Sperm/egg interaction in freshwater fish: influence of environment on fertilization process

Interakce spermií a jiker u sladkovodních ryb: vliv prostředí na fertilizační proces

Vitaliy Kholodnyy

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Czech Republic, Vodňany, 2020

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. Influence of environment on freshwater fish gametes' encounter guidance

1.2. Kholodnyy, V., Gadêlha, H., Cosson, J., Boryshpolets, S., 2020. How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish. Reviews in Aquaculture 12, 1165–1192.

My share in this work was about 70%.

1.1. Influence of environment on freshwater fish gametes' encounter guidance

Although it is generally believed that Darwinian evolution is associated with all life forms on our planet, and many issues do not matter for dispute anymore, the mechanisms behind genetic adaptation and natural selection are still essential for investigation in this respect. Reproduction is a key process for entire life matter, and allows passing genetic information from parents to progeny and secures both variability and stability of species. Sexual reproduction relies on the fertilization of female ova by male spermatozoa. For successful fertilization, the spermatozoa have a certain "window of opportunity" and must reach the egg within a limited time, which depends on the features of both counterparts: the female and male gametes. The regulatory mechanisms that govern this process highly likely are the key components of different strategies of reproduction, and spawning modes in particular, and correspondingly a spermatozoon behavior in different species.

During the last decades the belief that fertilization occurs randomly, via simple diffusion of a large number of spermatozoa and the eggs acting only as a passive acceptor of male genotype (Parker, 1993), was significantly modified. As one of the alternative theories a guidance hypothesis was proposed (Eisenbach and Giojalas, 2006). The latter is successful in explaining different aspects of reproduction such as the directed fusion of sperm-egg and specific mechanisms for sperm selection. The idea about spermatozoa guidance is supported *e.g.* by the ability of male gametes to sense and react to changes in the environment, from the fluid viscosity and background flows to the pH, ion concentration, and even temperature (Fechner et al., 2015). Theoretical support of the potential spermatozoa guidance and navigation is provided by biologically inspired mathematical models (Cosson, 2015, details to be found below in this chapter). In recent years, the non-random spermatozoa performance was confirmed empirically in several species of mammals and seawater aquatic animals (detailed in **Chapter 1.2**). Interestingly, the biased behavior of spermatozoa caused by the agents associated with females for fish species was only clearly demonstrated in pacific herring spermatozoa via chemotaxis only (Cherr et al., 2008; Yanagimachi et al., 2013).

The lack of studies on the guidance of fish spermatozoa is despite empirical evidence of possible chemotactic response for selected fish species, reported almost half a century ago in fat minnow (Suzuki, 1958) and lamprey (Kille, 1960). Recent studies on the rosy barb, black flounder, barfin flounder, and herring also presented some evidence of possible chemoattraction response (Amanze and Iyengar, 1990; Yanagimachi et al., 2013). Nevertheless, there are no studies to date on the direct demonstration of any type of guidance mechanism of freshwater fish spermatozoa. This can be attributed to the biological challenges associated with their specialized motility, *e.g.* very limited period of motility, typically less than 1 minute; the large size of eggs; and the existence of specialized fertilization site, the micropyle, which should be reached during the short motility period. Under these conditions, ***reproductive success is chronically limited to the ability of spermatozoa to find the egg and reach the fertilization site, and thus they are under the need of mechanisms that increase sperm-egg encounter.***

Spermatozoa activation and rise in their motility traits in the vicinity of the eggs were first observed in marine invertebrates about 100 years ago (Lillie, 1912), and female fluids like egg jelly or ovarian fluid (OF) were supposed to cause these phenomena. During the release of the spawned eggs through oviducts into fresh or saltwater, ovarian fluid still surrounds the eggs of many externally fertilizing female fish (Rosengrave et al., 2008), creating the coat for the female gametes. The composition of the OF varies among the species, generally, it contains ions and different substances of protein nature, sugars and lipids in the different ratios (*e.g.* Lahnsteiner et al., 1995, more details to be found below in this chapter). Numerous reports exist about differences in sperm behavior after being activated in OF (or its mixtures with

water) instead of only water. In particular, higher velocities of spermatozoa in OF comparing to water were found in guppy *Poecilia reticulata* (Gasparini and Pilastro, 2011) or lake trout *Salvelinus namaycush* (Butts et al., 2012). Percentage of motile cells was higher when activated in ovarian fluid in brown trout *Salmo trutta* f. *fario* (Lahnsteiner 2002) and in lake trout *Salvelinus namaycush* (Butts et al., 2012). A lot of evidence was found about prolonged longevity of sperm in ovarian fluid, *e.g.* in Arctic charr *Salvelinus alpinus* (Turner and Montgomerie, 2002). Generally, a "pro-kinetic effect" of ovarian fluid on sperm cells was found in numerous species, and it is of great biological importance because spermatozoa motility properties, swimming speed in particular, was shown to be the key determinant in male fertilization success under conditions of sperm competition in a variety of species, *e.g.* lake trout (Butts et al., 2012). However, there is no clear understanding of the mechanisms of such enhancement provided by ovarian fluid. Some authors claim that only ionic composition is responsible for improved motility traits, showing that there was no significant difference between the motility traits assessed in OF and the saline solution in various salmonids (Lahnsteiner, 2002; Rosengrave et al., 2009; Hatef et al., 2009). Nevertheless, the ovarian fluid is likely the only candidate for providing the trigger or control of fish gametes encounter in natural conditions.

It seems very likely that spermatozoa after being activated should find their way to egg driven by some factors in OF or released by egg *per se*. These phenomena could underlie the success of fertilization and prevention of crossbreeding (Yoshida et al., 2013). It was stated previously that only a small proportion of sperm is good for fertilization in mammals because of its integrity and/or presence of morphological and genetic abnormalities (Cohen, 1975). Considering this, the randomness of egg and sperm contact could not be the strategy for proper stability and variability of living matter. There is a lot of examples in nature showing that several cells can recognize a molecule of some substance, find the direction of increasing concentration of the substance, and move there. In spermatozoa, the earliest observations of such behavior were made in medusan *Spirocodon saltatrix* (Dan, 1950). Such substances could improve the outcome of fertilization because of contributing to sperm guidance to the egg. To date, there are only a few candidate chemoattractants found in fishes (details could be found below in the chapter).

Thus, there are almost no studies on the direct demonstration of any type of chemotactic behavior of freshwater fish spermatozoa, this is caused in particular by the above-mentioned conditions of reproduction. The study of the complex of the phenomena including motility activation and progress, kinetic and tactic effects, possible selection, and promotion of spermatozoa, as well as the potential signaling associated with eggs, could be the elements in a system of gamete guidance. The understanding of the mechanisms behind sperm guidance and spermatozoa selection in freshwater fish species can be important not only for fundamental biology but may help to elucidate the impact of the traditional aquaculture practice of artificial fertilization on progeny quality and species sustainability.

Collectively, considering the peculiarities of the reproduction process, we **hypothesize** that the only source of stimuli or signaling during the reproduction of freshwater fish may come from the egg or OF. These stimuli would affect the behavior of sperm cells, rendering both chemokinetic and chemotactic effects and finally the outcome of fertilization. Thus, the **aim** of the study is to find the evidence or absence of guidance behavior in three representatives of freshwater fishes, the rainbow trout *Oncorhynchus mykiss*, sterlet *Acipenser ruthenus,* and common carp *Cyprinus carpio*, and to clear up the potential mechanism of sperm guidance in this species. The above-raised issues, in particular the effects of fish maternal fluids on the chemokinetic and chemotactic performance of the fish spermatozoa, will be considered in **Chapters 2**, **3**, and **4** respectively for each of the three mentioned freshwater fish species.

These species are popular aquaculture objects and there are numerous investigations on their gamete physiology, which allow to combine existing data with newly found insights and to reveal new peculiarities in the reproductive physiology of these species. At the same time, the chosen fishes represent quite distant taxonomical groups, which use various spawning behavior. Thus, the comparative studies of their reproductive strategies may contribute to developmental biology as well. We will try to get a view of the interplay of sperm-egg (ovarian fluid) interaction and reproductive (spawning) behavior of our model fishes in the General Discussion of **Chapter 5.**

Coming from the stated above the objectives of the thesis are:
1. To analyze available knowledge on physicochemical factors responsible for sperm-egg encounters in the case of externally fertilizing fish species.
2. Scrutinize the role of the ovarian fluid during spermatozoa motility in three species of freshwater fishes as well as its potential for sperm attraction via chemical signaling.
3. Explore the effect of external factors that are biologically relevant during the fertilization process.
4. Analyze how the differences in reproductive strategies in fish could affect (or be affected by) the features of sperm-egg interaction.

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1.2. How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish

How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish

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Abstract

The lifespan of spermatozoa from externally fertilizing freshwater fish ranges from a few seconds to several minutes, depending on the species. External factors, such as temperature, background flows and ion composition, play an important role in fertilization success. Specific mechanisms guiding spermatozoa appear to be essential to maximize the sperm–egg encounter under these strenuous conditions. Although some existing data support the hypothesis that both the ovarian fluid and the eggs may release chemoattractants that significantly affect spermatozoa behaviour and the fertilization outcome, this hypothesis is still open to debate, as the existence of freshwater fish spermatozoa chemotaxis has yet to be demonstrated; in addition, specific mechanisms supporting spermatozoa guidance and gamete selection have not been elucidated. Is the natural selection of gametes determined by a combination of different physicochemical phenomena? Alternatively, is the natural selection of species-specific gametes biased towards the species-specific guidance mechanisms of their natural landscape? These questions have received more attention as new studies have revealed potential, distinct guidance mechanisms in freshwater fish reproduction. In this review, we discuss the empirical studies supporting different hypotheses about freshwater fish gamete guidance and highlight the synergistic combination of experiments and biomathematical modelling to explore these questions. Finally, we discuss the challenges in understanding the mechanisms behind sperm guidance in freshwater fish species, and we suppose that knowledge about the mechanisms that underlie spermatozoa selection and guidance in freshwater fish species may elucidate the impact of the traditional aquaculture practice of artificial fertilization on progeny quality and species sustainability.

Key words: egg, flagellar dynamics, freshwater fish, guidance, ovarian fluid, spermatozoa.

Introduction

Darwinian evolution permeates all life forms on our planet. For most species from Animalia, natural selection begins with the successful swapping of genetic material following fertilization, where an embryo is formed and developed, and if the embryo is sufficiently healthy, it grows and reaches maturity to reproduce before dying (Pelegri *et al.* 2017). This selection is perhaps one of the most fundamental components of life. The very first stage of natural selection takes place even before the gametes meet. Among the different species of fish, for example, some are adapted to fertilize eggs either internally or externally. The exact evolutionary pressures for particular adaptations resulting in the existence of these two modes of fertilization are unknown thus far. Nevertheless, the selective pressure for external fertilizers is undeniably higher for both gametes; and they pass through convoluted processes and challenges in a harsh and uncharted territory outside the body. Successful fertilization results from the sum of strict step-by-

V. Kholodnyy *et al.*

step phenomena leading to the fusion of the male and female gametes. Each individual step triggers the next step, and if a step has a weak performance, the fertilization outcome may be dramatically affected.

In most externally fertilizing freshwater fish species, both males and females spawn synchronously by generating sufficient muscular contractions to ultimately produce an underwater plume of gametes containing approximately 10 billion spermatozoa and 3 million eggs (Wootton & Smith 2014). Nevertheless, the short lifespan of either spermatozoa or eggs, as well as the environmental conditions (e.g. flow), makes the reproductive success quite time-limited by sperm availability, and the gametes are under selection for mechanisms that may control sperm–egg encounters. The mechanisms involved in these processes are sophisticated and combine various molecular, chemical and physical factors (Zimmer & Riffell 2011; Zaferani *et al.* 2018). Egg signalling and sperm response could be adapted, for instance, to meet one or many specific environmental constraints, such as chemical diffusivity, pH, fluid and flow properties, surface interactions and other external factors (Hart 1990; Iwamatsu 2000). Likewise, these external selective stresses are likely to drive gamete evolution and determine fitness within natural habitats.

Freshwater fish species that reproduce externally constitute a group of perfect model organisms for reproduction studies. *In vivo* investigations with internally fertilizing species often suffer from the unavoidable translation between natural and *in vitro* conditions. Freshwater fish species allow a close inspection of the selective pressures mediating reproduction and sperm–egg fusion, including the consequences of changes in the environment, adaptivity or even extinction potential given external perturbations. This is particularly relevant now, as external environmental stresses are rapidly changing, from global warming and pollution to other detrimental aspects of our industrial society. These environmental changes may dramatically decrease the natural stocks of fish, as has been the case for sturgeons (Acipenseridae and Polyodontidae; Bronzi *et al.* 2011), or other commercially important freshwater fish species (Ficke *et al.* 2007). The taxonomical position of fish, as well as the breadth of their taxonomic tree, offers a large variety of reproductive strategies for successful fertilization and may be used for a comprehensive analysis of evolutionary and developmental adaptations. Indeed, more research on freshwater fish reproduction is currently needed. This research will positively impact both commercial and endangered species and will affect the prospects for more sustainable fish production, including the standardization of gamete quality, disease control and genetic improvement; these findings will also eventually reduce the current environmental impact. In particular, the knowledge about the mechanisms that underlie spermatozoa selection and

guidance in freshwater fish species may help elucidate the impact of traditional artificial fertilization practices on the quality and sustainability of progeny.

Although several excellent reviews have been compiled covering different aspects of fish reproduction (Hart 1990; Taborsky 1998; Coward *et al.* 2002; Cosson 2004; Alavi & Cosson 2005, 2006; Yoshida *et al.* 2008; Bobe & Labbé 2010; Schulz *et al.* 2010; Dzyuba & Cosson 2014; Browne *et al.* 2015; Smith & Wootton 2016), there is still a need for an analysis with a focus on the biophysical and biochemical aspects of freshwater gamete guidance. In this review, we explore the scientific developments thus far covering the underlying physicochemical factors responsible for sperm–egg encounters in the case of externally fertilizing fish species. We highlight the gaps present in our understanding of freshwater sperm guidance, and thus, the urgent need for substantial further research. Indeed, to date, empirical evidence of the guidance mechanisms in many fish species is lacking, and knowledge on the processes by which spermatozoa find the egg in these species is scarce. Most of the studies on reproduction and the established regularities and mechanisms in external fertilizers are traditionally and predominantly dealing with marine invertebrates, mainly sea urchins, for example *Arbacia punctulata*, which may be attributed to their ready availability, relatively convenient handling and processing (Kaupp *et al.* 2008), as well as the simplicity of their internal structure, especially that of their flagellum (Gibbons & Grimstone 1960). In our review, we discuss the new directions of investigation needed and highlight the synergistic combination of interdisciplinary research exploring experimental investigations, advances in imaging technology, mathematical data analysis and in-silico predictions of virtual sperm–egg meeting models. We address the future challenges of observational and theoretical studies in understanding the external selective pressures for sperm–egg encounters in freshwater fish.

This review is organized as follows: in the first section, we present an overview of the basic morphological and physiological features of freshwater fish gametes, including the maturation conditions of gamete activation, and the environmental effects on sperm motility, that is, the conditions crucial for the proper function of fish gametes that are apparently necessary for the sperm–egg encounter; in the second section, we introduce the feasibility of the specific mechanisms underlying the guidance of spermatozoa towards the eggs in the light of freshwater fish reproductive behaviour and the theories on gamete encounters. Then, after hypothesizing the need for specific female factors to control sperm cell behaviour, the attention is focused on the most likely 'conductor' of female factors, ovarian fluid and its effects on the fertilization process, including chemotactic and chemokinetic effects on the behaviour of spermatozoa, as well as the effect on fertilization outcome. The

next part of this section is devoted to the specific site where the sperm cell could enter the egg, the micropyle: the existing data on its physical and chemical cues for the male gametes are presented. After a description of specific spermatozoa behaviour observations, the sections are concluded by summing up the specific features of the male gametes that enable their response to changes in environment while approaching the female gamete, either of a chemical or physical nature. The next section introduces the reader to the biophysical and mathematical models aimed at clarifying the guidance mechanisms for fish spermatozoa. Finally, we present some findings on post-copulative female control over fertilization, the so-called cryptic female choice, which may confirm the data on the specific encounters of gametes, and make the conclusion summing up the egg–sperm interactions framework.

Freshwater fish gametes

Structure of 'aquasperm' and eggs

Externally fertilizing fish species, which represent the vast majority of fish species, release both gametes into the water for fertilization. The gamete contact with the external aqueous environment activates the spermatozoa motility (Morisawa 1985). However, the aqueous medium is a hostile environment for both gametes, and thus, the lifespan of activated sperm is unusually short. Upon release, the osmotic shock activates the spermatozoa in the very first period, and the osmotic shock also continuously damages the cell during the following seconds or minutes of contact with water. Thus, the male gamete is under pressure to find the egg as quickly as possible. This scenario is aggravated by the existence of only one site at the egg surface where the spermatozoa can potentially enter the egg, the micropyle. This is a diminutive opening, with a diameter of only a few micrometres, and the spermatozoa have to find this opening within this short motility period in an environment that is constantly moving and changing (Jamieson 1991).

In general, the spermatozoa of different externally fertilizing freshwater fish share a stereotypic structure, the so-called 'aquasperm' (Fig. 1). The vast majority of spermatozoa has no acrosome and the head contains a round nucleus with homogenous, condensed chromatin. The flagellum has the typical 9 + 2 axonemal structure with nine microtubule doublets cylindrically arranged around a central pair of microtubules. The whole axonemal complex is further surrounded by the plasma membrane. In several species, the membrane has longitudinally arranged folded structures forming fins (Jamieson 1991). The total length of the flagellum is tens of micrometres. Among the 57 freshwater species for which such data exist (Liao *et al.* 2018, Supplement S1), *Barbus grypus* has the shortest flagellum, 14.20 μm, while *Acipenser dabryanus* has the longest,

70.40 μm; in other popular model species, such as *Cyprinus carpio* and *Oncorhynchus mykiss*, the flagellum lengths extend to 45.5 and 37.3 μm respectively.

The eggs of externally fertilizing freshwater fish are covered with a dense vitelline envelope or chorion; in *C. carpio*, this is 10.0–10.2 μm thick and consists of four highly proteinaceous layers (Linhart *et al.* 1995). Such a structure is most commonly arranged as a multilayer across species, with the exception of salmonids, which only possess a single layer (Brivio *et al.* 1991). The dense envelope has a specialized narrow opening called the micropyle (Fig. 1), which is hypothesized to have co-evolved with the reduced structure of the spermatozoa. The micropyle is key to permit a non-acrosomal sperm to enter the egg without requiring an acrosomal reaction (Jamieson 1991). For acipenserids, the multiple micropyles and acrosomal spermatozoa can be considered as a transitional case, and their taxonomical position supports the idea stated in the previous sentence (Jamieson 1991). The inner layer of the chorion, the oolemma, is often considered a part of the primary envelope of the egg *per se* (Iwamatsu 2000; Fig. 1d). Other structures of eggs include the nucleus, which is located close to the micropyle opening, and the cortical alveoli, located in a gel-like layer close to the oolemma (Fig. 1b). The latter participates in the post-activation and fertilization transformation of the chorion and in the formation of the perivitelline space between the oolemma and the chorion. The majority of the intra-oocyte space is occupied by yolk, offering a source of nutrients for the future developing embryo (Hart 1990). The diameters of the eggs among the 57 freshwater species mentioned above varied from 0.42 mm in *Prochilodus lineatus* and 0.7 mm in *Brachydanio rerio* up to 6.55 mm in *Oncorhynchus tshawytscha* (Liao *et al.* 2018, Supplement S1). We further direct the reader to the following contributions on fish gamete structure and physiology (Jamieson 1991; Linhart *et al.* 1995; Coward *et al.* 2002; Babin *et al.* 2007). More details in fish gametogenesis may be found in the reviews by Babin *et al.* (2007), Lubzens *et al.* (2010) and Schulz *et al.* (2010).

Maturation of gametes

Before the gametes will start to encounter each other, they should mature inside the parental body. Spermatozoa with regular structures could be easily found in the testes; however, the 'normal' structure does not mean that the cell is fully functional. In many fish species, for example, in salmonids, the motility of spermatozoa with a testis origin cannot be initiated (Morisawa 1986). The cells need to be transmitted through the epididymal (testicular) tracts to mature (Morisawa *et al.* 1993). It is likely that the process involves the acquisition and/or exposition of specific receptors, ionic channels, minor changes in membrane
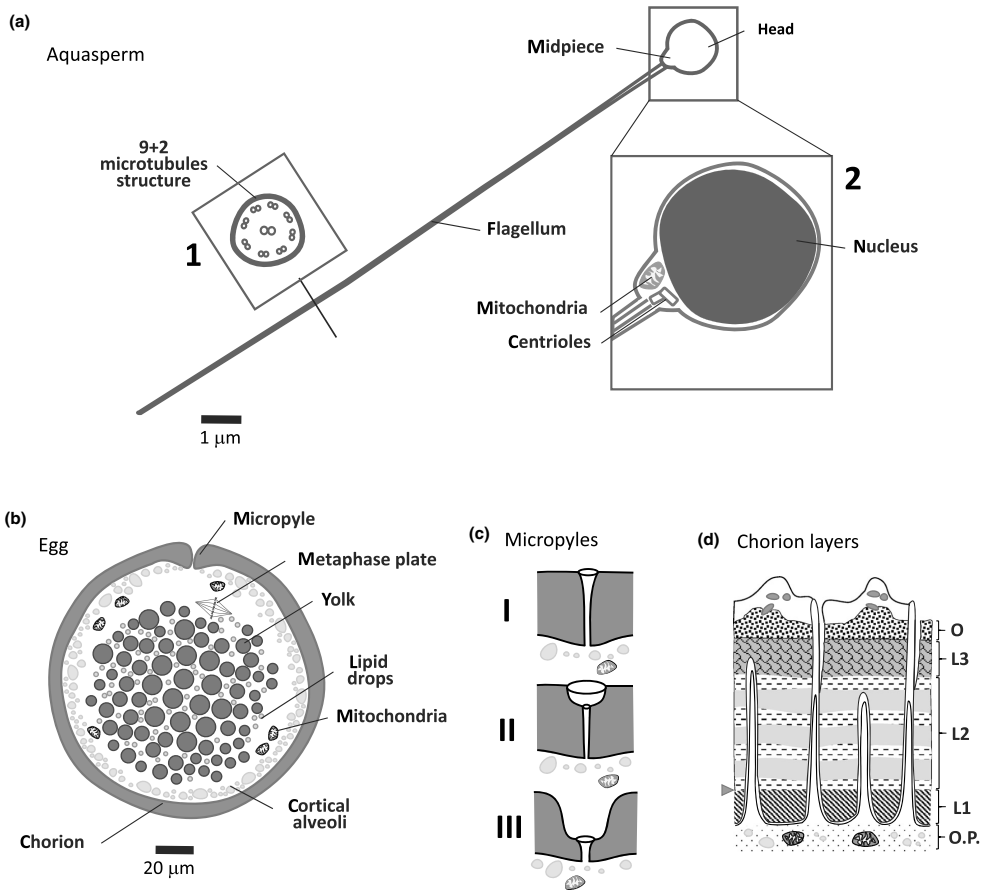
V. Kholodnyy *et al.*

(a) Aquasperm

9+2 microtubules structure

Midpiece

Head

Flagellum

1

2

Nucleus

Mitochondria

Centrioles

1 μm

(b) Egg

Micropyle

Metaphase plate

Yolk

Lipid drops

Mitochondria

Cortical alveoli

Chorion

20 μm

(c) Micropyles

I

II

III

(d) Chorion layers

O

L3

L2

L1

O.P.

Figure 1 Schematic representation of typical gametes from externally fertilizing fish. (a) Carp spermatozoa (according to electronic micrographs by Verma *et al.* (2009)); cross section of flagellum (1) shows 9 + 2 microtubular structure covered with membrane; head and midpiece (2) contain large rounded nucleus, mitochondria and centrioles. (b) Carp egg structure (according to description of Linhart *et al.* (1995)). (c) Three main types of fish egg micropyle (adapted from Yanagimachi *et al.* (2017)). (d) Multilayer structure of fish egg chorion (exemplified by white sturgeon *Acipenser transmontanus*, figure adapted from Murata *et al.* (2014)): O – outer layer of egg envelope; L1, L2 and L3 – layers of chorion with different structure; O.P. – ooplasm with mitochondria and cortical alveoli; the arrowhead shows the border between chorion and oocyte plasma membrane.

composition, effect on motility apparatus (Dzyuba *et al.* 2014; Ciereszko *et al.* 2015; Gallo & Tosti 2015) and/or a combination of these factors: in other words, the sperm cells must gain the ability to respond to the external signals for its activation.

To accomplish successful fertilization, the fundamental structures of mature oocytes are built and assembled during oogenesis (Iwamatsu 2000). In addition to the male gamete, its female counterpart undergoes several transformations to acquire 'fertilizability'. These processes are triggered by an increase in the luteinizing hormone levels in the plasma and the subsequent switch of the steroidogenic pathway to the production of maturation-inducing steroids. The latter bind with oocyte membrane receptors and activate maturation (metaphase)-promoting factor, so-called MPF, which results in meiosis resuming, after which the egg acquires the ability for cortical reaction and then the ability to interact with the spermatozoon (Nagahama & Yamashita 2008).

Spermatozoa and egg activation

After the process of maturation is completed, the gametes are ready to be released outside the fish body. Spermatozoa of teleost fish are generally quiescent in the male body (i.e. in seminal plasma; Morisawa *et al.* 1983). They become motile once released into freshwater or sea water in the case of external fertilization or when ejaculated into the female genital tract during internal fertilization. Usually, motility is initiated by the change in environmental conditions (compared with the conditions of the seminal plasma), both chemical (concentration of organic and non-organic substances, especially ions) and non-chemical (osmolality or temperature) (Billard 1978; Morisawa 1994; Alavi & Cosson 2005, 2006; Fig. 2).

After being released into the environment, spermatozoa experience a change in external osmolality (a rise in marine species and a decrease in freshwater species), followed by a readjustment of internal ionic concentration by osmo-regulative processes in the membrane. After a while, the internal ionic concentration reaches values where dynein-ATPase activity is optimal and the motile velocity is high (Cosson 2004). Later, in the motility period, the ATP content becomes lower because its renewal by mitochondrial phosphorylation is slower than its utilization rate; this process combined with a further readjustment of the internal ionic concentration leads to a gradual decrease, and thereafter, the cessation of dynein activity; these events cause a full arrest of flagellar waves several minutes after motility initiation (Cosson 2004).

Despite the fact that the necessary condition for motility activation in most fish is a change in the osmolality of the medium (Morisawa & Suzuki 1980; Morisawa *et al.* 1983; Linhart *et al.* 1999), the specific ionic composition could contribute to different patterns of motility. This differs among representatives of various families, genera and even within individual species, depending on the occupied ecological niche and habitat (Elofsson *et al.* 2006; Beirão *et al.* 2015); moreover, the differences are often considered as a precondition for preventing crossbreeding (Yoshida *et al.* 2013).

The spermatozoa of some teleosts, for example, salmonids, acquire motility due to a decrease in the K^+ content and an increase or decrease in the osmolarity of the fluid surrounding the spawned spermatozoa (Morisawa 1994). The effects of the potassium ion concentration and changes in the activity of the potassium channels were also found to affect sperm motility traits in common carp (*C. carpio*; Morisawa *et al.* 1983; Redondo-Müller *et al.* 1991; Krasznai *et al.* 1995). These effects were even more prominent in rainbow trout (*O. mykiss*) sperm (Alavi & Cosson 2006) or in burbot (*Lota lota*) sperm (Dziewulska & Pilarska 2018). Activation of spermatozoa motility at low potassium concentrations in external medium has been associated with the hyperpolarization of the plasma membrane resulting from a K^+ outflux (Tanimoto & Morisawa 1988), which in turn induces an intracellular Ca^{2+} concentration rise due to a release from the internal stores (Boitano & Omoto 1991). These phenomena are followed by the increased synthesis of cAMP by adenylate cyclase, and finally, flagellar motion is initiated (Boitano & Omoto 1991). Previously, it was
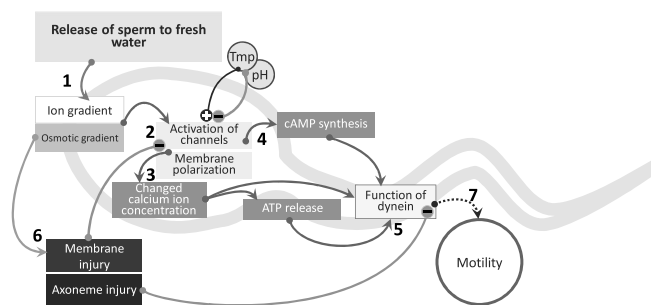

Figure 2 Motility activation and its support in fresh water fish spermatozoa (simplified scheme, details in the text): 1. Release of spermatozoa to the fresh water results in appearance of osmotic/ionic gradient on plasma membrane. 2. The gradient allows the activity of membrane channels and membrane polarization (these depend also on various environmental conditions, temperature (Tmp) and pH in particular). 3. Operation of channels increase the internal concentration of calcium ions (due to influx of external ions and its following release from the internal stores), which take part in regulation of enzymes and the release and synthesis of ATP. 4. Activity of membrane channel cascade involves the synthesis of cyclic monophosphates, which act as mediators and affect function of dyneins. 5. Function of dynein motors in the flagella is controlled by presence of ATP, cyclic monophosphates and calcium ions. 6. The gradients on the membrane affect its integrity and consistency of axoneme. 7. The motility is a balance between activity and injury of cell structures caused by gradients on the membrane.

V. Kholodnyy *et al.*

demonstrated that the activation of trout sperm motility is also accompanied by a transmembrane Ca^{2+} influx (Cosson *et al.* 1989). A recent discovery of a cyclic nucleotide-gated K^+ channel (CNGK) in zebrafish (*Danio rerio*) sperm that mediates a cGMP-induced hyperpolarization (Fechner *et al.* 2015) is helpful for linking these processes together. A similar channel was previously found in invertebrates, for example, in the sea urchin (*Strongylocentrotus purpuratus*; Su & Vacquier 2002). Although the mechanism of motility activation in zebrafish spermatozoa is quite different from that of sea urchin sperm (osmolarity- versus pH-dependence respectively), the principal CNGK function, which is to provide a hyperpolarization event that triggers a Ca^{2+} signal, was evolutionarily conserved. Intracellular alkalization, a key mechanism for the control of sperm motility function in many species, was shown to activate CNGK, thereby triggering a Ca^{2+} signal and a motility response in zebrafish spermatozoa (Fechner *et al.* 2015). A specific cation channel, CatSper, was previously found to regulate the Ca^{2+} ion concentration in mammalian spermatozoa depending on cAMP presence and was associated with the hyperactive motility of the cells (Garbers 2001). Interestingly, the positive reaction to CatSper antibodies was recently discovered in the spermatozoa of rainbow (steelhead) trout (*O. mykiss*, anadromous form), Pacific herring (*Clupea pallasii*), flounders (*Verasper moseri* and *Pleuronectes schrenki*) and medaka (*Oryzias latipes*), but not in goldfish (*Carassius auratus*), loach (*Misgurnus anguillicaudatus* and *Lefua nikkonis*) or zebrafish (*D. rerio*; Yanagimachi *et al.* 2017).

The initiation and duration of sperm motility in common carp (*C. carpio*) was also shown to depend on intra- and extracellular pH (Márián *et al.* 1997). The effect of pH on salmonid spermatozoa was not universal; some scholars reported the absence of sperm motility initiation following the alkalization of external media (Baynes *et al.* 1981), whereas in other studies, the rise of the pH level above the 'physiological' level was shown to increase the per cent of motile cells (Dziewulska & Domagała 2013). Changes in the internal pH of trout sperm were shown to depend strongly on the external pH level, and these changes were mediated by the plasma membrane potential rather than by Na^+/H^+ or K^+/H^+ exchange (Hamamah & Gatti 1998). Moreover, the increase in internal pH at a constant external pH causes plasma membrane depolarization and prevents rainbow trout (*O. mykiss*) sperm motility (Gatti *et al.* 1990). An analysis of the internal pH of *C. carpio* spermatozoa was carried out using a fluorescent probe coupled with flow cytometry (Márián *et al.* 1997). In carp, the changes in the internal pH were associated with the Na^+/H^+ exchanger: the activity of the latter results in a rise of the internal pH of sperm cells and is totally blocked by a high external osmolarity (Márián *et al.* 1997). Changes in the

pH in the physiological range were shown to be important for the initiation of motility but had little effect on motility progress (Alavi & Cosson 2005). Thus, the changes in the Na^+, K^+, Ca^{2+} and H^+ concentrations of the ionic milieu represent key factors of membrane hyperpolarization control due to osmotic pressure changes (Boitano & Omoto 1991; Takai & Morisawa 1995; Krasznai *et al.* 1998). Nevertheless, only the presence of Ca^{2+} ions at a minimal concentration inside the spermatozoa was shown to be an indispensable condition for motility initiation in all fish species (Tanimoto & Morisawa 1988) that have been studied so far.

Generally, the duration of motility is a trade off between the amount of the energy stores possessed by a sperm cell and the amount of osmotic damage experienced by the cell. The latter is more critical in freshwater fish species, whereas the former is important for marine (Cosson 2004). The longevity and velocity of spermatozoa depend on the temperature of the medium (Billard *et al.* 1995; Dadras *et al.* 2017); the higher the temperature, the faster the metabolic exhaustion of energetic resources and the shorter the duration of motility and *vice versa*. This consideration is of course adequate in the temperature limits within which the motility is possible and, in addition, it should also be recognized that a higher temperature will, to some extent, promote the recovery of macro-energetic compounds due to the increase in reaction rates. Under the natural conditions of spawning, the duration of progressive motility in freshwater fish varies from only half a minute in some salmonids (e.g. 29 s in *Salvelinus namaycush*) to several minutes in acipenserids (373 s in *A. dabryanus*). The usual longevity for freshwater cyprinid sperm is approximately 1 min (54 s for *Tinca tinca*, 55 s for *C. carpio* and 75 s for *Hypophthalmichthys molitrix*; Liao *et al.* 2018). The reported initial average path velocities (~10 s post-activation) also vary over a wide interval: from 35 $\mu m\ s^{-1}$ in cyprinid *Rutilus rutilus*, up to 183 $\mu m\ s^{-1}$ in *O. mykiss* and 192.0 $\mu m\ s^{-1}$ in *Acipenser baerii* (Liao *et al.* 2018), but again, those values are highly temperature-dependent.

During the motility period, the motile flagella change the pattern and pass through several stages (Boryshpolets *et al.* 2018). The motility starts with fully developed waves along the whole flagellum with a constant amplitude and a high frequency. During the first stages following activation, the sperm cells can change the mode of motion from helical to planar. Due to the appearance of asymmetrical waves, spermatozoa can change the direction of motion. The beating frequency gradually drops down along with the decrease in the internal ATP concentration; the waves in the posterior part of the flagellum tip decrease their amplitude and disappear after a certain period of time, leading to full motility arrest (Boryshpolets *et al.* 2018).

The fact of sperm motility activation *per se* does not mean that the male gametes could reach the target without any signals controlling the direction of their movement or other traits of motility. Nevertheless, several studies have reported correlations between sperm motility and fertilizing ability in fish (Moccia & Munkittrick 1987; Liley *et al.* 2002; Bozkurt *et al.* 2011; Sheng *et al.* 2014). This could be explained by conditions of artificial fertilization using a very high sperm per egg ratio, which rarely occurs in natural conditions, while less than 0.1% of spermatozoa are involved in fertilization. Freshwater fish spermatozoa were shown to be highly sensitive to any change in environmental conditions, the latter activating and regulating their motility parameters. In addition, several studies have indicated specific motile sperm behaviour when the sperm tend to swim in a close vicinity to a surface (Cosson *et al.* 2003; Woolley 2003; Riedel *et al.* 2005; Elgeti *et al.* 2010; Boryshpolets *et al.* 2013), a feature that was associated with the physical phenomenon of sperm propagation and water properties at a micrometre distance from surfaces (this will be discussed later). This phenomenon may allow spermatozoa to remain near the surface of the egg and helps the spermatozoa find the fertilization site (Ishimoto *et al.* 2016).

The contact of the released egg with aqueous solutions other than ovarian fluid also triggers a process called spontaneous activation in some fish species (e.g. in cyprinids and salmonids), even in the absence of sperm cells (Renard *et al.* 1990; Coward *et al.* 2002). This causes changes in the appearance of the vitelline membrane and its permeability due to the release of cortical granule content (cortical reaction). If this process is initiated, the egg rapidly loses its ability to be fertilized. This process was particularly shown in zebrafish; if no spermatozoa enter the egg during the 30-s period post-activation, later fertilization will not be possible (Lee *et al.* 1999).

Several investigations have even shown that entry of spermatozoa into fertilizable teleost eggs may not be in a sufficient condition to induce activation (Hart 1990). Eggs of medaka (*O. latipes*) could be activated by some artificial stimulants, for example, sodium oleate and saponin, in the presence of calcium ions (Yamamoto 1954).

Another way to provoke artificial activation is the so-called 'prick-activation' by the tip of a microneedle (Hart 1990). The use of the latter approach allowed to reveal that the elevation of the internal calcium ion concentration can activate the fish egg (Yamamoto 1962b). Further studies have shown that the activation of eggs is accompanied/initiated by an increase (abrupt or wave-like depending on the species) in calcium ion concentration inside the egg, a phenomenon that was observed for the first time in *O. latipes* eggs by Gilkey *et al.* (1978). Finally, the following three models were proposed: the reaction associated with the

introduction of calcium ions by a spermatozoon was explained by the 'calcium bomb' model; the signalling cascade activation after contact between a spermatozoon and a specific receptor on the egg surface was explained by the membrane receptor model; and the introduction of Ca^{2+} releasing factor after fusion with a spermatozoon was the soluble sperm factor model (Coward *et al.* 2002). Interestingly, the process of activation can be postponed if the eggs stay in the ovarian fluid for a long period of time and, in this case, no cortical reaction is observed for at least several hours (Billard *et al.* 1986).

