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Sperm competition and the alternative reproductive tactics of Chinook salmon (Oncorhynchus tshawytscha)

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Sperm competition and the alternative reproductive tactics of Chinook salmon

(*Oncorhynchus tshawytscha)*

By

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: my first data chapter was co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Ian Butts. My second data chapter was co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Daniel Heath. In each case, my collaborators provided valuable feedback, helped with the project design and statistical analysis, and provided editorial input during the writing of each manuscript; however, in both cases the primary contributions have all been by the author. Both Chapter Two and Chapter Three have been prepared as manuscripts, and will be submitted to Behavioral Ecology and Sociobiology and Biology Letters for publication, respectively.

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ABSTRACT

Sperm competition is an important determinant of male reproductive success. This thesis examined sperm competition in the context of the alternative reproductive tactics (jacks and hooknoses) of Chinook salmon (*Oncorhyncus tshawytscha)*. I found that jacks had relatively larger gonads and had higher sperm velocity than hooknoses. I also examined competitive fertilization success of the two tactics using a more realistic spawning microenvironment, in the presence of ovarian fluid. I found a significant increase in sperm velocity when activated in ovarian fluid compared to river water for both reproductive tactics and jacks were more successful at siring offspring in sperm competition than hooknoses in water but not in ovarian fluid. I found a significant positive relationship between sperm velocity and competitive fertilization success in water but not ovarian fluid. These results have implications for studies of sperm competition in taxa that do not take into account the female role in reproduction.

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CHAPTER 1: GENERAL INTRODUCTION

Darwin's (1871) theory of sexual selection can be understood as two selection pressures: intrasexual selection, where males typically compete with one another for access to females and intersexual selection, where females typically choose to mate with certain males based on their preference for specific traits. This theory was developed to explain a class of traits known as secondary sexual characteristics including size differences, bright colouration, weapons, elaborate songs and other displays that would in theory be opposed by natural selection because they would reduce the survivorship of the bearer. Although sexual selection was able to explain a lot of the elaborate secondary sexual characters seen in nature, it did not originally anticipate that sexual selection would proceed even after copulation occurred. Darwin's original version of sexual selection theory only considered the behavioural processes taking place prior to copulation, overlooking the events that occur post-copulation (Birkhead 1998). Since Darwin's time, Parker (1970; 1998) identified that males commonly compete with each other post-copulation, where ejaculates from different males may compete for access to a female's eggs, a phenomenon known as sperm competition. Sperm competition is now acknowledged as a dominant selective force responsible for influencing many aspects of male reproductive anatomy, physiology, and behaviour (reviewed in Birkhead and Moller 1998; Simmons 2001; Birkhead et al. 2009). Another key modification to Darwin's theory of sexual selection also occurred when it was realized that females are not passive participants in sperm competition and instead they attempt to bias paternity in favour of certain males using post copulatory processes, collectively known as cryptic female choice (reviewed in Eberhard 1996). The term cryptic female choice describes events

occurring post-copulation that determine the extent to which a female affects a male's reproductive success (Thornhill 1983).

Sperm competition

Sperm competition is known to be one of the main determinants of reproductive success between males. It occurs post-copulation when the ejaculates from multiple males compete for access and fertilization of a given set of ova (Parker 1970). As a result, there are evolutionary forces acting to enhance specific sperm traits in order to attain maximum reproductive fitness (Birkhead and Moller 1998). Males may respond to sperm competition by enhancing the competitive ability of their ejaculates by either increasing sperm number or by augmenting the quality of their sperm. There are two mechanisms that explain these responses to sperm competition: the fair raffle and the loaded raffle (Parker 1990a). Parker et al. (1990) developed a model in order to distinguish between the processes of the fair and loaded raffles.

$$P_2 = \frac{N_2}{N_1 + N_2} = \frac{rS_2}{S_1 + S_2}$$

P_2 is defined as the paternity of the second male, r is the measure of loading, N_1 and N_2 are the number of offspring sired by male 1 and male 2, respectively, and S_1 and S_2 are the numbers of sperm transferred to the female by the first and second male, respectively. When r > 1 the second male has competitively superior sperm while when r = 1 the sperm from each male are competitively equal, and when r < 1 the second male has competitively inferior sperm (see Neff and Wahl 2004)

Sperm competition mechanisms

The 'fair raffle' states that sperm number determines fertilization success and that the male contributing a larger amount of sperm (relative to his competitors) will have higher reproductive success and hence fitness (Parker 1990a). This hypothesis predicts that each sperm has an equal chance of fertilizing the female's ova (i.e. $r = 1$) and that sperm competition will select for males to increase sperm production. For example, across fishes, Stockley (1997) found that many species experiencing sperm competition invest more in spermatogenesis, having higher gonadosomatic indexes and increased sperm density.

In contrast, the 'loaded raffle' hypothesis states that the variation in sperm quality (i.e. $r < 1$ or $r > 1$) is responsible for variation in male competitive fertilization success. This hypothesis predicts that each sperm has an unequal chance of fertilizing the ova and individual sperm quality will give some sperm (e.g. faster sperm) an advantage over others (Parker 1990a). Sperm quality is influenced by several traits, including sperm velocity, motility, longevity, and morphology (Snook 2005). For example, sperm velocity has shown to be primary determinant of competitive fertilization success in Atlantic salmon (*Salmo salar*; Gage et al. 2004). Gage et al. (2004) performed competitive in vitro fertilizations altering only the number of sperm contributed from each male. Gage and colleagues found that sperm number did not play a significant role in competitive fertilization success in this species and that sperm quality (i.e. velocity) was the most important factor in sperm competition success.

Cryptic female choice

Cryptic female choice is another form of post-copulatory sexual selection occurring when a female biases paternity towards a particular male using certain processes or structures after mating with more than one individual (Eberhard 1996). There are many ways in which females can alter a males' chance at fertilization success including sperm transport to storage or fertilization sites, rejection or removal of sperm or mating plugs, remating and offspring production (Eberhand 1996). Most of the work on cryptic female choice has focused on internal fertilizing species (hence the name "cryptic"; the interaction between sperm, eggs and ovarian fluid could not be directly observed), mainly insects, including black field crickets (*Teleogryllus commodus*; Bussiere et al. 2006), arctiid moths (*Utetheisa ornatrix*; Curril and LaMunyon 2006), yellow dung flies (*Scathophaga stercoraria*; Ward et al. 2008) and flour beetles (*Tribolium castaneum*; Bloch Qazi 2003).

More recently, evidence for cryptic female choice has been found in studies focused on the ovarian fluid that accompanies egg release in externally fertilizing fish species. Ovarian fluid has been shown to increase sperm activity compared to sperm activity in water alone (see Table 1.1). Studies where sperm from multiple males was activated with ovarian fluid from multiple females showed that a particular male's sperm activity varied differentially among females (e.g. for some females sperm velocity was faster, with other females sperm velocity was slower) in the rainbow trout (*Oncorhynchus mykiss;* Dietrich et al. 2008), the arctic charr (Urbach et al. 2005) and Chinook salmon *(Oncorhynchus tshawytscha;* Rosengrave et al. 2008). These results suggest ovarian fluid has the potential to be a mechanism of cryptic female choice, although none of the

studies to date have shown that patterns of paternity vary after activation in ovarian fluid versus its absence.

Alternative reproductive tactics

To date, research surrounding the topic of sexual variation has mainly focused on the differences observed between sexes (e.g. sexual dimorphism between male and female birds, Dunn et al. 2001). However, recently more attention has been given to the variation observed within a sex, referred to as alternative reproductive tactics (Gross 1996). Alternative reproductive tactics can be defined as different traits that have been selected in two divergent ways in order to maximize fitness within a sex (reviewed in Brockmann 2001). In general, alternative reproductive tactics are characterized by a discrete, bimodal distribution of traits such as size dimorphisms, colour polymorphisms and behavioural alternatives (reviewed in Brockmann 2001; Taborsky 2008). These alternative reproductive tactics are more commonly found in males and reveal themselves in terms of significant behavioural, physiological, morphological and life history differences (Gross 1996; Taborsky 1998; Knapp and Neff 2008).

Alternative reproductive tactics typically have two types of males: guards and sneaks (Taborsky 1997). Guard males usually have primary access to females by either defending resources attractive to the female or defending the female herself. They achieve this by often having larger body sizes, teeth and/or other morphological structures that help them exclude other males (reviewed in Knapp and Neff 2008). Sneaker males take advantage of the guard males' efforts and employ more covert tactics; they dart into mating events, steal fertilization and then hurry away usually undetected

(although guard males on occasion will catch and kill sneaker males) (Knapp and Neff 2008). These sneaker males often mature precociously and have a much smaller body size compared to guard males (Gross 1996)

Alternative reproductive tactics are common to many taxa, including insects (e.g. Blackenhorn 1994; Simmons et al. 2000), amphibians, (e.g. Hettyey and Roberts 2006; Castellano et al. 2009) and birds (e.g. Lank 1995; Widemo 1998; Tuttle 2003). In particular, the sneak/guard alternative reproductive tactic complex is very common in fish (e.g. Gage et al. 1995; Neff et al. 2003). It is hypothesized that alternative reproductive tactics are more common in fishes compared to other taxa because of three factors (outlined in Knapp and Neff 2008). First, the majority of fishes employ external fertilization, creating more opportunities for disfavored males to access female eggs covertly via sneak fertilizations and consequently generating more opportunities for sperm competition (Taborsky 2008). Second, fishes have indeterminate growth that can cause an immense variation in body size, selecting for divergence in reproductive tactics (Taborsky 2008). Third, fishes exhibit a large distribution of parental care roles (e.g. no parental care, shared care, uniparental) creating opportunities for males to take advantage of this variation by adopting alternative reproductive tactics (Knapp and Neff 2008).

Sneak/guard hypothesis

Parker (1990b; 1998) developed the sneak/guard hypothesis of sperm competition recognizing that many mating systems have asymmetries in their sperm competition risk and the probability of encountering sperm competition. The sneak/guard hypothesis assumes that sneaker males participate in a small proportion of matings and therefore

face sperm competition each time they mate. In contrast, guard males face sperm competition in only a portion of their matings but it is not possible for them to predict when competition will occur. The sneak/guard hypothesis posits that the strategy used by each reproductive tactic will reflect their respective sperm competition risk. Having a guaranteed risk, sneaker males are predicted to invest relatively more energy into spermatogenesis compared to guard males. Whereas guard males are predicted to expend the majority of their reproductive effort on secondary sexual characteristics, monopolizing resources that attract females (or monopolize females themselves) in order to reduce sperm competition (Parker 1990b). Overall, the sneak/guard hypothesis predicts that the sneak reproductive tactic will invest more in competition related traits (e.g. larger gonads to produce more sperm and sperm quality metrics related to competitive fertilization success) because of the certainty of sperm competition risk (Parker 1998; Table 1.2).

Chinook salmon

The Chinook salmon is a large, externally fertilizing fish exhibiting an anadromous and semelparous mating system (Healey 1991). Chinook salmon are found along the pacific coast of North America (from California to Alaska), in parts of Asia (from the Anadyr River to Amur river in Russia) and in the Great Lakes (Major et al. 1978; Urawa et al. 1998; Crawford 2001). Chinook salmon reach sexual maturity between the ages of 2-4 years and the season (fall or spring) during which they spawn will vary depending on which river system they spawn in (Healey 1991). Upon returning to their natal stream, guard males attempt to achieve superior spawning positions by competing for access to females. The guard males are referred to as 'hooknoses' (because

of their hooked kype (snout), sensu Gross 1985) and possess secondary sexual characteristics such as large body size and a hump on their backs (Fleming and Reynolds 2004) (see Figure 1.1a). Sperm competition is intense in this species, as a sneaking alternative reproductive tactic exists. The smaller precocious sneaker males are called 'jacks' (Heath et al.1994) (see Figure 1.1b). Jacks' smaller size allows them to elude aggressive hooknoses by swimming under a spawning female while she is releasing eggs (Fleming and Reynolds, 2004). Both types of alternative reproductive male tactics provide only their sperm (i.e. genes) and no parental care (or other resources) to females (Healey 1991).

Spawning usually occurs at the head of a riffle in about a meter of water because of the high subsurface flow that occurs there (Chapman 1943). Chinook salmon have the largest eggs of all salmonidae and therefore their eggs are more sensitive to oxygen levels in the water (Rounsefell 1957). After choosing a location for oviposition (releasing of eggs), female Chinook dig depressions in the gravel of the stream floor using oscillating movements with their tails. The females deposit groups of eggs in these depressions and then cover them with gravel once males fertilize them with their sperm. This process is repeated four to five times upstream comprising one single redd (Berejikian et al. 2000). The alternative reproductive tactics in this species are fixed. Upon the completion of spawning both jacks and hooknoses undergo rapid senescence leading to death, leaving no possibility for a change in reproductive tactic (Heath et al. 1994)

Overview of the thesis

The objective of my thesis was to investigate investment patterns and sperm competition between the alternative reproductive life histories of the Chinook salmon. Chapter two focuses on the reproductive investment patterns of the two alternative reproductive tactics in Chinook salmon (jacks and hooknoses) by assessing gonadal investment and sperm quality traits. In chapter three, I perform competitive in vitro fertilization trials and paternity analyses (using microsatellites) in river water to explore which sperm traits are important determinants of competitive fertilization success in this species. I also replicated the in vitro competitive fertilization trials and paternity analyses using a solution of female ovarian fluid to more accurately represent the spawning environment and investigate whether the presence of ovarian fluid affects the outcome of sperm competition between the alternative reproductive tactics.

