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Stickleback sperm saved by salt in ovarian fluid

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Summary

Sperm of the three-spined stickleback *Gasterosteus aculeatus* display a prolonged motility in the presence of ovarian fluid. The ovarian fluid prolongs sperm motility in freshwater from approximately 1 min to several hours, a trait that possibly gives the stickleback its unusual ability to spawn in waters of all salinities. The aim of the study was to look for factor(s) within the ovarian fluid responsible for prolonging sperm motility as well as to investigate the possible biological importance of the ovarian fluid under natural conditions. To that end, we measured the ionic content (Na^+, Cl^-, Ca^{2+} and K^+) of the ovarian fluid and prepared ionic artificial ovarian fluids. We also prepared a mannitol solution with the same osmolality as the ovarian fluid in order to distinguish between the ionic and osmotic effect. We found that the ionic artificial fluids were equally effective as the natural ovarian fluid in prolonging sperm motility and survival over a range of concentrations, whereas the mannitol solution was far less effective. By insertion of natural ovarian fluid or ovarian fluid from which macromolecules had been removed by ultra filtration in nests it was found that macromolecules help by retaining ions. We also found that ovarian fluid in water, at concentrations as low as 0.75 and 1.56%, prolonged sperm motility and that the concentration of ions (Na^+) present in the nest 15 min after spawning corresponded to at least 3% ovarian fluid. Previous fertilisation experiments have shown that it takes at least 15 min for stickleback sperm to fertilise all eggs in a batch. This indicates that the role of ovarian fluid in prolonging the sperm motility is biologically relevant and that the effect is exerted by the fluid's ionic content.

Key words: sperm motility, fish, teleost, ionic concentration, osmolality.

Introduction

Sperm of most externally fertilising fishes have a brief, often less than two minutes, period of motility. This brevity can either be due to the change in osmolality occurring when the sperm moves from the seminal fluid into water (Billard, 1978; Morisawa, 1994), and/or be the result of a limited energy supply (Christen et al., 1987). In some fishes, such as rainbow trout *Oncorhynchus mykiss* (Yousida and Nomura, 1972), Arctic charr *Salvelinus alpinus* (Turner and Montgomerie, 2002), and in the marine sculpins, elkhorn sculpin *Alcichthus alcicornis* (Koya et al., 1993) and Gilbert's Irish lord *Hemilepidotus gilberti* (Hayakava and Munehara, 1998), the period of sperm motility can be prolonged by the presence of ovarian fluid which is expelled with the eggs. We have found this to be true also for the sperm of the three-spined stickleback *Gasterosteus aculeatus*, where ovarian fluid prolonged the period of motility from 30–60 s in freshwater alone to up to 7 h in freshwater with one fourth ovarian fluid added (Elofsson et al., 2003). The motility period of three-spined stickleback sperm is also longer in brackish water compared to freshwater (Elofsson et al., 2003). However, the biological significance of the presence of ovarian fluid has yet to be established in the three-spined stickleback and in other fishes.

The aim of this study was to examine whether the effect of ovarian fluid on sperm motility is of biological significance, and if so, which component(s) in the ovarian fluid are responsible for this effect.

Materials and methods

Fish

Adult three-spined sticklebacks *Gasterosteus aculeatus* L. were caught in the following habitats: freshwater (a pond in Umeå; 63°50′N; 20°19′E, salinity 0%), brackish water (Öresund at Scania in the south of Sweden, 55°25′N; 12°51′E, salinity 1%) and seawater (Tjärnö Marine Laboratory on the Swedish west coast; 58°52′N; 11°10′E, salinity 3%) and transported to the Department of Zoology, Stockholm University. Freshwater (FW), brackish (BW) and seawater (SW) sticklebacks were all used in analyses of plasma and ovarian fluid. BW sticklebacks were used in all other experiments. The main reason for the use of BW fish in

spawning experiments carried out in FW, was the limited availability of FW sticklebacks. However, marine sticklebacks in nature often migrate into freshwater to spawn.

Fish were brought to breeding condition by exposure to a temperature of 20°C and a photoperiod of 16 h:8 h L:D. The fish were held in 200 l aquaria filled with tapwater (for FW) or tapwater to which Aqua Medic seawater aquarium salt (Bissendorf, Germany) had been added, to reach a salinity of 0.5 and 3.0% for the BW and SW fish, respectively. The aquaria were provided with sand and algae, and the fish were fed daily with red midge larvae. The study was carried out with the permission from the Northern Stockholm Animal Research Ethical Committee, permit number N 179/00 and N 350/02.