Sperm–egg interaction and sperm guidance

Reproductive strategies and gametes' behaviour

A common feature of all externally fertilizing freshwater fish is that they expel their gametes into the aqueous environment. Nevertheless, the exact way that the representatives of various species do this differs depending on their reproduction strategies.

Several species adopt spawning in pairs, for example, *Salmo trutta*; in some species, the established pairs are followed by smaller subordinate males, such as in *Hucho hucho* or *Oncorhynchus kisutch*; in many cyprinids, the spawning is polyandrous, for example, in *C. carpio*, where one female is courted by several males who simultaneously release their sperm in the area around the egg batch; and some species utilize group spawning, where each male in the group fertilizes the eggs of many females, such as in *Perca fluviatilis* (Stockley *et al.* 1997). Other differences include the environment of spawning: these could be streams or shallow still-water (e.g. in *O. mykiss* and *C. carpio* respectively) or the eggs could be layered into male-built nests, similar to the spawning of *Gasterosteus aculeatus*, onto grass substrates, similar to the spawning of *Sander lucioperca* or *C. carpio* or fertilized in open water similar to the spawning of *H. molitrix*.

These different types of behaviour affect the 'scenery' of the fertilization process and thus could change the mechanisms of gamete encounter. It is generally accepted now that polyandry, which is taxonomically widespread, is potentially advantageous for females and allows the choice of the best sire for their offspring due to the arising sperm competition among two or more males to fertilize a given, limited set of ova (Parker 1998; Simmons & Fitzpatrick 2012). The initial studies on sperm competition were based mainly on the hypothesis of so-called 'fair raffle', when success of a particular male depends only on a number of spermatozoa it could provide to the 'fertilization lottery' (Parker 1993). These suppositions led to a suggestion that males will prefer to invest less in each spermatozoon along with the increase in their number ('tiny' sperm); nevertheless, this was not confirmed in comparative studies, where

V. Kholodnyy *et al.*

sperm size was found to be highly labile across the taxa (Pitnick *et al.* 2009). Later, Parker *et al.* (2010) introduced the models that assessed the interrelations between spermatozoa size (mass) and number in various conditions of spermatozoa competition and postulated the existence of a 'trade off' (or balance) between these parameters and the dependence of this balance on the risk of sperm competition. Spermatozoa velocity and longevity are generally supposed to be important traits for the outcome of the fertilization process (especially for external fertilizers; e.g. Gage *et al.* 2004; Geßner *et al.* 2017), as well as egg size/number and finally spawning conditions. Nevertheless, the recent analysis conducted by Liao *et al.* (2018) of relations between these parameters across several freshwater fish species showed that the importance of sperm velocity is overestimated due to differences between laboratory and natural conditions; here, the authors found a strong correlation of ejaculate characteristics with egg number and water turbulence that was typical for the spawning site of particular species.

The abovementioned analysis by Liao *et al.* (2018) may be one among many confirming the complexity of factors that could affect the outcomes of sperm competition, for example, the existence of female post-mating control, socalled 'cryptic female choice' (Thornhill 1983; which we will discuss later in this review), or other environmental factors. Altogether, these may be considered as a part of the so-called guidance hypothesis (Eisenbach & Giojalas 2006). The latter is successful in explaining different aspects of reproduction, such as sperm–egg fusion and specific spermatozoa selection mechanisms. This hypothesis finds confirmation in several species, for example, invertebrates and mammals, where sperm cells use various sensing mechanisms, including chemotaxis, rheotaxis and thermotaxis, to gather physical or chemical cues to spot the egg (Fechner *et al.* 2015). These mechanisms are highly sensitive to changes in the environment, thus driving a high degree of selectiveness among the total sperm population. Spermatozoa chemotaxis is based on the ability of male gametes to sense special molecules called chemoattractants, which leads to the modulation of their motility characteristics depending on variations in the chemoattractant concentration (Miller 1973; Armon & Eisenbach 2011). Likewise, during thermotaxis, the spermatozoa are able to sense small changes in temperature, which can induce further variations in the swimming direction. Thermotaxis typically guides cells to warmer temperatures, where the cells are less prone to tumbling effects, thus achieving smoother and more linear swimming paths (Boryshpolets *et al.* 2013). In addition to the above-mentioned guiding mechanisms, a guidance cue can be provided by the changes in sperm swimming behaviour caused by the direction of fluid flow, namely, by the passive reorientation of a tilted conical helix, representing the flagellum in shear flow (Ishimoto & Gaffney 2015). It is worth noting that these three mechanisms may act independently or in a constructive combination (Cosson 2015; Eisenbach *et al.* 2015).

Strikingly, although spermatozoa chemotaxis was first observed in external fertilizers, such as in sea urchins (Lillie 1912), empirical and theoretical investigations on the spermatozoa guidance mechanisms in fish species, especially freshwater species, are still very scarce in the literature (Cosson 2015). This fact is more intriguing because there is empirical evidence of a possible chemotactic response for selected fish species, and studies of that phenomenon were performed more than half a century ago in fat minnow *Sarcocheilichthys variegatus* (Suzuki 1958), lamprey *Lampetra fluviatilis* and *L. planeri* (Kille 1960) and rainbow trout *O. mykiss* (Hartmann *et al.* 1947). More recent studies on rosy barb *Barbus conchonius*, black flounder *Pleuronectes obscurus*, barfin flounder *V. moseri*, herring *C. pallasii*, and steelhead trout *O. mykiss* (anadromous form) also presented evidence of possible chemoattraction response, in particular found in the micropyle region of fish eggs (Amanze & Iyengar 1990; Yanagimachi *et al.* 2013, 2017). Since the purpose of spermatozoa motility is to fertilize the egg, one can hypothesize that the fluids surrounding the egg or the substances released by the egg itself could somehow affect sperm behaviour.

Interactions between sperm and ovarian fluid

Spermatozoa activation and rise in motility traits in the vicinity of the eggs were first observed in marine invertebrates approximately 100 years ago (Lillie 1912), and specific 'female fluid', the egg jelly, was supposed to be the main factor for this change (the egg jelly that surrounds and sticks to the eggs was observed to 'attract' spermatozoa in sea urchins). The ovarian fluid bathes the mature oocytes in the ovarian cavity of fish (van den Hurk & Peute 1979). The cells lining the ovarian cavity were found to be secretory cells that were active in the medaka *O. latipes* (Yamamoto 1962a). During the release of the spawned eggs through the oviducts into freshwater or saltwater, ovarian fluid still surrounds the eggs of many externally fertilizing female fish (Rosengrave *et al.* 2008), creating a 'protection' coat for the female gametes. In the case of salmonids, which do not have an oviduct and the eggs pass through the body cavity prior to spawning, there was a doubt about the ovarian nature of the fluid, and the fluid was often called coelomic. However, these doubts were not confirmed by simple analysis of other possible sources of the fluid, for example, the coelomic cavity (the absence of a reasonable amount of fluids in the coelomic cavity and no secretory activity of coelomic cells; Lahnsteiner *et al.* 1995).

Ovarian fluid origin and composition

The ovarian fluid composition varies among species and generally contains ions (Table 1) and different substances made from different ratios of proteins, sugars and lipids. Sodium and chloride are usually the major ions present in the ovarian fluid of most fish species. Other ions include calcium, magnesium and potassium. The ion ratio is similar among relative species populating similar conditions but varies significantly between marine and freshwater inhabitants. As shown in the data in Table 1, the ovarian fluid of salmonid and cyprinid fish is alkaline. This feature helps to stabilize the microenvironment around the egg, especially in acidic waters (Lahnsteiner *et al.* 1995). The inorganic composition of the ovarian fluid of the family Salmonidae is adapted to egg storage and prolongs the fertilization period in natural and artificial conditions (Lahnsteiner *et al.* 1995). Moreover, in salmonids, the low-potassium level and alkaline pH of ovarian fluid activate sperm motility (Morisawa *et al.* 1983). The osmolality of ovarian fluid is adequate to prevent the activation, cortical reaction and swelling of the eggs before fertilization (Billard *et al.* 1974; Billard & Cosson 1988). The ovarian fluid was often reported to be more viscous than water (e.g. Rosengrave *et al.* 2009a). This phenomenon is believed to protect the egg batches from being washed away by water flow (or to at least delay the process), such as the case for salmonids (McDowell 2000). In addition, a higher viscosity could contribute to partly slowing down the diffusion process, thus holding the ion concentration in the vicinity of the egg surface (Elofsson *et al.* 2003).

The organic composition of the ovarian fluid is characterized by high levels of protein, free amino acids, glucose, lactate, phospholipids and cholesterol. In salmonids, that is, rainbow trout, lake trout, charr, Danube salmon and Caspian brown trout (*O. mykiss*, *S. namaycush*, *Salvelinus alpinus*, *H. hucho* and *S. trutta caspius* respectively), the total protein concentration amounts to 1.17 ± 0.20; 1.46 ± 0.23; 0.95 ± 0.28; 2.78 ± 0.15; and 2.23 ± 0.45 mg mL^{-1} respectively (Lahnsteiner *et al.* 1995; Bahrekazem *et al.* 2009). In a cyprinid representative, bleak *A. alburnus*, proteins are also a main component of the ovarian fluid, and their concentration equals 1.58 mg mL^{-1} (Lahnsteiner *et al.* 1997). The amount of proteins reaches a similar range in the ovarian fluid of sturgeons: 2.98 ± 0.35; 2.41 ± 0.30; and 3.57 ± 1.41 in sterlet (*Acipenser ruthenus*), Russian and Siberian sturgeons (*Acipenser gueldenstaedtii* and *A. baerii*) respectively (Siddique *et al.* 2016b). The total amount of protein in the ovarian fluid may vary significantly, even in the same fish during the spawning season; for example, in the turbot *Scophthalmus maximus* at the start of the spawning season, the protein content amounts to 3.96 ± 0.05 mg mL^{-1}, decreases to 0.54 ± 0.01 in the

mid-season and levels up to 7.63 ± 0.55 in the late season (Jia *et al.* 2015). The proteomic analysis of rainbow trout ovarian fluid revealed more than 50 different molecular species (Nynca *et al.* 2015); the predominant proteins of *O. mykiss* ovarian fluid were associated with binding and catalytic activity; the other proteins (approximately 15%) were related to the immune system, proteolysis, carbohydrate and lipid binding and metabolism, cell structure and cell shape. A significant activity of some enzymes was found in the ovarian fluid of various species. In particular, high levels of acid phosphatase, alkaline phosphatase and aspartate aminotransferase were found in *S. maximus* (45.28 ± 4.51; 13.73 ± 3.50; and 40.59 ± 1.20 μg mL^{-1} protein, respectively, in the early spawning season; Jia *et al.* 2015). These enzymes were reported to have important functions during follicular development and egg maturation. In *O. mykiss*, *S. namaycush*, *S. alpinus*, and *H. hucho*, the alkaline phosphatase was also the most active among the investigated enzymes present in the ovarian fluid, followed by lactate dehydrogenase, 13-D-glucuronidase, protease and acid phosphatase, while the glucose-6-phosphate dehydrogenase activity and α-glucosidase activities were completely missing (Lahnsteiner *et al.* 1995). Hydrolases, hydrogenases, glucose, lactic acid and other organic acids have been identified in *C. carpio* ovarian fluid (Ginsburg 1968). In another cyprinid, bleak *A. alburnus*, the most active enzymes in the ovarian fluid were alkaline and acid phosphatases and protease, and the fluid contained a significant amount of glucose and cholesterol, as well as some galactose, glycerol, phosphatidylcholine and choline (Lahnsteiner *et al.* 1997). Fluctuations in the organic components of the ovarian fluid in related species are generally higher than those of inorganic components and may indicate a dynamic organic metabolism (Lahnsteiner *et al.* 1995).

Thus, the ovarian fluid contains some compounds that were shown to activate motility and control its progression. The differences in the fluid composition among species could differently affect the characteristics of sperm motility, for example, cause a chemokinetic effect.

Effects of ovarian fluid on sperm kinetics

As already mentioned, ovarian fluid is a maternally derived liquid that surrounds the egg mass inside the female fish and is expelled during spawning (Rosengrave *et al.* 2008). It represents 10–30% of the weight of the egg batch in Salmonidae (Lahnsteiner 2002). After being released with the eggs, the ovarian fluid contacts the surrounding medium (freshwater or saltwater), creates a protective coat around the batch and changes the basic properties of the adjacent water environment, creating a peculiar milieu for subsequent fertilization.

V. Kholodnyy *et al.*

Table 1 Ions in ovarian fluid of some fish species

Species	Osmolality, mmol kg⁻¹	Ion content (mM)					pH	Reference
		Na⁺	K⁺	Ca²⁺	Mg²⁺	Cl⁻		
Oncorhynchus tshawytscha	278–322	156–172	2.7–4.4	2.1–5.5	0.35–2.5	100–119	8.43 ± 0.13	Rosengrave et al. (2009a)
Salmo trutta caspius							8.32 ± 0.12	Bahrekazem et al. (2009)
Salmo trutta	268.2 ± 7.7	106.6 ± 10.7	1.7 ± 0.4	0.58 ± 0.07			8.6 ± 0.1	Lahnsteiner et al. (1995)
Salmo alpinus	256.4 ± 16.2	111.0 ± 13.6	1.9 ± 0.5	0.61 ± 0.10			8.6 ± 0.1	
Hucho hucho	290.3 ± 4.4	142.2 ± 11.7	2.2 ± 0.7	0.6 ± 0.1			8.8 ± 0.1	
Oncorhynchus mykiss	291.6 ± 12.9	134.7 ± 7.4	2.7 ± 0.2	0.45 ± 0.04			8.4 ± 0.1	
Oncorhynchus mykiss		145	4.0	4.5	0.5			Holtz et al. (1977)
Scophthalmus maximus (mid-season)		206.67 ± 3.53	10.67 ± 1.07	2.65 ± 0.18		165.33 ± 2.67	8.01 ± 0.01	Jia et al. (2015)
Gasterosteus aculeatus L.Freshwater/brackish water/salt water populations	208 ± 25; 246 ± 26; 314 ± 47	136 ± 12; 150 ± 11; 203 ± 30	2.12 ± 1.32; 3.53 ± 1.72; 3.67 ± 0.90	1.58 ± 0.34; 0.16 ± 0.32; 2.64 ± 0.71		102 ± 18; 136 ± 71; 71 ± 36		Elofsson et al. (2006)
Ctenopharyngodon idella		741.0	0.45	6.38	2.58		8.1	Linhart et al. (1995)
Carassius gibelio (April)	237.0 ± 27.12	129 ± 5.2	2.1 ± 0.24	0.54 ± 0.42	0.66 ± 0.15	135.4 ± 3.6	8.2 ± 0.2	Taati et al. (2010)
Alburnus alburnus	190.0 ± 6.28	171.58 ± 25.83	2.93 ± 0.57	0.63 ± 0.11			8.61 ± 0.10	Lahnsteiner et al. (1997)
Acipenser ruthenus	208.43 ± 9.20	104.68 ± 7.74	6.11 ± 0.55	0.92 ± 0.17	0.63 ± 0.04	89.80 ± 6.41	7.92 ± 0.03	Siddique et al. (2016b)
Acipenser baerii	213.50 ± 8.03	126.37 ± 6.19a	5.42 ± 0.42	0.87 ± 0.06	0.67 ± 0.06	98 ± 5.33	7.98 ± 0.03	
Acipenser gueldenstaedtii		123.01 ± 5.86a	4.39 ± 1.06	0.96 ± 0.24	0.57 ± 0.07	94.0 ± 5.72	7.96 ± 0.04	
Leuciscus idus	321.7 ± 14.5	149.0 ± 12.2	2.8 ± 1.9	1.6 ± 0.3	1.0 ± 0.3	113.6 ± 12.3	8.1 ± 0.1	Siddique et al. (2016a)
Esox lucius	291.2 ± 8.7	149.6 ± 12.4	6.2 ± 2.5	1.9 ± 0.4	0.8 ± 0.3	110.8 ± 13.6	8.2 ± 0.3	

One of the most interesting features of ovarian fluid is its effect on sperm swimming performance in many fish species. There are numerous reports about differences in sperm behaviour after being activated in ovarian fluid (or its mixtures with water at different ratios) instead of water *per se*. In particular, higher velocities of spermatozoa in ovarian fluid compared with water were found in the guppy *Poecilia reticulata* (Gasparini & Pilastro 2011), the lake trout *S. namaycush* (Butts *et al.* 2012), the Arctic charr *S. alpinus* (Turner & Montgomerie 2002; Urbach *et al.* 2005), the steelhead *O. mykiss* (Woolsey *et al.* 2006) or in the brown trout *S. trutta f. fario* (Lahnsteiner 2002). The per cent of motile cells increased when activated in ovarian fluid from *S. trutta f. fario* (Lahnsteiner 2002) and from *S. namaycush* (Butts *et al.* 2012). Much evidence has been found regarding the higher longevity of sperm in ovarian fluid, for example, in brown trout *S. trutta f. fario*, lake trout *S. namaycush*, three-spined stickleback *G. aculeatus*, marine sculpin *Hemilepidotus gilberti* and Arctic charr *S. alpinus* (Hayakawa & Munehara 1998; Lahnsteiner 2002; Turner & Montgomerie 2002; Elofsson *et al.* 2003; Butts *et al.* 2012). In other words, ovarian fluid provokes a kinetic effect on sperm cells and is of great biological importance.

Sperm motility in general and swimming speed in particular were shown to be key determinants in male fertilization success under conditions of sperm competition in a variety of species, for example, lake trout (Butts *et al.* 2012) and salmon (Butts *et al.* 2017). However, there is no clear understanding about the mechanisms of such enhancement provided by ovarian fluid. Some authors claim that only the ionic composition is responsible for better motility traits, showing that there was no significant difference between the motility traits assessed in ovarian fluid and the saline solution in various salmonids (Lahnsteiner 2002; Hatef *et al.* 2009; Rosengrave *et al.* 2009b). The effect of various ion concentrations was not the same. Each ion had a contributory and prohibitory concentration for sperm motility. In particular, it is known that the presence of Ca^{2+} ions is obligatory for sperm motility activation in the Pacific herring *C. pallasi* (Cherr *et al.* 2008); these ions were critical for spermatozoa entry into the micropyle in several species (Yanagimachi *et al.* 2013, 2017). At the same time, excessive Ca^{2+} inhibited sperm motility in the marine Atlantic cod *Gadus morhua* (Beirão *et al.* 2015) and in the chinook salmon *O. tshawytscha* (Rosengrave *et al.* 2009b). In some other marine fish, specific extracellular ions were reported not to affect the motility initiation of sperm at all, and only the increased osmolality was essential for the intracellular release of Ca^{2+} (Morita *et al.* 2003). In other studies, the effects of ovarian fluid on sperm motility were associated with its protein and carbohydrate composition (Yoshida & Nomura 1972) or pH (Ciereszko *et al.* 2010).

Proteinaceous or peptidic sperm-activating factors have been identified in ovarian fluid or among the substances released by the eggs of sea urchins (several species; Suzuki 1990; Suzuki & Yoshino 1992), jellyfish (*Hippopodius hippopus*; Cosson *et al.* 1986), starfish (*Asterias amurensis*; Nishigaki *et al.* 1996), herring (*C. pallasi*; Morisawa *et al.* 1992; Pillai *et al.* 1993; Oda *et al.* 1995) and newts (*Cynops pyrrhogaster*) (Watanabe *et al.* 2010) (Table 2). The release of several glycoproteins mostly originating from the chorion after contact with water was shown in *A. ruthenus* eggs and was associated with the potential chemotactic effects of these substances (Niksirat *et al.* 2017; more about these potential chemotactic agents released by the egg below in the next section). In contrast, the sperm-activating factors in the coral *Montipora digitata* (Coll *et al.* 1994) and ascidians *Ciona intestinalis* and *Ciona savignyi* (Yoshida *et al.* 2002) are small organic compounds: unsaturated fatty alcohols in the former and sulphated sterols in the latter. Some studies have shown that the activating factors were expelled by the egg itself into the water, for example, in the Pacific herring *C. pallasii* (Pillai *et al.* 1993) or by a specific structure covering the animal pole of the egg of the jellyfish *H. hippopus* (Cosson *et al.* 1986). Thus, the above survey of the literature shows that the ovarian fluid considerably affects the sperm motility traits in aquatic species, particularly in fish. Often, these chemokinetic effects could not be easily isolated from the other specific chemoattractive reactions observed in the presence of egg-associated agents, including ovarian fluid.

Ovarian fluid and substances released by the egg as attractants

It seems very likely that after being activated, spermatozoa should find their way to the egg driven by constitutively present factors in the ovarian fluid or released by the egg. These phenomena could underlie the success of fertilization and the prevention of crossbreeding (Yoshida *et al.* 2013), as well as provide intraspecific sperm selection (Evans *et al.* 2012). It was previously stated that only a small proportion of the sperm population is competent for fertilization in mammals because of the differences in their integrity and/or the presence of morphological and genetic abnormalities (Cohen 1975). Under the assumption that this fitness theory is true in teleost fish, Amanze and Iyengar (1990) stated that the randomness of egg and sperm contact could not be the only proper strategy for the stability and variability of living beings, and other mechanisms are highly probable. Moreover, in fish, this is aggravated by the fact that only one site per egg (in vast majority of species, except sturgeons where there are several) is present for a spermatozoon to enter the egg and that the fertilization period after the activation of gametes in/by water is relatively short (Amanze & Iyengar 1990).

V. Kholodnyy *et al.*

Table 2 Identified and potential agents with chemokinetic and chemoattractant effects in animals with external fertilization

Species	Substance	Origin	Class	MW	Attractant/ activator	Reference
Corals						
Montipora digitata	dodeca-2,4-diynol	Egg	Fatty alcohol	243.4	Attraction	Coll *et al.* (1994)
Lobophytum crassum	(—)-epi-thunbergol	Egg	Cembranoid diterpene	278.48	Attraction	Coll *et al.* (1995)
Ascidians						
Ciona intestinalis/Ciona savignyi	SAAF (sperm-activating and -attracting factor) 3,4,7,26-tetrahydroxycholestane-3,26-disulphate	Egg	Sulphated steroud	297.13	Activation, attraction	Yoshida *et al.* (2002)
Sea urchins						
Hemicentrotus pulcherrimus/ Stronglyocentrotus purpuratus	Speract (SAP, sperm-activating peptide) Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly	Egg jelly	Peptide	892	Activation, attraction	Hansbrough and Garbers (1981) and Suzuki (1990)
Arbacia punctulata	Resact (SAP, sperm-activating peptide) Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH₂	Egg jelly	Peptide	1245	Activation, attraction	Ward *et al.* (1985)
Cuttlefish						
Sepia officinalis	SepSAP (SAP, sperm-activating peptide) Pro—Ile—Asp—Pro—Gly—Val—CONH₂	Egg mass	Peptide	596.6	Attraction	Zatylny *et al.* (2002)
Starfish						
Asterias amurensis	Several isomeric sperm-activating peptides, Asterosaps	Egg jelly	Peptide	3773–4003	Activation, attraction	Nishigaki *et al.* (1996)
Jellyfish						
Hippopodius hippopus	–	Egg-associated structure	Protein	25 000	Attraction	Cosson *et al.* (1986)
Abalone						
Haliotis rufescens	L-tryptophan	Egg	Amino acid	204.23	Attractant	Riffell *et al.* (2002)
Newt						
Cynops pyrrhogaster	SMIS (sperm motility-initiating substance)	Egg jelly	Protein	34 000	Activation	Watanabe *et al.* (2010)
Frog						
Xenopus laevis	Allurin	Whole-egg jelly	Acidic protein	21 073	Attractant	Olson *et al.* (2001)
Herring						
Clupea pallasii	SMIF (sperm motility-initiating factor)/MISA (micropylar sperm attractant)	Egg (micropyle area)	Polypeptide	105 000	Activation, attraction	Pillai *et al.* (1993); Yanagimachi *et al.* (2017)
	HSAPs (herring sperm-activating proteins)	Egg		~7700	Activation	Oda *et al.* (1995)
Rainbow trout						
Oncorhynchus mykiss	Astaxantin	Ovarian fluid	Karotenoid	597	Attraction	Hartmann *et al.* (1947)
	Not identified	Ovarian fluid	Not identified	1000–3000	Kinetic effect	Yoshida and Nomura (1972)

There are many examples in nature showing that several cell types exhibit a specific behaviour in the presence of a gradient of certain chemical agents, that is, they can recognize specific molecules, find the direction of an increasing concentration of these molecules and move preferentially towards their source. Such features were observed

in microorganisms, such as bacteria and amoebae, and in mammalian immune cells, such as leucocytes (Kretschmer & Collado 1980). In spermatozoa, the earliest observations of such behaviour were made in sea urchins (Arbacia) and marine worms (Nereis; Lillie 1912), and further observations were made later in the medusa *Spirocodon saltatrix* (Dan 1950) and in the rainbow trout *O. mykiss* (Hartmann *et al*. 1947). Such chemoattractants are scarcely described in fish (Cosson 1990, 2015). Generally, the candidate substances thought to be clearly distinguished from the factors causing chemokinesis and/or sperm trapping (Eisenbach & Giojalas 2006) usually use one or two criteria: on one hand, the spermatozoa should move directionally to the source of the chemoattractant, and on the other hand, the receptors associated with the attraction should exhibit saturation at a certain concentration level; once saturated, the reaction of cells to the attractant will cease (peak-like dependence). Some substances that activate and affect spermatozoa motility and are chemoattractant candidates are present in the ovarian fluid or secreted by the eggs (Table 2). Such substances could improve the outcome of fertilization because of their contribution to sperm guidance to the egg. The known sperm attractants (or candidate attractants) in different species of marine or freshwater external fertilizers were mostly of a proteinaceous or peptidic nature (Table 2); this phenomenon is particularly true in sea urchins and cuttlefish (Ward *et al*. 1985; Zatylny *et al*. 2002; Guerrero *et al*. 2010). An investigation on the Pacific herring *C. pallasii* showed the presence of a glycoprotein sperm attractant around the micropyle opening (Yanagimachi *et al*. 2013). Smaller molecules serve as chemoattractants in the red abalone *Haliotis rufescens* (amino acid L-tryptophan; Riffell *et al*. 2002) and the rainbow trout (carotenoid astaxanthin; Hartmann *et al*. 1947). There are only scarce studies on the direct demonstration of any type of chemotactic behaviour of freshwater fish spermatozoa, probably because of the complications in the experimental design due to their particular spawning conditions, that is, the low osmolality of the environment results in rapidly developing osmotic shock that leads to the loss of the ability of gametes to fertilize (male) or to be fertilized (female) within tens of seconds following their activation by contact with the surrounding water (Hoysak & Liley 2001).

Influence of ovarian fluid on the fertilization success
It is obvious from the literature described above that ovarian fluid improves sperm motility in most fish species. Therefore, it is very probable that the presence of ovarian fluid is necessary for successful fertilization because sperm motility, swimming speed in particular, was reported as a crucial factor for male impact under conditions of sperm competition in a variety of species, such as lake trout (Butts *et al*. 2012). Indeed, in fertilization assays with the Caspian

brown trout *S. trutta caspius*, in conditions of sperm competition, embryonic eyeing stage rates were higher after the activation of sperm in ovarian fluid (and in saline solution with the same ionic composition as ovarian fluid) than in freshwater (Hatef *et al*. 2009). Lahnsteiner (2002) showed that ovarian fluid improved the fertilization outcome compared with the fertilization outcome in water, and the effect was associated with an elevation of sperm motility, the number of fertilizable eggs, and sperm–egg contact. Nevertheless, there were only slight but significant differences in the fertilization rates in ovarian fluid compared with those in an artificial fertilization solution of an ionic nature, and these effects only occurred if a low sperm-to-egg ratio was used. Not less important is the protective effect provided by the ovarian fluid to the eggs. In salmonids, the eggs lose their fertility after 1 min of contact with water (Billard 1983), whereas in the ovarian fluid, they remain fertile for more than 10 min (Lahnsteiner 2002). The stabilizing effect of ovarian fluid on gamete physiology depends on the dilution ratio of the ovarian fluid in water. The extent of stabilization was significantly reduced at a dilution ratio of 1:1 and it was completely lost at a ratio of 1:8, (Lahnsteiner 2002). In natural conditions, the effect of the ovarian fluid could be significant since it surrounds the eggs and their micropyle, and its volume is significantly large (e.g. up to 30% of the total egg batch volume in salmonids; Lahnsteiner *et al*. 1995) in many fish species; as a rule, the release of sperm and eggs by the two partners is simultaneous (Hart 1990; Yanagimachi *et al*. 1992). Thus, it is clear that in salmonids, the ovarian fluid improves the fertilization rates in comparison to water *per se* due to its effect on sperm motility and egg fertilizability. Moreover, in these species, ovarian fluid could also compensate for suboptimal environmental conditions (Lahnsteiner 2002). Unfortunately, there is a lack of experimental data on the use of ovarian fluid during the fertilization of fish eggs in other species. It is highly probable that the species utilizing other reproduction strategies would have other outcomes of egg fertilization depending on the presence/absence of ovarian fluid.

The ability of sperm cells to react to changes in the environment, for example, the fluid viscosity, background flows, pH, ion concentration and even temperature, makes the simplistic view of random fertilization unlikely during sperm navigation. Successful fertilization will not be possible if the spermatozoon does not reach the micropyle, which is why the characteristics of its area and male gamete behaviour could be critical to the entire process.

Interactions with the micropylar region of the egg

As stated above, the fish eggs possess a specific site, the micropyle, that is a narrow opening inside the egg chorion

V. Kholodnyy *et al.*

that allows the spermatozoon to pass through the dense membrane and reach the ovum. This is the only site where egg–spermatozoon contact is possible because the chorion is normally impermeable to sperm cells. It was shown that the area around the micropyle in many species is not flat but is slightly depressed and may have various groove ridges or surface microvilli-like projections (Kudo 1980; Hart & Donovan 1983; Amanze & Iyengar 1990). Moreover, an analysis of the overall structure of the micropyle opening allowed to categorize three types of micropyle openings (Fig. 1c). The first type is characterized by a man-hole-like canal opened directly onto the chorion surface with no (or almost no) depression of the surrounding area (this type is common for marine fish, e.g. *C. pallasii*); the second type has a funnel-like canal and a shallow saucer-like pit (typical for salmonids); and the third type is characterized by a deeply depressed sink-hole-like depression in the chorion, which is connected to a short canal (typical for cyprinids; Jamieson 1991; Yanagimachi *et al.* 2017). Fish spermatozoa, similar to the spermatozoa of many other animals, possess thigmotactic behaviour, that is, the ability to follow the chorion surface or any other surface (Cosson *et al.* 2003; Woolley 2003; we will discuss this behaviour in more detail later). Taking this into account, the special shape of the micropyle region could facilitate the orientation of spermatozoa and its penetration into the canal. In particular, it was found in the rosy barb *B. conchonius* that the micropyle area in the eggs of this species has 7–10 ridges, and the vast majority of spermatozoa appearing in this region travelled along the grooves to appear in the micropylar vestibule (Amanze & Iyengar 1990). Several studies with mathematical simulations of the hydrodynamic forces arising during the swimming of the spermatozoa along a surface showed that these are strong enough to retain spermatozoa in the close vicinity of that surface (Riedel *et al.* 2005; Elgeti *et al.* 2010). Riedel *et al.* (2005) stated that even the 'large-scale coordination of cells can be regulated hydrodynamically, and chemical signals are not required'. Ishimoto *et al.* (2016) made a limitation to that statement and studied the swimming pattern of a virtual turbot spermatozoon. The authors have found that virtual sperm cells can follow the surface to search the micropyle, but this swimming will be very sensitive to geometrical parameters, for example, the curvature of the surface. In the case of turbot-sized eggs (more precisely, all the eggs with radii smaller than 1.8 mm), the 'guidance cue' will not be strong enough to retain the sperm cell (Ishimoto *et al.* 2016). This could be partly confirmed by the findings made by Iwamatsu *et al.* (1993) who reported no effect of the micropyle area structure on medaka *O. latipes* sperm traits (velocity or rotation direction) if the canal was occluded artificially or following fertilization. Iwamatsu *et al.* (1993) concluded that some other factors, presumably

of a chemical nature, are involved in the spermatozoa guidance; nevertheless, the role of the peri-micropylar depression was not excluded. The existence of harmonic assemblage involving both chemical and physical agents associated with the micropylar region was shown in numerous studies by Yanagimachi *et al.* (1992, 2013, 2017). In particular, a glycoprotein bound to the chorion around the micropyle was found in several fish species, including herring (*C. pallasii*) flounders (*P. obscurus*, *V. moseri* and *P. schrenki*), steelhead trout (*O. mykiss*), and medaka (*O. latipes*), which guided the spermatozoa into the canal (Yanagimachi *et al.* 2017). The interaction of spermatozoa with the micropyle was strongly dependent on Ca^{2+} presence in all the mentioned species. In other studied species, such as goldfish (*C. auratus*), loach (*M. anguillicaudatus* and *L. nikkonis*), and zebrafish (*D. rerio*), no such glycoprotein was found, nor was any Ca^{2+} dependence for the process of spermatozoa approach to the micropyle vestibule. Interestingly, the latter species were exclusively freshwater species, and the shape of their micropyle was significantly different from that of the other fish, where chemotactic behaviour was present (Yanagimachi *et al.* 2017).

The variety of signalling features developed by female cells, including the factors contained in ovarian fluid and the influence exerted by the micropyle area of the egg, could not piece together the entire system of guidance/navigation without a consideration for how the sperm cell senses these signals.

Intracellular mechanisms of specific spermatozoa responses

Many scholars hypothesize that the membranes of external fertilizers' spermatozoa (among which marine invertebrates were studied the most) have special receptors that specifically react to the presence of particular substances and initiate the cascade of intracellular responses resolving into changes in motility traits and trajectory. In particular, Matsumoto *et al.* (2003) found a receptor-type guanylyl cyclase in the starfish *A. amurensis* sperm flagellum that bound asterosap (a peptide with chemoattractant effect) and provoked a cGMP-mediated increase in internal Ca^{2+} concentration. A receptor to a chemoattractant named 'speract' was revealed earlier in the sperm flagella of the sea urchins *S. purpuratus* and *Lytechinus pictus* (Cardullo *et al.* 1994). The general signalling pathway for marine invertebrates (exemplified by *A. punctulata* and another chemoattractant, 'resact') was described by Kaupp *et al.* (2008): the chemoattractant molecule binds and activates the receptor of the guanylate cyclase family, initiates the synthesis of cGMP and mediates the opening of the K^+-channel, the hyperpolarization of the membrane and the rise in the

intracellular calcium ion concentration. The activation is transient, and the receptor then undergoes phosphorylation and remains in a resting state until the next binding with a chemoattractant. The resulting changes in the intracellular Ca^{2+} concentration trigger symmetric/asymmetric flagellar beating and control the trajectory of sperm cells in the gradient of the chemoattractant. For example, spermatozoa of the sea urchin *A. punctulata* will swim in to circles until sensing the resact molecule, which will result in rapid, specific 'turn-and-run' trajectories to approach the source of the attractant molecules (Kaupp *et al.* 2008). Similar behaviour of mammalian spermatozoa is well known as hyperactivation (Suarez & Ho 2003).

It was hypothesized by Garbers (1989), that the similarity (and even evolutionary conservation) in the intracellular domains of revealed receptors (also taking into account the known examples of chemoreceptors in bacteria and other receptors) and the variation in their binding structures should provide equal biological responses while keeping specificity to the effector molecules. The idea about the similarity between mechanisms controlling the fertilization process was also substantiated by Yoshida *et al.* (2008), that is, the dependence of various steps of the process on internal Ca^{2+} concentration/presence and a role of cyclic nucleotides (such as cAMP or cGMP) as mediators. These hypotheses allow us to assume that there are receptors sensing the chemoattractants in externally fertilizing fish, whereas their existence has not yet been shown experimentally as clearly as the receptors have in marine invertebrates. In particular, in the herring *C. pallasii*, it was shown that several specific components, including a chemoattractant substance, K^+, Ca^{2+}, cAMP and a calcium-selective channel (CatSper analogue), were involved in spermatozoa chemotactic behaviour (Yanagimachi *et al.* 2017). The authors presumed the existence of receptors to the chemoattractant and that the receptors most likely possess a trypsin-like activity because the chemotactic behaviour of herring spermatozoa was blocked by serine protease inhibitors. It was also reported by the same research group that the spermatozoa activity of the trout *O. mykiss* and the success of fertilization depended on the presence of cAMP and external Ca^{2+}, as well as on the rise in internal Ca^{2+} concentration; at the same time, no involvement of cAMP in the process of sperm penetration into micropyle was revealed in *C. auratus* (Yanagimachi *et al.* 2017). Thomas *et al.* (2004, 2005) found a receptor in the spermatozoa midpiece of the Atlantic croaker *Micropogonias undulatus*; the receptor initiated the rapid rise in intracellular Ca^{2+} and cAMP concentrations as well as increased the spermatozoa activity in the presence of specific steroids, and these effects also contribute to oocyte maturation in this species. This receptor could be qualified as a candidate for the described 'sensing' cascade, although unfortunately, the authors have not studied any chemotactic activity of the mentioned steroid.

