Ejaculate traits and ovarian fluid as a potential mechanism for cryptic female choice in chinook salmon (*Oncorhynchus tshawytscha*)

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GENERAL ABSTRACT

Marine and freshwater environments support numerous species of teleost fish with a wide and diverse range of reproductive strategies. Despite the considerable interest in fish reproduction, our knowledge regarding ejaculate traits and factors affecting them is limited. Using computer-assisted sperm analysis (CASA) I measured ejaculate traits (sperm swimming speed, motility, path trajectory, longevity and concentration) from sexually mature chinook salmon (*Oncorhynchus tshawytscha*) activated in freshwater and ovarian fluid. I also looked at these ejaculate traits in relation to measures of male quality (body condition) and investment into reproduction (relative testes mass). Furthermore, I determined the chemical composition of seminal and ovarian fluid and looked at the effect these fluids have on sperm behaviour.

A considerable amount of intraspecific variation existed in all ejaculate traits measured, and investment into reproduction (relative testes mass) was dependent on male body condition, as males in better condition had relatively larger testes. However, these males did not have superior quality ejaculates or ejaculates with a higher density of spermatozoa; hence the potential reproductive advantage of having larger relative testes in this species remains unknown and requires further investigation. In addition, a positive relation between sperm longevity and sperm swimming speed was observed defying the expected trade-off between ejaculate traits according to theory. There was also a weak negative trend in our data between body condition and sperm swimming speed, linearity, and longevity.

All sperm traits measured were greatly enhanced when activated in a solution containing ovarian fluid (a viscous fluid which is excreted with the egg batch during spawning) from female chinook salmon. Interestingly, sperm swimming speed activated in fresh water only accounted for < 12% of the observed variation in mean sperm swimming speed in ovarian fluid. This result suggests the sperm traits measured in fresh water are not relevant to those same traits measured in ovarian fluid, so caution should be applied when

comparing the potential for individual males to fertilize ova when sperm traits are activated in water, especially in studies of sperm competition in an externally fertilising species.

Sperm competition between males is known to strongly influence sperm and ejaculate traits, but less is known about female sperm choice after copulation via a process called cryptic female choice (CFC). In CFC, females may have the ability to favour the sperm of one male over another and bias fertilisation accordingly. To test whether ovarian fluid could act as a mechanism of CFC in an externally fertilising fish species, I measured sperm traits from each male activated in the ovarian fluids from different females. I found that mean sperm swimming speed, longevity, and path trajectory differed significantly among males, but most importantly, the pattern of within-male variation in these traits also varied significantly among males in response to different females' ovarian fluids. This result suggests that ovarian fluid may be a mechanism of CFC whereby females differentially enhance the swimming speed of sperm from different males. In addition, I found that sperm longevity was negatively correlated with variation in [Ca^{2+}] and [Mg^{2+}] concentration in the ovarian fluid, while percent motility increased with increasing concentration of [Mg^{2+}].

These observations provide a possible chemical basis for cryptic female choice whereby female ovarian fluid differentially influences the behaviour of sperm from different males and thus their fertilisation success. This finding is particularly exciting, as we may have uncovered a potential mechanism of CFC in an externally fertilising species, which is poorly understood. In addition, results from this study suggest new directions for genetic studies to provide direct evidence for CFC. For example, does sperm selection via ovarian fluid promote favoured genetic combinations that enhance male reproductive success?

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Chapter 1

General Introduction

1.1 SPERMATOZOA DIVERSITY

Spermatozoa are by far the most diverse cells in the animal kingdom (Cohen 1977) with sperm size and shape varying considerably both between (Pitnick et al. 1995; Balshine et al 2001; Calhim et al. 2007; Lüpold et al. 2009; Pitnick et al. 2009) and within species (Ward 1998; Morrow and Gage 2001; Birkhead et al. 2005; Harris et al. 2007). Why such diversity has arisen is puzzling, as sperm types share a common function – to fertilise ova. Teleost spermatozoa similarly exhibit a broad range of varying structural features making it impossible to depict a common sperm type (Stoss 1983; Jamieson 1991; Lahnsteiner et al. 1995; Stockley et al. 1997). Additionally, there is considerable variation in sperm characteristics, such as sperm swimming speed, quantity, size and longevity among fish taxa (Gage et al. 1995; Stockley et al. 1997; Gomendio et al. 1998; Neff et al. 2003; Schulte-Hostedde and Millar 2004; Hettyey and Roberts 2006). However, the cause and significance of the observed ejaculate diversity and function is now just beginning to be understood (Petersen and Warner 1998; Cosson et al. 1999; Coward et al. 2002; Linhart et al. 2005; Snook 2005). Two post-copulatory selective forces have been identified as major contributors that strongly influence ejaculate traits: sperm competition (Parker 1970), and cryptic female choice (Thornhill 1983; Eberhard 1996).

1.2 EXTERNAL FERTILISATION IN FISHES

The processes leading up to the activation and ultimate fertilisation of fish oocytes are very different from those same processes in well-studied taxa such as mammals. In fishes, there is a vast array of fertilisation environments across taxa (fresh, salt and brackish water), and an enormous variety of reproductive modes that effect fertilisation

either inside or outside of the female reproductive tract (internally or externally) (see Hoar 1969; Jobling 1995). External fertilisation whereby females and males shed gametes into the water during spawning, and fertilisation occurs externally, is by far the most common mode of fertilisation in teleosts. In many fish species, this entire process can occur within a couple of seconds once gametes have been expelled into the aquatic environment (Kobayashi and Yamamoto 1981; Hoysak and Liley 2001; Liley et al. 2002; Yeates et al. 2007).

Most teleost sperm, unlike the mammalian sperm, lack an acrosome and sperm must reach the plasma membrane of the egg by swimming though a hole in the chorion called the micropyle, and then fuse with the oocyte plasma membrane initiating fertilisation (Ginsberg 1963; Amanze and Iyengar 1990; Billard and Cosson 1992; Yanagimachi et al. 1992). Once fish spermatozoa begin to swimming in the external aqueous environment they typically move in a more curved trajectory pathway than mammalian sperm (Kime et al. 2001), and as in other vertebrate systems, energy for movement comes from ATP stored or generated in the mitochondria (Christen et al. 1987; Burness et al. 2004).

In salmonids spermatozoa are immotile within the testes and sperm ducts prior to release into the water (Stoss 1983), due to a high concentration of potassium ions within the seminal fluid (Morisawa and Suzuki 1980; Morisawa 1985; Cosson et al. 1999). During spawning spermatozoa are released from the sperm duct into the water and become motile upon contact with fresh water and/or the ovarian fluid that surrounds the salmonid egg. In Salmonidae, the decrease in extra-cellular K^+ activates sperm motility (Morisawa and Suzuki 1980), and this period of motility is incredibly brief (*c.* 30 – 40 s) (Ginsburg 1968: Billard 1986; Perchec et al. 1996) as osmotic shock caused from the effect of freshwater on sperm membranes slows and eventually stops sperm movement (Huxley 1930; Christen and Billard 1987; Morisawa 1994; Perchec et al. 1996).

Unlike mammals, the female reproductive system of salmonid fish does not contain an oviduct (Hoar 1969). Mature oocytes are released from the follicles and are held in the peritoneal cavity bathed in ovarian fluid (Billard 1992; Billard et al. 1995). During

spawning the eggs are released via the genital pore and are accompanied by ovarian fluid. In Salmonidae this fluid comprises 10-30% of the total egg mass (Lahnsteiner et al. 1999b) and is known to increase both sperm viability (the percentage of living sperm) (Scott and Baynes 1980; Lahnsteiner et al. 1997; Turner and Montgomerie 2002) and fertilisation success (Litvak and Trippel 1998). When eggs are exposed to water the unfertilized ova are activated immediately and remain fertilizable for up to forty seconds before osmotic swelling blocks the micropyle and prevents fertilisation from occurring (Ginsberg 1963; Billard and Cosson 1992).

In salmonids, the first spermatozoa to find and enter the micropyle will fertilise the egg (Kobayashi and Yamamoto 1998) and thus there should be strong selection to promote this opportunity among males. For females, eggs unfertilised within forty seconds of release are wasted, thus it is in the female's best interest to ensure that first, her eggs are fertilised and second, that sperm from the best possible male can fertilise the maximum number of her eggs. One might envisage a system whereby chemoattractants are used to draw sperm towards the egg micropyle (Yanagimachi et al. 1992; Iwamatsu et al. 1993), potentially with some form of mechanism to select for sperm from males with compatible genotypes or avoiding males with incompatible genes (Zeh and Zeh 1996; Zeh and Zeh 1997; Tregenza and Wedell 2002).

1.3 FACTORS INFLUENCING EJACULATE TRAITS

Sperm competition

In 1970, Geoff Parker illustrated that sexual selection can continue to occur after mating, and can occur at the gamete level (Parker 1970). This process was coined "sperm competition" being the competition between the ejaculates of different males to fertilise the ova of a particular female. In a wide range of species, females copulate (or spawn) with more than one male within a single reproductive bout and this leads to competition between ejaculates of rival males (Parker 1970; Birkhead and Møller 1998). This competition among ejaculates is now recognised as one of the most powerful evolutionary forces influencing male reproductive behaviour (Birkhead and Møller, 1998;

Birkhead and Pizzari, 2002), and much of the diversity of ejaculate traits and testes morphology in many species, including fish, has been attributed to this intense competition (Ball and Parker 1996; Stockley et al. 1997; Birkhead and Møller 1998; Snook 2005; Montgomerie and Fitzpatrick 2009).

Geoff Parker proposed two theoretical models namely a 'fair raffle' and a 'loaded raffle' for analysing sperm competition, as it has now become clear that males do not always compete on a level playing field (Parker 1990a, 1990b). In the fair raffle all sperm compete on equal terms, i.e. competiting ejaculates are physiological and energetically equal. In this raffle sperm number is important for fertilisation success, thus, an increase in the number of spermatozoa in the ejaculate will result in a greater chance of fertilisation success (Parker 1990a). This is in contrast to the loaded raffle whereby spermatozoa in sperm competition have unequal chances of succeeding in fertilising an egg. Competiting ejaculates will display differing qualities and one male's sperm will have a competitive advantage over the other. In a loaded raffle it is expected that sperm quality will determine the outcome of competition. Therefore, a male who produces a high quality ejaculate with superior sperm, for example, faster swimming sperm, will be more likely to outcompete a male of a similar reproductive role with an ejaculate of lower quality, such as, slower swimming sperm.

Sperm-competition game theory predicts that, across species, male ejaculate expenditure should increase with sperm-competition risk (the probability that a female will mate with another male (reviewed Parker et al. 1997). For example breeding systems whereby males exhibit alternative reproductive phenotypes, such as sneakers and guarders, some males will face a higher risk of sperm competition. For instance, sneaks will always be subject to sperm competition but guarders will be subject to sperm competition with low probability, dependent on the number of sneaks (Parker 1990b). For example, in Atlantic salmon ejaculates of parr face a higher risk of sperm competition than territorial males and parr produced greater numbers of spermatozoa per ejaculate and there is also some indication that parr sperm have higher longevity (Gage et al. 1995; Vladic et al. 2002).

Among species with external fertilisation, it has been observed that fishes show one of the widest ranges of sperm competition intensity of any animal group (Peterson and Warner 1998; Taborsky 1998). Sperm from competing ejaculates race against each other to find the micropyle of the egg. All things being equal, the fastest swimming sperm should reach the egg first, thus having the greatest chance of fertilisation success. In salmonids fertilisation occurs over a very short time, as only 5 – 10 s is required for fertilisation to occur in sockeye salmon (*Oncorhynchus nerka*) (Hoysak and Liley 2001).

It is therefore predicted that sperm traits such as sperm swimming speed and sperm number are under direct selection when sperm competition is at play (Parker 1993; Parker et al. 1996; Ball and Parker 1996). Previous studies in external fertilising fishes have documented the effects of sperm competition on sperm swimming speed; e.g. Atlantic salmon (*Salmo salar*) (Gage et al. 2004), bluegill (*Lepomis macrochirus*) (Burness et al. 2004; Schulte-Hostedde and Burness 2005), walleye (*Sander vitreus*) (Casselman et al. 2006) and Arctic charr (*Salvelinus alpinus*) (Rudolfsen et al. 2006; Liljedal et al. 2008). For example, in Atlantic salmon, males with the fastest swimming sperm fertilised a greater proportion of eggs in two-male competition trials (Gage et al. 2004). Additionally, recent work by Fitzpatrick et al. (2009) within a single family (Cichlidae) showed that species experiencing greater levels of sperm competition have faster-swimming sperm, and that sperm competition also selected for an increase in sperm number, size and longevity.

It is also likely that several components of the seminal fluid can also participate in sperm competition through their effects on spermatozoa speed (Poiani 2006; Cornwallis and O'Connor 2009), and a variety of components of the seminal fluid in vertebrates and invertebrates have been observed to enhance spermatozoa swimming speed (Davis et al. 1989; Costa et al. 1994; Chapman 2001; Mungan et al. 2001). Recent research in rainbow trout has demonstrated that seminal plasma proteins interact with spermatozoa and positively affect sperm motility (Lahnsteiner 2007). Presently, the relationship between seminal fluid composition and sperm function has been investigated in only a few fish species, including Atlantic salmon (Hwang and Idler 1969), rainbow trout (Lahnsteiner et

al. 1998), common carp (*Cyprinus carpio*), common bleak (*Alburnus alburnus*) (Kruger et al. 1984; Lahnsteiner et al. 1996; Alavi et al. 2004), Persian sturgeon (*Acipenser persicus*) (Alavi et al. 2004), and burbot (*Lota lota*) (Lahnsteiner et al. 1997). In the Persian sturgeon the percentage of motile sperm cells and the total duration of sperm motility increased when the potassium ion level decreases and the sodium ion level and osmolality increases in the seminal fluid (Alavi et al. 2004). Also in the common bleak sodium and potassium levels in the seminal fluid have statistically significant positive and negative relations, respectively, with the percentage of motile sperm cells (Lahnsteiner et al. 1996).

Cryptic female choice

Female mate choice is an important evolutionary process that can occur before or after mating. Precopulatory female mate choice is facilitated in some species as males have developed advertising strategies, such as exaggerated male ornaments and behavioural displays to help influence a females mating decision (Darwin 1871; Andersson 1994). Such male traits work to advertise the quality of the male, and therefore benefit females who choose to mate with that male (Darwin 1871; Andersson1994; Møller and Jennions 2001). Evidence is now growing to suggest that females are also capable of controlling who fertilises her eggs via a postcopulatory process called cryptic female choice (CFC) (Thornhill 1983; Eberhard 1996). CFC is the ability of a female to favour the sperm of one male over another and bias fertilisation accordingly (Thornhill 1983; Eberhard 1996). This process is referred to as "cryptic" as it is not readily observed (Eberhard 1996; Birkhead and Pizzari 2002). It is notoriously difficult to establish whether or not CFC is occurring (Birkhead 1998; Birkhead 2000; Kempenaers et al. 2000; Pitnick and Brown 2000). However, evidence regarding CFC is provided by Edvardsson and Arnqvist (2000), whereby they manipulated the female perception of copulatory courtship behaviour in red flour beetles (*Tribolium castaneum*). This study found that the female perception of male copulatory courtship behaviour controls the fate of sperm competition over fertilisations within the female, demonstrating that copulatory courtship is under selection by cryptic female choice. Through CFC it is possible that females exert preferences for male sperm traits to determine fertilisation. Moreover, females may rely

on CFC to exclude fertilisation by particular males, thereby ensuring against infertility, genetic incompatibility, or genetic similarity (Bishop et al. 1996; Sherman et al. 2008).

The potential mechanisms that females could use to bias sperm use in favour of certain males and against others are often unclear, although, a number of potential physiological and biochemical mechanisms have been identified in a range of species with internal fertilisation (Eberhard 1996; Birkhead and Pizzari 2002). For example, a female can physically block or eject certain sperm from her reproductive tract (Pizzari and Birkhead 2000) in favour of a certain male "type". There are typically fewer possible mechanisms of CFC in species with external fertilisation. In fish species, where sperm competition is widespread (Taborsky 1994), a female may indeed require some method of sperm selection post-copulation as spermatozoa from many males are racing against each other to fertilise her egg batch.

So far there have been four main potential mechanisms of CFC identified in species with external fertilisation. First, female European waterfrogs (*Rana lessonae* and *Rana exculenta*) have been observed to control the numbers of eggs laid with different males once fertilisation has occurred (Reyer et al. 1999). Second, the egg itself may discriminate among sperm (Carre and Sardet 1984; Zeh and Zeh 1997; Skarstein et al. 2005) as the teleost egg has structures that may facilitate sperm discrimination, such as the micropyle - the only entrance for the sperm into the egg (Kamler 1992). In addition, a chemical sperm guidance system may also exist via some chemo-attractant provided by the egg surface or micropyle area (Amanze and Iyengar 1990). Moreover, sperm attraction to the egg could be influenced by major histocompatibility complex (MHC) dependent diploid or haploid influences on sperm phenotype as sperm may contain MHC antigens in their cell surface that reveal aspects of their genome (Wedekind et al. 2004; Skarstein et al. 2005). Third, sperm selection may occur following fusion of the gametes during the formation of the second polar body because the second maturation division is completed only after the sperm has penetrated the egg, and differential discarding of the egg's second polar body after fertilisation has occurred (Wolgemuth 1983; Wedekind et al. 1996; Pitcher and Neff 2006). While this mechanism has yet to be observed in a

teleost, in mice the last meiotic division in the egg was influenced by the type of sperm that entered (Agulnik et al. 1993). Finally, ovarian fluid could have a selective effect on sperm if its effects on sperm behaviour differ among competing males (Turner and Montgomerie 2002; Wedekind et al. 2004; Urbach et al. 2005; Dietrich et al. 2008).

1.4 OVARIAN FLUID AND CRYPTIC FEMALE CHOICE

Maternally derived ovarian fluid has the potential to influence sperm behaviour and the outcome of sperm competition. For example, previous research has shown that motility parameters of the spermatozoa are enhanced when activated in the ovarian fluid from externally fertilising fish species (Billard 1983, 1992; Turner and Montgomerie 2002). Spermatozoa activated in ovarian fluid swam faster in brown trout (*Salmo trutta f. fario)* (Lahnsteiner 2002), Atlantic cod (*Gadus morhua*) (Litvak and Trippel 1998) and rainbow trout (Oncorhynchus mykiss) (Wojtczak et al. 2007; Dietrich et al. 2008). The duration of sperm motility (longevity) was prolonged in brown trout (Lahnsteiner 2002), the three-spined stickleback (*Gasterosteus aculeatus*) (Elofsson et al. 2003), and in Arctic charr when sperm were activated in ovarian fluid (Turner and Montgomerie 2002). In addition, sperm from Arctic charr also swam faster and were more motile in ovarian fluid (Turner and Montgomerie 2002).

Recent research has demonstrated that sperm swimming speed in the ovarian fluid from female Arctic charr is dependent on a particular female-male combination (Urbach et al. 2005). Consequently, ovarian fluid may play an important role at fertilisation, as it seems possible that positive action towards spermatozoa of certain males could occur. Just how ovarian fluid interacts with sperm is unknown, but its positive effects on sperm function have been attributed to the composition of the ovarian fluid (Lahnsteiner et al. 1995; Lahnsteiner 2002; Turner and Montgomerie 2002; Cosson 2004; Elofsson et al. 2006). Females may differentially affect the swimming speed of sperm from different males as the composition of the ovarian fluid might vary among females to promote fertilisation by preferred males. Lahnsteiner et al. (1995) found intraspecific variation in composition

of the ovarian fluid of four salmonid species, and suggested that variation in the chemical composition of the ovarian fluid between females affected sperm traits from some males differentially. Potentially, some substance in the ovarian fluid protects the spermatozoa (Billard 1983), such as the presence of protein or carbohydrate in the ovarian fluid (Yoshida and Nomura 1972; Lahnsteiner 2002). Turner and Montgomerie (2002) suggested that some component of ovarian fluid can influence ATP metabolism and hence increase sperm longevity and swimming speed. Recently, Wojtczak et al. (2007) showed that the pH of the ovarian fluid enhances the motility of rainbow trout spermatozoa. It is therefore possible that changes in the composition of the ovarian fluid could play an important role in female sperm selection via CFC, whereby female ovarian fluid differentially influences the sperm behaviour of different males, resulting in prejudiced paternity.

1.5 CHINOOK SALMON - AN IDEAL STUDY SPECIES

Salmonid spawning behaviour lends itself to intense levels of sperm competition behaviour (Gross 1985; Fleming 1998; Hoysak and Liley 2001) as males congregate and typically fight for close access to females, thereby competing to fertilize eggs (Fleming 1998). As salmonids have external fertilisation, milt and eggs /ovarian fluid are easy to obtain and the manipulation of gametes is straightforward. Even though the gametes are short lived, they can be stored appropriately and activated some time after collection, subsequently, sperm can be directly observed and behaviour can be measured (Trippel and Neilson 1992; Gage et al. 1995; Leach and Montgomerie 2000; Rurangwa et al. 2004). The influence of ovarian fluid on sperm behaviour can also be easily measured in externally fertilising fishes (Turner and Montgomerie 2002; Elofosson et al. 2003; Urbach et al. 2004; Wojtczak et al. 2007). Moreover, there is a large body of research pertaining to the biology, behaviour, husbandry and genetics of salmonids (Groot and Margolis 1991; Healey 1991; Quinn and Bloomberg 1992; Fleming 1998; Hurst et al. 1999; Quinn et al. 2001).

The Pacific salmon (*Oncorhynchus* spp.) are anadromous, living part of their life in the ocean and returning to breed in freshwater streams (Fleming 1998). They are generally considered to be entirely semelparous, as they invest all their resources in a single breeding episode before dying (Groot and Margolis 1991). Anadromy is generally associated with high growth rate and early age at maturation: 2 years for males and 3 years for females (Unwin et al. 1999). A small proportion of male mature at the end of their first year of life and are called 'precocious'. These smaller sized fish can survive into a second year and reinitiate gonad development both in the wild and under hatchery conditions (Flain 1971). Thus, there are two phenotypes on male chinook salmon: mature parr (precocious) and anadromous adults. Following migration to a suitable spawning area, females select and defend a territory, building a series of nests. A dominant male (closest to the female) attempts to monopolize access to the female, directing aggression towards subordinate and precocious (sneaker) males who are typically further away from the spawning female (Fleming and Gross 1994). A male's proximity to a female during spawning allows for closer / quicker access to eggs during oviposition (Gross 1985; Foote et al. 1997), and potentially provides a greater chance of fertilization success (Schroder 1981). Spawning areas are typically teeming with conspecific males, and both subordinate and precocious males may attempt sneak fertilizations.

Chinook salmon (*Oncorhynchus tshawytscha*) were first introduced to New Zealand from fall-run Sacramento River (California, USA) stock in the early 1900s (McDowall 1994), and self-sustaining populations were established on the east coast of the South Island by about 1915. Today, this salmonid species is widely distributed, occurring in many of New Zealand's streams and rivers. In the late 1970s increasing interest in commercial salmon farming has led to reestablishment of hatcheries on many New Zealand salmon rivers (Unwin and Glova 1997). Chinook salmon are now commercially farmed throughout the South Island in seawater net-pens and freshwater ponds.

The chinook salmon used in this research were obtained from a hatchery-reared broodstook pool generated from semi-wild returns. These fish are the descendants of juvenile fish collected from all of the major chinook salmon-producing rivers found in

the central South Island of New Zealand. Each year gametes were harvested from wild returns of this stock released into the Kaiapoi River, as well as other major South Island Rivers. The fish used in this research were surplus fish of this same stock (Metcalf and Gemmell 2006).

1.6 HOW THIS THESIS CONTRIBUTES TO AN UNDERSTANDING OF EJACULATE CHARACTERISTICS, SPERM COMPETITION AND CRYPTIC FEMALE CHOICE.

Thesis objectives

There were five main goals of my thesis research. First, to examine the effects of sperm competition and CFC on ejaculate traits, I had to measure ejaculate traits from a population of sexually mature male chinook salmon. As previous research on fish species has observed considerable variation in ejaculate traits, it was imperative that we assessed the degree of variation in ejaculate traits within our study population and measure any external effects that might impact such traits. In addition, morphological measurements of the fish were used in combination with the ejaculate traits to explore the theoretical predictions under models of sexual selection and sperm competition (Andersson 1994; Parker 1998). Second, I examined the effects of ovarian fluid on sperm behaviour. Prior work in other fish species had shown that sperm traits are greatly enhanced when activated in a solution containing ovarian fluid (Litvak and Tripped 1998; Lahnsteiner 2002; Turner and Montgomerie 2002; Wojtczak et al. 2007; Dietrich et al. 2008). Therefore, I measured and compared sperm traits from sexually mature male chinook salmon activated in both fresh water and a solution containing ovarian fluid. Third, I explored the possibility that ovarian fluid’s influence on sperm behaviour could be a mechanism of CFC (Eberhard 1996). To do this, I measured and compared how ovarian fluid from different females affects the sperm behaviour of different males. Fourth, I investigated whether the chemical composition of ovarian fluid varied among female chinook salmon, and if so, whether or not this maternal fluid could provide a chemical basis for CFC. In addition, I measured the chemical composition of seminal fluid in this species, as this is previously unknown in New Zealand chinook salmon, and the

composition analysis in other salmonid species has so far been scarce (Aas et al. 1991; Linhart et al. 2000; Alavi and Cosson 2005; Lahnsteiner et al. 1998). I also explored relationships between the composition of the ovarian and seminal fluid on sperm behaviour, as this is generally understudied in fish species. Fifth, I use my data to address the lack of knowledge regarding ejaculate traits and factors that may influence these traits in an externally fertilising species. I place my findings of this research in the context of postcopulatory sexual selection – sperm competition and cryptic female choice.

Outline of thesis structure

This thesis has been written as a series of four, separate, but interrelated, scientific manuscripts. Three manuscripts have already been published (see below) and the fourth is submitted to Canadian Journal of Zoology. Consequently, there is some overlap between chapters, particularly in the methods sections.

Chapter 2

Ejaculate traits in chinook salmon (*Oncorhynchus tshawytscha*) in relation to male body condition, size and investment into sperm competition

Patrice Rosengrave, Robert Montgomerie, Victoria Metcalf, Katherine McBride, Neil Gemmell
Submitted to Canadian Journal of Zoology

To establish base-line data on ejaculate traits from sexually mature two-year-old chinook salmon I measured; sperm motility, pattern of motility, velocity, and proportion of motile sperm cells, using computer-assisted sperm analysis system (CASA). This system provides an objective measurement of sperm velocity and movement and can be used to assess the quality of fish sperm (Toth et al. 1997; Lahnsteiner et al 1998; Kime et al. 2001; Burness et al. 2004; Rurangwa et al. 2004; Locatello et al. 2006). In addition we measured sperm longevity and density, and morphological measurements including; body size, relative testes mass and overall body condition. We then examine our results in relation to theoretical predictions underlying sperm competition (Parker 1990; Ball and Parker 1997; Parker 1998). I compare our findings with previous studies in fish species,

and discuss the importance of these findings in the context of variation in ejaculate traits and implications for sperm competition, and discuss whether traits involved in sperm competition depend on male body condition. In addition, the results from this study will add to the growing body of knowledge relating to ejaculate traits in fish.

Chapter 3

<u>Sperm traits in chinook salmon depend upon activation medium: implications for studies of sperm competition in fishes</u>

Patrice Rosengrave, Robert Montgomerie, Victoria Metcalf, Katherine McBride, Neil Gemmell
Published in 2009 *Canadian Journal of Zoology* 87: 920-927

In this chapter I measure and compare sperm traits of sexually mature chinook salmon in fresh water and dilute ovarian fluid at two time intervals post-activation using CASA. In addition, I assess the repeatability of sperm traits measured in fresh water versus dilute ovarian fluid solution and discuss the relevancy of sperm traits measured in fresh water. Furthermore, I also consider the implications of using sperm traits measured in water as an index of male ejaculate quality, as they may not be representative of those measured under natural spawning conditions where ovarian fluid is present.

Chapter 4

<u>A mechanism for cryptic female choice in Chinook salmon</u>

Patrice Rosengrave, Neil Gemmell, Victoria Metcalf, Katherine McBride, Robert Montgomerie
Published in 2008 *Behavorial Ecology* 19 (6):1179-1185

In this chapter I test whether ovarian fluid could act as a potential mechanism of cryptic female choice in chinook salmon. Using CASA we measured sperm traits from each male activated in the ovarian fluids from different females. I discuss the observed significant interaction between sperm traits and ovarian fluid in the context of cryptic female choice.

Results from this study add to the paucity of research regarding known mechanisms of cryptic female choice in externally fertilsing species.

Chapter 5

Chemical composition of seminal and ovarian fluids of Chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits

Patrice Rosengrave, Harry Taylor, Robert Montgomerie,Victoria Metcalf, Katherine McBride, Neil Gemmell
Published in 2008 *Comparative Biochemistry and Physiology, Part A.* 152: 123-129

In this chapter I determine the concentration of the major inorganic ions using atomic absorption spectroscopy, osmolality, and pH of ovarian and seminal fluid of sexually mature chinook salmon. I also look at the composition of these fluids and how they influence the sperm traits measured using CASA. I compare our analyses of the chemical composition of ovarian and seminal fluid with previous studies, and discuss the results in regards to the possible chemical basis for cryptic female choice in this species.

Chapter 6

General Discussion

In this final chapter, I have summarised my findings within previous chapters and discuss in the context of cryptic female choice and sperm competition.

This is the first study to investigate ejaculate traits using CASA within a fish population in New Zealand, and therefore adds greatly to our knowledge of ejaculate traits in chinook salmon, and externally fertilising fish species in general. Importantly, we have revealed a possible mechanism of CFC in an externally fertilising species. This result is particularly exciting as it is generally unknown if and how CFC might be exerted in external fertilisers. Results from this study suggest new directions for research on sperm competition and for future genetic studies to provide direct genetic evidence for CFC. As data on ejaculate traits in externally fertilising species, and potential factors influencing

these traits are limited, I envisage our findings will substantially broaden our knowledge in this field. In addition, we highlight the maternal effects that ovarian fluid has on the fertilisation process.

1.7 References

Aas GH, Refstie T, Gjerde B. 1991. Evaluation of milt quality of Atlantic salmon. Aquaculture. 95:125-135.

Agulnik S, Sabantsev ID, Orlova GV. 1993. Effect of sperm genotype on chromatid segregation in female mice heterozygous for aberrant chromosome 1. Gent Res. 61:97-100.

Alavi SMH, Cosson J. 2005. Sperm motility in fishes. I. Effects of temperature and pH: A review. Cell Biol Int. 29:101-110.

Alavi SMH, Cosson J, Karami M, Abdolhay H, Amiri BM. 2004. Chemical composition and osmolality of seminal fluid of *Acipenser persicus*; their physiological relationship with sperm motility. Aquacult Res. 35:1238-1243.

Amanze D, Iyengar A. 1990. The micropyle: a sperm guidance system in teleost fertilization. Development. 109:495-500.

Andersson M, 1994. Sexual selection. Princeton, New Jersey: Princeton University Press.

Ball MA, and Parker GA. 1996. Sperm competition games: External fertilization and "adaptive" infertility. J Theor Biol. 180:141-150.

Ball MA, and Parker GA. 1997. Sperm competition games: inter- and intra-species results of a continuous external fertilization model. J Theor Biol. 186:459-466.

Balshine S, Leach BJ, Neat F, Werner NY, Montgomerie R. 2001. Sperm size of African cichlids in relation to sperm competition. Behav Ecol. 12: 726-731.

Billard R. 1983. Effects of ceolomic and seminal fluids and various saline diluents on the fertilizing ability of spermatozoa in the rainbow trout, *Salmo gairdnerii*. J Reprod Fert. 68:77-84.

Billard R. 1986. Spermatogenesis and spermatology of some teleost fish species. Reprod Nutr Develop. 26:877-920.

Billard R, Cosson J, Perchec G, Linhart O.1995. Biology of sperm and artificial reproduction in carp. Aquaculture. 129:95-112.

Birkhead TR. 1998. Cryptic female choice: criteria for establishing female sperm choice. Evolution. 52:1212–1218.

Birkhead TR. 2000. Defining and demonstrating postcopulatory female choice—again. Evolution. 54:1057-1060.

Birkhead TR, Møller AP. 1998. Sperm competition and Sexual Selection. San Diego:Academic Press.

Birkhead TR, Pizzari T. 2002. Postcopulatory sexual selection. Nat Rev Genet. 3:262-273.

Birkhead TR, Pellatt EJ, Brekke P, Yeates R, Castillo-Juarez H. 2005. Genetic effects on sperm design in the zebra finch. Nature. 434: 383-387.

Bishop JDD, Jones CS, Noble LR. 1996. Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum* II. Investigation of male mating success using RAPD markers. Proc R Soc Lond B. 263:401-407.

Burness G, Casselman SJ, Schulte-Hostedde AI, Moyers, CD, Montgomerie R. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav Ecol Sociobiol. 56:65-70.

Calhim S, Immler S, Birkhead TR. 2007. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. PloS ONE. 2(5): e413.

Carre D, Sardet C. 1984. Fertilisation and early development in *Beroe ovata*. Dev Biol. 105:188-195.

Casselman SJ, Schulte-Hostedde AI, Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci. 63: 2119-2125.

Chapman T .2001. Seminal fluid-mediated fitness traits in Drosophila. Heredity. 87:511-521.

Christen R, Billard R. 1987. Trout sperm motility. The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. Eur J Biochem.166:667-671.

Cohen, J. 1977: Reproduction. London: Butterworths.

Cornwallis CK, O'Connor EA. 2009. Sperm: seminal fluid interactions and the adjustment of sperm quality in relation to female attractiveness. Proc R Soc B. 276:3467-3475.

Cosson J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult Int. 12:69-85.

Costa M, Canale D, Filicori M, D'Lddio S, Lenzi A .1994. Lcarnitine in idiopathic asthenozoospermia: a multicenter study. Italian study group on carnitine and male infertility. Andrologia. 26:155-59.

Coward K, Bromage NR, Hibbitt O, Parrington J. 2002. Gamete physiology, fertilization and egg activation in teleost fish. 12: 33-58. Rev Fish Biol Fisheries.

Darwin C. 1871. The descent of man, and selection in relation to sex. London: John Murray.

Davis NS, Di Sant'Agnese PA, Ewing JF, Mooney RA. 1989. The neuroendocrine prostate: Characterization and quantitation of calcitonin in the human gland. J Urol. 142:884-888.

