Universidad de León

Facultad de C.C. Biológicas y Ambientales

Dpto. Biología Molecular

Área Biología Celular

Sperm quality in marine teleosts: applications to broodstock management and sperm storage in two farmed species *Solea senegalensis* and *Sparus aurata*

Calidad del semen en teleósteos marinos: aplicaciones a la gestión de reproductores y al almacenamiento del semen de dos especies cultivadas *Solea senegalensis* y *Sparus aurata*

By

José Beirão Santos

Thesis presented for the obtention of PhD grade

Memoria presentada como parte de los requerimientos para optar al título de Doctor

León, 30 de Mayo de 2011

José Beirão received a PhD grant SFRH / BD / 31990 / 2006

financed by the POPH – QREN (Tipologia 4.1)

and co-funded by the European Social Fund

and by national funds from the

Portuguese Ministry of Science and Technology.

FCT Fundação para a Ciência e a Tecnologia

MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Uma mosca sem valor pousa com a

mesma alegria na careca de um doutor

como em qualquer porcaria

António Aleixo (1899-1949)

Contents / Índice

Summary 7
Introduction 21
The importance of control reproduction for aquaculture and its limitations 23
Sperm quality 24
Basic quality parameters 25
Morphology 25
Sperm motility 25
Motility activation 26
Motility measurement 26
ATP 28
Mitochondria 28
Metabolism 29
Viability 29
DNA 30
Apoptosis 30
Seminal plasma composition 31
Sperm membrane composition 31
Factors affecting sperm quality 33
Nutrition 33
Husbandry conditions and abiotic/biotic factors 35
Hormonal treatments 36
Broodstock selection 36
The use of sperm cryopreservation to overcome some reproductive constraints 37
Improving cryopreservation protocols 37
The effect of cryopreservation on plasma membranes 40
Solea Senegalensis - a promising species to aquaculture with reproductive constraints 41
Sparus aurata - an excellent aquaculture model for sperm cryopreservation studies of marine teleosts 42

References ... 43

Objectives ... 53

Chapter 1 WILD CAPTIVE *SOLEA SENEGALENSIS* SPERM QUALITY ANALYSIS AND IMPROVEMENT ... 57

1.1.Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level ... 59

Abstract ... 63

1.Introduction ... 64

2.Material and Methods ... 65

2.1.Chemicals ... 65

2.2.Broodstock husbandry conditions and sampling ... 65

2.3.Sperm quality analysis ... 66

2.4.Statistical analysis ... 68

3.Results ... 68

3.1.Motility ... 68

3.2.Hyperosmotic test ... 71

3.3.Apoptosis assay ... 72

3.4.DNA integrity ... 74

4.Discussion ... 75

Acknowledgements ... 78

References ... 79

1.2.Changes in *Solea senegalensis* sperm quality throughout the year ... 83

Abstract ... 87

1.Introduction ... 88

2.Material and Methods ... 89

2.1.Broodstock husbandry conditions ... 89

2.2.Sampling ... 89

2.3.Sperm analysis ... 90

2.4.Statistical analysis ... 91

3.Results ... 91

3.1.Sperm production and motility evaluation ... 91

3.2.DNA fragmentation and apoptosis ... 93

3.3.Sperm membrane resistance to seawater ... 94

3.4.Correlations between the sperm parameters ... 95

4.Discussion ... 97

Acknowledgments ... 100

Bibliography ... 100

1.3.Sperm quality of *Solea senegalensis* could be improved through diet ... 103

Abstract ... 107

1.Introduction ... 108

2.Material and methods ... 109

2.1.Fish and rearing conditions ... 109

2.2.Feeding regime ... 110

2.3.Samplings: sperm and blood ... 110

2.4.Sperm quality analysis ... 111

2.5.Glutathione peroxidase assay ... 113

2.6.Data analysis ... 113

3.Results ... 113

4.Discussion ... 118

Acknowledgments ... 122

References ... 122

Chapter 2 EVALUATION AND IMPROVEMENT OF SPERM CRYOPRESERVATION IN MARINE TELEOSTS (*SPARUS AURATA*) ... 129

2.1.Effect of cryopreservation on fish sperm subpopulations ... 131

Abstract ... 135

Introduction ... 136

Material and methods ... 137

Gametes ... 137

Sperm motility ... 137

Fertilization trials ... 138

Cryopreservation trials 138

Data analysis 139

Results 139

Fertilization trials 139

Cryopreservation trials 140

Discussion 144

Acknowledgments 151

References 151

2.2.Detection of early damage of sperm cell membrane in Gilthead seabream (*Sparus aurata*) with the nuclear stain YO-PRO 155

Summary 159

Introduction 160

Material and Methods 160

Results and Discussion 161

Acknowledgments 163

References 164

2.3. Improving sperm cryopreservation with antifreeze proteins: effect on seabream plasma membrane lipids 165

Abstract 169

1.Introduction 170

2.Material and Methods 172

2.1.Sampling and cryopreservation procedure 172

2.2.Motility and Viability analysis 172

2.3.Head plasma membrane (HM) and flagella isolation 173

2.4.Lipids extraction and analysis 173

2.5.Statistical analysis 174

3.Results 175

4.Discussion 179

Acknowledgments 182

References 183

Final Discussion 187

Wild captive *Solea senegalensis* sperm quality analysis and improvement 189

Evaluation and improvement of sperm cryopreservation in marine teleosts (*Sparus aurata*) 193

References 198

Conclusions 201

Acknowledgments 209

Appendix 211

Resumen 7
Introdución… 21
La importancia del control de la reproducción para la acuicultura y sus limitaciónes............ 23
Calidad del sémen 24
Composición de la membrana espermática.. 31
Factores que afectan la calidad espermática.. 33
El uso de la criopreservación para solucionar los problemas de la reproducción …….............. 37
Solea Senegalensis – una especie prometedora con algunas limitaciones reproductivas....... 41
Sparus aurata – un modelo excelente para estudios sobre la criopreservación de semen de teleósteos marinos.. 42
Bibliografía 43
Objectivos.. 53
Chapter 1 CALIDAD Y MEJORA DEL SEMEN DE *SOLEA SENEGALENSIS CAUTIVO*S.................... 57
1.1.Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level.. 59
Abstract 63
1.Introduction 64
2.Material and Methods 65
3.Results 68
4.Discussion.. 75
Acknowledgements.. 78
References.. 79
1.2.Changes in *Solea senegalensis* sperm quality throughout the year 83
Abstract 87
1.Introduction 88
2.Material and Methods 89
3.Results 91
4.Discussion.. 97

Acknowledgments 100
Bibliography 100
1.3.Sperm quality of *Solea senegalensis* could be improved through diet 103
Abstract 107
1.Introduction 108
2.Material and methods 109
3.Results 113
4.Discussion 118
Acknowledgments 122
References 122
Chapter 2 EVALUACIÓN Y MEJORA DE LA CRIOPRESERVACIÓN DE SEMEN DE TELEÓSTEOS MARINOS (SPARUS AURATA) 129
2.1.Effect of cryopreservation on fish sperm subpopulations 131
Abstract 135
Introduction 136
Material and methods 137
Results 139
Discussion 144
Acknowledgments 151
References 151
2.2.Detection of early damage of sperm cell membrane in Gilthead seabream (*Sparus aurata*) with the nuclear stain YO-PRO 155
Summary 159
Introduction 160
Material and Methods 160
Results and Discussion 161
Acknowledgments 163
References 164
2.3. Improving sperm cryopreservation with antifreeze proteins: effect on seabream plasma membrane lipids 165

Abstract 169
1.Introduction 170
2.Material and Methods 172
3.Results 175
4.Discussion 179
Acknowledgments 182
References 183
Discusión Final 187
Calidad y mejora del semen de *Solea senegalensis* cautivos 189
Evaluación y mejora de la criopreservación de semen de teleóstes marinos (*Sparus aurata*) 193
Bibliografía 198
Conclusiones 201
Agradecimentos 209
Apendice 211

Summary / Resumen

In aquaculture like in any other animal production industry, optimal broodstock husbandry management is a key issue for reproduction control. Even so, most approaches to this subject are still very empiric, the majority of cultivated species show some reproductive dysfunctions and in several species fish are captured from the wild for broodstock repopulation. Correct reproduction control implies control of gametogenesis and the development of appropriate techniques for its evaluation. In this context male reproductive status and the evaluation of spermatogenesis and sperm quality of cultivated species are of utmost importance. Sperm quality is the ability of a sperm sample to fertilize the egg and produce normal offspring. Nevertheless for aquaculture and research purposes, easiest and more rapid parameters/methods are needed, whereas researchers require complementary methodological approaches to understand the basic factors affecting sperm quality.

There is not a single parameter predictive of sperm quality but the assessment of some parameters together could give a consistent idea of the sample quality. Thus, in the present work we have tested the applicability of some new tools for sperm quality analysis and at the same time we have improved some of the more commonly applied ones. Sperm motility, besides being easy to monitor, also reflects several attributes of sperm functionality and for this reason is often used as a quality measurement. In recent years computer analysis (CASA) has been applied for an objective evaluation of this parameter. CASA software analyzes several parameters of each spermatozoon individually and thus a huge amount of data is produced from each sample. Complete use of the data rendered by the CASA software implies the application of an elaborated statistical analysis such as cluster analysis, grouping spermatozoa with similar characteristics. While motility is the decisive factor on which spermatozoa achieve fertilization, DNA integrity is a decisive factor for normal embryonic development and in this context, the comet assay technique measures chromatin integrity in each single cell. DNA damage is usually correlated with the apoptosis process, and although this issue is not as extensively studied in fish sperm as in mammals, it could help explain the low observed sperm quality in some captive species and/or low sperm quality after some storage procedures. One of the easiest ways to measure cell apoptosis is the detection of the plasma membrane destabilization process that occurs in early steps of apoptotic process, through specific markers. In addition to the apoptosis process, membrane integrity is often assessed for sperm quality measurement, by the application of hydrophilic dyes such as propidium iodide (PI). A cell with an injured membrane will be unable to perform its functions, such as the reception of stimulus that triggers motility activation or gamete fusion. Furthermore, sperm plasma membrane composition, because of its unique function, is different from the somatic cells, presenting a high content in polyunsaturated fatty acids which increases membrane fluidity and allows the normal sperm function and mechanisms such as gamete fusion. All sperm plasma membrane components and their interaction are essential for the correct cell function and thus alterations

on them can seriously affect the spermatozoa functionality. In this sense, it is also important to evaluate sperm membrane composition.

In the present work we were interested in developing better tools for sperm evaluation of cultivated marine teleost by means of: giving some clues about potential changes on broodstock husbandry conditions, mainly through dietary supplementation for improving sperm quality; and studying the effect that the cryopreservation technique (the easiest way to store sperm for long periods) has on sperm quality, mainly the effects on sperm motility and membrane composition. To achieve these goals we focus our attention on Senegalese sole, *Solea senegalensis,* sperm quality in captivity and tried to improve male reproductive performance. In this species, sperm quality seems to be one of the factors preventing good quality spawn from being obtained, which impairs *S. senegalensis* mass production. Additionally, we also focus our attention on gilthead seabream, *Sparus aurata*, using this species as a model for sperm cryopreservation in marine teleosts, due to high availability of sperm, knowledge of reproductive biology and its importance for the Mediterranean aquaculture industry.

In a **first** work we analyzed sperm quality of a *S. senegalensis* broodstock using different cellular approaches during the reproductive season. Sperm motility was measured with CASA and a clustering procedure was applied that allowed the detection of four distinct subpopulations. These subpopulations varied with time after activation and the fast and linear subpopulation (probably responsible for fertilization) just 60 s after motility activation was nearly absent. Also, we measured cell plasma membrane resistance to seawater hyperosmolarity and noticed that there was a fast decrease in percentage of cells with intact membrane after sperm motility activation (no more than 12% viable cells 45 s after motility activation). This fact seemed to be modulating sperm subpopulation composition of the different males. Sperm plasma membrane resistance to seawater hyperosmolarity was negatively correlated with mean percentage of DNA fragmentation presented by the sperm cells (measured with the comet assay). Nonetheless, the majority (64.1%) of the spermatozoa in the different males presented DNA fragmentation below 30%, which is considered relatively low when compared with other marine teleosts. We also measured the percentage of apoptotic cells, using the fluorescent probe Annexin-V FITC. The apoptotic population ranged from 6 to 20%, their presence in the ejaculate indicating some spermiation deregulation.

Since in *S. senegalensis* males spermiate all year round, in a **second** work we applied a similar approach monthly throughout the year to better understand *S. senegalensis* sperm quality limitations. There was a tendency for the semen to attain a quality peak between the beginning and the middle of the first spawning season (March-May), followed by a pronounced decrease, achieving the lowest values during the months with the highest water temperature, and

recovering slightly in the second spawning season (September-October). In this sense, the percentage of progressive motile sperm and sperm velocity showed a decrease from March to July (when the highest temperatures were observed), followed by a slight increase in August and October. DNA fragmentation values showed highest values between the two spawning seasons (summer months) and decreased to the end of the year. The percentage of apoptotic cells was lowest in March and highest in November. The percentage of cells resistant to seawater exposure was constantly low throughout the year and in summer months presented values lower than 5% of cells with intact membrane just 15 s after motility activation. However, there were two peaks of cells with intact membrane 15 s after motility activation in May and October, related with the two spawning seasons.

The low sperm plasma membrane resistance to seawater hyperosmolarity observed in the two works can be part of a semen selection process to positively choose cells with better performance, eliminating overripe cells, and preventing them from achieving fertilization. Additionally, *S. senegalensis* presents a reproductive behavior that allows the sperm to achieve oocytes in a short period after ejaculation. The different males present in the broodstocks reached their sperm quality peak at different times of the year and of the spawning seasons, which will result in an unequal contribution for the next generation since females only spawn in specific periods.

In the **third** work we designed a strategy to improve *Solea senegalensis* sperm quality through dietary improvement, feeding three broodstocks on different diets supplemented with fatty acids (docosahexaenoic acid (DHA)) and antioxidants (selenium (Se) and vitamin E (vitE)). Our aim was to improve *S. senegalensis* sperm quality, especially its membrane resistance to seawater hyperosmotic shock, to which they are exposed after ejaculation. In the males fed on the supplemented diet (DHA plus antioxidants) there was an increase in sperm motility parameters (percentage of progressive spermatozoa and curvilinear velocity (VCL)). These motility parameters were positively correlated with both polyunsaturated fatty acids (PUFAs) and DHA proportion in the sperm. Fish fed on DHA only did not show any improvement because of the harmful effects of the generation of reactive oxygen species, causing the high lipid peroxidation values observed in the sperm of the males fed on this diet. This value was positively correlated with the amount of lysophosphatidyl-choline and sphingomyelin. Glutathione peroxidase activity, measured as an indirect contribution of Se, was significantly higher in blood plasma of males fed on DHA plus antioxidants. The addition of the antioxidants to the diets circumvented the higher lipid peroxidation. Opposed to what was initially proposed, cell resistance to seawater hyperosmotic shock was not improved. There was an increase in cholesterol and in several unsaturated fatty acids (DHA, oleic acid, linoleic acid, etc.) in sperm from males fed on DHA plus antioxidants, and at the same time, there was a decrease in the proportion of saturated fatty acids. The components added to the diets were clearly

incorporated in the semen composition and positively affected *S. senegalensis* sperm quality traits. Improvement of motility could reduce the time required for fertilization, thus minimizing the effect of the high sensitivity of cells to seawater.

In the **fourth** work we studied the effect of different cryopreservation protocols on fish sperm motile subpopulations using *S. aurata* sperm and applying a Two-Step Cluster analysis to the data rendered by CASA. We identified 3 different sperm subpopulations. The fast and linear subpopulation (SP3) was the better correlated with the hatching rate. This same subpopulation was the best represented 15 s after activation and was also the one showing a greater decrease in time, being the least represented after 60 s. There was a significant variation in the subpopulation composition between the different cryopreservation protocols (three cryoprotectants (dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG)) each one at two different concentrations; two packaging volumes (0.5 ml straws, and 1.8 ml cryovials); and different freezing rates (1, 2, 3, 4 and 8 cm above the liquid nitrogen surface for the straws and 1, 2 and 4 cm for the cryovials)). The different composition in subpopulations obtained between the protocols pointed out which were the less efficient freezing/thawing procedures. According to the analysis of the subpopulations, variation through time after motility activation, samples frozen in straws with 5% DMSO and in cryovials with 10% DMSO at 2 and 1 cm from the liquid nitrogen, respectively, produced the best results (closer to the control). Samples cryopreserved with cryovials presented a significant decrease in SP3 compared with the same treatment in straws. Clustering analysis allowed the detection of fish sperm subpopulations according to their motility pattern and showed that sperm composition in terms of subpopulations was differentially affected by the cryopreservation protocol depending on the cryoprotectant used, freezing rates and packaging systems.

In the **fifth** work we proposed to test a new tool for fish sperm, YO-PRO 1 nucleic acid stain, to detect early plasma membrane destabilization for the evaluation of spermatozoa viability after the freezing ⁄ thawing procedures. Our results showed that instead of the two sperm subpopulations detected with the traditional evaluation with the PI ⁄ SYBR 14 assay, the application of YO-PRO 1 together with PI allowed the detection of a third subpopulation of cells permeable to YO-PRO 1 but not to PI. This subpopulation was composed by cells with a disturbed membrane which were non-permeable to PI, but whose permeability was already compromised, thus YO-PRO 1 stain was more sensitive than PI. There was an increase in the YO-PRO 1 subpopulation after sperm cryopreservation and, as observed by flow cytometry, these changes in membrane permeability and⁄ or integrity were also accompanied by spermatozoa morphometric modifications.

After detecting this membrane destabilization in the **sixth** and last work we decided to study the effects that the cryopreservation process has on the head plasma membrane (HM) and

flagella lipidic composition. Simultaneously we attempted to stabilize membrane composition by the addition of antifreeze proteins- AFPs (AFPI and AFPIII). The addition of AFPIII (1μg/mL) significantly increased straight line velocity (VSL) and the percentage of viable cells compared with samples only cryopreserved with 5% DMSO. The same concentration of AFPI did not produce any significant improvement in sperm quality. Also, we observed different effects depending on the cryoprotectant solution used (whether AFPI and AFPIII were used or not) on the phospholipid and fatty acids composition of the spermatozoa. Freezing with 5% DMSO alone increased the phosphatidyl-serine (PS) content, increased the saturated fatty acids and decreased the unsaturated fatty acids (mainly PUFA) in both HM and flagella. Nevertheless flagella lipidic composition was not so affected as the HM by the cryopreservation procedure. On the other hand, AFPs, especially AFPIII, seemed to have interacted with unsaturated fatty acids avoiding changes in lipidic profiles during cryopreservation and stabilizing the plasma membrane organization both in head and flagella. Also it was observed that flagella had a higher amounts of unsaturated fatty acids and higher amounts of phosphatidyl-serine and phosphatidyl-ethanolamine than the HM. This will probably confer higher fluidity to the flagella and explain its lower degree of lipid modifications after cryopreservation. Finally the HM lipid composition but not the flagella composition was correlated with sperm quality parameters such as viability, motility and velocity, suggesting that mainly the HM lipid composition influenced sperm performance. These sperm quality parameters were positively affected by the higher amount of unsaturated fatty acids. Our data support the hypothesis of a direct interaction of AFPs with specific hydrophobic moieties of the membrane lipids.

En la acuicultura, como en cualquier otra industria animal, el control del ciclo reproductor es clave para su desarrollo. Sin embargo, y a pesar de su importancia, muchos de los conocimientos en esta área han sido obtenidos de forma empírica. Además, la mayoría de las especies de teleósteos cultivadas presenta algún tipo de desregulación y, en muchos casos, existe la necesidad de capturar individuos salvajes para componer los lotes de reproductores. El correcto manejo de la reproducción necesita de un control de la gametogénesis y de un desarrollo de técnicas apropiadas para su evaluación. En este contexto, el status reproductivo de los machos y la evaluación de la calidad seminal de las especies cultivadas es de vital importancia. La calidad del semen se mide habitualmente por la capacidad de una muestra para fertilizar los huevos y producir descendencia viable. Sin embargo, tanto para la acuicultura como para la investigación, es preciso desarrollar parámetros que sean sencillos y que puedan medirse con rapidez. En el caso concreto de la investigación, se requieren, además, metodologías más complejas que permitan evaluar diferentes factores relacionados con la citofisiología del esperma para complementar así el conocimiento sobre los factores que afectan a la calidad seminal. No existe un parámetro que, aislado, sea indicador de la calidad seminal, pero la evaluación conjunta de varios de ellos puede dar una buena idea de la calidad de una muestra. De este modo, en el presente trabajo hemos evaluado la aplicabilidad de algunas nuevas herramientas para el análisis de la calidad seminal y, a la vez, hemos probado algunos de los métodos de análisis más comunes.

La movilidad espermática puede ser sencilla de analizar y es el resultado de otros aspectos que revelan la funcionalidad del semen y, por esa razón, suele ser usada como medida de calidad. En las últimas décadas, han sido utilizados programas informáticos de análisis de imagen específicos para la movilidad del semen (CASA), que permiten una evaluación objetiva de esta actividad. El *software* CASA analiza diversos parámetros de cada espermatozoide individualmente y, por esta razón, de cada muestra analizada se obtiene un gran número de datos. El aprovechamiento al máximo de los datos obtenidos implica la aplicación de análisis estadísticos elaborados, como el análisis de clusters, que reúne en un mismo conjunto espermatozoides con características semejantes. Mientras la movilidad es un factor decisivo para alcanzar el huevo y fertilizar, la integridad del ADN es decisiva para el correcto desarrollo del embrión. La utilización del ensayo cometa permite medir la integridad de la cromatina de cada célula individualmente. El daño en el ADN puede, además, estar relacionado con el proceso de apoptosis y, aunque en semen de peces esta cuestión no esté tan estudiada como en mamíferos, su evaluación puede ayudar a comprender la baja calidad del semen presentada por algunas especies en cautividad, así como el descenso que se produce en la calidad como consecuencia del almacenamiento del esperma. Una de las técnicas más sencillas para identificar la apoptosis se basa en la detección de la desestabilización de la membrana que ocurre al principio del proceso, al aplicarse marcadores específicos. La integridad de la

membrana es también un indicador de la calidad del semen y suele realizarse mediante la aplicación de fluorocromos hidrofílicos intercalantes, como el ioduro de propidio (IP). Una célula con la membrana dañada es incapaz de realizar sus funciones, ya sea la recepción de los estímulos que activan la movilidad o la fusión de los gametos. Por otra parte, la membrana plasmática del espermatozoide tiene una composición específica distinta de la de las células somáticas, con un elevado contenido en ácidos grasos poliinsaturados, que facilitan la fluidez y la organización necesarias para que se produzca la fusión de los gametos. Todos sus componentes, así como su interacción, son fundamentales para el correcto funcionamiento de la membrana, de modo que posibles modificaciones en la composición, aunque no afecten a la integridad, pueden tener consecuencias importantes. Por ello, el estudio detallado de la composición de la membrana puede aportar datos de interés.

Los objetivos del presente trabajo han sido: desarrollar y mejorar las herramientas para la evaluación de la calidad seminal en teleósteos marinos importantes para la acuicultura mediterránea; conocer algunas de las claves que explican la baja calidad del semen producido por algunas especies en cautividad y tratar de mejorarla mediante cambios en la alimentación de los reproductores; y proponer mejoras en los métodos de criopreservación con vistas a reducir sus efectos nocivos. Para lograr estos objetivos se ha utilizado *Solea senegalensis*, como ejemplo de especie que presenta problemas reproductivos en cautividad, y *Sparus aurata*, como modelo de un teleósteo marino que completa su ciclo reproductor y permite buena disponibilidad de semen para los estudios de criopreservación.

En el **primer** trabajo hemos analizado la calidad del semen de reproductores de *S. senegalensis* aplicando distintas técnicas a lo largo de la época reproductiva. La movilidad del semen fue medida mediante CASA y se aplicó un análisis de clusters, que permitió la detección de cuatro subpoblaciones distintas. Estas subpoblaciones variaron a lo largo del tiempo tras la activación de la movilidad. La subpoblacion con movimiento rápido y lineal (probablemente la responsable de la fertilización) casi había desaparecido a los 60 s. Se evaluó también la resistencia de la membrana plasmática a la hiperosmolaridad del agua de mar, detectándose un rápido descenso del porcentaje de células que presentan la membrana intacta después de su dilución en este medio (menos de 12% de células viables tan solo 45 s después de la activación de la movilidad). Este hecho parece haber modulado la composición de las subpoblaciones espermáticas de los distintos machos. La resistencia de la membrana plasmática de los espermatozoides a la hiperosmolaridad del agua de mar tuvo una correlación negativa con la fragmentación media del ADN (medida con el ensayo cometa). Sin embargo, la mayoría (64,1%) de los espermatozoides de los distintos machos analizados presentaron una fragmentación del ADN inferior a 30%, lo cual es considerado relativamente bajo si se compara con las observaciones hechas en otros teleósteos marinos. Asimismo, se analizó el porcentaje de

células apoptóticas, usando el marcador fluorescente Annexina-V FITC. La población apoptótica varió entre 6 y 20%. Su presencia en el eyaculado indica algún tipo de desregulación de la espermiación.

Los machos de *S. senegalensis* presentan producción seminal a lo largo de todo el año, por lo que en el **segundo** trabajo hemos aplicado una evaluación semejante, mensualmente a lo largo de un año. En este trabajo se observó una mejor calidad del semen durante la primera época de puesta anual (marzo-mayo), seguida de un fuerte descenso, alcanzándose los valores más bajos durante los meses con más altas temperaturas de agua y recuperándose ligeramente en la segunda época de puesta (septiembre-octubre). En concreto, el porcentaje de espermatozoides progresivos y la velocidad espermática presentaron un descenso de marzo a julio, seguido por un pequeño aumento en agosto y octubre. Los valores de fragmentación del ADN fueron más elevados entre las dos épocas de puesta (verano) disminuyendo después hacia el final del año. El porcentaje de células apoptóticas presentó valores bajos en marzo y elevados en noviembre, mientras que el de células resistentes a la exposición del agua de mar fue reducido a lo largo de todo el año, especialmente en los meses de verano, cuando llegó a presentar valores inferiores a 5% de células, con la membrana intacta 15 s tras la activación de la movilidad. Sin embargo, hubo dos picos (mayo y octubre) con mayor porcentaje de células resistentes, relacionados con las dos épocas de puesta.

La baja resistencia de la membrana plasmática de los espermatozoides a la hiperosmolaridad del agua de mar, observada en los dos trabajos, puede explicar los bajos porcentajes de fecundación que se observan en las puestas obtenidas en cautividad. Su correlación negativa con la fragmentación del ADN puede indicar un mecanismo de selección de las células con mejores características, al eliminar aquellas menos competentes. *S. senegalensis* presenta un comportamiento reproductivo que le permite que los espermatozoides alcancen los oocitos poco tiempo después de la eyaculación. Los distintos machos presentes en el lote estudiado presentaron su pico de calidad seminal en distintos momentos del año e, incluso, en distintos momentos de la época de reproducción. Este hecho supone una distinta contribución por parte de los machos a la siguiente generación.

En el **tercer** trabajo nos planteamos una estrategia para mejorar la calidad del semen del *S. senegalensis* a través de la mejora de la dieta: se alimentaron tres lotes de reproductores con distintos piensos enriquecidos con ácidos grasos poliinsaturados (ácido docosahexaenoico (DHA)) y antioxidantes (selenio (Se) y vitamina e (vitE)). En los machos alimentados con el pienso enriquecido con DHA y antioxidantes hubo un incremento en los parámetros de movilidad (porcentaje de espermatozoides móviles y velocidad curvilínea (VCL)). Estos parámetros de movilidad estuvieron correlacionados positivamente con la proporción de ácidos grasos poliinsaturados (PUFAs) y DHA en el semen. Los peces alimentados solamente con DHA no presentaron ninguna mejora debido a los efectos negativos provocados por el incremento

de especies reactivas de oxígeno, que causaron un aumento de la peroxidación lipídica. Este valor estuvo positivamente correlacionado con la concentración seminal de lisofosfatidilcolina y esfingomielina. La actividad de la glutatión peroxidasa (medida indirecta de la concentración de Se) fue significativamente superior en el plasma sanguíneo de los machos alimentados con DHA y antioxidantes. La adición de antioxidantes a las dietas impidió el aumento de la peroxidación lipídica. El semen de machos alimentados con DHA y antioxidantes presentó un aumento en el colesterol y en diversos ácidos grasos insaturados (DHA, ácido oleico, ácido linoleico, etc) y una disminución de los ácidos grasos saturados. Los componentes añadidos a las dietas fueron claramente incorporados en la composición del semen y han afectado positivamente los parámetros de calidad del *S. senegalensis*, a pesar de que el objetivo de incrementar la resistencia de la membrana plasmática al choque hiperosmótico no fue conseguido. La mejora de los parámetros de movilidad puede reducir el tiempo necesario para la fertilización, disminuyendo el tiempo de exposición al agua de mar y sus efectos dañinos.

En el **cuarto** trabajo se ha estudiado el efecto de los distintos protocolos de criopreservación sobre la estructura de las subpoblaciones espermáticas identificadas mediante el análisis de la movilidad y usando semen de *S. aurata* y aplicando un análisis de clusters a los datos obtenidos con el CASA. Hemos identificado tres subpoblaciones distintas. La subpoblacion rápida y lineal (SP3) fue la que mejor correlacionó con las tasas de eclosión. A la vez, esta misma subpoblacion fue la mejor representada a los 15 s después de activar la movilidad, pero también fue la que más disminuyó con el tiempo, pues a los 60 s fue la subpoblación menos representada. Se detectó una variación significativa en la proporción de espermatozoides en cada subpoblación entre los distintos protocolos de criopreservación (tres crioprotectores (dimetil sulfóxido (DMSO), etilenglicol (EG) y propilenglicol (PG)), cada uno con dos concentraciones distintas, dos envases distintos (pajuelas de 0,5 ml y criotubos de 1,8 ml), y distintas rampas de congelación (1, 2, 3, 4 y 8 cm sobre la superficie del nitrógeno líquido para las pajuelas y 1, 2 y 4 cm para los criotubos)). La diferente composición seminal, en términos de subpoblaciones obtenidas, entre los distintos protocolos de criopreservación, indicó cuáles fueron los protocolos menos eficientes. De acuerdo con el análisis de subpoblaciones y su variación a lo largo del tiempo tras activar la movilidad, las muestras congeladas en pajuelas con DMSO 5% a 2cm del nitrógeno líquido y en criotubos con DMSO 10% a 1cm, produjeron los mejores resultados (más cercanos al control). Las muestras criopreservadas con criotubos presentaron un descenso significativo de la SP3 en comparación con los mismos tratamientos con pajuelas. El análisis de clusters, por un parte, ha permitido detectar distintas subpoblaciones en el semen de la *S. aurata*, de acuerdo con su patrón de movilidad, y por otra, ha demostrado que la composición en términos de subpoblaciones es afectada por el protocolo de criopreservación, en dependencia del crioprotector usado, la rampa de congelación y el envase.

En el **quinto** trabajo nos propusimos evaluar una nueva herramienta para el semen de peces: el marcador de ácidos nucleicos YO-PRO 1, con el fin de detectar espermatozoides que han sufrido una desestabilización en la membrana plasmática, tras el procedimiento de criopreservación. Nuestros resultados han demostrado que, además de las dos subpoblaciones espermáticas detectadas con la tradicional prueba IP/SYBR 14, la aplicación del YO-PRO 1, conjuntamente con el IP, ha permitido la detección de una tercera subpoblación de células permeables al YO-PRO 1 pero no al IP. Esta subpoblación está compuesta por células con la membrana desestabilizada pero no permeable al IP, lo cual el YO-PRO 1 es un indicador más fino de procesos tempranos de alteración en la permeabilidad original. Se observó un incremento de la subpoblación de YO-PRO 1 en el semen criopreservado y la citometría de flujo reveló que los cambios en la estabilidad e integridad de la membrana plasmática fueron acompañados por cambios morfométricos.

Una vez detectada esta desestabilización de la membrana durante la congelación, en el **sexto** y último trabajo, se evaluaron los efectos de la criopreservación sobre la composición lipídica de la membrana plasmática de la cabeza (HM) y del flagelo, analizándose la proporción colesterol/ fosfolípidos, las clases de fosfolípidos y las especies moleculares de ácidos grasos. Al mismo tiempo se analizó el posible efecto estabilizador de las proteínas anticongelantes (AFPs) AFPI y AFPIII. En comparación con las muestras criopreservadas solamente con DMSO 5%, la adición de AFPIII (1µg/mL) mejoró significativamente tanto la velocidad lineal (VSL) como el porcentaje de células viables. La misma concentración de AFPI no produjo ninguna mejora significativa en dichos parámetros de calidad seminal. Por lo que se refiere a la composición de la membrana, esta mostró un contenido distinto en fosfolipidos y ácidos grasos, en dependencia del protocolo de criopreservación usado (sea con AFPI o AFPIII o tan solo con DMSO 5%). La criopreservación solo con DMSO 5% incrementó el contenido en fosfatidilserina y en ácidos grasos saturados y disminuyó la proporción de ácidos grasos insaturados (especialmente PUFA) en ambos dominios estudiados: HM y flagelo. No obstante, la composición lipídica de los flagelos no fue tan afectada por la congelación como la de la membrana de la cabeza. Por otro lado, las AFPs han mostrado un efecto estabilizador en la organización de la membrana de ambos dominios, cabeza y flagelo, y las muestras congeladas con ellas, en especial la AFPIII, no han mostrado diferencias con la composición del semen fresco. En cuanto a las diferencias entre la membrana de la cabeza y el flagelo, este último mostró un mayor contenido en fosfatidilserina y fosfatidiletanolamina y en ácidos grasos insaturados, lo que probablemente podría proporcionar una mayor fluidez al flagelo a la vez que podría explicar su menor sensibilidad a la criopreservación. La composición lipídica de la membrana plasmática de la cabeza, y no la de los flagelos, tuvo correlación con los parámetros de calidad seminal, tales como viabilidad, movilidad y velocidad. La correlación de estos parámetros con la proporción de ácidos grasos insaturados fue positiva. Nuestros datos apoyan la hipótesis de una interacción directa de las AFPs con regiones hidrofóbicas específicas de los lípidos de membrana.

Introduction

THE IMPORTANCE OF CONTROL REPRODUCTION FOR AQUACULTURE AND ITS LIMITATIONS

The control of reproduction is a key issue in aquaculture. Thus, obtaining new larvae for tank repopulation with precise timing depends on the correct and careful planning of broodstock management. In one way or another all fish species held in captivity exhibit some degree of reproductive dysfunction either caused by stress associated to captivity or inappropriate regulation of the environment (Mañanós *et al*., 2008a). For this reason, for some cultivated species, brood fish are often captured from the wild. Dysfunctions observed in broodstocks depend on the species, varying from the total absence of spawning to significant reductions in the quantity and quality of gametes produced. In this way one of the limiting factors every time one new species is proposed for aquaculture is obtaining gametes of proven quality. The use of high quality gametes is vital to ensure the production of offspring with high survival rates that meets the aquaculture market demand (Alavi *et al*., 2007).

It is crucial for the development of protocols to control maturation and spawning in fish species, to precisely understand its reproductive strategy (sexual maturation, size at first maturity, spawning behavior, cytophysiology, etc) and reproductive cycle in relation to environmental conditions (Mañanós *et al*., 2008a). Moreover, as explained by Alavi *et al*. (2007), conditions affecting the reproduction of each species are highly species-specific. For this reason an increasing number of publications exist on the study of reproductive biology of fishes as well as in the analysis of gamete quality and sperm analysis. This fact has enabled a continuing increase in the number of cultured species and their mass production, hence reducing pressure on natural fish stocks.

Nevertheless, the selection of individuals for broodstocks is based on phenotypical characteristics such as fast growing rather than good reproducer, and more often than not there is no control over the individuals that are contributing to the spawns. Recently, some studies have been carried out using genetic tools to study the loss of genetic variability in cultivated species (McDonald *et al*., 2004; Porta *et al*., 2006). In this way, the evaluation of both male and female reproductive performance is important. Unfortunately the aquaculture industry has been more focused on the quality of eggs and larvae rather than sperm, even though it also affects fertilization success as much as larvae quality (Rurangwa *et al*., 2004). Thus the male reproductive status is as important as the female reproductive status and the development of tools for evaluating spermatogenesis and sperm quality, as well as studies on male sperm quality of cultivated species, are of utmost importance.

SPERM QUALITY

Sperm is the result of a maturation process (spermatogenesis) where several haploid cells are produced aiming to transmit paternal genetic information to the next generation. Failure to produce high quality sperm will decrease the possibility to obtain viable offspring with these paternal genes.

More consensually sperm quality is **defined** as the ability of a sperm sample to fertilize and produce normal offspring. According to Cabrita *et al*. (2008a) five events will affect the success of a spermatozoon to achieve its goal: a) the ability of the spermatozoon to reach the egg, b) its capacity to cross the egg envelopes or enter the micropyle, c) recognition of the oolema and fusion of both plasma membranes, d) correct activation of the egg metabolic pathways and e) contribution to the future embryo with an undamaged genome. Assessment of sperm quality should then inform about these endpoints. Testing fertilizing capacity is difficult and time-consuming, and it is thus easier for both fish farmers and researchers to use different parameters that allow a more rapid evaluation. Some authors have tried to relate fertilization with other parameters that are more or less easy to monitor, as has been reviewed by several authors (Bobe and Labbé, 2010; Cabrita *et al*., 2008a; Rurangwa *et al*., 2004). Nevertheless, because quality does not rely on a particular characteristic of the milt, there is not a single predictive parameter. However the assessment of some parameters together could give us a consistent idea of the sample quality in order to predict its fertilizing ability. Moreover sperm quality is very variable between species and so are the parameters to be used in each species for the quality evaluation.

The evaluation of sperm samples is important when testing the maturation level of males, for the identification of males with better reproductive performance, for situations where *in vitro* fertilization is needed, to monitor the best broodstock rearing conditions or in the case of specific biotechnological applications, as is the case of cryobanking. As explained by Rurangwa *et al*. (2004), several fish farms used a pool of sperm from different males to perform artificial inseminations, avoiding prior evaluation of samples. This practice will often mask the effect of poor semen quality presented by some males, which will ultimately lead to the risk of severe bottleneck and inbreeding and finally production of homozygous strains of low fitness. Understanding the difference in sperm quality between males will hence help a proper planning of the artificial insemination process. Moreover, identification of bad quality males would significantly improve broodstock management, discarding non-productive individuals. The quality requirements for specific biotechnological applications, as is the case of cryobanking, deserve special mention. The development of protocols for semen storage, either fresh storage at 4ºC or cryopreservation, requires careful monitoring of sperm quality. Sperm quality assessment is also useful for ecotoxicology, both as an environmental marker or to study the effects of pollutants on fish reproductive system.

Basic quality parameters

Standard parameters that enable a fast analysis are: semen volume, spermatozoa concentration and spermatocrit, subjective assessment of motility, pH and osmolarity. These aspects could easily cover the needs of fish farmers since no expensive equipment is needed and all parameters are fast and easily assessed, helping the operator to detect the general status of the samples and discard bad quality ones. Moreover, in some species a correlation has been established between some of these variables and fertilization rate, as is the case of *Hippoglossus hippoglossus* (Tvedt *et al*., 2001). Nevertheless, several other parameters could impair sperm quality and its fertilizing capacity, likewise a more precise evaluation is required for experimental purposes, since there is the need to understand the putative causes behind variations in these parameters, as well as a more objective evaluation of some of them, particularly cell motility.

Morphology

Sperm from teleost do not possess acrosomes, which is compensated for by the presence of micropyles in the fish egg envelope. The sperm head in external fertilizers is usually almost spherical with a collar formed by an extrusion of the plasmalemma as midpiece and an indentation of the nucleus. The centrioles plus a few mitochondria are located in the midpiece. The axoneme typically presents an arrangement of nine pairs of peripheral microtubules and one pair of central tubules (Cosson, 2007).

