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Short communication

Sperm trait differences between wild and farmed Chinook salmon (*Oncorhynchus tshawytscha*)

Sarah J. Lehnert [a,*], Daniel D. Heath [a,b], Trevor E. Pitcher [b]

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

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ABSTRACT

The expansion of salmon aquaculture, coupled with fish escaping from those sites, has raised concerns about the possible impacts of escaped farmed fish on wild fish populations. The potential for hybridization through reproductive interactions between escaped farmed and wild salmon can have significant impacts on the fitness and genetic composition of the natural population. Reproductive success of farmed male salmon in the wild will depend on their ability to compete for mates; however, it will also depend on their relative sperm performance, given that sperm competition is known to contribute to salmonid reproductive success. Farming practices, including the hormonal sex-reversal of females to create homogametic (XX) males, may have effects on sperm traits in salmon. We therefore analyzed sperm traits of XX farmed, XY farmed and wild Chinook salmon males during the spawning season. No significant difference was found between XX and XY farmed males for all sperm traits, except sperm density, which was significantly higher in XY males than XX males. XX and XY farmed males had significantly higher sperm motility and sperm velocity compared to wild males. In addition, wild males had lower sperm longevity and sperm density compared to farmed males. Our results indicate that farming practices may lead to increased sperm performance in Chinook salmon males. While we did not evaluate reproductive success resulting from spawning interactions in the wild, our results do highlight the potential for substantial introgression resulting from male–male competition between farmed and wild Chinook salmon in the wild.

1. Introduction

Salmon aquaculture is an economically important industry; however, there are increasing concerns about the potential impacts of interactions between farmed and wild fish (Hindar et al., 1991; Naylor et al., 2005; Skaala et al., 1990). These interactions are of major concern when considering escapes from aquaculture sites, because the unnatural and controlled aquaculture setting provides an especially different environment for fish to evolve in compared to the wild, resulting in phenotypic and genetic differences in the farmed populations (Heath et al., 2003; Skaala et al., 1990). The genetic changes occurring in aquaculture involve the loss of genetic diversity as well as the divergence of farmed stocks from the original wild population (Hindar et al., 1991; Skaala et al., 1990). Additionally, homogametic male fish (XX males) are used for commercial production of all female stocks, and if such fish escape and reproduce successfully in the wild they would skew the sex ratio in the wild population. Hybridization through reproductive interactions between escaped farmed and wild salmon is an immediate threat to the fitness and genetic composition of natural populations (Hindar et al., 1991; McGinnity et al., 2003; Naylor et al., 2005). For example, McGinnity et al. (2003) showed that farmed-wild hybrid offspring have lower survival compared to wild offspring, and that competition from farmed and hybrid offspring reduces wild smolt production in Atlantic salmon (*Salmo salar*).

The potential for hybridization between wild and farmed salmon will depend on numerous factors, although primarily on the reproductive success of escaped farmed individuals in the wild (Fleming et al., 1996). The effect of artificial rearing on salmon reproductive behavior and success has been widely studied showing, under experimental conditions, farm-raised, transgenic and hatchery salmon have reduced competitive and reproductive success compared to wild salmon (Berejikian et al., 2001; Fitzpatrick et al., 2011; Fleming and Gross, 1993; Fleming et al., 1996; Moreau et al., 2011; Weir et al., 2004). Although artificially reared males and females both experience lower reproductive success when in competition with wild fish, the lower reproductive success is more pronounced in males relative to females (Fleming and Gross, 1993; Fleming et al., 1996). Specifically, males show less aggression and partake in fewer spawning events than wild males; as well, they display inappropriate mating behavior resulting in females denying access to the oviposition site (Fleming

* Corresponding author at: Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Ave., Windsor, Ontario, Canada N9B 3P4. Tel.: +1 519 253 3000x4739; fax: +1 519 971 3616.
E-mail address: lehnert@uwindsor.ca (S.J. Lehnert).

