations of that species that live in allopatry (Geyer & Palumbi 2003). This can relate to sympatric divergence in traits such as mate recognition (Höbel & Gerhardt 2003; Waage 1979) and timing of reproduction (Hillis 1981; Marshall & Cooley 2000). RCD is thought to result through direct selection to limit hybridisation, and is closely linked to reinforcement often with confusion over the two terms. Reinforcement is the process by which prezygotic isolation evolves as a direct result of selection against hybrids. It describes the mechanism by which natural selection against unfit hybrids strengthens prezygotic isolation (Coyne & Orr 2004; Geyer & Palumbi 2003). RCD is often thought of as the pattern resulting from the reinforcement mechanism (Rundle & Schluter 1998). In some cases authors reserve the term reinforcement for events that led up to species divergence, and RCD to those that strengthen isolation after species have split and already become "good" species; i.e. they are different processes that result from the same mechanism of selection (Butlin 1987). However, those distinctions will not be made here. RCD is identified in a number of cases, but only a few clearly demonstrate the role of selection (Geyer & Palumbi 2003). As discussed, sea urchins are isolated due to gametic mate recognition as a result of divergent amino acid sequences of sperm proteins. The sea urchin *Echinometra oblonga* is found in mixed sympatric assemblages with an as yet unnamed closely related congener, *E. sp C.* Individuals of *E. oblonga* sympatric with *E. sp. C* show much higher divergence at

sperm binding protein alleles compared with individuals in allopatric populations, that are shown to share, in some cases identical, allele sequences with allopatric *E sp. C* (Geyer & Palumbi 2003). This divergence is driven by positive selection on sperm binding alleles, and is an example of RCD that is suggestive of reinforcement (Geyer & Palumbi 2003). However, specific knowledge on the fitness of hybrids is needed in this case before conclusions on whether selection is acting to limit hybridisation can be drawn, as RCD could also arise as a secondary effect of environmental adaptation (Coyne & Orr 2004; Noor 1999), opposed to directly selecting against unfit hybrids.

The CSP between salmon and trout observed in this study could be another example of RCD arising from reinforcement in an external fertiliser, mediated through OF rather than sperm egg recognition proteins. As salmon and trout share overlapping habitats with very little segregation (Armstrong et al. 2003; Heggberget et al. 1988), there is probably limited potential that CSP would arise as a by-product of environmental adaptation. If OF also plays a role in fertilisation it is unlikely to differ greatly between the two species. The place environmental selection could potentially influence divergence between sperm and OF, is in the part OF may play in helping to prevent eggs and sperm being dispersed from the females nest (Rosengrave et al. 2008). The high viscosity of salmonid OF (Rosengrave et al. 2008; Turner & Montgomerie 2002) may help impede the dislodgement of eggs from redds in fast flowing water, creating a more stable nest environment to allow more efficient fertilisation. OF in the abalone species, *Haliotis rufescens w*as shown to create a low rate laminar shear flow that allowed for faster sperm swimming speeds, increased encounter rates and increased fertilisation relative to water (Riffell & Zimmer 2007). In humans, sperm are also subject to fluid shear flow in the reproductive tract and only swim effectively at very low shear flows (Winet et al. 1984). However, if nesting sites of salmon and trout differed in surrounding water velocity, different selection pressures may act on OF to shape how viscous it is between species, with sperm naturally selected to swim in their own species OF. This could possibly result in incompatibility between the swimming efficiency of a male's sperm and heterospecific OF. Yet, for this to happen salmon and trout would have to have differences in the flow speed of water where females nest. While there are subtle differences in the water velocity at nest sites between salmon and trout there is also a large amount of overlap. Mean velocity of river water at redds are very similar between the two species (Armstrong et al.

2003), making it unlikely that differential spawning habitat is currently driving gametic divergence in these species.

