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Sperm motility in *Solea senegalensis*: effect of temperature, salinity, pH and ovarian fluid

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(…) O sonho é ver as formas invisíveis
Da distância imprecisa, e, com sensíveis
Movimentos da esp'rança e da vontade,
Buscar na linha fria do horizonte
A árvore, a praia, a flor, a ave, a fonte -
Os beijos merecidos da Verdade. (…)

(…) Deus quere, o homem sonha, a obra nasce. (…)

Fernando Pessoa, Mensagem

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List of Abbreviations

µl - Microlitre
ALH - Amplitude of lateral displacement
BCF - Beat cross frequency
E25 - Artificial sea water diluted with 25% of *Epinephelus marginatus* ovarian fluid
E50 - Artificial sea water diluted with 50% of *Epinephelus marginatus* ovarian fluid
Kg - Kilogram
g - Gram
L - Litre
h - Hour
LIN - Linearity
m - Meter
ml - Millilitre
mm - Millimetre
OF - Ovarian fluid
PM - Progressive motility
s- Second
S25 - Artificial sea water diluted with 25% of *Solea senegaslensis* ovarian fluid
S50 - Artificial sea water diluted with 50% of *Solea senegaslensis* ovarian fluid
SP - Subpopulation
STR - Straightness
TM -Total motility
VCL - Curvilinear velocity
VSL - Straight line velocity
WOB - Wobble

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RESUMO/ABSTRACT

Resumo

A análise da mobilidade seminal é uma ferramenta importante para reprodução em aquacultura. Esta é uma técnica *in vitro* que auxilia a estabulação, manutenção e selecção de lotes de reprodutores. A análise de mobilidade seminal pode tornar-se potencialmente uma ferramenta para o melhoramento das condições do ambiente de fertilização. A utilização do software CASA (Computer Assisted Sperm Analysis) revolucionou a descrição e quantificação específica da mobilidade seminal. A maioria da informação recolhida sobre mobilidade de sémen de peixes baseia-se em espécies de água doce, pelo que é crucial conhecer as condições óptimas de activação da mobilidade de espermatozóides para novas espécies de de água salgada de interesse em aquacultura tal como *Solea senegalensis*. A optimização das condições de fertilização desta espécie é particularmente importante já que os lotes de reprodutores em cativeiro podem desenvolver disfunções reprodutoras. Este trabalho teve como objectivo realizar a avaliação das condições óptimas de activação da mobilidade do sémen em *S. senegalensis* em termos de temperatura, salinidade e pH. O segundo objectivo foi realizar a avaliação da influência de fluido ovárico homólogo (*S. senegalensis*) e heterólogo (*Epinephelus marginatus*) na mobilidade seminal de *S. senegalensis*. Deste modo foram realizados dois conjuntos de experiências: 1) mobilidade de sémen de 7 machos analisado através do CASA em diferentes temperaturas, salinidades e pH, 2) mobilidade de sémen de 8 machos activados na presença de diferentes concentrações de fluido ovárico. Os parâmetros do CASA foram registados e posteriormente analisados através de médias e cluster analysis. Concluiu-se que temperaturas mais elevadas (20 ºC) e baixas salinidades (25 ‰ e 30 ‰) da solução de activação ocorre um melhoramento das características de mobilidade seminal, tal como a velocidade. A presença de fluido ovárico em baixas concentrações melhora as características da mobilidade seminal assim como a longevidade dos espermatozóides. O fluido ovárico é consequentemente um factor que estimula a mobilidade seminal que tem sido negligenciado em estudos anteriores. Este estudo demonstrou que durante a época de reprodução a temperatura da água (20 ºC) e a salinidade (25 ‰ e 30 ‰) no tanque são os principais factores que melhoram a activação da mobilidade do sémen, sendo consequentemente uma contribuição importante para compreender a dinâmica do processo de fertilização em *S. senegalensis*.

Palavras-chave: mobilidade, *Solea senegalensis*, “Computer Assisted Sperm Analysis” (CASA), qualidade seminal, fluido ovárico, subpopulações espermáticas

Abstract

The analysis of fish sperm motility is an important functional tool to assist reproduction in aquaculture. It is an *in vitro* technique that has an important role in broodstock selection, management and establishment as well to potentially improve fertilization environmental conditions. The use of CASA (Computer Assisted Sperm Analysis) systems revolutionized motility quantification and description to a scientific level. Most of the knowledge collected about fish sperm motility is based on fresh water species, but it is crucial to assess spermatozoa optimal activation conditions for new species, such as *Solea senegalensis*. The optimization of the fertilization conditions of this species is particularly important since broodstocks maintained in captivity developed several reproductive dysfunctions. This work aimed to perform an assessment of the optimal motility activation conditions in *S. senegalensis* sperm, in terms of temperature, salinity and pH. The second objective was to assess the influence of homologous (*S. senegalensis*) and heterologous (*Epinephelus marginatus*) ovarian fluid on *Solea senegalensis* sperm motility solution. For those reasons two sets of experiments were performed: 1) sperm motility from 7 males was analysed with CASA in different temperature, salinity and pH conditions, 2) sperm motility from 8 males in the presence of different ovarian fluid concentrations. CASA parameters were recorded and analysed afterwards by mean values and cluster analysis. Higher temperatures (20 ºC) and low salinities (25 ‰ and 30 ‰) of the activation solution improved sperm motility characteristics such as velocity. The presence of ovarian fluid in low concentrations improved sperm motility characteristics and sperm longevity. The ovarian fluid can be thus an important factor that stimulates sperm motility which has been neglected in previous studies. This study demonstrated that during the breeding season water temperature (20 ºC) and salinity (ranging between 25 ‰ and 30 ‰) in the tank are the main factors improving the activation of sperm motility, being therefore an important contribution to understand the fertilization dynamics in *S. senegalensis*.

Keywords: motility, *Solea senegalensis*, Computer Assisted Sperm Analysis (CASA), sperm quality, ovarian fluid, sperm subpopulations

INTRODUCTION

1. Introduction

During the past years the overexploitation of fisheries resulted in an increase of aquaculture production to fulfil the market demands on marine products of a global exponentially growing human population. However, the domestication of cultured species requires an intense scientific and technological effort. One of the most sensitive stages of the fish life cycle is the reproduction period, which in many species has several constraints. The optimization of reproductive performance on husbandry broodstock is essential to obtain high quality fry, which is imperative to allow the aquaculture industry to produce high quality fish. Fish aquaculture production, as fish recruitment in the wild, depends directly on the quality and quantity of eggs available during the reproductive season (Lahnsteiner *et al*., 2009). Egg quality is determined by intrinsic and extrinsic factors, including the fertilization and incubation environment (Brooks *et al*., 1997). As in other vertebrates, embryos result from the fusion between oocyte and sperm, however traditional quality studies focused only on egg quality, since it was attributed to the female the responsibility to produce the egg substances necessary for normal larval development (Lahnsteiner *et al*., 2009). In the last years the quality of gametes of both female and male parents were admitted to be a contributing factor for fry viability, although in different ontogeny times and ways. The importance of male gamete quality in terms of undamaged genome, the male genetic compatibility with the female and the recent discover of specific mRNAs present in spermatozoa are important factors that contribute to the knowledge that sperm plays a crucial role in fertilization and embryo development (Cabrita *et al*. 2009; Miller *et al.*, 2005; Simmons, 2005). Until recently, spermatozoa contribution to fertilization process relied only on the fact that sperm needs to reach the oocytes and deliver the genomic content, which must be maintained undamaged to avoid abortions or embryonic malformations. The recent discover of specific mRNAs in mammalian sperm proposed that these transcripts might play an important role in genomic imprinting, conditioning sperm quality and even participating in the zygote development (Miller *et al.*, 2005). Another issue is the genetic compatibility that may be involved in fertilization selection. Stickleback females (*Gasterosteus aculeatus*) choose males that complement their own alleles, producing offspring with an optimal number of alleles (Aeschlimann *et al*., 2003; Reusch *et al*., 2001). It is then clear the importance of the spermatozoa genetic heritage or “male genetic factor” on egg quality, as well as male and sperm quality on reproductive performance.

Sperm quality has been a focus of research since it can be used as a biomarker of the male status (Cabrita *et al.*, 2009; Chauvaud *et al*., 1995). As in eggs, there is no consensus about the most suited sperm quality assessment technique. Nevertheless, several sperm parameters have been used to determine sperm quality with indubitable value for male characterization and broodstock selection, enabling the improvement of the quality of male broodstock as a mean to increase reproduction success. The study of sperm quality is essential to understand the overall dynamic of fertilization process in fish. This will allow for an improvement of the fertilization conditions, aiming for higher fertilization and hatching rates as well as reliable fry production being an important tool to apply in hatcheries and research.

1.1 Fish sperm quality

The use of high quality sperm is imperative for aquaculture purposes to ensure viable offspring when in contact with good quality eggs and appropriate environment (Kjørsvik *et al*., 1990). Cabrita *et al*. (2009) described the requirements for high sperm quality as the ability of the spermatozoa to reach the egg and the capacity to cross the egg envelopes or enter through the micropyle. It is also the ability to recognize the oolema and the fusion of both plasma membranes, the capacity to perform correct activation of the egg metabolic pathways and finally, sperm must have an undamaged genome with genetic compatibility with the egg genome (Simmons, 2005). The studies describing milt characteristics revealed high individual variability variations in different parameters (Dreanno *et al*., 1998). Consequently, as the definition of high quality sperm relies on its overall “fitness” and not on individual traits, an objective quality biomarker is difficult to institute (Cabrita *et al*., 2009).

The use of high quality sperm in fish farms is beneficial to increase the effectiveness of artificial fertilization, since low quality samples can be discarded. Also, the frequent sperm analysis of the male broodstock enables the identification of males with better reproductive traits, which in conjunction with genetic analysis allows for the selection of good breeders. Furthermore, it facilitates the storage of sperm, through short or long term storage improving the management of sperm in aquafarms (Cabrita *et al*., 2009; Rurangwa *et al*., 2004).

1.2 Factors affecting sperm quality

High quality sperm is primarily dependent of factors such as the paternal genetic heritage (Simmons, 2005), the spermiation period and sperm storage conditions in the testes, as well as the favourable environmental conditions during motility activation (Billard, 1986). Cultured species are very susceptible to husbandry management conditions, stress, disease, nutrition disorders, water quality and exposure to pollutants, which may impair spermiation and sperm quality. Sperm quality in many species changes along the reproductive season, such as in European sea bass (*Dicentrarchus labrax*), where two weeks after the beginning of spermiation period the sperm concentration decreased (Dreanno *et al*., 1999b). Morphological, biochemical and sperm motility traits changed at the end of the reproductive season in *Pagrus pagrus*, *Dicentrarchus labrax*, *Hippoglossus hippoglossus* and in some freshwater species (Billard *et al*., 1993; Dreanno *et al*., 1999b; Mylonas *et al*., 2003). In *Solea senegalensis* this variation was not so evident, but during female spawning season sperm quality was improved

(Cabrita *et al*., 2006). The stripping frequency also affects sperm quality if a recovery period is not respected, which is different between species (Cabrita *et al*., 2009).

The quality of sperm can be affected by the environmental conditions which males are exposed to. Due to the reproductive dysfunctions occurring in the F1 generation in some species, aquaculture breeders are sometimes collected from the wild. As a consequence their background is unknown, including their life-history with possible exposure to toxics that may affect their reproductive performance (Van der Oost *et al*., 2003). The male exposure to contaminants (heavy metals, organochlorides, carbamates, tributyltine) may cause hazardous effects on gonadal development, maturation and spermiation. In terms of sperm quality, the main effects are loss of sperm motility, velocity, viability, fertility and metabolic activity (Kime *et al*., 1996; Rurangwa *et al.*, 2002). In fresh water species such as rainbow trout (*Oncorhynchus mykiss*) a shift from warm to cold water improved sperm quality (Labbé *et al.*, 2001). However, in Senegalese sole (*Solea senegalensis*) an increase of water temperature during summer period resulted in a sperm volume decrease (Cabrita *et al*., 2009). Fish nutrition is known to affect the composition of seminal plasma and spermatozoa, such as plasma membrane phospholipids and cholesterol levels and distribution. For example, *S. senegalensis* males fed with mussels had higher cholesterol in spermatozoa plasma membrane than fish fed with polychaete (Cabrita *et al*., unpublished data). The same findings were reported in rainbow trout (Labbé *et al*., 1995).

Sperm handling is another factor that may affect sperm quality. In hatcheries that perform artificial fertilization it is a common procedure to maintain sperm in short or long term storage to optimize gametes management. These techniques can decrease sperm quality (Suquet *et al*., 2000) due to the negative effects of some procedures such as cooling, freezing or thawing, which may affect spermatozoa membrane integrity and fertilizing capacity (Parks and Graham, 2003). Consequently it is important to ensure the proper functioning of cell metabolism and maintenance of cell function through the development of adequate protocols and extenders, which can be monitored by sperm quality techniques.

1.3 Sperm quality evaluation

A quality assessment must be reliable and fast to be useful in commercial aquaculture (Cabrita *et al*., 2009). The most common milt quality biomarkers, such as spermatocrit, sperm density, osmolarity and pH of the seminal plasma, chemical composition of the seminal plasma, enzymatic activity, ATP concentration, motility, as well as fertilizing ability have been determined in several species (Billard *et al*., 1995a,b; Billard and Cosson, 1992; Ciereszko and Dabrowski, 1993, 1994; Chowdhury and Joy, 2001; Fauvel *et al*., 1998; Geffen and Evans, 2000; Lahnsteiner *et al*., 1996, 1998; Rurangwa *et al.,* 2004). For a more exhaustive analysis, the evaluation of other cell functions should be assessed. However when whole-milt quality is assessed it disregards the individual spermatozoa status, which can be disadvantageous when sperm from multiple males is mixed. Individual spermatozoa-based measurements are more discriminatory techniques to analyse membrane integrity, sperm morphology, ultrastructure, and sperm motility characteristics (Rurangwa *et al*., 2004). Cell viability is another assay that measures spermatozoa individual status and usually is related with plasma membrane integrity and resistance. The level of DNA fragmentation in nucleus can be used to assess the status of the DNA integrity. Single cell gel electrophoresis or comet assay is one of the methods used to perform this analysis (Lee and Steinert, 2003). Mitochondria function can be determined by measuring membrane potential, enzymatic activity or ATP levels. Their impairment can be responsible for the presence of non motile spermatozoa (Ogier *et al*., 1997). Fluorescent probes such as rodamine 123 or JC1 have been used to assay changes in mitochondria membrane potential in rainbow trout (Ogier *et al*., 1997), and in gilthead seabream (Cabrita *et al*., 2005).

Sperm motility is the most studied parameter of sperm quality assessment in fish due to its correlation with fertility (Rurangwa *et al*., 2001). Although it is an incomplete physiological analysis and needs other quality assays to guarantee the status of spermatozoa, it may reveal the probability of fertility success and is useful to analyse the effects of different treatments (Kime *et al*., 2001).

Sperm motility studies are adequate for sperm quality assessment purposes in aquaculture, since fish sperm has several characteristics which facilitate this type of analysis. Fish are generally external fertilizers that possess immotile spermatozoa in the seminal fluid, consequently it is easy to trigger its motility with a competent medium (Cosson *et al*., 2008a,b). All these facts support that sperm motility analysis is an advantageous technique for fish sperm quality assessment (Cosson *et al*., 2008a,b).

1.4 Sperm motility activation mechanisms

Spermatozoa from teleost fish are flagellated single cells adapted to external fertilization that undergo a long period of spermatogenesis in a safe environment, surrounded by seminal plasma and sertoli cells in the testes, with physicochemical conditions similar to the body environment (Billard, 1986; Schulz *et al*., 2002). Under these conditions, spermatozoa are immotile in the testes and only acquire motility when in contact with the external medium.

