A mechanism for cryptic female choice in chinook salmon

Patrice Rosengrave,[a] Neil J. Gemmell,[b] Victoria Metcalf,[c] Katherine McBride,[a] and Robert Montgomerie[d]
[a]School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand, [b]Centre for Reproduction and Genomics, Department of Anatomy and Structural Biology, University of Otago, PO Box 913, Dunedin 9054, New Zealand, [c]Agriculture and Life Sciences Division, Lincoln University, PO Box 84, Lincoln, Canterbury 7647, New Zealand, and [d]Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada

Female mate choice after copulation or spawning is cryptic when a female differentially influences the fertilization success of sperm from different males. We tested whether ovarian fluid could act as a potential mechanism of cryptic female choice (CFC) in chinook salmon (*Oncorhynchus tshawytscha*) by comparing how ovarian fluid from each of 7 females affected the sperm behavior of 11 different males. Using computer-assisted sperm analysis, we measured sperm velocity, motility, longevity, and linearity from the ejaculates of each male activated in the ovarian fluid from each female. Mean sperm swimming speed, path trajectory, and longevity differed significantly among males, and within males depended on the female's ovarian fluid in which it was activated. Most important, the pattern of within-male variation in these traits also varied significantly among males in response to different females' ovarian fluids. As sperm velocity is known to be a prime determinant of fertilization success in externally fertilizing fishes. This finding suggests that variation in the composition of female ovarian fluid may be a mechanism for CFC, whereby females differentially enhance the swimming speed of sperm from different males. Thus, female ovarian fluid can alter relative male fertilization success when there is intense sperm competition, as there is in this and other group spawning fish species. *Key words:* chinook salmon, cryptic female choice, *Oncorhynchus tshawytscha*, ovarian fluid, sperm traits, sperm velocity. *[Behav Ecol 19:1179–1185 (2008)]*

Cryptic female choice (CFC) is defined as mate choice that occurs after mating or spawning (Thornhill 1983; Eberhard 1996). It has recently been suggested that CFC may be widespread when females rely on sperm selection to increase the genetic quality of their offspring (Jennions and Petrie 2000; Neff and Pitcher 2005). Nonetheless, the concept remains controversial as it has proved to be notoriously difficult to establish whether or not CFC has actually occurred (Birkhead 2000; Kempenaers et al. 2000; Pitnick and Brown 2000; Birkhead and Pizzari 2002).

The mechanisms underlying CFC have not been well studied, although a number of potential physiological and biochemical mechanisms have been identified in a range of species with internal fertilization (Birkhead 2000; Pitnick and Brown 2000; Birkhead and Pizzari 2002). For example, ejaculate manipulation can occur when a female ejects semen from her reproductive tract (Pizzari and Birkhead 2000). In species with external fertilization, females may control the numbers of eggs laid in the presence of different males (Reyer et al. 1999), or the egg itself may discriminate among sperm by biochemical means (e.g., Zeh and Zeh 1997). Sperm selection by the ovum may also be an important mechanism of CFC. For example, in the comb jelly, *Beroe ovata,* the egg pronuclei is able to choose among the sperm of different males once they have entered the egg (Carre and Sardet 1984). In externally fertilizing teleost fishes this may occur after fusion of the gametes during the formation of the second polar body as the second maturation division in many fish is completed only after the sperm has penetrated the egg (Wolgemuth 1983). In mice, this meiotic division in the egg was influenced by the type of sperm that entered (Agulnik et al. 1993). Finally, in externally fertilizing teleosts, the ovarian fluid that is released by the female with her eggs during spawning is known to influence sperm behavior and could be a mechanism of CFC if its effects on sperm behavior differ among competing males during multimale spawning events (Turner and Montgomerie 2002; Urbach et al. 2005; Nordeide 2007).

