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The effects of rival seminal plasma on sperm velocity in the alternative reproductive tactics of Chinook salmon

Jason A. Lewis [a, *], Trevor E. Pitcher [a, b]

[a] Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Avenue, Windsor, Ontario, N9B 3P4, Canada
[b] Department of Biological Sciences, University of Windsor, 401 Sunset Avenue, Windsor, Ontario, N9B 3P4, Canada

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ABSTRACT

Sperm competition is prevalent and intense in many animal mating systems, and is a major force driving evolution of such mating systems. The objective of this study was to determine the effect of seminal plasma on sperm velocity of male Chinook salmon (*Onchorhynchus tshawytscha*), which possesses a mating system with male alternative reproductive tactics and intense sperm competition. Male Chinook salmon either adopt a small, precocious sneaking tactic (jack) or a large, dominant tactic (hooknose). To test whether the seminal plasma can effect sperm velocity amongst sperm competitors, two experiments were done whereby males were paired based upon the alternative tactic each male adopted, with the first experiment consisting of jack-hooknose pairs (N = 16) and the second experiment consisting of jack-jack and hooknose-hooknose pairs (N = 12 and 14, respectively). Within each pair, milt of each male was manipulated such that seminal plasma was removed and swapped between the males in each pair and sperm velocity was measured. Jack seminal plasma caused a significant decrease (~11.9%) in hooknose sperm velocity while causing a significant increase in jack sperm velocity (~7%), while alternatively, hooknose seminal plasma had no affect on sperm velocity of jack or other hooknose males. This study shows that rival seminal plasma may affect the outcome of sperm competition between males; males adopting a sneaking tactic, that spawn in a disadvantageous mating position, may be able to compensate for this deficit by being more competitive through the effects of their seminal plasma on their competitor's sperm velocity.

1. Introduction

Sperm competition occurs when sperm from multiple males compete to fertilize a female's eggs [1]. 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 [2–4]. Sperm competition is especially prevalent in species in which male alternative reproductive tactics are present due to males from each tactic having unequal opportunities to fertilize eggs (e.g. Refs. [5–7]). In such species, the males often have different traits, that can take the form of morphological, behavioural, and life history differences, selected to maximize reproductive success [8].

The most prevalent alternative reproductive tactics across taxa is the existence of the sneak-guard dichotomy in males (see Ref. [9] for a taxonomic review). Sneaker males usually have small body size and use covert techniques to sneak into mating events between guard males and females to obtain reproductive opportunities. Whereas guard males are typically large in body size and have more pronounced secondary sexual characteristics to aid in asserting behavioural dominance over other males and females, including fighting off other males while protecting and monopolizing females. Parker [10] 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 are presumed to 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. Whereas the guard males are presumed to 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

* Corresponding author.
E-mail address: lewis11a@uwindsor.ca (J.A. Lewis).

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empirical studies. For example, in Atlantic salmon (*Salmo salar*), precocious parr (sneaker male) had larger testes, ejaculate volume, and number of sperm cells (all relative to body size) in addition to having more motile, longer living sperm [11,12], which were shown to provide greater fertilization success per spawning event than the anadromous (guard) males [12].

Most of the studies to date that examine sperm competition dynamics have focused on either differences in sperm number or sperm quality [2]. 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 [13] 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 male *Drosophila melanogaster* can alter the amount of seminal plasma in an ejaculate depending on the level of sperm competition risk [14]. It is important to note that most of this evidence stems from studies done on insects, but there is little known about whether seminal plasma can have similar effects in other taxa.

