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Polyandry and sperm competition in the alternative reproductive tactics in Chinook salmon (Oncorhynchus tshawytscha)

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Polyandry and sperm competition in 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: both of my data chapters were co-authored with my supervisor, Dr. Trevor Pitcher. In each case, my co-author 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, with Chapter Two being submitted to the Journal of Evolutionary Biology for publication and Chapter Three being planned to submit for publication to an undecided journal.

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ABSTRACT

Chinook salmon (*Oncorhynchus tshawytscha*) exhibit male alternative reproductive tactics (ARTs), in which males are either large, dominant hooknoses, or small precocious jacks. Multiple mating (or polyandry) is common by females, which partly explains the intense sperm competition. I examined whether females benefit genetically by mating with multiple males thus promoting sperm competition and whether males can use seminal plasma to influence the potential outcome during sperm competition. I found that polyandrous females do indeed benefit genetically compared to monandrous (singly mated) females through an increase in offspring hatching success. The benefits received by polyandrous females varied significantly depending on the ARTs used during sperm competition trials, with crosses involving a jack and a hooknose producing the offspring with the highest hatching success. I also found that jack seminal plasma decreases hooknose sperm velocity, with potential implications on the outcome of sperm competition between the two tactics.

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CHAPTER 1: GENERAL INTRODUCTION

Darwin first formalized the term 'sexual selection', defined originally as sexually dimorphic traits that maximize the success of individuals in reproduction are selected upon to explain why animals exhibited a suite of traits (now known as secondary sexual characteristics) that clearly would decrease their survival (counter to the logic of natural selection), such as: bright coloration, large ornamentation, and elaborate songs. However, Darwin failed to grasp the wide-ranging scope of sexual selection, as he believed that all selection occurred prior to copulation. Pre-copulatory sexual selection entails both intersexual selection (i.e. male-male competition); in which males compete and fight for dominance and ultimately access to females, and intrasexual selection (i.e. female choice); where females choose which male they prefer; typically based on these elaborate secondary sexual characteristics (reviewed in Andersson, 1994). More recently, the field has come to realize that sexual selection also occurs after copulation (see reviews Birkhead, 2010; Andersson & Simmons, 2006; Birkhead & Pizzari, 2002). Post-copulatory selection also encompasses both intersexual selection (i.e. sperm competition between males) and intrasexual selection (i.e. cryptic female choice), however, the processes take place after copulation has occurred (reviewed in Birkhead & Møller, 1998).

Sperm competition

Parker (1970) was the first to define sperm competition as "*competition within a single female between the sperm from two or more males for the fertilization of the ova*" and has since expanded this definition to include external fertilizers as "*competition*

between the sperm from two or more males for the fertilization of a given set of ova" (Parker, 1998). An extensive review done by Parker (1970) whereby he presents evidence in a number of insect species to support his findings of sperm competition shows that because females mate with more than one male and that sperm storage organs exist that allow sperm to stay viable for a long period of time, the chance for sperm competition to occur is very high and as a result males have adapted a number of strategies to try and outcompete other males that mate with the same female. These strategies include sperm precedence through the displacement of sperm from the first male to mate by the second male (especially prevalent in *Drosophila melanogaster*; e.g. Lefevre & Jonsson, 1962) and mating plugs, which are formed in the genital track of females from secretions by the male (seminal plasma or accessory gland) that work to prevent another male from depositing their sperm in the female and possibly outcompete the previous male's sperm (Parker, 1970).

Parker (1990a) developed mathematical models to explain ways in which males can enhance their ejaculates and overcome sperm competition to increase success against competitors: the fair raffle and the loaded raffle. The fair raffle assumes that every sperm cell in the ejaculate has an equal chance to fertilize the eggs (hence "fair"), therefore in species where a fair raffle exists, the male with the most sperm cells in their ejaculate will have a greater chance to outcompete other males and have higher success (Parker, 1990a). For example, Holman *et al.* (2011) show that in the leaf-cutting ant (*Atta colombica*), sperm use by females, after sperm was stored from multiple males, was determined by sperm numbers alone, which suggests there are no differences in sperm competitive ability among males. In contrast, the loaded raffle assumes that each sperm cell does not

have an equal chance to fertilize eggs, but instead that there is variation of sperm quality within an ejaculate, and therefore male's with greater sperm quality will be selected for, as they have a greater chance to outcompete other males and have higher fitness (Parker, 1990a). For example, it was shown that bluegill (*Lepomis macrochirus*) exhibit a loaded raffle because sperm from two alternative reproductive tactics (see below) have a competitive advantage over the third alternative tactic and because of differences in mating position between the different tactics, this must be due to differences in sperm quality (Stoltz & Neff, 2006).

Cryptic female choice

Cryptic female choice is the ability of a female to bias paternity towards a particular male when mating with multiple males and sperm competition provides the female with ejaculates from multiple males to 'choose' from. This bias can be accomplished using a variety of mechanisms such as inhibiting sperm storage and transport to fertilization sites within the genital tract, removal of mating plugs formed by males, and the act of remating with other males (Eberhard, 1996). Similar to research on sperm competition, all of the early work on cryptic female choice has focused on insects (red flour beetle, *Tribolium castaneum*, Edvardsson & Arnqvist, 2000; black field cricket, *Teleogryllus commodus*, Bussiere *et al*., 2006; soldier fly, *Merosargus cingulatus*, Barbosa, 2009), which is why it was termed 'cryptic', as fertilization occurs inside the female's genital tract and therefore cannot be observed.

More recently, studies of cryptic female choice have been focused on externally fertilizing species such as fish. Unlike in insects, it is thought that because of the gametes

interacting in the environment and not inside the female, the only mechanism a female can use to bias paternity is through her ovarian fluid, which accompanies the eggs upon release. It has been shown across many fish species that ovarian fluid increases sperm activity (sperm longevity, percent motility, and sperm velocity) when compared to the absence of ovarian fluid: Arctic charr, *Salvelinus alpinus*, Turner & Montgomerie (2002); Rainbow trout, *Oncorhynchus mykiss*, Woolsey *et al.* (2006); Atlantic cod, *Gadus morhua*, Litvak & Trippel (1998); Chinook salmon, *Oncorhynchus tshawytscha*, Rosengrave *et* al. (2009). Additionally, there has been evidence of potential paternity biasing by females using their ovarian fluid to influence sperm velocity, such that in the presence of one female's ovarian fluid, a male's sperm is faster, but in the presence of a different female's ovarian fluid the same male's sperm is slower (Rainbow trout, Dietrich *et al*., 2008; Arctic charr, Urbach *et al*., 2005; Chinook salmon, Rosengrave *et al*., 2008).

Potential genetic benefits of polyandry

Mechanisms of sperm competition and cryptic female choice are especially prevalent in species where polyandry occurs, that is where females mate with multiple males during a single mating event. Polyandry is common across the entire animal kingdom (Jennions & Petrie, 2000; Simmons, 2005) but until recently the reason why was not clear. The act of mating is very costly in a number of ways (Rowe, 1994) so why do females from many taxa choose to mate with more than one male? In species where males offer direct benefits, such as more parental care or nuptial gifts it's clear why polyandry has evolved (Simmons, 2001). However, in species where males offer no direct benefits to the female (i.e. only provide females with sperm for fertilization), an explanation for polyandry remains elusive. There are a number of hypotheses to why

polyandry in these non-resource based mating systems exists (reviewed in Simmons, 2001; Newcomer *et al*., 1999), including (among others) two not mutually exclusive hypotheses: (i) the *intrinsic male quality hypothesis* suggests that polyandrous females can improve their chances of mating with a male of high genetic quality (presumably producing more fit offspring) compared to monandrous (single mating) females. This potential benefit of multiple mating can be achieved by both pre-copulatory (i.e. females choosing males of high genetic quality determined by their secondary sexual characteristics) and post-copulatory (i.e. through sperm competition between the males or cryptic female choice) mechanisms (e.g. Madsen *et al*., 1992); and (ii) *the good sperm hypothesis*, where females have greater fitness, through increased offspring quality, because offspring were sired by males of superior sperm quality and are better sperm competitors, thus a positive relationship should exist between offspring quality and respective sire sperm competitiveness (Yasui, 1997). These hypotheses are not mutually exclusive, in that they both state that sperm competition should benefit the female through increased offspring fitness, and by mating with multiple males, she can at least ensure that a greater proportion of resulting offspring will be sired by the male with the higher sperm competitiveness. However, there is limited evidence showing support for the good sperm hypothesis (reviewed in Evans & Simmons, 2008), but there is evidence that polyandrous females obtain genetic benefits, through increases offspring quality, compared to monandrous females. For example, Simmons *et al*. (2001) showed that female Australian field crickets (*Teleogryllus oceanicus*) had greater hatching success of their eggs when mated with multiple males compared to when females were mated to a single male multiple times. Additionally, in yellow dung flies (*Scathophaga stercoraria*)

it was shown that male's sperm that were able to displace previous male's sperm and thus sire more offspring as the second male to mate produced offspring with lower developmental time, and because development time and offspring size are not related, these offspring are deemed to be of higher fitness (Hosken *et al*., 2003).

Alternative reproductive tactics

Alternative reproductive tactics are when traits have been selected in two divergent ways for individuals within a sex (mostly males) to obtain reproductive success (Taborsky *et al*., 2008). In general, this divergence creates a discontinuous, bimodal distribution of traits, which can be seen as size dimorphisms, colour polymorphisms, and behavioural alternatives, that allow individuals to allocate resources to alternative ways of achieving the same end goal of reproductive success (reviewed in Brockmann, 2001; Taborsky *et al*., 2008).

Alternative reproductive tactics are common across all taxa, including, insects (e.g. dung beetles, *Onthophagus acuminatus*; Emlen, 1997), amphibians (e.g strawberry poison frog, *Oophaga pumilio*; Meuche & Proehl, 2011), and mammals (e.g. meerkat, *Suricata suricatta*; Young *et al*., 2007), but in particular, it is very common among fish species. Knapp & Neff (2008) hypothesize why this is the case by outlining three factors (also see Taborsky, 2008): (1) the majority of fishes have external fertilization, which allows greater access to a female's eggs compared to internal fertilizers and offers greater opportunity for sneak fertilizations and therefore generate higher sperm competition between males (Knapp & Neff, 2008; Taborsky, 2008). (2) Fishes have indeterminate growth, which can cause there to be immense variation among body size between

individuals, which can be selected for to create alternative tactics within these individuals (Knapp & Neff, 2008; Taborsky, 2008). (3) Fishes exhibit a large variation in parental care provided to offspring, including offering no parental care, shared care by both parents, or care provided by only one of the parents, which can create opportunities for males to use this variation to adopt alternative tactics (Knapp & Neff, 2008; Taborsky, 2008).

Sneak-Guard hypothesis

One of the most prevalent alternative reproductive tactic systems across the animal kingdom is the sneak-guard dichotomy in males. The sneaker male is most often smaller in body size, matures precociously, and attempts to mate with a female by sneaking into mating events. Whereas the guard males are typically larger in body size, mature at an older age, and are behaviorally dominant in mating events, by monopolizing resources that are sought out by the female, or by guarding the female herself. Parker (1990b) developed the Sneak-Guard model to help explain the evolution of these alternative reproductive tactics in the context of polyandry and sperm competition. This model assumes that the sneaker male will face sperm competition each time it mates, whereas the guard male will only face sperm competition in a proportion of its matings, and in addition, guard males cannot predict when sperm competition will occur, as they are often unaware when a sneaker male sneaks into a mating event (Parker, 1990b). Therefore, the model predicts that each tactic will adopt a strategy to reflect their respective sperm competition risk. Sneaker males, due the guaranteed risk of sperm competition, should invest relatively more energy into their gonads compared to guard males. On the other hand, guard males, due to their unpredictable, lower risk of sperm

competition, should invest more energy into their body size and other secondary sexual characteristics to be able to monopolize resources or females (Parker, 1990b). The sneak-guard model is now widely supported by the literature and has been supported in a number of studies. For example, in Atlantic salmon (*Salmo salar*), the precocious parr (sneaker male), relative to body size, had larger testes, ejaculate volume, number of sperm cells and also the sperm were more motile and lived longer, which all give a greater fertilization success than the anadromous (guard) male (Gage *et al*., 1995; Vladic & Jarvi, 2001).

Study system: Chinook salmon

The Chinook salmon is a large, externally fertilizing fish exhibiting an anadromous and semelparous mating system (Healey, 1991). Male Chinook salmon exhibit sneak-guard alternative reproductive tactics, where males either adopt the guard tactic, which are referred to as hooknoses due to their large 'hooked' snout (kype), or the sneaking tactic, referred to as jacks (Berejikian *et al*., 2000). Chinook salmon are external fertilizers and mating occurs seasonally in rivers. This is a non-resource based mating system in which males only provide sperm (i.e. genes) to females and offer no parental care to offspring or offer any direct benefit to females (Flannery *et al*., 2013). Females dig nests, and a series of these nests comprise a single red; this is accomplished by using an oscillating motion with their caudal fin to make depressions in the gravel, where eggs can be deposited when the female wants to mate. Females have been shown to prefer to mate with the larger hooknose males, as they seem to delay spawning when only smaller jack males are present (Berejikian *et al*., 2000). Hooknoses mature at age 3-4 (in the Great Lakes) and use their larger body size to have primary access to females, entering

the nesting area first during spawning events and fighting off other males that come near spawning females (Flannery *et al*., 2013; Berejikian *et al*., 2000). Jacks mature precociously at age 2 (in the Great Lakes) and adopt satellite positions either upstream or downstream from nesting sites, where they use their small body size and burst speed to sneak into spawning events between females and hooknoses. Berejikian *et al*. (2010) recently examined Chinook salmon spawning behavior in semi-natural spawning channels and observed that 40% of spawning events involved only one hooknose male, while the rest included two to five males, including both hooknoses and jacks. In addition, jacks were observed to participate in spawning almost exclusively through sneaking and sired approximately 20% of offspring (Berejikian *et al*., 2010).

Overview of the thesis

The objective of this thesis was to explore polyandry and the resulting sperm competition dynamics and its implications on both females and the two male alternative reproductive tactics in Chinook salmon. Chapter two focuses on how sperm competition can influence female fitness, specifically looking at whether a female benefits genetically, through increased offspring quality, by mating with multiple males and to determine if these benefits are tactic-specific. I used an *in vitro* maternal half-sib breeding design to create families that had one or two potential sires in all possible combinations using both male alternative reproductive tactics and measured the resulting offspring hatching success to gauge offspring quality. In chapter three, I specifically wanted to determine if the seminal plasma from each tactic has any effect on sperm performance from males adopting the same or the opposite tactic. This was done by using computer assisted sperm analysis software and conducting an *in vitro* experimental manipulation of male

ejaculates to create treatments that had sperm in the presence of the seminal plasma from another male; from the same or the opposite tactic. These were compared to control treatments to determine if sperm competitiveness is affected by another male's seminal plasma.

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CHAPTER 2: TACTIC-SPECIFIC GENETIC BENEFITS OF POLYANDRY IN CHINOOK SALMON

1. Introduction

The evolution of multiple mating by females (or polyandry) remains an important question for evolutionary biologists (Taylor *et al*., 2014; Simmons, 2001; García-González & Simmons, 2005). Although the benefits of polyandry have been well documented in mating systems where females derive direct benefits from multiple mating (Ridley, 1988; Arnqvist & Nilsson, 2000), the maintenance of polyandry in species where females gain no material benefits are less clear. Several hypotheses exist that propose mechanisms for indirect genetic benefits to females of multiple mating (Jennions & Petrie, 2000; Tregenza & Wedell, 2000; Neff & Pitcher, 2005) including the good sperm hypothesis (Yasui, 1997). It predicts that polyandrous females will have a selective advantage over monandrous females in terms of higher viability of offspring because offspring will be sired by males with competitively superior sperm and it also predicts a positive correlation between a male's sperm competitiveness and the viability of both sons and daughters. To date, there is limited evidence for the good sperm hypothesis (see Evans & Simmons, 2008). For example, Hosken *et al*. (2003) showed that males which were highly competitive in sperm competition produced offspring that had a faster development rate in the yellow dung fly (*Scatophaga stercoraria*). Also, Fisher *et al*. (2006) showed that in the brown antechinus (*Antechinus stuartii*), males that have high paternity (i.e. were competitively superior in sperm competition) sired offspring that have greater survival.

The highly polyandrous Chinook salmon (*Oncorhynchus tshawytscha*) is an ideal system to test the good sperm hypothesis for several reasons. First, multiple mating by females is common, and additionally, the presence of male alternative reproductive tactics (ARTs) make the system interesting to study any possible tactic-specific effects of polyandry. The ARTs include large guard type males (known as "hooknoses", due to the curved snout) and precocious sneaky males (known as "jacks") (see Berejikian *et al*., 2010; Butts *et al*., 2012; Flannery *et al*., 2013). Jacks have a smaller body size, which allows them to hide to elude aggressive hooknose males and employ a sneaking tactic to steal fertilizations from hooknoses. Second, a predictor of sperm competition has already been established; sperm velocity is positively correlated with male paternity success in sperm competition (Flannery, 2011). Third, sperm and eggs can be easily extracted from individuals, allowing for powerful maternal half-sib study designs that control for maternal effects (see Simmons, 2005).

