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Egg-sperm interaction in sturgeon: role of ovarian fluid

Vitaliy Kholodnyy · Borys Dzyuba · Hermes Gadêlha · Jacky Cosson · Sergii Boryshpolets

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Abstract Fertilization of freshwater fish occurs in the environment which negatively affects a lifespan of gametes mostly due to the osmotic shock; therefore, male gametes should reach the female gamete, as soon as possible. The existence of mechanisms controlling the encounter of gametes would be highly expedient in this case. By analogy with other species for which guidance was demonstrated, it is likely that this control may be performed by ovarian fluid or substances released by eggs. The aim was to study the effect of ovarian fluid and egg-released substances on spermatozoa behavior in sterlet. It was found that the presence of a particular concentration of ovarian fluid (30% solution in water) had an inhibiting effect on spermatozoa motility initiation. Lower concentrations of the ovarian fluid improved the longevity of spermatozoa and did not affect their trajectories. Test of chemotactic response (using a microcapillary injection of fluids into the suspension of motile spermatozoa) showed no effect of ovarian fluid on spermatozoa behavior, while at the same time, the attracting effect of the egg-conditioned medium was evident (i.e., due to some substances released from the eggs during their contact with freshwater). The results of the fertilization test showed that the presence of ovarian fluid prevented the eggs from losing the fertilizing ability due to the contact with water, as well as promoted the spermatozoa to fertilize the eggs during a longer period of time. Thus, the combined physicochemical action of "female factors" affects sterlet gametes during fertilization and may be involved in the guidance and selection mechanisms.

Keywords Sperm motility · Fertilization · Ovarian fluid · Egg water · Chemotaxis · *Acipenser ruthenus*

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V. Kholodnyy (✉) · B. Dzyuba · H. Gadêlha · J. Cosson · S. Boryshpolets
University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25, Vodňany, Czech Republic
e-mail: vkholodnyy@frov.jcu.cz

H. Gadêlha
Department of Engineering Mathematics, University of Bristol, Bristol BS8 1UB, UK

Introduction

In externally fertilizing fish, eggs and sperm are released into the water and thereafter fertilization usually occurs without any participation of parents. The aqueous environment is adverse and deleterious for the gametes; therefore, the lifespan of activated spermatozoa is quite short, especially in freshwater, where the fish male gametes should reach their target, the female gamete, as soon as possible because the spermatozoa become damaged within several minutes mostly due to an osmotic shock (Morisawa 1985; Hart 1990), and with the eventual contribution of other factors, e.g., oxidative

stress (Dzyuba et al. 2015). Under such conditions, reproductive success is chronically limited by the ability of spermatozoa to find the egg and reach a fertilization site (micropyle) (Amanze and Iyengar 1990). Two general explanations have been proposed: (1) external fertilization in fish involves specific triggers and controls over spermatozoa behavior and (2) external fertilization conforms to a model of fair raffle behavior, i.e., it occurs through release and simple dispersion of a large number of spermatozoa. Another postulation is the gamete guidance hypothesis (Eisenbach and Giojalas 2006), which is based on the ability of sperm cells to sense and react to the changes of the environment, from a fluid viscosity and background flows to pH, ion concentration, and even temperature. Currently, the widely accepted hypothesis is the "cryptic female choice" (Firman et al. 2017) in which complex of female-driven processes shift the fertilization success towards specific males. Recently, Kekäläinen and Evans (2018) introduced a "gamete-mediated mate control", postulating that the encounter of gametes is mediated by a complex chemical dialogue between gametes of both sexes.

Ovarian fluid (OF), which surrounds fish eggs during spawning, is the best candidate to provide control over fish male gametes guidance, as well as to create an environment for the cryptic female choice or the gamete-mediated mate control. In several species, it was shown that the composition of ions, proteins, amino acids, sugars, among other factors is ideal for supporting and protecting eggs and sperm against the deleterious effect of freshwater. The ovarian fluid of brown trout was found to significantly prolong the time period during which either female or male gamete could be fertilized or fertilize, respectively (Lahnsteiner 2002). It was shown as well (e.g., in stickleback, rainbow trout, Chinook salmon) that some unidentified agents that are contained in ovarian fluid or released by the eggs could significantly affect the behavior of male gametes and, consequently, influence the outcome of fertilization (Elofsson et al. 2003b; Wojtczak et al. 2007; Johnson et al. 2014).

Generally, the way how the gametes are activated, as well as the process of how they encounter, may differ in representatives of various taxa due to the evolutionary changes and indeed the reproduction strategy employed by each species (Kholodnyy et al. 2020). In this respect, the sturgeons are good model species to study the evolution of gamete interaction mechanisms as the Acipenceriformes is one of the early diverged order of extant fishes. This taxonomic position of sturgeons is associated with a very specific structure of the reproductive organ; in particular, testes have a tight connection with kidneys and spermatozoa are getting matured (acquiring the ability to move and fertilize) when mixing with hypotonic urine (Dzyuba et al. 2019). This causes the low osmolarity of seminal fluid in sturgeons, and its variability from 50 to 100 mOsm/l, depending on the rate of dilution with the urine. This specific feature is supposed to be a reason for the high osmotolerance of sturgeon spermatozoa comparing with other freshwater fish species (Bondarenko et al. 2013; Dzyuba et al. 2014). The activation of motility in sturgeon spermatozoa is potassium dependent; i.e., there is a certain concentration of K^+ ions, which block its initiation (Alavi et al. 2019). In addition, the acipencerid gametes possess specific morphological features, i.e., presence of an acrosome in spermatozoa (an extremely rare feature in externally fertilizing fishes) (Psenicka et al. 2009) or multiple micropyle on the animal pole of the egg (other fish have only one micropyle) (Debus et al. 2002).

Thus, we suppose that specific features of gamete physiology in sturgeons may lead to taxa-specific egg-sperm interaction mechanisms. That is why our study is focused on finding peculiarities for the effect of ovarian fluid on spermatozoon physiological activity of sterlet *Acipenser ruthenus* during the reproductive process having in mind to ascertain the role of "female factors" during fertilization. To do this, the sperm cell behavior will be investigated in different media including those containing ovarian fluid or "egg-conditioned medium" (i.e., medium containing substances released by eggs during incubation in water). The study of the behavior of spermatozoa will include the assessment of changes in the motility traits (velocity and path linearity), presence or absence of chemotactic response, and will be discussed in a view of the composition of the used media. In addition to motility and chemotaxis assays, the study includes in vitro fertilization procedure.

Materials and methods

Ethics statement

Manipulations with animals were performed according to authorization for breeding and delivery of experimental animals (reference number: 56665/2016-MZE-17214 17OZ19180/2016-17214, valid from the 4

October, 2016 for 5 years) and the authorization for the use of experimental animals (reference number: 2293/2015-MZE-17214 16OZ22302/2014-17214, valid from 22 January 2015 for 5 years) issued to the Faculty of Fisheries and Protection of Waters, the University of South Bohemia by Ministry of Agriculture of the Czech Republic.