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and Gross, 1993; Fleming et al., 1996). In addition to those behaviors, Webb et al. (1991) reported that escaped farmed and wild Atlantic salmon spawn in different reaches of the river, further reducing the likelihood of hybridization. Nevertheless, escaped farmed salmon do successfully reproduce and hybridize with wild fish (Crozier, 2000; Lura and Sægrov, 1991). In a study of 16 Scottish rivers, escaped Atlantic salmon females contributed up to 7% of the fry in some rivers (Webb et al., 1993), furthermore the experimental release of farmed Atlantic salmon in a Norwegian river revealed that 55% of farm escapes contributed 19% of the genes to the next generation of adult salmon (Fleming et al., 2000). While behavioral interactions play a key role in breeding success, salmonids are external fertilizers allowing several males to simultaneously fertilize the eggs of a single female. Consequently, relative sperm performance will also be an important contributing factor to the reproductive success of farmed salmon in the wild (Gage et al., 2004). This is because subdominant males can offset behavioral inferiority through enhanced sperm traits (Birkhead and Møller, ~~1988~~; Hutchings and Myers, 1988). Farmed males could achieve higher fertilization success by having faster swimming sperm, as Gage et al. (2004) found males with higher sperm velocity had greater fertilization success even when competing male had a greater number of sperm.

Gamete quality is an important factor in evaluating the risk associated with farm escapes and it is also important to ensure high fertilization rates under farm production breeding, yet few studies have tested the effects of farm rearing on sperm traits in fishes. The effect of farming on reproductive traits in penaeid prawns has been extensively studied (Alfaro and Lozano, 1993; Pratoomchat et al., 1993; Rendon Rodriguez et al., 2007). Research shows captive rearing can negatively impact sperm traits in prawns, including an increased percentage of abnormal spermatozoa, reduced number of sperm in spermatophores, reduced percentage of viable sperm (Leung-Trujillo and Lawrence, 1987), and the degeneration of the male reproductive tract (Talbot et al., 1989). The effect of farming on sperm traits in fishes has been studied by Skjæraasen et al. (2009) where sperm traits were compared between wild and farmed cod (*Gadus morhua*). They showed that wild males had a higher percentage of motile sperm, sperm velocity and spermatocrit compared to farmed males at the beginning of the spawning season; whereas, at the end of the spawning season sperm velocity was still higher in wild males, but there were no differences in other traits. Greater sperm velocity observed in wild cod relative to farmed was also shown in a second study (Butts et al., 2011) indicating that higher sperm quality in wild males may be a common phenomenon in this species. On the other hand, a study on haddock (*Melanogrammus aeglefinus*) found no difference in sperm velocity or spermatocrit between wild and farmed males throughout the spawning season (Rideout et al., 2004). All of those studies examined farmed fish populations only ~~removed~~ one generation from the wild, thus highlighting the need for studies examining sperm traits in a more intensively farmed species, several generations removed from the wild, to assess the true impacts of farming on sperm traits in fishes.

A common practice used in salmonid aquaculture to reduce the early maturation of males is the hormonal sex-reversal of females to create homogametic (XX) males (Heath et al., 2002). XX males produce sperm that only bears the X chromosome and milt from these males can be used to fertilize eggs and produce all female production stock (Devlin et al., 1991). The hormonal manipulation associated with sex-reversal can have negative impacts on testes development and sperm traits in teleosts, including a decrease in sperm density and motility in *Betta splendens* (Kirankumar and Pandian, 2002), deformed testis in Eurasian perch (*Perca fluviatilis*) (Rougeot et al., 2002), and incomplete sperm duct development in salmonids (Geffen and Evans, 2000; Johnstone et al., 1979). However, normal gonadal development and sperm duct formation have been demonstrated in XX males from various species, including northern pike (*Esox lucius*) (Luczynski et al., 2003) and Chinook salmon (*O. tshawytscha*) (Heath et al., 2002). As well, studies report no difference in sperm traits between XX and XY males for Eurasian perch (Rougeot et al., 2004) and Coho salmon (*Oncorhynchus kisutch*) (Fitzpatrick et al., 2005), and no difference in testicular sperm density or ATP concentrations between XX and XY male rainbow trout (*O. mykiss*) (Geffen and Evans, 2000). Although sex-reversal is prevalent in aquaculture, few comparative studies on sperm traits of XX and XY males exist for salmonids (Fitzpatrick et al., 2005; Geffen and Evans, 2000), particularly for species with morphologically normal gonads and functional sperm ducts.

