Chapter

# Fish Sperm Physiology: Structure, Factors Regulating Motility, and Motility Evaluation

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## Abstract

For reproduction, most fish species adopt external fertilization: their spermatozoa are delivered in the external milieu (marine- or freshwater) that represents both a drastic environment and a source of signals that control the motility function. This chapter is an updated overview of the signaling pathways going from external signals such as osmolarity and ionic concentration and their membrane reception to their transduction through the membrane and their final reception at the flagellar axoneme level. Additional factors such as energy management will be addressed as they constitute a limiting factor of the motility period of fish spermatozoa. Modern technologies used nowadays for quantitative description of fish sperm flagella in movement will be briefly described as they are more and more needed for prediction of the quality of sperm used for artificial propagation of many fish species used in aquaculture. The chapter will present some applications of these technologies and the information to which they allow access in some aquaculture species.

**Keywords:** flagellum, sperm energetics, sperm signaling, sperm motility, osmolarity

## 1. Introduction

The main function of a spermatozoon is to convey the male genome remotely to the female one, which occurs in case of fish by swimming in the external milieu, marine or freshwater. Spermatozoa must access, bind, and penetrate an egg, for successful fertilization. Therefore, most of the physiological activity of fish spermatozoa is motility oriented. These processes include as a prerequisite the activation of spermatozoon motility. In case of fish species, spermatozoa stored in the seminal plasma are immotile during transit through the genital tract of most externally fertilizing teleost and chondrostean. Motility is induced immediately following the release of spermatozoa from the male genital tract into the aqueous environment. External trigger agents for the initiation of motility depend on the species' reproductive behavior that is mostly controlled by the aquatic environment (fresh or salt water). Triggering signals include osmotic pressure, ionic and gaseous components of the external media, and, in some cases, egg-derived substances used for sperm guidance. Environmental factors influencing fish spermatozoon motility have received a large attention: these extensive studies led to several mechanisms of activation for freshwater and marine fish spermatozoa. However, after reception of the signal, a transduction pathway initiated by these mechanisms must lead the information to the flagellar motility apparatus (axoneme). This review presents

the current knowledge with respect to (1) membrane reception of the activation signal and its transduction through the spermatozoon plasma membrane via the external membrane components such as ion channels or aquaporins; (2) cytoplasmic trafficking of the activation signal; (3) final steps of the signaling, including signal transduction to the axonemal machinery, and activation of axonemal dynein motors and regulation of their activity; (4) signaling involved in guidance processes that control the sperm/egg approach and meeting; and (5) pathways supplying energy for the short flagellar motility period of fish spermatozoa. For each step in this signaling process, quantitative methods were developed to evaluate the quality of the sperm samples that are used for aquaculture propagation of many fish species. These methods as well as examples of their usefulness for application to fish artificial reproduction are presented in the last part of this chapter.

## 2. Fish sperm structure and spermatogenesis

The main traits of the flagellar mechanics of spermatozoa and the main factors that regulate their motility have been described in details in a recent review chapter by Cosson et al. [1].

### 2.1 Structure of fish spermatozoa: a brief presentation

Compared to mammalian spermatozoa, the structure of a fish spermatozoon is qualified as "simple sperm", mostly because the flagellum structure does not include additional columns flanking the motor part (axoneme) that are present in mammal sperm. In teleost fish, spermatozoa generally have no acrosome (in contrast to chondrostean such as sturgeon), and the impenetrable chorion presents a micropyle that gives access to the membrane of the oocyte. Spermatozoa often show a spherical nucleus with homogenous, highly condensed chromatin, a nuclear fossa, a midpiece of variable size with or without a cytoplasmic channel, and one or two long flagella [2]. Moreover, fish spermatozoa can be classified into two forms, aqua sperm and intro-sperm, according the external or internal mode of fertilization, respectively [2].

The main components present in a fish spermatozoon [3, 4] are: the head is occupied mostly by the nucleus with paternal DNA material. In most fish species, the head has an almost spherical shape (diameter of 2–4 μm). In some case such as sturgeon, paddlefish, and eel spermatozoa, the shape of head is elongated (up to 9 μm long and 2 μm wide) [5–7], the midpiece is mostly composed of the centrioles, and the mitochondria (usually from 2 to 9 per each spermatozoon) is generating energy (ATP) for motility [8]. In a mature spermatozoon, because the protein synthesis machinery is absent, no gene expression occurs. The centriolar complex of midpiece consists of the proximal and the distal centrioles, which forms the basal body of the flagellum, used for anchoring the flagellum to the head of the sperm cell.

The flagellum is a highly conserved organelle during evolution, and there are very few differences between molecular composition of sperm flagella relatively to that in protists [1, 10]. Fish sperm flagellar length varies from 20 to 100 μm, depending on species. Flagellar bending is generated by a highly organized cylindrical system of microtubules, called the axoneme, emanating from the basal body [11]. In turn, the canonical "9 + 2" axoneme consists of nine pairs of peripheral microtubular doublets and one central pair of singlet microtubules. This structural arrangement [12] is illustrated in **Figure 1**. Such axonemal pattern is highly conserved and almost identical among eukaryotic cilia and flagella from protozoans to human. Nevertheless, in Anguilliformes and Elopiformes sperm flagella present a "9 + 0" pattern lacking central microtubules [6, 7, 13].