Fish broodstock, gamete, and fluid collection

The experiments were performed using mature sterlets (6–7 years, 2–3 kg) kept at the Genetic Resource Centre of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Altogether, we have used 10 females: 5 for collecting the ovarian fluids for the motility experiments and fluid composition analysis and 5 females for the in vitro fertilization test; and 17 males: 7 for the analysis of spermatozoon motility traits, 5 individuals for collecting sperm for the chemotactic tests, and 5 males were used for the in vitro fertilization test

Before starting the experiment (February–March 2018, February 2019), the fish were transferred to indoor tanks equipped with a temperature-controlled water recirculation system and constant air supply. The temperature of the water was stepwise increased from 2 to 14 °C by 1 degree per day. Before the sperm collection (36 h prior to stripping), the males were treated by an intramuscular injection of homogenized carp pituitary in 0.9% (w/v) NaCl (Sigma-Aldrich, USA) solution (4 mg/kg body weight). The females were injected with homogenized carp pituitary in 0.9% (w/v) NaCl solution twice: 36 h (0.5 mg/kg of body weight) and 24 h prior to stripping (4.5 mg/kg of body weight) (Dettlaff et al. 1993). Sperm was collected by catheterization from the urogenital papilla to 100-ml cell culture containers and stored on ice (4 °C) until use. Ovulated eggs were collected into dry plastic bowls and stored at 15 to17 °C. The ovarian fluid was collected using a pipette, centrifuged to remove debris, and stored on ice in closed plastic tubes or was frozen and stored at − 80 °C if not used on the same day. Fresh eggs were used for preparing egg-conditioned medium ("egg water") to estimate the specific effect on the behavior of spermatozoa caused by eggs themselves (Pillai et al. 1993): the eggs were thrice washed by 0.9% NaCl solution and then placed to the equal amount of distilled water. After 15 min of incubation at the room temperature with periodic gentle shaking, the medium was collected and stored at 4 °C or frozen and stored at − 80 °C. Collection of substances, which may be released from the egg membrane to water, was the main goal of the incubation (Niksirat et al. 2017); thus, only the media which had no destroyed eggs was collected for further use. Osmolarity, pH, protein, and ion content in the ovarian fluid and the egg water were assessed. Osmolarity was measured using a freezing point osmometer Osmomat 3000 (Gonotec GmbH, Germany) and was expressed in milliosmoles per liter. Concentrations of sodium and potassium ions were measured by potentiometry using ion-selective electrodes (Bayer HealthCare, Tarrytown, NY, USA). Calcium ion concentration was measured by absorption photometry applying the o-cresolphthalein complexone method (Moorehead and Biggs 1974). The ion concentration is expressed in millimoles per liter of medium. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, USA) and shown in milligram per milliliter. The measurements of the protein and ion contents were done in the range of standard calibration curves, appropriate to the used method.

Motility observation and recording

Sperm suspensions from 7 males were carefully mixed with 40 μl of tested solutions (around 0.1 to 0.5 μl were introduced depending on spermatozoa concentration to have 50–300 spermatozoa in the vision field) and motility (if present) was recorded post-activation using ISAS digital camera (PROISER, Spain) set at 25 frames/s and microscope (UB 200i, PROISER, Spain) with phase contrast at 17 °C (controlled by cooling stage (Semic, Poland)). The records were done in three replicates. The duration of the records was 5 min.

The records were analyzed with ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) using CASA plugins (Wilson-Leedy and Ingermann 2007; Purchase and Earle 2012). The analysis was performed if the decline in the percentage of motile cells did not reach 10% (to have an adequate number of cells for performing motility analysis). Values of spermatozoa velocity, the linearity of the spermatozoa trajectories, and the pattern of motility (1–2-s tracks of individual spermatozoa in the vision field) were obtained.

Media used for spermatozoa activation

Motility of spermatozoa was tested in the distilled water, 10 mM Tris HCl (Sigma-Aldrich, USA) buffered solutions of NaCl (10, 20, 30, 40, 50 mmol/l), ovarian fluid and its dilutions with water (10, 20, 30, 40, and 50%), and the egg water (Table 1).

Sperm chemotaxis tests

Sperm from 5 males was used in these experiments. The chemotactic reaction of spermatozoa was assessed by analysis of cell behavior following the injection of tested fluids with glass microcapillaries into the medium with activated cells under the microscope, which is a sort of accumulation assay conventionally applied for simple spermatozoa chemotaxis analysis. To do this, glass microcapillaries (G100, Narishige, Japan) were pooled (PC-100 puller, Narishige) to get microneedles with tips of ~ 20 μm outer diameter, which were additionally cut by a microgrinder (EG-401, Narishige) to have uniform tip openings. The microcapillary was filled with test fluid and assembled to a microinjector (CellTram Vario, Eppendorf, Germany), then fixed on a holder (Narishige), and adjusted above a specimen glass on a microscope table. The microinjector pressure was applied to ensure the slow discharge of the fluid. A drop of the activation medium (40 μl) was placed on the glass, spermatozoa were activated in the drop, and the microneedle with discharging fluid was introduced. The behavior of the spermatozoa near the tip of microcapillary was observed directly under the microscope and video-recorded for 2 min. The resulting records were then processed by CASA plugin for ImageJ to get the tracks of spermatozoa, and these patterns of motility were thereafter analyzed.

In vitro fertilization

The in vitro fertilization was performed in February 2018 (and additional tests were done in February 2019). Eggs from 5 females were pooled in equal ratio, as well as the ovarian fluid from the same individuals. Pooled sperm from 5 males with motility 80% and higher was used. Two types of test were done: the first, to check if the OF protects the eggs while being in the water before contact with the spermatozoa and the second if the OF presence has the effect on spermatozoa performance. The experimental groups are shown in Table 2. In all cases, 2 g of eggs (approx. 135 eggs) was fertilized by 3 μl of sperm (spermatozoon concentration was 4.06×10^8/ml) mixed in 8 ml of water from the hatchery supply system. The temperature of the water was at 17 °C. In some cases, the eggs were washed with 0.9% NaCl solution three times during 10 s to remove the ovarian fluid. In the first series, the eggs (washed/non-washed, with/without OF) were put to the plastic beaker and poured with the test solution, and

Table 1 Solutions used for sterlet spermatozoa activation and/or chemotaxis tests

Solution		Used for activation/chemotaxis (injected fluid) tests	Osmolarity, mOsm/l	pH
Distilled water		Activation/chemotaxis	~ 0	
10 mM Tris HCl buffer		Activation	10	8
Ovarian fluid		Activation/chemotaxis	200	~ 8
Ovarian fluid in water	50%	Activation/chemotaxis	100	~ 8
	40%	Activation	80	~ 8
	30%	Activation	60	~ 8
	20%	Activation	40	~ 8
	10%	Activation	20	~ 8
NaCl solution (Tris buffer)	50 mmol/l	Activation	100	8
	40 mmol/l	Activation	80	8
	30 mmol/l	Activation	60	8
	20 mmol/l	Activation	40	8
	10 mmol/l	Activation	20	8
Egg-conditioned medium		Activation/chemotaxis	48	7

Table 2 Effect of ovarian fluid on fertilization performance in sterlet: experimental design