Dietrich GJ, Wojczak M, Slowińska M, Dobosz S, Kuźmiński H, Ciereszko A. 2008. Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss* Walbaum) spermatozoa. J Appl Ichthyol. 24:503-507.

Eberhard WG .1996. Female control: sexual selection by cryptic female choice. Princeton: Princeton University Press.

Edvardsson M, Arnqvist G. 2000. Copulatory courtship and cryptic female choice in red flour beetles *Tribolium castaneum*. Proc R Soc Lond B. 267:559-563.

Elofsson H, McAllister BG, Kime DE, Mayer I, Borg B. 2003. Long lasting stickleback sperm: is ovarian fluid a key to success in fresh water? J Fish Biol. 63:240-253.

Elofsson H, Van Look KJW, Sundell K, Sundh H, Borg B. 2006. Stickleback sperm saved by salt in ovarian fluid. J Exp Biol. 209:4230-4237.

Fitzpatrick JL, Montgomerie R, Desjardins JK, Stiver KA, Kolm N, Balshine S. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc Nat Acad Sci. 106:1128-1132.

Flain M. 1971. Repeated spawning and survival of male quinnat salmon in New Zealand. NZ J Mar Freshw Res. 5:519-521.

Fleming IA. 1998. Pattern and variability in the breeding system of Atlantic salmon (*Salmo salar*), with comparisons to other salmonids. Can J Fisheries Aquat Sci. 55(1): 59-76.

Fleming IA, Gross MR. 1994. Breeding competition in a Pacific salmon (coho: *Oncorhynchus kisutch*): measures of natural and sexual selection. Evolution. 48: 637-657.

Foote CJ, Brown GS, Wood CC. 1997. Spawning success of males using alternative mating tactics in sockeye salmon, *Oncorhynchus nerka*. Can J Fish Aquat Sci. 54:1785-1795.

Gage MJG. 1994. Association between body size, mating pattern, testis size and sperm lengths across butterflies. Proc R Soc Lond B. 258:247-254.

Gage MJP, Stockley P, Parker GA. 1995. Effects of alternative male mating strategies in characteristics of sperm production in the Atlantic salmon (*salmo salar*): Theoretical and empirical investigations. Phil Trans R Soc Lond B. 350:391-399.

Gage MJG, MacFarlane C, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr Biol. 4:44-47.

Ginsburg AS. 1963. Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. J Embro Exper Morph.11:13-33.

Gomendio M, Harcour AH, Roldan ERS. 1998. Sperm competition in mammals. In Birkhead TR, Møller AP, editors. Sperm competition and sexual selection. London: Academic Press. p 667-751.

Groot C, Margolis L. 1991. Pacific salmon life histories. Vancouver: University of British Columbia Press.

Gross MR. 1985. Disruptive selection for alternative life histories in salmon. Nature. 313: 47-48.

Harris WE, Moore AJ, Moore PJ. 2007. Variation in sperm size within and between ejaculates in a cockroach. Funct Ecol. 21:598-602.

Healey MC.1991. Life history of Chinook Salmon. In: Groot C, Margolis L, editors. Pacific Salmon Life Histories. Vancouver: University of British Columbia Press. p. 311-394.

Hettyey A, Roberts JD. 2006. Sperm traits of the quacking frog, *Crinia georgiana*: intra- and interpopulation variation in a species with a high risk of sperm competition. Behav Ecol Sociobiol. 59:389-396.

Hoar WS. 1969. Reproduction. In: Hoar WS, Randall DJ, editors. Fish Physiology (III). New York: Academic press. p.1-72.

Hosken DJ, Garner TWJ, Blanckenhorn WU. 2003. Asymmetry, testis and sperm size in yellow dung flies. Funct Ecol. 17:231-236.

Hoysak DJ, Liley NR. 2001. Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. J Fish Biol. 58:1286-1300.

Hurst CD, Bartlett SE, Davidson WS, Bruce IJ. 1999. The complete mitochondrial DNA sequence of the Atlantic salmon, *Salmo sala*r. Gene. 239: 237-242.

Huxley JS. 1930. The maladaptation of trout spermatozoa to freshwater. Nature. 25.

Hwang PC, Idler DR. 1969. A study of major cations, osmotic pressure, and pH in seminal components of Atlantic salmon. J Fish Res Can. 26:413-419.

Iwamatsu T, Ishijimia S, Nakashima S. 1993. Movement of spermatozoa and changes in micropyles during fertilization in Medaka eggs. J Exp Zool. 266:57-64.

Jamieson BGM. 1991. Fish evolution and systematics: evidence from spermatozoa.Cambrideg: Cambridge University Press.

Jobling M. 1995. Environmental Biology of Fishes. London: Chapman and Hall.

Kamler E. 1992. Early life history of fish: an energetics approach. London: Chapman and Hall.

Kempenaers B, Foerster K, Questiau S, Roberyson BC, Vermeirssen ELM. 2000. Distinguishing between female sperm choice versus male sperm competition: a comment on birkhead. Evolution 54(3):1050–1052

Kime DE, Van Look KJW, McAllister BG, Huyskens G, Rurangwa E, Ollevier F. 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol C.130:425-433.

Kobayashi W, Yamamoto TS. 1981. Fine structure of the micropylar apparatus of the chum salmon egg, with a discussion of the mechanism for blocking polyspermy. J Exper Zool. 217:265-275.

Kruger JC, Smith Gl, Van Vuren JHJ, Ferreira JT. 1984. Some chemical and physical characteristics of the semen of *Cyprinus carpio* and *Oreochromis mossambicus.* J Fish Biol. 24:263-272.

Lahnsteiner F. 2002. The influence of ovarian fluid on the gamete physiology in the Salmonidae. Fish Physiol Biochem. 27: 49-59.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1995. Fine structure and motility of spermatozoa and composition of the seminal fluid in the perch. J Fish Biol. 47: 492-508.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1996. Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiol Biochem.15:167-179.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1997. Sperm motility and seminal fluid composition in the burbot, *Lota lota*. J Appl Ichthyol.13:113-119.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture. 163:163-181.

Lahnsteiner F, Berger B, Weismann T. 1999. Sperm Metabolism of the teleost fishes *Chalcalburnus chalcoides* and *Oncorhynchus mykiss* and its relation to motility and viability. J Exp Zool. 284:454–465.

Lahnsteiner F. 2007. Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (*Oncorhynchus mykiss*) Animal Repro Sci. 97:151-164.

Leach B, Montgomerie R. 2000. Sperm characteristics associated with different male reproductive tactics in bluegills (*Lepomis macrochirus*). Behav Ecol Sociobiol. 49:31-37.

Liley NR, Tamkee P, Tsai R, Hoysak DJ. 2002. Fertilization dynamics in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and sperm concentration and motility on in vitro fertilization. Can J Fish Aquatic Sci. 59: 144-152.

Liljedal S, Folstad I. 2003. Milt quality, parasites, and immune function in dominant and subordinate Arctic charr. Can J Zool. 81:221-227.

Liljedal S, Rudolfsen G, Folstad I. 2008. Factors predicting male fertilization success in an external fertilizer. Behav Ecol Sociobiol. 62:1805–1811.

Linhart O, Rodina M, Cosson J. 2000. Cryopreservation of sperm in common carp *Cyprinus carpio*: sperm motility and hatching success of embryos. Cryobiology. 41:241–250.

Linhart O, Rodina M, Gela D, Kocour M, Vandeputte M. 2005. Spermatozoa competition in common carp (*Cyprinus carpio*): what is the primary determinant of competition success? Reproduction. 130: 705-711.

Litvak MK, Trippel EA. 1998. Sperm motility pattern of Atlantic cod (*Gadus morhua)* in relation to salinity: effects of ovarian fluid and egg presence. Can J Fish Aquat Sci. 55:1871-1877.

Locatello L, Rasotto MB, Evans JP, Pilastro A. 2006. Colourful male guppies produce faster and more viable sperm. J Evol Biol. 19:1595-1602.

Lüpold S, Linz GM, Birkhead TR. 2009. Sperm design and variation in the New World blackbirds (Icteridae). Behav Ecol Sociobiol. 63:899-909.

McDowall RM. 1994. The origins of New Zealand's stocks of chinook salmon. Mar. Fish. Rev. 56:1-7.

Metcalf VJ, Gemmell NJ. 2006. Sexual genotype markers absent from small numbers of male New Zealand *Oncorhynchus tschawytscha*. J Fish Biol. 68(A):136-143.

Møller AP, Jennions MD. 2001. How important are direct fitness benefits of sexual selection? Naturwissenschaften. 88: 401-415.

Montgomerie R, Fitzpatrick J. 2009. Testes, sperm, and sperm competition. In: Jamieson BGM, editor. Reproductive biology and phylogeny of fishes (Agnathans and Bony Fishes), Part B. Enfield science publishers. p 1-53.

Moore PJ, Harris WE, Montrose VT, Levin D, Moore AJ. 2004. Constraints on evolution and post-copulatory sexual selection: trade-offs among ejaculate characteristics. Evolution. 58:1773-1780.

Morisawa M. 1994. Cell signaling mechanism for sperm motility. Zool. Sci. 11:647-662.

Morisawa M. 1985. Initiation mechanism of sperm motility at spawning in teleosts. Zool. Sci. 2:605-615.

Morisawa M, Suzuki K. 1980. Osmolality and potassium ions: Their roles in initiation of sperm motility in teleosts. Science. 210:1145-1147.

Morrow EH, Gage MJG. 2001. Consistent significant variation between individual males in spermatozoal morphometry. J Zool Lond. 254:147-153.

Mungan NA, Mungan G, Basar MM, Baykam M, Atan A. 2001. Effect of seminal plasma calcitonin levels on sperm motility. Arch Androl. 47:113-117.

Neff BD, Peng F, Gross MR. 2003. Sperm competition and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). Behav Ecol. 14:634-641.

on polymorphisms in sea urchins. Proc Natl Acad Aci USA. 96:12632-12637.

Parker GA. 1970. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 45:525-567.

Parker G A. 1990a. Sperm competition games: raffles and roles. Proc R Soc B. 242:120-126.

Parker GA. 1990b. Sperm competition games: sneaks and extra-pair copulations. Proc R Soc Lond B. 242:127-133.

Parker GA. 1993. Sperm competition games: sperm size and sperm number under adult control. Proc R Soc Lond B. 253:245-254.

Parker GA. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection. San Diego: Academic Press. p. 3-54.

Parker GA, Ball MA, Stockley P. Gage MJG. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proc R Soc Lond B. 263:291-1297.

Parker GA, Ball MA, Stockley P. Gage MJG 1997. Sperm competition games: a prospective analysis of risk assessment. Proc R Soc Lond B. 264: 1793-1802.

Perchec G, Cosson MP, Cosson J, Jeulin C, Billard R. 1996. Morphological and kinetic changes of carp (*Cyprinus carpio*) spermatozoa after initiation of motility in distilled water. Cell Motil. Cytoskeleton. 35:113-120.

Petersen CW, Warner RR.1998. Sperm competition in fishes In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection. San Diego: Academic Press. p. 435-463.

Pitcher TE, Neff BD. 2006. MHC class IIB alleles contribute to both additive and non-additive genetic effects on survival in Chinook salmon. Mol Ecol. 15:2357-2365.

Pitnick S. 1996. Investment in testes and the cost of making long sperm in *Drosophila*. Am. Nat.148:57-80.

Pitnick S, Brown WD. 2000. Criteria for demonstrating female sperm choice. Evolution. 54:1052-1056.

Pitnick S, Spicer GS, Markow TA. 1995. How long is a giant sperm? Nature. 375:109.

Pitnick S, Hosken DJ, Birkhead TR. 2009. Sperm diversity. In: Birkhead TR, Hosken DJ, Pitnick S, editors. Sperm biology: an evolutionary perspective. London: Elsevier. p. 69-149.

Pizzari T, Birkhead TR. 2000. Female fowl eject sperm of subdominant males. Nature. 405:787-789.

Poiani A. 2006. Complexity of seminal fluid: a review. Behav Ecol Sociol Biol. 60:289-310.

Quinn TP, Bloomberg S. 1992. Fecundity of chinook salmon (*Oncorhynchus tshawytscha*) from the Waitaki and Rakaia rivers, New Zealand. NZ J Mar Freshwater Res.26:429-434.

Quinn TP, Kinnison MT, Unwin MJ. 2001. Evolution of chinook salmon (*Oncorhynchus tshawytscha*) populations in New Zealand: pattern, rate, and process. Genetica. 112-113:493-513.

Reyer HU, Frei G, Som C. 1999. Cryptic female choice: frogs reduce clutchsize when amplexed by undesired males. Proc R Soc Lond B. 266:2101-2107.

Rudolfsen G, Figenschou L, Folstad I, Tveiten H, Figenschou M. 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc R Soc B. 273:325-332.

Rurangwa E, Kime DE, Ollevier F, Nash JP. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture. 234:1-28.

Schroder SL.1981. The role of sexual selection in determining the overall mating patterns and mate choice in chum salmon. PhD Thesis, University of Washington, Seattle, WA, USA.

Schulte-Hostedde AI, Millar JS. 2004. Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (*Tamias amoenus*): implications for sperm competition and reproductive success. Behav Ecol Sociobiol. 55:272-277.

Schulte-Hostedde AI, Burness G. 2005. Fertilization dynamics of sperm from different male mating tactics in bluegill (*Lepomis macrochirus*). Can J Zool. 83:1638-1642.

Schulte-Hostedde AI, Millar JS, Hickling GJ. 2005. Condition dependence of testis size in small mammals. Evol Ecol Res. 7:143–149.

Scott AP, Baynes SM. 1980. A review of the biology, handling storage of salmonid spermatozoa. J Fish Biol. 17:707-739.

Sherman CDH, Wapstra E, Uller T, Olsson M. 2008. Males with high genetic similarity to females sire more offspring in sperm competition in Peron's tree frog *Litoria peronii*. Proc R Soc B. 275:971-978.

Skarstein F, Folstad I, Ronning HP. 2005. Spawning colouration, parasites and habitat selection in *Salvelinus alpinus*: initiating speciation by sexual selection? J. Fish Biol. 67:969-980.

Snook RR. 2005. Sperm in competition: not playing by the numbers. Trends Ecol Evol. 20:46–53.

Stockley P, Gage MJG, Parker GA, Møller AP. 1997. Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. Am Nat. 149:933-954.

Stoss J. 1983. Fish gamete preservation and spermatozoon physiology. In: Hoar WS, Randall DJ, Donaldson EM, editors. Fish Physiology (9). London: Academic Press. p.305-350.

Taborsky M. 1994. Sneaker, satellites, and helpers: parasitic and cooperative behavior in fish reproduction. Adv Study Behav. 23:1-100.

Taborsky M, 1998. Sperm competition in fish: `bourgeois' males and parasitic spawning. Trends Ecol Evol 13:222-227.

Thornhill R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am Nat. 122:765-788.

Toth GP, Ciereszko A, Christ SA, Dabrowski K. 1997. Objective analysis of sperm motility in the Lake sturgeon, *Acipenser fulvescens*: activation and inhibition conditions. Aquaculture. 154: 337-348.

Trippel EA, Neilson JD. 1992. Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (*Gadus morhua*) and associated hatching success. Can J Fish Aquat Sci. 49:2118-2127.

Turner E, Montgomerie R. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol. 60:1570-1579.

Urbach D, Folstad I, Rudolfsen G. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol. 57:438-444.

Unwin MJ, Glova GJ. 1997. Changes in life history parameters in a naturally spawning population of chinook salmon (*Oncorhynchus tshawytscha*) associated with releases of hatchery-reared fish. Can J Fish Aquat Sci. 54:1235.1245.

Unwin MJ, Kinnison MT, Quinn TP. 1999. Exceptions to semelparity: postmaturation survival, morphology, and energetics of male chinook salmon (*Oncorhynchus tshawytscha*). Can J Fish Aquat Sci. 56:1172-1181.

Vladic TV, Jarvi T. 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. Proc R Soc Lond B. 268: 2375-2381.

Ward PI. 1998. Intraspecific variation in sperm size characters. Heredity 80:655-659.

Wedell N, Gage MJG, Parker GA. 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol. 17:313-320.

Wedekind C, Müller R. 2004. Parental characteristics versus egg survival: towards an improved genetic management in the supportive breeding of lake whitefish. Annales Zoologici Fennici. 41:105–115.

Wedekind C, Chapuisat M, Macas E, Rülicke T. 1996. Non-random fertilization in mice correlates with the MHC and something else. Heredity. 77:400-409.

Wedekind C, Walker M, Portmann J, Cenni B, Müller R, Binz T. 2004. MHC-linked susceptibility to a bacterial infection, but no MHC- linked cryptic female choice in whitefish. J Evol Biol. 17:11-18.

Wojtcza M, Dietrich GJ, Słowińska M, Dobosz S, Kuźmiński H, Ciereszko A. 2007. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Aquaculture. 270:259-264.

Wolgemuth DJ. 1983. Synthetic activities of the mammalian early embryo: molecular and genetic alterations following fertilization. In: Hartmann JF, editor. Mechanism and control of animal fertilization. London: Academic Press. p.415-452.

Yanagimachi R, Cherr GN, Pillai MC, Baldwin JD. 1992. Factors controlling sperm entry into the micropyles of salmonid and herring eggs. Devel Growth Diff. 34:447-461.

Yeates S, Searle J, Ward RG, Gage MJG. 2007. A two-second delay confers first-male fertilization precedence within in vitro sperm competition experiments in Atlantic salmon. J Fish Biol. 70:318-322.

Yoshida T, Nomura M. 1972. A substance enhancing sperm motility in the ovarian fluid of the brown trout. B Jpn Soc Sci Fish. 30:1073.

Zeh JA, Zeh DW. 1996. The evolution of polyandry I: Intragenomic conflict and genetic incompatibility. Proc R Soc Lond B. 263:1711-1717.

Zeh JA, Zeh DW. 1997. The evolution of polyandry II: Post-copulatory defences against genetic incompatibility. Proc R Soc Lond B. 264:69-75.

Chapter 2

EJACULATE TRAITS IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) IN RELATION TO MALE BODY CONDITION, SIZE AND INVESTMENT INTO SPERM COMPETITION

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2.1 ABSTRACT

Ejaculate traits (sperm swimming speed, motility, path trajectory, longevity and sperm concentration) and measures of male quality (body condition and size), along with investment into reproduction (relative testes mass) were studied within a single male alternative reproductive tactic (wherein large males spawn in dominance-based hierarchies) in chinook salmon (*Oncorhynchus tshawytscha*). We show that males in better condition have relatively larger testes. However, having larger testes relative to body size did not correspond with a high spermatozoa concentration in the ejaculate or superior quality spermatozoa. In addition, there was also a weak negative relation between body condition and sperm swimming speed, linearity and longevity. Moreover, there were none of the trade-offs between ejaculate traits predicted by theory, with a positive relation observed between sperm longevity and swimming speed. Additionally, smaller males tended to have higher sperm concentration in their ejaculate compared to larger males, possibly in response to a greater risk of sperm competition.

Keywords: Sperm swimming speed, body condition, testes, chinook salmon, sperm competition, spermatocrit.

2.2 INTRODUCTION

Ejaculates can be complex, and are more than just the sum of sperm numbers present, as they are the product of many interacting components that include: sperm density, velocity, motility and morphology, as well as the seminal fluid (Snook 2005; Poiani 2006; Roldan 2007). These ejaculate traits are thought to function to promote fertilisation and thus to maximize a male's paternity, and it is noteworthy that a striking pattern of variation in ejaculate traits has been observed within and among different animal taxa (Ward et al. 1998; Snook 2005; Locatello et al. 2007; Burness et al. 2008). It is especially puzzling that such intra-specific variation in ejaculate traits has arisen, given strong directional selection expected within each species due to the pressures of sperm competition and the female reproductive environment in which ejaculates must compete. This observed variation in ejaculate components is now just beginning to be investigated (Vladic and Jarvi 2001; Birkhead et al. 2005; Snook 2005; Locatello et al. 2007; Roldan 2007; Burness et al. 2008).

Externally fertilising fish species have ejaculate traits that are highly variable between males within a single population (Morrow and Gage 2001), and recent studies have uncovered a number of factors that explain much of the observed variation; male alternative reproductive tactics (Uglem et al. 2001; Locatello et al. 2007), body size (Skinner and Watt 2007) and condition (Burness and Montgomerie 2004; Urbach et al. 2007; Burness et al. 2008), social status (e.g. Rudolfsen et al. 2006), stage of the breeding season (Methven and Crim 1991), and the presence and composition of ovarian fluid (Turner and Montgomerie 2002; Urbach et al. 2005; see Chapter 3 and 5). In externally fertilising species, two strong selective forces are likely to play an important role in the observed variation in ejaculate traits. These have been identified as the fertilization environment created by the female (Urbach et al. 2005; Simmons et al. 2009; see Chapter 3), and sperm competition (Birkhead and Møller 1998).

Sperm competition occurs when the ejaculates from two or more males compete to fertilise a female's eggs (Parker 1970), and it is very common among externally-

fertilising fish species (Petersen and Warner 1998; Taborsky 1998). This intense male-male competition among ejaculates is now recognised as one of the most powerful evolutionary forces shaping male physiology, morphology and behaviour (Birkhead and Møller 1998; Simmons 2001; Birkhead and Pizzari 2002). Ejaculate traits that are actually or predicted to be influenced by selection pressure via sperm competition include sperm velocity, motility, density, longevity, morphology and energetics (Parker 1990; Ball and Parker 1996, Stockley et al. 1997; LaMunyon and Ward 1998; Donoghue et al. 1999; Gage et al. 2004; Denk et al. 2005; Garcia-Gonzalez et al. 2005, 2007; Casselman et al. 2006; Fitzpatrick et al. 2007; Thomas and Simmons 2007; Fitzpatrick et al. 2009). In fishes, sperm velocity has been identified as an important sperm trait that can predict male fertilisation success (Burness et al. 2004; Gage et al. 2004; Rudolfsen et al. 2008). Thus, faster swimming sperm may provide males with a competitive advantage as they will reach the egg first (Ball and Parker 1996; Parker 1990). Additionally, a recent comparative study in cichlid fish, demonstrated that sperm competition promotes the evolution of longer and faster sperm (Fitzpatrick et al. 2009).

Theory and empirical evidence from studies of sperm competition suggest that male ejaculate traits associated with intense male-male competition are energetically demanding and thus costly for a male (Dewsbury 1982; Olsson and Madsen 1998; reviewed in Simmons 2001). As a result, such traits should be influenced by body condition (Rowe and Houle 1996; Kotiaho 2000; Kotiaho et al. 2001; Cotton et al. 2004; Tomkins et al. 2004) such that only those males in good condition should be able to produce high quality ejaculates. Recent studies have revealed that this is indeed the case (Simmons and Kotiaho 2002; Burness et al. 2008). In perch (*Perca fluviatilis*), sperm density was positively correlated with body condition (Wirtz and Steinmann 2006), while in the lake whitefish (*Coregonus clupeaformis*) males in good body condition were able to invest more into reproduction as they had larger relative testes mass (Burness et al. 2008). In Atlantic cod (*Gadus morhua*) fertilisation success was positively associated with male body condition (Rakitin et al. 1999) and in Alpine lake whitefish (*Coregonus sp.*) eggs fertilised by males in good condition had a higher survival rate (Wedekind and Müller 2004). However, only a few studies have considered the relationship between

body condition and ejaculate investment (relative testes mass) and/or sperm traits (Rakitin et al. 1999; Casselman and Montgomerie 2004; Urbach et al. 2007; Burness et al. 2008) in externally fertilising fishes.

In this study we look at several ejaculate traits (sperm density, longevity, velocity, motility and swimming path trajectory) within a single male reproductive tactic (i.e., males that spawn in dominance based hierarchies) of male chinook salmon to examine intraspecific variation. Theory predicts a trade-off will exists between ejaculate traits involved in sperm competition (Ball and Parker 1996; Parker 1998), due to the need to differentially allocate limited resources to traits that enhance reproductive success (Dewsbury 1982). For example, sperm longevity is expected to involve a trade-off with sperm velocity as both depend upon energetic resources (Ball and Parker 1996; Levitan 2000; Snook 2005). However, it is likely that sperm competition may select on a number of traits jointly rather than selecting on individual traits (Moore et al. 2004; Snook 2005), as it is thought that measures of single ejaculate traits are poorly correlated with male fertility (Rodriguez-Martinez 2007).

In Pacific salmonids (genus *Oncorhynchus*), the natural spawning environment swarms with conspecific males leading to intense levels of sperm competition, as several males compete to fertilise the eggs from a single spawning female (Gross 1985; Fleming and Gross 1994; Quinn 1999). This intense competition between males results in dominance-based spawning hierarchies, wherein males that are positioned closest to the female are in favourable positions, while those males further away occupy unfavourable positions with respect to their opportunity to fertilise that female's eggs (Fleming and Gross 1994; Quinn and Foote 1994). Dominance is generally associated with body size, with larger-boded males obtaining more favourable spawning positions within the spawning hierarchy (Berejikian and Tezak 2005; Labonne et al. 2009).

Most studies on the effects of sperm competition on ejaculate traits have been done on species with male alternative reproductive tactics (MARTs), in which the sperm traits from these different reproductive tactics are compared (Leach and Montgomerie 2000;

Vladic and Jarvi 2001; Neff et al. 2003; Schulte-Hostedde and Burness 2005a; Locatello et al. 2007). However, there is a paucity of information regarding ejaculate traits and the effects of sperm competition on these traits within a single MART, as it generally assumed that all individuals adopting the same tactic experience the same level of sperm competition (Pitcher et al. 2009). Yet, within a single MART, males will vary in distance from a female during spawning — some males will be close to a spawning female and others further away (Stolz and Neff 2006). A recent study within a single MART in coho salmon (*Oncorhynchus kisutch*)—large hooknose males that spawn in dominance-based hierarchies—demonstrated that males did in fact experience different levels of sperm competition, resulting in a potential trade-off between investment into secondary sexual characteristics and sperm quality (Pitcher et al. 2009).

In addition, we also examine the relations among body condition, ejaculate investment (relative testes size), body size (length) and ejaculate traits. We predicted that ejaculate investment would depend upon body condition – as males in good condition should have both larger testes mass relative to their body size, and higher quality ejaculates, than males in poorer condition. We also tested the prediction that smaller males—occupying disfavoured roles with respect to proximity to females during spawning—would have ejaculates of higher quality compared to larger males, to compensate. Sperm competition theory suggests that males in disfavoured roles (proximity to females during spawning) will invest more into ejaculate quality than dominant males (Parker 1990; Ball and Parker 1996; 2000). For example, males in disfavoured roles may compensate by having larger gonads for their body size (Taborsky 1998; Liljedal and Folstad 2003), or, as demonstrated in Arctic charr (*Salvelinus alpinus*), males that become subdominant produce ejaculates with a higher sperm cell density along with higher initial sperm swimming speeds (Rudolfsen et al. 2006).

2.3 METHODS

From 24 April to 18 May 2004, we obtained milt samples from sexually mature two-year-old male chinook salmon (n=60) from a hatchery-reared broodstock generated from

semi-wild returns. All fish were released into the wild and then returned to the hatchery to spawn. The fish were reared at the National Institute of Water and Atmospheric Research Silverstream Hatchery, Canterbury, New Zealand. All of the study fish were housed in a common raceway at the hatchery and maintained using standard husbandry procedures (Pennell and Barton 1996).

Milt samples were collected on different days during the spawning season. On each day when milt was sampled, each male was netted and dried around the cloaca to avoid premature activation of sperm cells by water/urine. Milt was then collected by applying gentle bilateral abdominal pressure. Milt samples were then immediately refrigerated at 4°C and transported to the University of Canterbury, where the behaviour of activated spermatozoa was video-recorded. From each of 60 males, sperm motility was recorded after activation in fresh water from the raceway in which they were housed. Each sampling day, males were stripped of milt in a haphazard order so that the amount of time between stripping milt and recording sperm behaviour would not confound our results. The ambient air temperature in the laboratory was set at the water temperature (12.0°C) of the holding raceway to control for variation in sperm swimming speed with varying water temperatures (Alavi and Cosson 2005). Sperm motility was initiated by adding 499 μL of freshwater to 1 μL of the milt sample. We immediately placed 10 μL of this solution on a glass slide and gently placed a cover slip over the sample for viewing and recording at 400X magnification with a negative phase-contrast microscope (Leica DMR). Upon activation, sperm behaviour was recorded using a high resolution digital videocam, and sperm longevity (DUR in s) for each milt sample was recorded using a stopwatch, starting at the contact of the milt with the activation solution, and ending when all progressive forward motility had ceased (e.g. Leach and Montgomerie 2000); spermatozoa that were still vibrating at the end of the period of propulsive sperm motility were considered to be immotile. Activation of sperm samples was done within 5 hours of collection.

Video recordings were later analysed using computer assisted sperm analysis (HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research, Beverly, MA, USA;

see Appendix 2.1 for set-up parameters for CASA). For each milt sample, we quantified the swimming paths of all spermatozoa in a field of view for 0.5 s at 10 s intervals postactivation. Sperm tracks that were clearly influenced by sample drift, and/or were incomplete were not analyzed. On average, 13 sperm tracks (range = 5 - 45 sperm tracks) were analyzed per milt sample. Hoysak and Liley (2001) have shown that the majority of fertilisations in salmonids are likely to occur within a few seconds after sperm activation. Moreover, in walleye (*Sander vitreus*), sperm swimming speed at 10 s post-activation was associated with male fertilisation success, whereas at 20 s post-activation it had no relation to fertilisation success (Casselman et al. 2006). Therefore, in this paper we report on sperm traits at 10 s post-activation only.

The average values of the following parameters for each male were calculated from the sperm tracks of each trial: average path velocity (VAP in $\mu m \cdot s^{-1}$), mean straight line velocity (VSL in $\mu m \cdot s^{-1}$), mean curvilinear velocity (VCL in $\mu m \cdot s^{-1}$), and linearity (LIN; the ratio of VSL/VCL expressed as a percentage). Like other studies, we used VAP as a measure of sperm swimming speed (e.g., Lahnsteiner et al. 1998; Burness et al. 2005; Casselman et al. 2006; Pitcher et al. 2009) because it is calculated from the smoothed path of the sperm moving through water in a trajectory that most closely mimics the path over which a spermatozoon would encounter an unfertilized ovum. LIN describes the path trajectory of the sperm through the solution. A circular trajectory, for example would have a low LIN, and a high LIN would indicate that the sperm cell is moving in a straight-line path. For each trial, we also measured the percentage of cells in the field of view that were progressively motile (MOT). We present VAP results as a measure of sperm velocity, as this velocity parameter is likely to best represent the speed over the trajectory the sperm will take, and to facilitate comparison with studies on other fish species (Lahnsteiner et al. 1998; Casselman et al. 2006; Fitzpatrick et al. 2007; Burness et al. 2008; Pitcher et al. 2009).

First, we determined sperm density (total number of spermatozoa$\cdot ml^{-1}$) for 20 males by counting sperm cells in a haemocytometer (improved Neubauer counting chamber, depth 0.1 mm) (Bouk and Jacobson 1976; Wirtz and Steinmann 2006) under 400X

magnification. Each milt sample was diluted (5µl:1.5 ml) in freshwater and the mean number of spermatozoa in each milt sample was quantified by counting the number of sperm from five squares on the haemocytometer, then multiplying it by the sample dilution factor and the initial volume. Three replicates were obtained from each milt sample and the mean sperm density was calculated for each male.

Second, we measured spermatocrit values (as described below) for the same 20 males to compare with the results from the haemocytometer. The spermatocrit for each milt sample was defined as the ratio of volume of white packed material to the total volume of semen x 100 (Rurangwa et al. 2004). Microhaematocrit capillary tubes (75 mm length and 1.1-1.2 mm diameter) were filled approx 70% full with semen, and one end of each tube was sealed with clay. Capillary tubes were then centrifuged for 10 min at 7500 rpm (5350 g). Measurements were replicated for every milt sample. If the spermatocrit for the first two measurements were within 5% they were accepted. If more than a 5 % difference between the two values another measurement was obtained for that milt sample (Rakitin et al. 1999). In addition, if there was not a clear interface between packed sperm cells and clear seminal fluid the sample was repeated. Typically, there was usually a sharp separation between sperm cells and seminal fluid after centrifugation. Spermatocrit was measured the same day the milt sample was collected, or from milt stored at 4 °C and analysed the next day.

There was a highly significant positive linear relationship ($SD = 2.36+1.60SC$, $r^2 = 0.95$, $P < 0.001$, $n=20$; Appendix 2.2) between spermatocrit (SC) and spermatozoa density (SD). A direct relationship between spermatocrit values and sperm count has also been observed for Atlantic salmon (Aas et al. 1991) and the spermatocrit method has been used for other salmonid species (Bouk and Jacobson 1976; Piironen 1985; Poole and Dillane 1998; Liley et al. 2002). Thus we used the spermatocrit to estimate sperm density as it can be measured more quickly (Hoysak and Liley 2001).

Following the last milt collection, on 18 May, all fish were killed and their standard length (total body length less caudal fin length; *SL*, ±1 mm) and body mass (*BM*, ±1 g)

measured, and both testes removed and weighed (±1 g). Soma mass (*SM*) was calculated by subtracting the total mass of the testes from body mass. Fulton's condition factor (K = mass/length^3 x 100; Rakitin et al. 1999) was calculated as a measure of body condition. This condition factor was chosen rather than other indicators of condition because it has been used in studies of Atlantic (Sutton et al. 2002) and chinook salmon (Pitcher and Neff 2007). The slope of the linear regression of log *SL* on log *BM* was 3.1 (n=60), confirming isometric growth ($BM = aSL^b$, with b = 3) in our study population. K was also not significantly correlated with *SL* (r = 0.18, P = 0.18, n =59).

All animals were collected and maintained according to the standards of the Animals Ethics Committee for the University of Canterbury, New Zealand.

2.4 STATISTICAL ANALYSES

We constructed multiple regression models that included four predictor variables for each ejaculate trait (VAP, LIN, MOT, DUR, sperm density). For each male these ejaculate traits were measured three to four times during the spawning season and we calculated an overall average for each male. The predictor variables were; (i) body condition (measured as Fulton's), (ii) ejaculate investment (measured as total testes mass), (iii) body size (measured as standard length), and (iv) soma mass. All of these predictor variables were measured towards the end of the spawning season.