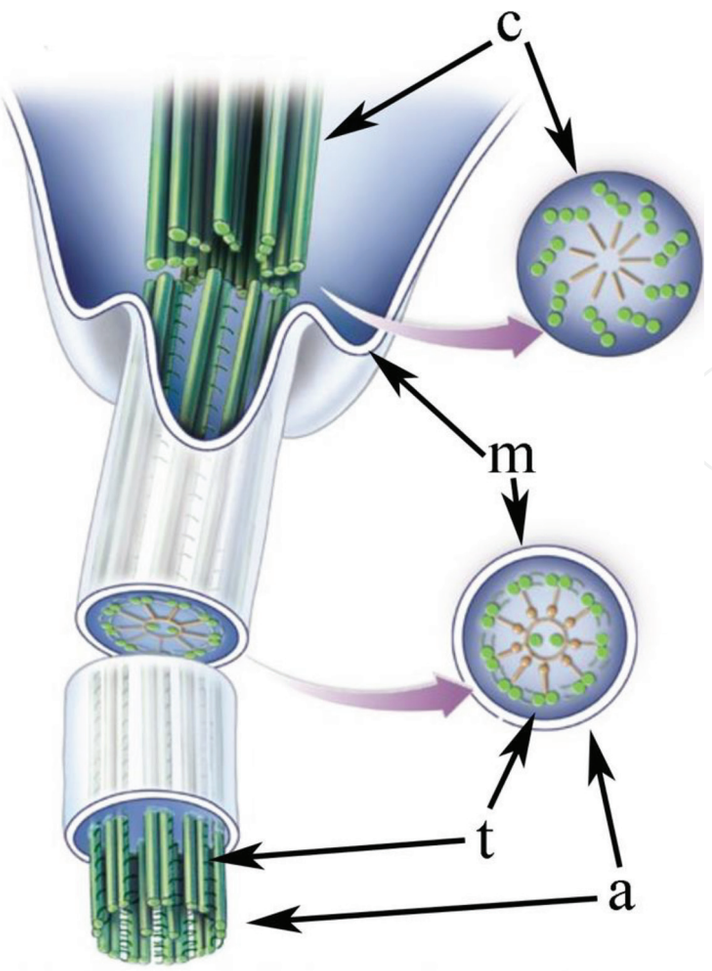


**Figure 1.**
*Schematic structure of the head-tail junction of a model spermatozoon: the artistic view applies to a teleost such as trout [9] as an example of sperm cell. m, membrane surrounding both the head (top) and the tail; a, axoneme, the mechanical part actuating the flagellum (bottom); t, microtubule doublets, the major scaffold of the 9 + 2 axoneme; c, centriolar complex, at the basal part of the axoneme, made of microtubule triplets. In many fish species, the mitochondria (not shown for simplification) are localized in this head-tail junction zone, and two centrioles are orthogonally assembled to form the centriolar complex.*

The structural connections between the nine peripheral outer doublets and the sheath surrounding the central pair occur through the radial spokes. The central pair of singlets is enclosed in this sheath of proteins forming a series of projections that are well positioned to interact with each of the spoke heads and regulate the wave propagation [14]. Each of the outer doublets is connected to adjacent pairs of doublets by nexin links, presenting elastic properties allowing to resist the free sliding of the microtubules; nexin is a dynein regulatory protein [15]. The peripheral doublets are strung with two rows of dynein arms along the entire length of microtubules. These dynein arms consist of macromolecular ATPase complex [16, 17] and represent the basic motor actuating the whole axoneme; they extend from an outer doublet toward an adjacent doublet [18]. Both the spokes and the dynein complex contain different calcium-binding proteins so as for flagella to be able to respond to regulation by free calcium concentration through altering their beating pattern [19, 20]. As briefly described above, axonemes are complex structures composed of at least 500 different protein components [21].

The bending process in an axoneme is caused by sliding between two adjacent doublets of outer microtubules that slide relatively to each other due to the motive force, generated by molecular dynein motor activity [16]. Due to enzymatic hydrolysis of ATP by the latter, which induces force generation of the power stroke of individual dynein molecules, the dynein arms interact with tubulin of the B tubule

from the adjacent doublet, causing a process of active sliding in a cooperative way [22]. Several local bending processes occur because this sliding activity is present in only some segments of the axoneme at a given time, while other segments remain inactive [4]. Wave propagation from head to tip provokes the translation of the whole spermatozoon in the opposite (forward) direction.

### 2.2 Spermatogenesis in fish

For obtainment of full efficiency of motility, all the elements of the flagellum must have been, during spermatogenesis, correctly assembled mostly as an elongated structure called axoneme playing the role of propelling engine, surrounded by the plasma membrane, and this device must be provided in energy in terms of ATP [8], the fuel common to many cell types that is mostly generated by mitochondrial respiration in case of fish sperm as detailed in paragraph 4.

Spermatogenesis is an important phase in case of fish spermatozoa because it is the ATP store that constitutes the main source of energy that will sustain the short but highly energy-demanding motility period [8].

A detailed description of fish spermatogenesis is well documented in many species. Briefly, spermatogenesis is a developmental process during which a small number of diploid stem cells (spermatogonia) produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome, and a structurally complete flagellum. Survival and development of those germ cells depend on their close contact with specific cells called Sertoli cells. Apart from their phagocytic role, the Sertoli cells change the growth factor expression, and subsequently, modulate germ cell proliferation/differentiation via complex mechanisms involving, in fish, both pituitary gonadotropins LH and FSH that stimulate gonadal sex steroid hormone production directly by the activation of another cell type called Leydig cells.

Fishes represent the largest and most diverse group of vertebrates. However, our knowledge on spermatogenesis in this group is limited to a few species used in basic research and/or in aquaculture biotechnologies such as guppy, catfish, cod, eel, medaka, salmon, tilapia, trout, and zebrafish. In an amniote vertebrates (fishes and amphibians), one observes a cystic type of spermatogenesis, which presents two main differences compared to higher vertebrates [23]. First, within the spermatogenic tubules, cytoplasmic extensions of Sertoli cells form cysts that envelope a single, clonally and hence synchronously developing group of germ cells deriving from a single spermatogonium. Second, the cyst-forming Sertoli cells retain their capacity to proliferate also in the adult fish. Sertoli cells are surrounding and nursing one synchronously developing germ cell clone. Different clones being in different stages of development generate a tubular compartment containing differently sized groups of germ cells in different stages of spermatogenesis.

So as for the distribution of spermatogonia in the germinal compartment, one can observe either a first type of restricted spermatogonial distribution, which is found in the higher teleost groups, such as in the order Atheriniformes, Cyprinodontiformes, and Beloniformes, where the distal regions of the germinal compartment are occupied by Sertoli cells surrounding early, undifferentiated spermatogonia. While the cells divide and enter in meiosis and the cysts migrate toward the region of the spermatic ducts located centrally in the testis, this is where spermiation occurs, i.e. the cysts open to release spermatozoa.

In a second type, where an unrestricted spermatogonial distribution that is considered a more primitive pattern found in less evolved taxonomic groups, such as in the order Cypriniformes, Characiformes, and Salmoniformes, occurs, spermatogonia are spread along the germinal compartment throughout the testis. The cysts do not migrate during their development. In addition, intermediate forms also

exist between restricted and unrestricted spermatogonial distribution, such as in Perciformes, tilapia, Pleuronectiformes, or Gadiformes.