Group			Incubation time, minutes	Details
Effect on eggs	Control conditions		0, 2, 5, 10, 30, 60	Non-washed eggs incubated in water then sperm added
	Washed eggs		0, 2, 5, 10	Eggs were thrice washed with 0.9% NaCl solution, incubated in water then sperm added
	Washed eggs and 10% of ovarian fluid added back		0, 2, 5, 10	Eggs were washed thrice washed with 0.9% NaCl solution, and ovarian fluid in an amount of 10% egg weight was added back; the eggs were incubated in water then sperm added
	Washed eggs and 50% of ovarian fluid added back		0, 2, 5, 10	Eggs were washed thrice washed with 0.9% NaCl solution, and ovarian fluid in an amount of 50% egg weight was added back; the eggs were incubated in water then sperm added
Effect on sperm	No ovarian fluid in the activating solution	Non-washed eggs Washed eggs	0, 2, 5	Sperm was activated in water and after 0, 2, 5 min post-activation added to washed/non-washed eggs
	2.5% ovarian fluid in water	Non-washed eggs Washed eggs	0, 2, 5	Sperm was activated in water with 2,5% OF (amount corresponds to 10% of egg batch weight) and after 0, 2, 5 min post-activation added to washed/non-washed eggs
	12.5% ovarian fluid in water	Non-washed eggs Washed eggs	0, 2, 5	Sperm was activated in water with 12.5% OF (amount corresponds to 50% of egg batch weight) and after 0, 2, 5 min post-activation added to washed/non-washed eggs

after a certain time (0, 2, 5, 10, 30, 60 min), the sperm was introduced. In the second series, the sperm was mixed with test solutions and after 0, 2, and 5 min added to the eggs in the plastic beakers; if the eggs were treated, the washing was performed keeping the same time limits before mixing with the sperm in all cases. In both series, the beakers were then placed onto shaker (around 100 rpm) and after a 2-min incubation, the eggs were transferred to glass Petri dishes. The dishes were thereafter settled into a tank with baskets for further incubation at 17 °C. The tank had a closed water circuit with aeration, UV-treatment, and temperature control. All fertilization trials were done in three replicates. Taking into account specific features of sturgeon fertilization and embryonic development, such as a risk of detrimental polyspermic fertilization, the possibility of parthenogenetic development, and only a short time period being available for the correct estimation of actual fertilization rate (Dettlaff et al. 1993), the outcome of in vitro fertilization procedure was assessed in 3 days by the amount of embryo reached neurulation stage: termed as embryonic development rate; i.e., the amount of eggs reached the neurulation stage divided by the total amount of eggs.

Statistical analysis

Measurements of the protein and ion contents in the OF and egg water were done in the samples obtained from 5 females. The data are presented as mean ± standard deviation (SD).

Assessment of the motility parameters in different activation media was conducted in triplicates for 7 males; the traits were obtained from the motility measurement from 50–300 spermatozoa per replicate per time point during 10–299 s post-activation with a 10-s increment. Curvilinear velocity (VCL) and path linearity (LIN) were chosen as the indices reflecting typical changes in the motility in various conditions. The motility parameters were then $\log_e$ transformed to ensure a normal distribution of the data, and analyses of interactive effects among variables were performed using Factorial ANOVA in Statistica v. 13 (TIBCO Software Inc., USA). Media and post-activation time were considered as independent variables and VCL or LIN as dependent ones. In case of significant interaction between independent variables (i.e., the difference in spermatozoa behavior in various media along motility time was present), we have conducted pair-wise analyses between several media. The data on VCL and LIN are presented as means with corresponding confidence intervals. The data for spermatozoa velocities in 5 media (water, 10 and 20% ovarian fluid, and NaCl solutions with the corresponding osmolarity, i.e., 20 and 40 mOsm/l) were used then to obtain linear regression dependencies in GraphPad Prism version 6 for Windows software (La Jolla, CA, USA), and the following parameters were obtained: slope (A), intercepts with x and y axes (B and C), coefficient of determination (R^2). The hypothesis for the equality of regression slopes was checked by the t test with the Bonferroni correction using Statistica software.

The embryonic development rate was assessed in three replicates per experimental point. The values of the percentage of developing embryos were expressed as the mean ± SD. The data were then processed by parametric ANOVA followed by Tukey's honest significant difference (HSD) to characterize differences among groups.

Statistical significance in all tests was considered at $P < 0.05$.

Results

Physicochemical characteristics of the ovarian fluid and egg water

The osmolarity of sterlet ovarian fluid was 200 ± 17 mOsm/l; pH was equal to 8.18 ± 0.16; it contained 1.70 ± 0.52 mg/ml total protein. Content of main ions was 1.13 ± 0.12 mmol/l Ca^{2+}; 7.43 ± 0.65 mmol/K^+; 113.60 ± 19.10 mmol/l Na^+; and 51.00 ± 9.54 mmol/l Cl^-.

Egg water had osmolarity of 48.4 ± 4.10 mOsm/l; pH 6.89 ± 0.16; contained 0.11 ± 0.10 mg/ml protein; ion content was 0.02 ± 0.01 mmol/l Ca^{2+}; 0.31 ± 0.17 mmol/K^+; 28.67 ± 2.94 mmol/l Na^+; and 26.00 ± 2.65 mmol/l Cl^-.

Motility of sterlet spermatozoa in the presence of ovarian fluid

Sterlet spermatozoa were fully activated in water (tap water or distilled water), but not in the ovarian fluid or NaCl solution with corresponding osmolarity (200 mOsm/l). In NaCl solution with 60–100 mOsm/l osmolarity, 30–50% of sperm cells were activated. The cells activated in these conditions had lower velocity

comparing with those in water and motility lasted shortly (Table 3, supplementary Fig. 6, video in Online Resource 1); no motility (or only a few motile cells) was present in 30–50% solution of ovarian fluid with the same osmolarity of medium. The shown concentration/osmolarity range was due to individual specificity of the males; i.e., in several males, this limit was lower (only single cells were activated in 30% of ovarian fluid and correspondingly 60 mOsm/l), and in others, the "border" concentration of ovarian fluid was higher (i.e., poor or no activation in solutions of 50% of ovarian fluid and 100 mOsm/l, respectively, and normal activation in solutions with lower ovarian fluid content, e.g., 30%). The following data on VCL and LIN are presented for the media, where spermatozoa motility in all males was initiated: water, 10 and 20% ovarian fluid in water; 20, 40, and 60 mOsm/l NaCl; and egg water.

The curvilinear velocity of the spermatozoa activated in the mentioned above media decreased with time (supplementary Fig. 6a), at the 10 s post-activation, it ranged from 100 to 120 μm/s depending on the media. There was a difference in the velocity decline rate across the different media: factorial analysis showed a significant interaction between media and time (supplementary Table 5). The pair-wise analysis showed the similarity for spermatozoa performance between several media: 20 vs 40 mOsm/l NaCl solutions and 10% ovarian fluid vs 20 or 40 mOsm/l NaCl solutions (see supplementary Table 5). The performance of spermatozoa in egg water was not uniform in all males; generally, the VCL of spermatozoa activated in egg water decreased faster compared with other media (except 60 mOsm/l NaCl solution), and in most males, the spermatozoa stopped in egg water after 3 min post-activation (the motility of spermatozoa from the same samples lasted longer in 60 mOsm/l NaCl). The dependencies for VCL over time for water, 10 and 20% ovarian fluid, and 20 and 40 mOsm/l NaCl can be well described with linear regression (R^2 is higher than 0.97 for VCL dependencies in all 5 media, Fig. 1, Table 4). The slope of the regression line for VCL in water was the highest among 5 analyzed media; i.e., the spermatozoa activated in water slowed over time faster compared with the other four media (10 and 20% ovarian fluid, 20 and 40 mOsm/l NaCl solutions). No significant differences in the slopes of the regression lines, i.e., in the changes of swimming velocities over time, were found among media with 10 and 20% ovarian fluid and 20 mOsm/l NaCl (Fig. 1, Table 4). In all the media, the percentage of motile cells declined over time; the velocity values were calculated for the motile cells if their amount was higher than 10% to ensure the adequate number of cells to perform CASA.

The changes in the linearity of swimming tracks did not differ in the spermatozoa activated in different media over time (supplementary Fig. 6b, Table 5), and altogether, the trajectories in different media tended to be more linear to the end of motility period. The tracks of activated sterlet spermatozoa were mostly straight and part of spermatozoa moved in ark-like trajectories (Fig. 2). No visible effect on these patterns was present if the activation medium contained either ovarian fluid or NaCl solution of the corresponding osmolarity, as well as if spermatozoa were activated in the egg water.

