



## Original Article

Post-spawning sexual selection in red and white Chinook salmon (*Oncorhynchus tshawytscha*)Sarah J. Lehnert,<sup>a</sup> Daniel D. Heath,<sup>a,b</sup> Robert H. Devlin,<sup>c</sup> Trevor E. Pitcher<sup>a,b</sup><sup>a</sup>Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario N9B 3P4, Canada, <sup>b</sup>Department of Biological Sciences, University of Windsor, Windsor, Ontario N9B 3P4, Canada, <sup>c</sup>Center for Aquaculture and Environmental Research, Fisheries and Oceans Canada, 4160 Marine Drive, West Vancouver, British Columbia V7V 1N6, Canada

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Post-copulatory processes, including sperm competition and cryptic female choice (CFC), can play important roles in the maintenance of polymorphisms. In Chinook salmon (*Oncorhynchus tshawytscha*), color morphs (red and white) exist due to genetic polymorphisms affecting carotenoid deposition in flesh, skin, and gametes. We investigated the role of post-spawning sexual selection in maintaining the polymorphism in a mixed population. First, we compared sperm velocity differences in water between morphs. Next, we measured color-based CFC via 2 methods: 1) sperm velocity in ovarian fluid and 2) in vitro competitive fertilization using paired red and white males. We found that red males had marginally faster sperm relative to white males in water, suggesting that carotenoid storage may affect sperm performance. However, ovarian fluid of red and white females influenced sperm velocity of red and white males differently, indicative of color-based CFC on sperm velocity. Furthermore, we found evidence of color-based CFC on paternity success during in vitro competitive fertilizations; however, sperm velocity in ovarian fluid did not predict results found under in vitro fertilization. Instead, in our study, sperm velocity in water was a significant predictor of fertilization success. When we accounted for this difference in sperm velocity (in water) between paired males, we partitioned the amount of variation in fertilization success that was attributed to individual level CFC (male pair  $\times$  female) and male competitiveness (male pair) as 43% and 16%, respectively. In conclusion, post-spawning sexual selection processes represent important mechanisms contributing to the maintenance of the color polymorphism in nature.

**Key words:** carotenoids, color polymorphism, competitive fertilization, cryptic female choice, ovarian fluid, sperm competition.

## INTRODUCTION

Sexual selection is an important evolutionary process that acts both before and after copulation (Birkhead and Pizzari 2002; Andersson and Simmons 2006). Although early sexual selection research focused on pre-copulatory mechanisms, researchers have recently shown the critical role of post-copulatory sexual selection in diverse evolutionary processes, such as speciation (Mendelson et al. 2007; Yeates et al. 2013), local adaptation (Palumbi 1999; Yeates et al. 2009), and the maintenance of genetic variation (Birkhead and Pizzari 2002; Gasparini and Pilastro 2011; Løvlie et al. 2013). Post-copulatory sexual selection includes both sperm competition and cryptic female choice (CFC). Sperm competition arises when sperm from more than 1 male compete to fertilize the eggs of a

female (Parker 1970), and the outcome can depend on sperm quality (Gage et al. 2004; García-González and Simmons 2005; Snook 2005; Gasparini et al. 2010; Beausoleil et al. 2012). Although sperm quality traits can be good predictors of fertilization success, CFC can also influence the outcome of sperm competition where females may bias fertilization in favor of a specific male that will confer the greatest fitness benefit for her offspring (Eberhard 1996; Birkhead 1998; Neff and Pitcher 2005). Potential mechanisms for CFC include egg–sperm recognition (Palumbi 1999; Yeates et al. 2009) and female-related chemical processes that mediate sperm performance or fertilization success (Rosengrave et al. 2008; Butts et al. 2012; Yeates et al. 2013).

Evidence for CFC has been demonstrated in many taxa and has been shown to discriminate among conspecifics to skew fertilization success in favor of males with specific genotypes (Palumbi 1999; Yeates et al. 2009; Butts et al. 2012; Løvlie et al. 2013) or

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phenotypes (Evans et al. 2003; Bussière et al. 2006). Additionally, CFC may operate as a form of reinforcement to reduce the risk of hybridization between closely related species (Yeates et al. 2013). While empirical evidence indicates that CFC is found in many species, the specific mechanisms by which CFC is achieved are less understood. Studies on CFC can be confounded by male effects, such as differences between males in sperm performance (Birkhead and Pizzari 2002; Evans et al. 2013). Although both sperm competition and CFC are important to our understanding of sexual selection, partitioning the relative effects of female, male, and their interaction on post-copulatory success is often difficult (Birkhead and Pizzari 2002; Evans et al. 2013). However, studies designed to evaluate CFC and sperm competition simultaneously can properly quantify post-copulatory success attributed to each process.

While post-copulatory sexual selection has many important evolutionary consequences, one of particular interest is its role in maintaining genetic variation in nature (Birkhead and Pizzari 2002). Chinook salmon (*Oncorhynchus tshawytscha*) display remarkable variation in flesh coloration, resulting from genetic polymorphisms that affect their ability to deposit dietary carotenoid pigments into their tissues (Withler 1986; Lehnert et al. 2016b). Consequently, within some populations of Chinook salmon, individuals exhibit flesh color that is white (unpigmented) or red (pigmented; Withler 1986), and the percentage of white individuals within a population can range from 0 to 100% of the population (Hard et al. 1989). Differences in flesh pigmentation also translate into differences in egg and spawning coloration (Withler 1986; Rajasingh et al. 2007; Lehnert et al. 2016b). Carotenoid pigments play an important role in salmonid fitness (Rajasingh et al. 2007) as carotenoids have been linked to salmon immune function (Amar et al. 2012), egg survival (Tyndale et al. 2008), mate choice (Fleming and Gross 1994; Skarstein and Folstad 1996; Craig and Foote 2001), and sperm quality (Ahmadi et al. 2006; Janhunen et al. 2009; Pitcher et al. 2009). However, despite this potential handicap to white Chinook salmon, both phenotypes persist in mixed populations in nature. Thus, in our study, we investigate post-spawning sexual selection as a possible mechanism that contributes to the maintenance of the color polymorphism.