Given that large numbers of farmed salmonids are known to escape from aquaculture sites (Naylor et al., 2005), studying sperm traits in wild and farmed salmon will provide insight into the potential for escaped males to hybridize with the wild population. Through the examination of sperm motility, velocity, longevity and density, we evaluate sperm performance of farmed fish relative to wild fish in Chinook salmon. In this study we compare sperm traits between XX farmed, XY farmed and wild (XY) males, allowing us to determine the impact of farming as well as sex-reversal on sperm traits in salmon. Additionally, competitive fertilization success is positively correlated with sperm velocity in salmonids (Gage et al., 2004; Lahnsteiner et al., 1998; Liljedal et al., 2008; Pitcher et al., unpublished data), allowing us to assess the potential reproductive success of escaping farmed male salmon in the wild based on their sperm characteristics.

2. Materials and methods

2.1. Fish type and origin

All Chinook salmon used in this study originate from river systems on Vancouver Island, British Columbia, Canada. Farmed salmon were obtained from an organic Chinook salmon farm, Yellow Island Aquaculture Ltd. (YIAL), Quadra Island, BC. The organic farming practices involve no use of pesticides or antibiotics and the fish are fed a diet that mimics that of wild salmon, which includes offshore fish protein and naturally derived carotenoid pigment. The farmed salmon males included both homogametic (XX) and heterogametic (XY) males. YIAL began producing homogametic males in 1985 from XX milt acquired from the Big Qualicum hatchery, Vancouver Island. In the years following, XX males were spawned with YIAL broodstock to create a monosex population. At YIAL, XX males are generated through the exogenous treatment with the androgen 17 α-methyltestosterone (400 μg L^{-1}) for 2 h at 520 ATUs (accumulated thermal units) and at 620 ATUs of development (Heath et al., 2002). All XX males in this study were 6 to 7 generations domesticated at YIAL and were bred in either the fall of 2005 or 2006 through mixed-milt spawning and were thus 4 or 5 years of age at time of sampling. All XY males at YIAL were descendant from gametes obtained from Robertson Creek and Big Qualicum hatcheries in 1985, and 4 generations later (1997), fish were mated in a full factorial cross with wild fish from Big Qualicum River (Bryden et al., 2004). All XY males used in this study are therefore up to 7 generations domesticated at YIAL but introgressed with wild genes 3 generations removed from the wild Big Qualicum stocks. The XY stock has been maintained by single male and single female crosses, and all XY males used in this study were bred in the fall of 2006 and were thus 4 years of age at the time of sampling. Both farmed male types were hatched and reared in fresh water until smolting when they were transferred to saltwater pens until sexual maturation. Mature XX and XY males were seined from saltwater pens and transferred to fresh water from October 4 to October 13 and October 14 to 18, 2010, respectively. Wild Chinook salmon were seined from the Quinsam River on October 21, anesthetized with CO_2 and transported approximately 1.5-hours by

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vehicle to YIAL in 700-L of oxygenated river water. No mortalities occurred as a result of transport. Wild males were presumed to be individuals spawned in the fall of 2007 and were thus 3 years of age at time of sampling. All farmed and wild males were kept in 2500-L freshwater holding tanks and sampled between October 14 and 22. Fish were anesthetized with buffered MS222, then weight (±10 g) and fork length measurements (±1 mm) were recorded.