7.2 Hybrid ecological and reproductive fitness

For reinforcement to be possible as the driving force behind the RCD in salmon and trout CSP, knowledge on the fitness of hybrids is needed. If producing hybrids does not confer fitness losses they will not be selected against, and reinforcement cannot lead to prezygotic isolation. Chapters 5 and 6 assessed the fitness of salmon-trout reciprocal hybrids. While both hybrids showed fitness relative to that of parent species at early life stages, particularly maternal salmon hybrids (Chapter 5), males of neither hybrid cross were able to backcross to brown trout females, and had very low paternity success with salmon females in competition with conspecific males (Chapter 6). Evidence from my work and previous studies (reviewed in chapter 6) suggest strong postzygotic isolation exists between these species. Pre-existing postzygotic isolation is one of the criteria needed to allow RCD as a result of reinforcement to develop between species (Coyne & Orr 2004). Natural selection will only act to limit hybridisation if the high costs to fitness exist when producing hybrids. In the case of salmon and trout, the cost of fitness appears to come in the form of wasted reproductive effort (Chapter 6). However, Marshall et al (2002) argued that in species with strong conspecific gamete precedence (CGP) and multiply mated females (like salmon and trout), selection for reinforcement would be weak in females. This is because the cost of mating with heterospecific males would be much reduced if they also mated with conspecific males, as the presence of CGP would mean that the majority of their progeny would be of conspecific, rather than hybrid, origin (Marshall et al. 2002). Yet, the fact that salmon and trout share spawning habitats so closely and CSP is not complete, (females still producing on average 37% hybrids when 'spawning' with salmon and trout males simultaneously (*In vitro* sperm competitions, S. Yeates unpublished data)), together with strong postzygotic isolation suggest that selection could be acting to limit hybridisation in these species through CSP. To effectively test whether reinforcement is playing a role in the RCD of salmon-trout CSP, *in vitro* spawning experiments like those carried out in chapter 4, with the manipulation of OF, need to be done with sympatric and allopatric populations of salmon and trout. This will

allow investigation into whether CSP is stronger in sympatric populations as opposed to allopatric populations of these species, which would be indicative of CSP as a population-level mechanism of reinforcement.

7.3. Consequences of salmon trout hybridisation

One of the main aims of my thesis was to assess the fitness of salmon and trout reciprocal hybrids in relation to parental species at early life history stages, in order to infer impacts of hybridisation on wild salmon and trout populations. This is important as Atlantic salmon are viewed with high conservation importance due to declining populations in the majority of their distribution (Parrish et al. 1998; WWF 2001), and are vulnerable to negative impacts on population growth. Hybridisation is one of a variety of factors to have a negative effect on salmon populations, and has been seen to be increasing (Hindar & Balstad 1994; Jansson & Ost 1997). Possible threats hybridisation pose to vulnerable Atlantic salmon include loss of local adaptation due to introgression and/or genetic swamping, reductions in effective population size due to wasted reproductive effort, and being out competed and replaced by ecologically fit hybrid progeny (reviewed in Chapters 1, 5 and 6 of this thesis).

The survival of hybrids in natural conditions could be an issue for pure salmon juveniles in declining populations that find themselves subject to hybridisation as a result of lack of conspecific mates. Hybrids that are ecologically fit at all or some life stages have the potential to generate ecological or evolutionary impacts on one or both of the parental species, thereby driving down pure species fitness via ecological or genetic loading. The Pecos pupfish, *Cyprinodon pecosensis*, is threatened with replacement by hybrids arising from breeding with a closely-related species, the sheapshead minnow, *C. variegates*. Hybrids have elevated swimming performance and faster growth, both of which increase food acquisition, reduce the threat of predation, and improve the gaining and holding of breeding territories (Rosenfield et al. 2004). Results of my study into the ecological fitness of salmon-trout hybrids (Chapter 5), suggest that F_1 maternal salmon hybrids had no detectable differences in fitness in relation to pure salmon and trout at any of the early life history stages examined, and may have a potential fitness advantage at hatch (Chapter 5).

Conversely, maternal trout hybrids suffered reduced fitness at some stages, yet had equal fitness to parent species at others, particularly under low density conditions. Importantly however, my results show that neither hybrid cross exceeded parental fitness at any life history stage measured in the study. This suggests it is unlikely that salmon-trout hybrids would be able to out-compete juvenile individuals of parental species, at early life history stages at least. However, if hybrids were shown to be sterile or unable to produce viable offspring themselves, they would represent wasted reproductive effort for their parents; regardless of how fit they were at other life history stages. The next step of my thesis was therefore to investigate the reproductive fitness of salmon and trout reciprocal F_1 hybrid males when backcrossing to salmon and trout eggs, and whether paternity could be gained in competition with salmon and trout males. This would allow insights into whether salmon-trout hybrids are capable of longer term, trans-generational fitness effects.