While sperm performance has been shown to be influenced by carotenoid pigments (Evans et al. 2003; Locatello et al. 2006; Pike et al. 2010; Tizkar et al. 2015), differences in sperm performance between red and white Chinook salmon males have yet to be evaluated. The persistence of the white phenotype in nature may indicate that white Chinook salmon have evolved compensatory mechanisms to increase their relative fitness despite lacking carotenoids. Therefore, by assessing sperm performance differences between red and white males, we can determine whether competitive differences exist between the phenotypes during reproduction that may contribute to the maintenance of the 2 morphs in nature. In addition to sperm performance, CFC may also influence fertilization success, and in salmon, CFC may be mediated by ovarian fluid (a viscous liquid that is expelled with the eggs during spawning; Urbach et al. 2005; Rosengrave et al. 2008; Butts et al. 2012; Yeates et al. 2013) and/or egg-sperm recognition (Yeates et al. 2009). Although CFC has not been examined in red and white Chinook salmon, a recent study showed that the red and white phenotypes do successfully interbreed in one population; however, under semi-natural conditions, 71% of mating events were found to be color assortative (Lehnert et al. 2016b). The potential fitness consequences of interbreeding between color morphs have not been established. CFC may act to reinforce pre-copulatory choice

(Evans et al. 2003; Parker 2009), although studies have also found that CFC can act antagonistically with pre-copulatory sexual selection (Danielsson 2001; Bussière et al. 2006). If there is a cost to interbreeding between morphs (i.e., hybrid breakdown) then CFC may bias paternity in favor of a male that has the same phenotype as the female, thus offering an evolutionary mechanism contributing to the maintenance of the morphs.

Chinook salmon are external fertilizers, thus sperm competition and CFC can be studied using in vitro experiments where sperm, eggs, and other reproductive components (i.e., ovarian fluid) can be easily manipulated. Furthermore, Chinook salmon have high fecundity and high volume of semen allowing factorial mating designs that allow the partitioning of male, female, and interaction effects on fertilization success (Evans et al. 2013). In this study, we first quantify differences between red and white Chinook salmon males in sperm velocity upon activation in fresh water (sperm quality). Next, given that the ovarian fluid of Chinook salmon females may mediate CFC (Rosengrave et al. 2016), we test for color-based CFC in red and white Chinook salmon females by quantifying relative sperm velocity in ovarian fluid (using microscopy). In addition, although sperm velocity in ovarian fluid may determine the outcome of post-copulatory sexual selection, to ultimately partition the relative contribution of each process (i.e., sperm competition and CFC) to fertilization success, we use in vitro competitive fertilization trials. Our study evaluates how post-spawning sexual selection operates in a species that exhibits genetic polymorphisms for carotenoid pigmentation, where the relative contribution of both sperm competition and CFC to post-spawning success is quantified. The results of our study will determine whether post-spawning sexual selection may be a mechanism contributing to the maintenance of the color polymorphism in nature.

## MATERIALS AND METHODS

### Fish collection

During the fall of 2013 and 2014, adult Chinook salmon were caught by seine from the Quesnel River, Likely, British Columbia, Canada (GPS coordinates: 52°36'28"N and 121°32'57"W). During both sampling seasons, fish collection occurred from September 13 to October 1. In 2013, we collected only male fish to measure sperm velocity in water, and in 2014, we collected male and female fish to measure sperm velocity and to evaluate CFC. After capture, fish were held in the river temporarily before being transported to the Quesnel River Research Center (QRRC). During transport, fish were placed in holding tanks with aerated river water and transported for approximately 15 minutes (5 km). Fish were then held in 3000 l freshwater tanks or semi-natural spawning channels (see Lehnert et al. 2016b for description) at 10 °C until sampling. All fish were sampled within 2 weeks of capture.

### Gamete collection

In both the fall of 2013 and 2014, live males were anesthetized in clove oil and sampled for semen, weight, color score (see below), and a fin clip for DNA extraction. The age of the fish was unknown, however, it is expected that males and females were approximately 4–5 years of age based on their size. Weights and lengths did not differ for males between sampling years ( $n = 48$ ;  $t$  tests,  $P$  values  $> 0.29$ ). Thus, across both years, red and white males did not differ significantly in weight or length ( $t$  tests,  $P$  values  $> 0.64$ ), as the mean ( $\pm$ standard error [SE]) weights of red and white males were

7.2 ( $\pm 0.57$ ) and 7.1 ( $\pm 0.66$ ) kg, respectively, and weights ranged from 3.3 to 13.3 kg for red males and 3.0 to 13.9 kg for white males. Additionally, mean ( $\pm$ SE) fork lengths for red and white males were 88.3 ( $\pm 2.2$ ) and 86.7 ( $\pm 2.5$ ) cm, respectively, where red males ranged in length from 70 to 107 cm and white males ranged from 71 to 110 cm. Semen samples were collected from males by first drying the fish and then applying gentle pressure to the abdomen. Semen was collected into a plastic bag, then sealed and kept at approximately 4 °C. In the fall of 2014, females were also sampled for eggs, ovarian fluid, color score, and a fin clip. Females were euthanized then wiped dry to remove excess water, and gametes were collected by applying pressure to the abdomen to remove both eggs and ovarian fluid. Eggs and ovarian fluid were kept covered in plastic containers at approximately 10 °C until fertilizations were performed (<6 hours). For both males and females, color was determined visually such that individuals were categorized as “red” (pigmented) or “white” (non-pigmented) based on external spawning coloration (Withler 1986; Lehnert et al. 2016b). Individuals were assigned as red when they exhibited external red skin pigmentation and individuals were assigned as white when they showed no external red pigmentation and were gray in color. Color assignment of females was also confirmed by egg color, and there were no cases where external color did not correspond to egg color. Additionally, in 2014, spectral readings of external spawning color were taken from fish post-mortem using a Jaz Spectrophotometer (Ocean Optics), where readings were taken in triplicate at 3 positions along the lateral body of the fish (see Lehnert et al. 2016b for details). Given that some fish had been deceased for several hours, we only analyzed individuals that showed no evidence of discoloration due to their mortality. Using the *pavo* package (Maia et al. 2013) in R software (R Core Team 2016), we calculated chroma (red saturation; S1.red color variable in *pavo*; see Montgomerie 2006) for each male. Red and white males ( $n = 9$  red and 10 white) differed significantly in chroma ( $t = 5.39$ ; degree of freedom = 17;  $P < 0.001$ ), where mean ( $\pm$ SE) chroma was 0.286 ( $\pm 0.007$ ) and 0.247 ( $\pm 0.003$ ) for red and white males, respectively.