In marine fish, when sperm is delivered into the seawater, during the fertilization process, along with eggs, cells make contact with an extremely hazardous medium. Sperm motility is then trigged by hyperosmotic shock combined with ionic exchange with the environment, causing a severe change in the sperm membrane potential (Morisawa *et al*., 1983). The change in the membrane potential promotes a rise in the intracellular ionic concentration, especially Ca^{2+} ions, and a pH rise resulting in flagellar beating, thus promoting motility activation (Cosson *et al*., 2008a,b). Once the movement is started, the cells have only few seconds or minutes to reach the oocytes and penetrate the micropyle before it closes up or the few mitochondrial ATP reserves in sperm get fully exhausted (Rurangwa *et al*., 2004). In *Sparus aurata* (Cabrita *et al*., 2006), *S. senegalensis* (Cabrita *et al*., 2007; Martínez-Pastor *et al*., 2008) and flounder (Oda *et al*., 1998), sperm motility is trigged by hyperosmotic solutions of sugar or other non-ionic compounds, revealing that motility is probably mediated by osmotic pressure receptors in the membrane (Cabrita *et al*., 2009). Also, it is known that the activation occurs when sodium ions of seawater are substituted by choline chloride (Cosson *et al*., 2008a). It is assumed that sperm motility activation in marine species occurs through a positive osmolarity gradient between the outside and the inside of the cells. This process was also proved to be reversible in a triggering/inactivation mechanism (Cosson *et al*., 2008a). In freshwater species, sperm motility is activated when in contact with the hypotonic external environment. Krasznai *et al*. (2000) proposed for carp semen a cell signalling cascade to promote sperm motility activation, where the hyposmotic shock causes the opening of K^+ channels due to the low ionic concentration in the environment, promoting an efflux of K^+ from the cell that hyperpolarizes the plasma membrane. This mechanism is followed by a plasma membrane depolarization and consequently an influx of Ca^{2+}, promoting flagellar beating.

In euryhaline, as in most species, osmolarity is considered to be the most important feature to trigger sperm motility. The sperm motility activation depends on the period of fish adaptation to the environment. Individuals adapted to freshwater environmental, hypotonicity is the triggering factor promoting motility activation, whether individuals adapted to saline conditions, motility is trigged by hypertonicity (Morita *et al*., 2003).

1.5 Factors affecting sperm motility: environmental and biological cues

Immediately after activation fish spermatozoa reveal the highest motility efficiency, declining progressively motility parameters throughout time. Initially all the ATP mitochondrial reserves are fully available but in fact the energy management throughout time and its expenditure can be modulated by the characteristics of the motility activation media (Cosson *et al.,* 2008a,b).There are several factors that affect sperm motility in fish which can be considered as environmental and biological factors.

1.5.1 Environmental cues

The temperature of the activation solutions is known to affect spermatozoa since it increases cell metabolism, causing an increase in velocity with quicker depletion of the scarce energetic resources, promoting an earlier motility cessation. On the other hand, lower temperature results in a prolongation of sperm motility with reduction in velocity and flagellar beating frequency, (reviewed in Cosson *et al*., 1985). The temperature affects differently the beating frequency of spermatozoa flagella, which is more or less physiological related to the adaptation of each species to natural environment conditions (Alavi and Cosson, 2005).

Osmolarity seems to be one of the most relevant factors in motility activation in several species. As mentioned previously, the hypertonicity of the environment triggers motility activation on marine fish sperm, while the opposite triggers motility in most freshwater species. This fact is widely reported for species such as Atlantic cod (*Gadus morhua*) (Westin and Nissling, 1991), European sea bass (*Dicentrarchus labrax*) (Dreanno *et al*., 1999b) and gilthead seabream (Billard, 1978). The salinity is not usually considered by itself an independent factor in sperm motility triggering because is associated with ionic composition such as Ca^{2+}, K^{+}, Mg^{2+}, Na^{+}.

The effect of pH from the activation solution is considered to have a low interference in sperm motility activation (Cosson, 2004). However it is known that the internal pH of the cell is a key factor for motility initiation. Gatti *et al*. (1990) reported that rainbow trout sperm plasma membrane potential is sensitive to external pH, consequently improving membrane hyperpolarization. Miura *et al*. (1992) demonstrated that a rise of external pH was associated with an increase of cAMP levels in masou salmon (*Onchorynchus masou*), promoting sperm motility activation. Nevertheless in species such as sea bass (*Dicentrarchus labrax*) (Billard *et al*., 1977), turbot (Chauvaud *et al*., 1995) and halibut (Billard *et al*., 1993) spermatozoa achieved motility activation in a wide range of pH with optimal motility values in a specific pH for each species, although with a tendency for slightly alkaline solutions.

1.5.2 Biological cues

The presence of ovarian fluid in the activation solution improves sperm longevity in freshwater species (Lahnsteiner *et al*., 1995; Turner and Montgomerie, 2002; Urbach *et al*., 2005; Wojtczak *et al*., 2007; Dietrich *et al*., 2008) and may enhance sperm motility characteristics such as speed, trajectory and motility pattern (Lahnsteiner *et al*., 1996). The same findings were observed by Litvak and Trippel (1998) in Atlantic cod (*Gadus morhua*). These facts imply an important female role in the fertilization process and in the characterization of sperm motility, being the ovarian fluid one of the main biological factors that may modulate sperm motility.

The mechanism through which ovarian fluid may affect sperm motility is not known, but may be related with its physico-chemical composition. Ovarian fluid composition is highly variable among species, but also between individuals, due to differences in females such as physiological status, maturation grade and egg quality (Lahnsteiner, 2002). It has several hormones, nutrients and metabolites that spermatozoa may metabolize (Lahnsteiner *et al*., 1996), thus also working as a substrate for spermatozoa physiological functions. Wojtczak *et al*. (2007) showed that in rainbow trout (*Oncorhynchus mykiss*), ovarian fluid pH improved sperm motility. Its characteristics may also modify the osmolarity of the fertilization microenvironment, affecting the duration of sperm motility (Lahnsteiner *et al*., 1995; Westin and Nissling, 1991;

Wojtczak *et al*., 2007). However these facts are related to the triggering of sperm motility and the real mechanism behind the influence of ovarian fluid is still unknown.

The role of ovarian fluid on sperm motility in some species might also be related to the presence of chemoatractants released by the eggs (Urbach *et al*., 1995). Chemotaxis is the modulation of the direction of movement of motile cells in response to a chemical gradient stimulus, resulting in approach to an attractant or retreat from a repellent. In species like herring the chemotaxis physiological role of ovarian fluid/egg is to attract as many spermatozoa as possible towards the egg in response to its chemoatractive factors (Yanagimachi *et al*., 1992). There are still few studies about chemoatractants composition and it is quite variable among species, but in herring (Ohtake, 2003) and corals (Coll *et al*., 1994) they were found to be low molecular weight proteins.

1.6 Methods for sperm motility analysis

Sperm motility can be determined using two assessment methods: subjective analysis and quantitative computer assisted methods. During subjective analysis of motility, an estimation of motile cells and velocity is made by an observer scoring the results in an arbitrary scale of criteria from 0 (immotile) to 5 (all spermatozoa highly motile) (Billard *et al.*, 1995). This method is usually used in fishfarms and in order to guarantee some accuracy of results, the observations should be performed by the same observer. One of the main problems in these nonlinear arbitrary scales is that data cannot be statistically analysed (Rurangwa *et al*., 2004). To reduce the subjectivity of the tests, recorded images of activated samples and further videotape analysis was performed by multiple observers to determine sperm motility, but this procedure expended too much time and was not practical (Kime *et al*., 2001). Sperm motility became easily verified and quantifiable especially since quantitative computer assisted methods were developed. Sperm motility analysis software such as Computer Assisted Sperm Analysis (CASA) software was developed for human tests and later for other mammalian species (Vantman *et al*., 1989; Farrell *et al*., 1998; Hirano *et al*., 2001). The first sperm motility study in fish was conducted by Cosson *et al*. (1985) using video recording with stroboscopic illumination. In the last years CASA systems were adapted to sperm motility in fish, thus allowing a more practical scientifically accurate assessment of motility parameters (Kime *et al*., 2001). The delay in the implementation of CASA analysis in fish sperm was due to its biological differences compared to mammalian sperm, since it has a short lifespan and high flagellar beating frequency after activation (Billard and Cosson, 1992). However, the use of this software has not yet been widely adopted by the Aquaculture industry, since although it is a very simple and practical analysis, it requires some investment.

CASA can generate several parameters related with spermatozoa type of movement, trajectory and velocity. In fish, the percentage of motile cell, progressive spermatozoa, straight line velocity (VSL), curvilinear velocity (VCL), and linearity are the most common parameters analysed to characterize a sperm sample. Computer sperm analysis generates thousands of data since it registers individual spermatozoa characteristics. Consequently, several authors have been studying the best method to interpret results. Mean values have been used to characterize samples, but due to the high heterogeneity of sperm samples, some characteristics may be hidden, overlooking the useful information of the individual variability. Due to this heterogeneity and the identification of different characteristics within an ejaculate, the possibility of using subpopulations in the interpretation of results has gained more value. The study of sperm subpopulations started originally on mammalian species such as gazelle (Abaigar *et al*., 2001), stallion (Quintero-Moreno *et al*., 2003) and red deer (Martínez-Pastor *et al*., 2005), among others. Nowadays, the presence of distinct sperm subpopulations within a sample is generally accepted (Martínez-Pastor *et al*., 2005, 2006). Although it is potentially a powerful tool, the use of sperm subpopulations analysis is still unusual in fish sperm motility studies, although several reports have characterized sperm subpopulations in gilthead seabream and Senegalese sole (Beirão *et al*., in press; Martínez-Pastor *et al*., 2008). The mechanism of sperm subpopulations formation in the testes is still unknown as well as its physiological role (Martínez-Pastor *et al*., 2005, 2008). The analysis performed with CASA software has the ability to typify the individual motility of each spermatozoon, thus allowing a characterization and inclusion in a group or subpopulation with common characteristics, identified by means of cluster analysis. Subpopulation analysis has the potential to locate significant differences between treatments in the same sample that would otherwise be shadowed since, unlike mean values, it considers the heterogeneity of the sample. Furthermore, several studies showed correlations not only between subpopulations and fertilization ability but also sperm quality, physiology, behaviour and genetics (Petrunkina and Topfer-Petersen, 2000; Thurston *et al.,* 1999).

1.7 Applications of sperm motility assays

Fish sperm motility analysis has been used in numerous ecotoxicological studies since it is a good biomarker of contaminants exposure. The organs and tissues located in the abdominal cavity concentrate preferably the toxics absorbed in food and environment and the reproductive system is known to be vulnerable to contaminants toxic levels (Xing *et al*., 2008). The use of motility assays as indicators of pollutant effects has been used in several fish species such as flounder (Chauvaud *et al*., 1995) and African catfish (Rurangwa *et al*., 2002). Furthermore, it is advantageous to use sperm motility analysis for ecotoxicological studies since no fish slaughter is needed and individual fish monitoring can be achieved (Kime *et al*., 1996).

Sperm short and long term storage is another procedure that may require the analysis of motility either at fish farms or for research purposes. It is a common procedure in hatcheries to store sperm in order to optimize gametes management (Suquet *et al*., 2000). In cryopreservation studies, motility analysis is fundamental to select the best protocols for sperm storage and to identify damage occurring during this process. Motility analysis was a useful tool to assess the effect of different diluents in sperm cryopreservation in trout (*Oncorhynchus mykiss*) (Cabrita *et al*., 2001), in turbot (Dreanno *et al*., 1997), African catfish (Viveiros and Komen, 2000) and common carp (Linhart *et al*., 2000), among others. Also sperm motility is used to optimize not only the extenders but also the cryopreservation techniques for non-studied species, since to control sperm quality after the use of this technique several parameters must be tested, such as freezing and thawing velocities, extender and cryoprotectant type and concentration (Dreanno *et al*., 1997; Rurangwa *et al*., 2004).

When controlled maturation and spawning is obtained by hormonal treatments and applied in industrial hatcheries, sperm motility analysis is the most practical technique to use as a tool for male broodstock selection but also to check individual samples for artificial fertilization (Devauchelle *et al*., 1988). The use of this method for the optimization of fertilization conditions is highly suitable, since it is an *in vitro* test that can be extrapolated to *in vivo* conditions.

1.8 *Solea senegalensis* reproduction

Solea senegalensis (Order Pleuronectiformes, Family Soleidae) is a marine teleost widely distributed from the Mediterranean to the Atlantic Ocean. The southern limit of distribution is Senegal, which lent the name to this species. It presents a benthic behaviour, living on sandy or muddy bottoms usually in coastal waters and estuaries. This species tolerates substantial changes in the environment, such as temperature and salinity, with high stress resistance. This species is gonochoric, with females reaching the maturation at age 3+. In the Portuguese coasts it has two reproduction periods per year, being the first one during the spring (March/April-June) and the second one during the autumn (September) (Dinis *et al*., 1999).

Although many achievements in *S. senegalensis* production have been accomplished in the past years, some production restrictions remain unsolved, mainly related with reproduction control. Some of the main bottlenecks in this species reproduction are variable egg production and quality, low fertilization rates and the dependence on wild broodstock. For these reasons the selection of good breeders that produce quality gametes is essential to overcome the reproductive dysfunctions and the low fertility rates obtained. In this species low fertility rates are observed not only due to variable egg quality, but also to the high variability in sperm quality within males (Cabrita *et al*., 2006).

The first attempts to induce spawning hormonally resulted in the production of eggs that either are not fertilized, fail to hatch or abort (Dinis *et al.*, 1999). The fluent males can produce motile sperm all year around during spermiation cycles (Cabrita *et al*., 2006). High spermiation periods are registered in mid March, May and during the second spawning period in October, coinciding with the female reproduction cycle (Anguis and Cañavate, 2005; Cabrita *et al*., 2006; Dinis *et al*., 1999; García-López *et al*., 2005, 2006), although it was observed in captivity that not all males revealed a peak of spermiation during this period. *S. senegalensis* sperm production is very low compared to other flatfish species and ranges from 50 to 110×10^6 spermatozoa in high spermiation periods to less than 20×10^6 spermatozoa, due to the low sperm volume (less than 80 µl individually) and concentration (Cabrita *et al*., 2006).

Like most marine teleost fish species with external fertilization *S. senegalensis* spermatozoa remain immotile until the contact with seawater. The spermatozoa lifespan of this species ranges from 1 to 1.5 minutes (Cabrita *et al*., 2006). Sperm motility in *S. senegalensis* is higher within the spermiation period that coincides with the females' reproductive cycle. During this period, Cabrita *et al*. (2006) registered 80% of spermatozoa showing progressive movement. Their results also showed that high temperatures during August seem to inhibit sperm motility by 50%.

In this species different sperm subpopulations could be identified in the same ejaculate according to motility and resistance to osmotic shock criteria. The variability of spermatozoa quality within the same ejaculate has been attributed to top cell aging (Martínez-Pastor *et al*., 2008). Beirão *et al*. (2008) identified some discrepancy in the maturation state among the males which can represent a problem in this species since during the female spawning season some males have highly variable sperm quality, especially in terms of cell damage. All studies in this species revealed a high level of spermatozoa sensitivity to the external environment; however the most adequate environmental conditions to spermatozoa were not studied yet. There is a lack of information about the factors that affect sperm motility in *S. senegalensis.* The modulation of the sperm motility activation solution can infer the reproductive natural conditions of this species, but most especially it can give indications about the proper husbandry conditions during the reproductive season to enhance the male potential to fertilization for aquaculture purposes.

OBJECTIVES

2. Objectives

The first objective was to perform an assessment of the optimal conditions, in terms of temperature, salinity and pH, for *S. senegalensis* sperm motility activation. This work aimed to reach some conclusions about the husbandry conditions that favour an improvement and prolongation on sperm motility in this species. A comparative analysis between multivariate cluster analysis and mean values with this data was performed in order to conclude which analytical tool is more suited to *S. senegalensis* sperm motility analysis.

The second objective of this work was to assess the influence of homologous and heterologous ovarian fluid on *Solea senegalensis* sperm motility. Additionally, ovarian fluid specificity has been evaluated as well as the effect of concentration on the electiveness of the activation solution.

MATERIAL AND METHODS

3. Material and Methods

3.1 Broodstock husbandry conditions

The experiments were carried out at the Ramalhete aquaculture station (Faro, Portugal) using *Solea senegalensis* males (1,144.6 ± 709.9 g) from an originally wild-captured broodstock established in captivity 3 years before the onset of experiments. The fish (n = 50, 25 females and 25 males) were kept in 4 round fiberglass tanks (3,000 L) with sand subtract in a semi-closed system with 500 L/h of water flow with aeration (Figure 1). The individuals were subjected to a natural photoperiod and the temperature and salinity fluctuated according to Ria Formosa's natural patterns (37º00´N, 7º56´W) (Figure 2). The tanks were indoors with overhead fluorescent daylight lamps (58 watts) with 200 Lux at water surface. Breeders were maintained at a density of 5 Kg/m^3 with a sex ratio of 1:2 (female:male). The fish were fed with 3% of biomass every day, alternating between frozen mussel and squid. Each individual was tagged with a PIT Tag (Trovan, NL) for individual monitoring. Experiments were performed during the reproductive season lasting from March to June.