Cell morphology and membrane ultrastructure could be indicative of different features such as maturation stage of the cell or injuries caused by environmental factors or manipulation. This aspect is often evaluated in fish sperm using scanning electron microscopy (SEM) and ⁄ or transmission electron microscopy (TEM) (Alavi *et al*., 2008; Gwo *et al*., 2005; Psenicka *et al*., 2008; Wei *et al*., 2007). Different studies have identified morphological and morphometric changes in head, flagella and midpiece as a result of cryopreservation in species such as *Pagrus major, Diplodus puntazzo* and *Macrozoarces americanus* (Liu *et al*., 2007b; Taddei *et al*., 2001; Yao *et al*., 2000).

More recently, ASMA (computer assisted morphology analysis) has been applied to analyze sperm morphology (Marco-Jimenez *et al*., 2006, 2008; Peñaranda *et al*., 2008; Tuset *et al*., 2008). This software has advantages over traditional SEM analysis because it provides objective faster automated evaluation of sperm morphometry, which together with statistical tools, allows spermatozoa populations with increased fertilizing ability and resistance to cryoinjury to be identified (Thurston *et al*., 2001).

Sperm motility

The most commonly used parameter to determine sperm quality is sperm motility, as it is the decisive factor on which sperm first achieve the oocyte. Although it does not indicate the real

status of the spermatozoa, this aspect in addition to being easy to study, reflects several other attributes of the sperm, such as plasma membrane function, ATP availability and hence mitochondria functionality, axoneme/flagellar structure and functionality etc. This parameter is not just used to evaluate sperm quality for productive purposes, but to study the effect of different broodstock treatments (e.g. hormonal treatment (Cabrita *et al*., 2011; Schiavone *et al*., 2006)), sperm manipulation (e.g. cryopreservation (Ding *et al*., 2009; Nascimento *et al*., 2010)) or reproductive strategies (Fitzpatrick *et al*., 2007; Rudolfsen *et al*., 2006). Moreover, several authors have successfully correlated sperm motility parameters with fertilization rate (Le Comber *et al*., 2004; Liu *et al*., 2007; Ottensen *et al*., 2009).

In fish with external fertilization, spermatozoa are immature while in the testis, and mature during migration through the sperm ducts. Spermatozoa motility is activated when in contact with freshwater/seawater due to ionic changes between the extra and intracellular medium or/and to some egg factors in the ovarian fluid as is the case of herring (Inaba, 2007; Kho *et al*., 2005; Ohtake, 2003). Sperm motility capacity is acquired in the sperm ducts as reviewed by Schulz *et al*., (2010) caused by an increase in semen pH and bicarbonate (HCO_3^-) levels that lead to an increase in intracellular cAMP levels. This increase will trigger the maturation of some proteins of the non-motile axoneme allowing the acquisition of sperm motility (Morisawa and Okuno, 1982; Schulz *et al*., 2010).

Several authors have reviewed the sequential events, mostly in salmonid species, that ultimately lead to microtubule sliding and flagellar beating (Inaba, 2007; Kho *et al*., 2005; Morisawa, 2008). Nonetheless several aspects are still unclear.

Motility activation

In external fertilizers sperm motility initiation is known to be dependent on osmotic or chemical changes detected by cell receptors between the seminal plasma and the external environment (Alavi and Cosson, 2006; Inaba, 2007). In the case of marine fish, spermatozoa are active as a response to the hyperosmolarity of the external medium. The rise in ionic strengths during transfer to the seawater changes the membrane potential and, as a consequence, Ca^{2+} and pH intracellular concentrations increase (Cosson, 2007). As reviewed by Inaba (2007), a number of signaling molecules and cytoskeletal elements are involved in sperm rapid response to these changes that lead to intracellular signal transduction and the activation of the motile machinery, the axoneme. The axoneme is activated via phosphorylation of subunits associated with molecular motor dyneins. Once these proteins are phosphorylated, ATP can be hydrolyzed by dinein-ATPase and flagellar beating takes place.

Motility measurement

The most simple and common method used to screen sperm motility is phase contrast or a dark field microscope to visually evaluate the percentage of motile sperm or the duration of motility.

Although this method has been used for a long time it is highly subjective and depends on observer expertise. Moreover, several aspects such as sperm density, drifting, sperm velocity etc. can cause an over or underestimation. The low reproducibility of this analysis often makes the results difficult to interpret. Hence there was the need of a rapid and sensitive method to obtain an accurate evaluation.

Since the late 90's the development of objective methods of computer assisted sperm analysis (**CASA** system) for fish sperm, has provided a tool to make motility evaluation much more accurate, providing more complete information on sperm movement (Ciereszko *et al*., 1996; Ravinder *et al*., 1997). The CASA system is based on a video recording of the sperm movement that tracks each individual cell. This technique was first introduced in 1979 in mammalian sperm (Dott and Foster, 1979), and recently an open source of CASA software was developed (Wilson-Leedy and Ingermann, 2007). The use of computerized analysis allows a more objective analysis of the different motility parameters with high repeatability and documented results. Several authors have correlated the data obtained with the CASA system with different aspects of sperm, like DNA integrity (Li *et al*., 2008) or with the external conditions, such as ovarian fluid composition (Dietrich *et al*., 2008; Rosengrave *et al*., 2009), activation solutions (Martinez-Pastor *et al*., 2008a), etc. These conditions will ultimately affect sperm ability to fertilize and thus in some cases CASA data was directly correlated with fertilization (Dietrich *et al*., 2010; Rurangwa *et al*., 2001). CASA systems quantify different sperm motility parameters, including some that could not be detected by visual inspection. The parameters rendered by the system could vary slightly according to the different software used. The most commonly used for fish sperm analysis have been revised by Rurangwa *et al*. (2004): the percentage of motile sperm (MOT) and the percentage of progressive spermatozoa (PRO) are good indicators of the numbers of motile sperm; some of the most used by fish sperm researchers are curvilinear velocity (VCL), which is the actual velocity along the trajectory and straight line velocity (VSL), the straight line distance between the start and end point of the track divided by the time of the track; VCL and VSL are identical when the trajectory is a straight line; angular path velocity (VAP) is the velocity along a derived smoothed path, the ratio of the net distance moved to the total path distance (VSL/VCL) is linearity (LIN) the most often used parameter to analyze the curvature of the trajectory, the ratio of the net distance moved to the smoothed path distance (VSL/VAP) is straightness (STR). Other parameters such as amplitude of the lateral displacement of the sperm head (ALH), percentage of wobble (WOB) or beat-cross frequency (BCF) have a small meaning for fish spermatozoa.

The fact that with CASA each spermatozoon is monitorized implies that an enormous amount of data is produced, but most of the studies only use the mean values rendered by the system. Recently, it has been pointed out that semen is not a homogeneous mixture of cells and plasma but is made of different sperm subpopulations, with a different genotype, maturation stage and

characteristics. This question has mostly been studied in mammals (Martinez-Pastor *et al*., 2005; Muiño *et al*., 2008, Ortega-Ferrusola *et al*., 2009) using clustering analyses and defining sperm subpopulations that help to understand some issues not perceptible when only using mean motility values. Martinez-Pastor *et al*. (2008a) and Le Comber *et al*. (2004) have clearly shown the coexistence within the same sample, in fish sperm, of distinct motility-based sperm subpopulations in *Solea senegalensis* and *Gasterosteus aculeatus,* respectively.

Another objective method is the use of stroboscopic light for image recording, using dark field microscopy and higher magnification lenses, allowing the detection of events on the axoneme, together with flagellar beating and motility duration. This method was first described by Cosson *et al*. (1985).

ATP

When spermatozoa are released in the water they face a huge energy demand to induce and sustain flagellar beating (Cosson *et al*., 2008a; Zietara *et al*., 2009). While they are capable of synthesizing ATP, through mitochondrial oxidative phosphorylation or lipid metabolism (Lahnsteiner *et al*., 2010a; Perchec *et al*., 1995; Zietara *et al*., 2009), this ATP production is not enough to sustain high ATP consumption once motility is activated, causing the short motility period, from some seconds to a few minutes, observed in fish sperm. The maintenance of an appropriate ATP concentration is important for modulating flagellar beating (Zietara *et al*., 2009). Most ATP consumption is linked to dynein ATP-ase located within the flagellar motile apparatus. Therefore, the ATP stores accumulated in quiescent spermatozoa are the main supply for sustaining motility after activation (Ingermann, 2007). Some studies have proposed ATP concentration as a good predictor of sperm fertilizing ability (Zilli *et al*., 2004). ATP and ADP measurements are usually done by luminescence (Burness *et al*., 2005; Cosson *et al*., 2008b; Mansour *et al*., 2003; Zilli *et al*., 2004).

Mitochondria

Mitochondria are responsible for ATP supply to the cell, thus their functionality, together with respiration measurements, are often used as an alternative way to evaluate sperm quality. Unlike mammalian spermatozoa, most fish species have a small number of rounded cristae mitochondria. Mitochondrial damage causes a decrease in motility as well as in ATP levels. Mitochondria displaying normal potential gradient have been studied by different authors with Rhodamine 123 (a cationic fluorescent probe) or other stains such as JC-1 (a lipophilic cation), that penetrate the cell and accumulate inside the mitochondria (De Baulny *et al*., 1997; He and Woods, 2004; Segovia *et al*., 2000). Flow cytometry is often used as a tool for sperm mitochondria status evaluation (Cabrita *et al*., 2005a; Guthrie *et al*., 2008; Liu *et al*., 2007). Mitochondrial functionality can also be measured through specific enzyme activity (Mansour *et al*., 2003).

Metabolism

In addition to ATP, already referred to, the study of enzymatic activity and metabolic constituents in both spermatozoa and seminal plasma reflects semen status (Cabrita *et al*., 2008a). Moreover, it is a determining factor in sperm motility duration (Mansour *et al*., 2003). Different metabolic pathways can be analyzed: oxidative phosphorilation, tricarboxylic acid cycle, lipid metabolism, glycolisis, ATP metabolism and respiration rate (Lahnsteiner *et al*., 1998; Lahnsteiner *et al*., 1999; Lahnsteiner *et al*., 2010a; Mansour *et al*., 2003). Enzymatic activity is more commonly measured through the consumption or production of NADH or NADPH using UV-spectrophotometry measurements.

Viability

Viability is one of the easiest ways to evaluate sperm quality. This term usually refers to plasma membrane integrity and functionality. Viability is considered a primary need for cell survival since a cell with an injured membrane will be unable to develop its functions. The easiest way of measuring cell membrane viability is by assessing plasma membrane impermeability to hydrophilic dyes. The simplest assays estimate viable cells using a light microscope after staining with vital dyes that only enter the cell through altered membranes, such as trypan blue (Lubzens *et al*., 1997) and eosin (Lahnsteiner *et al*., 1996). Other dyes such as Hoescht 33258 (pentahydrate, bis-benzimide) requires the use of a fluorescent microscope to perform the same procedure (Cabrita *et al*., 1998). More recently, the use of dual fluorescent staining SYBR-14 and propidium iodide together with flow cytometry enabled a faster and more accurate evaluation of plasma membrane integrity (Cabrita *et al*., 2005a; Horváth *et al*., 2008). Flow cytometry allows the evaluation of different cell parameters, in addition to those detected with probes fluorescence, such as size and shape. The use of flow cytometry for purposes other than sperm membrane integrity, such as mitochondria status, apoptosis and DNA damage, was reviewed by Martinez-Pastor *et al*. (2010).

Additionally, some authors use these probes to test sperm cell membrane resistance to osmotic and mechanical stress occurring due to osmotic variations followed by sperm release into fresh or seawater (Cabrita *et al*., 1998; Marian *et al*., 1993). According to Cabrita *et al*. (2008a), marine species suffer a lower degree of damage when compared to freshwater species, and their survival time is not a determining factor for fertilization success. Even so, low membrane resistance could indicate suboptimal membrane functionality.

Another way of measuring cell membrane viability is to analyze the presence of cytoplasmic components in the seminal fluid (enzymes, metabolites, etc), as this indicates cell membrane rupture (Lahnsteiner *et al*., 1998; Zilli *et al*., 2004).

DNA

The main purpose of gametes is to transmit the genetic information to the next generation. Thus, it is of utmost importance to ensure that genetically damaged spermatozoa do not achieve fertilization, as observed by Pérez-Cerezales *et al*. (2010a) affecting progeny survival. Several studies analyze sperm DNA in mammalian species, whilst in fish spermatozoa this is quite a new field. Reports on fish sperm analyzing DNA integrity apply the comet assay, SCGE (single cell gel electrophoresis) and refer to the damage that cryopreservation causes to sperm (Cabrita *et al*., 2005a; Labbé *et al*., 2001; Pérez-Cerezales *et al*., 2009; Zilli *et al*., 2003) but also to damage caused by genotoxic effects (Ciereszko *et al*., 2005). This assay is also used in ecotoxicology studies (Dietrich *et al*., 2007; Dietrich *et al*., 2010). This technique measures the migration of damaged DNA strand fragments from the immobilized nucleus in an electrophoresis gel. The smaller the DNA strands the faster they migrate, leading to the occurrence of a comet-like tail preceding the nucleus DNA (head of the comet). Different parameters might be obtained from the analysis of the comet images using specific software: the percentage of tail DNA (% DNAt) and the Olive Tail Moment (Mt), which includes both length and intensity of DNA in the tail, are the most consensual ones (Lee and Steinert, 2003; Kumaravel and Jha, 2006). Other reports use techniques such as SCSA (sperm chromatin structure assay), which measures the susceptibility of sperm nuclear DNA to acid-induced *in situ* denaturation (Pérez-Cerezales *et al*., 2006) or TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP-biotin end-labelling), which allows the direct detection of DNA breaks (Ma *et al*., 2010). Only recently, Pérez-Cerezales *et al*. (2009 and 2010a) applied more complete comet techniques to study sperm DNA, combining comet assay with specific endonucleases. The high degree of chromatin condensation with proteins and the presence of antioxidants in the seminal plasma usually protect DNA from injuries. Additionally, the eggs can repair alterations in the chromatin to a certain extent (Pérez-Cerezales *et al*., 2010a). As explained by Bobe and Labbé (2010), the susceptibility of fish sperm to DNA destabilization will depend on the nuclear proteins present. For example, *Dicentrarchus labrax* sperm nuclei contain only protamines whereas *Sparus aurata* contains only histones, and *Oncorhynchus mykiss* both histones and protamines.

Apoptosis

Programmed cell death is a common phenomenon during spermatogenesis (Sakkas and Alvarez, 2010), however, according to studies conducted in mammals these apoptotic cells should be phagocytized while in the testis. A similar phenomenon was described for *S. aurata* apoptotic spermatogonia in postspawning (Chaves-Pozo *et al*., 2005). Thus, the presence of apoptotic cells in the ejaculate could indicate deregulation of the normal sperm maturation process. This topic has been largely studied in mammals sperm (Khan *et al*., 2009; Marti *et al*., 2008; Martinez-Pastor *et al*., 2008b; Ortega-Ferrusola *et al*., 2008) and in human spermatozoa

cell apoptosis was negatively correlated with sperm motility (Zhang *et al*., 2008). Nevertheless, as far as we know, there is no published bibliography concerning the presence of apoptotic cells in fish ejaculate (except those present in this thesis). Several apoptosis markers, such as Annexin V and Yo-Pro 1, and different methods such as TUNEL, immunohistochemistry, etc, have been used in both mammals and fish to study apoptosis in different types of reproductive cells (Cal *et al*., 2010; Chaves-Pozo *et al*., 2005; Corriero *et al*., 2009; Jeong *et al*., 2009; Ortega-Ferrusola *et al*., 2008). Annexin V, one of the most commonly used markers to detect apoptosis in the ejaculate, is a calcium-dependent binding protein that has high affinity to phosphatidylserine. This phospholipid is translocated to the outer leaflet of the plasma membrane during the early process of apoptosis due to loss of membrane asymmetry, allowing the detection of cells under the apoptosis process (Peña *et al*., 2003). Annexin V is usually employed with propidium iodide to detect different spermatozoa cell populations present in ejaculate. In teleost fish, apoptosis was studied in *S. aurata* and *Scophthalmus maximus* during spermatogenesis in the testis and only observed at the beginning of spermatogenesis or during the postspawning period (Cal *et al*., 2010; Chaves-Pozo *et al*., 2005).

Seminal plasma composition

Seminal plasma plays a crucial role supporting spermatozoa and physico-endocrinological function after release of sperm from the testis into the sperm duct, and subsequently to the aquatic environment (Alavi *et al*., 2007). Its composition reflects the conditions in which spermatogenesis occurs, such as possible ageing processes, metabolic alterations, contamination or other factors that affect sperm quality (Cabrita *et al*., 2008a). Inorganic compounds normally determined in seminal plasma that are usually involved in the process of sperm motility activation are: sodium, potassium, chloride, calcium and magnesium. Organic compounds normally detected in the seminal plasma could be related with energy metabolism, like glucose and other metabolites and several enzymes, or associated with anti-oxidant capacity, for example vitamins. Moreover, the presence of some compounds in the seminal plasma could reflect cell lysis as previously mentioned in the section on viability. Seminal plasma composition has been related with motility (Butts *et al*., 2010; Lanes *et al*., 2010) and fertilization rate (Lahnsteiner *et al*., 1998).

SPERM MEMBRANE COMPOSITION

Plasma membrane is the main structure responsible for extracellular communication and extracellular environment signal detection. Although it is often not directly analyzed, membrane is a fundamental structure, and its composition and functionality are indirectly measured when analyzing sperm motility, viability, morphology, etc. In fish with external fertilization, sperm membrane plays a major role in detecting the transition from the seminal

plasma environment to the freshwater/seawater environment, sensing the ionic changes that lead to motility activation. Within teleosts (lacking acrosome), sperm membrane will also be responsible for gamete fusion. Some components involved in sperm adhesion to the egg micropyle were described in *O. mykiss* (Yu *et al*., 2002). The sperm membrane structure is highly sensitive to environmental stress and alterations can lead to spermatozoa malfunction (Cabrita *et al*., 2010; Muller *et al*., 2008).

Membranes are mainly composed by lipids, mostly phospholipids, and proteins. Phospholipids are arranged in a bilayer responsible for the membrane structure and together with cholesterol will determine membrane fluidity. Phosphatidyl-choline (PC) is the main phospholipid representing usually more than 50%, but other phospholipids like phosphatidyl-ethanolamine (PE) (30%) and phosphatidyl-serine (PS) (10%), present in the inner leaflet, correspond to a large fraction (Bobe and Labbé, 2010). Other phospholipids usually present are phosphatidyl-inositol (PI), sphingomyelin (SM) and lyso-phosphatidyl-choline (LPC).

The hydrophobic tail of phospholipids consists of long fatty acid hydrocarbon chains. Fatty acids can be either saturated or unsaturated, depending on the double bonds and this fact affects membrane fluidity (Giraud *et al*., 2000; Labbé *et al*., 1995). Unsaturated fatty acids resemble saturated fatty acids, except that the chain has one or more double bonds between carbon atoms. If they have only a single double bond they are monounsaturated fatty acids (MUFA), whereas if they contain more than one double bond they are called polyunsaturated fatty acids (PUFA). Compared with somatic cells, sperm phospholipids are richer in PUFA (Bell *et al*., 1997; Waterhouse *et al*., 2006). Moreover it is usually accepted that the majority of marine species have a much higher level of unsaturated fatty acids than freshwater ones. As referred to by Kopeika and Kopeika (2007), the polyunsaturated /saturated fatty acid ratio in PE is twice as high in marine species as in freshwater species. Even so, the sperm composition in fatty acids is highly species-specific as noticed by Bell and his colleagues (1997) and highly affected by diets (Bell *et al*., 1996; Pustowka *et al*., 2000). Additionally, some authors have observed that fatty acids can affect sperm functions, such as motility and fertilization capacity (Lahnsteiner *et al*., 2009). The main saturated fatty acids are C14 (commonly known as myristic acid), C16 (palmitic acid), and C18 (stearic acid); the main MUFAs are C16:1n7 (palmitoleic acid), C18:1n9 (oleic acid), C18:1n7 (vaccenic acid), C20:1n9 (eicosenoic acid); the main PUFAs are C20:3n6 (linoleic acid(LA)), C20:4n6 (arachidonic acid (ARA)), C20:5n3 (eicosapentaenoic acid (EPA)), C22:5n6 (docosapentaenoic acid (DPA)) and C22:6n3 (docosahexaenoic acid (DHA)).

Another essential structural component of the plasma membranes, already referred to, is cholesterol**.** It is formed by a rigid tetracyclic ring structure with a hydroxyl group at one end and a short hydrocarbon tail at the other. Cholesterol regulates the lipid chain order and molecular organization of the membranes, and hence controls many membrane properties and

functions, such as formation of specific domains, fluidity, permeability to water and other molecules, lipid phase transition, etc (Muller *et al*., 2008; Wassall and Stillwell, 2009).

Other main components of the membranes are proteins that together with the lipids are responsible for selective permeability to water and ions. Proteins also actively participate in osmolarity regulation through ion channels and aquaporins in the case of water (Zilli *et al*., 2009). Moreover, proteins are responsible for cell signaling (Li *et al*., 2009). Compared to mammals few studies exist on fish sperm proteins, and most of them, as reviewed by Li *et al*. (2009), only refer to plasma proteins or all spermatozoa proteins rather than focusing on membrane proteins. Nonetheless, some of the detected proteins were membrane structural proteins. Based on the fish sperm membrane proteins already documented, most of them seem to be involved in sperm motility initiation, like ion channels, in exocytosis, in egg activation or in membrane stabilization (Krasnai *et al*., 2003; Li *et al*., 2010a; Vines *et al*., 2002). Nevertheless, several of the proteins isolated from fish sperm membrane remain unidentified (Li *et al*., 2009).

Therefore, the study of sperm membrane composition is of utmost importance in fishes with external fertilization because of the hazardous external medium the spermatozoa have to sustain after ejaculation. In this sense several authors have investigated plasma membrane content in phospholipids, cholesterol and lipoproteins and their interaction to understand how its composition will affect sperm quality and influence its ability to successfully fertilize eggs (Bell *et al*., 1997; Drokin, 1993; Labbé *et al*., 1995; Labbé *et al*., 1996; Lahnsteiner *et al*., 2009; Muller *et al*., 2008).

FACTORS AFFECTING SPERM QUALITY

Fish maturation and spawning is highly regulated by environmental factors. In order to maximize offspring survival to nature variations, fishes have evolved in a way to reproduce when food availability is higher. In this sense they have developed the ability to use environmental cues (temperature, photoperiod, etc) to know when to invest in reproduction (Bromage *et al*., 2001; Mañanós *et al*., 2008a). Hence, several factors affect sperm quality in different ways (from food availability and its quality to social aspects or numerous abiotic factors). Most of these factors are not well characterized and now that interest in the aquaculture industry is increasing, research is being developed concerning these issues. Understanding the factors that affect gamete quality is vital for the development and expansion of the aquaculture industry.

Nutrition

It is widely accepted that gonadal development and fecundity are affected by diet composition and some specific nutritional requirements. Nevertheless, the analysis of broodstock nutrition

effects on gamete quality are still at an early stage, and most studies only refer to the quality of spawn, rather than gamete quality.

As explained by Izquierdo *et al*. (2001), lipid and fatty acid composition of the broodstock diet are the main factors affecting reproduction success and survival of offspring. Many PUFAs are essential fatty acids that cannot be synthesized by fishes, and must therefore be provided in the diet. It is commonly accepted that the increase in polyunsaturated fatty acids (PUFAs) improves sperm fluidity (Blesbois *et al*., 2005; Ladha, 1998) and hence its resilience to osmotic effects when sperm is ejaculated. Fatty acids and cholesterol enriched diets could change the composition of sperm, as observed by Labbé *et al*. (1995) in *O. mykiss*, where fish fed on a fish oil diet displayed higher n-3/n-6 fatty acid ratio in sperm membranes than did those receiving corn oil. Pustowka *et al*. (2000) also observed differences in *O. mykiss* sperm membrane fatty acid and cholesterol composition according to the diet, and Asturiano *et al*. (2001) observed an increase in PUFAs present in the sperm of *D. labrax* fed on an enriched diet, and also an increase in male reproductive performance. Similar results were obtained by other authors in different fish species (Nandi *et al*., 2007; Vassallo-Agius *et al*., 2001), yet in some cases improved sperm quality was not observed (Henrotte *et al*., 2010). Generally, fresh water species need both 18:2(n-6) and 18:3(n-3) fatty acids in the diet, while marine species need more elongated and unsaturated fatty acids, or highly unsaturated fatty acids (HUFA) such as EPA and DHA (Izquierdo *et al*., 2001). These HUFAs will be important in fish maturation and steroidogenesis. Usually cuttlefish, squid and krill are identified as valuable diet components containing these HUFAs (Izquierdo *et al*., 2001).

Generally the addition of PUFAs to the diets leads to an increase in membrane sensitivity to lipid peroxidation (Surai *et al*., 1998). Moreover, due to the high metabolism rate presented by spermatozoa a significant production of reactive oxygen species (ROS) can be generated (Edens and Brake, 2006). A complex antioxidant defense system is present in the semen, which is highly affected by the diet (Mansour *et al*., 2006) and variable during the reproductive season (Liu *et al*., 1997). In reproductive tissues several enzymes (e.g. glutathione peroxidase and superoxide dismutase) and compounds (e.g. ascorbic acid, glutathione and α-tocopherol) are involved in the antioxidant system (Dabrowsky and Ciereszko, 2001). A balance between ROS production and the antioxidant system must be established in order not to affect sperm quality. The antioxidant effect of vitamins C (ascorbic acid) and E (tocopherol) can provide important protection to sperm cells during spermatogenesis and fertilization, by reducing the risk of lipid peroxidation (Izquierdo *et al*., 2001). Therefore, many authors have added antioxidant components such as vitamins to the diet (Canyurt and Akhan, 2008; Lee and Dabrowski, 2004; Mansour *et al*., 2006; Rainis *et al*., 2007). Vitamins cannot be synthesized by fish, thus their addition to the diet could be limiting (Ciereszko and Dabrowski, 1995). Both ascorbic acid and α-tocopherol concentration in seminal fluid reflect their concentration in the broodstock diet

(Dabrowski and Ciereszko, 2001; Lee and Dabrowski, 2004; Liu *et al*., 1997). Ascorbic acid antioxidant function provides protection for cells by reducing the risk of membrane lipid peroxidation and DNA damage, and at the same time increases sperm production and motility (Dabrowski and Ciereszko, 2001). In the same way, a deficiency of ascorbic acid can reduce sperm concentration, motility and fertilizing ability (Ciereszko and Dabrowski, 1995). Another component that could affect the antioxidant capacity is the trace element selenium (Se), since the selenoprotein glutathione peroxidase (GPx) plays a crucial role in male gamete quality (Edens and Brake, 2006; Shalini and Bansal, 2008; Surai *et al*., 1998). Even so, there are no studies on its effect on fish sperm quality. Widely studied in mammals and poultry reproduction, it is established that both deficiency as well as excess Se impair sperm quality. Most studies reflect that an Se deficiency will affect spermatogenesis causing morphological alterations (affecting head morphology and midpiece integrity), improper chromatin condensation, which makes sperm DNA more vulnerable to ROS attack and increased DNA breaks (Shalini and Bansal, 2008) and impaired sperm motility that will finally lead to fertilization failure (Bertelsmann *et al*., 2010; Edens and Brake, 2006; Marin-Guzman *et al*., 2000; Shalini and Bansal, 2008; Surai *et al*., 1998).

Husbandry conditions and abiotic/biotic factors

Since spermatogenesis in nature is highly regulated by abiotic factors, its control on captivity will have a considerable effect on gamete quality (Mylonas *et al*., 2010). Several factors such as temperature, photoperiod, spawning substrate and biotic factors have been studied, although the seasonally-changing pattern of day length and temperature are most probably the responsible for the cueing and timing of reproduction in the majority of fish (Bromage *et al*., 2001). Both temperature and photoperiod are often manipulated to accelerate or delay gonadal maturation, so that fish spawn at a convenient time (Bromage *et al*., 2001). Yet the effect on the male gamete quality of these factors and other biotic and abiotic factors is barely known (Mylonas *et al*., 2010).

Any cause of stress can decrease fish sperm production or/and its quality. Usually, stress is associated with captivity conditions caused by handling, transportation, stocking density and the daily routine in the fishfarm (e.g. noise). This issue is even more relevant in animals captured from the wild, which are usually more prone to suffering from stocking conditions than those produced in captivity (Bobe and Labbé, 2010; Mylonas *et al*., 2010). Other factors such as stripping frequency and territorial behavior during the reproductive season could also be a cause of stress. Additionally because of high stocking conditions, cultivated fishes are more vulnerable to diseases and that will obviously affect their gamete quality (Rurangwa *et al*., 2004). In-depth knowledge on the reproductive biology of the species being cultivated will help the fish farmer to reproduce the natural conditions in captivity and hence decrease stress associated to it.

Semen characteristics do not just vary between species, but also between stocks and throughout the reproductive season. Several studies analyze sperm quality during the reproductive season in order to establish the best period to collect samples either for *in vitro* manipulation or storage by cryopreservation (Babiak *et al*., 2006; Butts *et al*., 2010; Lanes *et al*., 2010; Mylonas *et al*., 2003; Peréz-Cerezales *et al*., 2010b; Robles *et al*., 2003). Sperm usually present higher quality in the middle of the reproductive season or during the time females are fully mature. In *S. maximus* and *H. hippoglossus* a decrease in sperm quality at the end of the reproductive season was observed caused by an ageing phenomenon (Babiak *et al*., 2006; Suquet *et al*., 1998).

Also, the presence of aquatic pollutants acting as endocrine-disrupting substances in the fish farm water affects reproduction in general and could lead to a decrease in sperm quality (Abascal *et al*., 2007; Dietrich *et al*., 2010; Jobling *et al*., 2002; Marchand *et al*., 2010).

Hormonal treatments

The reproductive cycle is regulated by a sequence of hormonal events controlled by the brain-pituitary-gonad axis. The success of reproductive maturation and obtaining high quality gametes depends on the correct functioning of all components of the axis throughout the reproductive cycle (Mañanós *et al*., 2008a; Mylonas *et al*., 2010). Husbandry conditions often result in the disruption of the brain-pituitary-gonad axis because of stress or inappropriate environmental conditions. In males, as explained by Zohar and Mylonas (2001), this aspect decreases milt production and quality. The use of hormonal treatments in cultured fish has permitted the reproduction in captivity of several fish species that do not do so spontaneously and the synchronization of gamete release in both sexes at a convenient time (Mylonas *et al*., 2010; Zohar and Mylonas, 2001). Spawning inductors are very efficient increasing sperm volume production and density, and in some reported cases also increase sperm motility (Mylonas *et al*., 2010). It seems that hormonal induction is favorable to sperm maturation in the testis and in the sperm ducts. In this sense, assessment of sperm quality is frequently used to optimize hormonal dosage and its timing. Nowadays different synthetic, less species-specific and highly potent agonists of the gonadotropin-releasing hormone (GnRHa) are available. Additionally, sustained-release delivery systems for controlled administration have been developed (Zohar and Mylonas, 2001), proving to be more effective in enhancing milt production compared to acute treatments (Mylonas *et al*., 2010).

Broodstock selection

Farmed fish are selected to be appealing to the consumer and grow at maximal rates and frequently some of those fish are included in the new broodstock although this selection does not offer any reliable cue about the reproductive performance of individuals in terms of sperm quality (Rurangwa *et al*., 2004). This broodstock selection is basically made on visual

observation, rather than using gamete analysis techniques or genetic tools. Additionally, the age of the broodstock could be a determining factor in sperm quality. Genetic tools have recently been used in selective breeding programs to help improve broodstock selection and to avoid loss of genetic variation. Nonetheless, in production, most genetic selection is still based on fast growth and flesh quality (Mylonas *et al*., 2010; Zohar and Mylonas, 2001) and as previously mentioned if some of those fish are included as breeders in a new stock, very little is known about the effects this could have on gamete quality (Bobe and Labbé, 2010).

THE USE OF SPERM CRYOPRESERVATION TO OVERCOME SOME REPRODUCTIVE CONSTRAINTS

Cryopreservation is the process of preserving cells, tissues or living organisms at sub-zero temperatures, typically -197ºC using liquid nitrogen (LN). First reports on sperm cryopreservation date back to 1776, when Spallanzani observed inhibition followed by activation of man and horse spermatozoa when exposed to cooling temperatures. Nonetheless, modern history only began in 1949 with Polge *et al*. (1949) publishing in Nature the cryopreservation of human sperm using glycerol as cryoprotectant. Nowadays, the application of sperm cryopreservation is common practice in mammals including humans, for industrial, conservational and medical purposes. Nevertheless, it is still far from being common practice in fishes, although cryopreservation is the simplest means of storing fish germplasm for long periods of time. Cryopreservation protocols are well established for several freshwater and marine species, from aquarium species to food industry species, to endangered ones. The benefits of fish sperm cryopreservation have been reviewed by several authors (Cabrita *et al*.,2010; Tiersch *et al*., 2007): 1) synchronization of gamete availability of both sexes; 2) sperm economy, when the existing volume of sperm is a limiting factor; 3) simplification of broodstock management and genetic improvement programs, when the animals must be manipulated for fertilization, sperm cryopreservation allows this manipulation to be reduced hence simplifying aquaculture procedures; 4) decreasing costs caused by the maintenance of live males and facilitating the transport of gametes between fish farms; and 5) germplasm storage for genetic selection in the case of valuable research or aquaculture strains and conservation programs.

Improving cryopreservation protocols

Although cryopreservation protocols have been established for several species (Cabrita *et al*., 2008a) and applied with different purposes, there is always a decrease in sperm quality. During the cryopreservation process there are four critical periods, reviewed by Cloud and Patton (2008): 1) cooling the cells to the point of ice formation; 2) formation of ice (temperature of the cells and their immediate surroundings increase because of the heat of fusion of water); 3) cooling through the critical period (-10 to -40ºC), movement of water out of the cells causing an increase in the concentration of intracellular salts; and 4) reduction to liquid nitrogen (LN_2)

temperature. All of this process is potentially destructive causing a decrease in sperm fertilization capacity. The decrease in sperm quality during cryopreservation could occur in different structures: cell membrane, mitochondria, chromatin structure, axoneme and other structural damage, etc. Most of this damage is caused between 0 and -40ºC because of volume changes, cryoprotectant exposure and toxicity and heat removal. Additionally, other causes affect sperm quality, e.g. cryoinjury, pH fluctuation, ice crystal formation, etc (Chao and Liao, 2001; Wolfe and Bryant, 2001; Woods *et al*., 2004). Although the decrease in sperm fertilizing ability can easily be overcome by increasing the volume of used sperm, the objective of sperm cryopreservation is to maintain the initial sperm characteristics. Besides, some damage caused in cryopreserved sperm may not limit the fertilization ability, in which case fertilization with DNA damaged sperm could occur and have further negative consequences during embryo development. Nevertheless, Labbé *et al*. (2001) did not observed any significant difference in the survival and abnormality rates between fertilizations with fresh and cryopreserved sperm in *O. mykiss*, and also Martínez-Páramo *et al*. (2009) using microsatellites in brown trout, proved that there were no significant differences in the male genetic contribution to the offspring using either fresh or cryopreserved sperm. Even so, Pérez-Cerezales *et al*. (2010a) demonstrated that spermatozoa with DNA damage are capable of fertilizing oocytes, and despite the fact that the damage can be repaired by an oocyte-repair mechanism, this system is inefficient when DNA damage is above a certain level. In this situation, an increase in the rate of abortions and some gene expression deregulation seems to occur (Herráez *et al*., 2010).

Moreover, the different sperm subpopulations in an ejaculate previously referred to will be differently affected by cryopreservation, hence cryopreservation should not be considered a homogeneous process (Taddei *et al*., 2001). In this sense, the success of cryopreservation protocols is affected by several different aspects, and their optimization is one of the important areas of research since it is highly species-specific.

Different aspects should be considered:

1) The majority of cryoinjuries are caused by water. **Cryoprotectants** are small compounds that enter cells and protect them during the dehydratation process, replacing the water content of the cell, in the case of permeating cryoprotectants such as dimethyl sulfoxide, or stabilizing the membrane during the cryopreservation process, in the case of non-permeating cryoprotectants such as sugars (Tiersch *et al*., 2007). Although these compounds are absolutely necessary for successful cryopreservation, above a particular concentration they are biochemically toxic for sperm cells. They are usually added at low temperatures to diminish their toxicity. Cryoprotectant toxicity as explained by Kopeika and Kopeika (2007) could be a result of an interaction with enzymes or caused by alterations in the cellular biomolecular environment. Thus, the specific type and concentration of cryoprotectant should be optimized to an equilibrium point to afford

the greatest protection during the freezing thawing process, while minimizing toxicity to the cells being cryopreserved (Chao and Liao, 2001).

2) The composition of **extenders** usually mimics that of seminal plasma. Nevertheless, several other compounds are used for example to act as membrane stabilizers, as is the case of bovine serum albumin (BSA) (Cabrita *et al*., 2008b) and egg yolk or LDL (Pérez-Cerezales *et al*., 2010b), or to prevent the harmful effect of reactive oxygen species adding antioxidants, such as uric acid (Lahnsteiner *et al*., 2010b). Once the sperm cell membrane seems to be able to exchange lipid components with the environment, according to some studies in mammal semen, specific lipids and fatty acids could be added to the extender to improve sperm survival to freezing/thawing (Cerolini *et al*., 2001). Additionally, antifreeze proteins (AFPs) have been incorporated in the extender composition in mammalian sperm cryopreservation (Younis *et al*., 1998; Prathalingam *et al*., 2006), to protect cells through modification of the ice crystals, preventing recrystallization and interacting with the plasma membrane at low temperatures (Inglis *et al*., 2006).
3) **Freezing** methodology and freezing curves should be carefully optimized, either using a thermocouple which monitors the temperature curves or using controlled biofreezers. As explained by Cloud and Patton (2008), the freezing rate must be slow enough to allow the movement of water out of the cells so that ice crystals do not form intracellularly, and rapid enough to prevent the increase in intracellular salt concentration from affecting cellular components. Also, the **thawing** process should be optimized: usually fast thawing is preferred to avoid recrystalization, which is damaging for the cells (Tiersch *et al*., 2007).
4) **Packing** volumes will define the diffusion of temperatures, and for some species it is important to preserve high amounts of sperm (Cabrita *et al*., 2001; Liu *et al*., 2006; Horvath *et al*., 2007). Conversely, in other cases it is important to preserve high quality samples rather than quantity (Robles *et al*., 2009; Yang and Tiersch, 2009). Usually small volume plastic straws or vials are used. Sperm packing volumes could go from 0.25 ml straws (because their geometrical structure will allow semen to freeze and thaw faster) to 5 ml straws (Cabrita *et al*., 2001). An increase in volume makes it difficult to achieve a homogeneous freezing/thawing process and temperature diffusion.

Consequently, methodology improvement is one of the important areas of research, taking into account all the previously mentioned points. Unfortunately, there is a lack of standardization and different laboratories often use different protocols for the same species. Recently, Cabrita *et al*. (2008) published a compilation of protocols for different species trying to summarize the knowledge acquired to date in marine and freshwater species.

The effect of cryopreservation on plasma membranes

During cryopreservation, the sperm plasma membrane is the structure most susceptible to suffering damage, and some of its components are lost during the process. Cold shock is caused by the change of membrane lipids from the liquid to the solid phase during the freezing process, from 10 to -16ºC (Chao and Liao, 2001). Differences in membrane organization, lipid-protein interactions, phospholipid asymmetry and lipid composition affect cell membrane permeability and hence its capacity to adjust rapidly to osmotic variations during the cryopreservation process (Cerolini *et al*., 2001; Muller *et al*., 2008; Waterhouse *et al*., 2006). Membrane phospholipids go through phase transition during cryopreservation, resulting in a spatial redistribution of the membrane components, alteration of the phospholipids/cholesterol ratio and aggregation of membrane proteins which could affect cell osmoregulation mechanisms (Drokin *et al*., 1998) and impair sperm egg recognizing mechanisms and plasma membrane fusion. Furthermore, sperm "freezability" is known to be dependent on the physico-chemical characteristics of plasma membrane such as membrane fluidity, which correlates with cold tolerance and cryopreservation (Giraud *et al*., 2000). It is usually accepted that, in marine teleosts, regarding freshwater species, the higher tolerance to cryopreservation is attributed to a higher cholesterol/phospholipid ratio present in plasma membrane (Kopeika and Kopeika, 2008). It seems that the increase in cholesterol/phospholipid decreases the temperature of membrane phase transition, which makes the membrane more stable during freezing. Also, as exposed by Kopeika and Kopeika (2008), cholesterol decreases the strength of interactions between long-chained saturated phospholipids, resulting in an increase in membrane fluidity. Even so, this is not true for all species, as observed by Labbé *et al*. (2001) when they tested this hypothesis in sperm from different mammalian species. The relation between saturated and unsaturated fatty acids also affects the cryoresistance of the membrane: a higher ratio of unsaturated/saturated membrane fatty acids usually improves membrane resistance to cold-shock (Waterhouse *et al*., 2006). However, membrane properties not only depend on the cholesterol or fatty acids content, but on its components in general and their interaction. Muller *et al*. (2008) showed that membrane fluidity detected by fluorescence anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH), is the most likely candidate to be a determinant of fish sperm cryotolerance. Moreover, during cryopreservation ROS are generated, affecting sperm membrane composition in terms of lipids and proteins (Cerolini *et al*., 2001; Morte *et al*., 2008), which could also lead to sperm membrane structure destabilization (Li *et al*., 2010b).