### Sperm velocity in red and white Chinook salmon

Sperm velocity was chosen as a measure of sperm quality as sperm velocity is the primary predictor of competitive fertilization success in salmonids (Lahnsteiner et al. 1998; Gage et al. 2004; Liljedal et al. 2008), including Chinook salmon (Flannery 2011). In the fall of 2013 and 2014, sperm velocity was assessed upon activation in fresh water (river water in 2013 and hatchery water [well water] in 2014) using video recordings (see Lehnert et al. 2012) of sperm recorded under a negative phase-contrast microscope (CX41 Olympus) with  $\times 10$  magnification and a CCD B/W video camera (at 50 Hz vertical frequency). Using HTM-CEROS sperm analysis system (CEROS version 12, Hamilton Thorne Research, Beverly, MA), the following parameters were used to assess sperm velocity: number of frames = 60, minimum contrast = 11, and minimum cell size = 3 pixels. Sperm velocity estimates were represented by the mean velocity of all individual motile sperm cells in the video. Sperm velocity was measured as average path velocity (VAP), which describes a smoothed path of the sperm cell's trajectory (Rurangwa et al. 2004). We chose VAP as our measure of sperm velocity because it is often used in other Chinook salmon studies (Rosengrave et al. 2008; Lehnert et al. 2012; Evans et al. 2013; Rosengrave et al. 2016) as well as studies on other salmonids (Lahnsteiner et al. 1998). Additionally, 2 other measures of sperm velocity, curvilinear velocity (VCL, defined as the average velocity along the actual path of the sperm

cell's trajectory), and straight line velocity (VSL, defined as the average velocity along a straight line connecting the start and end points of the sperm cell's path) were highly correlated with VAP when we examined their relationship for videos of sperm velocity measures in water (Pearson correlation,  $P$  values  $< 0.001$ ;  $n = 95$  videos) thus we present only VAP in our results. In 2013, 2 video recordings were taken for each male, and in 2014, 2 video recordings were taken if time permitted where 60% of data points were from replicated videos (replication addressed in statistical analyses). Sperm velocity was evaluated at 5 seconds post-activation in fresh water, as a previous study found that the majority (80%) of fertilization occurs within 5 seconds of sperm and egg association in salmon (Hoysak and Liley 2001). Sperm velocity was measured for different males over multiple days during the spawning season in both sampling years. In 2013, sperm velocity was recorded on 8 dates between September 18 and 30, and different males were sampled on each of these dates ( $n = 28$  males in total). In 2014, sperm velocity was recorded on 3 dates between September 18 and October 2, where the same males were sampled on multiple dates if possible ( $n = 20$  males in total). In this case, 1 male was sampled on September 18, a total of 15 males were sampled on September 27, and 12 males were sampled on October 2. In total, 20 different males were sampled in 2014, as 8 of the same males were sampled on both September 27 and October 2.

### CFC in red and white Chinook salmon

#### *Sperm velocity in ovarian fluid*

In the fall of 2014, sperm velocity (VAP) was assessed in both fresh water (described above) and in diluted ovarian fluid of 8 females (4 red and 4 white) on 2 sampling dates: September 27 and October 2. Ovarian fluid was collected from females through lethal sampling (as described above), where 4 different females were sampled on each date. If possible, sperm from all males was collected on both dates; however, in some cases, males could not be sampled on both dates due to differences in ripening (i.e., reproductive status). Ovarian fluid was diluted to 20% in hatchery water and used to activate sperm under the microscope. Although the concentration of ovarian fluid in wild spawning events is unknown, ovarian fluid represents <30% of the combined mass of eggs and fluid in salmonids (Lahnsteiner et al. 1995); thus, it is likely that the concentration of ovarian fluid in nature would be low. Therefore, we chose a dilution of 20%, which has been used in a previous study in salmonids (Butts et al. 2012). Sperm velocity in ovarian fluid was measured using the same protocol and same males (from 2014) as described above. A total of 19 males were used, where each male was activated in the ovarian fluid from a minimum of 4 females. In total, our analysis involved 104 male  $\times$  female ovarian fluid combinations, where sperm from 10 to 15 males was activated in the ovarian fluid of each female ( $n = 8$  females). Again, we chose to only present VAP as a sperm velocity metric, as VAP was highly correlated with VCL and VSL (Pearson correlation,  $P$  values  $< 0.001$ ) when using all 104 data points (male  $\times$  female combinations) for sperm velocity in ovarian fluid. However, given that Yeates et al. (2013) found that ovarian fluid can have a strong effect on the straightness of sperm trajectory, we have included analyses for VSL and sperm path straightness (STR, calculated from VSL/VAP) in the supplementary materials (Table S1).

#### *Competitive fertilizations*

In addition to sperm velocity in ovarian fluid, we also examined CFC through competitive fertilization success of males under in



vitro sperm competition in 2014. Eight males were used to create 4 male pairs where 1 male was red and 1 male was white within each pair. The male pairs included the same 8 males that were tested for sperm velocity in water and ovarian fluid as described above. Although a greater number of males would be ideal, given that fish were captured from a wild population during a low escapement year (28% lower than in 2013; R. Bailey DFO Stock Assessment, personal communication), logistical, and biological constraints (i.e., low population density, equal color and sex ratios, maturation stage, and holding space) reduced our ability to incorporate more individuals within the short time frame necessary to have all gametes for testing available simultaneously (i.e., within a 4-day period). Each male pair competed to fertilize the eggs of 8 females (4 red and 4 white females), resulting in a total of 32 competitions (4 male pairs  $\times$  8 females). Eggs were separated from ovarian fluid using a sieve and each female's eggs were divided into batches for fertilization. The number of eggs per batch depended on the number and size of a female's eggs, where the mean ( $\pm$ SE) number of eggs per batch was 135.1 ( $\pm$ 5.98) eggs. After the eggs were separated, ovarian fluid was measured then divided and poured onto egg batches. Given that different females had different volumes of ovarian fluid, different volumes of water were added to activate sperm and eggs (ranging from 20 to 100 ml) to ensure that ovarian fluid represented 20% of the total volume added for each competitive fertilization.