Ovarian fluids

In order to construct artificial ovarian fluid (AOV) with ionic concentrations in line with the natural ovarian fluid (OV) and to compare OV of sticklebacks from different environments, OV was collected with a pipette after the egg batch had been expelled by gentle abdominal pressure on fully ripe females. The fluid was immediately frozen and stored at –70°C until analysis. Ovarian fluid concentrations of Na^+, K^+ and Ca^{2+} were analysed by flame emission spectroscopy (Eppendorf ELEX 6361 Hamburg, Germany) using an internal Li standard, Cl^- was analysed by amperometric titration (radiometer CMT10 Copenhagen, Denmark) and osmolality was assessed by freezing point determination (Advanced instruments 3MO; Norwood, MA, USA). All ion measuring apparatus were calibrated before the start and continuously re-calibrated after every 10th sample during the measurements, according to the manufacturers manual, allowing a 1% error. Furthermore, the flame photometer used for analyses of the positive ions was used with an internal lithium standard in every sample to avoid drifting. The interassay variations, expressed as CV (coefficient of variation) during the period of analyses of the present study were 0.37% for Na^+, 2.26% for K^+, 1.03% for Cl^- and 1.32% for osmolality.

In order to study the effects of removal of macromolecules (cut off molecular mass: 30 kDa) on ionic composition, six pools of ovarian fluid were collected, each from several BW females, and stored frozen. Part of each pool was ultra filtered using a micropartition system (Amicon MPS-1, Danvers, MA, USA), in order to remove proteins and other large molecules. Levels of ions and osmolality in ovarian fluid was then measured in the unfiltered and ultrafiltered portions.

Sperm testing fluids

Based on the above analysis of ion concentrations in the natural ovarian fluid of fish from the brackish water habitat, two types of artificial ovarian fluid were made, AOV1 and AOV2. AOV1 contained all the investigated ions, whereas AOV2 only contained NaCl. Both of these fluids were based on Millipore-filtrated water, AOV1 containing 150 mmol l^{-1} Na^+, 158 mmol l^{-1} Cl^-, 4 mmol l^{-1} K^+ and 2 mmol l^{-1} Ca^{2+}, and AOV2 containing only 150 mmol l^{-1} NaCl. Further, a solution containing 245 mmol l^{-1} mannitol (Sigma Chemical Co., St Louis, MO, USA) was prepared in order to test the effect of osmolality alone. The osmolality of this test solution corresponded to the osmolality of ovarian fluid in brackish water fish (245 mosmol kg^{-1}).

Sperm motility was tested in a range of concentrations of natural *versus* artificial ovarian fluid as well as in AOV1 *versus* mannitol solution. As stickleback sperm cannot be stripped by gentle abdominal pressure, the fish were killed by destruction of the brain, the throat was cut and the testes dissected out. Milt was obtained by excision and mincing of the testes. Approximately 1 μl of milt was diluted in 1 ml of the desired medium. Sperm of six randomly chosen BW males were tested individually in each of the following media; seven dilutions of OV (0.75, 1.56, 3.13, 6.25, 12.5, 25 and 50%), four dilutions of AOV1 (3.13, 6.25, 12.5 and 25%), two dilutions of AOV2 (12.5 and 25%) and one control group, in which sperm were diluted in Millipore-filtrated water alone. Since no differences were found between the AOV1 and AOV2 of the first high concentrations tested, 25% and 12.5%, no further dilutions of AOV2 were made in order to reduce the number of fish for ethical reasons. Two pair-wise tests were carried out, using six males in each, where sperm diluted in 25% of the above mannitol solution were compared to sperm diluted in 25% AOV1 and Millipore filtrated water.

Plasma samples

Ten fishes from each of the three habitats were anaesthetized and blood was collected in heparinized capillary tubes (75 mm KEBO Lab., Stockholm, Oslo, Copenhagen) from the severed peduncle. The tubes were centrifuged and the plasma of the 10 fish was pooled in order to get a sufficient amount for analysis. The samples were immediately frozen at –70°C and subsequently analysed as described for the ovarian fluids.

Quantitative analysis of sperm motility

The general testing procedure was similar to that previously described (Kime et al., 1996) and adapted for sticklebacks (Elofsson et al., 2003). Tubes containing the sperm suspension from BW males were kept in a water bath at 20°C. Consecutive samples of the sperm suspensions were taken: immediately (i.e. approximately 20 s) 2, 5, 10 and 30 min and 1, 3, 6, 10 and 24 h after dilution. At each sampling event ~0.6 μl of the sample was placed into a well on a Multitest slide (12-well; ICN, Basingstoke, UK), covered with a coverslip and video recorded using a Sony CCD black and white video camera (XC-75CE, Japan) connected to a Leitz DMR (Leica, Wetzlar, Germany) microscope with a 40× negative phase-contrast objective. At each sampling point, sperm motility was recorded for 1 min. The recordings of sperm movement were analysed at the Institute of Zoology, Zoological Society of London, using CASA (Computer-Assisted Sperm Analysis) on a Hobson Sperm Tracker (Hobson Vision Ltd, Baslow, Derbyshire, UK).