Similarly to the facts mentioned previously, and also for cryopreservation purposes, it is possible to modulate sperm membrane quality through broodstock nutrition (Pustowka *et al*., 2000), or stocking conditions (Labbé and Maisse, 1996) to better withstand the cryopreservation procedure. Labbé and Maisse (1996 and 2001) working with *O. mykiss* tested

the effect of thermal-acclimation and salinity on the fatty acids and cholesterol/phospholipids profile, and concluded that salinity did not affect these parameters, but thermal acclimation did and also improved sperm resistance to cryopreservation. On the other hand, Pustowka *et al*. (2000) observed that feeding *O. mykiss* broodstocks with specific diets could increase levels of cholesterol and MUFA, thus increasing sperm cryoresistance. In addition, it is possible to improve cryopreservation protocols to reduce membrane damage by adding specific components to the extender as mentioned above.

SOLEA SENEGALENSIS **- A PROMISING SPECIES TO AQUACULTURE WITH REPRODUCTIVE CONSTRAINTS**

Since the 80's, several studies have been carried out in Senegalese sole (*Solea senegalensis*) farming (Dinis *et al*., 2008). Even so, efforts to introduce this species in the aquaculture industry in recent years have been impaired by difficulties in controlling reproduction, poor egg quality and low fertilization rates (Cabrita *et al*., 2006). Temperature and photoperiod seem to be the key factors triggering gonadal maturation (Oliveira *et al*., 2008), although the effect of the manipulation of abiotic factors on sperm quality is yet to be studied in depth. Some attempts have been made to increase the low sperm volume (quality) produced by this species using hormonal therapies such as GnRHa treatments (Agulleiro *et al*., 2007; Cabrita *et al*., 2011; Guzmán *et al*., 2008), changing the feeding regime (Anguís *et al*., 2008) and testing the effect of female-to-male communication (Cabrita *et al*., 2011).

Usually *S. senegalensis* spawns are obtained naturally in the tank in two periods (late spring to the beginning of summer and early autumn), when temperatures are similar. Recent reports described the collection of eggs by stripping after hormonal induction, allowing the performance of artificial fertilization and controlling the quality of both oocytes and sperm (Mañanós *et al*., 2008b). Additionally recent studies have shown that low-quality spawns (low rate of fertilization) in Senegalese sole broodstocks might be related to poor and highly variable semen quality (Cabrita *et al*., 2006; Beirão *et al*., 2008; Martinez-Pastor *et al*., 2008a). Limited information on sole gamete quality leads to difficulties in controlling the reproductive cycle of this species. Similar to its relative *Solea solea*, *S. senegalensis* has a very low gonadosomatic index when compared with that of other fish species, with very small variations during spermatogenesis (García-López et al., 2006). They present asynchronous germ cell maturation and low sperm concentration, volume, and production throughout the year. All these facts might be related to reproductive behavior, since it seems that during courtship, males only release sperm when in contact with the eggs and the genital ducts are held closely together (Duncan *et al*., 2008). Also, the existence of a high sensitivity sperm population to hyperosmotic conditions, causing cell lysis when activated with seawater (Martinez-Pastor *et al*., 2008a), was

observed. These authors have suggested that this might be due to an ageing process occurring in sperm cells.

SPARUS AURATA **- AN EXCELLENT AQUACULTURE MODEL FOR SPERM CRYOPRESERVATION STUDIES OF MARINE TELEOSTS**

Gilthead seabream (*Sparus aurata*) is the main cultivated species in the Mediterranean area, with a peak production in 2008 of over 130,000 tons, according to FAO data (FAO, 2011). Its reproductive cycle is well controlled and usually manipulated with water temperature and photoperiod, obtaining production throughout the year. Besides the diversity of studies on different aquaculture aspects of *S. aurata* biology, from nutritional (Pinto *et al*., 2009) to pathological aspects (Ibarz *et al*., 2010), *S. aurata* reproductive biology has also been extensively studied (Chaves-Pozo *et al*., 2005; Lahnsteiner, 2006). In our case, we should make reference to the different sperm biology studies: from morphology studies (Maricchiolo *et al*., 2007; Marco-Jimenez *et al*., 2008), to metabolism studies (Lahnsteiner *et al*., 2010a) or sperm motility activation studies (Zilli *et al*., 2009), most of which have served as models for other marine teleost species.

Successful cryopreservation protocols have already been developed for *S. aurata* (Cabrita *et al*., 2005a; Fabbrocini *et al*., 2000). However, cryopreservation protocols, as previously mentioned, always imply a loss of sperm quality. Both studies by Fabbrocini *et al*. (2000) analyzing post-thaw motility, and Cabrita *et al*. (2005a) analyzing post-thaw membrane integrity, resistance to hyperosmotic shock and mitochondrial functionality, reported a loss of quality caused by cell damage.

References

Abascal F., Cosson J., Fauvel C. 2007. Characterization of sperm motility in sea bass: the effect of heavy metals and physicochemical variables on sperm motility. J. Fish Biol. 70: 509–522

Agulleiro M.J., Scott A.P., Duncan N., Mylonas C.C., Cerda J. 2007. Treatment of GnRHa-implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comp. Biochem. Physiol. A 147(4): 885-892

Alavi S.M.H. and Cosson J. 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: A review. Cell Biol. Int. 30(1): 1-14

Alavi S.M.H., Linhart O., Coward K., Rodina M. 2007. Fish spermatology: Implications for aquaculture management. In: Fish spermatology. Eds: Alavi S.M.H., Cosson J., Coward K., Raffie G. Alpha Science Intl Ltd, Oxford, UK, 397- 460

Alavi S.M.H., Psenicka M., Policar T., Linhart O. 2008. Morphology and fine structure of *Barbus barbus* (Teleostei : Cyprinidae) spermatozoa. J. Appl. Ichthyol. 24(4): 378-381

Anguís V., Salas-Leiton E., Martín I., Chereguini O. 2008. Influence of the feeding on sperm quality of first generation (f1) *Solea senegalensis* during an annual cycle in two facilities of Spain. Aquaculture Europe 08. Krakow, Poland, September 15-18

Asturiano J.F., Sorbera L.A., Carrillo M., Zanuy S., Ramos J., Navarro J.C., Bromage N. 2001. Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture 194(1-2): 173-190

Babiak I., Ottesen O., Rudolfsen G., Johnsen S. 2006. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 65(8): 1587-1604

Beirão J., Cabrita E., Soares F., Herráez M.P., Dinis M. T. 2008. Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: comet analysis and Annexin V–Fluorescein staining. J. Appl. Ichthyol. 24(4): 508-513

Bell M.V., Dick J.R., Thrush M., Navarro J.C. 1996. Decreased 20:4n-6/20:5n-3 ratio in sperm from cultured sea bass, *Dicentrarchus labrax*, broodstock compared with wild fish. Aquaculture 144(1-3): 189-199

Bell M.V., Dick J.R., Buda C. 1997. Molecular speciation of fish sperm phospholipids: Large amounts of dipolyunsaturated phosphatidylserine. Lipids 32(10): 1085-1091

Bertelsmann H., Keppler S., Holtershinken M., Bollwein H., Behne D., Alber D., Bukalis G., Kyriakopoulos A., Sieme, H. 2010. Selenium in blood, semen, seminal plasma and spermatozoa of stallions and its relationship to sperm quality. Reprod. Fertil. Dev. 22(5): 886–891

Blesbois E., Grasseau I., Seigneurin F. 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. Reproduction 129(3): 371-378

Bobe J. and Labbé C. 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165 (3): 535-548

Bromage N., Porter M., Randall C. 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. Aquaculture 197(1-4): 63-98

Burness G., Moyes C. D., Montgomerie R. 2005. Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). Comp. Biochem. Physiol. A 140(1): 11-17

Butts I.A.E., Litvak M.K., Trippel E.A. 2010, Seasonal variations in seminal plasma and sperm characteristics of wild-caught and cultivated Atlantic cod, *Gadus morhua*. Theriogenology 73(7): 873-885

Cabrita E., Alvarez R., Anel L., Rana K.J., Herráez, M.P. 1998. Sublethal damage during cryopreservation of rainbow trout sperm. Cryobiology 37(3): 245-253

Cabrita E., Robles V., Alvarez R., Herráez M.P. 2001. Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. Aquaculture 201(3-4): 301-314

Cabrita E., Robles V., Cuñado S., Wallace J. C., Sarasquete C., Herráez M.P. 2005a. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5ml macrotubes. Cryobiology 50(3): 273-284

Cabrita E., Robles V., Rebordinos L., Sarasquete C., Herráez M. P. 2005b. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50(2): 144-153

Cabrita E., Soares F., Dinis M. T. 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975

Cabrita E., Robles V., Herráez M.P. 2008a. Sperm quality assessment. In: Methods in reproductive aquaculture: Marine and freshwater species. Biology series. Eds: Cabrita E., Robles V., Herráez M.P. CRCPress (Taylor and Francis group), London, UK, 93-147

Cabrita E., Engrola S., Conceição L.E.C., Pousão-Ferreira P., Dinis M.T. 2008b. Successful cryopreservation of sperm from sex-reversed dusky grouper, *Epinephelus marginatus*. Aquaculture 287(1-2): 152-157

Cabrita E., Sarasquete C., Martínez-Páramo S., Robles V., Beirão J., Pérez-Cerezales S., Herraéz, M.P. 2010. Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichtyol. 26(5): 623-635

Cabrita E., Soares F., Beirão J., García-López A., Martínez-Rodríguez G., Dinis M.T. 2011. Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. Theriogenology 75(1): 1-9

Cal R., Terrones J., Vidal S., Martínez P., Piferrer F. 2010. Differential incidence of gonadal apoptosis in triploid-induced male and female turbot (*Scophthalmus maximus*). Aquaculture 307(3-4): 193-200

Canyurt M.A. and Akhan S. 2008. Effect of dietary vitamin E on the sperm quality of rainbow trout (*Onchorynchus mykiss*). Aquac. Res. 39(9): 1014-1018

Cerolini S., Maldjian A., Pizzi F., Gliozzi T.M. 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121(3): 395-401

Chao N. and Liao C.I. 2001. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 197(1-4): 161-189

Chaves-Pozo E., Mulero V., Meseguer J., Ayala A.G. 2005. An overview of cell renewal in the testis throughout the reproductive cycle of a seasonal breeding teleost, the gilthead seabream (*Sparus aurata* L.). Biol. Reprod. 72(3): 593-601

Ciereszko A. and Dabrowski K. 1995. Sperm quality and ascorbic-acid concentration in rainbow-trout semen are affected by dietary vitamin-C - an across-season study. Biol. Reprod. 52(5): 982-988

Ciereszko A., Toth G.P., Christ S.A., Dabrowski K. 1996. Effect of cryopreservation and theophylline on motility characteristics of lake sturgeon (*Acipenser fulvescens*) spermatozoa. Theriogenology 45(3): 665-672

Ciereszko A., Wolfe T.D., Dabrowski K. 2005. Analysis of DNA damage in sea lamprey (*Petromyzon marinus*) spermatozoa by UV, hydrogen peroxide, and the toxicant bisazir. Aquat. Toxicol. 73(2): 128-138

Cloud J.G. and Patton S.J. 2008. Basic principles of fish spermatozoa cryopreservation. In: Methods in reproductive aquaculture: Marine and freshwater species. Biology series. Eds: Cabrita E., Robles V., Herráez M.P. CRCPress (Taylor and Francis group), London, UK, 237-250

Corriero A., Medina A., Mylonas C.C., Bridges C.R., Santamaria N., Deflorio M., Losurdo M., Zupa R., Gordin H., de la Gandara F., Rios A.B., Pousis C., De Metrio G. 2009. Proliferation and apoptosis of male germ cells in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) treated with gonadotropin-releasing hormone agonist (GnRHa). Anim. Reprod. Sci. 116(3-4): 346-357

Cosson J.J. 2007. Methods to analyze the movements of fish spermatozoa and their flagella. In: Fish spermatology. Eds: Alavi S.M.H., Cosson J., Coward K., Raffie G. Alpha Science Intl Ltd, Oxford, UK, 63-102

Cosson J., Groison A., Suquet M., Fauvel C., Dreanno C., Billard R. 2008a. Marine fish spermatozoa: racing ephemeral swimmers. Reproduction 136(3): 277-294

Cosson J., Groison A. L., Suquet M., Fauvel C., Dreanno C., Billard R. 2008b. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24 (4): 460-486

Cosson M.P., Billard R., Gatti J.L., Christen R. 1985. Rapid and quantitative assessment of trout spermatozoa motility using stroboscopy. Aquaculture 46(1): 71-75

Dabrowski K. and Ciereszko A. 2001. Ascorbic acid and reproduction in fish: endocrine regulation and gamete quality. Aquac. Res. 32(8): 623-638

De Baulny B.O., LeVern Y., Kerboeuf D., Maisse G. 1997. Flow cytometric evaluation of mitochondrial activity and membrane integrity in fresh and cryopreserved rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Cryobiology 34(2): 141-149

Dietrich G.J., Zabowska M., Wojtczak M., Slowinska M., Kucharczyk D., Ciereszko A. 2007. Effects of different surfactants on motility and DNA integrity of brown trout (*Salmo trutta fario*) and common carp (*Cyprinus carpio*) spermatozoa. Reprod. Biol. 7(2): 127-42

Dietrich G.J., Wojtczak M., Slowinska M., Dobosz S., Kuzminski H., Ciereszko A. 2008. Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss* Walbaum) spermatozoa. J. Appl. Ichthyol. 24(4): 503-507

Dietrich G.J., Dietrich M., Kowalski R.K., Dobosz S., Karol H., Demianowicz W., Glogowski J. 2010. Exposure of rainbow trout milt to mercury and cadmium alters sperm motility parameters and reproductive success. Aquat. Toxicol. 97(4): 277-284

Ding S.Y., Ge J.C., Hao C., Zhang M.S., Yan W.H., Xu Z.Q., Jianlin P.L., Chen S.L., Tian Y.S., Huang Y.H. 2009. Long-term cryopreservation of sperm from Mandarin fish *Siniperca chuatsi*. Anim. Reprod. Sci. 113(1-4): 229-235

Dinis M.T., Soares F., Conceição L., Cañavate J.P., Anguis V., Manchado M., Infante C., Pousão-Ferreira P., Engrola S., Herrera M., Hachero I., Navas J.I., Fernandez, J.R.L. 2007. Manual de Cultivo de Lenguado y otros peces planos. Junta de Andalucía

Dott H.M. and Foster G.C.A. 1979. Estimation of sperm motility in semen, on a membrane slide, by measuring the area change frequency with an image analyzing computer. J. Reprod. Fertil. 55(1): 161-166

Drokin S.I. 1993. Phospholipids and fatty-acids of phospholipids of sperm from several fresh-water and marine species of fish. Comp. Biochem. Physiol. B 104(2): 423-428

Drokin S., Stein H., Bartscherer H. 1998. Effect of Cryopreservation on the Fine Structure of Spermatozoa of Rainbow Trout (*Oncorhynchus mykiss*) and Brown Trout (*Salmo trutta F. fario*). Cryobiology 37(3): 263-270

Duncan N., Carazo I., Chereguini O., Norambuena F., Mañanós E., Estevez A. 2008. Observations of spawning in wild caught and captivity reared broodstock. In: Report of the IV Workshop on the cultivation of Soles, Faro, Portugal, 7-8

Edens F.W. and Brake J. 2006. The role of selenium in poultry reproduction. World's Poult. Sci. J. 12th European Poultry Conference. Verona, Italy, September 10-14

Fabbrocini A., Lavadera S.L., Rispoli S., Sansone G. 2000. Cryopreservation of seabream (*Sparus aurata*) spermatozoa. Cryobiology 40(1): 46-53

FAO. 2011. Cultured Aquatic Species Information Programme. URL: www.fao.org/fishery/culturedspecies/Sparus aurata/en

Fitzpatrick J.L., Desjardins J.K., Milligan N., Montgomerie R., Balshine S. 2007. Reproductive-tactic-specific variation in sperm swimming speeds in a shell-brooding Cichlid. Biol. Reprod. 77(2): 280-284

García-López A., Fernandez-Pasquier V., Couto E., Canário A. V. M., Sarasquete C., Martínez-Rodríguez, G. 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen. Comp. Endocrinol. 147(3): 343-351

Giraud M.N., Motta C., Boucher D., Grizard G. 2000. Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. Hum. Reprod. 15(10): 2160-2164

Guthrie H.D., Woods L.C.III, Long J.A., Welch G.R. 2008. Effects of osmolality on inner mitochondrial transmembrane potential and ATP content in spermatozoa recovered from the testes of striped bass (*Morone saxatilis*). Theriogenology 69(8): 1007-1012

Guzmán J.M., Norberg B., Ramos J., Mylonas C.C., Mañanos E.L. 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). Gen. Comp. Endocrinol. 156 (2): 285-297

Gwo J.C., Chiu J.Y., Lin C.Y., Su Y., Yu S.L. 2005. Spermatozoal ultrastructure of four Sparidae fishes: *Acanthopagrus berda*, *Acanthopagrus australis*, *Lagodon rhomboids* and *Archosargus probatocephus*. Tissue Cell 37(2): 109-115

He S.Y. and Woods C. 2004. Effects of dimethyl sulfoxide and glycine on cryopreservation induced damage of plasma membranes and mitochondria to striped bass (*Morone saxatilis*) sperm. Cryobiology 48(3): 254-262

Henrotte E., Kaspar V., Rodina M., Psenicka M., Linhart O., Kestemont P. 2010. Dietary n-3/n-6 ratio affects the biochemical composition of Eurasian perch (*Perca fluviatilis*) semen but not indicators of sperm quality. Aquac. Res. 41(9): e31-e38

Herráez M.P., Pérez-Cerezales S., Martínez-Páramo S., Beirão J., Martínez-Pastor F., Gutiérrez-Adán A. 2010. Altered gene expression in embryo obtained with DNA-cryodamaged sperm. 1st International Congress on Controversies in cryopreservation of stem cells, reproductive cells, tissue & organs. Valencia, Spain, May 27-30

Horváth A., Miskolui E., Mihalffy S., Osz K., Szabo K., Urbányi B. 2007. Cryopreservation of common carp (*Cyprinus carpio*) sperm in 1.2 and 5 ml straws and occurrence of haploids among larvae produced with cryopreserved sperm. Cryobiology 54(3): 251-257

Horváth A., Wayman W.R., Dean J.C., Urbányi B., Tiersch T.R., Mims S.D., Johnson D., Jenkins J.A. 2008. Viability and fertilizing capacity of cryopreserved sperm from three North American acipenseriform species: a retrospective study. J. Appl. Ichthyol. 24(4): 443-449

Ibarz A., Padros F., Gallardo M. A., Fernández-Borras J., Blasco J., Tort L. 2010. Low-temperature challenges to gilthead sea bream culture: review of cold-induced alterations and 'Winter Syndrome'. Rev. Fish. Biol. Fish. 20 (4): 539-556

Inaba K. 2007. Molecular mechanisms of the activation of flagellar motility in sperm. In: Fish spermatology. Eds: Alavi S.M.H., Cosson J., Coward K., Raffie G. Alpha Science Intl Ltd, Oxford, UK, 267-280

Ingermann R.L. 2007. Energy metabolism and respiration in fish spermatozoa. In: Fish spermatology. Eds: Alavi S.M.H., Cosson J., Coward K., Raffie G. Alpha Science Intl Ltd, Oxford, UK, 241-266

Inglis S.R., Turner J.J., Harding M.M. 2006. Applications of type I antifreeze proteins: Studies with model membranes & cryoprotectant properties. Curr. Protein Pept. Sci. 7(6): 509-522

Izquierdo M.S., Fernández-Palacios H., Tacon A.G.J. 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197(1-4): 25-42

Jeong Y.J., Kim M.K., Song H.J., Kang E.J., Ock S.A., Kumar M., Balasubramanian S., Rho G.J. 2009. Effect of alpha-tocopherol supplementation during boar semen cryopreservation on sperm characteristics and expression of apoptosis related genes. Cryobiology 58(2): 181-189

Jobling S., Coey S., Whitmore J.G., Kime D.E., Van Look K.J.W., McAllister B.G., Beresford N., Henshaw A.C., Brighty G., Tyler C.R., Sumpter J.P. 2002. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Reprod. 67(2): 515-524

Khan D.R., Ahmad N., Anzar M., Channa A.A. 2009. Apoptosis in fresh and cryopreserved buffalo sperm. Theriogenology 71 (5): 872-876

Kho K.H., Morisawa M., Choi K.S. 2005. Cell signaling mechanisms of sperm motility in aquatic species. Journal of Microbiology and Biotechnology 15(3): 665-671

Kopeika E. and Kopeika J. 2007. Variability of sperm quality after cryopreservation in fish. In: Fish spermatology. Eds: Alavi S.M.H., Cosson J., Coward K., Raffie G. Alpha Science Intl Ltd, Oxford, UK, 347-396

Krasznai Z., Morisawa M., Krasznai Z.T., Morisawa S., Inaba K., Bazsane Z.K., Rubovszky B., Bodnar B., Borsos A., Marian T. 2003. Gadolinium, a mechano-sensitive channel blocker, inhibits osmosis-initiated motility of sea- and freshwater fish sperm, but does not affect human or ascidian sperm motility. Cell Motil. Cytoskel. 55(4): 232-243

Kumaravel T.S., Jha A.N. 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat. Res.-Genet Toxicol. Environ. Mutagen. 605(1-2): 7-16

Labbé C., Maisse G., Muller K., Zachowski A., Kaushik S., Loir M. 1995. Thermal-Acclimation and Dietary Lipids Alter the Composition, but Not Fluidity, of Trout Sperm Plasma-Membrane. Lipids 30(1): 23-33

Labbé C. and Maisse G. 1996. Influence of rainbow trout thermal acclimation on sperm cryopreservation: Relation to change in the lipid composition of the plasma membrane. Aquaculture 145(1-4): 281-294

Labbé C. and Maisse G. 2001. Characteristics and freezing tolerance of brown trout spermatozoa according to rearing water salinity. Aquaculture 201(3-4): 287-299

Labbé C., Martoriati, A., Devaux A., Maisse G. 2001. Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Mol. Reprod. Dev. 60(3): 397-404

Ladha S. 1998. Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. J. Membr. Biol. 165(1): 1-10

Lahnsteiner F., Berger F., Weismann T., Patzner R. 1996. The influence of various cryoprotectants on semen quality of the rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation. J. Appl. Ichthyol. 12(2): 99–106.

Lahnsteiner F., Berger B., Weismann T., Patzner R.A. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture 163(1-2): 163-181

Lahnsteiner F., Berger B., Weismann T. 1999. Sperm metabolism of the teleost fishes Chalcalburnus chalcoides and *Oncorhynchus mykiss* and its relation to motility and viability. J. Exp. Zool. 284(4): 454-465

Lahnsteiner F. 2006 Carbohydrate Metabolism of Vitellogenic Follicles and Eggs of *Serranus cabrilla* (Serranidae) and *Mullus barbatus* (Mullidae) and of Embryos of *Sparus aurata* (Sparidae). Fish Physiol. Biochem. 32(2): 131-139

Lahnsteiner F., Mansour N., McNiven M.A., Richardson G.F. 2009. Fatty acids of rainbow trout (*Oncorhynchus mykiss*) semen: Composition and effects on sperm functionality. Aquaculture 298(1-2): 118-124

Lahnsteiner F., Mansour N., Caberlotto S. 2010a. Composition and metabolism of carbohydrates and lipids in *Sparus aurata* semen and its relation to viability expressed as sperm motility when activated. Comp. Biochem. Physiol. B 157(1): 39-45

Lahnsteiner F., Mansour N., Plaetzer K. 2010b. Antioxidant systems of brown trout (*Salmo trutta f. fario*) semen. Anim. Reprod. Sci. 119(3-4): 314-321

Lanes C.F.C., Okamoto M.H., Bianchini A., Marins L.F., Sampaio L.A. 2010. Sperm quality of Brazilian flounder *Paralichthys orbignyanus* throughout the reproductive season. Aquac. Res. 41(9): e199-e207

Le Comber S.C., Faulkes C.G., Van Look K.J.W., Holt W.V., Smith C. 2004. Recovery of sperm activity after osmotic shock in the three-spined stickleback: Implications for pre-oviposition ejaculation. Behaviour 141: 1555-1569

Lee K.J. and Dabrowski K. 2004. Long-term effects and interactions of dietary vitamins C and E on growth and reproduction of yellow perch, *Perca flavescens*. Aquaculture 230(1-4): 377-389

Lee R.F. and Steinert S. 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat. Res.-Rev. Mutat. Res. 544(1): 43-64

Li P., Wei Q., Liu L. 2008. DNA integrity of Polyodon spathula cryopreserved sperm. J. Appl. Ichthyol. 24(2): 121-125

Li P., Hulak M., Linhart O. 2009. Sperm proteins in teleostean and chondrostean (sturgeon) fishes. Fish Physiol. Biochem. 35(4): 567-581

Li P., Rodina M., Hulak M., Gela D., Li Z.H., Linhart O. 2010a. Physico-biochemical parameters and protein profiles of sperm from beluga. J. Appl. Ichtyol. 26(5): 753-755

Li P., Hulak M., Koubek P., Sulc M., Dzyuba B., Boryshpolets S., Rodina M., Gela D., Manaskova-Postlerova P., Peknicova J., Linhart O. 2010b. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio* L. Theriogenology 74(3): 413-423

Liu Q.H., Li J., Zhang S.C., Ding F.H., Xu X.Z., Xiao Z.Z., Xu S.H. 2006. An efficient methodology for cryopreservation of spermatozoa of red seabream, *Pagrus major*, with 2-mL cryovials. J. World Aquacult. Soc. 37(3): 289-297

Liu Q.H., Li J., Zhang S.C., Xiao Z.Z., Ding F.H., Yu D.D., Xu X.Z. 2007a. Flow cytometry and ultrastructure of cryopreserved red seabream (*Pagrus major*) sperm. Theriogenology 67(6): 1168-1174

Liu Q.H., Li J., Xiao Z.Z., Ding F.H., Yu D.D., Xu X.Z. 2007b. Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus major*). Aquaculture 263(1-4): 20-25

Liu L., Ciereszko A., Czesny S., Dabrowski K. 1997. Dietary ascorbyl monophosphate depresses lipid peroxidation in rainbow trout spermatozoa. J. Aquat. Anim. Health 9(4): 249-257

Lubzens E., Daube N., Pekarsky I., Magnus Y., Cohen A., Yusefovich F., Feigin P. 1997. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks - strategies in research and application. Aquaculture 155(1-4): 13-30

Ma S., Diogo P., Martínez-Páramo S., Dinis M.T., Sarasquete C., Cabrita E. 2010. Effect of antioxidant supplementation in fish sperm extenders on post-thaw sperm quality and DNA fragmentation. Aquaculture Europe 10. Oporto, Portugal, October 5-8

Mansour N., Lahnsteiner F., Berger B. 2003. Metabolism of intratesticular spermatozoa of a tropical teleost fish (*Clarias gariepinus*). Comp. Biochem. Physiol B 135(2): 285-296

Mansour N., McNiven M.A., Richardson G.F. 2006. The effect of dietary supplementation with blueberry, alpha-tocopherol or astaxanthin on oxidative stability of Arctic char (*Salvelinus alpinus*) semen. Theriogenology 66(2): 373-382

Mañanós E., Guzmán J.M., Ramos J., Mylonas C.C., Zohar J., Peleteiro J.B., Chereguini O., Duncan N., Sarasquete C., Riaza A. 2008a. Hormonal stimulation of ovulation, spermiation and spawning in cultured Senegalese sole (*Solea senegalensis*) broodstock. In: Report of the IV Workshop on the cultivation of Soles, Faro, Portugal, 16-17

Mañanós E., Duncan N., Mylonas C.C. 2008b. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In: Methods in reproductive aquaculture: Marine and freshwater species. Biology series. Eds: Cabrita E., Robles V., Herráez M.P. CRCPress (Taylor and Francis group), London, UK, 3-80

Marchand M.J., Pieterse G.M., Barnhoorn I.E.J. 2010. Sperm motility and testicular histology as eproductive indicators of two feral fish species from a currently DDT sprayed area, South Africa. 2nd International Workshop on the Biology of Fish Gametes. Valencia, Spain, September 9-11

Marco-Jiménez F., Pérez L., de Castro M.P.V., Garzón D.L., Peñaranda D.S., Vicente J.S., Jover M., Asturiano J.F. 2006. Morphometry characterisation of European eel spermatozoa with computer-assisted spermatozoa analysis and scanning electron microscopy. Theriogenology 65(7): 1302-1310

Marco-Jiménez F., Peñaranda D. S., Pérez L., Viudes-de-Castro M.P., Mylonas C.C., Jover M., Asturiano J.F. 2008. Morphometric characterization of sharpsnout sea bream (*Diplodus puntazzo*) and gilthead sea bream (*Sparus aurata*) spermatozoa using computer-assisted spermatozoa analysis (ASMA). J. Appl. Ichthyol. 24(4): 382-385

Marian T., Krasznai Z., Balkay L., Balazs M., Emri M., Bene L., Tron L. 1993. Hypoosmotic Shock Induces an Osmolality-Dependent Permeabilization and Structural-Changes in the Membrane of Carp Sperm. J. Histochem. Cytochem. 41(2): 291-297

Maricchiolo G., Genovese L., Laura R., Micale V., Muglia U. 2007. Fine structure of spermatozoa in the gilthead sea bream (*Sparus aurata* Linnaeus, 1758) (Perciformes, Sparidae). Histol. Histopath. 22(1): 79-83

Marin-Guzman J., Mahan D.C., Whitmoyer R. 2000. Effect of dietary selenium and vitamin E on the ultrastructure and ATP concentration of boar spermatozoa, and the efficacy of added sodium selenite in extended semen on sperm motility. J. Anim. Sci. 78(6): 1544-1550

Marti E., Perez-Pe R., Colas C., Muiño-Blanco T., Cebrián-Pérez J.A. 2008. Study of apoptosis-related markers in ram spermatozoa. Anim. Reprod. Sci. 106(1-2): 113-32

Martínez-Páramo S., Pérez-Cerezales S., Gómez-Romano F., Blanco G., Sánchez J.A., Herráez M.P. 2009. Cryobanking as tool for conservation of biodiversity: Effect of brown trout sperm cryopreservation on the male genetic potential. Theriogenology 71(4): 594-604

Martínez-Pastor F., Garcia-Macias V., Alvarez M., Herráez M.P., Anel L., de Paz P. 2005. Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process. Biol. Reprod. 72(2): 316-327

Martínez-Pastor F., Cabrita E., Soares F., Anel L., Dinis M.T. 2008a. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 135(4): 449-59

Martínez-Pastor F., Fernandez-Santos M. R., del Olmo E., Dominguez-Rebolledo A. E., Esteso M. C., Montoro V., Garde J. J. 2008b. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod. Fertil. Dev. 20(5): 547-556

Martínez-Pastor F., Mata-Campuzano M., Alvarez-Rodriguez M., Alvarez M., Anel L., de Paz P. 2010. Probes and Techniques for Sperm Evaluation by Flow Cytometry. Reprod. Domest. Anim. 45: 67-78

McDonald G.J., Danzmann R.G., Ferguson M.M. 2004. Relatedness determination in the absence of pedigree information in three cultured strains of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 233 (1-4): 65-78

Morisawa M. and Okuno M. 1982. Cyclic-Amp Induces Maturation Of Trout Sperm Axoneme To Initiate Motility. Nature 295(5851): 703-704

Morisawa M. 2008. Adaptation and strategy for fertilization in the sperm of teleost fish. J. Appl. Ichthyol. 24(4): 362-370

Morte M., Rodrigues A., Soares D., Rodrigues A., Gamboa S., Ramalho-Santos J. 2008. The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: Seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. Anim. Reprod. Sci. 106(1-2):36-47

Muino R., Tamargo C., Hidalgo C.O., Pena A.I. 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. Anim. Reprod. Sci. 109(1-4): 27-39

Muller K., Muller P., Pincemy G., Kurz A., Labbé C. 2008. Characterization of sperm plasma membrane properties after cholesterol modification: Consequences for cryopreservation of rainbow trout spermatozoa. Biol. Reprod. 78(3): 390-399

Mylonas C.C., Papadaki M., Divanach P. 2003. Seasonal changes in sperm production and quality in the red porgy *Pagrus pagrus* (L.). Aquac. Res. 34(13): 1161-1170

Mylonas C.C., Fostier A., Zanuy S. 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165(3): 516-534

Nandi S., Routray P., Gupta S.D., Rath S.C., Dasgupta S., Meher P.K., Mukhopadhyay P.K. 2007. Reproductive performance of carp, *Catla catla* (Ham.), reared on a formulated diet with PUFA supplementation. J. Appl. Ichthyol. 23(6): 684-691

Nascimento A.F., Maria A.N., Pessoa N.O., Carvalho M.A.M., Viveiros A.T.M. 2010. Out-of-season sperm cryopreserved in different media of the Amazonian freshwater fish pirapitinga (*Piaractus brachypomus*). Anim. Reprod. Sci. 118(2-4): 324-329

Ohtake H. 2003. Sperm-activating proteins obtained from the herring eggs. Fish Physiology and Biochemistry 28(1-4): 199-202

Oliveira C., Vera L.M., López-Olmeda J.F., Guzmán J.M., Mañanós E., Ramos J., Sánchez-Vázquez F.J. 2008. Monthly day/night changes and seasonal daily rhythms of sexual steroids in Senegal sole (*Solea senegalensis*) under natural fluctuating or controlled environmental conditions. Comp. Biochem. Physiol. A 152(2): 168-175

Ortega-Ferrusola C., Sotillo-Galan Y., Varela-Fernández E., Gallardo-Bolaños J.M., Muriel A., González-Fernández L., Tapia J. A., Peña F. J. 2008. Detection of "Apoptosis-Like" changes during the cryopreservation process in equine sperm. J. Androl. 29(2): 213-221

Ortega-Ferrusola C., García B.M., Rama V.S., Gallardo-Bolaños J.M., González-Fernández L., Tapia J.A., Rodríguez-Martinez H., Peña F.J. 2009. Identification of Sperm Subpopulations in Stallion Ejaculates: Changes after Cryopreservation and Comparison with Traditional Statistics. Reprod. Domest. Anim. 44(3): 419-423

Ottesen O.H., Babiak I., Dahle G. 2009. Sperm competition and fertilization success of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture 286(3-4): 240-245

Peña F.J., Johannisson A., Wallgren, M., Rodríguez-Martinez H. 2003. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 60(4): 677-689

Peñaranda D.S., Pérez L., Fakriadis G., Mylonas C.C., Asturiano J.F. 2008. Effects of extenders and cryoprotectant combinations on motility and morphometry of sea bass (*Dicentrarchus labrax*) spermatozoa. J. Appl. Ichthyol. 24(4): 450-455

Perchec G., Jeulin C., Cosson J., Andre F., Billard R. 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108: 747-753

Pérez-Cerezales S. 2006. Estabilidad de la cromatina de los espermatozoides de neomachos de trucha arco-iris (*Oncorhynchus mykiss*) durante su almacenamiento a corto y largo plazo. Tesina. Facultad de CC Biológicas. Unversidad de León

Pérez-Cerezales S, Martínez-Páramo S, Cabrita E, Martínez-Pastor F, de Paz P, Herráez M. 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71(4):605-613

Pérez-Cerezales S., Martínez-Páramo S., Beirão J., Herráez M.P. 2010a. Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 139(6): 989-997

Pérez-Cerezales S., Martínez-Páramo S., Beirão J., Herráez M.P. 2010b. Evaluation of DNA damage as a quality marker for rainbow trout sperm cryopreservation and use of LDL as cryoprotectant. Theriogenology 74(2): 282-289

Pinto W., Figueira L., Dinis M.T., Aragão C. 2009. How does fish metamorphosis affect aromatic amino acid metabolism? Amino Acids 36(2): 177-183

Polge C., Smith A.U., Parkes A.S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164(4172): 666

Porta J., Porta J.M., Martínez-Rodriguez G., Alvarez M.C. 2006. Genetic structure and genetic relatedness of a hatchery stock of Senegal sole (*Solea senegalensis*) inferred by microsatellites. Aquaculture 251(1): 46-55

Prathalingam N.S., Holt W.V., Revell S.G., Mirczuk S., Fleck R.A., Watson P.F. 2006. Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw. Theriogenology 66(8): 1894-1900

Psenicka M., Alavi S.M.H., Rodina M., Cicova Z., Gela D., Cosson J., Nebesarova J., Linhart O. 2008. Morphology, chemical contents and physiology of chondrostean fish sperm: a comparative study between Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*). J. Appl. Ichthyol. 24(4): 371-377

Pustowka C., McNiven M.A., Richardson G.F., Lall S.P. 2000. Source of dietary lipid affects sperm plasma membrane integrity and fertility in rainbow trout *Oncorhynchus mykiss* (Walbaum) after cryopreservation. Aquac. Res. 30(3): 297-305

Rainis S., Gasco L., Ballestrazzi R., Zoccarato I. 2007. Effects of vitamin E and phosphatidylcholine on qualitative and quantitative parameters of rainbow trout (*Oncorhynchus mykiss*) milt. Ital. J. Anim. Sci. 6(4): 339-350

Ravinder K., Nasaruddin K., Majumdar K.C., Shivaji S. 1997. Computerized analysis of motility, motility patterns and motility parameters of spermatozoa of carp following short-term storage of semen. J. Fish Biol. 50(6): 1309-1328

Robles V., Cabrita E., Cuñado S., Herráez M.P. 2003. Sperm cryopreservation of sex-reversed rainbow trout (Oncorhynchus mykiss): parameters that affect its ability for freezing. Aquaculture 224(1-4): 203-212

Robles V., Cabrita E., Herráez M.P. 2009. Germplasm Cryobanking in Zebrafish and Other Aquarium Model Species. Zebrafish 6(3): 281-293

Rosengrave P., Taylor H., Montgomerie R., Metcalf V., McBride K., Gemmell N.J. 2009. Chemical composition of seminal and ovarian fluids of chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. Comp. Biochem. Physiol. A 152(1): 123-129

Rudolfsen G., Figenschou L., Folstad I., Tveiten H., Figenschou M. 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc. R. Soc. B-Biol. Sci. 273(1584):325-332

Rurangwa E., Volckaert F.A.M., Huyskens G., Kime D.E., Ollevier F. 2001. Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*). Theriogenology 55(3): 751-769

Rurangwa E., Kime D.E., Ollevier F., Nash J.P. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234(1-4): 1-28

Sakkas D. and Alvarez J.G. 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil. Steril. 93(4): 1027-1036

Schiavone R., Zilli L., Vilella S., Fauvel C. 2006. Human chorionic gonadotropin induces spermatogenesis and spermiation in 1-year-old European sea bass (*Dicentrarchus labrax*): Assessment of sperm quality. Aquaculture 255(1-4): 522-531

Schulz R.W., de Franca L.R., Lareyre J.J., Legac F., Chiarini-Garcia H., Nobrega R.H., Miura T. 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165(3): 390-411