In fishes, there are only two studies that examine the effects on seminal plasma on the outcome of sperm competition [15,16], furthermore, only one of these studies examine the effects in a mating system that exhibits alternative reproductive tactics [15]. Within male Arctic charr (*Salvelinus alpinus*), it was found that the percentage of motile sperm was significantly higher in the presence of another male's seminal plasma, than in the male's own seminal plasma, however there was no such effect on sperm velocity [16]. This result may have little biological relevance however, because sperm velocity, and not percent motility, is the best predictor of fertilization success in Arctic charr [17] and other salmonid species [18–20]. Locatello et al. [15] showed that in the grass goby (*Zosterisessor ophiocephalus*), a species with a sneak-guard alternative reproductive tactic mating system, there was a tactic-specific effect of seminal plasma on a rival male's sperm performance. In the seminal plasma of the guard males, sneaker males showed an increase in sperm velocity of approximately 9%, which consequently resulted in a 10% increase in their fertilization success. Conversely, the presence of sneaker seminal plasma decreased the sperm velocity of guard males by approximately 7%, which in turn caused a 9% reduction in fertilization success.

Chinook salmon (*Oncorhynchus tshawytscha*) exhibit the sneak-guard alternative reproductive tactic in males, where the large, dominant hooknoses (i.e. guards) have priority in mating positions with females, while the small, precocious jack males (i.e. sneakers) adopt the sneaking tactic [21–23]. 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 to 5 males present, including both jacks and hooknoses [24]. Previous work has shown that jacks have relatively larger testes and their sperm swims faster in river water compared to hooknose sperm [25], which supports the theoretical work done by Parker [10], suggesting that the sneaker (jack) should invest more into spermatogenesis instead of other traits. However, energetic 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 velocity, an important metric for competitive fertilization success (e.g. Refs. [18,19]). Based on sperm competition theory [10], it can be hypothesized that, due to the asymmetry in sperm competition risk between tactics, jacks should be selected to be more competitive, which can happen in a number of ways: (1) jack seminal plasma decreases hooknose sperm velocity, (2) hooknose seminal plasma increases jack sperm velocity, and/or (3) jack seminal plasma increases another jack's sperm velocity. We tested these hypotheses using two experiments, the first used pairs of males that adopted different tactics (between-tactic) and the second used pairs of males adopting the same tactic (within-tactic). In both of these experiments, seminal plasma was swapped between males in each pair to examine the effect of seminal plasma on sperm velocity of other males.

2. Materials and methods

2.1. Fish collection

Male Chinook salmon from both alternative reproductive tactics were collected, using standard electroshocking techniques, from the Credit River (Mississauga, Ontario, Canada; 43°35′N, 79°42′W) between September 30 and October 11 in 2013 (experiment one; Hooknose: N = 16, mean ± S.E. mass = 7.7 kg ± 0.5 kg, range = 5.1–11.0 kg; Jack: N = 16, mean ± S.E. mass = 2.2 kg ± 0.2 kg, range = 1.3–3.6 kg) and September 29 and October 9 in 2014 (experiment two; Hooknose: N = 28, mean ± S.E. mass = 8.0 kg ± 0.3 kg, range = 4.6–11.4 kg; Jack: N = 24, mean ± S.E. mass = 2.0 kg ± 0.1 kg, range = 0.4–3.4 kg).

2.2. Milt collection

Milt (sperm and seminal plasma) was collected from all males 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 river water temperature (~11 °C) until analysis took place (2 to 3 h later).

2.3. Experimental design

There are three treatment groups for each of these experiments: (1) control, (2) sham control and (3) tactic-swap. The control treatment is milt that has not been centrifuged, while the sham control treatment is milt that has been centrifuged, but the resulting separate sperm cells and seminal plasma were immediately recombined. By comparing these two treatments, the effect of centrifugation on the sperm cells can be determined. The tactic-swap treatment is the main experimental treatment in which seminal plasma is swapped between males in each pair, which for experiment one contained a jack male and a hooknose male, therefore deemed the between-tactic swap experiment, and for experiment two contained males from the same tactic, both jack-jack pairs and hooknose-hooknose pairs, therefore deemed the within-tactic swap experiment. For experiment one, N = 16 jack-hooknose pairs were used, and for experiment two, N = 12 jack-jack pairs and N = 14 hooknose-hooknose pairs were used. For both experiments, males were only used once, so each pair contains a unique set of males.

