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SHORT COMMUNICATION

Effects of ovarian fluid and genetic differences on sperm performance and fertilization success of alternative reproductive tactics in Chinook salmon

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Abstract

In many species, sperm velocity affects variation in the outcome of male competitive fertilization success. In fishes, ovarian fluid (OF) released with the eggs can increase male sperm velocity and potentially facilitate cryptic female choice for males of specific phenotypes and/or genotypes. Therefore, to investigate the effect of OF on fertilization success, we measured sperm velocity and conducted *in vitro* competitive fertilizations with paired Chinook salmon (*Oncorhynchus tshawytscha*) males representing two alternative reproductive tactics, jacks (small sneaker males) and hooknoses (large guarding males), in the presence of river water alone and OF mixed with river water. To determine the effect of genetic differences on fertilization success, we genotyped fish at neutral (microsatellites) and functional [major histocompatibility complex (MHC) II ß1] markers. We found that when sperm were competed in river water, jacks sired significantly more offspring than hooknoses; however, in OF, there was no difference in paternity between the tactics. Sperm velocity was significantly correlated with paternity success in river water, but not in ovarian fluid. Paternity success in OF, but not in river water alone, was correlated with genetic relatedness between male and female, where males that were less related to the female attained greater paternity. We found no relationship between MHC II ß1 divergence between mates and paternity success in water or OF. Our results indicate that OF can influence the outcome of sperm competition in Chinook salmon, where OF provides both male tactics with fertilization opportunities, which may in part explain what maintains both tactics in nature.

Introduction

Sperm competition occurs post-copulation when sperm from two or more males simultaneously compete with one another to fertilize a female's eggs (Parker, 1970). Sperm competition is known to be one of the primary factors determining fertilization success differences among males, creating selective pressures on males to enhance specific sperm traits to maximize fitness (Montgomerie & Fitzpatrick, 2009). Sperm quality metrics (such as velocity, motility, density and morphology) have been shown to be determinants of competitive reproductive success (i.e. sperm competition success) in many taxa including birds (e.g. Denk *et al.*, 2005; Bennison *et al.*, 2015), mammals (e.g. Malo *et al.*, 2005; Firman, 2014), amphibians (e.g. Dziminski *et al.*, 2009), invertebrates (e.g. Lüpold *et al.*, 2012; Hansen *et al.*, 2015) and fishes (e.g. Gage *et al.*, 2004; Beausoleil *et al.*, 2012; Devigili *et al.*, 2015). In addition, sperm competition is not the only factor contributing to fertilization

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success, as recent studies have revealed the active role that females play in post-copulatory processes (e.g. Lüpold *et al.*, 2013; Alonzo *et al.*, 2016). For example, experimentally controlled matings of red junglefowl (*Gallus gallus*) showed that cryptic female choice [differential use of sperm from one male over another (Eberhard, 1996)] favoured sperm from major histocompatibility complex (MHC)-dissimilar males and this selection for MHC-dissimilar males' sperm occurred within the female reproductive tract (Løvlie *et al.*, 2013). Indeed, in several taxa from invertebrates to mammals, studies have demonstrated that cryptic female choice can bias fertilization in favour of males with specific genotypes (Palumbi, 1999; Schwensow *et al.*, 2008; Gasparini & Pilastro, 2011; Løvlie *et al.*, 2013).

In externally fertilizing fish species, including Atlantic salmon (*Salmo salar*) (Gage *et al.*, 2004), Chinook salmon (*Oncorhynchus tshawytscha*) (Rosengrave *et al.*, 2008; Lehnert *et al.*, 2016a), walleye (*Sander vitreus*) (Casselman *et al.*, 2006), arctic charr (*Salvelinus alpines*) (Liljedal *et al.*, 2008) and Atlantic cod (*Gadus morhua*) (Skjæraasen *et al.*, 2009), sperm velocity is a significant predictor of competitive fertilization success. However, this relationship between sperm velocity and competitive fertilization success is often only examined in water, despite the fact that in natural spawning situations sperm and eggs interact in a medium that is not entirely composed of water (Rosengrave *et al.*, 2009). In externally fertilizing fish species, egg release is accompanied by the simultaneous expulsion of ovarian fluid (Lahnsteiner *et al.*, 1999). Lahnsteiner *et al.* (1995) investigated the composition of ovarian fluid from four salmonid species, rainbow trout (*O. mykiss*), arctic charr, lake trout (*S. namaycush*) and Danube salmon (*Hucho hucho*), to quantify their inorganic (pH, osmolarity, K^+, Na^+, Ca^{2+}) and organic (protein, free amino acids, glucose, lactate, phospholipids, cholesterol and various enzymes) components. Many of these organic and inorganic components have a significant effect on sperm activity, such as sperm velocity, where they typically increase the velocity of sperm compared to its activation in water in externally fertilizing fishes including Atlantic cod (Litvak & Trippel, 1998), rainbow trout (Wojtczak *et al.*, 2007; Dietrich *et al.*, 2008) and arctic charr (Turner & Montgomerie, 2002; Urbach *et al.*, 2005). Therefore, understanding the role that ovarian fluid plays in determining reproductive success under sperm competition is critical to our understanding of sexual selection. A recent study in Chinook salmon demonstrated that sperm velocity in ovarian fluid can predict fertilization success using experimental *in vitro* competitive fertilizations, where the male with faster sperm in the female's ovarian fluid sired more eggs than the rival male (Rosengrave *et al.*, 2016). Thus, this study clearly demonstrated that the bias in competitive fertilization success mediated by the female's ovarian fluid [i.e. cryptic female choice (CFC)] is adaptive, as male sperm velocity in ovarian fluid was also correlated with embryo survival (Lewis & Pitcher, 2016; Rosengrave *et al.*, 2016).