We then performed hierarchical partitioning (HP) to select the most important predictor variables that influenced ejaculate traits using the 'hier.-part package' version 1.0-3 (Walsh and Mac Nally 2008) in the R statistical application (R Development Core Team 2008). This analysis enabled us to assess the amount of variation contributed by each of the four predictors (Mac Nally 2002; Walsh and Mac Nally 2004; Appendix 2.3). HP quantifies the independent and joint contribution of each predictor variable and helps to avoid the problem of multi-colinearity (Quinn and Keough 2002; Appendix 2.4). Predictor variables for each sperm trait that accounted for < 10% of the independent variation were not included in multiple regression models (Appendix 2.3). We also

evaluated regression models using an information-theoretic (IT) approach (Burnham and Anderson 1998; 2004) using the MuMIn package in R. We present model weight (w_i), a measure of a model's relative probability of being the best model, given the data, compared with alternative models (Burnham and Anderson 1998). In addition, we calculated Δ_i as the difference between the AICc for the ith model in the set and the AICc of the best model. These Δ_i values are used to gauge the relative plausibility of each model. Models with Δ_i <2 are as well supported as the best model, those with Δ_i = 2–7 are less well supported, while those with Δ_i >7 have no support from the data (Burnham and Anderson 1998).

Correlation analyses were used to explore linear relations between all ejaculate traits measured and to explore the relation between testes mass and sperm density. Assumptions underlying all models were verified by analyzing residuals for normality (Shapiro-Wilk tests; p >0.05). DUR was $\log^{10}$ transformed to normalise residuals. In our analyses we identified one outlier that we excluded from all analyses, as that male had unusually large testes for his body size (>2 standard deviations above the predicted values for this trait). Removal of this male from the analyses did not alter our conclusions.

2.5 RESULTS

Intraspecific variation and relations between ejaculate traits

There was considerable variation among all ejaculate traits measured as demonstrated by the coefficients of variation ranging from 23.6-32.9% (Table 2.1). This was not surprising as similar patterns have also been observed in other fish species that experience high levels of sperm competition (Leach and Montgomerie 2000; Vladic and Jarvi 2001; Burness et al. 2005; Rudolfsen et al. 2006). Mean spermatocrit was similar to those reported for Atlantic salmon (*Salmo salar*) (mean spermatocrit 23.4%) (Aas et al. 1991) and brown trout (*Salmo trutta caspius) (*spermatocrit ranged from 25 – 52%) (Hatef et al. 2007). The three measures of sperm swimming speed (VAP, VCL, and VSL) were all significantly positively correlated (Table 2.2). In addition, there were also significant

positive correlations between DUR and VSL, DUR and VAP (Fig. 2.1, Table 2.2), and between LIN and VSL (Table 2.2).

As predicted, males in good body condition (*K*) had heavier testes for their body size than males in poorer body condition (partial $r = 0.39$, $n = 59$, $P < 0.0001$, controlling for body length; Fig. 2.2). In a separate multiple regression analysis that included length (*SL*) and condition (*K*) as predictor variables, the effect of condition ($F_{1,57} = 10.3$, $P = 0.002$), and body length ($F_{1,57} = 48.8$, $P < 0.0001$) were both significant. There was no relation between sperm density (spermatocrit) and testes mass (partial $r = 0.18$, $n = 59$, $P = 0.85$, controlling for body size; Fig. 2.3). In addition, there were no significant relations between relative testes mass and the other ejaculate traits measured; VAP, $r = –0.08$, $n = 56$, $P = 0.56$; LIN, $r = –0.18$, $n = 57$, $P = 0.18$; DUR, $r = 0.01$, $n = 59$, $P = 0.96$; MOT, $r = –0.03$, $n = 56$, $P = 0.85$), and all correlations were weak.

Table 2.1. Descriptive statistics for ejaculate and morphological traits from sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*).

Trait	n	Mean	CI	CV	Range
Ejaculate					
Spermatocrit (%)	59	22.5	20.9-24.1	26.7	7.4-39.5
DUR (s)	59	61.5	56.5-66.3	31.2	27.8-124
VAP (µm•s^{-1}) at 10 s postactivation	56	47.1	44.6-50	23.8	23.7-77.9
VSL (µm•s^{-1}) at 10 s postactivation	56	29.8	27.1-32.3	32.9	9.3-61.7
VCL (µm•s^{-1}) at 10 s postactivation	56	70.7	64.9-76.6	31.4	38.1-149.5
MOT (%) at 10 s postactivation	56	53.8	50.1-56.9	23.4	28.3-81.3
LIN (%) at 10 s postactivation	57	45.1	41.6-48.1	27.7	17-74.3
Morphological					
Fulton's *K*	59	1.6	1.5-1.6	6.2	1.3-1.9
Testes mass (g)	59	49.6	39.7-59.6	43.3	9.1-110
Standard length (*L*s) (mm)	59	335.7	326.4-346.4	11.4	230-425
Somatic weight (g)	59	573.1	563.2-583.1	35.3	169.6-1224.2
body mass (g)	59	622.8	612.8-632.8	35.2	179.6-1334.6

CI, 95% confidence interval; CV, coefficient of variation; DUR, mean sperm swimming duration; VAP, mean smoothed path velocity (sperm swimming speed); VCL, mean curvilinear velocity; VSL, mean straight-line velocity, LIN, mean sperm path linearity; MOT, mean percentage of progressively motile cells.

Table 2.2. Correlations between ejaculate traits from sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*) (n=59). In bold are significant correlations (P < 0.05).

	DUR		LIN		VAP		VSL		VCL		MOT	
	r	P	r	P	r	P	r	P	r	P	r	P
SC	0.11 n=(58)	0.17	-0.25 (n=57)	0.06	-0.18 (n=55)	0.19	-0.27 (n=55)	0.05	-0.05 (n=55)	0.74	-0.13 (n=56)	0.36
DUR			0.22 (n=57)	0.12	**0.32** (n=56)	**0.02**	**0.35** (n=56)	**0.008**	0.14 (n=56)	0.3	0.11 (n=55)	0.43
LIN					0.2 (n=55)	0.15	**0.65** (n=55)	**<0.0001**	-0.24 (n=56)	0.08	0.17 (n=55)	0.2
VAP							**0.76** (n=56)	**<0.0001**	**0.85** (n=56)	**<0.0001**	0.1 (n=56)	0.46
VSL									**0.39** (n=56)	0.003	0.19 (n=56)	0.17
VCL											-0.09 (n=56)	0.5

SC, spermatocrit (%), DUR, mean sperm swimming duration (s); LIN, mean sperm path linearity (%); VAP, mean smoothed path velocity (µm•s^{-1}); VSL, mean straight-line velocity (µm•s^{-1});VCL, mean curvilinear velocity (µm•s^{-1}); MOT, percentage of progressively motile cells.

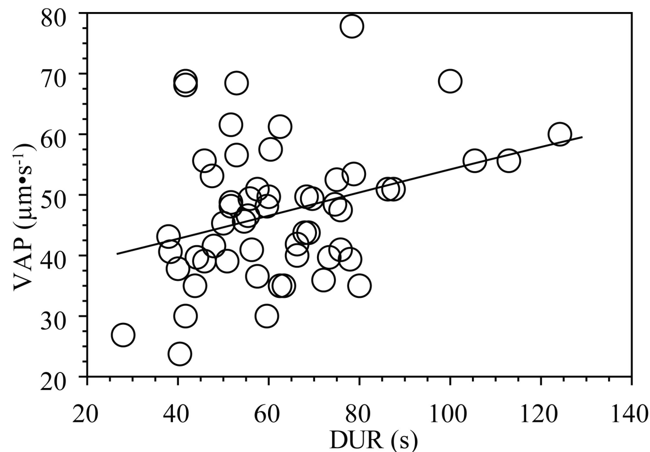

Figure 2.1. Relation between mean sperm swimming duration (DUR s) and average sperm swimming speed (VAP µm•s^{-1}) 10 s post-activation. In this plot, VAP = 35.5 + 0.2DUR (r = 0.32, P = 0.02, n = 56).

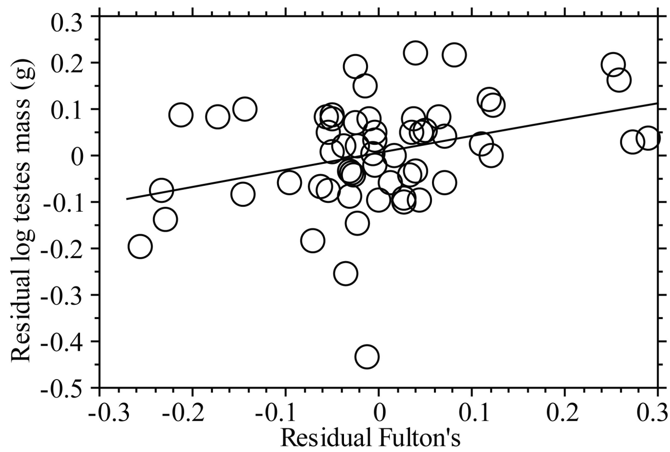

Figure 2.2. Partial regression plot showing that testes mass from 59 sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*), increased with body condition (Fulton's *K*), controlling both variables for body length. In this plot, y = 3.4 x 10^{-4} + 0.4*x* (*r* = 0.34, P = 0.009, n = 59).

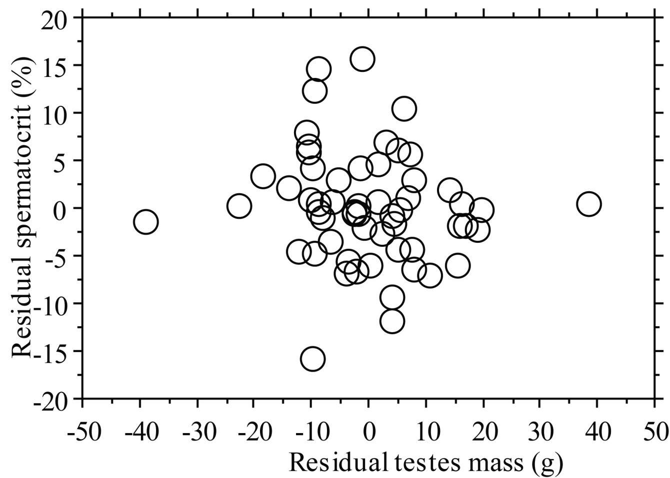

Figure 2.3. Partial regression plot between average sperm density per ejaculate (spermatocrit) and testes mass from 59 sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*), controlling both variables for body length.

Table 2.3. Corrected Akaike Information Criterion (AICc) for regression models that are most strongly supported by the data (Δ_i < 2) to predict sperm traits; mean sperm swimming speed (VAP µm•s^{-1}), mean path trajectory (LIN %), mean percentage of progressively motile cells (MOT %)10 seconds post-activation, and mean sperm swimming duration (DUR s), along with the spermatocrit (SC %) of sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*) (n = 59). Weights (w_i) are a measure of the weight of evidence in favour of that particular model over all the others.

	R^2	AIC_c	Δ_i	w_i
VAP				
Fulton's	0.07	438.40	0.00	0.342
NULL	0.00	440.00	1.67	0.148
MOT				
NULL	0.00	455.20	0.00	0.294
Somatic weight (g)	0.02	456.10	0.86	0.192
Standard length (mm)	0.02	456.10	0.92	0.185
Testes mass (g)	0.01	456.80	1.62	0.131
LIN				
Fulton's	0.12	454.50	0.00	0.489
DUR				
NULL	0.00	27.10	0.00	0.292
Fulton's	0.03	27.25	0.16	0.270
SC				
Standard length (mm)	0.06	2666.00	0.00	0.244
Standard length (mm) and testes mass (g)	0.09	2666.00	0.32	0.208
Somatic weight (g)	0.05	2667.00	1.02	0.146
NULL	0.00	2668.00	1.68	0.105
Standard length (mm) and somatic weight (g)	0.07	2668.00	1.83	0.098
Somatic weight (g) and testes mass (g)	0.07	2668.00	1.97	0.091

Using the IT approach, the top linear models (Δ_i <2) to predict both VAP and LIN at 10s post-activation, as well as DUR, included only body condition (Fulton's *K*) as a predictor (Table 2.3). Each of these variables (LIN, VAP and DUR) were negatively related to body condition (Fig. 2.4). For VAP the model containing body condition as a predictor was more than twice likely as the null model (evidence ratio = 2.30), with a 34% probability that this model is true given the data (w_i = 0.342; Table 2.3). For DUR there was no evidence to favour the null model over the next best model containing body

condition as a predictor (evidence ratio = 1.08), and there is only a 29% probability that this model is true given the data (w_i = 0.292; Table 2.3). The best linear model to predict spermatocrit contained only body length (; r = -0.23, P = 0.08, n = 59; Fig.2. 5), but there is only a 24% probability that this model is true given the data (w_i = 0.244; Table 2.3). There was also little evidence to prefer this model over the next model, containing body size (standard length) and testes mass (evidence ratio = 1.17; Table 2.3). For the percentage of motile cells at 10 s post-activation (MOT), the best model is the null model but it has only a 29% probability that it is true, given the data (Table 2.3), and it is only 1.5 times more likely than the next model, with somatic mass as a predictor.

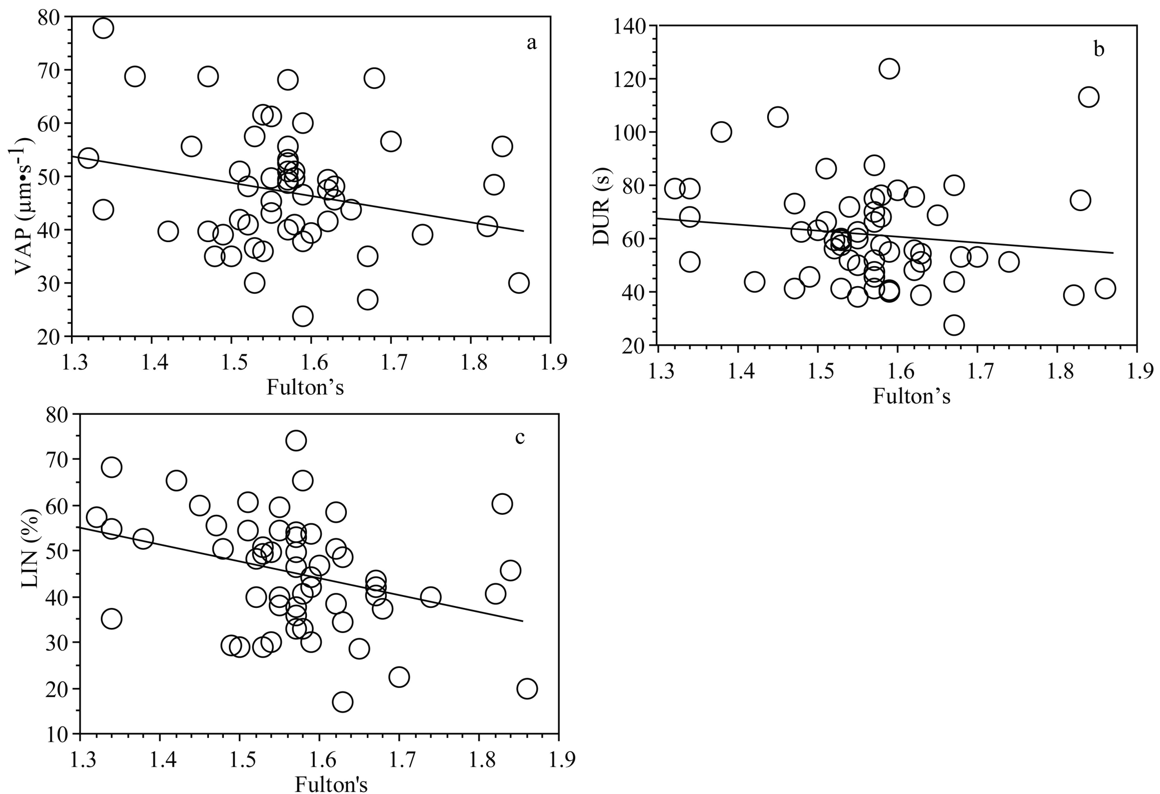

Figure 2.4. Relations between body condition (Fulton's *K*) and (a) average sperm swimming speed (VAP µm•s^{-1}) 10 s post-activation (VAP = 86.1-24.8*K*, n = 56), (b) mean sperm swimming duration (DUR s) (DUR = 98-23.3*K*, n = 59), and (c) mean sperm path linearity (LIN %) at 10 s post-activation (LIN = 103.7-37.3*K*, n = 57) for two-year-old sexually mature male chinook salmon (*Oncorhynchus tshawytscha*).

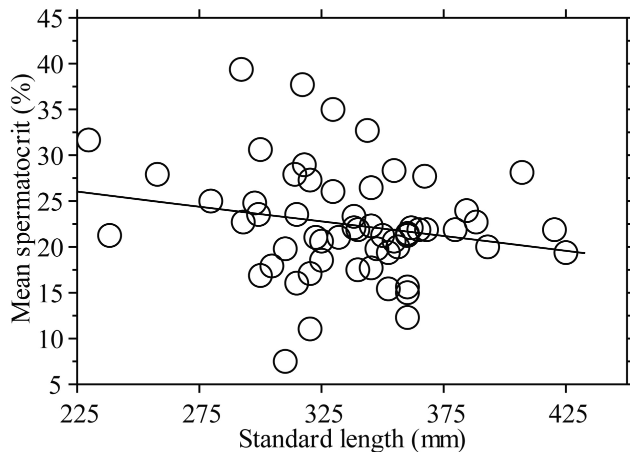

Figure 2.5. Relation between mean sperm density (spermatocrit, SC) and body size (standard length, SL) over the spawning season for 58 sexually mature male two-year-old chinook salmon (*Oncorhynchus tshawytscha*). Regression is SC=34.6-0.04*SL*. (r = 0.21, P = 0.12).

Overall, the relation between LIN and condition had the highest evidence of support (w_i = 0.489; Table 3) with condition explaining about 12% of the variation in LIN (r = –0.35, P = 0.009, n = 57; Fig. 2.3c). Negative relations between condition and both VAP (r = –0.25, P = 0.06, n = 56; Fig. 2.3a) and DUR (r = –0.14, P = 0.30, n=59; Fig. 2.3b), but both relations are weak.

2.6 DISCUSSION

Testes mass was a condition dependent male trait within a single MART of chinook salmon, with males in better condition having relatively larger testes (Fig. 2.2). However, males with larger testes did not have superior ejaculates with a higher quality or density of spermatozoa (Fig. 2.3). Additionally, there was a pattern of negative relations between body condition and sperm traits (Fig. 2.4). This result may be biologically important as it suggests that only those males in good condition prior to spawning are able to allocate more energy into ejaculate quality, resulting in poorer body condition at the time of spawning (Casselman and Montgomerie 2004). The theoretically predicted trade-off between sperm longevity and swimming speed was not observed as both traits were

positively correlated (Fig. 2.1, Table 2.2). In addition, there was a trend that smaller-sized males may compensate for their disfavoured role during spawning by increasing the sperm concentration in their milt (Table 2.3 and Fig. 2.5).

As predicted, males in good body condition had relatively larger testes (Fig. 2.2), suggesting that testes size (adjusted for body size), a male reproductive trait that is typically involved in sperm competition, depends on male condition. A similar relation between body condition and relative testes mass has also been reported in lake whitefish (*Coregonus clupeaformis*) (Burness et al. 2008). Furthermore, a positive relation between relative testes size and body condition has been found for several small mammals (Schulte-Hostedde and Millar 2004; Schulte-Hostedde et al. 2005b), and the Hawaiian fruitfly (*Drosophila grimshawi*) (Droney 1998). Additionally, in the dung beetle (*Onthophagus taurus*) males in good condition had larger testes along with shorter sperm (Simmons and Kotiaho 2002), while in the red deer (*Cervus elaphus*) the size and complexity of antlers was associated with relative testes mass and sperm velocity (Malo et al. 2005).

Presumably males with relatively larger testes have some kind of reproductive advantage over males with smaller size testes. In Atlantic cod (*Gadus morhua*); two experiments demonstrated that fertilisation success was positively associated with male body condition (*K*) when equal volumes of semen from each male were used (Rakitin et al. 1999). And in mammals, males with relatively larger testes have high quality ejaculates; more sperm, greater motility and higher ejaculate volume (Ginsburg and Huck 1989; Stockley and Purvis 1993; Gomendio et al. 1998). Moreover, male mammals with absolute larger testes also have higher reproductive success than males with smaller testes (Preston et al. 2003; Schulte-Hostedde and Millar 2004). Conversely, the results of our study showed that those males with relatively larger testes did not have ejaculates of superior quality; such as, faster swimming sperm that lived longer, or ejaculates with a higher sperm density.

Typically testes size is a good measure of investment into reproductive tissue, as fish testes consist mainly of spermatogenic cells (Billard 1986), and relative testes size is commonly used as an index of sperm production in an ejaculate (Andersson 1994; Gomendio et al. 1998; Wedell et al. 2002; Malo et al. 2005). For this reason testes size and sperm production are considered to be interchangeably (Pitnick et al. 2001; Byrne et al. 2002; Newlon et al. 2003; Malo et al. 2005), and it is generally assumed that bigger testes enable males to produce more spermatozoa (Møller 1988; 1989; Gage 1995; Parker and Ball 2005). As a successful outcome in regards to sperm competition may depend on a number of ejaculate traits, a greater number of spermatozoa should enable males to win more fertilisations (Parker 1982; Ball and Parker 1996). This has been demonstrated in the male bucktooth parrotfish (*Sparisoma radians*) as male fertilisation success increased significantly with the number of sperm released during natural spawning (Marconato and Shapiro 1996). However, in this study, having larger relative testes did not equate to an increase in sperm density per ejaculate (Fig. 2.3).

It would seem reasonable that a male with larger testes would be able to release more spermatozoa per ejaculate during spawning. There are a few potential explanations for why we did not find a correlation between relative testes size and sperm density. First, in this study we did not measure sperm size, and relationships have been observed between testes size, sperm size and number. For example, among *Drosophila*, species with large testes produce fewer but longer sperm (Pitnick and Markow 1994; Pitnick 1996), and a recent comparative study in New World Blackbirds (Icteridae) reports that long sperm require more space within the testes, and therefore influence the rate of sperm production (Lüpold 2009). Second, salmonids have lobular testes made up of spermatogenic tissue and a testicular gland (Billard 1986), so differences in testes size may not reflect sperm production capacity. For example, the portion of the testes occupied by the testicular gland varied greatly among blenniid fishes (Giacomello et al. 2008). The lack of a relation between testes size and sperm density in this species warrants further investigation to determine if larger testis do have more sperm producing tissue, and are therefore capable of producing sperm at a faster rate.

There were negative relations between body condition and sperm swimming speed (VAP), linearity (LIN) and longevity (DUR) (Fig. 2.4). The top models to explain variation in (VAP) and (LIN) at 10 s post-activation, as well as (DUR), were those models that included only body condition, but this accounted for <12% of the independent variation in these traits (Table 2.3). Moreover, the weight of evidence in favour of these models for each trait over the next best models was low (see weights, Table 2.3). At best our results suggest a potentially biologically important pattern in our data may exist as similar negative relations between body condition and sperm velocity have been observed in the Alpine whitefish (*Coregonus fatioi*) (Urbach et al. 2007), and in lake whitefish (Casselman and Montgomerie 2004). It has been suggested that males in good condition prior to spawning are able to allocate more energy into ejaculate quality, which then manifests as poorer body condition at the time of spawning (Casselman and Montgomerie 2004). However, further experimental work to determine how varying the body condition (e.g., manipulated via diet) of individual males influences ejaculate traits. Moreover, body condition may also effect the chemical composition of the seminal fluid which in turn may also affect sperm function.

According to theory, sperm longevity is expected to involve a trade-off with sperm velocity, as they both depend on energetic resources (Parker 1993; Ball and Parker 1996; Levitan 2000; Snook 2005). We did not observe this predicted trade-off in chinook as a significant positive correlation was observed between sperm longevity and sperm swimming speed 10 s post-activation (Table 2.2; Fig. 2.1). The same positive relation between these two traits was also observed within another single MART in coho salmon (*Oncorhynchus kisutch*) (Pitcher et al. 2009), and in four other externally fertilising fish species (Gage et al 2002; Kortet et al 2004; Casselman et al 2006; Burness et al 2008). Conversely, no relations between sperm longevity and velocity have been observed in Atlantic salmon (Gage et al. 2004) and two gobiid species (Locatello et al. 2007), along with a recent study across 29 species of fish in the cichlid family (Fitzpatrick et al. 2009). These varying results lead us to question the generality of the theoretically predicted trade-off between sperm longevity and swimming speed (Ball and Parker 1996).

Within a single MART, our finding supports the prediction that males in disfavoured spawning roles (smaller sized typically further away from a spawning female) invest more into ejaculate quality than males in favoured spawning roles (larger-sized dominant males closer to a spawning female) (Ball and Parker 1996), as a negative relation (although not statistically significant) was observed between spermatocrit and body size (Fig. 2.5). The best model explaining the variation in sperm density included body length as the sole predictor (Table 2.3). Our results do contrast with one other study undertaken within a single MART in coho salmon (*Oncorhynchus kitsutch*) whereby no relation was observed between sperm density and body size, but in that study a relationship was observed between spawning colouration and sperm velocity (Pitcher et al. 2009). Support of the results in this study are found in studies on fish species whereby sperm characteristics are compared between males with different mating tactics e.g. sneakers (smaller males), dominant males (larger). Male morphs that experienced the highest magnitude of sperm competition in bluegill sunfish (*Lepomis macrochirus*) (Leach and Montgomerie 2000), bluehead wrasse (*Thalassoma bifasciatum*) (Schärer and Robertson 1999) and in sockeye salmon (*Oncorhynchus nerka*) (Hoysak and Liley 2001) had a higher sperm concentration in their milt than males that experienced less sperm competition.

An increase in sperm density per ejaculate in smaller size males within a single MART may be an important general adaptive response to sperm competition. In Pacific salmonids, the spawning area is typically teeming with conspecific males leading to intense sperm competition (Gross 1985). Larger-sized males participate in more spawning events and obtain better positions in spawning hierarchies compared to smaller size males (Fleming and Gross 1993, 1994; Dickerson et al. 2002). Consequently, smaller-sized males, in a disfavoured spawning role, experience a higher risk or intensity of sperm competition (Parker 1998), and possibly in response to this greater risk they produce ejaculates with a higher concentration of spermatozoa. Having ejaculates with a greater concentration of spermatozoa will enable smaller males to win more fertilisations as sperm density is known to influence fertilisation success in externally fertilising fish species (Marconato et al. 1995; Marconato and Shapiro 1996; Rakitin et al. 1999; Hoysak

and Liley 2001; Liley et al. 2002). Theory predicts that sperm competition should select for maximum sperm number (Parker 1998), and within a single MART, sperm competition may be acting solely on sperm density as selective pressure for high quality ejaculate traits may act equally on all males, irrespective of their body size.

In conclusion, our findings emphasize the need for further research regarding the interaction of ejaculate traits. Males in good body condition invested more into reproduction as these males had bigger testes (Fig. 2.2). However, the reproductive advantage that these males may have is unexplained and warrants further investigation, as males with bigger testes did not have superior quality ejaculates or ejaculates with a higher density of spermatozoa. Further study is also required to determine the impact of body condition on sperm traits that may be involved in sperm competition, and the relation between body condition and testes size, physiology and function on sperm production. In addition, our results do not show the theoretically predicted trade-off between sperm longevity and sperm swimming speed (Fig. 2.1), questioning our understanding of energetic requirements/allocation for these ejaculate traits and factors that influence ejaculate quality.

2.7 ACKNOWLEDGEMENTS

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2.8 REFERENCES

Andersson M. 1994. Sexual selection. Princeton: Princeton.University Press.

Aas GH, Refstie T, Gjerde B .1991. Evaluation of milt quality of Atlantic salmon. Aquaculture. 95:125-132.

Alavi SMH, Cosson J. 2005. Sperm motility in fishes. I. Effects of temperature and pH: A review. Cell Biol Int. 29:101-110.

Ball MA, Parker GA. 1996. Sperm competition games: external fertilization and ‘‘adaptive’’ infertility. J Theor Biol. 180:141-150.

Ball MA, Parker GA. 2000. Sperm competition games: A comparison of loaded raffle models and their biological implications. J Theor Biol. 206:487-506.

Berejikian BA, Tezak EP. 2005. Male size effects on fertilization success: lack of evidence in chinook spawning under experimental conditions. Env Biol Fishes. 72:235-240.

Billard R. 1986. Spermatogenesis and spermatology of some teleost fish species. Reprod Nutr. Develop. 26:877-920.

Birkhead TR, Møller AP. 1998. Sperm Competition and Sexual Selection. San Diego: Academic Press.

Birkhead TR, Pellatt EJ, Brekke P, Yeates R, Castillo-Juarez H. 2005. Genetic effects on sperm design in the zebra finch. Nature. 434:383-387.

Bouk GR, Jacobson J. 1976. Estimation of salmonid sperm concentration by microhematocrit technique. Trans Am Fish Soc. 105:534-535.

Burnham KP, Anderson DR. 1998. Model selection and inference: A practical information-theoretical approach. New York: Spriner-Verlag.

Burnham KP, Anderson DR. 2004. Mulitmodel Inference. Understanding AIC and BIC in model selection. Sociological Methods and Research. 33:261-304.

Burness G, Moyes CD, Montgomerie, R. 2005. Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). Comp Biochem Physiol A. 140:11-17.

Burness G, Schulte-Hostedde AI, Montgomerie R. 2008. Body condition influences sperm energetic in lake whitefish (*Coregonus clupeaformis*). Can J Fish Aquat Sci. 65:615-620.

Burness G, Casselman SJ, Schulte-Hostedde AI, Moyes CD, Montgomerie R. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav Ecol Sociobiol. 56:65-70.

Byrne PG, Roberts JD, Simmons LW. 2002. Sperm competition selects for increased testes mass in Australian frogs. J Evol Biol.15:347-355.

Casselman SJ, Montgomerie R. 2004. Sperm traits in relation to male quality in colonial spawning bluegill. J Fish Biol. 64:1700-1711.

Casselman SJ, Schulte-Hostedde AI, Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci. 63: 2119-2125.

Cotton S, Fowler K, Pomiankowski A. 2004. Heightened condition dependence is not a general feature of male eyespan in stalk-eyed flies (Diptera: Diopsidae). J Evol Biol. 17(6):1310-1316.

Denk AG, Holzmann A, Peters A, Vermeirssen ELM, Kempenaers B. 2005. Paternity in mallards: effects of sperm quality and female sperm selection for inbreeding avoidance. Behav Ecol. 16:825-833.

Dewsbury DA. 1982. Ejaculate cost and male choice. Am Nat. 119:601-610.

Dickerson BR, Quinn TP, Willson MF. 2002. Body size, arrival date, and reproductive success of pink salmon, *Oncorhynchus gorbuscha*. Ethol Ecol Evol. 14: 29-44.

Donoghue AM, Sonstegard TS, King LM, Smith EJ, Burt DW. 1999. Turkey sperm mobility influences paternity in the context of competitive fertilization. Biol Reprod. 61:422-427.

Droney DC. 1998. The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*. Funct Ecol. 12:920-928.

Fleming IA, Gross MR. 1993. Breeding success of hatchery and wild coho salmon (*Oncorhynchus kisutch*) in competition. Ecol Appl. 3:230-245.

Fleming IA, Gross MR. 1994. Breeding competition in a Pacific salmon (coho: *Oncorhynchus kisutch*): measures of natural and sexual selection. Evolution 48: 637-657.

Fitzpatrick JL, Desjardins JK, Milligan N, Montgomerie R, Balshine S. 2007. Reproductive-Tactic-Specific variation in sperm swimming speed in a shell-brooding cichlid. Biol reprod. 77: 280-284.

Fitzpatrick JL, Montgomerie R, Desjardins JK, Stiver KA, Kolm N, Balshine S. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc Nat Acad Sci. 106:1128-1132.

Gage MJG. 1995. Continuous variation in reproductive strategy as an adaptive response to population density in the moth *Plodia interpunctella*. Proc R Soc Lond B. 261:25-30.

Gage MJG, Macfarlane C, Yeates S, Shackleton R, Parker GA. 2002. Relationships between sperm morphometry and sperm motility in the Atlantic salmon. J Fish Biol. 61:1528-1539.

Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative velocity is the primary determinant of fertilization success. Curr Biol. 14:44-47.

García-González F, Simmons LW. 2005. Sperm viability matters in insect sperm competition. Curr Biol. 15:271-275.

García-González F, Simmons LW. 2007. Shorter sperm confer higher competitive fertilization success. Evolution. 61:816-824.

Giacomello E, Neat FC, Rasotto MB. 2008. Mechanisms enabling sperm economy in blenniid fishes. Behav Ecol Sociobiol. 62:671–680.

Ginsberg JR, Huck UW. 1989. Sperm competition in mammals. Trends Ecol Evol. 4:74-79.

Gomendio M, Harcourt AH, Roldan ERS. 1998. Sperm competition in mammals. In: Birkhead TR, Møller AP, editors. Sperm Competition and Sexual Selection. San Diego: Academic Press. p. 667-751.

Gross MR. 1985. Disruptive selection for alternative life histories in salmon. Nature. 313:47-48.

Hatef A, Niksirat H, Amiri BM, Alavi SMH, Karami M. 2007. Sperm density, seminal plasma composition and their physiological relationship in the endangered Caspian brown trout (*Salmo trutta caspius*). Aquacult Res. 2007: 1175-1181.

Hoysak DJ, Liley NR. 2001. Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. J Fish Biol. 58:1286-1300.

Kortet R, Vainikka A, Rantala MJ, Taskinen J. 2004. Sperm quality, secondary sexual characters and parasitism in roach (*Rutilus rutilus* L.). Biol Linnean Soc. 81:111-117.

Kotiaho JS, Simmons LW, Tomkins JL. 2001.Towards resolution of the lex paradox. Nature. 410:684–686.

Kotiaho JS. 2000. Testing the assumptions of conditional handicap theory: costs and condition dependence of a sexually selected trait. Behav Ecol Sociobiol. 48:188-194.

Labonne J, Augery M, Parade M, Brinkert S, Prevost E, Heland M, Beall E. 2009. Female preference for male body size in brown trout, *Salmo trutta*: is big still fashionable? Anim Behav. 77:129-137.

Lahnsteiner F, Berger B, Weismann T, Patzner A. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture. 163:163-181.

LaMunyon CW, Ward S. 1998. Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. Proc R Soc Lond B. 265:1997-2002.