No less remarkable are the assumptions and speculations about the driving force and mechanisms of thigmotactic behaviour of the spermatozoa. As mentioned above, spermatozoa preferentially follow the vicinity of any surface (Woolley 2003), including that of the chorion in the case of fish eggs; this phenomenon is why the sperm cells do not require any chemical signals to coordinate this swimming (Riedel *et al.* 2005); however, sperm behaviour may depend on geometric characteristics of the followed surface (mainly the curvature radius; Ishimoto *et al.* 2016). Several scholars have tried to elucidate the mechanism of such behaviour in cells. In particular, Hernandez-Ortiz *et al.* (2005) stated that the motion of flagellated cells near the surfaces could be described by relatively simple hydrodynamic models; their mathematical simulations revealed that at low concentrations, the flagellated cells, for example, the spermatozoa, tend to move towards the confining walls. Berke *et al.* (2008) showed that motile cells approaching the surface are attracted to the surface due to hydrodynamic forces and reorient and swim in their close vicinity in a parallel direction. However, Kantsler *et al.* (2013) found that hydrodynamic forces play only a secondary role in these superficial interactions and that the contact of the flagella with the walls could be the main factor. The model by Elgeti *et al.* (2010) accounts equally for both the 'driving' forces of superficial attraction, that is, a hydrodynamic attraction of the midpiece region caused by low pressure between this part of the cell and the 'wall' and the thrust motions of the head to the surface due to a repulsion of the tail away from the surface. The latter model allowed the authors to explain the circular approach to the chemoattractant source. Experimental studies in fish spermatozoa showed that the cell changes the helical pattern of flagellar beating to a planar pattern, which provides higher efficiency during propagation on a surface and *vice versa* (Boryshpolets *et al.* 2013). More details about spermatozoa features associated with reaction to external signals can be found in the book edited by Cosson (2015), including the limitation of modelling approaches. More examples of combined observations and the biophysical or mathematical modelling of 'guided'-sperm swimming will be presented in the next section.

Biomathematical modelling and in-silico investigations on sperm guidance

A variety of mathematical models devoted to different aspects of sperm swimming behaviours have been developed to date. It would be a difficult task to review this wealth of advances here; thus, we direct the reader to review on the topic (Lauga & Powers 2009; Gaffney *et al.* 2011).

V. Kholodnyy *et al.*

Founding studies, such as the pioneering work of Gray and Hancock (1955) shaped the research that is still carried out today, mainly by constructively combining theory and empirical observations. In this section, we discuss selected examples of how such interdisciplinary work has been used thus far to advance our understanding in swimming spermatozoa research. Despite the ongoing success of combining observations and theory on the realm of spermatozoa swimming, the latter have been mostly focused on model organisms, such as sea urchin and selected species of mammalian spermatozoa. To date, the interdisciplinary applications of these cross-fertilizing interdisciplinary methods have been overlooked in fish reproduction research. This current gap in the literature thus represents a vast and fruitful environment for multidisciplinary interactions.

From a biophysical, mathematical and chemical standpoint, sperm guidance is an incredibly complex system. The latter involves multilayered interactions at different length and time scales to achieve reproductive biological function. To date, no attempt has been made to model the complex cascade of interactions described in the sections above required for freshwater fish reproduction. Here, we present few examples of the successive union of theoretical and observational studies that lead to important discoveries that would not otherwise be possible. We hope that this will encourage cell biologists, sperm physiologists and experts in fish reproduction to interact more with mathematicians, physicists, engineers and computer scientists (the list is not exhaustive) for fresh and interdisciplinary collaborations.

From the flagellar beating to the molecular motor coordination during guidance

Mathematical models attempt to incorporate key biophysical interactions driving the sperm flagella movement. The aim was to achieve predictive power and gain a deeper understanding of ubiquitous mechanisms involved in the process, as well as to test hypotheses, among others. No mathematical model can ever describe the full complexity, and assumptions are made at every stage in order to reduce this complexity and enable its investigation. Models serve as a mere approximation of observations, as good, or as bad, as the model assumptions made. Typically, the level of theoretical detail depends on the underlying question, the methods to test the hypothesis, the available empirical parameters, among other factors. The level of detail needed is not known *a priori*, but adding complexity in an *ad hoc* manner may lead to unnecessarily complex system and cloud our understanding of the phenomenon. Thus, choosing the right level of complexity could be a challenging task. For example, although all spermatozoa swimming on the planet Earth are subject to gravitational forces and planetary movements, these forces have very little impact on the

sperm swimming simply because of their micrometric scale. Thus, adding gravitational terms to the governing equation of sperm movement, although strictly correct, will only add insignificant corrections and obscure model interpretations.

We briefly introduce the main biophysical interactions relevant for spermatozoa swimming and guidance. For instance, since sperm navigate through the fluid, hydrodynamic interactions are critical to understanding cell propulsion. Likewise, the sperm flagellum is a flexible structure powered by molecular motors deeply embedded in the flagellar scaffold; thus, solid mechanics, the elasticity of the tail and molecular motor dynamics are equally important to understand flagellar modulation, coordinated swimming and guidance. We briefly describe each of these interactions and provide a few examples of how the theoretical modelling was exploited together with experiments to study sperm guidance in other model organisms.

We begin by discussing the spermatozoa flagellum hydrodynamics. One of the most popular models still in use was first described by Gray and Hancock (1955), the so-called resistive-force theory (RFT). This powerful mathematical approximation allows simple measurements of the hydrodynamic forces experienced by the sperm flagellum directly from imaging experiments. Physically, without the hydrodynamic friction, sperm cannot move anywhere. The RFT captures the main contribution of the hydrodynamic drag exerted by the fluid on a small cylindrical section of the flagellum (Fig. 3). The perpendicular motion of this tiny section of the tail 'feels' the hydrodynamic drag almost twice as much as if the cylinder moved tangentially. In other words, the hydrodynamic friction is anisotropic and depends on the direction in which the small element of the tail is moving, unlike a sphere moving in a fluid that 'feels' the hydrodynamic friction in the same way regardless of the direction in which the sphere is pushed. Gray and Hancock showed that the portion of the undulating tail movement that contributes to the progressive sperm swimming is the portion that moves perpendicularly to the swimming direction (see Fig. 3). The tail velocity is linearly related to the hydrodynamic forces. Thus, if the flagellar velocity is known from video recordings of a swimming spermatozoa, the hydrodynamic forces along the tail can be easily measured (Gaffney *et al.* 2011). This proportionality between force and velocity underpins the so-called low Reynolds number regime or small-scale fluid mechanics (Purcell 1977), that is, when inertial forces are not present. The spermatozoa are so small that the hydrodynamic friction far exceeds any other force present, even for very low viscosity fluids such as sea water. We direct the reader to a few reviews and historical texts on the topic (Lighthill 1975; Purcell 1977; Lauga & Powers 2009). Today, RFT approximation is widely used in all fields of science, from the
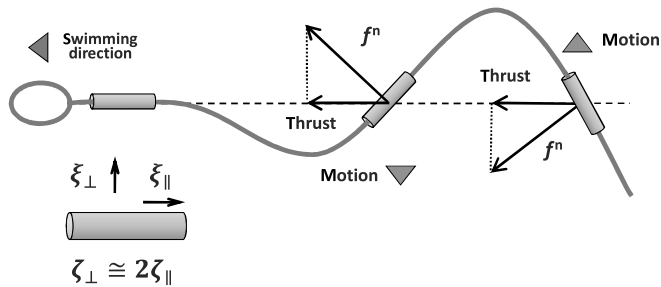
- 28 -

Figure 3 Resistive force theory based on the hydrodynamic forces experienced by motile spermatozoa as a sum of local contribution of small 'rod-like' sections along the flagellum. The drag for perpendicular to the flagellum, f', and contributes to the total thrust in the swimming direction. The drag coefficient in the rod-like element is anisotropic, thus the hydrodynamic friction perpendicular, $\zeta_\perp$ is almost twice the tangential viscous $\zeta_\parallel$.

dynamics of polymers in viscous fluids to buckling of biofilaments and microfilaments and the swimming of microorganisms, including theoretical studies on sperm guidance (Friedrich & Jülicher 2007, 2008; Zimmer & Riffell 2011; Ramírez-Gómez *et al.* 2017).

It follows from low Reynolds number hydrodynamics that if the flagellar wave is known, for instance, from flagellar tracking algorithms of video microscopy imaging (Smith *et al.* 2009a), the total hydrodynamic forces and torques can be inferred indirectly (Friedrich *et al.* 2010; Gaffney *et al.* 2011; Ishimoto *et al.* 2017, 2018); without the necessity of using calibrated micromanipulators to measure the flagellar forces *in-situ* (Lindemann *et al.* 2005). We describe below the general framework on how this may be achieved.

The internal flagellar forces are generally unknown. However, they must match the external forces as all forces must be in balance, following Newton's laws. The external forces arise solely from the external fluid friction for free-swimming sperm (Brokaw 2002). On the other hand, internal forces are split between a 'passive' and an 'active' part. The passive contribution is given by the elastic components of the flagellar structure, and the 'active' forces arise from the molecular motor activity that drives the motion (Hines & Blum 1978; Brokaw 1991; Lindemann 1994b). While the elastic properties of the flagellum are still a matter of debate (Gadêlha *et al.* 2013; Sartori *et al.* 2016; Coy & Gadêlha 2017; Moreau *et al.* 2018), it is not uncommon to invoke the simplest elastic theory for filamentous structures that relates bending deformations (the flagellar waveform) with elastic torques (Antman 2005). Given that both elastic and hydrodynamic contributions are shape-dependent and can be measured directly from imaging experiments, once the elastic and hydrodynamic forces are known in each video frame of the flagellar movement, the molecular motor forces and coordination along the flagellum may be

inferred by subtracting the elastic contribution from the total external forces. An example of this step-by-step procedure is described by Gaffney *et al.* (2011).

The theoretical approach described above illustrates how the motor activity deep inside the flagellum may be readily inferred from video microscopy by simply 'observing' how the flagellar waveform is modulated over the course of time. While a similar framework was successfully employed for spermatozoa of different species (Riedel-Kruse *et al.* 2007), ranging from mammals to sea urchins, no attempt has been made to exploit this in freshwater fish spermatozoa during the critically short guidance period. The powerful combination between experiments and theory continues to shed new light on how sperm coordination takes place without requiring electron tomography and motor fixation techniques (Lin & Nicastro 2018). Indeed, a recent example within the context of sperm guidance employed a similar framework to demonstrate chemotaxis in species of sea urchin spermatozoa believed to not respond chemotactically (Ramírez-Gómez *et al.* 2017).

Predicting sperm swimming trajectories to study guidance mechanisms

Other popular route for theoretical studies prescribes waveform of the flagellar beat, in which the kinematic characteristics of the tail, amplitude, frequency and wave-number, are imposed by model assumptions (Johnson 1980; Smith 2009; Smith *et al.* 2009b; Friedrich *et al.* 2010; Jikeli *et al.* 2015). By employing RFT, the hydrodynamic forces and torques may be evaluated at every point along the flagellum and balanced with the total hydrodynamic drag experience by the sperm head to predict the swimming trajectory of a sperm cell (Johnson & Brokaw 1979). The sperm head trajectory is thus a result of the total balance of hydrodynamic forces (and torques) acting on the head. This provides a

V. Kholodnyy *et al.*

way to numerically simulate the sperm head movement for a given flagellar waveform imposed. Another possibility is to consider that the flagellar waveform is not fixed but rather arises dynamically as a result of the balance between internal and external forces and torques (Gadelha *et al.* 2010; Sartori *et al.* 2016; Oriola *et al.* 2017). In this case, the hydrodynamic and elastic contributions are coupled, giving rise to the so-called spermatozoa elastohydrodynamics. The elastohydrodynamics of the flagellum is thus further coupled with molecular motor internal activity. In this bottom–up approach, both the shape of the flagellar beat and the sperm head trajectories are outputs of the model. The elastohydrodynamic formulation is typically utilized to test hypotheses about the flagellar elastic contribution or the molecular motor control hypothesis (Gadelha *et al.* 2010; Sartori *et al.* 2016; Oriola *et al.* 2017), such as curvature control (Brokaw 2002; Sartori *et al.* 2016), sliding filament control (Brokaw 1991; Riedel-Kruse *et al.* 2007; Oriola *et al.* 2017) and the geometric-clutch control (Lindemann 1994a).

An excellent example of the constructive combination between experiments and theory applied in the context of sperm guidance was reported by Jikeli *et al.* (2015). Their idea is simple and intuitive, and the mathematical implementation is straightforward. Nevertheless, the predictive power gained is excellent. Symmetric waveforms force sperm head trajectories to move in straight-line (on average). Asymmetric waveforms tend to move the heads along a circular motion (Friedrich & Jülicher 2007). Under increasing concentrations of chemoattractants, the mathematical model assumes that the waveform responds by increasing the flagellar asymmetry. The numerical results found an excellent match with complex sperm head trajectories measured from experiments, demonstrating how the flagellar waveform is capable of inducing flagellar asymmetry in a coordinated fashion to find steeps in the chemoattractant landscape. In another study by Ramírez-Gómez *et al.* (2017), both the flagellar waveform and the calcium activity were recorded for different species of sea urchin spermatozoa. Using a similar hydrodynamic modelling framework, they unveiled spatiotemporal correlations between flagellar waveforms and calcium oscillations. They reported that internal oscillations synchronize perfectly with the chemotactic gradient landscape and that this synchronization is linked with the diameter of circular sperm trajectories in sea urchin spermatozoa. As a result, the diameter of the swimming trajectory constrains the chemotactic gradient that a given species may detect (Ramírez-Gómez *et al.* 2017). Using this information, they found chemotaxis in new species of sea urchin spermatozoa that was previously believed to not be respondent to chemotaxis by simply adjusting the

chemical gradient. Once again, the framework described above has yet to be applied to freshwater fish spermatozoa during guidance.

Other biophysical interactions may also be incorporated in the modelling framework to investigate, for example, guidance under external fluid flows and the potential for rheotaxis (Kantsler *et al.* 2014; Bukatin *et al.* 2015). In this case, a much simpler phenomenological mathematical model was developed, in which the sperm flagellum is neglected all together (Hernandez-Ortiz *et al.* 2005). Instead, each sperm head is considered an 'active particle', and the motion is solely arising from the balance of forces between the sperm head drag and a 'phantom' flagellum force.

We finish this section with another fine example of simple but yet powerful mathematical model used to understand the rheotactic response of mammalian spermatozoa (Bukatin *et al.* 2015). In this case, the spermatozoa speed is assumed to be ballistic, always point towards the direction they are initialised if no external fluid flow is present. Under the action of an external fluid flow, the swimming direction tends to slowly align against (or in favour of) the flow direction via an effective hydrodynamic torque potential acting on the sperm head. A comparison of the model with the experiments allowed for the inference of such sperm rheotactic parameters across the population of spermatozoa. This modelling framework is now used as a testbed of hypotheses for different external fluid flow conditions. This simple yet powerful framework was not used in the realm of freshwater fish spermatozoa guidance, although perfectly suited for experiments where the external flow may be varied experimentally, and the sperm head trajectories are easily accessible from the experiments. Indeed, such a modelling framework could test the effectiveness of sperm guidance when perturbed by oscillatory and other complex external flows in freshwater fish reproduction.

Manifestations of post-copulative female control over the fertilization process

In the case of species with internal fertilization, the females could choose a particular male for mating and perform in such a way to select the proper genetic material for fertilization purposes. Such a direct choice is hardly possible in external fertilizers because even in the species with stable pairs, the spawning pair could be accompanied by some random male (a so-called sneaker e.g. in the three-spined stickleback *G. aculeatus* (Taborsky 1998) or 'parasitic' males in the chinook salmon, *O. tshawytscha* (Butts *et al.* 2017)), which will 'add' its sperm into the competition for egg fertilization. There is a strong belief that externally fertilizing females have gained a mechanism that would

promote the sperm of genetically preferable males to encounter their eggs, the so-called 'cryptic female choice'. The latter is considered a part of a post-copulatory sexual selection characteristic both for internal and external fertilizers, and this post-copulatory sire choice is thought to follow the same models of female preferences as pre-copulatory mate choice (Pitnick & Hosken 2010; Firman *et al.* 2017). There are also suppositions that the gamete level (and gamete-mediated) control over fertilization may have even preceded the widely recognized pre-copulatory sexual selection in the course of evolution and was an inevitable result of evolved syngamy (Parker 2014; Beekman *et al.* 2016; Kekäläinen & Evans 2018).

Several attempts were made to support these ideas in fish. It was shown in the ocellated wrasse *Symphodus ocellatus* that the presence of female ovarian fluid enhanced sperm velocity, motility, straightness and chemoattraction in conspecific males, that is, the spermatozoa of certain males were selected at the individual gamete level through some characteristics that allowed female choice to affect the paternity (Alonzo *et al.* 2016). A strong effect on sperm swimming speed, longevity and path trajectory in males of the chinook salmon *O. tshawytscha* was provided by the presence of ovarian fluids from different females (Rosengrave *et al.* 2008); this influence was attributed to the differences in the chemical composition of the ovarian fluid. Sperm swimming speed was chosen as the most important sperm motility trait triggering fertilization success and contributing to cryptic female choice. A relationship between the composition of the ovarian fluid and sperm function was found in a hybridization test between the Atlantic salmon *S. salar* and the brown trout *S. trutta* (Yeates *et al.* 2013). Ovarian fluid has been shown here to promote fertilization by the conspecific sperm of salmon and trout. The promotion occurred only if sperm competition was possible, and it was stressed that cryptic female choice is closely associated with the control of the 'usual' sperm motility traits, that is, the improvement in velocity or the longevity of 'proper' spermatozoa (Yeates *et al.* 2013). Improving the spermatozoa velocity in a manner that was dependent on the compatibility of the sperm with the ovarian fluid of a particular female was also found in another salmonid, the lake trout *S. namaycush* (Butts *et al.* 2012). In an earlier study, Yeates *et al.* (2009) found that the fertilization of eggs from the Atlantic salmon *Salmo salar* with sperm from males differing in major histocompatibility (MH) complex genes resulted in the promotion of males with MH similarity over males with MH differences; this promotion was associated with female-driven control against hybridization with close species. In this study, the authors have not determined whether the effect was caused by substances present in the ovarian fluid or released by the egg. The influence of female 'identity' on spermatozoa velocities and the

outcome of fertilization was also found by Geßner *et al.* (2017) in a fertilization trial performed with *O. tshawytscha*; it was found that the observed effects were strongly associated with female–male relatedness. Moreover, it was found that some of the differences in fertilization outcome that could not be explained by changes in velocities were significantly associated with relatedness by gene, presumably responsible for non-random gamete fusion (Geßner *et al.* 2017).

It was also found that ovarian fluid could not only improve some features of specific sperm cells but also inhibit the 'unwanted' features. In particular, the study in the internally fertilizing guppies *P. reticulata* showed that ovarian fluid may slow down the male gametes when mating with sisters occurs but that the ovarian fluid does not slow down the unrelated male gametes (Gasparini & Pilastro 2011).

Nevertheless, the opinion about the existence of the ovarian fluid-mediated selection of sperm is not common. Lahnsteiner (2002) showed no changes in sperm motility of the brown trout *S. trutta f. fario* if the ovarian fluid from different batches was used. Despite the evidence for ovarian fluid–sperm interactions, Evans *et al.* (2013) did not find female–male interaction effects on the progeny in the sperm competition experiment performed in the chinook salmon, *O. tshawytscha* (the design involved 10 various crosses between genetically uniform batch of eggs mixed with ovarian fluid from one of two females and competitive sperm from one focal and two rival males). The investigators revealed the relative paternity success of particular males by determining that the average fertilization capacities of their ejaculates were higher than the fertilization capacities of the others; these results were due to their higher spermatozoa swimming velocity and, as a result, better competitive abilities.

Most of these observations on the existence or absence of specific sperm selection mechanisms were made empirically, and only a few attempts were undertaken to find the driving force of such phenomena. One of the reasons for this could be the complexity of the potential cue used for the selective control of conspecific male spermatozoa traits in external fertilizers (Lymbery *et al.* 2017). In other words, in contrast to sperm-activating agents, which vary only slightly among relative species and are thought to be conserved during evolution (Jagadeeshan *et al.* 2015), the proper parental genotype could not be selected by the female counterpart using only a particular single molecule, but rather, the specific variety of molecules, a 'molecular key', will differently affect the spermatozoa signalling pathway traits (Lymbery *et al.* 2017). Nevertheless, the importance of post-copulative female control over fertilization outcome for external fertilizers, freshwater fish in particular, where premating female choice is difficult or severely
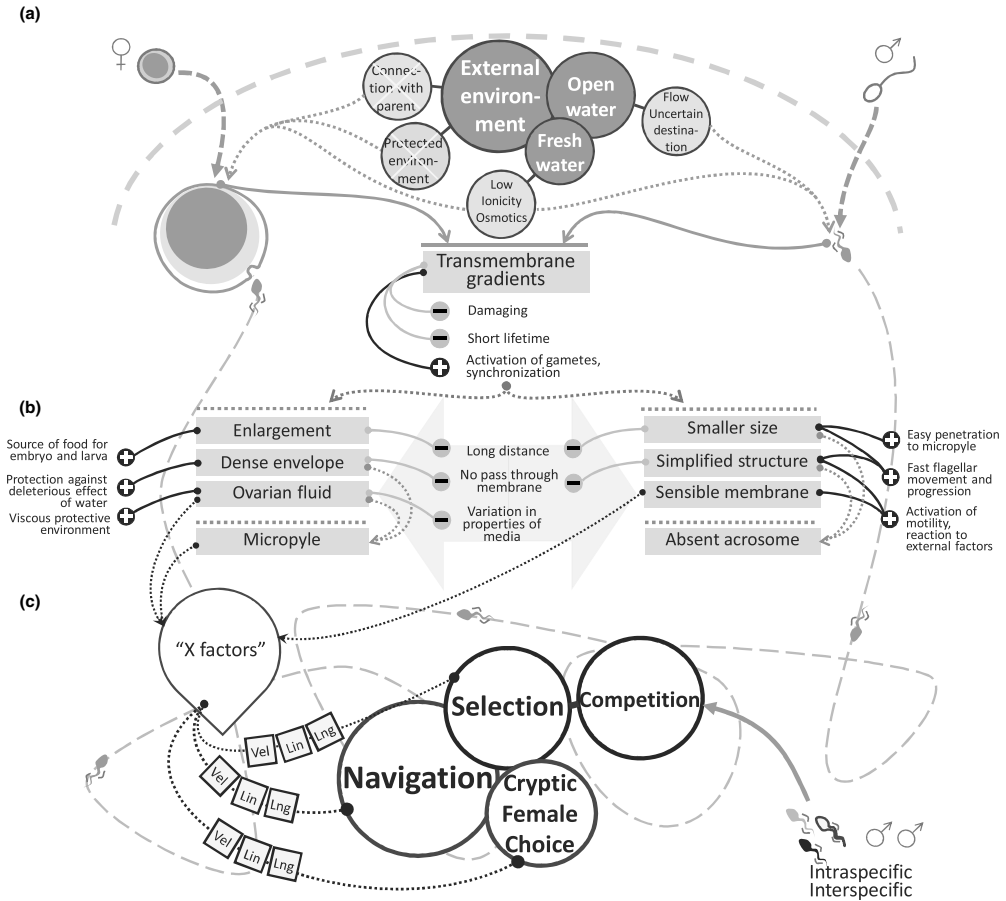
V. Kholodnyy *et al.*

(a)

Figure 4 Hypothetic scheme of sperm guidance/selection in freshwater fish. (a) Due to evolution, the water medium outside the parental organism became the place where gametes meet, and where the resulting new organism developed. After being released into an external environment, the male and female gametes of fish interact with freshwater, which has an extremely low osmolality compared with the osmolarity of body fluids, and these conditions could be damaging to living cells, thus limiting their lifetime to several minutes. At the same time, the conditions of the medium are the indispensable part of rapid motility activation in spermatozoa, which are immotile before being released (usually during tens of milliseconds). The eggs also undergo activation after contact with freshwater. (b) During evolution, the features of this specific and harsh environment caused the appearance of denser chorionic envelopes and the production and release of ovarian fluid together with the eggs, which can prolong their lifetime. The enlargement of the egg could be associated with the need to provide substrates for embryo/larva development outside of the parental body. These features resulted in the appearance of a specific canal, providing the possibility of passing the egg chorion, that is, micropyle. At the same time, the evolution of freshwater fish spermatozoa resulted in a simplification of their structure and a decrease in size, providing higher mobility and the ability to react very rapidly to changes in the environment due to an extremely sensitive membrane. (c) After being released into the environment, eggs perform a passive role, whereas the tiny and fast spermatozoa have to find and fertilize the egg through the micropyle. During this time, the spermatozoon meets several external factors that activate and sustain short-term motility ('X-factors'). When the spermatozoon comes close to the egg, the spermatozoon could be guided by the surface, and this approach can be supported by ovarian fluid. This fluid may affect spermatozoa motility (velocity = 'Vel', linearity = 'Lin', longevity = 'Lng') and protect the spermatozoa from osmotic damage because the osmolality and viscosity of the ovarian fluid is higher than those of freshwater. Additionally, ovarian fluid may contain some chemical agents acting as attractants, and these agents could be released from the micropyles (the final target for attraction), which affect sperm motility and cause sperm accumulation. Collectively, the ability of spermatozoa to react to these different external factors, for example, by changes in motility traits, could be a basis for sperm selection and could be involved in sperm competition and cryptic female choice.

- 32 -

constrained, is believed to be high and could be an agent of evolutionary exaggeration and diversification in highly polyandrous species, which make up the majority of broadcast spawners (Firman *et al*. 2017). In wider sense, this control may be even termed 'gamete-mediated mate control' because it requires 'a complex chemical dialogue between the gametes from both sexes' (Kekäläinen & Evans 2018). Finally, the process may lead either to the directional selection of a particular individual phenotype beneficial to the offspring, or a non-directional one aimed to 'check' the genetic compatibility of mates to avoid inbreeding or hybridization (Kekäläinen & Evans 2018).

Conclusions

Evolutionary evolved external fertilization in water entailed the appearance of a broad set of adaptations that cover the whole process from the preparation of the gametes for the existence in the external environment to the support of the development of a new organism (Fig. 4). This set includes, among others, the evolution of a dense protective shield around the egg with a minute opening, the micropyle, allowing the penetration of the spermatozoa through it. In addition, fertilization in the water allowed the existence of a sophisticated system of spermatozoa motility initiation and further support of propagation using the 'power' of external factors, for example, ionic content or osmolarity of the medium, as triggers and controls. Many broadcast spawners inhabiting the sea use the water medium to deliver the chemical signals to the male gamete, allowing to find its female counterpart (or *vice versa*, the female to choose the proper male genotype). A variety of studies in these marine organisms allowed the scholars to uncover the amazing membrane-associated system of receptors, channels and other molecules, making possible the precise guidance of the spermatozoa on its way to the egg. Freshwater fish are quite unique among all external fertilizers due to the specific features of the environment, that is, the extremely low osmolality, which has negative effects on the cells. This circumstance makes the need for a specific promotion of cell encounters even more apparent. The eggs of many externally fertilizing freshwater fish species are released into the external milieu surrounded by a coat of ovarian fluid with a composition (content of ions, proteins, amino acids, sugars, etc.) ideal for supporting and protecting eggs and sperm against the deleterious effect of freshwater. The data presented here support the idea that the properties of ovarian fluid and/or the specific compounds contained in it or released by the eggs could significantly affect the behaviour of male gametes and consequently influence the outcome of fertilization in terms of the number of fertilized oocytes. Moreover, there are clear indications that these factors may affect the choice of genetic material from a specific parent.

This choice may be made by the support of sperm motility traits on a certain level, the attraction or repulsion of gametes with some pre-defined qualitative characteristics and the targeted promotion of sperm with the proper genetic material to encounter the egg. The specific mechanisms of this selection in externally fertilizing fish are still unclear, which makes further research in the field highly promising. The efforts of scholars could be applied in particular to the identification of active agents triggering gamete encounters and the systems of signal perception and conduction. Mechanisms of gamete encounters may also be predicted and/or explained by mathematical and biophysical modelling of spermatozoa behaviour guided or not by female triggers. All the phenomena, that is, motility activation and progress, kinetic and tactic effects, possible selection and the promotion of gametes could be elements in a harmonic pattern of gamete guidance in fish. Together with well-studied marine invertebrates, the latter will be an important piece in the whole 'puzzle' of evolutionary developmental biology. Uncovering the mechanisms will contribute not only to the fundamental physiology of reproduction but also to the optimization of artificial reproduction technologies. For instance, considering the features of gamete encounters, including post-copulative female effects, may help to control the quality of the progeny and will allow us to estimate the impact of the aquaculture practice on the sustainability of the involved species. Finally, we believe that guidance during fertilization is a rule, not a fortune, at least because guidance is highly expedient during external fertilization in terms of supporting the variability and stability of living matter.

Acknowledgements

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V. Kholodnyy *et al.*

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CHAPTER 2

DOES THE RAINBOW TROUT OVARIAN FLUID NAVIGATE THE SPERMATOZOON ON ITS WAY TO THE EGG?

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My share in this work was about 70%.

**Does the rainbow trout ovarian fluid navigate the spermatozoon
on its way to the egg?**

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Fertilization of freshwater fish occurs in an environment which may affect negatively the gametes, therefore the existence of specific mechanism guiding and triggering the encounter of gametes would be highly expedient in these conditions. More than likely that the only source of signaling may come from the egg or ovarian fluid (OF). In this light we aimed to explore how freshwater fish sperm behavior is affected by OF, using rainbow trout as a model. The study was performed with gametes obtained from farmed fish during the natural fertilization cycle. Sperm behavior (motility parameters and patterns, calcium ions content) was assessed in various conditions (presence of OF or its molecular weight fractions, various activation media, etc.). It was found that the presence of OF affected significantly the behavior of rainbow trout spermatozoa, in particular, kept high velocity for a longer time. There was a change in the pattern of motility from tumbling, observed in water, to directed straightforward moving in OF. The effect may be associated partially with osmotic properties of the fluid and with its calcium content. Different molecular weight fractions of OF affected the kinetics and the motility patterns of spermatozoa variously. Rainbow trout OF rendered a trapping effect on activated male gametes. The effect depended on the osmotic properties of the activating media. Different molecular weight fractions from OF cause non-uniform tactic behavior of the cells. The most significant trapping effect was rendered by a low molecular fraction and the possible chemotactic agent was thermostable. The interaction of the spermatozoa with the attracting agents was accompanied by their "turn-and-run" behavior involving the asymmetric flagella beating and a kind of calcium ion concentration bursts in the bent flagella area. Collectively, ovarian fluid is an important part of the rainbow trout fertilization process, nevertheless, the fine machinery of the effect needs to be ascertained.

Keywords: sperm motility, fertilization, ovarian fluid, chemotaxis, Oncorhynchus mykiss

Introduction

Sexual reproduction grounds on the fertilization of female ova by male spermatozoa. For successful fertilization, the spermatozoa must reach the egg in a due time after ovulation. Externally fertilizing freshwater fish spermatozoa are activated after direct contact with freshwater and its motility is only sustained for a very limited period, typically tens of seconds (Hart 1990). Freshwater fish eggs are incredibly large, on the millimeter scale, and possess a specialized fertilization site, namely micropyle, which is to be found by the diminutive sperm cell (few tens of microns long) during its short motility period in an environment that is constantly moving and changing. Under these conditions, reproductive success is chronically limited to the ability of spermatozoa to find the egg and reach the fertilization site, and thus they are depending on mechanisms that increase sperm-egg encounter. What are these mechanisms based on? Generally, the ideas explaining these vary from the predominance of a "male factor", (*e.g.* "fair raffle" concept when the success of a particular male depends only on a number of spermatozoa it could provide to the "fertilization lottery" (Parker, 1993)) to the existence of specific female post-mating control (*e.g.* "cryptic female choice" (Thornhill, 1983)), or finally the combination of many factors, including environmental ones (*e.g.* guidance hypothesis (Eisenbach and Giojalas, 2006)). There are numerous assumptions that the gametes' encounter is controlled by stimuli or signaling which come from the egg or accompanying female fluids, and confirmations were found across many taxa, including marine invertebrates, mammals, and in few fish species (Morisawa and Yoshida, 2005; Yanagimachi et al., 2017). These stimuli may affect the behavior of sperm cells, rendering both chemokinetic and chemotactic effects, and finally the outcome of fertilization. First observations on sperm activation and rise in its motility traits in the vicinity of the eggs were made in marine invertebrates about 100 years ago (Lillie, 1912), and "egg jelly", a substance surrounding the eggs, was believed to be a reason for that. In the fish, such a female effect provider can be the ovarian fluid (OF), which bathes mature fish oocytes in the ovarian cavity (van den Hurk and Peute, 1979) and surrounds the eggs of many externally fertilizing female fish during the release of the spawned eggs through the oviducts into the water (Rosengrave et al., 2008). The composition of the OF varies among the fish species, generally it contains ions and different substances of protein nature, sugars and lipids in different ratio (Lahnsteiner et al., 1995; Rosengrave et al., 2009b; Nynca et al., 2015).

There are several reports about the existence of chemokinetic reaction of fish male gametes, *i.e.* the changes in swimming velocity and trajectories of spermatozoa depending on the presence of ovarian fluid in the media (*e.g.* Lahnsteiner, 2002; Dietrich et al., 2008; Kanuga et al., 2012) scrutinized recently by Myers et al. (2020) in their meta-analysis. Interestingly, that most of the studies, represented in this analysis were done in salmonids, which is not surprising, considering their popularity as a research object for fish reproduction studies, and preconditioned by its wide usage in aquaculture and consequently high market value. The meta-analysis of Myers et al. (2020) showed the overall enhancing effect of ovarian fluid on the velocity of the salmonid spermatozoa, pointing, however, high heterogeneity of the data. Nevertheless, there is still very little information beyond these kinetic observations. It is not clear if the ovarian fluid of salmonid fishes renders any chemotactic effect, or how it may trigger the success of egg fertilization, and there is no clear understanding about the mechanisms of motility enhancement provided by ovarian fluid.

In our study, we aimed to perform comprehensive testing of interaction of spermatozoa and ovarian fluid, as well as to find the evidence or absence of chemotactic behavior in one of the representative of freshwater spawning fishes of Salmonidae family, the rainbow trout *Oncorhynchus mykiss*, and to clear up the potential mechanism of gametes' encounter guidance in this species.

To reach our goal we will resolve the following issues:

- How the presence of ovarian fluid in the activation medium affects the spermatozoa motility traits including velocity and linearity of motion?
- Whether the ovarian fluid has a chemotactic effect on spermatozoa?
- Are the changes in the spermatozoa motility accompanied by characteristic changes in calcium ion concentration inside the flagellum?
- How the presence of ovarian fluid affects the outcome of *in vitro* fertilization of eggs?

Materials and methods

Ethical 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 October 4, 2016, for 5 years) and the authorization for the use of experimental animals (Reference number: 2293/2015-MZE-17214 16OZ22302/2014-17214, valid from 22nd January 2015 for 5 years) issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia by Ministry of Agriculture of the Czech Republic.

Fish broodstock. Gametes and fluids collection

The experiments were performed in rainbow trouts *Oncorhynchus mykiss* (2–3-year-old, 0.6–1 kg weight) in spring and autumn seasons in 2017, 2018, and 2019. Fish were obtained from a fish farm in Bušanovice, Czech Republic and Oshino Trout Hatchery, Yamanashi Prefectural Fisheries Technology Center, Oshino, Yamanashi, Japan (in autumn 2018), and kept in an indoor aquarium at 11 °C. Ovarian fluid and sperm were obtained during the natural spawning period from strains that spawn in spring or autumn. Sperm was collected by stripping, stored during analysis on ice, and used for 6 hours. The ovarian fluid (OF) was drained off from the eggs with a sieve, centrifuged to remove debris and stored at 4 °C in plastic tubes or frozen at -80 °C when long term storage was required. Only ovarian fluid from non-overripen eggs was used in conventional motility analyses. Fertilization test, presented in the paper, was done in February 2019 using albino rainbow trout females and males and normal color males; ovarian fluid from albino females was collected and stored separately.

Preparation of media

The evaluation of chemokinetic properties of the ovarian fluid was done in series of activating media (Table 1): with varying concentration of OF (from 100 to 2%); with various concentrations of NaCl (Sigma Aldrich, USA), to mimic osmotic pressure of corresponding dilutions of ovarian fluid; with various molecular weight fractions of ovarian fluid; thermotreated ovarian fluid (ovarian fluid was boiled at 100 °C for 5 minutes); rainbow trout blood serum (collected from the blood centrifuged at 3000 g for 5 minutes); bovine serum albumin (Sigma Aldrich, USA) 1 mg/ml solution in water. Molecular weight cut-off (MWCO) fractions of the ovarian fluid were prepared using Amicon Ultra centrifugal filters with 3, 10, 30, 50, and 100 kDa filters (Merck Millipore Ltd., Ireland). Stepwise centrifugation through the filters was done starting from 100 kDa. Processing was performed at 4 °C till 10-time concentrating of the fluid above the filter (10% of the initial volume). The volume of the collected fluids was recovered with isotonic NaCl (0.9% w/v) solution. The fluid passed through the filter was transferred to a next filter with lower MWCO, and the procedure was repeated until the lowest MW filter was used.