Therefore, the development of spermatogenic cells strictly depends on their interaction with the somatic elements of the testis, among which Sertoli cells play a crucial role. During fish spermatogenesis, Sertoli cells are formed by mitosis just in time and in exactly the number required. This tailored Sertoli cell proliferation was first described in the guppy [24]. So far, we observe that spermatogenesis is a highly organized and coordinated process, in which diploid spermatogonia proliferate and differentiate to form spermatozoa in their final morphology. The duration of this process is usually shorter in fish than in mammals. In principle, this process can be divided, from a morpho-functional point of view, in three different phases: (i) the mitotic or spermatogonial phase with the different generations of spermatogonia, (ii) the meiotic phase with the primary and secondary spermatocytes, and (iii) the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating, without further proliferation, into flagellated spermatozoa.

Analysis of the role of hormones reveals a complex process. Three steps at which reproductive hormones play a critical regulatory role are (i) the balance between self-renewal and differentiation of spermatogonial stem cells, (ii) the transition from type A spermatogonia to rapidly proliferating type B spermatogonia, and (iii) the entry into meiosis. During later developmental stages, on the other hand, the endocrine system seems to ensure a permissive rather than stimulatory role, enabling Sertoli cells and possibly other somatic cells to generate a microenvironment that germ cells require to proceed through meiosis and spermiogenesis [25]. In case of fish, three types of spermiogenesis have been described, based on the orientation of the flagellum relatively to the nucleus and on whether or not a nuclear rotation occurs.

### 2.3 Flagellum genesis

The main organelle in the flagellum, the axoneme, is resulting from the progressive assembly of groups of elements synthesized in the cell body and then transported to the flagellar compartment and delivered to the flagellar tip to elongate it thanks to the motility of specific transporters called intra-flagellar transport (IFT) along the internal side of the flagellar membrane [21]. The trafficking is ensured by two important molecular motors, belonging to the dynein group (retrograde, meaning from tip to base of the flagellum) and to the kinesin group (anterograde, meaning from the cell body to the flagellum tip) [26].

## 3. Successive steps leading to fish sperm motility

### 3.1 The maturation step

Maturation is the step following the end of spermatogenesis and that provides to the spermatozoon its ability to respond to motility-activating factors [27]. Signals for maturation are quite various among fish species. Sperm maturation is also regulated by the endocrine system.

Examples of sperm maturation have been studied in details in different fish species: salmonids, cyprinids, and sturgeons. In salmonids, the group of Morisawa, in Japan, demonstrated, in particular in case of salmon, that maturation is mainly under control of cAMP and pH of the water where fish are transiting during migration [28]. In carp, results from Redondo et al. [29] indicate that an ionic equilibration across the sperm membrane is the main factor responsible for maturation. In case of sturgeon species, it was shown that spermatozoa are not able to become

activated at simple contact with external water [30]; sperm cells need a transient contact with urine, the latter getting mixed with milt prior to ejaculation [31] which is enough to render spermatozoa fully motile.

### 3.2 The activation step per se

This is a very brief event lasting a fraction of a second [32, 33]. Among several kinds of activating signals, one can mention (1) osmolarity, the most common, (2) specific ions such as $K^+$ in a few species (salmonids and sturgeons as examples), and (3) other signaling molecules such as $CO_2$ in few cases [34]. The osmolarity signal depends on the external medium where fish delivers its sperm: marine fish spermatozoa activate when coming in contact with sea water, a salty solution of high osmolarity, while freshwater fish species shed their sperm in a very low-osmolarity medium. In both cases, the spermatozoa sustain a large stress that consists in a jump from seminal fluid ranging an osmolarity 300 mOsmol/kg to either sea water (around 1100 mOsmol/kg) or freshwater (maximum 50 mOsmol/kg).The environmental osmotic pressure appears consistently to be the main factor involved in fish motility activation among species [35, 36]. In a few fish species, osmolarity is acting in synergy with another factor such as specific ions [35]. In some marine species such as herring, activation of spermatozoa requires egg-derived substances. Two types of sperm-activating factors have been identified in Pacific herring, *Clupea pallasii*, eggs: a water-soluble protein released into the surrounding water [37] and a water-insoluble sperm motility-initiating factor localized in the vicinity of the micropylar opening of eggs [38].

### 3.3 The perception of the signal by the sperm membrane

Freshwater fish sperm cells when released into the surrounding water can increase their cytoplasmic volume in response to osmotic stress. In case of carp spermatozoa, the cell volume increases several times as a result of the influx of water [39]. Results of Cabrita et al. [40] show that small change of cell volume occurs in response to hypo-osmotic shock. The comparative study of Bondarenko et al. [41] puts forward a large species specificity regarding the osmotic reaction (swelling) among freshwater species and demonstrates that there is no change of trout sperm volume measured during the motility period. Altogether, the volume change, if any, represents a long-term osmotic reaction rather than the immediate signal for motility activation given its time delay (several tens of seconds) relative to the briefness of motility appearance (less than a second according to Prokopchuk et al. [33].

A series of experiments by Takei et al. [42] aims to better explore the respective roles of $K^+$ ions and osmolarity; this paper proposes a mechanism involving aquaporins and volume changes as a response to osmolarity stress. However, the volume changes measured by the authors are of low amplitude: the engendered volume difference is so low that it cannot be responsible of a physiological role in motility control. Furthermore, the time scale and the volume change measurements obtained at 5 min after motility activation correspond to time scales that are not fit with the previously published results of Prokopchuk et al. [33] showing that the motility response occurs about 100 ms after reception of the activation signal of fish spermatozoa. In addition, previous results by Bondarenko et al. [41] demonstrate that no significant volume change follows the motility activation of trout spermatozoa, a situation that contrasts with that of carp.

In many marine teleost species, hypertonicity induces the motility of spermatozoa. Nevertheless, an increase in external osmolality is sometimes not the only condition for motility activation of marine fish spermatozoa: in case of herring, this

activation also needs the contact of sperm with egg-derived substances, facilitating fertilization [37, 38, 43, 44]. Sperm-activating factors are two types of in the Pacific herring, *Clupea pallasii*: a water-soluble herring egg protein [37, 43, 44] and water-insoluble initiating factor, from the vicinity of the micropylar opening of the egg [38]. In another group of marine fish species collectively named flatfishes, the main signal perceived by the sperm membrane is the $CO_2$ concentration [34] that is high in the genital tract but very low in sea water. The intracellular equilibrium between $CO_2$ and bicarbonate constitutes the second step in the control of intracellular ionic concentration that leads to regulation of flagellar motility [33].

