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ORIGINAL ARTICLE

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Effects of ovarian fluid on sperm velocity in Arctic charr (*Salvelinus alpinus*)

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Abstract Numbers of studies in externally fertilizing fish species provide evidence for an effect of ovarian fluid on sperm motility characteristics such as duration of forward mobility, velocity or percent motile sperm cells. Yet, because of variations among females in the quality of their ovarian fluid, such effects might differ between individuals. Additionally, ovarian fluid from different females could also be expected to affect each ejaculate differently, resulting in cryptic female choice. In this study on Artic charr (*Salvelnius alpinus*), sperm velocity from several males was measured in the diluted ovarian fluid of several females according to a fully balanced crossing design. This design allowed us to estimate variations among females in the effect of their ovarian fluid on the velocity of sperm from different males, and to detect variations among males in the ability of their sperm to swim in ovarian fluid. Sperm velocity was estimated by computer-assisted sperm analysis. Average velocity was found to vary among females, with some females having constantly higher velocity measurements in their ovarian fluid, and among males, indicating that some males had overall faster sperm in ovarian fluid than others. Moreover, variation in sperm velocity was shown to depend on individual female-male interactions. Our results document that females vary in the effect of their ovarian fluid on sperm velocity and that their ovarian fluid may stimulate sperm velocity according to individual characteristics of males. This latter result suggests a potential mechanism for cryptic female choice.

Keywords Arctic charr · Sperm velocity · Ovarian fluid

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Introduction

In externally fertilizing fish species, egg discharge is accompanied by the simultaneous release of the ovarian fluid (i.e. coelomic or peritoneal fluid) in which the eggs are stored in the coelomic cavity. In the salmonids for instance, the amount of ovarian fluid released together with the eggs is about 10–30% of the total egg volume (Lahnsteiner et al. 1999b). Besides its pheromonal function in the spawning fish (Scott and Vermeirssen 1994), ovarian fluid has been found to improve the fertilization rate, prolong the egg fertilizability during short-term storage and influence sperm motility characteristics (Lahnsteiner 2002).[1] Effects of ovarian fluid on sperm motility characteristics are described in terms of prolonged mobility periods and increased velocity. Prolonged mobility periods have been observed in the freshwater bullhead, *Cottus gobio* (Lahnsteiner et al. 1997), the marine sculpin *Hemilepidotus gilberti* Jordan, Starks (Hayakawa and Munehara 1998), the brown trout *Salmo trutta f. fario* (Lahnsteiner 2002) and in the three-spined stickleback, *Gasterosteus aculeatus* (Elofsson et al. 2003a). Yet, ovarian fluid did not increase the mobility period in fifteen-spined sticklebacks, *Spinachia spinachia* (Elofsson et al. 2003b) and in sockeye salmon, *Oncorhynchus nerk* (Macfarlane 2002). Increased mean and maximum velocity has been observed in Atlantic cod *Gadus morhua* (Litvak and Trippel 1998). Positive effects of ovarian fluid on sperm motility characteristics have

[1] Throughout the literature cited in this study, the terms sperm motility (characteristics) or sperm movement encompass several components of sperm motion such as duration or longevity (i.e. mobility period), percent motile sperm cells and sperm trajectory. Yet the present study focuses only on sperm velocity, i.e. sperm swimming speed.

also been documented in Arctic charr (*Salvelinus alpinus*). That is, variations in ovarian fluid concentrations influence percent motile sperm cells, sperm longevity (duration of forward mobility), velocity and swimming trajectories, with all of these variables generally increasing as the concentration of ovarian fluid increases from 0 to 50% (Turner and Montgomerie 2002).