Table 1. *Solutions used for rainbow trout spermatozoa activation and/or chemotaxis tests.*

Solution		Used for activation/ chemotaxis (injected fluid) tests	Osmolarity, mOsm/l	pH
Distilled water		activation/chemotaxis	~0	–
Tap water		activation/chemotaxis	~0	–
10mM Tris HCl buffer		activation	10	8
Ovarian fluid		activation/chemotaxis	290	~8
Ovarian fluid in water	50%	activation/chemotaxis	150	~8
	20%	activation/chemotaxis	60	~8
	10%	activation/chemotaxis	30	~8
	5%	activation/chemotaxis	15	~8
	2%	activation/chemotaxis	5	~8
Ovarian fluid in isotonic NaCl solution	50%	activation/chemotaxis	290	~8
	20%	activation/chemotaxis	290	~8
	10%	activation/chemotaxis	290	~8
	5%	activation/chemotaxis	290	~8
	2%	activation/chemotaxis	290	~8
NaCl solution (+Tris buffer)	150 mmol/l	activation/chemotaxis	300	8
	75 mmol/l	activation/chemotaxis	150	8
	30 mmol/l	activation/chemotaxis	60	8
	15 mmol/l	activation/chemotaxis	30	8
EGTA supplement to water	5 mmol/l	activation/chemotaxis	10	–
Ca^{2+} supplement to water	0.2, 1, 2, 5 mmol/l	activation/chemotaxis	1, 3, 6, 15	–

Additional experiments were performed with checking of the cross effect of calcium ion concentration and osmolarity of the activation media. For doing this the set of media was prepared with varying osmolarity (0, 30, 60, 150, 300 mOsm/l, done with NaCl and 10 mM Tris buffer (Sigma Aldrich), pH 8, where appropriate) and calcium content (0, 0.2, 1, 2, and 5 mmol/l made with an appropriate amount of $CaCl_2$ (Sigma Aldrich) or 2 mmol/l EGTA (Sigma Aldrich)). The "0" mOsm/l media was the distilled water with either 2 mmol/l EGTA or corresponding $CaCl_2$ concentration, *i.e.* the osmolarity in this media was, in reality, higher than 0, the real values are shown in Table 1.

Appropriate solutions were assessed for osmolarity, pH, protein, and ion content. Osmolarity was measured using a freezing point osmometer Osmomat 3000 (Gonotec GmbH, Germany) and expressed in mOsm/l. 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 mmol/l of the medium. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, USA) and shown in mg/ml. The measurements of the protein and ion contents (Moorehead and Biggs 1974) were done in the range of standard calibration curves, appropriate to the used method.

Motility observation and recording

Sperm suspensions (around 0,1 μl) were carefully mixed for 5 s with 40 μl of tested solutions and motility (if present) was recorded for 1 min post-activation using ISAS digital camera

(PROISER, Spain) set at 25 frames/s and microscope (UB 200i, PROISER, Spain). Video records were analyzed to estimate spermatozoa motility traits using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA) and following plugins: CASA and CASA modified for multiple analyses (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%. Values of spermatozoa velocity, linearity of the spermatozoa trajectories and well as a pattern of motility (the 1–2 sec tracks of individual spermatozoa in the vision field) were obtained.

Sperm chemotaxis tests

Series of experiments were done to assess the response of the spermatozoa to the injection of various test fluids (see Table 1) into activating media using glass microcapillaries, being 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, Japan) to get microneedles with tips of 20 µm size, which were additionally cut by a microgrinder (EG-401, Narishige, Japan) to have uniform tip openings. The microcapillary was filled with test fluid and assembled to a microinjector (CellTram Vario, Eppendorf), then fixed on a holder (Narishige, Japan) 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 minutes. 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.

Ca imaging

For imaging of intracellular calcium ion content ($[Ca^{2+}]_i$), semen was suspended in 4–5 volumes of immobilization solution, *i.e.* artificial seminal fluid (Morisawa and Morisawa 1988) containing 0.05% Cremophor EL (Dojindo, Japan) and 20 µM Fluo-4 AM (Dojindo), and incubated for dye loading at 10 ºC during 2 h. Then the sperm was activated in an observation chamber and microinjector with test liquids was introduced. Fluorescence signals emitted by the swimming sperm were captured by a microscope equipped with fluorescence illumination and a digital CCD camera (Shiba et al., 2008). The videos were then analyzed and the fluorescence of individual cells was tracked. The typical fluorescent response observed in the spermatozoa during the motility is represented in a series of heat map images.

In vitro fertilization

The *in vitro* fertilization assays were performed in February 2019. The fertilization was done with the eggs, collected from 3 albino females and two mixed sperm specimens from 5 albino males or 5 normal colored males. The experimental groups are shown in Table 2. The experimental design was aimed to check if the presence of ovarian fluid may change the outcome of the *in vitro* fertilization. In all the cases 5 g of eggs (on average 79 eggs) were fertilized by 0.5 ml of sperm mixed in 8 ml of water from the hatchery supply system at 11 ºC. The concentration of spermatozoa in the sperm was 2.15×10^{10}/ml, *i.e.* around 140,000 spermatozoa per egg in the fertilization medium. In some cases, the eggs were washed with 0.9% NaCl solution three times during 10 s to remove the ovarian fluid. The eggs (washed/non washed) were put to the plastic beaker, poured with test solution (water, isotonic NaCl solution, or ovarian fluid) and the sperm was added. The beakers were then placed onto shaker (around 100 rpm) and after 1 minute-incubation the eggs were rinsed and transferred to glass Petri dishes.

Table 2. *Effect of ovarian fluid on fertilization performance in rainbow trout, albino vs. conventional color fish: experimental design.*

Washing of eggs	Sperm activation medium	Male fish color	Procedure
Intact albino fish eggs	Tap water	Albino	5 g of eggs were placed into the plastic beaker and supplemented with 8 ml of water together with 0.5 ml sperm, either from albino or from the normal color male. In the case of mixed sperm, the albino and normal color male sperm were mixed and 0.5 ml were taken from the mixture.
		Normal color	
		Albino+normal color	
Albino fish eggs washed thrice with isotonic saline	Tap water	Albino	
		Normal color	
		Albino+normal color	
Albino fish eggs washed thrice with isotonic saline	NaCl 0.9% saline	Albino	
		Normal color	
		Albino+normal color	
Intact albino fish eggs	100% ovarian fluid	Albino	
		Normal color	
		Albino+normal color	

The dishes were transferred into the tank with baskets for further incubation at 11 °C. The tank had a closed water circuit with aeration, UV-treatment, and temperature control. All fertilizations were done in three replicates. The outcome of *in vitro* fertilization was assessed in 11 days by the amount of developing embryos (the embryo development rate is the amount of developing embryo divided by the total amount of eggs); simultaneously the share of albino embryos was counted. The number of the hatched larva was counted as well, the hatching rate (number of hatched larva/number of eggs) did not differ significantly from the number of developing embryos, and thus only the latter is presented.

Statistical analysis

Assessment of motility parameters in different activation media was conducted in triplicates for 18 males in case of water, ovarian fluid and its dilutions, media with various osmolarity based on sodium chloride; 5 males in case of MWCO fractions of ovarian fluid; 3 males for blood serum and thermotreated ovarian fluid; 16 males (8 albino and 8 normal color males) for checking the specific effect of ovarian fluid among populations; sperm samples from 8 males were used for experiments with combined effects of calcium concentrations and osmolarity. Curvilinear velocity (VCL) and path linearity (LIN) were obtained from the motility measurement from 50–300 spermatozoa per replicate per time point during 10–59 seconds post-activation with 3 s increment. The motility parameters were then $\log_e$ transformed to ensure the normal distribution of the data, and analysis of interactive effects between variables was 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), a pair-wise analysis was conducted between several media. The data on VCL and LIN are presented as means with corresponding confidence intervals. The data for spermatozoa velocities in several media (water, isotonic NaCl saline, ovarian fluid and dilutions in water or isotonic saline, dilutions of isotonic saline with water, MWCO fraction of ovarian fluid, blood serum and thermotreated ovarian fluid) 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 or the goodness-of-fit (R^2). The hypothesis for the equality of regression slopes was checked by the t-test with Bonferroni correction using Statistica software.

The fertilization tests were done in three replicates per experimental point. The values of the percentage of developing embryos were expressed as the mean ± standard deviation (± SD); the number of albino embryos was estimated simultaneously and its share among total fertilized embryo was calculated. 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 $P < 0.05$. All the numbers, presented in the text are average ± standard deviation if others not stated.

Results

Basic physico-chemical characteristics of ovarian fluid

The ovarian fluid makes 10–30% of egg batch volume in rainbow trout. It is isosmotic, 299.1 ± 3.7 mOsm/l with pH 8.3 ± 0.15, and total protein content of 1.16 ± 0.11 mg/ml. Main ions in the ovarian fluid are 1.28 ± 0.28 mmol/l Ca^{2+}, 2.32 ± 0.17 mmol/l K^+, and 164 ± 15 and 134 ± 20 mmol/l for Na^+ and Cl^-, correspondingly.

Spermatozoon kinetics

The first set of experiments was done to evaluate the chemokinetic properties of the rainbow trout ovarian fluid. To do this, series of activating media were prepared: with varying concentrations of ovarian fluid; with various concentrations of NaCl, to mimic osmotic pressure of corresponding dilutions of ovarian fluid; with various molecular weight fractions of ovarian fluid; *etc*. The motility of spermatozoa was recorded during one minute post-activation in these media and then analyzed using CASA with obtaining motility traits (velocity, path linearity). The data on curvilinear velocity (VCL) and path linearity were chosen as representative ones.

Figure 1 shows the data on the curvilinear velocity of spermatozoa activated in various media depending on time post activation, the dots are the averaged data and the lines are the linear regressions. The indices characterizing the linear regressions are in Table 3, *i.e.* the goodness-of-fit, the slopes of the curves, and the intercepts with the time and velocity axes. The descriptive statistics data in terms of mean and 0.95 confidence intervals are represented in supplementary Figure 7, and the essential results of pair-wise factorial analysis of the data are in the supplementary Table 4. Rainbow trout spermatozoa were fully activated either in hypotonic media (water) or in isotonic media (ovarian fluid or saline) and their combinations. There was no significant difference found in the curvilinear velocity between males of autumn and spring strains for all the tested media (data not shown), thus all the following indices are the combination obtained from both strains.
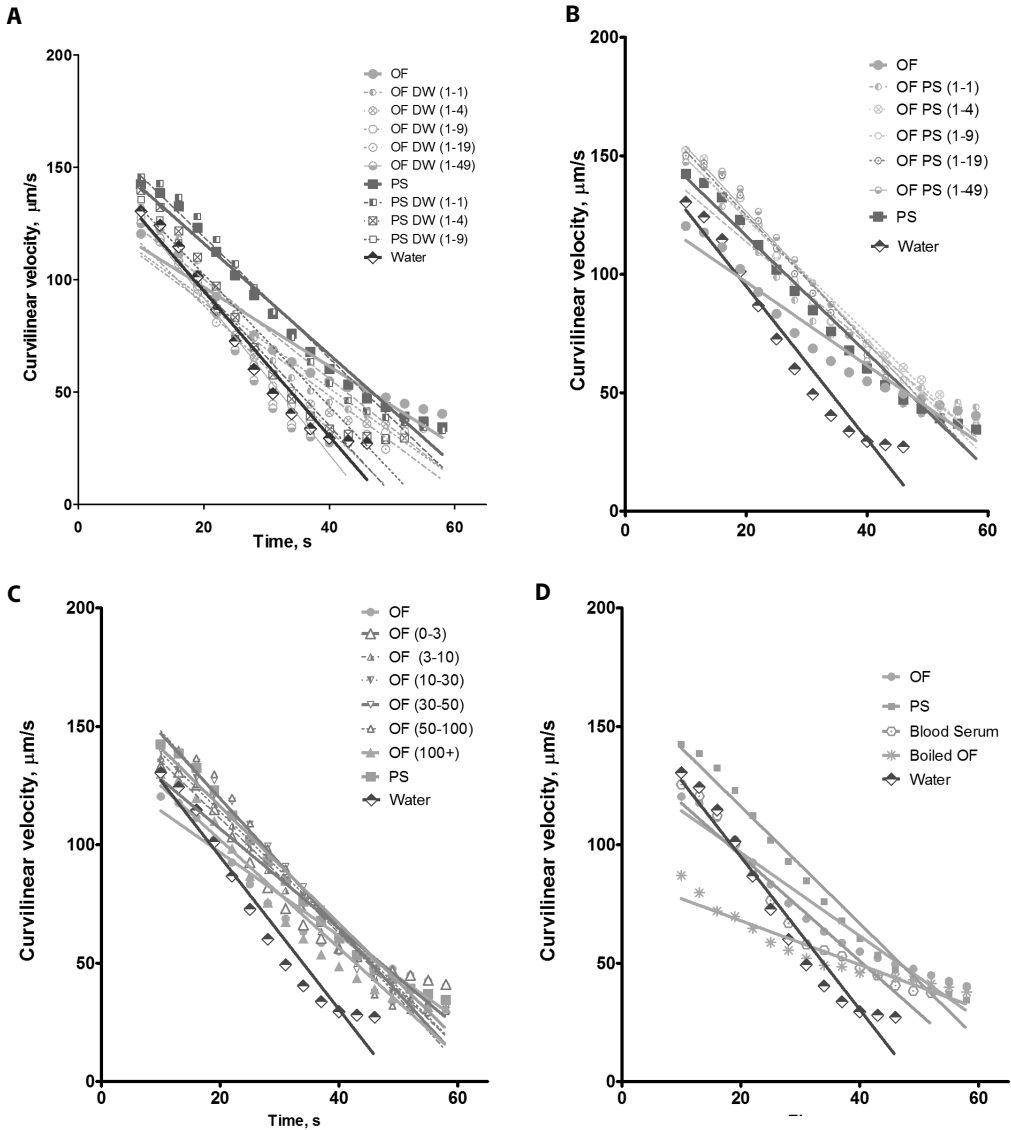
Figure 1. *The curvilinear velocity of rainbow trout spermatozoa in various activation media depending on time post-activation, data divided to four sets: A – velocity in water, ovarian fluid, NaCl solution isotonic to ovarian fluid (physiological solution, PS, 290 mOsm/l); and their dilutions with water (1-1; 1-4; 1-9; 1-19 and 1-49); B – motility in ovarian fluid diluted with isotonic NaCl solution (in comparison with water and isotonic saline); C – motility in MWCO fractions of ovarian fluid, and D – in blood serum and thermotreated ovarian fluid (compared with water, ovarian fluid, and isotonic saline). Markers are mean experimental values obtained from averaging data from 5–18 males. Lines represent linear regressions of experimental dependencies, the parameters of the fitting, values of slopes and intercepts are shown in the supplementary Table 3.*

Curvilinear velocity in the aqueous dilutions of the ovarian fluid

Presence of ovarian fluid improved the longevity of spermatozoa, at the same time the initial velocity was lower (Figure 1A, supplementary Table 3), comparing the case of water as an activation medium: estimated initial VCL in water was around 160 μm/s, and almost 132 μm/s in the ovarian fluid (supplementary Table 3). The decrease of ovarian fluid content in the mixture with water overturned its effects: starting from the 10% dilution there was no significant difference comparing with water as an activation medium in terms of the velocity changes in time. The curvilinear velocity of spermatozoa in water decreased sharply and the characteristic intercept with the time axis obtained from regression analysis was 49 seconds, and the presence of ovarian fluid rose this index to 75 seconds. It is worth to note, that the curves of average velocity changes during motility period have three slopes. The first reflects slow velocity change in the first ~15 seconds, fast changes in the second middle part, which length depends on the medium, *i.e.* it ends at around 35 seconds in water and 50 or even later in ovarian fluid, and the last part of the curve shows slow changes of velocity at the end of motility period recorded in few left spermatozoa. Linear regression follows the most essential middle slope which has most data points, and the intercepts with the axes may not reflect exact values of initial velocity and longevity, nevertheless may serve as numeral characteristics allowing to compare different treatments. Exact analysis of the velocity change curves was beyond the scope of this paper and maybe performed separately.

Curvilinear velocity in the isotonic saline and the saline supplemented with the ovarian fluid

Using of NaCl isotonic medium had an improving effect on initial velocity as well as longevity of spermatozoa. The dilution of isosmotic media entailed the disappearance of significant differences with motility traits obtained in water in terms of velocity changes, and the gradual decrease in estimated longevity of spermatozoa. If the spermatozoa were activated in dilutions of ovarian fluid with isosmotic medium, the resulting velocity changes did not differ significantly from the isosmotic medium indices (Figure 1B, supplementary Table 3) and the traits differed from the case of ovarian fluid if it was diluted more than twice.

Curvilinear velocity in the MWCO fractions of ovarian fluid

There were no significant differences between MWCO fractions of ovarian fluid and NaCl isosmotic medium in terms of velocity changes in time; only fraction with MW lower than 3 had significant differences with water. No significant differences in velocity changes comparing to ovarian fluid were found with low molecular (0–3 and 3–10 kDa) and high molecular (100+ kDa) fractions. Interestingly, that thermally treated ovarian fluid dramatically decreased the initial velocity of spermatozoa and the longevity in this activation medium was the longest among all the studied medium.

Path linearity and patterns of spermatozoon motility in ovarian fluid, isotonic solutions, and MWCO fractions

The trajectories of the spermatozoa obtained in various media at 10–12 seconds of the motility period are shown in Figure 2. The descriptive statistics on spermatozoa path linearity along the motility period in terms of mean ± 0.95 confidence interval is shown in the supplementary Figure 8. Spermatozoa activated in water move in tight circles during the initial period of motility (Figure 2A). Trajectories of most spermatozoa recorded in ovarian fluid and NaCl isotonic solution are straight or arc-like with a greater proportion of straight moving cells in ovarian fluid; which could be seen in Figure 2 and is reflected in higher initial average linearity on the corresponding graph in supplementary Figure 8A; pair-wise factorial analysis of changes in linearity in time between these two media showed a significant difference (p<0.0001).

Twice dilution of ovarian fluid and NaCl isotonic solution did not change significantly the path linearity, while further dilution of both activation media dramatically decreased the number of spermatozoa moving straightforward. Interestingly, the pattern of motility in diluted ovarian fluid differs from the saline medium with the same osmolarity: in the ovarian fluid, many cells move in complex coil-like trajectories (arrowheads in Figure 2A).
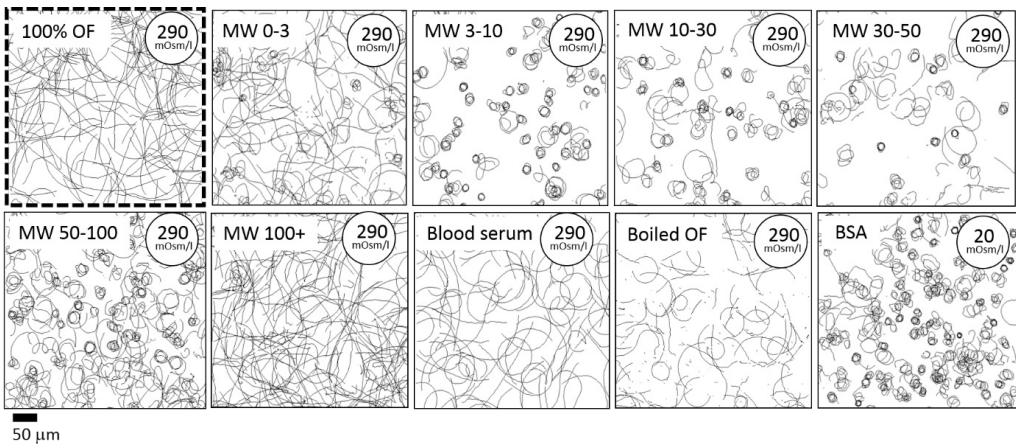
A
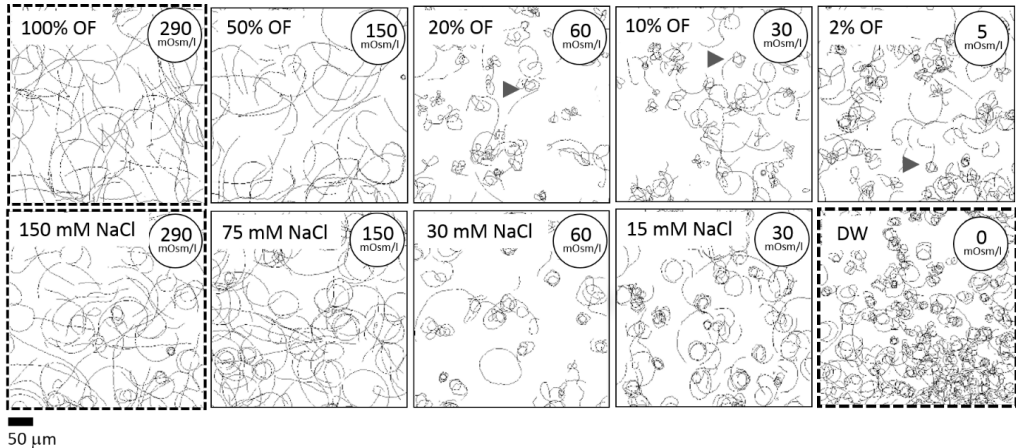
B

Figure 2. *The pattern of motility of rainbow trout spermatozoa in various activation media: A – trajectories in distilled water (DW), ovarian fluid (OF), NaCl solution isotonic to ovarian fluid (290 mOsm/l); and their dilutions with water (1-1; 1-4; 1-9; 1-19 and 1-49); B – motility in MWCO fractions of ovarian fluid (MW 0-3, 3-10, 10-30, 30-50, 50-100 and 100+; numbers denote molecular weight in kDa), blood serum, thermotreated ovarian fluid, and bovine serum albumin (BSA) solution 1 mg/ml in water. Each track corresponds to the trajectory of individual spermatozoa during 2 seconds starting from 10 seconds post activation. Numbers in circles show the osmolarity of the tested activation media.*

Dilution of ovarian fluid with isotonic saline significantly affects the pattern and path linearity only in the case of 10% ovarian fluid dilution and lower (Figure 2A, supplementary Figure 8B). Among the MWCO fractions, only the 100+ kDa fraction shows the same pattern of motility and path linearity as ovarian fluid. The traits in the other MW fractions (0–3, 3–10, 10–30, 30–50, and 50–100 kDa) do not differ significantly from the isotonic NaCl solution.

The same motility pattern and path linearity as in ovarian fluid were found in blood serum and thermally treated ovarian fluid. In the aqueous solution of 1 mmol/l BSA, the spermatozoa had the same trajectories as in water.

We have found clear differences in spermatozoa motility patterns, observed in ovarian fluid and the media containing its dilutions (Figure 2). The more ovarian fluid was present in the medium – the more straightforward was the path of spermatozoa. In the hypotonic medium, the spermatozoa moved in tight circles. There was found the pattern of so-called explorative behavior of spermatozoa or 'turn-and-run' (Kaupp, 2012) usually associated with an effect of a chemotactic agent (examples shown by arrowheads).

The pattern of spermatozoon motility in the same osmotic conditions simulated by sodium chloride dilutions differs from the case of ovarian fluid dilutions (Figure 2). There were no "explorative movements" observed in similar osmolarities made by sodium chloride. The motion of significant part of cells in isotonic saline was not straight or arc-like. The cells tend to move more circular.

Motility of spermatozoa in depending on calcium concentration and osmolarity of the activation medium

One of the suppositions about the role of ovarian fluid in the reproduction of salmonids is to provide a proper environment for the gametes interaction (Lahnsteiner, 2002). The osmolarity of the media is an important agent, triggering the motility onset and affecting its course. Another important agent involved in the motility phenomenon of fish spermatozoa is ionic calcium. The next series of experiments were done to assess the combined action of alternating external calcium ion concentration and osmolarity on motility traits of rainbow trout spermatozoa, and compare these with corresponding indices found in the case of ovarian fluid. Figure 3 shows the typical tracks recorded in 10–12 seconds post-activation of spermatozoa in series of calcium ion concentrations, *i.e.* "0" (2 mmol/l EGTA), 0.2, 1, 2, and 5 mmol/l; and osmolarity of the media: "0", 30, 60, 150 and 300 mOsm/l (made by varying NaCl concentration in 10 mmol/l Tris buffer pH 8, except the case of "0" mOsm/l medium, which was the distilled water with 2 mmol/l EGTA or corresponding $CaCl_2$ concentration, *i.e.* the osmolarity in this media was, in reality, higher than 0). These indices include the most likely natural range of osmolarities and calcium ion concentrations met by rainbow trout spermatozoa after releasing by a male. The supplementary Figure 9 and 10 show the dependence of changes in curvilinear velocity and path linearity along the motility period depending on osmolarity and calcium concentration in the activation medium. It is clear that the motility pattern depends both on the concentration of external calcium and the osmolarity of the activation medium. In the absence of external calcium, the spermatozoa are poorly activated in 0 mOsm/l medium, the activated spermatozoa move in circular trajectories. Interestingly, the motility in the 30 mOsm/l medium was almost absent in all the studied males. In the Ca-free media with 60, 150, and 300 osmolarities the spermatozoa are activated and most of them have straight trajectories. This observation is supported by the graphs describing path linearity in supplementary Figure 10A. The changes in the curvilinear velocity of spermatozoa had no significant differences in the first part of the motility period, and then the cells in media with higher osmolarity had a longer period of motility and higher velocity after 30 seconds of motility period. The presence of 0.2 mmol/l calcium dramatically changes the pattern of motility of spermatozoa. The majority of them move in tight circles in media with 0 and 30 mOsm/l osmolarity, and with a rise in osmolarity the trajectories "uncoil" to some extent, and became more straight in 300 mOsm/l medium. This diversity is obvious as well in the linearity dependence in supplementary Figure 10B. In terms of VCL, the cells in the media with 60, 150 and 300 mOsm/l osmolarity had higher velocity and were motile longer

comparing to 0 mOsm/l medium. The presence of 1 mmol/l Ca in the activation media entails the straightening of the trajectories in most cases even in case of 0 mOsm/l medium. Further rise of calcium ion concentration up to 2 and 5 mmol/l did not lead to significant changes comparing to the case with 1 mmol/l concentration. The velocity graphs show the enhancing effect of media with 60, 150 and 300 mOsm/l osmolarity in terms of higher VCL and a longer period of motility in case of 1 mmol/l calcium, and 150 and 300 mOsm/l osmolarity in case 2 and 5 mmol/l calcium.
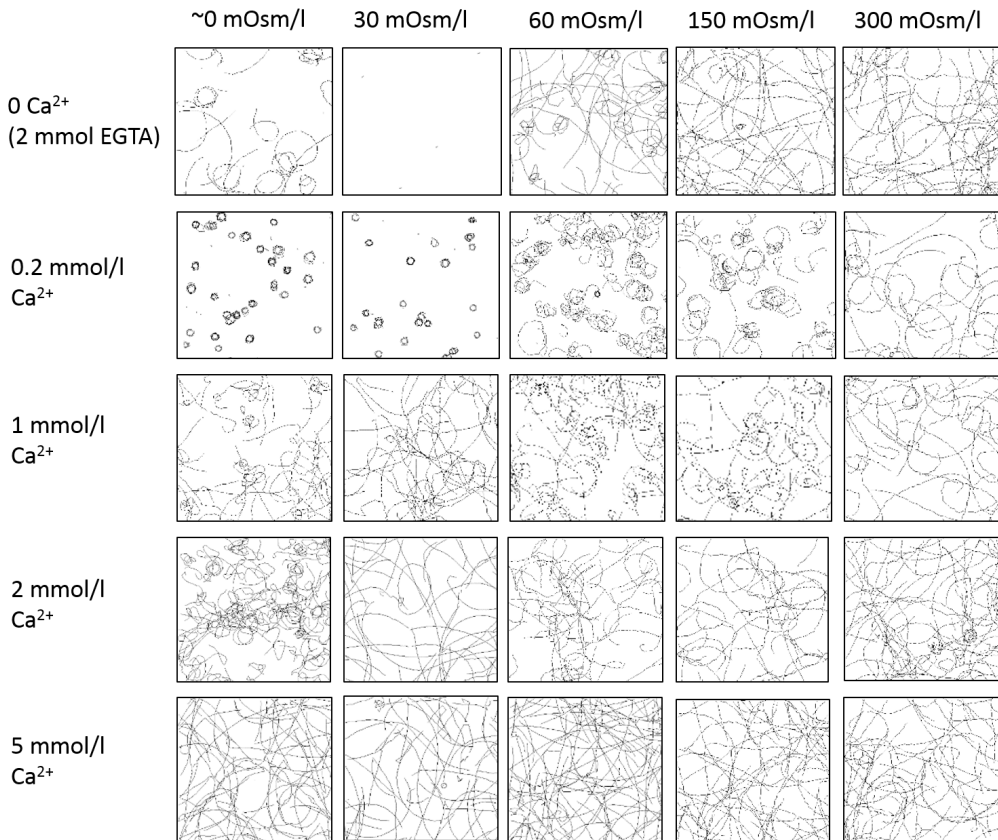

Figure 3. *The pattern of motility of rainbow trout spermatozoa in the activation media with various osmolarity and calcium content: 30, 60, 150, 300 mOsm/l, prepared with NaCl and 10 mM Tris buffer, pH 8; and calcium content 0, 0.2, 1, 2, and 5 mmol/l prepared with an appropriate amount of $CaCl_2$ or 2 mmol/l EGTA in case of 0 mmol/l Ca medium. The "0" mOsm/l media was the distilled water with either 2 mmol/l EGTA or corresponding $CaCl_2$ concentration, i.e. the osmolarity in this media was, in reality, higher than 0, the real values are shown in Table 1. Each track corresponds to the trajectory of individual spermatozoa during 2 seconds starting from 10 seconds post activation.*

Chemotactic tests. Attraction/trapping of cells

The behavior of spermatozoa in the media with variating concentrations of substances (gradients) may differ from the case with their uniform distribution. Such conditions may be created in an accumulation test with microcapillary, *i.e.* using glass capillary to introduce various test solutions into the bulk volume of medium containing activated spermatozoa.
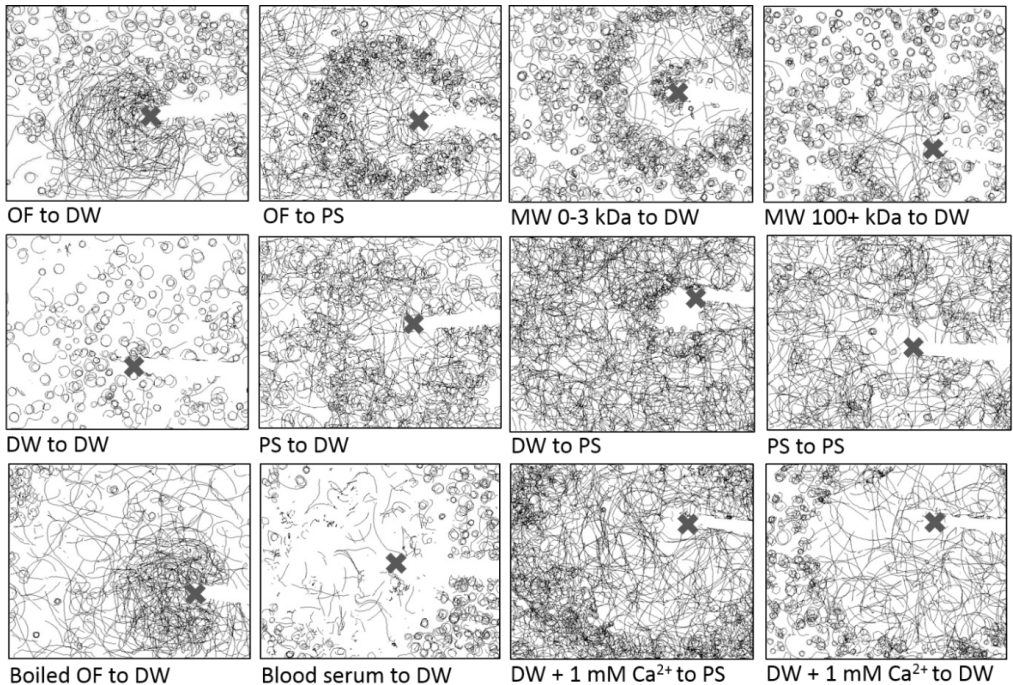
Figure 4. *Swimming tracks of rainbow trout spermatozoa activated in various media near the tip of microcapillary filled with test fluids: ovarian fluid (OF); distilled water (DW); isotonic NaCl saline (PS, 290 mOsm/l); molecular weight cut-off (MWCO) fractions; thermotreated (boiled) ovarian fluid; blood serum; distilled water with added 1mM CaCl$_2$. Each track represents 2 seconds of motility (15–17 s post activation). The cross shows the tip of the microcapillary.*

Typical motility patterns of spermatozoa in our microcapillary tests are presented in Figure 4 and Video in Online resource 1. No changes in the usual tumbling behavior of spermatozoa activated in water were found if the water was injected by the microcapillary. If the capillary was filled with ovarian fluid the male gametes, which got into contact with the injected "cloud", changed their behavior for a straight-line pattern. Moreover, the affected cells abruptly changed the direction of motion if reached the border of the cloud, *i.e.* they were trapped in the ovarian fluid, and "positive taxis" was present. Injection of ovarian fluid into the suspension of spermatozoa activated in isotonic NaCl resulted in the appearance of the "tumbling layer" of spermatozoa around the cloud, and the part of cells which entered the cloud moved straight. Changes in the behavior of spermatozoa and trapping were observed as well if isotonic saline was injected to water as an activation medium, nevertheless, no changes were seen if activation media was changed to the same NaCl isotonic saline, which may suggest the presence of a sort of "osmotaxis" in our experimental conditions. Interestingly, that injection of distilled water into the spermatozoa activated in isotonic saline caused the "negative taxis": the cells avoided entering the hypoosmotic area, tended to stay in the medium with higher osmolarity and performing abrupt turns on the borders of the "cloud" with water. Introduction of thermotreated ovarian fluid entailed the appearance of two areas with different density of spermatozoa: more dense cloud around the tip of capillary and surrounding "loose" area with straight moving cells. Low molecular MWCO fraction caused a quite bright response in the behavior of spermatozoa, activated in water: some cells swirled on the border of injected

cloud, some spermatozoa moved straight without leaving the area, and some cells gathered close to the microcapillary tip. Other MWCO fractions did not differ significantly from the isotonic saline in effect caused to the behavior of spermatozoa. Surprisingly, the blood serum caused inhibition of motility of cells was observed in the injected cloud.