### 3.4 The transduction of the signal across the membrane and the cytoplasm

In fish spermatozoa, external signals triggering sperm motility activation are acting at the level of spermatozoon plasma membrane, hyperpolarization/depolarization of membrane, and ion channels or aquaporins activity, but this topic is still challenging because of the scarcity of experimental results in this area. As mentioned above, it is clear that water transport itself is not a main process involved in fish sperm motility activation. Nevertheless, in the presence of aquaporins in the head and flagella plasma membrane of the seawater fish, gilthead sea bream (*Sparus aurata*) spermatozoa and their involvement in cAMP-mediated phosphorylation of axonemal proteins were established [45, 46]. In this species, the water efflux via aquaporins would determine a reduction in the cell volume, which would raise the intracellular concentration of ions. This would lead to the activation of adenylyl cyclase and motility initiation by cyclic AMP-dependent protein phosphorylation and dephosphorylation [46]. Such cascade of events remains hypothetical because of the timing of such process compared to the extreme briefness (less than 0.1 s) of the reaction of the axoneme activation [33].

The presence of different ion channels was described in sperm plasma membrane [34]. Cytosolic pH could be considered as another participant of signaling pathways, as it is known to be one of the parameters influencing sperm motility [47, 48]. Environmental conditions that inhibit spermatozoa motility can decrease the intracellular pH, resulting in a more acidic cytoplasm in nonmotile spermatozoa than in motile spermatozoa [49]. The decrease of internal pH in sperm would directly affect flagellar movement through inhibition of dynein activity. The involvement of the Na+/H+ exchangers in sperm motility activation process was reported for *Cyprinus carpio* [50] and was proposed for *Brycon henni* [51]. The former authors suggest that the regulation of the exchangers depends on osmolality conditions [52]. According to Krasznai et al. [53], an opening and closing of $K^+$ channels in the plasma membrane of the spermatozoon under hypo-osmosis-induced initiation of sperm motility is resulting in a remarkable local hyperpolarization or depolarization of the spermatozoon plasma membrane. Such transient depolarization may open $Ca^{2+}$ channels, resulting in an influx of $Ca^{2+}$ and activation of the flagellar motility of carp sperm.

The involvement of $K^+$ and $Ca^{2+}$ transport through ion channels at the plasma membrane of spermatozoa in the triggering of the motility initiation has also been shown for rainbow trout (*Salmo gairdneri*) spermatozoa [54]. As for $Na^+$ channels, Tanimoto and Morisawa [54] supposed that $Na^+$ channels do not play an important role in sperm motility in rainbow trout, although they did not exclude its possible involvement in sperm motility control, for example, through $Na^+$-$H^+$ exchange.

Altogether, the precise mechanisms of regulation of ion channel activity and their participation in the hyperpolarization of the spermatozoon membrane, which is associated with the activation of sperm motility [35, 53], remain poorly understood. What are the next-step processes occurring at the level of membrane leading the subsequent activation of the axoneme?

### 3.5 Transduction and reception of the signal at the axoneme level

Among other processes, an increase of intracellular concentration of ions could lead to the activation of adenylyl cyclase, which in turn would determine the motility initiation by a cAMP-dependent protein phosphorylation and dephosphorylation mechanism [46]. It is known that, in mammals, protein tyrosine phosphorylation of several proteins is upregulated by reactive oxygen species (ROS). ROS (especially $H_2O_2$) may enhance tyrosine phosphorylation through the selective suppression of tyrosine phosphatase activity [55] or activation of adenylyl cyclase, thus producing a higher cAMP level and leading to the subsequent activation of the serine/threonine kinase A [56].

Cyclic AMP is an important factor in the activation process of fish spermatozoa. The link between cAMP concentration increase and motility initiation at the axoneme level was mainly investigated in Salmonidae. It involves a complex series of phosphorylation and dephosphorylation events. This includes the cAMP-dependent phosphorylation of the 15 kDa movement-initiating phosphoprotein [57, 58] of a PKA [59] and of the 22 kDa dynein light chain [60]. Protein phosphorylation is also regulated by proteasomes [60, 61]. The precise dependence between protein phosphorylation and microtubule sliding and movement initiation is still under investigation. A $Ca^{2+}$-mediated and/or cAMP-dependent phosphorylation signaling mechanism through the radial spoke/central pair system of the axoneme has been proposed [62, 63].

The potential role of reactive oxygen species (ROS) generated at the contact of sperm with aerobic condition such as the external medium at ejaculation and the molecular mechanisms by which these reactive metabolites exert their biological activity has been put forward by Baker and Aitken [64]. A gas, NO, was also observed to enhance motility of fathead minnow spermatozoa [65]. Nevertheless, the mechanism by which NO affects sperm motility is probably unrelated to osmolality, because of its very low active concentration range.

### 3.6 The motility period

During the period after spermatozoa comes in contact with an activating medium, the ion concentrations inside the sperm cell are rebalanced, and osmotic pressure affecting the sperm membrane becomes harmful for sperm integrity, limiting the period of motility to a short interval [32]. These phenomena are much faster and more obvious in freshwater species, in which sperm motility usually does not last for more than 0.5–2 min [32].

**Video 1** can be viewed at https://vimeo.com/310085381.

Brook trout (*Salvelinus fontinalis*) spermatozoa recorded while swimming in a swimming medium composed of low osmolarity (10 mM Tris at pH 8.0 + 10 mM $CaCl_2$). Remark the briefness of motility (around 30 s duration at 10°C) (courtesy of Dr. Galina Prokopchuk).

In case of sperm collection for artificial reproduction or in vitro studies, particular care should be taken to avoid precocious activation of motility: one should avoid any contact of sperm cells with external water [66] or urine during stripping [67, 68]. Any assessment of motility parameters should be started as soon as possible after sperm activation, bearing in mind that the earliest period of motility (the most efficient one) should be characterized. Fish spermatozoa are usually characterized by a very high initial velocity (up to 200 μm $s^{-1}$) due to the high flagellar beat frequency (up to 100 Hz; [32]); this "most active" period lasts only a few seconds immediately after contact with the activating medium.