Leach B, Montgomerie R. 2000. Sperm characteristics associated with different male reproductive tactics in bluegills (*Lepomis macrochirus*). Behav Ecol Sociobiol. 49: 31-37.

Levitan DR. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegates.* Proc R Soc Lond B. 267: 531-534.

Liley NR, Tamkee P, Tsai R, Hoysak DJ. 2002. Fertilization dynamics in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and sperm concentration and motility on in vitro fertilization. Can J Fish Aquatic Sci. 59: 144-152.

Liljedal S, Folstad I. 2003. Milt quality, parasites, and immune function in dominant and subordinate Arctic charr. Can J Zool. 81:221-227.

Locatello L, Pilastro A, Deana R, Zarpellon A, Rasotto MB. 2007. Variation pattern of sperm quality traits in two gobies with alternative mating tactics. Funct Ecol. 21:975-981.

Lüpold, S Linz GM, Rivers JW, Westneat DF, Birkhead TR. 2009. Sperm competition selects beyond relative testes size in birds. Evolution. 63: 391-402.

Mac Nally R. 2002. Multiple regression and inference in ecology and conservation biology: further comments on identifying important predictor variables. Biodivers Conserv. 11:1397-1401.

Mac Nally R, Walsh CJ. 2004. Hierarchical partitioning public-domain software. Biodivers Conserv. 13:659-660.

Malo AF, Roldan ERS, Garde J, Soler AJ. 2005. Antlers honestly advertise sperm production and quality. Proc R Soc Lond B. 272:149-157.

Marconato A, Tessari V, Marin G. 1995. The mating system of *Xyrichtys novacula* L.: sperm economy and fertilization success. J. Fish Biol. 47:291-301.

Marconato A, Shapiro DY. 1996. Sperm allocation, sperm production and fertilization rates in the bucktooth parrotfish. Anim Behav. 52: 971-980.

Methven DA, Crim LW. 1991. Seasonal changes in spermatocrit, plasma sex steroids and motility of sperm from Atlantic halibut *Hippoglossus hippoglosuus*. In: Scott AP, Sumpter JP, Kine DE, Rolfe MS, editors. Proceedings of 4th International symposium of reproductive physiology of fish. p.170.

Møller AP. 1988. Testis size, ejaculate quality and sperm competition in birds. Biol J Linn Soc. 33:273-283.

Møller AP. 1989. Ejaculate quality, testes size and sperm production in mammals. Funct Ecol. 3:91-96.

Møller AP. 1998. Sperm competition and sexual selection In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection. London: Academic. p. 55-90.

Moore PJ, Harris EW, Montrose TV, Levin D, Moore AJ. 2004. Constraints on evolution and postcopulatory sexual selection: trade-offs among ejaculate characteristics. Evolution. 58:1773–1780.

Morrow EH, Gage MJG. 2001. Consistent significant variation between individual males in spermatozoal morphometry. J Zool Lond. 254:147-153.

Neff BD, Fu P, Gross MR. 2003. Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). Behav Ecol. 14: 634-641.

Newlon AW, Yund PO, Stewart-Savage J. 2003. Phenotypic plasiticity of reproductive effort in a colonial ascidian, *Botryllus schlosseri*. J Exp Zool A 297(A):180-188.

Olsson M, Madsen T. 1998. Sexual selection and sperm competition in Reptiles. In: Birkhead TR, Møller AP, editors. Sexual selection and sperm competition. London: Academic Press.

Parker GA. 1970. Sperm competition and its evolutionary consequences in the insects. Biol Rev. 45:525–567.

Parker GA. 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J. Theor. Biol. 96:281-294.

Parker GA. 1990. Sperm competition games: raffles and roles. Proc R Soc B. 242:120-126.

Parker GA. 1993. Sperm competition games: sperm size and sperm number under adult control. Proc R Soc Lond B. 253:245-254.

Parker GA. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection. London: Academic Press. p. 3-54.

Parker GA, Ball MA. 2005. Sperm competition, mating rate and the evolution of testis and ejaculate sizes: a population model. Biol Lett. 1:235-238.

Pennell W, Barton BA. 1996. Principles of salmonid culture. Amsterdam: Elsevier.

Petersen CW. Warner RR.1998. Sperm competition in fishes. In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection. San Diego: Academic Press p. 435-463.

Piironen J. 1985. Variation in the properties of milt from the Finnish landlocked salmon (*Salmo salar m. sebago* Girard) during a spawning season. Aquaculture 48:337-350.

Poole WR, Dillane MG. 1998. Estimation of sperm concentration of wild and reconditioned brown trout, *Salmo trutta* L. Aquacult Res. 29:439-445.

Pitcher TE, Neff BD. 2007. Genetic quality and offspring performance in Chinook salmon: implications for supportive breeding. Cons Genet. 8:607-616.

Pitcher TE, Doucet SM, Beausoleil JMJ, Hanley D. 2009. Secondary sexual characters and sperm traits in coho salmon *Oncorhynchus kisutch.* J Fish Biol. 74:1450-1461.

Pitnick S. 1996. Investment in testes and the cost of making long sperm in *Drosophila*. Am Nat. 148:27-80.

Pitnick S, Markow TA. 1994. Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. Am Nat. 143:785-819.

Pitnick S, Miller GT, Reagan J, Holland B. 2001. Males' evolutionary responses to experimental removal of sexual selection. Proc R Soc Lond B. 268:1071-1080.

Poiani A. 2006. Complexity of seminal fluid: a review. Behav Ecol Sociol Biol. 60:289-310.

Preston BT, Stevenson IR, Pemberton JM, Coltman DW, Wilson K. 2003. Overt and covert competition in a promiscuous mammal: the importance of weaponry and testes size to male reproductive success. Proc R Soc Lond B. 270:633-640.

Quinn TP. 19991. Variation in pacific salmon reproductive Behaviour associated with species, sex and levels of competition. Behaviour 136:179-204

Quinn TP, Foote CJ. 1994. The effects of body size and sexual dimorphism on the reproductive behavior of sockeye salmon, *Oncorhynchus nerka*. Anim Behav. 48: 751-761.

Quinn GP, Keough MJ. 2002. Experimental design and data analysis for biologists. Cambridge: Cambridge University Press.

R Development Core Team. 2008. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org. [accessed January 2010]

Rakitin A, Ferguson MM, Trippel EA. 1999. Sperm competition and fertilization success in Atlantic cod (*Gadus morhua*): effect of sire size and condition factor on gamete quality. Can J Fish Aquat Sci. 56:2315-2323.

Rodriguez-Martinez H. 2006. Can we increase the estimative value of semen assessment? Reprod Domest Anim. 4(2): 2-10.

Rolden ERS. 2007. Focus on determinants of male fertility. Reprod. 134:1-2.

Rowe L, Houle D. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc R Soc Lond B. 263:1415-1421.

Rudolfsen G, Figenschou L, Folstad I, Tveiten H, Figenschou M. 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc R Soc Lond B. 273:325-332.

Rudolfsen G, Figenschou L, Folstad I, Kleven O. 2008. Sperm velocity influences paternity in the Atlantic cod (*Gadus morhua* L.). Aquacult Res. 39:212-216.

Rurangwa E, Kime DE, Ollevier F, Nash JP. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture. 234:1-28.

Sutton SG, Bult TP, Haedrich RL. 2000. Relationships among fat weight, body weight, water weight, and condition factors in wild Atlantic salmon parr. Trans Amer Fish Soc. 129:527-538.

Schärer L, Robertson DR. 1999. Sperm and milt characteristics and male versus female investment in the Carribean reef fish *Thalassoma bifasciatum*. J Fish Biol. 55:329-343.

Schulte-Hostedde AI, Millar JS. 2004. Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (*Tamias amoenus*): implications for sperm competition and reproductive success. Behav Ecol Sociobiol. 55: 272–277.

Schulte-Hostedde AI, Burness G. 2005. Fertilization dynamics of sperm from different male mating tactics in blugegill (*Lepomis macrochirus*). Can J Zool. 83:1638-1642.

Schulte-Hostedde AI, Millar JS, Hickling GJ. 2005. Condition dependence of testis size in small mammals. Evol Ecol Res. 7:143–149.

Simmons LW. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton: Princeton University Press.

Simmons LW, Kotiaho JS. 2002. Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. Evolution. 56:1622-1631.

Simmons LW, Roberts JD, Dziminski MA. 2009. Egg jelly influences sperm motility in the externally fertilizing frog, *Crinia Georgiana*. J Evol Biol. 22: 225-229.

Skinner AMJ, Watt PJ. 2007. Phenotypic correlates of spermatozoon quality in guppy, *Poecilia reticulata*. Behav Ecol.18: 47-52.

Snook RR. 2005. Sperm in competition: not playing by the numbers. Trends Ecol Evol. 20:46-53.

Stockley P, Purvis A. 1993. Sperm competition in mammals: a comparative study of male roles and relative investment in sperm production. Funct Ecol. 7: 560-570.

Stockley P, Gage MJG, Parker GA, Møller AP. 1997. Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. The Am Nat. 149:933-954.

Stoltz JA, Neff BD. 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. J Evol Biol. 19:1873-1881.

Taborsky M. 1998. Sperm competition in fish: ‘‘bourgeois’’ males and parasitic spawning. Trends Ecol Evol. 13:222-227.

Thomas ML, Simmons LW. 2007. Male crickets adjust the viability of their sperm in response to female mating status. Am Nat. 170:190-195.

Tomkins JL, Radwan J, Kotiaho JS, Tregenza T. 2004. Genic capture and resolving the lek paradox. Trends Ecol Evol. 19:323-328.

Turner E, Montgomerie R. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol. 60:1570-1579.

Uglem I, Galloway TF, Rosenqvist G, Folstad I. 2001. Male dimorphism, sperm traits and immunology in the corkwing wrasse (*Symphodus melops L.*). Behav Ecol Sociobiol. 50: 511-518.

Urbach D, Folstad I, Rudolfsen G. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol. 57:438-444.

Urbach D, Bittner D, Lenz TL, Bernet T, Wahli T, Wedekind C. 2007. Sperm velocity in an Alpine whitefish: effects of age, size, condition, fluctuating asymmetry and gonad abnormalities. J Fish Biol. 71: 672-683.

Vladic TV, Jarvi T. 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. Proc R Soc Lond B. 268:2375-2381.

Walsh C, Mac Nally R. 2008. hier.part: hierarchical partitioning. R package version 1.0-3, URL http://cran.r-project.org/web/packages/hier.part/index.html. [accessed January 2010].

Ward PI. 1998. Intraspecific variation in sperm size characters. Heredity. 80:655-659.

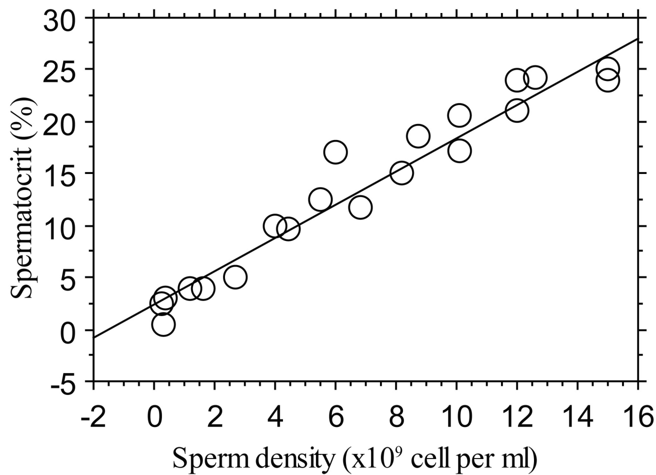
Wedekind C, Müller R. 2004. Parental characteristics versus egg survival: towards an improved genetic management in the supportive breeding of lake whitefish. Annales Zoologici Fennici. 41:105-115.

Wedell N, Gage MJG, Parker GA. 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol. 17:313-320.

Wirtz S, Steinmann P. 2006. Sperm characteristics in perch *Perca fluviatilis* L. J Fish Biol. 68:1869-1902.

Appendix 2.1. Set- up parameters for computer-assisted sperm analysis for chinook salmon (*Oncorhynchus tshawytscha*)

CASA set-up parameters	
Number of frames acquired	25
Frame rate	50 Hz
Contrast (acceptable brightness)	32
Minimum cell size (number of pixels in order to be counted)	18
STR threshold – if STR below this value cell is not progressive	50 (μm/s)
Speed slow – VAP cutoff	6 (μm/s)
Speed medium – progressive min VAP	20 (μm/s)
VSL slow – VSL low cutoff	6 (μm/s)
Magnification	4.05
Frequency of video (Hz)	50



Appendix 2.2. Relation between sperm density and spermatocrit for two-year old male chinook salmon (*Oncorhynchus tshawytscha*). In this plot, y = 2.36+1.60*x* (r = 0.95, P <0.0001, n =20).

Appendix 2.3. Results from hierarchical partitioning analyses. The independent contribution of amount of variation that each predictor variable (phenotypic traits) explains for each sperm trait. Those phenotypic traits in bold were removed from models for each sperm trait.

Phenotypic trait	VAP	MOT	LIN	DUR	Spermatocrit
Standard length (mm)	20	39	10	16	48
Fulton’s	50	**7**	66	60	**4**
Somatic weight (g)	23	41	16	14	30
Testes mass (g)	**7**	13	**8**	**10**	18

DUR, sperm longevity (s); LIN, sperm path linearity (%); VAP, mean smoothed path velocity (µm s^{-1}); Spermatocrit (%), MOT, percentage of progressively motile cells (%).

Appendix 2.4. Pearson correlation coefficient for predictors used in multiple regression models. In bold are significant correlations (P < 0.05).

	Standard length (mm)	Testes mass (g)	Fulton's
Somatic weight (g)	**0.96**	**0.63**	**0.35**
Standard length (mm)		**0.98**	0.17
Testes mass (g)			**0.33**

Chapter 3

SPERM TRAITS IN CHINOOK SALMON DEPEND UPON ACTIVATION MEDIUM: IMPLICATIONS FOR STUDIES OF SPERM COMPETITION IN FISHES

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3.1 ABSTRACT

Sperm traits of externally-fertilizing fish species are typically measured in fresh (or salt) water, even though the spawning environment of their ova contains ovarian fluid. In this study we measured sperm traits of chinook salmon (*Oncorhynchus tshawytscha* (Walbaum, 1792)) in both fresh water and dilute ovarian fluid at 10 s and 20 s postactivation, using a computer-assisted sperm analysis (CASA) system. Spermatozoa swam faster, and had both higher percent motility and a straighter path trajectory for a longer period of forward motility when activated in ovarian fluid compared to activation in fresh water. Comparing sperm activity of 10 males in water versus ovarian fluid, we found a weak but significant correlation for sperm swimming speed at 10 s postactivation ($r = 0.34$, $P = 0.01$), but not for any other sperm traits measured. Most important, across males, mean sperm swimming speed in water accounted for < 10% of the observed variation in mean sperm swimming speed in ovarian fluid. Thus, we argue that sperm traits measured in fresh water are not particularly relevant to those same traits during normal spawning in this species. We suggest that sperm performance measured in fresh water should be used with caution when comparing the potential for individual males to fertilize ova, especially in studies of sperm competition in externally fertilising species.

Keywords: chinook salmon, sperm competition, ovarian fluid, sperm velocity

3.2 INTRODUCTION

In salmonids, and most other externally-fertilizing fishes, the period during which spawned ova can be fertilized is brief. For example, in sockeye salmon *Oncorhynchus nerka* (Walbaum 1792) 25% of fertilisation occurs within 0.5 s of sperm and egg release, and 80% occurs within 5 s (Hoysak and Liley 2001). Thus, any factor that enhances a sperm's ability to make its way to an unfertilized ovum — for example, by increasing sperm swimming speed during the short interval after sperm activation — would be expected to increase a male's fertilisation success (Gage et al. 2004).

In several species of externally fertilising fishes, ovarian fluid (also called coelomic fluid) is expelled with the egg batch, and has been documented to increase both sperm velocity and the period of forward motility (Litvak and Trippel 1998; Lahnsteiner 2002; Turner and Montgomerie 2002; Elofsson et al. 2003a; Woolsey et al. 2006; Hatef et al. 2009). In salmonids, ovarian fluid comprises 10-30% of the volume of the spawned egg mass (Lahnsteiner 2002), and because ovarian fluid is more viscous than water (Hirano et al. 1978; Turner and Montgomerie 2002; see chapter 5), it tends to remain near the ova after a spawning event. Hence, it seems likely that salmonid spermatozoa encounter ovarian fluid of increasing concentrations as they approach an unfertilized ovum (Turner and Montgomerie 2002), and that both sperm activation and egg fertilisation occur in an environment containing ovarian fluid (Litvak and Trippel 1998).

Previous research in a few fish species has documented that the presence of ovarian fluid in the sperm-activating solution influences sperm behaviour in a concentration-dependent manner (Lahnsteiner 2002; Turner and Montgomerie 2002). For example, as the concentration of ovarian fluid was increased experimentally from 0-50%, Arctic charr *Salvelinus alpinus* (L., 1758) sperm longevity, motility and swimming speed were all enhanced (Turner and Montgomerie 2002). Similarly, in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), the percentage of motile sperm and the duration of sperm motility were both higher when sperm were activated in an aqueous solution of 50%

ovarian fluid compared to activation in fresh water (Woolsey et al. 2006). In Atlantic cod *Gadus morhua* (L., 1758), sperm activated in ovarian fluid also swam faster, and a higher percentage were motile for a longer period, compared to sperm activated in sea water (Litvak and Trippel 1998).

This enhancing effect of ovarian fluid on sperm behaviour is important because several studies of fishes have found a strong association between sperm motility and fertilisation success (Gage et al. 2004; Linhart et al. 2005; Schulte-Hostedde and Burness 2005; Casselman et al. 2006; Rudolfsen et al. 2008a; Liljedal et al. 2008). Thus, in Atlantic salmon *Salmo salar* (L. 1758), sperm velocity was the primary determinant of fertilisation success during sperm competition, controlling for sperm number — in controlled experiments, males with faster sperm had higher rates of fertilisation than males with slower sperm (Gage et al. 2004). In salmonids and other fish species, a reduction in sperm motility and any delay in sperm release relative to the release of ova results in a decrease in reproductive success (Munkittrick and Moccia 1987; Bencic et al. 2000; Casselman et al. 2006). Even a 2 s delay of sperm release in Atlantic salmon caused significant reductions in paternity; with second males achieving only 30% fertilization success rather than the expected 50% (Yeates et al. 2007). Potentially, this maternal influence via ovarian fluid can play an important part in the fertilisation process.

In chapter 4, we found that when sperm from chinook salmon were activated in ovarian fluid, sperm traits were variable and depended upon individual male x female combinations. Those results suggested that ovarian fluid could be a mechanism of cryptic female choice whereby females favour sperm from certain males (Thornhill 1983; Eberhard 1996) by enhancing their sperm behaviour (swimming speed, swimming path trajectory, etc.). As sperm swimming speed is a good predictor of male fertilisation success (Lahnsteiner et al. 1998; Levitan 2000; Gage et al. 2004), any effect that ovarian fluid has on this trait is expected to influence male fertilisation success.

The effects of ovarian fluid on different aspects of sperm motility have only been explored in a handful of fish species to date: Arctic charr (Turner and Montgomerie 2002;

Urbach et al. 2005); fifteen-spined stickleback *Spinachia spinachia* (L.) (Elofsson et al. 2003b); three-spined stickleback *Gasterosteus aculeatus* (L.) (Elofsson et al. 2003a, 2006); Atlantic cod (Litvak and Trippel 1998); and rainbow trout (Dietrich et al. 2008). In the present study, the effects of ovarian fluid on four key sperm traits known or suspected to influence male fertilization success — sperm velocity, duration of sperm motility, swimming path trajectory, and progressive motility — were examined in sexually mature, male chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792), using computer assisted sperm analysis (CASA). We have previously reported on the data with respect to activation in ovarian fluid to examine the interaction between sperm traits and ovarian fluid in the context of cryptic female choice (see chapter 4). Our primary goal in the present study was to document the relations between sperm traits measured in water and those same traits measured in ovarian fluid. Thus, we sought to determine whether traits typically measured in water in studies of sperm behaviour are correlated with those measured in natural spawning conditions when ovarian fluid is present, as is almost universally assumed (Gage et al. 2004; Linhart et al. 2005; Rudolfsen et al. 2008b).

3.3 METHODS

From 3-14 May 2004, we took gametes from female and male chinook in a hatchery-reared broodstock generated from semi-wild returns. Milt samples were obtained from ten live, haphazardly chosen sexually mature two-year-old males (no precocial males were sampled) from a captive population of 77 males. On each male-sampling day, one or two haphazardly chosen, sexually mature, three-year-old females were killed with a stroke to the head, and their egg batch was expelled, through an incision in the abdomen, into a clean bowl. Ovarian fluid was then gently pipetted from each egg batch. Milt and ovarian fluid samples were stored separately, immediately refrigerated at 4°C, and transported to the University of Canterbury where sperm behaviour was video-recorded. All animals were collected and maintained according to the standards of the Animals Ethics Committee for the University of Canterbury, New Zealand.

Sperm behaviour was recorded on videotape for each of 10 males' sperm activated both in an aqueous solution of 50% ovarian fluid from each of 7 females, and in fresh water (i.e., 0% ovarian fluid) from the raceway in which the fish were housed. As not all experiments were conducted on the same day during the spawning season, we controlled experimentally for any seasonal variation in sperm traits as has been reported during the spawning season of some fish species (Rideout et al. 2004; Cruz-Casallas et al. 2007). To control for such seasonal variation, we collected milt from each male every day that females were sampled during the spawning season (3, 7, 10, 12 and 14 May). On each of these days, sperm behaviour was quantified in both fresh water and an aqueous solution of ovarian fluid. As a result, we recorded the behaviour of sperm from each of the 10 males in water on 5 different days to compare with their sperm behaviour when activated in the ovarian fluid of the one or two different females sampled on each of those days**.** We did an initial titration of ovarian fluid to determine the effects of different concentrations of ovarian fluid on the duration of sperm longevity (DUR s) (Appendix 3.1)

For each male on each sampling day, we recorded the behaviour of sperm activated in 0% and in 50% ovarian fluid solutions until no spermatozoa showed any progressive motility (see chapter 4 for detailed methods). The longest period between milt collection and activation of sperm samples was no greater than 5 h, and it has been our experience that this has no effect on sperm traits when the samples are kept at a similar temperature to the raceway water (approximately 12ºC), and in containers that were large enough to allow oxygen exchange.

Videotapes of sperm recordings were analysed using computer assisted sperm analysis (HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research, Beverly, MA, U.S.A). For each milt sample, we quantified the swimming paths of all spermatozoa in a field of view for 0.5 s at both 10 s and 20 s post-activation, and conducted this procedure twice for each milt sample. We were unable to get accurate sperm motility recordings prior to 10 s post-activation that were not subject to sample drift. We sampled at these

two time intervals to include the time frame wherein most fertilisation occurs. In sockeye salmon, the majority of fertilisations take place within 10 s post-activation, but some fertilisation still occurs 20 s post-activation (Hoysak and Liley 2001). On average, 13 spermatozoa were tracked per trial (range = 5-33, n = 154 trials). For each male, the means of the following variables were calculated from the sperm tracks sampled during each trial: average path velocity (VAP in $\mu m \cdot s^{-1}$), straight line velocity (VSL in $\mu m \cdot s^{-1}$), curvilinear velocity (VCL in $\mu m \cdot s^{-1}$), and linearity (LIN; the ratio of VSL to VCL, expressed as a percentage). Like other studies, we used VAP to measure of sperm swimming speed (e.g., Lahnsteiner et al. 1998; Casselman et al. 2006; Burness et al. 2008) because it describes a smoothed path along which each spermatozoon travels. Linearity (LIN) describes the path trajectory of the spermatozoa through the solution. For each trial, we also measured overall sperm motility (MOT) as the percentage of cells in the field of view that were progressively motile at >20 $\mu m \cdot s^{-1}$ (see Lahnsteiner et al. 1998 for a similar criterion). Sperm longevity (DUR) for each milt sample was also recorded (see chapter 4 for detailed methods).

3.4 Statistical analyses

Statistical analyses were performed using R version 2.7.1 (R Development Core Team, 2008) with the linear mixed-effects package lme4 (Bates et al. 2008). Mixed effects general linear models were fitted to response variables with treatment (0% vs. 50% ovarian fluid) as a fixed effect, and both female (n = 7) and male (n = 10) identities as random effects, in a repeated measures design, using the maximum likelihood method of estimation. The mean of the two replicates for each male activated in each female's ovarian fluid was used for these analyses. To test the statistical significance of fixed (treatment) effects, models were fitted with and without the relevant predictor variables, and then compared using log-likelihood ratio tests (LLR χ^2).

To calculate correlation coefficients that were not biased by pseudoreplication, we performed randomization tests as follows. First, we randomly selected data having unique male x female combinations without replacement such that each male and female was

represented only once in the selected dataset (n = 7) — note that each randomly selected dataset thus included only 7 of the 10 possible males. Then, we calculated the Pearson product-moment correlation coefficient for these randomly selected data. We repeated this procedure 1000 times on the original dataset, and calculated the correlation coefficient each time. We report both the median correlation coefficients from these randomization tests, as well as the LLR χ^2 tests of significance from the models using the full dataset, as described above.

To assess the repeatability of all measurements, we calculated intraclass coefficient coefficients (ICC) between the replicate trials using the psy package (Falissard 2008) in R. Due to skewness of data, bias-corrected accelerated (BC_a) 95 % confidence intervals were calculated from 1000 bootstrapped replicates using the boot package (Canty 2005) in R. Assumptions underlying all models were verified by analyzing residuals (Shapiro-Wilk tests; $p > 0.05$ in each case, so no transformations were needed). Data are presented throughout as mean ± SE unless otherwise stated.

3.5 RESULTS

Sperm traits

Maximum sperm longevity (DUR) for the 10 males increased significantly, almost 4-fold, from 70 s ± 2.6 (n = 50) when activated in fresh water (0% ovarian fluid) to 263 s ± 9 (n = 70) in 50% ovarian fluid from the seven females (LLR χ^2 = 215.0, df = 1, P < 0.0001; Fig. 3.1a). Sperm swimming speeds (VAP) were significantly higher in ovarian fluid than in freshwater at both 10 s (LLR χ^2 = 108.1, df = 1, P < 0.0001) and 20 s post-activation (LLR χ^2 = 71.6, df = 1, P < 0.0001; Fig. 3.1b). Sperm swimming speed also declined significantly between 10 s and 20 s post-activation in both 0% (LLR χ^2 =51.9, df = 1, P < 0.0001) and 50% (LLR χ^2 = 119.5, df = 1, P < 0.0001) ovarian fluid solutions (Fig. 3.1b), consistent with other studies on the effects of ovarian fluid on sperm swimming speed in fishes (Leach and Montgomerie 2000; Elofsson et al. 2003b; Rudolfsen et al. 2006; Fitzpatrick et al. 2007; Rudolfsen et al. 2008b).

Correlations between VAP and DUR were not significant at either 10 s or 20 s post-activation in either 0% (Fig. 3.2) or 50% ovarian fluid (Table 1). The analysis at 10 s post-activation in 0% ovarian fluid was strongly influenced by one outlier (Fig. 3.2a), and the correlation is significant with that outlier removed (median r = 0.30, LLR χ^2 = 5.9, df =1, P = 0.02).

Ovarian fluid significantly increased the mean percentage of progressively motile sperm cells (MOT) at both 10 s and 20 s post-activation, compared to activation in fresh water (10 s: LLR χ^2 = 198.2, df =1, P < 0.0001; 20 s: LLR χ^2 = 195.9, df =1, P < 0.0001; Fig. 3.1c). MOT in 0% and 50% ovarian fluid declined significantly between 10 s and 20 s after activation in both media (water: LLR χ^2 = 6.2, df =1, P = 0.01; 50% ovarian fluid: LLR χ^2 = 14.7, df =1, P = 0.0001; Fig. 3.1c).

MOT was positively correlated with VAP at 20 s post-activation in 50% ovarian fluid (Table 3.1), but not at 10 s post-activation in the ovarian fluid solution, or in water at either time interval post-activation. None of the correlations between MOT and DUR in either water or 50% ovarian fluid solution were significant at either 10 s or 20 s post-activation (Table 3.1).

Ovarian fluid had a significant effect on mean sperm path linearity (LIN) at both 10 s and 20 s post-activation (10 s: LLR χ^2 = 94.3, df =1, P < 0.0001; 20s: LLR χ^2 = 27.6, df =1, P < 0.0001; Fig. 3.1d), compared to activation in water. As with the other measured sperm traits, LIN in both 0% and 50% ovarian fluid solutions decreased significantly over time (water: LLR χ^2 =5.5, df = 1, P < 0.02; ovarian fluid: LLR χ^2 = 81.6, df = 1, P < 0.0001; Fig. 3.1d).

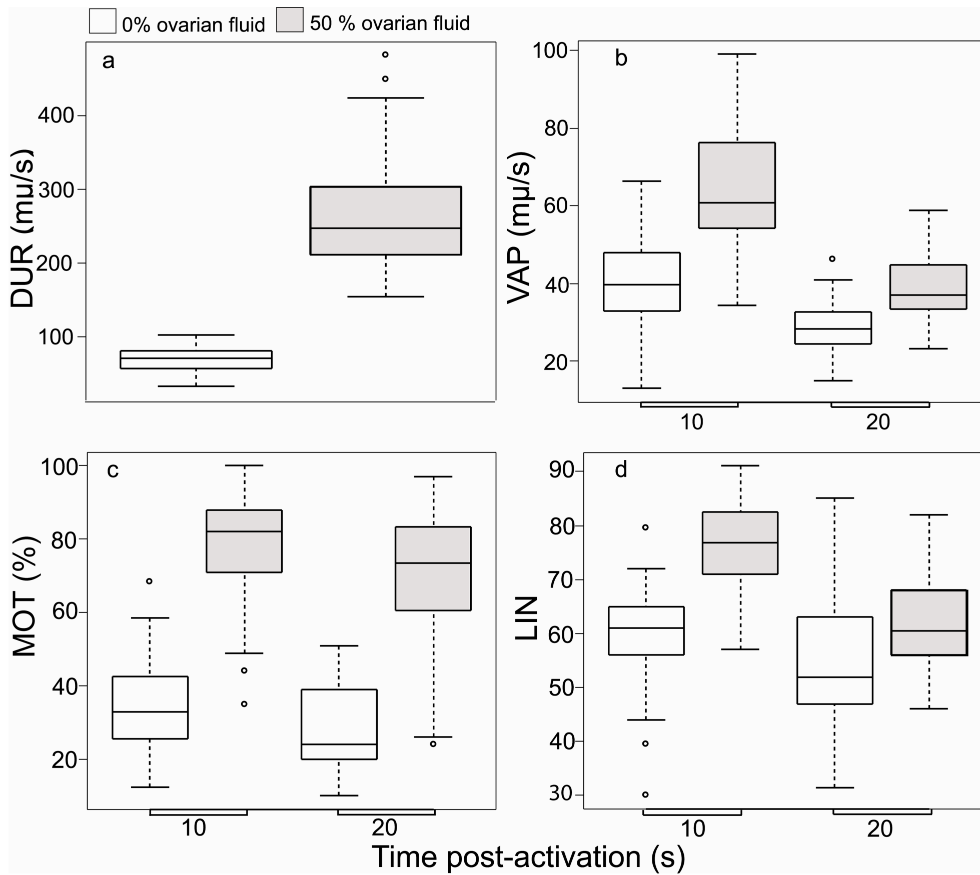

Figure 3.1. Sperm traits from male chinook salmon (*Oncorhynchus tshawytscha*): (a) sperm longevity (DUR s), (b) sperm swimming speed (VAP mμ/s), (c) percent sperm motility (MOT %), and (d) path linearity (LIN %) at 10 s and 20 s postactivation. Box plots show 10th, 25th, 50th (median), 75th and 90th percentiles as horizontal lines, and all data outside this range (○). Data are means from replicate samples on five different days of sperm from 10 sexually mature two-year-old males activated in 0% ovarian fluid (fresh water; n = 50 sample means) compared to 50% ovarian fluid taken from seven different females (n = 70 sample means).

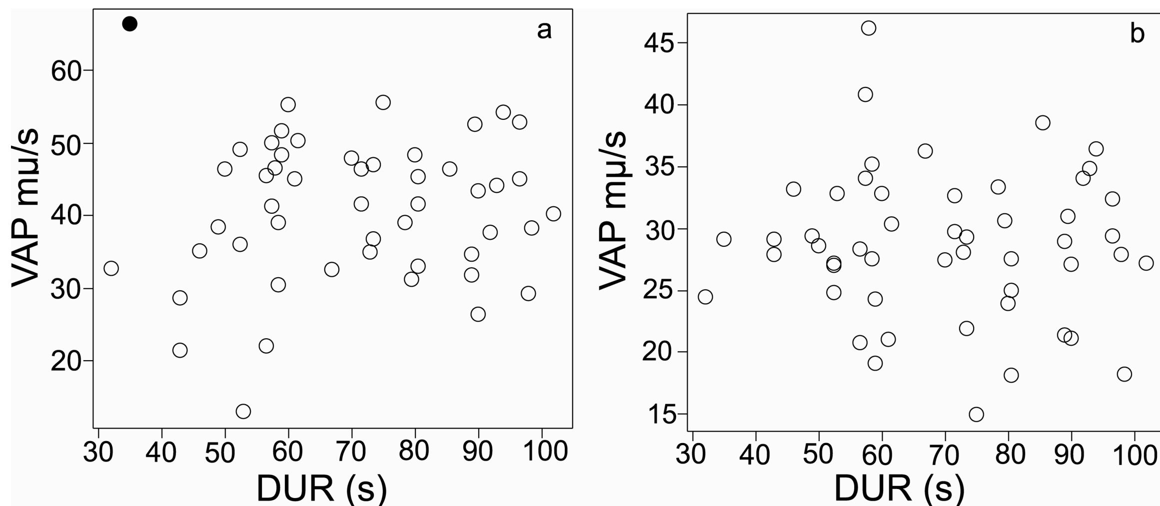

Figure 3.2. Relations between average sperm swimming speed (VAP mμ/s) and sperm longevity (DUR s), of chinook salmon (*Oncorhynchus tshawytscha*) at (a) 10 s and (b) 20 s postactivation in water (n=50 sample means). ●, outlier.