Given these recent findings, we investigated the effects of ovarian fluid on sperm velocity and ultimately competitive fertilization success using alternative reproductive tactics of Chinook salmon. In Chinook salmon, where fertilization occurs externally, multiple males can simultaneously compete for fertilization of a female's eggs. Due to this intense competition, different males can adopt different tactics to ensure successful fertilization. In Chinook salmon, the alternative reproductive tactics include large guarding-type males [known as 'hooknoses', due to the curved snout, sensu (Gross, 1985)] and precociously maturing sneaking males (known as 'jacks') (see Berejikian *et al.*, 2010; Butts *et al.*, 2012a; Flannery *et al.*, 2013 for details). Jacks have a smaller body size and cryptic coloration, which allows them to hide to elude aggressive hooknose males and employ a sneaking tactic to steal fertilizations from hooknoses. Their inferior spawning position causes them to enter the nest soon after the onset of spawning resulting in a delay in contact with the female's eggs (Berejikian *et al.*, 2010). Jacks invest disproportionately more into their gonads and they have faster sperm velocity in river water compared to hooknoses (Flannery *et al.*, 2013).

The primary aim of this study was to examine the effect of the presence of ovarian fluid on the outcome of sperm competition using *in vitro* competitive fertilization trials where sperm were activated with and without ovarian fluid. In Chinook salmon, inorganic ions in the ovarian fluid are known to affect sperm velocity of hooknoses; sperm swim faster when activated in ovarian fluid compared to water (Rosengrave *et al.*, 2009). However, no studies have investigated the effects of ovarian fluid on sperm velocity and competition outcome between the male alternative reproductive tactics in Chinook salmon, although recent work in the ocellated wrasse (*Symphodus ocellatus*) demonstrated that ovarian may influence fertilization success of alternative reproductive tactics (Alonzo *et al.*, 2016). We therefore used a paired design to compete sperm simultaneously from pairs of males, one from each alternative reproductive tactic (i.e. one jack and one hooknose), to fertilize eggs, with or without ovarian fluid. Microsatellite markers were then used to assign paternity to the offspring from *in vitro* competitive fertilization trials. Paternity was used to determine the relative competitive fertilization success for paired males in the presence and absence of ovarian fluid. We predicted that ovarian fluid would increase the sperm velocity of both alternative reproductive tactics of Chinook salmon, and we examined whether these differences in sperm velocity (between fertilizations in water and ovarian fluid) would influence the outcome of the

paternity and thus affect sexual selection pressure on the two alternative reproductive tactics.