Chemotaxis test

There were no changes in the behavior of the spermatozoa activated in water if the same water was the test media injected through microcapillary (Fig. 3, video in Online Resource 2). Diluted ovarian fluid (50% with water) did not affect the tracks of activated sterlet spermatozoa as well. However, in the case of undiluted ovarian fluid, part of the cells were "arrested" after entering the injected ovarian fluid "cloud" or their

Table 3 Motility of sterlet spermatozoa in ovarian fluid, its dilutions, and in NaCl solutions with osmolarities corresponding to a particular dilution of ovarian fluid

Ovarian fluid dilution	100% ovarian fluid	30-50% ovarian fluid	15% ovarian fluid	0% (distilled water)
Osmolarity	200 mOsm/l	60–100 mOsm/l	30 mOsm/l	~ 0
NaCl concentration in the solution	100 mmol/l	30–50 mmol/l	15 mmol/l	0% (distilled water)
Motility in NaCl solution /diluted ovarian fluid, %	0/0	30–50/0–20% (short duration of motility, individual specificity)	100/100	100/100

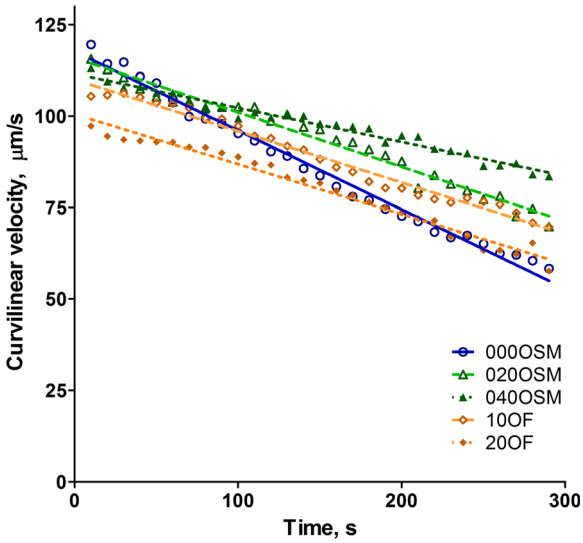

Fig. 1 Curvilinear velocity of sterlet spermatozoa in the presence of ovarian fluid depending on time post-activation. Activation media for spermatozoa were: distilled water (000OSM), distilled water mixed with 10%, or 20% of ovarian fluid (10OF and 20OF respectively); and NaCl solutions with 20 and 40 mOsm/l osmolarity (020OSM, 040OSM, respectively). Markers are mean experimental values obtained from averaging data from 7 males. Lines represent linear regressions of experimental dependencies; the parameters of the fitting, values of slopes, and intercepts are shown in Table 4

velocity was decreased. Nevertheless, other trapping or chemotaxis-like behavior was not observed. If the capillary contained the egg water, the spermatozoa tend to "follow" (or to stay inside) the injected "cloud" showing the chemotaxis-like behavior (see Fig. 3 and video in Online Resource 2). "Turn-and-run" loops were present in the behavior of some spermatozoa approaching the "borders" of the injected egg water cloud (video in Online Resource 2) which represent one of the characteristics of chemotactic behavior (Kaupp et al. 2008).

Effect of the ovarian fluid on in vitro fertilization

Effect on eggs There were no changes in the embryonic development rate during 10 min of incubation in water in the eggs covered by ovarian fluid (Fig. 4). Additional experiments allowed us to find out that eggs in water retained the ability to be fertilized up to half an hour. In the eggs without ovarian fluid, the embryonic development rate dropped starting from 0 point, and after 5 min of incubation of ovarian fluid-deprived eggs in water, only single cells were successfully developing on the third day post-fertilization. In the group with recovered ovarian fluid "coat" in the amount of 10% of egg batch, the fertilizability improved throughout the entire observation term. In the group where the introduced ovarian fluid made 50% of egg batch, the amount of developing eggs did not significantly differ from the group with "normal" fertilization conditions.

Effect on sperm Pre-incubation of spermatozoa in water before the introduction to the non-washed eggs during 2 min did not change the embryonic development rate (Fig. 5). If the spermatozoa stayed in water during 5 min, the embryonic development rate fell down to 5%. If the eggs were deprived of ovarian fluid, the embryonic development rate was dramatically lower even in the case of spermatozoa pre-incubated in water during 2 min, and almost no ovarian fluid-free eggs were found to develop after being fertilized by the spermatozoa 5 min following activation in water. The embryonic development rate was almost the same as in the previous group if the spermatozoa were activated in the medium with 2.5% of ovarian fluid; only the rate for washed eggs and 2 min of incubation was significantly higher compared with the previous group. The presence of 12.5% of the ovarian fluid in the spermatozoa activation

Table 4 Parameters of linear regression lines for curvilinear velocity dependencies of sterlet sperm activated in different media: distilled water (000OSM), distilled water mixed with 10% or 20% of ovarian fluid (10OF and 20OF respectively); NaCl solutions with 20 and 40 (020OSM, 040OSM, respectively). Data A, B, and C are mean ± SD; the different superscripts denote significant differences ($P < 0.05$)

Medium	R^2	P	A ± SD (slope of regression line)	B ± SD (intercept with y (VCL) axis)	C (intercept with x (time) axis)
000OSM	0.9875	< 0.0001	-0.2164 ± 0.0252^{a}	117.7 ± 4.3	544.1
020OSM	0.9724	< 0.0001	-0.1491 ± 0.0261^{b}	116.0 ± 4.5	777.4
040OSM	0.9719	< 0.0001	-0.0932 ± 0.0164^{c}	111.6 ± 2.8	1197
10OF	0.9821	< 0.0001	-0.1408 ± 0.0197^{b}	110.0 ± 3.4	781.0
20OF	0.9763	< 0.0001	-0.1368 ± 0.0221^{b}	100.5 ± 3.8	735.0