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Table 1.1 Summary of the existing literature examining the effects of ovarian fluid on sperm traits in fishes. A plus sign (+) indicates that the sperm trait was significantly increased in value; a minus sign (-) indicates that the sperm trait was significantly decreased in value; and a zero (0) indicated that there was no significant effect on the sperm trait. Blank spaces indicate that the trait was not examined. Velocity is the distance at which the sperm swim per unit of time. Motility is the percentage of sperm in movement a short time after activation. Longevity is measure as the time at which 95 percent of sperm are no longer motile.

Common Name	Genus species	Velocity	Motility	Longevity	References*
Arctic charr	*Salvelinus alpinus*	+	+	+	1
Atlantic cod	*Gadus morhua*	+	+		2
Rainbow trout	*Oncorhynchus mykiss*	[a]+, [b]+	[a]0, [b]+	[b]+	[a]3, [b]4
Brown trout	*Salmo trutta*		[a]+	[a]+, [b]+	[a]5, [b]6
Chinook salmon	*Oncorhynchus tshawytscha*	+	+	+	7
Three-spined stickleback	*Gasterosteus aculeatus*	+	+	+	8
Fifteen-spined stickleback	*Spinachia spinachia*	0	0	0	9

*1 (Turner and Montgomerie 2002), 2 (Litvak and Trippel 1998), 3 (Dietrich et al. 2008), 4 (Wojtczak et al. 2007), 5 (Hatef et al. 2009), 6 (Lahnsteiner 2002), 7 (Rosengrave et al. 2009), 8 (Elofsson et al. 2003a), 9 (Elofsson et al. 2003b)

Table 1.2 Summary of the existing literature that examines reproductive investment between the alternative reproductive tactics of various taxa.

Common Name	Genus species	Tactic	Reproductive investment	References
Fishes				
Atlantic salmon	*Salmo salar*	Anadromous (guard) Parr (sneak)	GSI: S>G Density: S>G Morphology: NS Longevity: S>G Motility: S>G	Gage et al. 1995
Atlantic salmon	*Salmo salar*	Anadromous (guard) Parr (sneak)	GSI: S>G Density: S>G Velocity: NS Longevity: S>G Motility: S>G	Vladic and Jarvi 2001
Atlantic salmon	*Salmo salar*	Anadromous (guard) Parr (sneak)	Morphology: NS	Vladic et al. 2002
Arctic charr	*Salvelinus alpinus*	Dominant (guard) Subordinate (sneak)	Density: S>G Velocity: S>G Motility: NS	Rudolfsen et al. 2006
Arctic charr	*Salvelinus alpinus*	Dominant (guard) Subordinate (sneak)	Velocity: S>G	Serrano et al. 2006
Arctic charr	*Salvelinus alpinus*	Dominant (guard) Subordinate (sneak)	Velocity: S>G	Haugland et al. 2009

Bluegill sunfish	*Lepomis macrochirus*	Parental (guard) Satellite (sneak) Sneaker	Density: S>G Morphology: NS Velocity: NS Motility: NS	Leach and Montgomerie 2000
Bluegill sunfish	*Lepomis macrochirus*	Parental (guard) Satellite (sneak) Sneaker	GSI: S>G Density: S>G Longevity: G>S	Neff et al. 2003
Bluegill sunfish	*Lepomis macrochirus*	Parental (guard) Satellite (sneak) Sneaker	Morphology: S>G Velocity: S>G Longevity: G>S	Burness et al. 2004
Mediterranean wrasse	*Symphodus acellatu*	Nesting (guard) Satellite (sneak) Sneaker	Density: S>G	Alonzo and Warner 2000
Three-spined stickleback	*Gasterosteus aculeatus*	Territorial (guard) Nonterritorial (sneak)	GSI: G>S Velocity: NS Motility: NS	Cote et al. 2009
Combtooth blenny	*Scartella cristata*	Nester (guard) Hole-dweller (guard) Sneaker	GSI: S>G	Neat et al. 2003
Round goby	*Apollonia melanostoma*	Dark morph (guard) Light morph (sneak)	GSI: S>G Density: S>G Morphology: NS Velocity: NS	Marentette et al. 2009

Grass goby	*Zosterisessor aphiocephalus*	Territorial (guard) Sneaker	Morphology: NS Velocity: NS Motility: NS	Locatello et al. 2007
Black goby	*Gobius niger*	Territorial (guard) Sneaker	Morphology: NS Velocity: S>G Motility: S>G	Locatello et al. 2007
Pygmy swordtail	*Xiphophorus nigrenis*	Large (guard) Small (guard)	GSI: NS Morphology: S>G Velocity: NS	Smith and Ryan 2010
Shell brooding cichlid	*Telmatochromis vittatus*	Pirate (guard) Territorial (guard) Satellite (sneak) Sneaker	Morphology: NS Velocity: S>G	Fitzpatrick et al. 2007
Shell brooding cichlid	*Lamprologus callipterus*	Nest male (guard) Dwarf male (sneak)	GSI: S>G	Schutz et al. 2010
Mammal				
Common shrew	*Sorex araneus*	Long ranging (guard) Short ranging (sneak)	Density: S>G GSI: NS	Stockley et al. 1994
Insect				
Dung beetle	*Onthophagus binodis*	Major (guard) Minor (sneak)	GSI: S>G Morphology: S>G	Simmons et al. 1999
Dung beetle	*Onthophagus taurus*	Major (guard) Minor (sneak)	GSI: NS Morphology: NS	Simmons et al. 1999

Wellington tree weta	*Hemideina crassidens*	Large (guard) Small (sneak)	GSI: NS Density: S>G	Clint 2008
Burrowing bee	*Hymenoptera anthophorini*	Major (guard) Minor (sneak)	GSI: NS	Simmons et al 2000
Amphibian				
Dragon lizard	*Ctenophorus pictus*	Red male (guard) Yellow male (sneak)	GSI: S>G	Olsson et al. 2009
Quacking frog	*Crinia georgiana*	Large (guard) Small (sneak)	Density: NS Morphology: NS Velocity: NS Longevity: NS	Hettyey and Roberts 2006

Figure Captions

Figure 1.1 Picture showing **(**a) a typical hooknose male Chinook salmon (*Oncorhynchus tshawytscha*) exhibiting secondary sexual characteristics such as a hooked kype (i.e. snout), large body size and humped back and (b) a typical jack male Chinook salmon exhibiting their smaller body size and female-like coloration.

Figure 1.1

A)

B)

CHAPTER 2: ALTERNATIVE REPRODUCTIVE TACTICS AND SPERM INVESTMENT PATTERNS IN THE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)[1]

SYNOPSIS

Alternative reproductive tactics are found in males of many taxa, however, little is known about how the different reproductive tactics adapt to sperm competition risk. The sneak/guard hypothesis of sperm competition was developed to describe the differences in sperm investment patterns that are observed in males with alternative reproductive tactics. In this study, we tested a prediction of the sneak/guard hypothesis, stating that sneaker males will have greater ejaculate expenditure than guard males as a result of higher competition intensity. We examined the reproductive investment strategies of hooknose (guard males) and jack (sneaker males) Chinook salmon (*Oncorhynchus tshawytscha*) in terms of gonadosomatic index, sperm velocity, sperm motility, sperm morphology, sperm longevity, and sperm density. We found that jacks had a significantly higher gonadosomatic index than hooknoses, sperm velocity varied significantly between the alternative reproductive tactics, with jacks having significantly faster sperm than hooknoses and sperm motility, longevity, density and head morphology all tended to be greater (albeit not significantly) in jacks compared to hooknoses. We interpret these results in light of the sneak/guard model of sperm competition that is based on differences in sperm competition risk.

[1] This chapter is the product of joint research with Dr. Trevor Pitcher and Dr. Ian Butts

INTRODUCTION

Sperm competition occurs when sperm from two or more males simultaneously compete to fertilize a set of female ova (Parker 1970). This form of competition is prevalent in nature and has been observed across a variety of taxa (reviewed in Birkhead and Moller 1998; Birkhead et al. 2009). In species where sperm competition occurs, males, with poor access to females (e.g., males with a smaller body size) may be selected upon for the development of different reproductive traits in order to maximize fitness (Taborsky 1998; 2008). Divergence in these reproductive traits can result in the evolution of alternative reproductive tactics and can manifest itself as size, structure, or color polymorphisms, as well as behavioural alternatives (Brockmann 2001). Of all vertebrates, fishes demonstrate the most widespread variability in alternative reproductive tactics for three primary reasons (reviewed in Knapp and Neff 2008). First, the majority of fishes employ external fertilization, creating more opportunities for disfavored males to access female eggs covertly via sneak fertilizations and consequently generating more opportunities for sperm competition (Taborsky 2008). Second, fishes have indeterminate growth, which results in immense variation in body size and consequently selects for divergence in reproductive tactics (Taborsky 1994; 2008). Finally, fishes exhibit a highly variable distribution of parental care roles creating opportunities for males to take advantage of this variation by adopting alternative tactics (Knapp and Neff 2008). The alternative reproductive tactics found in fish generally consist of two different male morphs. Larger males employ a guarding tactic, which usually involves active courting of females. In contrast, smaller males practice a more covert tactic by sneaking into mating events to steal fertilizations from guard males (Taborsky 1997).

Parker (1990; 1998) proposed the sneak/guard hypothesis of sperm competition to predict the different sperm investment patterns observed in species with alternative reproductive tactics. This hypothesis makes two testable predictions. First, sneaker males will invest relatively more energy in spermatogenesis than guard males because they always face sperm competition risk owing to the fact that they always spawn in the presence of another male. In contrast, guard males will invest relatively less in spermatogenesis because they are allocating more energy to the development of secondary sexual characteristics because they do not always face sperm competition (sneaker males will not necessarily be present for every spawning bout). For example, in bluegill sunfish *Lepomis macrochirus*, cuckolders (i.e. sneaker males) were present in only 10.3% of spawning bouts between females and parental males (i.e. guard males; Fu et al. 2001). Second, the sneak/guard hypothesis predicts that sneakers will sire more offspring per spawning bout than guard males because of their greater investment into spermatogenesis, resulting in sneaker males possessing faster sperm or more sperm. In the bluegill sunfish, Fu et al. (2001) used paternity analyses to show that sneaker males fertilized 78% of the eggs in a bout when in competition with guard males. This outcome is likely a result of the fact that increased sperm competition has selected for greater investment in sperm related traits in sneaker males. For example, in Atlantic salmon (*Salmo salar)*, precocious parr (i.e. sneaker males) invested relatively more in their testes; sperm density was greater, their sperm lived longer and were more motile than anadromous males (i.e. guard males) (Gage et al. 1995; Vladic and Jarvi 2001).

Chinook salmon have large guard-type males known as “hooknoses” (derived from the overstated kype, which develops at maturity), and small precocious sneaker

males known as “jacks” (Heath et al. 1994). Chinook salmon are external fertilizers, semelparous and exhibit a promiscuous non-resource based mating system. Mating occurs seasonally in streams; females compete for oviposition sites in order to dig nests (redds) using an oscillating motion with their tails (Healey 1991). Once the nest is complete the females will deposit their eggs in a series of nests comprising a single redd (Berejikian et al. 2000). Males provide only their sperm (i.e. genes) and no parental care to offspring (or material benefits to the female). Hooknoses mature after several years of leaving their natal streams (age-3 and age-4, Berejikian et al. 2010) and are characterized by a larger body size and hooked snout (Fleming and Reynolds 2004). Hooknoses have primary access to females, enter the nesting area first during spawning events, exhibit courtship behaviours, and chase off other males that come near spawning females. Jacks develop precociously and reach sexual maturity after a year of leaving their natal stream (age-2, Berejikian et al. 2010). Jacks have been observed to have similar colouration to females during spawning (Berejikian et al. 2010; Pitcher, T.E. unpublished data), hold positions upstream (or downstream) of the courting pair, are often chased off and attacked by hooknoses (occasionally resulting in death for the jack) and sneak into the nesting area from satellite positions when spawning occurs between a female and a hooknose. In a recent study examining Chinook salmon spawning behavior in semi-natural spawning channels containing hooknoses, jacks and females, 40% of the spawning events involved only one hooknose male, while the rest of the spawning events included 2 to 5 males (including both hooknoses and jacks) (Berejikian et al. 2010). In addition, hooknoses had superior access and position during spawning because they entered the

nest first during spawning events (i.e. prior to jacks) and jacks participated almost exclusively in spawning events by sneaking into the nest from satellite positions and sired approximately 20% of all progeny (Berejikian et al. 2010).

In this study, we use Chinook salmon from Lake Ontario to test the sneak/guard hypothesis by examining reproductive investment patterns of their alternative reproductive tactics. We predicted that jacks, who always face sperm competition when spawning occurs, would invest relatively more into spermatogenesis compared to hooknose males. In order to test this prediction, gonad and sperm related traits were measured in both hooknoses and jacks, including their relative testes investment, sperm motility, velocity, longevity, density and morphology.