Next, given that cryptic female choice can be adaptive (Rosengrave *et al.*, 2016), we explored hypotheses to explain variation in competitive fertilization success in river water and ovarian fluid. Here, we examined patterns of paternity as they related to genetic differences (at neutral and functional markers) between mates. In fishes, many studies have examined mate choice in relation to the highly polymorphic immune genes known as the major histocompatibility complex (MHC) genes (Neff *et al.*, 2008; Yeates *et al.*, 2009; Gasparini *et al.*, 2015; Lehnert *et al.*, 2016b). Previous studies on Chinook salmon paternity patterns showed that females mate nonrandomly at the MHC class II gene, appearing to select mates that produced offspring with greater genetic diversity as measured by amino acid divergence (Neff *et al.*, 2008; also see Pitcher & Neff, 2006; Garner *et al.*, 2010). Because male sperm velocity varies significantly depending on which female's ovarian fluid is present (Rosengrave *et al.*, 2008), the composition of female ovarian fluid may be a mechanism for cryptic female choice (Evans *et al.*, 2013; Rosengrave *et al.*, 2016), whereby females differentially enhance the swimming speed of sperm from genetically favourable males (i.e. more dissimilar MHC males). Although no studies in fishes have directly demonstrated that ovarian fluid affects sperm velocity based on MHC genotype, competitive fertilization success can be influenced by MHC similarity between mates (Gasparini *et al.*, 2015). Additionally, studies have demonstrated that ovarian fluid can influence sperm velocity based on genetic relatedness between mates (Gasparini & Pilastro, 2011; Butts *et al.*, 2012b). Therefore, to test these hypotheses, we examined the relationship between patterns of paternity and (i) relatedness [estimated using microsatellites (Queller & Goodnight, 1989)] and (ii) amino acid divergence between individuals at the MHC class II gene (see Landry *et al.*, 2001). Ultimately, our study will determine whether females bias competitive fertilization success in favour of one alternative reproductive tactic, and whether genetic relatedness and MHC divergence between the mates can explain the variation observed in competitive fertilization success.

Materials and methods

Sperm competition experiment

In the fall of 2011, Chinook salmon were caught using standard electroshock techniques from a large wild spawning population in the Credit River, flowing from Lake Ontario (Mississauga, Ontario, N 43°35′, 3W 79°42′) (see Pitcher & Neff, 2006, 2007; Butts *et al.*, 2012a; Flannery *et al.*, 2013 for details). Based on an examination of otoliths, hooknose males and females in our study population sexually mature at 3–5 years of age, whereas jacks sexually mature at 2 years of age (T.E. Pitcher, unpublished). Individuals were found haphazardly in flowing water approximately 0.6–1.2 m in depth and temperatures around 11 °C. After drying the individual, we applied gentle pressure to the individuals' abdomen to collect either the milt or eggs (and accompanying ovarian fluid). Milt, eggs and ovarian fluid were kept in a cooler that approximated the temperature of the river water (~ 11 °C) for transport back to the laboratory for sperm velocity analysis and competitive *in vitro* fertilization trials. Fin clips were collected from all adults and preserved in 95% ethanol for paternity assignment genotyping associated with the competitive fertilization trials.

Our competitive fertilizations included a total of 14 triads (each one consisting of a jack, hooknose and female), which were replicated in river water and ovarian fluid. Although we were able to use unique males for each triad, due to a limited number of females ($n = 8$), the same females were used for multiple triads in some cases. For the 14 triads, four females were used twice (i.e. in two triads) and one female was used three times, and three females were used only once. The eggs collected from females were placed in a sieve and the ovarian fluid was collected. Males from the two tactics were paired so that the difference in storage time of their respective milt samples was minimized. River water (250 mL) from the collection site was then poured over the eggs and 200 μL of milt from each of the two males was applied simultaneously to flowing water using pipetters. The individual holding both pipetters was blind to the identification (jack or hooknose) of the male's sperm samples. Fertilizations were replicated in a paired design (i.e. same males, same female, same milt volume, same time since collection of gametes and same egg number) with the same technique, except in the second set of competitive fertilization trials an ovarian fluid solution was used as the fertilization medium [dilution ratio of ovarian fluid to river water of 1:1, which is the same concentration as previous studies in Chinook salmon (Rosengrave *et al.*, 2008, 2016)], using ovarian fluid from the female within the respective triad. At the completion of the fertilization protocols, each set of eggs were haphazardly placed into a cell in a vertical stack incubation tray. These trays were then placed in an incubation stack located at Ringwood Fish Culture Station (Stouffville, ON) to ensure that oxygenated water would flow over the eggs. Eggs remained in the incubation stack until they were eyed-up (i.e. when the embryo had developed to the point you could see an eye spot). A subset of the eyed-up eggs ($n = 48$) from each of the competitive *in vitro* fertilization pairs ($n = 14$ triads in water, $n = 14$ triads in the ovarian fluid solution) were collected from each triad and preserved in 95% ethanol for subsequent DNA extraction and paternity analysis

(see below). Eggs were collected at the eyed-egg stage given that very limited egg mortality has occurred prior to this stage. Thus, the eyed-egg stage was sampled to avoid confounding estimations of true fertilization success due to differential embryo viability from the different alternative reproductive tactics (García-González, 2008). In total, 1344 eyed-eggs were assayed for paternity analysis.