Values of all motility parameters decrease rapidly immediately after initiation of fish sperm flagella movement [36, 69], which is why any motility parameter must

refer to a precise time point after activation for intra- or interspecies comparisons [36, 69, 70]. Generally, the duration of motility is a trade-off between the level of energy stocks possessed by a cell and the process of osmotic damage experienced by this cell. The latter is more critical in freshwater fish species, and the former is important for marine fish [36]. At a precise time point, most spermatozoa have very similar characteristics [69, 70].

**Video 2** can be viewed at: https://vimeo.com/310086859.

Carp (*Cyprinus carpio*) spermatozoa recorded while swimming in two different media successively; the first part of the record shows the carp sperm population activated in a swimming solution composed of 45 mM NaCl +5 mM KCl + Tris at pH 8.0, while the second part shows spermatozoon activation in a low-osmolarity medium (distilled water + Tris at pH 8.0). Remark the curling of the flagellar tip which limits the duration of motility at low osmolarity (courtesy of Dr. Volodymyr Bondarenko).

## 4. Energetic of sperm motility

Storage of energy mostly results from mitochondrial respiration that generates ATP. Energy metabolism also involves other compounds such as creatine phosphate that contributes to the maintenance of the intracellular energy level in connection with ATP.

### 4.1 Mitochondrial respiration

In many fish species, measurement of respiratory activity presents difficulties because of the low oxygen consumption of spermatozoa, in contrast to model species such as sea urchin [71]; in addition, the low respiratory activity remains almost unchanged when fish spermatozoa are transferred into motility-activating solutions [72], while it is about 50-fold increased when sea urchin sperm is transferred into sea water [71]. Efficient respiration needs to be coupled in mitochondria to ATP production via the ATP synthase [73]. For estimation of the full respiratory capacity of mitochondria, it is useful to apply diffusible "uncouplers" such as CCCP or FCCP (carbonylcyanide-4-trifluromethoxy-phenylhydrazone): these compounds are diffusible through the membranes and allow full rate of electron transfer in the electron chain of mitochondria without restriction due to its control by ATP synthase. The effects of respiratory inhibitors such as oligomycin [73] or KCN and their relationship with ATP stores of fish sperm were studied in details by Dreanno et al. [74]. Mitochondrial inhibitors have little effect in case of trout [75–78] or turbot [79] spermatozoa. Respiration rate in quiescent fish spermatozoa (before motility activation) needs to be only minimal but enough to maintain this ATP level prior to ejaculation. Such low but substantial respiration is enough for basal metabolism to maintain ionic exchanges and balances across the plasma membrane [8, 76, 79].

### 4.2 Generation and storage of ATP

Generation of ATP by mitochondria occurs by electron transfer along the mitochondrial respiratory chain that generates a proton gradient across the inner mitochondrial membrane. Dissipation of the proton gradient occurs by passage of $H^+$ through a specific ATP synthase localized at the inner part of the mitochondrial membrane [8, 73]. ATP thus accumulated is transported out of the mitochondrion by a translocase (ATP-ADP exchanger) toward the flagellar compartment; then, ATP molecules diffuse along the axoneme possibly assisted by a carrier device called energy shuttle as explained in Section 4.4.

The energy stored in fish sperm prior to and used during the motility period was evaluated in several fish species [80–82]. This was described in turbot, for example [79, 83, 84], sea bass [85] perch [86], bluegill [87, 88], trout [71, 89, 90], carp [39, 91], sturgeon [92, 93], and catfish [94]. Values for spermatozoa of other fish species can also be found in Cosson [8, 32, 95], Dzyuba et al. [30], and Ingermann [96].

### 4.3 Consumption of ATP during the motility period

ATP is the only high-energy compound that is hydrolyzed by the motor protein of the axoneme called dynein ATPase [16, 17, 97]. Dyneins are macromolecular assemblies of more than 1 MDa molecular weight linearly bound to each microtubule doublet of the axoneme and which function is mechanochemical. Its role it to collect the chemical energy generated by hydrolysis of an ATP molecule so as to induce a trans-conformation that constitutes the elementary step of movement generation. Rows of dyneins positioned along each microtubule amplify in a cooperative way this elementary movement so as to provoke the sliding between adjacent microtubules of the axoneme. Differential sliding generates bending, and the bending waves that propagate along the flagellum (usually from the head to the tip of the axoneme) result in a translational movement of the whole sperm cell [21].

In case of fish spermatozoa, dynein molecules have an intense activity: this fast activity results in a beat frequency (up to 80–100 Hz) much higher than in species other than fish. As a result, ATP is hydrolyzed at high speed, and the ATP store is rapidly decreasing, becoming partly exhausted at the end of the motility period [32].

### 4.4 Other energetic molecules assisting ATP maintenance

Following its synthesis by mitochondria, ATP should be transported and distributed all along the flagellum so as to supply chemical energy to sustain the mechanical energy generated by the dynein motors that are distributed all around the flagellar axoneme. Theoretical considerations lead to postulate the presence of a distribution system that ensures a constant ATP concentration at any point along the axoneme [98, 99]. Such shuttle system involves an additional high-energy compound and assistance of enzymatic system that was shown to be present in fish sperm cells [90] and is detailed below.

Even though the ATP molecule is the most common high-energy compound used as a fuel for many cell functions, including motility [8], several other high-energy molecules are present in living cells such as creatine phosphate or arginine phosphate. In fish, creatine phosphate (CrP) is the main high-energy compound that was characterized in spermatozoa of several fish species as a complement of ATP [74, 79, 85]. In the last decades, many studies have demonstrated the decrease of the ATP concentration inside the fish sperm cells during the motility period [39, 67, 74, 76, 79, 85, 90, 91, 93, 100]. A more restricted number of studies have investigated the concentration of ATP related compound such as ADP, AMP, CrP, and others [74, 79, 85, 100]. All these compounds are part of an intracellular network under control of different enzymes that are able to transfer high-energy phosphate bonds from one to another (see Figure XX in Chapter 1 of Cosson [21]), for example, the equilibrium ATP< = >ADP + Pi is catalyzed by enzymes called ATPases. In another example, ADP + CrP < = >ATP + Cr is controlled by enzymes called creatine kinases. One creatine kinase is mitochondrial, while a second one is in the flagellum and distributed all along the axoneme (**Figure 1**). The mitochondrial creatine kinase delivers CrP that diffuses along the flagellum, both creatine kinases being present in trout sperm cells. The rate of diffusion of CrP molecules is higher than that of ATP [99]. Such an arrangement of catalytic activities and substrates

