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Marine fish spermatozoa: racing ephemeral swimmers

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Abstract:

After a long period of spermatogenesis (several weeks to months), marine fish spermatozoa are delivered at male spawning in seawater (SW) at the same time as ova. In some fish species, as the ova micropyle closes quickly after release, these minute unicells, the spermatozoa, have to accomplish their task of reaching the micropyle within a very brief period (several seconds to minutes), for delivery of the haploid male genetic information to the ova. To achieve this goal, their high-performance motile equipment, the flagellum, must fully activate immediately on contact with the SW and then propel the sperm cell at an unusually high initial velocity. The cost of such 'hyperactivity' is a very rapid consumption of intracellular ATP that outstrips the supply. The spermatozoa become rapidly exhausted because mitochondria cannot compensate for this very fast flagellar energy consumption. Therefore, any spermatozoon ends up with two possibilities: either becoming exhausted and immotile or reaching the egg micropyle within its very short period of forward motility (in the range of tens of seconds) before micropyle closure in relation to both contact of SW and cortical reaction. The aim of the present review is to present step by step the successive events occurring in marine fish spermatozoa from activation until their full arrest of motility. The present knowledge of activation mechanisms is summarized, as well as a description of the motility parameters characterizing the motility period. As a complement, *in vitro* results on axonemal motility obtained after demembranation of flagella bring further understanding. The description of the sperm energetic content (ATP and other high energy compounds) and its evolution during the swimming period is also discussed. A general model aiming to explain all the successive cellular events occurring immediately after the activation is presented. This model is proposed as a guideline for understanding the events governing the sperm lifespan in the marine fish species that reproduce through external fertilization.

Keywords: marine fish, spermatozoon, motility, cell energetics, ions, osmolality, cell activation

Introduction

Marine fish spermatozoa are flagellated unicells, which have to deal with an extremely hostile environment right at delivery in seawater. During whole spermatogenesis, they have a safe environment, both surrounded and nourished by Sertoli cells and endocrine cells (Leidig cells) combined with non harmful fluid (seminal plasma). During spermatogenesis, sperm cells are prepared for accomplishing their fertilizing task for which they need to fully exploit their swimming ability immediately and as fast as possible to encounter the egg.

Most knowledge on sperm movement developed by simple flagella comes from studies on the classical model of sea urchin spermatozoa (Gibbons 1981a) and on mammals for structurally more complicated sperm cells. Nevertheless, some characteristics of fish sperm show original features: motility duration (Billard 1978; Billard and Cosson 1988 ; 1992), motility initiation (Morisawa 1985 & 1994; Cosson et *al*. 1995a) or motility pattern (Boitano & Omoto 1992; Cosson *et al*. 1997). Most studies were carried out in fresh water fish species, less information concerns marine sperm characteristics. In the latter, motility activation occurs immediately after contact with seawater, a high osmolarity medium compared to seminal fluid (SF). The initial velocity is very high right at activation, but motility duration lasts for periods ranging only 40 sec. to 20 min. as an energetic consequence of the high velocity.

Marine fish spermatozoa present unique features with which to study specific aspects of sperm movement: 1- Brood fish is easily available for part of the year in farmed species. 2- Fish sperm is easy to collect and to save for short term ; marine fish sperm are easy to cryopreserve. 3- Sperm of fishes with external fertilization being immotile in the SF, transfer in a swimming competent medium fully triggers its motility, which scores are currently used for selection of males, or for evaluation of cryopreservation results. A correlation between sperm motility and ability to fertilize the eggs has been established in many marine fish species. 4- Fish sperm cells are homogenous: all spermatozoa can be activated at the same time and then swim with very similar characteristics at a certain time point post-activation, an advantage for biochemists. 5- In many fish species, the flagellum is 50-60 µm long with a ribbon shape (presence of fins) instead of cylindrical : thus, the flagellum appears brighter by dark-field, which allows clear visualization of wave shapes. Attenuation (so-called dampening) of waves gradually invades the whole length of the flagellum during motility. At the last period before full stop, the waves are restricted to one third of the flagellum combined with a drastic decrease of flagellar beat frequency leading to a decrease of translation efficiency (Cosson *et al*. 1997). 6- In several fish species, spermatozoa follow linear tracks: the flagellar bending is symmetrical probably because of the absence of Ca^{2+} sensitivity of the axoneme.

Despite all these original features, few detailed studies on marine fish spermatozoa flagellar motility behavior were conducted. In the present paper, we aim to gather the present knowledge on sperm movement characteristics of marine fish, with special emphasize on their high velocity capacities. A relationship between ionic effects, osmolality and transcience/abortion of motility is established. Using this indicator of the local and temporal ionic concentration, we propose a model in which at transfer from SF to SW, the extra cellular osmolality makes the intracellular ionic concentration of sperm rapidly evolving during the course of the motility phase; as a consequence the flagellar axoneme immediately activates but becomes gradually exposed to an increasing intracellular ionic environment preventing the development of distal waves and further leading to their full arrest. For general information on fish sperm, the reader is advised to consult the folowing review papers : Stoss 1983 ; Billard *et al*. 1994 & 1995 ; Inaba 2003 ; Alavi & Cosson 2005 & 2006 ; Lahnsteiner & Patzner 2007) and a book, Fish Spermatology (Alavi *et al*. 2007).

Discussion

Spermatozoa of marine fishes reproducing by external fertilization are delivered in sea water (SW) at spawning. Oubviously these individual unicells have to cope immediately with SW (a medium external to the wheedling fish fluid) which is very harmful due to its high ionic concentration constituting a high osmolarity medium compared to the sperm cytoplasm.

The seminal fluid osmolality prevents sperm motility in the genital tracks.

In marine fish, the seminal fluid (SF) osmolality is much lower than that of SW and low enough to prevent motility (Table 1a). In addition, the sperm cells concentration in the semen is usually high (see Table 1a) which thus contributes to sperm immotility by local exhaustion of O_2 and generation of high CO_2 concentration in milt. In some species, spermatozoa have the ability to swim in seminal SF, sometimes only transiently. Motility also occurs when SF is slightly diluted by SW, which may accidentally happen at sperm collection. In turbot sperm, a 10% dilution of SF is enough to allow full motility. Contamination by urine may also accidentally happen when collecting sperm (Perchec-Poupard *et al*. 1998 ; Perchec *et al*. 1995) : in turbot (Dreanno *et al*. 1998), such urine contamination leads to deleterious effects to spermatozoa.

The ability for spermatozoa to swim is eventually dependent of their maturation in the ducts, prior to ejaculation. This maturation is frequently controlled in an hormonal manner and by adjustment of ionic concentration in the SF; such maturation can be induced *in vitro* (Redondo-Muller *et al*. 1991). This sperm maturation process studied in turbot (Suquet unpublished) and in sea bass (Dreanno *et al*. 1999) is not dependent on internal ATP stores.

The sperm immotility in SF suffers of a few exceptions : in sharks (dogfish, *Triakis scyllia*) spermatozoa are immotile in testis but become progressively motile in the spermiduct during their descent in the epididymal duct ; nevertheless ejaculated sperm are immotile while motility is fully triggered only at contact with SW (Minamikawa and Morisawa 1996).

Transfer from seminal fluid into seawater triggers full motility

Hypertonicity induces the motility of spermatozoa in marine teleosts : in cod, *Gadus morhua* macrocephalus (Westin & Nisling 1991), in flounders, *Limanda yokohamae* and *Kareius bicoloratus* (Morisawa & Suzuki 1980), in sea bass, *Dicentrarchus labrax*, in sea bream, *Sparus auratus* (Billard 1978), in gray mullet, *Mugil cephalus*, in *Trachurus mediterraneus*, in *Mullus barbutus*, in *Boops boops*, in *Diplodus sargus* (Lahnsteiner & Patzner 1998) and in the goby, *Gillichthys mirabilis* (Weisel 1948). Demonstration that osmolality is a key factor for motility activation comes from experiments using hypertonic sugar solutions (1 M and without any ions) instead of SW as triggering solution: this is shown in halibut (Billard *et al*. 1993), turbot (Chauvaud *et al*. 1995), sea bass (Dreanno *et al*., 1999b), tuna (Cosson *et al*. 2008), cod and hake (Cosson *et al*. 2007), sea bream (Cosson *et al*. 2008) as well as in mullet (Lee *et al*. 1992). When Na^+ ions of SW are substituted by choline (choline chloride 0.5 M), motility activates as well (Cosson *et al.* 2008). Experimental conditions can visually demonstrate that the SW activation process is fully and immediately reversible. Native turbot spermatozoa briefly exposed to condition where no motility was possible (4-fold diluted SW, immobilization medium (IM), or media below 300 mOsm/kg) are able to immediately initiate full motility upon transfer in a swimming medium (SM), i.e. SW. At transfer of the same sperm cells from SM back to IM, motility fully stops within a fraction of a second.

Tolerance of motility towards osmolality depends on species : motility is initiated at osmolality (in mOsm./L) of 300 or above in turbot (Dreanno *et al*. 1999c ; Chauvaud *et al*. 1995) ; 400 in sea bass (Villani & Catena 1991 ; Dreanno *et al*. 1999b) ; 333 to 645 in tilapia, *Sarathoredon melanotheron* (Linhart *et al*. 1999) but, according to recent results

(Legendre *et al*. 2007), 300 to 970 for fishes reared in SW (450 to 1600 for fishes reared in twice the salinity of SW), 480 in Atlantic croaker (Vizziano *et al.* 1995). In halibut, NaCl solutions with osmolalities 350 to 1200 mOsm/L also permit motility (Billard *et al*. 1993). At 300 mOsm./L, a lower percentage of turbot spermatozoa are activated (70% compared to 90% in control at 1100 mOsm./L) but velocity is same as in control. In sea bass, activation also occurs at osmolality lower than SW; at 630 mOsm./L there is no change of initial velocity but at 40 sec, flagella produce only oscillations without resulting in any efficient forward displacement. SF separated from turbot milt (316 +/- 1.5 mOsm./L) is permissive to motility in many samples, but CO_2 concentration is the main factor preventing motility in SF with total CO_2 concentration of 8.97 +/- 1.53 mmol./L , or 8.66 +/- 1.48 meq./L of HCO3 (at a pH of 7.58 +/- 0.03), pCO_2 being 6.74 +/- 1.15 (Dreanno 1998).

