REVIEWS IN Aquaculture

Reviews in Aquaculture (2020) **12**, 1165–1192 doi: 10.1111/raq.12378

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

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Received 5 March 2019; accepted 22 July 2019.

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-

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 predominately 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

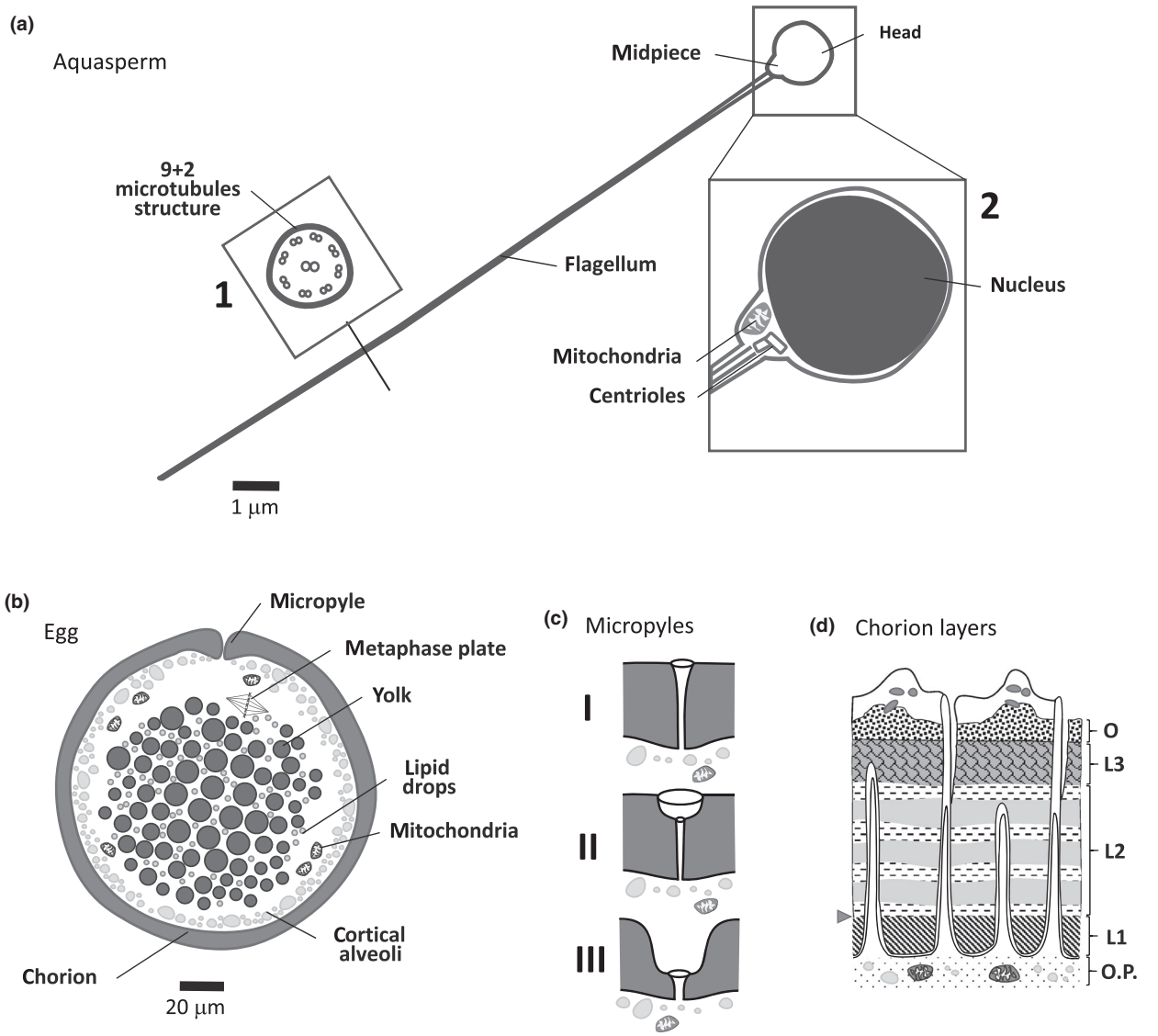

Figure 1 Schematic representation of typical gametes from externally fertilizing fish. (a) Carp spermatozoa (according to electronic micrographs by Verma *et al*. (2009)); cross section of flagellum (1) shows 9 + 2 microtubular structure covered with membrane; head and midpiece (2) contain large rounded 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