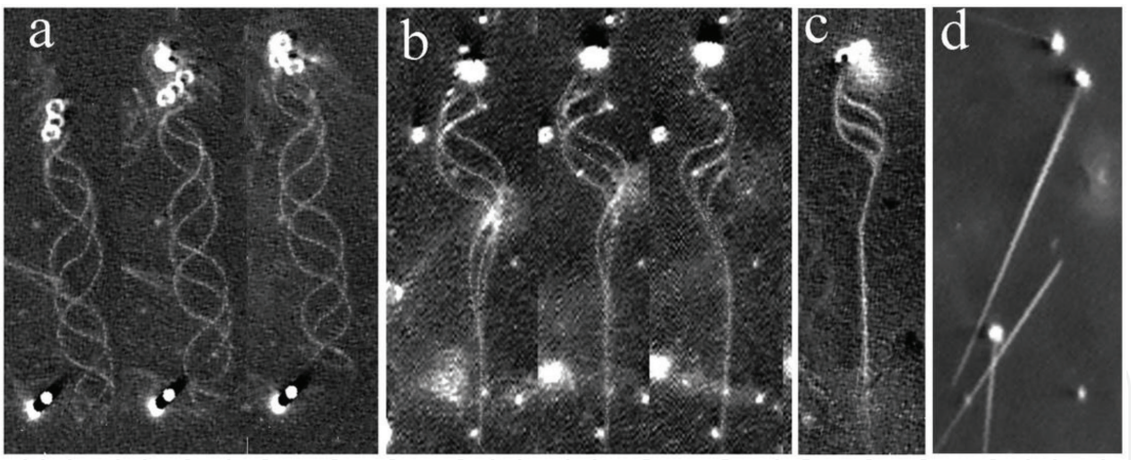


**Figure 2.**
*Evolution of the flagellar shape of fish spermatozoa during the motility period. From left to right, successive positions of a turbot (Scophthalmus maximus) spermatozoon video recorded at different time points post-activation: (a) right after transfer in sea water, (b) 2 min later, (c) more than 3 min later, (d) after full stop. Dark field microscopy with stroboscopic illumination (150 Hz); 100× objective lens [32].*

constitutes an intracellular network ensuring the correct production and distribution of energy in fish sperm cells (**Figure 1**) and is called the "ATP shuttle" [99].

A local axonemal paralysis appears in sperm flagella of many species during the motility period: this is an indication that the renewal of ATP is not fully efficient, and the production of ATP from CrP in this distal portion of the flagellum is too slow, while the proximal portion, that is close to the mitochondrion, can use directly the ATP still produced by the latter. Stiffening of the distal flagellar tip was observed in vivo (but not in reactivated sperm) in two sea urchin species [101] but also in trout sperm (Cosson, unpublished) after application of thiourea, an inhibitor of respiratory phosphorylation (**Figure 2**).

## 5. Control of fish sperm physiology by other factors

Changes in the environment of fish spermatozoa impose other chemicals as well as physical constrains: temperature is controlling sperm physiology at many levels such as membrane permeation, enzymatic activities, or energetic metabolism [102]. Fish species are exposed to a large variety of temperature conditions, especially during their reproduction period; thus, an optimal choice of temperature is a key parameter for controlling the best conditions for best adapted sperm physiology.

Viscosity of the swimming medium is also an important factor influencing the physiology of fish spermatozoa. According to physical laws, especially microfluidics, progression of the sperm cell occurs because of the friction of the flagellum against the external milieu, water being a quite viscous fluid. Higher viscosity is encountered by fish spermatozoa when getting close to the fluids surrounding the egg, which modifies and thus controls the swimming physiology of the sperm cells [21, 103] including female cryptic choice of sperm cells by the egg [104].

Among other factors that play a signaling role for fish physiology are some molecules that control the sperm/egg interplay at fertilization; these molecules are commonly called chemoattractants and are able to finely tune the motility function of fish spermatozoa so as to optimally guide sperm cells to egg which ultimately results in an increase of fertility success [21]. In case of fish, the spermatozoon must localize the entrance point at the surface of the egg so as to penetrate and deliver the male genome [105]. Ultimately, the male genome will combine with that of the female so as to constitute the zygote.

## 6. An update of modern technologies allowing fish sperm quality assessment

### 6.1 CASA systems

Evaluation of fish sperm swimming performances needs at first good quality video records so as to measure the distance covered by each sperm head for a time period corresponding, for example, to the time separating two successive video frames, such as 20 ms for the European video standard (**Figure 3**).

Several works in the last decade have reported the use of CASA systems to assess sperm motility in fish [106]. Based on the integration by the computer of successive positions of the moving head of spermatozoa in consecutive frames of video records to calculate the trajectories and their characteristics, CASA describes different parameters of sperm swimming linked to velocity, for example, VCL or instant speed (frame to frame displacement) along the real track, VAP or velocity along a smoothed track, VSL or progressive velocity following the straight line from the origin to the end of the track during the corresponding period of time and other parameters linked to the wobble of sperm head such as Mean angular displacement (MAD), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) and linearity. Lastly, the ratio between average path and straight-line path is used to describe the straightness of the trajectories (reviewed in Rurangwa et al. [107]).

The CASA system recently developed by Wilson-Leedy and Ingermann [108] as a plug-in to image J software freely available from NIH site (http://rsb.info.nih.gov/ij/plugins/casa.html) has been tested in different species including zebrafish [109]. More recently, it was improved and adapted to trout sperm motility by Purchase and Earle [110].