In sea bass, solutions of 150 to 300 mOsm./L reversibly prevent motility but full activation occurs at 1100 mOsm./L (Dreanno *et al*. 1999b ; Fauvel *et al*. 1998). In cod, sperm motility does not activate in a 2 fold dilution of SW by fresh water; therefore this solution can be used as a short term diluent. The motility is activated by transfer in artificial SW solutions with osmolalities from 700 to 1550 mOsm/L. Sperm is not active in SF but when the SF is diluted 1:4 by SW, full flagellar activation occurs (Cosson et al. 2008). In hake, a diluent made of SW :DW in a 1 :4 ratio does not activate motility, but allows further full activation by undiluted SW (Cosson *et al*. 2007).

A general rule for sperm of marine species is that the osmolality gradient must be positive between outside and inside sperm cells to trigger motility. Although a brief and transient activation is observed, when the amplitude of the difference of osmotic pressure (OP) is too low : it consists of stochastic initiation of a few flagellar waves during brief time period (about 1 sec.) in any individual spermatozoon intercalated with very long resting periods. Similar process probably exists in cases where semen is contaminated by low quantities of SW; motility is transiently but not fully activated, therefore such sperm do not exhaust their ATP content as discussed later in details in this review. Such transient activation already demonstrates the reversibilty of the activation process but this reversibility can also be appreciated in the more direct experiments which follow (Cosson et al. 2008). Sperm cells were diluted in an immobilizing solution and injected through a tiny micropipette (20 µm diam. and connected to a syringe providing low pressure) into a 100 µl drop of SW settled in between glass slide and coverslip. By observation with a microscope focused on the boundary between the SW and the injected immobilization solution (IS), each individual spermatozoon can be seen alternatively active or inactive depending whether it is in contact SW or IS respectively. This shows that activation and inactivation periods follow each other which demonstrates both full reversiblity and immediacy of the triggering/inactivating mechanism. Using a similar experimental design (Fig 1), one can deliver a gentle but local flow of SW through a tiny micropipette (2 µm opening) to any portion of the flagellar length of a sperm cell, the later being stuck by its head to a second holder micropipette (3-4 µm opening) itself immersed in a drop of IS. This way, local activation and inactivation can thus be visualized successively at any site located along the flagellum.

Nevertheless, the general model based on osmolality control of marine fish sperm motility activation has exceptions. Combined with osmolality, some species with external fertilization control sperm motility by CO_2 via carbonic anhydrase (Inaba et al 2003) as observed in many flatfish species. Other species like herring control sperm motility through the contact with some sperm initiation factor secreted by the egg of the same species. Both examples are further detailed in this review.

An ambiguity is encountered in the case of the sperm of the wolffish (*Anarhichas lupus*) which is motile on stripping and remains as such for several days : motility is restricted to a 200-500 mOsm/L osmolality range. It has been suggested that motility is adapted to the local

environment of the egg which in this species presents osmolality lower than the surrounding SW (Kime & Tveiten 2002) which could be related to the fact that fertilization seems to be close to « internal » in this species (Pavlov 1994; Pavlov *et al*. 1997).
Concerning pH, it is of low influence on motility and therefore pH is considered as a critical factor controlling motility. Values of pH in SF of several species are presented in table 1a.

Immediately after activation, marine fish sperm swim with very high efficiency
For any spermatologist observing marine spermatozoa through a microscope right after activation by SW, it is striking that, by any appreciation parameter (even just « visually ») all spermatozoa appear very « fast » swimmers. This impression is confirmed by many quantitative assessments of motility parameters (Cosson 2007a). Experimental values of the initial sperm speed of forward displacement in various species confirm a high velocity (expressed in µm/sec): 150-180 in halibut (Billard *et al*. 1993 ; Veirmeirssen *et al*. 2004), 160 in fugu (Takai & Morisawa, 1995), 130 (Cosson *et al*. 2007) or 65-100 (Trippel & Morgan 1994) in cod, 130 in hake (Cosson *et al*. 2007 ; Groison *et al*. 2007), 215-230 in tuna (Cosson *et al*. 2008), 220 in turbot (Dreanno *et al*. 1999c ; Cosson *et al*. 1997) and 120 (straight line velocity) in sea bass (Dreanno *et al*. 1999a & b), similar to values published by Abascal *et al*. (2007). However, these velocity characteristics may be modulated by sperm micro environment and particularly pH and osmolality of the swimming medium. Some discrepancies (as observed for cod sperm) probably come from the temperature at which measurements were obtained (Alavi & Cosson 2005), 22°C in the case of Cosson *et al*. (2007) compared to 2°C in the case of Trippel and Morgan (1994). Biophysical prediction is that the velocity value is more than doubled when the temperature is rised by 10°C.
These high velocity values are a consequence of the high flagellar beat frequency (BF) ranging 50 to 70 Hertz depending of species. Such high BF values are reminiscent of the so-called « hyperactivation » process occurring to mammalian spermatozoa in the vicinity of ova, which consists in change of activity,. However, in case of fish, spermatozoa present movement characteristics much less chaotic, i.e. much more propulsive in a straightforward manner, probably corresponding to different need and function.

The motility period is limited to minute range duration for marine fish's sperm.
Even though, sperm motility has a longer duration for marine fishes compared to fresh water species. This is mostly related to osmotic shock leading to membrane damage. The total duration of flagellar activity, including progressive and non progressive forward motility is quite short, ranging minutes to tens of minutes, which is very low compared to mammalian sperm or invertebrates spermatozoa (sea urchin or oyster sperm) which can swim for several hours.
The total duration of motility can be estimated either by visual microscopic observation of movement until full cessation of activity or by extrapolation to zero of the curve representing the percentage of motility vs time obtained by CASA (Computer Assisted Sperm Analysis ; Cosson, 2007a). The latter estimation is easiest in case of a linear decrease but more difficult in case of exponential or sigmoidal decrease. Table 1b gives values for the two types of estimation. By contrast to motility durations described in table 1b, eel spermatozoa can swim for more than 20 min with little change in their motility characteristics (Gibbons *et al*. 1985). The same is true in congers spermatozoa (Cosson et al. 2008).
It has been demonstrated that the duration of motility is temperature dependent and species-specific, varying from approximately 1 min for *Limanda yokohamae* and *L. herzensteini*; 2 min for *V. moseri*; 5 min for *P. maximus* and 7-10 min for *V. variegatus,* to an hour in cod (Trippel & Morgan 1994) at 2°C in the latter case. In hagfish, it was observed that sperm motility can last for periods up to 10 min. (Morisawa 1995).

Motility duration is also limited by damage appearing during the motility period. These damages appear in SW during the motility phase: they can be observed at high microscopical magnification. Either cytoplasmic blebs emerge anywhere along flagellar length, eventually impairing the propagation of waves (f to i in fig. 2A) or a curling process (i in fig. 2A) may develop at the flagellar tip which obviously shortens the efficient part for waves propagation along the flagellum. Such curling is reminiscent of the naturally occurring curling (helicoidal) observed in intact eel flagella (Gibbons *et al*. 1985). Such blebs or curling damages usually result from local membrane defects engendered by osmotic stress and they are usually reversible by reversing the osmolality of the surrounding solution to correct values (Perchec *et al*., 1996). Such damages may occur when milt happen to be contaminated by urine at collection by stripping (Dreanno *et al*. 1998 ; Perchec *et al*. 1998).

Most motility parameters are decreasing during the motility period.
Several methods to measure motility parameters have been recently described, some of which, called CASA, enable automatic and statistical analyses (Cosson 2007a). Most of the parameters used to characterize motility decline within tens of seconds to few minutes depending on species and this general decrease leads to eventual full arrest of spermatozoa (Cosson *et al*. 1997 ; 1999). When plotted as a function of time, the percentage of motile cells, the beat frequency and the velocity show differences between species in the time scale but general tendency is exhibited in all cases : a high initial value followed by a decline during the motility period.
Additional methods, designed to describe the details of waves parameters were initially developed to study sperm flagella of invertebrates (Brokaw 2004) and were adapted to marine fish spermatozoa (Cosson 2004 ; 2007a). In sperm cells from marine fishes, the initiation of the flagella waves occurs just at the junction between head and tail, therefore waves propagation occurs from head to flagellar tip, leading to opposite forward movement of the spermatozoon, head first (Cosson 2007b). Waves velocity along the flagellum ranges 2 to 3 times the translational velocity of the spermatozoon itself.

Wave shape is changing during the motility period:
In fish spermatozoa, the wave shape is of the type "arc sine" i.e. linear segments intercalated between 2 curvatures, similar to what occurs in tunicate sperm flagella (Brokaw 1991b) or sea urchin. At initiation of motility, several successive waves occupy the whole flagelar length. The wave shape is resulting partly from the physical constraints imposed by the external milieu and can be affected by one important physical parameter, the viscosity : this is observed when methyl cellulose (MeCell ; Brokaw 1968), a high molecular weight polysaccharide (nonpermeant through membranes) is included in the SM, SW in this case. When MeCell is included at 0.4 to 1% (w/v), viscosity increases from 10.86 (pure SW) to 76.3 centipoise (1% w/v MeCell in SW). This results in a strong decrease of velocity due to a large increase in the number of curvatures along the flagellum as illustrated in figure 3: from 5-6 in normal SW (fig. 3c) the number of curvatures rises up to 10-11 (fig. 3f) in viscous solution ; figure 3b shows exponential increase of viscosity when increasing of MeCell concentration ; the later obviously leads to decrease of other parameters : wave length or amplitude and consequently velocity (not detailed). As similar effects of MeCell are observed neither with turbot demembranated spermatozoa (Cosson *et al.* 2008) nor with sea urchins or tunicates sperms (Brokaw 1966), MeCell effects could result from the ribbon shaped flagella specific to native fish spematozoa due to the presence of fins which greatly increase the surface of viscous interaction with the surrounding medium. Thus, viscosity increase leads to an increase of waves number in native spermatozoa of cod and sea bass (Cosson *et al.* 2008). Such viscous medium mimicks the situation occurring for sperm cells