Segovia M., Jenkins J. A., Paniagua-Chavez C., Tiersch T.R. 2000. Flow cytometric evaluation of antibiotic effects on viability and mitochondrial, function of refrigerated spermatozoa of Nile tilapia. Theriogenology 53(7): 1489-1499

Shalini S. and Bansal M. P. 2008. Dietary selenium deficiency as well as excess supplementation induces multiple defects in mouse epididymal spermatozoa: understanding the role of selenium in male fertility. Int. J. Androl. 31(4): 438-449

Suquet M., Dreanno C., Dorange G., Normant Y., Quemener L., Gaignon J.L., Billard R. 1998. The ageing phenomenon of turbot spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization, and storage capacities. J. Fish Biol. 52(1): 31-41

Surai P., Kostjuk I., Wishart G., Macpherson A., Speake B., Noble R., Ionov I., Kutz E. 1998. Effect of vitamin E and selenium supplementation of cockerel diets on glutathione peroxidase activity and lipid peroxidation susceptibility in sperm, testes, and liver. Biol. Trace Elem. Res. 64(1-3): 119-132

Taddei A.R., Barbato F., Abelli L., Canese S., Moretti F., Rana K.J., Fausto A.M., Mazzini M. 2001. Is cryopreservation a homogeneous process? Ultrastructure and motility of untreated, prefreezing, and postthawed spermatozoa of *Diplodus puntazzo* (Cetti). Cryobiology 42(4): 244-55

Thurston L.M., Watson P.F., Mileham A.J., Holt W.V. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. J. Androl. 22(3): 382-394

Tiersch T.R., Yang H., Jenkins J.A., Dong Q. 2007. Sperm cryopreservation in fish and shellfish. Soc Reprod Fertil Suppl 65: 493-508

Tuset V.M., Dietrich G.J., Wojtczak M., Slowinska M., de Monserrat J., Ciereszko A. 2008. Comparison of three staining techniques for the morphometric study of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Theriogenology 69(8): 1033-1038

Tvedt H.B., Benfey T.J., Martin-Robichaud D.J., Power J. 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 194(1-2): 191-200

Van der Walt L. D., Van der Bank F. H., Steyn, G. J. 1993. The suitability of using cryopreservation of spermatozoa for the conservation of genetic diversity in African catfish (*Clarias gariepinus*). Comp. Biochem. Physiol. A 106a(2): 313-318

Vassallo-Agius R., Watanabe T., Yoshizaki G., Satoh S., Takeuchi Y. 2001. Quality of eggs and spermatozoa of rainbow trout fed an n-3 essential fatty acid-deficient diet and its effects on the lipid and fatty acid components of eggs, semen and livers. Fish. Sci. 67(5): 818-827

Vines C.A., Yoshida K., Griffin F.J., Pillai M.C., Morisawa M., Yanagimachi R., Cherr G. N. 2002. Motility initiation in herring sperm is regulated by reverse sodium-calcium exchange. Proc. Natl. Acad. Sci. U.S.A. 99(4): 2026-2031

Wassall S.R. and Stillwell W. 2009. Polyunsaturated fatty acid-cholesterol interactions: Domain formation in membranes. Biochim. Biophys. Acta-Biomembr. 1788(1): 24-32

Waterhouse K.E., Hofmo P.O., Tverdal A., Miller R.R. 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction 131(5): 887-894

Wei Q., Li P., Psenicka M., Alavi S.M.H., Shen L., Liu J., Peknicova J., Linhart O. 2007. Ultrastructure and morphology of spermatozoa in Chinese sturgeon (*Acipenser sinensis* Gray 1835) using scanning and transmission electron microscopy. Theriogenology 67(7): 1269-1278

Wilson-Leedy J.G. and Ingermann R.L. 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67(3): 661-672

Wolfe J. and Bryant G. 2001. Cellular cryobiology: Thermodynamic and mechanical effects. Intl. J. Refrig. 24(5): 438-450

Woods E., Benson J., Agca Y., Critser J. 2004. Fundamental cryobiology of reproductive cells and tissues. Cryobiology 48(2): 146–156

Yang H. and Tiersch T.R. 2009. Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and Xiphophorus. Comp. Biochem. Physiol. C 149(2): 224-232

Yao Z., Crim L. W., Richardson G.F., Emerson C.J. 2000. Motility, fertility and ultrastructural changes of ocean pout (*Macrozoarces americanus* L.) sperm after cryopreservation. Aquaculture 181(3-4): 361-375

Younis A.I., Rooks B., Khan S., Gould K.G. 1998. The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa. J. Androl. 19(2): 207-214

Yu S., Kojima N., Hakomori S., Kudo S., Inoue S., Inoue Y. 2002. Binding of rainbow trout sperm to egg is mediated by strong carbohydrate-to-carbohydrate interaction between (KDN)GM3 (deaminated neuraminyl ganglioside) and Gg3-like epitope. Proc. Natl. Acad. Sci. U.S.A. 99(5): 2854-2859

Zhang H.B., Lu S.M., Ma C.Y., Wang L., Li X., Chen Z.J. 2008. Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation. Asian J. Androl. 10(2): 227-235

Zietara M.S., Biegniewska A., Rurangwa E., Swierczynski J., Ollevier F., Skorkowski E.F. 2009. Bioenergetics of fish spermatozoa during semen storage. Fish Physiol. Biochem. 35(4): 607-614

Zilli L., Schiavone R., Zonno V., Storelli C., Vilella S. 2003. Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology 47(3): 227-235

Zilli L., Schiavone R., Zonno V., Storelli C., Vilella S. 2004. Adenosine triphosphate concentration and beta-D-glucuronidase activity as indicators of sea bass semen quality. Biol. Reprod. 70(6): 1679-1684

Zilli L., Schiavone R., Chauvigné F., Cerdà J., Storelli C., Vilella S. 2009. Evidence for the Involvement of Aquaporins in Sperm Motility Activation of the Teleost Gilthead Sea Bream (*Sparus aurata*). Biol. Reprod. 81(5): 880-888

Zohar Y., Mylonas C.C. 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197(1-4): 99-136

Objectives

Given the growing importance of the marine Mediterranean aquaculture industry and the great benefits that may result from an optimal management of the broodstocks, our aim is to contribute to this field focusing on the quality and storage of male gametes. Problems related to male reproductive performance are very different in species with a relatively high degree of domestication such as gilthead seabream (*Sparus aurata*) or in those still showing reproductive problems when reared in captivity, such as Senegalese sole (*Solea senegalensis*). This study aims to apply methods of sperm analysis providing more detailed information about sperm quality and focused on two major objectives.

1. Contribute to a better knowledge of *S. senegalensis* sperm quality in captivity and try to improve male reproductive performance. This objective was divided into two parts:

1.1. The study of *S. senegalensis* sperm quality, performing an exhaustive analysis of sperm either focusing in the main reproductive season and throughout the year using different cytophysiological approaches (sperm motility, DNA integrity, cell viability, presence of apoptotic cells and plasma membrane resistance to seawater hyperosmotic conditions) and analyzing possible correlations between parameters as well as interindividual variability.

1.2. According to the results obtained in the previous studies, to try to modulate sperm quality through the development of specific diets with particular lipidic components and antioxidant elements and evaluate their effects using different parameters, focusing specially on lipid sperm composition.

2. Increase knowledge of marine teleosts sperm cryopreservation and improve cryopreservation protocols using *S. aurata* as representative of species without sperm quality problems in captivity. This objective was divided into three parts:

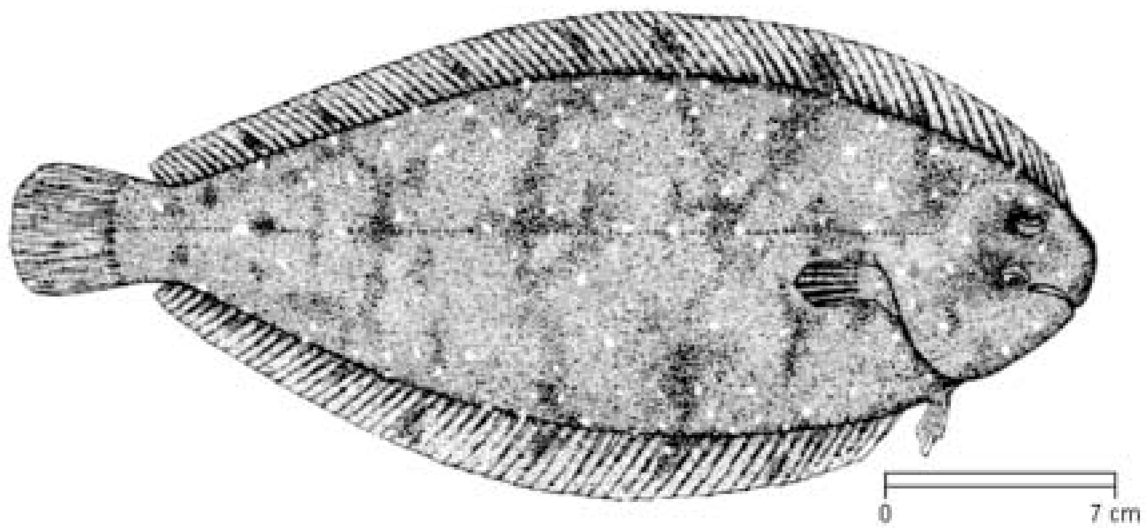
2.1. Analyze the effect of different cryopreservation treatments on the structure of spermatozoa subpopulations identified according to their motility characteristics as an alternative to the use of mean motility values provided by the Computer assisted sperm analysis systems (CASA).

2.2. Test the applicability of the nucleic acid stain YO-PRO 1 to assist in optimizing fish sperm cryopreservation protocols and detect early damage to the sperm cell membrane.

2.3. Study the putative beneficial effects of adding antifreeze proteins (AFPs) to cryopreservation extenders on post-thaw quality, evaluating the potential modifications of sperm plasma membrane composition in depth.

Chapter 1

Wild captive *Solea senegalensis* sperm quality analysis and improvement



FAO

Chapter 1.1.

Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level

J. Beirão[a], F. Soares[b], M.P. Herráez[a], M.T. Dinis[b], E. Cabrita[c]

[a] Department of Molecular Biology, Faculty of Biology, University of León, León, Spain
[b] Center for Marine Sciences-CCMAR, University of Algarve, Faro, Portugal
[c] Institute of Marine Sciences of Andalusia- ICMAN, Spanish National Research Council, Puerto Real, Cádiz, Spain

Theriogenology 72 (2009) 1251–1261
doi:10.1016/j.theriogenology.2009.07.021

Abstract

Sperm quality seems to be one of the reasons for the reproduction constraints faced by Senegalese sole (*Solea senegalensis*) aquaculturists. Previous studies in this species indicated that the sperm quality of individuals kept in culture varies throughout the year and that different sperm subpopulations can be identified in ejaculates according to the motility pattern of spermatozoa. Aiming to better understand factors affecting sole sperm quality in captivity, sperm of eleven males was assessed during the reproductive season using different parameters: motility characteristics using CASA analysis; cell plasma membrane resistance to seawater hyperosmolarity; DNA fragmentation with single-cell gel electrophoresis; and early apoptosis, labeled with Annexin-V FITC. CASA motility data were treated using multivariate analysis in order to identify the presence of different spermatozoa subpopulations according to their motility pattern. Four distinct sperm subpopulations were obtained: Subpop1 that includes fast linear spermatozoa; Subpop2 made up of fast non-linear spermatozoa; Subpop3 including slow linear spermatozoa; and Subpop4 containing slow non-linear spermatozoa. The sperm subpopulation structure varied with time after activation and with male. Low cell resistance to the seawater hyperosmotic conditions was noticed. The Annexin-V assay allowed the identification of an apoptotic population ranging from 6 to 20%. A high percentage of cells (64.1%) showed a DNA fragmentation level below 30%, but these values varied significantly ($p<0.05$) between males. DNA fragmentation appears to be related to cell membrane resistance to hyperosmotic conditions faced by the cells when in contact with seawater. This condition seems to modulate the composition of the motile sperm population and performance after activation. This phenomenon could be related with the spermatozoa maturation process.

Keywords: Senegalese sole; Sperm subpopulations; Annexin-V; Hoechst 33258; DNA fragmentation

1. Introduction

The massive production of the Senegalese sole (*Solea senegalensis*) is mainly impaired by the inexistence of control over the reproduction, poor egg quality and low fertilization rates [1]. Moreover spawns are obtained naturally in the tank and few experiments were able to collect eggs by stripping and perform artificial fertilization, controlling the quality of oocytes and sperm [2]. Recent studies have shown that low quality spawns (low rate of fertilization) in Senegalese sole broodstocks might be related to poor and highly variable semen quality [1,3-4]. Limited information on sole gamete quality leads to difficulties in controlling the reproductive cycle of this species. Similar to its relative *Solea solea*, *S. senegalensis* has a very low gonadosomatic index when compared to other fish species, with very small variations during spermatogenesis [5]. They present asynchronous germ cell maturation, low sperm concentration, volume and production throughout the year. All these facts might be related to reproductive behavior, as the males only release sperm when in contact with the eggs and the female [1].

Sperm quality monitoring is an important step in the selection of male breeders and might provide some clues to sperm problems. Any quantifiable parameter that directly correlates with the fertilization capacity of sperm could be potentially used as a measure of sperm quality. Rurangwa and co-workers [6] and Cabrita et al. [7] have reviewed in fish the different parameters that may be related with sperm fertilization ability such as motility, spermatocrit, sperm density, osmolarity, pH and seminal plasma constituents, spermatozoa morphology and ultrastructure, sperm viability and membrane integrity. In a previous work, Cabrita and co-workers [1] measured some of these parameters in sole throughout the year and characterized sperm quality and production in this species. Moreover, Martinez-Pastor et al. [3] examined sperm motility in Senegalese sole using computer assisted sperm analyses (CASA), and noticed the existence of different sperm subpopulations within the same sample. Computer assisted sperm analysis (CASA) allows the quantification of different motility-associated parameters in a simple and rapid way and could allow sperm fertilizing ability to be predicted [6-8]. In recent years several authors have used these programs to assess fish sperm quality in different species [9-11].

Beside sperm motility, membrane integrity and its resilience to the external conditions has been used to assay fish sperm quality. In the study of sole sperm motility Martinez-Pastor et al. [3] found a low cell resistance of sperm to seawater in some cell population after motility activation. This fact could be related with seawater hyperosmolarity relatively to the seminal plasma, indicating the importance of designing a test to evaluate sperm cell membrane resistance to this type of damage. Cells presenting a certain degree of damage might not be able to achieve fertilization due to the effects of osmotic stress.

Cell apoptosis is a necessary process during spermatogenesis, however the presence of apoptotic cells in the ejaculate could indicate dysregulation of the normal sperm maturation process. In human spermatozoa cell apoptosis is negatively correlated with sperm motility [12]. This parameter can be measured using Annexin-V, a calcium-dependent binding protein that has high affinity to phosphatidylserine (PS) in the presence of Ca^{2+}. This phospholipid is translocated to the outer leaflet of the cell plasma membrane during early apoptosis [13].

DNA stability is an index of sperm quality [7]. While DNA fragmentation is part of the apoptotic process, it can also be promoted by other factors, leading to apoptosis by itself. DNA integrity can be analyzed using a single-cell gel electrophoresis, commonly known as comet assay, allowing the identification of DNA strand breaks and alkali labile sites by measuring the migration of DNA from the immobilized nucleus [14]. Both techniques proved to be useful in the analysis of sperm quality in *Solea senegalensis* [4] and could help in better understanding which factors affect sole sperm quality.

Only a more complete understanding of the semen features responsible for its low quality observed during the reproductive season can help us to find better solutions. Hence, bearing in mind the knowledge obtained so far in previous studies, and using the above-mentioned assays, we decided to study Senegalese sole semen quality during the reproductive season, and to analyze how these parameters are correlated.

2. Material and methods

2.1. Chemicals

All the chemicals were obtained from Sigma-Aldrich (Madrid, Spain), unless otherwise indicated. Chemicals were reagent grade or higher.

2.2. Broodstock husbandry conditions and sampling

The individuals used in the experiments were captured in the wild and maintained for 2 years at the Ramalhete Experimental Station (37º00’22N, 7º58’03W), Faro, Portugal. Fishes (1:2, female:male) were kept in 3000 L round fiberglass tanks with sand substrate and aeration. Photoperiod simulates the environmental conditions in the area. Temperature (10.9º to 26.4ºC) and salinity (36.6 ± 0.8 ppt) were natural for the season. Water exchange was 500 L/h.

Sperm samples (n=11) were obtained from fluent males during the reproductive season, from March to May. Fishes were anesthetized in a seawater tank with 300 ppm 2-phenoxyethanol. To obtain the semen, the urogenital pore was cleaned from mucus, faeces and water and a

syringe without needle was used to collect the semen by gently pressing the testes in the fish blind side. The sperm was placed in microcentrifuge tubes and stored at 7ºC for further analysis (no longer than 3 hours). Samples contaminated with urine and seawater were discarded.

2.3. Sperm quality analysis

2.3.1. Motility

Sperm motility was assessed using the CASA system (ISAS-Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon E-200, Nikon, Tokio, Japan) with a 10× negative phase contrast objective. Samples were previously diluted in a 300 mOsm/kg sucrose solution (2 µL of sperm in 25 µL of sucrose), in order to obtain 300-500 cells per field. For sperm activation, 5 µL of artificial seawater (1100 mOsm/kg) was added to 1 µL of the cell suspension (final osmolarity 874 mOsm/kg) and motility was recorded at 15, 30, 45 and 60 seconds post-activation. Image sequences were saved and analyzed afterwards. Each sample was analyzed thrice. The parameters rendered by ISAS were as follows: the percentage of motile cells within the sample and for each individual spermatozoa, VCL (velocity according to the actual path; µm/sec), VSL (velocity according to the straight path; µm/sec), VAP (velocity according to the smoothed path; µm/sec), LIN (linearity; %), STR (straightness; %), WOB (wobble; %), ALH (amplitude of the lateral displacement of the sperm head; µm), and BCF (beat-cross frequency; Hz). These parameters have been described elsewhere [6,15]. The software settings were adjusted to sole spermatozoa: 25 images per second; 1 to 90 μm^2 for head area; and VCL > 10 µm/s to classify a spermatozoon as motile.

2.3.2. Hyperosmotic test

To assess cells membrane resistance to seawater we used Hoechst 33258, a membrane-impermeable nucleic acid stain that positively identifies cells with membrane damage. In this assay 1 µL of the sperm was activated with 9 µL artificial seawater (final osmolarity of 913 mOsm/kg) and then fixed at different time intervals post-activation (15, 30 and 45 sec) with 4% glutaraldehyde in 1% NaCl for 1 min (1 µL of activated semen: 9 µL of glutaraldehyde solution) (418 mOsm/kg). Fixation was stopped by dilution 1:4 in 1% NaCl, obtaining a final osmolarity of 326 mOsm/kg. Previously, the osmolarity of the fixation solution was tested to assure that this procedure did not affect cells viability. All the procedure was performed at 7ºC. The cells were stained with 2 µL of Hoechst 33258 (200 µg/mL) in 50 µL of the semen suspension for 5 min at 7ºC in the dark. After the incubation period, three slides were observed with a fluorescence microscope (Olympus IX 81; Olympus, Tokio, Japan) with UV light (330 – 380 nm). At least 100 cells were counted per slide. Cells were classified as non-viable when showing blue fluorescence and viable when not stained. The percentage of viable cells was recorded as percentage of cells resistant to hyperosmotic shock.

2.3.3. Apoptosis assay

Cell viability and the occurrence of apoptotic cells were evaluated with Annexin-V FITC (Fluorescein) assay conjugated with propidium iodide (PI). For this assay, 1 µL semen was diluted in 100 µL of a non-activating buffer (1% NaCl with 2.5 mM of $CaCl_2$) and double stained with 1 µL of Annexin-V FITC (50 µg/mL) for 20 min and with 1.5 µL PI (1 mg/mL; Fluka, Madrid, Spain) for 5 min at 7ºC in the dark. After incubation, one drop of the suspension was placed on a slide and immediately observed under the fluorescence microscope (Olympus IX 81) with blue excitation (450 – 480 nm). The same field was then observed with phase contrast to count all cells. Cells were differentiated in Annexin+/PI- (green stained); Annexin+/PI+ (green and red stained); Annexin-/PI+ (red stained); and Annexin-/PI- (non-stained cells). At least 100 cells were counted on each slide and three slides were observed per sample. Results were expressed as percentage of viable cells (non-stained cells), Annexin-V stained cells (Annexin+/PI- and Annexin+/PI+) and PI stained cells (Annexin+/PI+ and Annexin-/PI+).

2.3.4. DNA integrity

DNA integrity was evaluated with the single-cell gel electrophoresis (comet assay) technique using the protocol described by Cabrita et al. [16] and adapted to sole sperm by Beirão et al. [4]. Briefly, pre-diluted semen in a non-activating solution was embedded on a 0.5% agarose slide. After cell lysis (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% Triton X-100, 1% lauril sarcosine) for 1 h at 4ºC, DNA was descondensed using dithiothreitol (10 mM, 30 min at 4ºC) added to the lysis buffer, and with lithium diiodosalicylate (4 mM, 90 min at room temperature). Electrophoresis was performed in an alkaline solution (0.3 M NaOH, 1 mM Na_2-EDTA) for 10 min at 25v (± 300 mA) at 4ºC. Before electrophoresis started, the slides where left in contact with the alkaline solution for 20 min. They were then neutralized using 0.4 M Tris solution at 4ºC for 5 min, drained and fixed in 96% methanol and stored until further observation. A control slide was used in each electrophoresis, generating DNA fragmented cells with 200 mM H_2O_2 (20 min at 4ºC).

Visualization of comets was carried out by pipetting 30 µL ethidium bromide (0.5 µg/mL) onto the slide. Observation was performed under a fluorescence microscope (Olympus IX 81) with blue excitation (450 - 480 nm) at 600× magnification coupled with a digital camera (Olympus U-CMAD 3, Japan) and with the help of the software image Cell F (Olympus). Two slides were used for each sample and at least 50 cells were recorded per slide.

The percentage DNA in tail provided by the software Komet 5.5 analysis (Kinetic Imaging, Nottingham, UK) was recorded as DNA fragmentation in each cell. Results were expressed as mean DNA fragmentation and as percentage of cells per class of DNA fragmentation (<10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70% and 70-100%).

2.4. Statistical analysis

All the statistical analyses were carried out using SPSS (Version 15.0). Eventually outliers were graphically identified and removed in the different assays.

For motility evaluation eleven samples with a total of 12981 sperm cells were used to run a cluster analysis using the 8 parameters rendered by the CASA system. We applied a Twostep Cluster Analysis procedure and we used the log-likelihood distances and the Schwarz's Bayesian criterion (BIC). A one-way ANOVA was applied followed by a Gabriel's pairwise test ($p<0.05$) to test for significant differences in each sperm motility descriptor between clusters.

A linear mixed model was applied ($p<0.05$) to test for differences in the evolution of sperm motility throughout time between males. Differences in the subpopulation distribution throughout time were detected with a One-way ANOVA followed by a SNK test (Student-Newman-Keuls) ($p<0.05$).

Comet assay, apoptosis assay and seawater hyperosmotic resistance results were expressed as means ± SD and percentile data were normalized through arcsine transformation. One-way ANOVA was conducted for the comet assay and apoptosis assay. Significant differences between males were analyzed with a multiple comparison procedure, SNK test (Student-Newman-Keuls) ($p<0.05$) for the comet assay and Gabriel's pairwise test ($p<0.05$) for the apoptosis assay due to an unequal sample size after the removal of some outlier values. In the seawater hyperosmotic resistance test significant differences were detected with a linear mixed model using the Bonferroni correction ($p<0.05$).

The study of linear relationships between the different parameters and the size of the different sperm subpopulations were tested with the Pearson parametric correlation ($p<0.05$ or $p<0.01$).The Pearson parametric correlation was also applied between the percentage of cells in the different subpopulations at different times after motility activation.

3. Results

3.1. Motility

All males showed a significant decrease in motility with time and at 60 sec the percentage of motile cells ranged from 4 to 21% (data not shown).

The cluster analysis identified four different clusters (subpopulations) (Table 1). Each subpopulation was characterized by a mean value of the descriptors rendered by CASA. The 1st subpopulation (Subpop1) included spermatozoa with high values of VCL, LIN and STR, hence they were labeled fast and linear spermatozoa. The 2nd subpopulation (Subpop2) included

spermatozoa with high values of VCL and low values of LIN and STR: fast non-linear spermatozoa. The 3rd subpopulation (Subpop3) included spermatozoa with low values of VCL and high values of LIN and STR: slow linear spermatozoa. The 4th subpopulation (Subpop4) included spermatozoa with low values of VCL, LIN and STR, hence slow non-linear spermatozoa.

Table 1- Cluster profiles according to the mean value for each of the descriptors rendered by CASA. Results are expressed as mean ± SD.

	Cluster			
Sperm motility descriptors	Subpop1	Subpop2	Subpop3	Subpop4
VCL (µm/s)	192.4 ± 46.7[a]	182.8 ± 39.2[b]	49.3 ± 30.5[c]	29.0 ± 20.5[d]
VSL (µm/s)	171.5 ± 46.6[a]	78.8 ± 38.2[b]	35.5 ± 25.0[c]	7.2 ± 6.0[d]
VAP (µm/s)	184.5 ± 46.2[a]	147.2 ± 38.5[b]	42.1 ± 28.3[c]	16.6 ± 15.3[d]
LIN (%)	89.1 ± 9.1[a]	42.7 ± 17.7[c]	70.1 ± 15.5[b]	24.7 ± 10.5[d]
STR (%)	92.9 ± 7.4[a]	53.1 ± 21.0[c]	83.1 ± 11.9[b]	47.3 ± 18.4[d]
WOB (%)	95.8 ± 4.1[a]	80.3 ± 10.1[c]	84.0 ± 11.5[b]	54.4 ± 16.5[d]
ALH (µm)	2.4 ± 0.9[b]	4.9 ± 1.4[a]	1.7 ± 0.7[c]	1.6 ± 0.8[c]
BCF (Hz)	7.0 ± 3.0[a]	4.6 ± 2.6[c]	5.2 ± 3.3[b]	3.1 ± 2.3[d]

Different superscripts within the same row indicate significant differences ($p<0.05$).

The evolution of the four spermatozoa subpopulations after motility activation for the eleven individuals is shown in Figure 1. At 15 sec the subpopulation distribution was highly variable. In some males linear subpopulations prevailed (male 1), in others non-linear (males 8 and 9) and others slow (male 5). At more advance times, slow subpopulations (Subpop3 and Subpop4) became more important and at 60 sec these subpopulations represented almost 100% of the motile spermatozoa. There were significant differences in subpopulation distribution between the different males at the different times ($p<0.05$) (Fig. 1).

Several correlations were obtained when the percentage of the different subpopulations at different times were analyzed (Table 2). Subpop1 presented a positive correlation with itself and Subpop2 and 3 at the subsequent times. Subpop2 always presented a positive correlation with Subpop3 at the subsequent time. At 15 and 30 sec, Subpop3 presented a positive correlation with Subpop4 at the subsequent time, whereas Subpop4, except for 15 sec, only presented a correlation with itself.

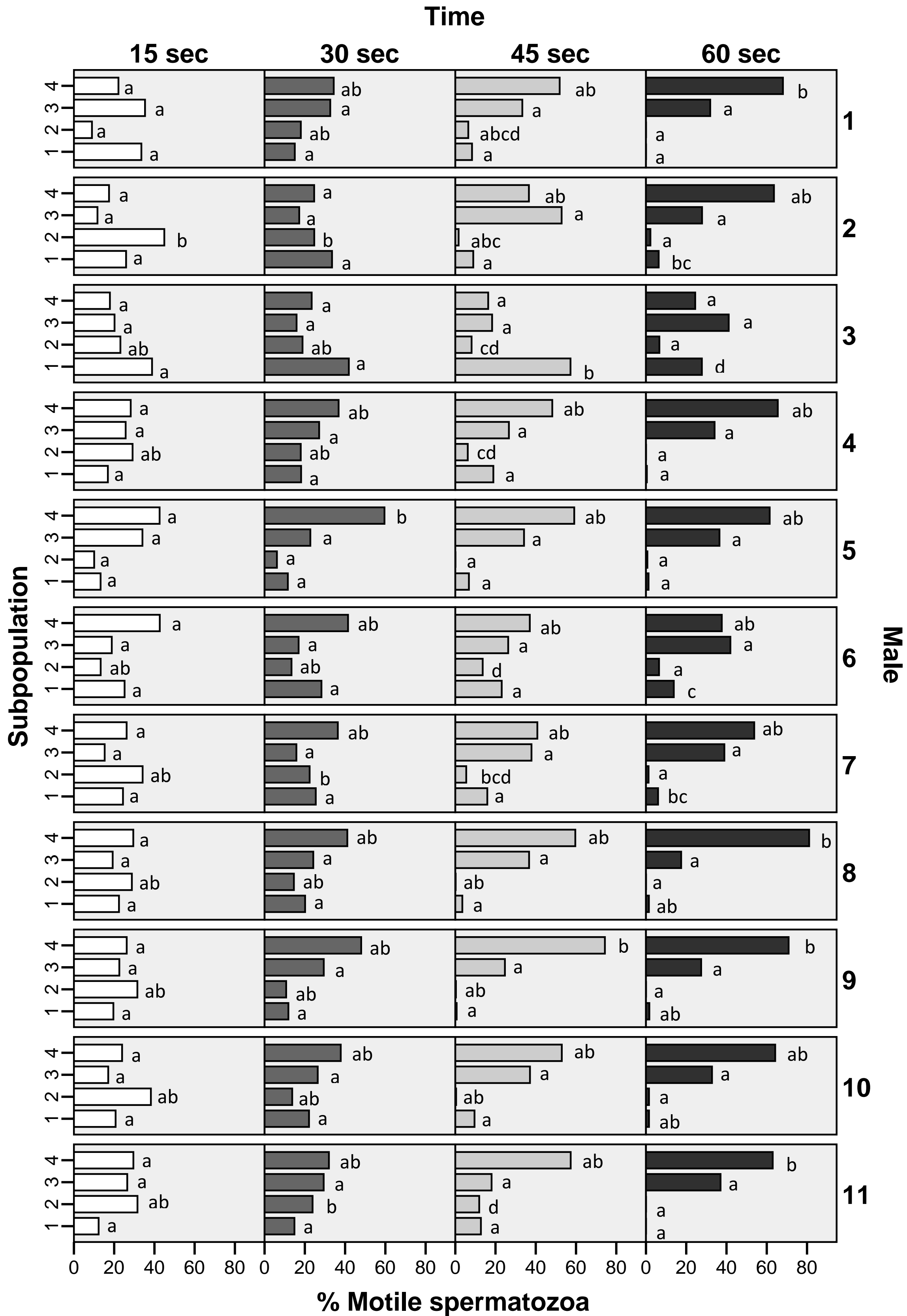

Time
15 sec
30 sec
45 sec
60 sec
Subpopulation
Male
% Motile spermatozoa

Fig. 1. (Previous page) Evolution of each subpopulation in each male through time: white bars for 15 seconds; dark grey for 30 seconds; light grey for 45 seconds; black for 60 seconds. The data was transformed as a percentage regarding the motile population in each sample. Significant differences between males for the different subpopulations at different times are indicated by different letters (p<0.05).

3.2. Hyperosmotic test

The percentage of cells with functional membrane decreased with time after activation with seawater (Table 3), with values at 15 sec varying from 32.9% to 3.4% and at 45 sec from 11.0% to 0.9%. Nevertheless resistance to seawater hyperosmotic shock was significantly different between males (p<0.05), as demonstrated by the linear mixed model, some of them (male 1) showing a higher percentage of resistant cells and others (males 4 and 9) a higher percentage of susceptible cells.

Table 2 - Correlations obtained between percentages of the different motility subpopulations at different times (15, 30, 45 and 60 sec).

Subpopulation / time	1/30	2/30	3/30	4/30	1/45	2/45	3/45	4/45	1/60	2/60	3/60	4/60
1/15	0.81**	0.78**	0.57	0.54	0.05	-0.27	0.78**	0.57	0.03	-0.07	0.07	0.45
2/15	0.76**	0.84**	0.75**	0.66*	-0.13	-0.31	0.83**	0.76**	-0.25	-0.31	-0.03	0.50
3/15	-0.09	0.13	0.83**	0.79**	-0.39	-0.47	0.14	0.78**	-0.57	-0.69*	-0.19	0.52
4/15	0.23	0.36	0.81**	0.96**	-0.37	-0.34	0.47	0.95**	-0.50	-0.53	0.00	0.74**
1/30	1	0.89**	0.30	0.28	0.39	0.10	0.88**	0.37	0.30	0.26	0.41	0.49
2/30		1	0.46	0.40	0.16	0.08	0.88**	0.52	-0.04	-0.11	0.30	0.62*
3/30			1	0.87**	-0.29	-0.38	0.41	0.93**	-0.47	-0.55	-0.16	0.57
4/30				1	-0.30	-0.35	0.46	0.96**	-0.40	-0.45	0.01	0.64*
1/45					1	0.66*	0.01	-0.32	0.88**	0.81**	0.84**	0.08
2/45						1	-0.12	-0.30	0.49	0.59	0.72*	0.07
3/45							1	0.57	-0.12	-0.12	0.21	0.59
4/45								1	-0.51	-0.53	-0.02	0.70*

Relationships with Pearson linear correlation (r) (n=11) significant correlations with p<0.05 are signed *, or p<0.01 with **.

Significant negative correlations were found between the percentage of cells with functional membrane at 15 sec after seawater hyperosmotic shock, and Subpop2 and 4 at the same time (p<0.01, r=0.82 and r=0.84, respectively) (Fig. 2F), as well as between Subpo3 and 4 at 30 sec (p<0.01, r=0.82 and r=0.78, respectively) (Fig. 2E). Similar significant negative correlations were obtained between the percentage of cells with functional membrane at15 sec after seawater hyperosmotic shock and the percentage of motile cells at 15, 30 and 45 sec (p<0.01, r=-0.81, r=-0.88, r=-0.80, respectively).

Table 3 - Results of the hyperosmotic test and apoptosis assay for each male. Results are expressed as mean ± SD.

MALE	Staining results ± SD					
	Non-stained	Annexin-V	PI	H 15 sec	H 30 sec	H 45 sec
1	59.40 ± 6.85[bc]	13.24 ± 1.43[ab]	35.47 ± 2.87[ab]	32.91 ± 6.98	21.07 ± 5.35	7.05 ± 2.24 [d]
2	26.61 ± 4.64[ab]	11.86 ± 0.96[ab]	67.19 ± 2.57[b]	7.66 ± 4.33	10.00 ± 1.29	3.45 ± 1.85 [abc]
3	23.17 ± 4.78[a]	14.21 ± 4.28[ab]	67.99 ± 6.88[b]	-	5.59 ± 2.78	5.36 ± 3.30 [abcd]
4	28.61 ± 4.37[ab]	7.89 ± 2.12[ab]	67.13 ± 2.32[b]	3.37 ± 0.68	2.13 ± 0.78	1.35 ± 0.50 [a]
5	41.26 ± 4.29[abc]	23.44 ± 3.43[b]	48.59 ± 4.87[ab]	22.45 ± 9.27	12.39 ± 1.83	3.21 ± 1.00 [cd]
6	66.26 ± 18.33[c]	11.19 ± 1.92[ab]	30.58 ± 9.38[a]	14.31 ± 5.68	8.66 ± 5.56	11.04 ± 1.55 [cd]
7	32.67 ± 6.47[abc]	10.84 ± 1.50[ab]	60.02 ± 4.31[ab]	11.62 ± 7.86	6.41 ± 1.99	0.95 ± 0.98 [abc]
8	29.17 ± 0.00[ab]	15.42 ± 2.65[ab]	62.50 ± 3.54[ab]	9.51 ± 0.42	7.30 ± 3.33	4.04 ± 1.94 [abc]
9	34.77 ± 11.38[abc]	15.44 ± 2.94[ab]	57.84 ± 5.00[ab]	7.36 ± 2.56	2.87 ± 2.61	2.40 ± 1.15 [ab]
10	48.64 ± 23.34[bc]	6.37 ± 1.20[a]	49.97 ± 11.79[ab]	15.98 ± 8.45	6.20 ± 1.42	4.11 ± 1.66 [abc]
11	49.91 ± 10.63[abc]	20.28 ± 3.29[b]	40.10 ± 5.41[ab]	26.79 ± 4.02	7.34 ± 2.53	5.44 ± 2.13 [cd]

Results of the hyperosmotic test at 15 (H 15 sec), 30 (H 30 sec) and 45 seconds (H 45 sec) refer to non-stained cells (viable). Non-stained (Annexin-/IP-), Annexin-V (Annexin+/PI- plus Annexin+/PI+), PI (Annexin-/PI+ plus Annexin+/PI+). For non-stained, Annexin-V and PI different letters in the same column mean significant differences (p<0.05). For the hyperosmotic test the significant differences, detected with the linear mixed model (p<0.05), are marked with different letters in the H 45 sec column.

3.3. Apoptosis assay

Three different cell populations were observed according to this assay (Table 3). Viable cells were always less than 50% except for male 1 and 6. Most of the males displayed more than 50% PI stained cells whereas Annexin-V stained cells ranged from 6% to 23%. The different cell populations were significantly different between males (p<0.05). Moreover there was a

significant positive correlation between viable cells and the percentage of cells with functional membrane 15 sec after the seawater hyperosmotic shock (r=0.69, p<0.05) (Fig. 2D).

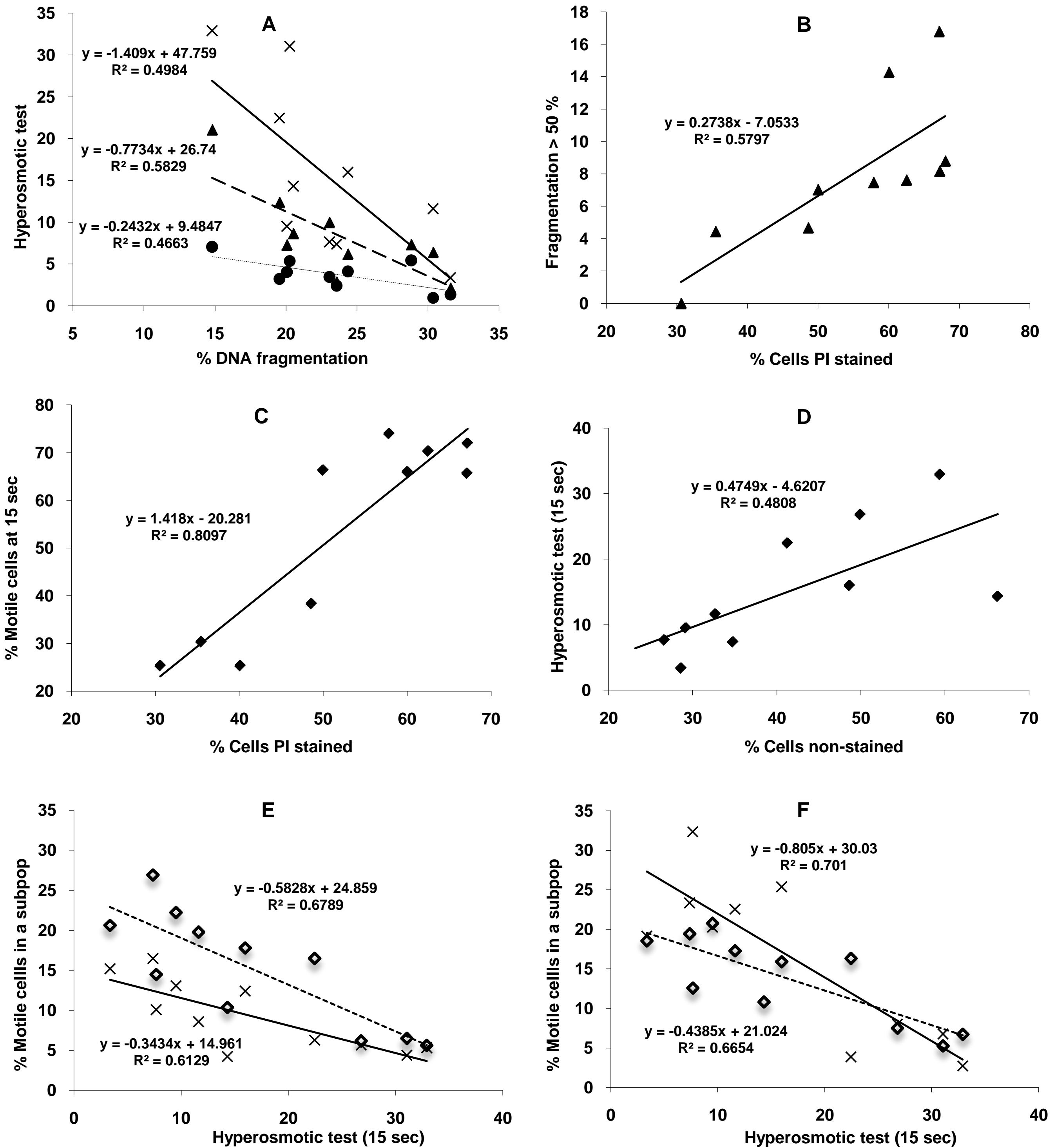


Fig. 2. (Previous page) (A) Correlation between the mean percentage of DNA fragmentation (comet assay) and the % of cells resistant to seawater hyperosmotic shock at 15 sec - X; 30 sec - ▲; and 45 sec - ● (n=11) (p<0.05). The solid line corresponds to the correlation for the 15 sec trial; dashed line to the correlation for the 30 sec trial; and dotted line to the correlation for the 45 sec trial. (B) Correlation between the percentage of cells PI stained (apoptosis assay) and cells with DNA fragmentation higher than 50% (comet assay) (n=10) (p<0.05). (C) Correlation between the percentage of cells PI stained (apoptosis assay) and percentage of motile cells at 15 sec after activation (n=10) (p<0.01). (D) Correlation between the percentage of viable cells (apoptosis assay) and % of cells resistant to seawater hyperosmotic shock at 15 sec (n=10) (p<0.05). (E) Correlation between the % of cells resistant to seawater hyperosmotic shock at 15 sec and: Subpop4 at 30 sec (◊ and dotted line); Subpop3 at 30 sec (X and solid line). Subpopulations obtained with motility data (n=11) (p<0.001). (F) Correlation between the % of cells resistant to seawater hyperosmotic shock at 15 sec and: Subpop4 at 15 sec (◊ and dotted line); Subpop2 at 15 sec (X and solid line). Subpopulations obtained with motility data (n=11) (p<0.001).