Thus, most of the above-mentioned studies provide evidences for an effect of ovarian fluid on different characteristics of sperm motility and point to the importance of adding a female dimension to the description of sperm motility (Litvak and Trippel 1998). Yet, pooling the ovarian fluid of several females (Turner and Montgomerie 2002) or using the ovarian fluid of only one individual (Litvak and Trippel 1998) does not allow for detection of variations among females in the effect of their ovarian fluid on sperm motility characteristics. In the salmonids, the composition of the ovarian fluid varies depending on the females' physiological status, on the egg quality and on the egg maturity grade (Lahnsteiner et al. 1999a; Lahnsteiner 2000). Therefore, ovarian fluid from different females could be expected to affect sperm motility characteristics in different ways (Lahnsteiner 2002). However, ovarian fluid from different females could also be expected to affect sperm from various males differently. In species subject to sperm competition, such mechanisms could result in so-called cryptic female choice.

In species with external fertilization, one form of cryptic female choice seems to be the control of the number of eggs laid with successive males (Reyer et al. 1999). Additionally, there might be some potential for discrimination among mates at the level of the egg itself (Eberhard 1996; Wedekind et al. 1996; Stockley 1999). Tunicate eggs, for instance, are found to resist fertilization longer by sperm with similar alleles on the fusibility locus than by sperm with different alleles (Scofield et al. 1982), while sea urchins (Echinodermata) have developed a "Lock-and-Key" system of recognition between a specific binding protein on the acrosomal process of sperm and a specific receptor located on the egg-envelop, to avoid inter-specific egg fertilization (Campbell 1993). In teleost fish, sperm cells usually have no acrosome (Kime et al. 2001; Breuckmann and Brucker 2002). Sperm discrimination based on a molecular recognition between the acrosome and the egg is thus not possible and the ovarian fluid could consequently be an important medium for sperm selection to occur. Yet, recent immunohistochemical detection, on the sperm head of the ovoviviparous swordtail (*Xiphophorus helleri*), of an acrosomal sperm antigen involved in the mammalian acrosome reaction (Breuckmann and Brucker 2002) opens the avenue for further discussion about a potential molecular recognition between eggs and sperm also in teleosts.

By examining sperm velocity of different male Arctic charr in the ovarian fluid of different female charr in a fully balanced design, this study aimed to investigate potential differences among females in the effect of their ovarian fluid on sperm velocity, and to determine whether the effect of any female's ovarian fluid varies according to the male from which the milt was collected. Additionally, this design allowed us to detect potential variations among males in the ability of their sperm to swim in ovarian fluid. Average sperm velocity was expected to vary between females and between males but also depending on each female-male combination.

Methods

Fish sampling

In late September 2002, nine ripe males and six ripe females were caught at night on one spawning ground in lake Fjellfrøsvatn, northern Norway. To avoid interactions between females and males until handling the next morning, the individuals were kept for the rest of the night in two separate cages according to sex. Once taken out of their cages, the individuals were handled at a rate of one fish every 2 min, keeping a short time lag between individual handling. After having been killed by a stroke to the back of the head, each fish was stripped for gametes by applying bilateral pressure from the anterior part of the abdomen towards the genital pore. To avoid contamination and activation of sperm during stripping, the area around the genital pore was dried carefully before milt collection. The same care was taken during egg collection. Milt samples were stored in Eppendorf tubes and kept in the laboratory at 10°C. This temperature corresponds to the water temperature in the lake at that period. Ovarian fluid was obtained from the egg batches with a Pasteur pipette and stored separately under the same conditions as the milt samples. In order to assess a potential effect of storage time on sperm velocity and ovarian fluid quality, time between gamete collection and use was recorded.

Sperm motility recording and analysis

To examine the effect of ovarian fluid on sperm velocity, we exposed pure milt to 1:2 water-diluted ovarian fluid (OF: water), assuming that under natural conditions, sperm reside within relatively undiluted ovarian fluid. This assumption seems to be met because of the sperm's characteristics: duration of forward mobility in salmonids is short and the cells are not able to swim round even half the circumference of the egg (Kime et al. 2001). Thus, to insure fertilization, males must shed sperm in the immediate vicinity of the ova where the ovarian-fluid concentration is likely to be high.