METHODS

Fish collection and body size measurements

We collected Chinook salmon (n = 45 hooknoses and 19 jacks) during the spawning season (2 to 6 October, 2010) using standard electroshock methods from a winter run in the Credit River (Mississauga, Ontario, Canada, N 43° 35', W 79°42'), which flows into Lake Ontario (see Pitcher and Neff 2006; 2007). Chinook salmon have been stocked in Lake Ontario for over 40 years (Crawford 2001). Chinook salmon were located upstream in turbid water ranging from 2 to 4 feet in depth. Water temperature at the time of collection was ~11°C. We humanely sacrificed the fish and obtained milt samples by applying pressure on the abdomen of each fish. The initial male ejaculate was

discarded and the external urogenital pore was wiped dry to avoid contamination from water, urine, feces, and blood. Milt samples were collected into 532 mL clear Whirl-pak sample bags (Nasco Ltd.) and were placed in coolers for motility, velocity, longevity, density and morphological analyses (see below). Total body mass (± 10 g), testes mass (± 5 g), and fork length (± 1cm) were recorded for each male. Finally, soma mass (body mass – testes mass) was calculated and used to calculate the gonadosomatic index (GSI = testes mass / soma mass), which provides a metric for reproductive investment.

Sperm trait assessment

A milt sample (1.5 µL) from each male was micropipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, MA, USA), covered with a glass coverslip (22 × 22 mm), and activated with 15 µL of 11°C river water (the approximate temperature of the river during spawning), less than four hours after collection. Water temperature was maintained at 11.0 +/- 0.5 °C using a HEC-400 Heat Exchanger, a BC-110 Bionomic Controller and an AS-3001 Stage Cooler (20/20 Technology Inc., Wilmington, NC, USA). Activated sperm were video recorded using a CCD B/W video camera module (XC-ST50, Sony Corporation) at 50Hz vertical frequency, mounted on a microscope (CX41, Olympus) that was equipped with a 10× negative-phase objective (see Pitcher et al. 2009). Video-recordings were analyzed using the HTM-CEROS sperm tracking software package (CEROS version 12, Hamilton Thorne research, Beverly, MA, USA), an objective tool for studying sperm motility in fish (see Kime et al. 2001; Rurangwa et al. 2004). We used the following recording parameters: number of frames = 60; minimum contrast = 11; minimum cell size = 3 pixels. The following parameters were measured for each male's sperm: motility (% of active sperm in the field of view showing propulsive

motility), average path velocity (average velocity on the smoothed cell path), straight-line velocity (average velocity on a straight line between the start and end points of the track), and curvilinear velocity (average velocity on the actual point-to-point track followed by the cell), and motility (% of active sperm in the field of view showing propulsive motility) at five, ten and fifteen seconds post-activation. These estimates correspond to the mean of all motile cells analyzed; that is, for each male, the motility and velocity of each individual sperm cell was measured but the estimate used in our final analyses corresponds to a mean across all individual sperm cells. Results were qualitatively similar for all three sperm velocity estimates and as such we present only results from the straight-line velocity, hereafter sperm velocity. Sperm longevity was estimated as the time from sperm activation until ~95% of the sperm cells within the field of view were no longer exhibiting progressive forward motion (Gage et al. 2004). Two observers (with no knowledge of the male's identities) measured longevity with high repeatability between measures for all of the males examined ($r^2 = 0.91$, $P = 0.001$, $n = 64$). Thus, we use the mean longevity across both observers in all of the analyses.

Sperm density was counted under a Zeiss Axiostar compound microscope at 400× magnification using an improved Neubauer haemocytometer (see Pitcher et al. 2009 for details). Milt (1.5 μL) from each male was first diluted in 500 μL of Cortland's saline solution (7.25 g/L NaCl; 0.38 g/L KCl; 0.47 $MgSO_4 \times 7H_20$; 0.4 g/L $Na_2HPO_4 \times H_20$; 1.0 g/L $NaHCO_3$; 0.22 g/L $MgCl_2$; 1.0 g/L $C_6H_{12}O_6$; adjusted to pH 7.8). To obtain homogenous milt-dilutent solutions, samples were mixed thoroughly using a pipettor. A sample of the sperm suspension (10 μL) was then micropippetted onto a haemocytometer that had been pre-covered with a coverslip. We counted the number of sperm from five

large squares on the haemocytometer. There are 25 of these large squares on the haemocytometer and each of these large squares has 16 smaller squares within it. Sperm were counted in the four large corner squares and the large centre one. The mean number of sperm per large square count (i.e. mean of the 5 counts) was multiplied by 25 (to obtain the mean per 5 x 5 large-square grid), by 10 (the depth of the chamber in um) and then by the initial volume of the sample to estimate the sperm density. Sperm densities are expressed as the total number of sperm per mL of a male's stripped ejaculate.

Sperm morphology

We examined sperm morphology for a subset of jacks and hooknoses (n=14 for each). Sperm morphology smears were prepared by diluting 1.5 µL of milt in a solution composed of Cortland's saline (200 µL) and glutaraldehyde (125 µL; G7526; Sigma-Aldrich, St. Louis, MO). For each male, 5 µL of this sperm solution was then pipetted onto a frosted tip microscope slide and prepared using Kwik-Diff (see Tuset et al. 2008). We prepared three separate smears for each male. The smears were allowed to air dry, then permanently sealed with Permount mounting medium (SP15; Fisher Scientific Inc.) and topped with a coverslip. Digital images of sperm heads and flagella were captured using an Olympus BX51 microscope equipped with an Olympus DP72 digital camera. A total of ~100 sperm heads were measured haphazardly for each smear with an oil immersion objective (100× magnification). Morphometric analyses of sperm heads (width and length) were performed using ImageJ analysis software (V. 1.41; developed by W. Rasband, National Institutes of Health, Bethesda, MD, USA). Sperm head width and length was measured using an ImageJ plug-in (Butts et al. in press). The mean sperm head trait (width or length) value from the three independent smears was used for

statistical analyses. In addition, flagellum length (n = 30 sperm per male) was manually measured from its insertion in the head to the end of the filament using ImageJ analysis software.

Statistical analyses

We used JMP (v.8.0.2; SAS Institute Inc., Cary, NC, U.S.A) and RMA (v. 1.17, http://www.bio.sdsu.edu/pub/andy/rma.html) statistical software to analyze data. Soma mass was a more useful measure than body mass in allometry analyses, particularly because the gonads represented a significant proportion of the total body mass (Tomkins and Simmons 2002). Soma mass and testes mass obtained from each male were log10 transformed; testes masses were regressed onto soma masses using a model II linear regression (i.e. a reduced major axis regression) to estimate the allometric slope (equal to the slope of the regression line, see Stotlz et al. 2005). Following Zar (1996), we used a model II linear regression rather than standard ordinary least regression because both the x and the y-variables are measured with error (Stoltz et al. 2005).

We used independent t-tests to compare gonadosomatic index, sperm longevity, sperm density and sperm morphometrics between the jacks and hooknoses. We used repeated measures ANOVAs to measure temporal changes (i.e. at 5, 10 and 15 seconds post-activation) in sperm activity variables (motility, sperm velocity) between jacks and hooknoses. When a non-significant first-order "reproductive tactic × post-activation time" interaction was detected, the model was re-run with the interaction effect removed and main effects were then re-interpreted. Residuals were tested for normality (Shapiro–Wilk test) and homogeneity of variance (plot of residuals vs. predicted values). Data were

transformed to meet assumptions of normality, and homoscedasticity when necessary. Soma mass, testes mass, sperm density, and sperm velocity estimates were $\log_{10}$ transformed while percentage data (sperm motility and gonadosomatic index) were arcsin square-root transformed. Alpha was set at 0.05 for main effects and interactions. Values are given as means ± SE.

RESULTS

Testes investment

Soma mass (hooknose: 7609.6 ± 289.9 g, jack: 2258.3 ± 108.8 g, $t_{62} = 17.87$, $P < 0.001$; Figure 1) and testes mass (hooknose: 413.3 ± 21.7 g, jack: 179.7 ± 13.3 g, $t_{62} = 7.13$, $P < 0.001$; Figure 2.1, Table 2.1) differed significantly between the two alternative reproductive tactics, soma mass was 30% larger in hooknoses and testes mass was 43% larger in jacks. For hooknoses, there was a significant positive linear relationship between testes mass and soma mass ($r^2 = 0.27$, $F = 15.83$, $P < 0.001$, $n = 45$, Figure 2.1). A similar, but non-significant, positive relationship was found between the testes mass and soma mass of jacks ($r^2 = 0.15$, $F = 2.93$, $P = 0.11$, $n = 19$, Figure 2.1). The gonadosomatic index of jacks was significantly greater than that of hooknoses (hooknose: 5.5 ± 0.3, jack: 8.1 ± 0.7, $t_{62} = 4.28$, $P < 0.001$, Figure 2.2, Table 2.1).

Sperm activity variables

We found no significant differences in sperm motility between the two alternative reproductive tactics (Figure 2.3 and Table 2.2). However, the mean motility values were

higher for jacks at all times post activation. Sperm velocity varied significantly among males from the two alternative reproductive tactics (Fig. 2.4 and Table 2.2), with jacks having faster sperm overall. Post-hoc univariate analyses of sperm velocity at each time post-activation showed significant differences between life histories at 5 seconds (t_{62} = 2.24, P = 0.029) and 10 seconds (t_{62} = 2.05, P = 0.047) but not at 15 seconds (t_{62} = 0.69, P = 0.49), post-activation (see Figure 2.4). Sperm velocity and sperm motility decreased significantly with time after activation occurred (Table 2.2). There was no interaction between life history and time for any of the sperm activity metrics (all P > 0.10), which suggests that sperm traits of both jacks and hooknoses decreased at similar rates. There was no significant difference in sperm longevity between hooknoses (20.7 ± 0.8s) and jacks (22.6 ± 0.9s; t_{62} = 1.55, P = 0.13, Figure 2.5, Table 2.2).

Sperm density

There was no significant difference in sperm density between hooknoses (4.9 x 10^7 ± 0.2 x 10^7) and jacks (5.3 x 10^7 ± 0.3 x 10^7; t_{62} = 1.13, P = 0.26, Figure 2.6, Table 2.2). Sperm density did not co-vary with gonad mass for jacks (r^2 = 0.01, n = 19, p = 0.77) or hooknoses (r^2 = 0.04, n = 45, p = 0.20).

Sperm morphology

There was no significant difference between hooknoses and jacks in terms of sperm head length (hooknose: 2.82 ± 0.02 μm, jack: 2.83 ± 0.02 μm; t_{26} = -0.59, P = 0.58); sperm head width (hooknose: 2.30 ± 0.01 μm, jack: 2.31 ± 0.02 μm; t_{26} = -0.50, P = 0.62) and flagellum length (hooknose: 26.97 ± 0.3 μm, jack: 26.10 ± 0.4 μm; t_{26} = 1.74, P = 0.09).

DISCUSSION

The sneak-guard hypothesis predicts that males employing the sneaking tactic will invest relatively more energy into spermatogenesis and have been selected to have higher sperm quality than males employing the guarding tactic as a result of greater sperm competition risk (Parker 1990; Parker 1998). Overall, our data supports this hypothesis as we found: (i) jacks invest significantly more of their somatic tissue into gonads compared to hooknoses, (ii) sperm velocity varied significantly between the alternative reproductive tactics, with jacks having faster sperm than hooknoses and (iii) sperm motility, longevity, density and head morphology all tended to be greater (albeit not significantly) in jacks compared to hooknoses.

Consistent with the sneak-guard hypothesis we found that jacks invest about 50% more of their somatic tissue into testes compared to hooknoses. Our finding that jacks had significantly larger relative investment in gonads is consistent with other studies of fishes with alternative reproductive tactics, including Atlantic salmon (Gage et al. 1995; Vladic and Jarvi 2001), bluegill sunfish (Neff et al. 2003), combtooth blenny, *Scartella cristata* (Neat et al. 2003), round goby, *Apollonia melanostoma*, (Marentette et al. 2009), and a shell brooding cichlid, *Lamprologus callipterus* (Schutz et al. 2010). The difference in relative testes investment likely relates to trade-offs with other investments such as spawning hierarchy defense, which is often related to body size investment. For example, larger hooknose males can obtain better spawning positions than smaller hooknose males, thereby reducing their sperm competition risk by having more favorable spawning

positions in terms of excluding other males and improving their proximity to females during spawning (see Berejikian et al. 2010). In contrast, it is unlikely jacks investing more into body size would benefit in terms of mating access to females, thus investing more energy into testes may improve their success at sperm competition, if larger investment in gonads led to more sperm. However, based on a post-hoc analysis examining the relationship between absolute and relative testes mass (GSI) and sperm density for both tactics, we found no evidence suggesting sperm density covaries with absolute (jacks: $r^2 = 0.01$, $F_{1,18} = 0.09$, $P = 0.767$; hooknoses: $r^2 = 0.04$, $F_{1,44} = 1.66$, $P = 0.204$) or relative (jacks: $r^2 = 0.16$, $F_{1,18} = 3.19$, $P = 0.092$; hooknoses: $r^2 = 0.01$, $F_{1,44} = 0.48$, $P = 0.491$) investment in gonads. Instead, investment in gonads may relate to the ability of males' to replenish their sperm stores and seminal plasma after several matings, a hypothesis that could be tested using a new method to measure ejaculate size in salmonids (see Fitzpatrick and Liley 2008).