No correlations were found between Annexin-V stained cells and the different subpopulations of motile cells, DNA fragmentation values or the percentage of cells resistant to the seawater.

3.4. DNA integrity

Mean DNA fragmentation varied between 15 and 32% and only male 1 and 11 were significantly different from the others (p<0.05) (Table 4). However, considering the percentage of cells at each level of DNA fragmentation, the results obtained were highly variable. Male 6 presented low variation in DNA fragmentation (from <10% to 50%), while males 4 and 11 had spermatozoa with more than 80% DNA fragmentation. Considering all the analyzed samples most of the cells (64.1%) presented a fragmentation level below 30% and around 8.3% of the analyzed cells had over 50% fragmentation. Nonetheless, only 19.7% showed less than 10% fragmentation. However this value was highly variable between males.

Significant negative correlations were obtained (p<0.05) between the percentage of cells resistant to seawater hyperosmotic shock and the mean percentage of DNA fragmentation (Fig. 2A) (r=0.71 at 15 sec, r=0.76 at 30 sec and r=0.68 at 45 sec). The curve corresponding to the 15 sec trial presented the highest slope, followed by the 30 sec curve. The 45 sec curve presented the least accentuate slope due to the low rate of viable cells.

There was a significant positive correlation between the percentage of cells stained with PI (non-viable cells) and the cells with more than 50% of fragmented DNA (r=0.76, p<0.05) (Fig. 2B). Moreover cells stained with PI also presented a positive correlation with motile cells at 15 sec (r=0.90, p<0.01) (Fig. 2C). A significant positive correlation was detected between Subpop1 at 15 sec and cells stained with PI (r=0.68, p<0.05).

Table 4 - Results of the comet assay for the 11 males.

Male	DNA Fragmentation (%) <10	10-20	20-30	30-40	40-50	50-60	60-70	70-100	Mean
1	38.52	20.74	19.26	13.33	3.70	2.96	1.48	0.00	14.80 ± 15.25^{a}
2	21.82	20.91	22.73	15.45	10.91	6.36	1.82	0.00	23.06 ± 15.56^{b}
3	28.00	21.60	12.80	16.80	12.00	7.20	1.60	0.00	20.26 ± 17.06^{b}
4	12.00	15.20	21.60	18.40	16.00	5.60	7.20	4.00	31.58 ± 18.88^{b}
5	19.63	32.71	21.50	14.02	7.48	1.87	2.80	0.00	19.54 ± 14.51^{b}
6	21.43	25.00	25.00	17.86	10.71	0.00	0.00	0.00	20.52 ± 12.17^{b}
7	14.29	14.29	20.95	21.90	14.29	9.52	4.76	0.00	30.36 ± 16.44^{bc}
8	27.12	22.03	18.64	19.49	5.08	5.08	2.54	0.00	20.06 ± 16.24^{b}
9	7.48	32.71	23.36	16.82	12.15	4.67	1.87	0.93	23.56 ± 15.35^{b}
10	17.19	25.78	25.00	16.41	8.59	3.91	1.56	1.56	24.36 ± 15.30^{b}
11	9.43	17.92	28.30	25.47	6.60	5.66	3.77	2.83	28.82 ± 16.09^{c}

Cells were grouped in classes according the percentage of DNA fragmentation determined for each cell. In the last column mean DNA fragmentation values ± SD for each male are represented, significant differences between males are signed with different letters ($p<0.05$)**.**

4. Discussion

It is consensual that a deeper knowledge of the reproductive biology of *S. senegalensis* is needed to overcome reproductive constraints faced by broodstocks maintained in captivity [1,5,17]. Sperm quality is one of the features responsible for these reproductive constraints. The choice of appropriate parameters to evaluate sperm quality could be a useful tool in the selection of high quality breeders.

Motility analysis, using computer assisted sperm analysis (CASA), has been one of the parameters used to classify spermatozoa quality. Most authors working with fish spermatozoa use few of the descriptors rendered by this software to describe the behavior of a motile population [10-11,18-20]. In this work we applied a similar multivariate statistical analysis to the one used by Martinez-Pastor and co-workers [3] working in the same species. These analyses allowed the classification of heterogeneous semen samples into homogeneous subpopulations with spermatozoa showing similar motility characteristics, using the overall CASA data [3]. Beside the large decrease in motility observed in sole sperm after activation (51.18% motile cells at 15 sec to 13.33% at 60 sec), there was an evolution in the motile

population pattern (subpopulations) through time. At 15 sec, fast spermatozoa represented 49.73% of the motile population, while at the 60 sec they only represented 7% of the motile population, corresponding to about 1% of the overall sperm population. Additionally, at that time, most of the spermatozoa presented a non-linear path. As was evidenced by some authors working in sperm competition in other fish species, sperm progressiveness [21] and velocity [22] influences fertilization success. Hence, males with slow and non-linear spermatozoa would probably be less efficient at achieving fertilization, making fecundation very unlikely to occur less than 1 min after semen ejaculation. Furthermore, as supported by the obtained correlations, there was a loss of motility (both speed and linearity) with time, spermatozoa within fast subpopulations migrating to slow subpopulations, and those within linear subpopulations to non-linear ones. Motility loss might be related to different parameters: ATP exhaustion, increasing internal osmolality derived of the osmotic shock [23], morphological changes in mitochondria, chromatin and midpiece [24], etc.

Similar to previous studies [4], sperm viability was relatively low, frequently below 50%, not just due to PI permeable cells, but also to the presence of apoptotic cells (Annexin-V stained). The average percentage of apoptotic cells (13.7%) was similar to that reported in our previous work [4] in the same species, and to data reported by other authors working with mammalian sperm [13,25-26], but to our knowledge there are no reports on the analysis of sperm apoptosis in other fish species. The presence of apoptotic cells in ejaculated spermatozoa is widely documented for mammalian sperm [13,25-27]. Although its origin is not well understood, it could be one of the reasons for poor fertility. Sakkas et al., [27] support that apoptotic cells present in the ejaculate have their origin in an apoptotic mechanism that has failed to eliminate these cells before ejaculation, while Paasch et al., [28] attribute this fact to the existence of immature cells that enter apoptosis after ejaculation. Some studies have also hypothesized stress as the cause of high levels of apoptotic cells [25]. In captive sole, environmental stress due to captivity or inappropriate husbandry conditions could have been one of the causes for these values. In our case since spermatozoa production was reported in this species throughout the year [1,5] we consider that apoptotic cells may be related to a normal mechanism to eliminate overripe spermatozoa, since in the testes several cell maturation stages have been identified [5]. However more studies are needed to confirm this hypothesis.

The hyperosmotic test revealed poor resistance of sole spermatozoa plasma membrane to seawater. In fact, after 45 sec exposure to seawater most samples had less than 10% viable cells and even at 15 sec after sperm exposure to seawater, there were a high number of cells affected by the hyperosmotic condition.

Significant positive correlation between cell viability after ejaculation and plasma membrane functionality (non-stained cells at 15 sec after seawater hyperosmotic shock) showed that samples presenting higher cell viability are also the ones that better sustain hyperosmotic

shock. It is well established that spermatozoa, including those of fish, are capable of regulating their volume under moderate osmotic stress [29], presenting different structures to deal with osmotic changes [30]. Sole spermatozoa must support intense osmotic stress when ejaculated, and our results reveal that a high percentage of cells are extremely sensitive to seawater. This fact might be related to poor development of osmoregulatory mechanisms in this species or to some kind of damage in captive breeders affecting membrane osmoregulatory mechanisms. Only resistant and motile spermatozoa could reach the oocyte and participate in the fertilization process. This phenomenon could eliminate a non-motile population at the beginning of the activation process, since it is likely that PI positive cells (non-viable), also present the motility activation mechanism damaged, because mechanisms occurring at the initiation of motility require a functional membrane [23].

Most of the analyzed cells presented some DNA damage according to the comet assay. When considering less than 10% DNA fragmentation as undamaged cells, only an average of 19.7% of the cells were classified in this category. However, when compared with other marine species, our mean value of DNA fragmentation (23.4%) was lower. In European seabass (*Dicentrarchus labrax*), a 32.7% DNA_t was determined [31] while in gilthead seabream (*Sparus aurata*), this value corresponded to 28.2% DNA_t [16]. The mean DNA fragmentation was negatively correlated with the percentage of cells resistant to seawater hyperosmotic shock. This means that cells with fragmented DNA also display weakened membranes, probably due to changes in permeability and/or PS translocation. Moreover cells with high percentage of DNA fragmentation (more than 50%) also correlated with cells stained with PI, suggesting that cells with high percentages of DNA fragmentation presented a damaged plasma membrane. This phenomenon was also reinforced by the fact that cells with damaged membranes suffered lyses more easily just after sperm activation promoting the selection of motile cells after activation. Thus, seawater hyperosmotic shock could be responsible for the selection of spermatozoa capable of fertilization. This hypothesis is supported by the fact that the percentage of cells stained with PI was positively correlated with the percentage of cells within Subpop1 (fast and linear subpopulation).

Several authors support that cell apoptosis determined by Annexin-V assay is related to cell DNA fragmentation [26,32]. However, as in our previous study [4], we were not able to find any correlation between these two assays, although both may be related. Since Annexin-V identifies cells in an early stage of apoptosis and DNA fragmentation only occurs in later stages, it is possible that Annexin+ cells do not always correspond to DNA fragmented cells. On the other hand DNA fragmentation could be produced by causes other than apoptosis [33]. The same happened with the lack of correlation between the apoptosis assay (Annexin-V) and the other studied parameters.

We obtained negative correlations between viable cells in hyperosmotic test at 15 sec, and Subpop2 and 4 at 15 sec, which were the subpopulations with a non-linear path. Moreover, there was also a negative correlation between these viable cells and Subpop3 and 4 at 30 sec (slow subpopulations). These correlations could indicate that cell resistance to seawater hyperosmotic conditions affected population structure after activation. This fact seemed to be supported by the above discussion: cell lysis after exposure to seawater eliminates damaged cells from the assays of cell motility and cell resistance to seawater hyperosmolarity. Therefore, putative cell lyses can also be part of a semen selection process to positively choose cells with better performance, eliminating overripe cells, and preventing them from achieving fertilization, similar to the mechanism above referred for the apoptotic cells. In the samples where this mechanism was not evident there were fewer viable cells and more cells within the populations with poorer motility performance. Babiak and co-workers [18] suggested the occurrence of cell lyses as part of an ageing process in Atlantic halibut, *Hippoglossus hippoglossus* L. sperm. A similar process could have occurred in overripe sole cells, presenting alterations in the cellular membrane and in chromatin condensation as described by Suquet et al. [34] for turbot, *Scophthalmus maximus*. Furthermore, due to the fact that this species presents asynchronous spermatogenesis [5], there might have been extraction of immature cells, caused by overpressure on the testes during the sperm extraction, which in natural fertilization conditions would not be ejaculated. The presence of immature cells, would explain the high proportion of cells with a non-linear path at 15 sec (Subpop2 and 4) observed in some males, as was proposed by Martinez-Pastor et al. [3].

We provide evidence that poor sole semen quality is related to low ability of the spermatozoa to sustain membrane integrity when exposed to seawater. This fact seems to be related to a maturation process in order to eliminate spermatozoa presenting high levels of DNA fragmentation. Moreover, it was shown that DNA fragmentation levels seem to be related to the structure of subpopulations within the ejaculate. This work showed that a complete annual follow of the sperm quality parameters is needed, together with biotic and feeding regimes manipulation, in order to improve Senegalese sole broodstock maintenance and to test if these parameters can be used to select males according to sperm characteristics.

Acknowledgements

The authors would like to thank Patricia Diogo, Vânia Santos, Isabel Batista, Emilio Mota and Diana Reis for their help during the experimental procedures. We are thankful to Felipe Martinez-Pastor for assistance with the multivariate analyses. This work was supported by a PhD fellowship (SFRH/BD/31990/2006) and REPROSOL 2006.71.001.443.9 project.

References

[1] Cabrita E, Soares F, Dinis MT. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 2006;261:967-975.

[2] Mañanos E, Gruzmán JM, Ramos J, Mylonas CC, Zohar J, Peleteiro JB, Chereguini O, Duncan N, Sarasquete C, Riaza A. Hormonal stimulation of ovulation, spermiation and spawning in cultured Senegalese sole (*Solea senegalensis*) broodstock. In: Report of the IV Workshop on the cultivation of Soles. Faro, Portugal; 2008. p. 16-17.

[3] Martinez-Pastor F, Cabrita E, Soares F, Anel L, Dinis MT. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 2008;135:449-59.

[4] Beirão J, Cabrita E, Soares F, Herraez MP, Dinis MT. Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: comet analysis and Annexin V–Fluorescein staining. J Appl Ichthyol 2008;24:508-513.

[5] Garcia-Lopez A, Fernandez-Pasquier V, Couto E, Canario AVM, Sarasquete C, Martinez-Rodriguez G. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen Comp Endocrinol 2006;147:343-351.

[6] Rurangwa E, Kime DE, Ollevier F, Nash JP. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 2004;234:1-28.

[7] Cabrita E, Robles V, Herráez P. Sperm quality assessment. In: Cabrita E, Robles V, Herraez P, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton: Taylor and Francis; 2008. p. 93-147.

[8] Kime DE, Van Look KJW, McAllister BG, Huyskens G, Rurangwa E, Ollevier F. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol C Toxicol Pharmacol 2001;130:425-433.

[9] Toth GP, Ciereszko A, Christ SA, Dabrowski K. Objective analysis of sperm motility in the lake sturgeon, *Acipenser fulvescens*: activation and inhibition conditions. Aquaculture 1997;154:337-348.

[10] Elofsson H, Van Look K, Borg B, Mayer I. Influence of salinity and ovarian fluid on sperm motility in the fifteen-spined stickleback. J Fish Biol 2003;63:1429-1438.

[11] Liu QH, Li J, Xiao ZZ, Ding FH, Yu DD, Xu XZ. Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus major*). Aquaculture 2007;263:20-25.

[12] Zhang HB, Lu SM, Ma CY, Wang L, Li X, Chen ZJ. Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation. Asian J Androl 2008;10:227-235.

[13] Peña FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003;60:677-689.

[14] Lee RF, Steinert S. Use of the single-cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat Res Rev Mutat Res 2003;544:43-64.

[15] Quintero-Moreno A, Miro J, Rigau AT, Rodriguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 2003;59:1973-1990.

[16] Cabrita E, Robles V, Rebordinos L, Sarasquete C, Herraez MP. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 2005;50:144-153.

[17] Agulleiro MJ, Anguis V, Canavate JP, Martinez-Rodriguez G, Mylonas CC, Cerda J. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture 2006;257:511-524.

[18] Babiak I, Ottesen O, Rudolfsen G, Johnsen S. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 2006;65:1587-1604.

[19] Wojtczak M, Dietrich GJ, Slowinska M, Dobosz S, Kuzminski H, Ciereszko A. Ovarian fluid pH enhances motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Aquaculture 2007;270:259-264.

[20] Li P, Wei Q, Liu L. DNA integrity of *Polyodon spathula* cryopreserved sperm. J Appl Ichthyol 2008;24:121-125.

[21] Rudolfsen G, Figenschou L, Folstad I, Tveiten H, Figenschou M. Rapid adjustments of sperm characteristics in relation to social status. Proc R Soc Lond B Biol Sci 2006;273:325-332.

[22] Casselman SJ, Schulte-Hostedde AI, Montgomerie R. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci 2006;63:2119-2125.

[23] Cosson J. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquac Int 2004;12:69-85.

[24] Dreanno C, Cosson J, Suquet M, Cibert C, Fauvel C, Dorange G, Billard R. Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. J Reprod Fertil 1999;116:113-125.

[25] Anzar M, He LW, Buhr MM, Kroetsch TG, Pauls KP. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 2002;66:354-360.

[26] Marti E, Perez-Pe R, Colas C, Muino-Blanco T, Cebrian-Perez JA. Study of apoptosis-related markers in ram spermatozoa. Anim Reprod Sci 2008;106:113-32.

[27] Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 1999;4:31-37.

[28] Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. Fertil Steril 2004;81: 802-809.

[29] Cabrita E, Robles V, Cunado S, Wallace JC, Sarasquete C, Herraez MP. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5ml macrotubes. Cryobiology 2005;50:273-284.

[30] Morisawa S. Ultrastructural studies of late-stage spermatids and mature spermatozoa of the puffer fish, *Takifugu niphobles* (Tetraodontiformes) and the effects of osmolality on spermatozoan structure. Tissue Cell 2001;33:78-85.

[31] Zilli L, Schiavone R, Zonno V, Storelli C, Vilella S. Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology 2003;47:227-235.

[32] Sharma RK, Said T, Agarwal A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. Asian J Androl 2004;6:139-148.

[33] Martínez-Pastor F, Fernández-Santos MR, Domínguez-Rebolledo A, Esteso MC, Garde JJ. DNA status on thawed semen from fighting bull: A comparison between the SCD and the SCSA tests. Reprod Domest Anim 2008;44:424-431.

[34] Suquet M, Dreanno C, Dorange G, Normant Y, Quemener L, Gaignon JL, Billard R. The ageing phenomenon of turbot spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization, and storage capacities. J Fish Biol 1998;52:31-41.

Chapter 1.2.

Changes in *Solea senegalensis* sperm quality throughout the year

J. Beirão[a], F. Soares[b], M.P. Herráez[a], M.T. Dinis[c], E. Cabrita[d]

[a]Dept. Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain.
[b]INIAP, IPIMAR-CRIP Sul, Av. 5 Outubro, 8700-305 Olhão, Portugal.
[c]Center for Marine Sciences-CCMAR, University of Algarve, 8005-139 Faro, Portugal.
[d]Institute of Marine Science of Andalusia-ICMAN, Spanish National Research Council-CSIC, Av Republica Saharaui 2, 11510 Puerto Real, Cádiz, Spain.

Animal Reproduction Science (accepted)
10.1016/j.anireprosci.2011.04.009

Abstract

Some of the Senegalese sole (*Solea senegalensis*) broodstock reproductive constraints are related to sperm quality. Although they present two defined spawning season (spring and autumn), males gave semen during all the year thus an exhaustive annual sperm analysis is important to determine the seasonal changes in semen quality. Sampling was performed monthly during one year, analyzing different cellular parameters to better understand sperm quality limitations obstructing sole mass production. The percentage of progressive motile cells and their linear velocity showed a decrease from March (beginning of the first spawning season) to July (when the highest temperatures were observed), followed by a slight increase in August and October (second spawning season). DNA fragmentation values showed highest values between the two spawning seasons and decreased to the end of the year. The percentage of apoptotic cells was lowest in March (beginning of the first spawning season) and the highest in November. The percentage of cells resistant to seawater exposure presented two peaks related with both spawning seasons. There was a tendency for the semen to attain a quality peak between the beginning and the middle of the first spawning season (March-May), followed by a pronounced decrease, achieving the lowest values during the months with the highest temperature. Also, the different males present in the broodstocks reach their sperm quality peak at different times, which will result in an unequal contribution for the next generation.

Keywords: sperm quality, Senegalese sole, DNA integrity, hyperosmotic shock, monthly variations

1. Introduction

In Senegalese sole (*Solea senegalensis*) natural spawning can be achieved in captivity with wild stocks (Imsland et al., 2003), however poor control over the reproductive cycle has obstructed the massive culture of this species. Temperature and photoperiod seem to be the key factors triggering gonadal maturation (Oliveira et al., 2008), although the effect of the manipulation of biotic factors on sperm quality has never been tested. Some attempts have been made to increase the low sperm volume and quality produced by this species using hormonal therapies such as GnRHa treatment (Agulleiro et al., 2007; Cabrita et al., 2011; Guzmán et al., 2008) or changing the feeding regime (Anguís et al., 2008).

Despite some reports in which eggs were obtained by stripping (Mañanos et al., 2008), in most cases females only spawn naturally in two periods (late spring to the beginning of summer and early autumn, when temperatures are similar), even though males spermiate throughout the year (Anguís and Cañavate, 2005; Cabrita et al., 2006; Garcia-López et al., 2006). According to these authors sperm could be obtained at any time without manipulating fish (hormonal stimulation or manipulation of biotic factors). Cabrita et al. (2006) demonstrated that sperm motility was variable during the year, with an improvement during the spawning seasons, however an explanation for this variation is missing. In a previous work, sperm samples with low resistance of the plasma membrane to seawater hyperosmolarity were noticed during the spawning season, and this low membrane resistance was correlated with DNA integrity (Beirão et al., 2009). There is no information whether this could be due to the assynchronization of individuals, to the presence of overripe cells, or to the fact that sperm presents these characteristics all year. Thus, an exhaustive sperm analysis throughout the year is important to determine the status of spermatozoa variation in broodstocks. This knowledge may be useful for monitoring males and optimizing broodstock maintenance through manipulation of biotic parameters or hormonal therapies and to determine the relevance of using these treatments in sperm production and quality.

In the present work we evaluated sperm quality in a broodstock based on the following parameters: 1) sperm motility; 2) DNA integrity; 3) cell viability and the presence of apoptotic cells and; 4) spermatozoa plasma membrane resistance to seawater hyperosmotic conditions. The analyses were performed during one year in a broodstock kept under natural temperature, photoperiod and salinity conditions.

2. Material and methods

2.1. Broodstock husbandry conditions

The individuals used in the experiments were captured in the wild and maintained for more than 2 years at the Ramalhete Experimental Station (37º00'N, 7º58'W), Faro, Portugal. Fishes (1:2, female:male) were kept in 6 round fiberglass indoor tanks (3,000 liter), with sand substrate and compressed air provided through air stones, stock density around 5 kg m^{-3} (mean individual weight 1.83 ± 0.43 Kg). The photoperiod, water temperature and salinity varied throughout the year according to the environmental conditions of the area (Fig. 1). In each tank, water flow rate was set to 500 L/h. All individuals were tagged with individual PIT Tags (Trovan, NL) in order to identify each specimen. The fish were fed daily with 3% biomass (wet weight basis), consisting of squid (*Loligo* sp.) and vitamin (Premix, Sorgal, Portugal) enriched mussels (*Mytilus* sp.).

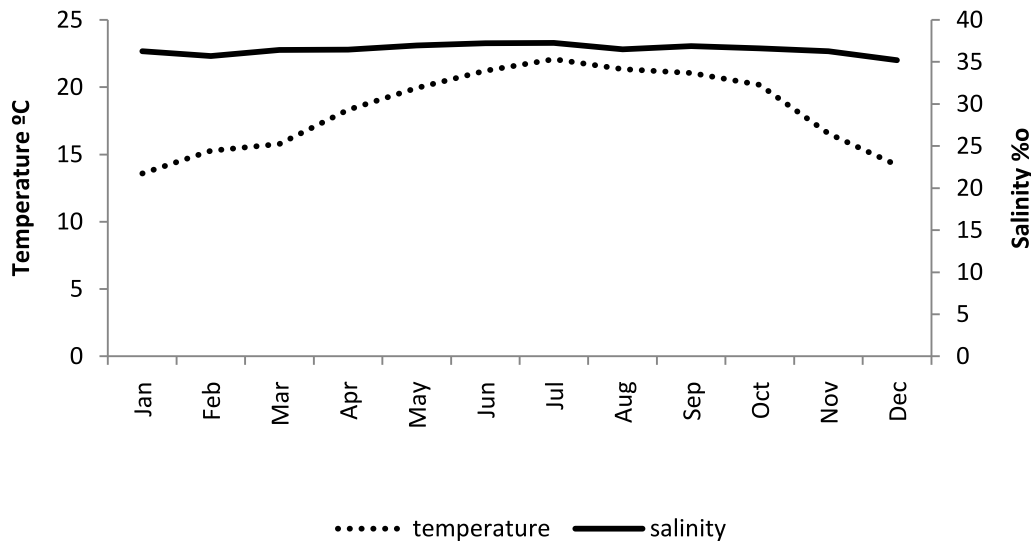


Fig 1 – Monthly variation in water temperature and salinity in the tanks between January and December 2007.

2.2. Sampling

All the males (n=29) were sampled monthly (January to December, excepting April) for fluency analysis and samples from spermiating males were collected. Fishes were caught in the tank and transferred to anesthesia (300 ppm 2-Phenoxyethanol, Fluka (Madrid, Spain)) in a smaller tank. Mucus, faeces and water were cleaned from the urogenital pore and a 1 mL syringe without a needle was used to collect the semen. The sperm was placed in 1.5 mL eppendorf

tubes and stored at approximately 7ºC for further analysis (no longer than 3 hours). Samples contaminated with urine or seawater were discarded.

2.3. Sperm analysis

2.3.1. Sperm production and motility evaluation

Individual semen production was quantified monthly based on cell concentration (evaluated using the CASA system) and collected volume, calculating the number of spermatozoa extracted. Sperm motility was assessed using the CASA system (ISAS-Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a 10 × negative phase contrast objective. One-µL of diluted samples (2 µL of semen in 25 µL of 300 mOsm/kg sucrose) was activated with 5 µL of artificial seawater (1100 mOsm/kg). Motility was recorded at 15, 30, 45 and 60 seconds post-activation. Each sample was analyzed thrice. CASA settings were adjusted to *S. senegalensis* spermatozoa according to Beirão et al. (2009). Percentage of motile spermatozoa (% Mot), percentage of spermatozoa with progressive movement (% Pro) and the curvilinear velocity (VCL, µm s^{-1}) were measured and registered.

2.3.2. DNA fragmentation and apoptosis

The single-cell gel electrophoresis (comet assay) technique was used to assess DNA strand breaks as described by Cabrita et al. (2005) and adapted to *S. senegalensis* sperm by Beirão et al. (2008). Comets were visualized in a fluorescent microscope (Olympus IX 81) with blue excitation (450 – 480 nm) at 600 × magnification coupled to a digital camera (Olympus U-CMAD 3) and using the image software Cell F (Olympus). The percentage of DNA in tail (corresponding to the DNA strand breaks migrated from the immobilized nuclear DNA) was analyzed with the software Komet 5.5 (Kinetic Imaging, Nottingham, UK) and recorded as DNA fragmentation in each cell. Results were expressed as mean DNA fragmentation.

The percentage of apoptotic cells and cell viability was evaluated using the Annexin-V FITC, Sigma-Aldrich (Madrid, Spain) assay conjugated with propidium iodide (PI), Fluka (Madrid, Spain). Briefly, 1 µL of pre-diluted semen was double stained with 1 µL of Annexin-V FITC (50 µg/mL) for 20 min and with 1.5 µL PI (1 mg/mL) for 5 min. After incubation, samples were observed in a fluorescence microscope (Olympus IX 81) with blue excitation (450 – 480 nm). The results were expressed as a percentage of viable cells (Annexin negative/PI negative) and apoptotic cells (Annexin positive/PI negative and Annexin positive/PI positive).

2.3.3. Sperm membrane resistance to seawater

Cell plasma resistance to seawater hyperosmolarity was assayed according to our previous work (Beirão et al., 2009). Briefly, 1µL of the semen was activated with 9 µL artificial seawater and fixed at different time intervals post-activation (15, 30, 45 and 60 seconds) with 4 % glutaraldehyde. The cells were stained by adding 2 µL Hoechst 33258 (200 µg/mL), to 50 µL sperm suspension. Three slides were observed under a fluorescence microscope (Olympus IX 81) with UV light (330 – 380 nm). Cells were classified as non-resistant to osmotic stress when showing blue fluorescence and resistant to osmotic stress when not stained. The results are presented as a percentage of cells resistant to seawater hyperosmolarity at different times after activation.

2.4. Statistical analysis

All the results are presented as mean values ± SEM (standard error of the mean).

Percentile data were normalized through arcsine transformation. Semen production, cell viability and cell apoptosis were analyzed with one-way ANOVA, in order to identify seasonal differences. Significant differences were detected with Gabriel's pairwise test, due to unequal sample size between the different months. DNA fragmentation data were analyzed with the non-parametric Kruskal-Wallis test. A general linear mixed model with Sidak's adjustment for multiple comparisons was applied to the motility and resistance to osmotic stress data, considering data through the time. $P<0.05$ was considered statistically significant.

Correlations between the distinct parameters throughout the year were analyzed with Pearson's parametric correlation ($p<0.001$). All statistics were conducted using the software SPSS 15.0 for Windows.

3. Results

3.1. Sperm production and motility evaluation

In *S. senegalensis* semen production was highest in February and March ($52.4 \pm 76.4 \times 10^6$ spermatozoa and $52.2 \pm 60.9 \times 10^6$ spermatozoa, respectively) (beginning of the first spawning season) as well as in October ($50.5 \pm 61.9 \times 10^6$ spermatozoa) (second spawning season) nevertheless there were no significant differences with the other months (Fig. 2). There was a high variability within males, all the months presenting males with more than 130 million cells and males with less than 10 million cells. The mean sperm volume collected from the males did not present any significant variation along the year (Table 1). Moreover, of the 29 males used in

this experiment, only four gave sperm during all the year, and in all months there were records of spermiating and non-spermiating males, there were at least 69% spermiating males in each month (Table 1). For all the analyzed parameters there was a high variability between males in all months (data not show).

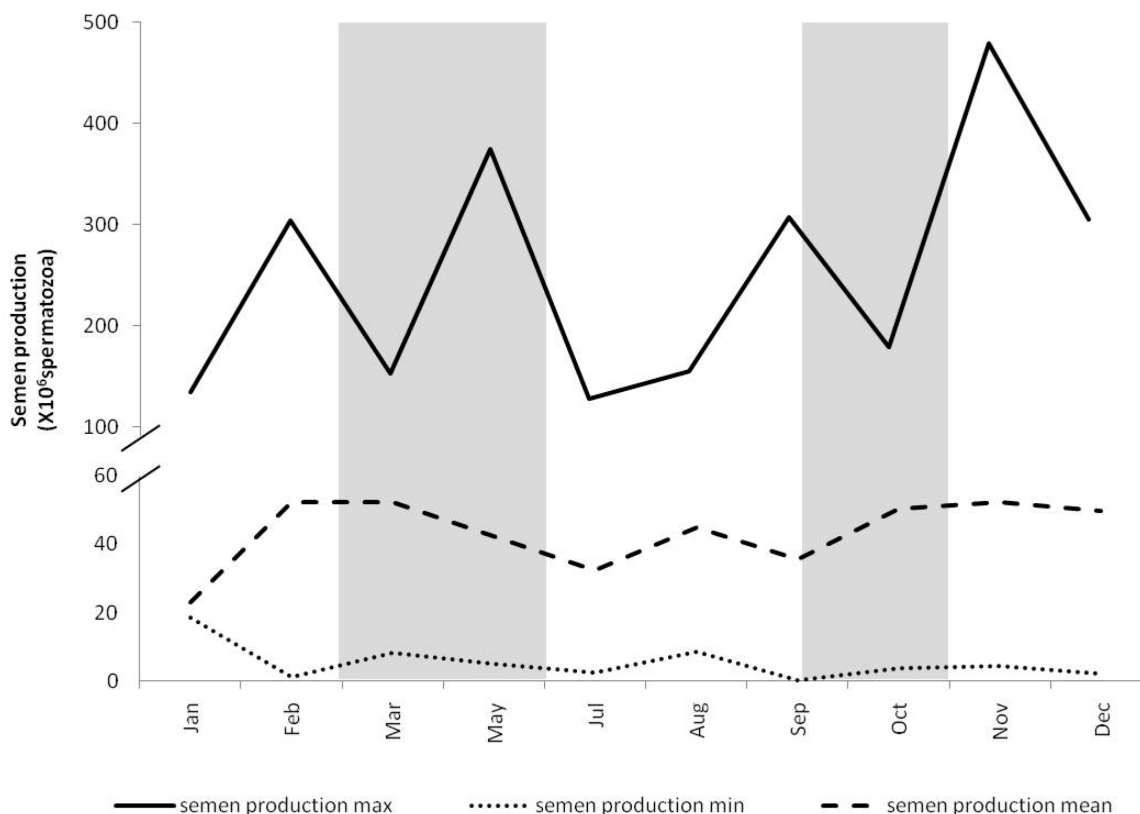


Fig 2 – Semen production monthly variation, maximum (solid line), mean (discontinuous line) and minimum values (dotted line) of semen production in cells × 10^6. Grey bars indicate usual *S. senegalensis* spawning seasons in the Ramalhete Experimental station, Faro, Portugal.

The percentage of motile cells, progressive cells and VCL values changed during the seasons (Fig. 3). In May there was a significant decrease in the percentage of motile cells at 15 s (32.3 ± 3.4 %), while it showed a peak in August (57.2 ± 4.0 %) and in October (68.4 ± 4.5 %). For the sperm VCL (Fig. 3C), the highest values were observed in February, March and May (132.5 ± 5.8 µm/s, 133.0 ± 6.8 µm/s and 129.3 ± 8.8 µm/s respectively at 15 s) decreasing significantly in June (88.72 ± 5.9 µm/s at 15 s). In the following months no significant differences in VCL values were observed until September, decreasing significantly in October (78.2 ± 4.7 µm/s at 15 s). For the percentage of progressive spermatozoa (Fig. 3B) there were also two significant

decreases, the first in June, with 9.7 ± 1.3 % progressive spermatozoa at 15 s, and the second in November, with 11.2 ± 1.3 % progressive spermatozoa at 15 s.

Table 1 – Monthly variation of the percentage of spermiating males, of the mean sperm concentration and of the mean sperm volume collected.

Month	% spermiating males	Sperm concentration ± SEM × 10^9 (cell/mL)	Sperm volume ± SEM (μL)
January	72.4	1.60 ± 0.14^{a}	23.6 ± 5.3^{a}
February	69.0	3.38 ± 0.60^{b}	19.5 ± 3.7^{a}
March	69.0	3.34 ± 0.35^{b}	22.7 ± 3.3^{a}
May	72.4	2.06 ± 0.35^{ab}	23.3 ± 3.5^{a}
June	86.2	-	23.0 ± 5.1^{a}
July	72.4	2.38 ± 0.24^{ab}	18.9 ± 3.4^{a}
August	75.9	2.43 ± 0.25^{ab}	25.0 ± 3.9^{a}
September	88.9	1.29 ± 0.56^{a}	29.3 ± 5.2^{a}
October	74.1	2.81 ± 0.40^{ab}	20.0 ± 4.0^{a}
November	74.1	2.33 ± 0.40^{ab}	24.6 ± 5.1^{a}
December	70.4	2.07 ± 0.34^{ab}	32.6 ± 8.6^{a}

Different superscript letters within the same column indicate significant differences.

3.2. DNA fragmentation and apoptosis

DNA fragmentation (Fig. 4A) values showed a continuous increase until June with 51.15 ± 4.6 % of mean DNA fragmentation; after June there was a progressive decrease in the mean DNA fragmentation values to the end of the year and in December the values were as low as 22.8 ± 4.8 %. The variation curve of the DNA fragmentation values along the year was similar to the water temperature values (Fig. 1 and Fig. 4A).

Cell viability (Fig. 4B) was relatively constant throughout the year (between 53.6 ± 2.6 % in January and 29.3 ± 3.7 % in July), while the percentage of apoptotic cells (Fig. 4B) had the lowest value in March (beginning of the first spawning season) with 10.5 ± 1.2 % and as the spawning season proceeded there was an increase in this parameter.

3.3. Sperm membrane resistance to seawater

The spermatozoa membrane resistance to seawater exposure (Fig. 5) was also affected by the months. The lowest values were observed during the summer months, from June to September (between 2.9 ± 0.6 % and 6.0 ± 1.1 % of cells with a functional membrane 15 s after motility activation), increasing significantly in October (14.8 ± 2.5 % of cells with a functional membrane at 15 s), the highest value was observed in May (18.1 ± 2.1 % of cells with a functional membrane at 15 s). In all months, the percentage of cells with a functional membrane decreased with time after seawater exposure.

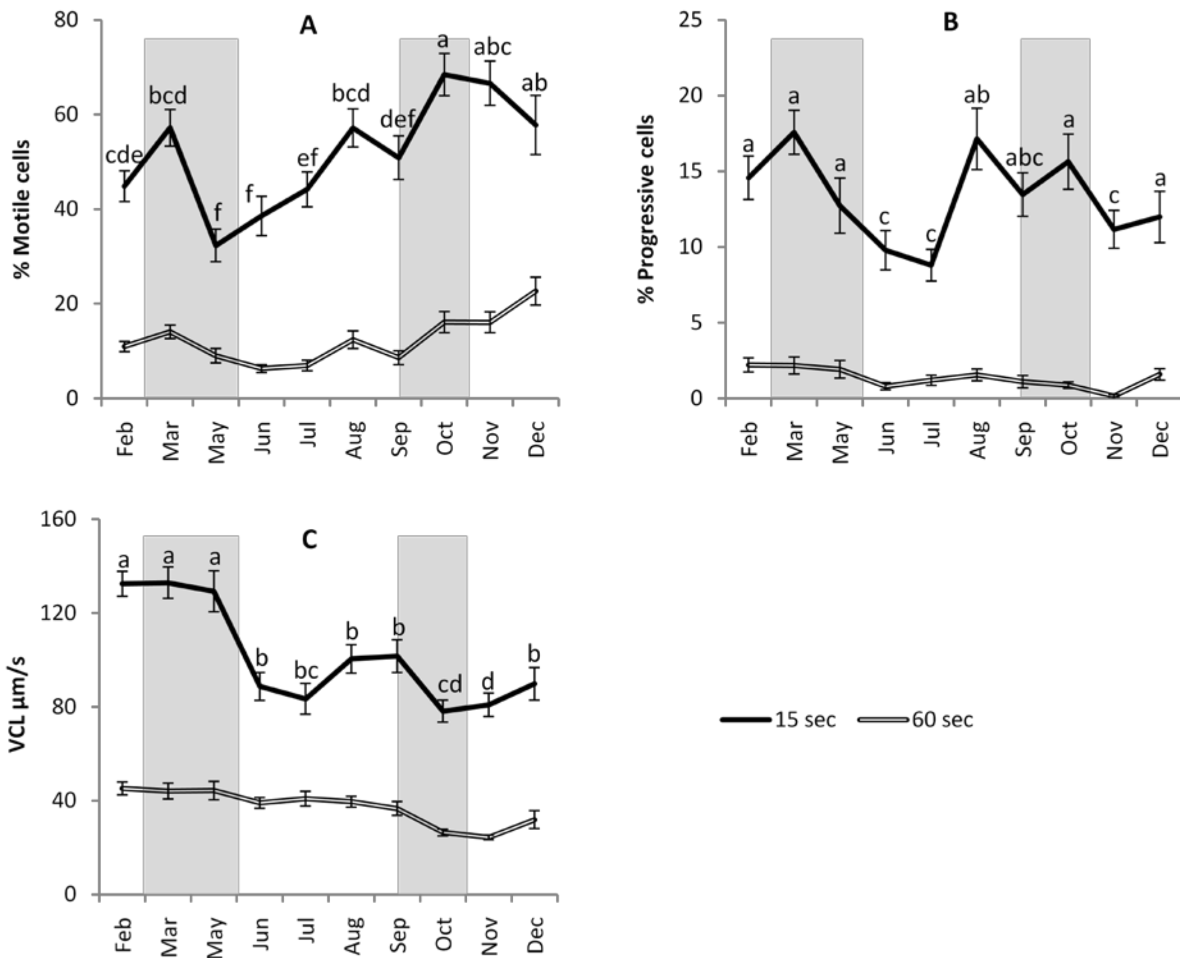


Fig 3 – A) Monthly variation in the percentage of motile cells 15 and 60 seconds after motility activation; B) monthly variation in the percentage of progressive cells 15 and 60 seconds after motility activation; C) monthly variation in curvilinear velocity 15 and 60 seconds after motility activation. Data corresponds to mean values ± SEM. Different letters mean significant differences between months obtained with the applied linear mixed model for the 15, 30, 45 and 60 seconds ($p<0.05$) (30 and 45 seconds data not show). Grey bars indicate usual *S. senegalensis* spawning seasons in the Ramalhete Experimental station, Faro, Portugal.

