

REVIEW

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Molecular mechanism regulating axoneme activation in marine fish: a review

Loredana Zilli*, Roberta Schiavone, Carlo Storelli and Sebastiano Vilella

* Correspondence: loredana.zilli@unisalento.it
Laboratory of Comparative Physiology-Department of Biological and Environmental Sciences and Technologies, University of Salento, Via Provinciale Lecce-Monteroni 73100 Lecce, Italy

Abstract

In many marine fish species, the spermatozoa are immotile in the testis and seminal plasma, and motility is induced when they are released in the aqueous environment. It is well known that the extracellular factors (hyperosmolality or sperm-activating peptides), controlling sperm motility in marine fish, act on the axonemal apparatus through signal transduction across the plasma membrane. To better understand the molecular mechanism regulating axoneme activation in marine fish, the present review examines the existing literature, with particular emphasis on protein phosphorylation/dephosphorylation process.

The present review suggests that: (1) there is no single model that can explain the molecular activation and regulation of sperm motility of the marine fish; (2) only in some species (puffer fish, tilapia, gilthead sea bream, and striped sea bream) protein phosphorylation/dephosphorylation has been shown to be involved in flagellar motility regulation; (3) only a few proteins were identified, which show a change in their state of phosphorylation following sperm activation.

A model of molecular mechanism controlling the activation of sperm motility in gilthead sea bream is being proposed here, which could be a useful model to clarify the sperm motility activation process in other species.

Keywords: Fish sperm, Sperm motility, Aquaporin, Teleost

Review

In fish with external fertilization, spermatozoa are usually immotile in the seminal tract (Stoss 1983). The seminal plasma, whose osmolality and composition depends on the species, protects and immobilizes spermatozoa until they are ejaculated and their motility is initiated (Morisawa 1985). Many environmental stimuli control sperm activation by triggering the different transduction pathways. In salmonids (Baynes et al. 1981; Billard 1983; Morisawa et al. 1983a; Stoss 1983; Morisawa 1985) and sturgeons (Gallis et al. 1991; Toth et al. 1997; Alavi et al. 2004), the reduction of the external K^+ concentration, upon dilution of semen, initiates sperm motility. Hypotonic exposure after dilution into freshwater is the trigger signal in non-salmonid freshwater fish (Morisawa and Suzuki 1980; Morisawa et al. 1983b; Stoss 1983; Morita et al. 2003; Krasznai et al. 2003b), while hypertonic exposure initiates sperm motility in many marine fishes (Morisawa and Suzuki 1980; Oda and Morisawa 1993; Detweiler and Thomas 1998; Krasznai et al. 2003a). It has been also reported that an egg-associated molecule triggers sperm activation in herring (Yanagimachi and Kanoh 1953; Yanagimachi 1957a, 1957b; Yanagimachi et al. 1992; Oda et al. 1998).

All these factors lead to the activation of the axoneme through signal transduction across the plasma membrane. Second messengers, such as cAMP and Ca^{2+} , play key roles in the initiation of sperm motility in fish (Morisawa and Okuno 1982; Krasznai et al. 2000; Morita et al. 2003; Zilli et al. 2008a), as well as mammals (Lindemann 1978; Tash and Means 1982; Okamura et al. 1985), sea urchin (Cook et al. 1994), mussel (Stephens and Prior 1992), and tunicate (Opresko and Brokaw 1983). The second messengers may trigger the dynein-mediated sliding of the axonemal outer-doublet microtubules through different mechanisms such as protein phosphorylation/dephosphorylation (Hayashi et al. 1987; Lindemann and Kanous 1989; Inaba et al. 1999; Nomura et al. 2000 Itoh et al. 2001; Zilli et al. 2008a), ADP—binding to dyneine (Lesich et al. 2008; Hayashi and Shingyoji 2009) or ionic strength (Cosson et al. 2008a). This review is focused on the molecular mechanisms that enable environmental stimuli to determine the activation of the axoneme, with emphasis on the role of proteins with phosphorylation/dephosphorylation activity.