Ovarian fluid is a maternally derived liquid that surrounds the egg mass inside the female fish and is expelled during spawning. In the Salmonidae, ovarian fluid comprises 10–30% of the total egg volume (Lahnsteiner 2002). When mixed with the spawning medium (fresh or salt water), ovarian fluid creates a chemical "microenvironment" for the sperm that differs from the surrounding freshwater medium. Previous studies have observed that sperm behave differently when activated in ovarian fluid compared with activation in pure water. For example, spermatozoa activated in ovarian fluid swam faster in brown trout (*Salmo trutta f. fario*) (Lahnsteiner 2002) and Atlantic cod (*Gadus morhua*) (Litvak and Trippel 1998), and also the duration of sperm motility (longevity) was prolonged in brown trout (Lahnsteiner 2002) and the 3-spined stickleback (*Gasterosteus aculeatus*) (Elofsson et al. 2003). In Arctic charr (*Salvelinus alpinus*), ovarian fluid increased sperm longevity, sperm swimming speed, and the percentage of motile sperm cells as well as affecting sperm trajectories, compared with sperm swimming in freshwater (Turner and Montgomerie 2002).

Two lines of evidence suggested to us that the composition of ovarian fluid might provide a mechanism for CFC in externally fertilizing fish. First, ovarian fluid influences sperm swimming speed, which in turn, is an important determinant of male fertilization success (Birkhead et al. 1999; Levitan 2000; Gage

et al. 2004; Rudolfsen et al. 2008). For example, using microsatellite DNA fingerprinting, Gage et al. (2004) demonstrated in Atlantic salmon (*Salmo salar*), that a male's relative sperm velocity was the most important factor affecting fertilization success during sperm competition. Second, there is evidence that the composition of ovarian fluid varies among females, particularly with respect to the constituents that are know to influence sperm behavior (Lahnsteiner et al. 1995; Wojtczak et al. 2007). Thus, a male's sperm might be expected to swim at different speeds in the ovarian fluid solution of different females.

A recent study of Arctic charr found a significant female (ovarian fluid) × male interaction on sperm swimming speed (Urbach et al. 2005), indicating that sperm swimming speed varied depending on the female from which the ovarian fluid was taken. They suggested that chemical variation in the composition of the ovarian fluid might indeed be a mechanism for CFC. In that study, however, sperm swimming speed was measured at 30 s after activation, long after most ova are fertilized in salmonids (Hoysak and Liley 2001) and other externally fertilizing fishes (e.g., Casselman et al. 2006) where most fertilization occurs within 10 s of sperm activation. By 30 s postactivation, the sperm of both salmonids (Christen and Billard 1987) and centrarchids (Burness et al. 2005) already has declining energy reserves and is swimming at less than its maximum speed. Thus, differences among males in sperm swimming speed at 30 s after activation might well be due to differences in longevity and the straightness of the sperm trajectory and not particularly relevant to fertilization success. Although the finding of male × female interaction by Urbach et al. (2005) is intriguing, it certainly warrants further study.

Just how ovarian fluid interacts with sperm is unknown, but its positive effects on sperm function have been attributed to the composition of the ovarian fluid (Lahnsteiner et al. 1995; Lahnsteiner 2002; Cosson 2004; Elofsson et al. 2006). The exact mechanism by which ions or inorganic compounds in the ovarian fluid influence the behavior of fish spermatozoa remains unclear. Lahnsteiner et al. (1995) found intraspecific variation in composition of the ovarian fluid of 4 salmonid species and suggested that variation in the chemical composition of the ovarian fluid between females differentially affected sperm traits from some males. It is therefore possible that intraspecific variation in the composition of the ovarian fluid could play an important role in female sperm selection via CFC, whereby female ovarian fluid differentially influences the sperm behavior of different males, resulting in prejudiced paternity.

Here we use the chinook salmon (*Oncorhynchus tshawytscha*), a fish with external fertilization and multiple-male spawnings, to investigate whether ovarian fluid could be the agent of CFC via its influence on sperm behavior and hence the sperm's ability to reach the egg. In this species, both sexes mate multiply during spawning (Berejikian et al. 2000). As a result, there is intense sperm competition among males (Fleming 1998), which can reduce the opportunities for precopulatory mate choice by females.

To investigate the possibility of CFC in Chinook, we used computer-assisted sperm analysis (CASA) to measure 4 key sperm traits known or expected to influence male fertilization success (sperm velocity, duration of sperm motility, swimming path trajectory, and progressive motility) in sperm from 11 male salmon activated in the ovarian fluid of each of 7 different females.