MOT and LIN were positively correlated in both water and ovarian fluid at both 10 s and 20 s post-activation, but at 20 s post-activation in water this correlation was not quite significant (Table 3.1). LIN and VAP were not significantly correlated at 10 s post-activation in water or 50% ovarian fluid, or at 20 s post-activation in water. However, the correlation between these two variables at 20 s post-activation in ovarian fluid was highly significant (Table 3.1).

There was no relation between either DUR, MOT, or LIN measured in fresh water versus 50% ovarian fluid solution at either time interval post-activation (Table 3.2, Fig. 3.2). However, there was a significant positive correlation between VAP measured in water versus 50% ovarian fluid at 10 s (Fig. 3.3), but not at 20 s post-activation (Table 3.2). Our measurements of VAP in both water and 50% ovarian fluid were significantly repeatable, with relatively high intraclass correlation coefficients (Table 3.3). Thus the method we used to measure sperm swimming speed resulted in relatively small measurement error, even though a small number of sperm were tracked per trial. All other sperm trait measurements were also significantly repeatable except both LIN and MOT measured in water at 10 s post-activation (Table 3.3). Interestingly, almost all variables measured were more repeatable (i.e., had higher intraclass correlations) when measured

in ovarian fluid compared to those same measurements when sperm were activated in water.

Table 3.1. Correlations between sperm traits from chinook salmon (*Oncorhynchus tshawytscha*), measured in 0% (water) versus 50% ovarian fluid solutions at both 10 s and 20 s post-activation.

Sperm trait	Sperm trait	Median r (LLR χ^2, *P*)
VAP at 10 s in 0%	DUR in 0%	0.11 (0.05, 0.82)
VAP at 20 s in 0%	DUR in 0%	–0.03 (0.3, 0.60)
VAP at 10 s in 50%	DUR in 50%	0.18 (0.1, 0.71)
VAP at 20 s in 50%	DUR in 50%	0.29 (0.6, 0.44)
MOT at 10 s in 0%	VAP at 10 s in 0%	0.34 (2.7, 0.10)
MOT at 20 s in 0%	VAP at 20 s in 0%	0.33 (2.9, 0.09)
MOT at 10 s in 50%	VAP at 10 s in 50%	0.14 (0.6, 0.44)
MOT at 20 s in 50%	VAP at 20 s in 50%	**0.55** (6.0, 0.01)
MOT at 10 s in 0%	DUR in 0%	–0.05 (0.003, 0.96)
MOT at 20 s in 0%	DUR in 0%	–0.05 (1.2, 0.28)
MOT at 10 s in 50%	DUR in 50%	0.30 (1.0, 0.32)
MOT at 20 s in 50%	DUR in 50%	–0.26 (0.1, 0.76)
MOT at 10 s in 0%	LIN at 10 s in 0%	**0.38** (8.2, 0.004)
MOT at 20 s in 0%	LIN at 20 s in 0%	0.28 (2.3, 0.13)
MOT at 10 s in 50%	LIN at 10 s in 50%	**0.34** (5.9, 0.01)
MOT at 20 s in 50%	LIN at 20 s in 50%	**0.42** (8.2, 0.004)
LIN at 10 s in 0%	VAP at 10 s in 0%	0.25 (3.2, 0.08)
LIN at 20 s in 0%	VAP at 20 s in 0%	−0.12 (0.4, 0.53)
LIN at 10 s in 50%	VAP at 10 s in 50%	0.06 (0.01, 0.14)
LIN at 20 s in 50%	VAP at 20 s in 50%	**0.66** (26.9, <0.0001)

Note: Correlation coefficients (median r) were determined by randomization methods, and tests of significance are from generalized linear models (see Methods). Significant correlation coefficients are shown in bold type. VAP is swimming speed in $\mu m \cdot s^{-1}$, DUR is longevity in s, MOT is percent motility, and LIN is path linearity (dimensionless).

Table 3.2. Correlations between sperm traits measured in water versus an aqueous solution of 50% ovarian fluid. Correlation coefficients (median r) were determined by randomization methods, and tests of significance from generalized linear models (see Methods). Significant correlation coefficients are shown in bold type. VAP is swimming speed in $\mu m \cdot s^{-1}$, DUR is longevity in s, MOT is percent and LIN is path linearity (dimensionless). VAP, LIN, and MOT were measured at 10 s and 20 s post-activation.

Sperm trait	Median r (LLR χ^2, *P*)
DUR	0.02 (1.6, 0.21)
LIN at 10 s	0.03 (0.3, 0.61)
LIN at 20 s	–0.01 (0.003, 0.95)
MOT at 10 s	–0.10 (0.04, 0.84)
MOT at 20 s	0.36 (0.5, 0.47)
VAP at 10 s	**0.34** (6.6, 0.01)
VAP at 20 s	0.19 (0.02, 0.88)

Table 3.3. Intraclass correlations of replicate values for sperm traits measured in 0% (water) and 50% ovarian fluid solution at both 10 s and 20 s post-activation. Sperm were from sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*) (n = 10 males), each measured in the ovarian fluid of 7 females. Bias corrected accelerated (BC_a) 95 % confidence intervals are provided. VAP is swimming speed in $\mu m \cdot s^{-1}$, DUR is longevity in s, MOT is percent motility and LIN is path linearity.

Sperm trait	Water (n = 50 sample means)		50% ovarian fluid (n = 70 sample means)	
	ICC	95 % CI	ICC	95% CI
DUR	0.65	0.33-0.84	0.61	0.44-0.76
VAP (10 s)	0.78	0.65-0.89	0.86	0.77-0.91
LIN (10 s)	0.27	–0.04-0.540	0.67	0.52-0.77
MOT (10 s)	0.20	–0.14-0.45	0.50	0.15-0.75
VAP (20 s)	0.90	0.80-0.95	0.73	0.62-0.83
LIN (20 s)	0.37	0.11-0.60	0.63	0.50-0.75
MOT (20 s)	0.49	0.27-0.70	0.62	0.45-0.78

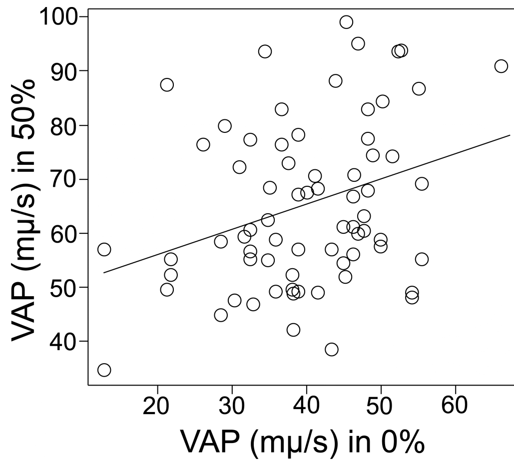

Figure 3.3. Relation between mean swimming speed (VAP mμ/s) of sperm from 10 sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*), activated in water versus ovarian fluid measured at 10 s postactivation (n = 70 sample means). Line is fitted by model II regression (y = 46.1 + 0.5*x*).

3.6 DISCUSSION

The results from this study show that spermatozoa swim faster; have a straighter path trajectory for a longer period of forward motility; and are more likely to be motile at both 10 s and 20 s post-activation when activated in an aqueous solution of 50% ovarian fluid compared to activation in fresh water. Thus, sperm deposited close to unfertilized ova, where the concentration of ovarian fluid is highest, may reach the site of fertilization even faster than expected from studies of sperm behaviour in fresh water. In addition, the consistently weak correlations that we discovered between sperm traits measured in fresh water and ovarian fluid (Table 3.2 and Fig. 3.3) suggest that studies measuring sperm traits in water do not actually mimic the natural spawning environment, contrary to expectations (e.g., Leach and Montgomerie 2000; Gage et al. 2004; Stoltz and Neff 2006; Rudolfsen et al. 2008b). Moreover, it also appears that sperm traits can be measured more reliably (i.e., with higher repeatability), and thus with less measurement error, when sperm is activated in an ovarian fluid solution (Table 3.3).

Our results demonstrate that ovarian fluid enhances all aspects of sperm movement in chinook salmon, and these results are consistent with previous studies in Arctic charr (Turner and Montgomerie 2002), Atlantic cod (with the exception of path linearity for which ovarian fluid had no effect; Litvak and Trippel 1998), three-spined stickleback (Elofsson et al. 2003a), and rainbow trout (Dietrich et al. 2008) in demonstrating the enhancing effects of ovarian fluid in external fertilisers. Sperm longevity was particularly influenced by ovarian fluid, as the overall average period of forward sperm motility was almost four times as long in 50% ovarian fluid solution compared to activation in fresh water (Fig. 3.1a). We previously found that mean longevity of sperm from the same males studied here was significantly negatively correlated with calcium and magnesium concentrations of the female's ovarian fluid, highlighting the importance that different ions and ion concentrations have on sperm longevity (see Chapter 5). It is difficult to see how such an increase in sperm longevity could be advantageous as most ova are fertilized within a few seconds of spawning (Hoysak and Liley 2001), and salmonid ova only remain fertilizable for about 40 s once expelled into the water (Ginsberg 1963; Billard and Cosson 1992). Thus, the increased longevity of sperm in ovarian fluid may have no adaptive consequence, resulting simply from the influence that ovarian fluid has on sperm energetics and swimming speed shortly after activation. Some previous research had suggested a tradeoff between sperm swimming speed and sperm longevity due to constraints on available energy for cell functioning (Stockley et al. 1997; Levitan 2000), but we did not find this tradeoff in chinook salmon when sperm are activated in either water or 50% ovarian fluid (Fig. 3.2 and Table 3.1).

As the time course of fertilisation during spawning in salmonids is extremely short, sperm swimming speed is likely to be a better predictor than sperm longevity of fertilisation success in this taxon (Ball and Parker 1996; Gage et al. 2004; Yeates et al. 2007). Therefore, we assume that sperm that swim faster immediately after activation would be most likely to fertilize ova (Burness et al. 2004). Ovarian fluid did in fact have a more pronounced effect on average sperm swimming speed at 10 s compared to 20 s post-activation—on average sperm swam 1.5 times faster in ovarian fluid than in water at 10 s post-activation. Even though ovarian fluid significantly increased overall mean

sperm swimming speed at 20 s post-activation compared to activation in fresh water, sperm traits measured that long after activation may not be relevant to a male's fertilisation success, similar to results reported for walleye *Sander vitreus* (Mitchill, 1818) (Casselman et al. 2006).

Interestingly, there was, at best, a weak positive correlation between sperm swimming speed 10 s post-activation measured in water versus ovarian fluid solution (Fig. 3.3). Even so, no more than 10% of the observed variation in swimming speed in ovarian fluid could be explained by variation in swimming speed in fresh water (Fig. 3.3). Similarly, in rainbow trout, two of nine males had significant positive relations between sperm motility in buffered salt solution versus ovarian fluid (Dietrich et al. 2008).

These results suggest that sperm traits measured in water do not accurately reflect the relative swimming speed of the sperm from different males in the presence of ovarian fluid. This has two important implications. Firstly, previously reported swimming speeds for fish spermatozoa activated in water underestimates sperm swimming speeds under natural spawning conditions (Litvak and Trippel 1998). This may be a particularly important consideration when modeling fertilization dynamics. Secondly, and most importantly, our results suggest that the use of overall sperm motility, sperm swimming speed, or path linearity measured in fresh water, as an index of a male's sperm quality and thus relative fertilization success, may be inaccurate. Indeed, our analysis suggests that some sperm traits measured in fresh water soon after activation are poor predictors of those same traits measured in an ovarian fluid solution, with a high proportion (90%) of variation remaining unexplained. Some of this unexplained variation is undoubtedly due to measurement error, as our repeatability analysis shows, but a large proportion still remains unexplained, presumably due to real differences between males in the response of their spermatozoa to the ovarian fluid of different females (see chapter 4). Thus, a sperm trait that predicts a male's fertilisation success (and the outcome of sperm competition) when measured in water may not necessarily predict fertilisation success when those same spermatozoa are activated in a solution containing ovarian fluid.

Because sperm traits measured in water are not representative of those measured under natural spawning conditions where ovarian fluid is present, we recommend that activating solutions used for assessing sperm motility contain ovarian fluid, though there needs to be more study to determine the most appropriate dilution. Studies that focus on sperm traits *per se* and the relation between those traits and fertilization success (e.g., in Atlantic salmon, Gage et al. 2004; Walleye, Casselman et al. 2006; Atlantic cod, Rudolfsen et al. 2008a) remain valid, even though they may underestimate the magnitude of such traits during normal spawning. On the other hand, studies that use sperm traits as a measure of male quality (Vladic and Jarvi 2001; Casselman and Montgomerie 2004; Burness et al. 2005; Rudolfsen et al. 2006; Stoltz and Neff 2006; Wedekind et al. 2007), however, may need to be re-evaluated in light of our findings.

3.7 ACKNOWLEDGEMENTS

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3.8 REFERENCES

Ball MA, Parker GA. 1996. Sperm competition games: external fertilization and "adaptive" infertility. J Theor Biol. 180:141-150.

Bates D, Maechler M, Dai B. 2008. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-28. Available from http://lme4.r-forge.r-project.org/ [accessed June 2008].

Bencic DC, Cloud JG, Ingermann RL. 2000. Carbon dioxide reversibly inhibits sperm motility and fertilizing ability in steelhead (*Oncorhynchus mykiss*). Fish Physiol Biochem. 23:275-281.

Billard R, Cosson M. 1992. Some problems related to the assessment of sperm motility in freshwater fish. J Exp Biol. 261:122-131.

Burness G, Casselman SJ, Schulte-Hostedde AI, Moyes CD, Montgomerie R. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav Ecol Sociobiol. 56:65-70.

Burness G, Moyes CD, Montgomerie R. 2005. Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). Comp Biochem Physiol. A. 140:11-17.

Burness G, Schulte-Hostedde AI, Montgomerie R. 2008. Body condition influences sperm energetics in lake whitefish (*Coregonus clupeaformis*). Can J Fish Aquat. Sci. 65:615-620.

Canty A. 2005. boot, Bootstrap R (S-Plus) Functions. R package version 1.2-24. Available from http://www.r-project.org [accessed June 2008].

Casselman SJ, Montgomerie R. 2004. Sperm traits in relation to male quality in colonial spawning bluegill. J Fish Biol. 64:1700-1711.

Casselman SJ, Schulte-Hostedde AI, Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat. Sci. 63: 2119-2125.

Cruz-Casallas PE, Medina-Robles VM, Velasco-Santamaria YM. 2007. Seasonal variation of sperm quality and the relationship between spermatocrit and sperm concentration in yamu *Brycon amazonicus*. N Am J Aquacult. 69:159-165.

Dietrich GJ, Wojtczak M, Slowińska M, Dobosz S, Kuźmińksi H, Ciereszko A. 2008. Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss* Walbaum) spermatozoa. J Appl Ichthyol 24:503-507.

Eberhard WG. 1996. Female control: sexual selection by cryptic female choice. New Jersey: Princeton University Press.

Elofsson H, McAllister BG, Kime DE, Mayer I, Borg B. 2003a. Long lasting stickleback sperm; is ovarian fluid a key to success in fresh water? J Fish Biol. 63:240-253.

Elofsson H, Van Look K, Borg B, Mayer I. 2003b. Influence of salinity and ovarian fluid on sperm motility in the fifteen-spined stickleback. J Fish Biol. 63:429-438.

Elofsson H, Van Look KJW, Sundell K, Sundh H, Borg B. 2006. Stickleback sperm saved by salt in ovarian fluid. J Exp Biol. 209:4230-4237.

Falissard B. 2008. psy, Various procedures used in psychometry. R package version 0.7. Available from http://www.r-project.org [accessed June 2008].

Fitzpatrick JL, Desjardins JK, Milligan N, Montgomerie R, Balshine S. 2007. Reproductive-tactic-specific variation in sperm swimming speeds in a shell-brooding cichlid. Biol Reprod. 77:280-284.

Gage MJ, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr Biol 14:44-47.

Ginsburg A. 1963. Sperm-egg association and its relationship to the activation of the egg in salmonid fishes. Embryo Exp Morphl. 11:13-33.

Hatef A, Niksirat, H, Alavi SMH. 2009. Composition of ovarian fluid in endangered Caspian brown trout, *Salmo trutta caspius*, and its effects on spermatozoa motility and fertilizing ability compared to freshwater and a saline medium. Fish Physiol Biochem. 35(4):351-565.

Hirano T, Morisawa M, Suzuki K. 1978. Changes in plasma and coelomic fluid composition of the mature salmon (*Onchorhynchus keta*) during freshwater adaptation. Comp Biochem Physiol. A. 61:5-8.

Hoysak DJ, Liley NR. 2001. Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. J Fish Biol. 58:1286-1300.

Lahnsteiner F. 2002. The influence of ovarian fluid on the gamete physiology in the Salmonidae. Fish Physiol Biochem. 27:49-59.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture. 163:163-181.

Leach B, Montgomerie R. 2000. Sperm characteristics associated with different male reproductive tactics in bluegills (*Lepomis macrochirus*). Behav Ecol Sociobiol. 49:31-37.

Levitan DR. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. Proc R Soc Lond B Biol. 267: 531-534.

Liljedal S, Rudolfsen G, Folstad I. 2008. Factors predicting male fertilization success in an external fertilizer. Behav Ecol Sociobiol. 62:1805-1811.

Linhart O, Rodina M, Gela D, Kocour M, Vandeputte M. 2005. Spermatozoal competition in common carp (*Cyprinus carpio*), what is the primary determinant of competition success? Reproduction. 130:705-711.

Litvak MK, Trippel EA. 1998. Sperm motility pattern of Atlantic cod (*Gadus morhua*) in relation to salinity: effects of ovarian fluid and egg presence. Can J Fish Aquat. Sci. 55:1871-1877.

Munkittrick KR, Moccia RD. 1987. Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*) semen: Effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma constituents. Aquaculture. 64:147-156.

R Development Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org [accessed June 2008].

Rideout RM, Trippel EA, Litvak MK. 2004. Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J Fish Biol. 65:319-332.

Rudolfsen G, Figenschou L, Folstad I, Tveiten H, Figenschou M. 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc R Soc Lond. B. 273: 325-332.

Rudolfsen G, Figenschou L, Folstad I, Oddmund K. 2008a. Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua* L.). Aquacult Res. 39:212-216.

Rudolfsen G, Müller R, Urbach D, Wedekind C. 2008b. Predicting the mating system from phenotypic correlations between life-history and sperm quality traits in the Alpine whitefish *Coregonus zugensis*. Behav Ecol Sociobiol. 62:561-567.

Schulte-Hostedde AI, Burness G. 2005. Fertilization dynamics of sperm from different male mating tactics in bluegill (*Lepomis macrochirus*). Can J Zool. 83:1638-1642.

Stockley P, Gage MJ, Parker GA, Møller AP. 1997. Sperm competition in fishes, the evolution of testis size and ejaculate characteristics. Am Nat. 149:933-954.

Stoltz JA, Neff, BD. 2006. Sperm competition in a fish with external fertilization, the contribution of sperm number, speed and length. J Evol Biol. 19:1873-1881.

Thornhill R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am Nat. 122:765-788.

Turner E, Montgomerie R. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol. 60:1570-1579.

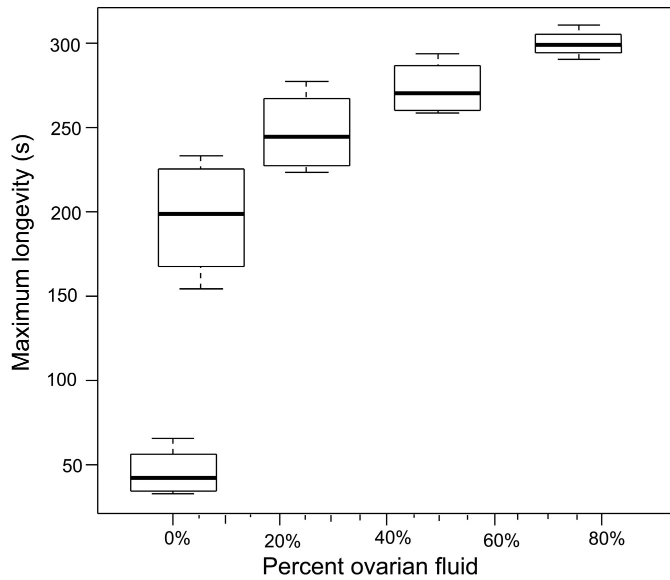
Urbach D, Folstad I, Rudolfsen G. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol. 57:438-444.

Vladić TV, Järvi T. 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. Proc R Soc Lond B. 268: 2375-2381.

Wedekind C, Rudolfsen G, Jacob A, Urbach D, Müller R. 2007. The genetic consequences of hatchery-induced sperm competition on a salmonid. Biol Cons. 137:180-188.

Woolsey J, Holcomb M, Cloud JG, Ingermann RL. 2006. Sperm motility in the steelhead *Oncorhynchus mykiss* (Walbaum), influence of the composition of the incubation and activation media. Aquacult Res. 37:215-223.

Yeates S, Searle J, Ward RG, Gage MJG. 2007. A two-second delay confers first-male fertilization precedence within in vitro sperm competition experiments in Atlantic salmon. J Fish Biol. 70:318-322.



Appendix 3.1. Maximum duration of forward motility (DUR s) of sperm, from sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*) (n = 8), after activation in different concentrations of ovarian fluid diluted in 12ºC fresh water. Box plots show 10th, 25th, 50th (median), 75th and 90th percentiles as horizontal lines.

Chapter 4

A MECHANISM FOR CRYPTIC FEMALE CHOICE IN CHINOOK SALMON

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4.1 Abstract

Female mate choice after copulation or spawning is cryptic when a female differentially influences the fertilisation success of sperm from different males. We tested whether ovarian fluid could act as a potential mechanism of cryptic female choice in chinook salmon (*Oncorhynchus tshawytscha*) by comparing how ovarian fluid from each of seven females affected the sperm behaviour of 11 different males. Using computer-assisted sperm analysis, we measured sperm velocity, motility, longevity and linearity from the ejaculates of each male activated in the ovarian fluid from each female. Mean sperm swimming speed, path trajectory, and longevity differed significantly among males, and within males depended upon the female's ovarian fluid in which it was activated. Most important, the pattern of within-male variation in these traits also varied significantly among males, in response to different females' ovarian fluids. As sperm velocity is known to be a prime determinant of fertilisation success in externally fertilizing fishes, this finding suggests that variation in the composition of female ovarian fluid may be a mechanism for cryptic female choice whereby females differentially enhance the swimming speed of sperm from different males. Thus, female ovarian fluid can alter relative male fertilisation success when there is intense sperm competition, as there is in this and other group spawning fish species.

Keywords: Cryptic female choice, chinook salmon, *Oncorhynchus tshawytscha*, ovarian fluid, sperm velocity, sperm traits.

4.2 Introduction

Cryptic female choice (CFC) is defined as mate choice that occurs after mating or spawning (Thornhill 1983; Eberhard 1996). It has recently been suggested that CFC may be widespread when females rely on sperm selection to increase the genetic quality of their offspring (Jennions and Petrie 2000; Neff and Pitcher 2005). Nonetheless, the concept remains controversial as it has proven notoriously difficult to establish whether or not CFC has actually occurred (Birkhead 2000; Kempenaers et al. 2000; Pitnick and Brown 2000; Birkhead and Pizzari, 2002).

The mechanisms underlying CFC have not been well studied, although a number of potential physiological and biochemical mechanisms have been identified in a range of species with internal fertilisation (Birkhead 2000; Birkhead and Pizzari 2002; Pitnick and Brown 2000). For example, ejaculate manipulation can occur when a female ejects semen from her reproductive tract (Pizzari and Birkhead 2000). In species with external fertilisation, females may control the numbers of eggs laid in the presence of different males (Reyer et al. 1999), or the egg itself may discriminate among sperm by biochemical means (e.g., Zeh and Zeh 1997). Sperm selection by the ovum may also be an important mechanism of cryptic female choice. For example, in the comb jelly, *Beroe ovata,* the egg pronuclei is able to choose amongst the sperm of different males once they have entered the egg (Carre and Sardet 1984). In externally fertilizing teleost fishes this may occur following fusion of the gametes during the formation of the second polar body, as the second maturation division in many fish is completed only after the sperm has penetrated the egg (Wolgemuth 1983). In mice this meiotic division in the egg was influenced by the type of sperm that entered (Agulnik et al. 1993). Finally, in externally fertilizing teleosts, the ovarian fluid that is released by the female with her eggs during spawning is known to influence sperm behaviour and could be a mechanism of CFC if its effects on sperm behaviour differ among competing males during multi-male spawning events (Turner and Montgomerie 2002; Urbach et al. 2005; Nordeide 2007).

Ovarian fluid is a maternally-derived liquid that surrounds the egg mass inside the female fish and is expelled during spawning. In the Salmonidae, ovarian fluid comprises 10-30% of the total egg volume (Lahnsteiner 2002). When mixed with the spawning medium (fresh or salt water), ovarian fluid creates a chemical "microenvironment" for the sperm that differs from the surrounding freshwater medium. Previous studies have observed that sperm behave differently when activated in ovarian fluid compared to activation in pure water. For example, spermatozoa activated in ovarian fluid swam faster in brown trout (*Salmo trutta f. fario*) (Lahnsteiner 2002), Atlantic cod (*Gadus morhua*) (Litvak and Trippel 1998), chinook salmon (see Chapter 3), and also the duration of sperm motility (longevity) was prolonged in brown trout (Lahnsteiner, 2002), chinook salmon (see Chapter 3) and the three-spined stickleback (*Gasterosteus aculeatus*) (Elofsson et al. 2003). In Arctic charr (*Salvelinus alpinus*) ovarian fluid increased sperm longevity, sperm swimming speed, and the percentage of motile sperm cells as well as affecting sperm trajectories, compared to sperm swimming in fresh water (Turner and Montgomerie, 2002).

Two lines of evidence suggested to us that the composition of ovarian fluid might provide a mechanism for cryptic female choice in externally fertilizing fish. First, ovarian fluid influences sperm swimming speed which in turn is an important determinant of male fertilisation success (Birkhead et al. 1999; Levitan 2000; Gage et al. 2004; Rudolfsen et al. 2008). For example, using microsatellite DNA fingerprinting, Gage et al. (2004) demonstrated, in Atlantic salmon (*Salmo salar*), that a male's relative sperm velocity was the most important factor affecting fertilization success during sperm competition. Second, there is evidence that the composition of ovarian fluid varies among females, particularly with respect to the constituents that are known to influence sperm behaviour (Lahnsteiner et al. 1995; Wojtczak et al. 2007). Thus a male's sperm might be expected to swim at different speeds in the ovarian fluid solution of different females.

A recent study of Arctic charr found a significant female (ovarian fluid) x male interaction on sperm swimming speed (Urbach et al. 2005), indicating that sperm swimming speed varied depending upon the female from which the ovarian fluid was

taken. They suggested that chemical variation in the composition of the ovarian fluid might indeed be a mechanism for cryptic female choice. In that study, however, sperm swimming speed was measured at 30 s after activation, long after most ova are fertilized in salmonids (Hoysak and Liley 2001) and other externally fertilizing fishes (e.g., Casselman et al. 2006) where most fertilization occurs within 10 s of sperm activation. By 30 s post activation, the sperm of both salmonids (Christen and Billard 1987) and centrarchids (Burness et al. 2005) already has declining energy reserves and is swimming at less than its maximum speed. Thus differences among males in sperm swimming speed at 30 s after activation might well be due to differences in longevity and the straightness of the sperm trajectory, and not particularly relevant to fertilization success. Although the finding of male x female interaction by Urbach et al. (2005) is intriguing, it certainly warrants further study.

Just how ovarian fluid interacts with sperm is unknown, but its positive effects on sperm function have been attributed to the composition of the ovarian fluid (Lahnsteiner et al. 1995; Lahnsteiner 2002; Cosson 2004; Elofsson et al. 2006). The exact mechanism by which ions or inorganic compounds in the ovarian fluid influence the behaviour of fish spermatozoa remains unclear. Lahnsteiner et al. (1995) found intraspecific variation in composition of the ovarian fluid of four salmonid species, and suggested that variation in the chemical composition of the ovarian fluid between females differentially affected sperm traits from some males. It is therefore possible that intraspecific variation in the composition of the ovarian fluid could play an important role in female sperm selection via CFC, whereby female ovarian fluid differentially influences the sperm behaviour of different males, resulting in prejudiced paternity.

Here we use the chinook salmon (*Oncorhynchus tshawytscha*), a fish with external fertilisation and multiple-male spawnings, to investigate whether ovarian fluid could be the agent of CFC via its influence on sperm behaviour, and hence the sperm's ability to reach the egg. In this species, both sexes mate multiply during spawning (Berejikian et al. 2000). As a result, there is intense sperm competition among males (Fleming 1998), which can reduce the opportunities for precopulatory mate choice by females.

To investigate the possibility of CFC in chinook, we used computer assisted sperm analysis (CASA) to measure four key sperm traits known or expected to influence male fertilization success (sperm velocity, duration of sperm motility, swimming path trajectory, and progressive motility) in sperm from eleven male salmon activated in the ovarian fluid of each of seven different females.

4.3 METHODS

Chinook salmon were obtained from a hatchery-reared population at the National Institute of Water and Atmospheric Research (NIWA) Silverstream Hatchery, Canterbury, New Zealand. All were descendants of juvenile fish collected the major chinook salmon-producing rivers as well as from several isolated land-locked populations on the central South Island of New Zealand (M. Unwin, pers. comm.). The two-year-old fish used in this study varied in both body length (mean±95% CL = 318±30 mm) and body mass (526±126 g). All fish were maintained in a hatchery raceway using standard husbandry procedures (Pennnell and Barton 1996; Unwin et al. 2004).

We studied haphazardly chosen, sexually mature, individually marked two-year-old male and three-year-old female salmon from 3-14 May 2004, during the spawning season. Milt (sperm and seminal fluid) samples from fish were collected on different days. On each day when milt was sampled, each male was netted and dried around the cloaca to avoid activation of sperm cells by water/urine. Milt was then collected (“stripped”) from the males by applying gentle bilateral abdominal pressure. On each sampling day, one or two females were killed with a stroke to the head, then their egg batch expelled and their ovarian fluid collected. Milt and ovarian fluid samples were immediately refrigerated at 4°C and transported to the University of Canterbury. The time from collection of the first to the last milt sample to be used for motility analysis was no greater than five hours.

Sperm motility recordings were obtained for each male (n = 11) activated in ovarian fluid from each female (n = 7). Each sampling day, milt samples were selected haphazardly

with respect to male identity and/or stripping order so that time since stripping would not confound our results. The ambient air temperature in the lab was set at the water temperature (12°C) of the holding raceway to control for variation in sperm swimming speed with varying water temperatures (Alavi and Cosson 2005). Ovarian fluid from each of the seven females was diluted to 50% by volume, using fresh water collected from the raceway. We used a 50% dilution of ovarian fluid as our activating solution as it seems likely that during a natural spawning, spermatozoa would encounter diluted ovarian fluid as the spermatozoa moves from pure freshwater into pure ovarian fluid at the egg surface. In addition, other studies have found that sperm swimming speed is maximized in dilutions close to 50% (Turner and Montgomerie 2002; Woolsey et al. 2006). Sperm motility was initiated by adding 499 µL of this ovarian fluid solution to about 1 µL of the milt sample. We then placed 10 µL of this fluid on a glass slide and gently placed a cover slip over the sample for viewing at 400X on a negative phase-contrast microscope (Leica DMR). Upon activation, sperm behaviour was recorded using a high resolution digital videocam, and sperm longevity (DUR in s) for each milt sample was recorded using a stopwatch, starting at the contact of the milt with the activation solution, and ending when all progressive forward motility had ceased (e.g. Leach and Montgomerie 2000). Vibrating spermatozoa at the end of the period of propulsive sperm motility were considered to be immotile. For each male x female combination we took two measurements using haphazardly collected milt and ovarian fluid samples for each trial.

Videotapes of sperm recordings were later analysed using computer assisted sperm analysis (HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research, Beverly, MA, USA). For each milt sample, we quantified the swimming paths of all spermatozoa in a field of view for 0.5 s at 10 s intervals post activation. Sperm tracks that were clearly influenced by sample drift, and/or were incomplete were not analyzed. On average, 13 sperm tracks were analyzed per trial (range = 5-33 sperm tracks, n = 154 trials). Hoysak and Liley (2001) have shown that the majority of fertilisations in salmonids are likely to occur within a few seconds after male ejaculation, and Yeates et al. (2007) demonstrated that a delay of only two seconds in sperm release by male Atlantic salmon caused a significant reduction in paternity. Moreover, in walleye (*Sander*

vitreus), sperm swimming speed at 10 s post-activation was associated with male fertilisation success, whereas at 20 s post-activation it had no relation to fertilisation success (Casselman et al. 2006). Therefore, in this paper we report on sperm traits at 10 s post-activation only.

The average values of the following parameters for each male were calculated from the sperm tracks of each trial: mean average path velocity (VAP in $\mu m \bullet s^{-1}$), mean straight line velocity (VSL in $\mu m \bullet s^{-1}$), mean curvilinear velocity (VCL in $\mu m \bullet s^{-1}$), and linearity (LIN; the ratio of VSL/VCL expressed as a percentage). Like other studies, we used VAP as a measure of sperm swimming speed (e.g., Lahnsteiner et al. 1998; Burness et al. 2005; Casselman et al. 2006). LIN describes the path trajectory of the sperm through the solution. A circular trajectory, for example would have a low LIN, and a high LIN would indicate that the sperm cell is moving in a straight-line path. For each trial, we also measured the percentage of cells in the field of view that were forwardly motile at >20 $\mu m \bullet s^{-1}$ (MOT) as an index of overall sperm motility (see Lahnsteiner et al. 1998 for a similar criterion).

4.4 STATISTICAL ANALYSES

Statistical analysis was performed using R, version 2.2.1 (R Core Development Team 2007) and the linear mixed-effects package lme4 (Bates 2007). A mixed effects general linear model was fitted using female and male as random effects. We included the day the experiment was conducted as a covariate in each model because there is evidence that sperm traits change over the spawning season in some fish species (Rideout et al. 2004; Cruz-Casallas et al. 2007). The main focus of our study was to test for an interaction between female and male identities on sperm longevity (DUR), average sperm swimming speed (VAP), linearity (LIN), and percentage of progressively motile cells (MOT) at 10 s post-activation using the maximum likelihood method of estimation. To test the statistical significance of one or more fixed or random effects a model was fitted with and without the explanatory variable using log likelihood ratio statistics (LLR χ^2). Assumptions

underlying all models were verified using residual plots. All p-values ≤0.05 were considered to be significant.