Next, we measured sperm velocity for all males. Sperm velocity for each male was video-recorded, within 6 h from the time of collection (stored at 11 °C), through a microscope and analysed with computer-assisted sperm-tracking software after approximately 0.1 μL of milt was activated with 15 μL of river water and again with ovarian fluid solution (50% dilution from the female in the respective triad), at 11 °C. Recordings were conducted using a CCD B/W video camera module at 25 Hz vertical frequency, mounted on an external negative phase-contrast microscope (CX41 Olympus) with a 10× magnification negative-phase objective (Pitcher *et al.*, 2009; Flannery *et al.*, 2013). Once recordings were completed, sperm velocity analysis was conducted using HTM-CEROS sperm analysis system version 12 (CEROS, Hamilton Thorne Biosciences, Beverly, MA, USA). Sperm velocity, measured as curvilinear velocity (VCL) (the actual point-to-track followed by the cell), was measured at five seconds post-activation in both water and the ovarian fluid solution. The sperm analysis software measures each sperm cell individually and generates an average velocity of all sperm cells combined. Sperm density was estimated using an 'improved Neubauer chamber' haemocytometer under 400× magnification following the protocol described in Pitcher *et al.* (2009). Estimated densities are expressed as the number of sperm cells per millilitre of stripped milt.

DNA was extracted, using a plate-based extraction method (Elphinstone *et al.*, 2003), from adult fin clips (two males and one female per triad) and tail tissue from developed embryos from eyed-eggs (n = 48 per replicate for river water and ovarian fluid trials). The unambiguous paternity of each offspring was determined using four polymorphic tetranucleotide repeat microsatellite markers: Ots107 (Nelson & Beacham, 1999), OtsG83b, OtsG432 (Williamson *et al.*, 2002) and RT-191 (Spies *et al.*, 2005). The loci were amplified using polymerase chain reaction (PCR) with the following protocol: denaturation for 2 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 45 s for primer annealing (see Table S1 for primer annealing temperatures and their accession numbers), 30s extension at 72 °C, then a final extension step of 1.5 min at 72 °C. Fluorescently labelled forward primers were used in the reaction; then, the PCR products were run and visualized by polyacrylamide gel electrophoresis using a Licor 4300 DNA Analyzer system. Allele sizes were called using Gene ImagIR version 4.05 software (Scanalytics Inc., Rockville, MD, USA). A single observer was able to determine paternity via the exclusion of one of the potential males; however, not all offspring could be assigned paternity in this manner using these particular loci. Paternity could be unambiguously assigned to an average of 45.3 ± 0.6 offspring (range: 37–48 offspring) of a possible 48 offspring per trial using the available microsatellite loci (n = 1268 assigned offspring in total). Relative fertilization success was measured for each male (in each replicate) by dividing the number of offspring sired by that male by the total number of offspring that could be unambiguously assigned to one of the two males.

Major histocompatibility genotyping

DNA was extracted from fin clips of adults used in the sperm competition experiment using the Wizard Kit (Promega Corp.) and then amplified at the MHC II ß1 locus using primers previously described by Miller *et al.* (1997). PCRs were performed using 32 cycles with an annealing temperature of 52.5 °C. Subsequent library preparation was performed following the same protocol described in Lehnert *et al.* (2016b), and the library was sequenced using the Ion Torrent Personalized Genome Machine (Life Technologies). Sequences were analysed using jMHC software (Stuglik *et al.*, 2011) to identify all sequence variants (i.e. potential alleles) that were detected at the MHC II ß1 locus with a perfect match to the primer set. MHC alleles were determined from jMHC output following the methods described by Galan *et al.* (2010) (see Supplementary Materials for more details). Within a triad, we calculated the amino acid divergence based on pairwise amino acid differences for the alleles between jack and female, and hooknose and female (following Landry *et al.*, 2001). Briefly, amino acid differences were determined for each pairwise allele combination using MEGA5 (Tamura *et al.*, 2011). For each male and female combination, the pairwise amino acid difference (D) between each allele of the female (A1A2) and the male (B1B2) was summed ($D_{A1B1} + D_{A1B2} + D_{A2B1} + D_{A2B2}$) to determine a divergence index for the pair (Landry *et al.*, 2001). The difference in the divergence index between jack and hooknose with the female in the triad was calculated.

Relatedness genotyping

In addition to MHC genotyping, DNA previously extracted from all adult fin clips were genotyped at 10 polymorphic microsatellite loci (see Table S1). PCRs and allele scoring were performed using the same methods as described above. Next, we used GenAlEx version 6.5 (Peakall & Smouse, 2012) to calculate relatedness within each triad using the Queller & Goodnight (1989) method. Within each triad, mean relatedness was calculated between female and jack, and female

and hooknose. The difference in the relatedness between jack and hooknose with the female in the triad was calculated.