Instrument settings optimised for the three-spined stickleback sperm were: search radius=7.5 μm; refresh time=1 s; thresholds=+25/–100; and filter weightings=1:3,

2:2, 3:1, 4:1. The following parameters were studied; straight line velocity, *VSL* (the straight line distance between the first and last point of the path of a sperm over time), longevity (⩾ the last time when motile sperm was observed) and percentage of motile sperm (the number of motile sperm divided by the sum of the motile plus immotile sperm within the analysis field).

Nest samples after spawning

In order to measure the concentration of ovarian fluid present in the nests at spawning, five BW males were gradually adapted to freshwater. The males were kept individually in 50 l half-filled aquaria where they were allowed to build nests. The five males were allowed to spawn with three females each. These females were kept in brackish water and were only put into the freshwater aquaria to spawn. If this did not happen within a few minutes, the female was removed and replaced with another female. After each individual spawning event, samples of approximately 200 μl were taken from the nests with a Finnpipette (200–1000 μl; Labsystems, Helsinki, Finland) through the entrances, at 0, 5 or 15 min after the eggs had been laid. After each sampling, the eggs were removed and the male was given time to rebuild his nest before the next female was introduced. Control samples of the ambient aquarium water were also taken. The samples were analysed for Na^+, K^+ and Ca^{2+} as described above.

Although the pipetting of samples of nest fluid was carried out carefully, it cannot be excluded that inflow of surrounding water can have diluted the samples. Thus, the ionic levels of nest fluid must be regarded as minimum values.

Effects of macromolecules on ovarian fluid retention within the nests

The effects of macromolecules (cut off molecular mass: 30 kDa) on the retention of ovarian fluid within the nests were tested. Ovarian fluid from ~40 BW females was pooled, and 2.5 ml of the fluid was ultra filtered as above (a separate batch from that used for the ionic measurement). 100 μl, i.e. approximately the amount that is possible to remove from a batch of eggs with a pipette, of the ovarian fluid ultra filtrate or natural ovarian fluid from the same batch was inserted into a newly built nest with a Finnpipette. After 5 or 15 min, samples of 200 μl were withdrawn from the nests as above and analysed for Na^+ content and osmolality. Nest and samples were chosen randomly, with eight in each category, making a total of 32 samples. Since there were no eggs in these nests and since there was no disturbance by the female, these samples are not comparable to the samples taken after natural spawning, but are only relevant to possible effects of macromolecules. A water sample was also taken from each aquarium in order to assess the level of Na^+ in surrounding freshwater.

Statistical analysis

Data were analysed using STATISTICA (StatSoft, Inc., 1998). Data were tested for normality using a Kolmogorov–Smirnov test and sperm motility variables (*VSL* and percentage of motile sperm) were $\log_{10}$ transformed to satisfy parametric assumptions. Longevity data were analysed with a Kruskal–Wallis ANOVA since they could not be transformed to fit parametric assumptions. In testing effects of concentrations of ovarian fluid and artificial ovarian fluids on sperm velocity and percentage of motile sperm, a one-way repeated measurement ANOVA was used with sampling events as the repeated measurement. In comparisons between natural and artificial ovarian fluids, a two-way repeated measurement ANOVA was used to examine both the effects of treatment and concentration. A two-way ANOVA was also used in order to test ultra-filtered ovarian fluid *versus* natural ovarian fluid in the nests. In the pair-wise mannitol tests, a Wilcoxon matched pair test was used for longevity data and Student's *t*-test for dependent samples for velocity and percentage of motile sperm data.

Results

Analysis of ovarian fluid and plasma

The concentrations of Na^+, Cl^-, Ca^{2+} and K^+ in the ovarian fluid and of Na^+, K^+ and Ca^{2+} in the plasma are shown in Table 1. Owing to technical problems, plasma Cl^- values are only available for FW fish. The highest ion concentrations of both ovarian fluid (Table 1A) and plasma (Table 1B) were found in the seawater population and the lowest concentrations in the freshwater population. SW sticklebacks had an osmolality of 314 mosmol kg^{-1} in ovarian fluid and 347 mosmol kg^{-1} in plasma. Brackish water sticklebacks had an osmolality of 246 mosmol kg^{-1} in the ovarian fluid and 301 mosmol kg^{-1} in the plasma and the corresponding values for test the freshwater sticklebacks were 208 and 266 mosmol kg^{-1}, respectively.

Ovarian fluid of sticklebacks adapted to the three different environments showed overall significant differences in osmolality (ANOVA, $F_{2,15}$=15.90, P<0.002), Cl^- ($F_{2,15}$=17.77, P<0.0001), Na^+ ($F_{2,15}$=16.67, P<0.0001), K^+ ($F_{2,15}$=4.03, P<0.04), and Ca^{2+} ($F_{2,15}$=11.81, P<0.0008). A subsequent multicomparison *post-hoc* test revealed that SW and FW fish differed in all measured parameters (Tukey's HSD, P<0.05), whereas between FW and BW living fish there were only differences in the levels of Cl^- and Ca^{2+} (P<0.05), and between BW and SW living fish there were differences in osmolality, Cl^- and Na^+ levels (P<0.05).