4.5 RESULTS

There were significant male, female, and female x male (i.e., ovarian fluid x sperm) interaction effects on sperm longevity (Table 4.1, Figure 4.1a). Thus average sperm longevity varied significantly among males, average sperm longevity (across males) varied significantly among female ovarian fluids, and the average sperm longevity of individual males was differentially affected by ovarian fluid in a pattern that varied significantly across males. Sperm longevity varied significantly within all males except males 4 ($p = 0.19$), 8 ($p = 0.07$), and 10 ($p = 0.06$) when compared across females (posthoc contrast analyses). For example, the sperm of male 1 swam longest in the ovarian fluid of female 1 but shortest with female 3; the sperm of male 3 swam for the shortest duration with female 1, but almost the longest with female 4 (Figure 4.1a). The sperm of male 5 swam longest in the ovarian fluid of females 2 and 6 but only at 60% of that duration with females 3 and 7 (Figure 4.1a).

Similar results were observed for average sperm swimming speed (VAP), as there were significant effects of female, male and female x male interaction (Table 4.1, Figure 4.1b). Thus average sperm swimming speed varied significantly among males, and there was significant variation among the average sperm swimming speed among females (ovarian fluids). The significant female x male interaction effect suggests that VAP varied within each female’s ovarian fluid according to individual male identity. Sperm swimming speed also varied significantly within each male (posthoc contrast analyses, all $p < 0.02$) depending upon the ovarian fluid in which it was activated. For example, the sperm from male 1 was among the fastest swimming sperm in the ovarian fluid of all 7 females whereas the sperm of male 3 was among the fastest with female 1, and among the slowest with female 7 (Figure 4.1b). The sperm of male 4 swam almost twice as fast with female 7 as it did with female 6 (Figure 4.1b). Interestingly, there was no correlation between average least squares (adjusted) means (controlling for date) for VAP and DUR across

male x female combinations (r = 0.06, p = 0.65, Figure 4.2a), suggesting that there was no trade off between these two traits. Thus, sperm did not swim faster at the expense of swimming duration, as has been suggested in other studies (Stockley et al. 1997; Levitan 2000).

Table 4.1. Summary of random effects from mixed effects general linear models predicting sperm traits (DUR, longevity; VAP, swimming speed; MOT, percent of motile cells; LIN, linearity of sperm trajectory) for two-year-old sexually mature male chinook salmon (*Oncorhynchus tshawytscha*) at10 s postactivation in a 50% ovarian fluid solution from seven different females.

Response	Predictors (n)	Percentage of variance explained	Chi-square	P
DUR	female (7)	24.7	32.6	**<0.0001**
	male (10)	9.9	10.8	**0.001**
	female x male	24.5	9.5	**0.002**
VAP	female (7)	11.3	19.0	**<0.0001**
	male (11)	25.0	38.7	**<0.0001**
	female x male	48.7	63.5	**<0.0001**
MOT	female (7)	10.1	7.3	**0.006**
	male (10)	1.3	0.4	0.54
	female x male	8.5	0.6	0.45
LIN	female	3.3	5.7	**0.02**
	male	8.7	14.5	**<0.0001**
	female x male	42.9	22.4	**<0.0001**

Significant ($P \leq 0.05$) random effects as determined by the likelihood ratio test are indicated in bold (df = 1 in each case).

There were significant female (LLR $\chi^2 = 11.9$, df = 1, p <0.001), and female x male interaction effects (LLR $\chi^2 = 4.8$, df = 1, p = 0.02) on the mean percent of progressive sperm cells (MOT) but the male effect was not significant (LLR $\chi^2 = 1.4$, df = 1, p = 0.23). However, there was significant variation only within male 1 (p <0.001, posthoc contrast analysis) suggesting that male 1 may have been responsible for the significant interaction effect due to the low motility of his sperm in the ovarian fluid of female 1 (Figure 4.1c). Indeed, when that male was not included in the analysis, the female x male

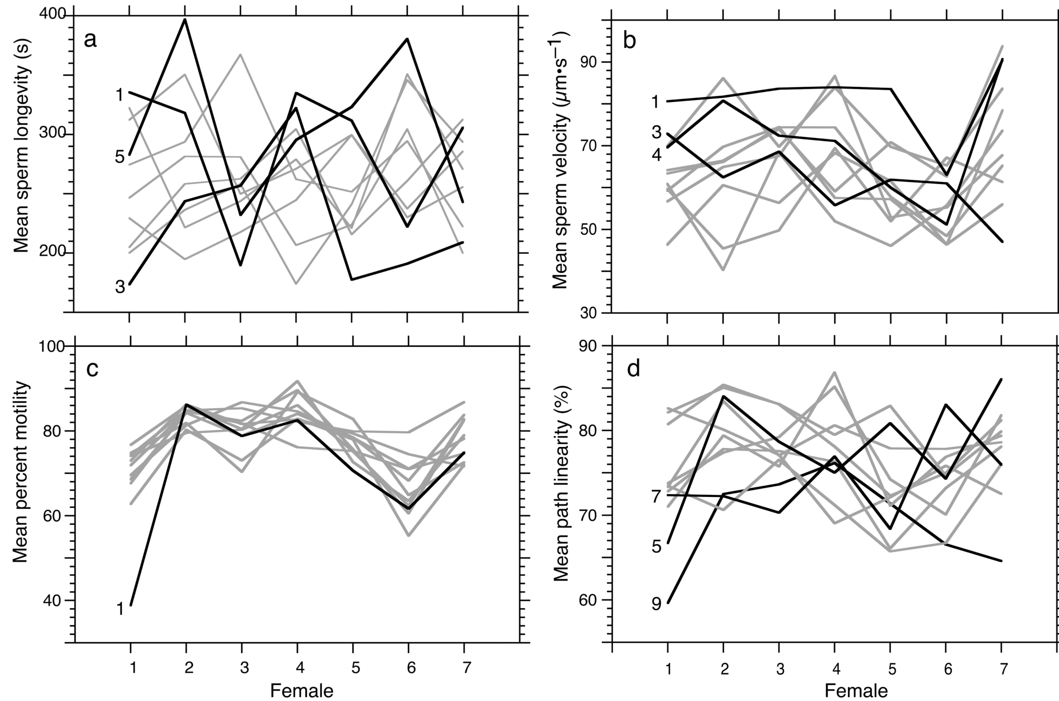

Figure 4.1. Least squares mean (controlling for date) sperm traits of male chinook salmon (*Oncorhynchus tshawytscha*) in the ovarian fluids of seven different females. Bold lines identified by male number are described in the text. (a) Sperm longevity (DUR; n = 10 males), (b) swimming speed (VAP; n = 11 males), (c) percentage of progressively motile cells (MOT ; n = 11 males), and (d) path linearity (LIN; n = 11 males). See Table 4.1 for statistics.

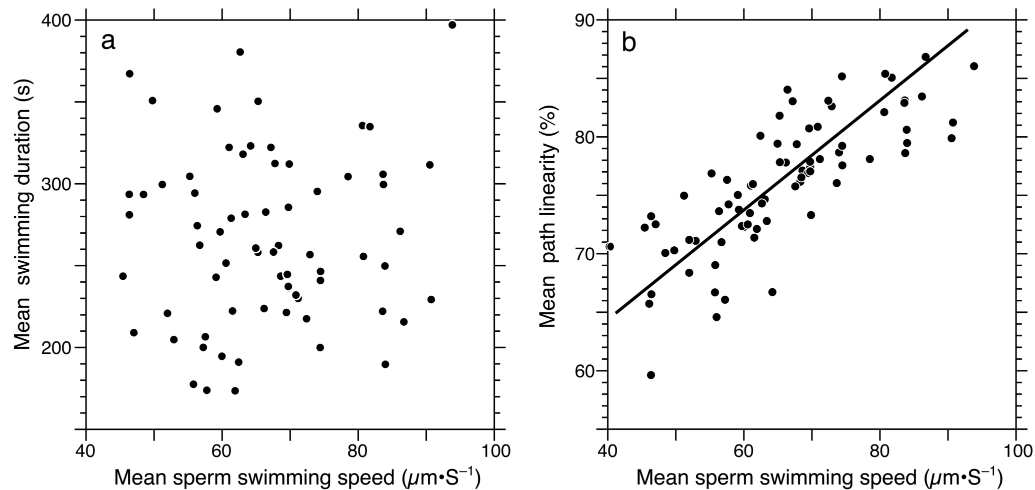

Figure 4.2. Relations between (a) sperm swimming duration and (b) sperm path linearity and sperm swimming speed in male chinook salmon (*Oncorhynchus tshawytscha*). Data points are least squares means from general linear models with day as a covariate. Line in (b) is model II regression (y = 45.8 + 0.5x).

interaction term was far from significant (Table 4.1). Thus the percent of sperm that were progressively motile does not in general seem to be affected by activation in the ovarian fluids of different females.

There were significant female, male, and interaction effects on mean sperm linearity (LIN), but mean linearity varied significantly only within males 5, 6, 7, 9, and 10 (Table 4.1, Figure 4.1d). The sperm of male 5, for example, had the straightest trajectory in the ovarian fluid of female 7 but one of the most curvilinear with female 1; male 7 was the most curvilinear with female 3 but the straightest with female 6; and male 9 was the most curvilinear with females 1 and 7 but about 25% straighter with female 4. LIN was positively correlated with VAP ($r = 0.79$, $p < 0.0001$, $n = 77$; Figure 4.2b), suggesting that variation in path linearity was simply due to changes in swimming speed.

4.6 DISCUSSION

The results from this study show that sperm swimming speed (VAP), longevity (DUR), and path trajectory (LIN) differed among males and were all differentially affected by the ovarian fluids from different females (Table 4.1, Figure 4.1). Thus we have uncovered a clear mechanism for cryptic female choice in this species, mediated by female ovarian fluid. Presumably the chemical composition of each female's ovarian fluid differentially affects male sperm performance during the brief fertilisation window after sperm activation. Sperm swimming speed is probably the most important variable influencing fertilization success in this species (see also Gage et al. 2004), and almost half of the variation in that variable (VAP) in our study was explained by male x female interaction (Table 4.1). Previous work on Arctic charr (Urbach et al. 2005) also found significant female x male interaction on sperm swimming speed well after this brief fertilization window.

To confirm that cryptic female choice is at play here, we now need studies that look at sperm traits in relation to male fertilization success during sperm competition in the presence of ovarian fluid from different females. We would predict, for example, that

male 1 would be most likely to fertilize the largest proportion of ova from female 5 in competition with the other 10 males (Figure 4.1b). In more extreme example, males 1 and 4 would be expected to vastly outcompete male 3 to fertilize the ova of female 7 but male 1 would be expected to outcompete both males 3 and 4 to fertilize the ova of female 5.

Our findings suggest that one or more components of a female's ovarian fluid differentially influence the sperm behaviour of males. CFC occurs when females favour the sperm of males with compatible genotypes irrespective of their phenotype (Zeh and Zeh 1996; Jennions and Petrie 2000), and is typically driven by genetic incompatibility between females and males (Birkhead 1998; Birkhead and Pizzari 2002). A male's 'quality' will thus vary from female to female, as potential mates will vary in the extent to which they are genetically compatible (Parker 2006), among other things. A number of potential molecules have been identified that influence male-female compatibility at fertilisation (reviewed by Vacquier 1998). It has been suggested that the major histocompatibility complex (MHC) may be a candidate for genotype-based recognition between the spermatozoa and the egg (Penn and Potts 1999; Birkhead and Pizzari 2002), as their MHC haplotype might be expressed on the surface of spermatozoa, thus enabling female recognition of sperm's alleles (Ziegler at al. 2005).

Evidence is gathering to suggest that fertilisation in many species is nonrandom and depends upon male and female compatibility (Wedekind et al. 1996; Marshall and Evans 2005; Dziminski et al. 2008). For example, fertilisation success was nonrandom with respect to male identity the Australian sea urchin (*Heliocidaris erythrogramma*), supporting the idea that females exercise cryptic female choice for compatible mating partners at a gamete level (Evans and Marshall 2005). Similarly, in a different sea urchin genus, *Echinometra*, ova exposed to experimental sperm mixtures show strong discrimination on the basis of the male's bindin genotype (Palumbi 1999), preferring sperm that carry the same bindin allele as the ovum.

Variation in the compositional "make-up" of a female's ovarian fluid (Lahnsteiner et al. 1995) might depend on the physiological status of the female (Lahnsteiner et al. 1999;

Lahnsteiner 2000). For example, salmonids can also hold their ovulated eggs in the body cavity for at least a week (Aegerter and Jalabert 2004), and post-ovulatory ageing of the eggs in the body cavity can affect the composition of the ovarian fluid (Rime et al. 2004). Some preliminary analyses looking at the chemical composition of the ovarian fluid for each of the seven females that we studied suggests that there may be significant variation in ion concentrations (see Chapter 5). For example, female 6 had a lower calcium concentration in her ovarian fluid compared to the other females, and we know that calcium ions are required in the external environment to initiate sperm motility (Morisawa and Morisawa 1986; Alavi and Cosson 2006). Other components of the ovarian fluid, such as proteins, may function as signaling molecules that have a chemokinetic or chemotactic effect on sperm. These peptide signaling molecules have been found on the surface of the unfertilized ovum in sea urchins (Neill and Vacquier 2004). Additionally, male and female reproductive proteins that bind each other to mediate fertilisation have been recognised in a handful of animal groups (Swanson and Vacquier 2002), and may potentially be found in both ovarian fluid and sperm membranes.

While the significant male effect on sperm performance (Table 4.1) suggests that some males may have generally superior quality spermatozoa that swim faster or for a longer duration, it appears that few, if any, males have sperm that performs well in the ovarian fluid of all females (Figure 4a, b). Some consistent differences among males is expected because sperm motility is partially controlled by mitochondrial genes that regulate sperm motility (Gemmell et al. 2004), that are thus likely to be responsible, at least in part, for differences in sperm swimming speed between males (Froman and Kirby 2005). Nonetheless, our results clearly show that the interaction between female ovarian fluid and male spermatozoa is responsible for more of the observed variation in sperm performance than male identity alone (Table 4.1).

In some externally fertilizing fishes there is undoubtedly a fertilization advantage to sperm swimming both faster and for longer duration, and it has been suggested that there is a tradeoff between these two traits resulting from the process of energy metabolism

(Stockley et al. 1997). Our results clearly show that there is no such tradeoff in chinook, at least in the presence of ovarian fluid (Figure 4.2a). Given that most fertilization in this species probably occurs within 10 s of sperm activation, it is presumably irrelevant that some sperm swim for 6 min or more, on average, in the presence of ovarian fluid (Figure 3.1a). More work will be needed to understand why sperm swimming speed and longevity appear not to be interdependent in this species.

Given the broad significance of sperm selection by females, and our limited knowledge regarding mechanisms of CFC in externally fertilising species, results from the present study suggest a promising area for further detailed investigation into the ability of females to favour the sperm of one male over another during a spawning event. In particular, study of the chemical components of ovarian fluid that influence sperm swimming speed and how those components vary within and among females is warranted. The implementation of competitive fertilisation experiments examining sperm traits in the presence of ovarian fluid from different females are particularly needed to confirm that cryptic female choice does indeed occur.

4.7 ACKNOWLEDGEMENTS

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4.8 References

Aegerter S, Jalabert B. 2004. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 231:59-71.

Agulnik S, Sabantsev ID, Orlova GV. 1993. Effect of sperm genotype on chromatid segregation in female mice heterozygous for aberrant chromosome 1. Gent Res. 61:97-100.

Alavi SMH, Cosson J. 2005. Sperm motility in fishes. I. Effects of temperature and pH: A review. Cell Biol Int. 29:101-110.

Alavi SMH, Cosson J. 2006. Sperm motility in fishes. II. Effects of ions and osmolality: A review. Cell Biol Int. 30:1-14.

Bates D. 2007. lme4: Linear mixed-effects models using S4 classes. R Packages version 0.99875-9.

Berejikian BA, Tezak EP, LaRae AL. 2000. Female mate choice and spawning behaviour of chinook salmon under experimental conditions. J Fish Biol. 57:647-661.

Birkhead TR. 1998. Cryptic female choice: Criteria for establishing female sperm choice. Evolution. 52:1212-1218.

Birkhead TR. 2000. Defining and demonstrating postcopulatory female choice — Again. Evolution. 54:1057-1060.

Birkhead TR, Pizzari T. 2002. Postcopulatory sexual selection. Nat Rev Genet. 3:262-273.

Birkhead TR, Martinez JG, Burke T, Froman DP. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc R Soc Lond B. 266:1759-1764.

Burness G, Moyes CD, Montgomerie R. 2005. Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochir*us). Comp Biochem Phys A. 140:11-17.

Carre D, Sardet C. 1984. Fertilisation and early development in *Beroe ovata*. Dev Biol. 105:188-195.

Casselman SJ, Schulte-Hostedde AI, Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci. 63:2119-2125.

Christen R, Billard R. 1987. Trout sperm motility. The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. Eur J Biochem. 166:667-671.

Cosson J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult Int. 12:69-85.

Cruz-Casallas PE, Medina-Robles VM, Velasco-Santamaria YM. 2007. Seasonal variation of sperm quality and the relationship between spermatocrit and sperm concentration in yamu *Brycon amazonicus*. N Am J Aquacult. 69:159-165.

Dziminski MA, Roberts JD, Simmons LW. 2008. Fitness consequences of parental compatibility in the frog *Crinia georgiana*. Evolution. 62:879-886.

Eberhard WG. 1996. Female control: sexual selection by cryptic female choice. Princeton: Princeton University Press.

Elofsson H, McAllister BG, Kime DE, Mayer I, Borg B. 2003. Long lasting stickleback sperm: is ovarian fluid a key to success in fresh water? J Fish Biol. 63:240-253.

Elofsson H, Van Look KJW, Sundell K, Sundh H, Borg B. 2006. Stickleback sperm saved by salt in ovarian fluid. J Exp Biol. 209:4230-4237.

Evans JP, Marshall DJ. 2005. Male-by-female interaction influence fertilization success and mediate the benefits of polyandry in the sea urchin *Helicidaris erythrogramma*. Evolution. 59:106-112.

Fleming IA. 1998. Pattern and variability in the breeding system of Atlantic salmon (*Salmo salar*), with comparisons to other salmonids. Can J Fish Aquat Sci. 55: (Suppl. 1), 59-76.

Froman DP, Kirby JD. 2005. Sperm mobility: Phenotype in roosters (*Gallus domesticus*) determined by mitochondrial function. Biol Reprod. 72:562-567.

Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr Biol. 14:44-47.

Gemmell NJ, Metcalf VJ, Allendorf FW. 2004. Mother's curse: the effect of mtDNA on individual fitness and population viability. Trends Ecol Evol. 19:238-244.

Hoysak DJ, Liley NR. 2001. Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. J Fish Biol. 58:1286-1300.

Jennions MD, Petrie M. 2000. Why do females mate multiply? A review of the genetic benefits. Biol Rev. 75:21-64.

Kempenaers B, Foerster K, Questiau S, Robertson BC, Vermeirssen ELM. 2000. Distinguishing between female sperm choice versus male sperm competition: A comment on Birkhead. Evolution. 54:1050-1052.

Lahnsteiner F. 2000. Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. Fish Physiol Biochem. 23:107-118.

Lahnsteiner F. 2002. The influence of ovarian fluid on the gamete physiology in the Salmonidae. Fish Physiol Biochem. 27:49-59.

Lahnsteiner F, Weismann T, Patzner RA. 1995. Composition of the ovarian fluid in 4 salmonid species: *Oncorhynchus mykiss, Salmo trutta* f *lacustris, Salvelinus alpinus* and *Hucho hucho*. Reprod Nutr Dev. 35:465-474.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture. 163:163-181.

Lahnsteiner F, Berger B, Weismann T. 1999. Sperm metabolism of the teleost fishes *Oncorhynchus mykiss* and *Chalcalburnus chalcoides* and its relation to motility and viability. J Exp Zool. 284:454-465.

Leach B, Montgomerie R. 2000. Sperm characteristics associated with different male reproductive tactics in bluegills (*Lepomis macrochirus*). Behav Ecol Sociobiol. 49: 31-37.

Levitan DL. 2000. Sperm velocity and longevity trade off each other and influence fertilisation in the sea urchin *Lytechinus variegatu*s. Proc R Soc Lond B. 267: 531-534.

Litvak MK, Trippel EA. 1998. Sperm motility pattern of Atlantic cod (*Gadus morhua)* in relation to salinity: effects of ovarian fluid and egg presence. Can J Fish Aquat Sci. 55:1871-1877.

Marshall DJ, Evans JP. 2005. The benefits of polyandry in the free-spawning polychaete *Galeolaria caespitosa*. J Evol Biol. 18:735-740.

Morisawa S, Morisawa M. 1986. Acquisition of potential for sperm motility in rainbow trout and chum salmon. J Exp Biol. 126:89-96.

Neff BD, Pitcher TE. 2005. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. Mol Ecol. 14:19-38.

Neill AT, Vacquier VD. 2004. Ligands and receptors mediating signal transduction in sea urchin spermatozoa. Reproduction. 127:141-149.

Nordeide JT. 2007. Is there more in 'gamete quality' than quality of the gametes? A review of effects of female mate choice and genetic compatibility on offspring quality. Aquacult Res. 38:1-16.

Palumbi SR. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc Natl Acad Aci USA. 96:12632-12637.

Parker GA. 2006. Sexual conflict over mating and fertilization: an overview. Phil Trans Roy Soc B. 361:235-259.

Penn DJ, Potts WK. 1999. The evolution of mating preferences and major histocompatibility complex genes. Am Nat. 153:145-164.

Pennell W, Barton BA. 1996. Principles of salmonid culture, Amsterdam: Elsevier.

Pitnick S, Brown WD. 2000. Criteria for demonstrating female sperm choice. Evolution. 54:1052-1056.

Pizzari T, Birkhead TR. 2000. Female fowl eject sperm of subdominant males. Nature. 405:787-789.

R Development Core Team 2007 R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria.

Reyer HU, Frei G, Som C. 1999. Cryptic female choice: frogs reduce clutchsize when amplexed by undesired males. Proc R Soc Lond B. 266:2101-2107.

Rideout RM, Trippel EA, Litvak MK. 2004. Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J Fish Biol. 65:319-332.

Rime H, Guitton N, Pineau C, Bonnet E, Bode J, Jalabert B. 2004. Post-ovulatory ageing and egg quality: A proteomic analysis of rainbow trout coelomic fluid. Reprod Biol Endocrin. 2:2-26.

Rudolfsen G, Figenschou L, Folstad I, Kleven O. 2008. Sperm velocity influences paternity in the Atlantic cod (*Gadus morhua L.*). Aquacult Res. 39:212-216.

Stockley P, Gage MJG, Parker GA, Møller AP. 1997. Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. Am Nat. 149:933-954.

Swanson WJ, Vacquier VD. 2002. The rapid evolution of reproductive proteins. Nat Rev Genet. 3:137-144.

Thornhill R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am Nat. 122:765-788.

Turner E, Montgomerie R. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol. 60:1570-1579.

Unwin MJ, Poortenaar CW, Rowe DK, Boustead NC, Porter MJR. 2004. Seasonal profiles in growth, energy reserves, gonad development, and plasma steroids in age 1+ cultured Chinook salmon (*Oncorhynchus tshawytscha*) females. New Zeal J Mar Fresh. 38: 29-41.

Urbach D, Folstad I, Rudolfsen G. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol. 57:438-444.

Vacquier VD. 1998. Evolution of gamete recognition proteins. Science. 281:1995-1998.

Wedekind C, Chapuisat M, Macas E, Rülicke T. 1996. Non-random fertilization in mice correlates with the MHC and something else. Heredity. 77:400-409.

Wojtczak M, Grzegorz JD, Słowińska M, Dobosz S, Kuźmiński H, Ciereszko A. 2007. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Aquacult. 270:259-264.

Wolgemuth DJ. 1983. Synthetic activities of the mammalian early embryo: molecular and genetic alterations following fertilization. In: Hartmann JF, editor.

Mechanism and control of animal fertilization. London: Academic Press. p. 415–452.

Woolsey J, Holcomb M, Cloud JG, Ingermann RL. 2006. Sperm motility in the steelhead *Oncorhynchus mykiss* (Walbaum): influence of the composition of the incubation and activation media. Aquacult Res. 37:215-223

Yeates S, Searle J, Ward RG, Gage MJG. 2007. A two-second delay confers first-male fertilization precedence within in vitro sperm competition experiments in Atlantic salmon. J of Fish Biol. 70: 318-322.

Zeh JA, Zeh DW. 1996. The evolution of polyandry I: Intragenomic conflict and genetic incompatibility. Proc R Soc Lond B. 264:69-75.

Zeh JA, Zeh DW. 1997. The evolution of polyandry II: Post-copulatory defences against genetic incompatibility. Proc R Soc Lond B. 264:69-75.

Ziegler A, Kentenich H, Uchanska-Ziegier B. 2005. Female choice and the MHC. Trends Immunol. 26:496-502.

Chapter 5

CHEMICAL COMPOSITION OF SEMINAL AND OVARIAN FLUIDS OF CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) AND THEIR EFFECTS ON SPERM MOTILITY TRAITS

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Chemical compositional of ovarian and seminal fluids via atomic absorption spectroscopy was carried out by Dr. Harry Taylor.
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5.1 ABSTRACT

The relationships between the compositions of ovarian and seminal fluids and sperm function are not well known in teleost fish species. The objective of the present study was to determine the concentration of the major inorganic ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), osmolality, and pH of ovarian and seminal fluid of sexually mature chinook salmon (*Oncorhynchus tshawytscha*), and to determine if the composition of these fluids influences sperm motility traits (swimming speed, duration of forward mobility, swimming path trajectory, and percent motility). Cation concentrations and osmolality were significantly different in the two fluids. The ionic composition of ovarian fluid differed among individual females, and also among samples collected at different times through the spawning season. Carbonate and bicarbonate were the principal buffer ions in ovarian fluid. Its viscosity was considerably greater than that of water and was shear-dependent. The duration of forward motility (longevity) of spermatozoa, swimming speed, percent motility, and swimming track linearity were measured using milt from 10 males activated in the ovarian fluid from 7 females whose ion concentrations were known. No significant correlations were observed between the composition of the seminal fluid and sperm traits. However, in ovarian fluid, sperm longevity was negatively

correlated with variation in [Ca^{2+}] and [Mg^{2+}], while percent motility increased with increasing [Mg^{2+}]. These observations provide a possible chemical basis for cryptic female mate choice whereby female ovarian fluid differentially influences the behaviour of sperm from different males, and thus their fertilization success.

Keywords: CASA; Chinook; Ovarian fluid; Seminal fluid; Spermatozoa

5.2 INTRODUCTION

The gametes of externally fertilizing fishes, such as salmonids, are released simultaneously into fresh or salt water during spawning. This potentially hostile aquatic environment induces physiological changes that result in the activation of both sperm motility and unfertilized ova (Jamieson 1991). The initiation of sperm motility in salmonids results from a decrease in external K^+ concentration once spermatozoa come into contact with fresh water (Morisawa 1994). Swelling and lysis of the sperm cells in the hypotonic water then limits the duration of sperm motility (Cosson 2004). Unfertilized ova are immediately activated once in contact with the fresh water which soon leads to closure of the micropyle due to osmotic swelling eventually preventing fertilisation from occurring (Billard and Cosson 1992). Moreover, the initiation of sperm motility, swimming speed and period of forward motility may also be influenced by the ion concentrations, pH (Stoss 1983; Wojtczak et al. 2007), viscosity (Brokaw 1966; Lauga 2007), and other components of the external media (Yoshida and Nomura, 1972) that spermatozoa encounter on their approach to the egg. As the short period of sperm motility and the closure of the micropyle limit the period during which fertilization is possible to about one minute (Billard et al. 1986), the properties of the ovarian and seminal fluids that are released with the gametes are expected to play an important role in the fertilization process.

Seminal fluid produced by the efferent duct provides an ionic environment that maintains the viability of spermatozoa after their release from the testis into the sperm duct (Morisawa and Suzuki 1980; Stoss 1983; Billard 1986). In salmonids and most other

teleosts (Billard 1986), spermatozoa are not motile within the testis, and sperm motility is believed to be stimulated primarily by dilution of potassium ions in the seminal fluid. In addition, sperm motility is also affected by the concentration of other cations such as sodium and calcium (Stoss 1983) in the female's ovarian fluid. Furthermore, the concentrations of ions in the seminal fluid prior to spawning might influence sperm motility following their release, by altering the intracellular ionic composition, pH, or osmolality (Scott and Baynes 1980; Billard and Cosson 1992). The correlation between seminal fluid composition and subsequent sperm motility has been investigated in only a few species: *Salmo sala* (Hwang and Idler 1969), *Oncorhynchus mykiss* (Lahnsteiner et al. 1998), *Cyprinus carpio* and *Alburnus alburnus* (Kruger et al. 1984; Lahnsteiner et al. 1996; Alavi et al. 2004), *Acipenser persicus* (Alavi et al. 2004), and *Lota lota* (Lahnsteiner et al. 1997). In the common bleak, *A. alburnus,* Na^+ and K^+ levels have statistically significant positive and negative relations, respectively, with the percent of motile cells (Lahnsteiner et al. 1996). In some teleost species, the percent of motile cells and duration of sperm motility increased when the K^+ ion levels decreased, and Na ion levels and osmolality increased in the seminal fluid (Billard and Cosson 1992; Lahnsteiner et al. 1996).

In female salmonids, ovarian fluid comprises 10 – 30% of the volume of the spawned egg mass (Lahnsteiner et al. 1995), and is formed by filtration from the blood plasma and the secretory activity of ovarian epithelia (Hirano et al. 1978). Several studies have investigated components of the seminal fluid in salmonids (see Alavi and Cosson 2006) but data on the composition of the ovarian fluid are more limited (see Hirano et al. 1978; Lahnsteiner et al. 1995; Wojtczak et al. 2007). In salmonids, this fluid has electrolyte concentrations similar, although not identical, to blood plasma, and has a rather lower protein concentration. Ovarian fluid also contains various nutrients, metabolites and hormones (Hirano et al. 1978; Lahnsteiner et al. 1995; Ingermann et al. 2002a). In several fish species, ovarian fluid has been found to increase sperm swimming speed and to prolong the period of forward motility of spermatozoa in comparison with activation in fresh water, sea water or a buffered solution (Arctic charr (*Salvelinus alpinus*)*,* Turner and Montgomerie (2002); Atlantic cod (*Gadus morhua*)*,* Litvak and Trippel (1998);

rainbow trout (*Oncorhynchus mykiss*), Dietrich et al. (2008); chinook salmon (*O. tshawytscha*), see Chapter 3)). As with seminal fluid, there have been few investigations of the relation between composition of ovarian fluid and sperm motility. A recent study investigated the effects of the pH of ovarian fluid of rainbow trout (*O. mykiss)* on sperm behaviour and found that the duration of sperm motility and sperm swimming speed increased with an increase in ovarian fluid pH (Wojtczak et al. 2007).

In chapter four we found that sperm swimming speed, path trajectory, and longevity differed significantly among males, and were all differentially affected by the ovarian fluid from different females, suggesting that variation in the composition of female ovarian fluid may be responsible. The aim of the present study was to determine to the composition of the ovarian and seminal fluids of chinook salmon (*O. tshawytscha)* with respect to the concentrations of the major inorganic ions (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}), osmolality, and pH. Carbonate and bicarbonate concentrations and the viscosity of ovarian fluid were also determined. It is generally believed that, in externally fertilizing fish species, sperm motility is the primary determinant of male fertilization success (Gage et al. 2004; Liljedal et al. 2008; Rudolfsen et al. 2008). Therefore, using computer-assisted sperm analysis (CASA), we investigated whether natural variability in the composition of ovarian fluid from individual females, and of the seminal fluid from individual males, influenced sperm motility traits (swimming speed, duration of forward mobility, swimming path trajectory, and percent motility).

5.3 METHODS

Collection of ovarian fluid and milt

Female and male chinook salmon were obtained from a hatchery-reared population at the National Institute of Water and Atmospheric Research (NIWA) Silverstream Hatchery, Canterbury, New Zealand. All were descendants of juvenile fish collected from the major chinook salmon-producing rivers and several isolated land-locked populations on the central South Island of New Zealand (M.J. Unwin, pers. comm.). Milt samples were collected during the 2004 spawning season (late March to early May) from 15 sexually

mature two-year-old male salmon. Fish were netted and dried to avoid activation of sperm by water or urine and milt was collected by applying gentle bilateral abdominal pressure. Seminal fluid was separated from the milt by centrifugation and transferred to screw-cap tubes. Ovarian fluid was also collected from mature three-year-old female salmon during the 2004 spawning season (n = 20) and on four occasions during the 2005 season (28 April, n = 16; 2 May, n = 4; 5 May, n = 10 and 11 May, n = 14). Fish were killed with a stroke to the head, the eggs were expelled into a dish and ovarian fluid was pipetted gently out of the egg batch and into screw-cap tubes with minimal head space to minimise air equilibration. We used ovarian fluid from egg batches that did not contain broken eggs as the breakdown of eggs can lead to changes in ovarian fluid pH (Lahnsteiner 2000). Seminal and ovarian fluids used for compositional analysis were frozen immediately and stored at -80ºC until processed. In the 2005 season, three aliquots of ovarian fluid were collected from each fish and taken separately through all storage and analytical procedures in order to identify compositional differences among individual fish (coefficients of variation of triplicate measurements were approximately 1 - 3% for osmolality and all ions except Mg^{2+} which was about 9%). For sperm motility analysis and some pH measurements (2004 samples), milt and ovarian fluids were immediately refrigerated at 4°C and transported to the University of Canterbury, where measurements were completed within five hours of collection.

Analytical procedures

The concentrations of sodium, potassium, calcium and magnesium in ovarian fluid were determined by Atomic Absorption Spectroscopy (Model Avanta, GBC Scientific Equipment Pty Ltd, Dandenong, Victoria, Australia) using an air:acetylene flame. For Na and K, 25-µL samples were diluted 200-fold and measured at 589.6 nm and 766.5 nm respectively, calibrated with a combined standard (Na 1.2 mmol L^{-1}; K 0.04 mmol L^{-1}). A burner angle of 45° was employed for Na analysis to reduce the sensitivity. Samples and standards contained 1.0 g L^{-1} Cs (chloride) for ionization suppression. For measurement of Ca and Mg, 100 µL samples were diluted 50-fold and measured at 422.7 nm and 285.2 nm respectively (a deuterium continuum lamp was employed for background correction for Mg). Samples and the combined standard (Ca 0.1 mmol L^{-1}, Mg 0.02 mmol L^{-1})

contained 2.5 g L^{-1} La (chloride) as a releasing agent, and 1 g L^{-1} HNO_3 which was necessary for complete recovery of Ca.