Calcium concentration changes in the flagellum

Changes in the internal concentration of ions, Na^+, K^+, Ca^{2+} and H^+ are thought to be the key factors of membrane hyperpolarization control due to osmotic pressure changes (Boitano et al., 1991; Takai and Morisawa, 1995; Krasznai et al., 1998). The presence of Ca^{2+} ions inside the spermatozoa was shown as an indispensable condition for motility initiation in all fish species (Tanimoto and Morisawa, 1988). Calcium ions take part in the complex membrane cascade controlling the motility of spermatozoa, and, what is most important, its direction (Kaupp and Strünker, 2017). It was shown, that the chemotactic response of ascidian spermatozoa, *i.e.* appearance of specific "turn-and-run" pattern, similar to observed by us, is associated with a burst-like rise in calcium ion concentration in the bent area of spermatozoa flagellum (Shiba et al., 2008).
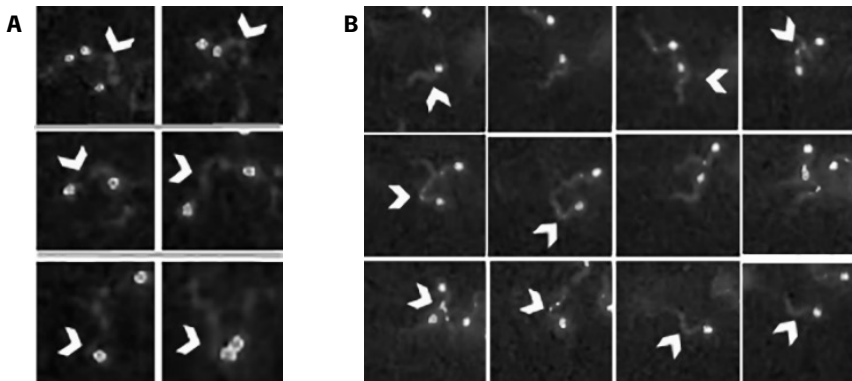

Figure 5. *Fluo-4 fluorescence in the sperm activated in water in the vicinity of the microcapillary filled with ovarian fluid or other fluids. A – Frame-by-frame sequence of cell performing tumbling (shown by arrowheads). B – Frame-by-frame sequence of cell performing straight run and turns (shown by arrowheads).*

The series of experiments similar to described above microcapillary tests were performed, which involved the fluorescent microscopy to observe the changes in calcium ion concentration in the cells (Fluo4 dye loading and further observation in the fluorescent microscopy at the excitation of 435 nm). Observation of cells which perform swirling moves did not show any changes in the Fluo-4 fluorescence in the flagella during motility (Figure 5A). The cells which did straight run and turn on the edge between the different media have bright "burst" of Fluo-4 fluorescence in the bent area when turning (Figure 5B), *i.e.* there was a local increase in calcium ion concentration during the change of motility direction, associated with the appearance of asymmetric flagellar waves.
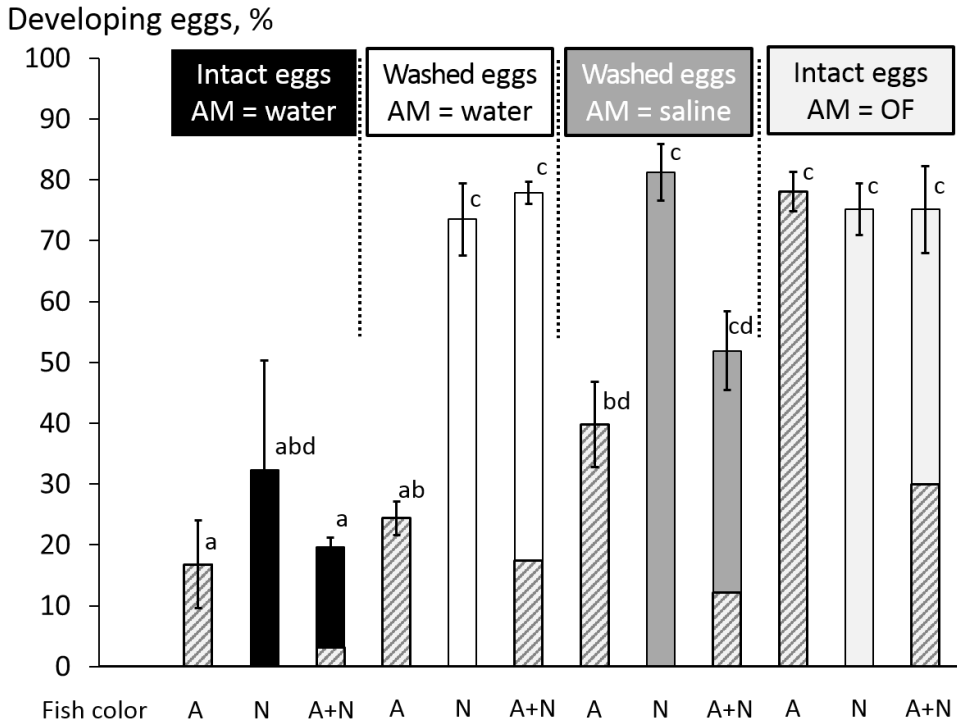
Developing eggs, %

Figure 6. *Albino rainbow trout egg embryo development rate after in vitro fertilization depending
on the presence of ovarian fluid around the eggs or in the activation medium, and male type (albino
or conventional color). Eggs were either non-washed to remove ovarian fluid ("Intact eggs") or washed
thrice with 0.9% NaCl solution ("Washed eggs"). Spermatozoa (0.5 µl) from albino males (A), normal
color males (N) or the mixed sample (A+N) were activated in 8 ml of water, isotonic NaCl saline or ovarian
fluid (AM = water, saline or OF, correspondingly) and added to the beakers with eggs, mixed and the
beakers were put to the shaker for 1 minute. Thereafter the eggs were rinsed, transferred to glass Petri
dishes, and put to incubators at 11 °C. In 11 days the developing embryos were counted, and fertilization
rate was calculated (fertilized eggs/total amount of eggs); as well as the color type of the embryos was
established. The striped bars show the albino embryos; plain color bars denote normal color embryos.
Data are mean ± SD, the different superscripts denote significant differences (P < 0.05)*

In vitro fertilization of eggs in the presence of ovarian fluid

It was shown that the presence of ovarian fluid increases the success of *in vitro* fertilization
in some salmonids, *e.g.* Caspian brown trout, *Salmo trutta caspius* (Hatef et al., 2007), our
preliminary tests confirmed this observation in rainbow trout. The presented here test was
performed to check the effect on ovarian fluid on outcome of fertilization in case if sperm
from related or non-related males was used: the albino males and females from the same
population, and conventionally colored males (the second population). The albino type is non-
dominant, *i.e.* fertilization of the egg from the albino female with the sperm from normal male
will result in normal color embryo/larva. It appeared during the preliminary motility tests, that
sperm from albino males had low motility in water, only up to 20%, however, the motility in the
ovarian fluid was higher than 95%. Sperm from normal color males had motility in water more
than 50% and more than 95% in the ovarian fluid. Fertilization of the intact (non-washed)
eggs in the water resulted in a quite low embryo count, less than 20% in the case on albino

males, and around 30% in normal males (Figure 6); in case of mixed sperm the outcome was about 20% fertilized eggs, and only 16% of them were albino embryos. Washing of the eggs and fertilization in water resulted in a significant rise of fertilization with sperm from normal males and no significant changes for the albino male group. The amount of fertilized eggs in the mixed group rose as well, the share of albino embryo stayed the same, nevertheless, their absolute number was higher compared to the case with intact eggs (but not higher than for the fertilization of washed eggs with only albino male sperm). Fertilization of washed eggs in the isotonic NaCl solution did now change significantly the outcome of fertilization comparing to the fertilization of washed eggs in the water. Using 100% ovarian fluid as an activation medium resulted in almost 80% amount of fertilized egg in all the cases, either in albino, normal males, or the mixed group. The share of albino embryos in the mixed group rose to about 40%. Analysis of the motility traits (VCL and LIN) of spermatozoa from albino and normal males in water, isotonic NaCl solution, and ovarian fluid showed the significant difference only between curvilinear velocity changes in the cells activated in water (pair-wise factorial analysis and supplementary Figure 11), in all other cases, *i.e.* path linearity in water, and velocity and linearity in isotonic saline and ovarian fluid, no significant differences in the effect of male origin were found.

Discussion

The present study showed that ovarian fluid renders chemokinetic effect on rainbow trout spermatozoa in terms of velocity, linearity, and longevity. The fluid had a chemotaxis-like or trapping effect on sperm cells, and the male gametes were able to abrupt changes in direction of motility due to the differences in the environmental conditions. These changes were accompanied by a burst-like rise in the calcium content in the bent area of flagella. And finally, the ovarian fluid had a positive effect on the fertilizing ability of spermatozoa, improving the non-optimal motility of the cells. We will discuss how all these fit the gametes' encounter guidance in this species and first how the spermatozoa change their motility traits after the contact with the ovarian fluid.

Kinetic changes in rainbow trout spermatozoa performance due to ovarian fluid
Successful fertilization depends on the ability of male gamete to carry the genetic material and fuse with a female gamete, thus, any change in kinetic characteristics of the spermatozoa is highly essential. In a current opinion one of the sperm characteristics, the spermatozoon velocity, contributes significantly to the success of fertilization. This is was shown even for the case of a single male, *i.e.* in the case of no competition present (in this case the outcome of fertilization correlates with a characteristic velocity of spermatozoa (Lahnsteiner et al., 1998)), and more clearly if the sperm competition is present, *i.e.* success of a particular male against rivals (in terms of the bigger relative genetic contribution of the male with faster spermatozoa (*e.g.* Levitan, 2000). Sperm competition is widespread in the externally fertilizing vertebrates, fishes in particular (Taborsky, 1998).
Salmonid fishes are typical examples of species with common and often intensive sperm competition. Their spawning tactics vary from spawning in pairs with the presence of parasitic or sneaker male or group spawning. The significance of spermatozoon velocity for the fertilization outcome in salmonids was reported widely (*e.g.* Gage et al., 2004; Liljedal et al., 2008); in particular, Gage et al. (2004) have found that relative sperm velocity is responsible for sperm competition success of a focal male comparing to its rival and stated that the increased velocity of the "winner's" spermatozoa allows the faster search of the spawning microenvironment and penetration to the egg micropyle comparing to the rival.

It was shown by several groups that the presence of ovarian fluid in the activation medium may positively affect the spermatozoa performance in these fishes by increasing the velocity of male gametes or their longevity (Litvak and Trippel, 1998; Lahnsteiner, 2002; Turner and Montgomerie, 2002; Rosengrave et al., 2009a; Butts et al., 2012). Interestingly, Butts et al., (2012) revealed in lake trout *Salvelinus namaycush* the rise in the curvilinear velocity of related (sibling) male spermatozoa after activating in 20% of ovarian fluid comparing to the gametes from the unrelated male; however, Rosengrave et al. (2009) have not found any specificity driven by individual differences but revealed the absence of correlations between kinetic characteristics recorded in water and ovarian fluid.

In our study, we have confirmed that the presence of ovarian fluid affects the kinetic characteristics of the rainbow trout spermatozoa comparing to activation in water. Nevertheless, we have not found the rise of the initial curvilinear velocity (at 10 s post activation), like it was reported by Turner and Montgomerie (2002) in Arctic charr *Salvelinus alpinus*, but the rate of velocity reduction was much smaller in the presence of ovarian fluid and its dilutions with water comparing with just water (significant difference disappeared in 5% aqueous solution of ovarian fluid, Figure 1A, supplementary Table 3A). The observed changes in the curvilinear velocity of spermatozoa activated in the isotonic saline differed significantly from that in the ovarian fluid. The initial velocity of the cells in the saline was higher than both in water and ovarian fluid. Nevertheless, the longevity of spermatozoa activated in the ovarian fluid was the highest compared to water and isotonic saline. This is in line with the "saving" effect of ovarian fluid reported by Elofsson et al. (2006), which found a similar positive effect on sperm longevity in three-spined stickleback *Gasterosteus aculeatus,* and associated it with solely ionic content of the fluid. Interestingly, the dilution of ovarian fluid with the isotonic saline entailed the absence of significant differences in velocity between diluted ovarian fluid and the saline.

In addition to the velocity, the direction of the motility plays an important part in the propagation of the spermatozoa. The ovarian fluid had a prominent effect on the path linearity of the male gametes, which was highest compared to activation in water or isotonic saline. A similar observation about a significant rise in path linearity in rainbow trout spermatozoa activated in ovarian fluid compared to the saline buffer was reported by Dietrich et al. (2008). Notably, with a decrease in the concentration of ovarian fluid, we observed the rise in the occurrence of specific trajectories, often called "turn-and-run" behavior. It was frequent even in the highly diluted ovarian fluid, *i.e.* 2%, (Figure 2A), as well as in 5 and 10%, but was not present in non-diluted ovarian fluid or its 50% dilution. Such behavior was not characteristic of the sperm activated in isotonic saline.

Among the molecular fractions of the ovarian fluid, only the 100+ kDa fraction had the motility pattern which had no differences from the ovarian fluid, *i.e.* the cells moved almost straightforward in both cases. In all other MW fractions, the trajectories were more round and the path linearity had significant differences both from ovarian fluid and 100+ kDa fraction (Figure 2B, supplementary Figure 8c).

Cross effects of calcium concentration and osmolarity on motility traits

The osmolarity of the medium is one of the essential drivers of freshwater fish spermatozoon motility, initializing the function of the membrane orchestra of channels and other molecules which trigger the motility (Alavi et al., 2019). No less important for the motility initiation and its progress are the calcium ions, which are an integral part of numerous physiological processes occurring in the spermatozoon (Alavi and Cosson, 2006). In the experiments on cross effects of osmolarity and calcium ion concentration, we varied these indices to see which of these isolated factors or their combination may be responsible for the chemokinetic

effects rendered on spermatozoa by the ovarian fluid. It is clear that these two indices affect path linearity both individually and in combination, *e.g.* in the absence of external calcium the rise in osmolarity of the activation medium from 0 to 300 mOsm/l entails the straightening of the spermatozoon trajectories, and rise in calcium concentration from 0 to 5 mmol/l in hypoosmotic medium causes the similar effect. Remarkably, that in case of intermediate concentration of calcium (0.2 mmol/l) the path linearity values and the patterns of trajectories were highly dependent on osmolarity of medium (Figure 4, and supplementary Figure 10). Collectively, the combination of calcium ion concentration and osmolarity which correspond to the ones in the ovarian fluid changed the motility traits similarly to the effect of ovarian fluid, nevertheless the effect of solely osmolarity was more significant.

Ovarian fluid cause attraction and trapping of spermatozoa
Microinjections of fluids into media with activated spermatozoa is one of the obvious ways to create the interfaces occurring naturally in the water around the eggs covered with ovarian fluid. This allows modeling the behavior of male gametes during interaction with the maternal fluids, which may differ from the motility in the uniform environment. In our experiments, the rainbow trout spermatozoa were able to react on the presence of this non-uniformity in the medium, by trapping in the area with more optimal conditions, *i.e.* osmolarity, pH, ionic content. Naturally, these conditions may be provided only by ovarian fluid, nevertheless, in the experiment, it is possible to investigate how these various constitutives affect the response of the spermatozoa and which of them are more critical. Our experiments showed that positive taxis and trapping were observed towards the ovarian fluid and its dilutions in rainbow trout spermatozoa activated either in water or isosmotic saline. This effect was even stronger towards low molecular weight fractions or thermotreated ovarian fluid. Interestingly, that in some cases shown in Figure 3, *i.e.* for ovarian fluid injected to isotonic medium or low molecular fraction of ovarian fluid injected to water the spermatozoa separated to populations, some of which showed a hyperactivation-like pattern of motility either on the borders of the injected cloud or near the tip of the microcapillary. Changes of motility pattern for a straightforward one and the trapping were observed as well in case of injection of isosmotic saline or even distilled water with 1 mmol/l calcium, which shows, that overall positive taxis towards ovarian fluid may be a complex of reactions of the spermatozoa to various triggers. Interestingly, the spermatozoa were able to abruptly change the direction of their movement, *i.e.* performing turn-and-run, if they met the interface between optimal and non-optimal environments. It may be exemplified by the trapping behavior in case of injected ovarian fluid or isotonic saline into the water with activated gametes when the cells tended to stay inside the optimal area, or *vice versa*, the "negative taxis" behavior in case of injected distilled water into isosmotic activation medium, when the male gametes "avoided" entering into the injected medium. This behavior allows us to conclude, that rainbow trout spermatozoa are highly sensitive to the environmental conditions and can change the direction of their motion to follow or to stay in the optimal conditions, which in the natural conditions are most likely created by ovarian fluid. Such behavior of freshwater fish spermatozoa is similar to earlier described triggered motility of sea invertebrates, *e.g.* sea urchin (Ward et al., 1985), ascidians (Yoshida et al., 1993) or squids (Hirohashi et al., 2013). The similarity of these sorts of behavior is even more spectacular considering that turn-and-run loops in rainbow trout spermatozoa are accompanied by asymmetric bending of the flagella and burst-like rise of calcium concentration in the asymmetric bend. The latter was found to mediate the chemotactic activity in the ascidian spermatozoa, and being its inherent part (Yoshida et al., 2018).

Ovarian fluid provides an optimal environment for fertilization

The fact of a positive effect of ovarian fluid on the outcome of *in vitro* fertilization in salmonids was reported for Caspian brown trout, *S. trutta caspius* (Hatef et al., 2009), brown trout *S. trutta* f. *fario* (Lahnsteiner, 2002). On contrary, Hugunin et al. (2008) recommended to wash-out the ovarian fluid before artificial fertilization in rainbow trout after finding the negative impact of ovarian fluid in "sub-fertile females", which they explained with "preventing fertilization through impeding sperm movement or recognition/contact with the egg". These discrepancies in the outcome of fertilization were associated by some authors with a post-copulative effect rendered by females to select the best sire, or "cryptic female choice". In particular, Butts et al. (2012) have found significant enhancement of spermatozoon performance after activation in ovarian fluid from related females. Wojtczak et al. (2007) have found that the differential effect of ovarian fluid from various females is mostly caused by various pH of the maternal fluids, nevertheless, the authors mention that 40% of the variability is caused by other factors, which may include ions, carbohydrates or proteins. Rosengrave et al. (2008) also found the differential effect of ovarian fluids from various females on spermatozoa performance in Chinook salmon *O. tshawytscha.* At the same time, there are opinions about the absence of cryptic female choice caused by the presence of ovarian fluid in the fertilization medium, *e.g.* in Arctic charr reported by Kleppe et al. (2018).

Our experiments have shown, that using of ovarian fluid as a fertilization medium significantly enhanced the fertilization outcome with the sperm, which showed low motility in the water (less than 20% motility in the albino male sample). This observation is in line with the opinions of Rosengrave et al. (2008) and Myers et al. (2020), which emphasized the inadequacy of pre-fertilization motility estimation in salmonids performed in water.

Interestingly, that wash-out of ovarian fluid from albino female eggs enhanced the fertilization outcome for the unrelated conventional colored males (Figure 6), if the activation medium was the water. If the ovarian fluid was used as the activation solutions the result of fertilization was maximal, as well as in case isosmotic saline was used.

Using ovarian fluid as a fertilization medium allowed to increase the percentage of embryos from related males, *i.e.* the ovarian fluid increased the chances of spermatozoa from related males to win the sperm competition. Surprisingly, we have not found any differences in curvilinear velocity and linearity between spermatozoa from albino and normal color males activated in isosmotic saline and ovarian fluid (supplementary Figure 11), only in water the motility traits differed in the initial period.

Collectively, we may conclude that rainbow trout ovarian fluid improves the spermatozoa motility: the cells move longer and with higher velocity, and highly straightforward. The ovarian fluid has a trapping effect on spermatozoa, and the male gametes are sensitive to changes in the environment being able to change motility direction to follow the conditions optimal for their functioning. Low molecular substances are mainly responsible for the effects of ovarian fluid on spermatozoa. And finally, the presence of ovarian fluid improves the outcome of fertilization by improving the performance of the sperm, showing non-optimal motility assessed in water, and may decrease the outcome of fertilization on non-related individuals.

Acknowledgments

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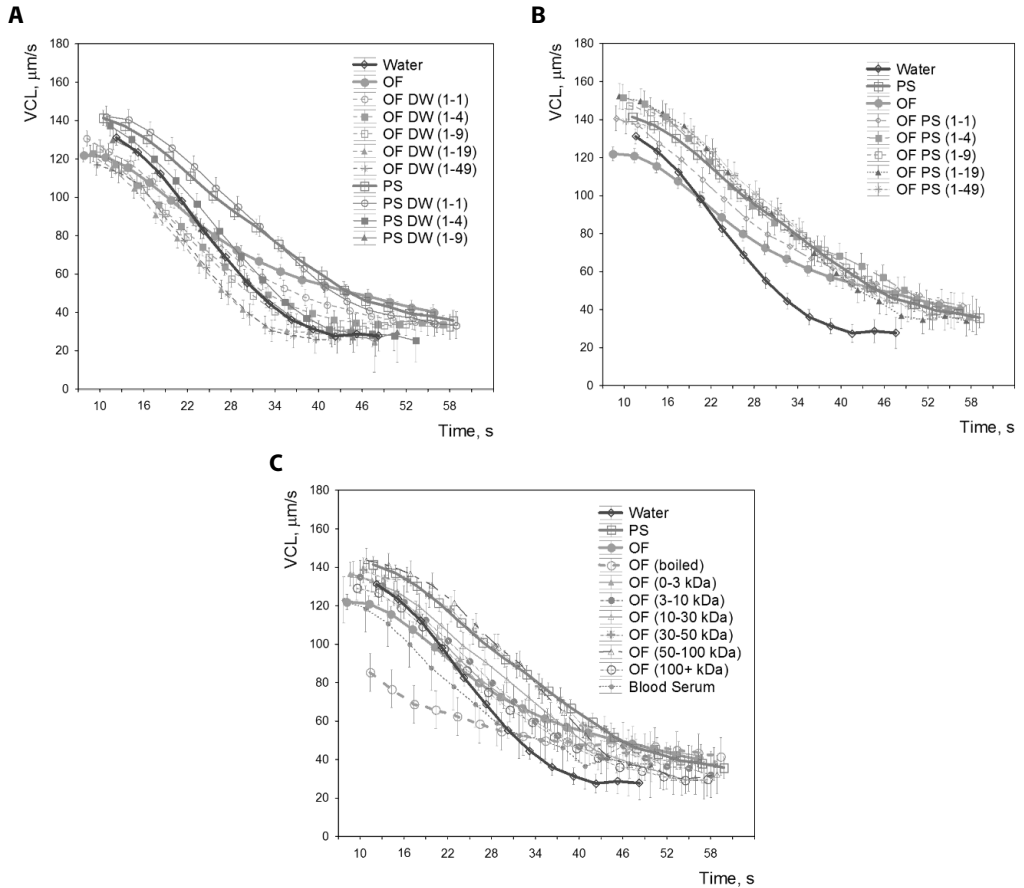
Annex

Figure 7 (supplementary). *Descriptive statistics data on curvilinear velocity of rainbow trout spermatozoa activated in the presence of ovarian fluid and other conditions, depending on time post-activation: A – velocity in water, ovarian fluid, NaCl solution isotonic to ovarian fluid (physiological solution, PS, 290 mOsm/l); and their dilutions with water (1–1; 1–4; 1–9; 1–19 and 1–49); B – motility in ovarian fluid diluted with isotonic NaCl solution (in comparison with water and isotonic saline); C – motility in MWCO fractions of ovarian fluid, blood serum and thermotreated ovarian fluid (compared with water, ovarian fluid, and isotonic saline). Data are mean values (average from 5–18 males), vertical bars denote 0.95 confidence interval.*

Figure 8 (supplementary). *Descriptive statistics data on the linearity of swimming paths of rainbow trout spermatozoa activated in the presence of ovarian fluid and other conditions, depending on time post-activation: A – velocity in water, ovarian fluid, NaCl solution isotonic to ovarian fluid (physiological solution, PS, 290 mOsm/l); and their dilutions with water (1–1; 1–4; 1–9; 1–19 and 1–49); B – motility in ovarian fluid diluted with isotonic NaCl solution (in comparison with water and isotonic saline); C – motility in MWCO fractions of ovarian fluid, blood serum and thermotreated ovarian fluid (compared with water, ovarian fluid, and isotonic saline). Data are mean values (average from 5–18 males), vertical bars denote 0.95 confidence interval.*
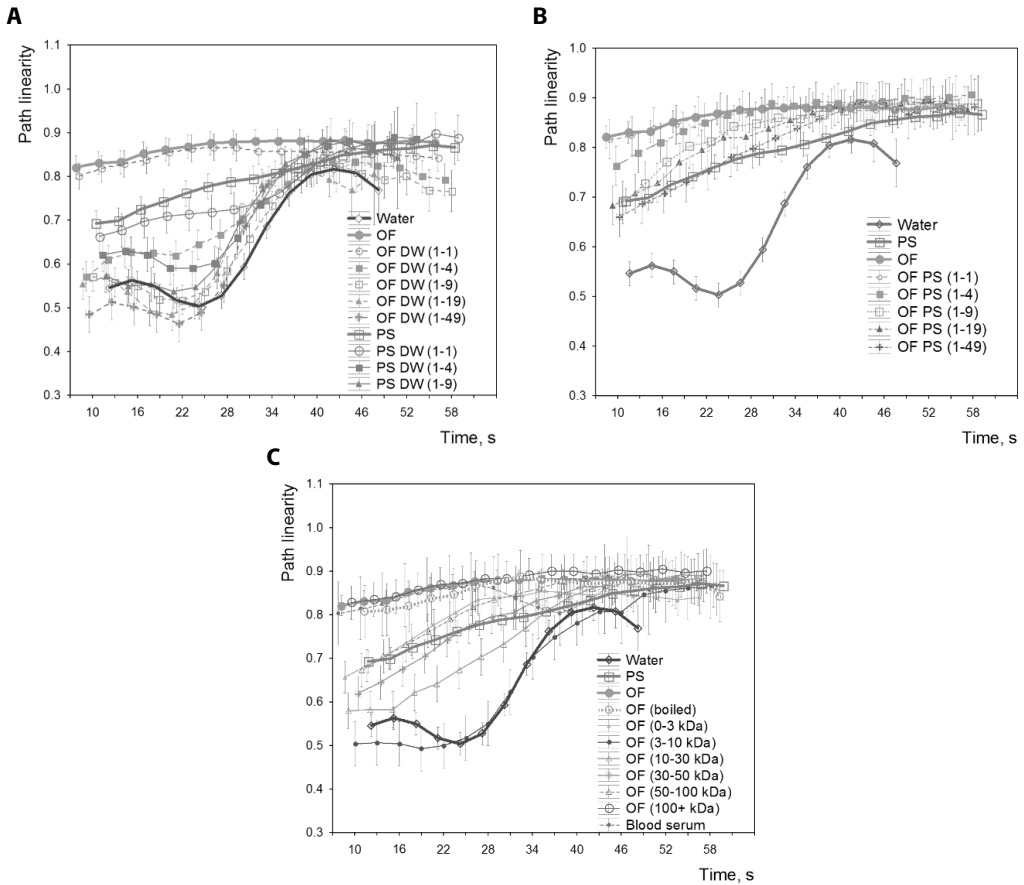
Curvilinear velocity, µm/s

Figure 9 (supplementary). *Curvilinear velocity of rainbow trout spermatozoa in the activation media with various osmolarity and calcium content: 30, 60, 150, 300 mOsm/l, prepared with NaCl and 10 mM Tris buffer, pH 8; and calcium content 0, 0.2, 1, 2, and 5 mmol/l prepared with the appropriate amount of CaCl$_2$ or 2 mmol/l EGTA in case of 0 mmol/l Ca medium. The "0" mOsm/l media was the distilled water with either 2 mmol/l EGTA or corresponding CaCl$_2$ concentration, i.e. the osmolarity in this media was, in reality, higher than 0, the real values are shown in Table 1. Data are mean values (average from 8 males).*
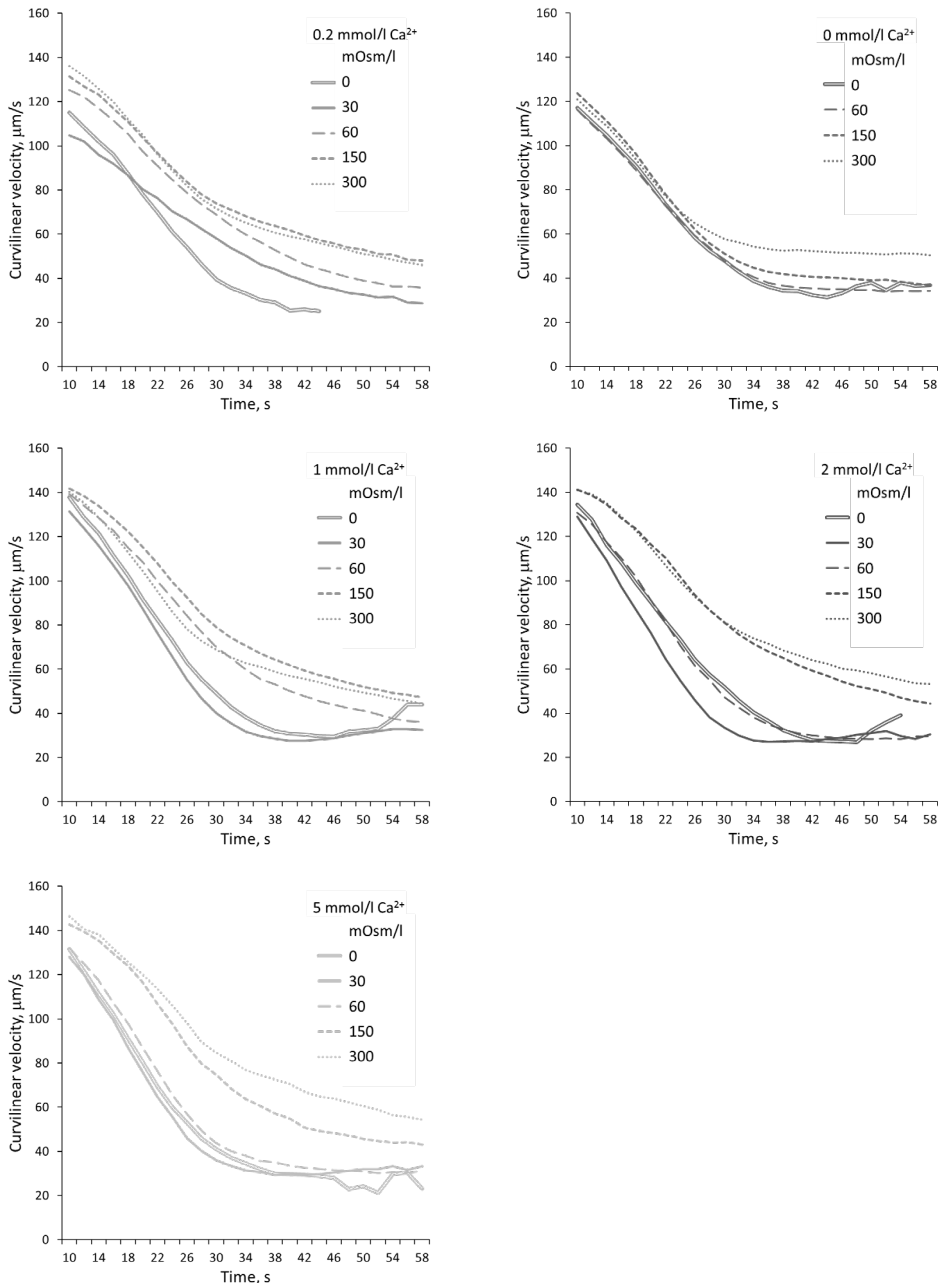
Path linearity
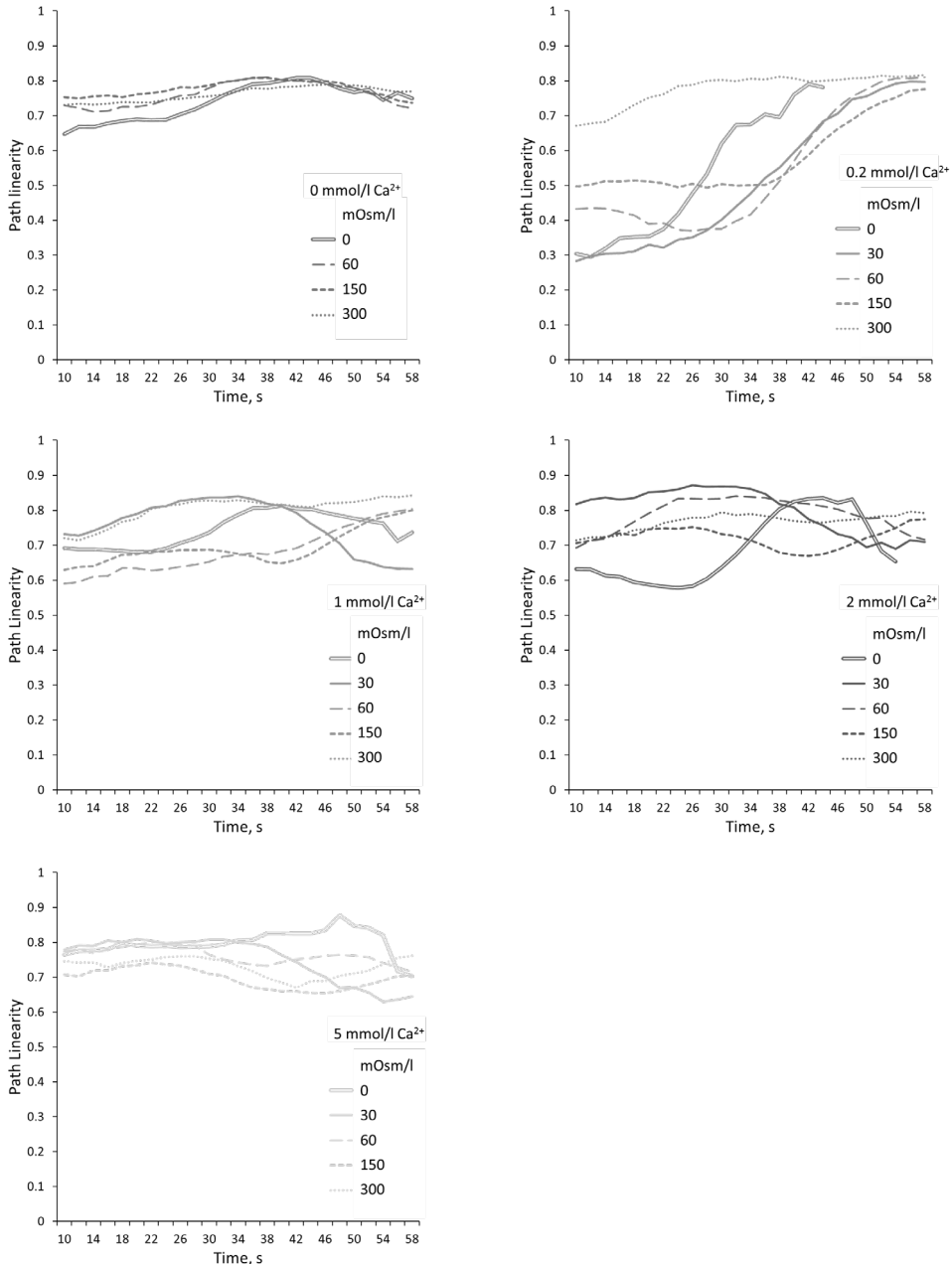
Figure 10 (supplementary). *Linearity of swimming paths of rainbow trout spermatozoa in the activation media with various osmolarity and calcium content: 30, 60, 150, 300 mOsm/l, prepared with NaCl and 10 mM Tris buffer, pH 8; and calcium content 0, 0.2, 1, 2, and 5 mmol/l prepared with an appropriate amount of CaCl$_2$ or 2 mmol/l EGTA in case of 0 mmol/l Ca medium. The "0" mOsm/l media was the distilled water with either 2 mmol/l EGTA or corresponding CaCl$_2$ concentration, i.e. the osmolarity in this media was, in reality, higher than 0, the real values are shown in Table 1. Data are mean values (average from 8 males).*
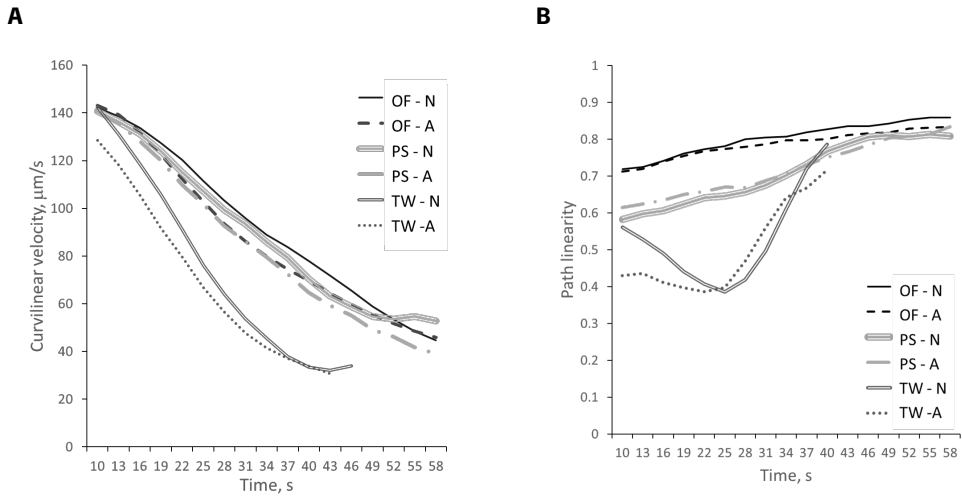
A

B

Figure 11 (supplementary). *Curvilinear velocity (A) and linearity of swimming paths (B) of rainbow trout spermatozoa from albino (A) and normal color (N) males activated in the water (TW, tap water); isotonic NaCl solution (PS, physiological solution, 290 mOsm/l), and ovarian fluid of albino females (OF). Data are mean values (average from 8 males).*

Table 3 (supplementary). *Parameters of linear regression lines for curvilinear velocity dependencies of rainbow trout spermatozoa activated in different media: distilled water (DW), ovarian fluid (OF); distilled water mixed with ovarian fluid (OF DW, in ratios 1:1; 1:4, 1:9, 1:19, 1:49); NaCl solutions (PS – physiological solution with the osmolarity of 290 mOsm/l, diluted with water in the same ratios as ovarian fluid: non-diluted and diluted 1:1, 1:4 and 1:9). Data A, B, and C are mean ± SD, the different superscripts denote significant differences (P < 0.05).*

Aqueous solutions of ovarian fluid and NaCl solutions

Medium	R2	p	A ± SD (slope of regression line)	B ± SD (intercept with y (VCL) axis)	C (intercept with x (Time) axis)
Water	0.9562	< 0.0001	-3.224 ± 0.721[a]	159.4 ± 21.7	49.43
OF	0.9399	< 0.0001	-1.761 ± 0.501 [b]	131.9 ± 18.6	74.94
OF DW (1-1)	0.9244	< 0.0001	-2.212 ± 0.633 [bc]	144.4 ± 23.4	65.31
OF DW (1-4)	0.8875	< 0.0001	-2.000 ± 0.637 [bc]	131.8 ± 23.6	65.88
OF DW (1-9)	0.8509	< 0.0001	-2.073 ± 0.776 [bc]	131.2 ± 28.7	63.31
OF DW (1-19)	0.9223	< 0.0001	-2.762 ± 0.768 [ac]	143.7 ± 24.5	52.03
OF DW (1-49)	0.9568	< 0.0001	-3.474 ± 0.661 [a]	161.3 ± 18.8	46.42
PS	0.9781	< 0.0001	-2.470 ± 0.382 [c]	165.6 ± 14.1	67.03
PS DW (1-1)	0.9652	< 0.0001	-2.696 ± 0.397 [ac]	173.0 ± 14.7	64.16
PS DW (1-4)	0.9321	< 0.0001	-2.933 ± 0.272 [ac]	161.0 ± 22.1	54.90
PS DW (1-9)	0.9274	< 0.0001	-3.046 ± 0.296 [ac]	156.9 ± 23.5	51.53

Ovarian fluid diluted in PS

Medium	R2	p	A ± SD (slope of regression line)	B SD (intercept with y (VCL) axis)	C (intercept with x (Time) axis)
Water	0.9562	< 0.0001	-3.224 ± 0.721 [a]	159.4 ± 21.7	49.43
OF	0.9399	< 0.0001	-1.761 ± 0.501 [b]	131.9 ± 18.6	74.94
OF PS (1-1)	0.9529	< 0.0001	-2.162 ± 0.304 [bc]	156.9 ± 11.3	72.59
OF PS (1-4)	0.9703	< 0.0001	-2.457 ± 0.272 [ac]	173.4 ± 10.1	70.58
OF PS (1-9)	0.9807	< 0.0001	-2.544 ± 0.206 [ac]	174.3 ± 7.6	68.51
OF PS (1-19)	0.9838	< 0.0001	-2.695 ± 0.219 [ac]	178.9 ± 8.1	66.36
OF PS (1-49)	0.9751	< 0.0001	-2.758 ± 0.279 [ac]	181.6 ± 10.3	65.84
PS	0.9781	< 0.0001	-2.470 ± 0.382 [c]	165.6 ± 14.1	67.03

MWCO fractions of ovarian fluid.