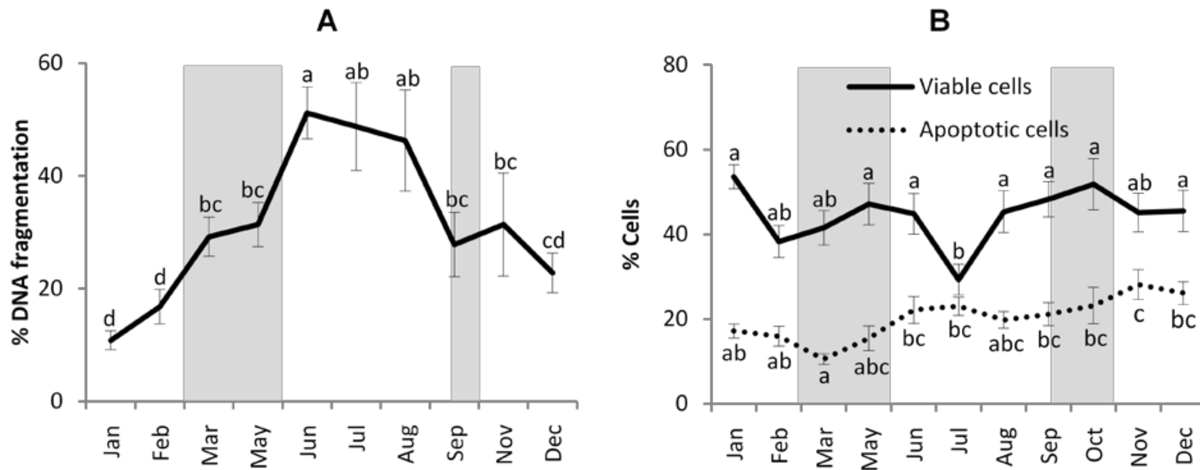


Fig 4 – A) Monthly variation in DNA fragmentation; B) monthly variation in cell viability (solid line) and percentage of apoptotic cells (dotted line). Data corresponds to mean values ± SEM. Different letters mean significant differences between months (p<0.05). Grey bars indicate usual *S. senegalensis* spawning seasons in the Ramalhete Experimental station, Faro, Portugal.

3.4. Correlations between the sperm parameters

From the different correlations obtained, only the stronger ones (with p<0.001 and R>I0.3I) are shown (Table 2). The percentage of apoptotic cells was negatively correlated with the percentage of motile and progressive spermatozoa at 15 seconds after motility activation. Moreover, motility at 15 seconds was also positively correlated with the percentage of viable cells. The mean DNA fragmentation was negatively correlated with the percentage of cells resistant to seawater hyperosmolarity 15 seconds after motility activation.

Table 2 – Most important obtained correlations

Correlation	Significance (p)	Pearson correlation (R)
% Apoptotic / VCL 15 s	<0.001	-0.3618
% Apoptotic / % Pro 15 s	<0.001	-0.3448
% Viables / % Motile 15 s	<0.001	0.3252
DNA Frag / % viable 15 s	<0.001	-0.3556

% Apoptotic – percentage of cells stained with Annexin-V FITC; VCL 15 s – curvilinear velocity 15 seconds after motility activation; % Pro 15 s – percentage of progressive cells 15 seconds after motility activation; % Viables –percentage of non-stained cells in the Annexin-V FITC assay; % Motile 15 s – percentage of motile cells 15 seconds after motility activation; DNA Frag – percentage of mean DNA fragmentation; %viable 15 s – percentage of cells with functional membrane 15 seconds after exposition to seawater.

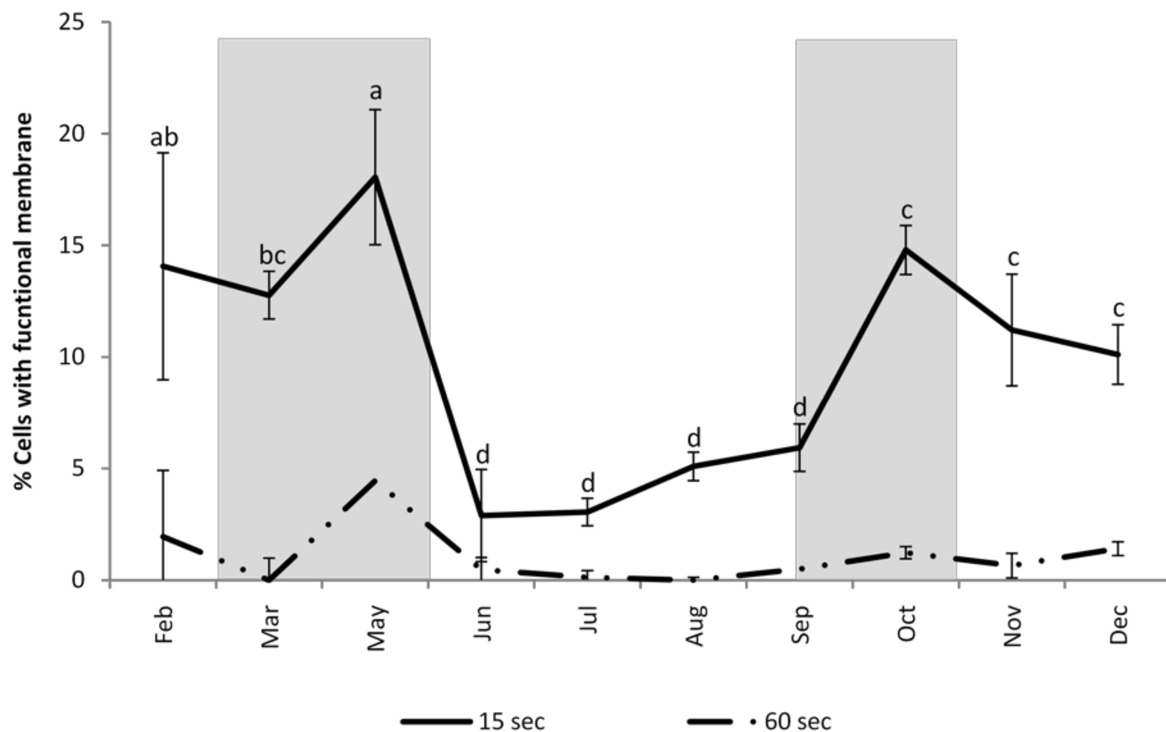


Fig 5 – Monthly variation in spermatozoa resistance to seawater hyperosmolarity at 15 and 60 seconds after motility activation. Data corresponds to mean values ± SEM. Different letters mean significant differences between months obtained with the applied linear mixed model for the 15, 30, 45 and 60 seconds ($p<0.05$) (30 and 45 seconds data not show). Grey bars indicate usual *S. senegalensis* spawning seasons in the Ramalhete Experimental station, Faro, Portugal.

4. Discussion

Despite the importance of male reproductive potential in recruitment success this question has not been studied in similar details as in females (Trippel, 2003). In *S. senegalensis* one of the factors responsible for the low and variable quality of the spawns seems to be low sperm quality, a fact already mentioned in previous studies (Beirão et al., 2009; Cabrita et al., 2006). In the present study we used several parameters to evaluate sperm quality, among which sperm fluency is one of the simplest used parameters to measure male reproductive potential and in this study there were spermiating males all-year-round, which was already reported and discussed by previous authors (Cabrita et al., 2006; Garcia-López et al., 2005, 2006).

On the other hand, the most commonly used parameter to evaluate male fertilization potential is motility (Trippel, 2003) and has been correlated with fertilization in several species (Rurangwa et al., 2004). Despite the differences in sperm motility observed amongst *S. senegalensis* males, in our study there were significant differences between the spawning seasons and the rest of the year. There were higher values of progressive spermatozoa with higher velocity (VCL) during the spawning season than during the other periods of the year. Variability in the percentage of motile cells observed within the spawning season could indicate some asynchrony in the maturation of males and females, which has already been described in other fish like the red porgy *Pagrus pagrus*, where according to Mylonas et al. (2003) males mature before females, presenting a decrease in gamete quality during the time the females are at their reproductive peak. Additionally, the decrease observed from March to May in the percentage of motile cells could represent a decrease in sperm quality during the spawning season, similar to other species such as seabass *(Dicentrarchus labrax)* (Rainis et al., 2003), turbot (*Scophthalmus maximus*) (Suquet et al., 1998) and Atlantic halibut (*Hippoglossus hippoglossus*) (Babiak et al., 2006). After motility activation there was a continuous and fast decrease in the values rendered by CASA until 60 seconds, this decrease is related to different factors, common to other marine fish spermatozoa, reviewed by Cosson et al. (2008): energy loss (ATP content decrease), increased intracellular ionic concentration, structural damages, etc. This decrease in motility parameters was very intense, and at 60 seconds motility values were similar along the year.

Regarding DNA integrity, there was an increase in DNA fragmentation during the spring spawning season reaching its maximum after the end of this period (June). This fact is probably related to some cell ageing phenomenon, meaning that cells extracted were already overripe or apoptotic. Suquet et al. (1998) observed in turbot an increase in chromatin decondensation in sperm undergoing an ageing process. Moreover, in our case DNA integrity might also be related with water temperature once it followed a similar pattern to this biotic parameter. In mammals the effect of temperature on sperm DNA integrity (Banks et al., 2005) has already been

demonstrated. A complete understanding of this and other biotic effects on *S. senegalensis* spermatogenesis has not yet been achieved. Additionally, cell apoptosis could be one of the causes of DNA fragmentation (Lee and Steinert, 2003; Pérez-Cerezales et al., 2009). Nonetheless, in our case, there was no correlation between cell DNA fragmentation and the percentage of apoptotic cells. This enforces the hypothesis that different factors are responsible for causing DNA destabilization. Other factors, such as reactive oxygen species, can affect DNA integrity (Sakkas and Alvarez, 2010) during cell ageing. *Solea senegalensis* has a group-synchronous testicular development (Garcia-López et al., 2006) and hence there is a higher probability of the appearance of overripe cells in the testicular lumen in the later months of the spawning season. In the same way, the lowest percentage of apoptotic cells throughout the year was observed in March (beginning of the first spawning), followed by a constant increase in apoptotic cells until the summer months.

Furthermore, there was a clear negative effect of artificial seawater on the *S. senegalensis* sperm membrane, a fact already observed in other marine fish (Gwo, 1995). Besides the low resistance of the cells to seawater hyperosmolarity (common to all samples in all months) the percentage of cells with a functional membrane after seawater exposure was clearly affected by the season. During the summer months the males registered the lowest percentage of cells resistant to seawater osmotic effects. If ruptures happen in *S. senegalensis* plasma membrane as observed by Suquet et al. (1998) in turbot spermatozoa towards the end of the reproductive season, they could be responsible for the decrease in cells plasma membrane resistance to seawater hyperosmolarity. Even during the spawning season, seawater hyperosmolarity seems to be a limiting factor of cell survival since the percentage of cells with a functional membrane decreased very rapidly during the 60 s of our assay. This would indicate that fertilization must occur immediately after gamete release. The observed low resistance of spermatozoa to seawater hyperosmolarity could act as a selection mechanism (causing a high percentage of cell lysis) to eliminate cells presenting high DNA fragmentation values, since both parameters were negatively correlated. Similarly, Babiak et al. (2006) observed aged spermatozoa lysis in halibut. Furthermore, low sperm membrane resistance to the seawater hyperosmotic effect is counteracted by *S. senegalensis* reproductive behavior. During spawning male and female swim in synchrony with the genital ducts held closely together (Duncan et al., 2008), increasing the probability of achieving fertilization by diminishing sperm scattering and the contact period with seawater. In the case of *S. senegalensis*, sensitivity to hyperosmolarity is lower when a non-ionic solution is used, as reported by Martínez-Pastor et al. (2008) using a 1100 mOsm/kg sucrose solution, supporting the hypothesis of the importance of the above-mentioned reproductive behavior and the close releasing of both male and female gametes.

Moreover, sperm velocity and the percentage of progressive cells 15 seconds after motility activation were negatively correlated with the percentage of apoptotic cells (cells stained with

Annexin-V FITC). Cells stained with Annexin-V FITC underwent membrane destabilization which could in part alter membrane permeability and affect cell osmotic control and thus affect motility and spermatozoa velocity. The same effect was previously reported in human sperm, where a negative correlation between apoptotic cells and motility was described (Zhang et al., 2008).

From the different analyzed parameters a common characteristic was high heterogeneity between the different males (demonstrated by the SEM obtained in all tested parameters) both during the year and the spawning seasons. This high heterogeneity between males in the semen quality had already been reported in previous works referring to different parameters (Beirão et al., 2009; Cabrita et al., 2006; Martínez-Pastor et al., 2008). Clearly, the different males are at their peak of semen quality at different moments of the spawning seasons and even in different periods of the year, denoting the existence of asynchronization within males and between males and females. In some species, this fact is attributed to male competition, for example in *Telmatochromis vittatus* and *Salvelinus alpinus*, where differences in ejaculate quality and spermatozoa speed have been identified as a result of sperm competition and male social status (Fitzpatrick et al., 2007; Rudolfsen et al., 2006). To our knowledge there is no information concerning male competition, dominance or other territorial behavior in *S. senegalensis*. In this case, semen quality variation will probably result in unequal contribution from males to the offspring once there is a high chance of just some males contributing to the following generation (Porta et al., 2006).

From a general point of view there was a tendency for the semen to attain a quality peak within the spawning season (March-May) followed by a pronounced decrease. This fact is in accordance with other species such as Atlantic halibut (Babiak et al., 2006) and turbot (Suquet et al., 1998). Even at this peak, sperm quality values were very low, mainly, cell membrane resistance to seawater hyperosmolarity. The lowest values for most parameters were observed during the months of June and July, corresponding to the months with highest temperature, indicating a possible effect of temperature on sperm quality and evidence of sperm ageing during this period. Although sperm can be collected during all the year, quality is highly variable, which means that manipulation of the spawning season needs to be controlled in both males and females. This could be especially important when collecting sperm outside of the natural spawning season for cryopreservation or artificial fertilization. Our results could be a useful tool in studies regarding manipulation of biotic and abiotic parameters.

Acknowledgements

The authors would like to thank Patricia Diogo, Vânia Santos and Emilio Mota for their help during the experimental procedures. This work was supported by a PhD fellowship (SFRH/BD/31990/2006) and REPROSOL 2006.71.001.443.9 project.

Bibliography

Agulleiro, M.J., Scott, A.P., Duncan, N., Mylonas, C.C., Cerda, J., 2007. Treatment of GnRHa-implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comp. Biochem. Phys. A 147(4), 885-892.

Anguís, V., Salas-Leiton, E., Martín, I., Chereguini, O., 2008. Influence of the feeding on sperm quality of first generation (f1) *Solea senegalensis* during an annual cycle in two facilities of Spain, in: Aquaculture Europe 08, Krakow, Poland.

Anguís, V., Cañavate, J.P., 2005. Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. Aquaculture 243(1-4), 133-145.

Babiak, I.,Ottesen, O., Rudolfsen, G., Johnsen, S., 2006. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 65(8), 1587-1604.

Banks, S., King, S.A., Irvine, D.S., Saunders, P. T. K., 2005. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. Reproduction 129(4), 505-514.

Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., Cabrita, E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72, 1251–1261.

Beirão, J., Cabrita, E., Soares, F., Herráez, M.P., Dinis, M.T., 2008. Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: comet analysis and Annexin V–Fluorescein staining. J Appl. Ichthyol. 24, 508–513.

Cabrita, E., Soares, F., Beirão, J., García-López, A., Martinez-Rodriguez, G., Dinis, M. T., 2011. Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. Theriogenology 75(1), 1-9.

Cabrita, E., Soares, F., Dinis, M.T., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261(3), 967-975.

Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., Herraez, M. P., 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50(2), 144-153.

Cosson, J., Groison, A., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008. Marine fish spermatozoa: racing ephemeral swimmers. Reproduction 136, 277-294.

Duncan, N., Carazo, I., Chereguini, O., Norambuena, F., Mañanós, E., Estevez, A., 2008. Observations of spawning in wild caught and captivity reared broodstock, in: Report of the IV Workshop on the Cultivation of Soles, Faro, Portugal, pp. 7-8.

Fitzpatrick, J.L., Desjardins, J.K., Milligan, N., Montgomerie, R., Balshine, S., 2007. Reproductive-tactic-specific variation in sperm swimming speeds in a shell-brooding Cichlid. Biol. Reprod. 77(2), 280-284.

García-López, A., Fernandez-Pasquier, V., Couto, E., Canario, A.V.M., Sarasquete, C., Martínez-Rodríguez, G., 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen. Comp. Endocr. 147(3), 343-351.

García-López, A., Martínez-Rodríguez, G., Sarasquete, C., 2005. Male reproductive system in Senegalese sole *Solea senegalensis* (Kaup): Anatomy, histology and histochemistry. Histol and Histopathol 20(4), 1179-1189.

Guzmán, J.M., Norberg, B., Ramos, J., Mylonas, C.C., Mañanos, E.L., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). Gen. Comp. Endocr. 156(2), 285-297.

Gwo, J.C., 1995. Ultrastructural study of osmolality effect on spermatozoa of 3 marine teleosts. Tissue Cell 27(5), 491-497.

Imsland, A.K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis*. Rev. Fish. Biol. Fisher. 13(4), 379-407.

Lee, R.F., Steinert, S., 2003.Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mut. Res.-Rev. Mutat. 544(1), 43-64.

Mañanos, E., Guzmán, J.M., Ramos, J., Mylonas, C.C., Zohar, J., Peleteiro, J.B., Chereguini, O., Duncan, N., Sarasquete, C., Riaza, A., 2008. Hormonal stimulation of ovulation, spermiation and spawning in cultured Senegalese sole (*Solea senegalensis*) broodstock, in: Report of the IV Workshop on the Cultivation of Soles, Faro, Portugal, pp. 16-17.

Martínez-Pastor, F., Cabrita, E., Soares, F., Anel, L., Dinis, M.T., 2008. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 135(4), 449-59.

Mylonas, C.C., Papadaki, M., Divanach, P., 2003. Seasonal changes in sperm production and quality in the red porgy *Pagrus pagrus* (L.). Aquac. Res. 34(13), 1161-1170.

Oliveira, C., Vera, L.M., López-Olmeda, J.F., Guzmán, J.M., Mañanós, E., Ramos, J., Sánchez-Vázquez, F.J., 2008. Monthly day/night changes and seasonal daily rhythms of sexual steroids in Senegal sole (*Solea senegalensis*) under natural fluctuating or controlled environmental conditions. Comp. Biochem. Phys. A 152(2), 168-175.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P., Herráez, M. P., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71(4), 605-613.

Porta, J., Porta, J.M., Martinez-Rodriguez, G., Alvarez, M.C., 2006. Genetic structure and genetic relatedness of a hatchery stock of Senegal sole (*Solea senegalensis*) inferred by microsatellites. Aquaculture 251(1), 46-55.

Rainis, S., Mylonas, C.C., Kyriakou, Y., Divanach, P., 2003. Enhancement of spermiation in European sea bass (*Dicentrarchus labrax*) at the end of the reproductive season using GnRHa implants. Aquaculture 219(1), 873-890.

Rudolfsen, G., Figenschou, L., Folstad, I., Tveiten, H., Figenschou, M., 2006. Rapid adjustments of sperm characteristics in relation to social status. Proc. R. Soc. Lond. B Biol. Sci. 273(1584), 325-332.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234(1-4), 1-28.

Sakkas, D., Alvarez, J. G., 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil. Steril. 93 (4), 1027-1036.

Suquet, M., Dreanno, C., Dorange, G., Normant, Y., Quemener, L., Gaignon, J.L., Billard, R., 1998. The ageing phenomenon of turbot spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization, and storage capacities. J. Fish Biol. 52(1), 31-41.

Trippel, E.A., 2003. Estimation of male reproductive success in marine fishes. J. Northwest Atl. Fish. Sci. 33, 81-113.

Zhang, H.B., Lu, S.M., Ma, C.Y., Wang, L., Li, X., Chen, Z.J., 2008. Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation. Asian. J. Androl. 10(2), 227-235.

Chapter 1.3.

Sperm quality of *Solea senegalensis* could be improved through diet

J. Beirão[a], F. Soares[b], P. Pousão-Ferreira[b], P. Diogo[c], J. Dias[c], M.T. Dinis[c], M.P. Herráez[a], E. Cabrita[d]

[a]Dept. Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain
[b]INIAP, IPIMAR-CRIP Sul, Av. 5 Outubro, 8700-305 Olhão, Portugal
[c]Center for Marine Sciences-CCMAR, University of Algarve, 8005-139 Faro, Portugal
[d]Institute of Marine Sciences of Andalusia- ICMAN, Spanish National Research Council, Av Republica Saharaui, 2, 11510 Puerto Real, Cadiz, Spain

Under review

Abstract

A strategy to improve *Solea senegalensis* sperm quality through diet was developed. Three broodstocks were fed on specific diets: control diet, acid docosahexaenoic (DHA) supplemented diet and DHA plus antioxidants (selenium and vitamin E) diet. During the spawning season fish were sampled for blood and semen. Glutathione peroxidase activity, measured as an indirect contribution of Se, was significantly higher in blood plasma of males fed on DHA plus antioxidants. Both percentage of progressive spermatozoa and sperm velocity were significantly higher in fish fed on DHA plus antioxidants, whereas fish fed only on DHA did not show any improvement. Sperm plasma membrane integrity before and after motility activation was not affected by the diets. Conversely, lipid peroxidation values were significantly higher in sperm of males fed on DHA diet. This value was positively correlated with the amount of lysophosphatidyl-choline and sphingomyelin. There was an increase in cholesterol (CHO) and in several unsaturated fatty acids in sperm from males fed on DHA plus antioxidants, and at the same time, there was a decrease in the amounts of saturated fatty acids. The addition of both DHA and antioxidants significantly affected *S. senegalensis* sperm lipidic composition and their synergic effects improved sperm quality traits.

Key words: *Solea senegalensis*; sperm quality; diet; docosahexaenoic acid; vitamin E; selenium; phospholipids; fatty acids

Abbreviations: AA, arachidonic acid; CASA, computer assisted sperm analysis; CHO, cholesterol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatography; GSH-Px, glutathione peroxidase; HPLC, high performance liquid chromatography; LPC, lysophosphatidyl-choline; MDA, malondialdehyde; % Mot, percentage of motile sperm; MUFA, monounsaturated fatty acids; PC, phosphatidyl-choline; PE, phosphatidyl-ethanolamine; PL, phospholipids; PI, phosphatidyl-inositol; % Pro, percentage of progressive sperm; PS, phosphatidyl-serine; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; Se, selenium; SM, sphingomyelin; VCL, spermatozoa curvilinear velocity; vitE, vitamin E

1.Introduction

Nutrition is a key factor controlling gonadal development and fecundity. According to Izquierdo et al., (2001) lipid and fatty acid composition of broodstock diet are the main factors affecting reproduction success and offspring survival. While membrane composition in phospholipids (PL) and cholesterol (CHO) determine sperm membrane fluidity, CHO regulates the lipid chain order and molecular organization of the membranes (Muller et al., 2008; Wassall and Stillwell, 2009). In this sense several authors have investigated sperm contents in PL, CHO and fatty acids and their interaction to understand how their composition will affect sperm quality and influence its ability to successfully fertilize eggs (Drokin, 1993; Labbé et al., 1995; Bell et al., 1997; Muller et al., 2008; Lahnsteiner et al., 2009; Henrotte et al., 2010). In the case of sperm compared with somatic cells, PLs have a high content in polyunsaturated fatty acids (PUFA) (Bell et al., 1997) and the PUFA of the n-3 and n-6 series cannot be synthesized by vertebrates and thus must be provided by the diet. It is commonly accepted that the increase in PUFA improves sperm membrane fluidity and hence its resilience to osmotic effects when sperm is ejaculated. This may be an important aspect in marine and freshwater fish with external fertilization. Moreover, some authors have observed that diet composition in fatty acids, CHO and PL could change the composition of the sperm (Labbé et al., 1995; Vassallo-Agius et al., 2001; Nandi et al., 2007) and affect males' reproductive performance (Asturiano et al., 2001). The addition of PUFA to the diet, usually leads to an increase in the sensitivity of the membranes to lipid peroxidation (Surai et al., 1998). Because spermatozoa have a high metabolic rate, a significant production of reactive oxygen species (ROS) can be generated (Edens and Brake, 2006). Although the production of ROS by spermatozoa is a normal physiological process, at certain levels it may causes lipid peroxidation, damage of midpiece, axonema and DNA, loss of motility and in the last instance, infertility (Lahnsteiner et al., 2010a). Hence, a complex antioxidant defense system is present in the semen, which is highly affected by the diet (Mansour et al., 2006). In reproductive tissues, several enzymes (e.g. glutathione peroxidase and superoxide dismutase) and compounds (e.g. ascorbic acid, glutathione and α-tocopherol) are involved in the antioxidant system. A balance between ROS production and the antioxidant system must be established in order not to affect sperm quality (Mansour et al., 2006). The antioxidant effect of vitamin E (tocopherol) can provide the sperm cells with important protection during spermatogenesis by reducing the risk of lipid peroxidation, protecting the biomembranes from degenerative damage (Izquierdo et al., 2001). Therefore, many authors have added antioxidant components such as vitamins to fish diets (Lee and Dabrowski, 2004; Mansour et al., 2006; Rainis et al., 2007; Canyurt and Akhan, 2008).

Also, the selenoprotein glutathione peroxidase (GSH-Px), plays a crucial role in male gamete quality, given that it catalyses the degradation of both lipid peroxides and hydrogen peroxide,

which are toxic for the sperm cells (Surai et al., 1998; Edens and Brake, 2006; Shalini and Bansal, 2007). Moreover, its important antioxidant activity was demonstrated in *Cyprinus carpio* by Li et al. (2010). Even so, there are no studies on the dietary effects of the trace element selenium (Se) on fish sperm quality. Selenium is usually added together with vitamin E (vitE), since according to studies by Diplock and Lucy (1973), vitE acts as a protector of selenium containing proteins.

Despite increasing research in Senegalese sole (*Solea senegalensis*) reproductive physiology (Forne et al., 2009; Diogo et al., 2010; Guzmán et al., 2011), poor egg quality and low fertilization rates are still impairing the massive culture of this species. Following our previous results where we demonstrated that spawns with low-quality (low rate of fertilization) in Senegalese sole broodstocks are in part related to poor and variable semen quality and to spermatozoa with high sensitivity to seawater hyperosmolarity, (Cabrita et al., 2006; Martinez-Pastor et al., 2008; Beirão et al., 2009; Beirão et al., 2011a), impairing sperm from several males to achieve fertilization, we decided to design a strategy to improve sperm quality. In this work we tested specific diets, enriched with docosahexaenoic acid (DHA), vitE and Se, aiming to improve sperm membrane resistance to seawater hyperosmolarity and hence sperm quality.

2. Material and methods

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (Spain) and were reagent grade or higher. Solvents were purchased from Romil (Spain).

2.1. Fish and rearing conditions

Individuals used in these experiments were captured in the wild, acclimatized and stocked in the facilities of the IPIMAR research station (37º02’N, 7º49’W) Olhão, Portugal. Fish (1:1, female:male) were kept in 18,000 L concrete outdoor tanks with a water depth of 1.2 m. The 83 individuals (males and females) used in this experiment, were equally distributed in 3 tanks and had a mean body weight of 1.55±0.37 Kg and mean total length of 52±4 cm. Each fish was tagged with an individual PIT Tag (Trovan, NL). Tanks were supplied with continuous aeration and water exchange was 30% h^{-1}. Each tank corresponded to a different treatment and contained between 13 and 16 males. Fish handling was in accordance with the European Union Directive (EEC, 1986) for the protection of animals used for experimental and other scientific purposes.

2.2. Feeding regime

Three experimental wet-dry feeds were formulated for Senegalese sole breeders (SPAROS, S.A.): a control diet, a control diet supplemented with DHA and a third diet supplemented with DHA, Se (0.25 mg/Kg) and vitE (800 mg/Kg) (DHA+antiox) (Table 1). Fishes were fed daily from December until the end of the experiment with 3% body weight of the corresponding wet-dry feed.

Table 1 – Composition of the experimental diets as percentage.

As fed basis	Control (%)	DHA (%)	DHA + antiox (%)
Fishmeal 67/10	20.00	20.00	20.00
Fishmeal 60/9	21.00	15.00	10.00
CPSP 90	10.00	10.00	11.00
CPSP G	10.00	18.00	17.00
Squid meal	5.00	5.00	8.50
Krill meal	0.00	2.50	2.50
Soybean meal 48	6.00	5.00	4.00
Sel-Plex (selenium)	**0.00**	**0.00**	**0.025**
Wheat DDGS	18.50	7.00	8.82
Fish oil	7.50	11.50	12.00
Krill oil	0.00	1.00	1.00
Vit & Min Premix	2.00	4.00	4.00
Vit E 50	**0.00**	**0.00**	**0.15**
Algatrium DHA	**0.00**	**1.00**	**1.00**
Theoretical composition			
Crude protein	54.83	54.75	54.77
Crude fat	16.11	22.97	22.88
Gross Energy	21.07	22.82	22.35

Control refer do control diet, DHA to diet supplemented with DHA and DHA + antiox to diet supplemented with DHA, Se and vitE.

2.3 Samplings: sperm and blood

Samplings were performed twice a month for all tanks during the reproductive season (April - June), collecting sperm and blood from each male. In each sampling individuals were anesthetized in a seawater tank with 300 ppm 2-phenoxyethanol and checked for sperm

fluidity. To obtain the semen, the urogenital pore was cleaned to eliminate mucus, feces, and water, and a syringe without a needle was used to collect the semen by gently pressing the testes on the fish blind side. The sperm samples were placed in microcentrifuge tubes and kept at 7ºC for further analysis. Samples contaminated with urine or seawater were discarded. From the uncontaminated samples belonging to the same treatment a pool was mixed and further analyzed. A total of 5 pools of sperm were analyzed for all assays for each treatment.

Blood samples were collected in the 1st, 3rd and 5th samplings by puncturing the caudal vein with a syringe (3000 IU heparin in 0.01 M PBS). Samples were centrifuged at 3000 × g, 10 min, 4ºC and plasma was stored at -20 ºC until analysis.

2.4. Sperm quality analysis

2.4.1. Motility

Concentration and motility of pooled sperm were evaluated using a computer assisted sperm analysis (CASA) system (ISAS Integrated System for Semen Analysis, Proiser, Valencia, Spain) coupled to a phase contrast microscope (Nikon E-200, Nikon, Tokyo, Japan) with a 10 × negative phase contrast objective. One-µL of diluted sperm (2 µL of semen in 25 µL of 300 mOsm/kg sucrose) was activated with 5 µL artificial seawater (1100 mOsm/kg). Motility was recorded at 15 and 30 seconds post-activation. Each sample was analyzed thrice. CASA settings were adjusted to Senegalese sole spermatozoa according to Beirão et al. (2009). From the parameters rendered by CASA the following were recorded and further analyzed: percentage of motile sperm (% Mot), percentage of progressive sperm (% Pro) and the curvilinear velocity (VCL, µm/s).

2.4.2. Viability and hyperosmotic test

Plasma membrane integrity before and after exposure to seawater hyperosmolarity was measured following the protocol by Beirão et al. (2009), using SYBR 14 and propidium iodide (PI) (LIVE/DEAD sperm viability Kit, Molecular Probes, Spain) to stain intact and damage cells, respectively. Briefly, 1 µL of the semen was activated with 9 µL of seawater and fixed with 4% glutaraldehyde at different times after activation (15 and 30 seconds). Cells were stained in the dark with SYBR 14 at a final concentration of 0.25 µM for 10 min, plus 5 min with 0.24 mM PI. Three slides were observed with fluorescence (450-490 nm) under the microscope (Nikon E-200, Nikon, Tokyo, Japan), and at least 100 cells were counted per slide: green stained cells were considered viable cells and red stained cells as membrane damaged cells. Results are presented as percentage of cells showing a functional membrane before and after exposure to seawater hyperosmolarity.

2.4.3. Lipid peroxidation assay

Sperm lipid peroxidation was measured by determining the malondialdehyde (MDA) production using the Bioxytech MDA-586 kit (OxisResearch, Spain) adapted for sperm analysis. For each measure, 30 µL of pooled semen was used and a calibration curve and a blank were prepared. Results are expressed as µM MDA per million cells.

2.4.4. Lipid analysis

Lipids were extracted from the sperm pools according the procedure by Folch et al. (1957), adjusted for sole sperm, and stored in 300 µL of isopropanol. Cholesterol was measured with the CHOD PAP Kit (Biolabo, France). For each measure a calibration curve was prepared with CHO.

Total PL were quantified by the procedure developed by Rouser et al., (1970). For PL purification, samples were passed through SEP-PAK silica gel cartridges (Waters, Spain), first 10 mL chloroform was pushed through the cartridge to eliminate neutral lipids, then 5 mL acetone was pushed through the cartridge to eliminate glycolipids and finally 10 mL methanol was pushed through to elute PL, which were collected in a glass vial and stored in chloroform/methanol (1/2, V/V).

For the detection and quantification of the different classes of PL, samples were analyzed in a HPLC (Waters 2695), equipped with an autoinjector and a UV detector (Waters 996) using a Waters Spherisorb column PSS838521 (Waters, Spain) (5 µm, 250 mm × 3.0 mm i.d.). The following mobile phase was used (acetonitrile/ methanol/ phosphoric acid, 130/5/1.5, V/V/V) at a constant flow rate of 1.0 mL/min. Peaks were identified and measured at 203 nm by comparison with standards. Results are presented as mg per mmol of PL.

For fatty acids analysis, methyl esters were obtained following the protocol by Berry et al. (1965) and analyzed with a gas chromatographer (GC) (Perkin Elmer Autosystem XL) equipped with a flame ionization detector and using a fused silica capillary column Omegawax 250 (Supelco, Spain) (30 m × 0.25 mm i.d. × 0.25 µm film thickness). Helium was used as the carrier gas and the following conditions were applied: initial temperature set at 50ºC for 2 min; warming to 200ºC at a rate of 7ºC/min; 2 min at 200ºC; warming to 220ºC at a rate of 0.5ºC and; 5 min at 220ºC. Three µL of sample were injected for each analysis. Fatty acids methyl esters standards mixture (C4 – C24:1) was used to identify and quantify the peaks and methyl nonanoate was used as internal standard. Results are represented as relative proportion to total fatty acids.

2.5. Glutathione peroxidase assay

Glutathione peroxidase (GSH-Px) activity was determined as an indirect contribution of Se since GSH-Px is a selenoprotein that incorporates the majority of Se present in the testes. Given that GSH-Px is also present in blood and because of the low sperm volume available we decided to test GSH-Px enzymatic activity in blood samples (plasma). The analysis was performed by spectrophotometry using the Ransel KIT (Randox, Ireland), measuring the absorbance values at time 0 and 4 min after the reaction started. Total protein was measured with the RC DC Protein Assay (Bio-Rad, Spain). Results are presented as IU GSH-Px per g of protein.

2.6. Data analysis

All statistics were conducted using the SPSS 15.0 software for Windows. With the exception of the chromatography data, all the analyses were performed in triplicate. Results are presented as mean values ± SEM (standard error of the mean).

Concentration data was non-parametric and hence was analyzed with a Kruskal-Wallis test. Percentile data was normalized through arcsine transformation. % Mot, % Pro, VCL, % of cells with functional membrane, MDA, CHO/PL ratio, PL classes and fatty acids data were analyzed with a General Linear Model (with the Bonferroni’s correction), considering sampling dates as covariable. GSH-Px activity was analyzed with a one-way ANOVA and Duncan’s pairwise comparisons. Differences were considered significant for $p<0.05$. Correlations between the distinct parameters were detected with Pearson’s parametric correlation coefficient or Spearman Rho in the case of cell concentration ($p<0.05$ or $p<0.01$).

3. Results

Sperm concentration was higher for the DHA+antiox treatment ($5.2\pm1.7\times10^9$ spermatozoa/mL), although there were no significant differences in comparison with the control ($2.5\pm0.4\times10^9$ spermatozoa/mL) (Fig. 1). Also, for the percentage of motile sperm there were no significant differences (Fig. 2A). On the other hand, for both the percentage of progressive sperm (Fig. 2B), and the VCL (Fig. 2C), the DHA+antiox treatment presented significantly higher values at 15 and 30 s post-

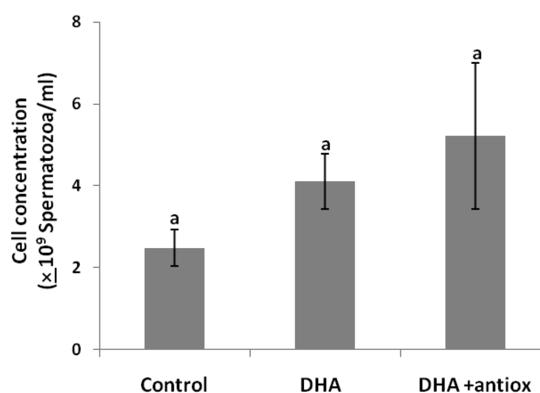


Fig. 1 – Sperm cell concentration (mean ± SEM) observed in pools from the different treatments: control diet, DHA (diet supplemented with DHA), DHA+antiox (diet supplemented with DHA, Se and vitE). Data correspond to. Different letters mean significant differences (p<0.05).

activation (20.7±2.2 and 19.5±2.0% of progressive cells and 149.4±11.0 and 131.5±11.0 µm/s for VCL at 15 and 30 s, respectively) when compared to the control (12.9±2.2 and 10.6±1.2% of progressive cells and 101.0±9.8 and 86.4±8.2 µm/s for VCL at 15 and 30 s, respectively). For the DHA treatment, no significant increase was observed with regard to the control for any of the motility parameters analyzed. Neither the percentages of viable cells nor cells resistant to osmotic shock presented significant differences between any of the two treatments and the control (Fig. 3).