The milt was exposed to the diluted ovarian fluid on a Micro slide (Assistent 50 Elka) using the following method. For each trial, a 0.5 µl aliquot of undiluted sperm was first isolated on a glass slide. The tip of a Finntip filled with 4.5 µl diluted ovarian fluid was then immediately dipped into this aliquot and the content of the Finntip was rapidly placed onto the slide. After having put an 18×18 mm cover-slide (Chance propper LTD, UK) on the approximately 4.5 µl mixture of milt and diluted ovarian fluid, the microscope was focused. To avoid a possible effect of cell density on the velocity of sperm cells, samples were screened for areas with approximately 50 sperm cells before focusing, as recommended by the European Society for Human Reproduction and Embryology (2001). This precaution additionally allowed us to standardize the recording procedures. Sperm activity was recorded for a total of 1.5 min from activation, i.e. from the precise moment the milt was exposed to the diluted ovarian fluid when dipping the Finntip into the milt sample. Records were made using a CCD B/W video camera module (Sony, XC-ST50 CE) attached to a CH30 Olympus microscope with a negative phase-contrast objective lens (×10 magnification). Activation was identified on the videotape by a vocal signal. This whole procedure was repeated twice for each female-male combination.

Video-recordings were analyzed using an HTM-CEROS sperm tracker (CEROS version 12, Hamilton Thorne Research, Beverly,

Mass.). Computer-assisted sperm analysis (CASA) has been shown to be an objective tool for studying sperm motility characteristics in fish (Kime et al. 1996, 2001; Elofsson et al. 2003a; Rurangwa et al. 2004). The image analyzer was set as follows: frame rate 50 Hz; no. of frames 10; minimum contrast 10; minimum cell size 2 pixels. The parameters assessed were: average path velocity (VAP: average velocity on the smoothed cell path), straight line velocity (VSL: average velocity on a straight line between the start and end points of the track), and curvilinear velocity (VCL: average velocity on the actual point-to-point track followed by the cell). Velocity estimates for each female-male combination correspond to the mean velocity of all motile cells analyzed. Thus, to avoid including, in the mean, measurements taken on sperm cells moving only because of drift or Brownian movement, threshold values for the only two optional settings defining static cells, i.e. VAP and VSL, were set at 20 μm/s for VAP and 30 μm/s for VSL. Sperm cells with velocity measurements below these limits were considered static and were thus not included in the analyses. Moreover, recordings where most cells obviously moved because of drift were not used. Published studies on sperm velocity in fish using similar sperm-tracking methods do not give clear information about which velocity parameter is best to use, and there is no consistency amongst studies (see Kime et al. 2001; Kime and Tveiten 2002; Elofsson et al. 2003a; Burness et al. 2004; but see Rurangwa et al. 2004). Since in this study there were neither eggs nor ovarian fluid gradients to attract or guide the sperm cells, cell trajectories were not expected to be linear, and final analyses were therefore performed on measures of the actual point-to-point track followed by the cells (VCL) obtained 20 and 30 s after activation. All females were combined with all males, resulting in a fully balanced design that allows testing for both main and interaction effects.

Statistical analyses

All statistical analyses were performed using the statistical software R, version 1.9 (Ihaka and Gentleman 1996). Linear mixed-effects models were first applied independently on velocity data obtained 20 and 30 s after activation to test for main and interaction effects of females and males on VCL. Because of haphazard fish sampling, females and males were defined as random predictors. Plots of the residuals were used to verify the assumptions of the parametric models. Convergence problems in the 20 s model hindered us in estimating a variance for the male effect and thus in considering data obtained 20 and 30 s after activation as repeated measurements in a single model. Consequently, only data of sperm velocity measured 30 s after activation were included in our final analyses. However, in spite of the above-mentioned problem in the 20 s model, female and interaction effects were similar in both data sets. Despite our efforts to standardize the density of cells to be analyzed per frame by focusing on areas with approximately 50 sperm cells, number of sperm cells per frame varied from 8 to 137 cells (42±2.03, mean±SE, n=108). Cell density was consequently included, together with time since stripping, as continuous fixed-effect predictor in the analyses. Restricted maximum likelihood (REML) was used for fitting the models. Likelihood ratio tests were applied for testing the significance of the random effects in the most parsimonious model, while a conditional F-test was used for fixed effects (Pinheiro and Bates 2000). Components of variance corresponding to the variance of each effect are given with their associate 95% confidence interval.