Sperm motility activation is mediated by an increase in intracellular calcium

In marine teleosts with external fertilization, three different mechanisms for motility initiation are known. (1) In flatfish species (Inaba et al. 2003), although osmolarity is the primary factor that regulates the initiation of sperm motility, the intracellular HCO_3^- concentration plays a key role in this process (higher levels of bicarbonate inhibits motility). The intracellular level of bicarbonate is controlled by a cytosolic carbonic anhydrase that convert intracellular HCO_3^- into CO_2 which lead to decrease in concentration of bicarbonate (CO_2 diffuses outside the spermatozoa); (2) in herring, *Clupea pallasii* spermatozoa motility is initiated by the presence of a sperm motility initiation factor (SMIF), a 105-kDa basic glycoprotein that is localized to the micropylar region of the herring egg (Yanagimachi and Kanoh 1953; Yanagimachi 1957a, 1957b; Yanagimachi et al. 1992; Pillai et al. 1993); (3) in several marine species (including sea bass *Dicentrarchus labrax*, tuna *Thunnus thynnus*, gilthead sea bream *Sparus aurata*, striped sea bream *Lithognathus mormyrus*, puffer fish *Tetraodontidae*, flounder *Paralichthys orbignyanus*, Atlantic croacker *Micropogonias undulatus*, hake *Merluccius merluccius*, and cod *Gadus morhua*) hyperosmolality regulates sperm activation (Morisawa and Suzuki 1980; Oda and Morisawa 1993; Detweiler and Thomas 1998; Krasznai et al. 2003a; Zilli et al. 2008a; Cosson et al. 2008a, 2008b). Moreover, it has been shown that calcium ions also play a key role in the initiation of sperm motility. Three different mechanisms of action have been proposed for physiological roles of Ca^{2+} : (a) Ca^{2+} acts directly on the axonemal structures (sea bass and tuna, Cosson et al. 2008a, 2008b); (b) Ca^{2+} regulates Ca^{2+} /calmodulin-dependent protein phosphorylation that in turn activates the axoneme (for example in puffer fish or seawater-acclimated euryhaline tilapia *Oreochromis mossambicus*) (Krasznai et al. 2003a; Morita et al. 2004); (c) Ca^{2+} leads to a cAMP-dependent protein phosphorylation that activates axoneme in gilthead sea bream and striped sea bream (Zilli et al. 2008a). In all suggested mechanisms, the hyperosmolality signal firstly increases intracellular Ca^{2+} concentration (Oda and Morisawa 1993), which is similar to what happens in freshwater fish (Cosson et al. 1989; Krasznai et al. 2000) and tunicates (Izumi et al. 1999). This increase could be due to a calcium influx across the plasma membrane, or to a calcium release from intracellular store, or to a cytosol concentration following massive water efflux by aquaporins.

Calcium influx across the plasma membranes has been demonstrated in spermatozoa of seawater-acclimated *Tilapia mossambicus*, (Linhart et al. 1999; Morita et al. 2004), sea

bass, and tuna (Cosson et al. 2008a, 2008b). In vertebrates and invertebrates, an ATP-driven Ca^{2+} pump and a $\text{Na}^+/\text{Ca}^{2+}$ exchangers (Wennemuth et al. 2003) together with many types of calcium channels (Wiesner et al. 1998; Arnoult et al. 1999; Serrano et al. 1999; Westebroek and Babcock 1999; Krasznai et al. 2000; Wennemuth et al. 2000; Jungnickel et al. 2001; Quill et al. 2001; Jagannathan et al. 2002; Nikpoor et al. 2004) allow the calcium flux throughout the plasma membranes of spermatozoa. Up to date, only one calcium transporter has been identified in the plasma membranes of fish spermatozoa. This is a reverse- $\text{Na}^+/\text{Ca}^{2+}$ exchange that causes an efflux of Na^+ and an influx of Ca^{2+} during ligand-induced motility initiation in herring sperm (Vines et al. 2002).