Ionic concentrations and osmolality of ultra filtered ovarian fluid and non-filtrated, i.e. natural ovarian fluid are shown in Table 2. Non-filtered fluid was similar to that from BW females in Table 1A. Osmolality was more than two times higher in ultra filtered ovarian fluid compared to natural (719 mosmol kg^{-1} in filtrated, 316 in the non filtrated), whereas the concentration of Na^+ ions was slightly lower (79%) in the ultra filtrated than in the natural ovarian fluid (ANOVA; $F_{1,10}$=11.66, P<0.007). No differences was found in concentrations of K^+, Cl^- and Ca^{2+} (ANOVA; K^+; $F_{1,10}$=2.19, P=0.170, Cl^-; $F_{1,10}$=0.63, P=0.447, Ca^{2+}; $F_{1,10}$=2.44, P=0.1496).

Table 1A. *Concentrations of Na^+, Cl^-, Ca^{2+} and K^+ in ovarian fluid of freshwater, brackish water and sea water sticklebacks*

Population	Osmolality (mosmol kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	K^+ (mmol l^{-1})	Ca^{2+} (mmol l^{-1})
FW: Freshwater	208±25	136±12	102±18	2.12±1.32	1.58±0.34
BW: Brackish water	246±26	150±11	136±7	3.53±1.72	2.16±0.32
SW: Seawater	314±47	203±30	171±36	3.67±0.90	2.64±0.71

Values are mean ± s.d., *N*=6.

Table 1B. *Concentrations of Na^+, Cl^-, K^+ and Ca^{2+} in plasma pools of 10 female sticklebacks*

Population	Osmolality (mosmol kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	K^+ (mmol l^{-1})	Ca^{2+} (mmol l^{-1})
Freshwater	266	157	134	3.37	3.68
Brackish water	301	164	–	5.17	1.92
Seawater	347	179	–	8.12	7.74

N=10 for each population.
Because of technical difficulties, Cl^- could not be measured for brackish and seawater species.

Nest sampling after spawning

The results of nest samplings are shown in Table 3. The concentrations of Na^+ ions were higher within the nest than in the surrounding water for at least 15 min after spawning. Samples taken from the nests 5 min after spawning contained ~16.8 mmol l^{-1} Na^+, corresponding to ovarian fluid from BW fish diluted to 11.2%, whereas 15 min after spawning Na^+ concentrations were ~4.8 mmol l^{-1}, which corresponds to the concentrations in ovarian fluid of brackish water fish, diluted to 3.2%

Effects of natural and artificial ovarian fluids at different concentrations

Sperm showed a strong response to natural ovarian fluid, both in longevity [Kruskall–Wallis ANOVA H (7, N=48)=46.29, P<0.001], VSL (ANOVA, $F_{7,40}$=143.02, P<0.001) and percentage motile sperm ($F_{7,40}$=1555.93, P<0.001) with higher ovarian fluid concentrations resulting in higher sperm motility values. As shown in Fig. 1A and Fig. 2A, even dilutions of ovarian fluid as low as 0.75% and 1.56% were effective in prolonging sperm motility. In ovarian fluid, sperm longevity was ⩾10 min in dilutions of 0.75, 1.56 and 3.13%, ⩾1 h in a dilution of 6.25% and ⩾10–24 h in 12.5%, 25% and 50%.

Table 2. *Concentrations of Na^+, K^+ and Ca^{2+} present in the nests after spawning*

Time after spawning (min)	Na^+ (mmol l^{-1})	K^+ (mmol l^{-1})	Ca^{2+} (mmol l^{-1})
0	19.38±12.46 (12.9%)	0.70±0.30	1.52±0.89
5	16.78±6.89 (11.2%)	0.55±0.25	1.01±0.31
15	4.78±0.80 (3.2%)	0.22±0.18	0.73±0.09
Ambient water	1.40±0.85	0.11±0.04	0.52±0.29

Values are means ± s.d.; *N*=5. Na^+ concentrations also given as a percentage of the concentration in ovarian fluid. The fish used in experiment were brackish water sticklebacks spawning in freshwater.

Sperm also showed a strong response to the two artificial ovarian fluids, both in longevity [AOV1: H (4, N=30)=28.67, P<0.001 and AOV2: H (2, N=18)=15.81, P<0.001], VSL (AOV1: $F_{4,25}$=76.05, P<0.001 and AOV2: $F_{2,15}$=221.95, P<0.001) and percentage of motile sperm (AOV1: $F_{4,25}$=1460.37, P<0.001 and AOV2: $F_{2,15}$=1848.67, P<0.001), with higher ion concentrations resulting in higher sperm motility values (Fig. 1B and Fig. 2B). In artificial ovarian fluid (AOV1), the longevity was ⩾15 min in a dilution of 3.25%, ⩾1 h in 6.25%, and ⩾10–24 h in 12.5 and 25%. In the other artificial ovarian fluid (AOV2), the longevity was ⩾10–24 h in dilutions of 12.5 and 25%.