in fluids such as ovary fluids or jelly like layers which surround eggs in some fish species.
A major and general change occurring to the wave shape of marine fish spermatozoa in sea water is the dampening process : it is accompanied in some species by asymmetry of beating. Flagellar waves are almost planar, i.e. each sine wave is « flat » and the successive waves are coplanar. The waves are also most of the time coplanar with the plane of observation, which is the focal plane of the objective lens and which usually coincides optically with the glass slide plane as well as that of the coverslip. An exception to waves flatness is found in eel spermatozoa in which waves are of corkscrew shape, i.e. helicoidal (Gibbons *et al*. 1985 ; Wooley 1997 ; 1998). Even though, eel spermatozoa swim with very high beat frequency 95 Hz, their original wave pattern of helicoidal shape is poorly efficient in terms of forward velocity. They mainly present a rolling motion at 19 Hz and flagella develop a 3D bending, recently detailed by Wooley (1998).
A more detailed analysis of the exact shape of the flagellum during motion can be obtained by the determination of the local curvature of the flagellum and a plotting of the curvature versus the distance on the flagellum (Cosson 2004).
The flatness of waves is not perfect, but waves slightly deviate from the plane : this was shown in sperm flagella of several species including marine fishes (Cosson *et al*. 2003). Such slight distortion is in shape of alternating helical segment and designed in such a way that each linear segment intercalated in between two successive curvatures generate a thrust which is not coplanar with the main beating plane : as a result, while swimming, sperm cells have the tendency to be pushed by their flagella toward surfaces and then to remain swimming in the vicinity of these surfaces. This presents an advantage for observers because sperm cells remain in focus very close to the glass slide plane (usually the focal plane of the microscope). But the drawback is that a majority of spermatozoa swim in the vicinity of these surfaces which leads to a bias when counting moving spermatozoa by automatic techniques such as CASA. Such an ability to swim in the vicinity of the egg surface can be a biological advantage for fertilization efficiency. Swimming close to surface frequently leads to the adhesion of sperm to glass ; in order to make sure that spermatozoa can swim *freely*, prevention of sticking to glass surfaces can be ensured by addition of BSA (bovine serum albumin at 0.1 to 0.5 %) or Pluronic F-127 (at 0.1% from Sigma).
Another type of wave distortion is also observed when turbot or sea bass sperm are exposed to pollutants such as mercury derivatives (see details in fig. 5).

The symmetry of flagellar waves and their dampening usually evolve during the motility period

Another characteristic of beating flagella is the ability to develop either symmetric or asymmetric ways of beating: waves follow each other and successive waves are called direct and reverse waves (Gibbons 1981). When both are of equal curvature, symmetrical movement is developed and forces the sperm cells to describe linear tracks. In case of unequal curvature, movement becomes consequently circular and sperm cells describe circles of corresponding diameter (Brokaw 1991a). This is observed in sea bass sperm flagella, *in vivo* as well as *in vitro* (Dreanno *et al*. 1999b) and in tuna sperm (this paper). In all cases, asymmetry of beating is related to Ca^{2+} regulation.
During the motility period, the wave pattern of fish spermatozoa rapidly evolves (fig. 2) successively from a fully beating pattern where waves propagate along the whole long of the flagellum, to a partially beating pattern where waves occupy only the portion of the flagellum proximal to the head, finally leading to full absence of any beating wave (Cosson *et al*. 1997 ; 1999). The blocking process occurs to the distal part of the flagella at precise time post activation (20-30 s or later depending of species). The distal part of the partially beating flagellum appears straight, rigid and devoid of any propagating wave (fig. 2). The

fully developed waves are initiated and propagated in the proximal segment of the flagellum over a distance covering one third to one fourth of the total length (fig. 4) . Such decrease of the wave amplitude along flagellum from proximal to distal part is called « wave dampening ». Flagellar wave dampening also occurs in invertebrates spermatozoa and was described more extensively by Tombes *et al*. (1987) in sea urchin flagella. In turbot spermatozoa, the wave dampening is also induced *in vitro* on demembranated flagella by the non adequate ionic strength (fig. 4) : in contrast, ionic concentration shows little effects on trypsin induced microtubule sliding (an *in vitro* measurement of dynein activity), on beat frequency and on proximal wave amplitude. In turbot sperm, this dampening is not related to Ca^{2+} induction of beat asymmetry as no sensitivity to this ion have been observed. Dampening occurs because waves persist preferentially in the portion of the flagellum close to the head. The observations of wave dampening obtained *in vivo* and *in vitro* on turbot sperm flagella (fig. 4) show traits common to sperm of most other fish species (Cosson *et al*. 1997 & 1999).

The rigidification process following dampening observed for turbot spermatozoa by Chauvaud *et al*. (1995) has been hypothetically related to the energy distribution in the flagellum at the end of the motility period (see paragraph on energetics): the ATP level would then be lower than initially, while that of ADP would be higher, as a consequence of ATP hydrolysis during the motility phase. Published results show that all the elements necessary for the functioning of a Phospho Creatine (Pcr) shuttle are present in turbot as well as in trout spermatozoa (Saudrais *et al*. 1998). This shuttle would allow a more homogeneous distribution of ATP along the flagellum. However as emphasized above, dampening also occurs *in vitro* in demembranated spermatozoa where no such shuttle can be active. In the case of turbot sperm, a similar sequence of wave dampening leading to full stiffening can also be precociously induced by CO_2 application (Dreanno *et al*. 1995 ; Inaba *et al*. 2003). Independently, it is also possible to trigger dampening *in vivo* in demembranated/reactivated flagella by adjustment of ionic concentration as detailed below. In the latter situation, ATP concentration is not responsible because, *in vitro*, it remains constant and at a value close to 1 mM as explained in a next paragraph.

Flagellar beat frequency and efficiency both decrease during the motility period

The beat frequency (BF) represents the number of waves generated every second ; the BF is directly in proportion to the activity of dyneins and therefore of the rate of ATP hydrolysis. In the six species described in this paper, the BF shows high values at initiation of motility, but decreases rapidly as a function of time.

Fish sperm advantageously exhibits a high homogeneity of movement in the sperm population at a given time point, i.e. the successive images of one single sperm cell are representative of the majority of the population but this is only true if considering any defined time point after activation. This is mainly obtained by the use of a double dilution procedure (first dilution of milt in a non swimming solution followed by a second dilution in the swimming medium), which allows homogeneity and synchrony in the motility initiation for the whole population of sperm cells.

Within a very brief period, i.e. less than 3-5 seconds, the minimal period of time required to achieve the second dilution and mixing, motility is initiated for almost 100% of the spermatozoa. This initial swimming period, which lasts 5 to 60 s depending on the species, is characterized by the high BF (up to 70 Hz) of fully developed waves proceeding throughout the whole flagellar length with an almost constant amplitude (fig. 2 & 4). These characteristics are similar to those of the sea urchin's sperm flagellum which are commonly used as a model for sperm movement studies (Gibbons 1981). In sea urchin, such behavior is constantly exhibited for very long periods of time, i.e. hours, with BF of 45 Hz. The wave

dampening features mentioned for turbot sperm as example occurs in all teleosts fish spermatozoa so far studied (Cosson *et al*. 1997 & 1999). After a first period post activation, fish sperm show a decrease not only in BF in the case of *O. mykiss* sperm (Cosson *et al*. 1985 ; 1991), for *A. baeri* sperm (Cosson *et al*. 1995b) and for *S. maximus* (Chauvaud *et al*. 1995) but also in wave amplitude (WA) in the distal portion of the flagellum (Cosson *et al*. 1999) as clearly demonstrated in turbot (fig. 2).
After a first period post activation, fish sperm show a decrease not only in BF but also in wave amplitude (WA) in the distal portion of the flagellum (Cosson *et al*. 1999 & fig. 4). The flagellar beat efficiency is a measurement of the propulsive efficiency or swimming performance : it represents a combination between the BF and the wave amplitude (WA). The combination of decrease in BF and WA leads to a faster decrease of the swimming performance (P), because P = BF x WA. This process is accelerated as the time progresses within the movement period, because waves travel in a more and more restricted part of the proximal flagellum, while a longer and longer distal part) becomes inactive and straight. This is clearly illustrated in turbot spermatozoa where the distal straight segment occupies 60 to 80% of the length or even the total length by the end of the translational motility period. This local paralysis may be paralleled by a curling process similar to that also observed in carp sperm (Perchec *et al*. 1996): the appearance of such distal loop represents an additional contribution to the rapid slow down of sperm cells ; it is followed by a full arrest. Nevertheless, both the rigidification and the curling are reversible processes: this reversal is obtained when sperm cells are transfered from SM (high OP) back to IM (low OP). The reversibility process is related to both reconstitution of energy stores and internal ionic concentration. A subsequent transfer in SW needs to be applied after a delay in IM, during which cells reload their ATP and their ionic levels to a normal value compatible with full motility (Cosson *et al*. 2008 ; Dreanno 1998). This delay probably involves both mitochondrial respiratory activity and ion pumping activities, the laters being also ATP dependent. Energy stores are allowed to reconstitute during the incubation in an opposite OP situation (sustaining no motilility) and this allows a second motility sequence to be triggered through a new transfer in SW. Sperm flagella exhibit a new full wave pattern with normal waves developed and high beat efficiency. The hypothesis of such a restauration of the initial energy store has been confirmed by direct measurement of the ATP concentration (see below) which shows a low value at the end of the first motility phase compared to that obtained after regeneration; this was observed for the sperm of *S. maximus* (Perchec *et al*. 1993 ; Suquet *et al*. 1994 ; Chauvaud *et al*. 1995) similarly to observations in fresh water species such as *O. mykiss* (Christen *et al*. 1987, Billard & Cosson 1992), or *Cyprinus carpio* (Perchec *et al*. 1993 & 1995a). A second activation of motility can also be induced in spermatozoa from *Ictalurus punctatus* (Guest *et al*. 1976).