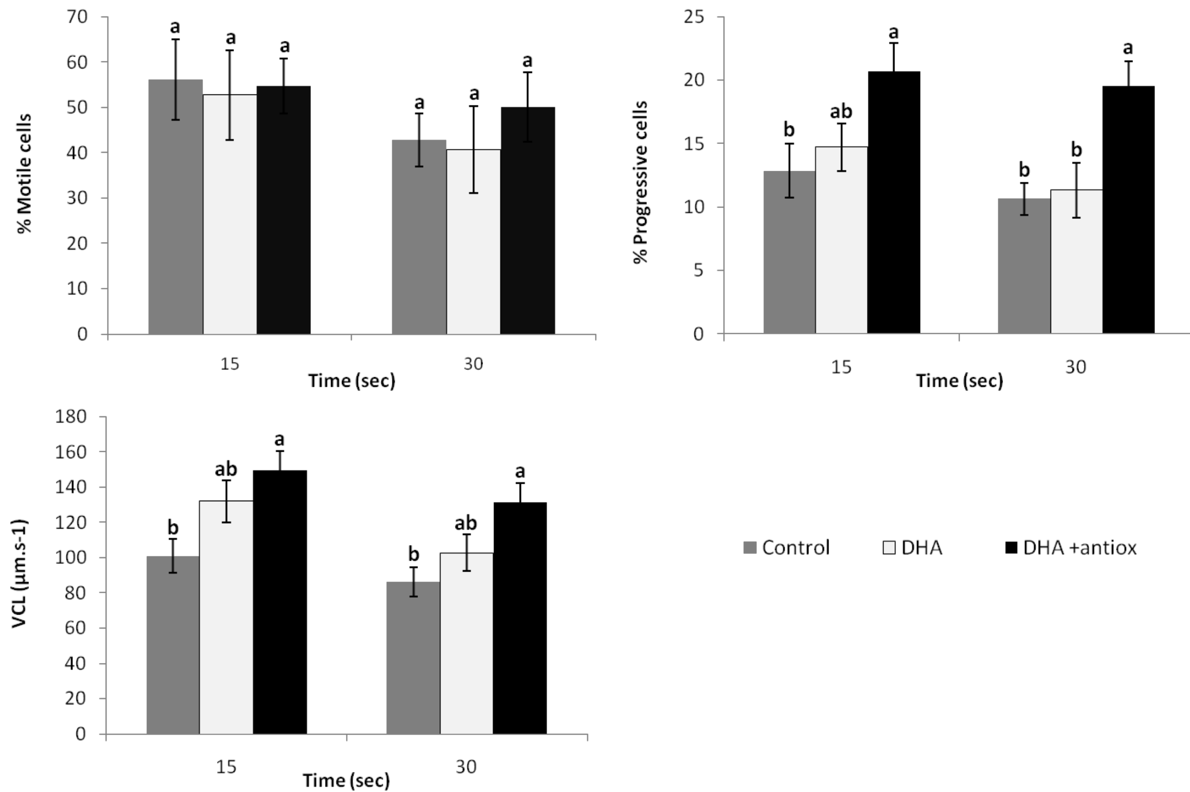


Fig 2 – Percentage of motile cells (A), percentage of progressive cells (B) and of VCL (C) for 15 and 30 seconds after motility activation. The different color bars represent the different diets, grey (control diet), white (diet supplemented with DHA) and black (diet supplemented with DHA, Se and vitE). Data correspond to mean values ± SEM. Different letters mean significant differences for the same time ($p<0.05$).

Lipid peroxidation determined through the MDA concentration in sperm showed significant higher values in the DHA treatment ($7.47±1.81×10^{-3}$) compared with both control ($3.24±0.007×10^{-3}$) and DHA+antiox treatment ($2.66 ±1.15×10^{-3}$) as shown in Fig. 4.

The ratio of CHO/PL increased significantly in sperm from males fed on DHA+antiox treatment ($59.9±2.1×10^{2}$) in comparison with the control ($47.0±2.3 × 10^{2}$) (Table 2), indicating an increase in the quantity of CHO in the sperm of fishes fed on the experimental diets. From the analyzed

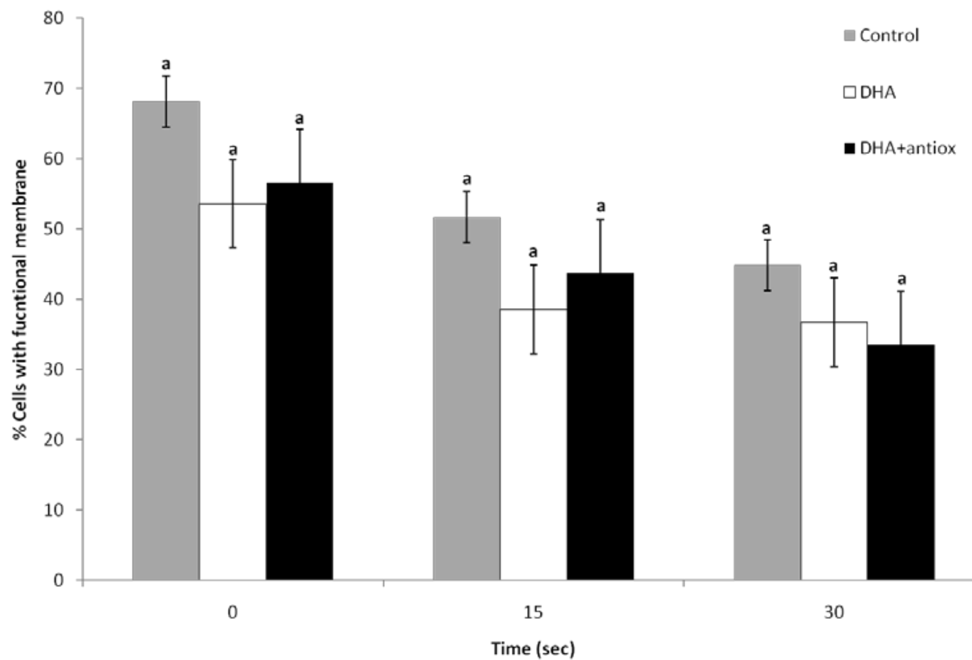


Fig. 3 – Percentage of cell with functional membrane before motility activation (viable cells) and 15 and 30 seconds after motility activation (percentage of cells resistant to the osmotic shock). The different color bars represent the different diets, grey (control diet), white (diet supplemented with DHA) and black (diet supplemented with DHA, Se and vitE). Data correspond to mean values ± SEM. Different letters mean significant differences for the same time ($p<0.05$).

PL, lysophosphatidyl-choline (LPC) and sphingomyelin (SM) were higher in the sperm of males fed on the DHA supplemented diet but this difference was only significant when compared to the DHA+antiox treatment (Table 2). Also, phosphatidyl-choline (PC) and phosphatidyl-ethanolamine (PE) increased in both DHA and DHA+antiox treatments but this increase was not significant. Phosphatidyl-serine (PS) and phosphatidyl-inositol (PI) were also detected but did not show significant differences between treatments. The fatty acids results are also expressed in Table 2. There was a significant decreased in the total saturated fatty acids of males fed on DHA (35.75±0.57) and DHA+antiox (36.49±0.77) when compared with the control diet (44.49 ± 4.05), these differences were perceptible in the saturate

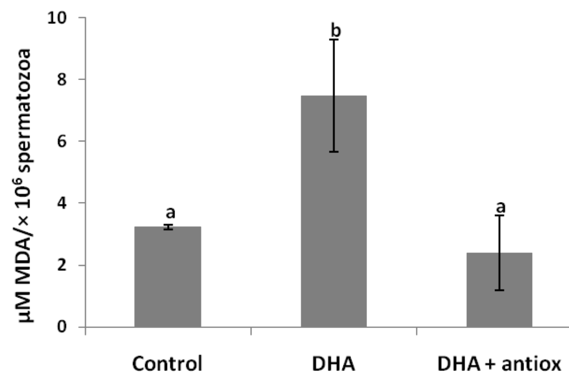


Fig. 4 – MDA concentrations (mean± SEM) from the pooled samples from the different treatments: control diet, DHA (diet supplemented with DHA), DHA+antiox (diet supplemented with DHA, Se and vitE). Different letters mean significant differences ($p<0.05$).

fatty acids C17, C18 and C24. On the other hand, the amount of MUFA and n6 fatty acids increased in males fed on the experimental (DHA and DHA+antiox) diets. As an example males fed on DHA+antiox had 8.58±1.01 % of oleic acid (C18:1n9) and 3.51±0.38 % of linoleic acid (C18:2n6), while males fed on the control diet only had 5.17±0.71 % and 2.03±0.29 %, respectively. As expected, there was an increase in the proportion of DHA in sperm from males fed on the supplemented diets, although this difference was only significant in samples from DHA+antiox treatment (37.26±0.39 %). Furthermore, the proportion of both eicosapentaenoic acid/arachidonic acid (EPA/AA) and DHA/EPA was also dependent on the diet provided. The EPA/AA proportion decreased significantly in the sperm from males fed on supplemented diets, because of the increase in the amount of AA. The proportion of DHA/EPA was significantly higher in sperm from males fed on DHA+antiox. The sperm fatty acids composition (DHA: EPA: AA) of the males fed on the diet supplemented with DHA+antiox was 207/13.11/1.

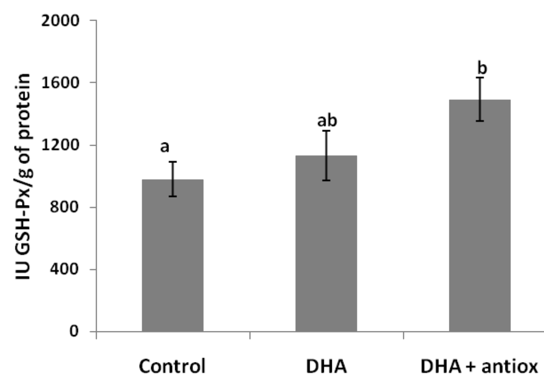


Fig. 5 – Glutathione peroxidase (GSH-Px) activity (mean ± SEM) in blood plasma samples obtained from individuals fed on different diets: control diet, DHA (diet supplemented with DHA), DHA+antiox (diet supplemented with DHA, Se and vitE). Different letters mean significant differences ($p<0.05$).

Several significant correlations were obtained between the analyzed parameters, the most important being represented in Table 3. Cell concentration, VCL and the percentage of cells showing progressive movement were positively correlated with the proportion of DHA in sperm. VCL and the percentage of cells with progressive movement were also positively correlated with the ratio of CHO/PL and the amount of PUFA. Moreover, the MDA concentration was positively correlated with the amount of LPC and SM detected.

Finally, GSH-Px activity in blood (Fig. 5) presented significantly higher values in the DHA+antiox treatment (1493.4±140.7 IU GSH-Px/g of protein) when compared with the control treatment (980.0±171.0 IU GSH-Px/g of protein).

In summary, DHA+antiox diet supplementation improved sperm progressiveness, velocity and increased the ratio of CHO/PL in the sperm in comparison with the control, while the higher lipid peroxidation observed in the DHA treatment returned to the control diet levels with the DHA+antiox. Sperm lipid composition was affected by the diets.

Table 2 – Cholesterol/phospholipids ratio, phospholipid classes (mg per mmol of phospholipids) and fatty acids (percentage) composition of the sperm of *S. senegalensis* fed with the three experimental diets. Control refer do control diet, DHA to diet supplemented with DHA and DHA + antiox to diet supplemented with DHA, Se and vE.

	Control	***DHA***	***DHA + antiox***
CHO/PL ×10²	**47.0 ± 2.3[b]**	**55.8 ± 2.1[ab]**	**59.9 ± 2.1[a]**
Phospholipids mg/mmol PL			
PI	53.76 ± 53.76[a]	90.02 ± 57.08[a]	142.52 ± 64.15[a]
PS	168.56 ± 63.39[a]	104.35 ± 16.87[a]	157.44 ± 32.14[a]
PE	27.69 ± 27.69[a]	150.03 ± 59.39[a]	151.37 ± 53.13[a]
PC	362.37 ± 111.11[a]	485.91 ± 138.52[a]	712.33 ± 67.20[a]
LPC	**152.30 ± 90.55[ab]**	**466.94 ± 49.51[a]**	**143.91 ± 65.26[b]**
SM	**114.68 ± 95.13[ab]**	**485.27 ± 34.55[a]**	**131.42 ± 48.01[b]**
Fatty acids (%)			
C10	0.05 ± 0.01[a]	0.03 ± 0.00[a]	0.03 ± 0.01[a]
C12	0.10 ± 0.05[a]	0.06 ± 0.02[a]	0.05 ± 0.02[a]
C14 (myristic acid)	0.70 ± 0.05[a]	0.59 ± 0.07[a]	0.97 ± 0.36[a]
C15	0.37 ± 0.02[a]	0.32 ± 0.02[a]	0.34 ± 0.03[a]
C16 (palmitic acid)	29.15 ± 1.08[a]	27.80 ± 0.65[a]	27.76 ± 0.93[a]
C16:1n7	1.01 ± 0.15[a]	1.14 ± 0.16[a]	1.14 ± 0.16[a]
C17	**0.65 ± 0.08[a]**	**0.42 ± 0.03[b]**	**0.51 ± 0.03[ab]**
C18 (stearic acid)	**14.75 ± 3.18[a]**	**6.50 ± 0.31[b]**	**6.54 ± 0.73[b]**
C18:1n9 (oleic acid)	**5.17 ± 0.71[b]**	**6.95 ± 0.35[ab]**	**8.58 ± 1.01[a]**
C18:2n6 (linoleic acid)	**2.03 ± 0.29[b]**	**2.94 ± 0.24[a]**	**3.51 ± 0.38[a]**
C18:3n6	0.15 ± 0.02[a]	0.11 ± 0.01[a]	0.15 ± 0.03[a]
C18:3n3	0.11 ± 0.01[a]	0.11 ± 0.03[a]	0.12 ± 0.03[a]
C20	0.29 ± 0.04[a]	0.20 ± 0.04[a]	0.25 ± 0.06[a]
C20:1n9	**0.40 ± 0.08[b]**	**0.64 ± 0.07[a]**	**0.75 ± 0.08[a]**
C20:2n6	0.76 ± 0.23[a]	1.06 ± 0.18[a]	0.71 ± 0.10[a]
C20:3n6	0.22 ± 0.02[a]	0.27 ± 0.04[a]	0.31 ± 0.03[a]
C20:3n3 (eicosatrienoic acid)	**7.94 ± 0.92[b]**	**9.74 ± 0.48[a]**	**10.00 ± 0.18[a]**
C20:4n6 (AA)	0.08 ± 0.02[a]	0.17 ± 0.05[a]	0.18 ± 0.03[a]
C20:5n3 (EPA)	2.18 ± 0.36[a]	1.96 ± 0.15[a]	2.36 ± 0.05[a]
C22	0.29 ± 0.06[a]	0.15 ± 0.02[a]	0.23 ± 0.03[a]
C22:1n9	0.12 ± 0.01[a]	0.11 ± 0.01[a]	0.13 ± 0.01[a]
C22:6n3 (DHA)	**26.19 ± 4.57[b]**	**35.16 ± 2.24[ab]**	**37.26 ± 0.39[a]**
C24	**0.28 ± 0.05[a]**	**0.15 ± 0.01[b]**	**0.22 ± 0.03a[b]**
Total Saturated	**44.49 ± 4.05[a]**	**35.75 ± 0.57[b]**	**36.49 ± 0.77[b]**
Total MUFA	**7.44 ± 0.58[b]**	**8.83 ± 0.46[ab]**	**10.58 ± 0.88[a]**
Total PUFA	40.99 ± 6.54[a]	51.77 ± 3.09[a]	54.69 ± 0.74[a]
Total n3	40.15 ± 4.95[a]	46.97 ± 2.76[a]	49.72 ± 0.46[a]
Total n6	**3.07 ± 0.24[b]**	**4.67 ± 0.19[a]**	**5.03 ± 0.42[a]**
n3/n6	12.06 ± 1.04[a]	10.03 ± 1.00[a]	10.01 ± 0.82[a]
EPA/AA	**35.39 ± 4.74[a]**	**16.00 ± 3.04[b]**	**15.44 ± 2.73[b]**
DHA/EPA	**12.07 ± 1.13[b]**	**18.29 ± 1.40[a]**	**15.80 ± 0.41a[b]**

Data correspond to mean values ± SEM. In bolt the parameters with significant differences, indicated by different letters ($p<0.05$).

Table 3 – Most important correlations obtained with Pearson's correlation or Spearman Rho in the case of cell concentration. Significant correlations with p<0.05 are signed*, or p<0.01 with **.

Obtained correlation	**(R)**
Cell concentration/DHA	0.656**
VCL 15 s/(Cho/Pho)	0.496*
% Pro 15 s/(Cho/Pho)	0.532*
VCL 15 s/DHA	0.777**
% Pro 15 s/DHA	0.646**
VCL 15 s/PUFA	0.769**
% Pro 15 s/PUFA	0.726*
MDA/LPC	0.709*
MDA/SM	0.755*

4. Discussion

Attempts made so far to increase low sperm volume and/or quality produced by Senegalese sole using hormonal therapies such as GnRHa treatments (Agulleiro et al., 2007) or testing the effect of female-to-male communication (Cabrita et al., 2010) have reported few advances and definitive protocols to improve sperm quality have yet to be established. In the present study we showed that it is possible to improve sperm sole quality through the incorporation of specific compounds in fish meal developing specific diets for this species during the reproductive period. Nevertheless, as theoretically proposed, cell resistance to the seawater hazardous external medium was not improved, even though there is a clear change in the lipidic components of the sperm. Unfortunately, we did not analyze separately seminal plasma and spermatozoon lipid composition because of the very low volume of sperm ejaculate by sole, but rather the overall lipids composition, thus we do not know if the observed variations in saturated and unsaturated fatty acids refer to seminal plasma or spermatozoa cells. This issue could explain the problem in detecting an improvement in membrane sperm resistance since it is not clear whether the incorporated lipids were absorbed into the plasma membrane.

Although the effects of experimental diets were not visible in plasma membrane viability and its resistance to hyperosmotic shock after motility activation, the motility parameters (% Pro and VCL) showed an improvement in sperm from males fed on the DHA+antiox diet, which probably increased the chances of spermatozoa to achieve fertilization, as observed by Casselman et al. (2006) for *Sander vitreus* and by our group for *Sparus aurata* (Beirão et al., 2011b). Nonetheless, in the case of *Solea* this issue must be further tested using artificial fertilization

techniques that are still under development for this species. Also, in *Barbus barbus* Alavi et al. (2009) observed an increase in sperm velocity in fishes fed on diets containing a higher PUFA content, while Asturiano et al. (2001) observed higher volumes of milt and higher percentage of larval survival in *Dicentrarchus labrax*. In the case of Senegalese sole, Anguís et al. (2008) observed some improvement in the sperm traits of F1 males after feeding with squid and mussels compared with males fed on commercial pellets and in the case of *Solea solea* the best reproductive performance in terms of spawn quality was obtained when broodstocks were fed on higher amounts of PUFA, specifically DHA (Cardinalleti et al., 2009).

Another parameter analyzed for sperm quality was cell concentration, and despite the fact that a slightly increase in sperm concentration was observed in fish fed on supplemented diets, this increase was not significant. This fact could have been related with the high variability between males referred to in previous studies (Beirão et al., 2009).

Several studies have shown a positive dietary effect of PUFAs and their incorporation in fish sperm composition (Labbé et al., 1995; Asturiano et al., 2001; Alavi et al., 2009; Henrotte et al., 2010). In the present experiment several lipidic components were affected by the diets. CHO usually acts as a rigidifying component of mammalian sperm membranes (Ladha, 1998). Muller et al. (2008) found that there was an increase in the resistance of the membrane to hypoosmotic stress in *Oncorhynchus mykiss* sperm, despite the fact that membrane fluidity decreased with increasing CHO. In our treatments, CHO/PL levels were higher in the DHA+antiox treatment than in control group, yet we could not detect whether this increase corresponded to plasma membrane cholesterol or to free cholesterol, explaining the fact why sperm membrane resistance was not affected by the change in the amount of CHO.

Also, in the present work there was an increase in the amount of DHA in the sperm composition, although this increase was only significant for the DHA+antiox diet: this could be explained by the increase in lipid peroxidation observed in the DHA treatment. In a similar study in chicken sperm, Cerolini et al. (2006) observed that n-3 fatty acids, particularly DHA incorporation was affected by vitE doses present in feeds, revealing that although DHA incorporation could be beneficial for some sperm functions, it needs to be incorporated with certain levels of antioxidants. As explained by Wassall and Sillwell (2009), DHA presence in plasma membrane increases fluidity, thus it would be expected to sustain hyperosmotic shock better. Once again we do not know if the increase in DHA refers to the cell or the seminal plasma, even though Lahnsteiner et al. (2009) in *O. mykiss* found that DHA was in much higher concentration in spermatozoa than in seminal plasma.

At the same time in our study there was an increase in several unsaturated fatty acids (total amount of MUFAs and n6) in the sperm of males fed on the supplemented diets, while there was a decrease in the amount of saturated fatty acids. The increase in MUFAs was specially caused by the increase in oleic acid (C18:1n9) and C20:1n9. The other unsaturated fatty acids

that showed an increase were eicosatrienoic acid (C20:3n3) and linoleic acid (C18:2n6). The latter in freshwater fish is the precursor of AA and some evidence of the same mechanism was observed in *D. labrax* (Asturiano et al., 2001). In our case there was an increase in AA in the sperm of males fed on supplemented diets, yet this increase was not significant, but it was reflected in the EPA/AA ratio (it decreased significantly in the experimental treatments). DHA, EPA and AA are all important in maintaining cell plasma membrane functions (Sargent et al., 1999), and despite the minor contribution of AA in fish sperm plasma membrane, it is also a key component of membranes and small variations would affect its function. As studied by Cerda et al. (1995) the deficiency of dietary lipids depresses testicular steroidogenesis and PUFAs (AA, EPA and DHA) can significantly affect prostaglandin production (Asturiano et al., 2000) and hence could have affected the different observed sperm traits of our individuals. As explained by Izquierdo et al. (2001), marine fish species need highly unsaturated fatty acids in their diet for correct maturation and steroidogenesis, and their presence increases fecundity and fertility. Thus the fact that velocity and progressiveness values were correlated with several other parameters such as the DHA and PUFA proportion showed that Senegalese sole sperm improvement and modulation is possible through diet.

On the other hand, the amount of saturated fatty acids was negatively affected by the supplemented diets, and in several studies in mammalian sperm the amount of saturated fatty acids has been negatively correlated with both sperm motility and viability (Aksoy et al., 2006; Am-In et al., 2011), which indicates that in some way saturated fatty acids are prejudicial for sperm quality.

Furthermore we could speculate that the possible increase in some free fatty acids (e.g. linoleic acid) in the semen could have been used as an energy resource as observed in *S. aurata* sperm by Lahnsteiner et al. (2010b) and thus explain, at least in part, the increase in sperm velocity.

Only a significant improvement in sperm quality was observed for the DHA+antiox diet rather than for the DHA diet, probably because of the harmful effects of the ROS on the spermatozoa causing the high observed lipid peroxidation values (MDA). The high levels of malondialdehyde produced due to lipid peroxidation could also produce a negative effect on other functions of the cell and even bring about an increase in DNA fragmentation. As demonstrated by Zhou et al. (2006) in *C. carpio* there is a decrease in spermatozoa motility as result of the oxidative damage in DNA and lipid peroxidation that modifies sperm lipids. Clearly, the increase in lipid peroxidation, observed in the males only fed on the supplemented DHA diet, was circumvented by the addition of Se and vitE to the diets as observed by the decrease in MDA results, since there were significantly higher values for the DHA treatment compared to the control and DHA+antiox treatments. The importance of antioxidants protecting sperm from lipid peroxidation has been highlighted by some authors (Pérez-Cerezales et al., 2009; Lahnsteiner and Mansour, 2010). As a consequence of lipid peroxidation, there was also an increase in LPC

and SM and both values were correlated with MDA levels. While the generation of LPC by PC breakdown by phospholipases has been described as a result of the deterioration of human sperm (Glander et al., 2002), cryopreservation procedures (Schiller et al., 2000) and in the membrane fusion process (Petcoff et al., 2008), the meaning of the increase in SM observed in our study is not clear. SM is very abundant in the myelin sheet of some nerve cell axons and erythrocytes in ruminant mammals, nevertheless in sperm cells its function is unknown. Some authors support that SM might play a role in membrane fusion (Rogasevskaia and Coorssen, 2006). To our knowledge there are no reports of the increase in SM as result of PUFA addition to the diets or increased lipid peroxidation, in fact feeding rats with PUFA rich diets, resulted in increased circulating sphingomyelinase activity (Drachmann et al., 2007), responsible for breaking SM. Nevertheless in chicken semen the addition of vitE to the diet resulted in a reduction in the proportion of SM (Surai et al., 1997), coinciding with our results. The other PL did not change significantly between treatments and the amounts were similar to those observed by Labbé et al. (1995) in *O. mykiss* sperm plasma membrane, by Drokin (1993) in spermatozoa from 10 different freshwater and marine fish species and by Henrotte et al. (2010) in *Perca fluviatilis*, PC being more abundant in marine species and PE in freshwater species. In our case PS was slightly higher, probably because its concentration is higher in seminal plasma, as is the case of human sperm (Grizard et al. 2000).

Furthermore, the increase in the % of Pro and VCL in the DHA+antiox treatment may also be related with the addition of Se in the diet, since in bovines fed on diets supplemented with Se there was an improvement in sperm motility (Siegel et al., 1980). Selenium supplementation has been widely studied in mammal and poultry reproduction, and it is established that both Se deficiency and excess can impair sperm quality, causing morphological alterations, improper chromatin condensation and reducing sperm motility, finally leading to fertilization failure (Surai et al., 1998; Edens and Brake, 2006; Shalini and Bansal, 2008). Selenium is a key component of the antioxidant enzyme glutathione peroxidase (GHS-Px). In this study, GHS-Px activity was significantly increased in blood samples of animals fed on DHA+antiox diet compared with the control diet, and at the same time there was a reduction in lipid peroxidation values and an increase in some sperm quality values. Li et al. (2010) showed in *C. carpio* sperm that, compared with other antioxidant enzymes, GHS-Px is the main antioxidant response to lipid peroxidation. Therefore it could be hypothesized that both control and DHA diet were deficitary in Se, causing a decrease in sperm quality. Nevertheless the amount of Se that allows an optimal reproductive performance should be optimized. Together with Se, vitE was also incorporated in the diet. Vitamin E is the major lipid soluble antioxidant present in cell membranes, playing a crucial role in breaking the chain of peroxidation. Several reports describe the effects of dietary vitE on male fish performance. Lee and Dabrowski (2004) working with *Perca flavescens* observed no improvement in sperm parameters. However this fact could be related to the low vitE doses tested (160 mg/Kg) compared with that used in our

study (800 mg/Kg). Conversely in other studies, dietary supplementation of vitE in *O. mykiss* broodstock (Rainis et al., 2007; Canyurt and Akhan, 2008) improved sperm concentration and motility parameters, and in *Salvelinus alpinus* (Mansour et al., 2006) there was a decrease in semen lipid peroxidation.

This work was the first approach to improving Senegalese sole sperm quality through fatty acids and antioxidant dietary enrichment. The results showed that this improvement is possible and that explain the low quality of sperm in some broodstocks established in captivity. Further studies are needed to have a more complete vision of the specific needs in fatty acids in this species, given that as pointed out by Sargent et al. (1999), different species of fish have different dietary needs according to their metabolic adaptations.

Acknowledgements

The authors would like to thank Dr Catherine Labbé from INRA (France) for providing the protocols for lipid extraction and processing, Francisca Martínez from LTI (University of León, Spain) for the help provided during lipids analysis and Catarina Oliveira for helping during sampling. J. Beirão was supported by a PhD grant from the Portuguese Ministry of Science and Technology (SFRH/BD/31990/2006) co-founded by POPH - QREN - Tipologia 4.1 (FEDER and MCTES) and E. Cabrita by a research contract (RYC -2007-01650) .

References

Agulleiro, M., Scott, A., Duncan, N., Mylonas, C., Cerda, J., 2007. Treatment of GnRHa-implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comp. Biochem Physiol. A 147, 885-892.

Aksoy, Y., Aksoy, H., Altinkaynak, K., Aydin, H., Ozkan, A., 2006. Sperm fatty acid composition in subfertile men. Prostaglandins Leukot. Essent. Fatty Acids 75, 75-79.

Alavi, S., Psenicka, M., Policar, T., Rodina, M., Hamackova, J., Kozak, P., Linhart, O., 2009. Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season. Fish Physiol. Biochem. 35, 683-693.

Am-In, N., Kirkwood, R., Techakumphu, M., Tantasuparuk, W., 2011. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology 75, 897-903.

Anguís, V., Salas-Leiton, Martín, E., Chereguini, O., 2008. Influence of the feeding on sperm quality of first generation (f1) *Solea Senegalensis* during an annual cycle in two facilities of Spain. Aquaculture Europe 08, Krakow, Poland, September 15-18

Asturiano, J., Sorbera, L., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J., Bromage, N., 2001. Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture 194, 173-190.

Asturiano, J., Sorbera, L., Zanuy, S., Carrillo, M., 2000. Effects of polyunsaturated fatty acids and gonadotropin on prostaglandin series E production in a primary testis cell culture system for the European sea bass. J. Fish Biol. 57, 1563-1574.

Beirão, J., Soares, F., Herráez, M., Dinis, M., Cabrita, E., 2009. Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. Theriogenology 72, 1251-1261.

Beirão, J., Soares, F., Herráez, M., Dinis, M., Cabrita, E., 2011a. Changes in *Solea senegalensis* sperm quality throughout the year. Anim. Reprod. Sci, *in press.*

Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S., Herráez, M., 2011b. Effect of cryopreservation on fish sperm subpopulations. Cryobiology 62, 22-31.

Bell, M., Dick, J., Buda, C., 1997. Molecular speciation of fish sperm phospholipids: Large amounts of dipolyunsaturated phosphatidylserine. Lipids 32, 1085-1091.

Berry, J., Cevallos, W., Wade, R., 1965. Lipid class and fatty acid composition of intact peripheral nerve and during wallerian degeneration. J. Am. Oil Chem. Soc. 42, 492-500.

Cabrita, E., Soares, F., Beirão, J., Garcia-López, A., Martínez-Rodriguez, G., Dinis, M., 2011. Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. Theriogenology 75, 1-9.

Cabrita, E., Soares, F., Dinis, M., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261, 967-975.

Canyurt, M., Akhan, S., 2008. Effect of dietary vitamin E on the sperm quality of rainbow trout (*Onchorynchus mykiss*). Aquac. Res. 39, 1014-1018.

Cardinaletti, G., Mosconi, G., Salvatori, R., Lanari, D., Tomassoni, D., Carnevali, O., Polzonetti-Magni, A., 2009. Effect of dietary supplements of mussel and polychaetes on spawning performance of captive sole, *Solea solea* (Linnaeus, 1758). Anim. Reprod. Sci. 113, 167-176.

Casselman, S., Schulte-Hostedde, A., Montgomerie, R., 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci. 63, 2119-2125.

Cerda, J., Zanuy, S., Carrillo, M., Ramos, J., & Serrano, R., 1995. Short-term and long-term

dietary-effects on female sea bass (*Dicentrarchus labrax*): seasonal changes in plasma profiles of lipids and sex steroids in relation to reproduction. Comp. Biochem. Physiol. C 111, 83-91.

Cerolini, S., Zaniboni, L., Maldjian, A., Gliozzi, T., 2006. Effect of docosahexaenoic acid and alpha-tocopherol enrichment in chicken sperm on semen quality, sperm lipid composition and susceptibility to peroxidation. Theriogenology 66, 877-886.

Diogo, P., Soares, F., Dinis, M., Cabrita, E., 2010. The influence of ovarian fluid on *Solea senegalensis* sperm motility. J. Appl. Ichthyol. 26, 690-695.

Diplock, A., Lucy, J., 1973. Biochemical modes of action of vitamin-E and selenium - Hypothesis. FEBS Lett. 29, 205-210.

Drachmann, T., Mathiessen, J., Pedersen, M., Hellgren, L., 2007. The source of dietary fatty acids alters the activity of secretory sphingomyelinase in the rat. Eur. J. Lipid Sci. Technol. 109, 1003-1009.

Drokin, S., 1993. Phospholipids and fatty-acids of phospholipids of sperm from several fresh-water and marine species of fish. Comp. Biochem. Physiol. B 104, 423-428.

Edens, F. W., Brake, J., 2006. The role of selenium in poultry reproduction. World's Poult. Sci. J. 62.

Folch, J., Lees, M., Stanley, G., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497-509.

Forne, I., Agulleiro, M., Asensio, E., Abian, J., Cerda, J., 2009. 2-D DIGE analysis of Senegalese sole (*Solea senegalensis*) testis proteome in wild-caught and hormone-treated F1 fish. Proteomics 9, 2171-2181.

Glander, H., Schiller, J., Suss, R., Paasch, U., Grunewald, S., Arnhold, J., 2002. Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines. Andrologia 34, 360-366.

Grizard, G., Sion, B., Bauchart, D., Boucher, D., 2000. Separation and quantification of cholesterol and major phospholipid classes in human semen by high-performance liquid chromatography and light-scattering detection. J. Chromatogr. B, 740, 101-107.

Gúzman, J., Cal, R., Garcia-Lopez, A., Chereguini, O., Kight, K., Olmedo, M., Sarasquete, C., Mylonas, C., Peleteiro, J., Zohar, Y., Mañanos, E., 2011. Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis of Senegalese sole (*Solea senegalensis*). Comp. Biochem. Physiol. A 158, 235-245.

Henrotte, E., Kaspar, V., Rodina, M., Psenicka, M., Linhart, O., Kestemont, P., 2010. Dietary n-

3/n-6 ratio affects the biochemical composition of Eurasian perch (*Perca fluviatilis*) semen but not indicators of sperm quality. Aquac. Res. 41, 31-38.

Izquierdo, M., Fernandez-Palacios, H., Tacon, A., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197, 25-42.

Labbé, C., Maisse, G., Muller, K., Zachowski, A., Kaushik, S., Loir, M., 1995. Thermal-acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma-membrane. Lipids 30, 23-33.

Ladha, S., 1998. Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. J. Membr. Biol. 1651, 1-10.

Lahnsteiner, F., Mansour, N., McNiven, M., Richardson, G., 2009. Fatty acids of rainbow trout (*Oncorhynchus mykiss*) semen: Composition and effects on sperm functionality. Aquaculture 298, 118-124.

Lahnsteiner, F., Mansour, N., 2010. A comparative study on antioxidant systems in semen of species of the Percidae, Salmonidae, Cyprinidae, and Lotidae for improving semen storage techniques. Aquaculture 307, 130-140.

Lahnsteiner, F., Mansour, N., Plaetzer, K., 2010a. Antioxidant systems of brown trout (*Salmo trutta f. fario*) semen. Anim. Reprod. Sci. 119, 314-321.

Lahnsteiner, F., Mansour, N., Caberlotto, S., 2010b. Composition and metabolism of carbohydrates and lipids in Sparus aurata semen and its relation to viability expressed as sperm motility when activated. Comp. Biochem. Physiol. B 157, 39-45.

Lee, K., Dabrowski, K., 2004. Long-term effects and interactions of dietary vitamins C and E on growth and reproduction of yellow perch, *Perca flavescens*. Aquaculture 230, 377-389.

Li, P., Li, Z., Dzyuba, B., Hulak, M., Rodina, M., Linhart, O., 2010. Evaluating the Impacts of Osmotic and Oxidative Stress on Common Carp (*Cyprinus carpio*, L.) Sperm Caused by Cryopreservation Techniques. Biol. Reprod. 83, 852-858.

Mansour, N., McNiven, M., Richardson, G., 2006. The effect of dietary supplementation with blueberry, alpha-tocopherol or astaxanthin on oxidative stability of Arctic char (*Salvelinus alpinus*) semen. Theriogenology 66, 373-382.

Martínez-Pastor, F., Cabrita, E., Soares, F., Anel, L., Dinis, M., 2008. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 135, 449-459.

Muller, K., Muller, P., Pincemy, G., Kurz, A., Labbé, C., 2008. Characterization of sperm plasma membrane properties after cholesterol modification: Consequences for cryopreservation of rainbow trout spermatozoal. Biol. Reprod. 78, 390-399.

Nandi, S., Routray, P., Gupta, S., Rath, S., Dasgupta, S., Meher, P., Mukhopadhyay, P., 2007. Reproductive performance of carp, *Catla catla* (Ham.), reared on a formulated diet with PUFA supplementation. J. Appl. Ichthyol. 23, 684-691.

Pérez-Cerezales, S., Martínez-Páramo, S., Cabrita, E., Martínez-Pastor, F., de Paz, P., Herráez, M., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71, 605-613.

Petcoff, D., Holland, W., Stith, B., 2008. Lipid levels in sperm, eggs, and during fertilization in *Xenopus laevis*. J. Lipid Res. 49, 2365-2378.

Rainis, S., Gasco, L., Ballestrazzi, R., Zoccarato, I., 2007. Effects of vitamin E and phosphatidylcholine on qualitative and quantitative parameters of rainbow trout (*Oncorhynchus mykiss*) milt. Ital. J. Anim. Sci. 6, 339-350.

Rogasevskaia, T., Coorssen, J., 2006. Sphingomyelin-enriched microdomains define the efficiency of native Ca^{2+}-triggered membrane fusion. J. Cell Sci. 119, 2688-2694.

Rouser, G., Fleischer, S., Yamamoto, A., 1970. 2 Dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*, *5*, 494-496.

Sargent, J., Bell, G., McEvoy, L., Tocher, D., Estevez, A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177, 191-199.

Schiller, J., Arnhold, J., Glander, H., Arnold, K., 2000. Lipid analysis of human spermatozoa and seminal plasma by MALDI-TOF mass spectrometry and NMR spectroscopy - effects of freezing and thawing. Chem. Phys. Lipids 106, 145-156.

Shalini, S., Bansal, M., 2008. Dietary selenium deficiency as well as excess supplementation induces multiple defects in mouse epididymal spermatozoa: understanding the role of selenium in male fertility. Int. J. Androl. 31, 438-449.

Siegel, R., Murray, F., Julien, W., Moxon, A., Conrad, H., 1980. Effect of *in vitro* selenium supplementation on bovine sperm motility. Theriogenology 13, 357-367.

Surai, P, Kostjuk, I., Wishart, G, Macpherson, A., Speake, B, Noble, R, Ionov, I., Kutz, E., 1998. Effect of vitamin E and selenium supplementation of cockerel diets on glutathione peroxidase activity and lipid peroxidation susceptibility in sperm, testes, and liver. Biol. Trace Elem. Res. 64, 119-132.

Surai, P.F., Kutz, E., Wishart, G.J., Noble, R.C., Speake, B.K., 1997. The relationship between the dietary provision of alpha-tocopherol and the concentration of this vitamin in the semen of chicken: Effects on lipid composition and susceptibility to peroxidation. J. Reprod. Fertil. 110, 47-51.