Results described in Chapter 6 found that both male maternal salmon and maternal trout hybrid parr were capable of producing viable sperm with comparable motility trait values to that of adult salmon and trout pure species males. This motile, viable sperm from both crosses was able to fertilise over 50% of both salmon and trout eggs compared with pure species fertilisation rates under equivalent conditions of over 70% for trout and 90% for salmon (figure 6.3.1). When competing with salmon males for salmon eggs, both reciprocal crosses were able to gain paternity, but average success was very low at less than 5% (figure 6.2.3a-b). When competing against trout males both hybrid male crosses failed to backcross at all, gaining no paternity. In previous studies, all backcrosses to brown trout have failed in the embryo stage (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004), and survival of offspring from hybrid F_1 males backcrossed to Atlantic salmon females has been very low (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004; Johnson & Wright 1986; Nygren et al. 1975; Wilkins et al. 1993). This, together with my results, shows a strong postzygotic reproductive isolation between salmon and trout, making introgression unlikely, though not impossible. Castillo et al (2008) found evidence of very low levels of introgression in Atlantic salmon populations in rivers that had been artificially stocked with brown trout. This introgression disappeared with the cessation of stocking (Castillo et al. 2008). So while introgression has been observed in salmon and trout, the threat of introgression to local adaptation and population fitness appears to be negligible, and the reproductive and

ecological fitness I have measured here confirm that finding, especially where competition exists.

It seems likely, therefore, that the larger threat posed by salmon-trout hybrids to wild populations is that of wasted reproductive effort, leading to reductions in population size as illustrated in figure 6.4.1. The consequences of wasted reproductive effort for Atlantic salmon populations could be severe, especially for the many declining populations. Individuals that spawn to produce functionally sterile salmon-trout hybrids are not contributing to the subsequent generation, lowering the effective population size. This is a problem that could become a real issue in Atlantic salmon conservation if hybridisation was to increase. Hybridisation between salmon and trout has been seen to be increasing in some places in Europe (Hindar & Balstad 1994; Jansson & Ost 1997), and is at risk of increasing further as the demand for food and natural resources rise, leading to continued growth in aquaculture and environment modification and disturbances to river flow (Hindar & Balstad 1994; Jansson & Ost 1997). Further to this, if those functionally sterile hybrids go on to mate themselves, their fertilisations will fail to result in viable backcrossed progeny (Chapter 6), again wasting the gametes of the pure species with which they have reproduced. It is known that small populations are at risk of losing genetic variation through random genetic drift, but this can be compounded by a small effective population size, leading to rapid loss of population genetic variation (Nunney & Elam 1994). By producing functionally sterile hybrids, salmon and trout populations are at risk of reducing their effective population size, (those individuals that can contribute to the next generation), by effectively removing gametes from the population. A small effective population size can lead to a reduction in heterozygosity (loss of genetic variation) which has been shown to have a negative relationship with fitness (Allendorf & Leary 1986), and exacerbate the risk of population extinction (Newman & Pilson 1997; Saccheri et al. 1998). If reproducing adults can survive an effective population size bottleneck and go on to breed again, then the genetic effect on the population will be much reduced (Nunney & Elam 1994). However, anadromous Atlantic salmon (particularly females) generally spawn only once in their lifetime. The huge energetic effort of migration coupled with the cessation of feeding during this time often leads to death after reproduction (Fleming 1996). This means that any reproductive effort that is wasted in a spawning season lowers the effective population size of that generation.

With Atlantic salmon declining across much of their range (Parrish et al. 1998; WWF 2001), the impact of the production of functionally sterile hybrids to vulnerable populations could be high.

7.4. Summary

The primary findings of this thesis have quantified the risks of hybridisation between salmon and brown trout under sperm competition, showing that some level of reproduction does occur at the level of the gamete. This isolation is not complete however, with hybridising males still able to gain a third of fertilisations under simultaneous sperm release. There is clear evidence that salmon and trout eggs are able to bias fertilisations toward conspecific sperm, even when heterospecific males have an advantageous position in the sperm release sequence (Yeates et al. 2007). Further to this, I have found strong evidence that this CSP is mediated by female OF, the first evidence for CFC via a reproductive fluid in an external fertiliser. The CSP mediated by OF is also an example of divergent gametic incompatibility in an external fertiliser. This reproductive character displacement has possibly arisen through selection against unfit hybrids as a mechanism of reinforcement. Experiments on F_1 hybrid males of salmon and brown trout in this thesis have shown them to be ecologically fit at early life history stages compared with pure species equivalents, but ultimately males show low reproductive fitness. This situation would select against their production, potentially driving the creation of the CSP I observe. More work that compares allopatric and sympatric populations of salmon and trout are needed to determine whether OF-mediated CSP is stronger in sympatry, providing evidence that this is a case of reinforcement.