Sperm velocity is the primary determinant of competitive fertilization success in salmonids (e.g. Gage et al. 2004; Rudolfsen et al. 2006). In our study, sperm velocity was faster in the sneaking tactic relative to the guarding tactic. Similar findings have been reported for other fish species with alternative reproductive tactics, including bluegill sunfish, (Burness et al. 2004), black goby, *Gobius niger* (Locatello et al. 2007), and a shell brooding cichlid, *Telamatochromis vittatus* (Fitzpatrick et al. 2007). Male salmon have a very short opportunity to fertilize eggs once their gametes are released into the aquatic environment. For example, in Sockeye salmon (*Oncorhynchus nerka*) 80% of the eggs are

fertilized within five seconds of gamete activation (Hoysak and Liley 2001) and a two second delay in sperm release significantly reduced fertilization success to 30% from an expected 50% in Atlantic salmon (Yeates et al. 2007). Therefore, in a competitive sperm-egg environment it would be advantageous to have faster swimming sperm, especially for jacks because of their suboptimal spawning position relative to the hooknoses (Berejikian et al. 2010). Currently, we do not have a mechanism to explain why jacks have faster sperm than hooknoses. Therefore, in order to fully understand the nature of this difference in sperm quality between the alternative reproductive tactics we need to explore biochemical and physiological properties of milt. Adenosine 5'-triphosphate (ATP) levels in sperm have been correlated with sperm velocity, motility and/or fertilizing ability (Lahnsteiner et al. 1998; Bencic et al. 1999; Zilli et al. 2004). In bluegill sunfish, a species with alternative reproductive tactics, sneaker males had 1.5 times more ATP in their sperm than parental males at times when sperm had significantly higher velocity (Burness et al. 2004). Future research should be undertaken to determine which biochemical and physiological properties of milt are responsible for affecting Chinook salmon sperm velocity.

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Table 2.1 Descriptive statistics (mean ± SE and range) for sperm related traits of both alternative reproductive tactics (hooknose (n = 45) and jacks (n = 19)) in Chinook salmon (*Oncorhynchus tshawytscha*).

		Hooknose males		Jack males	
Sperm traits		Mean (SE)	Range (min-max)	Mean (SE)	Range (min-max)
Motility (%)	5s	84.0 ± 2.6	21.9 – 99.0	89.6 ± 2.3	61.7 – 100.0
	10s	77.1 ± 2.6	30.7 – 98.6	84.5 ± 4.0	44.2 – 98.4
	15s	72.8 ± 2.5	28.8 – 99.7	81.8 ± 3.9	49.6 – 97.3
Sperm velocity (μm/s)	5s	55.1 ± 3.1	24.1 - 108.5	68.0 ± 5.1	34.2 - 102.9
	10s	35.2 ± 1.8	16.3 - 77.3	41.2 ± 2.6	22.9 - 57.7
	15s	28.6 ± 1.2	14.6 - 48.2	31.4 ± 2.9	11.7 - 69.9
Longevity (s)		20.7 ± 0.8	12.5 - 40	22.6 ± 0.9	16.5 - 28.5
Density (sperm per ml of milt)		$4.9x10^7$± $0.2x10^7$	$2.3x10^7$ - $8.6x10^7$	$5.3x10^7$ ± $0.3x10^7$	$3.4x10^7$ - $8.9x10^7$

Table 2.2 Repeated measures ANOVAs examining the effect of male reproductive tactic and time since sperm activation on motility, sperm velocity of sperm recorded at different time periods (5, 10 and 15 seconds) post-activation. None of the interaction terms (time x reproductive tactic) were significant (all $P > 0.10$).

Sperm trait	Effect	Test statistic	P value
Motility (%)	Tactic	$F_{1,61} = 3.85$	$P = 0.06$
	Time	$F_{2,61} = 16.76$	$P < 0.001$
Sperm velocity (*u*m/s)	Tactic	$F_{1,61} = 4.38$	$P = 0.04$
	Time	$F_{2,61} = 154.93$	$P < 0.001$

Figure captions

Figure 2.1 The allometric relationship between testes mass and soma mass for the alternative reproductive tactics (jacks = closed circles (n = 19) and hooknoses = open circles (n = 45)) in Chinook salmon (*Oncorhynchus tshawytscha)*.

Figure 2.2 Mean ± SE of the gonadosomatic index (GSI) for the two alternative reproductive tactics in Chinook salmon (*Oncorhynchus tshawytscha*), jacks (black bar, n = 19) and hooknose (open bar, n = 45) Chinook salmon (*Oncorhynchus tshawytscha)*. GSI is testes mass as a % of soma mass. Asterisks (***) indicate differences between alternative reproductive tactics at $p<0.001$.

Figure 2.3 The percentage of motile sperm (means ± SE) is reported at 5, 10 and 15 seconds post-activation for both of the alternative reproductive tactics (jacks = closed circles and hooknoses = open circles) in Chinook salmon (*Oncorhynchus tshawytscha*).

Figure 2.4 Sperm velocity (means ± SE) is reported at 5, 10 and 15 seconds post-activation for both of the alternative reproductive tactics (jacks = closed circles and hooknoses = open circles) in Chinook salmon (*Oncorhynchus tshawytscha)*. Asterisk (*) indicate differences between alternative reproductive tactics at $p<0.05$ and NS indicates no significant difference.

Figure 2.5 Sperm longevity (means ± SE) is reported for both alternative reproductive tactics (jacks = black bar and hooknoses = open bar) in Chinook salmon (*Oncorhynchus tshawytscha)*. NS indicates no significant difference between alternative reproductive tactics.

Figure 2.6 Sperm density (means ± SE) for both alternative reproductive tactics (jacks = black bar and hooknoses = open bar) in Chinook salmon (*Oncorhynchus tshawytscha)*. NS indicates no significant difference between alternative reproductive tactics.

Figure 2.1

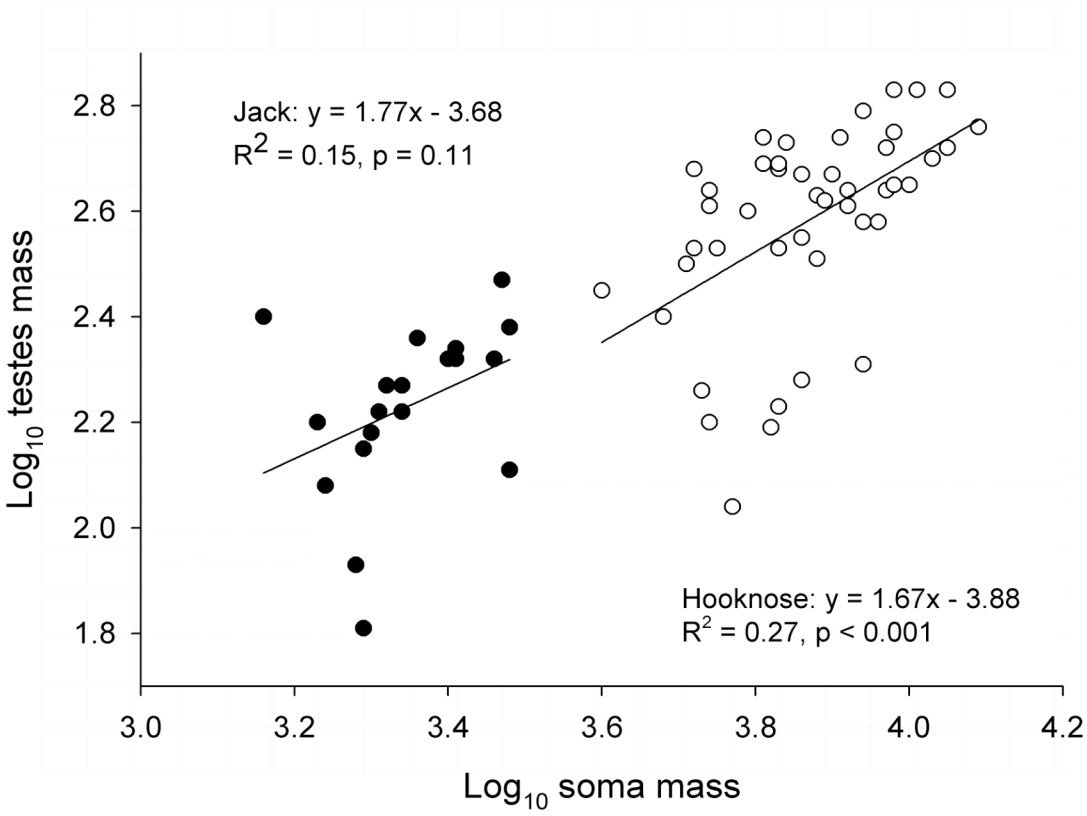

Jack: y = 1.77x - 3.68
R2 = 0.15, p = 0.11
Hooknose: y = 1.67x - 3.88
R2 = 0.27, p < 0.001
Log10 testes mass
Log10 soma mass
2.8
2.6
2.4
2.2
2.0
1.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2