Comparison between natural and artificial ovarian fluids

Natural and artificial ovarian fluids were compared at dilutions of 25 and 12.5%. There were no differences in sperm response between the natural and the two artificial ovarian fluids tested at a 25% dilution either in *VSL* ($F_{2,15}$=2.95, P=0.08) or percentage motile sperm ($F_{2,15}$=0.11, P=0.89). The same pattern was observed for *VSL* ($F_{2,15}$=0.35, P=0.70) and percentage of motile sperm ($F_{2,15}$=0.93, P=0.41) using a 12.5% dilution. Comparisons between the natural ovarian fluid and AOV1 at all dilutions (3.13, 6.25, 12.5 and 25%) and sampling times showed that there were no differences in sperm response between the two fluids either regarding *VSL* ($F_{1,40}$=0.78, P=0.383) or percentage of motile sperm ($F_{1,40}$=1.30, P=0.261).

In terms of longevity, sperm diluted in AOV1 to 3.13% survived for 5 min longer than those in a 3.13% dilution of natural ovarian fluid [H (1, N=12)=11.00, P=0.001], but this difference was not found in any of the other dilutions tested.

Effects of mannitol solution

Sperm were motile for approximately 2 min in Millipore

Table 3. *Concentrations of Na^+, Cl^-, K^+ and Ca^{2+} in the ultra and non filtrated ovarian fluid from brackish water sticklebacks*

Ovarian fluid	Osmolality (mosmol kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	K^+ (mmol l^{-1})	Ca^{2+} (mmol l^{-1})
Ultrafiltrated	719±12.0	148.7±2.5	126.0±5.2	1.96±0.36	1.76±0.30
Non filtrated	–	187.2±27.5	130.2±11.8	2.44±0.73	2.22±0.64

Values are means ± s.d., *N*=6.

filtrated water, ⩾30 min in mannitol solution and ⩾10 h in artificial ovarian fluid. Hence, there were significant differences in sperm longevity between the media (Wilcoxon matched pair tests; mannitol solution *versus* Millipore filtrated water: N=6, Z=2.201, P=0.028, and mannitol solution *versus* artificial ovarian fluid: N=6, Z=2.201, P=0.028). There were, however, no differences (P>0.05) in percentage motile sperm between mannitol solution and Millipore water at times 0 and 2 min as well as between mannitol solution and artificial ovarian fluid at times 0, 2, 5, 10, 15 and 30 min. See Fig. 2C.

Sperm velocity also differed between the media; see Fig. 1C. Immediately after dilution, sperm in mannitol solution had a higher velocity than sperm in Millipore filtrated water (17.53±1.64 μm s^{-1} *versus* 6.52±1.16 μm s^{-1}, Student's t-test for dependent samples; t=5.058, d.f.=5 P=0.0039). The difference between the media remained after 2 min (t=4.88, d.f.=5, P=0.0045) and after 5 min all motility had ceased in Millipore-filtrated water.

Sperm velocity was higher in the artificial ovarian fluid than in the mannitol solution from 5 min and onwards. Before 5 min the velocity were the same in AOV1 and mannitol solution (19.9±3.4 μm s^{-1} in AOV1 and 12.5±2.0 μm s^{-1} in mannitol; t=2.47, d.f.=5, P=0.056). After 5 min, however, sperm velocity was significantly lower in the mannitol solution than in the artificial ovarian fluid (17.2±1.9 μm s^{-1} *versus* 9.8±2.2 μm s^{-1}, t=3.44, d.f.=5, P=0.018) and continued to be so after 15 min (16.4±1.7 μm s^{-1} *versus* 3.3±0.7, t=5.89, d.f.=5, P=0.002), see Fig. 1C. Whereas almost all sperm were completely immotile in mannitol solution after 30 min (0.63±0.21 μm s^{-1}), the motility persisted in the artificial ovarian fluid for at least 10 h, when the velocity was still 8.86±2.51 μm s^{-1}.

Effects of proteins on ovarian fluid retention within the nest

The ultra filtered ovarian fluid and natural ovarian fluid was introduced into nests and sampled after 5 and 15 min. Na^+ levels and osmolality, at both time points, were significantly higher in the nests where natural ovarian fluid was inserted compared to the nest where ovarian fluid ultra filtrate was inserted (Table 4; two way ANOVA; Na^+: $F_{1,28}$=164.14, P<0.001, osmolality: $F_{1,28}$=735.60, P<0.001). There were no differences in Na^+ levels or osmolality between the two time points within either natural ovarian fluid or ultra filtered ovarian fluid (Na^+: $F_{1,28}$=2.74, P=0.109, osmolality: $F_{1,28}$=1.31, P=0.263).