Medium	R2	p	A ± SD (slope of regression line)	B SD (intercept with y (VCL) axis)	C (intercept with x (Time) axis)
Water	0.9562	< 0.0001	-3.224 ± 0.721 [a]	159.4 ± 21.7	49.43
OF	0.9399	< 0.0001	-1.761 ± 0.501 [b]	131.9 ± 18.6	74.94
OF (0-3)	0.9361	< 0.0001	-2.099 ± 0.401 [bc]	148.7 ± 14.8	70.85
OF (3-10)	0.9791	< 0.0001	-2.419 ± 0.182 [abc]	159.5 ± 6.8	65.93
OF (10-30)	0.9877	< 0.0001	-2.478 ± 0.175 [ac]	163.6 ± 6.5	66.01
OF (30-50)	0.9754	< 0.0001	-2.740 ± 0.297 [ac]	174.3 ± 11.0	63.61
OF (50-100)	0.9708	< 0.0001	-2.814 ± 0.333 [ac]	176.1 ± 12.3	62.60
OF (100+)	0.9482	< 0.0001	-2.283 ± 0.364 [abc]	147.7 ± 13.5	64.69
PS	0.9781	< 0.0001	-2.470 ± 0.382 [c]	165.6 ± 14.1	67.03
Blood Serum	0.9306	< 0.0001	-2.222 ± 0.293 [abc]	139.8 ± 9.8	62.93
Boiled OF	0.9057	< 0.0001	-0.9299 ± 0.134 [b]	86.54 ± 4.8	93.06

Table 4 (supplementary). *Factorial analysis (pair-wise) of interactive effects of media and time as independent variables, and dependent variables curvilinear velocity (VCL) and linearity (LIN) for rainbow trout spermatozoa motility activated in different media (Log-transformed for VCL, and Logit-transformed for LIN): distilled water, distilled water mixed with ovarian fluid (OF DW, in ratios 1–1; 1–4, 1–9, 1–19, 1–49); NaCl solutions (PS – physiological solution with the osmolarity of 290 mOsm/l, diluted with water in the same ratios as ovarian fluid, 1–1, 1–4 and 1–9).*

Index		Index	
VCL	All media	F	7.23
		P	<0.0001
	DW vs OF DW (1-1)	F	22.06
		P	<0.0001
	DW vs OF DW (1-4)	F	12.73
		P	<0.0001
	DW vs OF DW (1-9)	F	2.07
		P	0.0184
	DW vs PS	F	45.36
		P	<0.0001
	DW vs PS DW (1-1)	F	26.94
		P	<0.0001
	OF vs PS DW (1-1)	F	16.7
		P	<0.0001
	OF vs PS DW (1-4)	F	33.19
		P	<0.0001
	OF vs PS DW (1-9)	F	29.00
		P	<0.0001
	OF DW (1-1) vs OF DW (1-4)	F	1.8
		P	0.0326
	OF DW (1-1) vs OF DW (1-9)	F	6.7
		P	<0.0001
	OF DW (1-4) vs OF DW (1-9)	F	2.4
		P	0.0024
	OF DW (1-1) vs PS DW (1-1)	F	3.4
		P	<0.0001
	OF DW (1-4) vs PS DW (1-4)	F	6.37
		P	<0.0001
	PS DW (1-1) vs PS DW (1-4)	F	8.7
		P	<0.0001
	OF vs OF PS (1-1)	F	0.5
		P	0.947013
	OF vs OF PS (1-4)	F	4.7
		P	<0.0001
	OF vs OF PS (1-9)	F	6.3
		P	<0.0001
	OF PS (1-1) vs OF PS (1-4)	F	1.3
		P	0.2153
	OF PS (1-1) vs OF PS (1-9)	F	1.9
		P	0.0213
	OF PS (1-4) vs OF PS (1-9)	F	0.6
		P	0.8824
	OF vs OF (0-3)	F	0.9
		P	0.5842
	OF vs OF (10-30)	F	11.1
		P	<0.0001
	OF vs OF (100+)	F	9.9
		P	<0.0001

LIN	All media	F	6.08
		P	<0.0001
	DW vs OF DW (1-1)	F	30.2
		P	<0.0001
	DW vs OF DW (1-4)	F	3.00
		P	0.0006
	DW vs OF DW (1-9)	F	6.02
		P	<0.0001
	DW vs PS	F	9.57
		P	<0.0001
	DW vs PS DW (1-1)	F	4.81
		P	<0.0001
	OF DW (1-1) vs OF DW (1-4)	F	20.94
		P	<0.0001
	OF DW (1-1) vs OF DW (1-9)	F	30.87
		P	<0.0001
	OF DW (1-4) vs OF DW (1-9)	F	1.72
		P	0.0417
	OF DW (1-1) vs PS DW (1-1)	F	20.20
		P	<0.0001
	OF DW (1-4) vs PS DW (1-4)	F	2.82
		P	0.0006
	PS DW (1-1) vs PS DW (1-4)	F	3.75
		P	<0.0001
	OF vs OF PS (1-1)	F	13.47
		P	<0.0001
	OF vs OF PS (1-4)	F	2.68
		P	0.0005
	OF vs OF PS (1-9)	F	4.80
		P	<0.0001
	OF PS (1-1) vs OF PS (1-4)	F	1.87
		P	0.0262
	OF PS (1-1) vs OF PS (1-9)	F	4.93
		P	<0.0001
	OF PS (1-4) vs OF PS (1-9)	F	0.39
		P	0.9828
	OF vs OF (0-3)	F	3.94
		P	<0.0001
	OF vs OF (10-30)	F	22.06
		P	<0.0001
	OF vs OF (100+)	F	0.81
		P	0.6730

CHAPTER 3

EGG-SPERM INTERACTION IN STURGEON: ROLE OF OVARIAN FLUID

My share in this work was about 70%.

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Egg-sperm interaction in sturgeon: role of ovarian fluid

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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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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 osmolary 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 step-wise 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.

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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	0, 2, 5	Sperm was activated in water and after 0, 2, 5 min post-activation added to washed/non-washed eggs
		Washed eggs		
	2.5% ovarian fluid in water	Non-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
		Washed eggs		
	12.5% ovarian fluid in water	Non-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
		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 short-ly (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

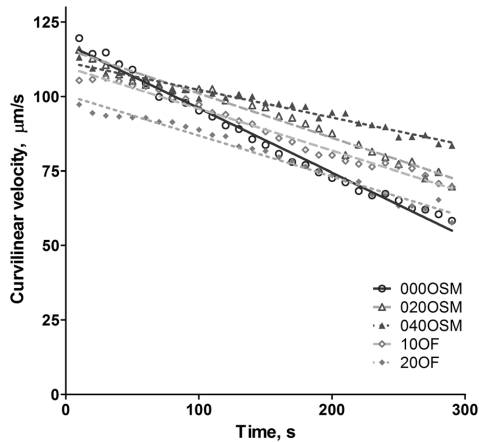| Ovarian fluid dilution	100% ovarian fluid	30-50% ovarian fluid	15% ovarian fluid	0% (distilled water)
Osmolarity	200 mOsm/l	60–100 mOsm/l	30 mOsm/l	~ 0
NaCl concentration in the solution	100 mmol/l	30–50 mmol/l	15 mmol/l	0% (distilled water)
Motility in NaCl solution /diluted ovarian fluid, %	0/0	30–50/0–20% (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 ($P < 0.05$)

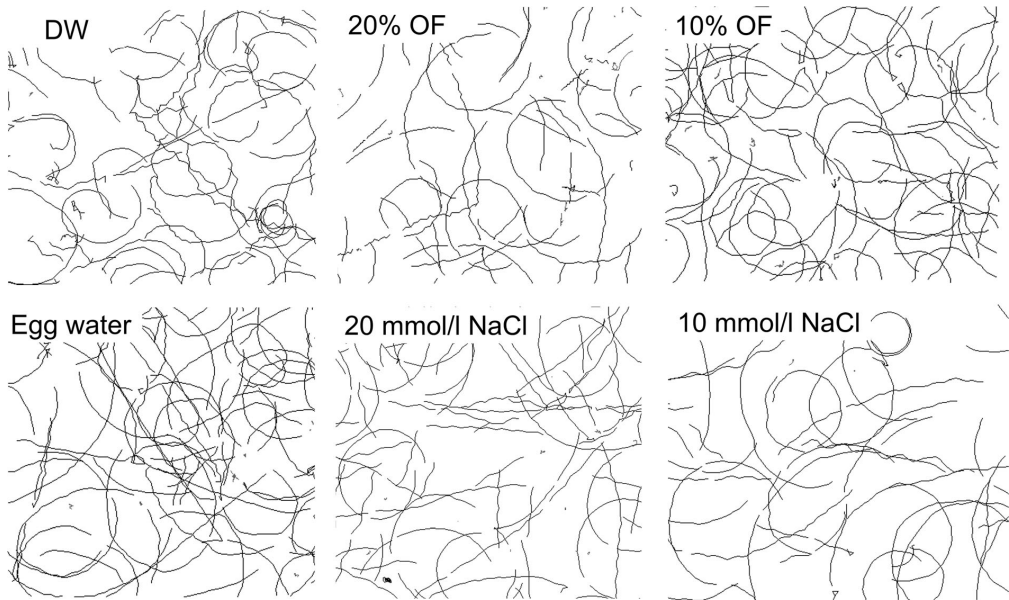| Medium	R^2	P	A ± SD (slope of regression line)	B ± SD (intercept with y (VCL) axis)	C (intercept with x (time) axis)
000OSM	0.9875	< 0.0001	− 0.2164 ± 0.0252[a]	117.7 ± 4.3	544.1
020OSM	0.9724	< 0.0001	− 0.1491 ± 0.0261[b]	116.0 ± 4.5	777.4
040OSM	0.9719	< 0.0001	− 0.0932 ± 0.0164[c]	111.6 ± 2.8	1197
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 spermatozoon 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
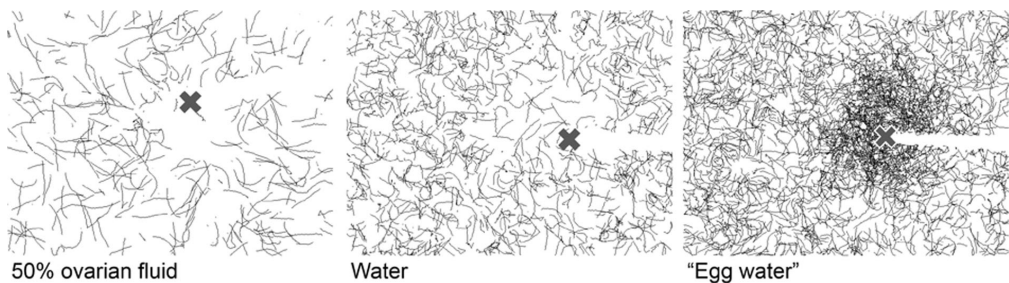

Fig. 3 Swimming tracks of sterlet spermatozoa activated in the water near the tip of microcapillary (cross) filled with fluids: 50% ovarian fluid solution in water; water, and egg water. Each track represents 1 s of motility (20–21 s post-activation)
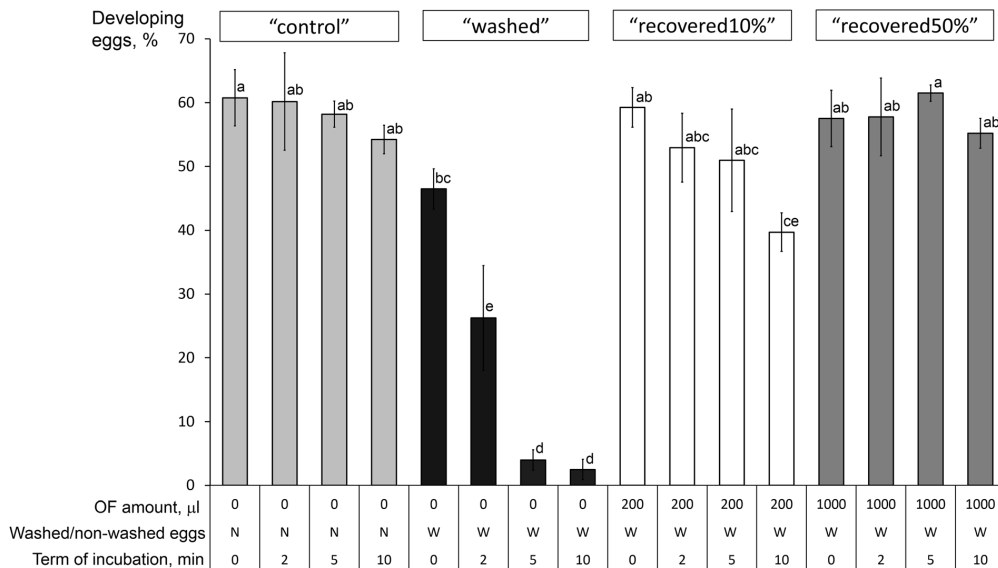
Fig. 4 Sterlet egg development rate depending on the presence of ovarian fluid around the eggs during in vitro fertilization. The eggs (2 g, approx. 135 eggs) were put into the plastic beaker, 8 ml of water was added, and incubation was performed during 0, 2, 5, or 5 min. Eggs were either non-washed 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).
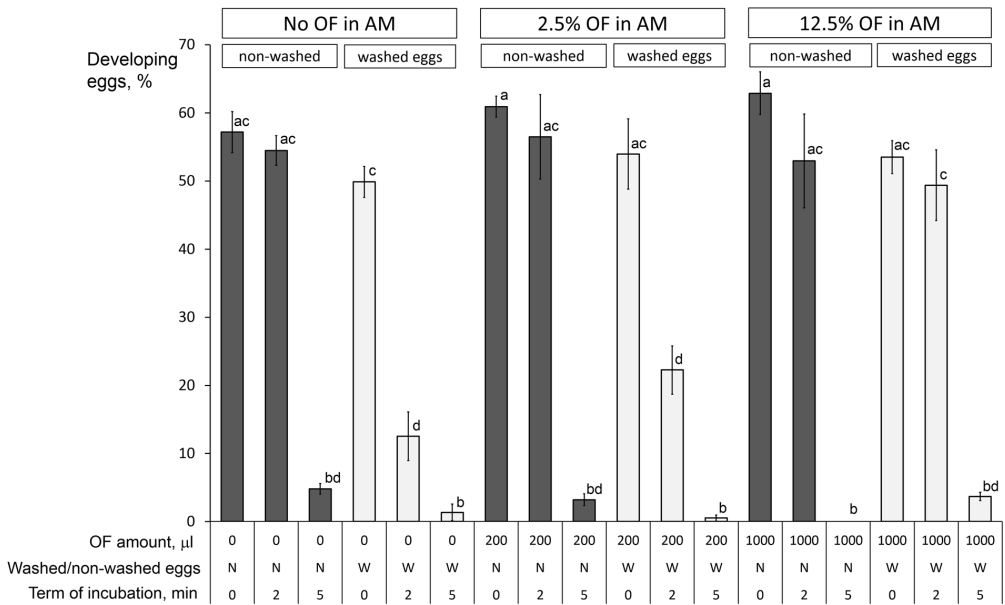
Fig. 5 Sterlet egg development rate depending on the presence of ovarian fluid around the eggs and in the spermatozoa activation medium during in vitro fertilization. The spermatozoa (3 μl) were mixed with 8 ml of water, water with 2.5 of 12.5% of ovarian fluid ("no OF in AM," "2.5% OF in AM," and "12.5% 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.

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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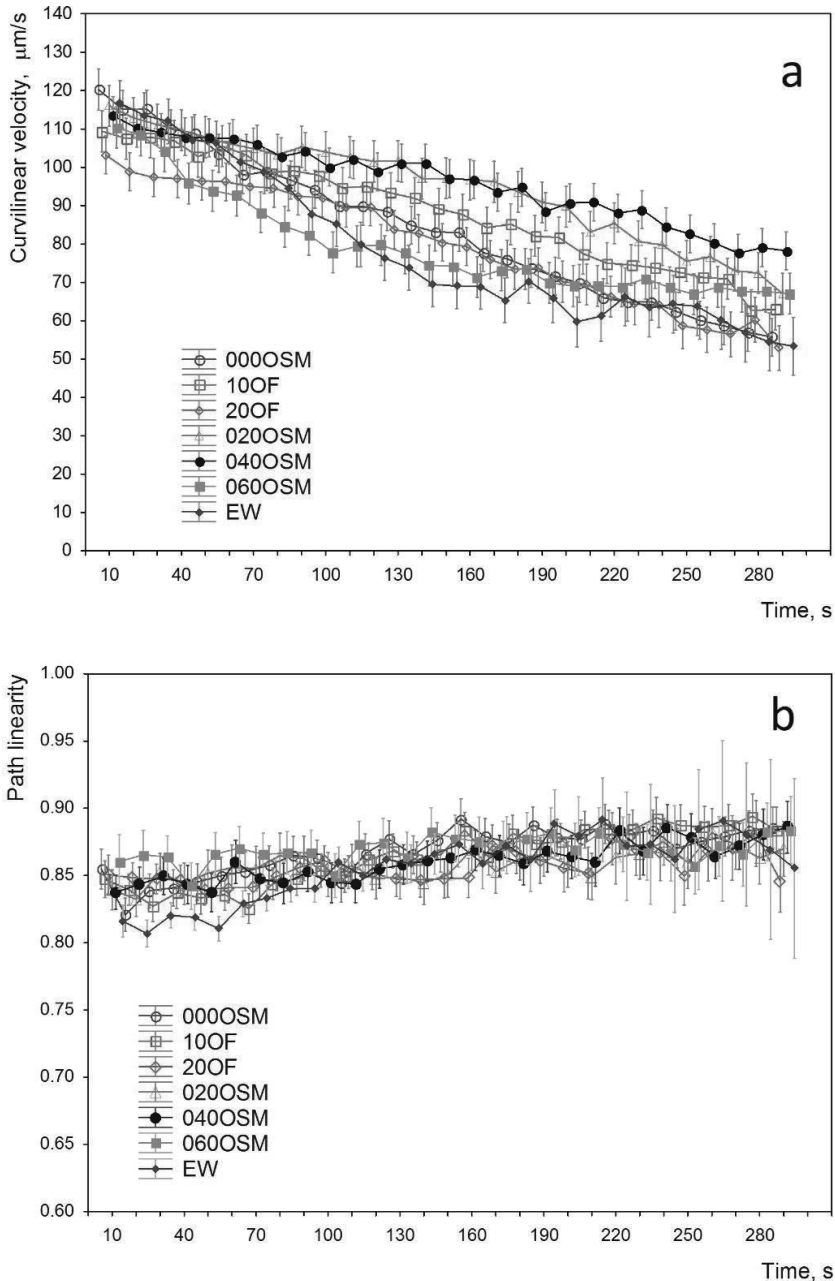
Appendix

Figure 6 *(supplementary). Curvilinear velocity (a) and linearity of swimming paths (b) 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); NaCl solutions with 20, 40 and 60 mOsm/l osmolarity (020OSM, 040OSM, and 060OSM, respectively); and egg water (EW). Data are mean values (average from 7 males), vertical bars denote 0.95 confidence interval.*

Table 5 (supplementary). *Factorial analysis of interactive effects of media and time as independent variables, and dependent variables curvilinear velocity (VCL) and linearity (LIN) for sterlet spermatozoa motility 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, 40 and 60 mOsm/l osmolarity (020OSM, 040OSM, and 060OSM respectively), and egg water (EW).*

Index			P
VCL	All media	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	000Osm vs. 10OF	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	000Osm vs 20OF	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	10OF vs 20OF	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	0.55
	000Osm vs 020 OSM	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	000OSM vs 040 OSM	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	020 OSM vs 040 OSM	Medium	0.01
		Time	< 0.0001
		Time × medium	0.11
	020OSM vs 10 OF	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	0.99
	040OSM vs 20OF	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	10OF vs 040 OSM	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	0.006
	EW vs 000OSM	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	0.038
	060OSM vs 000OSM	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	< 0.0001
	EW vs 060OSM	Medium	0.0038
		Time	< 0.0001
		Time × medium	< 0.0001
LIN	All media	Medium	< 0.0001
		Time	< 0.0001
		Time × medium	0.99

COMMON CARP SPERMATOZOA PERFORMANCE IS SIGNIFICANTLY AFFECTED BY OVARIAN FLUID

Kholodnyy, V., Dzyuba, B., Gadêlha, H., Cosson, J., Boryshpolets, S.
Common carp spermatozoa performance is significantly affected by ovarian fluid.
Manuscript

My share in this work was about 70%.

Common carp spermatozoa performance is significantly affected by ovarian fluid

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Abstract

During evolution, the fish adopted versatile spawning tactics which allowed them to use effectively sperm competition and prevent fertilization by inappropriate male gametes. Ovarian fluid (OF) affects significantly the performance of spermatozoa in several fish species, mainly salmonids, and likely plays an important part in the optimal sustenance of reproductive tactics. Common carps utilize different spawning behavior compared to salmonids, and this raises the question of how this may affect the interaction between male gametes and maternal fluids in this species. The results of our study show that the differences in the spawning process of common carp are accompanied by the specific features of OF-sperm interaction. The presence of common carp OF in the activation medium causes the decrease of the curvilinear velocity of spermatozoa compared to that in the OF-free medium and significantly alters the motility pattern from straightforward motility observed in the water to the hyperactivation-like tumbling in a swimming medium complemented by 50% OF. Chemotactic test (the microcapillary sperm accumulation test) showed a significant response of carp spermatozoa on the OF introduction into the medium with activated spermatozoa. The attraction phenomenon depended on the presence of external calcium ions: the spermatozoa lost the ability to react on the attractant if the calcium was absent in the activating medium. The carp spermatozoa were shown to be responsible for "osmotaxis". Thus, the environmental conditions which accompany the encounter of gametes, in particular presence of OF, calcium ion content, and osmolarity, significantly affect the performance of male gametes in common carp in terms of changes in velocity, path linearity and ability to respond to the external signals, *e.g.* attractants.

Keywords: *sperm motility, fertilization, ovarian fluid, chemotaxis, Cyprinus carpio*

Introduction

All externally fertilizing freshwater fish expel their gametes into the aqueous environment. Nevertheless, there are differences in the way how spawning occurs. Several species adhere to spawning in pairs (*e.g.* gobies *Valenciennea* (Whiteman and Côté 2004)), but most fish are polyandrous. In particular, there are species where smaller subordinate males join or follow the established pair (*e.g. Hucho hucho* or *Oncorhynchus mykiss*). More complex is the case when one female is courted by several males which ejaculate simultaneously around released eggs (*e.g. Cyprinus carpio*). The extreme case is the group spawning when each male in the group fertilizes the eggs of many females (*Perca fluviatilis*) (Stockley et al., 1997). The other differences between spawning procedure among different species are associated with the spawning environment: this could be a stream or shallow still-water (*e.g.*

in *O. mykiss* and *C. carpio*, correspondingly), the eggs could be laid into built by male nests like in *Gasterosteus aculeatus*, onto grass substrates like in *Sander lucioperca* or *C. carpio* or fertilized in free water like in *Hypophthalmichthys molitrix*. There are significant differences in the appearance of expelled egg batches as well as amount and viscosity of released ovarian fluid, *e.g.* typical egg batch of *O. mykiss* contains around 30% of low viscous ovarian fluid and several thousands of eggs with 5-6 mm size (Lahnsteiner, 2002); *C. carpio* eggs are much smaller (around 1 mm), the batch contains several hundreds thousands of eggs and is released only with a little amount of viscous ovarian fluid; sturgeons, *e.g. Acipenser ruthenus*, expel 2–3 mm big eggs with plenty of ovarian fluid, containing proteinaceous fibers. The spermatozoa of rainbow trout can be activated in isotonic potassium depleted media, while carp and sturgeon spermatozoa are activated only if the medium is hypoosmotic to the seminal fluid (Morisawa, 1985). All these different reproduction strategies vary across taxa and appeared during evolution. They are likely affecting the 'scenery' of the fertilization process and are associated with the mechanisms of gamete collision. Differences in reproductive strategies are probably leading to peculiarities in the spermatozoa activation mechanism in the above-mentioned species (or *vice versa*).

It is quite logical that ovarian fluid which is a maternally-derived liquid that surrounds the egg mass inside the female fish and is expelled during spawning (Rosengrave et al., 2008), is the most obvious candidate for providing the control over the behavior of spermatozoa and the state of eggs. After the release with the eggs, it creates the specific environment around them which significantly differs from the properties of a regular aqueous environment (Kholodnyy et al., 2020). It was shown in many fish species that ovarian fluid affects sperm swimming performance. In particular, higher velocities of spermatozoa in ovarian fluid compared to water were found in the guppy *Poecilia reticulata* (Gasparini and Pilastro, 2011), the lake trout *S. namaycush* (Butts et al., 2012), the Arctic charr *S. alpinus* (Turner and Montgomerie, 2002; Urbach et al., 2005), the steelhead *O. mykiss* (Woolsey et al., 2006) or in the brown trout *S. trutta* f. *fario* (Lahnsteiner, 2002). The percentage of motile cells increased when activated in ovarian fluid from *S. trutta* f. *fario* (Lahnsteiner, 2002) and *S. namaycush* (Butts et al., 2012). Much evidence has been found regarding the higher longevity of sperm in ovarian fluid, *e.g.,* in brown trout *S. trutta* f. *fario,* lake trout *S. namaycush*, three-spined stickleback *Gasterosteus aculeatus*, marine sculpin *Hemilepidotus gilberti* and Arctic charr *S. alpinus* (Hayakawa and Munehara, 1998; Lahnsteiner, 2002; Turner and Montgomerie, 2002; Elofsson et al., 2003; Butts et al., 2012). Thus, the ovarian fluid affects the performance of sperm cells in several freshwater fish species, and in this way, it may be one of the founding stones supporting the reproductive strategies. It is highly likely, that influence of ovarian fluid on spermatozoon motility traits is taxa specific, however, no enough data are available now to do general conclusions in this field, and they are generally limited by Salmonidae (Myers et al., 2020), thus the studies involving representatives of other taxa would benefit to our better understanding of fish reproductive physiology and evolutionary developmental biology.

The present study aimed to reveal the features of sperm-egg interaction in common carp (including the effect of ovarian fluid on spermatozoa activation and performance, the potential chemotactic effect of female fluids and effect of ovarian fluid on *in vitro* fertilization outcome), and to discuss how these may be associated with the reproductive behavior of common carp.

Materials and methods

Ethical 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 October 4, 2016, for 5 years) and the authorization for the use of experimental animals (Reference number: 2293/2015-MZE-17214 16OZ22302/2014-17214, valid from 22nd January 2015 for 5 years) issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia by Ministry of Agriculture of the Czech Republic.

Fish brood-stock. Gametes and fluids collection

The experiments were performed in mature common carps (5–6 years, 4–5 kg) kept in the ponds at the Genetic Resource Centre of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Before the experiments (May 2017-2019) the male and female fish suitable for stripping were selected and stocked separately in the indoor tanks with controlled water, constant air supply, and temperature of 18–22 °C. Additional experiments were performed in July 2018 and 2019 with 2-year-old males (1–2 kg weight) kept in closed recirculation system tanks equipped with air supply, with a water temperature of 18–22 °C.

Before sperm collection (24 h before stripping) the males were treated by an intramuscular injection of homogenized carp pituitary in 0.9% (w/v) NaCl solution (1 mg/kg body weight). Females were injected with homogenized carp pituitary in 0.9% (w/v) NaCl solution twice: 24 h (0.4 mg/kg of body weight) and 12 h before stripping (2.1 mg/kg of body weight) (Linhart, 2003). Sperm was collected by stripping into plastic containers and stored on ice until the use (not more than one hour). Ovulated eggs were collected into dry plastic bowls and stored at 15–17 °C. Ovarian fluid was collected using a fine-meshed plastic sieve, centrifuged to remove debris and stored on ice in closed plastic tubes or was frozen and stored at -80 °C if not used the same day.

Osmolarity, pH, protein, and ion content in the ovarian fluid were assessed. Osmolarity was measured using a freezing point osmometer Osmomat 3000 (Gonotec GmbH, Germany) and was expressed in mOsm/l. 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 protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, USA).

Motility observation and recording

Sperm motility was assessed by computer-assisted cell motility analysis. Each sperm suspension (around 0,1 µl) was carefully mixed for 2 s with 40 µl of tested solutions and motility (if present) was recorded for 1 min post-activation using ISAS digital camera (PROISER, Spain) set at 25 frames/s and microscope (UB 200i, PROISER, Spain). Video records were analyzed to estimate spermatozoa motility traits using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA) and following plugins: CASA and CASA modified for multiple analyses (Wilson-Leedy and Ingermann, 2007; Purchase and Earle, 2012). The analysis was performed until the percentage of motile cells did not decrease down to 10% (if it happened during the recorded first minute of motility). Values of spermatozoa velocity (curvilinear velocity, VCL), linearity of the spermatozoa trajectories as well as the pattern of motility (the 1–2 s tracks of individual spermatozoa in the vision field) were chosen as indices representing the motility.

Media used for spermatozoa activation.

Motility of spermatozoa was tested in distilled water, NaCl solutions, solution mimicking the ionic content of ovarian fluid ("OF ionic buffer": 10 mmol/l Tris buffer pH 8.2; 90 mM NaCl; 3.66 mM KCl; 1.41 mM CaCl), ovarian fluid and its dilutions with water (2, 5, 10, 20, 30, 40 and 50%), molecular weight cut-off (MWCO) fractions of ovarian fluid (0–3; 3–10, 10–30, 30–50 and 100+ kDa) (Table 1). The motility tests showed, that spermatozoa agglutinate in the presence of particular concentrations of ovarian fluid in the activating solution. Thus, additional tests to check the conditions of the agglutination were done: the solutions with concentrations of ovarian fluid, causing the agglutination were supplemented with NaCl to vary the osmolarity of the medium (Table 1).

The collected ovarian fluid was used to prepare MWCO fractions using Amicon Ultra centrifugal filters with 3, 10, 30, 50 and 100 kDa filters (Merck Millipore Ltd., Ireland): the ovarian fluid was stepwise passed through the MWCO filters starting from the 100 kDa down to 3 kDa filter; the processing (centrifugation at 3000 g and 10 °C) was done until 90% of fluid was passed through the filter; the collected fraction volumes were recovered by NaCl isotonic solution; the passed-through fraction was processed by next filter.

Sperm chemotaxis tests

The chemotactic reaction of spermatozoa was assessed by analysis of cell behavior following injection of tested fluids with glass microcapillaries into the medium with activated cells under the microscope, which is a variant of the accumulation assay conventionally applied for simple spermatozoa chemotaxis analysis (see the list of media in Table 1). To do this, glass microcapillaries (G100, Narishige, Japan) were pooled (PC-100 puller, Narishige) to get microneedles with tips of 20 μm external diameter, which were additionally cut by a microgrinder (EG-401, Narishige) to obtain 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, Japan) and adjusted above a specimen glass slide on a microscope stage. 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 slide, 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 minutes. The resulting records were then processed by CASA plugin for ImageJ to get the track of individual spermatozoon, and these motility patterns were thereafter subjected to analysis.

Table 1. *Solutions used for carp spermatozoa activation, chemotaxis and/or agglutination tests.*

Solution		Used for activation/chemotaxis (injected fluid)/agglutination tests	Osmolarity	pH
Distilled water		activation/chemotaxis	0	
10mM Tris HCl buffer		activation	10	8.2
Ovarian fluid		activation/chemotaxis	270	8.5
Ovarian fluid in water	50%	activation/chemotaxis/agglutination	145	8.5
	20%	activation/chemotaxis/agglutination	60	8.5
	10%	activation/chemotaxis/agglutination	30	8.5
	5%	activation/chemotaxis/agglutination	15	8.5
	2%	activation/chemotaxis/agglutination	3	8
NaCl solution (Tris HCl buffer)	75 mmol/l	activation/chemotaxis	150	8.2
	30 mmol/l	activation/chemotaxis	60	8.2
	20 mmol/l	activation/chemotaxis	40	8.2
	10 mmol/l	activation/chemotaxis	20	8.2
Molecular weight cut-off fractions of ovarian fluid (50% solutions with water)	0–3 kDa	activation/chemotaxis	290	8.5
	3–10 kDa	activation/chemotaxis	290	8.5
	10–30 kDa	activation/chemotaxis	290	8.5
	30–50 kDa	activation/chemotaxis	290	8.5
	100+ kDa	activation/chemotaxis	290	8.5

Fertilization assay

The *in vitro* fertilization assays were performed in May 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 in water 80% or higher was used. The tests were done to check if the presence of ovarian fluid in combination with spermatozoa post-activation time changes the outcome of the *in vitro* fertilization. The experimental groups are shown in Table 2. In all cases, 1g of eggs (approx. 800 eggs) were fertilized by 1ml of sperm dispersed into 4ml of water from the hatchery supply system. The temperature of the water was 17 °C. The concentration of spermatozoa in the sperm suspension was $4.06×10^{10}$/ml, *i.e.* around 50,000 per egg in the fertilization medium. In some cases, the eggs were pre-washed with 0.9% NaCl solution three times during 10 s to remove the ovarian fluid. The eggs (washed/non washed, with/without OF) were put into a plastic beaker, poured with a test solution. The sperm was mixed with test solutions and after 0, 15, and 30 s added to the eggs in the plastic beakers. The beakers were then placed onto shaker (100 rpm) and after 1 minute of incubation, the eggs were transferred to glass Petri dishes. The dishes were thereafter settled into a tank with baskets for further incubation at 18 °C. The tank had a closed water circuit with aeration, UV-treatment, and temperature control. All fertilization experiments were done in three replicates. The outcome of fertilization was assessed after 1 day by the rate of developing embryos (the embryo development rate is the amount of developing embryo divided by the total amount of eggs). The number of hatched larvae was counted too, and due to the absence of significant difference with the number of developing embryos, only the latter will be presented in the paper.

Table 2. *Effect of ovarian fluid on fertilization performance in common carp: experimental design.*

Group	Sperm pre-incubation time, seconds	Details
Intact eggs	0, 15, 30	Spermatozoa were activated in tap water and added to non-treated eggs
Washed eggs	0, 15, 30	Eggs were thrice washed with 0.9% NaCl solution. Spermatozoa were activated in water and added to the eggs
Washed eggs and 10% of ovarian fluid added back	0, 15, 30	Eggs were washed thrice with 0.9% NaCl solution. Ovarian fluid in an amount of 10% egg weight was added back. Spermatozoa were activated in water and added to the eggs
Washed eggs and 50% of ovarian fluid added back	0, 15, 30	Eggs were washed thrice with 0.9% NaCl solution, and ovarian fluid in an amount of 50% egg weight was added back, the sperm was activated in water and added to the eggs

Statistical analysis

Assessment of motility parameters in different activation media was conducted in triplicates for 10 males in case of ovarian fluid dilutions, media with various osmolarity based on sodium chloride, and 3 males in case of MWCO fractions of ovarian fluid. Motility traits were obtained from the motility records from 50–300 spermatozoa per replicate per time point during 10–59 seconds post-activation with 3 s increment, curvilinear velocity (VCL), and path linearity (LIN) were chosen as representative indices and will be presented in the results. The motility parameters were log_e transformed to ensure a normal distribution of the data, and analysis of interactive effects between variables was 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 motility index changes in various media along motility time was present), we have conducted pair-wise analysis between several media. The data on VCL and LIN are presented as means with corresponding confidence intervals. The data for spermatozoa velocities in 8 media (water, 2, 5, 10, 20 and 50% ovarian fluid, 20 and 40 mOsm/l NaCl solutions) 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 Bonferroni correction using Statistica software.

The fertilization tests were done in three replicates per experimental point. The values of the percentage of fertilized embryos were expressed as the mean ± standard deviation (± 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

Physico-chemical characteristics of ovarian fluid

Common carp females release a scarce amount of viscous ovarian fluid: 1–3% of egg batch. It is almost isosmotic, 268 ± 20 mOsm/l with pH 8.52 ± 0.24 and total protein content of 2.24 ± 0.06 mg/ml. Main ions in the carp ovarian fluid comprise 2.02 ± 0.20 mmol/l Ca^{2+}, 4.78 ± 0.14 mmol/l K^+, and 152.6 ± 15.6 and 108.0 ± 5.7 mmol/l for Na^+ and Cl^-, correspondingly.

Motility of common carp spermatozoa in the presence of ovarian fluid

Common carp spermatozoa gain full activity after contact with water. Generally, the carp male gametes are not active in ovarian fluid or sodium chloride solution of the same osmolarity as ovarian fluid.