To test the good sperm hypothesis in the polyandrous Chinook salmon, we used a split-clutch fertilization protocol and produced eggs fertilized by either a single male (from each tactic) or multiple males (using two males from each or both tactics). We reared the resulting clutches through to emergence and examined offspring viability, as measured by hatching success, in the different crosses. As per the good sperm hypothesis, we predicted that polyandrous crosses should have offspring with superior hatching success compared to monandrous crosses and a positive relationship between sperm velocity and hatching success.

2. Materials and methods

Spawning Chinook salmon were collected between September 29, 2014 and October 10, 2014 from the Credit River (Mississauga, Ontario, Canada; 43°35'N, 79°42'W), which flows into Lake Ontario. Hooknoses (N =30, mean (+/- SE) mass = 8.3 kg +/- 0.32 kg, range = 5 – 11.4 kg), jacks (N =30, mean mass = 2.0 kg +/- 0.12 kg, range = 0.35 – 3.2 kg) and females (N =15, mean mass = 7.0 kg +/- 0.33 kg, range = 4.95 – 8.85 kg) were collected by standard electrofishing techniques. Small body size and absence of secondary sexual characteristic (e.g. hooked snout and large teeth) were used to distinguish jacks from hooknoses. Gametes were collected by gently applying abdominal pressure on each individual, being careful there was no contamination with water, urine or feces. Gametes were kept in a cooler at the river water temperature (~11^{o}C) until analysis and fertilization occurred (up to four hours later).

A total of 61, 289 eggs were collected from fifteen females (mean = 4093 eggs per female, range = 3532 – 4270 eggs per female) and were placed in a strainer to separate the eggs from the ovarian fluid. Eggs from each female were then divided into 400 ml clear, plastic containers (mean +/- SE = 204 ± 3.9; range = 81 – 440 eggs), with each container of eggs representing a different cross. For the monandrous crosses, 200 μL of sperm from two jacks (J_1 & J_2) and two hooknoses (H_1 & H_2) were used to individually fertilize subsets of eggs (N = 4 crosses) and for the polyandrous crosses, 100 μL of sperm from two of the same jacks and hooknoses were then added to the eggs simultaneously in all possible combinations, resulting in two within-tactic (J_1 x J_2 (N = 1) and H_1 x H_2 (N = 1)) and four between-tactic (J_1 x H_1, J_1 x H_2, J_2 x H_1, and J_2 x H_2; N = 4) crosses, with all these crosses being replicated twice resulting, in 20 (10 x 2) crosses per female (see Fig.

2.1). A different set of four males (two jacks and two hooknoses) was used for each of the fifteen females, with no male being used again in crosses involving a different female. It should be noted that this design provides crosses that are both common in the wild (H, H x H, and H x J) and crosses that are rare (J, J x J), if they occur at all, in the wild (Berejikian *et al*., 2010). A 25% ovarian fluid solution (50mL of ovarian fluid : 150mL river water) was used to activate the sperm; sperm was pipetted into stream of ovarian solution mixture as it was poured into eggs. Eggs were then placed into a recirculating incubation system held at 11^{o} C. The incubation system contained two stacks of incubation trays, with each stack containing up to 8 trays that were each divided into 16 cells per tray so crosses could remain separate. Eggs were left undisturbed for a week after they were fertilized. After that, daily checks were performed where the number of non-viable eggs were counted and removed from each cell of the divided incubation trays. Non-viable eggs were placed in 5% acetic acid solution (see Hoysak & Liley, 2001) to determine if eggs were fertilized or not. If the eggs turned completely clear, they were deemed to be unfertilized, while fertilized eggs had a visible, small white mass inside the egg. Unfertilized eggs (n = 177) were removed from data analyses to avoid any confounding effects related to difference in fertility among males.

For each male, sperm velocity was assessed using a sperm sample (1.5 μl) that was pipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, Beverly, MA, USA), covered with a glass coverslip (22 x 22 mm), and activated with 15 μL of the 25% ovarian fluid solution, using the ovarian fluid of the female that the male was paired with during fertilization. Activated sperm were video recorded using a CCD B/W video camera module (XC-ST50, Sony, Japan) at 50 Hz vertical frequency, mounted on a

microscope (CX41 Olympus, Melville, NY, USA) that was equipped with a 10x negative-phase objective (see Flannery *et al*., 2013). Video-recordings were analyzed using the HTM-CEROS sperm tracking software package (CEROS version 12, Hamilton Thorne) (see Pitcher *et al*., 2009 for details). Curvilinear sperm velocity (VCL; average velocity on the actual point-to-point track followed by the cell) at 5s post-activation was the metric used to represent sperm velocity and these estimates correspond to the mean of all motile cells analyzed.

All data were analyzed using R software v. 2.15.1 (R development Core Team 2012). To investigate whether offspring hatching success differed between monandrous and polyandrous crosses and whether there was any tactic-specific effects, generalized linear mixed models (GLMM) were used for binomial data (eggs that hatched were scored as 1, eggs that did not hatch were scored as 0) with a logit-linked function using the "glmer" function in the lme4 package in R (Bates *et al*., 2009). Cross type (monandry or polyandry) was the fixed factor in the model while female identity, replicate, and tray identities were random factors. Models were compared with and without each random factor to determine which factors significantly contributed to the variance observed for offspring hatching success, and the percent variance explained was calculated for each factor. Next, models were compared with and without the fixed factor to determine the effect of treatment on hatching success. To investigate whether male alternative reproductive tactic had an effect on hatching success in both monandrous and polyandrous matings a similar approach as above was used except the model included tactic-specific crosses as the fixed factor. Models were compared as described above and Tukey post-hoc analyses were performed to compare differences between cross types. To

examine whether there was a positive relationship between sperm competitive ability and viability of the offspring, a GLMM was used with hatching success of monandrous crosses as the dependent variable, sperm velocity as a fixed factor, and female identity as a random factor. Models were compared with and without male sperm velocity to determine significance. Due to technical difficulties not all males were used for this test because sperm velocity could not be measured for one jack male and four hooknose males. All data are presented as means ± standard errors.

3. Results

Offspring from polyandrous crosses had significantly higher hatching success than offspring from monandrous crosses ($\chi^2 = 73.42$, $p < 0.001$; Fig. 2.2a). All three random factors (female ID, tray, and replicate) in the model were significant (see Table 2.1). When examining tactic-specific differences within the polyandry and monandry crosses, we found a significant tactic effect ($\chi^2 = 315.53$, $p < 0.001$; Fig. 2.2b) and again the three random factors were significant (see Table S1). Post-hoc analysis showed that there was a significant difference (all $p < 0.02$) between all cross types, with the exception of one comparison between the crosses involving a single jack and two jacks ($p = 0.86$; see Fig. 2.2b). Both jack and hooknose sperm velocity was significantly related to hatching success while controlling for any female identity effects (jack: $\chi^2 = 5.9$, p = 0.01, Fig. 2.2a; hooknose: $\chi^2 = 8.4$, p = 0.004; Fig. 2.2b).

4. Discussion

Our experiment provides support for the good sperm hypothesis in the Chinook salmon mating system. The first prediction of the good-sperm hypothesis is that polyandrous females should benefit more than monandrous females through increased

offspring fitness (Yasui, 1997). Our data supports this prediction; polyandrous females had higher hatching success than monandrous females (74.4% and 69.5% respectively). By contrast, McNamara *et al*. (2014) found no support for the good-sperm hypothesis in field crickets (*Teleogryllus oceanicus*), as offspring from polyandrous matings did not show an increase in immunocompetence compared to offspring from monandrous matings. A meta-analysis revealed that there is a small but significant positive effect of polyandry on embryo viability, as measured by hatching success (Simmons, 2005). However, elucidating the mechanism for this increase in hatching success was not possible for most of the studies because they did not control for maternal effects. Our experiment using an external fertilizing species and a maternal half-sib experimental design allowed us to control for these potentially confounding maternal effects (also see Purchase *et al*., 2007).

Only one study to date has examined tactic-specific effects of monandry and polyandry in relation to offspring quality. Johnson & Brockmann (2013) found that polyandrous female horseshoe crabs (*Limulus polyphemus*) garner genetic benefits, through increased offspring developmental success, by mating with sneaker males compared to when mated with guard males whereas monandrous females, who only mate with guard males, received no additional genetic benefits when mated with sneaker males. Our study also found tactic-specific effects of polyandry on offspring viability; of the crosses that occur most commonly in the wild (i.e. H, HxH and HxJ; Berejikian *et al*., 2010), polyandrous crosses had significantly higher hatching success than the monandrous cross. In addition, for the experimental crosses that rarely (J) or never occur (J x J) in the wild (Berejikian *et al*., 2010), the polyandrous crosses with two jacks did not

have significantly higher hatching success than the monandrous jack crosses, although both of these cross types had significantly higher hatching success compared to the monandrous and polyandrous hooknose crosses. Over all crosses, the polyandrous hooknose-jack cross had the highest hatching success, and this may occur because this cross type possesses the broadest genetic continuum of all of the crosses because the two male tactics are from different age classes; hooknoses (and females) are four years old whereas jacks are two years old (Flannery *et al*., 2013). In addition, although hooknoses and females do not differ significantly, jacks and hooknoses differ significantly in terms of their major histocompatibility (MH) genes (Helou, 2010). In theory females could be employing post-spawning mechanisms, including effects of ovarian fluid (e.g. Rosenrave *et al*., 2008), to bias paternity to the more genetically superior or compatible male of the dyad to increase embryo viability (Neff & Pitcher 2005). For example, Pitcher and Neff (2006) found (in the same Credit River population of Chinook salmon) that MH class IIB alleles contribute to both additive (good genes) and non-additive (compatible genes) genetic effects on viability, including hatching success and early juvenile survivorship (also see Pitcher and Neff 2007).

The second prediction of the good sperm hypothesis is that there is a positive relationship between sperm quality metrics associated with sperm competition success and offspring viability. We found that there was a positive relationship between sperm velocity, a metric correlated with sperm competition success in Chinook salmon (Flannery, 2010), and hatching success for both hooknoses and jacks, suggesting females could potentially use post-spawning processes to bias fertilization towards males of intrinsically higher genetic quality. In a salmonid species (rainbow trout; *Oncorhynchus*

mykiss), DNA integrity is positively correlated with sperm velocity (Dietrich *et al*., 2005), suggesting that the promotion of sperm competition, which is determined by sperm velocity in salmonids (e.g. Gage *et al*., 2004; Flannery *et al*., 2013), by females could potentially select for sperm with higher DNA integrity and thus higher hatching success. For example, Pérez-Cerezales *et al.* (2010) found that although DNA-damaged sperm of rainbow trout can still fertilize eggs, embryo development, and thus survival, is decreased due to damage of spermatozoa DNA.

Our experimental fertilization trials and correlational data support the benefits of polyandry via the good sperm model, although patterns of paternity and the additive genetic variance of sperm competitiveness were not examined in this study. We suggest these are potentially beneficial avenues for future research in order to partition the benefits of polyandry among cryptic female choice and sperm competition mechanisms.

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Table 2.1 Binomial models for hatching success from monandrous and polyandrous crosses and cross types involving both reproductive tactics of Chinook salmon (*Oncorhynchus tshawytscha*). Female ID, tray and replicate were treated as random effects in the model. Variance components were estimated using restricted maximum likelihood, and the significance of variance components were tested using likelihood ratio tests. Note that $\pi^2/3 \approx 3.3$ is the underlying residual variance of a binomial model with logit link function (Nakagawa & Schielzeth, 2010; Johnson & Brockmann, 2013).

		Variance Components		Likelihood ratio test		
	Effect (n)	Variance	St.Dev	χ^2	P	Phenotypic Variance (%)
Effect of monandry or polyandry on hatching success	Female ID (15)	0.64	0.80	2027	< 0.001	15.8
	Tray (20)	0.10	0.32	230.8	< 0.001	2.5
	Replicate (2)	0.001	0.036	5.094	0.02	0.02
	Error	$\pi^2/3$				81.7
Effect of cross type on hatching success	Female ID (15)	0.64	0.80	1933	< 0.001	15.9
	Tray (20)	0.084	0.29	229.1	< 0.001	2.1
	Replicate (2)	0.0014	0.038	5.642	0.02	0.03
	Error	$\pi^2/3$				82.0

Figure Captions

Figure 2.1 Experimental design for the creation of the different monandrous and polyandrous crosses of Chinook salmon (*Oncorhynchus tshawytscha*). For monandrous crosses, sperm from a single male from each tactic (Jack (J) or Hooknose (H)) was used to fertilize a portion of a female's eggs, and this was done with two unique, separate males (J_1, J_2, H_1, and H_2). For the polyandrous crosses, sperm from two males from each of these four males was added simultaneously in all combinations, resulting in two within-tactic treatments (J_1 x J_2 and H_1 x H_2) and four between-tactic treatments (J_1 x H_1, J_1 x H_2, J_2 x H_1, J_2 x H_1). This was done for 15 different females, using a different set of four males for each female. This design was done in full replication, resulting in 20 crosses (10 crosses x 2) per female.

Figure 2.2 Hatching success (%) from (*a*) monandrous or polyandrous crosses and (*b*) tactic-specific crosses (J = jack; H = hooknose) of Chinook salmon (*Oncorhynchus tshawytscha*). Bars without a common letter differed significantly ($p < 0.05$, see text for details). Data are presented as means ± standard errors.

Figure 2.3 Relationship between hatching success (%) of monandrous crosses for particular male and female Chinook salmon (*Oncorhynchus tshawytscha*) crosses and each male's sperm velocity (um/s) for (*a*) hooknose males and (*b*) jack males (raw data are presented for clarity, see text for details).

Figure 2.1

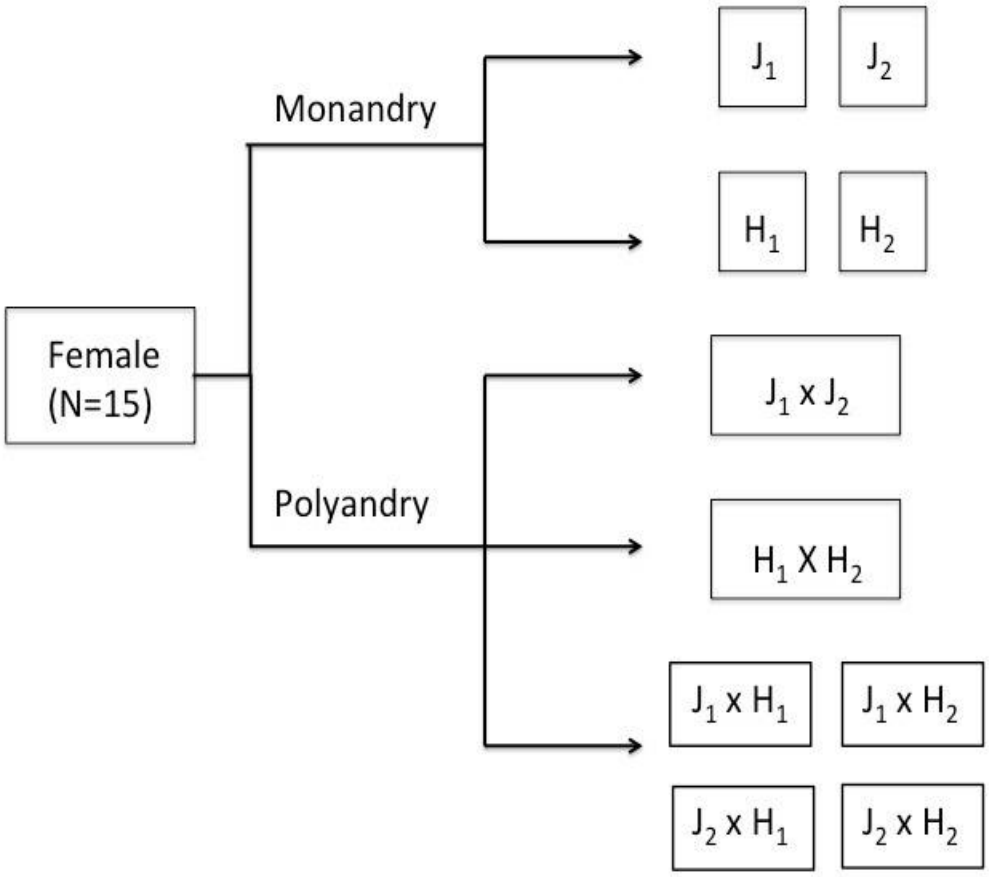
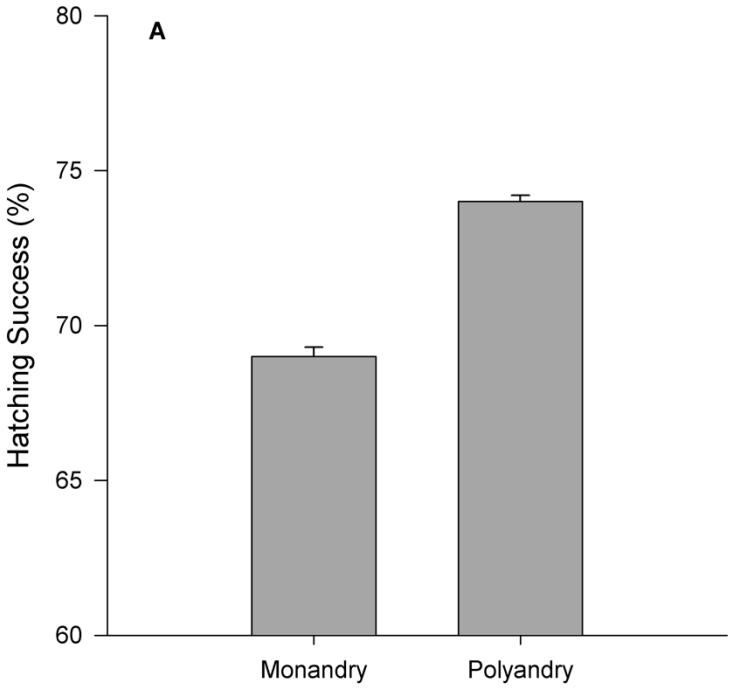