The osmolality of undiluted ovarian fluid samples was measured by vapour pressure osmometry (Model 5520, Wescor, Logan, Utah) and chloride was measured using an electrometric chloride titrator (Model CMT10, Radiometer, Copenhagen).

Measurements of the pH of milt and ovarian fluids were made on freshly collected samples in 2004 at the time of the sperm motility analyses. Total CO_2 concentration ($C\text{co}_2$) and pH were also measured at controlled temperature and $P\text{co}_2$ on five ovarian fluid samples collected in 2005. The samples (0.5 mL) were tonometered for >90 min at 15°C with humidified 1.0% CO_2 in N_2 ($P\text{co}_2$ = 1.0 kPa = 7.5 mm Hg) (Cameron Instruments Inc., Dual Equilibrator and Mass Flow Controller) to a stable pH. $C\text{co}_2$ and pH measurements were then repeated on the same samples tonometered with 0.2% CO_2 ($P\text{co}_2$ = 0.2 kPa) and with humidified air ($P\text{co}_2$ approximately 0.04 kPa). pH was measured directly in the tonometer using a combination glass electrode and pH meter (Philips PW9145) calibrated at 15°C (BDS Colorkey buffers). $C\text{co}_2$ measurements were made using the method of Cameron (1971). Samples (10 µL) were injected into a cell containing 10 mmol L^{-1} HCl thermostatted at 40°C. The CO_2 electrode signal was displayed on a pH meter (Radiometer PHM 84) and calibrated by bracketing ovarian fluid samples between 10 µL standards of 10 mmol L^{-1} $NaHCO_3$. Concentrations of HCO_3^- and CO_3^{2-} in ovarian fluid were calculated from $C\text{co}_2$ and pH values using the Henderson-Hasselbalch Equation after subtraction of dissolved [CO_2]. For these calculations, the solubility of CO_2 at 15°C (αco_2 = 0.0535 mmol L^{-1} mm Hg^{-1}) was estimated from an empirical formula derived for Rainbow trout plasma (Boutilier et al. 1984). The second dissociation constant of carbonic acid at 15°C (pK_2 = 9.46) was obtained using a formula for sea water of equivalent ionic strength (Roy et al. 1993).

Viscosity (mPa s = cps) was measured on 0.5 mL samples of ovarian fluid at 15°C using a cone plate viscometer (Wells-Brookfield model DV-II, cone spindle CP-40 radius 2.4 cm, 0.8°). After temperature equilibration, the viscosity was determined after 1 min at

each of four rotation speeds (60, 30, 12, and 6 rpm corresponding respectively to shear rates of 450, 225, 90, and 45 s^{-1}). Calibration was checked using silicone oil viscosity standards and water.

Sperm motility analysis

The effects of ovarian fluid on sperm motility traits were examined on the samples collected in 2004, for each male (n = 10) activated in the ovarian fluid from each female (n = 7). Ovarian fluid was diluted to 50% using fresh water from the raceway that housed the fish. We used a 50% dilution of ovarian fluid as our activating solution, as it seems likely that during the natural spawning period, spermatozoa will encounter diluted ovarian fluid as the spermatozoa moves from pure freshwater into pure ovarian fluid surrounding the egg, and ovarian fluid at this dilution has close to maximum effect of sperm motility (see Appendix 3.1). One µL of milt was thoroughly mixed (for approximately 3 s) with 499 µL of the 50% ovarian fluid, and then pipetted onto a glass slide. A cover slip was then gently placed over the sample for viewing at 400X on a negative phase-contrast microscope (Leica DMR). As sperm swimming speed is dependent upon water temperature (Alavi and Cosson 2005), the ambient air temperature in the laboratory was set at that of water in the raceway (12°C). Upon activation, sperm behaviour was recorded onto high resolution digital video. Sperm longevity (DUR, s) for each milt sample was recorded using a stopwatch starting at the contact of the milt with the activation solution and ending when all forward motility had ceased (e.g., Leach and Montgomerie 2000). Vibrating spermatozoa at the end of the period of progressive sperm motility were considered to be non-motile. For each male x female combination we took two measurements using haphazardly collected milt and ovarian fluid samples for each trial, and then used the mean of these two measurements for further analyses.

The effects of the composition of seminal fluid on sperm traits were examined for different males (n=12) from those in the ovarian fluid experiments described above, but using the same methods for activating and recording sperm motility. Replicate sperm motility measurements were taken of each male's milt activated in fresh water on the same day that the pH levels of the seminal fluid were analysed.

Videotapes of sperm recordings were later analysed using computer assisted sperm analysis (HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research, Beverly, MA, USA). For each milt sample, we quantified the swimming paths of all spermatozoa in the field of view for 0.5 s at 10 s intervals post-activation. On average 15 sperm tracks within the field of view were analyzed for each milt sample, (range = 7 - 41). The average values of the following parameters for each male were calculated from the sperm tracks recorded during each trial: mean average path velocity (VAP, μm s^{-1}), mean straight line velocity (VSL, μm s^{-1}), mean curvilinear velocity (VCL, μm s^{-1}), and linearity (LIN; ratio of VSL/VCL, expressed as a percentage). As in other studies, we used VAP as a measure of sperm swimming speed (e.g., Burness et al. 2005; Casselman et al. 2006; Lahnsteiner et al. 1998). We also measured overall sperm motility (MOT) as the percentage of cells in the field of view that were progressively motile at >20 μm s^{-1} (see Lahnsteiner et al. 1998, for a similar criterion).

5.4 STATISTICAL ANALYSES

Mean values of the ionic concentrations, pH and osmolality were compared between seminal and ovarian fluids using independent t-tests. Correlation analyses were used to measure the association between sperm traits and the ionic concentrations, pH and osmolality of the ovarian fluid and seminal fluid. A type II regression analyses equation was used to fit a line to significant correlation analyses as x and y are subject to errors (Dytham 2003). Differences between ovarian fluid samples collected on different dates during the spawning season with respect to ion concentrations and osmolalities were examined using single factor analysis of variance (ANOVA) on fish mean values. For differences among individual fish, a single factor ANOVA was employed using the triplicate samples for each fish. Statistical significance was set at the $P < 0.05$, mean ± standard deviation (SD), is given unless otherwise stated (SEM, the standard error of the mean).

5.5 RESULTS

The ionic composition and the osmolality of the ovarian and seminal fluids are shown in Table 5.1. Sodium and chloride ions were the major osmolytes in both seminal and ovarian fluids but there were highly significant differences between the two fluids (t-tests comparing samples from 2004: seminal fluid samples n=15; ovarian fluid samples n=20, apart from Na^+ n = 13) in the mean concentrations of all four inorganic cations, [K^+] (t = 26, P <0.0001), [Ca^{2+}] (t = -16, p < 0.001), [Mg^{2+}] (t = 4, p = 0.0002), [Na^+] (t = 8, p < 0.0001), but not of [Cl^-] (t = 1, *p* = 0.24). The sum of cations and chloride in ovarian fluid samples indicated an anion deficit of about 64 mmol L^{-1}. This difference was largely explained by bicarbonate and carbonate ions (see below). The mean osmolality of all ovarian fluid samples was 292 ± 7 mmol kg^{-1} (Table 5.1). The osmolality of seminal fluid was more variable (265 ± 32 mmol kg^{-1}) and was significantly hypo-osmotic to ovarian fluid collected in the same (2004) season (t= -3, *P* = 0.0026). Differences in seminal fluid osmolality primarily reflected differences in the concentrations of sodium, potassium and chloride which were positively correlated with osmolality ([Na^+], r = 0.42, P = 0.12; [K^+], r = 0.82, *p* = 0.0002; [Cl^-], r = 0.95, *p* < 0.0001; [Ca^{2+}], r = -0.07, P = 0.81; [Mg^{2+}]; r = -0.17, P = 0.56). In the ovarian fluid, there was a statistically significant correlation between [Ca^{2+}] and [Mg^{2+}] (r = 0.63, p = 0.0030).

There were statistically significant differences in ovarian fluid composition between sampling days during the spawning season with respect to osmolality and all ion concentrations except [K^+] and [Mg^{2+}]. In addition, there were also statistically significant differences in ion concentrations among individual fish (Table 5.2). The mean pH of fresh seminal plasma and ovarian fluid samples collected in 2004, and measured soon after transport to the laboratory, were identical (8.43 ± 0.13 and 8.43 ± 0.22, respectively; Table 5.3). For ovarian fluid tonometered at 15°C and $P\text{co}_2$ = 1.0 kPa in N_2, the mean pH was 8.18 ± 0.03 increasing to pH 8.78 ± 0.05 at $P\text{co}_2$ = 0.2 kPa. After air-equilibration, pH rose further to 8.88 ± 0.08. Substantial pH change occurred within minutes after changing $P\text{co}_2$, but a new stable value was achieved only after more than an

hour in the tonometer, indicating that carbonic anhydrase activity was absent from ovarian fluid.

Mean C_{CO_2} in the tonometered ovarian fluid was 40.8 ± 3.5 mmol L^{-1} at P_{CO_2} = 1.0 kPa, decreasing to 38.9 ± 2.6 mmol L^{-1} at 0.2 kPa, and to 37.4 ± 2.6 mmol L^{-1} in air (Table 5.3). These changes were small and not statistically significant. Dissolved CO_2 (including H_2CO_3) comprised <1% of total CO_2 in each case. At the highest P_{CO_2} (1 kPa), HCO_3^- comprised 94% and CO_3^{2-} 5% of the total. These proportions were 82%:17% at 0.2 kPa, and to 79%:21% in air-equilibrated ovarian fluid. The sum of inorganic ion concentrations and C_{CO_2} in this subset of samples was 327 ± 1 mmol L^{-1}. Their mean osmolality was 299 ± 2 (n = 5) mmol kg^{-1}, from which an osmotic coefficient of 0.91 ± 0.01 was calculated. This is close to the theoretical values for salt solutions of this concentration (El Guendouzi et al. 2001) and indicates that all of the major osmolytes were accounted for.

Ovarian fluid exhibited non-Newtonian rheology, its viscosity at 15°C decreasing from 4.2 ± 1.1 mPa s to 2.7 ± 0.4 mPa s as the shear rate was increased from 45 s^{-1} to 450 s^{-1} (Fig. 5.1). No hysteresis was observed on reversal of the sequence. In the same apparatus, the viscosity of pure water was nearly constant (1.12 to 1.19 mPa s) and agreed with documented values (Weast 1975).

Correlations between the compositions of seminal fluid (10 males) activated in each of the seven females ovarian fluid prior to activation and subsequent sperm motility traits are shown in Table 5.4, with the statistically significant correlations shown in Fig. 5.2. There was a significant negative relation between the $[K^+]$ of seminal fluid and mean sperm swimming speed (VAP) at 10 seconds post-activation (Fig 5.2a), but this analysis was strongly influenced by one outlier, and with that outlier removed the relation is far from significant (r = 0.081, p = 0.81). Mean sperm longevity (DUR) was significantly negatively correlated with the $[Ca^{2+}]$ (slope = –59.14 ± 15.38, P = 0.01, Fig. 5.2b), and $[Mg^{2+}]$ (slope = -349.50 ± 73.69, P = 0.005) of ovarian fluids (Fig. 5.2c). In contrast, there was a significant positive relation between the mean percent of progressively motile

cells at 10 s post activation (MOT) and the [Mg^{2+}] of the ovarian fluid (slope = 76.20 ± 18.18, $P = 0.009$) in which the sperm were activated (Fig. 5.2d).

Table 5.1. Concentrations of principal inorganic ions (mmol L^{-1}), and osmolality (mmol kg^{-1}), of the seminal and ovarian fluids of chinook salmon (*Oncorhynchus tshawytscha*) collected during the 2004 and 2005 spawning seasons.

Sample and Date collected	N	R	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Osmolality
Seminal Fluid								
April - May 2004	**15**	**1**	**110 ± 26**	**37.3 ± 6.0**	**0.65 ± 0.22**	**1.15 ± 0.41**	**109 ± 17**	**265 ± 32**
Ovarian Fluid								
April - May 2004	20 [1]	1	167 ± 4	3.3 ± 0.3	3.7 ± 0.7	0.67 ± 0.25	114 ± 4	290 ± 11
28 April 2005	16	3	164 ± 3	3.4 ± 0.4	3.9 ± 0.6	0.76 ± 0.48	113 ± 2	295 ± 4
2 May 2005	4	3	168 ± 2	3.2 ± 0.1	4.8 ± 0.7	0.72 ± 0.13	110 ± 3	295 ± 2
5 May 2005	10	3	162 ± 2	3.6 ± 0.4	4.1 ± 0.5	0.77 ± 0.15	110 ± 5	295 ± 3
11 May 2005	14	3	162 ± 3	3.5 ± 0.4	3.5 ± 0.4	0.73 ± 0.13	109 ± 4	290 ± 4
All Ovarian Fluid samples	**64 [2]**	**3**	**164 ± 4**	**3.4 ± 0.4**	**3.8 ± 0.7**	**0.73 ± 0.29**	**112 ± 4**	**292 ± 7**

Values are means ± standard deviation for *n* fish sampled on the dates shown. *R* refers to the number of replicate measurements on separately stored and analyzed sub-samples from each fish.
[1] N = 13 for Na^+ values.
[2] N = 57 for Na^+ values.

Table 5.2. Results from separate one-way ANOVAs for the effects for the effects of collection date (n=4) and individual fish identity (n = 44, 3 replicates) on ion concentrations and osmolalities of ovarian fluid (2005 samples; in Table 5.1).

Dependent variable	Date		Fish ID	
	$F_{3, 40}$	p	$F_{1, 42}$	p
[Na]	7.7	**0.003**	9.4	**0.004**
[K]	1.1	0.37	0.4	0.54
[Ca]	6.83	**0.0008**	5.1	**0.03**
[Mg]	4.2	**0.05**	3.0	0.09
[Cl]	3.0	**0.041**	7.1	**0.01**
Osmolality	5.68	**0.0024**	19.7	< **0.0001**

Significant ($P \leq 0.05$) are indicated in bold.

Table 5.3. Measurements of pH and total CO_2 and calculated $[HCO_3^-]$ and $[CO_3^{2-}]$ (mmol L^{-1}) in seminal fluid (SF) and ovarian fluid (OF) samples of chinook salmon (*Oncorhynchus tshawytscha*) tonometered at15°C with air and with two partial pressures of CO_2 in N_2.

Sample ID	Sample P_{CO_2}	pH	Total CO_2	$[HCO_3^-]$	$[CO_3^{2-}]$
SF May 2004 (n = 14)	Fresh (not tonometered)	**8.43** ± 0.13			
OF May 2004 (n = 20)	Fresh (not tonometered	**8.43** ± 0.22			
OF April 2005 (n = 5)	~0.04 kPa (air)	**8.88** ± 0.08	**37.4** ± 2.6	**29.5** ± 2.1	**7.9** ± 1.4
OF April 2005 (n = 5)	0.2 kPa in N_2	**8.78** ± 0.05	**38.9** ± 2.6	**32.0** ± 2.3	**6.8** ± 0.8
OF April 2005 (n = 5)	1.0 kPa in N_2	**8.18** ± 0.03	**40.8** ± 3.5	**38.4** ± 3.2	**2.1** ± 0.3

Values are means ± standard deviation.

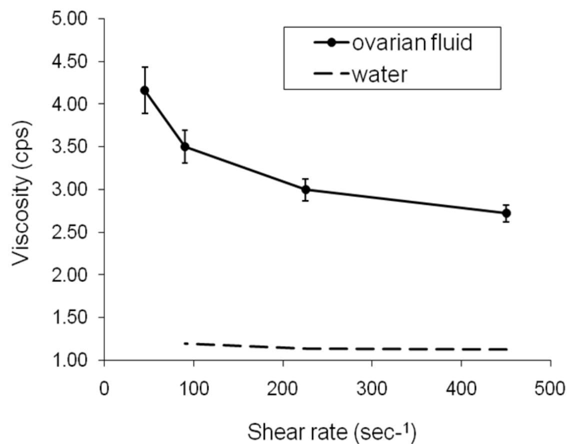

Figure 5.1. Mean (± SEM) viscosity of ovarian fluid decreased as shear rate increases for ovarian fluid samples from 16 sexually mature three-year-old female chinook salmon (*Oncorhynchus tshawytscha*).

Table 5.4. Correlation coefficients (r) between chinook salmon (*Oncorhynchus tshawytscha*) sperm motility traits and the pH, ion concentrations and osmolality of their own seminal fluids and the ovarian fluids from seven females. Longevity (DUR s) mean sperm swimming duration, (VAP $\mu m \cdot s^{-1}$) mean sperm swimming speed, mean percentage of progressively motile cells (MOT %), and mean linearity of sperm trajectory (LIN %) were measured at 10 s post-activation. Seminal fluid and sperm samples for the seminal plasma analyses were obtained from 12 sexually mature two-year-old male chinook salmon during the 2004 spawning season. For the ovarian fluid analyses, sperm traits from 10 different two-year-old chinook males from the 2004 spawning season were activated in the ovarian fluids from seven sexually mature three-year-old females.

Sperm traits	pH	Cl^-	Na^+	K^+	Ca^{2+}	Mg^{2+}	Osmolality
Seminal Plasma							
DUR (s)	0.47	-0.06	-0.26	-0.24	-0.28	-0.02	0.12
VAP (mμ/s)	0.44	-0.39	-0.46	**-0.60***	0.01	-0.14	-0.33
LIN (%)	0.09	0.07	-0.18	0.12	0.04	0.24	0.11
MOT (%)	0.33	0.04	-0.16	-0.20	-0.29	0.03	0.08
Ovarian Fluid							
DUR (s)	0.08	-0.66	-0.23	-0.03	**-0.86**	**-0.91**	0.48
VAP (mμ/s)	-0.28	0.26	-0.02	0.54	0.29	0.57	-0.08
LIN (%)	0.08	-0.08	-0.26	0.45	0.10	0.59	0.13
MOT (%)	0.19	0.20	-0.06	0.35	0.54	**0.88**	-0.09

Significant correlations ($P \leq 0.05$) are indicated in bold
*non-significant after removal of an obvious outlier (Figure 5.2a, see text)

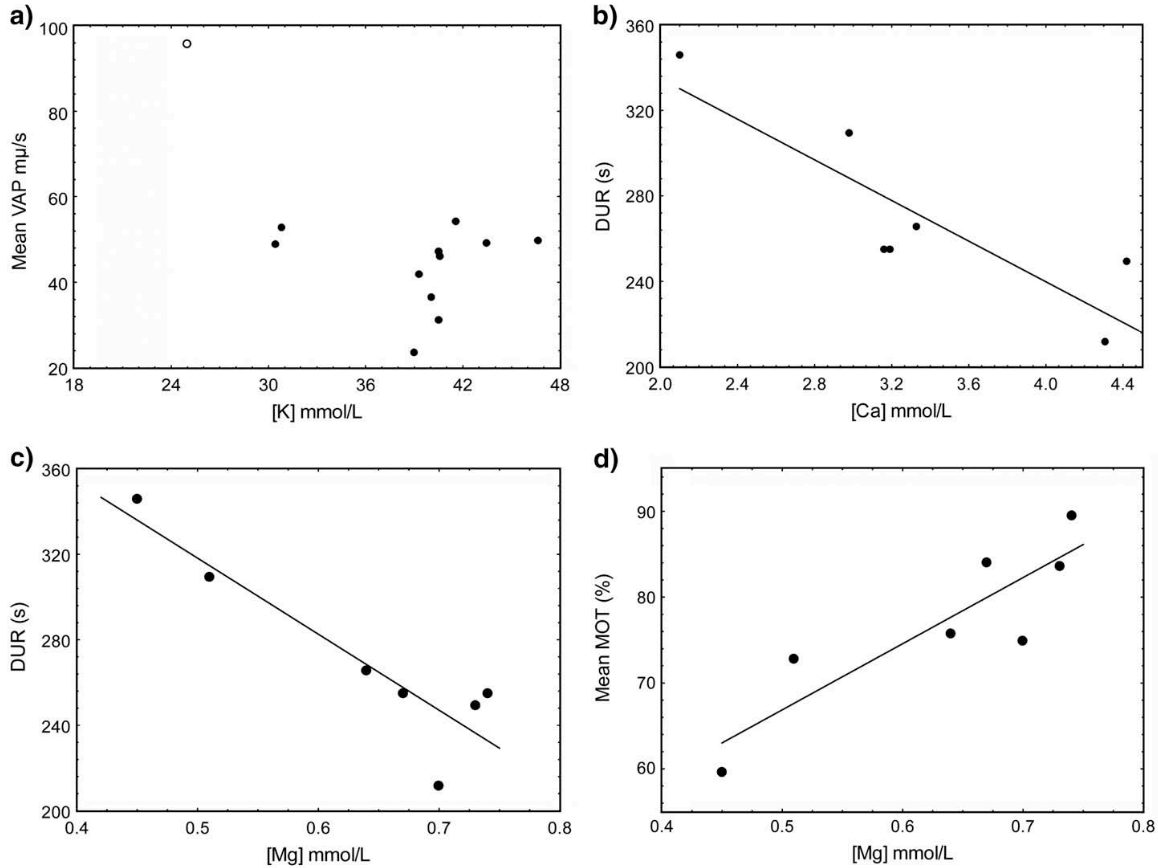

Figure 5.2. (a) Relationship between mean potassium concentrations in the seminal fluid from sexually mature two-year-old male chinook salmon (*Oncorhynchus tshawytscha*) (n=12 males) and average sperm swimming speed (VAP mμ/s) activated in fresh water 10 s post-activation. Outlier as indicate (○).(b) Relationship between mean sperm longevity (DUR s) from 10 sexually mature two- year-old males and mean [Ca^{2+}] concentrations in the ovarian fluid of seven sexually mature three-old-female chinook salmon. Model II regression line is fitted by y = 430.1-59.1x, r^2 = 75. (c) Relationship between mean sperm longevity (DUR s) from 10 sexually mature two year old males and mean [Mg] concentrations in the ovarian fluid of seven sexually mature three old female chinook salmon. Model II regression line is fitted by y = 492.1-349.5x, r^2 = 8. (d) Relationship between mean sperm motility (MOT %) from 10 sexually mature two-year-old males and mean [Mg^{2+}] concentrations in the ovarian fluid of seven sexually mature three-old-female chinook salmon. Model II regression line is fitted by y = 28.9 + 76.2x, r^2 = 77.

5.6 DISCUSSION

Ion concentrations and osmolality of the seminal fluid of *O. tshawytscha* were similar to those reported for *Salmo gairdnieri* (Morisawa et al. 1983), *Salmo salar* (Aas et al. 1991) and *O. mykiss* (Lahnsteiner et al. 1998). The composition of *O. tshawytscha* ovarian fluid was also comparable to that reported for other salmonids (*Salvelinus alpinus*, *Salmo trutta* and *Hucho hucho*, Lahnsteiner et al. 1995; *O. keta,* Hirano et al. 1978), although there were small differences among these salmonids in the mean values of individual ion concentrations. Lower values for [Na^+], and [K^+], and much lower values for [Ca^{2+}] reported by Lahnsteiner et al. (1995) probably reflect the use of ion specific electrodes, which sense only the free ions, in that study. Atomic absorption spectroscopy employed here, and by Hirano et al. (1978), measured total metal concentrations, including that bound to proteins and complexed in other ways. The mean concentrations of sodium, calcium, magnesium, chloride and osmolality of the ovarian fluid were generally similar to their concentrations in the blood plasma of this fish in freshwater (Holmes and Donaldson 1969). In contrast, [K^+] (3.4 ± 0.4 mmol L^{-1}) was three to four times higher in the ovarian fluid than in the plasma in our fish, as previously observed for freshwater acclimated *O. keta* (Hirano et al. 1978).

The discrepancy between the sum of cations and chloride concentration in ovarian fluid in the present study was largely accounted for by high concentrations of carbonate and bicarbonate. A similar anion deficit was apparent in the ovarian fluid of *O. keta* (Hirano et al. 1978). This indicates that the principal buffer of *O. tshawytscha* ovarian fluid is the CO_2-bicarbonate-carbonate system, as suggested by the relatively low concentrations of other potential buffers such as phosphate and proteins (Ingermann et al. 2002a). These ions were not measured in seminal fluid but a similar discrepancy between the concentrations of cations and chloride suggests that it was also buffered by bicarbonate.

The ovarian fluid was distinctly alkaline, a property noted in several other salmonids and in cyprinids (Hirano et al. 1978; Lahnsteiner et al. 1995, 2001; Wojtczak et al. 2007). Lahnsteiner et al. (1995) highlighted the importance of alkalinity of the ovarian fluid

under natural spawning conditions, as this would tend to stabilise the micro-environment around the egg, especially in acidic waters. Importantly, pH of the ovarian fluid in our fish was dependent on the partial pressure of dissolved carbon dioxide, rising sharply as $P\text{co}_2$ decreased (Table 5.2). Therefore, unless efforts are taken to control $P\text{co}_2$, loss of CO_2 to the atmosphere during sample processing could increase the apparent variability of the pH of salmonid ovarian fluids (e.g. Wojtczak et al. 2007). Aegerter and Jalabert (2004) noted large changes in the pH of *O. mykiss* ovarian fluid stored in contact with the air and cautioned against the use of pH as an egg quality indicator unless measurement protocols are carefully standardised. The CO_2 dependence of ovarian fluid pH (Table 5.2) indicates that its mean $P\text{co}_2$ soon after collection was about 0.7 kPa, which approximates the venous $P\text{co}_2$ of salmonids (Stevens and Randall 1967).

Carbonates buffer poorly between pH 7.5 and 8.0, consistent with observations of the buffer capacity of *O. tshawytscha* ovarian fluid (Ingermann et al. 2002a). However, the $[HCO_3^-]/[CO_3^{2-}]$ equilibrium would buffer effectively between pH 8.5 and 9.0 where salmonid sperm motility is maximal (Wojtczak et al. 2007). Diffusion of CO_2 from these fluids into air during artificial fertilisation would tend to elevate pH into this range.

The ovarian fluid was more viscous than water, as has been noted for the ovarian fluid of other salmonids (Hirano et al. 1978; Turner and Montgomerie 2002). High viscosity might impede dislodgement of the eggs from the redd by flowing water (McDowell 2000), and would maintain high ionic concentrations close to the egg surface. Additionally, it would provide low shear, laminar flow conditions adjacent to the eggs, properties which have been shown to be necessary for successful fertilisation in another external fertiliser, the red abalone (*Haliotis rufescens*) (Riffell and Zimmer 2007). Viscosity was markedly shear rate dependent (Fig. 5.1) and was highly variable among individuals (range 3.2 – 6.9 cps at 45 s^{-1} for the 16 fish represented by Fig. 5.1). Lauga (2007) pointed out that the rheological properties of non-Newtonian fluids theoretically can be tuned to allow selection of appropriately motile spermatozoa and proposed such a mechanism within the cervical mucus of the female reproductive tract. Possibly rheological differences among individual fish ovarian fluids likewise contribute to

differential fertilization success and perhaps could provide a basis for cryptic female mate choice.

The ovarian fluid is nearly iso-osmotic to seminal fluid, and thus to the sperm cells (Table 5.1; Lahnsteiner et al. 1995). Thus activation in an ovarian fluid solution is believed to prolong sperm motility compared to activation in fresh water, despite an apparent requirement for higher energy expenditure in the more viscous medium (Hirai et al. 1997). Turner and Montgomerie (2004) suggested that components of the ovarian fluid might also influence ATP metabolism such that the rate and duration of energy production are increased, but this deserves further study.

The ionic composition of the ovarian fluid varied among individual females with the largest relative differences being observed in [K^+], [Ca^{2+}] and [Mg^{2+}] (see ranges in Table 5.1). Lahnsteiner et al. (1995) also found considerable intraspecific variation in the composition of the ovarian fluid in four salmonid species. Intraspecific variation in ovarian fluid composition could result partly from variation in post-ovulatory maturation within the coelomic cavity, in the physiological status of the female, and in egg quality (Lahnsteiner et al. 1999, 2000). Aegerter and Jalabert (2004) observed progressive changes in the osmolality, pH and protein concentration of ovarian fluid following ovulation in *O. mykiss.* Correspondingly, we noted significant differences in the ionic composition of *O. tshawytscha* ovarian fluid collected at different periods during the spawning season. Such seasonal variation could be due to changes in a female's physiological status over the spawning season, along with changes in egg quality and maturity which will all affect the composition of the ovarian fluid (Lahnsteiner et al. 1999; Lahnsteiner 2000; Aegerter and Jalabert 2004). Such variation in the quality of a female's ovarian fluid could be expected to affect sperm motility, fertilisation success and, perhaps, the development and survival of embryos.

Seminal fluid [K^+] was ten times that of ovarian fluid, but the [Na^+] of seminal fluid was only two thirds that of ovarian fluid, in agreement with previous research in salmonids (Scott and Baynes 1980). High [K^+]/[Na^+] ratio in the seminal fluid is expected as this

inhibits sperm motility in the reproductive tract (Billard 1986; Billard et al. 1995; Morisawa et al. 1983). The [Ca^{2+}] of seminal fluid was less than one fifth that in ovarian fluid, possibly reflecting calcium bound to protein at higher concentration in the latter (Ingermann et al. 2002a). Seminal fluid osmolality was more variable than that of ovarian fluid and, on average, was hypo-osmotic to ovarian fluid and blood plasma. Correlations of seminal fluid osmolality with [Na^{+}], [K^{+}] and [Cl^{-}] (but not with [Ca^{2+}] or [Mg^{2+}]) could indicate contamination with dilute urine from the adjacent urinary bladder (Holmes and Stainer 1966; Wood et al. 1999) although care was taken to avoid this. However, similarly variable osmolality and ion concentrations were observed in *O. mykiss* seminal fluid when urine contamination was rigorously excluded (Lahnsteiner et al. 1998). Ion reabsorption in the sperm ducts is therefore a possible explanation of the hypotonicity. As fertilisation success is markedly dependent on seminal fluid electrolytes and pH (Lahnsteiner et al. 1998; Bencic et al. 2000; Ingermann et al. 2002a, b), the possible significance of modifications to the seminal fluid composition prior to spawning also deserve further study.

There were significant negative correlations between the duration of forward motility (DUR) of sperm and the [Ca^{2+}] and [Mg^{2+}] of ovarian fluid from seven females (Fig 5.2b and 5.2c). Conversely, the mean percent of progressively motile sperm (MOT) at 10 s post-activation increased as [Mg^{2+}] in the ovarian fluid increased (Fig. 5.2d). Ovarian fluid [Ca^{2+}] and [Mg^{2+}] were significantly correlated with each other, perhaps reflecting variation in protein concentration. Although positive correlation coefficients for [Ca^{2+}] with swimming speed (VAP) and (MOT) (Table 5.4) were not significant, the sperm from all males swam slowest and exhibited the lowest percent motility in the ovarian fluid of one female that had the lowest [Ca^{2+}] at 2.10 mmol L^{-1}. Wojtezak et al. (2007) found that that the percentage of motile sperm, as well as the swimming speed and duration of motility of the spermatozoa from a single male rainbow trout were positively correlated with the pH of the ovarian fluid from 31 different females. We did not find these relations to be significant, possibly due to our smaller sample size (n = 7 females) and the narrower pH range observed in ovarian fluid, and the relation between sperm VAP and ovarian fluid pH was negative (Table 5.4).

After correction for an outlier (Fig. 5.2a), there were no statistically significant correlations between sperm motility traits and any component of seminal fluid (Table 5.4). Nonetheless, correlations between VAP and both [Cl-] and [Na+] were both negative and reasonably large, and the small sample size would have resulted in low power to detect a real relation. Kusa (1950) found that a high potassium concentration in the seminal fluid of chum salmon (*O. keta)* inhibited sperm motility resulting in a decrease in fertilisation success, but the correlation with [K+] in our study was small and far from significant (Table 5.4, Fig. 5.2a). The effect of spermatozoa sensitivity to [K^+] in the seminal fluid may also vary through the reproductive season (see Alvai and Cosson 2006). In contrast, Alavi et al. (2004) observed no statistically significant correlations between seminal fluid composition and sperm motility traits for *Acipenser persicus*. There appears to be considerable inter- and intra-specific variability in the ionic composition of seminal fluid in fish (see Alavi and Cosson 2006). This may imply that different ions and ion concentrations are involved in regulating and initiating sperm motility for different fish species (Billard and Cosson 1992; Scott and Baynes 1980).

Recent studies have shown that the ovarian fluids from chinook salmon, rainbow trout and arctic charr of individual females differentially enhance the sperm mobility characteristics of different males (e.g., swimming speed; Urbach et al. 2005, Dietrich et al. 2008, see Chapter 4).Thus differences in ovarian fluid composition provide a mechanism of cryptic female choice, whereby fertilisation is biased towards certain males (Thornhill 1983; Eberhard 1996). The present study lends support to this hypothesis. Intraspecific variations in the ionic composition of seminal and ovarian fluids, and differences in ovarian fluid through the spawning season, are large enough to differentially enhance sperm motility and longevity. However, whether such selection operates during natural mating still remains to be demonstrated.

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5.8 REFERENCES

Aas HG, Refstie T, Gjerde B. 1991. Evaluation of milt quality of Atlantic salmon. Aquaculture. 95:125-135.

Aegerter S, Jalabert, B. 2004. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 231:59-71.

Alavi SMH, Cosson J. 2005. Sperm motility in fishes. I. Effects of temperature and pH: A review. Cell Biol Int. 29:101-110.

Alavi SMH, Cosson J. 2006. Sperm motility in fishes. II. Effects of ions and osmolality: A review. Cell Biol Int. 30:1-14.

Alavi SMH, Cosson J, Karami M, Mojazi Amiri B, Akhoundzadeh MA. 2004. Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: effects of pH, dilution rate, ions and osmolality. Reproduction. 128:819-828.

Bencic DC, Cloud JG, Ingermann RL. 2000. Carbon dioxide reversibly inhibits sperm motility and fertilizing ability in steelhead (*Oncorhynchus mykiss*). Fish Physiol Biochem. 23:275-281.

Billard R. 1986. Spermatogenesis and spermatology of some teleost fish species. Reprod Nutr Dev. 26:877-920.