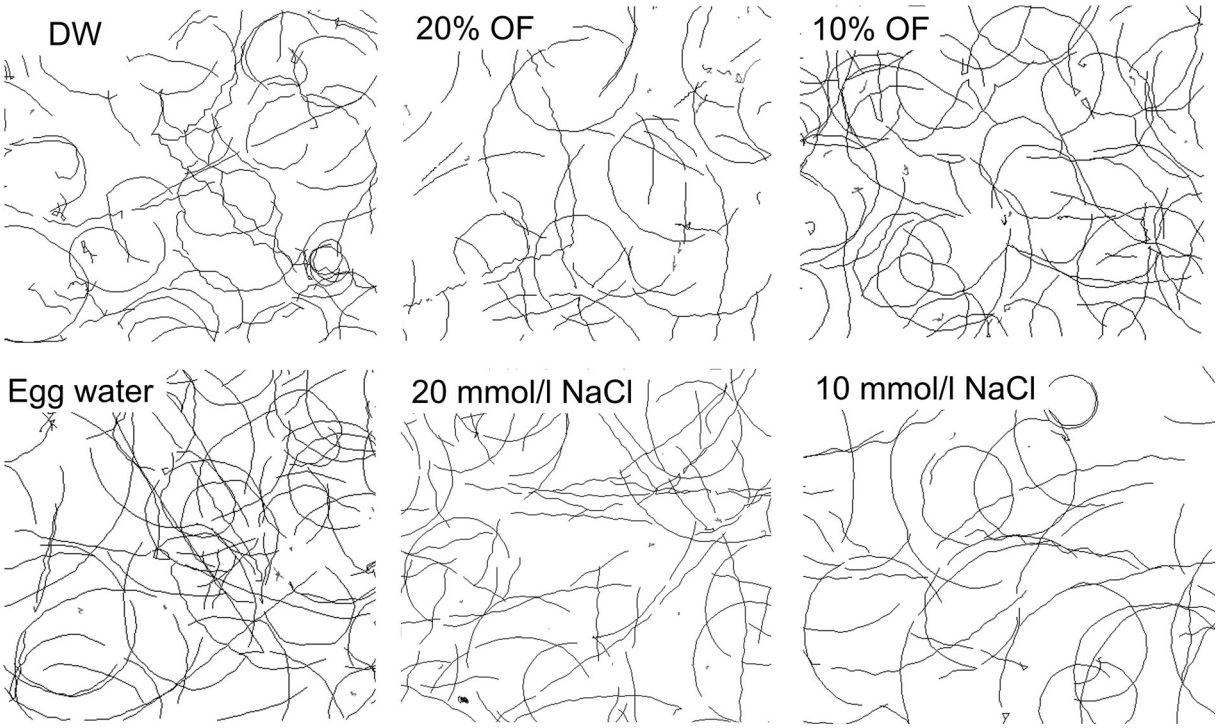

Fig. 2 Pattern of motility of sterlet spermatozoa in various activation media: DW, distilled water; 20% OF and 10% OF, ovarian fluid diluted in water; egg water; 20 and 10 mmol/l NaCl solution in water (40 and 20 mOsm/l osmolarity respectively). Each track corresponds to the trajectory of individual spermatozoa during 2 s starting from 10 s post-activation

medium did not change significantly the rate of developing embryos in case of non-washed eggs but improved the rate for washed eggs.

Discussion

A male gamete of externally fertilizing freshwater fishes is quiescent inside the parent body (or rather in its seminal fluid) and contact with an external aqueous environment activates its motility due to a difference in osmolarities of extra- and intracellular fluids and/or due to a various content of particular ions (mostly potassium, e.g., in salmonids) (Morisawa 1994). This difference in osmolarities outside and inside the spermatozo on being a requisite of its functional activity is nevertheless the reason for a short lifespan of the male gamete due to an osmotic shock which is continuously damaging the cell. In most externally fertilizing freshwater fish species, the fertility of the egg after the release

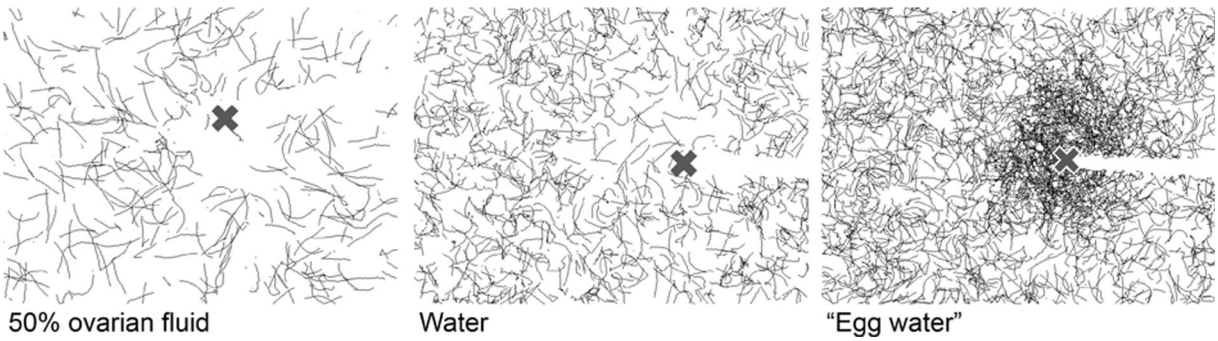

Fig. 3 Swimming tracks of sterlet spermatozoa activated in the water near the tip of microcapillary (cross) filled with fluids: 50% ovarian fluid solution in water; water, and egg water. Each track represents 1 s of motility (20–21 s post-activation)

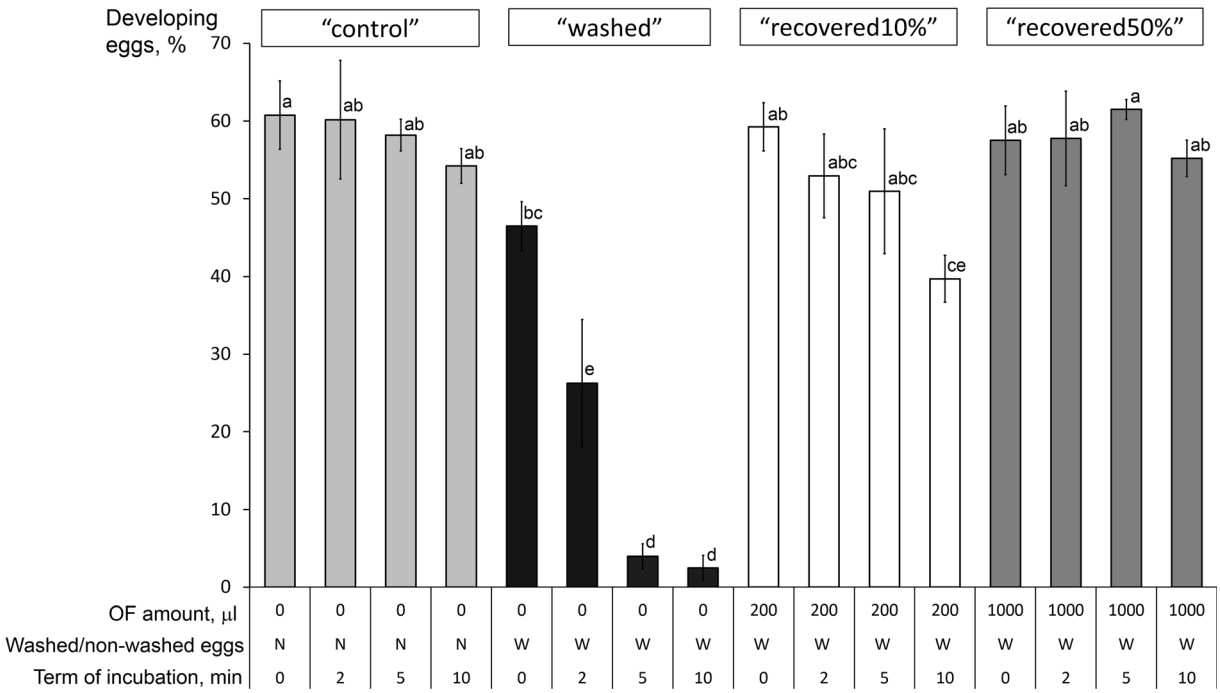

Fig. 4 Sterlet egg development rate depending on the presence of ovarian fluid around the eggs during in vitro fertilization. The eggs (2 g, approx. 135 eggs) were put into the plastic beaker, 8 ml of water was added, and incubation was performed during 0, 2, 5, or 5 min. Eggs were either non-washed to remove ovarian fluid ("control" group) or washed thrice with 0.9% NaCl solution ("washed" group); washed eggs were as well mixed with ovarian fluid in an amount of 10% of egg weight ("recovered 10%" group) or 50% of egg weight ("recovered 50%" group), i.e., 200 or 1000 µl of ovarian fluid. After incubation, 3 µl of sperm was added to the beakers with water and eggs, mixed with water, and the beakers were put to the shaker for 2 min. Thereafter, the eggs were transferred to glass Petri dishes and put to incubators at 17 °C. In 3 days, the eggs reached the neurulation stage were counted, which made the fertilization rate (fertilized eggs/total amount of eggs). Data are mean ± SD; the different superscripts denote significant differences ($P < 0.05$)

to water is also limited to few minutes because of the cortical reaction which leads to micropyle closure—the process is also called activation (Hart 1990). Thus, the short lifespan of either spermatozoa or eggs, as well as the environmental conditions (e.g., flow), makes the reproductive success quite time restricted, and the gametes are under selection for mechanisms that may control sperm-egg encounters.