2.4. Treatment preparation

To separate the milt into its components of sperm cells and seminal plasma, 1000 μL of milt was placed in a 1.7 mL Eppendorf tube and centrifuged (accuSpin Micro 17, Fisher Scientific) at 300 × g for 10 min [26]. The resulting separate seminal plasma and sperm components were carefully pipetted out and placed in separate Eppendorf tubes in a chilling block set at 11 °C

(approximate river water temperature). Based on preliminary data from a subset of males (N = 19, mean ± S. E = 24.4± 3.4%, range = 6.7–70.4% percent seminal plasma), 25% seminal plasma (i.e. 25% of the milt was seminal plasma while the residual 75% was composed of sperm) was used in the creation of the two manipulated treatments (sham control and tactic-swap). 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 alternate (experiment 1) or same tactic (experiment 2).

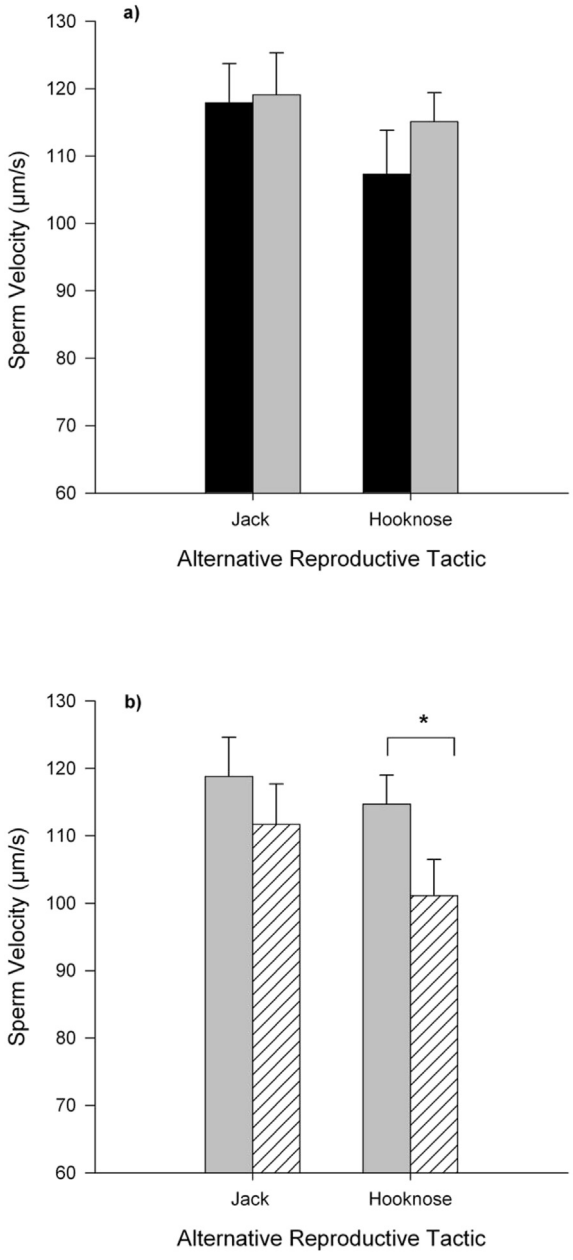

Fig. 1. Mean (±standard error) sperm velocity for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing (a) sperm that was not centrifuged (control; black bars) and sperm in own seminal plasma after being spun in centrifuge (sham control; grey bars) and (b) sperm in own seminal plasma after being spun in centrifuge (sham control; grey bars) and sperm in alternate tactic's seminal plasma (between-tactic; hashed bars). An asterisk (*) signifies a significant post-hoc test ($p < 0.05$).