Female
(N=15)
Monandry
Polyandry
J1
J2
H1
H2
J1 x J2
H1 X H2
J1 x H1
J1 x H2
J2 x H1
J2 x H2

Figure 2.2


A
Hatching Success (%)
80
75
70
65
60
Monandry
Polyandry

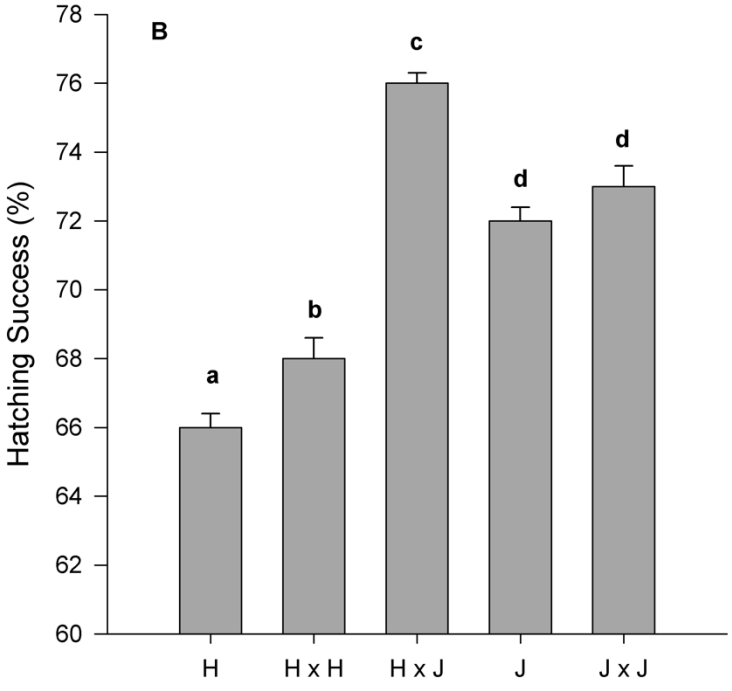
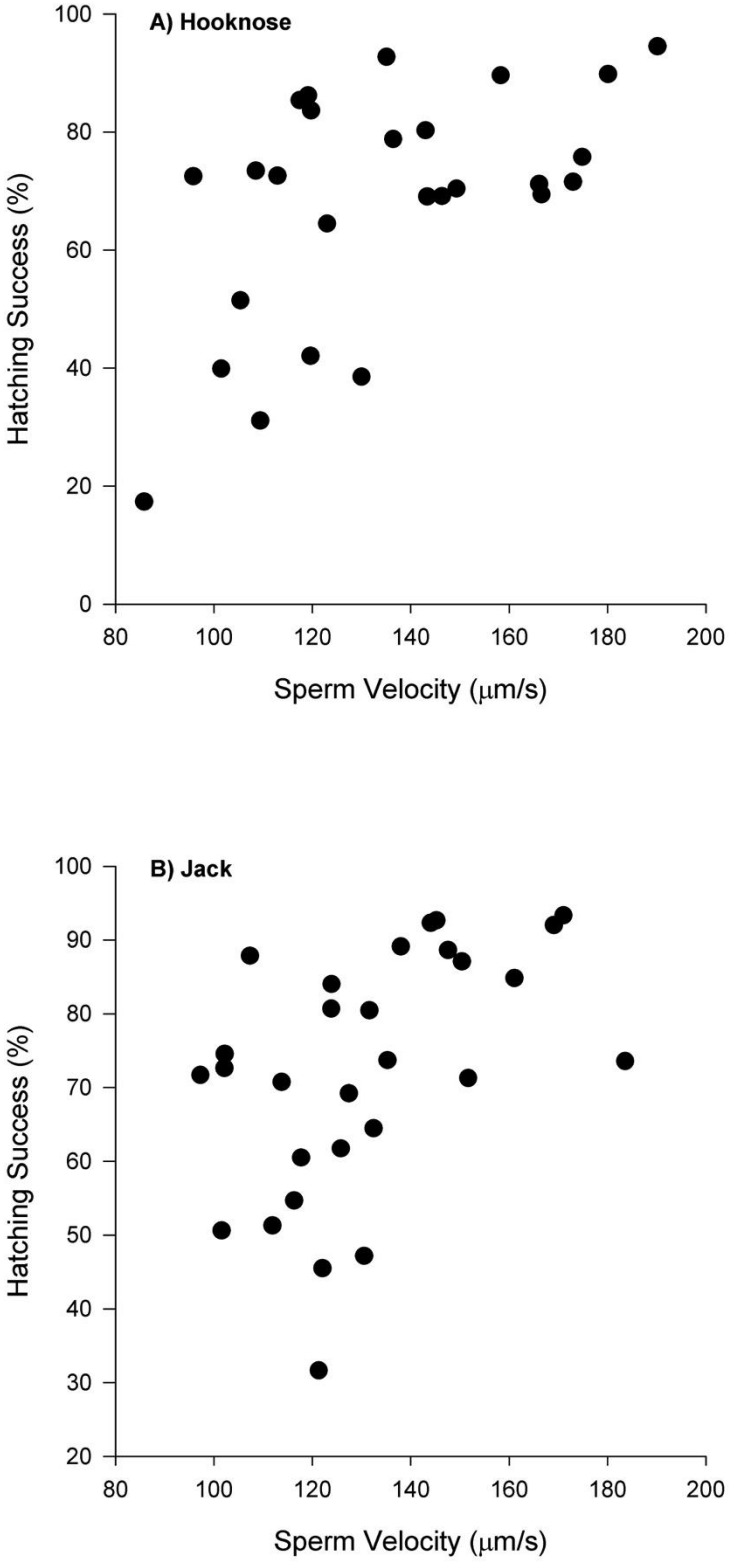

B
Hatching Success (%)
78
76
74
72
70
68
66
64
62
60
a
b
c
d
d
H
H x H
H x J
J
J x J

Figure 2.3


A) Hooknose
Hatching Success (%)
Sperm Velocity (μm/s)
B) Jack
Hatching Success (%)
Sperm Velocity (μm/s)

CHAPTER 3: THE EFFECTS OF OWN AND FOREIGN SEMINAL PLASMA ON SPERM PERFROMANCE IN THE ALTERNATIVE REPRODUCTIVE TACTICS OF CHINOOK SALMON

1. Introduction

Sperm competition occurs when sperm from multiple males compete to fertilize a female's eggs (Parker, 1970). This form of post-copulatory competition is a taxonomically widespread phenomenon and a powerful evolutionary force that has shaped the evolution of male mating behaviour, morphology and physiology (Birkhead & Moller, 1998; Simmons, 2001; Birkhead & Pizzari, 2002). Sperm competition is especially prevalent in species in which male alternative reproductive tactics are present due to male's having unequal opportunities to fertilize eggs (e.g. Stoltz & Neff, 2006; Taborsky, 1998; Burness *et al*., 2004). In such species, often the males have had different traits selected for to maximize reproductive success (Taborsky *et al*., 2008). These traits can take the form of morphological, behavioural, and life history differences (Taborsky *et al*., 2008).

A common alternative reproductive tactic system seen across taxa is the sneak-guard dichotomy in males (see Oliveira *et al*., 2008 for a taxonomic review). Sneaker males usually have small body size and uses covert techniques to sneak into mating events between guard males and females to obtain reproductive success. Whereas the guard males are typically large in body size and have more pronounced secondary sexual characteristics to aid in asserting dominance over other males and females, including fighting off other males while protecting and monopolizing females. Parker (1990) developed mathematical models for sneak-guard mating systems to help explain their

evolution in the context of sperm competition. Those models assume that there is a difference in sperm competition risk and perception of such risk between the two alternative tactics. Sneaker males have high sperm competition risk and accurate 'knowledge' of this risk because every time they mate there will be at least one other male (i.e. guard male(s)) present as well. Whereas the guard males have lower sperm competition risk because sneakers do not participate in all mating events and their "knowledge" of risk is less reliable because they are often unaware of the presence of sneaker males. These models have been supported in a number of empirical studies. For example, in Atlantic salmon (*Salmo salar*), the precocious parr (sneaker male), relative to body size, had larger testes, ejaculate volume, number of sperm cells and also the sperm were more motile and lived longer, which all provide greater fertilization success per spawning event than the anadromous (guard) male (Gage *et al*., 1995; Vladic & Jarvi 2001).

Most of the studies to date that examine sperm competition dynamics have focused on either differences in sperm number or sperm quality (see Birkhead & Moller 1998). However, sperm only make up a portion of the ejaculate and other components, such as seminal plasma (or fluid) can have effects on the outcome of sperm competition. For example, in the stalk-eyed fly (*Cyrtodiopsis whitei*), Fry & Wilkinson (2004) found that males had a dramatic decrease in fertilization success in the presence of the seminal plasma from other males. It has also been shown that males can alter the amount of seminal plasma in an ejaculate depending on the level of sperm competition risk (Wigby *et al*., 2009). It is important to note that most of this evidence stems from studies done on

insects, there is little known about whether seminal plasma can have similar effects in other taxa.

In fish, there are only two studies that examine the effects on seminal plasma on the outcome of sperm competition, and only one of these studies examine the effects in a mating system that exhibits alternative reproductive tactics. Within the subordinate males of Arctic charr (*Salvelinus alpinus*), it was found the percent of motile sperm was significantly higher in the presence of another male's seminal plasma, than in the male's own seminal plasma or in lake water, however there was no such effect on sperm velocity (Rudolfsen *et al*., 2015). This result may have little biological relevance however, because it is sperm velocity, and not percent motility, that is the best predictor of fertilization success in Arctic charr (Liljedal *et al*., 2008). Locatello *et al*. (2013) showed that in the grass goby (*Zosterisessor ophiocephalus*), a species with a sneak-guard alternative reproductive tactic mating system, there is a tactic-specific effect of seminal plasma on a rival male's sperm performance. Sneaker males were shown to increase their sperm velocity by approximately 9.3 % and thus causing an increase in their own fertilization success (by ~10%) by exploiting the guard male's seminal plasma, and also, the presence of sneaker seminal plasma caused a decrease in guard male's sperm velocity by approximately 6.8%, which in turn caused a decrease in fertilization success (of ~9%) (Locatello *et al*., 2013).

Chinook salmon (*Oncorhynchus tshawytscha*) exhibit the sneak-guard alternative reproductive tactic in males, where the large, dominant hooknose's (i.e. guards) have priority in mating positions with females, while the small, precocious jack males (i.e. sneakers) adopt the sneaking tactic (Berejikian *et al*., 2000; Heath *et al*., 1994; Heath *et*

al., 1996). This alternative reproductive tactic mating system with external fertilization allows females to mate with multiple males simultaneously and thus promotes intense sperm competition between males. It has been shown that in 40% of spawning events, only one hooknose is present, while in the other 60% there is anywhere from 2-5 males present, including both jacks and hooknoses (Berejikian *et al*., 2010). Previous work has shown that jacks have relatively larger testes and their sperm swims faster in river water compared to hooknose sperm in river water (Flannery *et al*., 2013), which supports the theoretical work done by Parker (1990) suggesting that the sneaker (jack) should invest more into spermatogenesis instead of other traits. However, investment into testes (as the model predicts) does not necessarily mean investment into just sperm cells; it could also be an investment into other components of the ejaculate, such as the seminal plasma. The objective of this study is to examine whether sperm competition is influenced by seminal plasma by examining sperm swimming performance, which included measures of sperm velocity, sperm path straightness, and percent of sperm motility because they are important for both competitive and non-competitive fertilization success (e.g. Gage *et al*., 2004). This was done by analyzing in vitro sperm swimming metrics when a male's sperm was in the presence of seminal plasma from a male of the opposite tactic or from a different male adopting the same tactic. This was done by the physical separation and manipulation of milt from males to swap out own seminal plasma for seminal plasma from other males. This design allows us, through two different experiments, to determine how seminal plasma from males adopting a different (experiment 1), and the same (experiment 2) tactic interact and if these males use seminal plasma as a mechanism to overcome intense sperm competition observed in Chinook salmon. Through sperm

competition theory (Parker, 1990), it can be hypothesized that, because of the asymmetry in sperm competition risk between tactics, jacks seminal plasma should be selected to impair a hooknose's sperm performance, and/or increase their own sperm performance in the presence of hooknose seminal plasma to be more competitive in sperm competition. Additionally, it is hypothesized that if there are any within-tactic effects of seminal plasma on sperm performance, it will only be found in hooknoses as they often compete against other hooknose's during sperm competition, so it would be beneficial for hooknose seminal plasma to cause a decrease in other hooknose's sperm performance, but there won't be an effect seen within jacks as they, presumably, rarely compete against other jack males in sperm competition.

2. Materials and Methods

Fish Collection

Male Chinook salmon were collected from the Credit River (Mississauga, Ontario, Canada; 43°35'N, 79°42'W) between September 30 and October 11 in 2013 (experiment 1; Hooknose: mean ± S.E. fork length = 87.3 cm ± 0.76 cm, range = 72 – 102 cm, mean ± S.E. mass = 7.7 kg ± 0.2 kg, range = 5.1 – 12.8 kg; Jack: mean ± S.E. fork length = 57.3 cm ± 0.6 cm, range = 46.8 – 68 cm, mean ± S.E. mass = 2.4 kg ± 0.09 kg, range = 1.3 – 3.6 kg) and September 29 and October 9 in 2014 (experiment 2; Hooknose: mean ± S.E. fork length = 87.4 cm ± 1.2 cm, range = 72.5 – 100 cm, mean ± S.E. mass = 7.8 kg ± 0.3 kg, range = 4.6 – 11.2 kg; Jack mean ± S.E. fork length = 52.7 cm ± 1.9 cm, range = 10 – 66.5 cm, mean ± S.E. mass = 2.0 kg ± 0.1 kg, range = 0.4 – 3.7 kg). Fish were collected by standard electrofishing and once caught, they were kept alive in pens that were placed in the river until length and weight were measured and milt was collected. Small body

size and absence of secondary sexual characteristic (e.g. hooked snout and large teeth) were used to distinguish jacks from hooknoses.

Milt Collection

Fish were humanely euthanized immediately before gametes were to be collected. Milt was collected in 532-mL clear whirl-pak sample bags (Nasco, Newmarket, ON, Canada) by gently applying abdominal pressure on the fish, being careful there was no contamination by water, urine or feces. The milt was then placed in a cooler at the water temperature of the river (11^{o}C) until analysis took place (two to three hours later).

Experimental Design

Experiment 1: There are three treatment groups for this experiment: (1) control, (2) sham control and (3) between tactic-swap. The sham control is used to determine if the centrifugation process to separate milt into its constituent parts (see below) has any effect on the sperm cells through comparison with the control treatment, which consists of milt that has not been centrifuged. The tactic-swap treatment is the main experimental treatment in which the effect of seminal plasma on the opposing alternative reproductive tactic's sperm performance was observed by swapping the seminal plasma of both tactics so that jack sperm cells are combined with hooknose seminal plasma and hooknose sperm cells are combined with jack seminal plasma. Fish were tested in jack-hooknose pairs (n = 16 unique pairs), so for each of the three treatments they were tested twice, once for the jack and once for the hooknose in each pair.

Experiment 2: A similar design was used as the first experiment, except instead of a between tactic-swap treatment, a within-tactic swap treatment was used. Fish were

tested in either jack-jack (n = 12) or hooknose-hooknose (n = 14) pairs, where fish were not used in more than one pair.

Treatment preparation

To swap the seminal plasma between fish (either within or between tactics), 1000 μL of milt was placed in an ependorf tube and centrifuged (accuSpin Micro 17, Fisher Scienctific) at 300xg for 10 minutes (Lahnsteiner *et al*., 2004). The resulting separate seminal plasma and sperm components were carefully pipetted out and placed in separate ependorf tubes in a chilling block set at 11^{o}C. For the sham control treatment, 75 μL of sperm was gently mixed with 25 μL of seminal plasma from the same male. For the tactic-swap treatments, 75 μL of sperm was gently mixed with 25 μL of seminal plasma of a male from the opposite (experiment 1) or same tactic (experiment 2). These concentrations were determined through preliminary data of spermatocrit values from a subset of males that resulted in males having ~25% seminal plasma (Mean ± S. E = 25.4 ± 3.4%; Range = 6.7 – 70.7%).

Sperm performance assessment

For each treatment in both experiments, a milt sample (1.5 μL) was pipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, Beverly, MA, USA), covered with a glass coverslip (22 x 22 mm), and activated with 15 μL of 11^{o}C river water (the approximate temperature of the river during spawning; maintained using the chilling block). Activated sperm were video recorded using a CCD B/W video camera module (XC-ST50, Sony, Japan) at 50 Hz vertical frequency, mounted on a microscope (CX41 Olympus, Melville, NY, USA) that was equipped with a 10x negative-phase objective (see Flannery *et al*., 2013). Video-recordings were analyzed using the HTM-CEROS

sperm tracking software package (CEROS version 12, Hamilton Thorne). We used the following recording parameters: number of frames captured in sequence with 1 s = 60 Hz; total number of sequential images captured for analysis = 60; minimum contrast = 11; minimum number of pixels that an object must be in order to be counted = 3. The following parameters were measured for each male's sperm: curvilinear velocity (VCL; average velocity on the actual point-to-point track followed by the cell, hereafter sperm velocity), straightness (STR; measure of the departure of the sperm cell path from a straight line), and percent motility (percent of motile sperm in the field of view showing propulsive motility) at 5s post-activation (see Hoysak & Liley, 2001). The sperm analysis software measures each sperm cell individually and generates an average of these cells for each treatment.