All fertilization trials were performed using 50  $\mu$ l of semen from each male within the male pair. We measured sperm density in all males during competitive fertilizations using the same protocol described in Lehnert et al. (2012). Based on sperm density estimates, the volume of semen used in the fertilization should be high enough to ensure fertilization as the sperm:egg ratio for all crosses ranged for 16,526 to 54,311 sperm cells per egg. Although sperm densities were not controlled for in competition, we consider sperm density effects in our analysis of competitive fertilization below. During fertilizations, sperm from paired males were pipetted simultaneously with hatchery water onto the eggs and ovarian fluid. Eggs, sperm, ovarian fluid, and water were mixed and left undisturbed for 2 minutes. Water was poured off the eggs, and eggs were transferred to vertical incubation trays. Eggs were left to incubate at 10 °C until the eyed-egg stage (250–500 accumulated thermal units), and on November 5 and 6, 2014, all eyed-eggs were counted and preserved in high salt preservative buffer (3.5M ammonium sulfate; 15 mM EDTA; 15 mM sodium citrate; pH 5.2) for DNA-based paternity analyses. Percent survival to the eyed-egg stage was calculated as the number of live eyed-eggs divided by the total number of eggs (dead and live). However, our survival estimates may be underestimates of actual survival because it is possible that some dead eggs were not fertilized, as we could not discriminate between unfertilized and fertilized dead eggs.

### Paternity analysis

DNA was extracted from parental fin clips and eyed-egg samples using a plate-based extraction method (Elphinstone et al. 2003). DNA was extracted from a total of 21 to 24 eggs per competition experiment, with the exception of one of the total 32 competitions where only four eggs survived; we thus excluded that competition from the analyses. Three microsatellite loci were genotyped to accurately differentiate between paired males for paternity assignment. In total, 6 microsatellite loci were chosen: OtsG68, OtsG78b, OtsG432 (Williamson et al. 2002), Ots211 (Greig et al. 2003), Omy325 (O'Connell et al. 1997), and Ots107 (Nelson and Beacham 1999). Polymerase chain reaction conditions included: a

5-minute denaturation step (94 °C), followed by 35–38 cycles of a 20-second denaturation step (94 °C), a 20-second annealing step (52.5 °C—OtsG68, OtsG78b; 54 °C—Omy325; 56 °C—OtsG432; 58 °C—Ots107; 60 °C—Ots211), and a 30-second extension step (72 °C), followed by a final extension of 3 minutes. All forward primers were fluorescently dye-labeled and polymerase chain reaction products were visualized using a LiCor 4300 DNA analyzer (LiCor Biosciences, Inc.). Fragment sizes (alleles) were scored using GENE IMAGIR 4.05 software (Scanalytics Inc.). Allele scores were used to determine paternity by exclusion of 1 male and positive inclusion of the other male at genotyped loci.

## Statistical analyses

### Sperm velocity in fresh water

All statistical analyses were performed in R software version 3.3.1 (R Core Team 2016) unless otherwise stated. Sperm velocity in water data for 2013 and 2014 were combined and red and white males ( $n = 48$  males) were compared using a linear mixed effects model in the *lme4* package (Bates et al. 2009). Linear models were compared using log-likelihood ratio test for random effects, whereas Kenward–Roger approximation test was used to examine the effect of the fixed factor. The model included the fixed factor of male color, with random factors of year, sampling date, and male ID. Male ID was included as a random factor because sperm velocity in water was recorded for 2 videos per male (if possible). Additionally, some of the same males were sampled over 2 of the sampling dates in 2014 (see above for description), where a total of 8 males were sampled on both dates and 12 males were sampled on only 1 date ( $n = 20$  different males in total). Therefore, all replicate videos were used as data points in the analysis. In 2013, sperm velocity measurements were replicated (i.e., 2 videos) for each male ( $n = 28$ ); however, not all males were replicated in 2014. In 2014, videos were recorded on 3 sampling dates (see above), and the percentage of males (with total males sampled) that were replicated on each date was 100% ( $n = 1$  total), 87% ( $n = 15$  total), and 25% ( $n = 12$  total) on September 18, 27, and October 2, respectively. We assessed repeatability of video replicates for sperm velocity using Pearson's correlation. Using 45 videos and their replicates, we found that replicates were highly correlated ( $r = 0.81$ ;  $P < 0.001$ ); therefore, we do not expect the lack of replication for some individuals to influence the overall results of our study. Assumptions of linear mixed models were assessed using diagnostic checks. Model residuals were assessed for normality using Shapiro–Wilk test and by examining Q–Q plots. Additionally, residual homogeneity was evaluated by plotting model residuals and fitted values. Assumption of linear mixed effect models were met, as model residuals met assumption of normality (Shapiro–Wilk test,  $W = 0.99$ ,  $P$ -value = 0.95) and homogeneity.

### Sperm velocity in ovarian fluid

Analyses were conducted using linear mixed effect models with male color, female color, and their interaction as fixed factors and sampling date, male ID, and female ID as random factors. When replicate videos were recorded for a male (within a female's ovarian fluid), velocities were averaged over replicate videos. Our analysis therefore involved a total of 104 male  $\times$  female combinations (data points). Diagnostic checks for linear mixed model revealed that model residuals met assumption of normality (Shapiro–Wilk test,  $W = 0.99$ ,  $P$ -value = 0.45) and homogeneity. In addition to average path sperm velocity (VAP), we conducted the same analyses for

VSL and sperm path straightness (STR) and these results are provided in the supplementary materials (see Table S1).

### Competitive fertilization success

After paternity of offspring from competitive fertilizations was determined for 31 crosses, offspring from each cross were coded as 1 if sired by a red male and 0 if sired by a white male ( $n = 727$  offspring). Using generalized linear mixed effects model (GLMM) with binomial distribution and logit link function, we used female color, male pair, and their interaction as fixed factors with sampling date and female ID as random factors. Using the GLMM, we could determine the main effect of female color, male pair, and their interaction on fertilization success, while controlling for confounding effects of date and female ID. The main effect that we were interested in was the interaction of female color and male pair, as a significant interaction effect would indicate color-based CFC where red and white male success within the pair differed by female color.