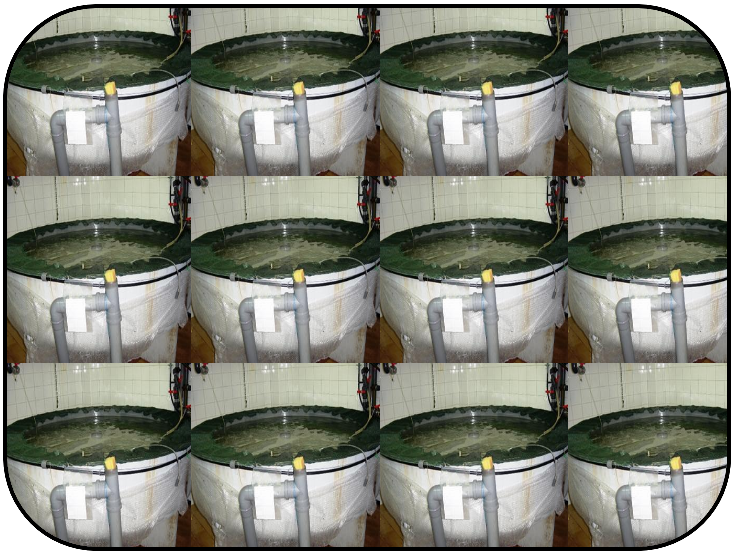


Figure 1 - *S. senegalensis* broodstock tank.

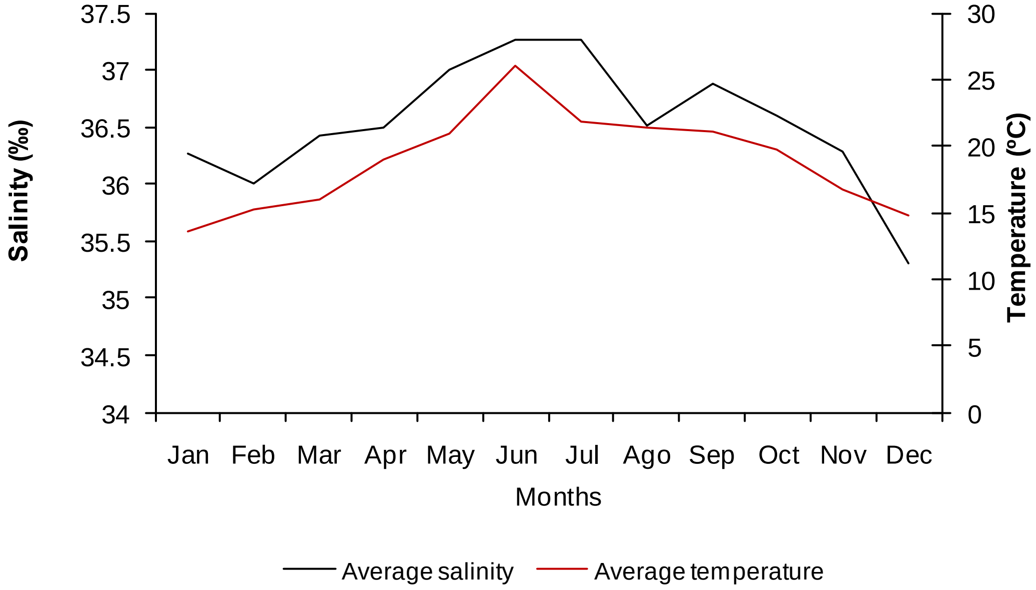


Figure 2 - Temperature and salinity fluctuations throughout the year at Ramalhete aquaculture station.

3.2 Sperm collection

The males were anaesthetized with 2-phenoxyethanol (300 ppm) in seawater (Figure 3 A) and the sperm was collected by testes pressure in the ventral side (blind side) of the fish. The urogenital pore was carefully cleaned to remove seawater, mucus and faeces that might contaminate the sample; afterwards the sperm was collected with a syringe (Figure 3 B). Also it was important to verify that no urine contaminated the sample, which is particularly difficult in this species due to low sperm volume. Thus all contaminated samples (more transparent and diluted) were discarded. The samples were maintained in microtubes at 7 ºC until analyse. This procedure was performed to all males, however after sperm motility analysis, 7 samples were selected for temperature, salinity and pH effect on sperm motility activation; and 8 samples were selected for ovarian fluid effect on sperm motility activation.

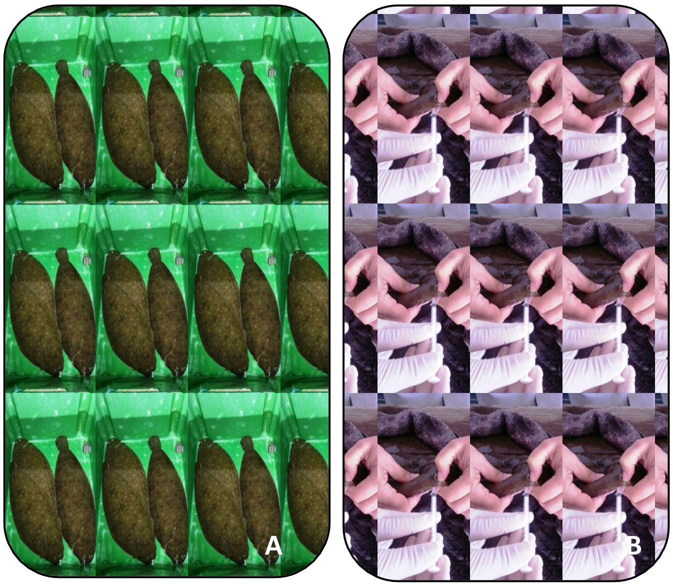


Figure 3 - A) *S. senegalensis* anaesthesia, B) sperm collection.

3.3 Ovarian fluid collection

The ovarian fluid was collected from two senegalense sole females (homologous ovarian fluid) and two dusky grouper (*Epinephelus marginatus*) females (heterologous ovarian fluid). Females of *S. senegalensis* were slaughtered using a lethal dose of 2-phenoxyethanol (3,500 ppm), and the gonads were excised. The gonad was pressed through a 50 µm mesh to separate the eggs from the fluid (Figure 4). The fluid was centrifuged (10 000 g, 20 min, 4 ºC) to eliminate the debris and was immediately stored at -80ºC. Grouper (*E. marginatus)* ovarian fluid was tested to check for species specificity during motility activation and fluid was collect from fish stripping.

Figure 4 - Ovarian fluid collection using a 50 µm mesh.

3.4 CASA system and parameters analysed

The spermatozoa were analysed with the software CASA (Computer Assisted Sperm Analysis) which consists in a image capture software (ISAS, Proiser, Valencia, Spain), connected with a firewire to a digital recording camera (Basler 312fc/c, Basler Vision Technologies, Ahrensburg, Germany), which in turn is attached to a optical phase-contrast microscope (Nikon E200, Tokyo, Japan). The image capture was performed with a 10x negative phase contrast objective. Sperm was activated in a Makler chamber covered with a special cover slip to capture only one field of cells. Image sequences were saved at 15, 30, 45 and 60 s post-activation and analysed afterwards.

CASA software settings were previously adjusted for analyzing fish spermatozoa namely: 30 frames/s for acquisition for 1 s acquisition time; 10–80 mm^2 for head area (this wide range was necessary for acquiring all spermatozoa). The parameters analysed for each spermatozoa where defined by Boyers *et al*. (1989) as VCL (curvilinear velocity, according to the actual path; µm/s), VSL (straight line velocity, according to the straight path; µm/s), VAP (velocity according to the smoothed path; µm/s), LIN (Linearity, VSL/VCLx100; %), STR (straightness, VSL/VAPx100; %), WOB (wobble, VAP/VCLx100; %), ALH (amplitude of lateral displacement of sperm head; µm), and BCF (beat-cross frequency; Hz). Furthermore, the percentage of total motility (TM; %) and progressive motility (PM; %) were assessed by mean values of all spermatozoa in each analysis.

3.5 Experimental design:

3.5.1 Effect of temperature, salinity and pH on sperm motility activation

Sperm (n = 7) was extracted from males using the methods previously described. Sperm was previously diluted with 300 mOsm/Kg sucrose solution (1:5) and activated with artificial seawater (1.5:5) in a Makler chamber in the microscope. The activation was performed with artificial seawater set at two temperatures (16 and 20 ºC), three salinities (25, 30 and 35 ‰) and three pH conditions (6, 7.4, and 9) (Figure 5). Motility parameters described previously, were recorded individually at 15, 30, 45 and 60 seconds after activation, and three replicates were done for each sample.

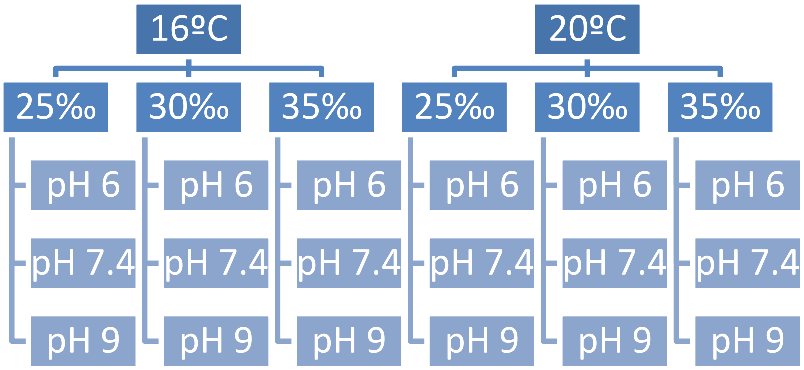


Figure 5 - Experimental design for each post-activation time.

3.5.2 Effect of homologous and heterelogous ovarian fluid concentration on motility activation

To study the influence of ovarian fluid in the activation of sperm motility in *S. senegalensis*, several concentrations of homologous ovarian fluid (*S. senegalensis*) and heterelogous (E*. marginatus*) ovarian fluid were tested. The ovarian fluid was collected as previously described and tested in the activation of sperm from 8 males. Previously, the ovarian fluid osmolarity was analysed with a cryo-osmometer (OSMOMAT 030, Gonotec); samples from *S. senegalensis* had 490 and 495 mOsm/Kg and samples from *E. marginatus* had 360 and 357 mOsm/Kg. For solutions preparation, artificial seawater was diluted with ovarian fluid in the following concentrations (v:v): 0:100, 25:75 50:50, 75:25, and 100:0 (seawater control). The osmolarity and pH of the several tested activation solutions are shown in Table 1. For motility activation, sperm was previously diluted with 300 mOsm/Kg sucrose solution (1:5) and activated with artificial seawater (1.5:5) as described previously. The spermatozoa were analysed with the software CASA and the described motility parameters were recorded individually at 15, 30, 45 and 60 seconds after activation.

Table 1 - Osmolarity and pH of activation solutions used in the sperm motility analysis. SW- seawater; OF- ovarian fluid.

		OF:SW dilution				
Ovarian Fluid	**Osmolarity/pH**	**0:100**	**100:0**	**25:75**	**50:50**	**75:25**
Homologous OF1	mOsm/Kg	1072	471	988	889	693
	pH	8.33	6.70	6.56	6.53	6.59
Homologous OF2	mOsm/Kg		514	1000	852	723
	pH		6.46	6.68	6.46	6.41
Heterelogous OF1	mOsm/Kg		383	906	737	550
	pH		8.07	8.32	8.15	8.23
Heterelogous OF2	mOsm/Kg		359	913	764	557
	pH		8.44	8.41	8.39	8.44

3.5.3 Effect of *S. senegalensis* ovarian fluid on individual male sperm motility activation

The effect of ovarian fluid on the activation of sperm was analysed. For that purpose, sperm from 8 males was activated with 25% of homologous ovarian fluid and with 100% seawater as described before. CASA parameters were recorded as previously described.

3.6 Data analysis and statistics

To perform mean values analysis for each frame, the individual spermatozoa data was extracted from the software to excel format and a macro was programmed to generate mean values for TM, PM, VCL, VSL and LIN. Whenever necessary the data was normalized and the adequate statistical analysis was performed. The effect of temperature, salinity and pH in sperm motility, in both mean values and subpopulation analysis, was submitted to three-way ANOVA multivariate statistical analysis (SNK $p < 0.05$). The effect of ovarian fluid in sperm motility, in both mean values and subpopulation analysis was submitted to one-way ANOVA. However the effect of ovarian fluid on individual male samples was submitted to independent samples Student’s t-test analysis.

To perform subpopulation analysis, a k-means cluster analysis was performed generating clusters that characterized different sperm subpopulations. Each spermatozoon was labelled within its subpopulation and the number of spermatozoa subpopulations for each treatment was identified. To establish subpopulations a two step cluster analysis with log likelihood distances and Schwarz´s Bayesian criterion (BIC) was performed to all CASA parameters to 80 033 spermatozoa in the first experiment and to 23 855 spermatozoa in the second one. The percentage of spermatozoa in each subpopulation was assessed for all treatments. All data (mean values and cluster analysis) were submitted to ANOVA multivariate statistical analysis (SNK $p < 0.05$) (SPSS 17.0).

RESULTS

4. Results

4.1 Effect of temperature, salinity and pH on sperm motility activation

The results gathered from the motility data of all spermatozoa parameters for each treatment and sampling time, resulted in mean values for the 7 selected male samples. The parameters described here are the most relevant descriptors for fish sperm motility analysis, namely TM, PM, VCL, VSL and LIN. A three-way ANOVA was performed for each post activation time for all parameters to analyse if there are interactions between the effects of temperature, salinity and pH. Since there were no interactions between the parameters analysed (Table 2) each factor could be analysed independently.

Temperature affected significantly all parameters except linearity (Figure 6 E) at 15 s post-activation. Its effect decreased in the following times affecting significantly TM ($p = 0.003$), PM ($p = 0.029$) and VCL ($p = 0.018$) at 30 s post-activation, TM ($p = 0.018$) and PM ($p = 0.006$) at 45 s post-activation and TM ($p = 0.048$) at 60 s post-activation.

Salinity affected significantly TM ($p = 0.043$) and LIN ($p < 0.001$) at 15 s post-activation and LIN ($p = 0.025$) at 30 s post-activation, however the effect of salinity was more pronounced in the last post-activation times. At 45 s post-activation, VCL ($p = 0.002$), VSL ($p = 0.004$) and LIN ($p < 0.001$) had significant differences among salinity treatments, as well as at 60 s post-activation (PM ($p = 0.019$), VCL ($p = 0.034$) and LIN ($p = 0.001$)).

The effect of pH was the less evident since only one significant different was observed for linearity ($p = 0.005$) at 30 s post-activation.

Table 2 - Statistical differences for temperature, salinity and pH for total motility (TM), progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL) and linearity (LIN) on *S. senegalensis* spermatozoa at 15, 30, 45 and 60 s post-activation, sustained by mean values of 7 males. The effect of the three tested conditions on motility parameters was detected by a three-way ANOVA (p < 0.05), significant differences are highlighted in bold.

Time	p < 0.05	TM	PM	VCL	VSL	LIN
15 s	Temperature	**0.006**	**0.016**	**0.042**	**0.017**	0.985
	Salinity	**0.043**	0.637	0.517	0.632	**<0.001**
	pH	0.367	0.975	0.865	0.816	0.577
	Temperature*Salinity	0.704	0.999	0.614	0.583	0.432
	Temperature*pH	0.992	0.821	0.874	0.896	0.434
	Salinity*pH	0.756	0.818	0.171	0.133	0.611
	Temperature*Salinity*pH	0.351	0.786	0.961	0.928	0.442
30 s	Temperature	**0.003**	**0.029**	**0.018**	0.057	0.731
	Salinity	0.598	0.428	0.442	0.374	**0.025**
	pH	0.487	0.397	0.433	0.153	**0.005**
	Temperature*Salinity	0.859	0.522	0.606	0.422	0.446
	Temperature*pH	0.897	0.972	0.960	0.997	0.494
	Salinity*pH	0.262	0.067	0.059	0.063	0.096
	Temperature*Salinity*pH	0.834	0.988	0.986	0.938	0.751
45 s	Temperature	**0.018**	**0.006**	0.057	0.065	0.072
	Salinity	0.323	0.087	**0.002**	**0.004**	**<0.001**
	pH	0.959	0.609	0.256	0.283	0.157
	Temperature*Salinity	0.697	0.968	0.310	0.261	0.575
	Temperature*pH	0.800	0.680	0.929	0.903	0.934
	Salinity*pH	0.704	0.626	0.362	0.362	0.645
	Temperature*Salinity*pH	0.560	0.413	0.379	0.379	0.573
60 s	Temperature	**0.048**	0.118	0.926	0.915	0.641
	Salinity	0.277	**0.019**	**0.034**	0.052	**0.001**
	pH	0.936	0.172	0.541	0.620	0.173
	Temperature*Salinity	0.997	0.889	0.62	0.644	0.221
	Temperature*pH	0.885	0.891	0.761	0.749	0.752
	Salinity*pH	0.809	0.583	0.607	0.515	0.579
	Temperature*Salinity*pH	0.846	0.871	0.983	0.970	0.785

4.1.1 Effect of temperature on sperm motility

The effect of temperature on *S. senegalensis* sperm motility was significantly different for total motility (TM) and progressive motility (PM) and both velocities analysed (VSL and VCL). No significant differences were observed in linearity of spermatozoa trajectory between 16 ºC and 20 ºC.