Statistical Analysis

Data was analyzed using SPSS statistical software analysis software (IBM Corp. Released 2013. IBM SPSS Statistics for Macintosh, Version 22.0. Armonk, NY: IBM Corp). Residuals were tested for normality (Shapiro-Wilkes test) and homogeneity of variance (plot of residuals vs. predicted values). Data are presented as mean ± SE. Effect of treatment on sperm performance of jacks and hooknoses in each of the two experiments was analyzed using an ANOVA for repeated measures (generalized linear model). The different treatments in both experiments were used as within-subject factor and the male tactic as between-subject factor. For each experiment, post hoc analysis of treatments within a single male tactic (e.g. jack sham control and jack seminal plasma swap) was performed using paired t-tests, while comparisons of treatments across male

tactics (e.g. jack seminal plasma swap and hooknose seminal plasma swap) were performed using independent t-tests.

3. Results

Experiment 1: Between-tactic manipulation

Centrifugation did not significantly affect sperm velocity (comparing control treatment with the sham control treatment) for males from either of the alternative reproductive tactics (repeated measures ANOVA: male tactic, $F_{1,26} = 1.09$, $p = 0.31$; treatment, $F_{1,26} = 1.13$, $p = 0.30$; tactic x treatment, $F_{1,26} = 0.62$, $p = 0.44$; Fig. A.1), however, this is not true for the other sperm performance metrics (see Appendix 3 for details for both experiments).

Comparison of sham control treatment and manipulated treatment show a significant effect on sperm velocity when sperm were activated in their own seminal fluid and that of a male of the opposing tactic (repeated measures ANOVA: male tactic, $F_{1,27} = 1.28$, $p = 0.27$; treatment, $F_{1,27} = 6.87$, $p = 0.014$; tactic x treatment, $F_{1,27} = 0.69$, $p = 0.42$; Fig. 3.1). Although the sperm of jack males were not significantly different in terms of velocity when exposed to hooknose males' seminal plasma and their own (jack sham control vs. tactic-swap treatment, paired t-test: $t_{13} = 1.1$, $p = 0.30$; Table 3.1; Fig. 3.1), hooknose males' sperm were slower when exposed to jack seminal plasma than when in their own seminal plasma (hooknose sham control vs. tactic-swap treatment, paired t-test: $t_{14} = 3.03$, $p = 0.009$; Fig. 3.1). However, there is no significant difference between these treatments for sperm straightness or percent motility (see Table 3.1; Fig. 3.2 and 3.3 respectively). A between-tactics comparison (comparing tactic-swap treatments between both tactics) showed that there was no difference between jack sperm velocity in

hooknose seminal plasma than hooknose sperm velocity in jack's seminal plasma (t-test: t = 1.32, p = 0.20 Fig. 3.1).

Experiment 2: Within-tactic manipulation

A comparison of sham control and manipulated treatments shows that there is no effect of seminal plasma of males adopting the same tactic on sperm velocity (repeated measures ANOVA: male tactic, $F_{1, 57} = 0.50$, p = 0.48; treatment, $F_{1, 57} = 0.12$, p = 0.73; tactic x treatment, $F_{1, 57} = 6.91$, p = 0.01; Table 3.2; Fig. 3.4), sperm straightness (repeated measures ANOVA: male tactic, $F_{1, 57} = 0.05$, p = 0.82; treatment, $F_{1, 57} = 0.001$, p = 0.97; tactic x treatment, $F_{1, 57} = 0.60$, p = 0.44; Table 3.2; Fig. 3.5), or percent motility (repeated measures ANOVA: male tactic, $F_{1, 57} = 0.02$, p = 0.88; treatment, $F_{1, 57} = 0.10$, p = 0.75; tactic x treatment, $F_{1, 57} = 1.7$, p = 0.20; Table 3.2; Fig. 3.6).

4. Discussion

The sperm competition hypothesis, which suggests that due to an asymmetry in sperm competition risk jack males should be selected to have seminal plasma to make them better competitors in sperm competition against hooknoses, is supported through the results of this study. We found that jack seminal plasma resulted in a decrease in sperm velocity, but not sperm straightness or percent motility, in hooknoses, however there is no effect on jack sperm performance in the presence of hooknose seminal plasma. In addition, we found no within-tactic effect of seminal plasma on sperm performance for either alternative reproductive tactic. These tactic-specific results, taken together, provide evidence that jack males may use seminal plasma to make themselves more competitive during sperm competition by causing a decrease in hooknose sperm velocity; a trait correlated with sperm competition success (Gage *et al*., 2004). In Chinook salmon, it has

been shown that, in river water, jack males outcompete and sire a greater proportion of offspring than hooknose males when in direct in vitro sperm competition (Flannery, 2011). Our finding of jack seminal plasma causing a decrease in hooknose sperm velocity of approximately 11.9% would cause a decrease in paternity of approximately 18 % when extrapolating from the data presented in Flannery (2011). However, because the between-tactic seminal plasma swap treatments for both jacks and hooknoses were not shown to be significantly different from each other, the effect of jack seminal plasma on hooknose sperm velocity at best levels the playing field between the two tactics during sperm competition.

A similar result has been found in the only other study done on fish that exhibit alternative reproductive tactics. Locatello *et al*. (2013) found that sneaker males use a two-fold mechanism to be more competitive in sperm competition with guard males: (1) sneaker seminal plasma causes a decrease in sperm velocity of guards, and (2) sneaker sperm velocity increases in the presence of guard seminal plasma. Although we didn't find an increase in jack sperm velocity in the presence of hooknose seminal plasma, both of these studies provide evidence that sneaker males increase their sperm competitive ability through seminal plasma interactions between reproductive tactics. Our study and Locatello *et al*. (2013) provide data suggesting an additional consideration to Parker's (1990) original sneak-guard model in which sneaker males do not only have to invest more into spermatogenesis than guard males, but an investment into seminal plasma and other ejaculate components could offer a competitive advantage during intense sperm competition between guards and sneakers.

A mechanism by which seminal plasma could be influencing sperm performance of other males and ultimately sperm competition would involve seminal plasma proteins. There is a large amount of literature on the study of seminal plasma (or fluid) proteins in insects (primarily *Drosophila spp.*) and their effects on male and female reproductive success. The majority of these diverse proteins in insects are produced by the accessory glands and have a wide range of fitness-related functions, such as sperm storage, sperm management and sperm competition, decreased female sexual receptivity and increases in egg production (reviewed in Chapman, 2001; 2008). For example, it was shown that one protein in particular, Acp36DE, is important in sperm competition in *Drosophila* because this protein is involved in displacing rival male's sperm (Chapman *et al*., 2000). Males that did not have the protein in their ejaculate sired a significantly lower numbers of offspring compared to males that did have the protein in their ejaculate due to their sperm being displaced by other males and thus being outcompeted (Chapman *et al*., 2000). Similar seminal plasma protein effects on sperm competition could be happening in Chinook salmon, as it has been shown that there are unique protein profiles found in each tactic's seminal plasma (Gombar *et al*., unpublished), which could provide a mechanism for how the seminal plasma has effects on sperm performance as shown in our study. It is important to note that in external fertilizers, especially fish, the effects of seminal plasma proteins are not expected to be as profound as seen in insects and other internal fertilizers because the seminal plasma is not directly transferred to females and with a very dynamic spawning environment, there is little time for interaction between sperm and seminal plasma. Nevertheless, determining if the effect of seminal plasma on sperm velocity is

due to specific proteins would be important to further our understanding of sperm competition.

Our results offer another interesting question that should be explored further; how does the seminal plasma from the different male tactics interact with ovarian fluid from females? Such a study would provide answers to the overall questions regarding which male tactic would outcompete the other in a more natural setting, as seminal plasma and ovarian fluid are present in the natural spawning micro-environment. Additionally, it could offer insight into more general sexual selection questions as it would show if there is any cryptic female choice occurring through ovarian fluid and if females are showing any kind of preference to either male tactic when it comes to sperm competition, or if females do not have a preference and instead allow males of both tactics equal opportunity during sperm competition to garner genetic benefits of multiple mating (see Chapter 2).

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Table 3.1 Summary of statistical analyses for the between-tactic seminal plasma swap experiment (experiment one) for three sperm performance metrics; including curvilinear sperm velocity, sperm straightness, and the percentage of motile sperm. Repeated measures ANOVAs were used to compare control treatments with sham control treatments as well as to compare sham control treatments to the manipulated between-tactic swap treatments. Models included treatment, male tactic (jack or hooknose) and the interaction of treatment x tactic. Paired t-tests were used to for post-hoc analysis of differences between treatments within a single tactic when the main model was significant.

		Repeated Measures ANOVA			Paired t-test			
					Jack		Hooknose	
Sperm Velocity (μm/s)	Control - Sham Control	Treatment	$F_{1, 26} = 1.1$	$p = 0.30$				
		Tactic	$F_{1, 26} = 1.1$	$p = 0.31$				
		Treatment x Tactic	$F_{1, 26} = 0.62$	$p = 0.44$				
	Sham Control - Manipulation	Treatment	$F_{1, 27} = 6.87$	$p = 0.014$	$t_{13} = 1.1$	$p = 0.30$	$t_{14} = 3.0$	$p = 0.009$
		Tactic	$F_{1, 27} = 1.28$	$p = 0.27$				
		Treatment x Tactic	$F_{1, 27} = 0.69$	$p = 0.42$				
Sperm Straightness	Control - Sham Control	Treatment	$F_{1, 26} = 7.8$	$p = 0.01$	$t_{12} = -1.65$	$p = 0.13$	$t_{14} = -2.4$``	$p = 0.03$
		Tactic	$F_{1, 26} = 0.35$	$p = 0.56$				
		Treatment x Tactic	$F_{1, 26} = 0.006$	$p = 0.94$				
	Sham Control - Manipulation	Treatment	$F_{1, 27} = 0.007$	$p = 0.93$				
		Tactic	$F_{1, 27} = 0.11$	$p = 0.75$				
		Treatment x Tactic	$F_{1, 27} = 0.34$	$p = 0.57$				
Motility (%)	Control - Sham Control	Treatment	$F_{1, 26} = 8.3$	$p = 0.008$	$t_{12} = 1.59$	$p = 0.14$	$t_{14} = 2.49$	$p = 0.026$
		Tactic	$F_{1, 26} = 0.003$	$p = 0.96$				
		Treatment x Tactic	$F_{1, 26} = 0.76$	$p = 0.39$				
	Sham Control - Manipulation	Treatment	$F_{1, 27} = 2.14$	$p = 0.16$				
		Tactic	$F_{1, 27} = 0.12$	$p = 0.74$				
		Treatment x Tactic	$F_{1, 27} = 0.059$	$p = 0.81$				

Table 3.2 Summary of statistical analyses for the within-tactic seminal plasma swap experiment (experiment two) for three sperm performance metrics; including curvilinear sperm velocity, sperm straightness, and the percentage of motile sperm. Repeated measures ANOVAs were used to compare control treatments with sham control treatments as well as to compare sham control treatments to the manipulated between-tactic swap treatments. Models included treatment, male tactic (jack or hooknose) and the interaction of treatment x tactic. Paired t-tests were used to for post-hoc analysis of differences between treatments within a single tactic when the main model was significant.

			Repeated Measures ANOVA		Paired t-test			
					Jack		Hooknose	
Sperm Velocity (μm/s)	Control - Sham Control	Treatment	$F_{1, 58} = 16.4$	$p < 0.001$	$t_{29} = 4.8$	$p < 0.001$	$t_{29} = 0.81$	$p = 0.43$
		Tactic	$F_{1, 58} = 0.26$	$p = 0.62$				
		Treatment x Tactic	$F_{1, 58} = 8.7$	$p = 0.005$				
	Sham Control - Manipulation	Treatment	$F_{1, 57} = 0.12$	$p = 0.73$				
		Tactic	$F_{1, 57} = 0.50$	$p = 0.48$				
		Treatment x Tactic	$F_{1, 57} = 6.9$	$p = 0.011$				
Sperm Straightness	Control - Sham Control	Treatment	$F_{1, 58} = 5.5$	$p = 0.022$	$t_{29} = -1.0$	$p = 0.33$	$t_{29} = -2.4$	$p = 0.025$
		Tactic	$F_{1, 58} = 0.042$	$p = 0.84$				
		Treatment x Tactic	$F_{1, 58} = 0.81$	$p = 0.37$				
	Sham Control - Manipulation	Treatment	$F_{1, 57} = 0.001$	$p = 0.97$				
		Tactic	$F_{1, 57} = 0.051$	$p = 0.82$				
		Treatment x Tactic	$F_{1, 57} = 0.60$	$p = 0.44$				
Motility (%)	Control - Sham Control	Treatment	$F_{1, 58} = 29.7$	$p < 0.001$	$t_{29} = 5.03$	$p < 0.001$	$t_{29} = 2.8$	$p = 0.009$
		Tactic	$F_{1, 58} = 0.11$	$p = 0.74$				
		Treatment x Tactic	$F_{1, 58} = 1.81$	$p = 0.18$				
	Sham Control - Manipulation	Treatment	$F_{1, 57} = 0.10$	$p = 0.75$				
		Tactic	$F_{1, 57} = 0.022$	$p = 0.88$				
		Treatment x Tactic	$F_{1, 57} = 1.7$	$p = 0.20$				

Figure Captions

Figure 3.1 Mean (± standard error) curvilinear velocity of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma after being spun in centrifuge (sham control; gray bars) and sperm in between-tactic's seminal plasma (manipulated; hashed bars). An asterix (*) signifies a significant post-hoc test ($p < 0.05$) when the main model was siginificant, otherwise no post-hoc tests were conducted.

Figure 3.2 Mean (± standard error) straightness (STR) of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma after being spun in centrifuge (sham control; gray bars) and sperm in between-tactic's seminal plasma (manipulated; hashed bars).

Figure 3.3 Mean (± standard error) percent motility of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma after being spun in centrifuge (sham control; gray bars) and sperm in between-tactic's seminal plasma (manipulated; hashed bars).

Figure 3.4 Mean (± standard error) curvilinear velocity of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma after being spun in centrifuge (sham control; gray bars) and sperm in combinations with within-tactic seminal plasma (manipulated; hashed bars).

Figure 3.5 Mean (± standard error) straightness (STR) of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma after being spun in centrifuge (sham control; gray bars) and sperm in combinations with within-tactic seminal plasma (manipulated; hashed bars).

Figure 3.6 Mean (± standard error) percent motility of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma after being spun in centrifuge (sham control; gray bars) and sperm in combinations with within-tactic seminal plasma (manipulated; hashed bars).