Figure 2.2

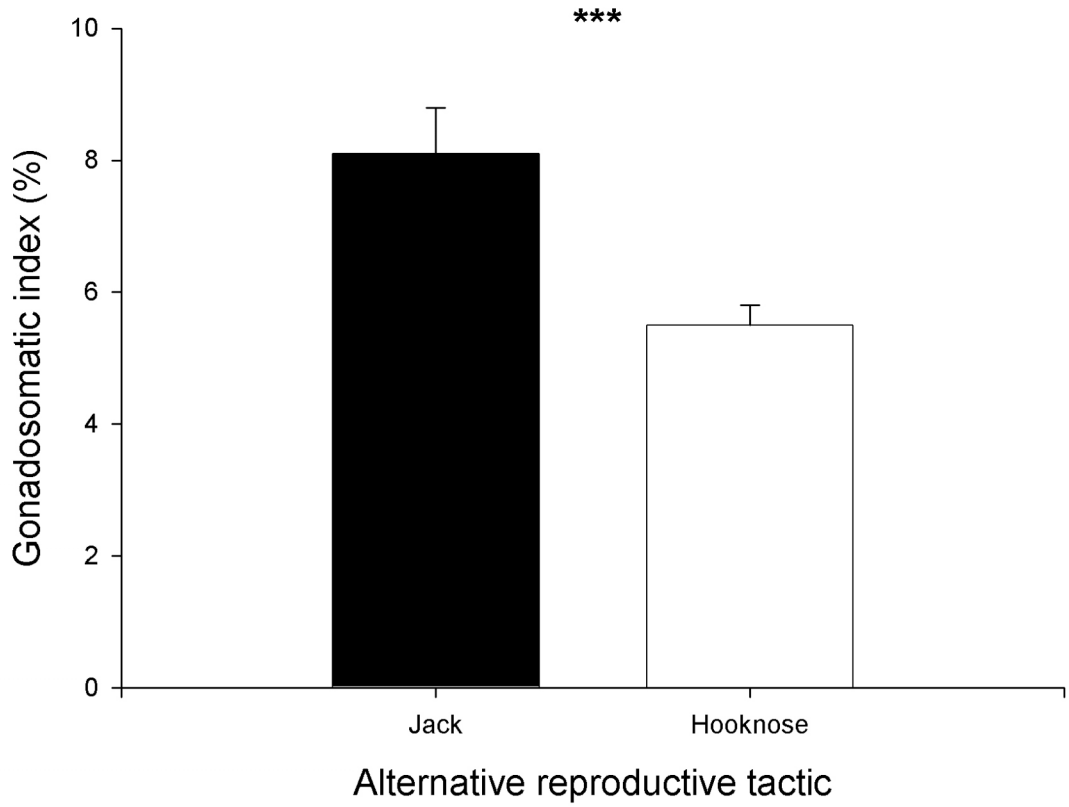

***
10
8
6
4
2
0
Gonadosomatic index (%)
Jack
Hooknose
Alternative reproductive tactic

Figure 2.3

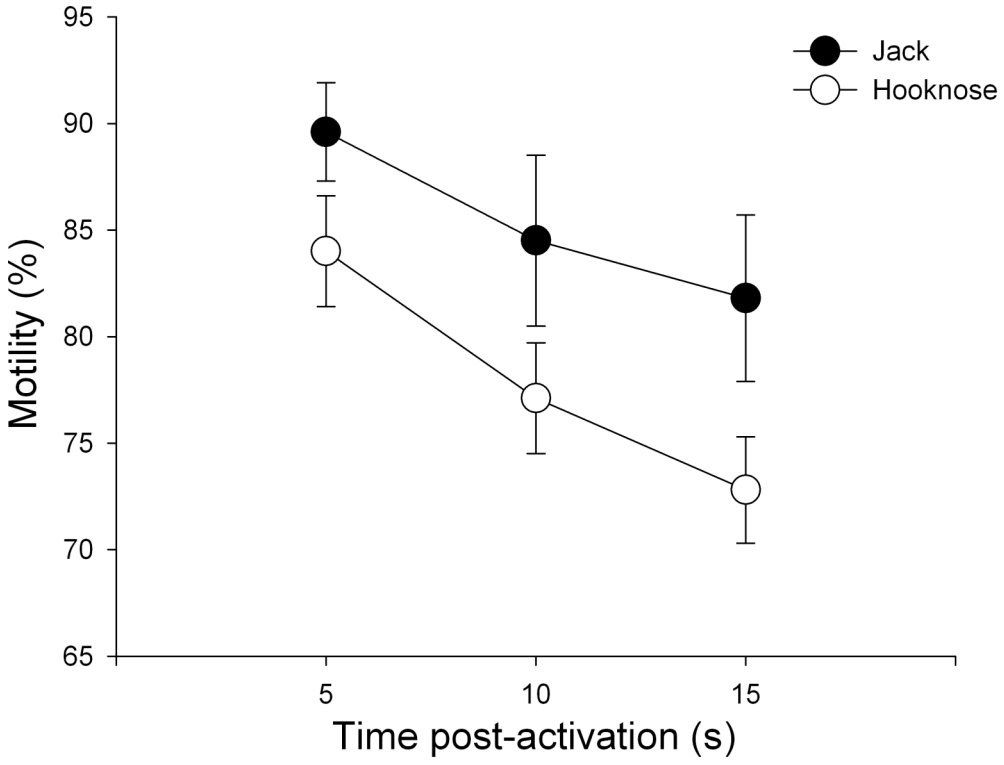

95
90
85
80
75
70
65
Motility (%)
5
10
15
Time post-activation (s)
Jack
Hooknose

Figure 2.4

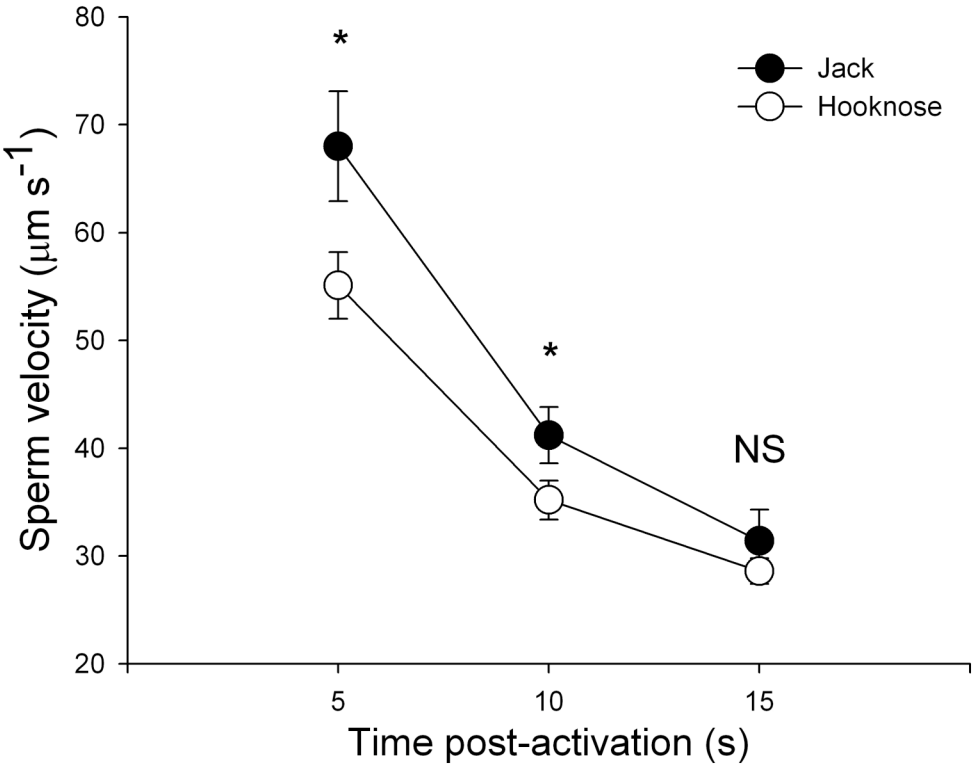

80
70
60
50
40
30
20
Sperm velocity (μm s-1)
5
10
15
Time post-activation (s)
*
*
NS
Jack
Hooknose

Figure 2.5

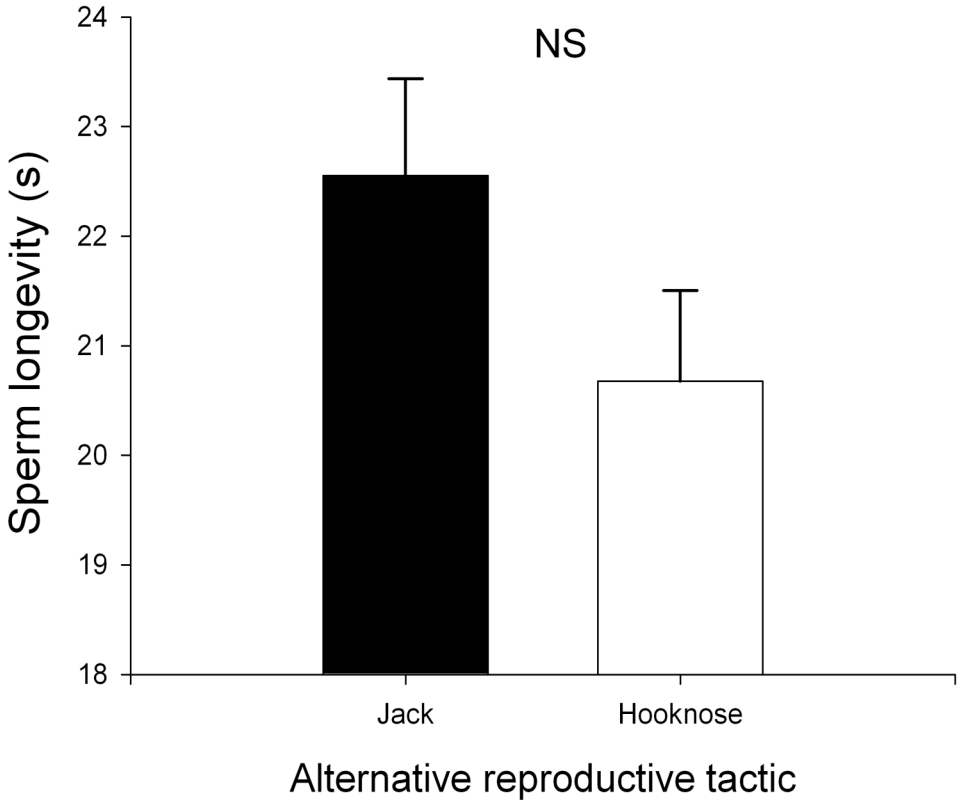
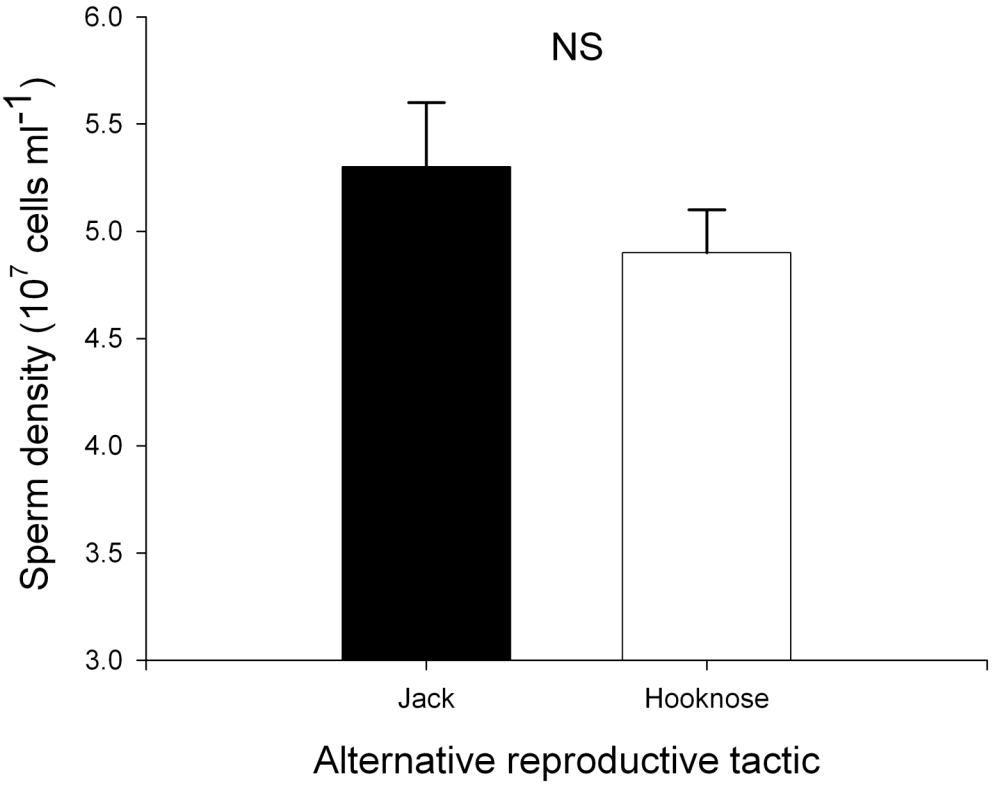

NS
Sperm longevity (s)
24
23
22
21
20
19
18
Jack
Hooknose
Alternative reproductive tactic

Figure 2.6


NS
Sperm density (10^7 cells ml^{-1})
6.0
5.5
5.0
4.5
4.0
3.5
3.0
Jack
Hooknose
Alternative reproductive tactic

CHAPTER 3: OVARIAN FLUID MEDIATES SPERM VELOCITY AND PATERNITY IN THE ALTERNATIVE REPRODUCTIVE TACTICS OF CHINOOK SALMON[2]

SYNOPSIS

In most teleost fish species, sperm competition is a key factor in determining male reproductive success, leading to selection on males to increase their ejaculate competitiveness. Sperm velocity has been shown to be the main determinant in fertilization success and ovarian fluid has also been shown to increase a males' sperm velocity. However, the role ovarian fluid play in fertilization success is unknown. In this study we measured sperm velocity as well as conducted in-vitro competitive fertilization trials with sperm from pairs of males representing both alternative reproductive tactics (jack and hooknose) and eggs from a single female, to examine the effects river water and ovarian fluid have on the outcome of paternity Chinook salmon (*Oncorhynchus tshawytscha*). We found that when sperm are competed in river water jacks sired significantly more offspring than hooknoses, however, in the presence of ovarian fluid there was no difference in the share of paternity between the two tactics. We also found that jack sperm velocity at five seconds post-activation was significantly correlated with paternity success in river water but not in ovarian fluid. These results suggest that either jacks' sperm are selected upon to perform better in water or that females are employing cryptic female choice (via their ovarian fluid) to bias paternity towards hooknoses.

[2] This chapter is the result of joint research with Dr. Trevor Pitcher and Dr. Daniel Heath.

INTRODUCTION

Sperm competition occurs post-copulation when sperm from two or more males simultaneously compete with one another to fertilize a set of a female's eggs (Parker 1970). For most teleost fish, sperm competition is known to be one of the primary factors determining reproductive success between males (Birkhead and Moller 1998; Simmons 2001), creating selective pressures on males to enhance specific sperm traits in order to maximize fitness. Sperm velocity has been shown to be the main determinant of competitive reproductive success (i.e. sperm competition success) in many fish species including, Atlantic salmon, *Salmo salar* (Gage et al. 2004), bluegill sunfish, *Lepomis macrochirus* (Burness et al. 2004), walleye, *Sander vitreus* (Casselman et al. 2006), arctic charr*, Salvelinus alpinus* (Liljedal et al. 2008), Atlantic cod, *Gadus morhua* (Rudolfsen et al. 2008; Skjaeraasen et al. 2009), green swordtail, *Xiphophorus helleri* (Gasparini et al. 2010), and the guppy, *Poecilia reticulata* (Boschetto et al. 2011). To date, all of these studies have examined the relationship between sperm velocity and sperm competition success in water, despite the fact that in natural spawning situations sperm and eggs interact in a medium that is not entirely composed of water, which may call into question the generality of these findings.

In externally fertilizing fish species egg release is accompanied by the simultaneous expulsion of ovarian fluid (Lahnsteiner et al. 1999). Lahnsteiner et al. (1995) investigated the composition of ovarian fluid from four salmonid species, rainbow trout *Oncorhynchus mykiss*, arctic charr*,* lake trout *Salmo trutta*, and Danube salmon *Hucho hucho* to quantify their inorganic (pH, osmolarity, K^+, Na^+, Ca^{2+}*)* and organic components (protein, free amino acids, glucose, lactate, phospholipids, cholesterol and

various enzymes). These organic and inorganic components have a significant effect on sperm activity such as sperm velocity, typically increasing the velocity of the sperm compared to its activation in water in fish species with external fertilization including, Atlantic cod (Litvak and Trippel 1998), rainbow trout (Wojtczak et al. 2007; Dietrich et al. 2008) and arctic charr (Turner and Montgomerie 2002; Urbach et al. 2005). Because there is evidence that sperm velocity is the primary determinant of competitive fertilization success (Gage et al. 2004) and that ovarian fluid affects sperm velocity (Rosengrave et al. 2008), understanding the role ovarian fluid directly plays in reproductive success under sperm competition is critical to our understanding of sexual selection. Therefore we investigated the effects of ovarian fluid on sperm velocity and ultimately competitive reproductive success using the alternative reproductive tactics of Chinook salmon.