The activated cells move in most media by trajectories close to straight (Figure 1, Video in Online resource 1). This pattern of motility was very similar in the solutions of sodium chloride with different osmolarities, the OF ionic buffer solution, or aqueous solutions of ovarian fluid. The exceptions were the solution of 50% ovarian fluid in water and the 0-3 kDa MWCO fraction of ovarian fluid (50% dilution with water). In these cases, the cells moved in circular trajectories.
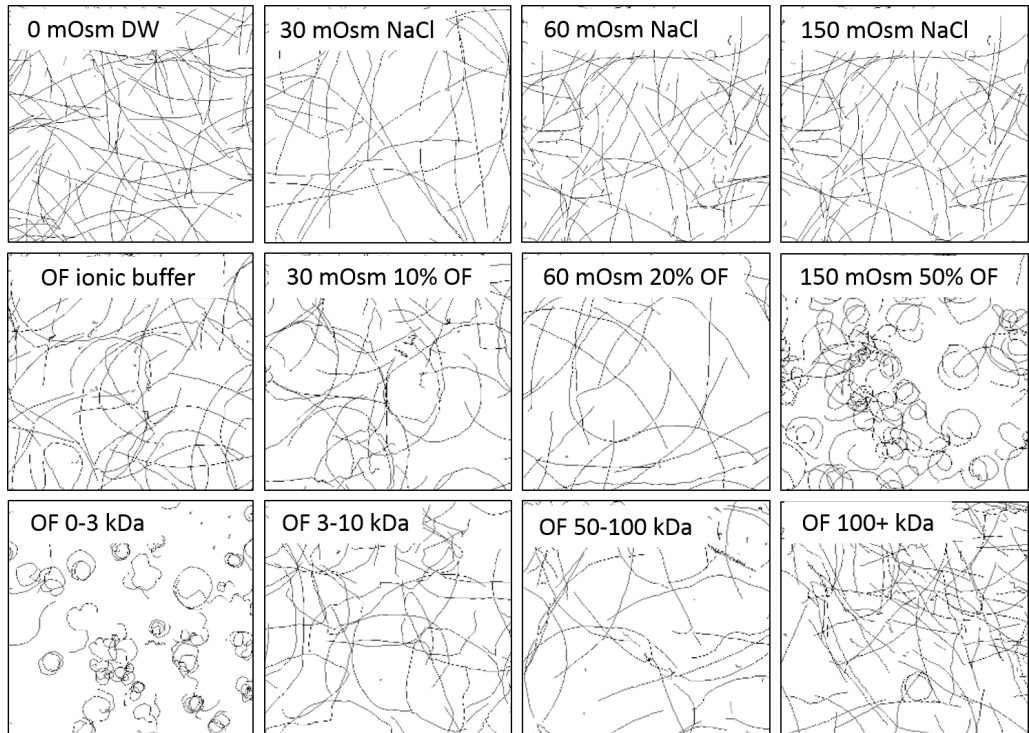

Figure 1. *The pattern of motility of common carp spermatozoa in various activation media, numbers in the circle denote the osmolarity of the test media: DW – distilled water; 50% OF, 20% OF and 10% OF – ovarian fluid diluted in water; 75, 30 and 15 mmol/l NaCl solution in water (150, 60 and 30 mOsm/l osmolarity respectively); OF ionic buffer – a solution containing calcium, potassium, sodium and chloride ions in the concentrations close to ovarian fluid; 50% dilution with water was used; OF 0–3 kDa, 3–10 kDa, 50–100 kDa, 100+ kDa – molecular weight cut-off fractions of ovarian fluid; 50% dilution with water was used for motility records. Each track corresponds to the trajectory of individual spermatozoa during 2 seconds starting from 10 seconds post activation.*

The majority of cells activated in water were motile less than one minute. The velocity of the cells decreased gradually during the motility period (Figure 2), as well as the percentage of motile cells (data not shown). The changes in velocity can be well described by linear regression lines (supplementary Figure 5, supplementary Table 3). The analysis of the linear regression allowed us to get the intercepts with axes, *i.e.* the approximated starting velocity and time of full stop of the spermatozoa (supplementary Table 3). In particular, the starting

curvilinear velocity (VCL) of carp spermatozoa activated in water is 123.3 ± 0.4 mm/s, and the estimated time of motility is 89 seconds. Adding of ovarian fluid into activation medium resulted in the drop of the initial velocity of spermatozoa and prolongation of motility, the more ovarian fluid was in the solution the slower and longer moved the cells.
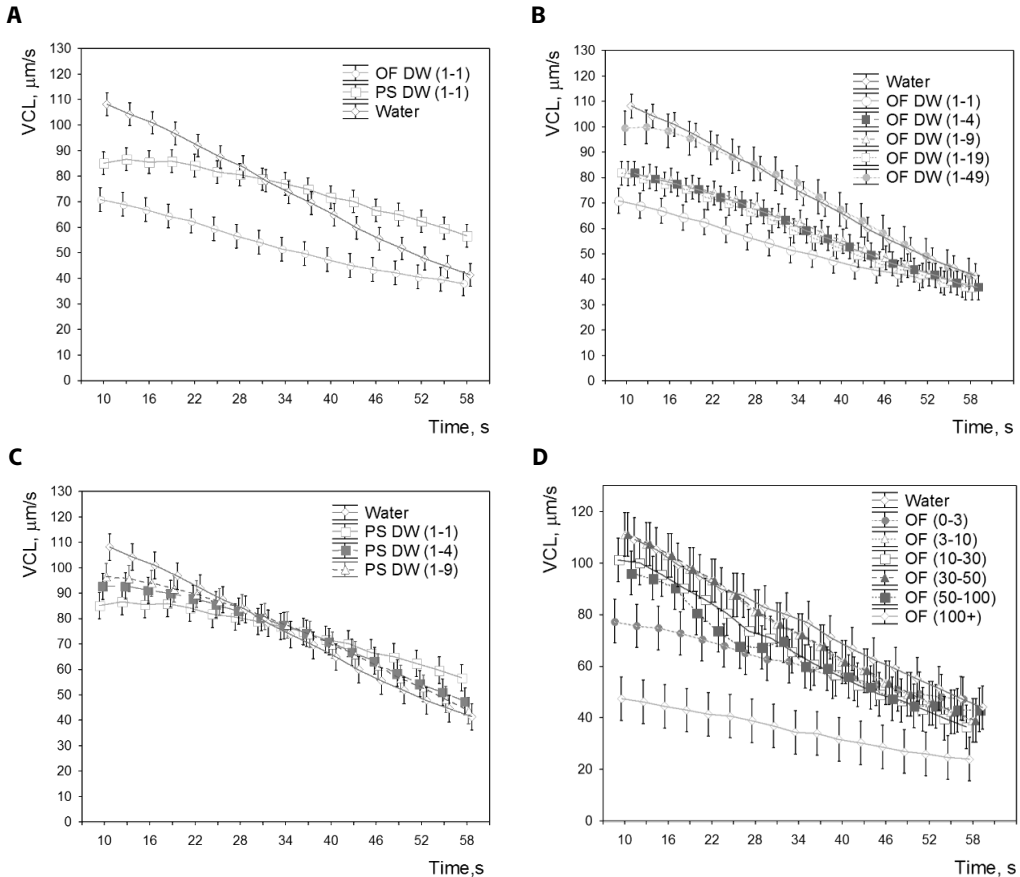

Figure 2. *The curvilinear velocity of common carp spermatozoa depending on time post-activation in various conditions: A – in 50% dilution of ovarian fluid, sodium chloride solution with corresponding osmolarity and water; B – dilutions of ovarian fluid 2-50%; D – sodium chloride solutions of descending osmolarity; D - molecular weight fractions of ovarian fluid. Activation media for spermatozoa were: distilled water, distilled water mixed with ovarian fluid (OF DW, in ratios 1:1; 1:4, 1:9, 1:19, 1:49); NaCl solutions (PS – a physiological solution with the osmolarity of 290 mOsm/l, diluted with water in the same ratios as ovarian fluid, 1:1, 1:4 and 1:9). Data are mean values (average from 7 males), vertical bars denote 0.95 confidence interval.*

The conducted factorial analysis (supplementary Table 4) showed a significant interaction between media and time, *i.e.* the changes in spermatozoa velocity during motility depended on the type of activating media. The character of VCL changes was different if comparing the dependencies obtained in water and 50% ovarian fluid, as well as water and 50% isotonic sodium chloride solution (Figure 2A, supplementary Table 4). In the case of 50% ovarian fluid solution in water the estimated initial VCL is 78.49 ± 0.53 mm/s and the period of motility is almost 103 seconds (supplementary Table 3). The motility traits in the 50% isotonic sodium

chloride solution according to linear approximation were as follows: the initial VCL was 98.12 ± 1.036 mm/s and period of motility almost 139 seconds. Further dilution of ovarian fluid (1:4 to 1:49) entailed the approximation of the VCL change dependences to the case of water, the additional dilution of the sodium chloride saline, i. e. 1:4 and 1:9, did not cause substantial changes in velocity curve character comparing to 1:1 dilution (Figure 2B and C). The effect of MWCO fractions of ovarian fluid on spermatozoa velocity was versatile (Figure 2D): the fraction 100+ kDa entailed the decrease of curvilinear velocity of carp spermatozoa, the effect of fraction 0–3 kDa was similar to the effect of 50% dilution of ovarian fluid the effect of other fractions on the velocity of spermatozoa did not differ significantly from the traits in water.

In the media with diluted ovarian fluid, we observed the phenomenon of spermatozoa aggregation: in the second part of the motility period the cells started to stick to each other (Video in Online Resource 2). The phenomenon was observed if the concentration of ovarian fluid was 2–10% and the osmolarity of the medium not higher than 60 mOsm/l.

Chemotactic and trapping behavior of spermatozoa

Injection of ovarian fluid into the suspension of spermatozoa activated in water (or Tris buffer) entails the immediate and strong effect of cells "gathering" around the capillary tip with the releasing test fluid (Figure 3A, Video in Online resource 3). The trajectories of the cells approaching the area around the ovarian fluid "cloud" have characteristic loops (Figure 3A). The biased behavior of spermatozoa is observed when diluting the ovarian fluid down to 2%. No reaction is observed if the injected test solution is the water (same as the activating medium) (Figure 3D). The agglutination (sticking) of the spermatozoa, similar to the one in motility tests with diluted ovarian fluid, was observed around the injected ovarian fluid cloud after 30-40 seconds of motility if the cells were activated in water (Video in Online resource 3).

There is no (or very insignificant) response of spermatozoa on introduced ovarian fluid if the activation was done in calcium-free medium (with introduced 5 mmol/l EGTA). In the media with 0.2 mmol/l Ca^{2+} the response was observed. Further rise of the Ca^{2+} concentration in the activation solution up to 5 mmol/l did not change the response significantly (Figure 3C).

The biased behavior of spermatozoa was present if the test media was one of MWCO fractions of ovarian fluid. The most significant effect was observed in the case of 0–3 kDa MWCO fraction (Figure 3E, video in Online resource 3). The characteristic agglutination of the spermatozoa was seen only in the case of 100+ kDa MWCO fraction of ovarian fluid (Video in Online resource 3).

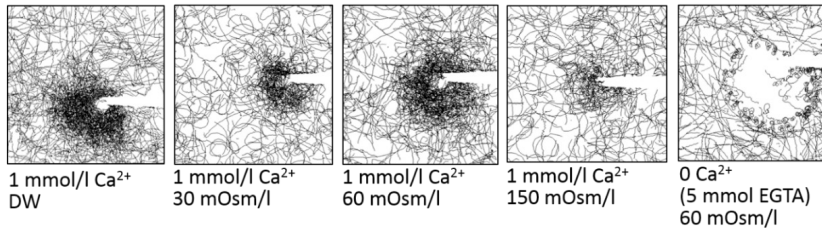**A.** Ovarian fluid injected to water
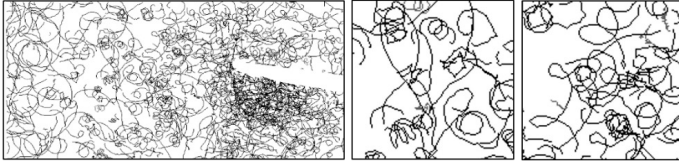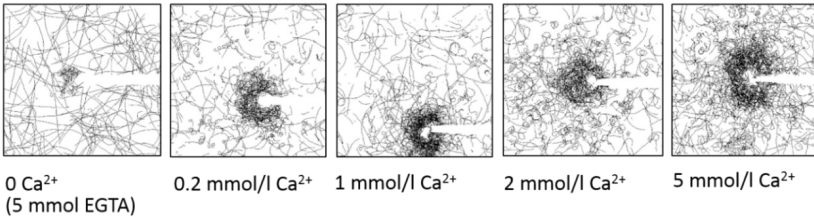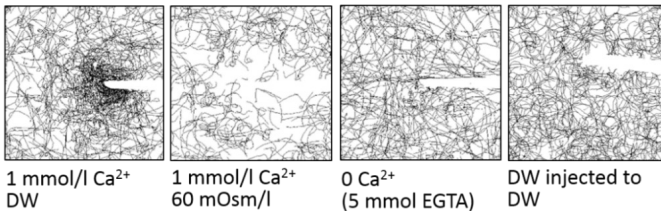

"Turn-and-run" loops

B. 20% OF (in water) injected to NaCl solutions with various osmolarity and calcium content

| 1 mmol/l Ca²⁺ DW | 1 mmol/l Ca²⁺ 30 mOsm/l | 1 mmol/l Ca²⁺ 60 mOsm/l | 1 mmol/l Ca²⁺ 150 mOsm/l | 0 Ca²⁺ (5 mmol EGTA) 60 mOsm/l |

C. 20% OF (in water) injected to solutions (water) with various calcium concentration

| 0 Ca²⁺ (5 mmol EGTA) | 0.2 mmol/l Ca²⁺ | 1 mmol/l Ca²⁺ | 2 mmol/l Ca²⁺ | 5 mmol/l Ca²⁺ |

D. Sodium chloride solution, 60 mOsm/l and 0.2 mmol/l Ca²⁺ injected to

| 1 mmol/l Ca²⁺ DW | 1 mmol/l Ca²⁺ 60 mOsm/l | 0 Ca²⁺ (5 mmol EGTA) | DW injected to DW |

E. MWCO fractions of OF injected to sodium chloride solution (30 mOsm/l) with 1 mmol/l Ca²⁺

| 0-3 kDa MWCO | 3-10 kDa MWCO | 10-100 kDa MWCO | 100+ kDa MWCO | 0-3 kDa MWCO AM: 0 Ca²⁺ (5 mmol EGTA) |
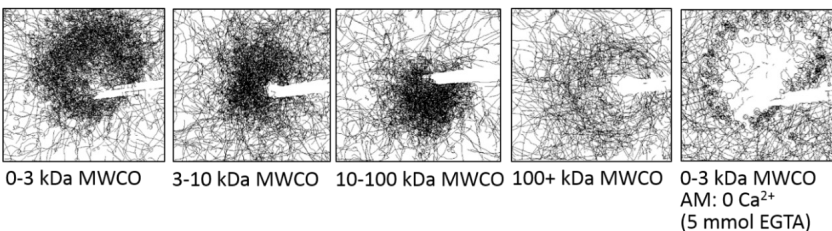
Figure 3. *Swimming tracks of carp spermatozoa activated in various media in the vicinity of the tip of a microcapillary filled with test fluids: A: ovarian fluid (OF) injected to water, as activation media; B: diluted ovarian fluid injected to solutions with various osmolarity; C: diluted ovarian fluid injected to water with various calcium content; D: sodium chloride solution (or water) injected to various media; E: molecular weight cut-off (MWCO) fractions injected to sodium chloride 30 mmol/l solution with 1 mmol/l Ca²⁺. Each track represents 2 seconds of motility (20–22 s post activation).*

The biased behavior of spermatozoa was observed also if the injected test solution was sodium chloride solution or OF ionic buffer. The cells were "trapped" in the cloud of injected fluid. The strength of the trapping was lower than for ovarian fluid. No signs of agglutination were found. The trapping reaction was present with the dilution of the injected test solution down to 30 mOsm/l with water as an activation medium. If the osmolarities of injected saline and the activation medium were the same, *e.g.* 60 mOsm/l in sodium chloride solution (Figure 3D), no spermatozoa response was present. In a similar case with the same osmolarity of injected diluted ovarian fluid and activation medium, *e.g.* 20% ovarian fluid as a test solution and 60 mOsm/l in sodium chloride solution as activation medium, the spermatozoa showed the trapping behavior (Figure 3D).

In vitro fertilization outcome depending on the ovarian fluid presence

If the untreated eggs were fertilized with sperm immediately after activation the success rate was almost 100% (Figure 4). No significant changes in developing embryo rate were found if the sperm was introduced 15 s post-activation in water. An increase of pre-activation time up to 30 s resulted in a significant drop of fertilizing ability of sperm. Removal of ovarian fluid by 3 times washing of the eggs did not change significantly the fertilization rate either in case of introducing the spermatozoa 0 and 15 seconds post-activation, or 30 seconds post-activation. Re-introduction of ovarian fluid into washed eggs did not change the rates of fertilization, only in the case of 30 s post activation time a slight rise was observed, nevertheless, it was not significant. The presence of 2% ovarian fluid in the activation medium did not change the rates of fertilization in all the post-activation time cases, comparing to the OF-free medium.

Discussion

It is believed that the placoderms, the predecessors of the modern fishes, were using internal fertilization, and the external fertilization arose in bony fishes later during evolution (Long et al., 2009). Change in the reproduction environment from internal fluids for the external water, which has conditions being far from physiological, entailed the development of numerous adaptations in the structure and function of fish gametes to counterbalance the negative impact of external media. Just to name a few, these include enlargement of the ovum and strengthening of its surrounding envelope, which became insuperable constraint for the male gamete to fuse with its female counterpart if there wasn't a micropyle, which is a simple (seemingly) but effective tool to ensure penetration of a single spermatozoon towards oocyte membrane (Jamieson, 1991). And *vice versa* in external fertilizers, the fish spermatozoon structure in external fertilizers became much more simple compared to that of internal fertilizers (Jamieson, 1991). This is often called aquasperm, and lacks acrosome, as well as has a lower number of mitochondria, comparing to the gametes from internally fertilizing species, *etc*. And last but not the least, the externally fertilizing fishes have a specific maternal fluid. This is ovarian fluid, which bathes the mature oocytes in the ovarian cavity of fish and is released during spawning together with the eggs through the oviducts into freshwater or saltwater, playing the role of a "protective coat" for the female gametes and creating an optimal environment for the gametes' encounters in many fish species (reviewed in Zadmajid et al., 2019; Kholodnyy et al., 2020). During the evolution, the fish adopted versatile reproductive (spawning) tactics which allowed them to use efficiently sperm competition tactics and prevent fertilization by inappropriate male gametes. The development of these tactics entailed the changes in the function of the testes and features of spermatozoa (Stockley et al., 1997). These features include the sensitivity of spermatozoa

to different conditions of the environment, *e.g.* ionic composition, which is under the control of membrane protein complexes (Alavi et al., 2019). It is highly likely, that ovarian fluid, which was shown to affect significantly the performance of spermatozoa in many fish species, plays an important part in the optimal sustenance of reproductive tactics.
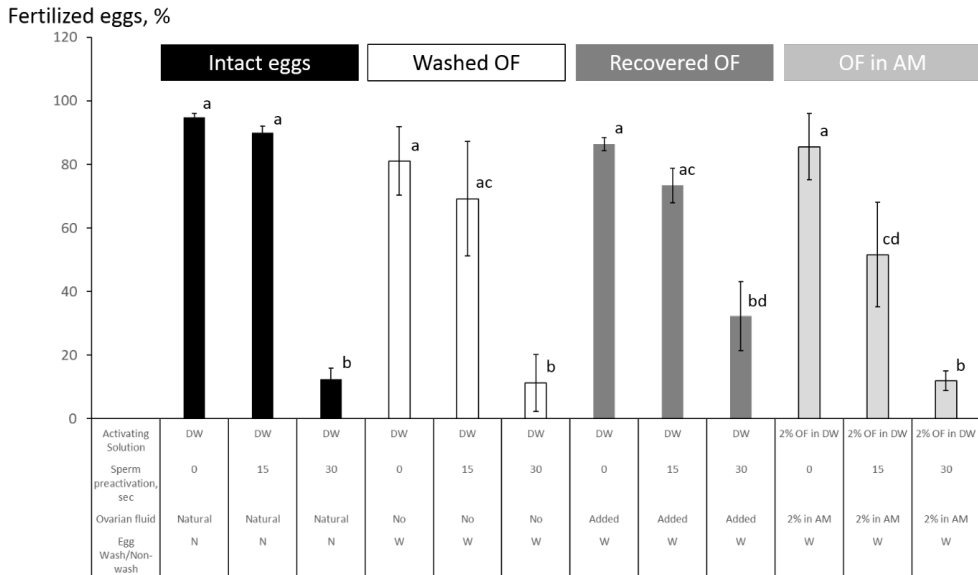

Figure 4. *Common carp egg fertilization rate depending on the presence of ovarian fluid around the eggs or in the sperm activation medium. Eggs were either non-washed to remove ovarian fluid ("Intact eggs") or washed thrice with 0.9% NaCl solution ("Washed OF" or "OF in AM" groups); washed eggs were as well mixed with ovarian fluid in an amount of 10% of egg weight ("Recovered OF" group), i.e. 100 µl of ovarian fluid. Spermatozoa (1 µl) were activated in 4 ml of water or 2% ovarian fluid in water ("OF in AM" group) and in 0, 15 or 30 s added to the beakers with eggs, mixed and the beakers were put to the shaker for 1 minute. Thereafter the eggs were transferred to glass Petri dishes and put to incubators at 18°C. In 3 days the developing embryos were counted, and the fertilization rate was calculated (fertilized eggs/total amount of eggs). Data are mean ± SD, the different superscripts denote significant differences (P < 0.05)*

We have shown recently that sterlet *A. ruthenus* and rainbow trout *O. mykiss* ovarian fluids affect differently the behavior of their respective spermatozoa (Chapters 2 and 3) which is quite impressive considering the differences in the spawning tactics between acipenserids and salmonids. For our best knowledge, by now there are no data on the egg (ovarian fluid) – sperm interaction in cyprinids, and our investigation on *C. carpio* will throw the light on this overlooked area.

Interestingly the common carps utilize absolutely different spawning behavior compared to the above two cases: they reproduce in shallow still water, *e.g.* ponds, small lakes, or quiet river which bottom is covered by plenty of water plants. Mostly the spawning act happens in big groups, where one or more females are followed by several males. During the intensive tail and body rattling of both males and females, the released eggs are intensively mixed with the water and ejaculated sperm, gravitate down and stick to the plants.

The results of our present study show that the spawning process of common carp is accompanied by the specific features of egg-sperm interaction and ovarian fluid.

In particular, the carps release a very little amount of viscous ovarian fluid which is isotonic and has a pH of 8.4. The amount of ovarian fluid *in O. mykiss* and *A. ruthenus* is significantly bigger than in *C. carpio* (Chapters 2 and 3). The value of pH is very close in all three species, as well as the content of calcium ions (pH 8.2, 8; and 8.41; Ca^{2+} 1.15; 1.2; and 1.5 mmol/l, in rainbow trout, sterlet, and common carp, correspondingly). The potassium ion content in common carp (3.6 mmol/l) is in between those in rainbow trout and sterlet (2.2 and 5.7 mmol/l, correspondingly).

Like typical representative of freshwater fishes, the carp spermatozoa are normally non-motile in isosmotic conditions (*e.g.* in seminal fluid or saline), and transfer to hypoosmotic conditions entails the activation of motility, which lasts around 1 minute in the water. Unlike our observations with sterlet (Chapter 3), we have not found any inhibiting effect of ovarian fluid on the activation in such osmotic conditions.

If the sperm cells were activated, no effect of osmolarity was found on motility patterns (Figure 1), the spermatozoa moved straight or according to wide arcs either in water and in the medium with osmolarity up to 150 mOsm/l. In addition to this, no effect of low concentrations of ovarian fluid on the straightness of spermatozoa motility was found as well. Only the presence of the ovarian fluid at 50% led to a significant change in the behavior of the spermatozoa: most started to move according to circular trajectories, the behavior which is similar to mammalian spermatozoon when hyperactivated in the vicinity of the egg (Cosson et al., 1999).

This absence of response of the motility patterns to the osmolarity of the medium and presence of ovarian fluid is quite similar to the case of sterlet spermatozoa, except the change of pattern in 50% ovarian fluid for carp. However, it was not possible to check the effects of this OF concentration in sterlet because of motility inhibition. And *vice versa*, the rainbow trout spermatozoa were highly responsive to the osmolarity of medium and presence of ovarian fluid: in our experiments, they moved according to circular trajectories in water (during the initial period of motility) but straightforwardly in the medium with a high content of the ovarian fluid.

Noteworthy only isolated low molecular fraction (0–3 kDa) of carp ovarian fluid caused the change of spermatozoa behavior from the straight-line to the circle pattern (Figure 1), but not the fractions with higher molecular weight. Moreover, the acting agent of this hyperactivation-like behavior is very likely not the ionic composition of this fraction or total ovarian fluid, because the same pattern was not recorded in the buffer, mimicking the content of essential ions in the OF (Ca^{2+}, K^+, Na^+, Cl^-), and having the same osmolarity and pH. In our previous experiments with rainbow trout, the spermatozoa had virtually the same motility pattern in the saline, which reflected the OF content of calcium, its osmolarity, and pH (Chapter 2).

The curvilinear velocity of carp spermatozoa was changed in the presence of ovarian fluid: generally, the more ovarian fluid was present in the activating medium, the slower moved the cells. This effect was rather not due to the changes in osmolarity of the medium since the changes in the velocity of spermatozoa in the media with higher osmolarity was little affected. Both higher osmolarity and presence of ovarian fluid prolonged the motility period of spermatozoa (supplementary Table 3 shows the estimated spermatozoon longevity according to a regression analysis). This carp ovarian fluid feature is quite specific considering the meta-analysis conducted recently by Myers et al. (2020), which concluded that the freshwater fish ovarian fluid enhances the velocity of spermatozoa. The authors mentioned, that their analysis is based on the data on the ovarian fluid effects available to the moment, which are quite scarce and mostly are limited to various representatives of Salmonidae.

Another specific feature of the interaction of common carp ovarian fluid and spermatozoa was the massive agglutination of the cells which was observed in the hypoosmotic media

(<60 mOsm/l) when 2–10% ovarian fluid was present (Video in the Online Resource 2). We suppose that this specific phenomenon may be associated with gradual swelling of spermatozoa in the low osmolarity conditions, which presumably causes the exposition of some molecules normally hidden in the membrane and following aggregation of the cells by some agent in the ovarian fluid. Molecular weight cut-off separation of the ovarian fluid showed that this potential agglutination agent is present in the fraction with MW higher than 100 kDa (only this fraction had such agglutinating effect). What may be the natural purpose of this phenomenon? Our tentative assumption is that this could be the mechanism "preventing" the cells already damaged by a low osmotic environment to approach the eggs, but of course, this needs to be confirmed, which is beyond the scope of this paper. Additional support of this idea will be mentioned below with the chemotaxis test.

Let us turn to the question of spermatozoon performance in the non-uniform medium, *e.g.* containing the volume of ovarian fluid included inside an aqueous environment. This situation can be created in a simple sperm accumulation test using a microcapillary injection of test fluids into the medium with motile spermatozoa. This allows to model the behavior of male gametes during approaching the potential attractants and reflect the naturally occurring processes. The ovarian fluid is usually the first candidate for containing such an attractant since the first reported cases of chemotaxis in externally fertilizing animals, namely experiment with egg jelly in sea urchin (Lillie, 1912). In our recent experiments with *O. mykiss* and *A. ruthenus*, we have found that rainbow trout ovarian fluid has a moderate attracting effect on spermatozoa, which is associated at least in part with the ability of the spermatozoa of this species to react on optimal osmotic pressure. In particular, the rainbow trout spermatozoa change their motility pattern for straight-line one when entering the area with ovarian fluid and "prefer" to stay in this optimal environment changing the direction of motility by "turn-and-run" if approaching the border of this area (Chapter 2). A similar effect was found in the case of sterlet spermatozoa, however not with ovarian fluid, but with the egg-conditioned medium obtained after incubation with washed integral (non-destroyed eggs) (Chapter 3). In case of common carp, it is obvious that ovarian fluid injected into the water medium containing activated spermatozoa caused strong attracting effect (Figure 4a, video in Online Resource 3) which overcome this in the similar test in rainbow trout and sterlet, and was observed even with highly diluted ovarian fluid (2% in water). Similarly to the situation in rainbow trout, one can observe a specific behavior of carp spermatozoa approaching the region of interest, *i.e.* "turn-and-runs". This is believed to reflect the process of sensing the attractant by the spermatozoon receptors in charge of the control of the motility direction (Kaupp, 2012). It is noteworthy that we can observe the formation a "belt" of agglutinated cells on the borders of injected ovarian fluid after 30–40 seconds post-activation (video in Online Resource 3), obviously in the area with the lower concentration of ovarian fluid, which caused the agglutination in the motility tests (*e.g.* video in Online Resourse 2). At the same moment, the cells which are inside the cloud, correspondingly in the volume with the higher concentration of the ovarian fluid proceed to move and show no signs of agglutination. This feature may again support the idea about a "negative selection" of the cells that may have been partly damaged by the surrounding hypoosmotic medium.

Interestingly, the chemotactic response was absent if the calcium ions were removed from the activating medium (Figure 4, video in Online resource 3). We suppose that the injected ovarian fluid did not attract the cells, or the latter lost the ability to approach the attractant by turn-and-runs. This kind of response of spermatozoa on external calcium was described in the marine broadcast spawners, *e.g. Ciona* (Yoshida et al., 2018), Siphonophores (Cosson et al., 1984) and in many other species (Cosson, 2015).

Like in our experiments with rainbow trout (Chapter 2), the carp spermatozoa showed the ability to "osmotaxis", *i.e.* injection of ionic buffer or just sodium chloride saline into the suspension of spermatozoa activated in water entailed the gathering of the cells and their trapping in the cloud. Turn-and-run loops were also present (Figure 4). This mode of response was seen until the difference between the activation medium and the injected test medium was at least 30 mOsm/l. No reaction to the injected saline or ionic buffer was present in the case of equal osmolarities (Figure 4). However, in the case of ovarian fluid even in case of equal osmolarity of the injected and the activation media, the positive reaction was still observed. This may indicate that the chemotactic response of the common carp spermatozoa is a sum of at least two components: the positive osmotaxis and the chemotaxis, caused by some low molecular weight agent.

Our *in vitro* fertilization tests were aimed to check both the effect of ovarian fluid presence on the outcome of fertilization and to evaluate how the spermatozoon post-activation time may affect the outcomes in the presence or absence of ovarian fluid. The latter was associated with the possible proof of the idea about "negative selection" of osmotically damaged spermatozoa by the agglutination, observed in the motility records. Unfortunately, we have not found any additional effect of removal of ovarian fluid in the experimental conditions we used, which may be caused either by the inefficiency of its removal from the eggs or non-optimal (too high) concentration of spermatozoa, which may mask the effects, essential for the *in situ* fertilization. In any case, the phenomena observed *in vitro* need to find a clear explanation in terms of fertilization.

Collectively we conclude that carp ovarian fluid significantly changes the motility traits of spermatozoa: the sperm cells change their motility pattern from straightforward in water to hyperactivation-like tumbling in presence of high concentration of ovarian fluid, they move significantly slower and longer compared to water. The ovarian fluid exhibits a strong trapping effect on spermatozoa, which combines osmotrapping and chemotaxis caused by a presently unidentified low molecular agent(s). This trapping is under control of external calcium ions' concentration: if these are absent, no response of spermatozoa could be detected. Ovarian fluid causes agglutination of male gametes provoked by low osmolarity and a relatively long period post activation. These features of the spermatozoon behavior allow supposing that the carp ovarian fluid may serve as the powerful "catcher" of spermatozoa in the course of the intensive mixing of the eggs and sperm during spawning. And finally, the comparison of the response of the freshwater fish male gametes (in our example, common carp, sterlet, and rainbow trout) to the presence of respective ovarian fluid allows to state that it is undoubtedly species-specific and depends on the mode of fish reproductive (spawning) behavior.

Acknowledgments

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Annex

Table 3 (supplementary). *Parameters of linear regression lines for curvilinear velocity dependency of carp spermatozoa activated in different media: distilled water (DW), distilled water mixed with ovarian fluid (OF DW, in ratios 1:1; 1–4, 1–9, 1–19, 1–49); NaCl solutions (PS – physiological solution with the osmolarity of 290 mOsm/l, diluted with water in the same ratios as ovarian fluid, 1:1, 1–4 and 1–9). Data A, B, and C are mean ± SD, the different superscripts denote significant differences (P < 0.05).*

Medium	R2	p	A ± SD (slope of regression line)	B ± SD (intercept with y (VCL) axis)	C (intercept with x (Time) axis)
Water	0.9989	<0.0001	-1.388 ± 0.01209[a]	123.3 ± 0.4476	88.78
OF DW (1-1)	0.9946	<0.0001	-0.7643 ± 0.01457[b]	78.49 ± 0.5396	102.7
OF DW (1-4)	0.9954	<0.0001	-1.001 ± 0.01762[c]	93.19 ± 0.6527	93.06
OF DW (1-9)	0.9931	<0.0001	-0.9604 ± 0.02063[d]	97.67 ± 0.7643	101.7
OF DW (1-19)	0.9940	<0.0001	-1.185 ± 0.02381[e]	108.6 ± 0.8818	91.61
OF DW (1-49)	0.9827	<0.0001	-1.288 ± 0.04415[f]	118.1 ± 1.635	91.68
PS DW (1-1)	0.9770	<0.0001	-0.7067 ± 0.02798[g]	98.12 ± 1.036	138.8
PS DW (1-4)	0.9801	<0.0001	-1.011 ± 0.03724[c]	110.2 ± 1.379	109.0
PS DW (1-9)	0.9818	<0.0001	-1.167 ± 0.04097[e]	113.9 ± 1.518	97.58

Table 4 (supplementary). *Factorial analysis of interactive effects of media and time as independent variables, and dependent variables curvilinear velocity (VCL) and linearity (LIN) for carp spermatozoa motility activated in different media: distilled water, distilled water mixed with ovarian fluid (OF DW, in ratios 1–1; 1–4, 1–9, 1–19, 1–49); NaCl solutions (PS – physiological solution with the osmolarity of 290 mOsm/l, diluted with water in the same ratios as ovarian fluid, 1–1, 1–4 and 1–9).*

Index			P
VCL	All media	Medium	<0.0001
		Time	<0.0001
		Time × medium	<0.0001
	DW vs OF DW (1–1)	Medium	<0.0001
		Time	<0.0001
		Time × medium	<0.0001
	DW vs OF DW (1–4)	Medium	<0.0001
		Time	<0.0001
		Time × medium	0.8260
	DW vs OF DW (1–9)	Medium	<0.0001
		Time	<0.0001
		Time × medium	0.8247
	DW vs OF DW (1–19)	Medium	<0.0001
		Time	<0.0001
		Time × medium	0.8260
	DW vs OF DW (1–49)	Medium	<0.0001
		Time	<0.0001
		Time × medium	0.9655
	DW vs PS DW (1–1)	Medium	0.0003
		Time	<0.0001
		Time × medium	<0.0001
	DW vs PS DW (1–4)	Medium	0.1212
		Time	<0.0001
		Time × medium	0.0016

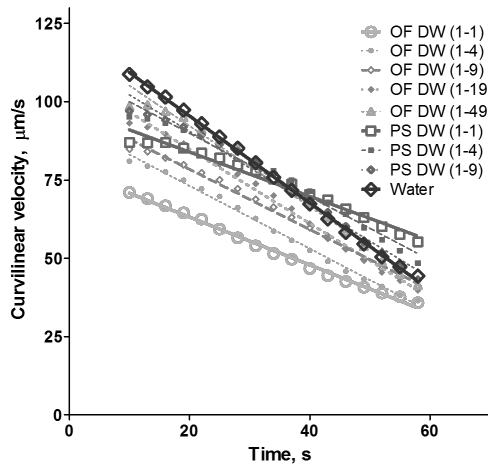| VCL			
DW vs PS DW (1–9)	Medium	0.1220	
	Time	<0.0001	
	Time × medium	0.0620	
OF DW (1–1) vs OF DW (1–4)	Medium	<0.0001	
	Time	<0.0001	
	Time × medium	0.0970	
OF DW (1–1) vs PS DW (1–1)	Medium	<0.0001	
	Time	<0.0001	
	Time × medium	0.0015	
OF DW (1–4) vs PS DW (1–4)	Medium	<0.0001	
	Time	<0.0001	
	Time × medium	0.7840	
PS DW (1–1) vs PS DW (1–4)	Medium	0.1464	
	Time	<0.0001	
	Time × medium	0.0147	

Figure 5 (supplementary). *Regression analysis of the curvilinear velocity of carp spermatozoa in the presence of ovarian fluid dependence on time post activation. Activation media for spermatozoa were: distilled water, distilled water mixed with ovarian fluid (in ratios 1–1; 1–4, 1–9, 1–19, 1–49); and NaCl solutions (PS – physiological solution with the osmolarity of 290 mOsm/l, i.e. close to ovarian fluid, was diluted with water in the same ratios as ovarian fluid, 1–1, 1–4 and 1–9). Markers are mean experimental values obtained from averaging data from 10 males. Lines represent linear regressions of experimental dependencies, the parameters of the fitting, values of slopes and intercepts are shown in supplementary Table 3.*

CHAPTER 5

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

General discussion

To be or not to be: that is the question not only for classic literature hero but for every living creature on planet Earth. This could be a motto of a natural selection, which combines the enormous variety of events, and in overall supports the existence of living matter. The first event in this selection complex happens as early the gametes start their encounter to meet each other. The male gamete should find its way to the female gamete using the sum of strict step-by-step phenomena which lead to a fusion of the gametes. Each of the steps triggers the next one, and if something goes wrong, the final target would never be reached (*i.e.* to-be-or-not-to-be issue will be not solved positively). This means that only cells that are compatible with the existing demands could meet, fuse, and give rise to a new organism. Different species of fish are adapted to fertilize eggs internally or externally. It is believed, that the earliest predecessors of fish, the placoderms, were internal fertilizers (Long et al., 2009) and the externally fertilizing bony fishes arose later. The exact pressures for this specific adaptation are unknown so far. Nevertheless, many structures and "tools" in the gametes were conserved during evolution along with arising of specific features (Darszon et al., 2020), and this fact allows to study reproduction issues across all the spectrum of taxa and create a logical system of interdependent components, and finally to better understand spermatozoon physiology.

Egg-sperm encounter in external fertilizers. Effect of environment and potential mechanisms

In externally fertilizing freshwater fish species, both male and female spawn synchronously to ultimately produce a plume of gametes underwater. The short lifespan of either spermatozoa or eggs, as well as the environmental conditions (*e.g.* water flow), makes the reproductive success quite time-limited by sperm availability, and the gametes are under selection for mechanisms that may control sperm-egg encounters. **Chapter 1** of the thesis discusses these sophisticated mechanisms, which combine various molecular, chemical, and physical factors. Egg signaling and sperm response could be adapted, for instance, to meet one or many specific environmental constraints, such as chemical diffusivity, pH, fluid and flow properties, and surface interactions, among other external factors (Hart, 1990; Iwamatsu, 2000).