Figure 3.1

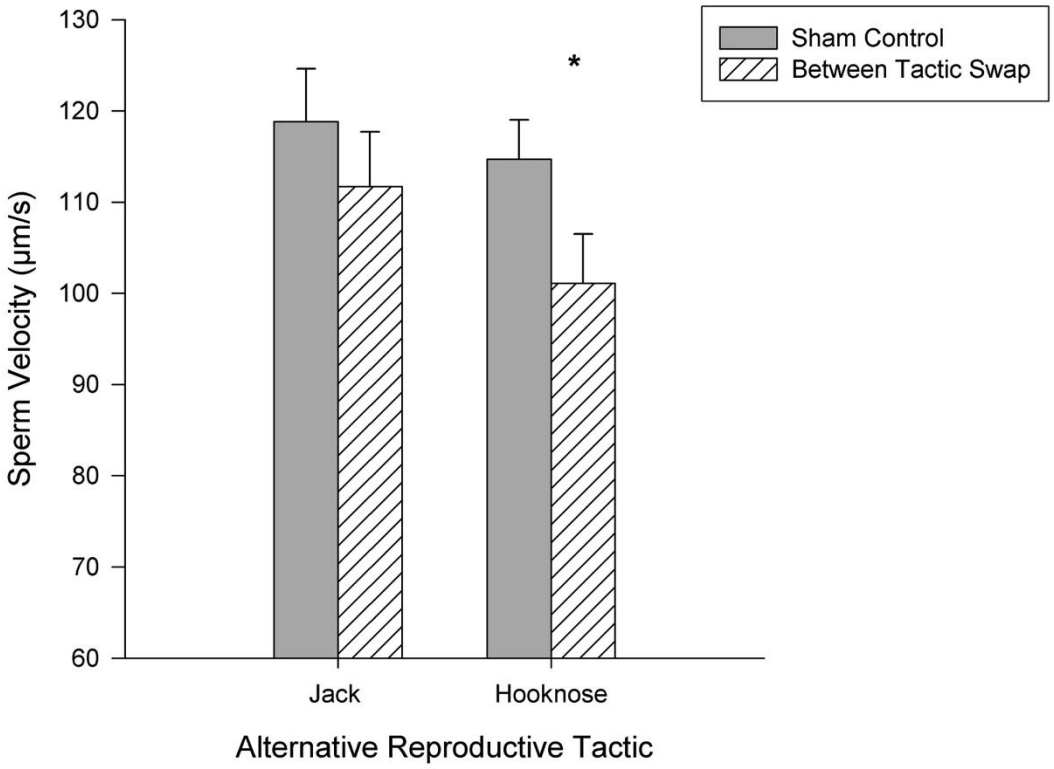

Sham Control
Between Tactic Swap
*
130
120
110
100
90
80
70
60
Sperm Velocity (µm/s)
Jack
Hooknose
Alternative Reproductive Tactic

Figure 3.2

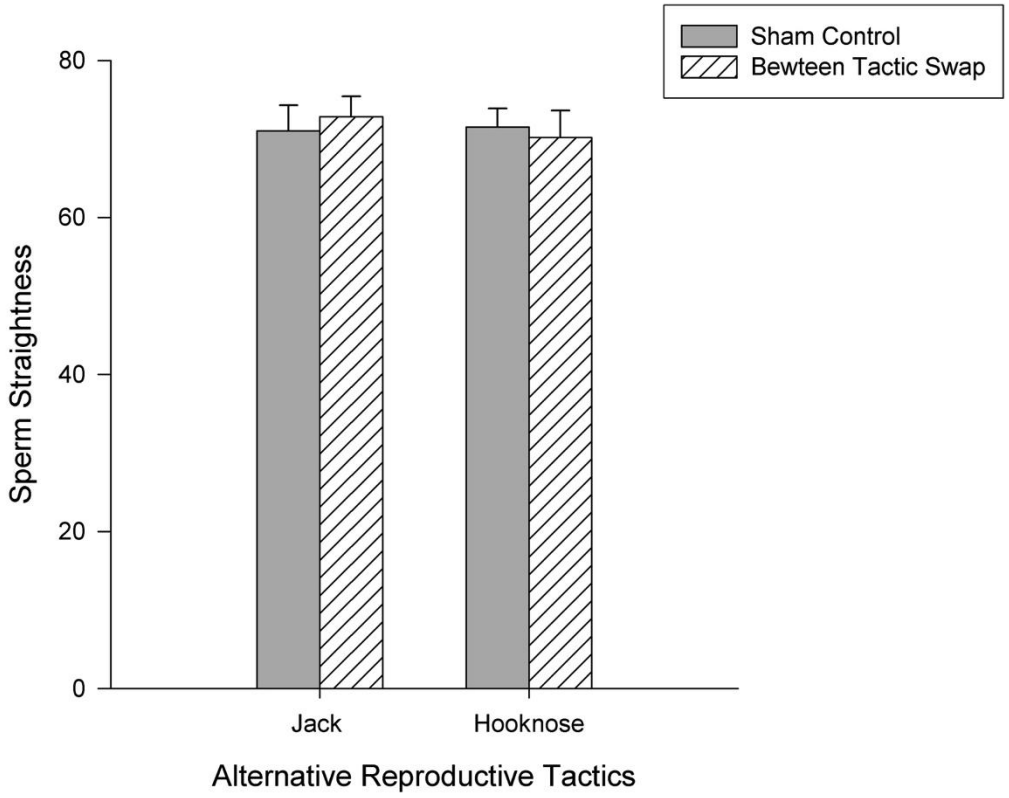

Sham Control
Bewteen Tactic Swap
80
60
40
20
0
Sperm Straightness
Jack
Hooknose
Alternative Reproductive Tactics

Figure 3.3

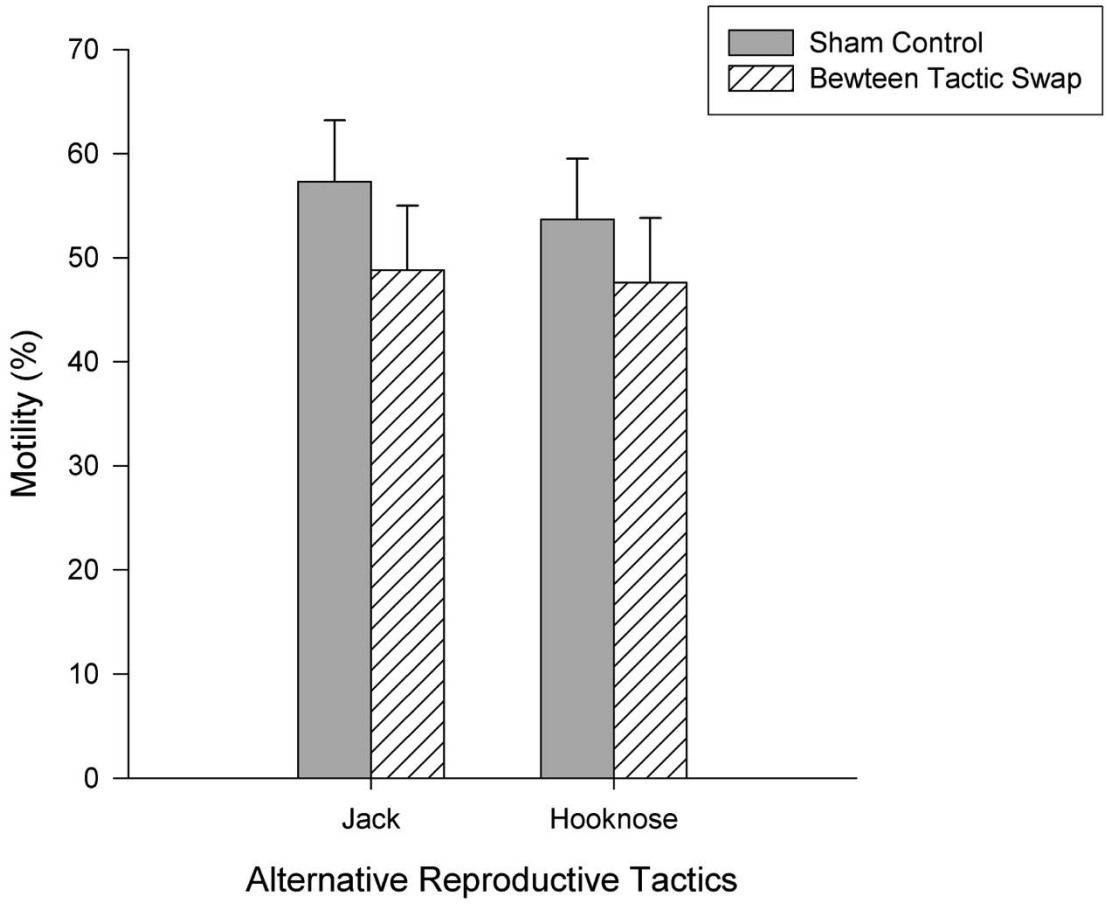

Sham Control
Bewteen Tactic Swap
70
60
50
40
30
20
10
0
Motility (%)
Jack
Hooknose
Alternative Reproductive Tactics

Figure 3.4

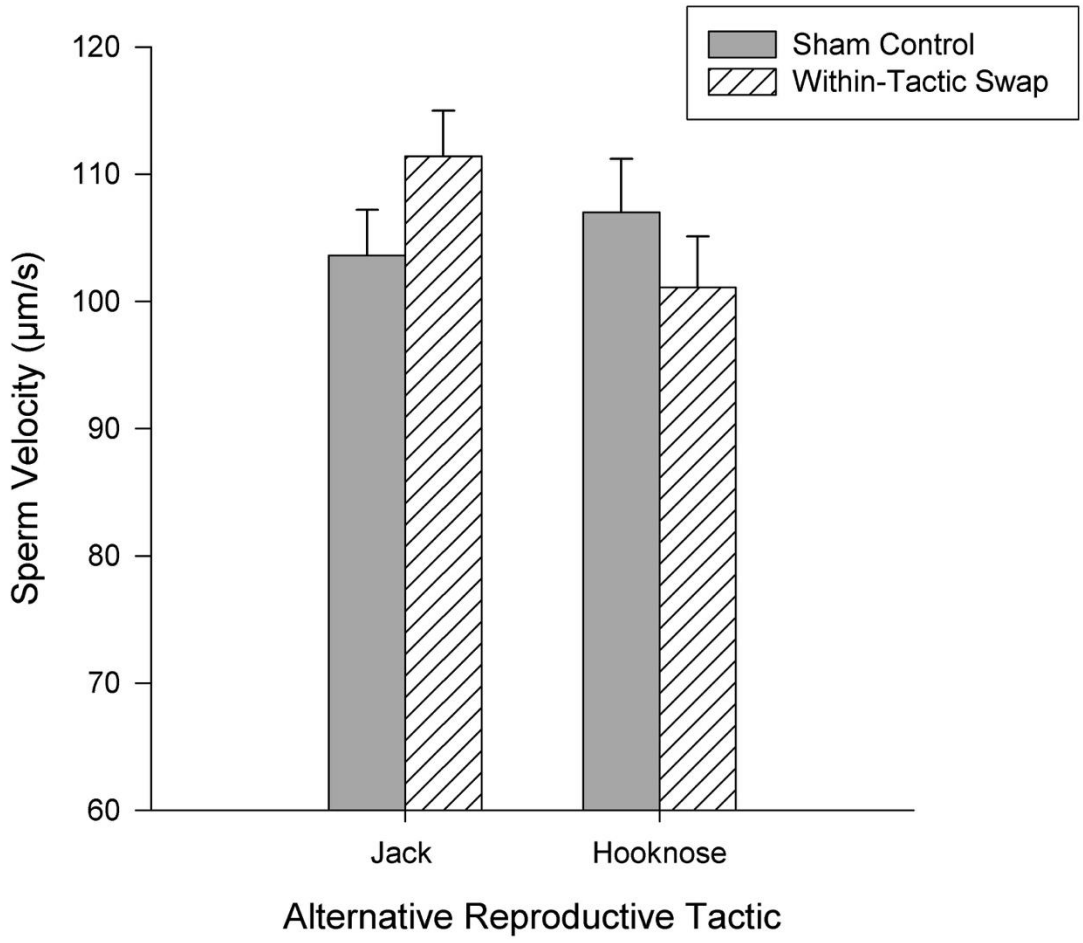

Sham Control
Within-Tactic Swap
120
110
100
90
80
70
60
Sperm Velocity (µm/s)
Jack
Hooknose
Alternative Reproductive Tactic

Figure 3.5

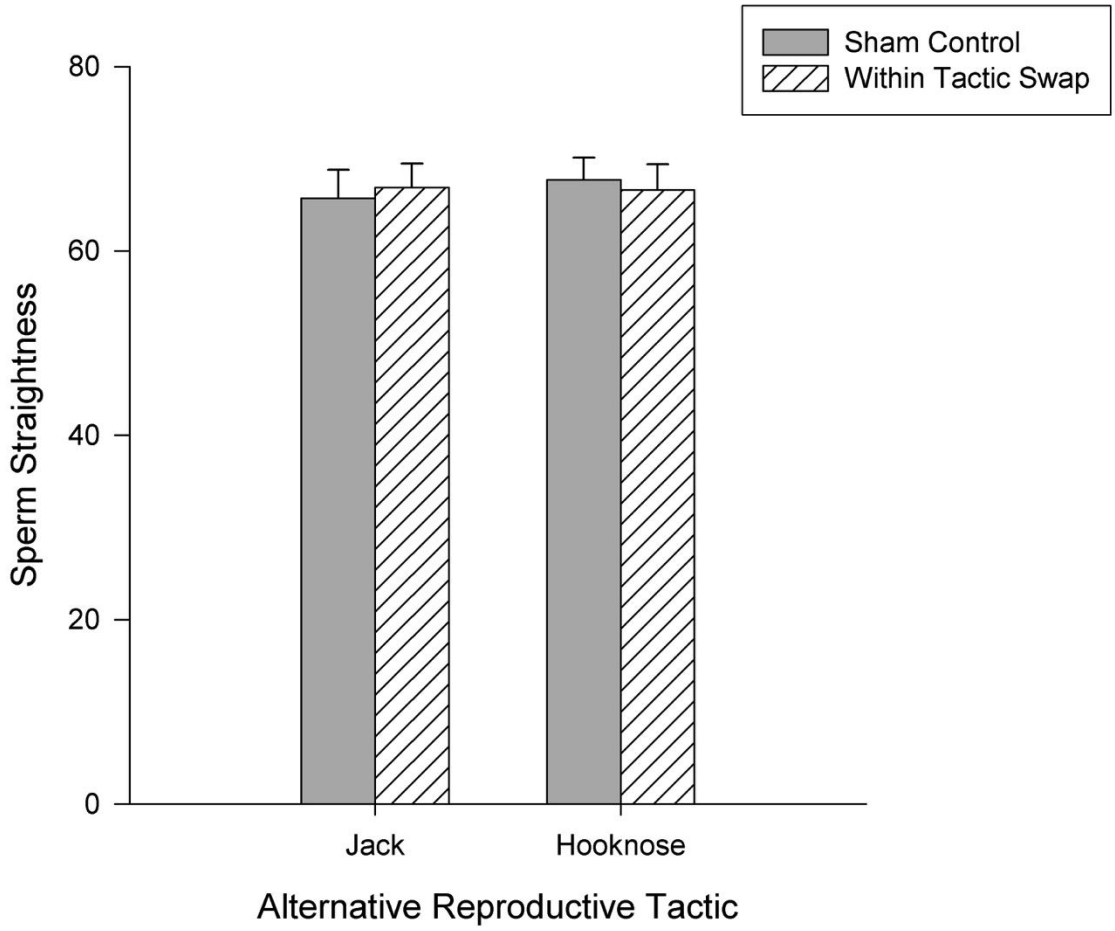
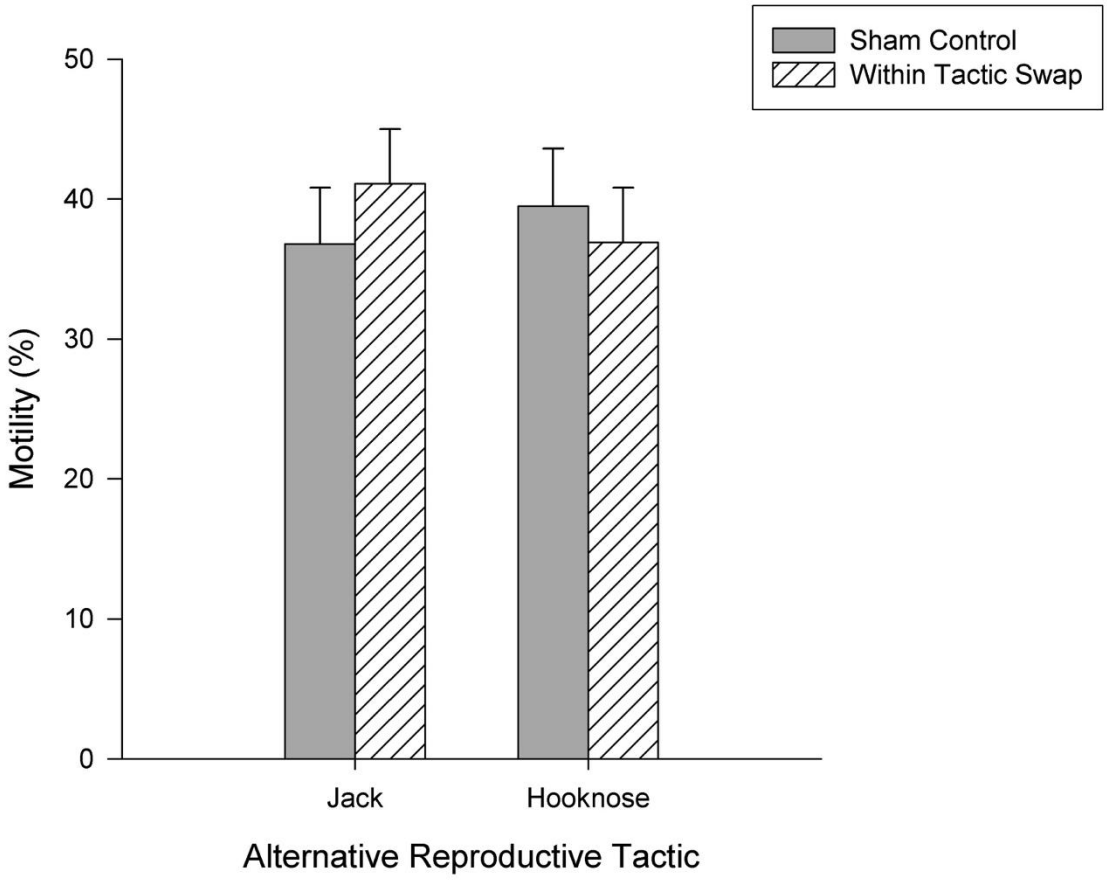

Sham Control
Within Tactic Swap
80
60
40
20
0
Sperm Straightness
Jack
Hooknose
Alternative Reproductive Tactic

Figure 3.6


Sham Control
Within Tactic Swap
50
40
30
20
10
0
Motility (%)
Jack
Hooknose
Alternative Reproductive Tactic

CHAPTER 4: GENERAL DISCUSSION

1. Summary

In this thesis I experimentally tested hypotheses related to the benefits of polyandry and sperm competition using male (from both alternative reproductive tactics) and female Chinook salmon (*Oncorhynchus tshawytscha*). The purpose of this chapter is to summarize the main conclusions and discuss the implications of my research so that specific directions of future research can be outlined to expand on the work done in this thesis.