Chinook salmon are an external fertilizing species in which their mating activities are short-lived, lasting <15s per bout (Fleming 1996). This intense and protracted spawning activity selects for a higher intensity of sperm competition, especially among their alternative reproductive tactics. The alternative reproductive tactics include a large guard type males (known as "hooknoses", due to the curved snout, sensu Gross 1985) and precocious sneaky males (known as "jacks") (Healey 1991). Jacks have a smaller body size, which allows them to hide in order to elude aggressive hooknose males and employ a sneaking tactic to steal fertilizations from hooknoses (Fleming and Reynolds 2004). Their inferior spawning position causes them to enter the nest soon after the onset of spawning resulting in a delay in contact with the female's eggs (Berejikian et al. 2010).

The aim of this study was to measure sperm velocity and the outcome of sperm competition using in vitro fertilizations where sperm were activated with and without ovarian fluid. We used a paired design to compete sperm simultaneously from pairs of males, one from each alternative reproductive tactic (i.e. one jack and one hooknose), to fertilize eggs from a single female, with or without ovarian fluid present. Microsatellite markers were then used to assign paternity to the offspring from the pairs of spawning males from the in vitro competitive fertilization trials in order to identify the relative competitive reproductive success (i.e. share of paternity) for each male in the presence and absence of ovarian fluid. We predicted that ovarian fluid would increase the sperm velocity of both alternative reproductive tactics of Chinook salmon and we examined whether these differences in sperm velocity (between fertilizations in water and ovarian fluid) would have an effect on the outcome of the paternity and thus affect sexual selection pressure on the males.

METHODS

Fish and gamete collection

All fourteen triads (each one consisting of a jack, a hooknose and a female) used in this study were wild-caught using standard electroshock techniques from the Credit River (Mississauga, Ontario, N 43° 35' W 79°42') from Oct 1-5, 2009. Individuals were found at random in flowing water approximately 2 to 4 feet in depth and temperatures around 11° C. Applying gentle pressure to the individuals' abdomen, we collected either the milt or eggs (and accompanying ovarian fluid). Milt, eggs and ovarian fluid were kept in a cooler that approximated the temperature of the river water (~11ºC) for transport back to

the lab for sperm velocity analysis and competitive in vitro fertilization trials. Fin clips were collected from all adults and preserved in 95% ethanol for paternity assignment genotyping associated with the competitive fertilization trials.

Competitive in vitro fertilization protocol

Female eggs (~350) were fertilized in small plastic containers within three hours from the time of collection. The eggs were collected from females, placed in a sieve, and the ovarian fluid was poured off and collected. Males were paired so that the difference in storage time of their respective milt samples was minimized. River water (250ml) from the collection site was then poured over the eggs and two hundred microliters of milt from each of the two males was applied simultaneously to flowing water using pipetters. The individual holding both pipetters was blind to the identification (jack or hooknose) of the male's sperm within. Fertilizations were replicated in a paired design (i.e. same males, same female, same milt volume and egg number) with the same technique, except in the second set of competitive fertilization trials an ovarian fluid solution was used as the fertilization medium (dilution ratio of ovarian fluid : river water of 1:1), using ovarian fluid from the female within the respective triad. At the completion of the fertilization protocols, each set of eggs was haphazardly placed into a well in a heath incubation tray. These trays were then placed in an incubation stack located at Ringwood Fish Culture Station (Stouffville, ON) to ensure that oxygenated water would flow over eggs. Fish remained in the incubation stack until they were strong eyed-up (i.e. when the fry had developed to the point you could see an eye spot). A subset of the strong eyed-up eggs (n = 48) from each of the competitive in vitro fertilization pairs (n = 14 in water, n = 14 in the ovarian fluid solution) was collected from each triad and preserved in 95% ethanol for

subsequent DNA extraction and paternity analysis (see below).

Sperm trait assessment

Sperm velocity was video recorded, within six hours from the time of collection, through a microscope and analysed with computer assisted sperm-tracking software after 1.5 uL of milt was activated with 15 uL of river water and again with ovarian fluid solution (from the female in the respective triad), at 11°C. Recordings were conducted using a CCD B/W video camera module at 25Hz vertical frequency, mounted on an external negative phase-contrast microscope (CX41 Olympus) with a 10X magnification negative phase objective (see Pitcher et al. 2009). Once recordings were completed, sperm velocity analysis was conducted using HTM-CEROS sperm analysis system Version 12 (CEROS, Hamilton Thorne Biosciences, Beverly MA, USA) set at the following parameters: number of frames=60; minimum contrast=11; minimum cell size= 3pixels. Sperm velocity, measured as the curvilinear velocity (the average velocity measured over the actual point-to-track followed by the cell) at 5, 10 and 15 seconds post-activation in both water and the ovarian fluid solution were used in all further analyses. The sperm analysis software measures each sperm cell individually and generates an average velocity of all sperm cells combined.

Paternity assignment

DNA was extracted from adult fin clips (2 males and 1 female per trial) and strong-eyed eggs (48 per trial). Fin clips or tissue collected from the developing egg samples were dried of ethanol and placed in 96 well plates with digestion buffer and proteinase K solution before being incubated at 37° C and agitated gently overnight.

Extraction was then performed using a Janus Automated Liquid Handling System (Perkin Elmer Life and Analytical Sciences, Dowers Grove, IL USA) following the protocol of Elphinstone et al. (2003). The unambiguous paternity of each offspring was determined using four polymorphic microsatellite markers: OTS 107 (tetranucleotide repeat motif), OTS G83b (tetranucleotide repeat motif), OMY 1191 (tetranucleotide repeat motif) and OTS G432 (tetranucleotide repeat motif). The loci were amplified using polymerase chain reaction with the following protocol: Denature for 2 minutes at 94° C, followed by thirty five cycles of 15 seconds at 94° C, 45 seconds annealing at 63° C (58° C for OTS-107; 54° C for OTS G432), 30 seconds extension at 72° C, then a final extension step of 1.5 minutes at 72° C. Fluorescently labelled primers were used and the product run by polyacrylamide gel electrophoresis using a Licor 4300 DNA Analyzer system. Allele sizes were called manually using Gene ImagIR (version 4.05) software. A single observer was able to determine paternity via the exclusion of one of the potential males; however, not all offspring could be assigned paternity in this manner using these particular loci. Paternity could be assigned to an average of 45.3 +/- 0.56 offspring (range: 37 and 48 offspring) of a possible 48 offspring per pair of trials using the available microsatellite loci. Relative fertilization success was measured for each male by dividing the number of offspring sired by that male by the total number of offspring that could be unambiguously assigned to one of the two males.

Competitive in vitro fertilization analyses

We used a paired design to compare the (i) sperm velocity (at 5, 10 and 15 seconds) and (ii) relative shares of paternity that hooknoses and jacks had in both river water and the ovarian fluid solution using a series of paired t-tests (in JMP version

5.0.1.2). We also used ordinary least linear regressions to examine the relationship between the difference of paternity (jack paternity (%) – hooknose paternity (%)) in relation to the difference in sperm velocity at 5, 10 and 15 seconds post-activation in both media (water and ovarian fluid solution).

RESULTS

We found a significant difference in sperm velocity in hooknoses between activation mediums at 5 (paired t-test: $t_{1,13} = -3.78$, $P = 0.002$), 10 (paired t-test: $t_{1,13} = -12.77$, $P<0.001$) and 15 (paired t-test: $t_{1,13} = -5.07$, $P<0.001$) seconds post-activation (see Figure 3.1a). We also found significant differences in sperm velocity for jacks at 10 (paired t-test: $t_{1,13} = -4.80$, $P<0.001$) and 15 (paired t-test: $t_{1,13} = -4.53$, $P=0.01$) seconds post-activation (see Figure 3.1b). There was no significant difference in jacks' sperm velocity in water and ovarian fluid at 5 seconds post activation (paired t-test: $t_{1,13} = -0.85$, $P = 0.41$, Figure 3.1b)

The relative share of paternity was significantly higher for jacks than hooknoses when eggs were fertilized using both males' sperm in river water (paired t-test: $t_{1,13} = -2.29$, $P=0.039$, Figure 3.2). However, there was no significant difference in the relative share of paternity between jacks and hooknoses when eggs were fertilized in the presence of ovarian fluid (paired t-test: $t_{1,13} = 0.63$, $P=0.54$, Figure 3.2).

Regression analyses showed that relative sperm velocity at 5 seconds post-activation in river water (jack minus hooknose sperm velocity) is significantly related to relative paternity (jack minus hooknose paternity) ($r^2= 0.29$, $P=0.048$, Figure 3.3),

whereas no significant relationship was found for the association between relative sperm velocity at 5 seconds post-activation in ovarian in relation to relative paternity (r^2=0.06, P=0.39). Regressions performed on the relationships between relative paternity and sperm velocity at 10 and 15 seconds post-activation in both activation mediums were non significant (all P>0.46).

DISCUSSION

In this experiment we found that sperm velocity was significantly faster for both alternative life histories when activated with an ovarian fluid solution versus river water. Jacks had a significantly higher relative share of paternity than hooknoses when sperm from both males were competed in river water while no significant difference was found in their relative share of paternity when eggs were competitively fertilized in the presence of ovarian fluid. We found that jacks had significantly higher sperm velocity compared to hooknoses at 5 seconds post-activation in river water but not at 5 seconds post-activation in the presence of ovarian fluid. We also found that sperm velocity at 5 seconds post-activation was significantly correlated with paternity success in river water but not in ovarian fluid, likely explaining the change jacks had in terms of competitive reproductive success between activation mediums.

Our finding that sperm velocity is higher in the ovarian fluid solution than river water is consistent with numerous studies looking at the effects of ovarian fluid on sperm velocity, including studies of Atlantic cod (Litvak and Trippel 1998), arctic charr (Turner and Montgomerie 2002; Urbach et al. 2005), rainbow trout (Wojtczak et al. 2007;

Dietrich et al. 2008) and most recently Chinook salmon (Rosengrave et al. 2008). Research done on the composition of ovarian fluid in salmonids has found that the concentration of some ions (K^+, Na^+ & Ca^{2+}) and the pH level are the main factors affecting sperm velocity (Wojtczak et al. 2007; Rosengrave et al. 2009). Unlike a previous study on Chinook salmon, we included the effects of ovarian fluid on sperm from both of the alternative reproductive tactics found in Chinook salmon, rather than just one of them (hooknoses; see Rosengrave et al. 2008).

We found that ovarian fluid affects jacks and hooknoses differently in terms of their sperm velocity and the outcome of sperm competition. Sperm velocity is significantly increased at all times post-activation (5, 10 and 15 seconds) in hooknoses while sperm velocity in jacks is only significantly increased at 10 and 15 seconds (not at 5 seconds post-activation). Our paternity analysis showed that jacks attain a higher relative fertilization success when in direct competition with hooknoses in river water, which is consistent with another study, in bluegill sunfish, that compete the alternative reproductive tactics using water as an activation medium (Fu et al. 2001). However, competition in the presence of ovarian fluid appears to "level the playing field" between the alternative reproductive tactics by disproportionately increasing the sperm velocity of hooknoses 23.7% compared to only 11.5% in jacks (especially at 5 seconds post-activation). We found that jacks had significantly higher sperm velocity at 5 seconds post-activation in river water but not in ovarian fluid. We also found that sperm velocity at 5 seconds post-activation was significantly correlated with paternity success in river water but not in ovarian fluid. A possible explanation for why jacks sired more offspring in river water than ovarian fluid.

There are two alternative explanations for the results of this experiment. First, females may be employing cryptic female choice (a form of post-copulatory sexual selection occurring when a female biases paternity using certain processes or structures after mating with multiple males; Eberhard 1996) in which their ovarian fluid creates an environment favourable for hooknose sperm to out compete jack sperm in those first 5 seconds post-activation. Second, jack sperm may have been selected upon to have higher initial velocities in water compared to hooknoses.

This experiment has potential implications for the study of cryptic female choice in externally fertilizing species. One area for future research is to look at the major histocompatibility complex (MHC) and determine if females are using their ovarian fluid to select males who are more genetically compatible to them in order to increase the genetic quality of their offspring. Studies on Chinook salmon have shown that there is potential for females to significantly increase the genetic quality (and thus fitness) of their offspring by choosing males based on their MHC genotype (Pitcher and Neff 2006; 2007). We also know that jacks' MHC's have been found to be more heterozygous than hooknoses (Heath, unpublished data), potentially making them less ideal genetic sources for females. Studies done in the three-spined stickleback found that males with intermediate instead of the maximum number of MHC alleles were less susceptible to parasites and were more likely to be chosen by females (Milinski 2003). Further investigation in the Chinook salmon is required to verify this possibility.

This selection may have occurred because jacks often have disadvantageous spawning positions and may be forced to release their gametes further away from the spawning female, as seen in the bluegill sunfish (Stoltz and Neff 2006), thus jacks release

their sperm in an area where there is a lower concentration of ovarian fluid (i.e. their sperm spends more time in water than a solution containing ovarian fluid). There may also be a less ovarian fluid in the spawning environment by the time the jack take part in spawning. Berejikian et al. (2010) found that jacks have delayed contact with the gametes because they enter spawning bouts later. Bluegill sneakers were found to spawn on average15 mm farther away than parentals (guard males) and release their sperm approximately 0.46s following the onset of spawning (Stoltz and Neff 2006). Any delay in release in gametes can have serious consequences for a male since fertilization occurs very rapidly in external fertilizers. For example, Hoysak and Liley (2001) found that 80% of fertilization occurs within the first 5 seconds following the release of gametes in the Sockeye salmon (*Oncorhynchus nerka*). Future studies should look at the natural spawning behaviour of the Chinook salmon to determine actual positioning and timing of gamete release.