Ovarian fluid and its composition

It was shown in several externally fertilizing species that specific "female fluid," egg jelly or ovarian fluid released together with eggs during spawning, affects the behavior of spermatozoa. It either activates and provides spermatozoa chemotaxis like in marine invertebrates of the *Ciona* genus (Yoshida et al. 1993), and Pacific herring *Clupea pallasii* (Yanagimachi et al. 2017), or supports the performance ("enhances the motility") of spermatozoa for a longer time like in rainbow trout *Oncorhynchus mykiss* (Wojtczak et al. 2007) or Arctic charr *Salvelinus alpinus* (Turner and Montgomerie 2002). It is believed that the composition of the ovarian fluid (in particular, its content in ions, proteins, amino acids, sugar) is ideal for supporting and protecting the eggs and sperm against the deleterious effect of freshwater and affects the behavior of male gametes and consequently influences an outcome of fertilization in several fish species (reviewed in Kholodnyy et al. 2020). The ovarian fluid surrounding the eggs in sturgeons, in particular those of the sterlet *Acipenser ruthenus*, makes up to 50% of the total egg volume. The fluid is viscous and contains fiber-like structures. The level of its pH as well as the content of the potassium and calcium ions found in this study is similar to the values published for other known fish species, while osmolarity is lower than in ovarian fluids of representatives of other studied freshwater fish families (see in Kholodnyy et al. 2020).

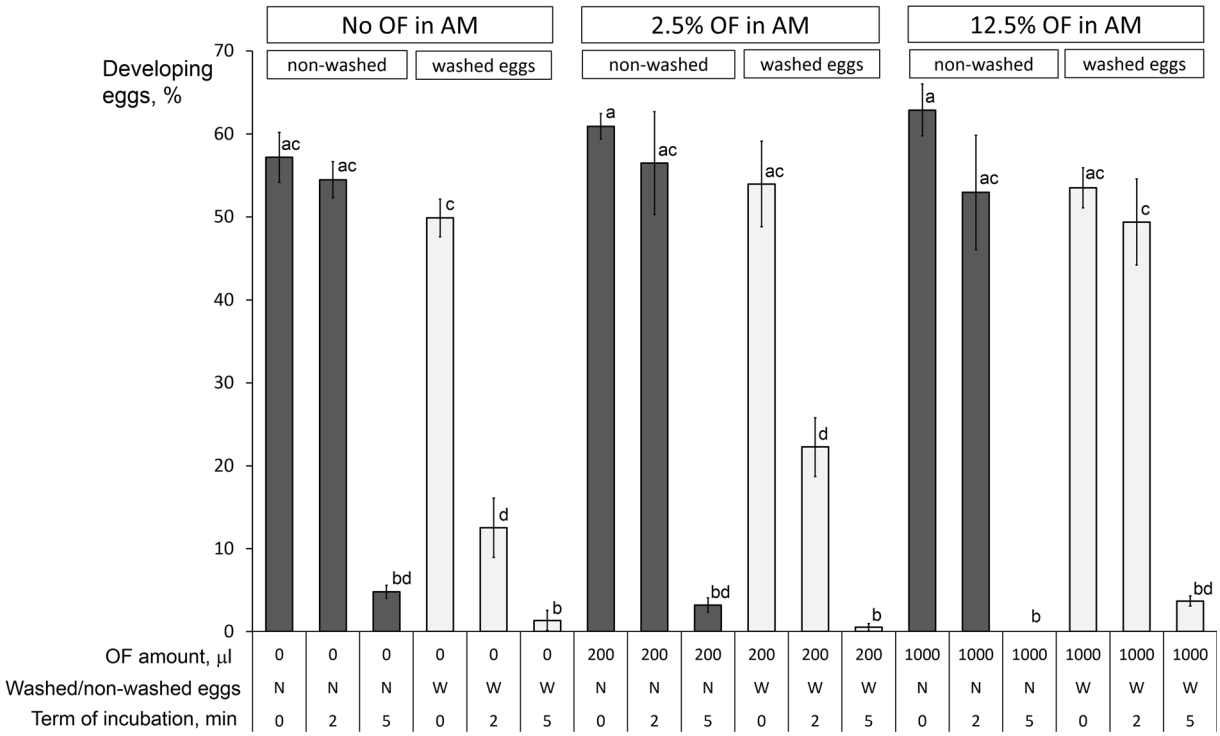

Fig. 5 Sterlet egg development rate depending on the presence of ovarian fluid around the eggs and in the spermatozoa activation medium during in vitro fertilization. The spermatozoa (3 µl) were mixed with 8 ml of water, water with 2.5 of 12.5% of ovarian fluid ("no OF in AM," "2.5% OF in AM," and "12.5% OF in AM" groups correspondingly). After 0, 2, or 5 min, the medium with activated spermatozoa was added to plastic beakers with 2 g of eggs either non-washed or washed thrice with 0.9% NaCl solution to remove ovarian fluid (washing was performed in such way that waiting time before mixing with spermatozoa was the same in all cases). The beakers were placed to shaker for 2 min and then, the eggs were transferred to Petri dishes and then to incubators. In 3 days, the eggs reached the neurulation stage were counted, which made the fertilization rate (fertilized eggs/total amount of eggs). Data are mean ± SD; the different superscripts denote significant differences ($P < 0.05$)

Motility of sterlet spermatozoa in the presence of ovarian fluid

The motility of sturgeon spermatozoa similarly to that of most freshwater fishes highly depends on the osmolarity of the environment (Cosson et al. 1999). Sterlet spermatozoa are not motile in ovarian fluid but are fully activated in freshwater (Table 3); there was found a certain osmotic limit (in the NaCl solution) in each particular male, at which the only single of spermatozoa are activated and are motile for a very short period up to several seconds, and no motility will be activated at osmolarities higher than this level. Nevertheless, it ranged across the studied males, from 60 to 100 mOsm/l, that we associate with the specific process of maturation of spermatozoa in the sturgeons (Dzyuba et al. 2019), i.e., mixing of sperm with urine, which entails the certain variation in several sperm parameters including the cell concentration and osmotic sensitivity. Nonetheless, the presence of ovarian fluid in the same osmotic conditions resulted in an additional inhibiting effect (Table 3). This may be associated with another fact about sturgeon spermatozoa motility initiation, which was reported as dependent on the potassium ion presence in the medium, e.g., in *Acipenser persicus* (Alavi et al. 2004). It was shown by the authors that the presence of 2 mM of KCl in the medium significantly decreased the amount of activated cells, and a concentration of 5 mM fully blocked the activation of the cells. In the present study, an "activation blocking" 30–50% solution of ovarian fluid contained around 2–3.5 mM K^+, which was similar to the inhibiting potassium level shown by Alavi et al. (2004). Nevertheless, a lower content of ovarian fluid (and lower potassium concentration, respectively) had no effect on motility percentage in our experiments (Table 3), unlike the results of Alavi et al. (2004), where the presence of 1 mM KCl still significantly affected the percentage of activated spermatozoa. This supports the

idea that potassium control of sterlet sperm motility is quite complex. In particular, Prokopchuk et al. (2016) pre-treated sturgeon (beluga *Huso huso*) spermatozoa in a hyperosmotic media and revealed that the sensitivity of spermatozoa to potassium ion presence was dramatically decreased, and an outflux of internal potassium was supposed to be a reason of this phenomenon. Moreover, the potassium sensitivity may be modified by changing the concentration of calcium ions in an activation media: Alavi et al. (2011) reported the overcoming of potassium ion inhibition of motility initiation if calcium ions were present in a concentration at least 0.25 of that of potassium (in our conditions the calcium/potassium ratio was twice less than this). Dzyuba et al. (2013) studied different modes of sturgeon spermatozoa activation (and re-activation) depending on the presence of calcium ions and various osmotic conditions and suggested that spermatozoa motility could be regulated by a combination of these factors. Altogether, we may point out that potassium control of motility activation in sturgeons may be modified in various osmotic and/or ionic conditions of activation media, and vice versa, the hypoosmotic trigger depends on the particular ionic composition.