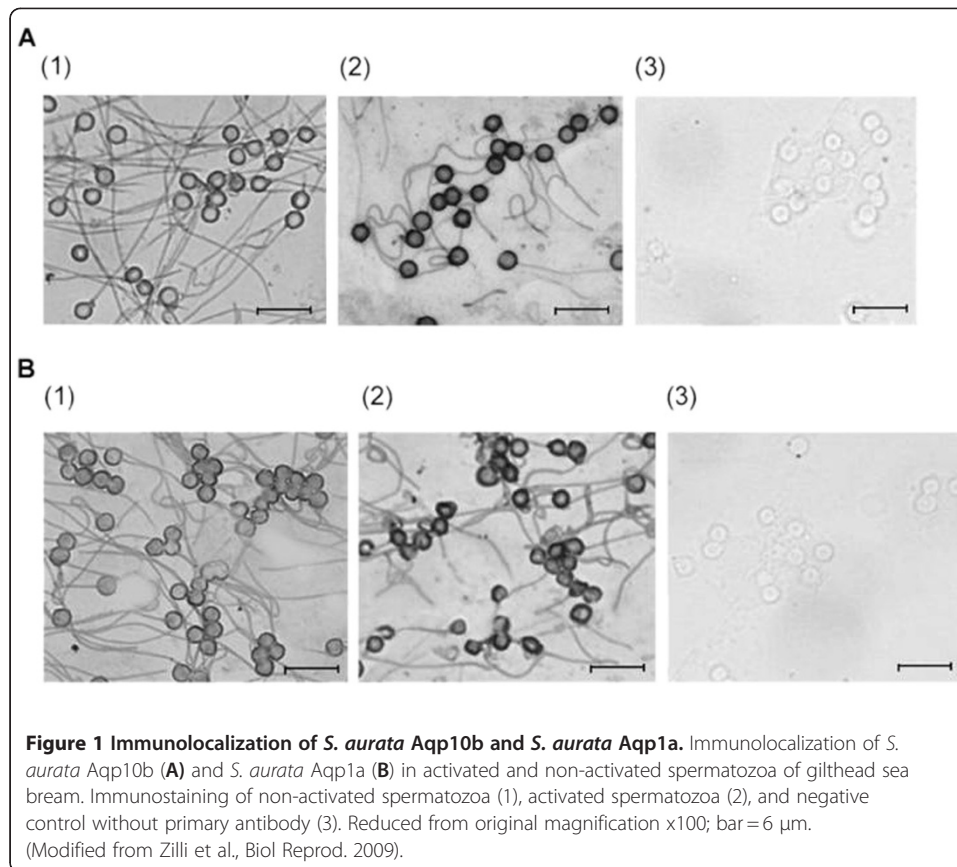
The increase of intracellular calcium concentration could be also a consequence of stretch-activated channel (SAC) activation, when changes in the osmotic pressure occur (Krasznai et al. 2003a; Cosson et al. 2008a, 2008b). It is known that SAC may modify the activity of certain membrane proteins (Vandorpe et al. 1994); therefore, they may increase the calcium membrane conductivity by direct influx of this ion (together with K^+) or by the activation of calcium channels as observed in carp (Krasznai et al. 2003b).

The increase of the intracellular (spermatozoa) calcium concentration, following hyperosmotic signal, could be also due to the release of Ca^{2+} from intracellular stores that has been demonstrated in puffer fish (Krasznai et al. 2003a). Although spermatozoa lack endoplasmic reticulum, it seems that the limited set of organelles that could work as intracellular Ca^{2+} stores (Naaby-Hansen et al. 2001; Ho and Suarez 2003; Publicover et al. 2007). The role of mitochondria in the calcium storage is still unclear. In sea urchin sperm, a Ca^{2+} ATPase (SPCA) has been localized into the giant mitochondrion in the midpiece, thus suggesting a possible role of this organelle as Ca^{2+} store (Gunaratne and Vacquier 2006).