Statistical analyses

All analyses were conducted in R software (R Core Team 2016). First, we tested the effect of activation medium on sperm velocity, where we used paired *t*-tests to determine the effect within each tactic. Next, using the results of genetic paternity, generalized linear mixed models (GLMMs) in the lme4 package (Bates *et al.*, 2009) were used to analyse paternity data. First, GLMMs with binomial distribution and logit-link function were used where the response variable was represented as paired numbers from each competition indicating the number of eggs sired by the jack and hooknose. The model included the fixed factor of activation medium (river water vs. ovarian fluid) to determine whether success of alternative tactics was influenced by the presence and absence of ovarian fluid. The model also included fixed variables representing sperm quality differences between paired males (two fixed continuous variables representing sperm quality: (1) sperm velocity differences between two males and (2) sperm density ratio of focal male to rival male). We also included the interaction between activation medium and difference in sperm velocity in the model, as it is possible that activation medium could differentially influence sperm velocity and thus influence fertilization success. Random effects of female ID and male pair were included in the model. Models were fit using Laplacian approximations for maximum likelihood. Significance of fixed effects were determined using the summary function in the lme4 package, where Wald's *z* tests were implemented to determine significance which is appropriate for GLMMs without overdispersion (Bolker *et al.*, 2009). Significance of random effects was determined using likelihood ratio tests (Bolker *et al.*, 2009). Significance levels were set at $P < 0.05$ for all fixed and random effects. If the fixed effects were significant in the model, reduced models were used to further examine these effects. Next, GLMMs with binomial distribution and logit-link function were used to determine whether differences in MHC amino acid divergence and genetic relatedness between mates within a triad had a significant effect on paternity success in river water or ovarian fluid. The response variable was coded in the same way as described above (paired number of eggs sired by jacks and hooknoses). GLMMs included the fixed effect of either difference in MHC divergence or difference in genetic relatedness while accounting for random effects of female ID and male pair. Significance of fixed and random effects was determined in the same way as described above. All full GLMMs in our analyses were tested for overdispersion using Pearson's chi-square test, and no models were significantly overdispersed ($P > 0.05$).

Results

Sperm competition experiment

We found a significant difference in sperm velocity for hooknoses between activation media; sperm were faster in the ovarian fluid solution compared to sperm activated in river water (paired *t*-test: $t_{13} = 3.78$, $P = 0.002$), where mean (± SE) velocity in water and ovarian fluid was 114.6 ± 6.2 and 141.7 ± 5.0 μm s^{-1}, respectively. However, there was no significant difference in jacks' sperm velocity in river water and the ovarian fluid solution (paired *t*-test: $t_{13} = 0.85$, $P = 0.41$), where mean (± SE) velocity in water and ovarian fluid was 123.8 ± 5.3 and 138.0 ± 14.9 μm s^{-1}, respectively.

Using genetic analyses, we were able to assign paternity to a total of 1268 eyed-eggs. Using generalized linear mixed models (GLMMs), we found no effect of sperm velocity ($z = -0.57$; $P = 0.57$) or sperm density ratio on the outcome of sperm competition ($z = -1.00$; $P = 0.31$). However, activation medium had a significant effect on the outcome of sperm competition between male tactics ($z = 2.89$, $P = 0.004$). Furthermore, the interaction between activation medium and sperm velocity was also marginally significant in the model ($z = 1.96$, $P = 0.0499$). The random effect of male pair was significant in the model ($\chi^2_1 = 46.48$; $P < 0.001$); however, female had no significant effect in the model ($\chi^2_1 = 0.02$; $P = 0.88$). Given the significant effect of activation medium on paternity success, we next investigated paternity differences between alternative tactics within each activation medium using paired *t*-tests. When sperm were competed in ovarian fluid, no significant difference in paternity was detected between the tactics (Fig. 1a; $t_{13} = -0.63$; $P = 0.54$). However, when sperm were competed in river water, the male tactic effect was significant (Fig. 1a; $t_{13} = -2.42$; $P = 0.03$), such that sperm from the jacks sired more offspring than sperm from the hooknoses. To further investigate the effect of activation medium on sperm velocity and its relationship with competitive fertilization success, we used linear regressions to test the relationship between sperm metrics and paternity success in water and ovarian fluid separately. We found that differences in sperm velocity between jacks and hooknoses predicted differences in paternity between males when activated in river water (Fig. 1b; $r^2 = 0.29$, $F_{1,12} = 4.83$; $P = 0.048$), but not in ovarian fluid (Fig. 1b; $r^2 = 0.06$, $F_{1,12} = 0.81$; $P = 0.39$).