The regulation of axonemal motility by ionic concentration can be observed *in vitro*
Complementary informations leading to the understanding if this specific *in vivo* behavior comes from experiment using demembranated sperm models : wave parameters of permeabilized models were measured in presence of various concentrations of ions. We observed mostly that *in vitro* waves pattern can mimic those of *in vivo* flagella depending of two kind of parameters : the general and non specific ions concentration so called ionic strength and the Ca^{2+} concentration. The ionic strength mostly controls two aspects of waves shape : activation and wave dampening, while the Ca^{2+} concentration mostly governs the asymmetry of beating , i.e. the circling of sperm cells or sperm models.
Below we detail these results on the *in vitro* sperm models of two species (turbot and sea bass) because they are crucially important to better understand two main features specific to fish sperm : the mechanism of activation and the briefness of motility, and finally give rise to

a common model allowing to explain both features.
In vitro waves pattern are shown in details in figure 4 : the wave amplitude, the wavelength as well as portion of the flagellum where waves fully develop are all controlled by the ionic concentration of the RM. Briefly, when increasing ionic concentration, waves activity parameters vary successively from zero to an optimal value, then decrease more and more down to zero again at much higher ionic concentrations. As *in vivo* motility can be triggered in no electrolyte solutions with osmolality higher than 300 mOsm/kg, a potent effect of osmolality on axonemal machinery was tested. When glucose, sucrose or mannitol were added at up to 500 mM to the reactivation solution (containing 75 mM KAc as major ions) neither distal blockage nor perturbation of the wave shape or of the frequency were observed. In contrast, media containing glucose from 10 to 500 mM but no KAc did not allow any flagellar motility. It is concluded that the effects observed *in vitro* are not due to a direct sensitivity of axonemes toward osmolality. In order to identify which element of the axonemal machinery is affected by ionic strength, experiments of microtubules sliding were conducted. When KAc concentration was varied from 25 to 250 mM, little effects of ionic strength were observed neither on the portion of the flagellum where sliding occurred (distal versus proximal) nor on the rate of sliding (Cosson *et al.* 2008) : dynein itself would not be the direct target of ionic effects. The same is concluded for the CO_2 effects, either in turbot (this paper) or in sea urchin sperm (Brokaw & Simonick 1976).
The variations of beat frequency of axonemes reactivated in presence of various ATP-Mg^{2+} concentrations indicate that the K_m for ATP and the corresponding V_m are unchanged when KAc concentration varies from 25 to 250mM. These results could tend to show that the axonemal component preferentially blocked by increasing ionic strength could be some dynein subspecies located in the distal portion of the axoneme, as observed in *Chlamydomonas* flagella (Piperno & Ramanis 1991). Nevertheless, our *in vivo* observations show that in some cases, only distal portions of the flagellum can be active (Cosson *et al.* 2008) do not favor such hypothesis.
In similar assays using demembranated turbot spermatozoa, *in vitro* reactivation medias made of KCl, KPropionate and NaCl gave equal results but KAcetate was preferred because sperm motility was more stable, as already stated for sea urchin spermatozoa in such media (Gibbons & Gibbons 1983; Gibbons *et al*. 1982). These assays also showed that K^+ ions, even at high concentrations, are not inhibitors of the reactivated movement. Absence of inhibitory effects of K^+ also holds for trout (Saudrais *et al*. 1998) and carp demembranated sperm models (Cosson & Gagnon 1988) and therefore cannot explain the K^+ blocking effect observed *in vivo* in trout. In turbot, other ions were also tested such as $NaHCO_3$ at concentrations from 2.5 to 50 mM. When combined with the optimum reactivation medium containing 50 to 75 mM KAc, no effect was observed with 1 to 5 mM $NaHCO_3$, but a progressive blockage of the axoneme in its the distal part was shown when higher $NaHCO_3$ concentrations were used. These results confirm an effect of the ionic strength on distal blockage of the axoneme as $NaHCO_3$ contributes more efficiently to the ionic strength (3 milliequivalents per molecule). The pH was controlled and it was little affected by addition of $NaHCO_3$. Results obtained *in vitro* with $NaHCO_3$ will be discussed later for comparison with *in vivo* effects of CO_2.
Results of the *in vitro* experiments with sea bass demembranated flagella show effects of Ca^{2+} ions combined with effects of ionic strength on the circularity of tracks, as seen in many species (Brokaw 1991a).

The energy available in marine fish spermatozoa is rapidly exhausted
For details about the energetics of fish spermatozoa, readers can find details in Gosh (1989)

and in the general review of Billard and Cosson (1990).

A common feature is the decrease in sperm ATP content during the motility period. Presently, most studies in marine fishes concern turbot (Dreanno *et al*. 1999c) and sea bass. In sea bass, ATP values of 1.22 µmol/mg of protein (Zilli *et al*. 2004) were observed. It is difficult to correlate with the previously published results of 90 nmole/10^9 spz by Dreanno *et al*. (1999 b) due to the difference of unit. Zilli *et al*. (2004) and Dreanno et al. (1997) have proposed to use ATP as a sperm quality marker for sperm used for cryoconservation.

In turbot the ATP content is dependent of an ageing phenomenon related to the maturity period (Suquet *et al*. 1997; Dreanno *et al*. 1999a). Usually, ATP content is around 200 nmoles.10^{-9} spermatozoa (Dreanno *et al*. 1999b). In turbot, inhibitors of respiration (KCN) or of ATP synthesis (oligomycin) have little effects on the internal ATP concentration.

The AEC (adenylate energy charge), a measurement of the percentage of energy present in ATP and ADP relative to adenylate compounds (Atkinson 1968), decreases from 90-95% at activation by SW to 50% at 1 min pot activation (Dreanno *et al*. 1999c)

NMR studies on turbot spermatozoa (Dreanno *et al*. 1999c & 2000) show that other energetic compounds such as creatine phosphate participate to the energetic balance during motility. The presence of creatine kinase was described in turbot spermatozoa and therefore a PCr shuttle is probably present in turbot as well as in trout spermatozoa (Saudrais 1996 ; Saudrais *et al*. 1998) allowing a more homogeneous distribution of ATP along the flagellum. This shuttle involves ATP/ADP and Creatine/Phosphocreatine is similar to that described in sea urchin sperm (Tombes *et al*. 1987). In turbot, the presence of CO_2 also affects the internal ATP level.

In hake sperm, preliminary results (Groison *et al*. 2007) indicate AEC initial value of 0.71 before motility activation, with a large individual variability from 0.17 to 0.96. In other species like cod and tuna, no information is available.

In turbot, the ATP concentration *in vivo* can be calculated from the ATP content per cell : assuming a volume of 16.10^{-9} µl per sperm cell (Christen *et al*. 1987) supposedly constant during motility, the results of Dreanno *et al*. (1999c) lead to 6 mM ATP and 2 mM ADP initial concentration ; in arrested spermatozoa the ATP drops down to 1.5 mM. The K_m of ATP for the dynein ATPase is about 150 µM and plateaus at 80 Hz (extrapolation of f_{max} ; Cosson et al. 2008). In halibut demembranated sperm flagella, K_m ATP is 170 µM with f_{max} 51 Hz (Billard *et al*. 1993). In eel K_m ATP is 150 µM with f_{max} 83 Hz (Gibbons *et al*. 1985). In eel spermatozoa, the dynein ATPase is located in axonemal inner arms only (Baccetti *et al*. 1979), outer arms being absent. In turbot, at the end of the motility period the intraflagellar ATP concentration drops down spectacularly but it is still high enough to sustain motility, while ADP (2 mM) is not high enough to fully inhibit the dynein activity by competition with ATP.

The respiration rate of marine fish sperm is boosted at activation. This was measured in few species because of the briefness of the motility period relative to the time period needed to obtain this respiration rate using an oxygen electrode. In turbot, this is possible because motility lasts long enough : initial respiration ranges 35 nmoles O2 per min per 10^9 spermatozoa but at transfer in SW, it reaches 135 (same units) then decreases to 40 at 2 min (Dreanno *et al*. 1999c). In cod, respiration at rest is of 1.5 to 3 (same units, Robitaille *et al*. 1987). The effect of respiratory inhibitors are detailled in Dreanno *et al*. (1999c) : FCCP or KCN do not affect respiration of swimming spermatozoa, oligomycin is of low inhibitory effect and KCN, Na N_3 or Na H CO3 do not affect motility or flagellar beat frequency.

Marine fish spermatozoa are able to sustain a second motility period after a certain period of rest, provided previously activated spermatozoa remain metabolically active. In turbot, reviving of spermatozoa rendered immotile by a first incubation in sea water can be obtained by allowing these cells to settle in an artificial SF (Cosson 2004). After a subsequent transfer

into sea water, spermatozoa reinitiate motility and swim similarly to the first activation by sea water. This second transfer needs to be applied after a delay, during which cells reload their ATP level (Cosson *et al*. 2008 ; Dreanno 1998). This delay probably involves both mitochondrial respiratory activity and ion pumping activities, the laters being both ATP- and motility- dependent.

The ultimate task for sperm is to meet an egg : how to attract sperm and guide it to micropyle. Even though one generally believes intuitively that sperm is attracted by egg, there are very few examples of the demonstration of such phenomenon, so called chemotaxis : this has been well established in sea urchins, in several jelly fishes, or in ascidians (Kaupp *et al*. 2006 ; Shiba *et al*. 2006 ; Bohmer *et al*. 2005). In fish, the only clear demonstration is in the pacific herring (*Clupea pallasi*) : spermatozoa are not active when delivered at spawning in SW but only when they happen to reach the egg chorion, more precisely the vicinity of the micropyle (Yanagimachi 1957 ; Yanagimachi *et al*. 1992 ; Griffin *et al*. 1996). The motility initiation is regulated by a reverse Na^+ /Ca^{2+} exchanger (Vines *et al*. 2002) but some components of the chorion are needed (Oda *et al*. 1995), the HSAP (Herring Sperm Activating Peptide) and the SMIF (Sperm Motility Initiation Factor). The HSAP has a protease inhibitor motifs (Oda *et al*. 1998) which binds to an endopeptidase of the flagellar membrane (Yoshida *et al*. 1999). The SMIF is a 105 kDa glycoprotein localized in the chorion part surrounding the micropyle which induces a trajectories switch from linear in « free » SW to helicoidal in the micropylar funnel. Such a specific sperm/egg behavior could be related to the spawning behavior and the male/female reproductive biology traits in this species (Stacey & Hourston 1982 ; Hay 1985).
Physicotaxis is necessary for physical guidance of sperm cells on the surface of eggs and eventually towards the micropyle. The first aspect of physicotaxis is the tendency for spermatozoa to swim on any surface, including the surface of an egg, which at this scale appears large and flat. This ability to swim in the close vicinity of surfaces is due to a slight deviation of the beating plane of sperm flagella which generates a thrust of small amplitude out of the main beat plane and lead to chiral shape in double successive helix inverted relative to each other (Cosson *et al*. 2003). The consequence is that spermatozoa at surfaces mostly follow 2D tracks instead of 3D ones when swimming in space in absence of any surface (example in turbot, Cosson and Corset, unpublished) : following the egg surface highly increases the chances for any spermatozoon to reach the micropyle. These chances are further improved by the presence of guidance grooves located on surface of some eggs. Such guidance tracks physically converge towards the micropylar funnel, resulting i*n fine* into a significant increase of sperm concentration in the close vicinity of the micropyle (estimated to 10 µm range in turbot eggs ; Cosson *et al*. 2008). This was also documented in some fresh water species such as *Puntius conchonius* (Amanze & Iyengar 1990) and medaka (Iwamatsu *et al*. 1993).
In addition to delivery of DNA information through the egg micropyle, the very ultimate task of the fish spermatozoon would be to induce a factor responsible for Ca^{2+} waves in the eggs, a necessary signal for egg activation (Coward *et al*. 2003).