Given that the results of competitive fertilization can be influenced by sperm velocity differences between males (Gage et al. 2004), we also calculated the difference in sperm velocity between paired males in both water and ovarian fluid. Therefore, within a male pair, velocity of the white male was subtracted from the velocity of the red male. Additionally, we considered that sperm density could also be important for fertilization success; therefore, we calculated the ratio of red:white sperm cells within each male pair during fertilizations. We used 3 GLMMs that included the fixed effects of male sperm differences (differential sperm velocity measures or density ratio) and female color in the model with the random effect of sampling date, male pair, female ID, and the interaction of male pair and female ID. We thus used 3 different GLMMs, where each model included a different measure (fixed effect) of male sperm differences. Therefore, 1 model included differential sperm velocity in ovarian fluid, the next model included differential sperm velocity in water and the final model included sperm density ratio within

the male pair. In this way, we could determine whether sperm differences between paired males significantly contributed to fertilization success. Next, if sperm differences (velocity or density) were a significant predictor of success in the model, we partitioned the variance in success attributed to random effects in the model that were associated with both post-spawning processes (CFC and male competitiveness). In our model, the variance associated with the interaction of male pair  $\times$  female ID represents the variation due to CFC at the individual level. The variance associated with male competition is represented by the variation caused by male pair. In some cases, sperm velocity was not recorded for all males due to unusable videos when flow affected velocity measures; nevertheless, the models for sperm velocity in ovarian fluid and water included 586 and 634 data points, respectively. For all GLMMs in our study, we used log-likelihood ratio tests to examine the effects of both fixed and random factors in the models. Additionally, all GLMMs were examined for overdispersion. Overdispersion was assessed by dividing residual deviation (rdev) by residual degrees of freedom (rdf) for the model, and if the ratio value was less than 1 then we concluded that the model was not overdispersed. None of the full GLMMs were overdispersed (rdev/rdf < 0.85).

## RESULTS

### Sperm velocity in red and white Chinook salmon

In 2013, we excluded data collected on 2 early sampling dates (September 18 and 19;  $n = 2$  red males and 1 white male), as all males on these dates had low sperm velocity (all replicate videos  $\leq 65$   $\mu\text{m}/\text{sec}$ ) and were likely not in full reproductive condition and thus not biologically relevant. Our analysis therefore involved a total of 45 males over the 2 years ( $n = 25$  in 2013 and  $n = 20$  in 2014). Linear mixed models revealed that red males had marginally faster sperm velocity in water relative to white males (see Table 1 and Fig. 1;  $F = 4.06$ ,  $P = 0.0506$ ;  $n = 45$  males). Sperm velocity did

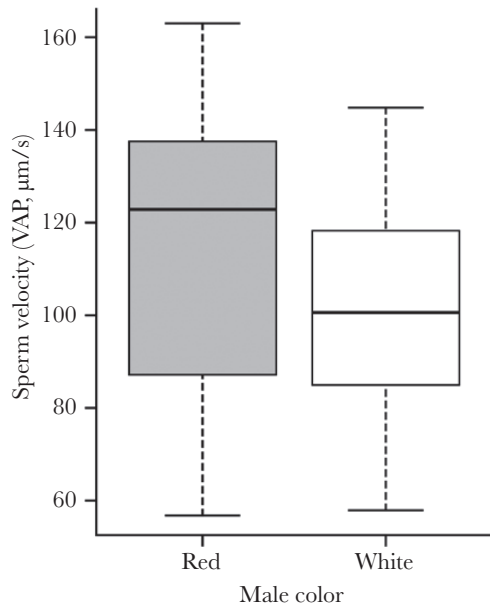
**Table 1**

**Results of linear mixed effect models for sperm velocity of red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when activated in water and in ovarian fluid of red and white Chinook salmon females**

Linear mixed effect models for sperm velocity

Sperm velocity (VAP) in water	Fixed effects	Estimate	95% CI	Kenward–Roger approx.	
				<i>F</i>	<i>P</i>
	Intercept	117.48	100.12, 134.84		
	Color	-12.65	-24.83, -0.47	4.06	0.0506
	Random effects	Variance	$\pm$ SD	% var	Log-likelihood ratio test
	Year ( $n = 2$ )	87.55	9.36	12.3	$\chi^2$ <i>P</i>
	Date ( $n = 9$ )	120.58	10.98	17.0	0.47 0.49
	Male ID ( $n = 45$ )	311.44	17.65	43.8	13.40 <b>0.0003*</b>
	Error	191.25	13.83	26.9	21.61 <b>&lt;0.001*</b>
Sperm velocity (VAP) in ovarian fluid	Fixed effects	Estimate	95% CI	Kenward–Roger approx.	
				<i>F</i>	<i>P</i>
	Intercept	125.40	114.44, 136.36		
	Male color	-16.49	-30.33, -2.66	0.74	0.40
	Female color	-10.87	-20.69, -1.05	0.24	0.64
	Interaction	19.19	9.19, 29.20	13.99	<b>0.0003*</b>
	Random effects	Variance	$\pm$ SD	% var	Log-likelihood ratio test
	Male ID ( $n = 19$ )	167.59	12.95	48.7	$\chi^2$ <i>P</i>
	Female ID ( $n = 8$ )	26.41	5.14	7.6	30.23 <b>&lt;0.001*</b>
	Error	150.18	12.26	43.6	4.18 <b>0.04*</b>

Fixed effects are presented with estimate parameters including 95% confidence intervals (CI) and statistical results of Kenward–Roger approximation test. Random effects are presented with variance components with standard deviations and percentage of the total variance (% var) as well as statistical results of log-likelihood ratio tests. Significant effects are indicated by bold font and an asterisk.



**Figure 1**

Box plot of sperm velocity (average path velocity, VAP) for red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when activated in water. Males ( $n = 45$ ) were sampled from the Quesnel River, British Columbia during 2 spawning seasons in 2013 ( $n = 13$  red; 12 white) and 2014 ( $n = 10$  red; 10 white). The difference between red and white males approached marginal significance ( $P = 0.0506$ ) based on linear mixed models (see Methods and Table 1 for details).

not differ significantly between years (Table 1;  $\chi^2 = 0.47$ ;  $P = 0.49$ ); however, random factors of male ID and date significantly contributed to the variance observed for sperm velocity (see Table 1; male ID:  $\chi^2 = 21.6$ ;  $P < 0.001$ ; date:  $\chi^2 = 13.4$ ;  $P = 0.0003$ ).