Results

Mixed-effects analyses showed that sperm velocity was not affected by the time elapsed from milt sampling until recording ($t_{1,105}$=0.23; P=0.82) and this variable was consequently removed from the model. Average sperm velocity slightly increased with cell density (β=0.1, $t_{1,106}$=2.97, P=0.004) but the variance in average sperm velocity accounted for by the number of sperm cells per frame analyzed was small (var=0.009, 95% C.I.=[0.001, 0.025]). There were significant female (l-ratio=8.35, df=1, P=0.004, var=22.83, 95% C.I.=[4.78, 108.90]) and male

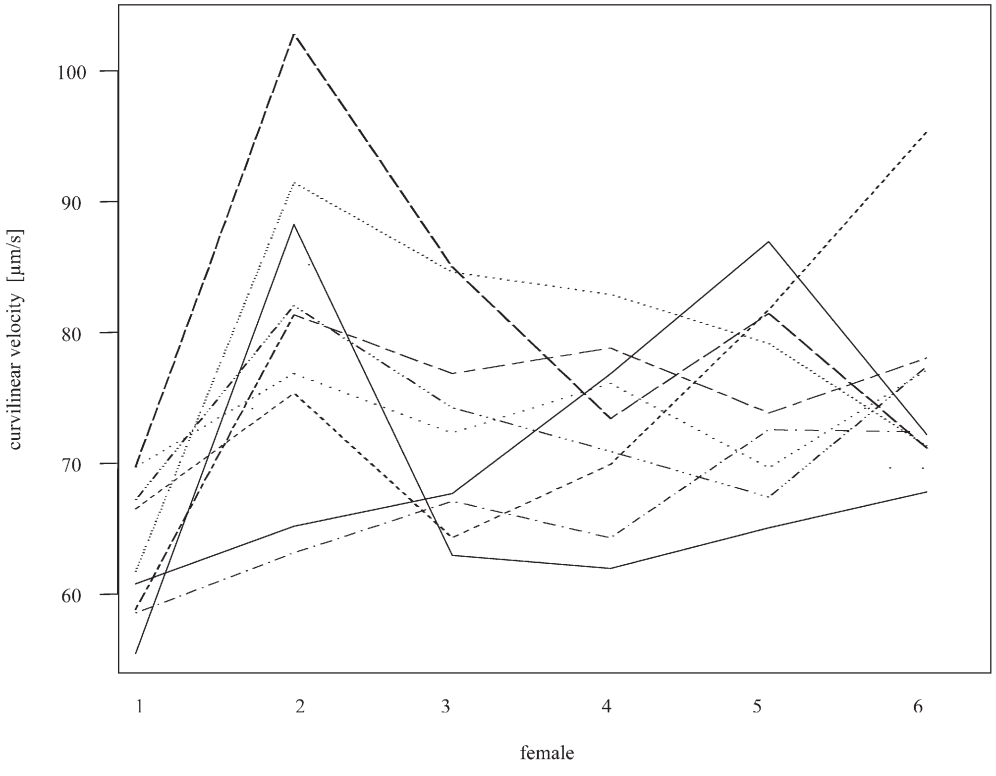

Fig. 1 Variation in Curvilinear Velocity (*VCL*) (μm/s) among males in every female. Each *line* represents the average values of one individual male over two replicates.

effects (l-ratio=3.82, df=1, P=0.05, var=14.19, 95% C.I.=[2.87, 70.25]) on sperm velocity, suggesting that some females have ovarian fluid in which sperm velocity of all males is higher and that some males perform better in the ovarian fluid of all females. Additionally, sperm velocity was also dependent on the particular female-male combinations, with a significant interaction effect (l-ratio=20.13, df=1, P<0.001, var=38.24, 95% C.I.=[20.76, 70.44]). That is, sperm behavior was affected by the combination of sperm from particular males with the ovarian fluid from particular females (Fig. 1). The residual variance was estimated at 26.94. The variance components indicate that the interaction term accounts for as much variance as the two main effects taken together.