Particularly, after the release from the parent body the male gamete contacts with the external hypotonic aqueous environment and the difference in the internal and external conditions entails its motility activation (Morisawa, 1985). In addition to the change in the osmolarity of the fluid surrounding the gametes, the spermatozoa of some teleosts, *e.g.*, salmonids, acquire motility due to a decrease in the K^+ content (Morisawa, 1994). It was found as well that the initiation of motility is associated with the changes in the internal pH (Alavi and Cosson, 2005). Basically, these phenomena are associated with the membrane hyperpolarization and function of membrane channels. In addition, the membrane hyperpolarization depends on the changes in the Na^+, K^+, Ca^{2+} and H^+ concentrations of the ionic milieu (Boitano and Omoto, 1991; Takai and Morisawa, 1995; Krasznai et al., 1998), from which the presence of Ca^{2+} ions at a minimal concentration inside the spermatozoa was shown to be an indispensable condition for motility initiation and progression in all fish species (Tanimoto and Morisawa, 1988).

After the motility activation, the osmotic shock will also continuously damage the cell during the following tens of seconds to several minutes. This period the spermatozoon should move in an environment that is constantly moving and changing and find the egg with the only possible site to enter, the micropyle, which is a diminutive opening with a diameter of only a few micrometers (Jamieson, 1991).

It was found, that the spermatozoon membranes of the externally fertilizing animals (from which the marine invertebrates are mostly studied to date) possess complex of receptors which react to the presence of particular substances and initiate the cascade of intracellular responses resolving into changes in motility traits and trajectory (Cardullo et al., 1994; Kaupp et al., 2006; Hirohashi et al., 2013) – the phenomenon generally defined as chemotaxis. The latter together with rheotaxis and thermotaxis (the ability of spermatozoon to react on the flow and temperature) are believed to contribute to the success of fertilization (Kaupp and Strünker, 2017). It is thus highly likely, that aforesaid external selective stresses determine species fitness within natural habitats.

Polyandry is advantageous for the reproduction success since it allows to choose the best sires for the offspring basing on sperm competition among two or more males to fertilize a limited amount of ova (Parker, 1998; Simmons and Fitzpatrick, 2012); moreover, this phenomenon was believed to be one of the important driving forces of spermatozoon evolution, in particular, the appearance of a "tiny" spermatozoa "acting" in huge numbers (Ball and Parker, 1996). This idea was based mainly on the hypothesis of so-called fair raffle when the success of a particular male depends only on a number of spermatozoa it could provide to the "fertilization lottery", and thus the relative "investment" of a male in every single gamete may be low (Parker, 1993). The tiny sperm hypothesis was not fully confirmed in comparative studies, because the sperm size was found to be highly labile across the taxa (Pitnick et al., 2009). Later Eisenbach and Giojalas (2006) proposed a guidance hypothesis, which associated the reproductive success with the existence of various sensing mechanisms in a spermatozoon to gather physical or chemical cues to spot the egg. And finally, not a few experimental findings allowed to hypothesize the existence of post-copulatory sexual selection mechanism, so-called "cryptic female choice", which is supposed to promote the sperm of genetically preferable males to encounter the eggs (Firman et al., 2017). In the externally fertilizing animals this female control may be provided "chemically" through the substances in the ovarian fluid, egg jelly or released by the egg itself, or "physically" through the specific structures on the egg surface (and/or the shape of micropyle) (Cosson, 2015).

Several attempts were made to support these ideas in fish. It was shown in ocellated wrasse *Symphodus ocellatus*, that presence of female ovarian fluid enhanced sperm velocity, motility, straightness, and chemo-attraction in certain males and in this way affecting the paternity (Alonzo et al., 2016). It was also found that ovarian fluid could not only improve some features of specific sperm cells but also inhibit the performance of the "unwanted" ones. In particular, the study in internally fertilizing guppies *Poecilia reticulata* showed that ovarian fluid may slow down the male gametes when mating with sisters occurs as compared with the features of unrelated male gametes (Gasparini and Pilastro, 2011). A recent attempt was made to analyze the existing data on the effect of ovarian fluid on sperm swimming performance in marine and freshwater teleosts (Myers et al., 2020): it was concluded that sperm velocity was enhanced by the presence of female ovarian fluid in the analyzed freshwater fish species, and such significant relationship was not found for marine fishes. The authors concluded as well that the spawning environment significantly affects the "power" of the observed effects, and importantly, that the effects varied depending on the fish phylogeny. However, this analysis was mainly based on salmonids and more data are needed to support the found relationships, especially taking into account the big variability in the existing fish spermatozoa motility traits, which may be caused *e.g.* by cryptic female choice. The opinion about the existence of ovarian fluid mediated selection of sperm is not common. Lahnsteiner (2002) showed no changes in sperm motility of brown trout *S. trutta* f. *fario* if the ovarian fluid from different batches was used. Thus, the effect of ovarian fluid and other egg-associated substances on freshwater fish spermatozoon performance and fertilization outcome is still an issue to explore.

Spawning strategies and egg-sperm interaction conditions

Externally fertilizing freshwater fish expel the gametes into a hypotonic aqueous environment. This similarity nevertheless does not entail the resemblance in their reproduction (spawning) strategies. Several species spawn in pairs (*e.g. Salmo trutta*), but most fish are polyandrous, with variations like a presence of smaller subordinate males which join/follow the established pair (*e.g. Hucho hucho* or *Oncorhynchus mykiss*), or the case when one female is courted by several males, which release the sperm simultaneously in the area around egg batch (*e.g. Cyprinus carpio)*; or finally, the group spawning, when each male in the group fertilize the eggs of many females (*Perca fluviatilis*) (Stockley et al., 1997). Very important differences include a quite diverse environment of spawning: this could be stream or shallow still-water (*e.g.* in *O. mykiss* and *C. carpio*, correspondingly), the eggs could be laid into built by male nests like in *Gasterosteus aculeatus*, onto grass substrates like in *Sander lucioperca* or *C. carpio* or fertilized in free water like in *Hypophthalmichthys molitrix*. All these different reproduction (spawning) strategies vary across taxa and are appeared during evolution. They are likely affecting the "scenery" of the fertilization process and may be interconnected with the mechanisms of gamete collision.

The present thesis represents the study of effects associated with ovarian fluid (and egg-derived substances) on fertilization performance in three freshwater species which are taxonomically distant: rainbow trout *O. mykiss*, order Salmoniformes (**Chapter 2**), sterlet *A. ruthenus*, order Acipenseriformes (**Chapter 3**), and common carp *C. carpio*, order Cypriniformes (**Chapter 4**), and discusses the interconnections between the features of sperm-egg interaction and peculiarities of the reproduction environment as well as differences in reproductive (spawning) strategies.

It was mentioned above, that many fish species differ in their spawning strategies, our chosen fishes are not the exclusion. Like most salmonids, the rainbow trout spawns in streams with a gravel bottom. The female digs a nest in the stones and release the eggs with ovarian fluid into it. The male accompanies her during the process and expels the sperm onto the egg batch. After the act, the female covers the nest with gravel. It is quite common, that the spawning pair will be joined by subordinate sneaker male(s). The sturgeons, sterlet in particular, also prefer the streaming waters with stone or gravel bottom, however, they do not build any nests. The females are escorted by several males and release the portions of eggs covered by viscous ovarian fluid onto or between the stones. The males apply the sperm onto egg batches and finally, the eggs densely stick onto the stones. The carps are spawning in shallow water, like ponds, small lakes, or quiet rivers rich in water plants. Mostly the spawning act happens in big groups, where one or more females are followed by several males. The released eggs are intensively mixed with the water and ejaculated sperm and stick to the plants.

The differences are very prominent both in the appearance of expelled egg batches as well as in an amount and viscosity of released ovarian fluid (Table 5.1.), *e.g.* typical egg batch of *O. mykiss* contains around 30% of low viscous ovarian fluid and several thousands of eggs with 5–6 mm size; *C. carpio* eggs are much smaller (around 1 mm) and the batch contains hundreds of thousands of eggs and only scarce amount of viscous ovarian fluid; sterlets *A. ruthenus* expel 2–3 mm big eggs with plenty of ovarian fluid, containing proteinaceous fibers. The osmolarity of ovarian fluid in rainbow trout is very close to "classic" isosmotic point and lower in sterlet. The lowest content of total proteins is in the rainbow trout ovarian fluid. The pH value is similar in all three species, as well as the calcium content. Potassium ion content varies from 2.2 mmol/l in trout to 5.7 mmol/l in sterlet.

Table 5.1. *Basic characteristics of the ovarian fluid in rainbow trout, sterlet, and common carp.*

Index	Rainbow trout	Sterlet	Common carp
Osmolarity, mOsmol/l	299.1 ± 3.7	200.8 ± 17.02	268.0 ± 20.43
pH	8.3 ± 0.15	8.18 ± 0.16	8.52 ± 0.24
Protein content, mg/ml	1.16 ± 0.11	1.70 ± 0.52	2.24 ± 0.06
[Ca^{2+}], mmol/l	1.28 ± 0.28	1.13 ± 0.12	2.02 ± 0.20
[K^+], mmol/l	2.32 ± 0.17	7.43 ± 0.65	4.78 ± 0.14
Amount of ovarian fluid in the egg batch, %	10–30	5–10	1–3

Differences in reproductive (spawning) strategies are probably associated with the peculiarities in the spermatozoa activation mechanism in mentioned species (or *vice versa*), *e.g.* spermatozoa of rainbow trout can be activated in isotonic potassium depleted media, while carp and sterlet spermatozoa are activated only if the activation medium is hypoosmotic.

The spermatozoa performance in the presence of ovarian fluid differs in the studied fishes significantly.

Egg sperm interaction in rainbow trout, sterlet, and common carp

Rainbow trout

The spermatozoa of rainbow trout change their motility pattern in the presence of ovarian fluid: the more ovarian fluid was present in the activation medium, the straighter was the path of spermatozoa. In the water, most spermatozoa moved circularly. Interestingly, that we have observed specific motility patterns in the diluted ovarian fluid which was similar to the so-called explorative behavior of sperm cells, or "turn-and-run" pattern usually associated with an effect of chemotactic agent presence (Kaupp, 2012). These changes in motility pattern were similar, but not equal in the same osmotic conditions created by sodium chloride solutions. In particular, no turn-and-run movements were found in these conditions, and the motion of a significant part of cells in the isotonic medium was not straight or arc-like. Both ovarian fluid and isosmotic activation medium significantly increased the longevity of activated spermatozoa, but ovarian fluid did not increase the initial velocity (measured at 10 s) of the spermatozoa, unlike reported observation in Chinook salmon by Rosengrave et al. (2009) (see **Chapter 2** for details).

In the chemotaxis microinjection test (sperm accumulation test) the rainbow trout sperm were reacting by positive taxis to the area filled with ovarian fluid, and similarly in the area with isotonic saline. The cells, which entered these areas became trapped – then they reached the border with water, they changed the direction of motion performing the turns. Interestingly, the opposite behavior, a negative taxis, was observed if the cells, activated in an isotonic solution, met the injected hypotonic solution. The spermatozoa avoided this area by performing similar turn-and-run loops (details in **Chapter 2**). A similar effect was observed in squid spermatozoa during the pH-taxis (Iida et al., 2017).

Remarkable observations were made when studying the changes of intracellular calcium concentrations in these spermatozoa using fluorescent Fluo-4 dye: the asymmetric bend in the flagella of these cells showed a short and bright increase of calcium concentration during turn-and-run activity (in terms of flashes of fluorescence), while no such changes were found neither during straightforward motility (flagella bends symmetrically) nor during tumbling *e.g.* in the water (flagella bends asymmetrically). Such a specific pattern of calcium concentration changes during explorative motion in the gradients of attractants was described in the spermatozoa of sea invertebrates (Alvarez et al., 2012; Yoshida et al., 2018), but, to the best

of our knowledge, never in the fish spermatozoa. This fact allows us to suppose the similarity of chemotactic responses in a wider spectrum of the externally fertilizing animals than it was established before.

Sterlet

Sterlet spermatozoa performance is significantly different from the above described. Unlike the rainbow trout, and like in the majority of freshwater fish the activation of sterlet spermatozoa highly depends on the osmolarity of the environment. There is a certain osmotic limit which will block the activation of the male gametes. It was found as well, that the presence of ovarian fluid rendered additional inhibiting effect in the same osmotic conditions, which we have associated with dependence of sturgeon spermatozoa motility initiation on the potassium ion presence in the medium (Alavi et al., 2004). Unlike rainbow trout, no specific effect of the presence of ovarian fluid in the activation medium was observed on the trajectories of motile spermatozoa, as well as no effect in the trajectories was present in the activation media with various osmolarities. In terms of the velocity of sterlet spermatozoa, the ovarian fluid caused its decrease (see details in **Chapter 3**).

The conducted sperm accumulation microcapillary test also found bright differences in sterlet sperm cell behavior comparing to the rainbow trout. Injection of diluted ovarian fluid into the suspension of activated spermatozoa did not cause any chemotactic or trapping effect. Moreover, the introduction of non-diluted fluid caused a significant decrease of spermatozoon velocity and even full inhibition of the motility of the gametes entered the area with the ovarian fluid. Noteworthy, that injection of egg conditioned medium (distilled water after incubation of washed non-destroyed eggs) entailed the bright response of the spermatozoa. Characteristic explorative turn-and-run loops were observed commonly around the injected attractant area. The found effect may be associated with the effect of egg-released substances as molecules attracting the spermatozoa in sturgeons, which is similar to the case of Pacific herring *Clupea pallasii*, where the proven chemoattractant was a glycoprotein released from the micropyle area of egg chorion (Pillai et al., 1993). This is highly likely considering the reported fact, that incubation of sterlet eggs in the water is accompanied by the massive release of a handful of proteins, including some glycoproteins, into the surrounding medium (Niksirat et al., 2017).

Common carp

Like typical representative of freshwater fishes and similarly to sterlet the carp spermatozoa are normally non-motile in isosmotic conditions and transfer to lower osmotic conditions activates them. Nevertheless, unlike the sterlet case, there was no additional inhibiting effect of ovarian fluid on the activation in the same osmotic conditions. In the case of already activated spermatozoa, no additional effects of osmolarity were apparent in terms of motility patterns. Low concentrations of ovarian fluid did not affect the straightness of spermatozoa motility; however, if its concentration reached 50% of the activating medium, the cells significantly change their behavior, *i.e.* the spermatozoa moved in circles. This behavior was earlier reported as similar to hyper-activation in mammal spermatozoa (Cosson et al., 1999). This specific behavior was found to depend on the molecular weight of the substances in the ovarian fluid: only the low molecular substances from ovarian fluid caused the "tumbling" effect. Noteworthy, that the patterns of motility recorded in the buffer, that mimic the ionic composition of the ovarian fluid, did not change significantly compared to the other solutions tested (see **Chapter 4** for details), *i.e.* the low molecular fraction of the ovarian fluid contains other agents, which cause the tumbling of spermatozoa. This low sensitivity to osmotic changes is similar to the case of sterlet (except the high concentration of ovarian

fluid), where the male gametes were also non-sensitive to changes in medium osmolarity and adding of ovarian fluid. And *vice versa*, the dependence of motility patterns in rainbow trout was opposite: the trout sperm was moving in circles in water but straightforward in the medium with a high content of the ovarian fluid.

The kinetic characteristics of carp sperm motility were significantly changed in the presence of ovarian fluid: the more ovarian fluid was present in the activating medium, the slower moved the cells. This effect was not due to the changes in osmolarity of the medium, because no such changes were found in the saline media with osmotic conditions similar to the diluted ovarian fluid.

The sperm accumulation microcapillary test performed in carp revealed a strong attraction effect rendered by the ovarian fluid, which overcame this in rainbow trout. Like in the above cases of attraction in sterlet (with the egg-conditioned medium) and rainbow trout (ovarian fluid) a specific explorative behavior of carp spermatozoa approaching the region of interest was observed massively (see **Chapter 4** for details). Interestingly, that the attraction phenomenon depended on the presence of external calcium ions: the spermatozoa lost the ability to react on the attractant if the ionic calcium was absent in the activating medium (*i.e.* supplemented with EGTA). Like in the case of rainbow trout the signs of "osmotaxis" of spermatozoa were found: the spermatozoa activated in water were trapped in the area with higher osmolarity.

In vitro fertilization tests

The *in vitro* fertilization tests performed with rainbow trout, sterlet and common carp showed the significant protective effect of the ovarian fluid rendered both to the eggs and spermatozoa. In particular, sterlet eggs coated by ovarian fluid survived the stay in the water without significant changes in fertilizability for the period of the half of an hour, and the spermatozoa were able to fertilize the eggs in the presence of ovarian fluid up to 5 minutes post motility activation, instead of 2 minutes in case if the ovarian fluid was removed. Interestingly, that *in vitro* fertilization test in the rainbow trout performed with the gametes from parents belonging to different strains showed the enhancing of fertilization with spermatozoa from males if the ovarian fluid from females of the same strain was present.

Ovarian fluid and the spawning tactics in rainbow trout, sterlet and common carp

The conducted comparison allows us to suppose that response of the freshwater fish male gametes to the presence of ovarian fluid is species-specific and depends on the mode of fish reproductive behavior (their reproductive or spawning strategies) (Table 5.2). More specifically, the rainbow trout females expel the eggs together with plenty of ovarian fluid, and the spermatozoa introduced into this cloud start to move straightforward and significantly longer. They are kept inside these areas with ovarian fluid and abruptly change the direction of movement in case of approaching the border of these areas. In the case of sterlet, we suppose that because of sturgeon females lay several batches of eggs covered by viscous ovarian fluid which preserve them for a significant time, this 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 to navigate 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. Common carp spermatozoa effectively follow the ovarian fluid concentration gradient, change the linear mode of motility

to explorative one, and even low concentrations of ovarian fluid prolong the activity period of spermatozoa; which altogether may be an efficient strategy in case of free submerging egg coated by a thin viscous layer of ovarian fluid.

Table 5.2. *Comparison of the effects of ovarian fluid rendered on spermatozoa performance in rainbow trout, sterlet, and common carp.*

Rainbow trout "The Kicker" OF	Sterlet "The Hugger" OF	Common carp "The Catcher" OF
Ovarian fluid enhances the spermatozoa motility: the cells move longer and more straightforward;	Ovarian fluid inhibits the spermatozoa motility. Arrested spermatozoa could be repeatedly activated after ovarian fluid dilution;	Ovarian fluid completely prevent spermatozoa motility;
The ovarian fluid has a trapping effect on spermatozoa;	Presence of a small amount of ovarian fluid prolongs the spermatozoa activity;	In diluted by water OF velocity of spermatozoa is significantly reduced, the cells move longer; and in circles.
Rainbow trout spermatozoa respond to osmotrapping;	The ovarian fluid has no chemotactic-like effects on spermatozoa;	The ovarian fluid has a trapping effect on spermatozoa;
Low molecular substances (including calcium ions) are mainly responsible for the effects of ovarian fluid on spermatozoa;	The low molecular agents released by eggs to the water attract spermatozoa;	Carp spermatozoa respond to osmotrapping;
Ovarian fluid saves the eggs while being in water;	Ovarian fluid saves the eggs while being in water;	Calcium ions trigger the ability of cells to respond to attractants;
The presence of ovarian fluid improves the outcome of fertilization.	The presence of ovarian fluid improves the outcome of fertilization.	The ovarian fluid causes adhesion of male gametes affected by low osmolarity;
		Low molecular substances are mainly responsible for the effects of ovarian fluid on spermatozoa.

The found peculiarities in the characteristics of the ovarian fluid and its interaction with the freshwater fish sperm are also in good concordance with already mentioned differences in characteristics of the preferred spawning sites. In particular, the amount of the ovarian fluid in rheophilic fishes like rainbow trout may be abundant to compensate the loss of the fluid due to the water flow and to have enough volume to "trap" the spermatozoa. And *vice versa*, in the case of common carp reproducing in the still shallow water, there is no need for an excess amount of expelled maternal fluid to fulfill its tasks.

Anyway, these findings suppose the existence of an amazing framework integrating the characteristics of the egg (ovarian fluid) – sperm encounter, its associations with fish spawning tactics, the taxonomic position of the species, and thus their evolutionary genesis. This construct was highly likely effective during the evolutionary change in the reproduction environment from internal fluids for the external water (the predecessors of the modern fishes are believed to be internal fertilizers (Long et al., 2009)). The external media obviously have the conditions being far from physiological and that is why numerous adaptations in the structure and function of fish gametes appeared to counterbalance the negative impact of external media, *e.g.* micropyle or simplified "aquasperm" structure (Jamieson 1991). And, that is important in terms of the present thesis, the externally fertilizing fishes produce the ovarian fluid which accompanies the expelled eggs and creates an optimal environment for the gametes' encounters in many fish species. The ovarian fluid thus became a part of versatile reproductive (spawning) tactics and allowed them to use efficiently sperm competition tactics and prevent fertilization by inappropriate male gametes. The development of these tactics involved the sensitivity of spermatozoa to different conditions of the environment, *i.e.* may be controlled with the features of the ovarian fluid.

Finally, the case of freshwater fish reproduction studied in this thesis can't be considered apart from sexual reproduction in general. Basically, the spermatozoon should be transferred from a male as close as possible to the oocyte, and the rest of the process is "just" an adaptation to the different environments. These adaptations were used during evolution by different species creating reproduction tactics and strategies accompanied by different spermatozoon behavior. The evolutionary challenge of studied here freshwater fish species was to accommodate the time-limited fertilization occurring out of the parents' bodies. The latter allows following the whole journey of spermatozoa to the egg in the various conditions, including virtually real ones, and, that is most important, to study the process of fertilization from different perspectives, making the conclusions about particular processes, which after all are the part of the universal system of sexual reproduction.

Conclusions of the study

Collectively, the following specific conclusions are made:
- The eggs of many externally fertilizing freshwater fish species are released into the external milieu surrounded by a coat of ovarian fluid with a composition supporting and protecting eggs and sperm against the deleterious effect of freshwater. The specific compounds contained in ovarian or released by eggs can significantly affect the behavior of male gametes and consequently influence the outcome of fertilization. The mechanisms which facilitate and trigger gametes' encounter are highly likely involved in natural selection (Chapter 1).
- The ovarian fluid saves the eggs while being in the water, prolongs the spermatozoa motility, and affects their performance in rainbow trout, sterlet and common carp, which is reflected in the enhancement of *in vitro* fertilization (Chapters 2, 3, 4).
- The way how the ovarian fluid affects the behavior of spermatozoa in rainbow trout, sterlet and common carp differ in these species and may be associated with their reproduction (spawning) strategy.
- The environmental conditions which accompany the encounter of gametes, in particular calcium ion content and osmolarity, significantly affect the performance of male gametes in spermatozoa in rainbow trout, sterlet and common carp in terms of changes in velocity, path linearity and ability to respond to the external signals, *e.g.* attractants.

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Sperm/egg interaction in freshwater fish: influence of environment on fertilization process

Vitaliy Kholodnyy

Fertilization of freshwater fish occurs in an environment which may affect negatively the gametes, therefore the fish male gametes should reach their counterpart, the female gamete, as soon as possible because spermatozoa become damaged within minutes or less due to osmotic shock. The existence of specific mechanisms triggering, supporting, and guiding the encounter of gametes would be highly expedient in these conditions. The eggs of many externally fertilizing freshwater fish species are released into the external milieu surrounded by a coat of ovarian fluid (OF) with a composition ideal for supporting and protecting eggs and sperm against the deleterious effect of freshwater. The existing data support the idea that the properties of OF and/or the specific compounds contained in it or released by the eggs could significantly affect the behavior of male gametes and consequently influence the outcome of fertilization in terms of the number of fertilized oocytes. The mechanisms which facilitate and trigger gametes' encounter are also highly demanded in terms of natural selection.

It was found that the presence of OF affected significantly the behavior of rainbow trout spermatozoa, in particular, their motility traits: higher velocity was supported for longer time and trajectories were straightened, comparing to those observed in water. In the microcapillary spermatozoon accumulation test (test of chemotactic response) the rainbow trout OF showed a trapping effect on activated male gametes which depended on osmotic properties of the activating media. Different molecular weight fractions from OF affected the tactic behavior of the cells in various ways. The most significant trapping effect was rendered by a low molecular fraction and the possible chemotactic agent was found to be thermostable. The trapped cells showed specific turn-and-run behavior accompanied by asymmetric bending of flagella and a burst-like increase of calcium concentration in the bent area. The *in vitro* fertilization test revealed the enhancement of spermatozoa performance, especially in the samples from the related individuals, which led to the higher embryo development rate.

The presence of a particular concentration of ovarian fluid (30% solution in water) had an inhibiting effect on sterlet spermatozoa motility initiation. Lower concentrations of the ovarian fluid improved the longevity of spermatozoa and did not affect their trajectories. Test of chemotactic response showed no effect of ovarian fluid on spermatozoa behavior, while at the same time the attracting effect of egg conditioned medium was evident (*i.e.* due to some substances released from the eggs during their contact with freshwater). The results of *in vitro* 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 for a longer period.

The presence of common carp ovarian fluid in the activation medium caused the decrease of the velocity of spermatozoa comparing to the OF-free medium and significantly altered the motility pattern from straightforward motility observed in the water to the tumbling in the medium with high OF content (50%). Introduction of OF (in the sperm accumulation test) entailed immediate and prominent chemotactic-like reaction of spermatozoa.

The environmental conditions which accompany the encounter of gametes, in particular presence of OF, calcium ion content and osmolarity, significantly affect the performance of male gametes in spermatozoa in rainbow trout, sterlet, and common carp in terms of changes in velocity, path linearity and ability to respond to the external signals, *e.g.* attractants. The conducted study allowed us to conclude that way how the ovarian fluid affects the behavior of spermatozoa in these species may be associated with their reproduction (spawning) strategy.

Czech summary

Interakce spermií a jiker u sladkovodních ryb: vliv prostředí na fertilizační proces

Vitaliy Kholodnyy

K oplození u sladkovodních ryb dochází v prostředí, které může negativně ovlivnit gamety. Z tohoto důvodu by se samčí gamety ryb měly co nejdříve dostat ke svému protějšku, samičí gametě, protože sperma se během osmotického šoku poškodí během několika minut nebo méně. Za těchto podmínek by bylo velmi výhodné, kdyby existoval specifický mechanizmus, který by spouštěl, podporoval a řídil vzájemné setkání obou gamet. Jikry mnoha druhů sladkovodních ryb s vnějším oplozením jsou uvolňovány do vnějšího prostředí obklopené pláštěm ovariální tekutiny (OT) se složením ideálním pro podporu a ochranu jiker a spermatu před škodlivým účinkem sladké vody. Stávající data podporují myšlenku, že vlastnosti OT a/ nebo specifických sloučenin obsažených v ní nebo uvolněných jikrami by mohly významně ovlivnit chování samčích gamet a následně ovlivnit výsledek oplodnění z hlediska počtu oplodněných oocytů. Mechanizmy, které by usnadňovaly a spouštěly setkání gamet, jsou také velmi žádoucí, pokud jde o přirozený výběr.

Zjistili jsme, že přítomnost OT významně ovlivnila chování spermií pstruha duhového, zejména znaky pohyblivosti spermií: vyšší rychlost byla udržována po delší dobu a trajektorie spermatu byly lineární, ve srovnání s těmi pozorovanými ve vodě. V testu akumulace spermií s mikro kapilárami (test chemotaktické reakce) ovariální tekutina pstruha duhového vykazovala „přitažlivý" účinek na aktivované samčí gamety, který závisel na osmotických vlastnostech aktivačního média. Různé frakce o různé molekulové hmotnosti OT ovlivňovaly „přitažlivé" chování buněk různým způsobem. Nejvýznamnější účinek zachytávání byl odhalen u nízkomolekulární frakce a bylo zjištěno, že možné chemotaktické činidlo je termostabilní. Zachycené buňky vykazovaly specifické chování tzv. *turn-and-run*, jež je doprovázené asymetrickým pohybem bičíku a výbušným zvýšením koncentrace iontů vápníku v ohýbané části bičíku. *In vitro* fertilizační test odhalil zvýšení výkonu spermií, zejména ve vzorcích od příbuzných jedinců, což vedlo ke zvýšení míry vývoje embryí.

Přítomnost ovariální tekutiny o zředěné koncentraci (30% roztok ve vodě) měla inhibiční účinek na iniciaci motility u jesetera malého. Nižší koncentrace ovariální tekutiny zvýšila životnost spermií a neovlivnila jejich trajektorie. Test chemotaktické odpovědi neprokázal žádný účinek ovariální tekutiny na chování spermií, zatímco současně byl patrný přitažlivý účinek roztoku inkubovaného jikrami (např. kvůli některým látkám uvolňovaných z jiker během jejich kontaktu se sladkou vodou). Výsledky *in vitro* fertilizačního testu ukázaly, že přítomnost ovariální tekutiny brání jikrám ve ztrátě fertilizační schopnosti v důsledku kontaktu s vodou, a také podporuje spermie, aby mohly oplodnit jikry během delší doby.

Přítomnost ovariální tekutiny u kapra obecného v aktivačním médiu způsobila snížení rychlosti spermií ve srovnání s médiem prostým OT a významně změnila vzorec pohybu z přímé motility pozorované ve vodě na krouživý pohyb v médiu s vysokým obsahem OT (50%). Vpichování OT (v testu akumulace spermií) znamenalo okamžitou a výraznou chemotaktickou reakci spermií.

Okolní podmínky, které doprovázejí setkání gamet, zejména přítomnost OT, obsah iontů vápníku a osmolarita, významně ovlivňují výkonnost samčích gamet u pstruha duhového, jesetera malého a kapra obecného, pokud jde o změny rychlosti, linearitu trajektorie a schopnosti reagovat na externí signály, např. atraktanty. Díky této provedené studii můžeme usuzovat, že způsob, jakým ovariální tekutina ovlivňuje chování spermií u těchto druhů, může být spojen s jejich reprodukční (výtěrovou) strategií.

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List of publications

Peer-reviewed journals with IF

Kholodnyy, V., Dzyuba, B., Gadêlha, H., Cosson, J., Boryshpolets, S., 2020. Egg-sperm interaction in sturgeon: role of ovarian fluid. Fish Physiology and Biochemistry. doi: 10.1007/s10695-020-00852-2 (in press). (IF 2019 = 2.242)

Kholodnyy, V., Gadêlha, H., Cosson, J., Boryshpolets, S., 2020. How do freshwater fish sperm find the egg? The physicochemical factors guiding the gamete encounters of externally fertilizing freshwater fish. Reviews in Aquaculture 12: 1165–1192. (IF 2019 = 7.772)

Yániz, J., Alquézar-Baeta, C., Yagüe-Martínez, J., Alastruey-Benedé, J., Palacín, I., Boryshpolets, S., **Kholodnyy, V.**, Gadêlha, H., Pérez-Pe, R., 2020 Expanding the limits of computer-assisted sperm analysis through the development of open software. Biology 9: 207. (IF 2019 = 3.796)

Dzyuba, V., Shelton, W.L., **Kholodnyy, V.**, Boryshpolets, S., Cosson, J., Dzyuba, B., 2019. Fish sperm biology in relation to urogenital system structure. Theriogenology 132: 153–163. (IF 2018 = 2.299)

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Xin, M., Sterba, J., Shaliutina-Kolesova, A., Dzyuba, B., Lieskovska, J., Boryshpolets, S., Siddique, M., **Kholodnyy, V**., Lebeda, I., Linhart, O., 2018. Protective role of antifreeze proteins on sterlet (*Acipenser ruthenus*) sperm during cryopreservation. Fish Physiology and Biochemistry 44: 1527–1533. (IF 2017 = 1.735)

Xin, M., Tučková, V., Rodina, M., **Kholodnyy, V.,** Dadras, H., Boryshpolets, S., Shaliutina-Kolešová, A., Linhart, O., 2018. Effects of antifreeze proteins on cryopreserved sterlet (*Acipenser ruthenus*) sperm motility variables and fertilization capacity. Animal Reproduction Science 196: 143–149. (IF 2017= 1.647)

Book chapters

Boryshpolets, S., **Kholodnyy, V.**, Cosson, J., Dzyuba, B., 2020. Fish Sperm Quality Evaluation After Cryopreservation. In: Betsy, J., Kumar S. (Eds), Cryopreservation of Fish Gametes. Springer Nature, 117–133.

Kholodnyy, V., Boryshpolets, S., Cosson, J., Dzyuba, B., 2020. Energetics of Fish Spermatozoa. In: Betsy, J., Kumar S. (Eds), Cryopreservation of Fish Gametes. Springer Nature, 69–116.

Abstracts and conference proceedings

Boryshpolets, S., **Kholodnyy, V.**, Gadêlha, H., Cosson, J., 2019. Guidance and selection during fertilization in fresh water fish: theory and practice. 7th International Workshop on the Biology of Fish Gametes, September 2–6, 2019, Rennes, France

Dzyuba, B., Dzyuba, V., Ninhaus-Silveira, A., Kahanec, M., Verissimo-Silveira, R., **Kholodnyy, V.**, Rodina, M., Boryshpolets, S., 2019. Steps towards understanding motility of morphologically complex sperm in cartilaginous fishes. In: Book of abstracts of 7th International Workshop on the Biology of Fish Gametes, September 2–6, 2019, Rennes, France.

Herrera, F., Bondarenko, O., Dzyuba, B., **Kholodnyy, V**., Boryshpolets, S., 2019. Osmoregulation in fish spermatozoa: involvement in motility activation and impact on short-term storage outcomes. 7th International Workshop on the Biology of Fish Gametes, September 2–6, 2019, Rennes, France.

Kholodnyy, V., Cosson, J., Boryshpolets, S., 2019. Does the rainbow trout ovarian fluid navigate the sperm on its way to the egg? 688. WE-Heraeus-Seminar: Physics and Physiology of Motile Cilia. January 27–30, 2019. Physikzentrum Bad Honnef, Germany.

Kholodnyy, V., Dzyuba, B., Gadêlha, H., Cosson, J., Boryshpolets, S., 2019. Egg-sperm interaction in sturgeon: role of ovarian fluid. 7th International Workshop on the Biology of Fish Gametes, September 2–6, 2019, Rennes, France.

Boryshpolets, S., **Kholodnyy, V**., Gadelha, H., Cosson, J., 2018. Gametes collision in fresh water fish: evidences of guidance and selection. XIII International Symposium on Spermatology. Stockholm, Sweden. May 9–13, 2018.

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Kholodnyy, V., Cosson J., Boryshpolets, S., 2017. Does ovarian fluid affect the fertilization in freshwater fish? 6th International Workshop on the Biology of Fish Gametes, September 4–7, 2017, Vodnany (Ceske Budejovice), Czech Republic.

Xin, M., Shaliutina-Kolešová, A., Siddique, M.A.M., Štěrba, J., Dzyuba, B., Boryshpolets, S., **Kholodnyy, V.,** Linhart, O., Ping, L., 2017. Protective role of antifreeze proteins during cryopreservation of sterlet (*Acipenser ruthenus*) spermatozoa. 6th International Workshop on the Biology of Fish Gametes, September 4–7, 2017, Vodnany (Ceske Budejovice), Czech Republic.

Training and supervision plan during study

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Ph.D. courses	Year
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Applied hydrobiology	2017
Fish reproduction	2018
Ichthyology and fish taxonomy	2018
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Scientific seminars	Year
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International conferences	Year
Kholodnyy, V., Cosson, J., Boryshpolets, S. Does ovarian fluid affect the fertilization in freshwater fish? In: Book of abstracts of 6th International Workshop on the Biology of Fish Gametes, September 4–7, 2017, Vodnany (Ceske Budejovice), Czech Republic, p. 27.	2017
Kholodnyy, V., Cosson, J., Boryshpolets, S. Chemotactic and chemokinetic properties of rainbow trout ovarian fluid. XIII International Symposium on Spermatology. May 9–13, 2018. Stockholm, Sweden	2018
Kholodnyy, V., Cosson, J., Boryshpolets, S. Does the rainbow trout ovarian fluid navigate the sperm on its way to the egg? 688. WE-Heraeus-Seminar: Physics and Physiology of Motile Cilia. January 27–30, 2019. Physikzentrum Bad Honnef, Germany. (Poster presentation)	2019
Kholodnyy, V., Dzyuba, B., Gadêlha, H., Cosson, J., Boryshpolets, S. Egg-sperm interaction in sturgeon: role of ovarian fluid. 7th International Workshop on the Biology of Fish Gametes, September 2–6, 2019. Rennes, France (Oral presentation)	2019

Foreign stays during Ph.D. study at RIFCH and FFPW	Year
Prof. Manabu Yoshida, Misaki Marine Biological Station, Tokyo University, Japan (2 months, Studying the Calcium fluorescent imaging in motile sperm cells)	2018

Pedagogical activities	Year
Leading of project entitled *Effects of ovarian fluids on fish sperm motility* at International Summer School at USB FFPW	2018
Leading of project entitled *Effects of environment on spermatozoa motility in freshwater fish* at International Summer School at USB FFPW	2019
Consultations concerning 1 bachelor and 1 doctoral study	2019
Laboratory training and practice leadership in new students and visiting students at USB FFPW in range of 76 teaching hours	2016–2019
Providing excursions at the Faculty Open Day during Vodňanské rybářské dny in range of 15 teaching hours	2017–2019

Curriculum vitae

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EDUCATION

2016 – present Ph.D., specialization Fishery; Faculty of Fisheries and Protection of Waters, University of South Bohemia, Ceske Budejovice, Czech Republic

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