The ability of spermatozoa to fertilize eggs : limitations to the fertilization process ?
In order to meet the egg, sperm should come in its vicinity, therefore the total averaged distance covered (called $\boldsymbol{D}$) by sperm cells is a key factor. $\boldsymbol{D}$ can be calculated from the change in velocity with time by use of the formula: $D = \int_{t_0}^{t_e} \left(-\frac{v_0}{t_0} \times t + v_0 \right) dt$
Integral calculation needs first a curve fitting of the plot of velocity vs time after activation.

In theory, this calculation must take into account the linearity index weighted according to the percentage of motility decrease. As tracks are usually not linear but circular, the efficient distance covered is even lower than that predicted by calculation. From the above formula, one can also predict that, when velocity decreases logarithmically with time (the most common case), 80 to 90% of the distance is covered by sperm during the first half of the motility period ($\boldsymbol{D}_{1/2}$).

A gross estimation of the distance covered by a spermatozoon is: 2.3 mm in sea bass (Dreanno *et al*. 1999b), 12 mm in turbot (Chauvaud *et al*. 1995), 14 mm in cod (this paper), 11 mm in hake (this paper), 10 mm in tuna and 9 mm in halibut (Billard *et al*. 1993). Trippel & Neilson (1992) estimated an initial velocity of 2 mm/min for turbot which leads to a total distance of 4-6 mm after integration during the motility period.

Compared to many other oviparous animal species, the egg size of marine fishes is relatively small but definitely big compared to the spermatozoon size. The egg diameter is (in mm) 1.02-1.39 (Carillo *et al*. 1995) and 1.16-1.89 in sea bass (Kjorsvik & Holmefjord 1995) and 1.2-1.8 (Miller *et al*. 1995) in cod, 3.00-3.80 in halibut (Kjorsvik & Holmefjord, 1995), 1,06 ± 0,10 (Groison et al, unpublished) in hake, 1.4-3.5 (Erhenbaun, 1905) or 1.01-1.07 in turbot and 1.0-1.2 in red tuna (Doumenge,1999).

Comparison of egg size relative to the distance covered by a spermatozoon leads to similar values : therefore any marine fish spermatozoon should be delivered in the close proximity of the ovocyte in order to reach its micropyle. This may explain a double reproductive strategy for marine fishes in order to accomplish reproductive task : 1) a very large excess of sperm cells relative to one egg and 2) a local delivery of sperm resulting from a close proximity between the two spawners, male and female.

Obviously, the fertilizing ability depends on the ratio of the number of active spermatozoa per egg but also of the time elapsed since motility activation (Fauvel *et al*., 1999). In turbot, after a 3 min. period of sperm swim, the number of swimmers decreases : the efficiency for fertilization decreases, reaching zero after tens of minutes. The fertilization ability of gametes is one among other traits of the reproductive biology of fishes : in this regard specific information concerning fish mating can be found in Rakitin *et al*. (2001) about cod in Murua & Motos (2006) about hake and more generally in Turner (1993).

Sperm movement specificities in some species : effects of Ca^{2+} and CO_2.

As seen in sea bass, tuna and tilapia (Morita et al., 2003), sperm motility relies both on external osmolality and on external Ca^{2+} concentration. Influx of Ca^{2+} across the plasma membrane is a common mechanism of $(Ca^{2+})_i$ signaling exploiting the limitless extracellular reservoir of Ca^{2+} , SW where it ranges 11 mM. In addition, some membrane vesicles containing calreticulin (a Ca^{2+} storage protein ; Ho & Suarez 2003) and a Ca^{2+} mobilization system (Ca^{2+} activated Ca channels ; Harper *et al*. 2004) were described. Selective localization of the various types of plasma-membrane channels that permit Ca^{2+} influx provides some flexibility. The expulsion of an excess of Ca^{2+} through the plasma membrane relies on the ATP-driven Ca^{2+} pumps and Na/Ca exchangers. A Ca^{2+}-ATPase pump is present in the mid piece region of sea urchin sperm, i.e. the mitochondrion (Gunaratne and Vacquier 2006). These data reveal a level of complexity in sperm Ca^{2+} signaling that rivals those of larger and more complicated cells.

Flagella can change their beating pattern (asymmetric or symmetric) in response to Ca^{2+} concentration perceived by the axoneme (Brokaw, 1991a) which contains several calcium binding proteins: calmodulin in the spokes (Yang *et al*. 2001), centrin (caltractin) at the attachment point of the I2 and I3 inner dynein heavy chains (DRC, Dynein Regulatory Complex ; LeDizet & Piperno 1995), all elements by which asymmetry of flagellar beating could be controlled by Ca^{2+} in marine fish sperm.

The main CO_2 effect is a blockage of axonemal motility both *in vivo* (Dreanno *et al*. 1995) and *in vitro* : this is specific to flatfishes, including turbot, due to the high concentration of Carbonic Anhydrase in their sperm flagella (Inaba *et al*. 2003). Both *in vivo* and *in vitro*, CO_2 controls dynein activity through a NaH CO_3 ionic effect similar to that of other ions (Dreanno *et al*., 1999d). In sea urchins sperm flagella, CO_2 was also shown to affect wave shape without blockage, therefore probably involving a different mechanism (Brokaw & Simonick 1976 ; Brokaw 1977).
Additional signaling such as protein phosphorylation were shown to be involved in flagellar motility regulation (Inaba 2003) but little is known in this respect in marine fish spermatozoa. In striped bass, flagellar activation seems to occurs through phosphorylation of some specific proteins via a cAMP-independent pathway (Shuyang *et al.* 2004) ; similar results were observed in tilapia (Morita *et al.* 2006). Viviparous fish like guppy (*Poecilia reticulata*), a fresh water species, adopts a strategy requiring cAMP signaling, but it needs application to the spermatophore of some chaotropic chemicals which destabilize specific flagellar protein(s) (Tanaka & Oka 2005) to reach motility activation.
Flagellar shape modifications, i.e. stiffening of distal part of fish sperm flagella, could result from a regulation by hydin, a central pair protein of cilia and flagella (Lechtreck & Witman 2007).

The osmolarity control of motility : towards a global explanatory model
Osmolatity effects on fish motility have been studied by Morisawa (1994) and Perchec-Poupard *et al*. (1997) in marine and fresh water species. Regarding osmolality adaptation of marine fish spermatozoa it is worth to remind the presence of membrane folding which develops in most species on both sides of the flagellar membrane. These fins are flanking the whole length of flagellum, ranging several µm width and obviously increases the ratio surface to volume of the flagellar organelle. They do not only contribute to the efficiency of the thrust generated by waves by increasing the flagellar surface used for the friction on the surrouding medium during movement; but they also contribute to a large increase of the membrane surface when compared to a simple cylindrical axoneme. A calculation applied to turbot flagellum leads the following : the surface of a flagellum in shape of a simple cylinder is about 34 millions square nm ; this is about 1/4 compared to surface of the same cylinder comprising fins (160 millions square nm). This « excess » of surface have large implications in the water exchange / osmotic regulation of sperm. At initiation of movement of marine fish spermatozoa in SW, the first signal received by the membrane is osmotic, followed by a water flux in either direction, which provokes local membrane distortions due to osmotic constraints. A significant increase of the membrane surface due to these fin-shaped creases (Cosson *et al*. 1999 ; Cosson 2007b) favors water exchange. The distortion ability of creases would lead to the blebs or coils observed on exposure to extreme osmotic situations (Cosson *et al*. 2000; Perchec *et al*. 1996).
Subsuming the above remarks on marine fish sperm, we have developed the following model to explain motility activation then inhibition resulting from non optimal internal ionic concentration, according to *in vitro* results. Sudden exposure of an animal cell to an extreme and drastic osmotic environment, i. e. SW, causes various reactions including volume and shape changes because, in contrast to vegetal cells, they are devoid of the constraints of a polysaccharide wall (Stein 2002). By the OP effect, sperm motility in marine fishes is induced by the hyper- osmotic shoc of the surrounding medium (Billard 1986; Billard *et al*. 1993; Gwo 1995 ; Chauvaud *et al*. 1995 ; Linhart *et al*. 1999; Krasznai *et al*. 2003; Cosson 2007b). Nevertheless sperm motility is triggered in turbot and other flatfish in iso- as well as in hyper- osmotic media (Suquet *et al*. 1994) relatively to the SF because of the extra control by CO_2. Motility occurs in a wide range of osmolalities, below or above that of sea water

(Chauvaud *et al.* 1995; Suquet *et al.* 1994; Billard *et al*. 1995) : optimal osmolality (in mOsm./Kg) is at 900-1100 in halibut (Billard *et al*. 1993), at 300-1100 in turbot, 333-645 in tilapia (for fishes rised in sea water, Linhart *et al*. 1999) but higher for tilapia fish rised in hypersalinity (Legendre *et al*. 2007) and at 480 in Atlantic croaker (Vizziano *et al.* 1995). A general model of marine fish sperm motility control by osmolality is proposed in figure 6, where turbot sperm is taken as example. It is based on results published by Chauvaud *et al*. (1995), Suquet *et al*. (1994) and Inaba *et al*. (2003) as well as additional *in vitro* result.

In some cases, resistance to very low osmolality is surprisingly high : turbot sperm can sustain dilution in distilled water and resist reversibly for several minutes. Osmolality is definitely a key factor for fish gametes released in the surrounding medium. In marine fishes oocytes, the permeability diffusion (P_d in µm/sec) was estimated in salmon to 1.68 at release in water but shown to decrease to 0 within 20 min from contact with water (Prescott 1955). This was confirmed in marine teleosts by use of radioactive tracers (Potts & Eddy 1973) where stretch receptors and aquaporins are supposed to play this regulatory role probably in contrast to that occurring in spermatozoa as discussed below.