In several fish species, the presence of ovarian fluid in an activation medium significantly affects the motility traits of male gametes. There are numerous reports of such changes in salmonid fishes. In particular, the linearity/straightness of spermatozoa trajectories in rainbow trout *O. mykiss* rises in solutions of ovarian fluid, as well as curvilinear velocity (Wojtczak et al. 2007; Dietrich et al. 2008); similar changes were found in Arctic charr *S. alpinus* (Turner and Montgomerie 2002), Chinook salmon *Oncorhynchus tshawytscha* (Rosengrave et al. 2009), and lake trout, *Salvelinus namaycush* (Galvano et al. 2013). The addition of the ovarian fluid to the activation medium caused the straightening of the trajectories and rise in velocity in freshwater and brackish water populations of three-spined stickleback *Gasterosteus aculeatus* (Elofsson et al. 2003a).

In several tested fish species, the average longevity of spermatozoa motility was found to rise significantly in the media containing ovarian fluid, e.g., in lake trout, *S. namaycush* (Galvano et al. 2013), whitefish *Coregonus lavaretus* (Dietrich et al. 2007), Angel fish *Pterophyllum scalare* Schultze (Faramarzi et al. 2011), and Caspian white fish *Rutilus kutum* (Golpour et al. 2015). Interestingly, such a significant "enhancing" effect of the ovarian fluid presence in the activation medium was found generally in the fishes spawning in freshwater. One of the main differences between marine and freshwater is the calcium ions content: in seawater, these ions are high in concentration and even after dilution (e.g., due to mixing with the ovarian fluid), it remains high which limits the possibility to use the modulation of calcium concentration as a factor to control linearity of sperm tracks in seawater (Alavi et al. 2019). The findings reported in marine fishes were not so univocal, e.g., the effects found in Atlantic cod *Gadus morhua* included the increase of spermatozoa swimming speed in the presence of ovarian fluid in seawater, but no differences in path linearity and even a decrease in the percentage of motile cells were found (Litvak and Trippel 1998). Diogo et al. (2010) reported highly variable effects of ovarian fluid on spermatozoa motility traits in Senegalese sole *Solea senegalensis*; the authors found some "enhancing" effect of heterologous maternal fluids on motility percentage at the end of spermatozoa motility period. The findings of the present study showed that in sterlet, the presence of ovarian fluid in concentrations lower than "activation blocking" one (as discussed above) did not affect significantly the pattern of swimming trajectories (Fig. 2). The presence of low concentrations of ovarian fluid (10 and 20%, as well as NaCl solutions with corresponding osmolarities) allowed to slow down the decline of the velocity of the spermatozoa: the slope of the regression line for sterlet spermatozoa VCL in water was the highest among 5 analyzed media (Fig. 1, Table 4). No significant differences in the slopes of the regression lines, i.e., in the changes of swimming velocities over time, were found between media with 10 and 20% ovarian fluid and 20 mOsm/l NaCl (Fig. 1, Table 4). Intercepts of the regression lines with the x axis allowed to range the tested media depending on the potential longevity of spermatozoa, taking into account conventionality of such estimation: the lowest value among 5 media was found in water, and the highest was for 40 mOsm/l NaCl solution (Table 4).

The swimming trajectories in the activating solutions with ovarian fluid were similar to that in sodium chloride solutions with the same osmolarities (which may serve as "osmotic control" to dilutions of ovarian fluid). The velocity of spermatozoa in the sodium chloride solutions with 20 and 40 mOsm/l osmolarities (similar to 10 and 20% ovarian fluid solutions) was also higher

than the ones recorded in water in the second half of motility period. Therefore, we may suppose that the presence of ovarian fluid in the activation solution for sterlet spermatozoa may affect the activation of male gametes due to osmolarity and potassium content higher than the optimal one for motility initiation, as well as possible modulation of motility initiation by the interactive effect of potassium and calcium ions mentioned above. In the activated cells, the additional effect of low concentration of ovarian fluid on spermatozoa performance consists of keeping higher velocity for a longer period.

Do the sterlet "female fluids" possess chemotactic features?

The ovarian fluid is believed to contain potential agents of "maternal control" over the spermatozoa in terms of chemotaxis and selection. The very first reports about chemotaxis in externally fertilizing animals associated with the observed phenomena with maternal fluid released together with eggs in sea urchin (Lillie 1912). Later, the role of the ovarian fluid as the chemotactic agent in sea urchin was confirmed and theory of sperm chemotaxis in externally fertilizing animals was built mainly using these species as model ones (Kaupp 2012). The substances released by the egg per se showed the chemotactic activity towards spermatozoa in many other externally fertilizing species, e.g., red abalone *Haliotis rufescens* (Riffell et al. 2002), ascidians *Ciona intestinalis* and *Ciona savignyi* (Yoshida et al. 2002), and Pacific herring *C. pallasii* (Cherr et al. 2008). In the present study, we have not found any signs of chemotactic activity of sterlet ovarian fluid when the tested fluid was introduced to the suspension of activated spermatozoa by capillary (Fig. 3, video in Online Resource 2); moreover, in the case of undiluted ovarian fluid, it acted even as a "trap" arresting the cells entering the area with the fluid (video in Online Resource 2). Not all the cells were arrested and this effect was temporary, disappearing after dilution of the ovarian fluid. So it could be associated with the sensitivity of sterlet spermatozoa to higher osmolarity and potassium content. No visible effect was found if the water was injected as a test medium (Fig. 3), suggesting no (or at least not significant) reaction of sterlet spermatozoa to the produced flow of a fluid (i.e., a rheotactic reaction, which may mask the reaction of the cells on some chemical gradient). When we introduced by a capillary the egg water (the medium which was the distilled water after 15-min incubation of washed non-destructed sterlet eggs in 1:1 volume ratio) through a capillary, we have found the bright response of the cells on the introduction of the egg water into the test suspension of activated spermatozoa; i.e., the cells followed the injected cloud, gradually accumulated inside it, and some kind of "turn-and-run" loops were seen in some spermatozoa on the "borders" of the injected egg water cloud (video in Online Resource 2) which are believed to be characteristic for chemotactic behavior (Kaupp et al. 2008). The found effect shows the potential role of egg-released substances as molecules attracting the spermatozoa in sturgeons. For the moment, the only one proved fish spermatozoon chemoattractant was found in Pacific herring *Clupea pallasii*, and it was a glycoprotein released from the micropyle area of egg chorion (Pillai et al. 1993). Incubation of sterlet eggs in the water entails the release of several proteins, including glycoproteins, into the surrounding medium (Niksirat et al. 2017), which may be the agents, causing the trapping effect in our experiments. In our experiments, the egg water contained a certain amount of substances of protein nature (which were absent in the distilled water, used for the preparation of the egg water), and some of these substances may cause the observed changes of spermatozoon behavior. Anyway, further investigations are needed to clear up the observed phenomenon in sterlet.