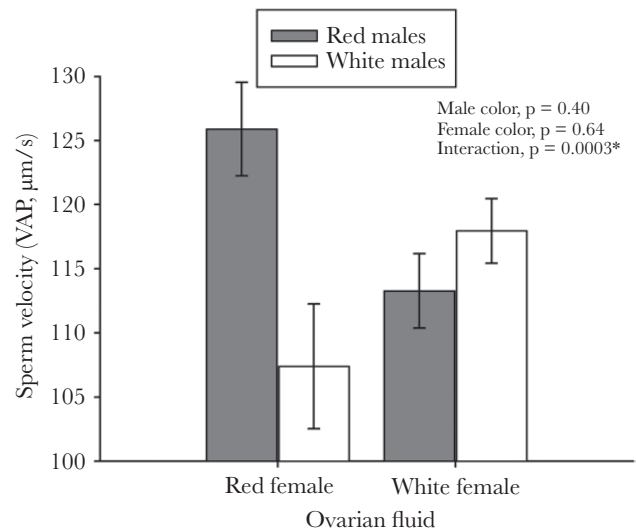
## CFC in red and white Chinook salmon

### Sperm velocity in ovarian fluid

Date did not contribute to the variance observed for sperm velocity (VAP) in ovarian fluid ( $P = 0.99$ ); therefore, it was excluded from the model to avoid over parameterization. For sperm velocity in ovarian fluid, we found a significant interaction of male color and female color (see Table 1 and Fig. 2;  $F = 13.99$ ;  $P = 0.0003$ ;  $n = 104$  male  $\times$  female combinations). Changes in mean sperm velocity for individual males when activated in the ovarian fluid of red versus white females are presented in supplementary materials (Fig. S1). Sperm velocity for red males was higher when activated in the ovarian fluid of red females and sperm velocity of white males was higher when activated in the ovarian fluid of white females (Fig. 2). Male ID (random factor) contributed significantly to the variance observed for sperm velocity (Table 1;  $\chi^2 = 30.23$ ;  $P < 0.001$ ). Female ID (random factor) was also significant in the model (Table 1;  $\chi^2 = 4.18$ ;  $P = 0.04$ ). The interaction detected between male color and female color (i.e., color-based CFC) was also significant when assessing straight line sperm velocity (VSL;  $F = 9.00$ ;  $P = 0.004$ ;  $n = 104$ ) in ovarian fluid but not for sperm path straightness (STR;  $F = 1.97$ ;  $P = 0.16$ ;  $n = 104$ ; see Table S1 for full results).

### Competitive fertilization success

Mean ( $\pm$ SE) eyed-egg survival from all 31 competitive fertilizations was 60.2% ( $\pm 4.35\%$ ) where survival ranged from 14.2 to 94.7%.

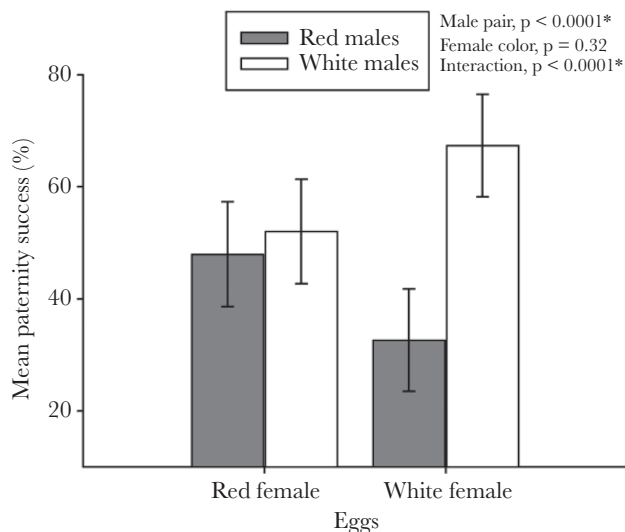


**Figure 2**

Mean ( $\pm$  standard error) sperm velocity (average path velocity, VAP) of red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when activated in ovarian fluid (20% dilution) from red and white Chinook salmon females. Data represent sperm velocities from 104 male  $\times$  female ovarian fluid combinations ( $n = 10$  to 15 males  $\times$  8 females). Significance ( $P$ -values) for fixed factors (male color, female color, and their interaction) in linear mixed effect models are presented in the right hand corner and full results are presented in Table 1.

Our estimates of survival may be underestimated, as we did not discriminate between dead eggs that were fertilized and those that were unfertilized. Nevertheless, the mean survival found in our study is similar to other studies on Chinook salmon, as Barnes et al. (2003) found mean survival to the eyed stage was 41–59% over different years and Pitcher and Neff (2006) found mean ( $\pm$ SE) egg survival to hatch was  $71 \pm 19\%$  (range: 13–99%). All paternity calculations were based on 21 to 24 eggs per competition that were genotyped and assigned to 1 male within the male pair (all genotyped eggs were successfully assigned to 1 male parent; see Table S2). We used a GLMM to assess paternity success as our response variable was binary where eggs sired by red males were coded as 1 and eggs sired by white males were coded as 0. Using GLMM, we found a significant interaction between female color and male pair (see Fig. 3 and Table 2;  $\chi^2 = 26.0$ ;  $P < 0.001$ ;  $n = 726$  eggs), indicating color-based CFC on male competitive fertilization success, where the paternity success of red and white males within a pair differed between female colors. We found that red and white males sired a similar number of offspring when competing for fertilization in the eggs of red females ( $n = 172$  red sired; 182 white sired); however, white males had significantly higher paternity compared to red males when competing for fertilization of eggs from white females ( $n = 123$  red sired;  $n = 250$  white sired), thus suggesting that the strength of color-based CFC is greater in white females compared to red females. Although we found no significant effect of female color on fertilization success overall ( $\chi^2 = 1.01$ ;  $P = 0.32$ ), we did find a significant effect of male pair ( $\chi^2 = 264.5$ ;  $P < 0.001$ ; see Table 2 for full results). Finally, we also found a significant random effect of female ID ( $\chi^2 = 25.3$ ;  $P < 0.001$ ), but no effect of sampling date ( $\chi^2 = 1.42$ ;  $P = 0.23$ ; Table 2).