In the case of male gametes, it is worth mentioning that mechanical activation could be the second signal in response to the first (osmotical) signal via the stretch activated channels located in the sperm membrane. It has been shown that a specific and reversible inhibitor of the stretch activated channels (SAC), gadolinium, is active on carp spermatozoa (Krasznai *et al.* 2003), and more generally in spermatozoa of several fish species including marine ones such as sea bass, turbot and tuna (Cosson et al. 2008), but inactive in sperm of other species apart from fishes (Kraznai *et al*. 2003). SACs are mechanosensitive channels, which increase the membrane conductivity to ions such as Ca^{2+} or K^+ when mechanical constraints induce distortion of this membrane (Yang & Sachs 1993). Mechanosensitivity is biologically important (Ingber 2006) especially considering that flagella and cilia are acting as mechanosensitive detectors : the signal is transduced through gene products of the PKD (Polycistic Kidney Disease) family (Pan *et al*. 2005). By proteomic analysis, the presence of a polycystin-2-like receptor was revealed in *Chlamydomonas* cilia (Pazour *et al.* 2005) and in metazoan cilia as well (Pazour & Rosenbaum 2002); in addition polycystin-2 is a "transient receptor potential", a cation channel with mechanosensory properties (Nauli *et al.* 2003). Mechanosensitivity is also a specific property of flagellar axonemes : no more beating cut-off pieces of axoneme that have lost coordination can be reinitiated by bending the flagellum with a microprobe (Lindemann and Rickemenspoel 1972). In fish spermatozoa, the same situation probably occurs when sperm are put in a medium limiting the initiation of motility. Activation by SW probably involves such mechanosensitivity: at first, mechanosensitive channels are activated which themselves mechanically activate the axoneme (Fig. 6).

The SACs may associate with other membrane proteins to modulate their activity (Vandorpe *et al*. 1994) ; those can be water channels (aquaporins), which are involved in the water transportation across membranes and may increase up to 1000 fold the diffusion rate of water molecules through membranes. In fish spermatozoa, the putative presence of aquaporins comes from observations where sperm motility is sensitive to low concentrations of inhibitors of aquaporins such as $HgCl_2$ (Abascal *et al*. 2007 ; Cosson *et al*. 1999). In turbot sperm, the effect of Hg Cl_2, supposedly targetted to aquaporins, is chronologically double : first, inhibiting initiation of motility and second, inducing a « twist » of the flagellum (fig. 5). Both aquaporins and polycystin-like receptors genes are present in a fish genome, the zebra fish.

Putting all together these features with the knowledge about the osmotic signal, we propose to involve SACs and aquaporins in the signaling pathway of fish sperm activation (Fig. 6). This paradigm proposes several steps : the very first signal perceived by the membrane is

osmotic; water exit would provoke a local membrane distortion or stretching. In this respect, the role of unusual creases shaped as fins (Cosson *et al*. 1999 ; Cosson 2007b) as discussed previously could be crucial in significantly increasing the membrane surface, this membrane "excess" favoring water exchange but also when distortions such as blebs appear on flagella exposed to extreme osmolalities (Cosson *et al*. 2000 ; Perchec *et al*. 1996). The SAC would respond immediately to this mechanical signal by increasing the local permeability, which would therefore allow ions such as Ca^{2+} or K^{+} and/or water to move rapidly in or out through channels or aquaporins. The triggering of an autocatalytic effect along flagellar membrane transmitted from place to place would explain why fish sperm activation proceeds in an extremely fast way (less than 20 msec. according to our estimations). *In fine*, the local stretching of membranes would be the signal perceived by the axoneme because of the mechanosensitivity of this micromachine.

Conclusions

The present review primarily focuses on the mechanisms by which sperm motility can be triggered by SW as encountered by fishes with external fertilization.

The *in vivo* and *in vitro* observations about ionic strength control of axonemal activities, are strengthened by observations of Billard (1978), by Lahnsteiner & Platzner (1998) and by Groison *et al*. (unpublished) which show that partly diluted SW supports longer sperm motility period than normal SW, as it leads to less harmful osmotical environment to sperm. Therefore our *in vitro* observations combined with ATP measurements have lead us to a possible general schematic flow chart explaining how changes in internal ionic concentration occurring in response to external osmolality could control fish sperm motility (Fig. 6). The response immediacy to the osmolality signal may be related to one major constraint endured by fish spermatozoa, which is to obey a reproduction strategy in which a very brief period of reaction is needed to achieve the task. They exhibit a hypermotile behavior (high but brief beat frequency) remarkably similar to the hyperactivated motility exhibited by mammalian spermatozoa in the vicinity of eggs before fertilization. The hypermotility of fish spermatozoa is demonstrated by a high velocity but a fast consumption of energy, which was stored during the spermatogenesis process. This strategy is probably dictated by another main constraint of many fishes, the short period of competence of the egg for fertilization in which the micropyle remains open only 10-20 seconds after contact with SW.

As complementary information, some video files about fish sperm motility are available at http://biodev.obs-vlfr.fr/~cosson/fishsperm/

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Figure legends

Figure 1 : Motility control of flagellar waves by local delivery of low osmolality medium. A turbot spermatozoon is clamped to a holding micropipet (upper right corner) and propagates waves in SW. A second micropipet (on right, with arrow inside) is approached to the middle part of the beating flagellum : in b, a pulse of non swimming solution locally delivered have stopped the tipward progression of waves. Bar scale (bottom b) corresponds to 10 µm.

Figure 2 : Photographs of moving spermatozoa of cod (2A), tuna (2B) and turbot (2C). Photographs were obtained using dark field microscopy and stroboscopic illumination. In fig. 2A, flashes every 3 millisecond (3 flashes per video image) head and flagellum appear in 3 successive positions. Cod sperm which motility was initiated by transfert in sea water since 14 sec. in a (waves propagating in the whole length of the flagellum); at 36 sec. in b and 58 sec. in c (waves propagating in the 3/4 of the flagellar length) ; at 2 min. in d (low amplitude waves only in the vicinity of the head); at 3 min. in e (full stop). In f to i, damages provoked by SW on cod spermatozoa : appearance of blebs (arrows) and curling of flagellar tip (arrow heads). Bar scale : 10 µm. In fig. 2B, swimming tuna cod spermatozoa spermatozoa : in a, 7 flashes applied during the 50 msec exposure ; in b, 3 flashes allowing visualization of wave propagation from base to tip ; in c, single flash showing flagellum in asymmetric shape ; in d, same but symmetric shape ; in f, wave dampening. Bar scale : 10 µm. In fig. 2C, swimming turbot sperm at high magnification : in a to c, three successive video images (3 flashes per image) of a spermatozoon activated since 23 sec ; in d to f, same at 63 sec. with the dampening of the waves appearing progressively during the motility period leading to full stop in g at 3 min. Bar scale : 10 µm.

Figure 3 : The effects of viscosity on the waves parameters of turbot sperm flagella. In a, plot of the number of twist along flagellum as a function of the concentration of methyl cellulose (MeCell). In b, the relationship between viscosity (in deciPoises as measured with a viscosimeter) and MeCell concentration. In c to f, successive images of an individual spermatozoon obtained every 3 msec. (from left to right in each pannel) at various MeCell concentrations : c = no MeCell in SW, d = 0.6% Me Cell, e = 0.8% and d = 1%. Bar scale : 10 µm.

Figure 4 : Wave shape of turbot sperm observed *in vivo* and *in vitro*. Upper pannel, from left to right, *in vivo*: a to d) just after the triggering of motility, fully developed waves occupy most of the length of the flagellum; their amplitude is constant and their frequency remain high ; e to g) at the middle of the motility phase, the frequency drops while the wave propagation becomes restricted to the proximal part of the flagellum and the very tip of the flagellum is in rigor; h to k) waves develop only in a very short portion close to the head ; l) No wave remains and the whole flagellum stops beating, it adopts a rigor aspect. Lower panel, *in vitro* : demembranated flagella reactivated by ATP-Mg^{2+} and exposed to increasing (from left to right) concentration of salt (K Acetate in this example) : the dampening of waves occupies a segment of increasing length on the distal flagellum. Bar scale : 10 µm. Bottom right : schematic representation of the progression of the wave shape in fish sperm flagella during the swimming period *in vivo* (upper part of the pannel) and *in vitro* (lower part of the panel).

Figure 5 : Effects of low concentrations of $HgCl_2$ on sea bass spermatozoa. After addition of spermatozoa in SW containing 200 µM $HgCl_2$, flagella stop movement. After 3-4 min. they undergo an unusual curling process, mainly a twisting of the flagellum (as observed by dark field microscopy) taking several seconds to reach completion then followed by partial

unfolding. From a to e, successive stages of this twisting shown by the red arrows, with timing in seconds at bottom. Bar scale : 10 µm.

Figure 6: Activation process and signal transduction in marine spermatozoa : general schematic representation of the interacting processes occurring during the motility period of a turbot spermatozoon. Sea water is of much higher osmolality compared to SF : the osmolality jump induces an osmolality reaction at the sperm membrane level ; water exits the sperm cell, a process accelerated by water pumps (aquaporins). As a consequence of water exit, internal ionic concentration increases and reaches optimal values for dynein motors activity. Beating of flagella is at maximal velocity but decreases with time because of 2 reasons : the ionic concentration becomes too high to sustain correct dynein activity and ATP concentration declines and becomes limiting for flagellar beating. After some period, flagellar activity stops because of these unfavorable conditions.

Table 1

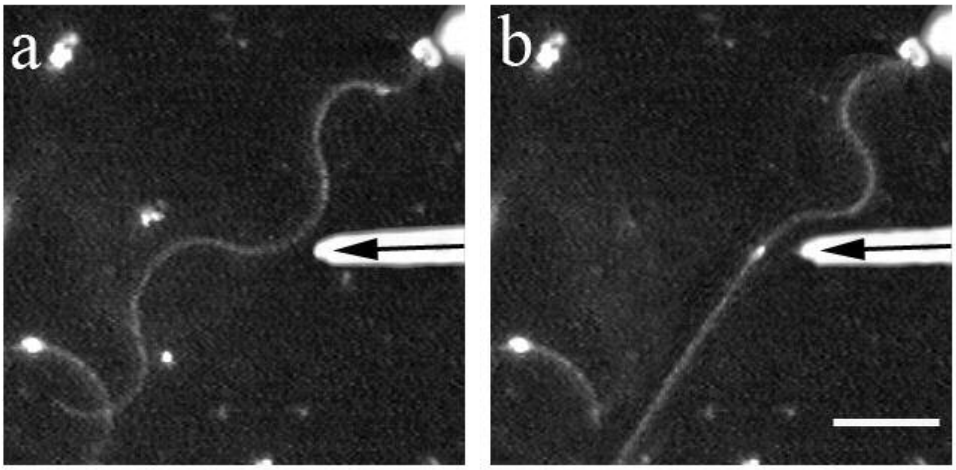
Table 1a : Sperm density, pH and osmolality of SF (Seminal Fluid) in several marine fish species

Species	Sperm density (10^9 ml^{-1})	pH	Osmolality (mOsmol. L^{-1})	References
turbot	2 to 9	nd	nd	Chauvaud *et al.* 1995 ; Suquet *et al.* 1998
turbot	nd	6.65 to 7.0	nd	Suquet *et al.* 1994 ; 2005 ; Dreanno *et al.* 1998
tuna testicular sperm	30-50	nd	nd	Doi *et al.* 1982 ; Mylonas *et al.* 2007
halibut	>100	nd	nd	Cosson *et al.* 2008
sea bass	60	nd	317	Fauvel *et al.* 1999 ; Dreanno *et al.* 1998 ; 1999b
sea bass	10-40	nd	400	Villani & Catena, 1991
hake	8.3+/-3	nd	nd	Cosson *et al.* 2008
cod	4.5 to 8 .7	7.9 to 8.4	360 to 380 400 to 417	Suquet *et al.* 2005 Hwang & Idler 1969 ; Litvack & Trippel 1998
sea bream	nd	7.8	365	Chambeyron & Zohar 1990
Tilapia (raised in sea water)			351	Linhart *et al.* 1999
Petromyzon marinus			250	Cieresko *et al.* 2002.