Another membrane protein involved in the sperm motility initiation in marine fish is aquaporin that could determine an increase of intracellular calcium as a consequence of cytosol concentration due to massive water efflux after hyper-osmotic signal (Cosson et al. 1999; Zilli et al. 2009). In particular, two kinds of aquaporins have been identified in gilthead sea bream spermatozoa: aquaporin1a (Aqp1a) and *S. aurata* aquaglyceroporin (Glp); the last one has been recently identified as Aqp10b (Zilli et al. 2009; Cerda and Finn 2010). Aqp1a and Aqp10b are localized in the plasma membrane of the head and flagellum of spermatozoa. Immunostaining technique demonstrated that the expression of aquaporins increases after motility activation, which suggests a possible recruitment of aquaporins into the plasma membrane from intracellular vesicles following hyperosmotic signal (Figure 1). Aqp1a and Aqp10b could play different roles during the process of sperm activation in sea bream. It is suggested that the Aqp1a mediates sperm activation, and Aqp10b involves in the maintenance of motility, as suggested for Aqp7 in human spermatozoa (Saito et al. 2004). The physiological role of aquaglyceroporins during sperm motility in vertebrates, however, is not well understood, although it is known that mammalian spermatozoa are able to use glycerol aerobically (Mann and White 1956; Aalbers et al. 1961) and that organic alcohols (including glycerol) induce protein phosphorylation for motility initiation in chum salmon *Oncorhynchus keta* (Morita et al. 2005).

Flagellar axoneme activation: final event in the mechanism of sperm motility activation in marine fish

The final event in the mechanism of sperm motility initiation is the activation of the axoneme. This is a microtubule-based, highly organized, and conserved structure composed



by more than 250 kinds of proteins. For motility activation, the activity of dynein (the molecular motor) has to be started (and regulated) to produce the coordinated sliding of microtubules in the axoneme (Brokaw 1989; King 2000).

In marine fish, the activation of axoneme is achieved by different mechanisms. In sea bass and tuna spermatozoa, the key factor to start the beating of the flagella is the variation of ionic strength (Alavi and Cosson 2006; Cosson et al. 2008a). In particular, Cosson et al. (2008a) proposed the following model. The water efflux due to the hyperosmotic shock could cause a local membrane distortion that activates SAC. The activation of the SAC could lead to the activation of water channels resulting in rapid release of water from the cells. The result of this process would increase the ionic strength of intracellular fluids leading to the activation of dynein. In flatfish, HCO_3^- ion appears to act directly on the axonemal machinery itself since it inhibits the movement of demembrated spermatozoa (Inaba et al. 2003).

In herring sperm, increasing concentration of calcium ions is the main factor that determines the activation of the axoneme. In particular, sperm motility initiation factor (SMIF) induces calcium influx by opening the voltage-gated calcium channels and activating a reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange (Vines et al. 2002). SMIF determines approximately fourfold increase in Ca^{2+} concentration that acts on the axoneme inducing motility (Pillai et al. 1993; Vines et al. 2002; Cherr et al. 2008).

In some fish species, protein phosphorylation/dephosphorylation is involved in flagellar motility regulation. In puffer fish and tilapia sperm, the activity of the flagellar axoneme is

regulated by Ca^{2+} /calmodulin-dependent protein phosphorylation, while in gilthead sea bream and striped sea bream by cAMP-dependent protein phosphorylation (Morita et al. 2003; Krasznai et al. 2003a; Zilli et al. 2008a, 2009). The major targets of protein phosphorylation/dephosphorylation causing the activation of sperm motility are structural components of dynein arms (inner and outer), kinases, and phosphatases anchored in the axoneme and in the radial spoke proteins (Dey and Brokaw 1991; Hamasaki et al. 1991; Porter and Sale 2000; Yang et al. 2001). Kinases and phosphatases are required for local control of motor activity (Porter and Sale 2000; Aparicio et al. 2007), and radial spoke proteins regulate inner arm dynein by phosphorylation/dephosphorylation (Smith and Lefebvre 1997; Porter and Sale 2000). However, only few proteins involved in the initiation of motility in marine fish spermatozoa have been identified. Morita et al. (2004) have demonstrated, using demembrated tilapia spermatozoa, that calcium not only initiates flagellar motility but also modulates the flagellar waveform. The same researchers identified a Ca^{2+} -binding protein (CaM) with MW of 18 kDa and pI 4.0 that regulates the flagellar motility in a calcium dependent manner by modifying both the sliding velocity and flagellar waveform (Morita et al. 2003, 2004, 2006). The same researchers also identified in tilapia spermatozoa a Ca^{2+} /CaM-dependent protein kinase IV (CaMKIV), localized along the flagellum and sleeve structure, that is involved in the activation and regulation of sperm flagellar motility through a Ca^{2+} /CaM-dependent phosphorylation of seven axonemal proteins. In gilthead sea bream, three proteins have been identified that change their phosphorylation state after sperm activation and play a role in the initiation of sperm motility (Zilli et al. 2008a, 2009):