During the motile period total motility was always significantly higher for 20 ºC (58.8%, 44.6%, 28.4%, and 16.6%, respectively) than for 16 ºC (43.6%, 32.5%, 22.2%, and 13.1%, respectively (Figure 6 A). Similarly, progressive motility was significantly improved with 20 ºC solutions (15.2%, 14.1%, and 9.9% respectively) than with 16 ºC solutions (11.3%, 9.6%, and 5.9% respectively) except at 60 s post-activation (Figure 6 B). Both sperm velocities were significantly improved by 20 ºC solution at 15 s (VCL - 76.2, VSL - 92.9 µm/s) and 30 s post-activation (VCL - 69.4%, VSL - 80.7%, compared with 16 ºC solution (VCL - 64.8%, VSL - 77.8% at 15 s post-activation and VCL - 57.3%, VSL - 66.9% 30 s post-activation) (Figure 6 C, D).

In all post-activation times it became evident that higher temperature improved significantly sperm motility. This improvement was not at the expense of premature motility loss. The results also show that it improved preferably parameters such as TM (p = 0.006), PM (p = 0.016), VCL (p = 0.042) and VSL (p = 0.017) (Table 2). Moreover, the effect of temperature seemed to improve significantly more parameters in the first recorded post-activation periods (such as TM, PM, VCL and VSL) than at the end of motility (such as TM and PM).

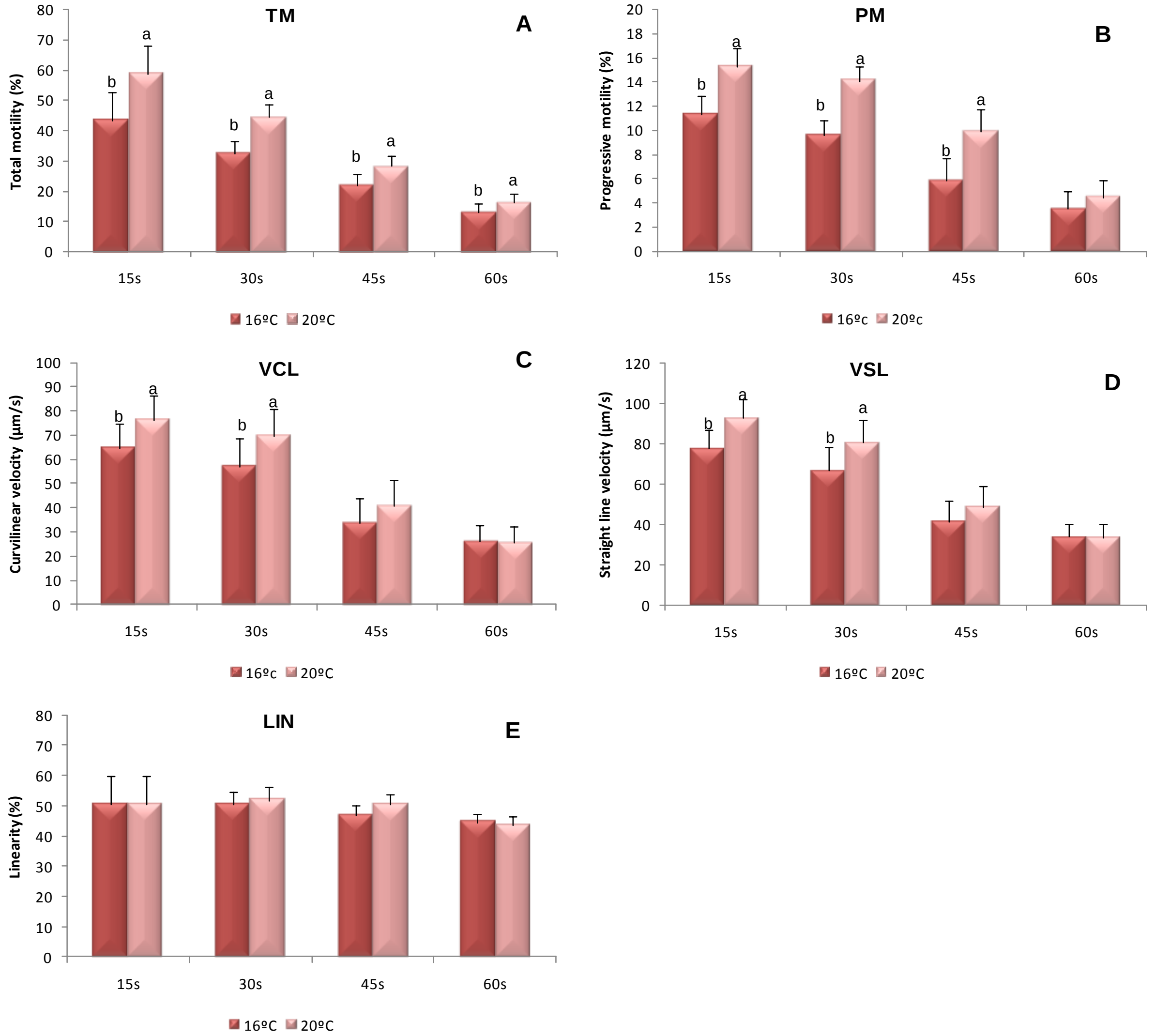


Figure 6 - Effect of temperature (16 ºC and 20 ºC) in *S. senegalensis* sperm motility at 15, 30, 45 and 60 s post-activation. Statistical differences (p < 0.05) between temperatures in each post-activation time are represented with letters. A - Total motility, B - Progressive motility, C- curvilinear velocity, D - straight line velocity, E - linearity.

4.1.2 Effect of salinity on sperm motility

The effect of salinity on *S. senegalensis* sperm motility could be observed in all the parameters analysed. In most of all parameters lower salinity improved sperm motility except for total motility where 30 ‰ (57.5%) and 35 ‰ (54.5%) were significantly higher than 25 ‰ (41.7%) at 15 s post-activation (Figure 7 A).

Progressive motility and both velocities (VCL, VSL) were improved with 25 ‰ solution in the last periods of motility. Progressive motility was significantly higher with 25 ‰ solution (5.6%) than 35 ‰ (2.9%) at 60 s post-activation although no differences were observed from 30 ‰ solution (3.5%) Figure 7 B). VCL and VSL at 45 s (VCL - 46.3 µm/s, VSL - 53.7 µm/s) post-activation were significantly higher comparing with 30 ‰ (VCL - 35.2 µm/s, VSL - 43.4 µm/s) and 35 ‰ (VCL - 30.6 µm/s, VSL - 38.9 µm/s). Curvilinear velocity had also significantly higher values with 25 ‰ (31.7 µm/s) than 30 ‰ (23.9 µm/s) and 35 ‰ (22.1 µm/s) at 60 s post-activation
(Figure 7 C, D).

The lowest salinity improved sperm linearity throughout the post-activation time (15 s – 55.4%, 30 s – 54.5%, 45 s – 55.0%, 60 s – 49.7%) comparing with 30 ‰ (15 s – 48.1%, 30 s – 49.7%, 45 s – 47.0%, 60 s – 41.4%) and 35 ‰ (15 s – 47.9%, 30 s – 49.5%, 45 s – 44.0%, 60 s – 41.5%) (Figure 7 E).

The use of low salinity (25 ‰) in the activation solution proved to enhance significantly parameters of sperm motility mainly linked with spermatozoa trajectories, such as LIN, throughout the motile period. Salinity was one of the important parameters tested for sperm motility, improving most of the motility parameters.

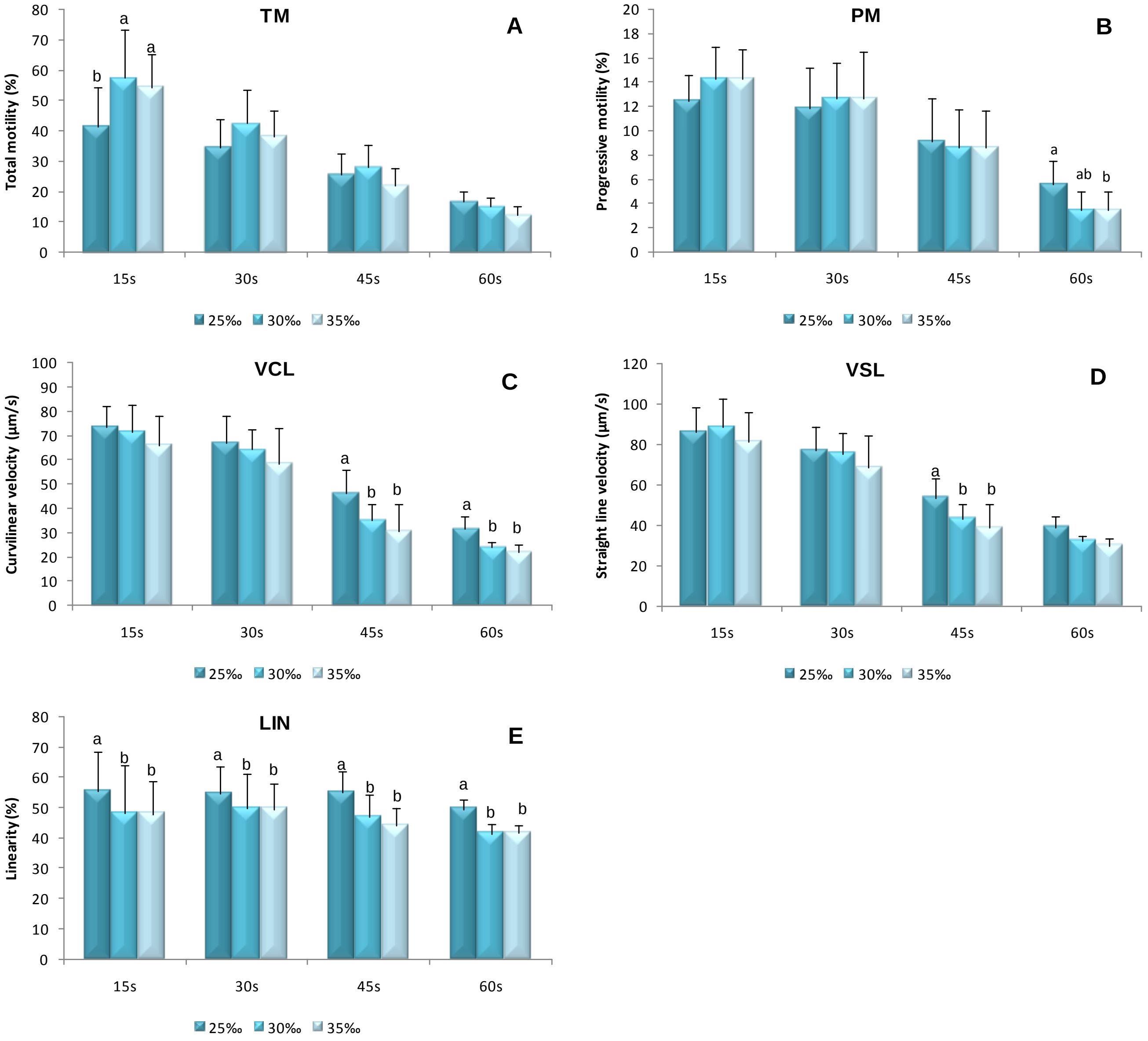


Figure 7 - Effect of salinity (25 ‰, 30 ‰ and 35 ‰) in *S. senegalensis* sperm motility at 15, 30, 45 and 60 s post-activation. Statistical differences ($p < 0.05$) between temperatures in each post-activation time are represented with letters. A - Total motility, B - Progressive motility, C- curvilinear velocity, D - straight line velocity, E - linearity.

4.1.3 Effect of pH on sperm motility

The effect of pH on *S. senegalensis* sperm motility was the less evident in this experiment, since no significant differences were detected in TM, PM, VCL and VSL (Figure 8 A,B,C,D). Nevertheless at 30 s post-activation, linearity was significantly lower with activation solution at pH 9 (48.8%) than pH 7.4 (51.6%) and pH 6 (53.4%) (Figure 8 E).

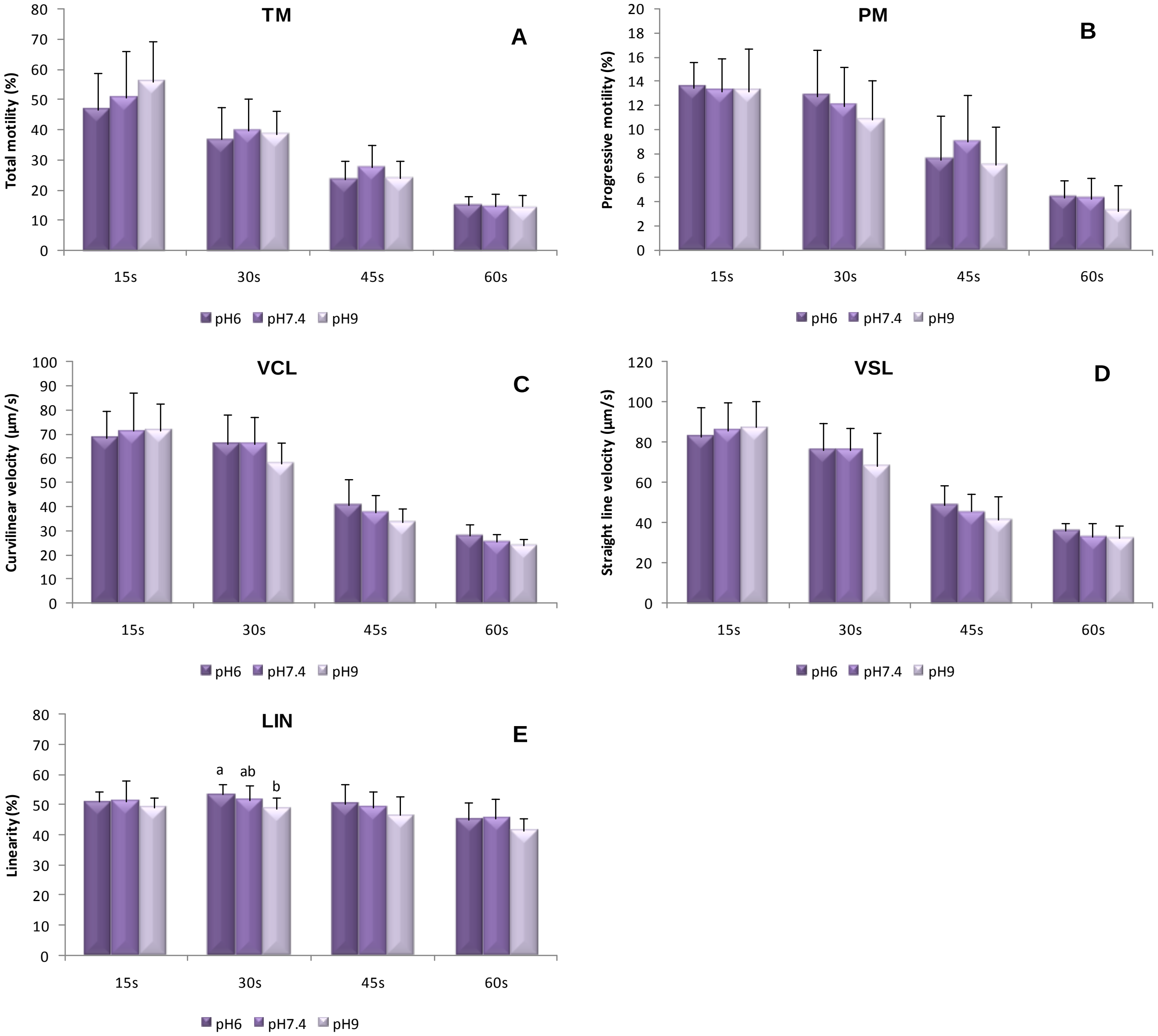


Figure 8 - Effect of pH (6, 7.4 and 9) in *S. senegalensis* sperm motility at 15, 30, 45 and 60 s post-activation. Statistical differences ($p < 0.05$) between temperatures in each post-activation time are represented with letters. A - Total motility, B - Progressive motility, C- curvilinear velocity, D - straight line velocity, E - linearity.