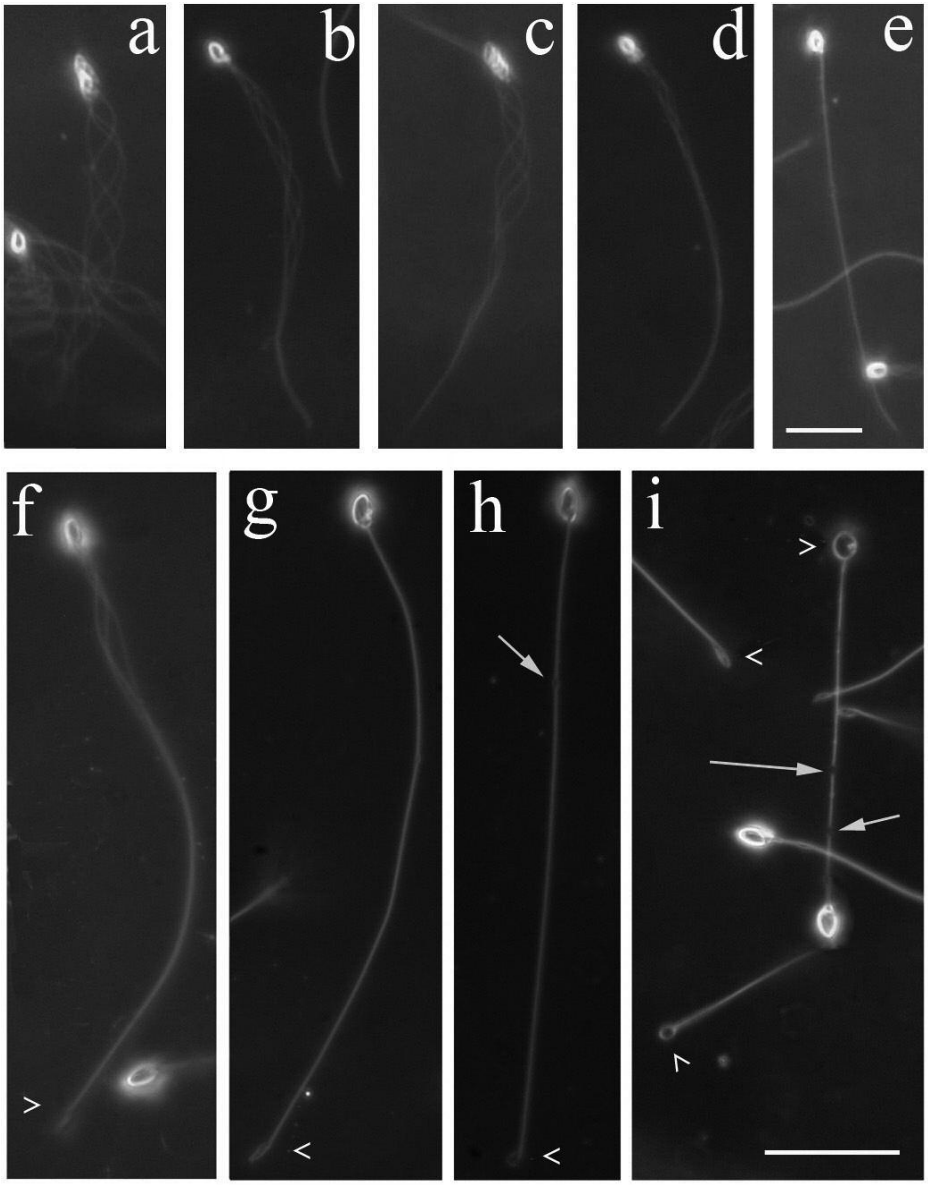
Table 1b : Sperm motility duration in sea water of various marine fishes

Species	Full immotility	Extrapolation
Halibut (Billard *et al.* 1993)	110-120 sec	100 sec
Turbot (Dreanno *et al.* 1999c)	600 sec	6-800 sec
Sea bass (Dreanno *et al.* 1999b)	50-60 sec	60 sec
Cod (Cosson *et al.* 2007)	7-800 sec	600 sec
Hake (Cosson *et al.* 2007)	4-500 sec	350 sec
Tuna (Cosson *et al.* 2008)	140 sec	125 sec

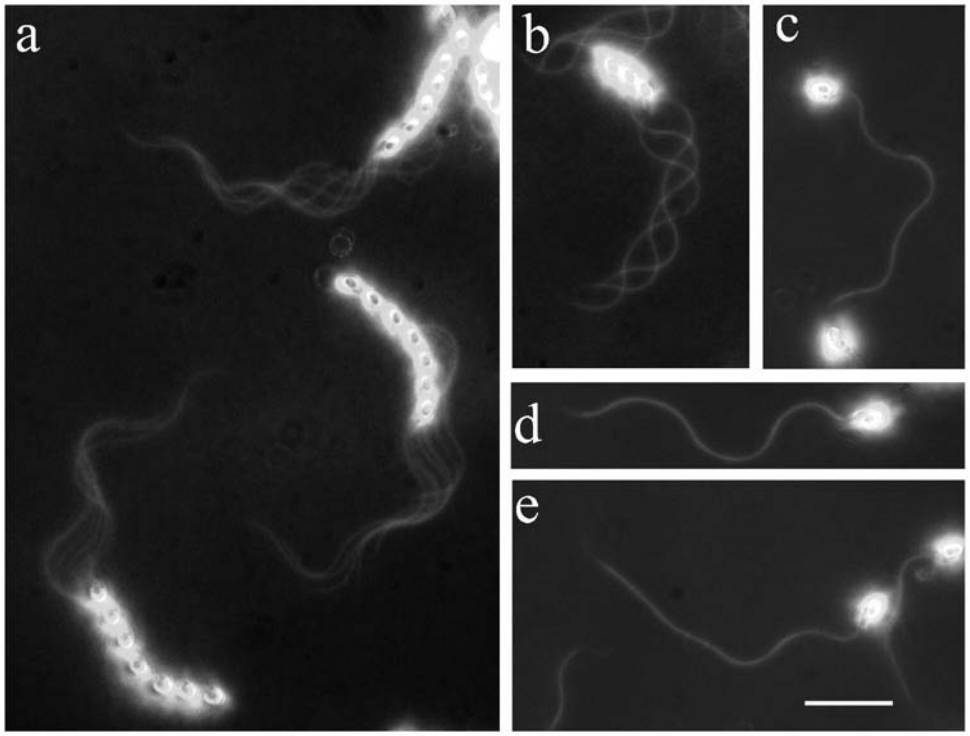
Full immotility refers to the time period at which no single sperm is seen active. Extrapolation means the intersection of the percent motility curve with the time abscissa.



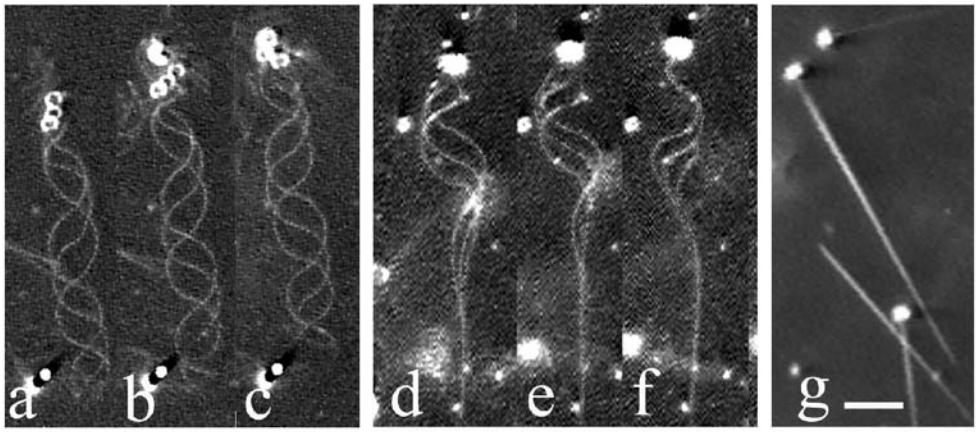
235x119mm (72 x 72 DPI)



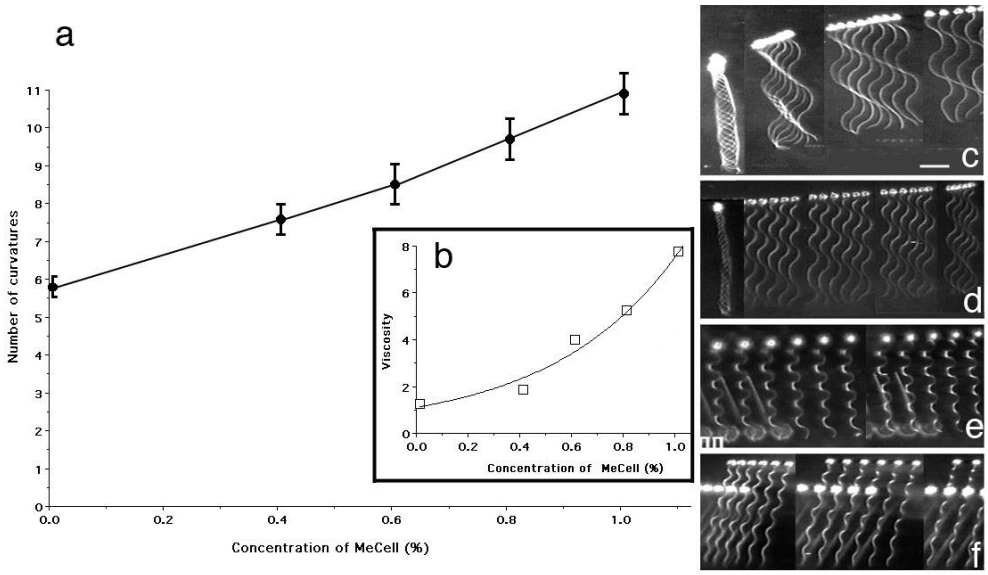
89x113mm (300 x 300 DPI)