METHODS

Chinook salmon were obtained from a hatchery-reared population at the National Institute of Water and Atmospheric Research Silverstream Hatchery, Canterbury, New Zealand. All were descendants of juvenile fish collected along the major chinook salmon–producing rivers as well as from several isolated land-locked populations on the central South Island of New Zealand (Unwin M, personal communication). The 2-year-old fish used in this study varied in both body length (mean ± 95% CL = 318 ± 30 mm) and body mass (526 ± 126 g). All fish were maintained in a hatchery raceway using standard husbandry procedures (Pennell and Barton 1996; Unwin et al. 2004) and were collected and maintained in this study according to the Animal Behavior Society/Association for the Study of Animal Behaviour guidelines for the treatment of animals in behavioral research.

We studied haphazardly chosen, sexually mature, individually marked 2-year-old male and 3-year-old female salmon from 3 to 14 May 2004, during the spawning season. Milt (sperm and seminal fluid) samples from fish were collected on different days. On each day when milt was sampled, each male was netted and dried around the cloaca to avoid activation of sperm cells by water/urine. Milt was then collected (stripped) from the males by applying gentle bilateral abdominal pressure. On each sampling day, 1 or 2 females were killed with a stroke to the head, then their egg batch expelled and their ovarian fluid collected. Milt and ovarian fluid samples were immediately refrigerated at 4 °C and transported to the University of Canterbury. The time from collection of the first to the last milt sample to be used for motility analysis was no greater than 5 h.

Sperm motility recordings were obtained for each male ($n =$ 11) activated in ovarian fluid from each female ($n = 7$). Each sampling day, milt samples were selected haphazardly with respect to male identity and/or stripping order so that time since stripping would not confound our results. The ambient air temperature in the lab was set at the water temperature (12 °C) of the holding raceway to control for variation in sperm swimming speed with varying water temperatures (Alavi and Cosson 2005). Ovarian fluid from each of the 7 females was diluted to 50% by volume, using freshwater collected from the raceway. We used a 50% dilution of ovarian fluid as our activating solution as it seems likely that during a natural spawning, spermatozoa would encounter diluted ovarian fluid as the spermatozoa moves from pure freshwater into pure ovarian fluid at the egg surface. In addition, other studies have found that sperm swimming speed is maximized in dilutions close to 50% (Turner and Montgomerie 2002; Woolsey et al. 2006). Sperm motility was initiated by adding 499 μL of this ovarian fluid solution to about 1 μL of the milt sample. We then placed 10 μL of this fluid on a glass slide and gently placed a cover slip over the sample for viewing at ×400 on a negative phase-contrast microscope (Leica DMR). On activation, sperm behavior was recorded using a high-resolution digital videocam, and sperm longevity (DUR in s) for each milt sample was recorded using a stopwatch, starting at the contact of the milt with the activation solution and ending when all progressive forward motility had ceased (e.g., Leach and Montgomerie 2000). Vibrating spermatozoa at the end of the period of propulsive sperm motility were considered to be immotile. For each male × female combination, we took 2 measurements using haphazardly collected milt and ovarian fluid samples for each trial.

Videotapes of sperm recordings were later analyzed using CASA (HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research, Beverly, MA). For each milt sample, we quantified the swimming paths of all spermatozoa in a field of view for 0.5 s at 10 s intervals postactivation. Sperm tracks that were clearly influenced by sample drift and/or were incomplete, were not analyzed. On average, 13 sperm tracks were analyzed per trial (range = 5–33 sperm tracks, $n = 154$ trials). Hoysak and Liley (2001) have shown that the majority of fertilizations

in salmonids are likely to occur within a few seconds after male ejaculation, and Yeates et al. (2007) demonstrated that a delay of only 2 s in sperm release by male Atlantic salmon caused a significant reduction in paternity. Moreover, in walleye (*Sander vitreus*), sperm swimming speed at 10 s postactivation was associated with male fertilization success, whereas at 20 s postactivation, it had no relation to fertilization success (Casselman et al. 2006). Therefore, in this paper, we report on sperm traits at 10 s postactivation only.

The average values of the following parameters for each male were calculated from the sperm tracks of each trial: mean average path velocity (VAP in $\mu m \cdot s^{-1}$), mean straight-line velocity (VSL in $\mu m \cdot s^{-1}$), mean curvilinear velocity (VCL in $\mu m \cdot s^{-1}$), and linearity (LIN; the ratio of VSL/VCL expressed as a percentage). Like other studies, we used VAP as a measure of sperm swimming speed (e.g., Lahnsteiner et al. 1998; Burness et al. 2005; Casselman et al. 2006). LIN describes the path trajectory of the sperm through the solution. A circular trajectory, for example, would have a low LIN, and a high LIN would indicate that the sperm cell is moving in a straight-line path. For each trial, we also measured the percentage of cells in the field of view that were forwardly motile at >20 $\mu m \cdot s^{-1}$ (MOT) as an index of overall sperm motility (see Lahnsteiner et al. 1998 for a similar criterion).