Major histocompatibility genes and relatedness

For adults used in our competitive fertilization trials, we found six MHC II ß1 alleles (see Table S2).

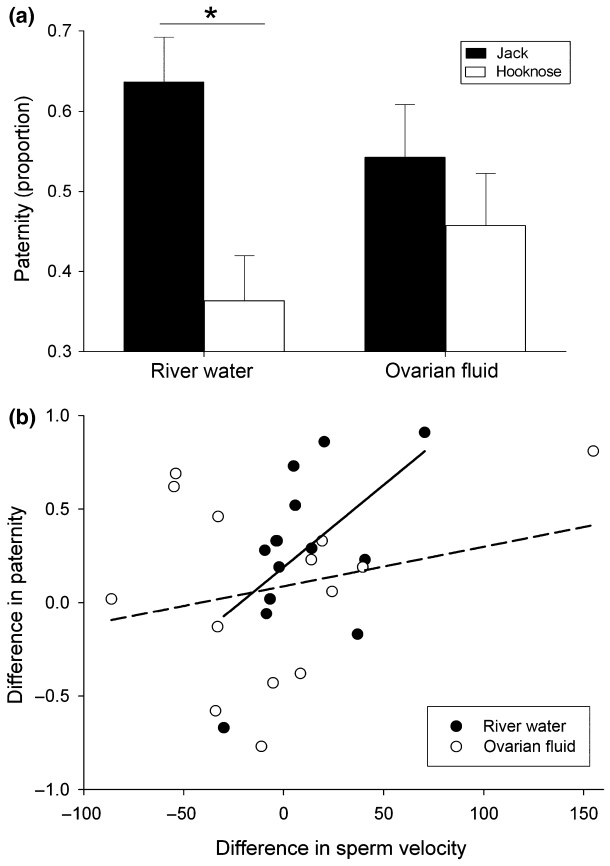

Fig. 1 (a) Mean competitive fertilization success (paternity) of hooknose (black bars) and jack (white bars) Chinook salmon (*Oncorhynchus tshawytscha*) shown as the mean ± SE proportion of total offspring sired in each activation medium (river water or ovarian fluid, $n = 14$ triads). Significant differences are indicated by asterisk (*) ($P < 0.05$). (b) The relationship between the difference in sperm velocity among pairs of males (jack values minus hooknose values within each pairing) and the difference in competitive fertilization success among pairs of males (paternity of jacks minus paternity of hooknoses) in river water (closed circles) and in the ovarian fluid solution (open circles). The lines reflect least squares regression analyses testing for linear relationships between differences in sperm velocity and differences in paternity among the males from the two tactics ($n = 14$ paired triads) activated in river water (solid line, $r^2 = 0.29$, $P = 0.048$, equation of the line: $y = 0.0088x + 0.19$) and an ovarian fluid solution (dashed line, $r^2 = 0.06$, $P = 0.39$, equation of the line: $y = 0.0021x + 0.09$).

Nucleotide sequences of the alleles were compared against the NCBI sequence database using BLAST (http://blast.ncbi.nlm.nih.gov), and all sequences matched 99–100% to MHC II ß1 alleles previously characterized by Miller & Withler (1996) and Pitcher & Neff (2006) (see Table S2). Alleles were comprised of 83 amino acids with seven variable sites, where the number of pairwise amino acid differences between alleles ranged from 1 to 7. Next, we assessed the relationship between MHC divergence and paternity success (in water and ovarian fluid) using GLMMs. We found that differences in MHC amino acid divergence (fixed effect) between mates within the triad did not predict paternity success in river water ($z = 0.27$; $P = 0.79$) or ovarian fluid ($z = -0.49$; $P = 0.62$) (see Fig. S1). The random effect of male pair was significant in both activation media (P values < 0.001); however, the random effect of female was not significant in either (P values > 0.99). For genetic relatedness, we found that differences in genetic relatedness (fixed effect) between males and the female within the triad did not predict paternity success in river water ($z = 01.53$; $P = 0.13$); however, difference in genetic relatedness was a significant predictor of success in ovarian fluid ($z = -2.24$; $P = 0.03$) (see Fig. S1). When sperm were activated in ovarian fluid, jacks attained higher paternity success when the difference in relatedness between the jack and female was greater relative to the difference between hooknose and female (i.e. less related individuals attained higher paternity success) (Fig. S1). The random effect of male pair had a significant effect on success in both activation media (P values < 0.001), whereas the random effect of female was not significant in either (P values > 0.46).