- (1) An A-kinase anchor proteins (AKAP) have the function of binding to the regulatory subunits (RI and RII) of protein kinase A (PKA) and confining the enzyme to discrete locations within the cell. Therefore, cAMP levels temporally regulate PKA, whereas the spatial regulation within the cell occurs through compartmentalization by binding to AKAP, thus assuring specificity of PKA function. The role of AKAP as a key regulator of sperm motility has been already established (Vijayaraghavan et al. 1997). In addition, a recent study demonstrated that phosphorylation of AKAP in human sperm results in tail recruitment of PKA and increase of sperm motility, providing evidence for a functional role of phosphorylation of AKAP (Luconi et al. 2004);
- (2) The acetyl-CoA synthetase activates acetate to acetyl-CoA, and provides the cell with the two carbon metabolite used in many anabolic and energy generation processes. Therefore, this enzyme could be activated in motile sperm to increase the level of ATP, which is necessary for flagellar movement;
- (3) A novel protein similar to phosphatase and actin regulator 3 of *Danio rerio* that may be a protein phosphatase inhibitor.

In striped sea bream, two proteins involved in the activation of sperm motility have been identified: myotubularin-related protein 1 and dual-specificity tyrosine phosphorylation-regulated kinase 3 (DYRK3) (Zilli et al. 2008a). The myotubularin-related protein 1 belongs to the protein-tyrosine phosphatase family, and DYRK3 is a protein kinase auto-phosphorylated on tyrosine residues belonging to the dual-specificity tyrosine phosphorylated and regulated kinase family. Many studies have demonstrated that the development and maintenance of motility is regulated by a complex balance

between kinase and phosphatase activities (Tash and Bracho 1994; Porter and Sale 2000, King 2000; Aparicio et al. 2007).

From the above report, it clearly emerges that there are different mechanisms of sperm motility initiation in marine fish. They are species-specific, and they reflect the adaptation to species life histories/environment, and only parts of which have been studied in the different species. The identified proteins that play a role in this mechanism in marine fish are summarized in Table 1, where possible homologues in freshwater fish, invertebrate, and mammalian have been also reported.

***S. aurata* spermatozoa as a model to study the molecular mechanism of sperm motility activation**

The molecular mechanism that determines sperm motility activation in gilthead sea bream has many similarities with the mechanisms observed in many animals (sea urchin, salmonids, and mammals). This is not surprising since that internal microtubule-based structures of the axoneme have been well conserved during evolution. For this reason, the identification of proteins that change their phosphorylation state following sperm motility activation and the understanding of signaling pathways among these in gilthead sea bream spermatozoa could be interesting to clarify this process in other species.