Next using GLMMs, we partitioned the effects of post-copulatory processes (i.e., male competitiveness and individual level CFC) on competitive fertilization success while accounting for sperm differences



**Figure 3** Mean (±standard error) paternity (percentage of eyed-eggs) of red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when in sperm competition to fertilize the eggs of red and white Chinook salmon females. Results are based on paternity of 727 genotyped eggs that were competitively fertilized in the presence of 20% ovarian fluid. Significance (*P*-values) for fixed factors (male pair, female color, and their interaction) in generalized linear mixed effect models are presented in the right hand corner with full results presented in Table 2.

**Table 2** Results of generalized linear mixed effect models testing post-copulatory processes affecting fertilization success (paternity) in red and white Chinook salmon (*Oncorhynchus tshawytscha*) males under sperm competition in the eggs of red and white females

Generalized linear mixed models for fertilization success

Model to test for color-based CFC on fertilization success	Fixed effects	Estimate	95% CI		Log-likelihood test	
					$\chi^2$	<i>P</i>
	Intercept	0.84	-0.57, 2.25			
	Female color	-2.02	-3.49, -0.56		1.01	0.32
	Male pair				264.5	<b>&lt;0.001*</b>
	MalePair (53–55)	0.69	-0.08, 1.46			
	MalePair (70–88)	-1.93	-2.66, -1.20			
	MalePair (80–84)	-3.50	-4.42, -2.58			
	Female color × male pair				26.03	<b>&lt;0.001*</b>
	FcolorW:MalePair (53–55)	2.44	1.31, 3.57			
	FcolorW:MalePair (70–88)	1.32	0.20, 2.44			
	FcolorW:MalePair (80–84)	2.62	1.35, 3.90			
	Random effects	Variance	SD	% var	$\chi^2$	<i>P</i>
	Female ID	0.51	0.71	11.7	25.28	<b>&lt;0.001*</b>
	Date	0.56	0.75	12.8	1.42	0.23
	Error	3.29		75.5		
	Fixed effect	Estimate	95% CI		$\chi^2$	<i>P</i>
Model to partition effects of post-copulatory processes on fertilization success while accounting for sperm velocity (VAP) differences between paired males in water	Intercept	-1.46	-3.24, 0.33			
	Female color	-1.08	-2.76, 0.60		1.63	0.20
	Difference sperm velocity in water	0.07	0.03, 0.11		8.28	<b>0.004*</b>
	Random effects	Variance	SD	% var	$\chi^2$	<i>P</i>
	Female ID	1.0e-08	1.0e-04	0.0	0	0.99
	Male pair	1.28	1.13	16.0	3.00	0.08
	Female ID × male ID	3.42	1.85	42.8	76.21	<b>&lt;0.001*</b>
	Error	3.29		41.2		

Fixed effects are presented with estimate parameters including 95% confidence intervals (CI). Random effects are presented with variance components with standard deviations and percentage of the total variance (% var). Significance of factors in models was determined by log-likelihood tests and significant effects are indicated by bold font and an asterisk.



## DISCUSSION

In our study, we examine whether post-copulatory sexual selection processes, specifically sperm competition and CFC, play an important role in the maintenance of a color polymorphism in Chinook salmon. Differences in carotenoid utilization are expected to influence sperm performance as suggested by studies in birds (Peters et al. 2004; Helfenstein et al. 2010) and fishes (Pike et al. 2009; Tizkar et al. 2015; but see Sullivan et al. 2014). For example, carotenoid levels have been positively associated with sperm velocity in mallard ducks (*Anas platyrhynchos*; Peters et al. 2004) as well as fertilization success in sticklebacks (*Gasterosteus aculeatus*; Pike et al. 2009). Indeed, our experiment demonstrated that red Chinook salmon males had higher sperm velocity in water relative to white males; however, we acknowledge that the difference only approached marginal significance ( $P = 0.0506$ ). Carotenoids can act as antioxidants, and thus may protect metabolically active sperm cells against oxidative damage (Blount et al. 2001; Costantini et al. 2010). White Chinook salmon consume carotenoids and are capable of circulating the pigments; however, unlike red individuals, white Chinook salmon have a reduced ability to store carotenoids in their tissues (see Ando et al. 1994). Chinook salmon in the Quesnel River migrate more than 800 kilometers to spawning grounds and normally carotenoids are mobilized from muscle tissue into the bloodstream to preserve vital functions during migration (Rajasingh et al. 2007). Consequently, white Chinook salmon could be at greater risk of oxidative damage given their lack of carotenoid stores when undertaking migration. Assuming oxidative stress reduces sperm performance (reviewed in Velando et al. 2008; Costantini et al. 2010), it is plausible that the small difference observed in sperm velocity in our study are attributed to differences in carotenoid availability during migration. However, these conclusions are speculative, as we did not assay differences in antioxidant capacity or carotenoid content in semen in our study and other factors could contribute to differences between morphs such as genetic differences linked to the polymorphism. The degree to which red and white males are in sperm competition is unknown; however, under experimental conditions, 33% of Chinook salmon females ( $n = 3/9$ ) spawned with both red and white males (Lehnert et al. 2016b). Given that both morphs spawn at the same time and in the same area in the Quesnel River, we would expect sperm competition to occur between morphs. Therefore, the lack of strong differences in sperm velocity between red and white males in water may suggest that both morphs can gain similar success under sperm competition, thus leading to the stable polymorphism seen in the natural population.