Billard R, Cosson M. 1992. Some problems related to the assessment of sperm motility in freshwater fish. J Exp Biol. 261:122-131.

Billard RG, Cosson G, Perchec G, Linhart O. 1995. Biology of sperm and artificial reproduction in carp. Aquaculture. 129:95-112.

Boutilier RG, Heming TA, Iwama GK. 1984. Physico-chemical parameters for use in fish respiratory physiology. In: Hoar WS, Randall DJ, editors. Fish Physiology, vol. 10A. Orlando: Academic Press. p. 403-430.

Brokaw CJ, 1966. Effects of increased viscosity on the movements of some invertebrate spermatozoa. J Exp Biol. 45:113-139.

Burness G, Moyes CD, Montgomerie R. 2005. Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochir*us). Comp Biochem Phys. A. 140:11-17.

Cameron JN. 1971. Rapid method for determination of total carbon dioxide in small blood samples. J Appl Physiol. 31:632-634.

Casselman SJ, Schulte-Hostedde AI, Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci. 63: 2119-2125.

Cosson J. 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquacult Int.12:69-85.

Dietrich GJ, Wojczak M, Slowińska M, Dobosz S, Kuźmiński H, Ciereszko A. 2008. Effects of ovarian fluid on motility characteristics of rainbow trout *Oncorhynchus mykiss* Walbaum) spermatozoa. J Appl Ichthyol. 24:503-507.

Dytham C. 2003. Choosing and using statistics. A biologist's guide. 2nd ed. Blackwell Publishing: MA. USA. p.192.

Eberhard WG. 1996. Female control: sexual selection by cryptic female choice. Princeton: Princeton University Press.

El Guendouzi M, Dinane A, Abdelfetah Mounir, A. 2001. Water activities, osmotic and activity coefficients in aqueous chloride solutions at T = 98.15 K by the hygrometric method. J Chem Thermodyn. 33:1059-1072.

Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr Biol. 14:44-47.

Hirai M, Cerbito WA, Wijayagunawardane MPB, Braun J, Leidl W, Ohosaki K, Matsuzawa T, Miyazawa K, Sato K. 1997. The effect of viscosity of semen diluents on motility of bull spermatozoa. Theriogenology. 47:1463-1478.

Hirano T, Morisawa M, Suzuki K. 1978. Changes in plasma and coelomic fluid composition of the mature salmon (*Onchorhynchus keta*) during freshwater adaptation. Comp Biochem Phys A. 61:5-8.

Holmes WN, Stainer IM. 1966. Studies on the renal excretion of electrolytes by the trout (*Salmo gairdneri*). J Exp Biol. 44:33-46.

Holmes WN, Donaldson EM. 1969. The body compartments and the distribution of electrolytes. In: Hoar WS, Randall DJ, editors. Fish Physiology, vol. 1. Academic Press: New York. p. 1-89.

Hwang PC, Idler DR. 1969. A study of major cations, osmotic pressure, and pH in seminal components of Atlantic salmon. J Fish Res Can. 26:413-419.

Ingermann RL, Bencic DC, Gloud JG. 2002a. Low seminal fluid buffering capacity corresponds to high pH sensitivity of sperm motility in salmonids. Fish Physiol. Biochem. 24:299-307.

Ingermann RL, Holcomb M, Robinson ML, Cloud JG. 2002b. Carbon dioxide and pH affect sperm motility of white sturgeon (*Acipenser transmontanus*). J Exp Biol. 205:2885-2890.

Jamieson BGM. 1991. Fish evolution and systematics: evidence from spermatozoa. Cambridge: Cambridge University Press.

Kruger JC, Smith Gl, Van Vuren JHJ, Ferreira JT. 1984. Some chemical and physical characteristics of the semen of *Cyprinus carpio* and *Oreochromis mossambicus*.J Fish Biol. 24:263-272.

Kusa M. 1950. Physiological analysis of fertilization in the egg of the salmon, *Onchorynchus keta*. I. Why are the eggs not fertilized in isotonic Ringer solution. Annot Zool Jpn. 24:22-28.

Lahnsteiner F. 2000. Morphological, physiological and biochemical parameters characterizing the over-ripening of rainbow trout eggs. Fish Physiol Biochem. 23: 107-118.

Lahnsteiner F, Weisman T, Patzner RA. 1995. Composition of the ovarian fluid in 4 salmonid species: *Onchorhynchus mykiss*, *Salmo trutta* f *lacustrus*, *Salvelinus lacustris* and *Hucho hucho*. Reprod Nutr Dev. 35:465-474.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1996. Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiol Biochem.15:167-179.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1997. Sperm motility and seminal fluid composition in the burbot, *Lota lota*. J Appl Ichthyol. 13:113-119.

Lahnsteiner F, Berger B, Weismann T, Patzner RA. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture. 163:163-181.

Lahnsteiner F, Weismann T, Patzner RA. 1999. Physiological and biochemical parameters of egg quality determination in lake trout, *Salmo trutta lacustris*. Fish Physiol Biochem. 20:375-388.

Lahnsteiner F, Urbanyi B, Horvath A, Weismann T. 2001. Biomarkers for egg quality determination in cyprinid fish. Aquaculture. 195:331-352.

Lauga E. 2007. Propulsion in a viscoelastic fluid. Phys. Fluids. 19, 083104 (13 pages).

Leach B, Montgomerie R. 2000. Sperm characteristics associated with different male reproductive tactics in bluegills (*Lepomis macrochirus*). Behav Ecol Sociobiol. 49:31-37.

Liljedal S, Rudolfsen G, Folstad I. 2008. Factors predicting male fertilization success in an external fertilizer. Behav Ecol Sociobiol. 62(1):1805-1811.

Litvak MK, Trippel EA. 1998. Sperm motility pattern of Atlantic cod (*Gadus morhua)* in relation to salinity: effects of ovarian fluid and egg presence. Can J Fish Aquat. Sci. 55:1871-1877.

McDowell RM. 2000. The Reed Field Guide to Freshwater Fishes. Auckland: Reed Books.

Morisawa M. 1994. Cell Signaling Mechanisms for Sperm Motility. Zool Sci. 11:647-662.

Morisawa M, Suzuki K. 1980. Osmolality and potassium ions: their roles in initiation of sperm motility in teleosts. Science 210:1145-1127.

Morisawa M, Suzuki K, Morisawa S. 1983. Effects of potassium and osmolality on spermatozoan motility of salmonid fishes. J Exp Biol. 107:105-113.

Noakes DL, Beamish RJ, Kent ML. 2000. On the decline of Pacific salmon and speculative links to salmon farming in British Columbia. Aquaculture 183:363-386

Riffell JA, Zimmer RK. 2007. Sex and flow: the consequences of fluid shear for sperm–egg interactions. J Exp Biol. 210:3644-3660.

Rowe S, Hutchings JA. 2003. Mating systems and the conservation of commercially exploited marine fish. Trends Ecol Evol. 18(11): 567-572.

Roy RN, Roy LN, Vogel KM, Vogel KM, Porter-Moore C, Pearson T, Good CE, Millero FJ, Campbell DM. 1993. The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperatures 0 to 45°C. Mar Chem. 44:249-267.

Rudolfsen G, Figenschou L, Folstad I, Kleven O. 2008. Sperm velocity influences paternity in the Atlantic cod (*Gadus morhua* L.). Aquacult Res. 39:212-216.

Scott AP, Baynes SM. 1980. A review of the biology, handling storage of salmonid spermatozoa. J Fish Biol. 17:707-739.

Stevens DE, Randall DJ. 1967. Changes of gas concentrations in blood and water during moderate swimming activity in rainbow trout. J Exp Biol. 46:329-337.

Stoss J. 1983. Fish gamete preservation and spermatozoan physiology. In: Hoar WS, Randall DJ, Donaldson EM, editors. Fish Physiology, vol. 9b, Orlando: Academic Press. p. 305-350.

Thornhill R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am Nat. 122:765-788.

Turner E, Montgomerie R. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol. 60:1570-1579.

Urbach D, Folstad I, Rudolfsen G. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol Sociobiol. 57:438-444.

Weast RC. 1975. CRC Handbook of Chemistry and Physics. 55th ed. Boca Raton: CRC Press.

Wojtczak M, Dietrich GJ, Słowińska M, Dobosz S, Kuźmiński H, Ciereszko A. 2007. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Aquaculture. 270:259-264.

Wood CM, Milligan CL, Walsh PJ. 1999. Renal responses of trout to chronic respiratory and metabolic acidoses and metabolic alkalosis. Am J Physiol. 277 (Regulatory Integrative Comp. Physiol.) 46:R482–R492.

Yoshida T, Nomura M. 1972. A substance enhancing sperm motility in the ovarian fluid of the brown trout. B Jpn Soc Sci Fish. 30:1073.

Chapter 6

GENERAL DISCUSSION

6.1 DISCUSSION

Ejaculates have multiple components that function to ensure male fertility and paternity by out-competition sperm from other males (Birkhead & Møller 1998; Evans and Simmons 2008), and on the whole, these male traits are poorly studied in many species. In this thesis, I examined ejaculate traits via computer-assisted sperm analysis (CASA) within a population of chinook salmon —a species introduced to New Zealand a little over 100 years ago. In general, as studies of other species have found (Froman et al. 1999; Levitan 2000; Morrow and Gage 2001a; Gage et al. 2002; Simmons and Kotiaho 2002; Gage and Freckleton 2003; Gage et al. 2004; Schulte-Hostedde and Millar 2004) a considerable amount of intraspecific variation existed in all ejaculate traits measured (Chapter 2 and 5). This result comes as no surprise as species typically experiencing intense postcopulatory sexual selection demonstrate a considerable amount of variation in ejaculate traits (Ward 1998; Morrow and Gage 2001b; Schulte-Hostedde and Millar 2004; Malo et al. 2006; Stoltz and Neff 2006).

Sperm traits varied considerably among males, and varied depending on the activating medium, as spermatozoa swim faster, for longer and in a straighter path trajectory when activated in ovarian fluid, compared to the same sperm traits activated in fresh water (Chapter 3). Most importantly, the pattern of within-male variation in these sperm traits also varied among males in response to different females' ovarian fluid (Chapter 4). This result suggests that in this species we have revealed a potential mechanism for cryptic female choice (CFC) — ovarian fluid (Chapter 4). In addition, we found that the chemical composition of the ovarian fluid varied among females, which could provide a chemical basis for CFC (Chapter 5). The question as to whether or not female animals can influence the fertilisation success of competing ejaculates has of late attracted broad

interest among evolutionary biologists (Birkhead 2000; Pitnick and Brown 2000; Birkhead and Pizzari 2002). Having discovered that ovarian fluid is a possible mechanism for CFC in an externally fertilising species has implications in the field of sexual selection, particularly with respect to predicting the outcome of sperm competition.

In chapter 2, our results serve to highlight the complexity of ejaculate traits and question the theoretical predictions of sperm competition (Parker 1993; Ball and Parker 1996). An assumption underlying sexual selection theory is that male traits associated with sperm competition should be costly to produce (Andersson 1994), and that there is a limit on resources available for reproduction (Dewsbury 1982; Olsson and Madsen 1998). Consequently, trade-offs are expected between ejaculate traits, either between different measures of sperm quality (e.g. sperm longevity, or swimming speed) or sperm quality and number (Parker 1993; Ball and Parker 1996; Moore et al. 2004). However, in this thesis (Chapter 2), the only significant relations between ejaculate traits (apart from positive relations between velocity parameters which is expected; Chapter 2, Table 2.2) was a positive relation between sperm longevity and sperm swimming speed which is the complete opposite from what is predicted (Table 2.2 and Fig. 2.1, Chapter 2).

In following with the theme that ejaculate traits are costly to produce (Dewsbury 1982; Olsson and Madsen 1998), we did reveal a trend in our data that male ejaculate traits that could potentially be under selection via sperm competition; sperm swimming speed (VAP), longevity (DUR) and sperm swimming path (LIN), may depend on body condition (Chapter 2). There were negative relations between body condition and VAP, LIN and DUR (Chapter 2, Fig. 2.4, Table 2.3). A similar pattern between body condition and sperm traits has also been observed in Alpine whitefish (Urbach et al. 2007) and in lake whitefish (Casselman and Montgomerie 2004). Potentially, only those males in good body condition prior to spawning are able to allocate more energy resources to produce high quality ejaculates, and this may come at a cost of reduced body condition at the time of spawning (Casselman and Montgomerie 2004; Urbach et al. 2007). Therefore, the observed negative relations between sperm traits and body condition are a consequence,

rather than a cause of variation in sperm traits (Casselman and Montgomerie 2004). The results presented here are based only on correlation analysis, consequently a cause an effect cannot be assigned with total confidence. Though, additional experimental work involving the manipulation of body condition via diet and effects on sperm traits, as suggested by Burness et al. (2008) would prove valuable.

The investment into male reproduction as represented by relative testes mass was dependent on male body condition, as males in good condition had relatively larger testes (Chapter 2). A similar result has been observed in another fish species, the lake whitefish (Burness et al. 2008). In this research, there was no indication for what the reproductive benefit would be for males with relatively larger sized testes, as these males did not have superior ejaculates or ejaculates with a higher density of sperm cells (Chapter 2, Fig 2.3). This was an interesting result as previous studies have shown that larger testes are typically associated with higher sperm production (Olar et al. 1983; Møller 1989; Gage 1994; Schärer et al. 2008) and relative testes size has often correlated with investment into sperm production, and has been used as a standard measure of investment into sperm production and the number of sperm present in each ejaculate (Andersson 1994; Gomendio et al. 1998; Wedell et al. 2002; Malo 2005). Only a handful of studies have tested whether traits that may be involved in sperm competition depend on male body condition (Droney 1998; Simmons and Kotiaho 2002; Casselman and Montgomerie 2004; Schulte- Hostedde and Millar 2004; Schulte-Hostedde et al. 2005; Wirtz and Steinman 2006; Urbach et al. 2007; Burness et al. 2008), signifying that further research into the effects of body condition on sperm traits is required.

In the following chapter, I measured and compared sperm traits in fresh water and a solution containing 50% ovarian fluid (Chapter 3). It became clear that the maternally derived ovarian fluid, which is expelled with the egg batch during spawning, plays a significant role in influencing the behaviour of sperm. All sperm traits measured were enhanced when activated in a solution containing 50% ovarian fluid compared to the same traits measured in fresh water (Chapter 3). For example, sperm longevity increased fourfold when activated in 50% ovarian fluid solution compared to fresh water (Chapter

3). These results come as no surprise as previous studies in other fish species, including salmonids, have also documented the positive effect that ovarian fluid has on sperm behaviour (Litvak and Trippel 1998; Lahnsteiner 2002; Turner and Montgomerie 2002; Elofsson 2003; Woolsey et al 2006; Dietrich et al. 2008).

However, it was surprising to observe that there were no significant relations when comparing sperm traits in water versus ovarian fluid, apart from a weak positive correlation between sperm swimming speed (VAP) 10 s post-activation (Chapter 3). This implies that sperm traits measured in water are not relevant to those same traits measured during normal spawning, whereby a certain amount of ovarian fluid will be present. Moreover, sperm traits can be measured more reliably when measured in a solution containing ovarian fluid (Chapter 3). These results suggest that interpretation of sperm swimming speed, pattern of motility and sperm longevity in essentially all previous studies may have been underestimated if the activating solution does not contain ovarian fluid (e.g., in Atlantic salmon, Gage et al. 2004; Casselman et al. 2006; Atlantic cod, Rudolfsen et al. 2008). Results from this chapter suggest that sperm performance measured in fresh water should be used with caution when these traits are to be used as an index of male quality.

Spermatozoal traits activated in water are commonly used to assess sperm quality. Some of these traits include; sperm velocity (Piironen and Hyvarinen 1983; Kime et al. 2001; Burness et al. 2004; Gage et al. 2004; Rideout et al. 2004), density (Tvedt et al. 2001; Rideout et al. 2004), and proportion of motile sperm (Vladic and Jarvi 2001; Burness, 2004). Ultimately, the true test of sperm quality is the actual ability of the sperm to successfully fertilise an egg. As we have discovered, sperm traits measured in water are quite different from those traits measured in ovarian fluid, therefore, I suggest the routine use of ovarian fluid in activating solutions to more accurately represent natural spawning conditions, in particular under sperm competition conditions. Moreover, the ability to accurately measure and use sperm traits as predictors of male reproductive success would prove useful both for aquaculture and conservation management programmes.

Not only did ovarian fluid enhance sperm traits, I also found evidence in this study to suggest that ovarian fluid could be a mechanism of CFC in this species, as sperm swimming speed, longevity and the pattern of motility varied significantly among males in response to different females' ovarian fluids (Chapter 4). For example, sperm from one male swam up to four times faster activated in one particular female's ovarian fluid, but slower in another female's ovarian fluid (Chapter 4, Fig. 4.1). As sperm velocity is known to be a key determinant of fertilisation success in domestic fowl (Birkhead et al. 1999), salmon (Gage et al. 2004; Yeates et al. 2007) and Arctic charr (Liljedal et al. 2008), this finding suggests that a female's ovarian fluid can differentially alter sperm function and thus potentially influence the reproductive success of one male over another under conditions of sperm competition.

The results from Chapter 4 are supported by research done in Arctic charr (Urbach et al. 2005) rainbow trout (Dietrich et al. 2008) and frogs (Simmons et al. 2009) which also verify the possibility of CFC via ovarian fluid and egg jelly. Most of the research into mechanism of CFC has focused on species with internal fertilisation (Birkhead 1998; 2000; Birkhead and Møller 1998; Pitnick and Brown 2000; Snook 2005), and very little is actually known about CFC in species with external fertilisation (Bishop et al. 1996; Palumbi 1999; Evans and Marshall 2005; Urbach et al. 2005; Simmons et al. 2009). Therefore, the results from this study have added to the paucity of research regarding CFC, and may help to explain the significant differences reported between expected and realised male reproductive success across taxa (Birkhead 1998; Coltman et al. 1999; Gemmell et al. 2001, Kempenaers 2007).

In Chapter 5 we observed a considerable amount of variation in the major inorganic ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), osmolality, and pH of ovarian and seminal fluid of chinook salmon. No significant correlations were observed between the composition of the seminal fluid and sperm traits. However, in the ovarian fluid, sperm longevity was negatively correlated with variation in [Ca^{2+}] and [Mg^{2+}], whereas the percentage of progressively motile cells was positively correlated with [Mg^{2+}]. While this result is suggestive of providing a possible chemical basis for CFC, no explicit tests of the

relationship between seminal and ovarian fluid composition, sperm function and fertilisation success were made.

6.2 FUTURE RESEARCH DIRECTIONS

The strong male x female interaction that ovarian fluid has on sperm behaviour such as; sperm swimming speed, path trajectory, and longevity is suggestive of CFC (Chapter 4), but not necessarily representative of CFC, as it is unknown to what degree the disparity in sperm swimming speed we observed in different ovarian fluids has on fertilisation success. However, our results from Chapter 4 are suggestive that females may be able to use ovarian fluid to bias fertilisation success among different males. For example, we observed that male 1 had the fastest swimming sperm in the ovarian fluid of female 1 (Chapter 4, Fig 4.1) and as sperm swimming speed is a key determinant of reproductive success (Gage et al. 2004; Liljedal et al. 2008), then presumably this male will fertilise the largest proportion of ova compared to the other males that had slower sperm swimming speeds in this female's ovarian fluid. Consequently, further experimental work is required to test that ovarian fluid can be a mechanism of sperm selection enhancing offspring fitness.

A genetic basis for CFC is likely with recent research demonstrating in Atlantic and chinook salmon respectively, as non-random selection for sperm occurs barring like major histocompatibility complex (MHC) class I (Yeates et al. 2009) and dislike MHC class II genotypes (Neff et al. 2008; Consuegra and Garcia de Leaniz 2009). Potentially the observed differences we observe in sperm motility in the ovarian fluids from different females may be due to CFC selection for compatible genotypes involving MHC class I and II loci, to ensure optimally fit offspring.

To determine if ovarian fluid can in fact mediate sperm selection, for example, favouring genetic combinations to enhance offspring fitness, would require further experimental work to test the following (i) the effects of a given female's ovarian fluid on a male's sperm traits, (ii) how the male x female interaction influences male reproductive success,

(iii) whether a particular male and female genotype combinations (MHC class I, II and multi-locus heterozygosity) are associated with less or greater sperm performance and hence male reproductive success, and (iv) whether any compositional difference among ovarian and seminal fluid might explain the differential function and fertilisation success. In addition, activating and recording sperm function in ovarian fluid that has had the chemical composition manipulated, may provide some insight into the mechanism whereby ovarian fluid can enhance or impair a male's sperm function.

6.3 CONSERVATION AND STOCK MANAGEMENT IMPLICATIONS: THE USE OF CASA AS A REPRODUCTIVE ASSESSMENT TOOL

Many salmonid populations are economically important and are routinely propagated in hatchery programs for conservation and/or harvest. As sperm velocity has been identified as the primary sperm trait predicting male fertilisation success in Atlantic salmon (Gage et al. 2004), Atlantic cod (Rudolfsen et al. 2008) and in bluegill (Burness et al. 2004) the assessment of this sperm trait using computer-assisted sperm analysis (CASA) will provide information that can be used as part of a reproductive assessment when monitoring sperm quality. As long-term storage of sperm in liquid nitrogen is a common technique used for genetic preservation, the evaluation of sperm motility and velocity using CASA post-thaw will aid in assessing sperm fertilisation capability. Furthermore, the use of CASA as a streamside assessment of sperm quality, will facilitate the optimization of breeding programmes, husbandry conditions, along with collection and storage methodologies of gametes (Rurangwa et al. 2004). Additionally, in light of our findings regarding the effects that ovarian fluid has on sperm behaviour (Chapter 2 and 4), the assessment of sperm traits from males activated in ovarian fluid from certain females, may be vital prior to the set-up of artificial fertilisation for breeding programmes.

In conclusion, results from this research draws attention to the complexity of ejaculate traits, and the fact that we still know very little about factors influencing them. As reproduction is the foundation on which a species survives, obtaining detailed knowledge of reproduction is fundamental to species conservation. Unfortunately though, there is a

general lack of detailed study in regards to ejaculate traits and influences on these traits in fish species. Detailed knowledge of male ejaculate traits will become increasingly important as fish population stocks worldwide (Myers and Worm 2003; Rowe and Hutchings 2003), including salmon populations (Fisher 1994; Nemeth and Kiefer 1999; Regetz 2003) are declining due to a combination of over-exploitation, pollution, climate change and freshwater habitat destruction (Noakes et al. 2000; Gewin 2004; Myers and Worm 2005; Cheek 2006).

Importantly, this research has highlighted the important processes that can occur after copulation the influence ejaculate traits; sperm competition and CFC. We have revealed that ovarian fluid may be a mechanism of CFC in this species whereby females may have the ability to influence the outcome of sperm competition via sperm selection. This exciting result leads the way for further experimental investigation to uncover the proximate and ultimate causes of CFC in an externally fertilising species.

6.4 REFERENCES

Andersson M. 1994. Sexual selection. Princeton: Princeton University Press.

Ball MA, Parker GA. 1996. Sperm competition games: external fertilization and ‘‘adaptive’’ infertility. J Theor Biol. 80:141-150.

Birkhead TR. 1998. Cryptic female choice: criteria for establishing female sperm choice. Evolution. 52:1212-1218.

Birkhead TR. 2000. Defining and demonstrating postcopulatory female choice—again. Evolution. 54:1057-1060.

Birkhead TR, Pizzari T. 2002. Postcopulatory sexual selection. Nat Rev Genet. 3:262-273.

Birkhead TR, Møller AP. 1998. Sperm competition and Sexual Selection. San Diego:Academic Press.

Birkhead TR, Martínez JG, Burke T, Froman DP. 1999. Sperm mobility predicts outcome of sperm competition in the domestic fowl. Proc R Soc Lond B. 266:1759-1764.

Bishop JDD, Jones CS, Noble LR. 1996. Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum* II. Investigation of male mating success using RAPD markers. Proc R Soc Lond B. 263:401-407.

Burness G, Casselman SJ, Schulte-Hostedde AI, Moyers, CD, Montgomerie R. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). Behav Ecol Sociobiol. 56:65-70.

Burness G, Schulte-Hostedde AI, Montgomerie R. 2008. Body condition influences sperm energetic in lake whitefish (*Coregonus clupeaformis*). Can J Fish Aquat Sci. 65:615-620.

Casselman SJ, Montgomerie R. 2004. Sperm traits in relation to male quality in colonial spawning bluegill. J Fish Biol. 64:1700–1711.

Casselman SJ, Schulte-Hostedde AI, Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci. 63: 2119-2125.

Cheek AO. 2006. Subtle sabotage: endocrine disruption in wild populations. Rev. Biol. Trop. 54(1):1-19.

Coltman DW, Bancroft DR, Roberston A, Smith JA, Clutton-Brock TH, Pemberton JM 1999. Male reproductive success in a promiscuous mammal: behavioural estimates compared with genetic paternity. Mol Ecol. 8:1199-1209.

Consuegra S, Garcia de Leaniz C. 2008. MHC-mediated mate choice increases parasite resistance in salmon. Proc R Soc Lond B. 275:1397-1403.

Dewsbury DA. 1982. Ejaculate cost and male choice. Am Nat. 119:601–610.

Dietrich GJ, Wojczak M, Slowińska M, Dobosz S, Kuźmiński H, Ciereszko A. 2008. Effects of ovarian fluid on motility characteristics of rainbow trout *Oncorhynchus mykiss* Walbaum) spermatozoa. J Appl Ichthyol. 24:503-507.

Droney DC. 1998. The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*. Funct Ecol. 12:920-928.

Elofsson H, McAllister BG, Kime DE, Mayer I, Borg B. 2003. Long lasting stickleback sperm: is ovarian fluid a key to success in fresh water? J Fish Biol. 63:240-253.

Evans JP, Marshall DJ. 2005. Male-by-female interactions influence fertilization success and mediate the benefits of polyandry in the sea urchin *Heliocidaris erythrogramma.* Evolution. 59(1): 106–112.

Evans JP, Simmons LW. 2008. The genetic basis of traits regulating sperm competition and polyandry: can selection favour the evolution of good- and sexy- sperm? Genetica.134:5-19.

Fisher FW. 1994. Past and present status of central valley Chinook salmon. Cons Biol. 8:870–873.

Froman DP, Feltmann AJ, Rhoads ML, Kirby JD. 1999. Sperm mobility: a primary determinant of fertility in the domestic fowl (*Gallus domesticus*). Biol Reprod. 61: 400–405.

Gage MJG. 1994. Association between body size, mating pattern, testis size and sperm lengths across butterflies. Proc R Soc Lond B. 258:247-254.

Gage JG, Freckleton RP. 2003. Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. Proc R Soc Lond B. 270:625-632.

Gage MJG, Macfarlane C, Yeates S, Shackleton R, Parker GA. 2002. Relationships between sperm morphometry and sperm motility in the Atlantic salmon. J Fish Biol. 61:528–1539.

Gage MJG, MacFarlane C, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr Biol. 4: 44-47.

Gemmell NJ, Bur TM, Boy IL, Amos W. 2001. Low reproductive success in territorial male Antarctic fur seals (*Arctocephalus gazella*) suggests the existence of alternative mating strategies. Mol Ecol. 10:451-460.

Gewin V. 2004. Troubled Waters: The Future of Global Fisheries. PLoS Biol. 2(4):0422-0427.

Gomendio M, Harcourt AH, Roldan ERS.1998. Sperm competition in mammals. In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection London: Academic Press p. 667–751.

Kempenaers B. 2007. Mate choice and genetic quality: A review of the heterozygosity theory. Adv Study Behav. 37:189-278.

Lahnsteiner F. 2002. The influence of ovarian fluid on the gamete physiology in the Salmonidae. Fish Physiol Biochem. 27:49-59.

Levitan DR. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. Proc R Soc Lond B. 267: 531-534.

Liljedal S, Rudolfsen G, Folstad I. 2008. Factors predicting male fertilization success in an external fertilizer. Behav Ecol Sociobiol. 62:1805–1811.

Litvak MK, Trippel EA. 1998. Sperm motility pattern of Atlantic cod (*Gadus morhua)* in relation to salinity: effects of ovarian fluid and egg presence. Can J Fish Aquat Sci. 55:1871-1877.

Malo AF, Roldan ERS, Garde J, Soler AJ. 2005. Antlers honestly advertise sperm production and quality. Proc R Soc Lond B. 272:149–157.

Møller AP. 1989. Ejaculate quality, testes size and sperm production in mammals. Funct. Ecol. 3:91–96.

Moore PJ, Harris EW, Montrose TV, Levin D, Moore AJ. 2004. Constraints on evolution and postcopulatory sexual selection: trade-offs among ejaculate characteristics. Evolution. 58:1773–1780.

Morrow EH, Gage MJG. 2001a. Consistent significant variation between individual males in spermatozoal morphometry. J Zool Lond. 254:147-153.

Morrow EH, Gage MJG. 2001. Sperm competition experiments between lines of crickets producing different sperm lengths. Proc Roy Soc Lond B. 268:2281-2286.

Myers RA, Worm B. 2003. Rapid worldwide depletion of predatory fish communities. Nature. 423:280-283.

Myers RA, Worm B. 2005. Extinction, survival or recovery of large predatory fishes. Phil Trans R Soc B. 360:13-20.

Neff BD, Garner SR, Heath JW, Heath DD. 2008. The MHC and non-random mating in a captive population of Chinook salmon. Heredity. 101:175-185.

Nemeth DJ, Kiefer RB. 1999. Snake River and summer Chinook salmon—the choice for recovery. Fisheries. 24:16-23.

Noakes DJ, Beamish RJ, Kent ML. 2000. On the decline of Pacific salmon and speculative links to salmon farming in British Columbia. Aquaculture 183: 363-386.

Olar TT, Amann RP, Pickett BW. 1983. Relationships among testicular size, daily production and output of spermatozoa, and extragonadal spermatozoal reserves of the dog. Biol. Reprod. 29:1114-1120.

Olsson M, Madsen T. 1998. Sexual selection and sperm competition in Reptiles. In: Birkhead TR, Møller AP, editors. Sexual selection and sperm competition. London: Academic Press.

Palumbi SR. 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc Natl Acad Aci USA. 96:12632-12637.

Parker GA. 1993. Sperm competition games: sperm size and sperm number under adult control. Proc R Soc Lond B. 253:245-254.

Parker GA. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. In: Birkhead TR, Møller AP, editors. Sperm competition and sexual selection San Diego: Academic Press. p. 3–54.

Piironen J, Hyvarinen H. 1983. Composition of the milt of some teleost fishes. J Fish Biol. 22:351-361.

Pitnick S, Brown WD. 2000. Criteria for demonstrating female sperm choice. Evolution 54:1052-1056.

Regetz J. 2003. Landscape-level constraints on recruitment of chinook salmon (*Oncorhynchus tshawytscha*) in the Columbia River basin, USA. Aquatic Cons Mar Freshwater Ecosyst. 13:35–49.

Rideout RM, Trippel EA, Litvak MK. 2004. Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. J Fish Biol. 65:319-332.

Rowe S, Hutchings JA. 2003. Mating systems and the conservation of commercially exploited marine fish. Trends Ecol Evol. 18:567-572.

Rudolfsen G, Figenschou L, Folstad I, Kleven O. 2008. Sperm velocity influences paternity in the Atlantic cod (*Gadus morhua* L.). Aquacult. Res. 39:212-216.

Rurangwa E, Kime DE, Ollevier F, Nash JP. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture. 234:1-28.

Schärer L, Da Lage JL, Joly D. 2008. Evolution of testicular architecture in the Drosophilidae: A role for sperm length. BMC Evol Biol. 8:143–143.

Schulte-Hostedde AI, Millar JS. 2004. Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (*Tamias amoenus*): implications for sperm competition and reproductive success. Behav Ecol Sociobiol. 55: 272-277.

Schulte-Hostedde AI, Millar JS, Hickling GJ. 2005. Condition dependence of testis size in small mammals. Evol Ecol Res. 7:143–149.

Simmons LW, Kotiaho JS. 2002. Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. Evolution 56: 1622–1631.

Simmons LW, Roberts JD, Dziminski MA. 2009. Egg jelly influences sperm motility in the externally fertilizing frog, *Crinia georgiana*. J Evol Biol. 22:225-229.

Snook RR. 2005. Sperm in competition: not playing by the numbers. Trends Ecol Evol. 20:46-53.

Stoltz JA, Neff BD. 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. J Evol Biol 19:1873–1881.

Turner E, Montgomerie R. 2002. Ovarian fluid enhances sperm movement in Arctic charr. J Fish Biol. 60:1570-1579.

Tvedt HB, Benfey TJ, Martin-Robichaud DJ, Power J. 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 194:191-200.

Urbach D, Folstad I, Rudolfsen G. 2005. Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*). Behav Ecol and Sociobiol 57:438-444.

Urbach D, Bittner D, Lenz TL, Bernet T, Wahli T, Wedekind C. 2007. Sperm velocity in an Alpine whitefish: effects of age, size, condition, fluctuating asymmetry and gonad abnormalities. J Fish Biol. 71: 672-683.

Vladic TV, Jarvi T. 2001. Sperm quality in the alternative reproductive tactics of Atlantic salmon: the importance of the loaded raffle mechanism. Proc R Soc Lond B. 268:2375–2381.

Ward PI. 1998. Intraspecific variation in sperm size characters. Heredity 80:655–659.

Wedell N, Gage MJ, Parker GA. 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol. 17:313–320.

Wirtz S, Steinmann P. 2006. Sperm characteristics in perch *Perca fluviatilis* L. J Fish Biol. 68:1869-1902.

Woolsey J, Holcomb M, Cloud JG, Ingermann RL, 2006. Sperm motility in the steelhead *Oncorhynchus mykiss* (Walbaum): influence of the composition of the incubation and activation media. Aquacult Res. 37:215-223.

Yeates S, Searle J, Ward RG, Gage MJG. 2007. A two-second delay confers first-male fertilization precedence within in vitro sperm competition experiments in Atlantic salmon. J Fish Biol. 70:318-322.

Yeates SE, Einum S, Fleming IA, Megens H, Stet RJM, Hindar K, Holt WV, Van Look KJW, Gage MJG. 2009. Atlantic salmon eggs favour sperm in competition that have similar major histocompatibility alleles. Proc R Soc Lond B. 276:559-566.

“So long, and thanks for all the fish”

The message left by the dolphins when they departed Planet Earth just before it was demolished to make way for a hyperspatial express route, as described in The Hitchhiker's Guide to the Galaxy by Douglas Adams.