In chapter two, I found that from a female's perspective, sperm competition, through the act of mating with multiple males simultaneously, is beneficial because it provides the female with greater fitness by increasing the quality of the offspring, as revealed by increased hatching success. By promoting sperm competition between males, a female can ensure that the majority of her offspring will be fertilized by the male with the highest sperm velocity, as this is a predictor of fertilization success (Flannery, 2011). However, these benefits of polyandry differ depending on the alternative reproductive tactic the males adopt, with the female receiving the greatest benefit by mating with a jack and a hooknose male simultaneously over all other combinations. My research further shows that males with faster moving sperm will also produce offspring of higher quality, specifically, higher hatching success, and therefore provide the female genetic benefits of mating with multiple males. The results from this data chapter show support for the good sperm hypothesis (Yasui, 1997) because firstly, polyandrous females received greater genetic benefits over monandrous females, and secondly, males with superior sperm quality produced offspring of higher quality, measured as hatching success.

In chapter three, I show that male's seminal plasma can potentially impact the outcome of sperm competition (and ultimately reproductive success) by altering the speed of rival males' sperm. These results exhibit tactic-specific effects as the impact of the seminal plasma on sperm velocity varies with the male alternative reproductive tactic. Jacks' seminal plasma causes a decrease in sperm velocity of hooknose males, but there is no effect of hooknose seminal plasma on jack male's sperm velocity. Finally, I found no effect of seminal plasma on sperm velocity of males adopting the same tactic.

2. Chapter Two

To determine if female Chinook salmon obtain genetic benefits of mating multiply, which might explain why polyandry is so prevalent, the good-sperm hypothesis (Yasui, 1997) was experimentally tested in this chapter. The good-sperm hypothesis has rarely been tested and to date has received limited support (but see Hosken *et al*., 2003; Fisher *et al*., 2006), but that could be mostly because most of the work on the potential genetic benefits of polyandry has focused on internal fertilizers, and these mating systems make it difficult to distinguish effects due to polyandry and possible confounding maternal effects (see Simmons, 2005). By using an external fertilizing species, I avoid this problem because maternal effects can be controlled through the use of a maternal half-sib experimental design.

I found that multiply mated females do accrue genetic benefits as offspring from polyandrous crosses had a higher hatching success than offspring from monandrous crosses. Furthermore, these benefits differed depending on the alternative reproductive tactic of the males for each cross. The use of jack males as the contributor of sperm show a general increase in offspring hatching success than that of using only hooknose males;

even a single jack male produced offspring with greater hatching success than crosses using either a single hooknose or two hooknose males. Even though a single jack male didn't differ from that of two jack males in terms of offspring hatching success, there seems to be some added benefit to the female by mating with a jack male. However, the cross that involved both a hooknose and a jack produced the offspring with the greatest hatching success, so if the effect of higher hatching success was only because of the jack, then we would expect the offspring from this cross to be worse than the cross involving one or two jack males.

One limitation from this study was that paternity of the offspring was not determined, so for the polyandrous crosses it is not known whether the paternity is biased towards one male or the other. Having this knowledge would allow me to more specifically comment on the tactic-specific effects I found. For example, if the cross involving a hooknose and jack (H x J) had the majority of the offspring sired by the jack, then this would fall in line with the results of the other crosses, with the jack males providing something added to the females to cause an increase in the benefits she receives. However, if the hooknose sired the majority of the offspring, then some other mechanism may be at play.

Future directions for this study, other than determining paternity of the offspring, would be to assess different fitness-related traits throughout the lifetime of the fish and determine overall fitness, including both survival and reproductive fitness. For example, immunocompetence challenges (see McNamara *et al.*, 2014) of the offspring would be an important trait to measure, as this would give insight into the quality of the offspring. MH (major histocompatibility) genotyping of the offspring and parents would allow us to

determine if there is any relationship between offspring and parental MH genes, which play a crucial role in the immune response as well as being a potential mate choice determinant (e.g. Landry *et al*., 2001). It would be ideal to allow the offspring to mature and also examine gamete quality to determine if they possess high quality gametes, which again can be linked back to parental gamete quality and would give a better indication of lifetime fitness benefits of polyandry. It would also be interesting to test the sexy-sperm hypothesis, which states that males that are superior in sperm competition will produce sons that are also superior in sperm competition. This would require offspring to be raised until maturity and genotyped to determine sire-offspring relationship in terms of sperm quality. Finally, the work completed in this chapter was done on hatchery-raised offspring, but it would be beneficial to attempt these experiments in a wild setting. This would require eggs to be incubated in native rivers and streams, where hatching success could be measured and give insight into the effects of polyandry when there is a more realistic selection pressure on eggs, instead of the very relaxed pressure experience through hatcheries. In a more extensive project, one could release tagged offspring back into the wild and monitor survival and returns to spawning grounds as the fish mature in the wild.

This chapter has potential implications for both the aquaculture industry and supportive breeding programs. It is important to note that often in these programs fertilization designs are often simplified and done with little scientific input, which could have implications on the quality of offspring produced, depending on the nature of the program (whether fish are for production only, or to be released to support wild populations). Campton (2004) suggests that sperm competition in these protocols,

specifically the common practice of combined milt from random multiple males to fertilize a set of eggs, will cause significant unequal genetic contributions from males (also see Wedekind *et al*., 2007). While this may be true, as some males are more certain to outcompete other males, the results from this chapter suggest that sperm competition may be beneficial by causing an increase in offspring viability as seen via increased hatching success, which is an obvious benefit to both aquaculture and supportive breeding programs. In addition, programs that are using Chinook salmon are most likely only using hooknoses when completing fertilization protocols, but the results from this chapter show there may be a great benefit to include jacks into these protocols, as it will lead to greater offspring hatching success of approximately 10% when comparing current hatchery protocols (a single hooknose = 66.6%) to the proposed new protocol (using a cross including both a jack and a hooknose = 76.3%), which could have significant positive implications on multi-million dollar per year programs.

3. Chapter Three

In this chapter, I attempt to determine if the alternative reproductive tactics within male Chinook salmon can influence the outcome of sperm competition by using seminal plasma as a means to alter rival sperm performance. This was done by swapping seminal plasma from males of the same and different alternative tactics in a paired design and completing an *in vitro* analysis of sperm performance. My experimental design allowed for the direct analysis of how sperm performance changed from natural (control) conditions to that of manipulated, where sperm cells were combined with foreign seminal plasma as there was no remnants of own seminal plasma in the manipulated treatments.

I found that there were tactic-specific effects of seminal plasma on sperm performance. The addition of jack seminal plasma caused a decrease in one metric of hooknose sperm performance, specifically, curvilinear velocity (which is correlated with sperm competition success), but hooknose seminal plasma had no effect on jack sperm performance. There was no effect on sperm performance when seminal plasma from males adopting the same tactic was added to the sperm of rival males. These findings demonstrate that the supposedly disadvantaged (at spawning; Berejikian *et al*., 2010) jack males can compensate for their less than ideal spawning positions, by at least leveling the 'playing field', with the hooknose males by taking advantage of seminal plasma components to alter the sperm velocity of rival males. The fact that there is no effect of jack seminal plasma on other jack males further shows that this may be a mechanism to aid in sperm competition and not just a general effect on all males, because multiple jacks rarely if ever compete against each other in sperm competition (Berejikian *et al*., 2010).

Future studies that can build upon the research completed in this chapter could be to determine the effect of adding female ovarian fluid has on the impact of seminal plasma. In order to determine the direct effects of seminal plasma, I chose to ignore female effects, but to determine what actually occurs at the site of fertilization it is important to include all components; sperm, egg, ovarian fluid, water, and seminal plasma. It is well known that the addition of ovarian fluid to the activation medium, instead of just water causes an increase in sperm performance metrics (Turner & Montgomerie, 2002; Woolsey *et al*., 2006; Litvak & Trippel, 1998; Rosengrave *et al*., 2009). But it is unknown if the addition of ovarian fluid will change the results found here, and it would give insight into possible cryptic female choice mechanisms. This

would provide the most realistic results of how all components of the spawning microenvironment interact. Another study that could be done to build upon the work done in chapter three, would be to create a treatment group that contain seminal plasma of both males in a pair with the sperm of just one of the males. This would provide knowledge with regards to how the seminal plasma of the two males interacts, as they would in natural spawning conditions. A study could be done in which the manipulated treatments used in this chapter are used to fertilize eggs in an *in vitro* sperm competition experiment, and eggs can be genotyped to determine paternity and give insight into how the effects of seminal plasma will have on fertilization and paternity success. And finally, to build upon the work in chapter three, a search for mechanisms needs to be done. One possible avenue for this is to look into the proteomics of seminal plasma, and try to determine if the results I have shown here are because of proteins in the seminal plasma interacting with sperm cells of other rival males. In insects this has been shown to be the case (reviewed in Chapman, 2001; 2008), and proteomics is becoming an advancing and promising field in studies on fish, with the ovarian fluid (Johnson *et al*., 2014) and seminal plasma (Gombar *et al*., unpubl. data) proteome being analyzed recently in Chinook salmon.

4. Conclusion

Although the study of sexual selection, and specifically sperm competition has been an important and popular field of science since the 1970's, it is still an ever evolving field as new approaches and techniques allow more sophisticated and in depth studies to be carried out. A major shift in this field has occurred from all work being done on internal fertilizing species, mainly insects, to now the use of externally fertilizing species,

such fishes, to answer new questions and even ones that have been left unanswered for many years. My thesis builds upon a large collection of existing studies of sperm competition, but for both of my chapters, studies of this kind have rarely been done on fish and by doing so I can find answers to questions that previously went unanswered and use these answers to guide future work that can help tackle some overarching questions to sexual selection theory that still puzzle scientists

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APPENDICES

APPENDIX 1: RAW DATA OF OFFSPRING HATCHING SUCCESS FROM MONANDROUS AND POLYANDROUS CROSSES FROM CHAPTER 2

Table A.1 Summary of hatching success of Chinook Salmon (*Oncorhynchus tshawytscha*) offspring from monandrous and polyandrous crosses involving both jack and hooknose males.

Female ID	Cross ID	Monandry/ Polyandry	Male ID #1	Male ID #2	Replicate	Hatching Success (%)
6	H	M	22	-	1	66.0
6	H	M	22	-	2	63.0
6	H	M	23	-	1	71.8
6	H	M	23	-	2	69.0
6	J	M	11	-	1	80.4
6	J	M	11	-	2	80.6
6	J	M	12	-	1	26.2
6	J	M	12	-	2	37.1
6	HxH	P	22	23	1	64.1
6	HxH	P	22	23	2	53.5
6	JxH	P	11	22	1	89.3
6	JxH	P	11	22	2	89.2
6	JxH	P	11	23	1	85.8
6	JxH	P	11	23	2	85.1
6	JxH	P	12	22	1	81.8

6	JxH	P	12	22	2	76.4
6	JxH	P	12	23	1	88.3
6	JxH	P	12	23	2	91.0
6	JxJ	P	11	12	1	84.8
6	JxJ	P	11	12	2	85.5
7	H	M	31	-	1	41.1
7	H	M	31	-	2	38.7
7	H	M	34	-	1	50.9
7	H	M	34	-	2	52.0
7	J	M	13	-	1	52.6
7	J	M	13	-	2	50.0
7	J	M	15	-	1	40.5
7	J	M	15	-	2	50.5
7	HxH	P	31	34	1	60.0
7	HxH	P	31	34	2	56.9
7	JxH	P	13	31	1	36.6
7	JxH	P	13	31	2	40.0
7	JxH	P	13	34	1	53.2
7	JxH	P	13	34	2	62.4
7	JxH	P	15	31	1	54.6
7	JxH	P	15	31	2	54.4
7	JxH	P	15	34	1	59.3
7	JxH	P	15	34	2	54.3
7	JxJ	P	13	15	1	54.0
7	JxJ	P	13	15	2	59.4
8	H	M	41	-	1	60.8

8	H	M	41	-	2	77.3
8	H	M	42	-	1	48.8
8	H	M	42	-	2	35.3
8	J	M	16	-	1	77.0
8	J	M	16	-	2	70.4
8	J	M	17	-	1	73.3
8	J	M	17	-	2	69.3
8	HxH	P	41	42	1	68.9
8	HxH	P	41	42	2	63.5
8	JxH	P	16	41	1	67.3
8	JxH	P	16	41	2	74.3
8	JxH	P	16	42	1	86.4
8	JxH	P	16	42	2	74.4
8	JxH	P	17	41	1	79.5
8	JxH	P	17	41	2	75.5
8	JxH	P	17	42	1	82.5
8	JxH	P	17	42	2	78.4
8	JxJ	P	16	17	1	62.4
8	JxJ	P	16	17	2	75.0
9	H	M	40	-	1	24.4
9	H	M	40	-	2	10.3
9	H	M	44	-	1	41.6
9	H	M	44	-	2	35.5
9	J	M	18	-	1	45.9
9	J	M	18	-	2	55.3
9	J	M	19	-	1	68.1

9	J	M	19	-	2	41.3
9	HxH	P	40	44	1	42.2
9	HxH	P	40	44	2	44.4
9	JxH	P	18	40	1	39.3
9	JxH	P	18	40	2	50.9
9	JxH	P	18	44	1	54.2
9	JxH	P	18	44	2	33.5
9	JxH	P	19	40	1	52.7
9	JxH	P	19	40	2	39.7
9	JxH	P	19	44	1	57.9
9	JxH	P	19	44	2	48.6
9	JxJ	P	18	19	1	70.2
9	JxJ	P	18	19	2	67.0
10	H	M	45	-	1	90.3
10	H	M	45	-	2	89.4
10	H	M	46	-	1	85.0
10	H	M	46	-	2	85.8
10	J	M	20	-	1	85.1
10	J	M	20	-	2	90.6
10	J	M	21	-	1	84.1
10	J	M	21	-	2	85.6
10	HxH	P	45	46	1	89.5
10	HxH	P	45	46	2	92.8
10	JxH	P	20	45	1	92.0
10	JxH	P	20	45	2	95.4
10	JxH	P	20	46	1	92.4

10	JxH	P	20	46	2	88.2
10	JxH	P	21	45	1	91.0
10	JxH	P	21	45	2	92.1
10	JxH	P	21	46	1	91.2
10	JxH	P	21	46	2	88.0
10	JxJ	P	20	21	1	90.6
10	JxJ	P	20	21	2	95.2
11	H	M	48	-	1	57.1
11	H	M	48	-	2	81.8
11	H	M	49	-	1	61.0
11	H	M	49	-	2	82.1
11	J	M	22	-	1	83.1
11	J	M	22	-	2	78.2
11	J	M	23	-	1	72.3
11	J	M	23	-	2	66.2
11	HxH	P	48	49	1	86.1
11	HxH	P	48	49	2	77.7
11	JxH	P	22	48	1	76.6
11	JxH	P	22	48	2	75.1
11	JxH	P	22	49	1	82.7
11	JxH	P	22	49	2	82.0
11	JxH	P	23	48	1	69.7
11	JxH	P	23	48	2	78.7
11	JxH	P	23	49	1	70.0
11	JxH	P	23	49	2	72.8
11	JxJ	P	22	23	1	65.8

11	JxJ	P	22	23	2	81.1
12	H	M	50	-	1	72.9
12	H	M	50	-	2	72.3
12	H	M	51	-	1	93.5
12	H	M	51	-	2	95.6
12	J	M	25	-	1	63.6
12	J	M	25	-	2	77.8
12	J	M	26	-	1	82.5
12	J	M	26	-	2	60.9
12	HxH	P	50	51	1	84.0
12	HxH	P	50	51	2	86.5
12	JxH	P	25	50	1	83.6
12	JxH	P	25	50	2	81.0
12	JxH	P	25	51	1	84.1
12	JxH	P	25	51	2	88.2
12	JxH	P	26	50	1	92.9
12	JxH	P	26	50	2	88.8
12	JxH	P	26	51	1	93.0
12	JxH	P	26	51	2	92.3
12	JxJ	P	25	26	1	78.9
12	JxJ	P	25	26	2	90.5
15	H	M	69	-	1	71.2
15	H	M	69	-	2	71.2
15	H	M	70	-	1	95.0
15	H	M	70	-	2	90.4
15	J	M	35	-	1	86.6

15	J	M	35	-	2	98.1
15	J	M	36	-	1	84.7
15	J	M	36	-	2	90.1
15	HxH	P	70	69	1	98.0
15	HxH	P	70	69	2	88.1
15	JxH	P	35	69	1	92.8
15	JxH	P	35	69	2	90.4
15	JxH	P	35	70	1	92.9
15	JxH	P	35	70	2	90.8
15	JxH	P	36	69	1	90.2
15	JxH	P	36	69	2	90.4
15	JxH	P	36	70	1	95.2
15	JxH	P	36	70	2	90.1
15	JxJ	P	35	36	1	92.2
15	JxJ	P	35	36	2	95.7
16	H	M	71	-	1	85.8
16	H	M	71	-	2	93.4
16	H	M	72	-	1	83.8
16	H	M	72	-	2	77.4
16	J	M	37	-	1	93.3
16	J	M	37	-	2	92.0
16	J	M	38	-	1	90.8
16	J	M	38	-	2	86.4
16	HxH	P	71	72	1	83.2
16	HxH	P	71	72	2	78.7
16	JxH	P	37	71	1	89.9