In addition to sperm velocity, CFC can influence fertilization as demonstrated in a wide range of taxa including insects, fishes, birds, and mammals (Eberhard 1996; Evans et al. 2003; Bussi ere et al. 2006; Yeates et al. 2009; L ovlie et al. 2013). In color polymorphic systems, color-based CFC has been demonstrated (Pryke et al. 2010). For example, in the Gouldian finch (*Erythrura gouldiae*), post-zygotic genetic incompatibilities have been detected when mating occurs between different head color morphs (Pryke and Griffith 2009), and consequently, post-copulatory processes have evolved to skew paternity in favour of more genetically compatible mates (Pryke et al. 2010). In salmon, ovarian fluid has been shown to be a potential mechanism for CFC (Urbach et al. 2005; Rosengrave et al. 2008; Butts et al. 2012; Yeates et al. 2013; Rosengrave et al. 2016). In our study, we demonstrate that ovarian fluid affects sperm velocity based on the interaction of male color and female color, where red males had faster sperm in the ovarian fluid of red females

relative to white females, and vice versa for white males. Therefore, our results show that ovarian fluid may be a mechanism for CFC in Chinook salmon and it operates based on male and female color. However, higher sperm velocity in ovarian fluid was not predictive of higher competitive fertilization success (performed in 20% ovarian fluid). The results of our study indicate that although ovarian fluid (at least at concentrations of 20%) may level the playing field for red and white males overall, sperm velocity changes via ovarian fluid may have limited influence on competitive fertilization success in Chinook salmon. Similar results have been demonstrated by Evans et al. (2013) in Chinook salmon, where they reported that sperm performance in ovarian fluid (10% dilution) differed from in vitro competitive fertilization success (but see Rosengrave et al. 2016). Sperm velocity differences in ovarian fluid mediated by female color may relate to protein differences in ovarian fluid and semen between color morphs, as protein composition of ovarian fluid is thought to be the primary mechanism for male–female interaction effects on sperm performance (Johnson et al. 2014).

Although sperm velocity in ovarian fluid has been demonstrated to be an important predictor of fertilization success in Chinook salmon (Rosengrave et al. 2016), in our study, we found that fertilization success was not correlated to sperm velocity in ovarian fluid. Nevertheless, under in vitro fertilization trials, we still found evidence of CFC based on male and female coloration (i.e., color-based CFC). In this case, paternity of red and white males was similar for competitive fertilization in the eggs of red females; however, white males sired significantly more white female eggs compared to red males when in competition. Our results suggest that the strength of color-based CFC may be greater for white females than red females. Previously, using semi-natural spawning channels, Lehnert et al. (2016b) found that red females showed a stronger preference for red males compared to white males (i.e., assortative mating), whereas white females showed little preference for male color. The study found that the percentage of offspring produced by color assortative mating by red females was 75.9%, whereas the percentage was only 55.3% for white females (Lehnert et al. 2016b). Interestingly, under in vitro competitive fertilization, we found the opposite, where red males sired 48.5% of red female offspring and white males sired 67.0% of white female offspring. Thus, it is plausible that red females employ pre-spawning processes to bias offspring paternity in favor of red males, whereas white females may use post-spawning processes to bias paternity in favor of white males. Differences between females in pre- and post-spawning decisions may in part provide a mechanism promoting the maintenance of the 2 morphs in nature.

In addition to color-based CFC, results for competitive fertilization success were partly driven by differences between males in sperm velocity in water (but not in ovarian fluid), as sperm velocity in water was a significant predictor of fertilization success, thus suggesting that the effects of CFC via ovarian fluid may be lost due to sexual conflict, where sperm competitiveness counteracts female choice mediated by the ovarian fluid. Additionally, the significant relationship between sperm velocity in water and fertilization success found in our study is comparable to previous studies in salmonids: Chinook salmon (Flannery 2011), Atlantic salmon (Gage et al. 2004), and Arctic charr (*Salvelinus alpinus*; Liljedal et al. 2008). Given that sperm velocity is an important driver of fertilization success, we would have expected red males to sire more eggs under competition rather than white males given their marginally higher sperm velocity in water. However, other mechanisms are likely operating to influence fertilization success (i.e., egg-sperm interactions), as



mechanisms of CFC (color-based and individual-based) not mediated by ovarian fluid appear to also be driving fertilization success in our study. When we accounted for differences in sperm velocity (in water) between paired males, individual level CFC (male pair  $\times$  female ID) and male competitiveness (male pair) were attributed to 43% and 16%, respectively, of the total variance in in vitro competitive fertilization success. While our study does not identify the specific mechanism for the identified individual level CFC, evidence of CFC in our study could be facilitated via egg–sperm recognition, which has been demonstrated in Atlantic salmon (Yeates et al. 2009). In teleosts, the egg has a single opening (micropyle) through which sperm must pass to achieve fertilization (Gilkey 1981). Little is known about a potential mechanism by which an egg could exert selection on sperm in salmon, but post-copulatory processes may continue to operate after entry of sperm into the egg (Yeates et al. 2009). One possibility is that egg–sperm level CFC may be driven by differences at major histocompatibility (MHC) genes (documented in Atlantic salmon (Yeates et al. 2009)), as red and white Chinook salmon differ significantly at MHC genes (Lehnert et al. 2016b).

In conclusion, we found only marginal differences in sperm velocity in water between red and white males, which may in part be important for allowing both red and white males to gain success under sperm competition events. Additionally, we found that sperm velocity was influenced by ovarian fluid on the basis of color suggesting a potential mechanism for CFC that could also contribute to the maintenance of the polymorphism. However, under in vitro sperm competition, we found that sperm velocity in ovarian fluid did not explain fertilization success, yet we still found evidence of color-assortative CFC on competitive fertilization success. Sperm velocity in water was a significant predictor of competitive success, potentially indicating evidence of sexual conflict, where sperm competitiveness can reduce effectiveness of ovarian fluid mediated CFC. Nonetheless, CFC and male competitiveness explained variation in fertilization success even when accounting for differences in sperm velocity between paired males, where individual level CFC (at the male  $\times$  female level) explained a greater proportion of the variance (2.7  $\times$  more) relative to male competitiveness. Our results suggest that egg–sperm interactions may be important for determining post-spawning success, which could provide females with an advantage in the evolutionary arms race between sexes. Post-copulatory processes can have important evolutionary consequences and our results from sperm performance in ovarian fluid and competitive fertilization success suggest that, despite the marginally lower sperm velocity of white males, mechanisms of CFC can help white males gain similar success to red males overall. We can conclude that post-spawning sexual selection contributes in part to the maintenance of the color polymorphism in the Quesnel River population.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.behco.oxfordjournals.org/>

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Lehnert et al. (2016a).

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