The results for hybrid fitness in this thesis suggest that the largest threat to declining populations of Atlantic salmon from hybridisation comes from reductions in effective population size, more than loss of local adaptation through introgression. My results, together with previous work (Galbreath & Thorgaard 1995; Garcia-Vazquez et al. 2004), have shown that there is strong postzygotic isolation between salmon and trout. This makes the threat genetic swamping from introgression of heterospecific genes negligible. Due to the low reproductive fitness of salmon and trout reciprocal male hybrids, any females that hatch

hybrid progeny will themselves suffer lower long-term fitness. Reductions in effective population sizes in already-declining Atlantic salmon populations could have serious negative effects on population fitness and survival. With hybridisation likely to increase through further aquaculture and habitat modification (Hindar & Balstad 1994), hybridisation could have a tangible effect on threatened Atlantic salmon, and should therefore be considered when trying to implement conservation plans.

The results of my thesis on aspects of hybrid fitness at early life history phases are in stark contrast with those from some studies, while being consistent with others. My study has clearly shown that maternal brown trout hybrids, while significantly smaller and taking longer to hatch have survival comparable to that of parental species and the reciprocal hybrid cross. In antithesis to my findings, results of the most recent studies have found survival at hatch and post hatch ranging from 0 to 1.95% in maternal trout hybrids (Álvarez & Garcia-Vazquez 2011; Garcia-Vazquez et al. 2002). By contrast, the results of older studies show survival at 68% (McGowan & Davidson 1992a) and occasionally higher (Chevassus 1979). The inconsistencies between my results and those from recent studies are unlikely to be due to inconsistencies in methods or relative gamete quality. Due to some salmon trout populations differing in peak spawning times there is a risk when generating hybrids artificially that the two species gametes will ripen at different times, possible weeks apart. In this case older gametes would have to be stored and run the risk of becoming over ripe. Improvements to fish culture and recognition of experimental control and non-confounded gamete storage techniques have improved since early studies, enabling the problem of overripe gametes to now be avoided. This has allowed superior and equivalent tests of hybrid fitness in more recent studies without confounds from unequal gamete ripening times. In addition, the differences seen between studies in hybrid fitness could be due to population-specific variance in genetic compatibility, perhaps as a result of sympatric and allopatric considerations already outlined. The fish used in my thesis originated from the northern-most range of both salmon and trout species, while fish in preceding studies used fish originating from species at the southern end of their European distribution (Álvarez & Garcia-Vazquez 2011; Garcia-Vazquez et al. 2002). The genetic structure of Northern European Atlantic salmon has been found to be generally distinct from that of modern southern European populations (Campos et al. 2008; King et al. 2001), as has that of

populations in North American and Europe (King et al. 2001; McConnell et al. 1995), providing evidence of genetic divergence. Further to this, it has been noted many times that some hybrid genotypes are fitter than others within the same hybrid zone, and even within the same brood or cohort (Arnold et al. 1999; Arnold & Hodges 1995; Burke & Arnold 2001; Kruuk et al. 1999; Parris 2001), making it likely that some hybrid genotypes will be fitter in some populations compared to others. It is therefore possible that different hybridising populations of salmon and trout have different levels of fitness expressed by their hybrids due to divergence in their genomes. As there is often variation in hybrid fitness and introgression occurring across and between hybrid zones, it is important to compare specific combinations in order to determine the importance of intrinsic and extrinsic factors influencing the dynamics of all possible hybrid crosses (Aboim et al. 2010). Genetic differentiation between geographically distinct populations could therefore be responsible for variation in relative hybrid fitness, as well as determining which cross is more prevalent. Both of these factors could have implications for the level of impact hybridisation will have on wild populations. These differences between and within populations may therefore make it impossible to achieve an all-encompassing assessment of relative hybrid fitness of salmon and trout hybrids for every natural population.

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