2.2. Sperm collection and measurements

After weight (mean weight ± S.E., 4.41 ± 0.16 kg) and length (mean length ± S.E., 71.0 ± 0.9 cm) measurements were taken, milt (sperm and seminal plasma) was stripped from individual males by applying gentle pressure to the abdomen. Any milt in contact with urine, water or other contaminants was not used. Milt was collected in plastic bags, stored at approximately 4 °C and analyzed immediately in the on-site laboratory. Sperm activated with 10 μL of fresh water were video recorded through a microscope and assessed with sperm-tracking software (see Pitcher et al., 2009). Video recordings were conducted using a negative phase-contrast microscope (CX41 Olympus) with 10× magnification objective mounted with a CCD B/W video camera (at 50 Hz vertical frequency). Sperm motility and velocity were measured at 5, 10 and 15 s post-activation using HTM-CEROS sperm analysis system (CEROS version 12, Hamilton Thorne Research, Beverly, MA, USA), an objective method for studying sperm motility in fish (Kime et al., 2001). The image analyzer was used with the following settings: number of frames = 60, minimum contrast = 20–30, and minimum cell size = 3 pixels. Sperm motility was defined as the percentage of motile sperm cells which was determined using this software by dividing the number of progressively motile sperm cells by the total number of sperm cells in the field of view at 5, 10 and 15 s post-activation. For each individual, three measures of sperm velocity were evaluated: The average path velocity (VAP in μm s^{-1}, defined as the average velocity along a smoothed cell path), the straight line velocity (VSL in μm s^{-1}, defined as the average velocity along a straight line connecting the start and end points of the cell's path) and the curvilinear velocity (VCL in μm s^{-1}, defined as the average velocity along the actual path that the cell travels). Velocity estimates represent the mean velocity of all individual motile sperm cells. All three sperm velocity measures described above, which are VAP, VSL and VCL, were significantly positively correlated at all time periods after activation (r^2 ranged from 0.20 to 0.88, all $p<0.003$, $N=43$), pooling male types. Given that all sperm velocity measures were correlated and yielded qualitatively similar results, all further velocity results will be based on VAP, which is commonly used in Chinook salmon and other *Oncorhynchus* spp. studies to represent sperm velocity (e.g. Lahnsteiner et al., 1998; Rosengrave et al., 2008) as it describes the smoothed path by which the sperm cell travels. Sperm longevity was also estimated from video tracks, and was considered the time from activation until approximately 95% of sperm cells within the field of view had ceased forward movement (see Gage et al., 2004). When assessing sperm motility, and sperm velocity and longevity, the total number of sperm cells in the field of view was on average (±S.E.): 79.3 ± 5.4, 70.7 ± 5.0 and 55.5 ± 4.8 at 5, 10 and 15 s post-activation, respectively.

An "improved Neubauer chamber" haemocytometer under 400× magnification was used to estimate sperm density (Pitcher et al., 2007, 2009). Briefly, the number of sperm cells in 5 of 25 larger squares was counted (each square subdivided for simplified counting). This count was used to estimate the number of sperm cells in all 25 squares, which was then multiplied by the depth of the chamber (10 μm) and then again by the initial volume of the sample. The estimated densities were expressed as the number of sperm cells per milliliter of stripped milt.

2.3. Statistical analyses

Temporal changes (5, 10 and 15 s post-activation) in sperm motility and velocity between XX, XY and wild males were analyzed using repeated measures ANOVAs followed by Tukey's test for post-hoc pairwise comparisons. The model was further decomposed into individual one-way ANOVAs coupled with Tukey's post hoc test at each time period to determine significant interactions. Sperm longevity, sperm density and Fulton's condition factor between XX, XY and wild males were analyzed using one-way ANOVAs followed by Tukey's post-hoc test to examine all pairwise comparisons.

All means are reported ±S.E. Data were tested for normality. Transformation of sperm motility and velocity data failed to improve normality, however, although assumptions of parametric tests were not fully met, the ANOVA is known to be robust enough to deal with these issues (Underwood, 1981). To verify this, non-parametric tests (Kruskal–Wallis) were also performed and yielded qualitatively similar results as parametric tests. Fish sample size varied across sperm performance metrics (XX N = 15–17, XY N = 8–11, Wild N = 20–26), as not all samples were usable for each trait examined due to video tracks displaying water flow causing inaccurate readings, or milt samples contaminated with water, blood and/or urine.

3. Results

3.1. Sperm motility

Percentage of motile sperm cells decreased significantly over time and differed significantly among male types (Fig. 1A; Repeated Measures ANOVA, $F=2.84$, $p=0.03$). XX and XY farmed males had significantly greater percentage of motile sperm compared to wild males ($p=0.0002$ and $p=0.003$, respectively), and there was no difference between XX and XY farmed males in percent motility ($p=0.99$).

3.2. Sperm velocity

Sperm velocity decreased significantly over time and differed significantly among male types (Fig. 1B; Repeated Measures ANOVA, $F=4.38$, $p=0.008$). Post-hoc tests revealed that XX and XY farmed male sperm velocity was significantly greater than that of wild males ($p=0.03$ and $p=0.04$, respectively), however no significant difference existed between XX and XY farmed males in sperm velocity ($p=0.45$).