Discussion

The results show that the average sperm velocity differed among ejaculates from different males and among ovarian fluids from different females but that the variation in sperm velocity was also explained by individual female-male interactions.

Sperm velocity was found to vary among males. Ejaculate characteristics might vary with male age: sperm concentration, for example, has been shown to be higher in the milt from precocious males than in that of adults of rainbow trout, *O. mykiss* (Liley et al. 2002) and sockeye salmon (Hoysak and Liley 2001). The differences in sperm velocity observed in our study could consequently be due to age differences between the sampled individuals. Additionally, ejaculate characteristics such as seminal plasma composition (Aas et al. 1991) or sperm motility characteristics (Billard et al. 1977; Büyükhatipoglu and Holtz 1984) also vary along the reproductive season. Thus, males at the beginning of their spawning season might differ from males at the end of it. Finally, differences at the gametic level, in terms of ejaculate size or sperm velocity for instance, might be expected in species adopting alternative mating tactics (Taborsky 1998) and are found in different fish species (Gage et al. 1995; Vladic et al. 2002). Since sperm velocity is positively related to fertilization success (Lahnsteiner et al. 1998; see Kime et al. 2001), sperm velocity should be higher in dominant males. Yet, sneakers fertilize more eggs than dominant males during sperm competition (Fu et al. 2001), suggesting that sperm from sneaky males might have higher velocity to counter the disadvantage of being ejaculated later or further from the egg (Leach and Montgomerie 2000). In bluegills (*Lepomis macrochirus*), disadvantaged males indeed have faster initial sperm velocity (Burness et al. 2004), but sperm cells from dominant males were shown to swim faster than sperm from sneaker males 45 s after activation (Leach and Montgomerie 2000; Burness et al. 2004). Individual male Arctic charr are known to adopt both dominant and sneaker's spawning behavior (Liljedal et al. 1999; Liljedal and Folstad 2003) and the differences in sperm velocity observed in our study could consequently be related to differences in reproductive tactics among individuals. Our experimental design does not, however, allow us to determine whether dominant males or sneakers had faster sperm, as the males were chosen randomly with no knowledge about their reproductive tactic. Yet, variations in male traits can also be observed among individuals within a same mating tactic, as in bluegills. The outlined mechanisms may, alone or in combination, have caused the observed differences in sperm velocity among males.

Unlike in brown trout (Lahnsteiner 2002), the ability of Arctic charr sperm to swim in ovarian fluid appeared to vary among females, independently of the males' identity. That is, females differed in the effect of their ovarian fluid on the average velocity of sperm cells. The ovarian fluid of Arctic charr contains a variety of compounds for the sperm to metabolize (Lahnsteiner et al. 1995). It also contains different ions and peptides shown to influence sperm motility characteristics in other species with external fertilization (Morisawa and Suzuki 1980; Lahnsteiner et al. 1995; Elofsson et al. 2003a). Thus, variations among females in the relative concentration of these different compounds could cause consistent variations in sperm velocity. Even if ovarian fluid is isoosmolar to sperm cells and thus increases sperm longevity compared to water (Billard et al. 1974), spawning females are at risk of not having their eggs fertilized because egg activation starts immediately after contact with water and leads rapidly to the closure of the micropyle (Billard 1988). Therefore, eggs of females able to promote sperm velocity might have better chances of being fertilized before undergoing irreversible modifications. Enhanced probability of successful fertilization for females able to increase sperm velocity is suggested by the positive correlation between fertilization rate and sperm velocity found in some fish species (Lahnsteiner et al. 1998; Kime et al. 2001). The importance for any given female to have all her gametes successfully fertilized is amplified by the potentially high costs of producing eggs and ovarian fluid. Composition of ovarian fluid was found to vary, depending on the females' physiological status, on the quality of their eggs and on their maturity grad (Lahnsteiner et al. 1999a; Lahnsteiner 2000). Yet, differences among females in their ability to trade reproductive investment over other costly activities may also result in differences in the composition of their ovarian fluid and explain differences among females in the effect of their ovarian fluid on sperm velocity.