2.5. Sperm performance assessment

For each treatment in both experiments, a milt sample (~0.1 μL) was pipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, Beverly, MA, USA), covered with a glass coverslip (22 × 22 mm), and activated with 15 μL of 11 °C river water (the approximate temperature of the river during spawning; 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 10× negative-phase objective. 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 curvilinear velocity (average velocity on the actual point-to-point track followed by the cell, hereafter sperm velocity) at 5s post-activation was the parameter used in the present study as sperm velocity is the primary determinant of fertilization success in salmonids [18]. The sperm analysis software measures each sperm cell individually and generates an average of these cells for each video.

2.6. Statistical analysis

Data were analyzed using SPSS statistical analysis software (IBM SPSS Statistics for Macintosh, Version 22.0. Armonk, NY: IBM Corp). The effect of the different treatments on sperm velocity of jacks and hooknoses in each of the two experiments was analyzed using one-way repeated measures ANOVAs (generalized linear model). For each experiment, two ANOVAs were used, with each ANOVA only comparing two treatments, the first comparing the control treatment with the sham control treatment to see the effect of centrifugation on sperm velocity, and the second comparing the sham control treatment with the manipulated treatment, to see the effect of foreign seminal plasma on sperm velocity. The different treatments in both experiments were used as within-subject factor (repeated measure) with two levels and the male tactic as between-subject factor. For each experiment, post hoc analysis of treatments within a single male tactic was performed using paired t-tests, while comparisons of treatments between two different male tactics were performed using independent t-tests as per [15].

3. Results

3.1. Experiment 1: between-tactic manipulation

Centrifugation did not significantly affect sperm velocity (comparing control treatment vs. the sham control treatment) for males from either of the alternative reproductive tactics (repeated measures ANOVA: male tactic, $F_{1,26} = 1.09$, $p = 0.306$; treatment, $F_{1,26} = 1.13$, $p = 0.297$; tactic x treatment, $F_{1,26} = 0.62$, $p = 0.437$; Fig. 1a).

Comparison of sham control treatment and manipulated treatment showed a significant effect on sperm velocity when sperm were activated in their own seminal plasma than the seminal plasma of a male of the alternate tactic (repeated measures ANOVA: male tactic, $F_{1,27} = 1.28$, $p = 0.267$; treatment, $F_{1,27} = 6.87$, $p = 0.014$; tactic x treatment, $F_{1,27} = 0.69$, $p = 0.415$; Fig. 1b). Although the sperm velocity of jack males was not significantly different when exposed to hooknose males' seminal plasma compared to their own (jack sham control vs. tactic-swap treatment; paired *t*-test: $t = 1.1$, $df = 13$, $p = 0.304$; Fig. 1b), hooknose male's 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 = 3.03, df = 14, p = 0.009; Fig. 1b). An across-tactics comparison (comparing tactic-swap treatments between both tactics) showed that there was no difference between jack sperm velocity in hooknose seminal plasma relative to hooknose sperm velocity in jack's seminal plasma (*t*-test: t = 1.32, df = 29, p = 0.196; Fig. 1b).

3.2. Experiment 2: within-tactic manipulation

Comparison of the control and sham control treatments shows an interactive effect between male tactic and treatment (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; Fig. 2a), with this effect only for jacks (paired *t*-test: t = 4.8, df = 29, p < 0.001; Fig. 2a) and not hooknoses (paired *t*-test: t = 0.81, df = 29, p = 0.425; Fig. 2a).

A comparison of sham control and manipulated treatments shows that there is a significant interaction effect between male tactic and treatment (repeated measures ANOVA: male tactic, $F_{1, 57} = 0.50$, p = 0.483; treatment, $F_{1, 57} = 0.12$, p = 0.730; tactic x treatment, $F_{1, 57} = 6.91$, p = 0.011; Fig. 2b). When seminal plasma was swapped between two jacks, there was a marginally significant increase in sperm velocity for jacks (paired *t*-test: t = −2.1, df = 27, p = 0.049; Fig. 2b), however, we found no difference when seminal plasma was swapped between hooknose males (paired *t*-test: t = 1.7, df = 30, p = 0.109; Fig. 2b). An across-tactics comparison (comparing manipulated treatments between both tactics) showed that there was no difference between jack sperm velocity in other jacks's seminal plasma than hooknose sperm velocity in other hooknose's seminal plasma (*t*-test: t = −1.8, df = 59, p = 0.070; Fig. 2b).