Effect of ovarian fluid on fertilization: effect on eggs

As it was mentioned above, the ability of the eggs to be fertilized (fertilizability) decreases dramatically during the first minutes after release from females' body in many freshwater fishes. In particular, only around 20% of *O. mykiss* eggs could be fertilized after 40 s in the freshwater, and only 5% retain this ability after 80 s (with 70% initial fertilization) (Liley et al. 2002). Similarly, less than 5% embryos were developed from the crucian carp *Carassius carassius* eggs fertilized after 90 s being in water (with more than 90% possible fertilization at earlier post-activation terms) (Zarski et al. 2014). This loss of fertilizability results from egg activation, the process associated with the release of cortical vesicles, the appearance of perivitelline space, and closure of micropyle, which makes impossible the penetration of spermatozoa (Hart 1990). If the eggs will be kept in the ovarian fluid after procuring from the female, they could retain the fertilizability for a much

longer period. Lahnsteiner (2002) showed that eggs of brown trout *Salmo trutta f. fario* remain fertile during stay in ovarian fluid for more than 10 min. Storage of unfertilized *O. mykiss* eggs in the ovarian fluid at 12–13 °C during 48 h did not change significantly the eyeing and hatching rate of embryos fertilized post-storage comparing with the eggs which were fertilized shortly after stripping (Goetz and Coffman 2000). The short-term storage of sturgeon eggs in the coelomic fluid does not change significantly their fertility (Dettlaff et al. 1993). This feature of ovarian was associated mainly with its ionic composition and osmolarity, preventing the activation of the egg, and allowed to develop artificial solutions for short-time (hours to days) storage of the eggs before fertilization (Goetz and Coffman 2000; Safarzadenia et al. 2013; Ribeiro et al. 2017). In the present study, we have found that in sterlet the coat of ovarian fluid around the eggs prevented their activation after being covered with water for a period of 30 min. Washing out the ovarian fluid led to an immediate drop in fertility in terms of embryo development rate starting from the very first moment of contact with water (Fig. 4). The procedure of washing itself did not affect significantly the eggs' fertility since the recovery of the ovarian fluid coat around the eggs resulted in a similar embryo development rate as in non-washed eggs (Fig. 4). This protective feature to some extent depended on the amount of ovarian fluid: if its volume was 10% of egg mass, there was a slight decline in fertilization rate to the end of the observation period, while if the volume of the recovered ovarian fluid coat was "natural," i.e., 50% of egg mass, there were no significant differences in the egg development rate index during the observation period (Fig. 4). This may indicate that viscosity and "fibrous" composition of ovarian fluid in sturgeons would allow to save its ionic composition in the layer adjacent to the egg batch (and correspondingly the osmolarity, higher than surrounding water) for a quite long period, enough to prevent the activation of the egg while the ovarian fluid is placed to contact with water and this effect remains for a longer period than in other fishes with less viscous ovarian fluid, e.g., salmonids.

Effect of ovarian fluid on fertilization: effect on spermatozoa

Spermatozoa of most freshwater fishes are able to fertilize the egg during the short time following their activation, e.g., in rainbow trout *O. mykiss*, only 10% egg fertilization rate could be achieved if sperms were introduced 40 s post-activation in water, and it was correlated with the percentage of motile cells (Liley et al. 2002). The presence of ovarian fluid in the activation medium allowed to prolong the motility period of the spermatozoa as well as the period when they may fertilize the eggs in Caspian brown trout, *Salmo trutta caspius* (Hatef et al. 2009). In our study, the "window of opportunity" for sterlet spermatozoa to fertilize the eggs was the period of the first 2 min post-activation if the eggs were not deprived of ovarian fluid; i.e., no significant differences were found between rates of developing embryos following fertilization with spermatozoa after 0- and 2-min incubation in water. After 5 min, this index dropped down to almost 5%. Interestingly, the removal of the ovarian fluid from the eggs caused a significant drop in fertilization rate with the spermatozoa introduced after 2 min of their incubation in water (the eggs were in the same conditions in all cases). We may suppose that the layer of ovarian fluid around the egg may support the longer activity of the cells and this is more obvious if the spermatozoa reach the egg not in the initial period of its motility (video in Online Resource 3 shows the spermatozoa approaching the surface of the egg surrounded by the residual layer of ovarian fluid and then following this surface under the "protective coat"). The addition of 2.5% of ovarian fluid into the sperm activation medium (corresponding to 10% of egg mass) did not change significantly the fertilization rates in all cases, except for a slight rise in washed eggs fertilized with 2 min pre-activated spermatozoa. The addition of 12.5% of ovarian fluid into spermatozoa activation medium resulted in the absence of significant differences in the fertilization rate of washed eggs between the "fresh" and pre-activated spermatozoa after 2 min of incubation. This amount of ovarian fluid obviously "neutralized" the absence of the protective layer around the egg.

Role of ovarian fluid in egg-sperm interaction in sterlet

What is the role of the ovarian fluid in the sterlet gamete interaction (and likely acipencerids in general)? Sterlet spermatozoa motility lasts longer compared with other freshwater species (Liao et al. 2018). One of the reasons for this phenomenon is that they are less sensitive to osmotic shock. It may be related to their specific "transient" structure of the urogenital system, which provides the maturation of spermatozoa particularly by mixing

with urine. The latter has lower "physiological" osmolarity (around 50 mOsm/l) and this may "prepare" the gametes to tolerate low osmolality of freshwater (Dzyuba et al. 2019). Nevertheless, the activation of spermatozoa depends on the medium osmolarity like in many other freshwater fishes, as well as it is controlled by potassium concentration decrease. The combination of the mentioned factors, longer motility, sensitivity to the medium osmolarity, and ion content together with the observed trapping of activated spermatozoa by viscous ovarian fluid may be potentially used in the reproduction strategy of the sturgeons. In particular, we may suggest that sturgeon females may lay several batches of eggs covered by viscous ovarian fluid which will preserve them for significant time and may "put on hold" spermatozoa from one or more males. Later on, the gradual dilution of the ovarian fluid layer will result in activation of the spermatozoa (the repeated activation of spermatozoa in the hypoosmotic medium after previous activation (and ceasing) in the hyperosmotic medium was described by Dzyuba et al. (2013)). The male gametes will be able then to approach the egg surface still protected by residual ovarian fluid and navigated by chemical signals released by the egg (presumably the micropyle). This may increase the probability of fertilization of the maximum amount of the eggs and increase the genetic diversity of the progeny. Nevertheless, these speculations need to be confirmed by further studies.

Conclusions

As a whole, we may conclude that in sterlet, the presence of ovarian fluid prevents the eggs from losing the fertilizing ability during the contact with water, by preventing their activation. This may prolong the time during which the eggs may be reached by spermatozoa. Moreover, the layer of ovarian fluid around the eggs promotes the spermatozoa to fertilize the eggs during a longer period of time. In other words, ovarian fluid may serve as a protector for the eggs and spermatozoa against the effect of freshwater during fertilization. It does not exhibit any chemotactic effect on the male gametes in our experimental conditions. At the same time, the attraction of spermatozoa may be provided by some substances released from the eggs during their contact with freshwater, and this issue requires further detailed investigation. Thus, the combined physicochemical action of "female factors" is important during the interaction of sterlet gametes and may provide support for guidance/selection mechanisms during fertilization. The obtained features of egg-sperm interaction in one of the acipenserid species may be a useful addition to the reproductive physiology of fishes and evolutionary developmental biology.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement Manipulations with animals were performed according to authorization for breeding and delivery of experimental animals (reference number: 56665/2016-MZE-17214 17OZ19180/2016-17214, valid from the 4 October, 2016 for 5 years) and the authorization for the use of experimental animals (reference number: 2293/2015-MZE-17214 16OZ22302/2014-17214, valid from 22 January 2015 for 5 years) issued to the Faculty of Fisheries and Protection of Waters, the University of South Bohemia by Ministry of Agriculture of the Czech Republic.

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