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Ovarian fluid enhances sperm velocity based on relatedness in lake trout, *Salvelinus namaycush*

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Abstract

Studying mate choice at the gamete level can provide valuable insights into proximate mechanisms that underlie the evolution of mating systems. The objective was to assess whether ovarian fluid enhances sperm performance based on relatedness of mates in lake trout, *Salvelinus namaycush*, an iteroparous salmonid. Twelve trios were used, each composed of a female and two male fish; one male was related (full sibling) to the female, whereas the other was unrelated. Sperm from each male was activated in hatchery water or ovarian fluid from each corresponding female. No significant difference in sperm velocity was detected between the related and unrelated male fish when activated in hatchery water. However, when sperm was activated in ovarian fluid, sperm velocity from the related male was significantly higher than that of the unrelated male fish. Overall, ovarian fluid enhanced sperm performance of related male fish and might act as part of a recognition system to select sperm of a specific genotype.

Keywords: Salmon; Inbreeding; Sperm competition; Cryptic female choice; Sexual selection; Mating system

1. Introduction

Contrary to conventional wisdom, recent empirical studies and theoretical models suggest that there might be a substantial benefit to mating incestuously [1]. For example, in a locally adapted population with a stable environment and limited genetic load, inbreeding could be beneficial in maintaining favorable gene combinations, as well as maximizing parental genetic fitness [2,3]. Studies demonstrating mate choice behavior for related mates do exist [4], although only a handful of studies showed biased prezygotic selection at the level of the gametes [5,6]. For example, in Peron's tree frog, *Litoria peronii*, male frogs with high genetic relatedness to female frogs fertilized a greater proportion of her eggs in sperm competition trials [6], whereas in the sea urchin, *Echinometra mathaei*, eggs select sperm with a bindin (protein involved in attaching sperm to the egg) genotype similar to their own, suggesting a strong linkage between female choice and male trait loci [5]. In many species, eggs also secrete substances that enhance sperm performance and/or attract sperm toward the vicinity of an egg, such as speract and other sperm-activating peptides from the egg jelly coat of the sea urchin, *Strongylocentrotus purpuratus* [7,8], L-tryptophan from eggs of red abalone, *Haliotis rufescens* [9,10], and herring sperm-activating proteins from eggs of Pacific herring, *Clupea pallasii* [11]. Although these egg-derived substances are extremely common among organisms (reviewed in [12]), very little is known about their ability to exert choice for sperm of a specific genotype. This is surprising considering that

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sperm are the haploid contribution; therefore, the female of the species can indirectly benefit by biasing in favor of some male sperm over others.

Using gametes from fish species with external fertilization provides an opportunity to further explore these potential gamete selection processes. For instance, sperm have measurable traits, such as velocity, that are related to competitive fertilization success [13–15]. Ovarian fluid is expelled with eggs batches and has been shown to enhance sperm performance compared with when activated in water alone [16–19]. When activated in ovarian fluid, sperm performance can also depend on individual male by female interactions [20,21], suggesting a possible role in cryptic female choice. For example, sperm–ovarian fluid interactions have been shown to mediate sperm competition success in relation to relatedness in the guppy, *Poecilia reticulata* [22].

The objective of this study was to assess whether ovarian fluid differentially affects sperm performance based on genetic relatedness in lake trout, *Salvelinus namaycush*, a long-lived, iteroparous salmonid. Lake trout spawn in nocturnal aggregations, where several male fish spawn simultaneously with a female, thus intense sperm competition and gamete selection are plausible in the wild [23].

2. Materials and methods

2.1. Genetic breeding design

The adult lake trout used in this experiment originated from Kingscote Lake (latitude, 45.2001; longitude, −78.2162) in Algonquin Park, Ontario. Wild spawn collections were carried out yearly from 1999 to 2002, with spawned adults given a finclip and released. Recaptured (previously clipped) fish within and between years were not used for further mating. The wild-origin offspring families (full and maternal half-sibling families) were individually reared at the Ontario Ministry of Natural Resources Codrington Fisheries Research Facility, located in Codrington, Ontario (latitude, 44.1468; longitude, −77.8045). Families were reared separately from fertilized eggs to age 2 y, when fish were marked to family using either finclips or family brands. Numbers of fish per family were equalized and periodically thinned to minimize hatchery selection. At age 2 y, families were raised together in 1000 L tanks (six families per tank) and periodically thinned. At first maturity, fish were marked with individual brands to enable tracking of individual-specific maturity and reproductive data. Two years before the sperm performance experiment, all families were moved to two 6000 L tanks and reared communally. These first-generation, wild-origin adults were used to assess the effects of relatedness on sperm performance in ovarian fluid. For the purposes of this experiment, only adults from full-sib families were used.