Considering all the conditions tested, the pH effect on sperm motility seemed to be the less important in *S. senegalensis* sperm motility activation comparing with the effect of temperature and salinity.

4.2 Ovarian fluid influence on *Solea senegalensis* sperm motility

4.2.1 Effect of homologous and heterelogous ovarian fluid concentration on motility activation

To analyse the sperm motility activation with ovarian fluid, the mean values of all spermatozoa captured with CASA of 8 males, for each treatment was recorded and analysed with a one-way ANOVA.

The homologous ovarian fluid had higher osmolarity, but lower pH comparing to heterelogous fluid (Table 1). Ovarian fluid by itself did not promote motility activation, since it had low osmolarity, which was very similar to seminal plasma. High concentrations of ovarian fluid (75%) promoted a deficient motility activation of spermatozoa, less than 10% of motile sperm, and consequently the analysis was discarded (data not shown). TM at 15 s post-activation was 63.6 ± 23.4% and at 60 s was 15.8 ± 11.9% when sperm was activated only with seawater (Figure 9 A). The presence of ovarian fluid in the activation solution in both concentrations (25% and 50%) produced significantly higher results for PM, VCL and VSL than control solution (100% seawater) (Figure 9 B, C, D). A supplementation of 25% homologous ovarian fluid produced the highest results (67.2 ± 27.1% of TM at 15 s), where heterelogous fluid in the same conditions resulted in a 59.1 ± 24.2% of TM. However at 60 s post-activation total motility was 32.0 ± 16.8% and 18.2 ± 8.9% for homologous and heterelogous fluid, revealing a motility prolongation compared to the control (Figure 9 A).

Linearity had unexpected results showing significant differences between all treatments, being the highest with homologous ovarian fluid 50% followed by heterelogous ovarian fluid 50% and the lowest with the control activation solution (Figure 9 F). At 60 s post-activation all samples with ovarian fluid had better results than the control in all variables (Figure 9 E).

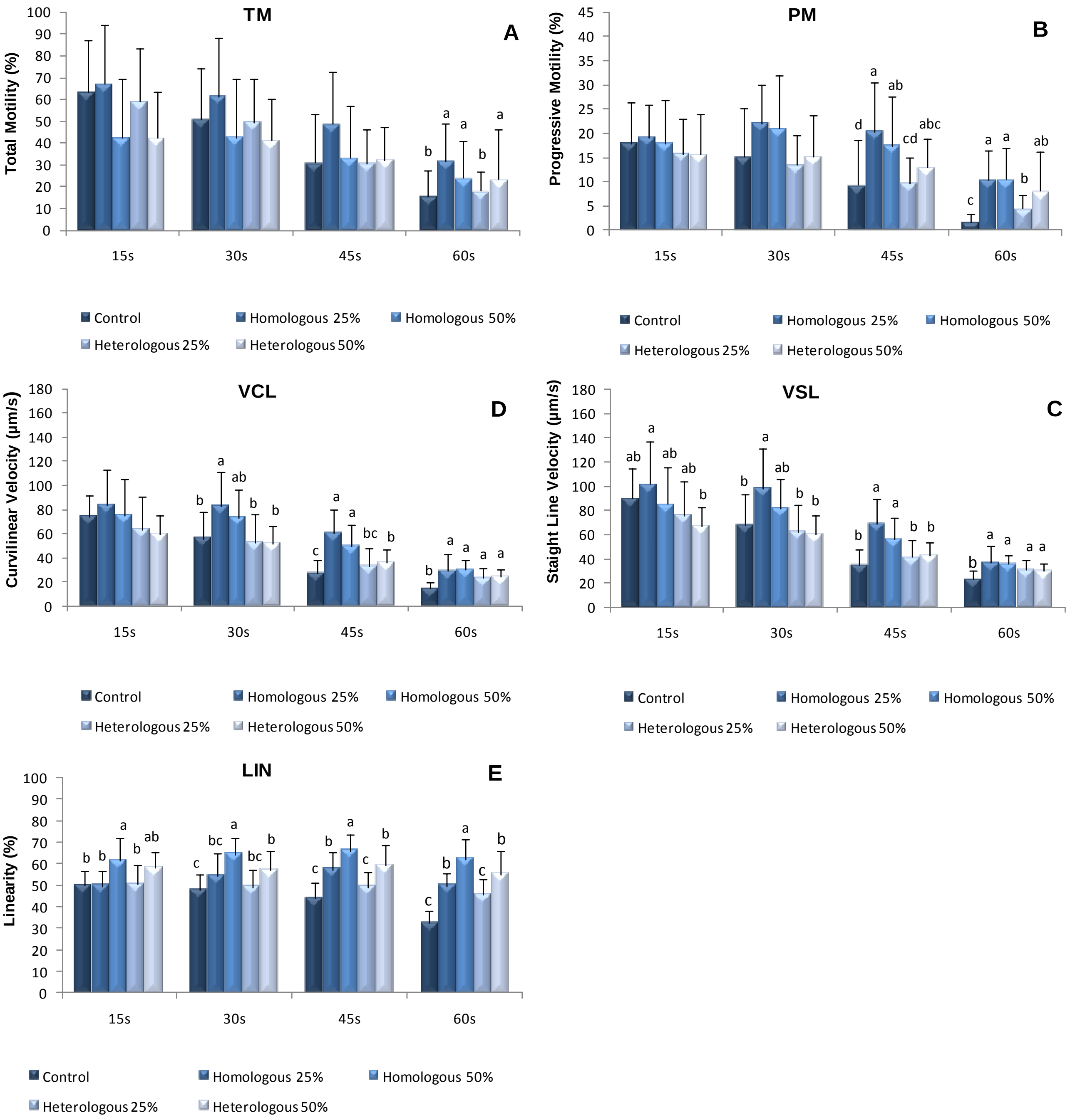
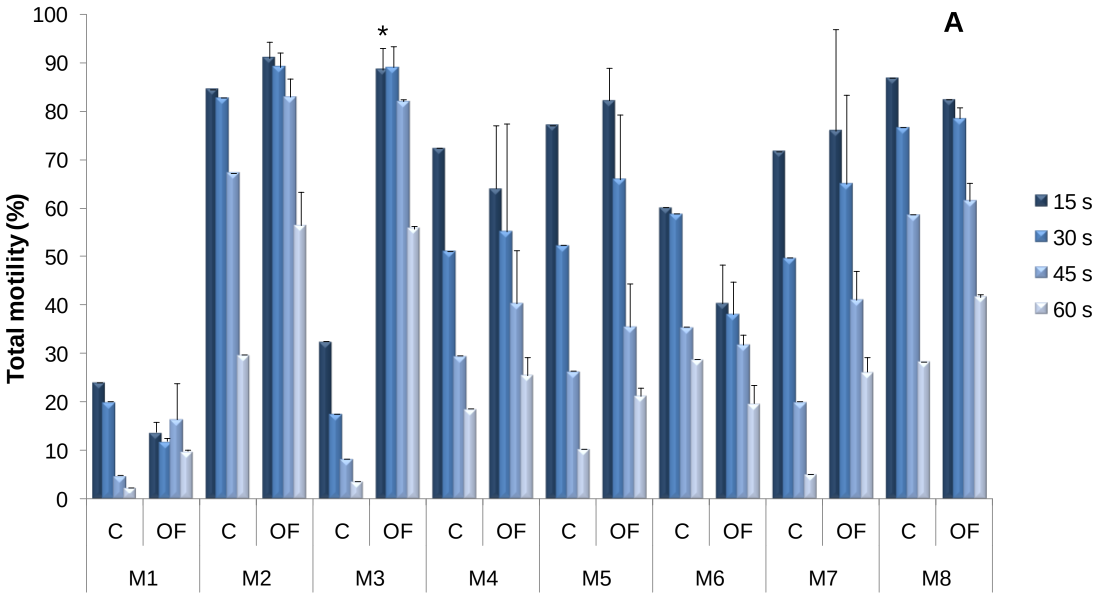


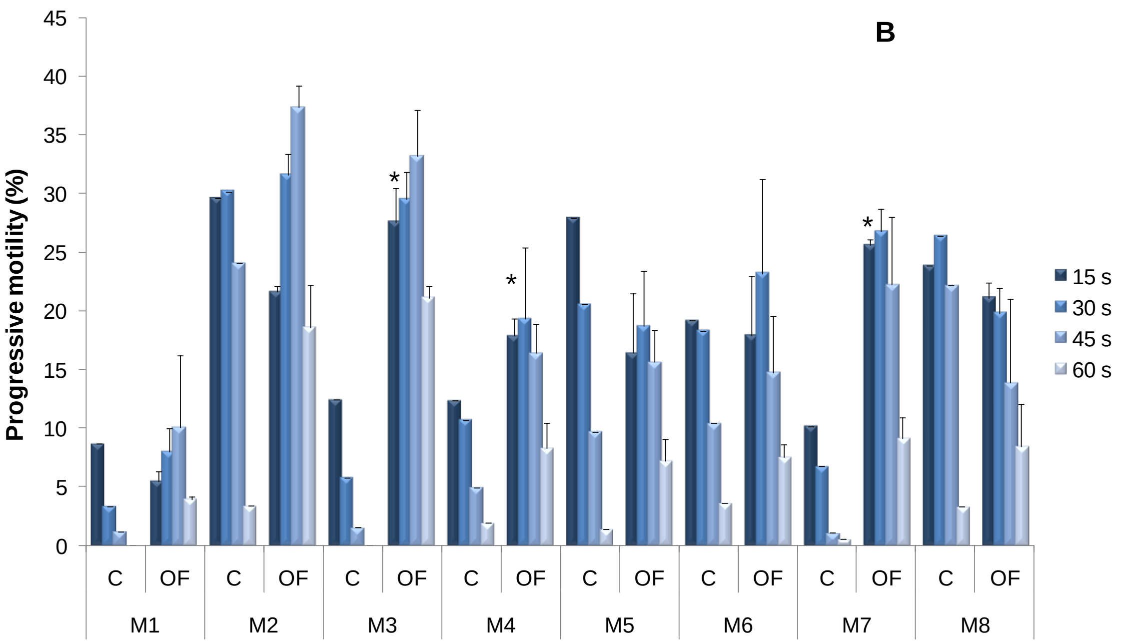
Figure 9 - Effect of homologous and heterelogous ovarian fluid concentration in motility activation parameters represented by data sustained by 8 males. Data were registered in intervals of 15 s during 1 min. Columns represent means, bars indicate standard deviation. Significant differences ($p < 0.05$) between treatments are represented with letters within each post-activation time. A - Total motility, B - Progressive motility, C- curvilinear velocity, D - straight line velocity, E - linearity.

In summary, *S. senegalensis* motility was positively affected by the presence of homologous and heterelogous ovarian fluid in the activation medium. Ovarian fluid concentration was inversely related with its effect on sperm motility activation. Spermatozoa longevity increased significantly in ovarian fluid treatments when compared with control solution.

4.2.2 Effect of *S. senegalensis* ovarian fluid on individual male sperm motility activation

To analyse individual responses to ovarian fluid, the best activation conditions obtained previously (25% of homologous fluid diluted in seawater) were used in the activation of sperm motility of 8 males. An independent Student’s t-test analysis was used to compare the two motility activation conditions in each male. Male 3 had a low motility sample that improved significantly TM, PM, VCL and VSL with the presence of ovarian fluid (Figure 10 A, B, C, D). This male showed the highest sperm motility improvement with ovarian fluid activation solution (control TM at 15 s is 32.4%, and ovarian fluid TM 15 s is 88.7%) (Figure 10 A). Although this male in control showed generally the lowest motility parameters, its linearity was the highest reported. During the motility period four males (1, 3, 4 and 7) had significantly higher motility parameters when activated with 25% ovarian fluid than control (Figure 10). Males 3, 4 and 7 had significantly higher PM with ovarian fluid (Figure 10 B) and males 1, 4 and 7 had significantly higher linearity with the same treatment (Figure 10 F). There was a variability of response to the presence of ovarian fluid, with half of the samplings revealing a significant improvement with ovarian fluid.




B
Progressive motility (%)
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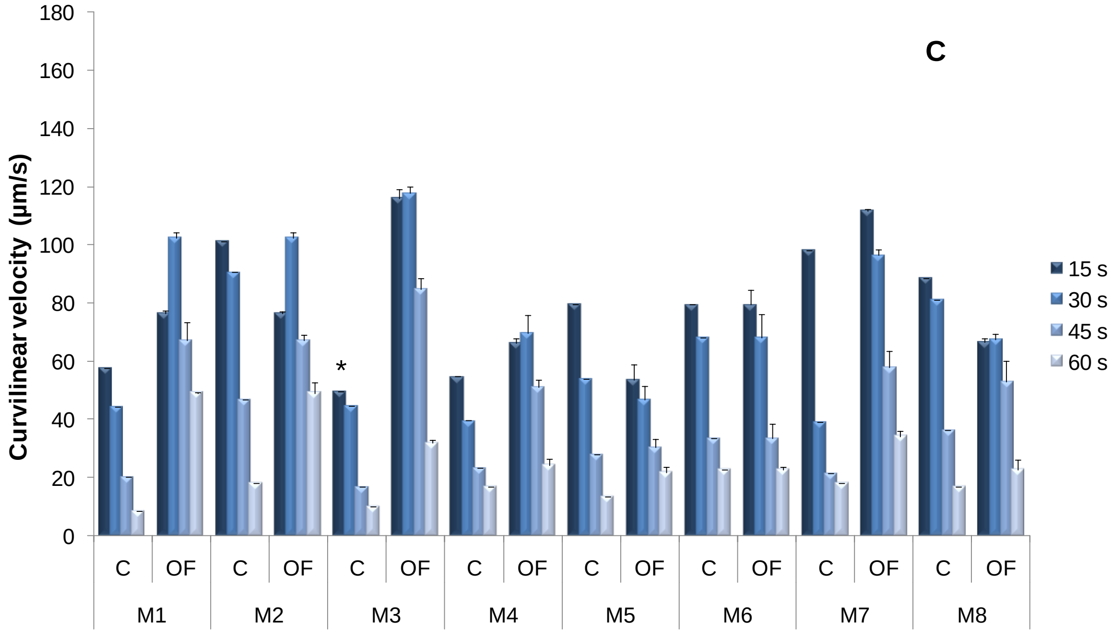
M7
M8


C
Curvilinear velocity (µm/s)
180
160
140
120
100
80
60
40
20
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*
15 s
30 s
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C
OF
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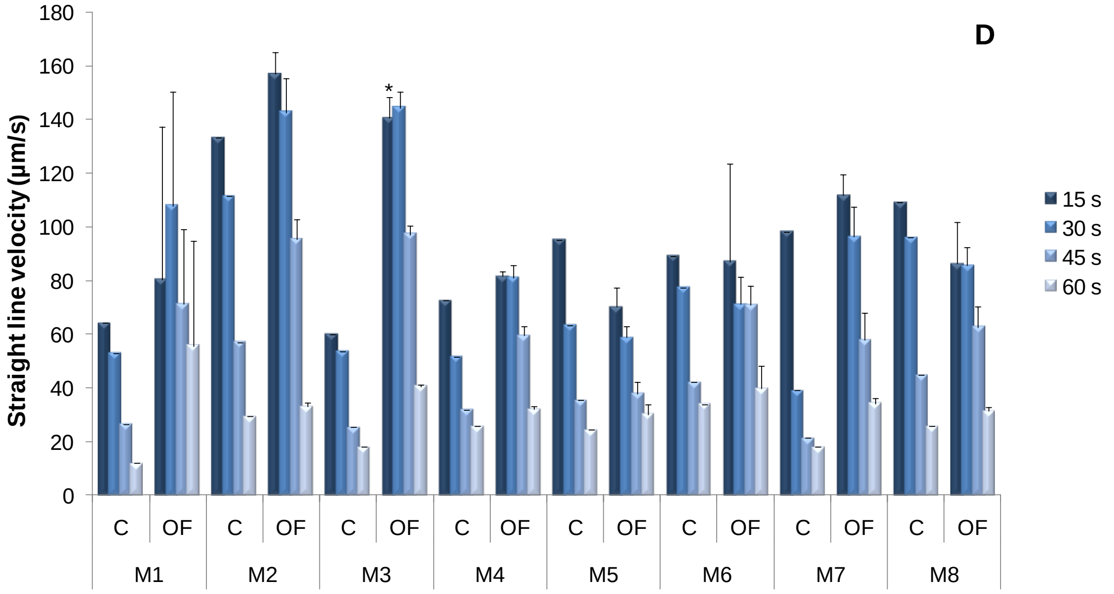