Discussion

Ions in ovarian fluid

We found ionic concentrations and osmolalities in ovarian fluid of sticklebacks of different habitats in the same range as previously reported for the elkhorn sculpin, *Alcichthys alcicornis* (Koya et al., 1993), the bleak, *Alburnus alburnus* (Lahnsteiner et al., 1995) and salmonids (Lahnsteiner et al., 1997b). The Na^+ and Cl^- levels in ovarian fluid from sticklebacks of unstated origin and environment reported in an earlier study (Thomopoulos, 1953) were within the range of our values, whereas the K^+ levels were higher (10 mmol l^{-1}) than we found in our study. The concentrations of ions (except Na^+ in seawater adapted sticklebacks) were slightly lower in the ovarian fluid than in plasma, but in both fluids, the ion levels increased with increasing salinity of the ambient water. This increase in ionic concentrations with increasing salinity was consistent with a previous study on salinity tolerance in sperm from sticklebacks adapted to different salinities (Elofsson et al., 2003). Sperm from SW-adapted sticklebacks showed higher salinity tolerance then sperm from FW-adapted fish.

Effects of natural and artificial ovarian fluid at different concentrations

As previously found (Elofsson et al., 2003), ovarian fluid had a motility enhancing effect on stickleback sperm. In the present study, this effect was found to exist already at concentrations as low as 0.75 and 1.56% of ovarian fluid, although the effect increased with a higher concentration of ovarian fluid.

There were, however, no differences in prolonging effects between the natural ovarian fluid and the two ionic artificial ovarian fluids. The two artificial ovarian fluids had an equally prolonging effect on sperm longevity, velocity and percentage motility as the natural ovarian fluid. The non-ionic mannitol solution, however, did not have the same sperm motility-extending effect as the artificial ovarian fluid at the same osmolality. Both longevity and velocity was substantially lower in mannitol solution than in the artificial or natural ovarian fluids. In the mannitol solution sperm remained motile for a maximum period of 30 min, whereas natural or artificial ovarian fluid with the same osmolality allowed sperm to stay motile for 10 h. This suggests that the osmolality of the ovarian fluid only explains part of the fluid's prolonging effect. It is instead the Na^+ and Cl^- ions in the fluid that produce the main effect.

That the ions of the ovarian fluid are at least partly responsible for prolonging sperm motility has previously been