2.2. Experimental design

Eggs (and their associated ovarian fluid) and milt (sperm and seminal plasma) were collected to form a series of 12 trios, each composed of a female and two male fish; one male fish was related to the female (full-sibling), and the other was unrelated. Individuals were anesthetized with tricaine methanesulfonate (MS-222; Syndel International, Vancouver, British Columbia, Canada) to minimize stress during handling and stripping of gametes. The urogenital pore was wiped dry, and gametes were obtained by applying slight pressure on the abdomen. To avoid contamination, the initial ejaculate was discarded in a standardized manner. Approximately 2 mL of ejaculate was then collected using sterilized Pasteur pipettes. Egg batches were collected using 500 mL dry polyethylene containers. Ovarian fluid was separated from the eggs using a sieve with 1.0 mm mesh. Samples were immediately placed in a cooler at 4 °C to 6 °C. For each of the 12 trios, sperm from the related and unrelated male fish were activated in a standardized water sample from the hatchery (natural stream source) and in an ovarian fluid solution composed of 20% ovarian fluid, diluted in the same hatchery water, from the corresponding female fish (hereafter referred to as ovarian fluid). A 20% ovarian fluid dilution was used in the activation medium because it seems likely that sperm will encounter low concentrations of ovarian fluid during a natural spawning event, because ovarian fluid comprises only 5% to 20% of the total egg volume in lake trout (Butts, Johnson, Wilson, and Pitcher, unpublished data). The relatedness of each male fish within each trio was unknown to the investigators at the time of sperm activation.

2.3. Sperm quality assessment

Milt (<0.2 μL) was micropipetted into a chamber of a 2X-CEL glass slide (Hamilton Thorne, Beverly, MA, USA) and covered with a coverslip (22 × 22 mm). Sperm were then activated with 15 μL of activation media. Sperm were video-recorded using a CCD black and white video camera (XC-ST50, Sony, Tokyo, Japan) module at 50 Hz vertical frequency, mounted on an external phase contrast microscope (CX41

Olympus, Melville, NY, USA) with a 10× negative-phase magnification objective [24,25]. When recorded, sperm traits were analyzed using the HTM-CEROS sperm analysis system (version 12, CEROS, Hamilton Thorne Biosciences, Beverly, MA, USA) set at the following parameters: number of frames = 60, minimum contrast = 11, photometer = 55 to 65, minimum cell size = 3 pixels [24,25]. The following sperm performance traits were evaluated: sperm velocity (defined as curvilinear velocity, see Supplementary data for other sperm velocity traits), linearity, motility, and longevity (defined as the time for approximately 95% of the sperm cells to become immotile [14]). Because sperm velocity is the primary determinant of competitive fertilization success in salmonids [14], this sperm metric was the focus of the present study; other sperm performance traits are presented in the Supplementary data. In salmonids, male fish have a brief opportunity to fertilize eggs when their gametes are released from the genital pore [26,27]. For instance, in Sockeye salmon, *Oncorhynchus nerka*, 80% of the eggs are fertilized within 5 s of gamete activation [26], and in Atlantic salmon, *Salmo salar,* a 2-s delay in sperm release reduced fertilization success to 30% from an expected 50% [27]. Therefore, to represent a more natural spawning event, sperm traits were analyzed at 5 s postactivation.

2.4. Statistical analyses

Statistical analyses were performed using SAS version 9.1. Sperm traits were analyzed using mixed-model repeated measures ANOVA containing the relatedness (related and unrelated), and activation medium (hatchery water and ovarian fluid; repeated factor) main effects, and the relatedness by activation medium interaction. Relatedness and activation medium were considered fixed factors and male identity was considered a random factor. Variation associated with each ovarian fluid sample was controlled for statistically by incorporating female as a random blocking factor. Significance levels were set at P = 0.05 for main effects and P = 0.10 for interactions to minimize the probability of type II error [28,29]. When interactions were detected or suspected, reduced models were run separately at each level of activation medium to facilitate their interpretation [30]. The reduced models involved only preplanned comparisons and did not include repeated use of the same data, so α-level corrections for *a posteriori* comparisons were not necessary. In the case of a nonsignificant interaction, main effects were interpreted. Treatment means were contrasted using the Tukey–Kramer Honestly significant difference test.