Statistical analysis was performed using the generalized linear mixed effects model package (lmer4) in R, version 2.2.1 (R Core Development Team 2007) and the linear mixed-effects package lme4. A mixed-effects general linear model was fitted using female and male as random effects. We included the day the experiment was conducted as a covariate in each model because there is evidence that sperm traits change over the spawning season in some fish species (Rideout et al. 2004; Cruz-Casallas et al. 2007). The main focus of our study was to test for an interaction between female and male identities on sperm longevity (DUR), average sperm swimming speed (VAP), linearity (LIN), and percentage of progressively motile cells (MOT) at 10 s postactivation using the maximum likelihood method of estimation. To test the statistical significance of one or more fixed or random effects, a model was fitted with and without the explanatory variable using log-likelihood ratio statistics (LLR χ^2). Assumptions underlying all models were verified using residual plots. All *P* values ≤0.05 were considered to be significant.

RESULTS

There were significant male, female, and female × male (i.e., ovarian fluid × sperm) interaction effects on sperm longevity (Table 1, Figure 1a). Thus, average sperm longevity varied significantly among males, average sperm longevity (across males) varied significantly among female ovarian fluids, and the average sperm longevity of individual males was differentially affected by ovarian fluid in a pattern that varied significantly across males. Sperm longevity varied significantly within all males except males 4 (*P* = 0.19), 8 (*P* = 0.07), and 10 (*P* = 0.06) when compared across females (post hoc contrast analyses). For example, the sperm of male 1 swam longest in the ovarian fluid of female 1 but shortest with female 3, the sperm of male 3 swam for the shortest duration with female 1 but almost the longest with female 4 (Figure 1a). The sperm of male 5 swam longest in the ovarian fluid of females 2 and 6 but only at 60% of that duration with females 3 and 7 (Figure 1a).

Similar results were observed for average sperm swimming speed (VAP) as there were significant effects of female, male, and female × male interaction (Table 1, Figure 1b). Thus, average sperm swimming speed varied significantly among males, and there was significant variation among the average

Table 1

Summary of random effects from mixed-effects general linear models predicting sperm traits (DUR, longevity; VAP, swimming speed; MOT, percentage of motile cells; LIN, linearity of sperm trajectory) for 2-year-old sexually mature male New Zealand chinook salmon at 10 s postactivation in a 50% ovarian fluid solution from 7 different females

Response	Predictors (n)	Percentage of variance explained	Chi-square	P
DUR	Female (7)	24.7	32.6	**<0.0001**
	Male (10)	9.9	10.8	**0.001**
	Female × male	24.5	9.5	**0.002**
VAP	Female (7)	11.3	19.0	**<0.0001**
	Male (11)	25.0	38.7	**<0.0001**
	Female × male	48.7	63.5	**<0.0001**
MOT	Female (7)	10.1	7.3	**0.006**
	Male (10)	1.3	0.4	0.54
	Female × male	8.5	0.6	0.45
LIN	Female	3.3	5.7	**0.02**
	Male	8.7	14.5	**<0.0001**
	Female × male	42.9	22.4	**<0.0001**

Significant ($P \leq 0.05$) random effects as determined by the likelihood ratio test are indicated in bold (df = 1 in each case).

sperm swimming speed among females (ovarian fluids). The significant female × male interaction effect suggests that VAP varied within each female's ovarian fluid according to individual male identity. Sperm swimming speed also varied significantly within each male (post hoc contrast analyses, all *P* < 0.02) depending on the ovarian fluid in which it was activated. For example, the sperm from male 1 was among the fastest swimming sperm in the ovarian fluid of all 7 females, whereas the sperm of male 3 was among the fastest with female 1 and among the slowest with female 7 (Figure 1b). The sperm of male 4 swam almost twice as fast with female 7 as it did with female 6 (Figure 1b). Interestingly, there was no correlation between average least squares (adjusted) means (controlling for date) for VAP and DUR across male × female combinations (*r* = 0.06, *P* = 0.65, Figure 2a), suggesting that there was no trade-off between these 2 traits. Thus, sperm did not swim faster at the expense of swimming duration, as has been suggested in other studies (Stockley et al. 1997; Levitan 2000).