Discussion

In this study, we examined sperm competition in alternative reproductive tactics of Chinook salmon in the presence and absence of a female's ovarian fluid. Alternative reproductive tactics are found in many taxa, where males can attain reproductive success by utilizing different strategies (reviewed in Oliveira *et al.*, 2008). Although competition among male tactics can be important for determining success, females can also influence reproductive outcomes using mechanisms such as cryptic female choice (CFC). This is particularly important in externally fertilizing fishes, where sperm from multiple males is simultaneously released to fertilize the eggs of a single female, and in this scenario, the female may have limited control over which males attempt to join in these spawning events. Therefore, females may evolve strategies to bias fertilization success in favour of specific males that will confer the greatest benefit to their offspring (e.g. Lewis & Pitcher, 2016; Rosengrave *et al.*, 2016). In our study, we found that ovarian fluid can indeed influence male sperm performance and this effect is tactic specific.

Our finding that sperm velocity is higher in the presence of ovarian fluid than in river water for hooknose males is consistent with numerous studies on the effects of ovarian fluid on sperm velocity in fishes (e.g. Litvak & Trippel, 1998; Turner & Montgomerie, 2002), including another study on Chinook salmon hooknose males (Rosengrave *et al.*, 2008). Hooknose sperm should be selected to function well in the presence of ovarian fluid

because hooknose males monopolize access to females, enter the nest first at the time of spawning and release their sperm in close proximity to where eggs and higher concentrations of ovarian fluid are expelled by females (Berejikian *et al.*, 2010). Unlike the previous studies on Chinook salmon that only examined hooknose males (e.g. Rosengrave *et al.*, 2008, 2016), we also examined sperm from males of the alternative reproductive tactic and found that sperm velocity of jacks was not significantly different in river water vs. ovarian fluid. This result could be explained by the fact that jack sperm must function well in both river water and in the presence of ovarian fluid because they enter spawning bouts later than hooknoses (Berejikian *et al.*, 2010) and as such are forced to release their gametes later and presumably in lower concentrations of ovarian fluid.

We found that the presence of ovarian fluid affects jacks and hooknoses differently in terms of their *in vitro* sperm competition success. Our paternity analyses showed that jacks attain higher fertilization success when in direct competition with hooknoses in river water, which is consistent with another study, in Chinook salmon, that competed the alternative reproductive tactics using water as an activation medium (Young *et al.*, 2013). However, sperm competition in the presence of ovarian fluid appears to 'level the playing field' between the alternative reproductive tactics by disproportionately increasing the sperm velocity of hooknoses by 23.7% compared to only 11.5% in jacks relative to when activation occurs in river water alone. We also found that differences in sperm velocity among individuals from the two tactics were significantly correlated with paternity success in river water, but not in ovarian fluid, providing a possible explanation for why jacks sired more offspring in river water than in ovarian fluid. One possible explanation for this result is that females may be employing CFC (Eberhard, 1996) in which their ovarian fluid creates an environment favourable for both hooknose males and jacks to fertilize the eggs of the female, potentially increasing the genetic variation in her offspring. The differences found between activation medium in our study are like a recent study in the ocellated wrasse using alternative male tactics, where the importance of a sperm trait (sperm number) is reduced in the presence of ovarian fluid (Alonzo *et al.*, 2016). However, rather than providing equal opportunities for both alternative tactics, Alonzo *et al.* (2016) suggest that the presence of ovarian fluid biases fertilization success in favour of dominant parental males relative to sneaker males.