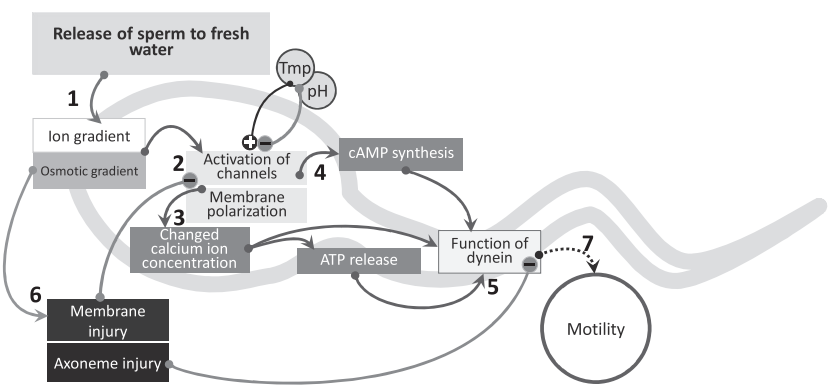

Figure 2 Motility activation and its support in fresh water fish spermatozoa (simplified scheme, details in the text): 1. Release of spermatozoa to the fresh water results in appearance of osmotic/ionic gradient on plasma membrane. 2. The gradient allows the activity of membrane channels and membrane 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.

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 fish (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

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, so-called '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.

Table 1 Ions in ovarian fluid of some fish species

Species	Osmolality, mmol kg^{-1}	Ion content (mM)					pH	Reference
		Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-		
Oncorhyncus 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	
Oncorhyncus mykiss	291.6 ± 12.9	134.7 ± 7.4	2.7 ± 0.2	0.45 ± 0.04			8.4 ± 0.1	
Oncorhyncus 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 ± 25246 ± 26314 ± 47	136 ± 12150 ± 11203 ± 30	2.12 ± 1.323.53 ± 1.723.67 ± 0.90	1.58 ± 0.340.16 ± 0.322.64 ± 0.71		102 ± 18136 ± 7171 ± 36		Elofsson *et al*. (2006)
Ctenopharyngodon idella		741.0	0.45	6.38	2.58		8.1	Linhart *et al*. (1995)
Carassius gibelio (April)		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	237.0 ± 27.12	171.58 ± 25.83	2.93 ± 0.57	0.63 ± 0.11			8.61 ± 0.10	Lahnsteiner *et al*. (1997)
Acipenser ruthenus	190.0 ± 6.28	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	208.43 ± 9.20	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	213.50 ± 8.03	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).

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-NH_2	Egg jelly	Peptide	1245	Activation, attraction	Ward *et al*. (1985)
Cuttlefish						
Sepia officinalis	SepSAP (SAP, sperm-activating peptide) Pro–Ile–Asp–Pro–Gly–Val–$CONH_2$	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

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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

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 manhole-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).

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 priory*, 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

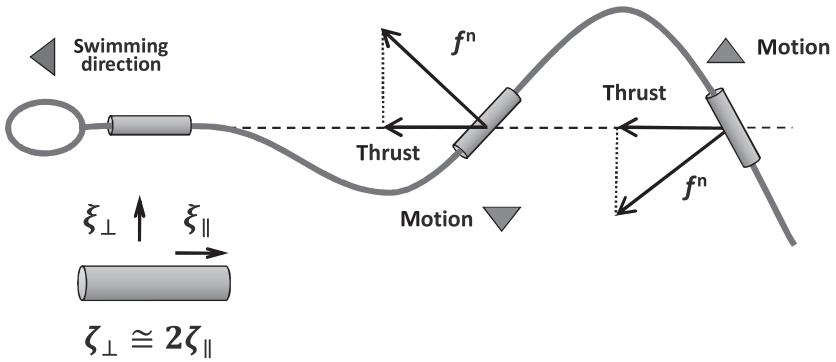

Figure 3 Resistive force theory based on the hydrodynamic forces experienced by motile spermatozoa as a sum of local contribution of small 'rod-like' sections along the flagellum. The drag for perpendicular to the flagellum, f^n, and contributes to the total thrust in the swimming direction. The drag 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 bio-filaments 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