One major theory to explain the evolution of alternative reproductive tactics is frequency dependent selection; requiring equal fitness to maintain both reproductive tactics (Gross 1996). Based on studies using water as an activation medium in competition trials (e.g. Neff et al. 2003), one would assume jacks would outcompete hooknoses eventually taking over the gene pool within the population. However, this experiment demonstrated that in the presence of ovarian fluid, which represents a more realistic spawning condition, both jacks and hooknoses achieved relatively equal paternity. These results have provided insight as to how these alternative reproductive tactics have been maintained in this population of Chinook salmon. Finally, our experiment has serious ramifications for studies investigating sperm competition success

in fishes; utilizing ovarian fluid in competition experiments is essential to obtain realistic data regarding their fertilization dynamics.

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Table 3.1 Summary of the mean (+/- standard deviation (SD)) sperm velocity (um/s at 5, 10 and 15 seconds post-activation) in river water (W) and ovarian fluid (OF) and sperm density for each pair of jack (J) and hooknose (HN) Chinook salmon (*Oncorhynchus tshawytscha*) used in this study.

Pair	Male	Velocity (um/s) 5 seconds				Velocity (um/s) 10 seconds				Velocity (um/s) 15 seconds				Density ($X10^7$)
		W	SD	OF	SD	W	SD	OF	SD	W	SD	OF	SD	
1	J	93.6	42.4	98	36.9	60.3	28.7	86.8	27.5	16.5	60.7	86.6	26.9	18.00
	HN	97.3	37.2	152	40.1	73.3	29	122.3	30.4	65.7	34.9	87.1	38.3	18.94
2	J	90.2	34.1	118.8	40.6	78.5	39.5	116.0	55.4	60.8	15.4	127.	43.5	18.33
	HN	98.8	39.3	151.8	99.5	68.4	27.4	114.7	37.3	45.1	16.4	84.2	29	15.05
3	J	131.1	45.3	152.7	37.1	79.6	37.8	132.5	30.6	44.7	13.9	103.	34	8.73
	HN	161	44.3	163.8	40.1	113.4	37.3	154.3	39.9	51.7	23.3	116.	35.4	20.89
4	J	150.1	17.4	170.9	40	77.0	21	103.1	21.5	41.9	8.4	71.1	16.2	2.32
	HN	144.3	41.6	131.4	67.7	80.6	23.5	122.7	41.5	52.3	24.9	96.9	35.1	8.82
5	J	144.8	50.4	107.7	53.1	72.4	19.6	110.2	35.1	51.7	11	94.8	26.1	12.30
	HN	107.9	47.5	141.7	64.8	48.2	20.6	117.7	24.2	0	0	60.5	11	10.33
6	J	153	31	154.7	37	75.3	34.2	76.6	42.7	0	0	78.4	12.6	9.64
	HN	82.6	28.5	140.9	44.2	59.0	14.8	95.8	34.2	40.3	3.8	68	30.1	12.87
7	J	123.3	56.4	131.5	42.6	61.1	17.9	90.1	37.9	60.9	16.5	51.6	15.1	11.92
	HN	130.1	33.8	123.1	62	27.2	27	115.1	45.2	37.5	1.3	84.2	31.1	5.80
8	J	133.3	47.5	111.1	42.5	89.3	39	82.4	44.5	47.9	31.6	88.4	52.6	7.83
	HN	119.3	37.6	166	31.5	49.0	21	116.4	25.2	23.9	0.9	82.8	37.4	17.72
9	J	119.1	39.8	94.1	36.5	64.4	25.4	61.7	26.5	65.5	49.4	96.3	24.5	6.24
	HN	114.1	31.3	126.8	54	76.1	23.2	110.2	40.9	44.4	5.4	64.8	32.6	19.69
10	J	116.3	25.7	304	218	79.4	29.2	144.9	76.1	52.2	16.6	94.5	34	3.70
	HN	96	51.1	149.2	46.8	86.6	31	140.9	28.3	66.4	45.9	103.	29.9	3.58
11	J	118.6	57.7	162.1	39.9	109.5	31.9	143.1	41	64.8	12.2	99.0	29.6	8.21
	HN	120.9	51.1	167.4	43.3	81.2	39.1	131.3	36.4	115.	80	87.1	35.6	4.64
12	J	92.1	43	159.7	29.5	104.1	25.6	124.7	30.3	122.	38.2	83.2	25.6	7.99
	HN	95.4	29.3	135.5	42.4	62.9	15.7	127.5	26.5	29.3	0	97.8	19.3	5.42
13	J	129.1	35.4	115	49.5	74.1	43.3	113.4	31.8	38.1	10.9	72.8	25.8	7.10
	HN	88.5	37	95.8	49.7	42.0	14.3	74.0	49.2	55.2	26.1	54.7	32.2	7.85
14	J	138.1	29.7	51.7	30.1	97.1	13.7	108.3	38.3	46.3	18.1	90.4	0	6.60
	HN	147.5	30.3	137.9	38.8	74.6	19.3	127.6	23.8	42.9	11.3	94.4	17.6	7.03

Table 3.2 Summary of the paternity results from river water (W) and ovarian fluid (OF) for each jack (J) and hooknose (HN) male used in this study (see text for details). Resolved paternity is the number of offspring assigned paternity to either the jack or hooknose of each pair out of a possible 48. Unresolved paternity is the number of offspring that couldn't be assigned paternity. Paternity is calculated as the percentage of the total offspring per trial pair that were sired by the male in question. Relative paternity is the difference between the % paternity of jack and hooknose of each pair (jack paternity – hooknose paternity).

Pair	Male	Resolved Paternity		Unresolved Paternity		Paternity (%)		Relative Paternity (%)	
		W	OF	W	OF	W	OF	W	OF
1	J	30	33	3	9	67	85	34	70
	HN	15	6			33	15		
2	J	22	21	1	0	47	44	-6	-12
	HN	25	27			53	56		
3	J	8	5	0	5	17	12	-66	-76
	HN	40	38			83	88		
4	J	35	28	2	1	76	60	52	20
	HN	11	19			24	40		
5	J	19	9	2	5	41	21	-18	-58
	HN	27	34			59	79		
6	J	41	29	5	1	95	62	90	24
	HN	2	18			05	38		
7	J	24	15	1	0	51	31	2	-38
	HN	23	33			49	69		
8	J	31	38	0	1	65	81	30	62
	HN	17	9			35	19		
9	J	39	35	3	0	87	73	74	46
	HN	6	13			13	27		
10	J	40	39	5	5	93	91	86	82
	HN	3	4			07	09		
11	J	22	12	11	6	59	29	18	-42
	HN	15	30			41	71		
12	J	32	25	0	1	67	53	34	6
	HN	16	22			33	47		
13	J	29	32	1	0	62	67	24	34
	HN	18	16			38	33		
14	J	30	21	1	7	64	51	28	2
	HN	17	20			36	49		

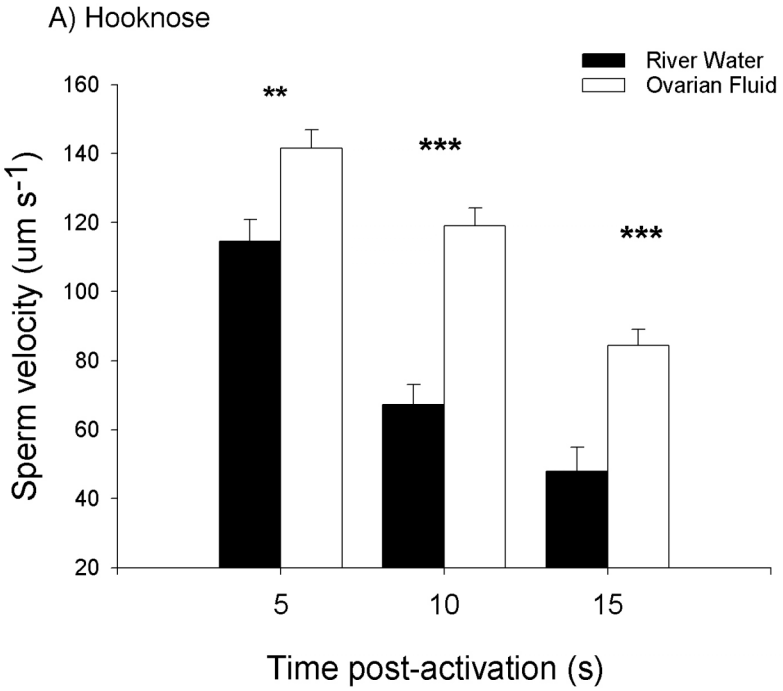
Figure Captions

Figure 3.1 (a) Comparison of sperm velocity of hooknoses (n = 14) between river water (black bars) and ovarian fluid (open bars) at 5, 10 and 15 seconds post activation. (b) Comparison of sperm velocity of jacks (n = 14) between river water (black bars) and ovarian fluid (open bars) at 5, 10 and 15 seconds post-activation. Asterisks (***), (**), and (*) indicates differences between alternative reproductive tactics at $p<0.001$, $p<0.01$, and $p<0.05$ respectively. NS Indicates no significant difference.

Figure 3.2 The competitive fertilization success of jacks (black bars, n = 14) and hooknoses (open bars, n = 14) shown as the percentage of the total offspring sired by each of the males in each activation medium. Asterisk (*) indicates difference between alternative reproductive tactics at $p<0.05$. NS indicates no significant difference.

Figure 3.3 The relationship between the relative sperm velocity (difference in sperm velocity among pairs of males, jacks minus hooknoses) and the difference in relative fertilization success among pairs of males (paternity of jacks minus paternity of hooknoses).

Figure 3.1


A) Hooknose
River Water
Ovarian Fluid
**
***
***
Sperm velocity (um s-1)
160
140
120
100
80
60
40
20
5
10
15
Time post-activation (s)

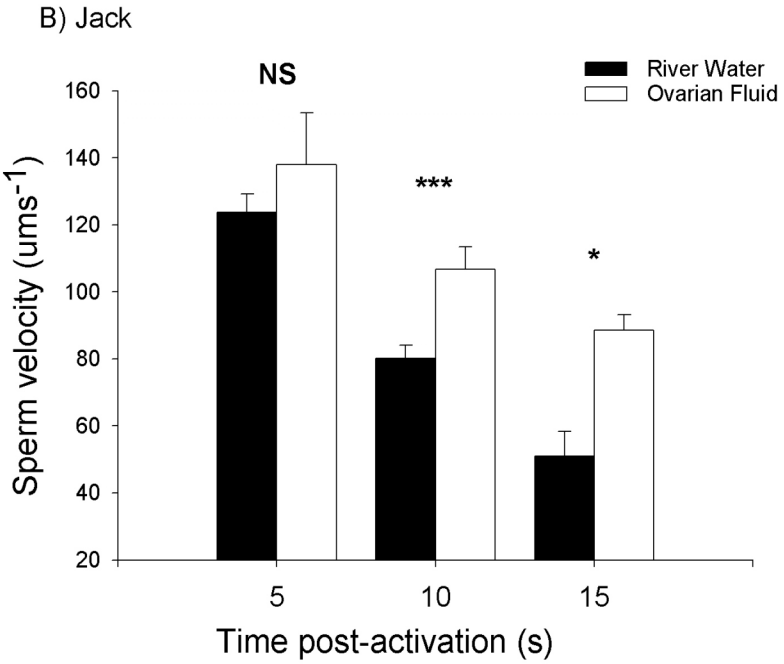

B) Jack
NS
River Water
Ovarian Fluid
***
*
Sperm velocity (ums-1)
160
140
120
100
80
60
40
20
5
10
15
Time post-activation (s)