3. Results

For 11 of the 12 trios, sperm velocity was higher for the related compared with the unrelated male fish, when sperm was activated in ovarian fluid (Fig. 1A). For the repeated measures ANOVA, the relatedness by activation medium interaction was significant ($F(1,22) = 3.77$; P = 0.065). Therefore, the model was revised into separate ANOVA models at each level of activation medium. When sperm was activated in hatchery water no significant difference in sperm velocity was detected between the related and unrelated fish ($F(1,11) = 0.59$; P = 0.460; Fig. 1B). When sperm was activated in ovarian fluid, however, the relatedness main effect was significant ($F(1,11) = 9.26$; P = 0.011), such that sperm from the related male fish swam faster than sperm from the unrelated fish (Fig. 1B).

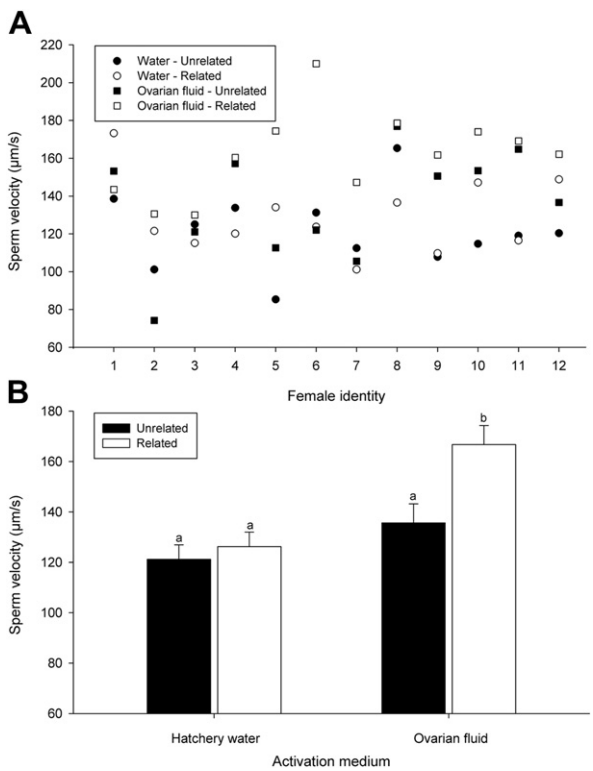

Fig. 1. The effects of ovarian fluid on sperm velocity from related (full sibling) and unrelated male lake trout, *Salvelinus namaycush*. (A) Mean sperm velocity is reported for related and unrelated male fish activated in hatchery water and in an ovarian fluid solution composed of water and 20% ovarian fluid. Separate trials were conducted with ovarian fluid from each female fish. (B) Results generated from a repeated measures ANOVA. Error bars represent SEM. Bars without a common letter differed (P < 0.05).

4. Discussion

To our knowledge, this was the first report that ovarian fluid differentially enhanced sperm performance in favor of related mates in fishes (see [22] for a study favoring unrelated sperm). Because the goal of reproduction is to maximize genetic fitness [3], passing on genes can be a tradeoff between maximizing relatedness with offspring and avoiding inbreeding depression [2]. As long-lived top predators in low productivity inland freshwater lakes which function as closed systems, lake trout are adapted to persist at low population numbers under isolated conditions [31]. If lake trout have evolved a higher tolerance for inbreeding, or underwent population-level inbreeding and purging of genetic load soon after deglaciation, matings between related adults would maximize their reproductive success or maintain locally adapted gene complexes. Therefore, based on the results from the present study, we inferred a potential ovarian fluid-based recognition system might have evolved to facilitate these processes and further promote a competitive fertilization advantage for genetically similar mates. Interestingly, in the Atlantic salmon, *Salmo salar*, male fish that were more genetically similar to a female at the major histocompatibility class I locus sired more eggs in sperm competition [32].

Currently, not much is known about how ovarian fluid favors sperm of a specific genotype. Perhaps there is a gamete navigation system to excite or enhance sperm via some chemoattractant provided by the ovarian fluid. For instance, sperm from the free-spawning red abalone, *Haliotis rufescens*, detect waterborne signaling molecules from conspecific eggs, and change their swimming behavior to increase the probability of successful contact [33]. Another possible explanation pertains to membrane-bound proteins, found on the surface of sperm and eggs [34]. These proteins are rapidly evolving to promote gamete recognition and facilitate fertilization [35]. Some of these proteins are even found in egg-derived compounds released with an egg batch and consequently influence sperm function before gamete contact [12].

5. Conclusions

Ovarian fluid not only enhanced sperm velocity in lake trout, but it also might have acted as a recognition system to select sperm based on genetic relatedness. Together, these findings suggested that evolutionary pressures acted on fish mating systems even before sperm penetrated an egg.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.theriogenology.2012.06.031.