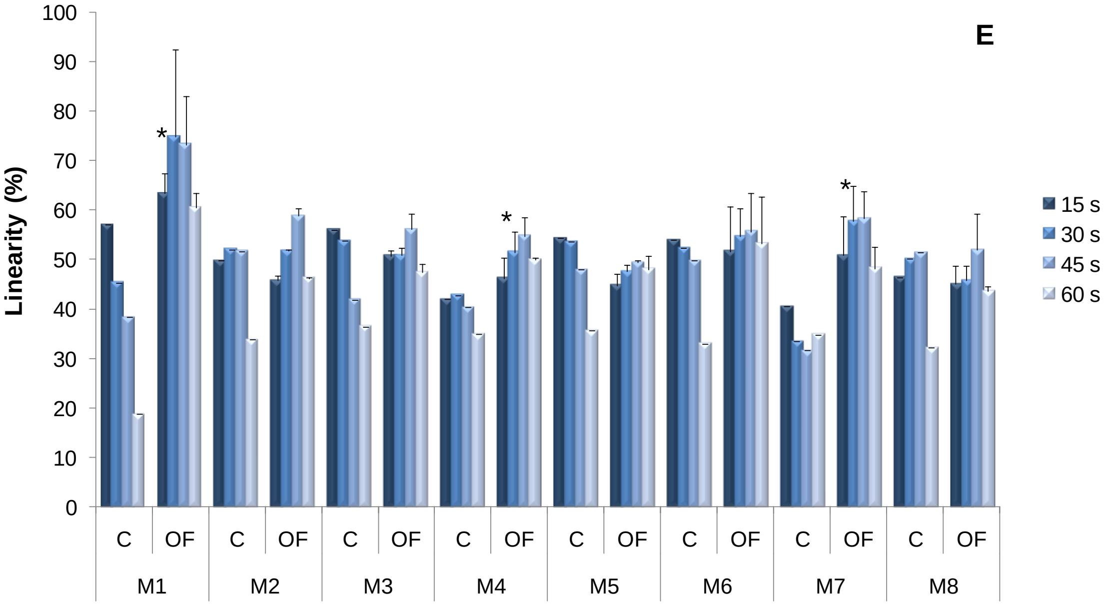

Figure 10 - Effect of *S. senegalensis* ovarian fluid on individual male motility activation registered in the 8 males (M1-M8). Motility parameters were registered in intervals of 15 s during 1 min for control (C; 100% seawater) and 25% of homologous ovarian fluid (OF, n = 2). Significant differences (Independent Student's t-test $p < 0.05$) between treatments (Control and OF) for each male are represented with (*). A - Total motility, B - Progressive motility, C- curvilinear velocity, D - straight line velocity, E - linearity.

4.3 Sperm subpopulation analysis

4.3.1. Sperm subpopulation analysis on sperm motility with different temperature, salinity and pH of the activation solution

The subpopulation analysis was performed through cluster analysis which required the use of all motile spermatozoa (n = 80 033), discarding the non motile spermatozoa. The cluster analysis used to assess sperm subpopulations was performed for each post-activation time.

Cluster analysis was applied at 15 s post-activation and four principal clusters or subpopulations were obtained. The fastest subpopulation (VCL - 178.7 µm/s, VSL - 148.5 µm/s, VAP - 165.0 µm) with the most linear path (LIN - 98.3%, STR - 99.7%, WOB - 98.9%), was thereby named subpopulation 1 (SP1). Subpopulation 2 (SP2) was characterized by fast spermatozoa (VCL - 172.2 µm/s, VSL - 64.0 µm/s, VAP - 128.0 µm) but with lower linearity (LIN - 40.3%, STR - 58.4%, WOB - 88.2%) (Table 3). The subpopulation 3 was slow (VCL - 40.1 µm/s, VSL - 26.4 µm/s, VAP - 32.1 µm) with high linearity (LIN - 79.2%, STR - 96.6%, WOB - 96.4%) and the subpopulation 4 was slow (VCL - 27.6, VSL - 7.3) with low linearity (LIN - 27.8) (Table 3).

When sperm was activated at 20 ºC, at 15 s post-activation the SP1 revealed significantly higher proportion of cells (p = 0.004) in the sample than with 16 ºC (Table 4). The activation solution with 25 ‰ produced significantly higher percentage of cells in SP1 (20.6%) than 30 ‰ (16.8%) and 35 ‰ (14.3%).

After 30 s of the spermatozoa motility activation, the cluster analysis revealed the presence of 3 major subpopulations. Subpopulation 1 was the fastest group (VCL - 182.9 µm/s, VSL - 126.0 µm/s, VAP - 161.8 µm) with high linearity (LIN - 86.6%, STR - 95.5%, WOB - 100.0%); subpopulation 2 was slow (VCL - 43.7 µm/s, VSL - 30.4 µm/s, VAP - 36.5 µm/s) with highly linear trajectory (LIN - 84.9%, STR - 98.1%, WOB - 98.8%) and subpopulation 3 had low velocity and was mainly curvilinear (VCL - 32.2 µm/s, VSL - 8.4 µm/s, VAP- 28.1 µm), with low linearity (LIN - 28.1%, STR - 54.0%, WOB - 70.5%) (Table 3). SP1 had a significant lower proportion of cells at 35 ‰ (18.8%) than at 25 ‰ (28.2%) and 30 ‰ (26.2%), but in SP3 at 25 ‰ significantly lower percentage of cells (39.7%) were found than at 30 ‰ (47.7%) and at 35 ‰ (48.4%). SP2 did not change significantly among treatments (Figure 11).

At 45 s post-activation only 2 subpopulations were detected by cluster analysis and significant differences were found for all treatments tested. Subpopulation 1 was slow (VCL - 22.6 µm/s, VSL - 7.1 µm/s, VAP - 13.5 µm) and nonlinear (LIN - 33.7%, STR - 64.5%, WOB- 69.4%), while subpopulation 2 was fast (VCL - 89.4 µm/s, VSL - 68.0 µm/s, VAP - 81.4 µm/s) and linear (LIN - 94.7%, STR - 99.8%, WOB - 99.4%) (Table 3). Subpopulation 2 had a significantly higher proportion of cells at 16 ºC (66.3%) than at 20 ºC (59.5%) and, inversely, subpopulation 1 had a significantly higher proportion of cells at 20 ºC (40.3%) than at 16 ºC (33.7%). With 25 ‰ of salinity a significant lower percentage of cells was found for subpopulation 2 (52.4%) than with 30 ‰ (65.1%) and 35 ‰ (71.0%), but in subpopulation 1 a significant higher percentage of cells was found for 25 ‰ (47.5%) than for 30 ‰ (34.6%) and 35 ‰ (29.0%). The same tendency was observed for pH treatments where subpopulation 2 had a significant higher percentage of cells for alkaline character, namely pH 9 (69.6%), than for pH 7.4 (60.1%) and pH 6 (58.8%) and subpopulation 1 expressed a higher proportion for pH 6 (41.2%) than for pH 7.4 (39.7%) and 9 (30.2%) (Figure 11).

Table 3 - Characterization of sperm subpopulations of sperm motility activated in different temperatures, salinities and pH treatments.

Subpopulations characterization					
	Motility Parameters	**SP1**	**SP2**	**SP3**	**SP4**
15 s	VCL (µm/s)	178.7 ± 57.6	172.2 ± 47.6	40.1 ± 24.0	27.6 ± 17.5
	VSL (µm/s)	148.5 ± 53.3	64.0 ± 33.1	26.4 ± 17.0	7.3 ± 5.6
	VAP (µm/s)	165.0 ± 55.7	128.0 ± 44.1	32.1 ± 19.5	16.1 ± 11.5
	LIN (%)	98.3 ± 2.8	40.3 ± 2.7	79.2 ± 2.7	27. 8 ± 1.4
	STR (%)	99.7 ± 2.3	58.4 ± 4.5	96.6 ± 2.5	53.5 ± 3.3
	WOB (%)	98.9 ± 1.5	88.2 ± 1.8	96.4 ± 2.9	68.3 ± 2.3
	ALH (µm)	3.0 ± 1.2	5.4 ± 1.5	1.7 ± 0.8	1.6 ± 0.7
	BCF (Hz)	6.3 ± 2.9	4.5 ± 2.5	3.1 ± 2.4	2.9 ± 2.1
30 s	VCL (µm/s)	182.9 ± 43.3	43.7 ± 28.3	32.2 ±29.7	
	VSL (µm/s)	126.0 ± 56.5	30.4 ± 20.2	8.4 ± 7.8	
	VAP (µm/s)	161.8 ± 43.8	36.5 ± 23.9	20.3 ± 22.9	
	LIN (%)	86.6 ± 9.0	84.9 ± 3.2	28.1 ± 1.7	
	STR (%)	95.5 ± 8.6	98.1 ± 2.7	54.0 ± 3.8	
	WOB (%)	100.0 ± 2.7	98.8 ± 3.0	70.5 ± 3.1	
	ALH (µm)	3.5 ± 1.6	1.7 ± 0.8	1.7 ± 1.0	
	BCF (Hz)	6.2 ± 2.8	3.5 ± 2.8	2.8 ± 2.1	
45 s	VCL (µm/s)	89.4 ± 53.6	22.6 ± 13.4		
	VSL (µm/s)	68.0 ± 46.8	7.1 ± 5.5		
	VAP (µm/s)	81.4 ± 50.7	13.5 ± 10.5		
	LIN (%)	94.7 ± 5.8	33.7 ± 3.6		
	STR (%)	99.8 ± 5.5	64.5 ± 5.4		
	WOB (%)	99.4 ± 2.3	69.4 ± 4.4		
	ALH (µm)	2.0 ± 1.0	1.4 ± 0.5		
	BCF (Hz)	6.4 ± 3.2	2.7 ± 2.2		
60 s	VCL (µm/s)	56.7 ± 36.1	21.5 ± 11.1		
	VSL (µm/s)	46.0 ± 31.8	6.2 ± 4.6		
	VAP (µm/s)	52.4 ± 35.1	11.6 ± 7.8		
	LIN (%)	86.9 ± 4.1	27.6 ± 3.1		
	STR (%)	92.8 ± 3.4	54.9 ± 5.2		
	WOB (%)	92.8 ± 2.4	54.1 ± 4.3		
	ALH (µm)	1.5 ± 0.5	1.3 ± 5.0		
	BCF (Hz)	6.3 ± 3.6	2.7 ± 2.4		

At the end of spermatozoa lifespan (60 s post-activation) two subpopulations were detected. The first subpopulation was fast (VCL - 56.7 µm/s, VSL - 46.0 µm/s, VAP - 52.4 µm) and linear (LIN - 86.9%, STR - 92.8%, WOB - 92.8%) and the second one was slow (VCL - 21.5 µm/s, VSL - 6.2 µm/s, VAP - 11.6 µm) and nonlinear (LIN - 27.6%, STR - 54.9%, WOB - 54.1%) (Table 3). At 60 s post-activation there were no significant differences in the percentage of cells of SP1 and SP2 among temperature and pH treatments (Figure 11). Nevertheless the lowest salinity had significant higher percentage of SP1 cells (34.0%) compared with 30 ‰ (22.4%) and 35 ‰ (24.3%). Contrarily, 35 ‰ solution produced significant higher percentage of cells in SP2 (73.0%) than 30 ‰ (59.5%) and 25 ‰ (55.6%) (Figure 11 D).

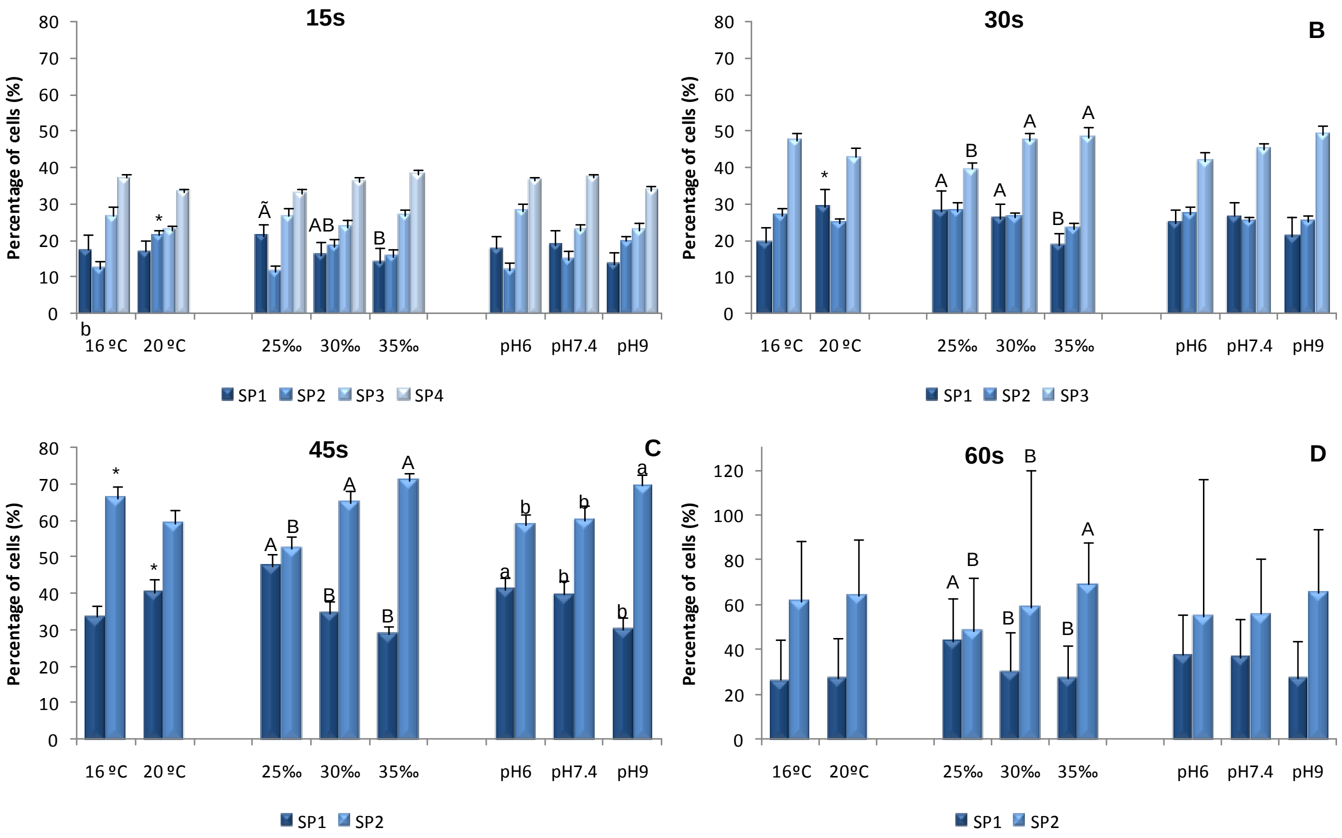

Figure 11 - Effect of temperature, salinity and pH in the percentage of cells in each subpopulation 15 s (A), 30 s (B), 45 s (C) and 60 s post-activation (D). SP1 - Subpopulation 1, SP2 - Subpopulation 2, SP3 - Subpopulation 3, SP4 - Subpopulation 4. Statistical differences ($p < 0.05$) between the percentage of cells in each subpopulation are represented with uppercase letters (salinity), lowercase letters (pH) and (*) (temperature).

Table 4 - Statistical differences between the percentage of cells in each subpopulation obtained for temperature, salinity and pH treatments, sustained by mean values of 7 males. Statistical differences (multivariate three way ANOVA, $p \leq 0.05$) are highlighted in bold. SP1 - Subpopulation 1, SP2 - Subpopulation 2, SP3 - Subpopulation 3, SP4 - Subpopulation 4.