3.3. Sperm longevity

Sperm longevity differed significantly among male types (Fig. 1 C; ANOVA; $F=4.10$, $p=0.020$). Post-hoc tests of sperm longevity showed significant differences between XX farmed and wild males ($p=0.03$), but no significant difference in sperm longevity between XX and XY farmed males ($p=0.97$) or XY farmed and wild males ($p=0.12$).

3.4. Sperm density

Sperm density differed significantly among male types (Fig. 1D; ANOVA; $F=6.39$, $p=0.003$), with XY farmed males having the greatest density of sperm cells per milliliter of milt. Post-hoc tests of sperm density showed significant differences between XY farmed and wild males ($p=0.003$) and XX and XY farmed males ($p=0.015$), but no significant differences between XX farmed and wild males ($p=0.94$).

3.5. Fulton's condition factor

A post-hoc examination of Fulton's condition factor for each of the groups was conducted, calculated as $K=(WL^{-3})\times 10^5$, where W

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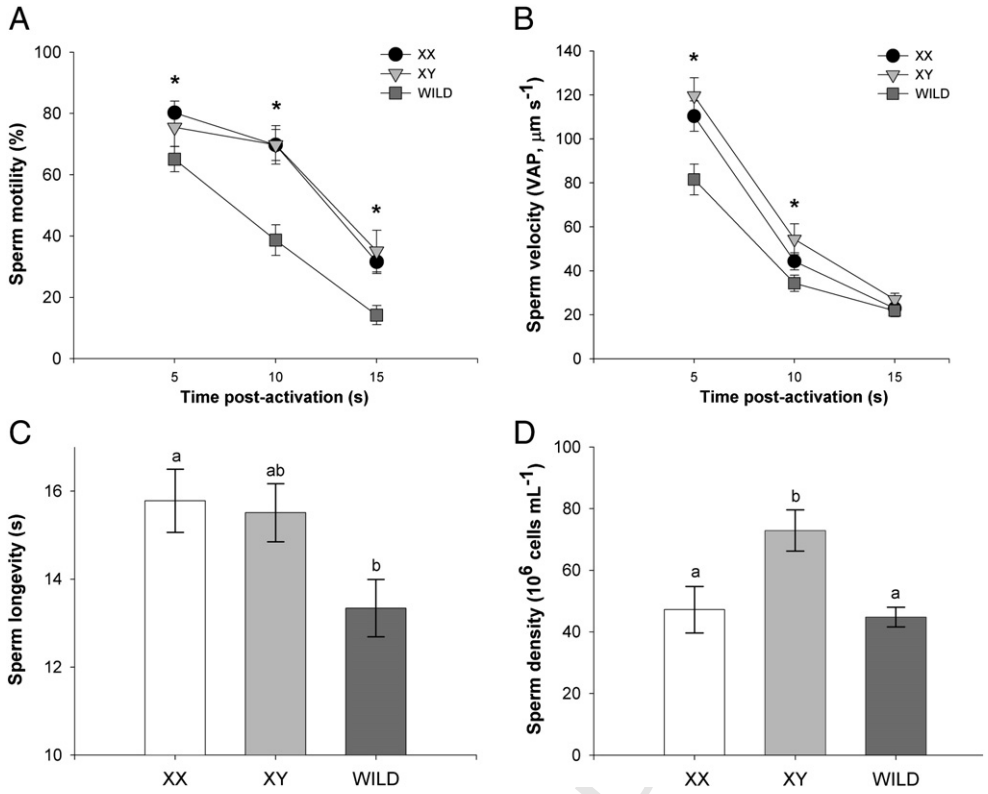

Fig. 1. Means (±standard error) of XX farmed, XY farmed and wild Chinook salmon (*Oncorhynchus tshawytscha*) males for sperm traits: (A) percent motility, (B) sperm velocity (VAP, see Materials and methods), (C) sperm longevity and (D) sperm density. Asterisks (*) over time periods and different letters over bars indicate significant differences between male types ($p<0.05$). Sample size varied over sperm traits (see ~~Table 1 and~~ Materials and methods for details). Q2

is weight (g) and *L* is fork length (mm). Condition factor was significantly different among male types (Fig. 2; ANOVA; $F=6.68$, $p=0.003$). XX and XY males had significantly higher condition factor than wild males ($p=0.021$ and $p=0.007$, respectively).