Figure 3.2

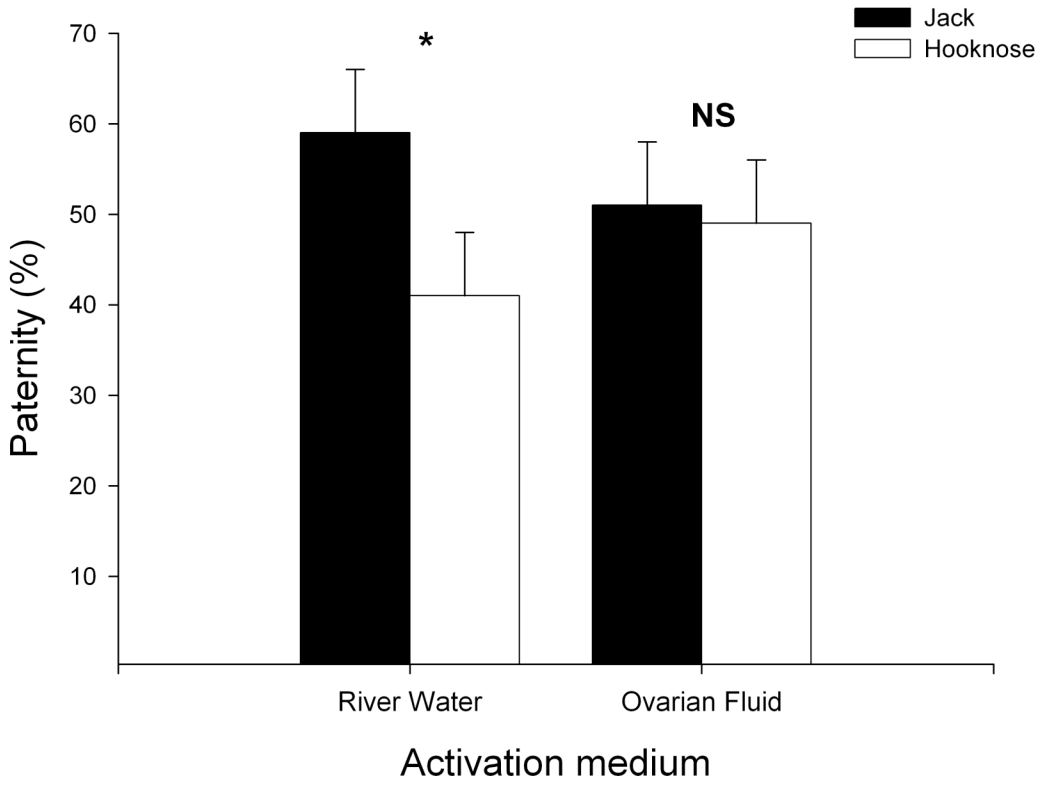
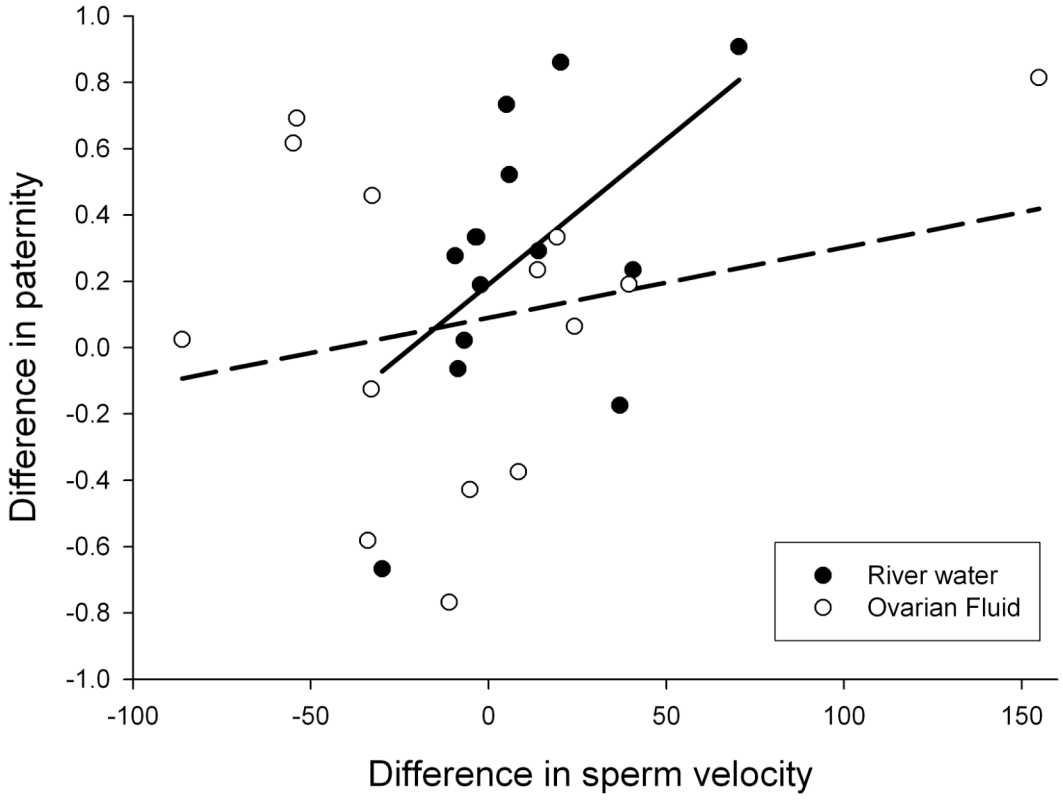

Jack
Hooknose
*
NS
70
60
50
40
30
20
10
Paternity (%)
River Water
Ovarian Fluid
Activation medium

Figure 3.3


1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Difference in paternity
-100
-50
0
50
100
150
Difference in sperm velocity
River water
Ovarian Fluid

CHAPTER 4: GENERAL DISCUSSION

Summary

In this thesis I examined sperm competition in the context of the alternative reproductive tactics (jacks and hooknoses) of Chinook salmon (*Oncorhyncus tshawytscha).* The purpose of this final chapter is to summarize the main conclusions of my research and indicate specific directions that future research should take in order to expand on the work done here. I found that jacks had relatively larger gonads and had higher sperm velocity (using river water as an activation medium) than hooknoses. I also examined competitive fertilization success of the two tactics using a more realistic spawning microenvironment (i.e. the inclusion of ovarian fluid). I found a significant increase in velocity when sperm were activated in ovarian fluid compared to river water for both reproductive tactics and jacks were more successful sperm competition than hooknoses in water but not in ovarian fluid. I found a significant positive relationship between sperm velocity and sperm competition success in water but not ovarian fluid. These results have significant implications for studies of sperm competition that do not take into account the female's role in reproduction.

Chapter Two

A prediction of the sneak/guard hypothesis (Parker 1990a) was tested in Chapter Two by comparing various reproductive investment metrics in the alternative reproductive tactics of Chinook salmon. The prediction is that males employing the sneaking tactic (i.e. jacks) will invest more energy into spermatogenesis than males employing the guard tactic (i.e. hooknoses), as a result of greater sperm competition risk

(Parker 1990a; Parker 1998). The gonadosomatic index as well as various sperm quality traits such as sperm longevity, velocity, motility, morphology and density were examined as surrogates of male reproductive investment in the two alternative reproductive tactics. I found that jacks have a significantly higher gonadosomatic index and faster swimming sperm (using river water as an activation medium) than hooknose males on average. Additionally, sperm motility, longevity, density and head morphology all tended to be greater (albeit not significantly) in jacks compared to hooknoses. Overall, my data provided general support the sneak/guard hypothesis.

Jacks have presumably been selected on for faster swimming sperm in order to increase their chances of siring more offspring. Sperm velocity is known to be the primary determinant of competitive fertilization success in salmonids (e.g. Gage et al. 2004). Greater velocity would allow sperm to cover more distance per unit time thus giving jacks a chance at reaching a females eggs before or at least at the same time as a hooknose despite their disadvantageous spawning position and delayed access to female eggs (Berejikian et al. 2010). Another reason for the importance of sperm velocity may be the necessity for teleost sperm to enter an egg using the micropyle, a channel through the membrane; higher velocity sperm increases their odds of entering the micropyle first (Kobayashi and Yamamoto 1981).

Future studies investigating the sneak/guard hypothesis in the Chinook salmon should focus on four main areas. The first would be to confirm that having a larger gonadosomatic index improves an individual's chance at increasing their fertilization success. To do this one would have to assess their natural ejaculate volume, to determine if jacks release more sperm on average than hooknoses during each spawning bout, as

well as their rate of milt production, to determine if they restore sperm quicker (Fitzpatrick and Liley 2008). These studies would provide more information regarding the potential benefit (increased sperm competitive success) of having a higher gonadosomatic index (Leach and Montgomerie 2000). Secondly, it would be ideal to observe the natural spawning behaviour of Chinook salmon. This could be accomplished by setting up cameras and video taping spawning behaviour in order to determine the proximity of males (both jack and hooknose) to the female as well as any possible delay in timing of gamete release (e.g. Stoltz and Neff 2006). The results of this study would allow researchers to accurately mimic the positioning and timing off gamete release in future competition trials. Thirdly, given that the distance sperm must travel to the egg is a major consideration one should consider the energy cost of transport by investigating the underlying mechanism that causes jack sperm to have a significantly higher velocity than hooknoses. Lastly, one should look for genes responsible for sperm quality metrics.

One possible caveat with the data from Chapter Two is that the sperm activity metrics (including sperm velocity, motility and longevity) were examined after they were activated in river water. However, a more natural spawning bout of Chinook salmon includes not only river water, but also ovarian fluid expelled by the female when eggs are being released. Several studies to date have suggested that ovarian fluid may affect sperm activity variables in fishes (e.g. Turner and Montgomerie 2002). As such, in Chapter Three I investigate the potential role of ovarian fluid on sperm activity variables and the ultimate success males have in sperm competition.

Chapter Three

Sperm competition between males to fertilize a given set of ova is considered to be a major determinant of male reproductive success in various mating systems (reviewed in Birkhead et al. 2009). Sperm competition leads to selection on males to increase the competitiveness of their ejaculates in order to reproduce successfully. There is evidence that sperm velocity is the primary determinant of competitive fertilization success in salmonids (Gage et al. 2004), however, studies of this kind do not take into account the actual spawning microenvironment (i.e. the inclusion of ovarian fluid). Because ovarian fluid affects sperm velocity (e.g. Rosengrave et al. 2009), understanding the role ovarian fluid directly plays in reproductive success under competition is critical to our understanding of sexual selection. Thus, in Chapter Three I investigated the effects of ovarian fluid on sperm velocity and ultimately competitive reproductive success between the alternative reproductive tactics of Chinook salmon.

In this experiment I found that sperm velocity significantly increased in the presence of ovarian fluid within both alternative reproductive tactics. However, ovarian fluid did not significantly alter jack sperm velocity at 5 seconds post-activation. Also, jacks had significantly higher paternity in river water than hooknoses but not when competed in the presence of ovarian fluid. I also determined that the differences between males in sperm velocity were positively related to competitive fertilization success in river water, but not in ovarian fluid at 5 seconds post-activation. These results confirm that sperm velocity is a critical determinant of competitive fertilization success in water, and as such, it appears this mating system follows the loaded raffle mechanism of sperm competition (Parker 1990b), at least in water. However, I also found that ovarian fluid

significantly increased hooknose sperm velocity at 5 seconds, the time at which 80% of fertilization is said to occur in salmonids (Hoysak and Liley 2001), which subsequently increased their competitive fertilization success enough to "level the playing field" between the alternative reproductive tactics. These results call into question the validity of the comparison of sperm traits between tactics in Chapter Two and more importantly, these results also challenge the fundamental mechanisms supported by sperm competition theory (that assume females are passive participants in reproduction) (Parker 1990b).

I am proposing two possible explanations for why ovarian fluid differentially affects jacks and hooknoses in terms of sperm velocity and ultimately paternity. The first explanation is that jack sperm may have been selected on for higher initial sperm velocity in water rather than ovarian fluid due to their inferior spawning position and timing of sperm release (Berejikian et al. 2010). Second, females may be employing a form of post-copulatory sexual selection called cryptic female choice (via ovarian fluid), where she biases sperm performance of certain males to perform better than others.

Future studies on sperm competition in the Chinook salmon should address these two potential explanations. First, one could use information from physics, biomechanics, and engineering to investigate any possible physiological differences between sperm from hooknoses and jacks. For example, I found no difference in sperm morphology between alternative reproductive tactics but there are other possibilities to explain the differences observed in sperm velocity such as flagellum wave propulsion, and the Reynolds number, which is the relative value of inertia by viscosity of the fluid where it is activated (Humphries et al. 2008)

Secondly, studies should focus on investigating the possibility of cryptic female choice. It is already known that ovarian fluid affects sperm traits and that these effects vary between males (Urbach et al. 2005; Dietrich et al. 2008; Rosengrave et al. 2008). Therefore, this study should be conducted by competing sperm from multiple pairs of males with the eggs of different females (including ovarian fluid) as well as measuring the between male differences ovarian fluid has on their sperm velocity. Genetic analysis on the parents and offspring would be require to determine if female ovarian fluid is influencing male sperm based on genetic compatibility (Milinski et al. 2005). The major histocompatibility complex (MHC) is a perfect candidate gene to investigate compatibility effects. The MHC is linked to the immune system, responsible for creating proteins that initiate immune responses to disease (Neff et al. 2008); and it is believed that females prefer to mate with males that differ from their own MHC genotype (Neff and Pitcher 2005). For example, female Atlantic salmon, *Salmo salar,* chose mates that will create highly heterozygotic offspring at the MHC loci (Landry et al. 2001). Strong evidence for cryptic female choice would be found if genetic analysis showed that the offspring from the most successful males had the highest heterozygosity at their MHC loci.

Conclusion

Sperm competition studies to date have rarely investigated the microenvironment where competition occurs. This is partly a result of the fact that many sperm competition studies are conducted on internal fertilizers where it is difficult to observe the competition between sperm from different males directly. However, in external fertilizing species, it is possible to experimentally manipulate the microenvironment

where competitive fertilization occurs. I was able to experimentally manipulate the presence and absence of ovarian fluid (which is part of the spawning microenvironment for externally fertilizing fishes) to test whether it affects the outcome of sperm competition between alternative reproductive tactics of Chinook salmon. Because the outcome of competition was not the same when ovarian fluid was included in the sperm competition trials, females are indeed playing a significant role in determining the outcome of paternity. This outcome strongly suggests there is significant amounts of sexual conflict between the sexes in Chinook salmon and this may help explain how females choose amongst males at the post-spawning stage. Finally, and likely most importantly, my results related to the inclusion of ovarian fluid have serious implications for studies investigating sperm competition theory. The models developed to date (e.g. Parker 1990a; 1990b; 1998), upon which sperm competition theory is founded, does not adequately take into account the role females play in the evolution of fertilization dynamics and as such sperm competition theory ought to be reevaluated.

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