Recent studies on Chinook salmon have found inconsistent results relating to ovarian fluid serving as an important mechanism for CFC (Evans *et al.*, 2013; Lehnert *et al.*, 2016a; Rosengrave *et al.*, 2016). Evans *et al.* (2013) found no evidence that ovarian fluid acts as a mechanism of CFC, whereas Rosengrave *et al.* (2016) demonstrated that ovarian fluid can mediate CFC in an adaptive way. Differences between the studies may reflect differences in ovarian fluid concentrations that were used, where Evans *et al.* (2013) used a concentration of 10%, whereas Rosengrave *et al.* (2016) used the same concentration as our study (50%) as well as 100% (no dilution). Nevertheless, in both studies, the mechanism of CFC was only examined using hooknose males with females; the authors did not examine fertilizations using males from the two alternative reproductive tactics. Males from the two alternative reproductive tactics may differ significantly in terms of how similar they are to females because hooknose males can often be from the same age as the spawning females (3–5 years old in our population), whereas jacks are younger (2 years) and always from a different age class cohort altogether. As such, there are likely genetic differences among the females and the two male alternative reproductive tactics providing a broader scope of genetic difference for CFC to act upon. Studies on Chinook salmon have shown that there is potential for females to significantly increase the genetic quality (and thus fitness) of their offspring by choosing males based on their MHC genotype (Pitcher & Neff, 2006, 2007; also see Lewis & Pitcher, 2016). For example, in Chinook salmon, studies have reported that females mate nonrandomly based on the MHC (i.e. precopulatory sexual selection), where females mate more often with males with more divergent genotypes relative to their own (Consuegra & Garcia de Leaniz, 2008; Neff *et al.*, 2008). However, in Atlantic salmon, under *in vitro* competitive fertilization (i.e. post-copulatory sexual selection), Yeates *et al.* (2009) found that males with more similar MHC genotypes experienced greater competitive fertilization success than males with dissimilar genotypes (also see Gasparini *et al.*, 2015). Females may demonstrate post-copulatory selection for more similar MHC genotypes as a mechanism to reduce the likelihood of interspecific hybridization; alternatively, females may prefer more similar mates to favour locally adapted genotypes. In our study, we found that paternity success in water and ovarian fluid was not related to amino acid divergence between mates at the MHC II ß1 locus; however, we did find that paternity success in ovarian fluid (but not water) was related to differences in genetic relatedness between mates. The presence of ovarian fluid resulted in males attaining higher paternity success when the male was less related to the female relative to the rival male. Our results are comparable to a study in guppies, where under sperm competition, males that were unrelated to the female experienced greater fertilization success compared to males that were related to the female (Gasparini & Pilastro, 2011). Ovarian fluid may thus act as a mechanism of inbreeding avoidance in salmon, which may be an important strategy given that salmon exhibit strong natal philopatry. Furthermore, our results may indicate that ovarian fluid acts as a mechanism of CFC that

biases fertilization in favour of an unrelated male regardless of that male's tactic.

In our study, we found that the presence of ovarian fluid during fertilization can alter outcomes of sperm competition in Chinook salmon, where paternity can be influenced by genetic relatedness between mates. We also found that the presence of ovarian fluid during fertilization helped both alternative male reproductive tactics attain similar fertilization success. In the absence of ovarian fluid, jacks sired a significantly greater proportion of the eggs; however, when ovarian fluid was present, jack and hooknose males showed no significant difference in paternity success. Therefore, females may influence paternity via ovarian fluid to increase genetic quality of their offspring, thus providing opportunities for both alternative reproductive tactics to fertilize their eggs. By having eggs fertilized by both hooknoses and jacks, females could be employing a 'bet-hedging' strategy (Yasui & Garcia-Gonzalez, 2016). Precocious maturation (or jacking) is heritable (Heath *et al.*, 1994, 2002); therefore, if a female has offspring sired by both a jack and a hooknose, this will increase the probability that some of her offspring will mature as jacks and others as hooknoses, and thus, her offspring will return to spawn during different years. Given that environmental conditions and consequently salmon returns can vary annually (Beamish & Bouillon, 1993; Mantua *et al.*, 1997; Cooke *et al.*, 2004; Schindler *et al.*, 2010), between-offspring variation in age at maturation could increase female fitness and thus explain the benefits of a female promoting the fertilization of her eggs by both alternative tactics via ovarian fluid. Finally, by equalizing reproductive opportunities for both male tactics, ovarian fluid may provide an important mechanism operating to maintain both alternative reproductive tactics in nature.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1 Sperm metrics, paternity success, genotypes and genetic differences for jack and hooknose Chinook salmon (*Oncorhynchus tshawytscha*).

Table S1 Polymorphic microsatellite loci amplified in Chinook salmon (*Oncorhynchus tshawytscha*) for paternity and relatedness (see Materials and Methods) with their source, accession number and annealing temperatures (T_a).

Table S2 MHC II ß1 alleles found in jack, hooknose and female Chinook salmon (*Oncorhynchus tshawytscha*) with their corresponding accession number and original reference.

Figure S1 The relationship between the difference in (A) genetic relatedness [Queller & Goodnight (1989) method, QGM] and (B) amino acid divergence at the major histocompatibility complex (MHC II ß1) between jack and hooknose Chinook salmon (*Oncorhynchus tshawytscha*) males when compared to the female and the difference in competitive fertilization success (paternity as proportion) among pairs of males in river water (closed circles) and in the ovarian fluid solution (open circles).

Data deposited at Dryad: https://doi.org/10.5061/dryad.46d37

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