There were significant female (LLR χ^2 = 11.9, df = 1, *P* < 0.001) and female × male interaction effects (LLR χ^2 = 4.8, df = 1, *P* = 0.02) on the mean percentage of progressive sperm cells (MOT), but the male effect was not significant (LLR χ^2 = 1.4, df = 1, *P* = 0.23). However, there was significant variation only within male 1 (*P* < 0.001, post hoc contrast analysis) suggesting that male 1 may have been responsible for the significant interaction effect due to the low motility of his sperm in the ovarian fluid of female 1 (Figure 1c). Indeed, when that male was not included in the analysis, the female × male interaction term was far from significant (Table 1). Thus, the percentage of sperm that were progressively motile does not in general seem to be affected by activation in the ovarian fluids of different females.

There were significant female, male, and interaction effects on mean sperm linearity (LIN), but mean linearity varied significantly only within males 5, 6, 7, 9, and 10 (Table 1, Figure 1d). The sperm of male 5, for example, had the straightest trajectory in the ovarian fluid of female 7 but one of the most curvilinear with female 1; male 7 was the most curvilinear with female 3 but the straightest with female 6; and male 9 was the most curvilinear with females 1 and 7 but about 25% straighter with female 4. LIN was positively correlated with
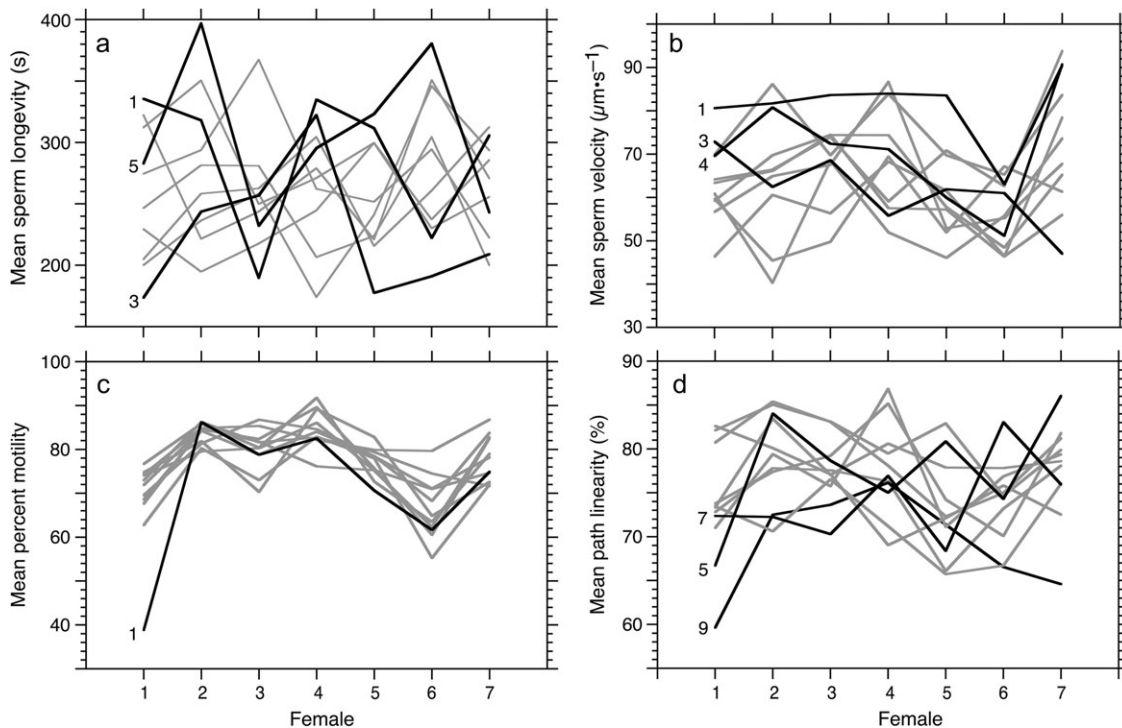
Figure 1
Least squares mean (controlling for date) sperm traits of male chinook salmon in the ovarian fluids of 7 different females. Bold lines identified by male number are described in the text. (a) Sperm longevity (DUR; $n = 10$ males), (b) swimming speed (VAP; $n = 11$ males), (c) percentage of progressively motile cells (MOT; $n = 11$ males), and (d) path linearity (LIN; $n = 11$ males). See Table 1 for statistics.