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Appendix A. Supplementary data

1. Supplementary results

In the main text, sperm velocity (defined as curvilinear velocity) was examined in detail, because it is the primary determinant of competitive fertilization success in salmonids. Presented below are additional analyses for other sperm performance traits: (1) principal component scores of three sperm velocity metrics (curvilinear velocity, average path velocity, and straight-line velocity); (2) average path velocity; (3) straight-line velocity; (4) linearity (calculated as straight-line velocity/curvilinear velocity); (5) percent sperm motility; and (6) sperm longevity (calculated as the time until 95% of the sperm cease progressive forward motion).

Principal components analysis was used to summarize variation in the three sperm velocity metrics (curvilinear velocity, average path velocity, straight-line velocity). One informative principal component axis was extracted that explained 93% of the variation in sperm velocity. Based on a repeated measures ANOVA model, there was a significant relatedness by activation medium interaction ($F(1,22) = 3.02$; $P = 0.096$). Therefore, the model was revised into separate ANOVA models at each level of activation medium. When activated in hatchery water, no significant difference in sperm velocity was detected between the related and unrelated males ($F(1,11) = 0.48$; $P = 0.504$; Supplementary Table 1). However, when activated in ovarian fluid, the relatedness effect was significant ($F(1,11) = 7.20$; $P = 0.021$), such that sperm from the related males swam faster than sperm from the unrelated males (Supplementary Table 1).

The relatedness by activation medium interaction had an effect on average path velocity ($F(1,22) = 3.22$; $P = 0.086$), therefore the model was revised into separate ANOVA models at each level of activation medium. No effect was detected between the related and unrelated males when activated in hatchery water ($F(1,11) = 0.83$; $P = 0.381$). However, when activated in ovarian fluid, sperm from the related males swam faster than sperm from the unrelated males ($F(1,11) = 8.05$; $P = 0.016$; Supplementary Table 1).

For straight-line velocity, the relatedness ($F(1,11) = 2.09$; $P = 0.176$), activation medium ($F(1,22) = 2.99$; $P = 0.098$), and relatedness by activation medium interaction ($F(1,22) = 2.24$; $P = 0.149$) were not significant; however, data followed the same trend as other velocity metrics, such that ovarian fluid favored related males (Supplementary Table 1).

For linearity, ovarian fluid significantly decreased the swimming trajectory of sperm ($F(1,22) = 4.61$; $P = 0.043$), while the relatedness ($F(1,11) = 0.19$; $P = 0.669$) and relatedness by activation medium effects were not significant ($F(1,22) = 1.36$; $P = 0.256$; Supplementary Table 1).

For sperm longevity, activation medium was significant ($F(1,22) = 73.07$; $P < 0.001$), such that sperm swam longer when activated in ovarian fluid. The other model terms had no effect on sperm longevity ($P \geq 0.244$; Supplementary Table 1).

The relatedness ($F(1,11) = 1.33$; $P = 0.274$), activation medium ($F(1,22) = 1.59$; $P = 0.220$) and relatedness by activation medium interaction had no effect on percent sperm motility ($F(1,22) = 2.36$; $P = 0.139$; Supplementary Table 1).

Supplementary Table 1
Summary of descriptive statistics (mean ± SEM) for sperm performance traits activated in hatchery water and ovarian fluid from related and unrelated males in lake trout, *Salvelinus namaycush*.

Sperm performance traits	Hatchery water		Ovarian fluid	
	Unrelated male	Related male	Unrelated male	Related male
$PCA_{velocity}$	−0.44 ± 0.22	−0.25 ± 0.22	−0.12 ± 0.30	0.80 ± 0.30
Average velocity (μm/s)	112.13 ± 5.78	118.54 ± 5.78	122.52 ± 8.32	148.79 ± 8.32
Straight-line velocity (μm/s)	89.11 ± 6.07	90.47 ± 6.07	90.44 ± 7.68	108.93 ± 7.68
Linearity (%)	73.00 ± 1.67	69.25 ± 2.75	65.54 ± 2.43	67.04 ± 2.71
Sperm longevity (s)	21.83 ± 1.04	20.68 ± 0.83	29.36 ± 1.19	28.57 ± 0.66
Motility (%)	51.81 ± 5.65	46.12 ± 4.59	53.73 ± 6.01	70.35 ± 6.49

Linearity is calculated as straight-line velocity/curvilinear velocity; sperm longevity is calculated as the time until 95% of the sperm cease progressive forward motion.
Abbreviation: $PCA_{velocity}$, principal components analysis based on the variation in the three sperm velocity metrics (curvilinear velocity, average path velocity, straight-line velocity).