4. Discussion

For the sperm traits examined, wild males generally had lower performance values than XX and XY farmed males, and no difference existed in sperm traits between XX and XY males, except in sperm density. Many sperm traits can be good indicators of fertilizing capacity, however, sperm velocity is known to be the primary variable affecting competitive fertilization success in salmonids, including Atlantic salmon (Gage et al., 2004), rainbow trout (Lahnsteiner et al., 1998), Arctic charr (*Salvelinus alpinus*) (Liljedal et al., 2008), Coho salmon (Pitcher et al., unpublished data) and Chinook salmon (Flannery, 2011). Sperm density can also be important in sperm competition, and sperm number is shown to increase with increasing intensity of sperm competition in fishes (Stockley et al., 1997). However, Gage et al. (2004) demonstrate the importance of sperm velocity in Atlantic salmon, as males with faster sperm had greater fertilization success even when competing males had more numerous sperm. Thus we suggest that our findings indicate XX and XY farmed males would have greater fertilization success when in sperm competition with wild males from the Quinsam River. Higher competitive fertilization success of farmed males may lead to a higher level of hybridization between escaping farmed fish and wild fish than expected based on the numbers of fish alone. Hybridization will allow gene flow from farmed stocks to the wild, likely resulting in a reduction of fitness in the wild population (McGinnity et al., 2003), perhaps increasing the likelihood for local population extirpation. However, the extent of hybridization may be reduced through behavioral inferiority in the farmed males, as many studies show that cultured salmon have reduced reproductive success when in competition with wild salmon (Berejikian et al., 2001; Fitzpatrick et al., 2011; Fleming and Gross, 1993; Fleming et al., 1996; Moreau et al., 2011; Weir et al., 2004).

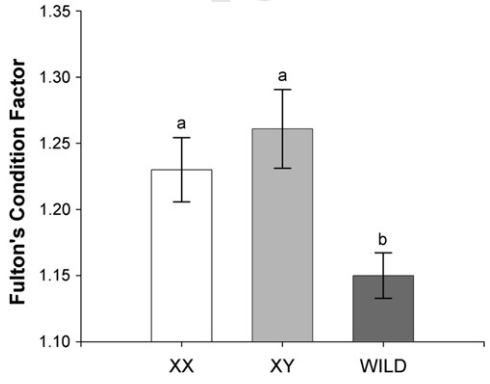

Fig. 2. Fulton's condition factor (mean±standard error) of XX farmed ($N=18$), XY farmed ($N=10$) and wild ($N=27$) Chinook salmon (*Oncorhynchus tshawytscha*) males. Fulton's condition factor was calculated as $K=(WL^{-3})\times 10^5$, where *W* is weight (g) and *L* is fork length (mm).

Our finding of little or no difference in sperm performance between XX and XY farmed males is consistent with other studies examining the effect of sex-reversing on sperm traits in closely related species such as Coho salmon (Fitzpatrick et al., 2005) and rainbow trout (Geffen and Evans, 2000). Unlike those species, all XX Chinook salmon have morphologically normal gonads and sperm ducts (Heath et al., 2002). Although our analyses should be replicated in other Chinook salmon broodstocks, we suggest that, based on our findings, there are no negative implications for fertilization success resulting from using sperm from XX males to fertilize production eggs.

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Only a few studies have examined the effect of farming on sperm traits in fishes. Skjæraasen et al. (2009) and Butts et al. (2011) reported that wild male cod had greater sperm performance compared to farmed cod, whereas Rideout et al. (2004) observed no difference in sperm traits between wild and farmed haddock. Our study provides the first sperm performance data for a farmed fish population several generations removed from the original wild stocks, which may provide an explanation as to why our results differ from previous studies. The greater sperm performance found in farmed Chinook salmon males may result from selective pressure on sperm competition from mixed-milt spawning in the aquaculture environment. The pooling of milt from several males to fertilize eggs can lead to a loss of genetic diversity in the population due to differences in sperm competitive ability among males being pooled (Campton, 2004; Neff et al., 2011). Mixed-milt spawning in Chinook salmon (Withler and Beacham, 1994) showed extreme variation in fertilization success of individual males, ranging between 5% and 88% when milt from three males was pooled. However, this only provides an explanation for the greater sperm performance observed in XX males, as XY males were not subjected to mixed-milt spawning at YIAL.