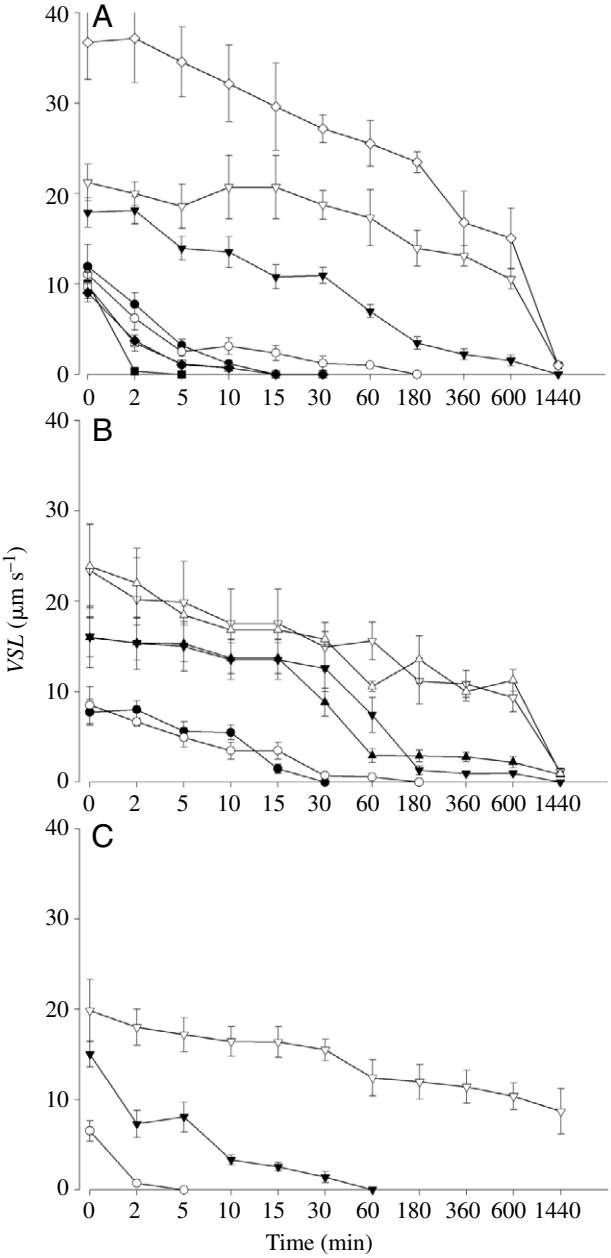

Fig. 1. (A) Straight line velocity (*VSL*) of stickleback sperm diluted in different concentrations of ovarian fluid: 0% (black squares) , 0.75% (white squares), 1.56% (filled diamonds), 3.125% (filled circles), 6.25% (open circles), 12.5% (downward pointing filled triangles), 25% (downward pointing open triangles), 50% (open diamonds). (B) *VSL* of stickleback sperm diluted in different concentrations of artificial ovarian fluid (AOV1 3.125% (filled circles), AOV1 6.25% (open circles), AOV1 12.5% (downward pointing filled triangles), AOV1 25% (downward pointing open triangles), AOV2 12.5% (upward pointing filled triangles) AOV2 25% (upward pointing open triangles). For A and B, *N*=6 for all groups. (C) *VSL* of stickleback sperm diluted in artificial ovarian fluid (AOV1) 25% (*N*=6) (downward pointing open triangles), Millipore filtrated water (*N*=6) (open circles), and mannitol solution (mean, *N*=12) (downward pointing filled triangles). Values are mean ± s.e.m. For statistics, see Results. Note that the scale of the *x*-axis is not linear.

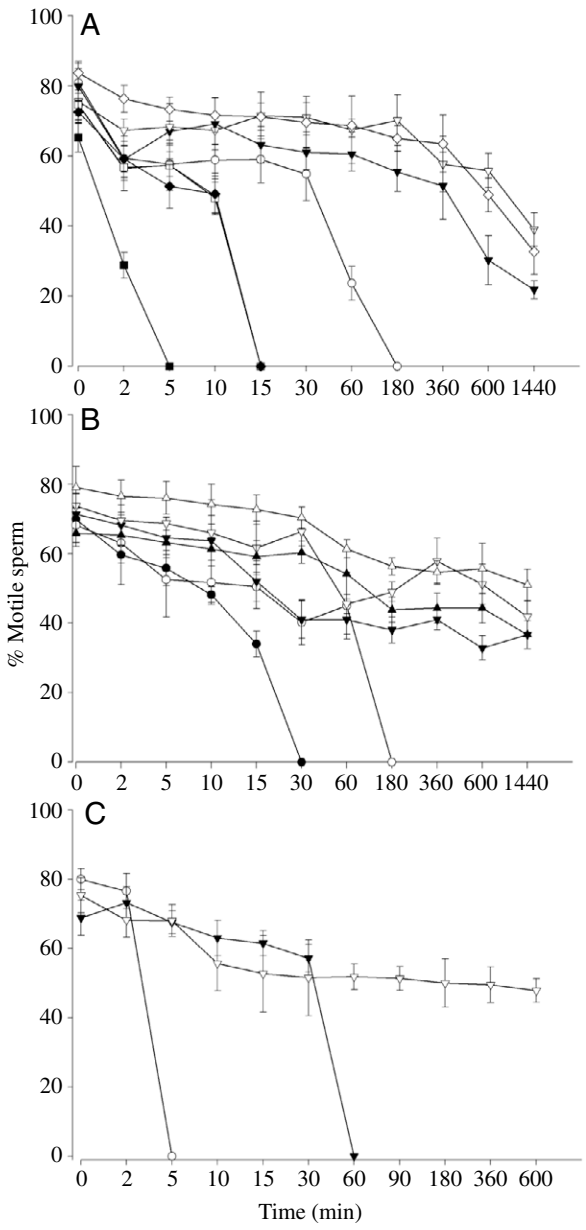

Fig. 2. (A) Percentage of motile stickleback sperm, when diluted in different concentrations of ovarian fluid: 0% (black squares), 0.75% (white squares), 1.56% (filled diamonds), 3.125% (filled circles), 6.25% (open circles), 12.5% (downward pointing filled triangles), 25% (downward pointing open triangles), 50% (open diamond). (B) Percentage of motile stickleback sperm diluted in different concentrations of artificial ovarian fluids. AOV1 3.125% (filled circles), AOV1 6.25% (open circles), AOV1 12.5 (downward pointing filled triangles), AOV1 25% (downward pointing open triangles), AOV2 12.5% (upward pointing filled triangles), AOV2 25% (upward pointing open triangles). For A and B, *N*=6 in all groups. (C) Percentage of motile stickleback sperm diluted in artificial ovarian fluid (AOV1) 25% (*N*=6) (downward pointing open triangles), Millipore filtrated water (*N*=6; open circles), and mannitol solution (mean, *N*=12) (downward pointing filled triangles). Values are means ± s.e.m. For statistics, see Results. Note that the scale of the *x*-axis is not linear.