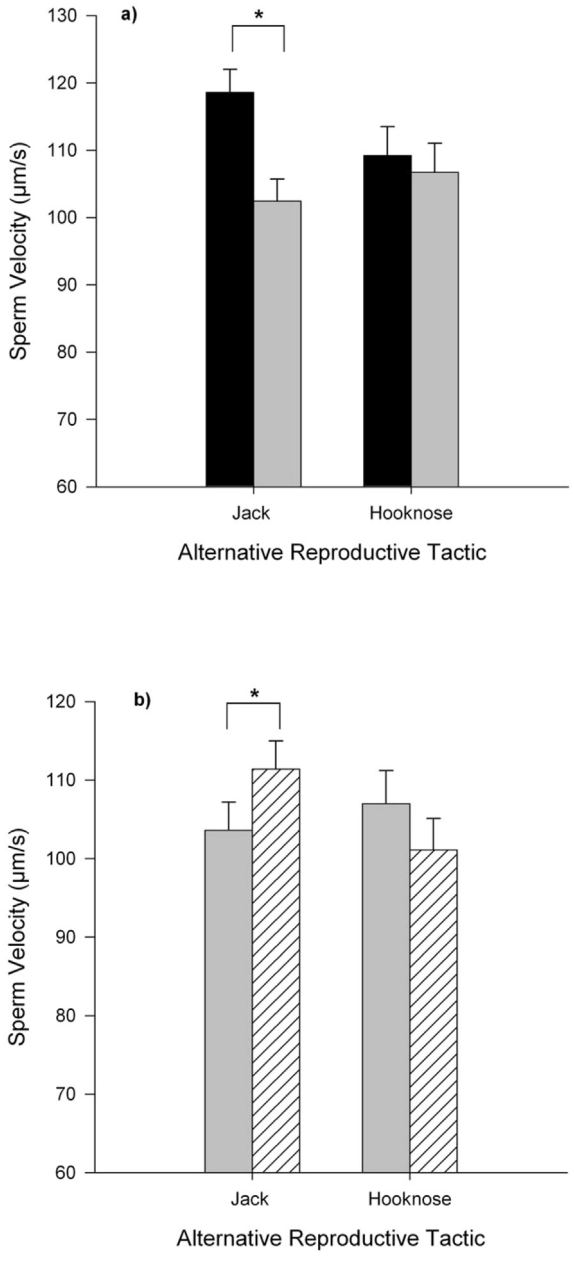

Fig. 2. Mean (±standard error) sperm velocity for both alternative reproductive tactics (jack and hooknose) in Chinook salmon (*Oncorhyncus tshawytscha*) comparing (a) sperm that was not centrifuged (control; black bars) and sperm in own seminal plasma after being spun in centrifuge (sham control; grey bars) and (b) sperm in own seminal plasma after being spun in centrifuge (sham control; grey bars) and sperm in the seminal plasma from a different male adopting the same tactic (within-tactic swap; hashed bars). An asterisk (*) signifies a significant post-hoc test (p < 0.05).