16	JxH	P	37	71	2	93.2
16	JxH	P	37	72	1	90.0
16	JxH	P	37	72	2	89.3
16	JxH	P	38	71	1	92.9
16	JxH	P	38	71	2	89.7
16	JxH	P	38	72	1	90.2
16	JxH	P	38	72	2	91.8
16	JxJ	P	37	38	1	91.1
16	JxJ	P	37	38	2	95.5
17	H	M	73	-	1	70.5
17	H	M	73	-	2	66.4
17	H	M	74	-	1	81.3
17	H	M	74	-	2	86.0
17	J	M	39	-	1	92.3
17	J	M	39	-	2	91.8
17	J	M	40	-	1	73.3
17	J	M	40	-	2	71.9
17	HxH	P	73	74	1	86.1
17	HxH	P	73	74	2	80.8
17	JxH	P	39	73	1	88.0
17	JxH	P	39	73	2	83.6
17	JxH	P	39	74	1	87.0
17	JxH	P	39	74	2	84.7
17	JxH	P	40	73	1	82.4
17	JxH	P	40	73	2	81.6
17	JxH	P	40	74	1	85.9

17	JxH	P	40	74	2	84.2
17	JxJ	P	39	40	1	83.9
17	JxJ	P	39	40	2	86.9
18	H	M	75	-	1	67.3
18	H	M	75	-	2	64.2
18	H	M	76	-	1	72.6
18	H	M	76	-	2	72.4
18	J	M	43	-	1	70.8
18	J	M	43	-	2	76.3
18	J	M	44	-	1	55.2
18	J	M	44	-	2	68.3
18	HxH	P	76	75	1	64.0
18	HxH	P	76	75	2	58.0
18	JxH	P	43	75	1	77.4
18	JxH	P	43	75	2	67.3
18	JxH	P	43	76	1	65.1
18	JxH	P	43	76	2	74.1
18	JxH	P	44	75	1	62.5
18	JxH	P	44	75	2	79.6
18	JxH	P	44	76	1	77.9
18	JxH	P	44	76	2	68.9
18	JxJ	P	43	44	1	74.9
18	JxJ	P	43	44	2	78.1
20	H	M	84	-	1	33.2
20	H	M	84	-	2	29.0
20	H	M	85	-	1	67.7

20	H	M	85	-	2	55.1
20	J	M	47	-	1	64.6
20	J	M	47	-	2	64.3
20	J	M	48	-	1	51.1
20	J	M	48	-	2	43.3
20	HxH	P	84	85	1	37.6
20	HxH	P	84	85	2	37.2
20	JxH	P	47	84	1	47.3
20	JxH	P	47	84	2	39.9
20	JxH	P	47	85	1	68.0
20	JxH	P	47	85	2	51.1
20	JxH	P	48	84	1	56.5
20	JxH	P	48	84	2	46.3
20	JxH	P	48	85	1	62.6
20	JxH	P	48	85	2	58.5
20	JxJ	P	47	48	1	42.2
20	JxJ	P	47	48	2	47.9
21	H	M	86	-	1	83.1
21	H	M	86	-	2	89.3
21	H	M	87	-	1	75.0
21	H	M	87	-	2	71.9
21	J	M	49	-	1	73.7
21	J	M	49	-	2	75.4
21	J	M	50	-	1	85.1
21	J	M	50	-	2	82.9
21	HxH	P	86	87	1	76.7

21	HxH	P	86	87	2	73.8
21	JxH	P	49	86	1	65.1
21	JxH	P	49	86	2	63.4
21	JxH	P	49	87	1	69.1
21	JxH	P	49	87	2	67.0
21	JxH	P	50	86	1	97.9
21	JxH	P	50	86	2	89.1
21	JxH	P	50	87	1	92.9
21	JxH	P	50	87	2	82.7
21	JxJ	P	49	50	1	96.8
21	JxJ	P	49	50	2	97.3
22	H	M	88	-	1	65.0
22	H	M	88	-	2	73.3
22	H	M	89	-	1	83.0
22	H	M	89	-	2	68.5
22	J	M	52	-	1	52.8
22	J	M	52	-	2	68.2
22	J	M	53	-	1	64.6
22	J	M	53	-	2	69.3
22	HxH	P	88	89	1	76.1
22	HxH	P	88	89	2	62.9
22	JxH	P	52	88	1	89.2
22	JxH	P	52	88	2	90.8
22	JxH	P	52	89	1	92.3
22	JxH	P	52	89	2	88.0
22	JxH	P	53	88	1	86.7

22	JxH	P	53	88	2	85.7
22	JxH	P	53	89	1	85.5
22	JxH	P	53	89	2	89.1
22	JxJ	P	53	52	1	61.7
22	JxJ	P	53	52	2	31.7
24	H	M	92	-	1	70.0
24	H	M	92	-	2	87.6
24	H	M	93	-	1	86.1
24	H	M	93	-	2	74.4
24	J	M	56	-	1	93.5
24	J	M	56	-	2	93.2
24	J	M	57	-	1	89.6
24	J	M	57	-	2	88.7
24	HxH	P	92	93	1	52.0
24	HxH	P	92	93	2	84.7
24	JxH	P	56	92	1	70.4
24	JxH	P	56	92	2	82.2
24	JxH	P	56	93	1	89.4
24	JxH	P	56	93	2	83.8
24	JxH	P	57	92	1	95.6
24	JxH	P	57	92	2	88.7
24	JxH	P	57	93	1	84.6
24	JxH	P	57	93	2	79.3
24	JxJ	P	56	57	1	89.4
24	JxJ	P	56	57	2	73.7

APPENDIX 2: RAW DATA OF OFFSPRING HATCHING SUCCESS FROM MONANDROUS CROSSES AND SPERM VELOCITY OF POTENTIAL SIRES USED TO CREATE THOSE OFFSPRING FROM CHAPTER 2

Table A.2 Sperm velocity of male Chinook salmon (*Oncorhynchus tshawytscha*) used in monandrous crosses and the resulting offspring hatching success from those crosses.

Male Tactic	Male ID	Female ID	Sperm Velocity (µm/s)	Hatching Success (%)
Hooknose	H22	6	123	64.5
Hooknose	H23	6	149.3	70.4
Hooknose	H31	7	101.5	39.9
Hooknose	H34	7	105.4	51.5
Hooknose	H40	9	85.85	17.4
Hooknose	H41	8	143.35	69.1
Hooknose	H42	8	119.65	42.1
Hooknose	H44	9	130	38.5
Hooknose	H45	10	180.1	89.8
Hooknose	H46	10	117.45	85.4
Hooknose	H48	11	166.6	69.4
Hooknose	H49	11	173	71.6
Hooknose	H50	12	112.95	72.6
Hooknose	H51	12	190.15	94.5
Hooknose	H69	15	166.1	71.2
Hooknose	H70	15	135.1	92.7
Hooknose	H71	16	158.3	89.6

Hooknose	H74	17	119.75	83.7
Hooknose	H76	18	95.85	72.5
Hooknose	H84	20	109.4	31.1
Hooknose	H86	21	119.15	86.2
Hooknose	H87	21	108.55	73.5
Hooknose	H88	22	146.35	69.1
Hooknose	H89	22	174.9	75.8
Hooknose	H92	24	136.45	78.8
Hooknose	H93	24	143	80.3
Jack	J11	6	131.65	80.5
Jack	J12	6	121.3	31.6
Jack	J13	7	111.9	51.3
Jack	J15	7	122.05	45.5
Jack	J16	8	135.3	73.7
Jack	J17	8	151.7	71.3
Jack	J18	9	101.55	50.6
Jack	J19	9	116.3	54.7
Jack	J20	10	107.3	87.9
Jack	J21	10	161.1	84.8
Jack	J22	11	123.85	80.7
Jack	J23	11	127.45	69.2
Jack	J25	12	113.8	70.7
Jack	J26	12	97.25	71.7
Jack	J35	15	144.1	92.3
Jack	J36	15	150.4	87.1
Jack	J37	16	145.2	92.6

Jack	J38	16	147.6	88.6
Jack	J39	17	169.1	92.0
Jack	J40	17	102.15	72.6
Jack	J43	18	183.6	73.6
Jack	J44	18	125.8	61.7
Jack	J47	20	132.45	64.5
Jack	J48	20	130.55	47.2
Jack	J49	21	102.2	74.6
Jack	J50	21	123.9	84.0
Jack	J52	22	117.7	60.5
Jack	J56	24	171.05	93.4
Jack	J57	24	138	89.1

APPRENDIX 3: RESULTS FROM THE COMPARISON OF CONTROL AND SHAM CONTROL TREATMENTS FROM CHAPTER 3

Experiment 1: Between-tactic manipulation

There was a significant difference between the control and sham control treatments for sperm straightness (repeated measures ANOVA: male tactic, $F_{1, 26} = 0.35$, $p = 0.56$; treatment, $F_{1, 26} = 7.8$, $p = 0.01$; tactic x treatment, $F_{1, 26} = 0.006$. $p = 0.94$) and percent motility (repeated measures ANOVA: male tactic, $F_{1, 27} = 0.003$, $p = 0.96$; treatment, $F_{1, 27} = 8.3$, $p = 0.008$; tactic x treatment, $F_{1, 27} = 0.76$, $p = 0.39$). Post-hoc analysis show that for sperm straightness, the sham control treatment is significantly higher than the control treatment in hooknoses only (paired t-test: Hooknose, $t_{14} = -2.4$, $p = 0.03$; Jack, $t_{12} = -1.65$, $p = 0.13$; Table 3.1; Fig. A.2) and for percent motility, the sham control is significantly lower than the control for hooknoses only (paired t-test: Hooknose, $t_{14} = 2.49$, $p = 0.026$; Jack, $t_{12} = 1.59$, $p = 0.14$; Table 3.1; Fig. A.3).

Experiment 2: Within-tactic manipulation

There was a significant difference between the control and sham control treatments for sperm velocity (repeated measures ANOVA: male tactic, $F_{1, 58} = 0.26$, $p = 0.616$; treatment, $F_{1, 58} = 16.4$, $p < 0.001$; tactic x treatment, $F_{1, 58} = 8.7$, $p = 0.005$), however, through post-hoc analysis, it had a negative effect on jack males (paired t-test: $t_{29} = 4.76$, $p < 0.001$; Fig. A.4) but no effect hooknose males (paired t-test: $t_{29} = 0.81$, $p = 0.425$; Fig. A.4). Similarly, there is a significant difference between control and sham control treatments for sperm straightness (repeated measures ANOVA: male tactic, $F_{1, 58} = 0.042$, $p = 0.84$; treatment, $F_{1, 58} = 5.5$, $p = 0.022$; tactic x treatment, $F_{1, 58} = 0.81$, $p =$

0.37) and percent motility (repeated measures ANOVA: male tactic, $F_{1, 58} = 0.11$, $p = 0.74$; treatment, $F_{1, 58} = 29.7$, $p < 0.001$; tactic x treatment, $F_{1, 58} = 1.81$, $p = 0.18$). Post-hoc analysis shows that there is significant increase in sperm straightness for hooknoses only (paired t-test: Hooknose, $t_{29} = -2.4$, $p = 0.025$; Jack, $t_{29} = -1.0$, $p = 0.33$; Table 3.2; Fig. A.5) and significant decrease in percent motility for both tactics (paired t-test: Hooknose, $t_{29} = 2.8$, $p = 0.009$; Jack, $t_{29} = -5.03$, $p < 0.001$; Table 3.2; Fig. A.6).

Figure Captions

Figure A.1 Mean (± standard error) curvilinear velocity of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma when not spun in centrifuge (control; black bars) and sperm after being spun in centrifuge (sham control; grey bars) in the between-tactic seminal plasma swap experiment (experiment one). An asterix (*) signifies a significant post-hoc test ($p < 0.05$) when the main model was significant, otherwise no post-hoc tests were conducted.

Figure A.2 Mean (± standard error) straightness (STR) of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma when not spun in centrifuge (control; black bars) and sperm after being spun in centrifuge (sham control; grey bars) in the between-tactic seminal plasma swap experiment (experiment one). An asterix (*) signifies a significant post-hoc test ($p < 0.05$) when the main model was significant, otherwise no post-hoc tests were conducted.

Figure A.3 Mean (± standard error) percent motility of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma when not spun in centrifuge (control; black bars) and sperm after being spun in centrifuge (sham control; grey bars) in the between-tactic seminal plasma swap experiment (experiment one). An asterix (*) signifies a significant post-hoc test ($p < 0.05$) when the main model was significant, otherwise no post-hoc tests were conducted.

Figure A.4 Mean (± standard error) curvilinear velocity of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma when not spun in centrifuge (control; black bars) and sperm after being spun in centrifuge (sham control; grey bars) in the within-tactic seminal plasma swap experiment (experiment two). An asterix (*) signifies a significant post-hoc test ($p < 0.05$) when the main model was significant, otherwise no post-hoc tests were conducted.

Figure A.5 Mean (± standard error) straightness (STR) of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma when not spun in centrifuge (control; black bars) and sperm after being spun in centrifuge (sham control; grey bars) in the within-tactic seminal plasma swap experiment (experiment two). An asterix (*) signifies a significant post-hoc test ($p < 0.05$) when the main model was significant, otherwise no post-hoc tests were conducted.

Figure A.6 Mean (± standard error) percent motility of sperm for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing sperm in own seminal plasma when not spun in centrifuge (control; black bars) and sperm after being spun in centrifuge (sham control; grey bars) in the within-tactic seminal plasma swap experiment (experiment two). An asterix (*) signifies a

significant post-hoc test ($p < 0.05$) when the main model was significant, otherwise no post-hoc tests were conducted.

Figure A.1

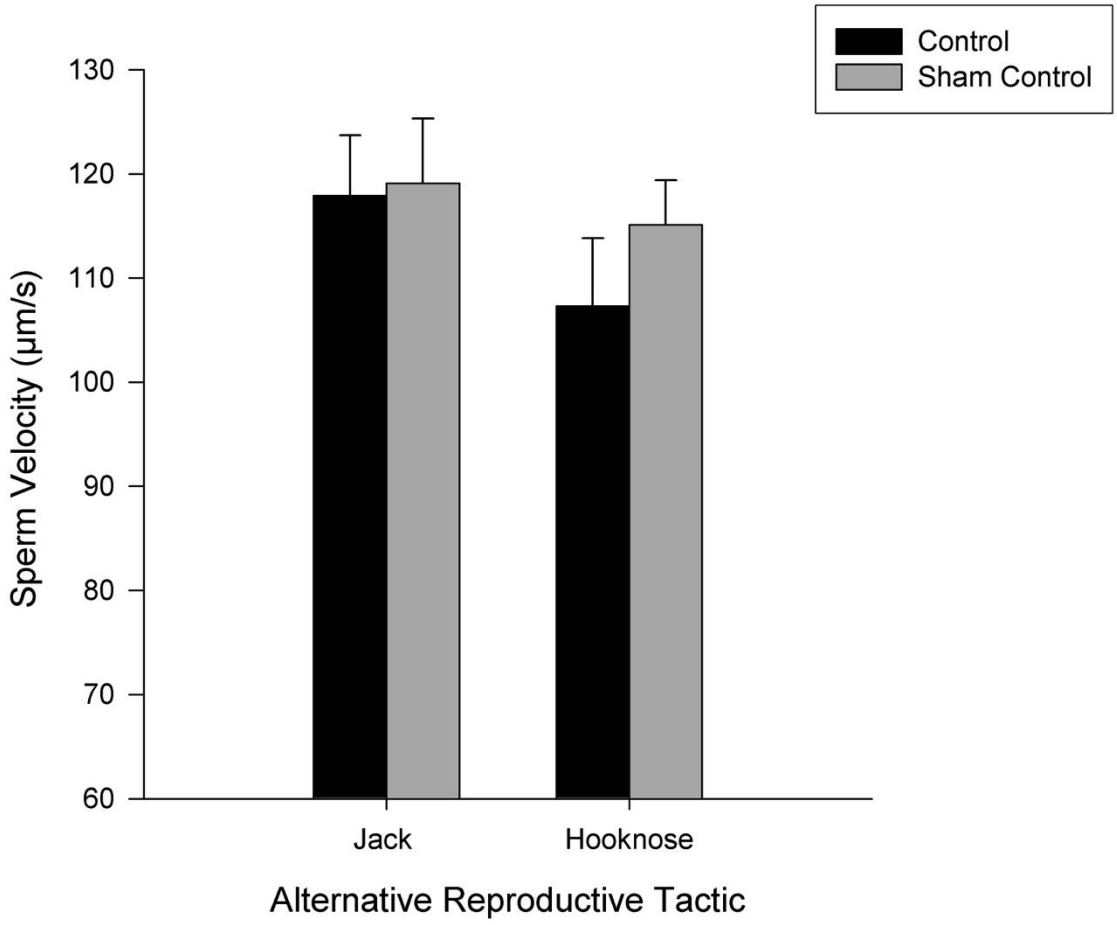

Control
Sham Control
130
120
110
100
90
80
70
60
Sperm Velocity (µm/s)
Jack
Hooknose
Alternative Reproductive Tactic