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

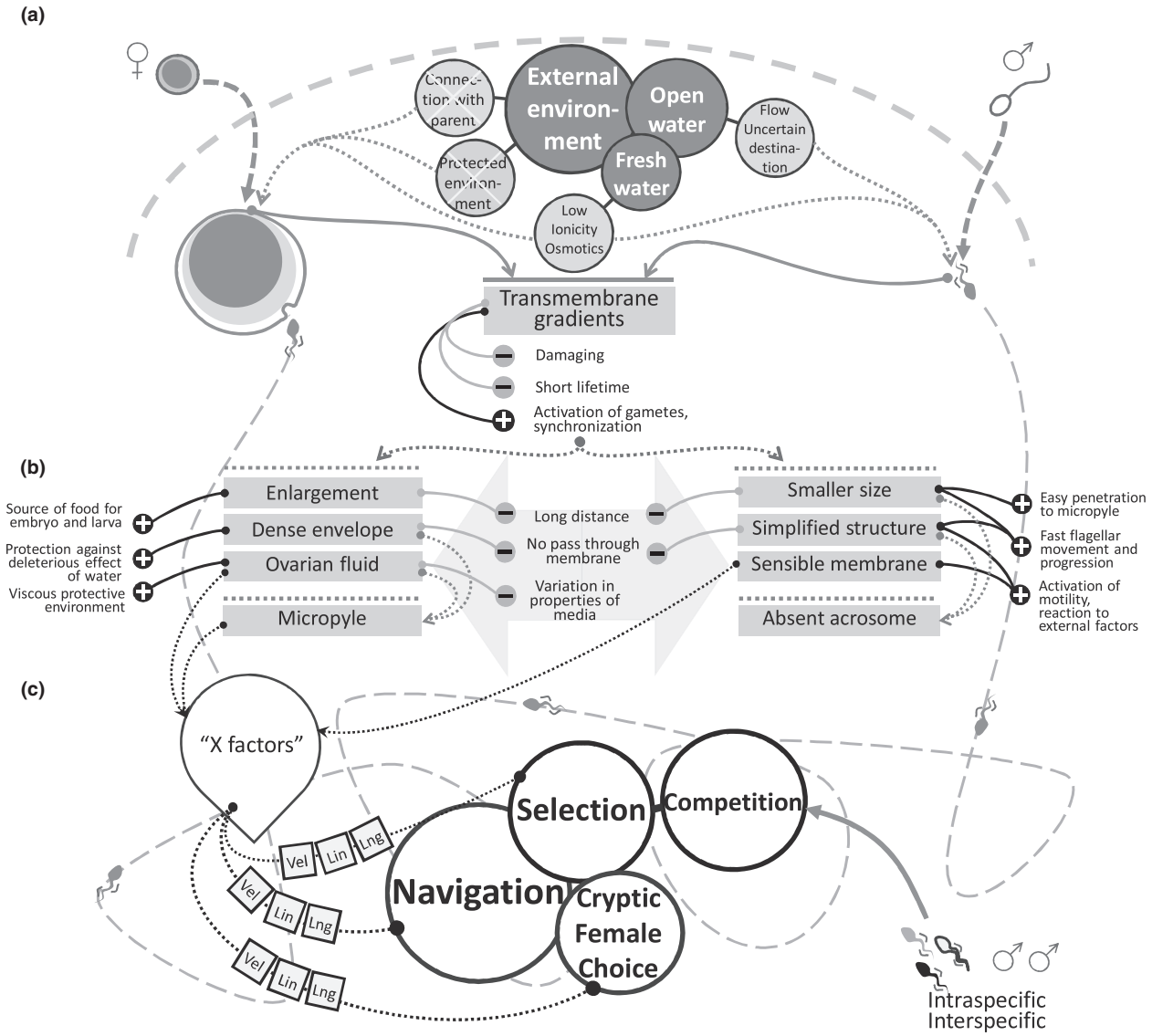

Figure 4 Hypothetic scheme of sperm guidance/selection in freshwater fish. (a) Due to evolution, the water medium outside the parental organism became the place where gametes meet, and where the resulting new organism developed. After being released into an external environment, the male and female gametes 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.

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 osmolarity, 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

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects 'CENAKVA' (LM2018099), by project Biodiversity (CZ.02.1.01./0.0/0.0/16_025/0007370 Reproductive and genetic procedures for preserving fish biodiversity and aquaculture), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (125/2016/Z and 013/2018/Z) and by the Czech Science Foundation (18-12465Y).

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