VAP ($r = 0.79$, $P < 0.0001$, $n = 77$; Figure 2b), suggesting that variation in path linearity was simply due to changes in swimming speed.

DISCUSSION

The results from this study show that sperm swimming speed (VAP), longevity (DUR), and path trajectory (LIN) differed among males and were all differentially affected by the ovarian fluids from different females (Table 1, Figure 1). Thus, we have uncovered a clear mechanism for CFC in this species, mediated by female ovarian fluid. Presumably, the chemical composition of each female's ovarian fluid differentially affects male sperm performance during the brief fertilization window after sperm activation. Sperm swimming speed is probably the most important variable influencing fertilization success in this species (see also Gage et al. 2004), and almost half of the variation in that variable (VAP) in our study was explained by male × female interaction (Table 1). Previous work on Arctic charr (Urbach et al. 2005) also found significant female × male interaction on sperm swimming speed well after this brief fertilization window.

To confirm that CFC is at play here, we now need studies that look at sperm traits in relation to male fertilization success during sperm competition in the presence of ovarian fluid from different females. We would predict, for example, that male 1 would be most likely to fertilize the largest proportion of ova from female 5 in competition with the other 10 males (Figure 1b). In more extreme example, males 1 and 4 would be expected to vastly outcompete male 3 to fertilize the ova of female 7, but male 1 would be expected to outcompete both males 3 and 4 to fertilize the ova of female 5.

Our findings suggest that one or more components of a female's ovarian fluid differentially influence the sperm be-

havior of males. CFC occurs when females favor the sperm of males with compatible genotypes irrespective of their phenotype (Zeh and Zeh 1996; Jennions and Petrie 2000) and is typically driven by genetic incompatibility between females and males (Birkhead 1998; Birkhead and Pizzari 2002). A male's "quality" will thus vary from female to female, as potential mates will vary in the extent to which they are genetically compatible (Parker 2006), among other things. A number of potential molecules have been identified that influence male–female compatibility at fertilization (reviewed by Vacquier 1998). It has been suggested that the major histocompatibility complex (MHC) may be a candidate for genotype-based recognition between the spermatozoa and the egg (Penn and Potts 1999; Birkhead and Pizzari 2002), as their MHC haplotype might be expressed on the surface of spermatozoa, thus enabling female recognition of sperm's alleles (Ziegler et al. 2005).

Evidence is gathering to suggest that fertilization in many species is nonrandom and depends on male and female compatibility (Wedekind et al. 1996; Marshall and Evans 2005; Dziminski et al. 2008). For example, fertilization success was nonrandom with respect to male identity of the Australian sea urchin (*Heliocidaris erythrogramma*), supporting the idea that females exercise CFC for compatible mating partners at a gamete level (Evans and Marshall 2005). Similarly, in a different sea urchin genus, *Echinometra*, ova exposed to experimental sperm mixtures show strong discrimination on the basis of the male's bindin genotype (Palumbi 1999), preferring sperm that carry the same bindin allele as the ovum.