Figure A.2

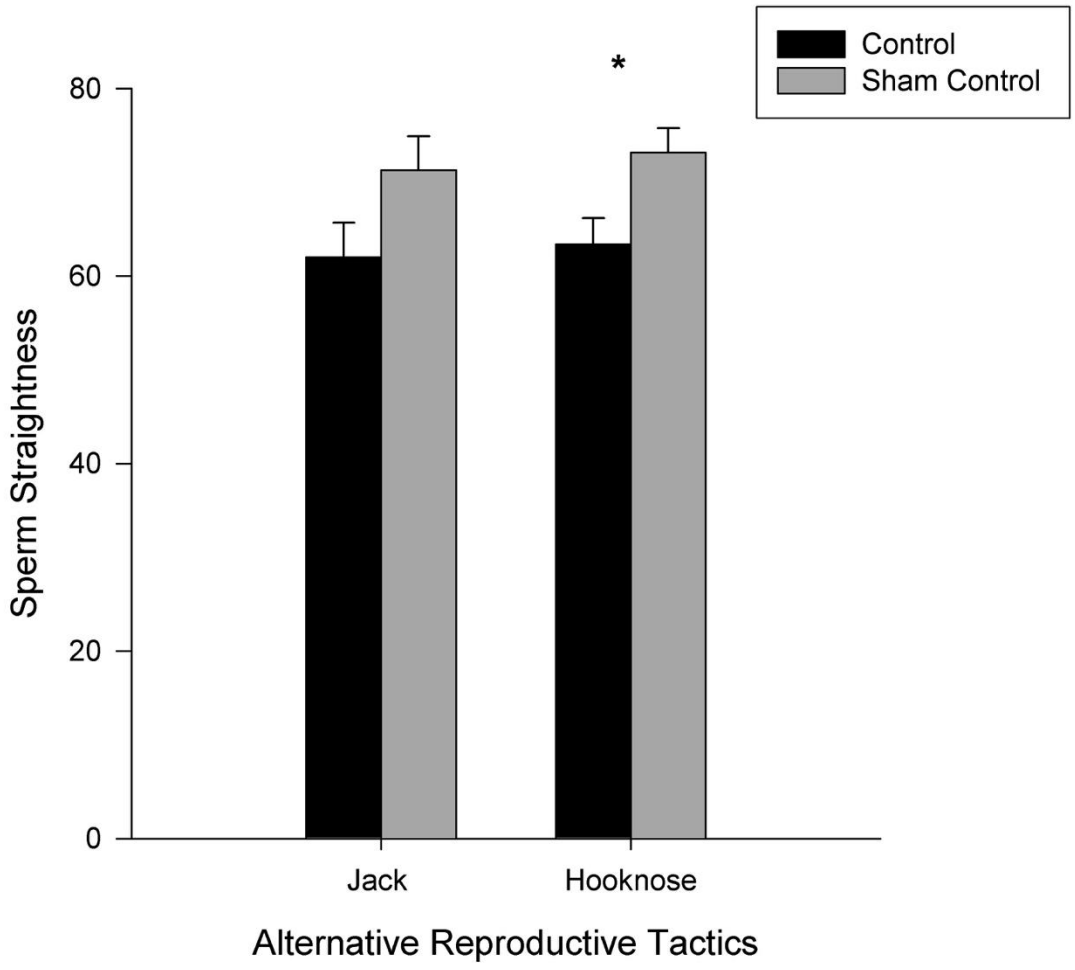

Control
Sham Control
*
80
60
40
20
0
Sperm Straightness
Jack
Hooknose
Alternative Reproductive Tactics

Figure A.3

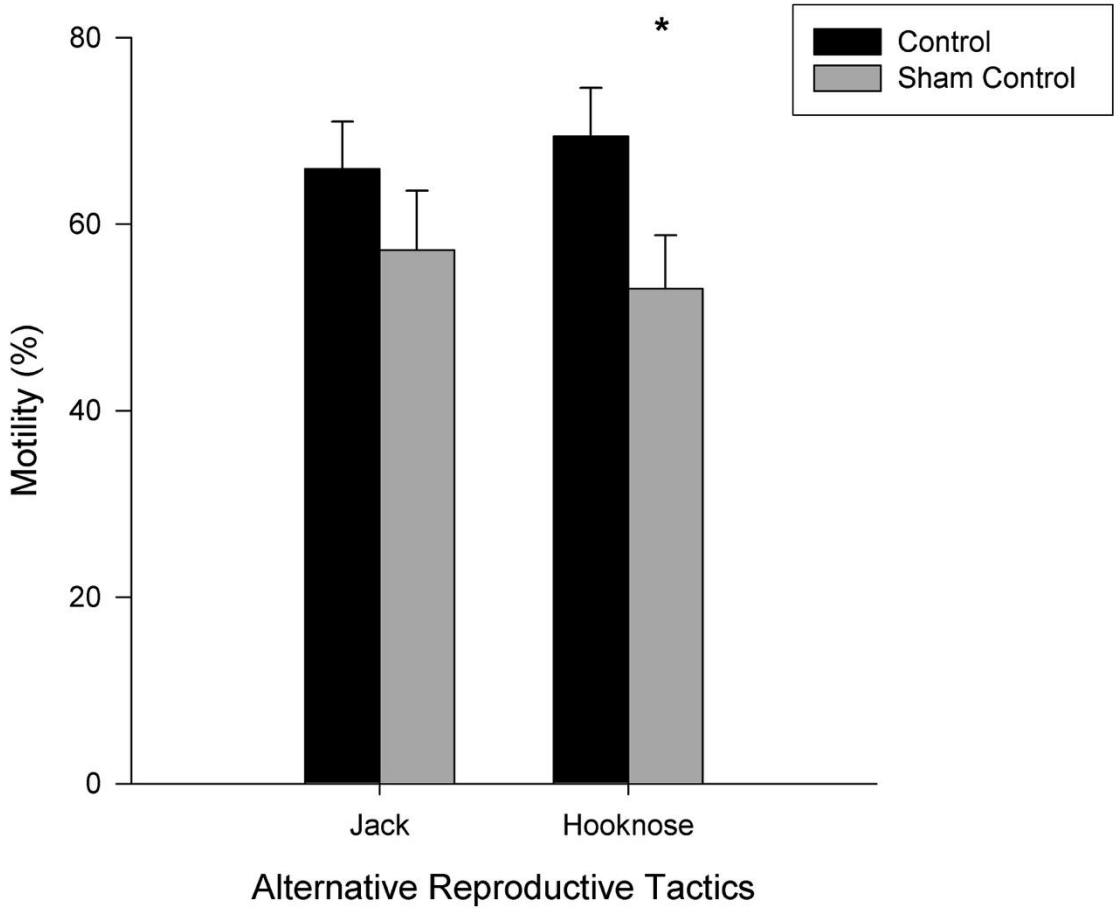

80
60
40
20
0
Motility (%)
*
Control
Sham Control
Jack
Hooknose
Alternative Reproductive Tactics

Figure A.4

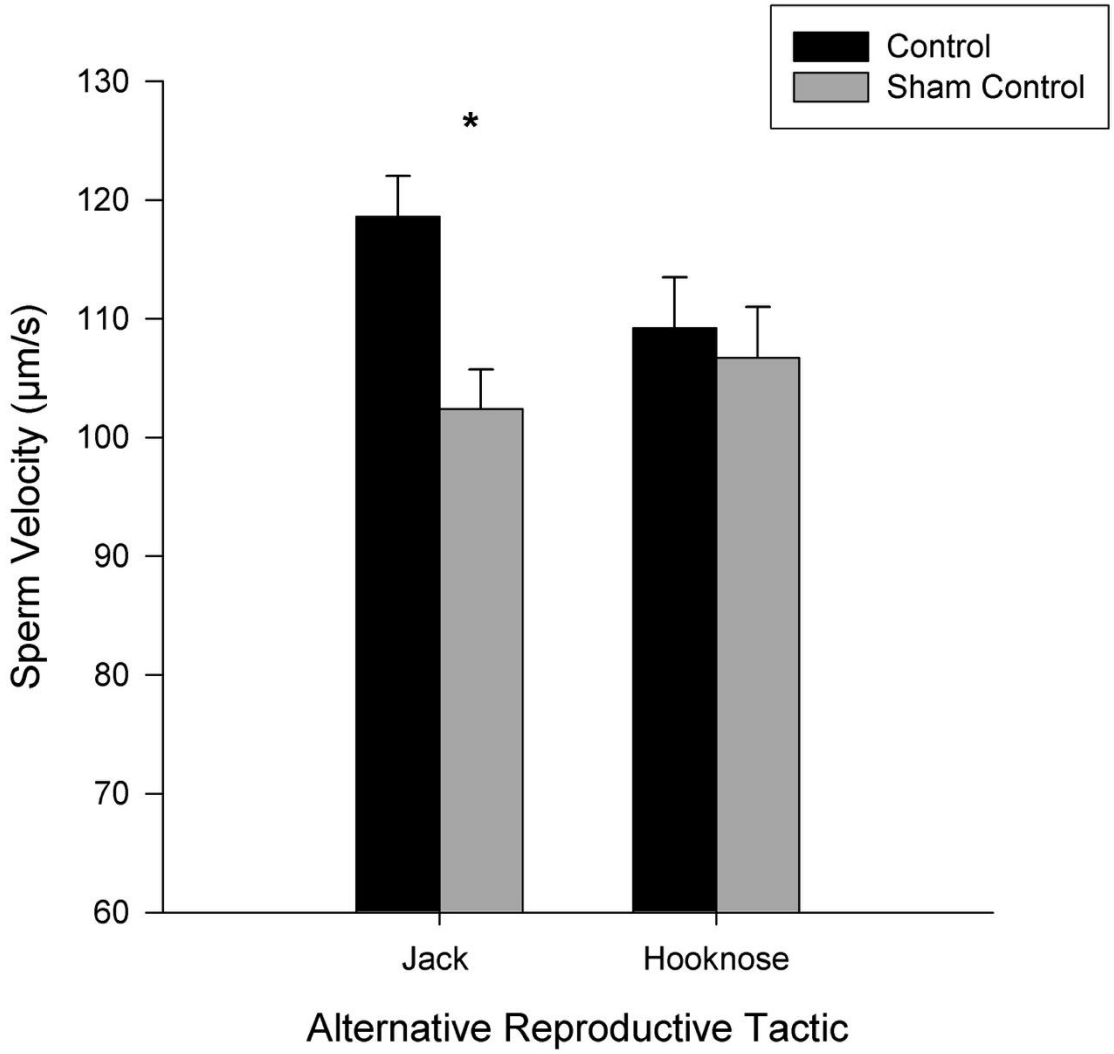

Control
Sham Control
*
130
120
110
100
90
80
70
60
Sperm Velocity (µm/s)
Jack
Hooknose
Alternative Reproductive Tactic

Figure A.5

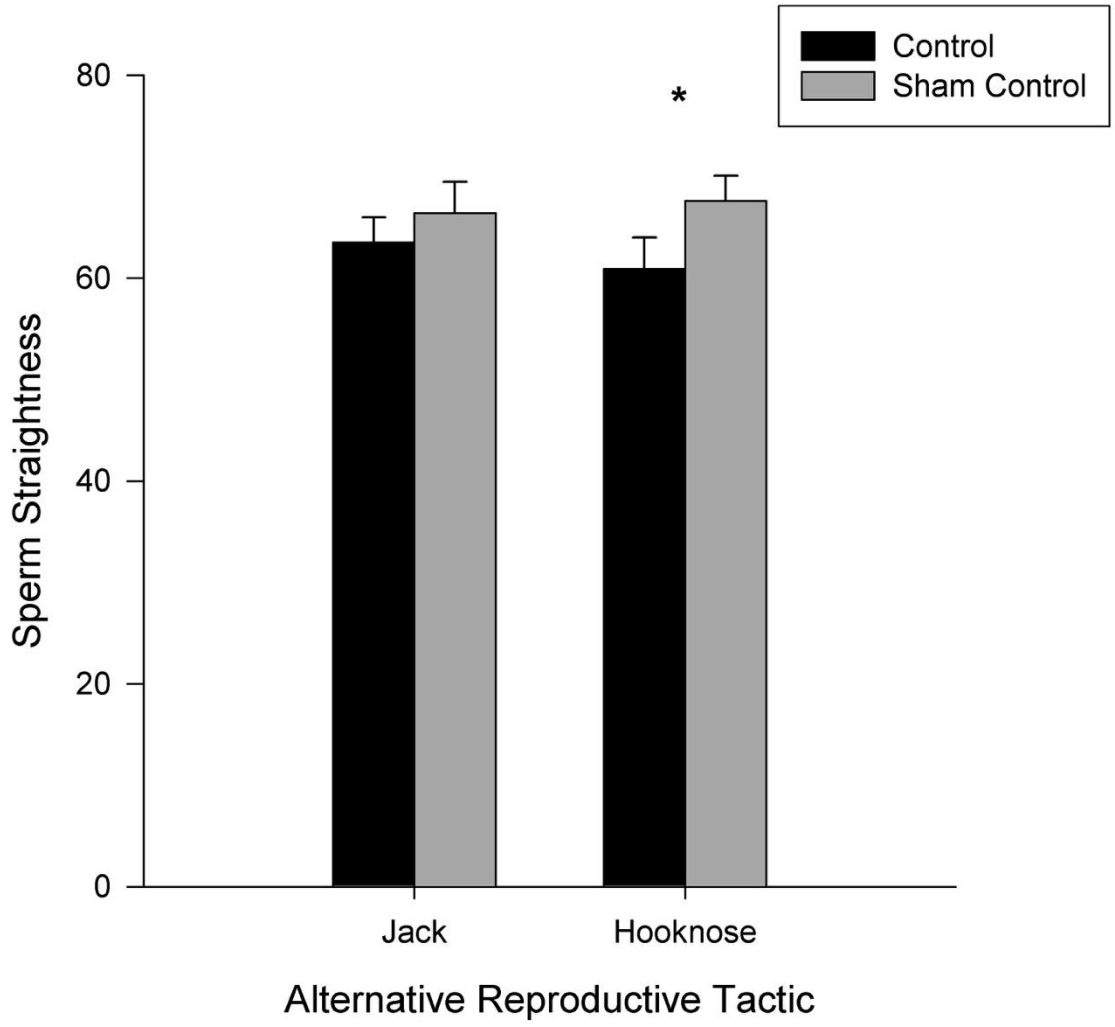
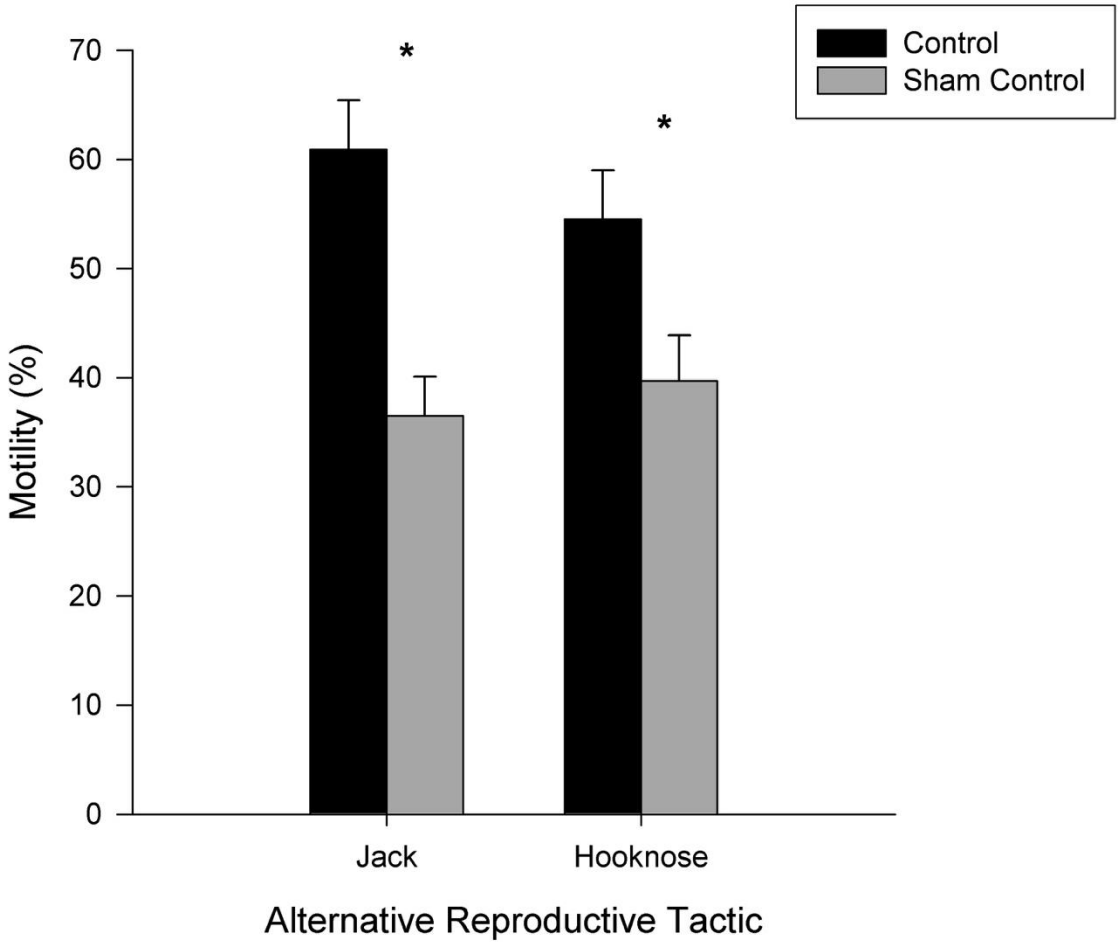

Control
Sham Control
*
80
60
40
20
0
Sperm Straightness
Jack
Hooknose
Alternative Reproductive Tactic

Figure A.6


Control
Sham Control
70
60
50
40
30
20
10
0
Motility (%)
*
*
Jack
Hooknose
Alternative Reproductive Tactic

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