Finally, the ability of sperm to swim in ovarian fluid appeared to vary within each female according to the male's identity. These observations suggest that the effect of ovarian fluid depends on some characteristics of the ejaculates and that females may discriminate among ejaculates from different males. The ovarian fluid might consequently act as an important factor mediating such particular form of cryptic female choice. Even if not yet fully accepted, the female counterpart to sperm competition, i.e. cryptic female choice (any post-copulatory ability of females to favor one male over another in a

process that cannot be directly observed), is supported by an increasing body of evidence (Thornhill 1983; Eberhard 1996). Cryptic female choice can be either directional or non-directional. In directional choice, females are expected to bias sperm use in favor of the male phenotypes that are also favored in mate choice, thus generating directional sexual selection. In non-directional choice, females are expected to favor sperm of males with compatible genotypes, regardless of their phenotype (Birkhead and Pizzari 2002). In this study, the observed female-male interaction effect on sperm velocity suggests potential cryptic female choice in ovarian fluid of female charr to be non-directional, favoring sperm from males with compatible genotypes.

A number of recent studies have investigated the possibility for females or their ova to select sperm on a genetic basis, and indications for genotype-dependent variations in fertilization success are found in several species (Bishop 1996; Olsson et al. 1996; Wilson et al. 1997; Wirtz 1997; Clark et al. 1999; but see Simmons et al. 1996; Stockley 1997). Prime candidates for a potential gene-based female choice of sperm are genes from the major histocompatibility complex (Brown 1997; Penn and Potts 1999; Birkhead and Pizzari 2002). The major histocompatibility complex (MHC) is a highly polymorphic gene complex responsible for disease resistance and immunological self/non-self recognition (Janeway 1993). Genes from the MHC complex have been shown to be expressed in the sperm membrane of several vertebrate species (Wedekind 1994; Martin-Villa et al. 1999; but see Hutter and Dohr 1998; Ziegler et al. 2002), and gene expression on the surface of spermatozoa is thought to facilitate a differential response by the female or the egg to sperm from different male genotypes (Birkhead and Pizzari 2002). Moreover, there is strong evidence that MHC-genes influence mate choice in vertebrates with internal (Penn and Potts 1999) and external fertilization (Bakker and Zbinden 2001; Landry et al. 2001; Skarstein et al. 2004). Finally, three mechanisms, i.e. inbreeding avoidance, heterozygosity increase and production of "moving targets" against rapidly co-evolving parasites, could make MHC-dependent mate choice adaptive (Penn and Potts 1999) and thus of importance in the context of post-ejaculatory female choice.

The results discussed here suggest that females, or their ovarian fluid, might have an important influence on the behavior of sperm cells during their approach to the egg. In a direct cost-benefit perspective, females should insure the successful fertilization of their eggs by positively influencing sperm velocity of all males. Yet, models of adaptive mate choice predicts females to select among males, either on the basis of condition-dependent signals of males' quality (Zahavi 1975; Andersson 1994) or, as more recently suggested, on the basis of genetic compatibility (Trivers 1972; see Mays and Hill 2004). Regardless of the genetic benefit females are aiming for, adaptive mate choice predicts females to favor particular males over others. Our study refines previous observations of a positive effect of ovarian fluid on average sperm velocity in Arctic charr (Turner and Montgomerie 2002) by suggesting that the ovarian fluid might not only serve females to insure fertilization but serve them also in increasing their fitness benefits through adaptive post-copulatory mate choice. However, demonstrating directional or non-directional cryptic female choice through ovarian fluid requires the eggs to be differentially fertilized by different sperm and sperm velocity to be related to fertilization success. Sperm competition experiments in Arctic charr have shown sperm velocity in water to be positively correlated with paternity. That is, males with faster sperm fertilized more eggs than males with slower sperm (Liljedal, S. personal communication), emphasizing the importance of sperm velocity in terms of fertilization. Yet, this study does not provide the necessary data to support the contention of cryptic female choice and our results only suggest that ovarian fluid can be a medium for such choice to occur.

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