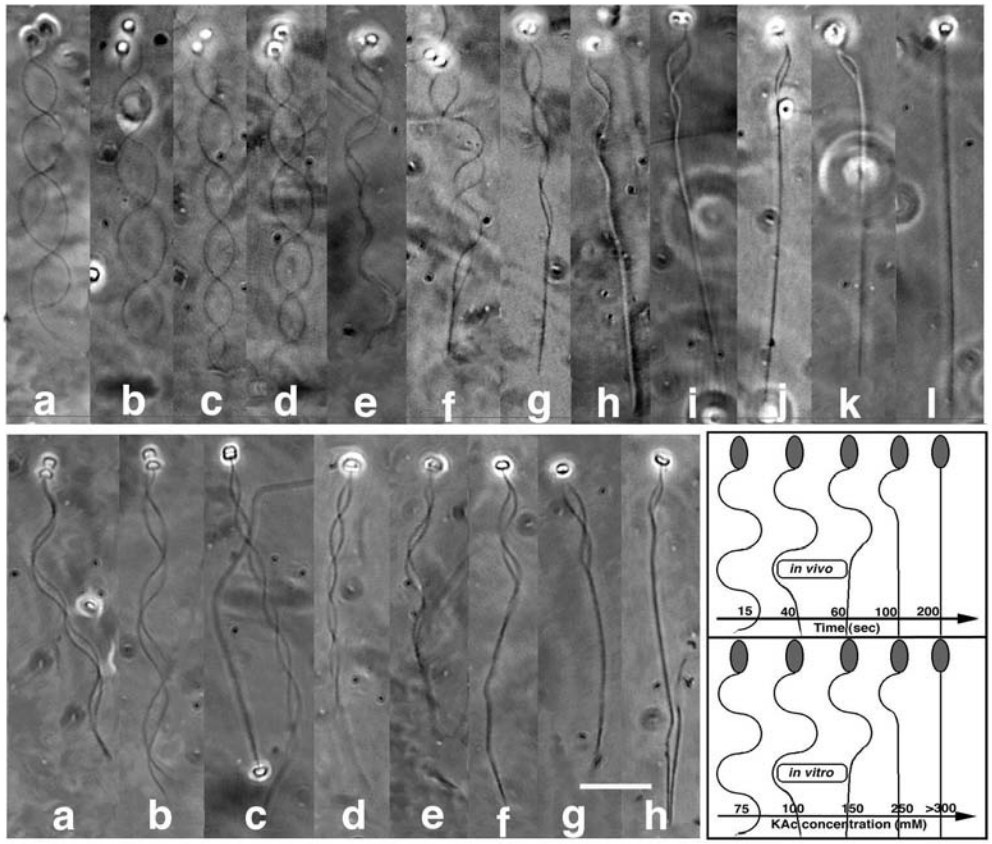
222x168mm (300 x 300 DPI)

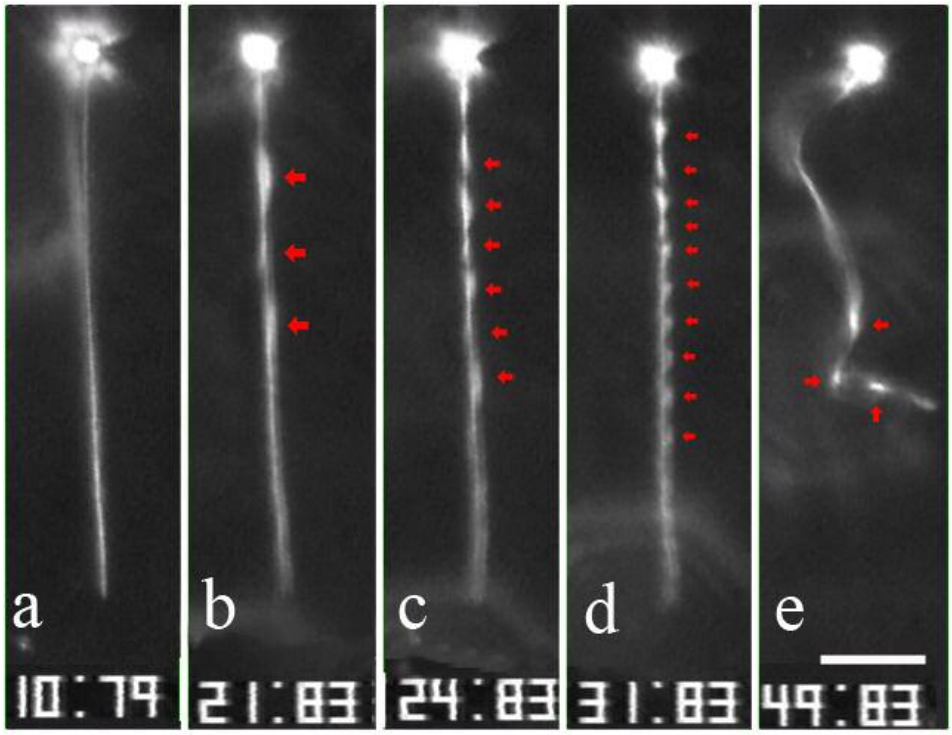


189x84mm (250 x 250 DPI)

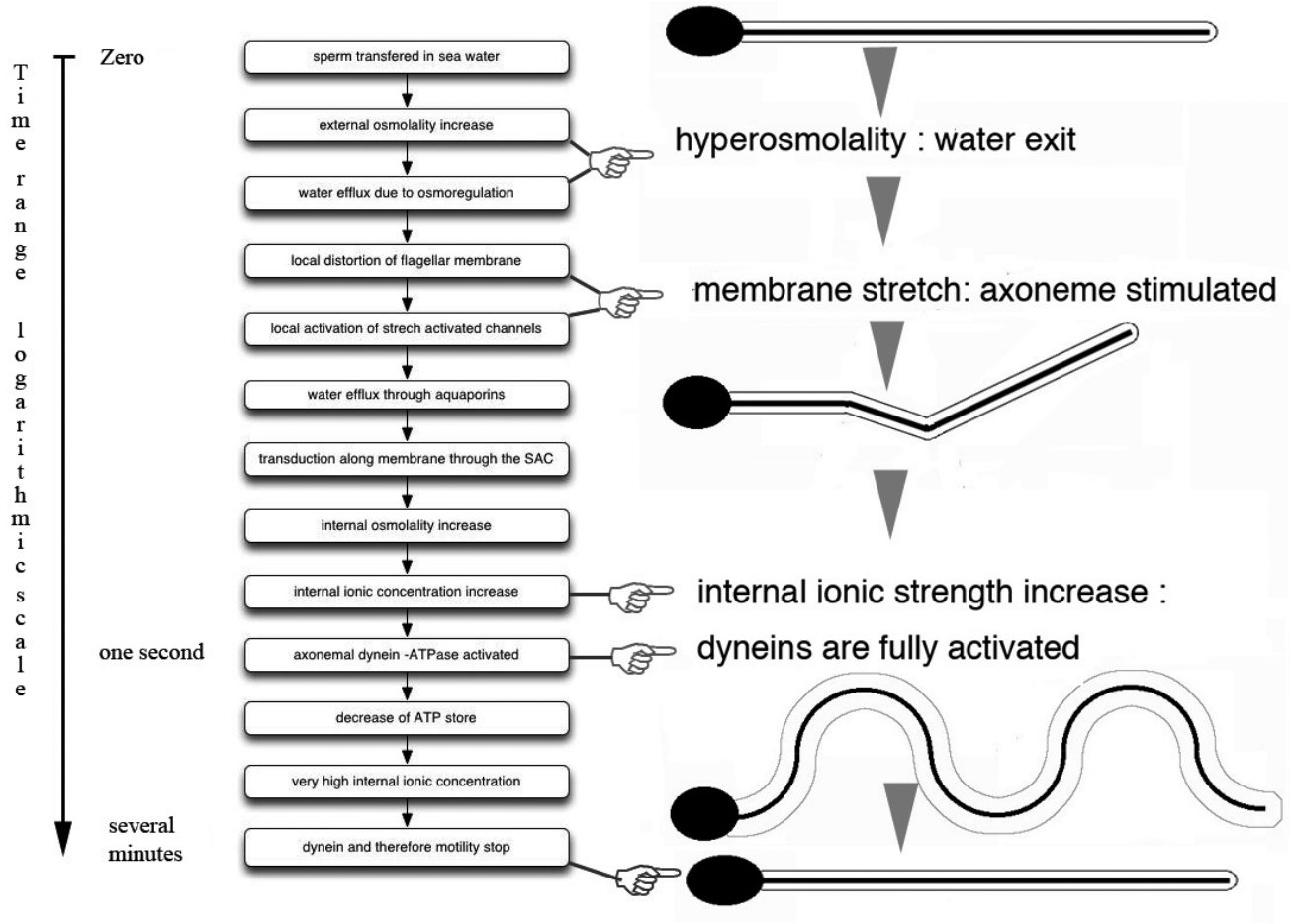


395x230mm (72 x 72 DPI)


a b c d e f g h i j k l
a b c d e f g h
in vivo
15 40 60 100 200
Time (sec)
in vitro
75 100 150 250 >300
KAc concentration (mM)



238x185mm (72 x 72 DPI)


Time range logarithmic scale
Zero
one second
several minutes
sperm transfered in sea water
external osmolality increase
water efflux due to osmoregulation
local distortion of flagellar membrane
local activation of strech activated channels
water efflux through aquaporins
transduction along membrane through the SAC
internal osmolality increase
internal ionic concentration increase
axonemal dynein -ATPase activated
decrease of ATP store
very high internal ionic concentration
dynein and therefore motility stop
hyperosmolality : water exit
membrane stretch: axoneme stimulated
internal ionic strength increase :
dyneins are fully activated