One important aspect for motility estimation of sperm quality by CASA is that the practical conditions employed to perform such tests are in several respects not reflecting the natural situation. Many broadcast spawners like salmon or trout

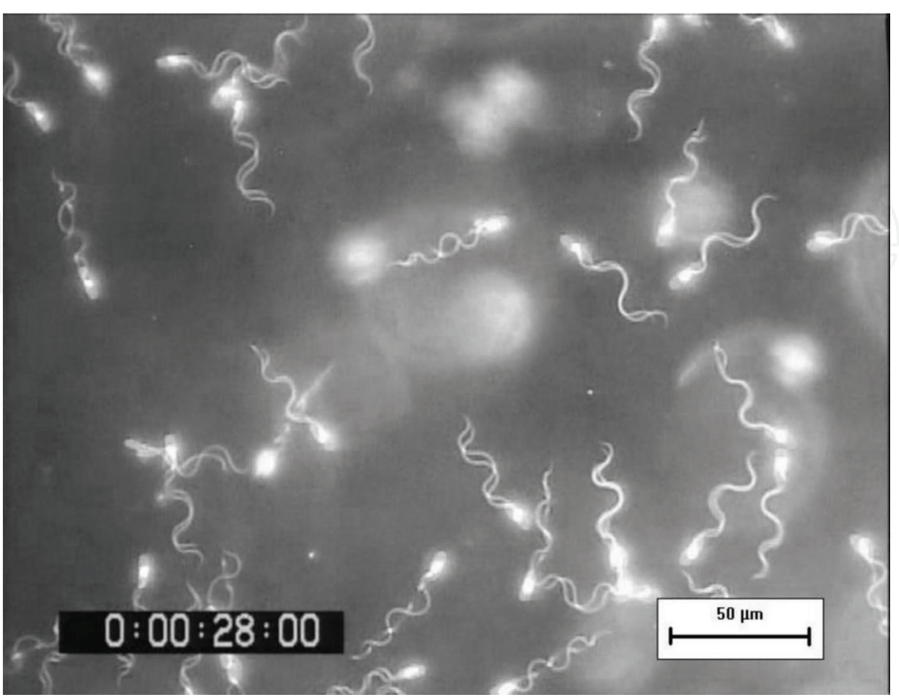


**Figure 3.**
*Video image of swimming sturgeon spermatozoa. Dark-field video microscopy with stroboscopic illumination. Flashes are every 10 ms. At bottom right is the indication from the stop-watch giving the time (in milliseconds) spent since motility activation.*

reproduce in highly turbulent water, which certainly influences the sperm/egg meeting chances and thus the fertilization success. Effect of such turbulent water shear at some optimal values was studied using biophysical methods by Crimaldi and Browning [111], and it was shown experimentally to increase the proportion of fertilized eggs in sea urchin [112].

Also, for practical reasons, CASA records are obtained in conditions where spermatozoa swim in the vicinity of glass surfaces: such situation was shown in literature to affect motility parameters [113, 114]. An important consequence of this is that the motility parameters mostly refer to a situation where sperm cells swim in a planar manner; it is well known since early studies [115] that when spermatozoa swim freely far from any surface, they adopt a helical trajectory. The latter was shown to decrease up to 10-fold the efficient gross velocity but surprisingly to increase around 6-fold the fertilization kinetics [116]. All these findings emphasize several limits of the application of these CASA systems when used to predict quality of sperm regarding the fertilization rate.

The comparative values of sperm velocity among fish species, including salmonid, can be found in Cosson [32] with respect to motility duration and ATP stores prior to activation.

### 6.2 Evaluation of flagella performances

Behavior of the flagellum determines the motility guideline of the spermatozoon, so the description of intrinsic flagellar wave properties is considered as one of the most informative methods for assessing and controlling sperm motility. In order to observe the detailed pattern of live flagella or of their major components, it was proposed to use phase contrast or dark field optical microscopy with high magnification (40×–100×) objective lenses, which, if applied with oil immersion, result in a bright image of the very small diameter object that constitutes a flagellum. To achieve complementary assessment, additional methods, such as stroboscopic illumination or high-speed video techniques, allow to record sperm during its motion and specially to obtain flagellar images of high quality and resolution. Multiflash stroboscopic illumination thus allows visualization on each frame of well-defined successive positions of a same moving spermatozoon at time intervals ranging in milliseconds. Alternatively, high-speed video recording provides higher spatial and temporal resolutions (up to several 1000 images/s). Serial frames individually selected from such video records allow to follow successive positions (every millisecond or less) of flagellum waves covering one or several full beat cycles [32].

**Video 3** can be viewed at https://vimeo.com/309942086.

Legend of the video: a sturgeon spermatozoon was recorded by phase-contrast video microscopy according to conditions similar to those described in **Figure 4** (courtesy of Dr. Bondarenko Volodymyr). Spermatozoa of Siberian sturgeon (*Acipenser baerii*) were activated by dilution in pond water and recorded 10 s after activation. The length of flagella is about 50 μm long. Image rate is 100-fold slower than real. Remark that some spermatozoa show abnormal shape.

Evaluation of sperm flagellum performances on a large variety of fish species leads to a series of predictions briefly summarized below (see **Figure 5**):

- The swimming velocity is linearly proportional to the flagellar beat frequency.
- The smaller the flagellum length, the lower the swimming velocity.
- The number of waves along the flagellum varies linearly with the flagellum length.

- The power output of the flagellum varies in proportion with the flagellar beat frequency.
- The flagellar length has no significant influence on the swimming velocity.
- The swimming efficiency increases in proportion with the swimming speed.
- The wave amplitude and the swimming velocity are linearly related.
- The swimming velocity is lowly dependent of the medium viscosity, but the latter greatly affects the wave shape.
- Waves of helical shape generate rotation of the sperm cell at a frequency that is in proportion with the flagellar beat frequency but less efficient than planar waves.

While the whole cell is moving from left to right, the flagellum wave progresses from right to left. The wavelength is defined as the distance "L" between two inflection points. The half wave amplitude is represented as "A" and the bend angle of each wave as "a". The number of waves generated every second is called the beat frequency. Such parameters are quantified and used to characterize fish sperm cells exposed to various swimming situations affecting their physiology.

Two other examples of high-speed video records of fish spermatozoa are presented below.