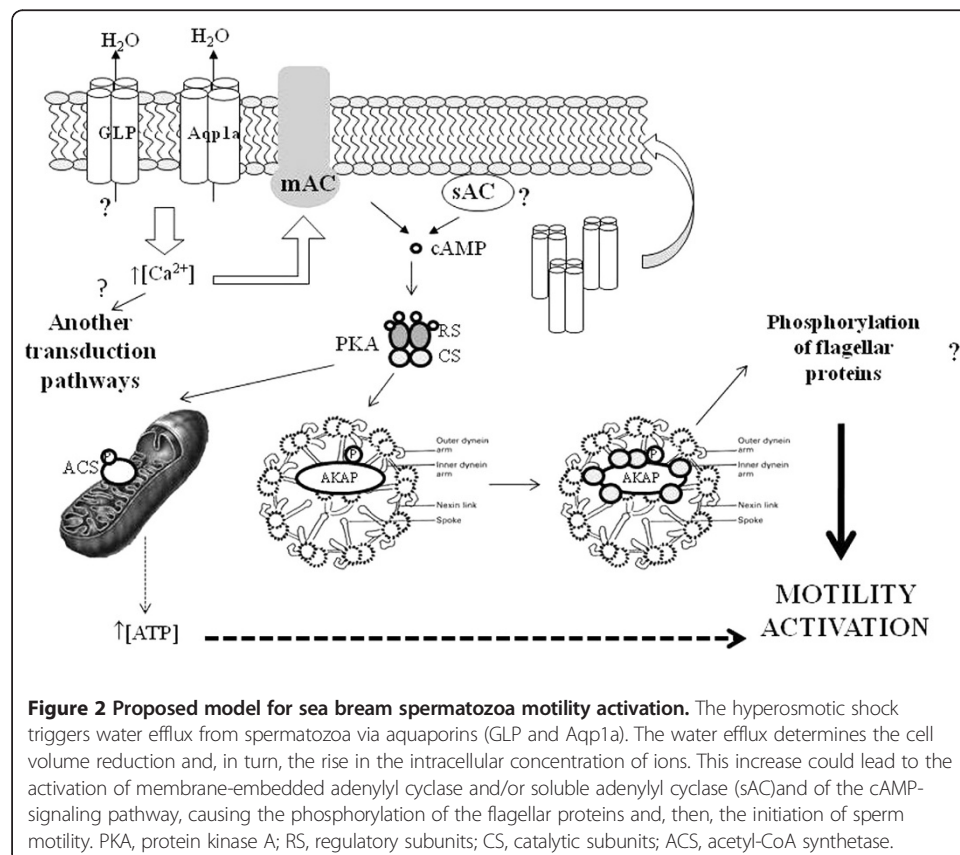
In *S. aurata*, a drastic change of the environmental osmolality is the signal that triggers sperm motility activation. The transduction of this event in axoneme activation requires action of many intracellular mediators. First event is the water efflux that leads to local distortions of the flagellar membrane that, in turn, activates water channels. Recent study (Zilli et al. 2011) confirms the important role of aquaporins in initiating sperm motility; in fact, when these proteins are inhibited by HgCl₂, the phosphorylation of some proteins (174 kDa protein of head; 147, 97, and 33 kDa proteins of flagella), following the hyper-

Table 1 Proteins involved in sperm motility activation and their homologues in fish, invertebrates, and mammals

Protein	Marine fish species	Fresh water fish species/ invertebrate and/mammalian homologues
Ca ²⁺ -binding protein	<i>O. mossambicus</i> (Morita et al. 2009)	<i>Homo sapiens</i> (Marín-Briggiler et al. 2005); <i>A. digitifera</i> (Morita et al. 2009); <i>Ciona intestinalis</i> (Nomura et al., 2000, 2004);
Ca ²⁺ /CaM dependent protein kinase IV	<i>O. mossambicus</i> (Morita et al. 2006)	<i>H. sapiens</i> (Marín-Briggiler et al. 2005); <i>A. digitifera</i> (Morita et al. 2009)
A-kinase anchor proteins	<i>S. aurata</i> (Zilli et al. 2008a)	<i>H. sapiens</i> (Carr et al. 2007; Luconi et al. 2011); <i>Bos taurus</i> , <i>Mus musculus</i> (Moss et al. 1999)
Acetyl-CoA synthetase	<i>S. aurata</i> (Zilli et al. 2008)	–
Novel protein similar to phosphatase and actin regulator 3 of <i>D. rerio</i>	<i>S. aurata</i> (Zilli et al. 2008)	–
Myotubularin-related protein 1	<i>L. mormyrus</i> (et al. 2008)	–
Dual-specificity tyrosine phosphorylation-regulated kinase 3	<i>L. mormyrus</i> (Zilli et al. 2008)	<i>Rattus norvegicus</i> (Becker et al. 1998)
Aquaporin	<i>S. aurata</i> (Zilli et al. 2009)	<i>H. sapiens</i> , <i>M. musculus</i> (Chen et al. 2011); <i>B. taurus</i> (Ma et al. 2011); <i>Macaca radiata</i> (Shayu et al. 2005)

osmotic shock, is also completely or partially inhibited. However, more than one transduction pathways could be activated when sea bream spermatozoa are ejaculated in seawater, since numerous proteins showed an HgCl₂ (Aqps)-independent phosphorylation state after sperm activation.