4. Discussion

Sperm competition theory, which suggests that due to an asymmetry in sperm competition risk, jack seminal plasma should be selected for to increase sperm competitiveness against other competing males, is supported through the results of our study. We found that the presence of jack seminal plasma on hooknose sperm resulted in a decrease in sperm velocity, a trait correlated with sperm competition success [18], however there was no effect on jack sperm velocity in the presence of hooknose seminal plasma. In addition, we found a marginally significant increase in jack sperm velocity in the presence of other jack male's seminal plasma, but there was no such within-tactic effect for hooknoses. Although we found an effect of the centrifugation process in experiment two with the sham control treatment being significantly lower than the control treatment for jacks, this does not alter the interpretation of the results as for the main experimental result, both treatments (sham control and within-tactic swap) underwent the same centrifugation process. These tactic-specific results, taken together, provide evidence that jacks may use seminal plasma as a mechanism to increase competitiveness during sperm competition with hooknoses, which are expected to be present during all mating events, and other jacks, which may also be present during the majority of mating events jacks attempt to sneak into [24]. In Chinook salmon, it has been shown that, in river water (same conditions used in the present study), jacks outcompete and sire a greater proportion of offspring than hooknoses when in direct *in vitro* sperm competition [27,28]. Based on the data presented in Flannery [27], our finding that jack seminal plasma decreases hooknose sperm velocity by approximately 11.9% would predict a subsequent decrease in hooknose paternity of approximately 18%. 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, but could be a mechanism contributing to the maintenance of the two alternate tactics in nature.

A similar result has been found in another study on fish with alternative reproductive tactics. Locatello et al. [15] 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 did not find an increase in jack sperm velocity in the presence of hooknose seminal plasma, both Locatello et al. [15] and the present study provide evidence that sneaker males increase their sperm competitiveness through seminal plasma interactions between reproductive tactics. Our study and Locatello et al. [15] provide data suggesting an additional consideration to Parker's [10] 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 could offer a competitive advantage during intense sperm competition between guards and sneakers.

The within-tactic effect of seminal plasma on sperm velocity in jacks that was found in the present study contradicts the results found in Locatello et al. [15] as they showed that male seminal plasma had no effect on sperm velocity of males that adopted the same tactic. Our study shows that the seminal plasma of jacks may operate in a self/non-self interaction as it affects sperm velocity of males from both tactics, and thus is not truly a tactic-specific interaction, which would result if only the sperm velocity of one tactic was affected. This effect could still be advantageous for sperm competition as the presence of other jack competitors, and thus other jack seminal plasma, will cause an increase in all the jacks sperm velocity. Although this may seem counterintuitive, it is important to note that the jacks main competitors are hooknoses and not necessarily other jacks, who are not always present. Therefore, jacks may be making the best out of a bad situation in being better competitors with hooknoses, but also providing an increase in sperm velocity to all jacks that may be present.

A mechanism by which seminal plasma could be influencing sperm velocity of other males and ultimately the outcome of sperm competition could relate to the proteins found within the seminal plasma. 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 and competition within the female reproductive tract, and increases in female egg production (reviewed in Refs. [29,30]). 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 within the female reproductive tract [31]. Males that did not have the protein in their ejaculate sired a significantly lower number 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 during sperm competition [31]. 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 [32], which could provide a mechanism for how the seminal plasma has effects on sperm velocity as shown in our study. For example, it was found that a number of proteins that may affect sperm motility were significantly higher in jack seminal plasma than hooknose plasma, such as sex hormone binding globulin (SHBG) and lactate dehydrogenase (LDH) [32]. Alternatively, there could be other components in the seminal plasma that could be responsible for the observed effect on sperm velocity, such as ions [33], which play a crucial role in the activation of sperm [34], however, it has been shown that there was no difference in osmolality between jacks and hooknoses in the same population of Chinook salmon as the present study [25]. It is important to note that in external fertilizers, especially fish, the effects of seminal plasma proteins or ions may not 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 may be 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 or ion would be important to further our understanding of sperm competition.

5. Conclusions

Sperm velocity is affected by seminal plasma of other males, however this effect is only seen within one of the alternative reproductive tactics in male Chinook salmon, as jack seminal plasma decreases hooknose sperm velocity and increases other jack's sperm velocity, but hooknose seminal plasma has no affect on male's sperm velocity from either tactic.

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