Time	p ≤ 0.05	SP1	SP2	SP3	SP4
15 s	Temperature	0.999	**0.004**	0.146	0.156
	Salinity	0.141	0.751	0.408	0.147
	pH	0.295	**0.019**	0.130	0.311
	Temperature*Salinity	0.577	0.315	0.965	0.906
	Temperature*pH	0.884	0.596	0.442	0.881
	Salinity*pH	0.424	0.193	**0.004**	0.846
	Temperature*Salinity*pH	0.324	0.127	0.594	0.909
30 s	Temperature	0.102	0.899	0.725	
	Salinity	0.253	0.526	0.365	
	pH	0.420	0.800	0.779	
	Temperature*Salinity	0.691	0.411	0.790	
	Temperature*pH	0.616	0.879	0.622	
	Salinity*pH	0.556	0.127	0.125	
	Temperature*Salinity*pH	0.996	0.478	0.823	
45 s	Temperature	**0.036**	**0.037**		
	Salinity	**< 0.001**	**< 0.001**		
	pH	**0.012**	**0.010**		
	Temperature*Salinity	0.686	0.653		
	Temperature*pH	0.920	0.890		
	Salinity*pH	0.671	0.691		
	Temperature*Salinity*pH	0.732	0.729		
60 s	Temperature	0.797	0.931		
	Salinity	0.993	0.959		
	pH	0.981	0.949		
	Temperature*Salinity	0.905	0.934		
	Temperature*pH	0.962	0.954		
	Salinity*pH	0.990	0.994		
	Temperature*Salinity*pH	0.996	0.999		

In summary, the conditions that improved sperm motility with mean values promote higher percentage of fast and linear subpopulations, namely high temperature and low salinity and pH. The slow subpopulations had higher proportion of cells in solutions with low temperature, and high salinity and pH. The differences observed among treatments during motility were more important at 30 s and 45 s post-activation.

4.3.2 Sperm subpopulation analysis on sperm motility with ovarian fluid activation solutions

Sperm motility activation with ovarian fluid resulted mainly in two subpopulations, except at 30 s post-activation. At 15 s post-activation the first subpopulation (SP1) showed high speed (VCL - 125.9 µm/s, VSL - 79.1 µm/s, VAP - 125.9 µm) and high linearity (LIN - 37.4%, STR - 48.0%, WOB - 56.4%. The slow subpopulation (SP2) had low swimming speed values (VCL - 30.2 µm/s, VSL - 8.4 µm/s, VAP - 18.5 µm) and very low linearity (LIN - 1.8%, STR - 3.5%, WOB - 2.4%) (Table 5). No significant differences were observed in each subpopulation among treatments (Figure 12 A).

After 30 s post-activation three major subpopulations were detected. The first subpopulation (SP1) was the fastest (VCL - 169.9 µm/s, VSL - 110.1 µm/s, VAP - 147.2 µm) with high linearity (LIN - 36.3%, STR - 44.6%, WOB - 58.0%). The second subpopulation (SP2) was slow (VCL - 26.6 µm/s, VSL - 20.6 µm/s, VAP - 23.6 µm) with a nonlinear path (LIN - 2.7%, STR - 1.7%, WOB - 1.4%). Finally, the third subpopulation (SP3) was slowest (VCL - 25.5 µm/s, VSL - 6.0 µm/s, VAP - 14.6 µm) and nonlinear (LIN - 0.8%, STR - 2.8%, WOB - 2.3%) (Table 5). The fastest subpopulation was significant higher at 30 s post-activation for 25% homologous OF (S25) (37.6%) being significant different from 25% heterelogous OF (E25) (20.9%) and 50% OF (E50) (23.6%). However no significant differences were found with control (26.8%) and 50% homologous OF (S50) (38.5%). SP2 was had a significant higher percentage of cells when activated with 50% heterologous OF E50 (33.3%) than 50% homologous OF (28.1%). SP3 was significant higher for control (34.2%) than 50% of any ovarian fluid treatment. (Figure 12 B).

After 45 s post-activation the motility values started to decrease. Once again, two subpopulations were detected. The fast subpopulation (SP1) had high speed (VCL - 92.7 µm/s, VSL - 71.6 µm/s, VAP - 84.6 µm) and high linearity (LIN - 49.6%, STR - 57.0%, WOB - 62.1%). The slow subpopulation (SP2) had slow speed values (VCL - 24.5 µm/s, VSL - 8.7 µm/s, VAP - 15.7 µm) and low linearity (LIN - 12.3%, STR - 29.1%, WOB - 33.1%) (Table 5). SP1 was significant higher for 25% and 50% homologous OF (48.9% and 51.1%) than control (23.8%). SP2 was significant higher for control (76.1%) compared with 25% homologous and heterologous OF (68.1% and 50.8%). However no differences were detected with 50% heterologous OF (Figure 12).

Table 5 - Characterization of sperm subpopulations of sperm motility activated with ovarian fluid.

		Subpopulations characterization		
	Motility Parameters	SP1	SP2	SP3
15 s	VCL (µm/s)	125.9 ± 72.5	30.3 ± 27.4	
	VSL (µm/s)	79.1 ± 54.9	8.5 ± 7.3	
	VAP (µm/s)	106.6 ± 63.5	18.5 ± 19.3	
	LIN (%)	37.4 ± 4.5	8.1 ± 1.8	
	STR (%)	48.0 ± 3.9	22.4 ± 3.5	
	WOB (%)	56.4 ± 1.2	31.0 ± 2.3	
	ALH (µm)	3.1 ± 1.7	1.7 ± 1.0	
	BCF (Hz)	5.3 ± 3.0	2.6 ± 2.0	
30 s	VCL (µm/s)	169.9 ± 41.7	47.1 ± 26.6	25.5 ± 20.7
	VSL (µm/s)	110.1 ± 53.3	32.1 ± 20.6	6.0 ± 5.3
	VAP (µm/s)	147.2 ± 41.3	39.2 ± 23.6	14.6 ± 14.5
	LIN (%)	36.3 ± 6.4	39.1 ± 2.7	5.4 ± 0.8
	STR (%)	44.6 ± 5.7	52.8 ± 1.7	18.4 ± 2.8
	WOB (%)	58.0 ± 1.3	53.9 ± 1.4	27.4 ± 2.3
	ALH (µm)	3.4 ± 1.6	1.8 ± 0.7	1.5 ± 0.8
	BCF (Hz)	6.1 ± 2.6	4.3 ± 3.2	2.5 ± 1.9
45 s	VCL (µm/s)	92.7 ± 47.4	24.5 ± 17.4	
	VSL (µm/s)	71.6 ± 42.0	8.8 ± 7.1	
	VAP (µm/s)	84.7 ± 45.2	15.6 ± 13.9	
	LIN (%)	49.6 ± 3.0	12.3 ± 4.5	
	STR (%)	57.0 ± 2.4	29.1 ± 4.8	
	WOB (%)	62.1 ± 0.6	33.1 ± 3.9	
	ALH (µm)	2.0 ± 0.9	1.4 ± 0.7	
	BCF (Hz)	7.1 ± 2.9	2.6 ± 2.2	
60 s	VCL (µm/s)	41.0 ± 24.9	20.0 ± 9.4	
	VSL (µm/s)	30.6 ± 21.7	4.9 ± 3.1	
	VAP (µm/s)	35.8 ± 24.3	9.6 ± 5.8	
	LIN (%)	44.9 ± 3.0	5.6 ± 1.2	
	STR (%)	56.5 ± 1.5	24.8 ± 3.6	
	WOB (%)	57.1 ± 1.6	21.4 ± 2.9	
	ALH (µm)	1.5 ± 0.5	1.3 ± 0.5	
	BCF (Hz)	5.5 ± 3.6	2.4 ± 2.0	

Finally at 60 s post-activation, spermatozoa reached the lowest motility values in the end of their lifespan and two subpopulations were identified. Subpopulation 1 (SP1) was the fastest with linear path (LIN - 41.0%, STR - 30.6%, WOB - 35.8%) and subpopulation 2 (SP2) was the slowest (VCL - 20.0 µm/s, VSL - 4.8 µm/s, VAP - 9.6 µm) with non linear path (LIN - 5.6%, STR - 24.8%, WOB - 21.4%) (Table 5). At this time the highest percentages of subpopulation 1 were detected in control (79.3%), being significantly higher compared with all the other treatments (S25 - 48.8%, S50 - 59.9%, M25 - 30.9), M50 - 38.6%). In SP2 the highest percentages recorded were for E25 (65.9%) and E50 (60.5%) which were significantly different from S25 (47.8%) and S50 (38.3%), however all treatments were significantly higher than control (19.7%) (Figure 12).

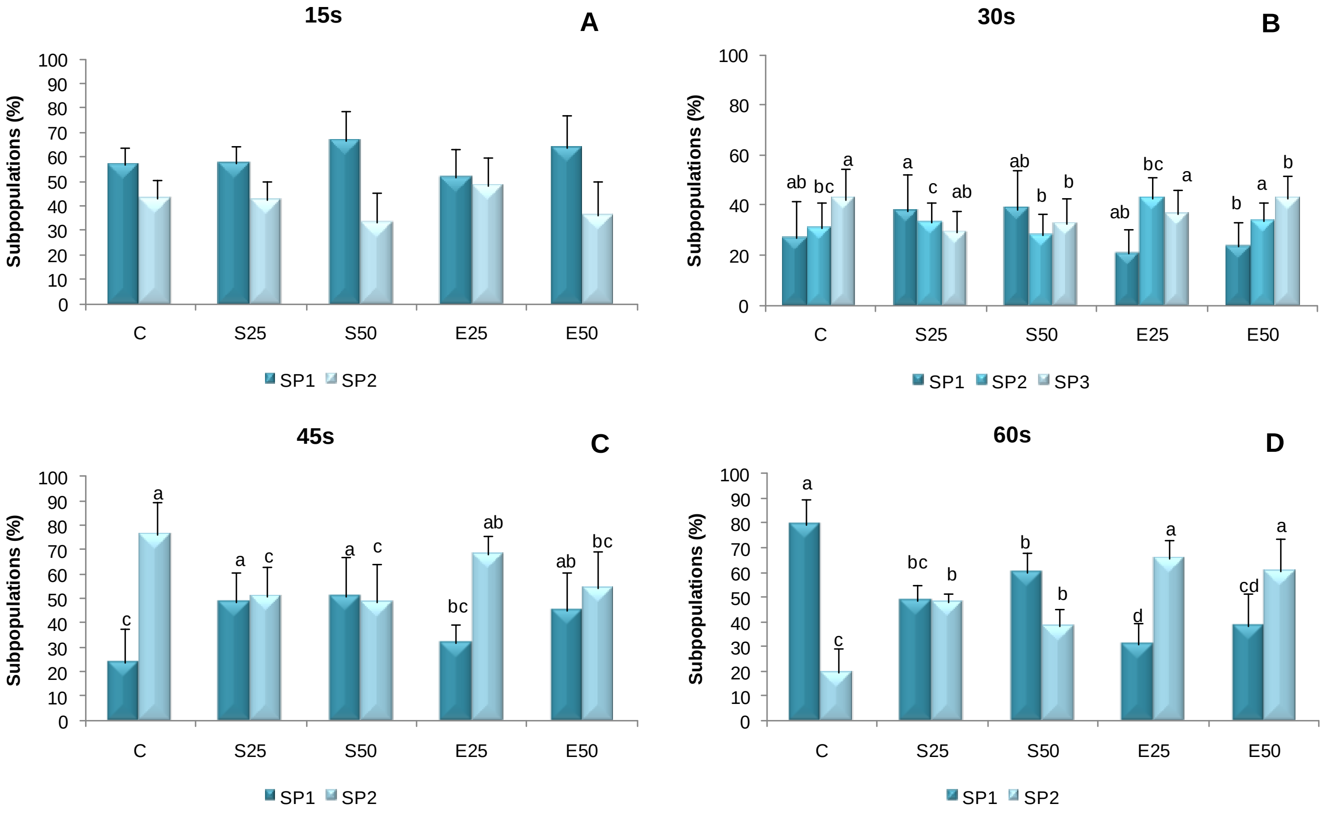

Figure 12 – Effect of ovarian fluid in the percentage of cells in each subpopulation 15 s (A), 30 s (B), 45 s (C), 60 s (D). Statistical differences of the percentage of cells in each subpopulation (SP) in sperm motility activated in the presence of sea water (control), 25% homologous ovarian fluid (S25) and 50% (S50), and 25% heterelogous ovarian fluid (E25) and 50% (E50). The differences between the percentage of cells in each subpopulation for all treatments are represented with letters. Significant differences ($p < 0.05$) were detected by one-way ANOVA.

DISCUSSION

5. Discussion

5.1 Effect of temperature, salinity and pH on sperm motility activation

In marine teleosts, sperm motility is activated in contact with sea water mainly through a positive increase in osmolarity and several factors are known to affect this process such as water pH and temperature (Alavi and Cosson 2005, 2006) and the proper salinity of seawater.

The effect of temperature on sperm motility has been scarcely studied in marine species. Nevertheless it has been reported that with the decrease of temperature flagellar beating frequency is lower and the duration of motility longer (Cosson *et al*., 2008a,b). As the ATP reserves are depleted throughout the motile period a gradual decrease of flagellar beating frequency and sperm motility occurs (Christen *et al*., 1987; Cosson *et al*., 2008a,b). This decrease of sperm motility was observed in carp (*Cyprinus carpio*) in the presence of lower temperatures of the activation solution (Billard *et al*., 1995b). Furthermore, higher water temperatures seem to result in higher sperm velocities, however at the expense of an earlier and acute decline of sperm velocity comparing with lower temperatures of the activation solution (Perchet *et al*., 1995). In the present study our data suggested that the activation solution set at 20 ºC improved total motility throughout time, and improved sperm velocity in the first seconds, compared to 16 ºC. Also it was evident that temperature affected preferably parameters linked with total and progressive motile cells, and velocity such as TM, PM, VCL and VSL.

Solea senegalensis spawning temperature conditions range between 16 ºC in the beginning of the reproductive season and 23 ºC at the end, with production of bigger eggs at nearly 20 ºC (19.8 ± 0.9 ºC) (Dinis *et al*., 1999). Males of this species produce sperm all year around (Cabrita *et al*., 2006), however the fact that sperm motility reaches higher performance at 20 ºC in the same thermal conditions of the reproductive period where females produce bigger eggs, it might indicate a physiological adaptation to exhibit a better sperm performance in the middle of the spawning season, enabling a synchronisation of sperm quality with female spawns.

The effect of salinity on sperm motility of marine fishes has received little attention, however there are thorough studies focusing on the specific ions that trigger sperm motility, such as K^+, Ca^{2+}, Mg^{2+} (Cosson, 2004), present in seawater and their influence on sperm motility activation. For some fish species such as salmonids (Huxley, 1930), guppy (*Poecilia reticulata*), rainbow trout (*O. mykiss*), pike (*Esox lucius*), gilthead seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) (Billard, 1978) it is known that the natural fertilization conditions are not the optimal conditions to enhance spermatozoa survival. In estuarine systems with brackish waters the salinity is considered a dominant abiotic factor to species distribution (Westin and Nissling, 1991), since in these environments to obtain successful spawning both sperm and eggs have to deal with low salinities, and both phenotypic plasticity (physiological adaptation to the environment) and genetic selection (natural selection) are two key elements (Nissling *et al.*, 2002). In the Baltic Sea the proportion of motile cells and duration of motility in cod (*Gadus morhua*) decreased significantly with the decrease of salinity (15 and 17 ‰) (Litvak and Trippel, 1998; Nissling and Westin, 1997; Westin and Nissling, 1991). High sperm motility could be obtained in the salinity range of 20-30 ‰, but the optimal salinity for the activation of motility in this species was 26 ‰. Although no tests were performed for lower salinities, this results are in agreement with our work where significantly higher sperm velocity and linearity were obtained with 25 ‰ and 30 ‰, since *S. senegalensis* is also a species that uses estuaries as preferable spawning areas (Cabral *et al.*, 2007; Vinagre *et al.*, 2008). Nevertheless, higher TM was obtained with 30 ‰ and 35 ‰ at 15 s post-activation. The same findings were found by Dreanno *et al.* (1999a) in sea bass, where low salinity and consequently low ionic strength, affected positively motility parameters linked with velocity and linearity. Also low salinities in *S. senegalensis* seemed to improve not only linearity but also swimming speed and progressive motility in the last seconds recorded. This improvement in motility will allow the sperm to have more changes to reach the oocytes during fertilization process. Our results were in accordance to the ones observed by Litvak and Tripel (1998) that found significant higher swimming speed values for 20 ‰ and 30 ‰ in cod.