As reported in Figure 2 in gilthead sea bream spermatozoa, the rapid water efflux across AQP's determines a reduction in cell volume with the increase in intracellular ionic concentration. It is known that adenylyl cyclase is activated by different mechanisms, such as membrane hyperpolarization (Beltran et al. 1996; Izumi et al. 1999) and/or increase in Ca²⁺ and HCO₃⁻ concentration (Visconti and Kopf 1998). The cAMP signaling pathway starts the activation of sperm motility by phosphorylation of some proteins. This post-transductional modification in sperm motility activation was recently (Zilli et al. 2011) confirmed by observation that a higher number of protein bands underwent a change of their phosphorylation state at flagella level with respect to the head level in gilthead sea bream. However, it must be underlined that the proposed model fits well to the gilthead sea bream sperm activation but cannot generalize to other marine fish species. A cAMP-dependent protein phosphorylation, involved in sperm motility activation, has been also demonstrated in chum salmon (Itoh et al. 2001) and trout (Hayashi et al. 1987). In many species, some proteins phosphorylated in PKA-dependent manner have been identified as the light (from 8 to 30 kDa) or heavy (approximately 500 kDa) chains of the outer arm dynein of sperm flagellum, such as the 21 kDa protein of chum salmon (Inaba et al. 1999), the 32 and the 500 kDa proteins in sea urchin spermatozoa (Bracho et al. 1998), the 21 and the 26 kDa proteins in ascidian



spermatozoa (Nomura et al. 2000), the 27 and the 20 kDa proteins of mussel spermatozoa (Stephens and Prior 1992), and the 18 to 20 kDa protein in *C. intestinalis* sperm (Dey and Brokaw 1991). In addition, in salmonid fish, a 48 kDa protein, phosphorylated in a cAMP-dependent manner, was identified as regulatory subunit of PKA (Itoh et al. 2003). In *S. aurata*, an AKAP protein that anchors the regulatory subunit of PKA for tethering of protein kinases in close proximity to their target proteins has been identified. Different types of AKAP have been found in spermatozoa, localized into the fibrous sheath of the principal piece (Moss and Gerton 2001). In mammals, it has been demonstrated that, among the proteins phosphorylated during epididymal maturation, there are several mitochondrial proteins (Aitken et al. 2007) and a protein phosphatase PP1 γ 2 (Chakrabarti et al. 2007). This is in agreement with a previous finding regarding proteins phosphorylated after motility initiation in gilthead sea bream spermatozoa that are precisely one mitochondrial protein (acetyl-CoA synthetase) and one protein that may be a protein phosphatase inhibitor; in addition in striped sea bream, two proteins were identified, a phosphatase and a kinase, that are involved in sperm motility activation (Zilli et al. 2008a). The activation of proteins of sperm mitochondria could be important to provide the energy for sperm motility; in fact, fish sperm quality is correlated with ATP content (Christen et al. 1987; Zilli et al. 2004). Mature spermatozoa are highly specialized cells, transcriptionally inactive and unable to synthesize new proteins; for this reason, protein phosphorylation/dephosphorylation has to rely on regulation of many processes that is greater than in many other types of cell (Urner and Sakkas 2003).

Conclusions

From this review emerges plainly that a complex universal mechanism for sperm motility initiation in marine fish does not exist, but there are different mechanisms that are species-specific, only parts of which have been studied in the different species. In particular, in some of these species (puffer fish, tilapia, gilthead sea bream, and striped sea bream), protein phosphorylation/dephosphorylation has been shown to be involved in flagellar motility regulation and present many similarities with the mechanisms of axoneme activation of marine invertebrate and mammal spermatozoa.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZL has made substantial contributions to conception and design. SR and SC have been involved in drafting the manuscript. VS has been involved in revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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