Variation in the compositional "make-up" of a female's ovarian fluid (Lahnsteiner et al. 1995) might depend on the physiological status of the female (Lahnsteiner et al. 1999; Lahnsteiner 2000). For example, salmonids can also hold their ovulated eggs in the body cavity for at least a week
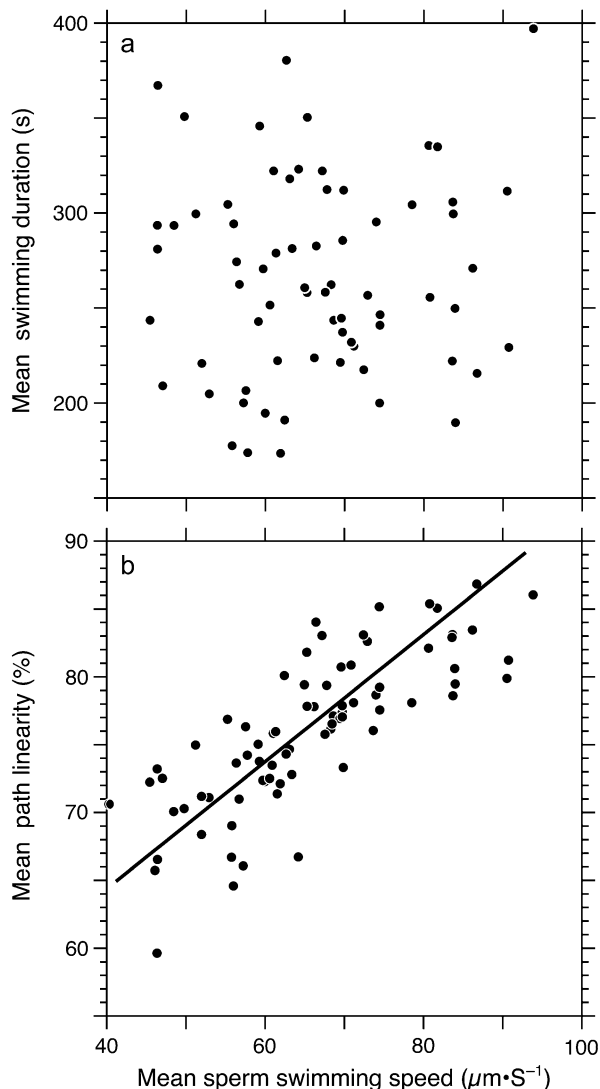
Figure 2
Relations between (a) sperm swimming duration and (b) sperm path linearity and sperm swimming speed in male chinook salmon. Data points are least squares means from general linear models with day as a covariate. Line in (b) is model II regression ($y = 45.8 + 0.5x$).

(Aegerter and Jalabert 2004), and postovulatory aging of the eggs in the body cavity can affect the composition of the ovarian fluid (Rime et al. 2004). Some preliminary analyses looking at the chemical composition of the ovarian fluid for each of the 7 females that we studied suggests that there may be significant variation in ion concentrations (Rosengrave P, unpublished data). For example, female 6 had a lower calcium concentration in her ovarian fluid compared with the other females, and we know that calcium ions are required in the external environment to initiate sperm motility (Morisawa and Morisawa 1986; Alavi and Cosson 2006). Other components of the ovarian fluid, such as proteins, may function as signaling molecules that have a chemokinetic or chemotactic effect on sperm. These peptide signaling molecules have been found on the surface of the unfertilized ovum in sea urchins (Neill and Vacquier 2004). Additionally, male and female reproductive proteins that bind each other to mediate fertilization have been recognized in a handful of animal groups

(Swanson and Vacquier 2002) and may potentially be found in both ovarian fluid and sperm membranes.

Whereas the significant male effect on sperm performance (Table 1) suggests that some males may have generally superior quality spermatozoa that swim faster or for a longer duration, it appears that few, if any, males have sperm that performs well in the ovarian fluid of all females (Figure 1a, b). Some consistent differences among males are expected because sperm motility is partially controlled by mitochondrial genes that regulate sperm motility (Gemmell et al. 2004) that are thus likely to be responsible, at least in part, for differences in sperm swimming speed between males (Froman and Kirby 2005). Nonetheless, our results clearly show that the interaction between female ovarian fluid and male spermatozoa is responsible for more of the observed variation in sperm performance than male identity alone (Table 1).

In some externally fertilizing fishes, there is undoubtedly a fertilization advantage to sperm swimming both faster and for longer duration, and it has been suggested that there is a trade-off between these 2 traits resulting from the process of energy metabolism (Stockley et al. 1997). Our results clearly show that there is no such trade-off in chinook, at least in the presence of ovarian fluid (Figure 2a). Given that most fertilization in this species probably occurs within 10 s of sperm activation, it is presumably irrelevant that some sperm swim for 6 min or more, on average, in the presence of ovarian fluid (Figure 1a). More work will be needed to understand why sperm swimming speed and longevity appear not to be interdependent in this species.

Given the broad significance of sperm selection by females, and our limited knowledge regarding mechanisms of CFC in externally fertilizing species, results from the present study suggest a promising area for further detailed investigation into the ability of females to favor the sperm of one male over another during a spawning event. In particular, study of the chemical components of ovarian fluid that influence sperm swimming speed and how those components vary within and among females is warranted. The implementation of competitive fertilization experiments examining sperm traits in the presence of ovarian fluid from different females is particularly needed to confirm that CFC does indeed occur.

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