The pH of the activation solution affects sperm motility in a low extent (Cosson, 2004), but it is generally accepted that the pH or ions present in the activation solution may polarize the cell membrane and stimulate motility of fish spermatozoa (Morisawa and Morisawa, 1988) by changing the Na^+/K^+ permeability (Boitano and Omoto, 1991; Gatti and Christen, 1985). The pH of the internal medium is one of the most important factors that affect sperm motility (Woolsey and Ingermann, 2003). The low internal pH may prevent sperm motility through pH sensitivities to cAMP levels (Miura *et al.*, 1992), membrane potential (Gatti *et al.*, 1990), and dynein ATPase activity (Woolsey and Ingermann, 2003). Since the cell internal pH gradient modifies rapidly approximately in parallel with the external pH when in contact with the activation medium (Woolsey and Ingermann, 2003) the onset of sperm motility is associated with one or more pH sensitive processes at the extracellular surface of the sperm (Ingermann *et al.*, 2008). An increase of external pH increases internal pH affecting thus the internal process that control sperm motility (Ingermann *et al.*, 2008). The external pH affects not only the sperm motility in salmonids (Miura *et al.*, 1992; Woolsey and Ingermann, 2003), but also the motility patterns (Woolsey and Ingermann, 2003). The alkaline character (pH = 8) of the activation solution seems to be the most adequate for species such as turbot (Chauvaud *et al.*, 1995), halibut (Billard *et al.*, 1993) and carp (Perchec *et al.*, 1995), although in species such as gilthead seabream (Chambeyron and Zohar, 1990) the external pH did not show any alterations on sperm motility. In *S. senegalensis* the pH of the activation solution had low effect on sperm motility, which is in accordance with the results obtained by Cosson (2004). Nevertheless after 30 s post-activation, significant differences were found revealing that pH 6 improved sperm linearity compared to pH 9, although no differences were observed with pH 7.4. Thus it seems that motility activation in *Solea senegalensis* is more affected by acidic pHs than alkaline, contrarily to the observed in some marine species (Billard *et al*., 1993; Chauvaud *et al*., 1995). As in gilthead seabream (Chambeyron and Zohar, 1990) pH did not affect sperm motility, although in this species the optimal pH is alkaline and in *S. senegalensis* a neutral or acidic pH of the solution might be the most adequate. The tendency observed to obtain better motility with neutral or slightly lower pH can be associated with the pH of females ovarian fluid, this matter will be discussed further, in the next chapter.

5.2 Ovarian fluid influence on *Solea senegalensis* sperm motility

5.2.1 Effect of homologous and heterologous ovarian fluid concentration on sperm motility activation

The *S. senegalensis* ovarian fluid is slightly acid, in opposition to other species reported which present an alkaline character, such as the heterelogous ovarian fluid used in this work and also the ovarian fluid from salmonids (Lahnsteiner *et al*., 1995; Wojtczak *et al*., 2007; Rosengrave *et al*., 2008). As stated previously *S. senegalensis* showed sperm motility activation enhanced with seawater solutions with lower pH (between 6 and 7.4). The best results in sperm motility in our work occurred with homologous ovarian fluid, possibly due to its acidic character, creating a pH beneficial to sperm motility in this species.

S. senegalensis is a flatfish with a benthonic behaviour that uses estuaries as preferable spawning areas (Cabral *et al*., 2007; Vinagre *et al*., 2008). These systems have a powerful input of fresh and acidic waters as well as anthropogenic pollution, which contributes to lower water pH (Schmitt *et al*., 2008). Additionally, the high organic matter characterizing these systems promotes redox reactions, thus creating an acidic environment. These facts may reveal a reproductive adaptation mechanism of *S. senegalensis* to the spawning area conditions since in other flatfish species such as turbot (Chauvaud *et al*., 1995) and halibut (Billard *et al*., 1993) a slightly alkaline pH enhances motility parameters.

Our data is consistent with previous studies where ovarian fluid is considered beneficial in fertilization due to sperm motility enhancement (Elofsson *et al*., 2006; Lahnsteiner *et al*., 1995; Lahnsteiner, 2002; Litvak and Trippel, 1998; Rosengrave *et al*., 2008; Turner and Montgomerie, 2002; Urbach *et al*., 2005; Wojtczak *et al*., 2007), though in salmonids it also promotes sperm motility activation (Lahnsteiner, 2002). In *S. senegalensis* pure ovarian fluid by itself was not enough to promote sperm activation since in this species ovarian fluid and seminal plasma have similar osmolarity, lacking the necessary increase in osmolarity to trigger the motility mechanism in marine fishes (Cosson *et al*., 2008a). However, our data showed a clear sperm motility improvement with a certain presence of ovarian fluid in the activation medium. The highest motility parameters registered were with homologous fluid, mainly at 25%. The most evident effect of the presence of ovarian fluid in the activation solution was the prolongation throughout time in most of all motility parameters. Usually treatments such as a decrease in water temperature of the activation solution may produce this same effect but at the expense of a decrease in some motility parameters such as VSL or VCL, due to slower consumption of ATP (slow metabolism). Our results revealed that the presence of ovarian fluid in the activation medium simultaneously enhanced both sperm velocity and duration of motility.

S. senegalensis is known to have high variability of sperm quality, with the occurrence of males with low sperm quality as evidenced by Cabrita *et al*. (2006). The presence of ovarian fluid may contribute to a higher fertilization potential in *S. senegalensis*. The role of ovarian fluid in the activation of spermatozoa is especially important in *S. senegalensis* since in previous studies, these sperm cells showed high sensitivity to hyperosmotic shock when in contact with sea water (Martínez-Pastor *et al*., 2008; Beirão *et al*., 2009). Ovarian fluid constituents may protect cells during exposure to a hazardous medium, as does seawater during sperm activation, reducing the percentage of shattered cells. Also, ovarian fluid lowers the osmolarity of the medium diminishing the harmful effect of seawater. This reduction in osmolarity still promotes an efficient activation and is still in the range of what Alavi and Cosson (2006) considered optimal to marine species. Throughout our analysis with homologous and heterelogous ovarian fluid, it became evident that spermatozoa was better stimulated with ovarian fluid of its own species, since motility values of heterelogous ovarian fluid activation were lower, but still promoted higher sperm longevity than seawater activation only. These facts may indicate that *S. senegalensis* spermatozoa recognize and react to the presence of ovarian fluid of its own species, probably due to its composition and characteristics.

S. senegalensis females produce a lower volume of ovarian fluid and viscosity comparing with heterelogous ovarian fluid from *E. marginatus*. Ovarian fluid viscosity provides close contact between gametes (Inaba *et al*., 2003; Mansour *et al*., 2009) and maintains ionic concentration around them, creating an enriched and stabilized fertilization microenvironment, avoiding gamete dispersion, essential in teleosts with external fertilization (Lahnsteiner, 2002), and specially in *S. senegalensis* that produces low sperm volumes.

The most beneficial concentration for *S. senegalensis* spermatozoa movement was 25% ovarian fluid in the activation solution and in salmonids it was reported to be 50% by Wojtczak *et al*. (2007). At high concentrations (75%) of ovarian fluid the analysis was difficult due to drifting caused by high viscosity, which also restricted spermatozoa movements and promoted a deficient activation due to low osmolarity. However, higher linearity results with homologous and heterelogous fluid at 50% were found. This is interesting, as in human spermatozoa it was reported that movement in a viscoelastic fluid promoted higher frequency of flagellar beating, smaller amplitude and wavelength, producing straighter paths (Lauga, 2007). These facts may explain our higher linearity results with 50% of ovarian fluid although other motility parameters were enhanced with 25% ovarian fluid.

The origin of the beneficial effects of ovarian fluid may not only be due to its physico-chemical characteristics such as pH, osmolarity and viscosity, but also to its inorganic composition (Lahnsteiner *et al*., 1995) and also sperm activating factors as reported in herrings (Ohtake, 2003). In herring it was observed that sperm motility is activated in the presence of seawater, promoting a linear trajectory that allows the spermatozoa to reach the egg. However in order to penetrate the micropyle sperm need to get in contact with the herring eggs (egg and ovarian fluid) which activates the release of some peptides from the eggs. Consequently an increase of Ca^{2+} concentration inside the cell produces curvilinear motility, which facilitates this penetration (Vines *et al*., 2002). All together these facts may be the origin of the effect of ovarian fluid on sperm motility, though the full mechanism is still unknown. It would be interesting to investigate if the origin of the sperm motility enhancement in the presence of ovarian fluid is due to lower expenditure of energy in the presence of a viscoelastic fluid or if sperm assimilate compounds of the ovarian fluid increasing its metabolism and production of ATP, or even if, like in other species such as herring (Ohtake, 2003) and sea urchin (Jantzen *et al*., 2001), the presence of chemoattractant substances improves sperm motility. More studies need to be conducted in order to determine the physiological bases behind this phenomenon.

5.2.2 Effect of *S. senegalensis* ovarian fluid on individual male sperm motility activation

Although the presence of ovarian fluid generally improves sperm motility in several species, its individual effect has been reported to be scattered and may affect only certain sperm samples. In this work, as observed by Lahnsteiner (2002) in salmonids, males with low sperm quality, ovarian fluid improved sperm motility. This fact is important in *S. senegalensis* males since Cabrita *et al*. (2006) reported not only highly variable sperm quality but also the incidence of males with less advantageous reproductive traits. Although these authors only determined sperm motility using semi-quantitative methods, based on their results they suggested the importance of breeder's selection according to individual sperm quality characteristics.

In our study, during the motility period, four males with low sperm quality enhanced their sperm characteristics in the presence of ovarian fluid, stimulating strongly sperm motility. However in the other four males analysed, this fact was not so evident, revealing different female-male physiological interactions. It is also interesting to note that not all motility parameters analysed were enhanced in these males. Generally, only the PM and LIN were enhanced significantly improving their spermatozoon trajectory quality. The effect of ovarian fluid was most impressive in male 3, improving TM, PM, VCL and VSL significantly.

As in the study by Urbach *et al*. (2005), sperm ability to swim in ovarian fluid solution depended on the male's identity and its sperm traits. Also, some studies with freshwater species proposed that ovarian fluid may benefit dominant males, since they release milt in closer contact with eggs than subordinate males (Liley *et al*., 2002).

Solea senegalensis ovarian fluid is difficult to obtain without using hormonal treatments, and more efficient collection methods should be developed, therefore few female samples were collected. Consequently, the influence of individual ovarian fluid in several sperm samples could not be evaluated as well as *S. senegalensis* ovarian fluid composition, due to low sample magnitude. This needs to be checked in further studies. Moreover, other sperm quality parameters such as spermatozoa resistance to osmotic shock may be important to determine, in the presence of activation solution containing ovarian fluid, thus resembling natural fertilization conditions as closely as possible.

5.3 Sperm subpopulation analysis

Recently new approaches to sperm motility analysis have been applied to fish sperm, and in particular to *S. senegalensis*, such as spermatozoa subpopulation through cluster analysis. Previous studies in this species revealed the presence of different sperm subpopulations in a sample which vary with post-activation time and treatments tested. Martínez-Pastor *et al*. (2008) and Beirão *et al*. (2009) detected the presence of four clusters in *S. senegalensis* at 15 s post-activation, representing potentially four sperm subpopulations. *S. senegalensis* sperm subpopulation dynamic is strongly preserved and highly motile spermatozoon lose its motility progressively being further along the post-activation period classified as slow subpopulations and consequently increasing the proportion of slow subpopulations in the last seconds (Martínez-Pastor *et al*., 2008). A similar behaviour was observed in our work on the study of sperm motility with different temperature salinity and pH, where four subpopulations were detected at 15 s post-activation. In this experiment the subpopulations decreased to three SP at 30 s post-activation and finally to two subpopulations at 45 and 60 s post-activation. The second fastest subpopulation at 15 s post-activation disappeared at 30 s post-activation, probably increasing the proportion of the slow subpopulations at this time. In the previous sperm subpopulations studies in this species cluster analysis was performed taking into account all the times recorded, whereas in our study each time was studied separately. The present study has the advantage to detect a more precise condition of each subpopulation at each time, but with less power of comparison between times post-activation times.

The improvement of sperm motility observed previously with 25 ‰ compared with 35 ‰ at 15 s post-activation was significant for the most rapid SP, while 20 ºC improved the second fastest SP. At 30 s post-activation 20 ºC improved the fastest subpopulation. At this time 35 ‰ produced a significantly smaller SP1 and 25 ‰ produced smaller SP3 which is the slowest subpopulation, since they are inversely related. At 45 and 60 s only 2 subpopulations were detected, corresponding to the 2 slower subpopulations present at 15 s post-activation. At 45 s post-activation all parameters produced significant different sperm subpopulations showing an inverse relation between the fastest and slowest SP for that time. Temperature 20 ºC, salinity 25 ‰ and pH 6 improved the proportion of the fastest subpopulation and 16 ºC, 35 ‰ and 30 ‰

and pH 9 improved the proportion of the slowest subpopulation. Finally, at 60 s post-activation low salinity promoted higher percentage of fast cells and the highest salinity produced significant higher percentage of slower cells, in accordance with previous data.

The analysis of motility using sperm subpopulations did not revealed clearly an effect of ovarian fluid treatments. At 15 s post-activation no significant differences were found among treatments, although it seemed that at 45 s S25 and S50 improved SP1 regarding control. However this tendency was not observed during all the experiment. The subpopulation analysis is still a new approach in fish sperm analysis, consequently few data is available, especially in treatments such as the ovarian fluid effect on sperm motility, which is still scarcely studied and understood.

The data of sperm motility analysis through CASA system is usually described through mean values, however the sperm subpopulation analysis brought the advantage of analysing each spermatozoon by its own characteristics allowing the classification of heterogeneous semen samples in homogeneous subpopulations. Sperm subpopulations can be related to treatments, male differences, sperm physiology, and sperm fertility (Abaigar *et al*., 1999; Davis *et al*., 1995; Martínez-Pastor *et al*., 2005; 2006; Quintero-Moreno *et al*., 2003). In this study both approaches were analysed providing deeper information about the treatments tested and allowing a comparison between both data analysis.

Generally the use of mean values produce discrepancies among the motility results for example the effect of a given treatment may increase the straight line velocity but decreases curvilinear velocity, becoming difficult to choose the best treatment as observed in the work of Dietrich *et al.*, 2008. To overcome this problem subpopulations are used, because the several descriptors of motility are clustered simplifying the choice of the best treatment. In the study of the effect of temperature, salinity and pH treatments it was observed a high coherence in the parameters results, not only in mean values but also in subpopulations. However the effect of ovarian fluid on *S. senegalensis* sperm motility was only clear in mean values analysis since the subpopulation analysis did not result in data with consistency in the several post-activation times. It was interesting to analyse the same treatments with mean values and sperm subpopulation analysis since it allowed seeing how treatments affected each subpopulation. Using this approach it was easier to see which treatments improved the subpopulations considered more appropriate to promote higher fertilization success with higher TM, PM, velocities and linearity as described by Martínez-Pastor *et al*. (2008). In conclusion, mean values were more useful to study the effect of experimental treatments on sperm motility activation. The complexity of the data treatment is very high and the development of software to facilitate the data processing to be potentially used in hatcheries is necessary.

CONCLUSIONS

6. Conclusions

With this study the basic optimal conditions to optimize sperm activation conditions in *Solea senegalensis* were assessed. Overall, temperature and salinity of the activation solution seemed to be the most important physico-chemical conditions that affect sperm motility in this species. Higher temperatures such as 20 ºC improved total sperm motility and velocity and lower salinities between 25 and 30 ‰ improved sperm velocity but also the linearity of the tracks, enhancing the spermatozoa potential to reach the egg and to obtain successful fertilization. The pH of the activation solution affected sperm motility in a lower extent. Nevertheless, there seemed to be a tendency to obtain higher values of motility for neutral and slightly acidic solution.

One of the most important effects observed was the presence of ovarian fluid in low concentrations on the activation solution, which not only improved sperm motility but also increased spermatozoa longevity. These low concentrations may be similar to those found in the natural fertilization environment, since ovarian fluid is diluted in the seawater during fertilization. The improvement observed on sperm motility with all the treatments tested seemed to be most important in the last seconds of motility, when sperm is less vigorous and consequently needs a more suited environment to survive. Also the presence of homologous ovarian fluid was more advantageous for *S. senegalensis* sperm motility. More studies need to be conducted to understand the mechanism of sperm motility improvement with ovarian fluid in this species.

The subpopulations analysis revealed that physico-chemical parameters seemed to affect preferably the fastest and the slowest subpopulations, but in the presence of ovarian fluid the intermediate subpopulations seemed to be enhanced to its full potential, remaining thus only two subpopulations with the fastest and the slowest spermatozoa. It would be constructive to study these experiments in assemble with cell viability tests to validate our data.

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ANNEX I

Annex I