The greater sperm performance of XX and XY farmed males may also be a consequence of differences in the relative spawning condition of the fish from each group. Fulton's condition factor (K), which reflects differences in fish body mass for a given body length such that higher values are presumed to indicate better condition, was greater for XX and XY farmed males compared to wild males (Fig. 2). Although the higher condition factor of farmed fish in comparison to wild fish can be attributed to diet, condition factor and sperm performance may also be a reflection of the male's spawning stage. During the spawning season, fish, especially anadromous species, are subjected to energetic costs that result in weight loss (Jonsson et al., 1997) and thus a reduction in condition factor, as well, the aging of sperm in fishes during the spawning season affects the quality of sperm (Rana, 1995). In many fish species, the spawning season is marked by a gradual increase followed by a gradual decrease in sperm motility (Munkittrick and Moccia, 1987; Suquet et al., 1998) and sperm density (Aas et al., 1991; Büyükhatipoglu and Holtz, 1984). However, other studies have shown an increase in sperm density or spermatocrit at the end of the spawning season (Rakitin et al., 1999; Rideout et al., 2004; Skjæraasen et al., 2009; Suquet et al., 1998). Although the pattern of changes in sperm traits over the spawning season is not known for Chinook salmon, the difference between farmed and wild males in condition and sperm performance may be an indication of their stage in the spawning process. However, we found no significant correlation between sperm velocity and condition factor ($p = 0.35$, $N = 43$), indicating that higher condition does not predict faster sperm. This suggests that our results are not an artifact of condition factor or spawning stage, but reflect fundamental differences in sperm performance between the Chinook salmon populations.

The differences observed between male types could be also attributed to the age of the individual males, as wild males were presumed to be younger than farmed males. It is possible that older males have greater sperm performance in Chinook salmon; however, previous studies of Pacific salmon species have found that younger males have similar or better sperm performance (Hoysak and Liley, 2001; Liley et al., 2002; Pitcher et al., unpublished data). Stress due to transportation may have also affected sperm performance of wild males, as a study on white bass, *Morone chrysops*, showed reduced motility in stressed individuals (Allyn et al., 2001), although these effects have not been examined in salmonids. Milt collection was completed immediately after transport for approximately half of the wild males, whereas the remaining wild males had 20-hours to recover prior to sampling. However, sperm velocity and sperm motility of wild males did not differ between sampling times (T-test; $p = 0.59$ and $p = 0.97$, respectively). Finally, we included only one wild and one farmed population in our analyses, thus raising the possibility of pseudoreplication (Hurlbert, 1984). Ideally, future studies should include multiple farmed and wild Chinook salmon populations to increase the generality of our results; however our study provides a valuable starting point for quantifying the hybridization risks associated with escaped farmed Chinook salmon on the spawning grounds.

In conclusion, our study shows that farmed males had greater sperm performance compared to wild males. Irrespective of condition factor, spawning stage and age, our data shows that if escaping farmed salmon males entered nearby rivers during the spawning season they would have an advantage in sperm competition with wild salmon. From an ecological perspective, the ability of farmed males to outcompete wild males can have significant impacts on natural populations, ranging from outbreeding depression and loss of genetic diversity to extirpation (Fleming et al., 2000; Hindar et al., 1991; McGinnity et al., 2003). However, despite sperm competition playing an important role in male–male interactions in salmonids, behavioral interactions are also critical for reproductive success (Fleming et al., 1996). While farmed Chinook salmon males may have greater sperm performance, it is possible that these farmed males have lost much of their behavioral ability to compete for mates and gain access to females due to domestication, and thus would not be reproductively successful in the wild. Currently, we are examining the semi-natural spawning competitions between wild and farmed Chinook salmon to test this possibility.

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