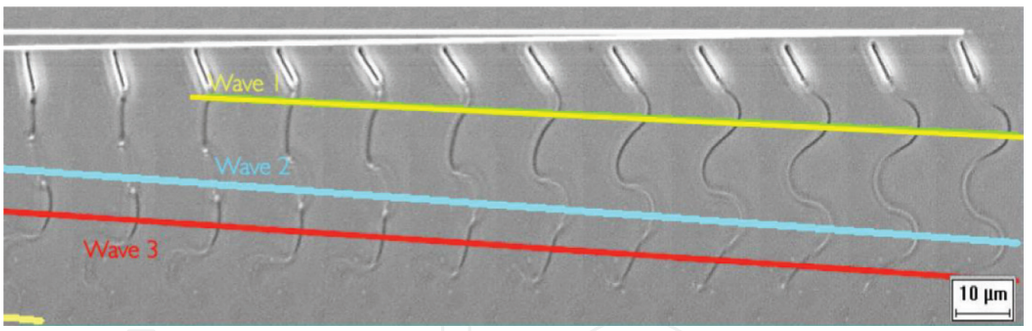


**Figure 4.**
*Successive positions of a sturgeon sperm flagellum recorded by high-speed video microscopy. Initial record was at 5000 images/s with a 100× phase-contrast lens and an Olympus high-speed video camera. In this panel from left to right, successive images collected every millisecond are presented so as to show the wave propagation of three successive waves and the minor progression of the head tip (white straight lines) during this short time period.*

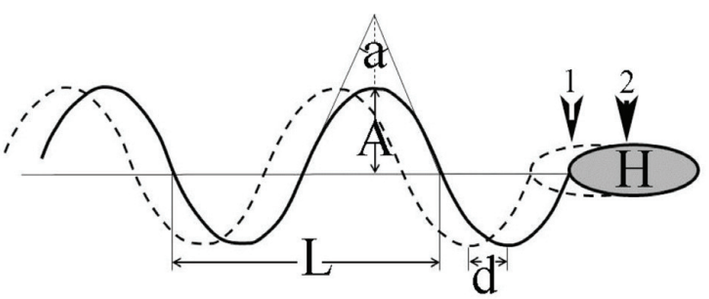


**Figure 5.**
*Flagellar parameters of a swimming fish spermatozoon. The sperm cell, such as that recorded according to **Figure 3**, is represented in two successive positions (1–2) separated by a short time period (0.5 ms, for example).*

**Video 4** can be viewed at https://vimeo.com/310100596.

Pangasius (*Pangasianodon hypophthalmus*) spermatozoa recorded by high-speed video combined with dark-field microscopy and was visualized 10-fold slower than normal. Sperm movement was recorded at 13 s post-activation in a 10% sea water solution. Length of flagellum is about 50 μm (courtesy of Dr. Galina Prokopchuk).

**Video 5** can be viewed at https://vimeo.com/310102833.

Tilapia (*Sarotherodon melanotheron heudelotii*) spermatozoon recorded by high-speed video microscopy and visualized 20-fold slower. Sperm movement was recorded at 18 s post-activation in 50% sea water containing bovine serum albumin (BSA at 0.5%) to prevent sticking to the glass slide. Notice the short sperm flagellum in this species and the swollen midpiece, mostly composed of mitochondria (courtesy of Dr. Galina Prokopchuk).

## 6.3 Biochemical methods

### *6.3.1 Respiration, oxidative stress, free radicals, and DNA damage*

Fish spermatozoa present a low respiratory rate that makes this evaluation quite delicate because of the sensibility of detection methods. Respiratory activity commonly uses oxygen electrodes that measure the oxygen concentration of a small volume of sperm suspension. These media are either preventing or activating motility and can be complemented by different effectors (substrates, uncouplers, or inhibitors) of respiration. In general, mitochondrial inhibitors have little effect on the motility of fish spermatozoa. Sperm oxygen consumption rate published in literature was presented in a comparative way for about 10 different fish species by Ingermann [96] and shows that values present a large variability from 1.4 to 70 nmoles $O_2$/min/$10^9$ spermatozoa depending on species.

The contact of fish sperm with the external milieu occurring at ejaculation leads to exposure of sperm cells to high concentration of oxygen, provoking different kinds of stress [117]. Among other chemicals responsible of stress, reactive oxygen species are highly aggressive [118, 119]. The oxidative stress can be evaluated by several methods. Results of these studies show that several protections against the oxidative stress are present in fish sperm cells and in the seminal fluid [31, 120].

Also, the presence of free radicals leads to DNA damage during stressing situations such as those due to application of cryo-techniques that influence the quality of the progeny [121]. Recent studies on genes and protein expression of fish sperm cells show that both are controlled by various factors of the external milieu such as salinity or timing during the reproductive season [122, 123]. Among other factors, the level of phosphorylation of specific proteins constitutes important signaling factors controlling function efficiency of fish spermatozoa [124, 125]. Proteomics represent a promising approach to study specific physiological situations encountered by fish spermatozoa [126].

### *6.3.2 Evaluation of energetic compounds concentration*

ATP content of sperm cells can be evaluated by several methods, including measurement in a single spermatozoon cell as recently shown by Chen et al. [127]. The most popular method for evaluation of the ATP content classically uses a coupling with the light-emitting system composed of luciferin and luciferase. A full evaluation of the storage of energy in fish sperm cells needs the determination not only of the internal content of ATP but also that of other energetic compounds that are

able to exchange high-energy phosphate bonds able to be transferred to ADP and allow to reconstitute the intracellular ATP store. Such evaluation was established in case of sturgeon sperm [100, 128] by the use of liquid chromatography combined to HPRS or in case of turbot or sea bass sperm where the adenine nucleotides' energetic balance was determined by $H^+$-NMR and $^{31}$P-NMR analysis, [74, 79] as well as in trout by $^{31}$P-NMR [89, 90]. All these results clearly point out to the fact that ATP level can be rescued by the CrP generated by the mitochondrial metabolism. This means that other phosphagen compounds are as important as ATP in the energy balance of fish sperm cells [129].

## 7. Conclusion

Fish sperm physiology is under control of various parameters of the external milieu: the latter is subjected to changes due to the different environmental conditions that sperm cells have to deal with such as (1) the ionic concentration of internal as well as external fluids, (2) the pH, (3) the osmolarity, (4) the temperature, and (5) specific molecules acting as signals such as chemoattractants that control the sperm-egg interaction at fertilization [21]. In fish spermatozoa, the interplay between the different actors results in a complex signaling network that exquisitely optimizes the various functions of fish spermatozoa, especially that of motility, in a large variety of situations. A better understanding of this complex network is important so as to decrease the effects of possible damage (osmotic, oxidative, etc.) when fish sperm cells are exposed to drastic conditions such as those imposed during application of cryopreservation methods.

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