Table 4. *Nest samples after insertion of ovarian fluid ultra filtrate or natural ovarian fluid*

Time after insertion (min)	Na^+ (mosmol kg^{-1})		Osmolality (mosmol kg^{-1})	
	Natural ovarian fluid	Ovarian fluid ultra filtrate	Natural ovarian fluid	Ovarian fluid ultra filtrate
5	21.6±7.2	5.4±3.3	64.8±22.9	9.3±7.9
15	20.1±5.4	5.2±4.2	62.2±19.1	9.6±3.4

Values are means ± s.d. of Na^+ concentrations and osmolality present in the nest after spawning. *N*=8.

suggested in a number of studies on fishes (Koya et al., 1993; Morisawa, 1994; Turner and Montgomerie, 2002; Cosson, 2004). Sperm motility in the brown trout *Salmo trutta* was stimulated by undiluted ovarian fluid and fluid with ionic composition similar to undiluted ovarian fluid had a similar effect (Lahnsteiner, 2004). The biological relevance of this is unclear, the effectiveness of natural ovarian fluid rapidly declined with dilution (only undiluted ionic fluid was tested). It is not known if the concentrations of ovarian fluid present around the eggs at spawning have any effect on sperm motility in the time span at which fertilisation occurs.

The mechanisms by which the ions exert their effect on stickleback sperm are still unclear, but sperm are sensitive to changes in their ionic milieu and alterations in ion concentrations and/or osmolality have been shown to regulate the ability of sperm to move (Takai and Morisawa, 1995; Morisawa, 1994; Cosson, 2004).

Nest samples

Sperm motility in the three-spined stickleback lasts for 1–2 min in Millipore-filtered water, as well as in natural FW (Elofsson et al., 2003). Nevertheless, fertilisation experiments show that it takes 15 min, or more, for all of the eggs in a stickleback batch to be fertilised (Zbinden, 2002). In freshwater alone, the longevity of the stickleback's sperm would therefore be insufficient. However, the results of the nest samples show that ions from the ovarian fluid remain in the nest for the critical period. The concentrations of Na^+ ions sampled in nests 5 and 15 min after spawning corresponds to, respectively, 11.2 and 3.2% of the concentration in natural ovarian fluid. Sperm could remain motile for a day in a 12.5% dilutions of natural ovarian fluid, for at least an hour in a 6.25% dilution and for at least 10 min in a 3.125% dilution. This indicates that total duration of sperm motility in the gradually diminishing salinity in the nest should last at least 15 min.

Thus, the ovarian fluid is of biological importance for the fertilization of stickleback eggs in freshwater. Whether this is also the case in other fishes is not known. Sperm of the freshwater bullhead sculpin *Cottus gobio* (Lahnsteiner et al., 1997a), like stickleback sperm, are motile for less than a minute in freshwater, but this is extended to several hours in the presence of ovarian fluid or in a Na^+Cl^- solution. Other sculpines such as the marine elkhorn sculpin *Alcichthus alcicorni* and Gilbert's Irish lord *Hemilepidotus gilberti*, also have a prolonged period of sperm motility in the presence of ovarian fluid (Koya et al., 1993; Hayakava and Munehara, 1998).

Effects of macromolecules on ovarian fluid retention within the nests

Besides ions, the stickleback's ovarian fluid also contains macromolecules, probably glycoproteins, making the fluid highly viscous. Although the results from the sperm motility tests in different AOVs does not suggest that these proteins affect sperm motility *per se*, the samples taken from the nests after ovarian fluid ultra filtrate or ovarian fluid had been inserted, show that these macromolecules may have other functions. The ultra filtration procedure separates the free fraction of different ions, i.e. the combination of free and low affinity complex bound ions (Toffaletti and Bowers, 1979), from the protein bound fraction. Thus, the approximately doubled osmolality of the ultra filtrated ovarian fluid compared to the non filtered fluid suggests that a large proportion of non-protein bound ions and smaller molecules were separated from the proteins during the ultrafiltration procedure. For Na^+, the concentration recovered in the ultra filtrate was 79% of that in the non filtered ovarian fluid, indicating a protein bound fraction of Na^+ only in the range of 20% within natural ovarian fluid. The removal of the macromolecules from the ovarian fluid resulted in a significantly lower levels (~25%) of Na^+ ions from the nests to the surrounding FW. These results together suggest a role for the ovarian fluid proteins in retaining ions within the ovarian fluid, not only by the actual binding to the proteins but also through low affinity complex and 'trapping' of ions within the highly viscous fluid. This effect of ovarian fluid macromolecules in retaining ions is probably beneficial for sperm motility in the three-spined stickleback, especially in freshwater.

To conclude, in this study we show that the effect of ovarian fluid in prolonging sperm motility in sticklebacks is of biological importance. The effects on sperm motility can be explained by the content of ions (Na^+ and Cl^-) in the fluid alone, but the macromolecules in the ovarian fluid have a role in retaining these ions within the nest.

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