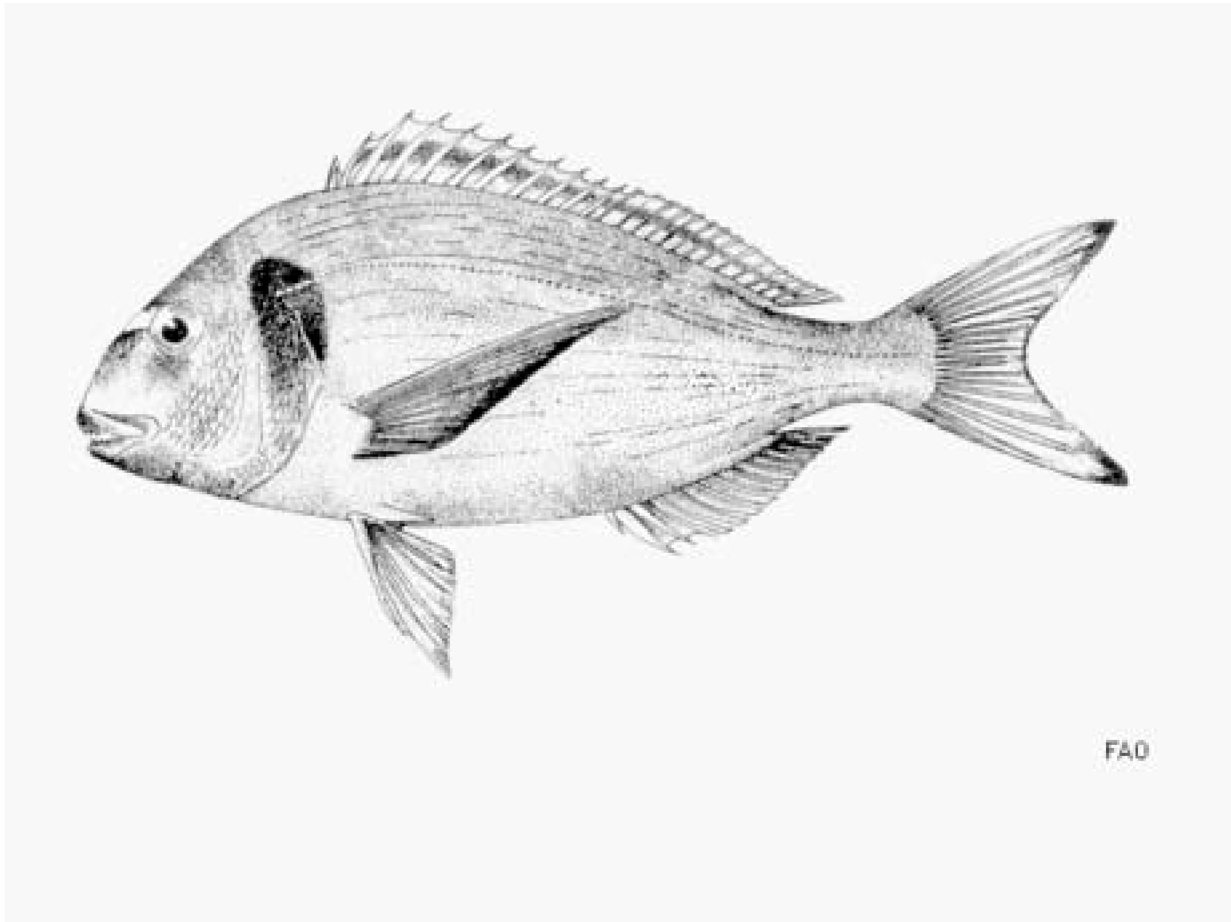
Vassallo-Agius, R., Watanabe, T., Yoshizaki, G., Satoh, S., Takeuchi, Y., 2001. Quality of eggs and spermatozoa of rainbow trout fed an n-3 essential fatty acid-deficient diet and its effects on the lipid and fatty acid components of eggs, semen and livers. Fish. Sci. 67, 818-827.

Wassall, S., Stillwell, W., 2009. Polyunsaturated fatty acid-cholesterol interactions: Domain formation in membranes. Biochim. Biophys. Acta-Biomembr. 1788, 24-32.

Zhou, B., Liu, W., Siu, W., O'Toole, D., Lam, P., Wu, R., 2006. Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. Aquat. Toxicol. 77, 136-142.

Chapter 2

Evaluation and improvement of sperm cryopreservation in marine teleosts (*Sparus aurata*)


FAO

Chapter 2.1.

Effect of cryopreservation on fish sperm subpopulations

J. Beirão[a], E. Cabrita[b], S. Pérez-Cerezales[a], S. Martínez-Páramo[c], M.P. Herráez[a]

[a] Dept. of Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain
[b] Institute of Marine Sciences of Andalusia – ICMAN, Spanish National Research Council, Av. Republica Saharaui, 2, 11510 Puerto Real, Cadiz, Spain
[c] Center for Marine Sciences – CCMAR, University of Algarve, 8005-139 Faro, Portugal

Cryobiology 62 (2011) 22–31

doi:10.1016/j.cryobiol.2010.11.005

Abstract

The evaluation of the motility data obtained with a CASA system, applying a Two-Step Cluster analysis, identified in seabream sperm 3 different sperm subpopulations that correlated differently with embryo hatching rates. Hence, we designed an experiment to understand the effect of the application of different cryopreservation protocols in these sperm motility-based subpopulations. We analyzed *Sparus aurata* frozen/thawed semen motility 15, 30, 45 and 60 s after activation, using CASA software. Different protocols were applied for cryopreservation: three different cryoprotectants (Dimethyl Sulfoxide (Me_2SO), Ethylene Glycol (EG) and Propylene Glycol (PG)) each at two different concentrations and two packaging volumes (0.5 ml straws, and 1.8 ml cryovials) were tested. Different freezing rates were evaluated corresponding to 1, 2, 3, 4 and 8 cm above the liquid nitrogen surface for the straws and 1, 2 and 4 cm for the cryovials. Motility parameters rendered by CASA were treated with a Two-Step Cluster analysis. Three different subpopulations were obtained: SP1 - slow non-linear spermatozoa, SP2 - slow linear spermatozoa and SP3 - fast linear spermatozoa. We considered SP3 as the subpopulation of interest and focused further analyses on it. Generally, SP3 was the best represented subpopulation 15 s after activation and was also the one showing a greater decrease in time, being the least represented after 60 s. According to the applied univariate general linear model, samples frozen in straws with 5% Me_2SO and in cryovials with 10% Me_2SO at 2 and 1 cm from the $LN_{2,}$ respectively, produced the best results (closer to the control). Clustering analysis allowed the detection of fish sperm subpopulations according to their motility pattern and showed that sperm composition in terms of subpopulations was differentially affected by the cryopreservation protocol depending on the cryoprotectant used, freezing rates and packaging systems.

Keywords: CASA, Cryopreservation, *Sparus aurata*, Sperm quality, Subpopulations

Introduction

The coexistence of different sperm subpopulations has recently been given considerable attention, mostly in mammals [1,19,22,23, 24,25,32]. These different sperm populations can be identified in terms of membrane resistance to osmotic shock [3], morphology [32], motility [21], etc. Motility is the most widely used parameter for studying subpopulations. Motility parameters are often measured with computer-assisted sperm analysis (CASA) for both fresh and cryopreserved sperm. Different authors usually try to correlate motility data with fertilization rates [16,18,28]. Nevertheless, most authors working with fish do not take full advantage of the CASA systems. Most motility analyses only rely on the mean values of few parameters from the several rendered by the CASA, considering the whole semen sample as homogeneous. In recent years, multivariate statistical analyses have been used with CASA data, generally in mammal semen [1,19,20,25], and in some cases with unfrozen fish sperm [16,21]. Martinez-Pastor et al. [21] and Le Comber et al.[16] have clearly shown coexistence within the same sample of distinct motility-based sperm subpopulations in *Solea senegalensis* and *Gasterosteus aculeatus*, respectively. Moreover, studies in mammals have shown that the cryopreservation process affects sperm subpopulations composition [19,22,23].

Successful cryopreservation protocols have already been developed for gilthead seabream (*Sparus aurata*) [6,10]. However, cryopreservation protocols always imply a loss of sperm quality. Both studies by Fabbrocini et al. [10] analyzing post-thaw motility and Cabrita et al. [6] analyzing post-thaw membrane integrity, resistance to hyperosmotic shock and mitochondrial functionality, reported a loss of quality caused by cell damage. Likewise, sperm cryopreservation has been successfully applied to several other marine fish species [30], however there are always differences in post-thaw semen quality depending on the protocols (cryoprotectants, extenders, freezing/thawing rates, etc.). Apart from the existence of different sperm subpopulations in an ejaculate, cryopreservation should not be considered a homogeneous process, as pointed out by Taddei et al. [31] and is highly dependent on the cryopreservation conditions.

After the identification of different subpopulations of spermatozoa in seabream (*S. aurata*) samples according to their motility characteristics and the observation that their fertilization ability might be different, we proposed applying this approach to identify more precisely the effect of different cryopreservation treatments on the structure of spermatozoa subpopulations, as an alternative to the use of mean values. For this purpose we cryopreserved gilthead seabream sperm with different cryoprotectants, freezing rates and packaging volumes and analyzed sperm subpopulations applying a Two-Step Clustering analysis to the different parameters rendered by the CASA system for each individual spermatozoa, at different times after motility activation.

Materials and methods

All chemicals were purchased from Sigma and were of reagent grade or higher.

Gametes

Sperm and eggs were obtained from breeders from the Maresa (Huelva, Spain) broodstock. Sperm was collected using a 1 ml syringe without a needle and applying abdominal massage. Samples contaminated with urine and seawater were discarded. Sperm motility (at least 50% motile cells), osmolality and pH were verified to assure sperm quality (acceptable values between 350 and 400 mOsm/kg and 7.5 and 8.0 for pH). Samples were maintained in 15 ml centrifuge tubes, at 7 ºC, and transported to our facilities at the University of León to be used in cryopreservation trials. Females were intramuscularly injected with GnRH 25 µg/kg 48 h before egg extraction to activate the ovulation process. Eggs were obtained by abdominal massage and collected in a plastic beaker. Egg quality was controlled by visual inspection to discard possible bad quality spawns. Fertilization trials were conducted in the ICMAN-CSIC facilities (Cadiz, Spain).

Sperm motility

Sperm motility was analyzed using a CASA system which consisted of an optical phase contrast microscope (Nikon Eclipse E-200, Tokyo, Japan), with an attached Basler 302 fs digital camera (Basler Vision Technologies, Ahrensburg, Germany). Images were captured with a 10× negative phase contrast objective and analyzed using the Integrated System for Semen Analysis (ISAS) software (Proiser, Valencia, Spain). Fresh or thawed sperm was prediluted in sucrose (1 µl semen: 25 µl sucrose 300 mOsm/kg) and 10 µl artificial seawater (30 g/l NaCl; 0.8 g/l KCl; 1.3 g/l $CaCl_2$; 6.6 g/l $MgSO_4$; 0.18 g/l $NaHCO_3$) was added to 1 µl of the diluted sperm to activate sperm motility. Motility was recorded 15, 30, 45 and 60 s after activation. CASA rendered the percentage of motile cells in the sperm sample as well as the following parameters for each analyzed sperm: VCL (velocity according to the actual path; µm/s), VSL (velocity according to the straight path; µm/s), VAP (velocity according to the smoothed path; µm/s), LIN (linearity;%), STR (straightness;%), WOB (wobble;%), ALH (amplitude of the lateral displacement of the sperm head; µm), and BCF (beat-cross frequency; Hz). These parameters have been described elsewhere [24,29]. The software settings were adjusted to fish spermatozoa: 25 images per second; 1 to 90 μm^2 for head area; and VCL >10 µm/s to classify a spermatozoon as motile. Image analyses were carefully reviewed in order to be sure that all records referred to sperm cells.

Fertilization trials

Sperm from 14 males presenting different sperm quality was used to perform fertilization trials. Pooled eggs from 2 females were separated in 42 batches (three for each sperm sample) of 1 g (approximately 1050 eggs). Each egg batch was fertilized with 25 µl of sperm and incubated in 100 ml beakers. The sperm concentration of each sample ($20.4 \pm 4.4 \times 10^9$ spermatozoa/ml) was measured with the CASA system in order to calculate the sperm/ egg ratio. Sperm was mixed with the eggs before activation with 2 ml of seawater. After 10 min the seawater volume was adjusted to 100 ml, and left to incubate at 18 ºC for 72 h in the dark. Following the incubation period the number of hatched larvae was counted. The hatching rate was calculated taking into account the total number of incubated eggs.

Cryopreservation trials

Semen from five pools of 25 ml (10 to 12 males each pool) was cryopreserved, each pool with 48 different cryopreservation protocols in a factorial design as follows: (a) three different cryoprotectants (CPAs) added to 1% NaCl (w/v) at two different concentrations each: Dimethyl sulfoxide (Me_2SO) at 5% and 10% (v/v) (final concentration 0.6 and 1.2 M, respectively), ethylene glycol (EG) and propylene glycol (PG) both at 10% and 15% (v/v) (final concentration 1.5 and 2.3 M for EG, and 1.2 and 1.8 M for PG); (b) two different packaging volumes: 0.5 ml straws and 1.8 ml cryovials; and (c) different distances from the liquid nitrogen (LN_2) surface (1, 2, 3, 4 and 8 cm for the straws and 1, 2 and 4 cm for the cryovials), providing different freezing rates. The sperm pools were diluted 1/6 (v/v) in the extender solution. For the different freezing rates floating devices (20 × 20 cm) were used inside a 40 × 30 × 30 cm styrofoam box with 5 cm of LN_2. A 4 min equilibration period was applied to all treatments, plus 10 min in the freezing device inside the styrofoam box. After this time both cryovials and straws were directly plunged in LN_2. The samples were thawed in a water bath for 30 s at 25 ºC for the straws and 2.5 min at 40 ºC for the cryovials.

Cooling and thawing rates were measured using a thermocouple, the temperature values were recorded placing the thermocouple sensor inside the straws or cryovials for all freezing/thawing protocols. Measures were performed thrice for all treatments. The cooling rates were calculated from 4 to - 20 ºC and from -20 to -100 ºC and the temperature reached after the 10 min freezing period was recorded. For the thawing rates we calculated the gradient from -196 to -20 ºC and from -20 to 4 ºC. The application of a two-way ANOVA revealed that only the distance to the LN_2, but not the used CPAs, significantly affected the freezing rates ($p < 0.05$) (data not shown). Therefore freezing and thawing rates were calculated for each distance and packaging volume with the mean values for the six cryoprotectant solutions.

Data analysis

All the statistics were conducted using the SPSS software (version 15.0). A total of 77,584 motile spermatozoa were analyzed for the fertilization trials and 160,891 motile spermatozoa were analyzed for the cryopreservation trials, based on a Two-Step Cluster analysis using the eight different parameters rendered by CASA. The Two-Step Cluster analysis procedure is an exploratory tool designed to reveal natural groupings (or clusters) within a data set that would otherwise not be apparent. Clustering was carried out using the log-likelihood distances and the Schwarz's Bayesian criterion (BIC). Once the clusters/subpopulations had been established, correlations between the hatching rate and the number of total motile cells or cells belonging to SP3, SP2 and SP1 per egg (detected subpopulation after Cluster analysis, see below) 15 s after motility activation, were analyzed using Pearson parametric correlation ($p < 0.01$). Significant differences in the SP3 size between treatments after freezing/thawing and interaction between the different parameters (cryoprotectant, freezing rate and packaging system) were analyzed using a univariate general linear model ($p < 0.05$) and the Duncan's multiple comparison test.

Results

Fertilization trials

The mean hatching rate for the 42 fertilization trials was 34.5 ± 19.3% with a mean 219 ± 102 motile spermatozoa per egg. The application of Two-Step Cluster analysis to the values obtained with CASA identified 3 clusters/subpopulations, which were characterized by the mean values of motility parameters and are presented in Table 1. SP1 showed low LIN as well as low VSL, therefore we considered this subpopulation to be a slow non-linear subpopulation. SP2 presented high LIN and low VSL, and was considered to be a slow linear subpopulation. Finally, SP3 displayed both high LIN and VSL, hence we considered this subpopulation a fast linear subpopulation. The analysis of correlation between the hatching rate and number of motile cells or cells within a subpopulation per egg, 15 s after motility activation, always rendered a positive correlation ($p < 0.01$), ($r = 0.77$ for motile cells, $r = 0.82$ for SP3 cells, $r = 0.66$ for SP1 cells and $r = 0.68$ for SP2 cells) (Fig. 1).

Table 1 – Mean values ± SD obtained from the clustering analysis for the 3 motile subpopulations (SP1, SP2 and SP3) for the eight descriptors given by CASA, for the Fertilization Trials and Cryopreservation Trials. The descriptors used to describe each subpopulation are marked in bold, being VCL (velocity according to the actual path; µm/s), VSL (velocity according to the straight path; µm/s), VAP (velocity according to the smoothed path; µm/s), LIN (linearity;%), STR (straightness;%), WOB (wobble;%), ALH (amplitude of the lateral displacement of the sperm head; µm) and BCF (beat-cross frequency; Hz).

	Subpopulations					
	Fertilization trials			Cryopreservation trials		
	SP1	SP2	SP3	SP1	SP2	SP3
VCL	**22.66 ± 12.96**	**28.87 ± 13.51**	**97.22 ± 34.29**	**23.90 ± 14.26**	**34.40 ± 14.99**	**115.04 ± 34.52**
VSL	5.00 ± 3.34	17.58 ± 9.65	65.84 ± 30.44	5.68 ± 4.33	25.24 ± 13.62	81.39 ± 33,04
VAP	11.65 ± 8.35	22.50 ± 11.42	86.03 ± 32.75	12.38 ± 9.87	28.96 ± 14.19	105.75 ± 32.49
LIN	**0.22 ± 0.09**	**0.61 ± 0.17**	**0.70 ± 0.21**	**0.24 ± 0.11**	**0.72 ± 0.16**	**0.73 ± 0.24**
STR	0.46 ± 0.18	0.78 ± 0.14	0.79 ± 0.19	0.49 ± 0.19	0.86 ± 0.12	0.78 ± 0.22
WOB	0.51 ± 0.16	0.78 ± 0.14	0.88 ± 0.11	0.51 ± 0.172	0.83 ± 0.13	0.92 ± 0.10
ALH	1.41 ± 0.59	1.40 ± 0.51	2.29 ± 1.00	1.43 ± 0.615	1.32 ± 0.41	2.19 ± 1.20
BCF	2.46 ± 1.92	3.55 ± 2.66	6.00 ± 2.95	2.75 ± 2.17	5.72 ± 3.58	5.66 ± 2.98

Cryopreservation trials

Measurement of the freezing curves showed that ice nucleation, revealed by an abrupt change in the slope of the registered temperatures, occurred later in the cryovials than in the straws, between 1 to 2 min after being placed in the LN_2 vapor for the cryovials (Fig. 2B) and 20 to 40 s for the straws (Fig. 2A). The freezing rates also varied extensively between the different treatments (Table 2): the faster freezing rate was obtained with straws at 1 cm (22.8 ± 2.8 ºC/min between 4 and -20 ºC) whereas the slower freezing rate was obtained with cryovials at 4 cm (2.9 ± 0.5 ºC/ min for the same range). The temperature reached after 10 min in the LN_2 vapor varied widely according to the distance from the LN_2 surface and the cryopreservation volume (Table 2) ranging from -176.50 ± 0.84 ºC in straws at 1 cm and -56.33 ± 10.46 ºC in cryovials at 4 cm. The final temperature reached after thawing was similar in straws (13.77 ± 3.42 ºC) and cryovials (10.94 ± 2.28 ºC). The thawing curves were significantly different, the thawing rate being much lower for cryovials than for straws (Fig. 2C and Table 2).

The average percentage of motile cells was 60.6 ± 12.2% in fresh sperm 15 s after activation. After thawing the higher values at 15 s were obtained with 5% Me_2SO at 1 cm (33.5 ± 13.8%) for the straws and with 15% EG at 2 cm (21.9 ± 15.3%) for the cryovials. The lower results were

obtained with 15% EG at 1 cm (7.4 ± 3.2%) for straws and under the same conditions, 15% EG at 1 cm (8.1 ± 11.2%) for cryovials.

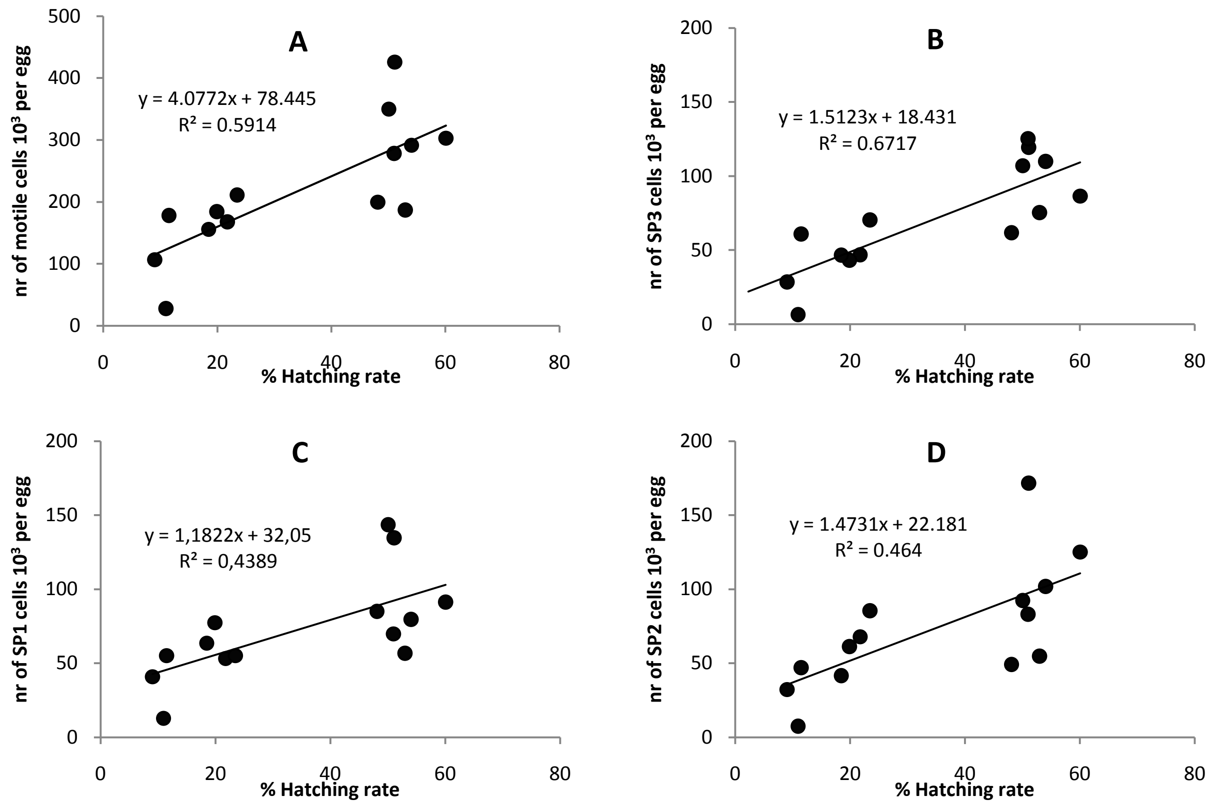


Figure 1 – (A) Correlation between the hatching rate and the number of motile cells per egg, 15 s after motility activation (n = 14) ($p < 0.01$). (B) Correlation between the hatching rate and the number of SP3 cells per egg, 15 s after motility activation (n = 14) ($p < 0.01$). (C) Correlation between the hatching rate and the number of SP1 cells per egg, 15 s after motility activation (n = 14) ($p < 0.01$). (D) Correlation between the hatching rate and the number of SP2 cells per egg, 15 s after motility activation (n = 14) ($p < 0.01$).

After applying the Two-Step Cluster analysis to the CASA values related to the thawed sperm cells, similar subpopulations to those in the fertilization trials were obtained. SP1, SP2, SP3 were characterized by the mean values of the motility parameters and are presented in Table 1 (subpopulations described above in Fertilization trials). In all the analyzed treatments as well as in the control samples, the percentage of SP3 decreased in time (Fig. 3). Less than 1% of the spermatozoa belong to SP3 60 s after activation and only the control samples showed a higher value (1.6%) (Tables 3 and 4). This decrease was less noticeable in SP1 and SP2. If only motile spermatozoa are considered, the relative sizes of the subpopulations are modified over time, increasing the relative size of SP1 and decreasing that of SP3, as shown in Fig. 3 in which mean

values for all treatments are represented. When compared with the control, most treatments promoted an increase of SP1 in comparison with SP3 (Table 3 and 4). Taking into consideration that the samples presenting better fertilization rates would be those with fast and linear spermatozoa (for a longer time), the evolution of SP3 was particularly analyzed.

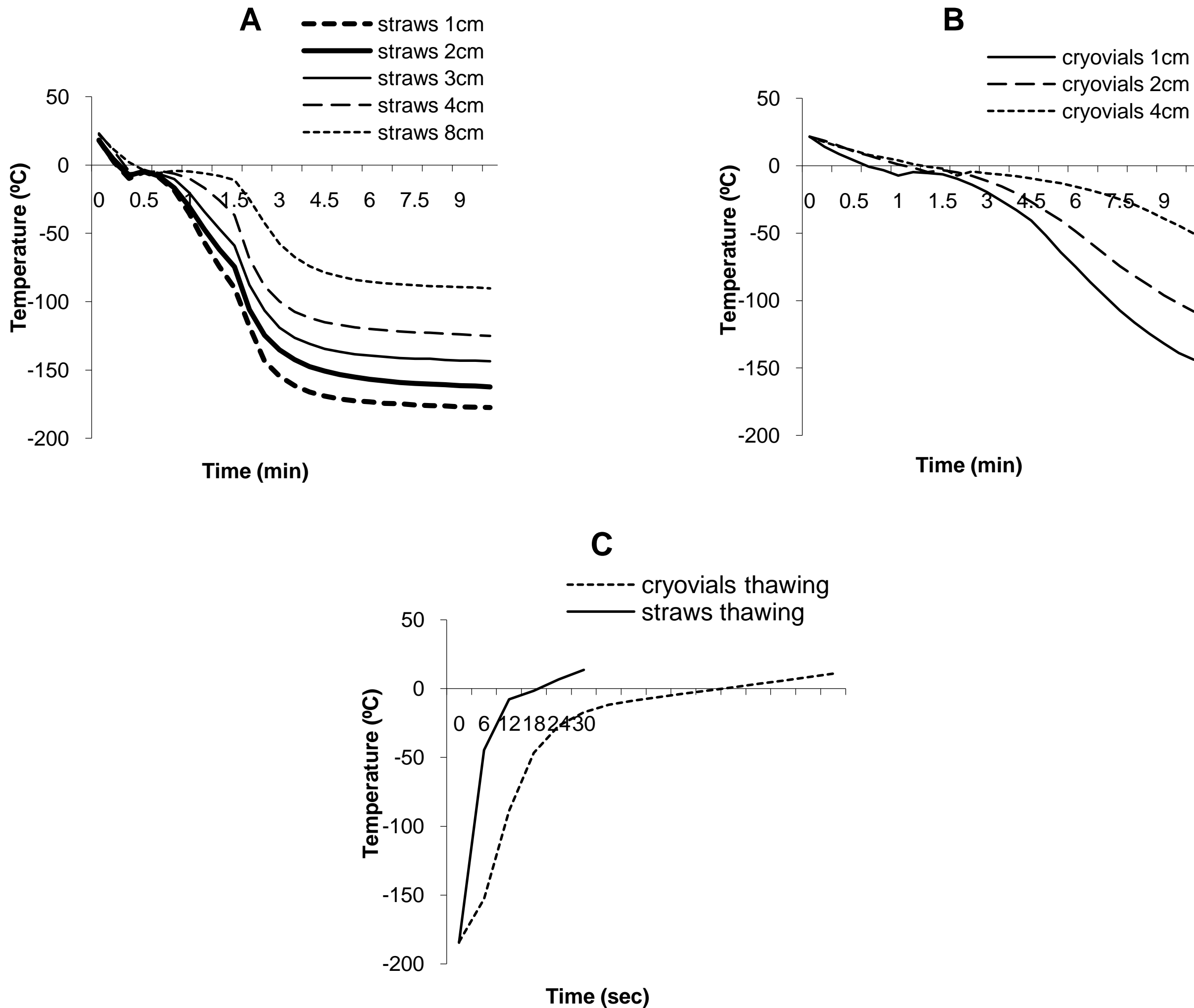


Figure 2 – Obtained freezing curves for straws (0.5 ml) cryopreserved at 1, 2, 3, 4 and 8 cm from the LN_2 (mean values for the 3 cryoprotectants). (B) Obtained freezing curves for cryovials (1.8 ml) cryopreserved at 1, 2 and 4 cm from the LN_2 (mean values for the 3 cryoprotectants). (C) Comparison between thawing curves for straws (0.5 ml) (25 ºC for 30 s) and cryovials (1.8 ml) (40 ºC for 150 s) (mean values for the 3 cryoprotectants).

All tested parameters: sperm packaging volumes, type of CPAs and concentration and freezing rates had a significant effect on SP3 evolution after activation ($p < 0.05$). Moreover, there was a significant interaction between sperm packaging and type of CPA ($p < 0.05$). In straws, samples frozen at 1 and 8 cm over the LN_2 showed lower percentage of spermatozoa within SP3,

indicating a suboptimal condition for freezing despite the results of average motility (Fig. 4). Dimethyl sulfoxide at 5% provided a high percentage of cells at SP3 15 s after activation (14.5% and 9.5% at 2 and 3 cm, respectively), which was significantly reduced after 60 s (0.5% and 0.4%). Subpopulation dynamics were different when 10% EG was used at the same freezing rates. Percentage of motile cells 15 s after activation was lower than with Me_2SO (11.6% and 6.1%, at 2 and 3 cm respectively), but cells continued to move fast and linear for longer, the size of SP3 being significantly more homogeneous during the analyzed period (0.91% and 0.61% at 60 s at 2 and 3 cm, respectively). According to the applied linear model, there were significant differences ($p < 0.05$) in the evolution of the percentage of cells from SP3 through time in the different treatments. In the cyopreservation protocols for the straws with 5% Me_2SO and 10% EG, both at 2 cm, the percentage of cells in SP3 was closer to the control fresh samples than with other cryopreservation procedures.

Table 2 – Lowest temperature after 10 min in the floating device and freezing and thawing rates for the tested cryopreservation volumes (mean values ± SD). Different letters means significant differences between data in each column.

		Freezing rates (ºC/min)		Thawing rates (ºC/min)	
	Lowest Temperature (ºC)	4ºC to -20ºC	-20ºC to -100ºC	-196ºC to -20ºC	-20ºC to 4ºC
Straws 1cm	-176.50 ± 0.84[a]	22.8 ± 2.8[a]	104.0 ± 12.1[a]		
Straws 2cm	-162.00 ± 1.10[b]	21.6 ± 3.3[a]	82.0 ± 17. 7[b]		
Straws 3cm	-142.83 ± 2.04[c]	18.3 ± 3.9[b]	71.7 ± 17.9[b]	1506 ± 159[a]	65 ± 5.4[a]
Straws 4cm	-125.00 ± 1.41[d]	15.7 ± 1.2[b]	48.6 ± 7.0[c]		
Straws 8cm	-89.33 ± 3.27[f]	10.0 ± 1.3[c]	-		
Cryovials 1cm	-151.17 ± 17.09[c]	6.6 ± 1.2[d]	19.8 ± 1.8[d]		
Cryovials 2cm	-110.50 ± 15.40[e]	5.1 ± 1.0[d]	16.7 ± 1.3[d]	235 ± 21[b]	13 ± 1.2[b]
Cryovials 4cm	-56.33 ± 10.46[g]	2.9 ± 0.5[e]	-		

Sperm frozen in cryovials presented a lower initial motility when compared with the straws, but the decrease in SP3 was less evident (Fig. 5). Dimethyl sulfoxide provided the best results in terms of percentage of cells within SP3 and was the only CPA giving more than 0.6% of spermatozoa within the SP3 after 60 s (10% Me_2SO at 1 cm). There were significant differences ($p < 0.05$) in the evolution of SP3 subpopulations through time in the different treatments performed with cryovials, 10% Me_2SO at 1 cm presenting closer results to the control samples.

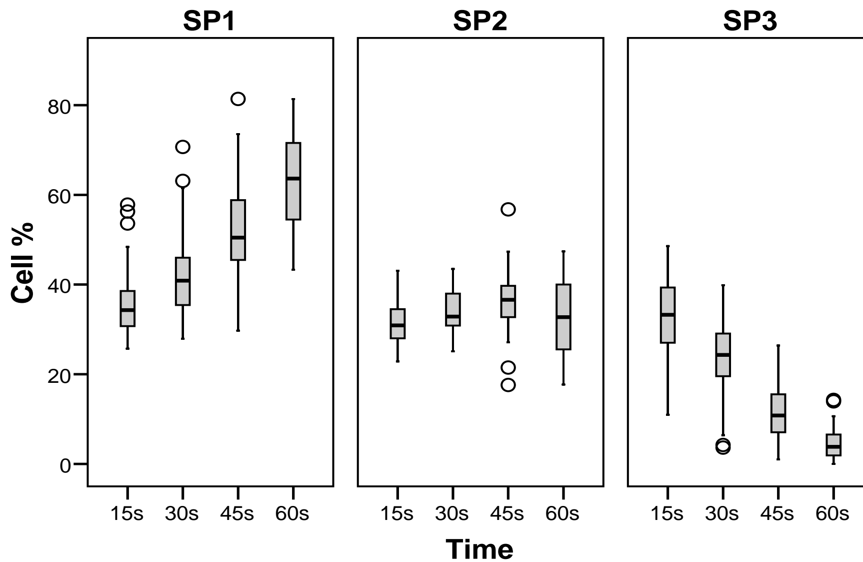


Figure 3 – Percentage of cells belonging to each subpopulation through time after motility activation, considering only the motile spermatozoa. Data are means of all the applied cryopreservation procedures. The box plot comprises from the 1st to the 3rd quartile, the whiskers indicate the most extreme values, horizontal line indicates the median and the dots outlier values.

Discussion

The existence of sperm subpopulations is neglected in most fish sperm studies, however their existence has already been stated in some species [2,4,16,21]. This is the first approach to the effect of cryopreservation on fish sperm subpopulations.

A similar pattern to the one described by Quintero-Moreno et al. [24] for mammals, consisting of three or four different subpopulations, also seems to exist in fish sperm, as shown by the data here presented and those reported in other fish species [2,16,21]. In the present work three well-defined subpopulations were identified. From them, SP3 presented fast and linear spermatozoa. As reported by Casselman et al. [7] in Sander vitreus, Gage et al. [12] in *Salmo salar* and Rudolfsen et al. [27] in *Gadus morhua*, fertilization success is correlated with spermatozoa swimming speed. Likewise in our case SP3 was the subpopulation best correlated with the hatching rate and was therefore given special attention in the cryopreservation trials. SP1, with slow non-linear spermatozoa and SP2, with slow linear spermatozoa, were also identified. Both SP1 and SP2 presented a lower correlation with the hatching rate, which could

Table 3 – Percentage of S. senegalensis sperm cells in each subpopulation (SP1, SP2 and SP3) afetr cryopreservation in cryovials

Cryoprotectant	Time/ Subpopulation distance from LN_2	15 sec SP1	15 sec SP2	15 sec SP3	Total 15 sec	30 sec SP1	30 sec SP2	30 sec SP3	Total 30 sec	45 sec SP1	45 sec SP2	45 sec SP3	Total 45 sec	60 sec SP1	60 sec SP2	60 sec SP3	Total 60 sec
Control		17.35	13.85	29.43	60.63	16.57	16.40	17.04	50.01	15.03	14.38	5.80	35.21	13.17	9.90	1.62	24.69
	1 cm [a]	**7.72**	**6.17**	**6.99**	**20.89**	**5.83**	**4.70**	**4.70**	**15.22**	**4.76**	**3.63**	**2.11**	**10.50**	**4.02**	**3.24**	**0.69**	**7.94**
	2 cm [ab]	5.29	5.61	5.38	16.28	4.35	3.05	2.83	10.23	3.65	2.58	1.46	7.69	2.79	1.87	0.35	5.01
10% Me_2SO	4 cm [ab]	5.81	6.08	5.78	17.67	4.24	3.06	3.56	10.86	3.15	2.66	1.09	6.90	2.76	2.05	0.51	5.32
	1 cm [ab]	4.57	3.42	1.99	9.98	3.01	2.29	1.70	7.01	2.06	1.43	0.76	4.25	1.49	1.40	0.28	3.17
	2 cm [b]	3.66	3.60	2.24	9.50	2.17	1.56	0.71	4.44	0.82	0.65	0.20	1.67	0.54	0.24	0.00	0.78
5% Me_2SO	4 cm [ab]	5.96	5.29	3.65	14.90	2.95	3.14	1.70	7.79	1.99	1.57	0.66	4.21	1.33	0.72	0.33	2.38
	1 cm [ab]	4.69	3.56	3.09	11.34	3.54	2.20	2.12	7.86	2.74	1.92	1.23	5.89	2.15	1.38	0.28	3.81
	2 cm [ab]	2.64	3.23	3.60	9.48	2.47	2.11	2.47	7.06	1.97	0.98	0.49	3.44	1.61	0.80	0.00	2.41
10% EG	4 cm [ab]	2.95	3.69	2.46	9.11	2.37	2.12	0.98	5.46	1.93	1.01	0.18	3.13	1.14	0.57	0.08	1.79
	1 cm [ab]	3.91	2.87	1.30	8.08	2.42	2.85	1.28	6.56	1.73	3.30	0.78	5.81	1.55	1.36	0.48	3.39
	2 cm [ab]	7.07	7.52	7.27	21.87	6.48	4.54	3.34	14.36	4.49	3.10	0.74	8.33	3.36	1.72	0.15	5.23
15% EG	4 cm [ab]	4.02	4.44	3.90	12.36	2.49	3.38	2.43	8.31	2.63	2.34	0.91	5.88	1.78	1.51	0.21	3.50
	1 cm [ab]	8.12	4.00	1.92	14.04	7.01	2.91	1.19	11.11	4.89	2.03	0.57	7.49	4.00	1.31	0.26	5.57
	2 cm [ab]	6.13	5.38	3.16	14.67	3.67	2.77	0.78	7.22	2.34	1.05	0.06	3.44	1.68	0.54	0.12	2.33
10% PG	4 cm [b]	7.29	6.87	1.85	16.01	6.75	4.88	0.44	12.07	4.90	1.43	0.33	6.66	3.82	1.14	0.10	5.06
	1 cm [b]	6.75	4.46	1.38	12.59	5.28	2.75	0.55	8.58	3.36	1.73	0.16	5.25	2.67	0.93	0.07	3.67
	2 cm [ab]	6.19	6.06	8.83	21.08	5.40	4.08	2.30	11.78	4.23	2.05	0.28	6.56	3.18	1.21	0.05	4.44
15% PG	4 cm [ab]	11.63	6.29	2.75	20.67	8.48	3.01	0.51	12.00	6.65	1.44	0.08	8.17	4.79	1.04	0.05	5.89

Different letters indicate significant differences ($p<0.05$) between the different treatments for the SP3 obtained with Univariate General Linear Model ($n=5$). Values closer to the control are marked in bolt.

indicate that they are represented by spermatozoa with a lower possibility of attaining fertilization when competing with spermatozoa present in SP3. Correlation between subpopulation size and fertilization ability has already been debated in mammals studies without a definite conclusion being reached [19,24,25]. Although our data indicated better correlation of SP3 with fertilization, they did not enable us to present conclusive results, which was not the aim of this study.

The origin and structure of these sperm subpopulations is not consensual, some authors support the idea that their origin is genetic and their structure is modified by maturation [1] and storage in the testis [21]. Moreover, Quintero-Moreno et al. [24], remark that a higher percentage of some subpopulations in the semen samples might be a sign of deteriorated semen quality. Likewise, in our study the general increase in the percentage of SP1 and SP2 in time after activation with respect to SP3 after cryopreservation, which was more evident in some treatments (for example 10% PG at 3 cm for straws and 15% PG at 4 cm for cryovials), was considered an index of occurrence of semen exhaustion and deterioration after activation. This fact could point out which are the less efficient freezing/thawing protocols: the lowest protection conferred to the spermatozoa the highest probability that the spermatozoa belong to SP2 or SP1 (slow subpopulations) instead of SP3 (fast linear subpopulation) after thawing. Clearly, cryopreservation protocols have an effect on the overall population structure, decreasing spermatozoa velocity and linearity. This observation contradicts that made by Lahnsteiner et al. [15], who analyzing motility in rainbow trout (*Oncorhynchus mykiss*), reported that cryopreservation did not have any effect on sperm velocity and only changed the motility pattern from a circular to a linear path. Nonetheless, similar studies reporting the effect of cryopreservation on mammalian sperm, showed dependence between the sperm subpopulation structure in a sample and the cryopreservation procedure applied [1].

Regarding the evolution of sperm subpopulations throughout time, cells belonging to SP3 might at later times be in SP1 or SP2 due to energy loss, displaying lower velocity and/or linearity. A similar hypothesis was proposed by Martinez-Pastor et al. [21] and our research team in previous works with Solea senegalensis [2]. Therefore, we consider that the percentage of SP3 at 15 s could be as important as the subpopulation evolution through time. For example, after freezing with 10% EG at 2 cm, SP3 size is more constant through time than after freezing with 5% Me_2SO at 2 cm. This means that sperm frozen with the first cryoprotectant would probably produce better fertility results, since the longer spermatozoa maintain straight and rapid motility the higher the probability of achieving fertilization, as discussed by Gage et al. [12] working with salmon. Moreover, data on the correlation between fertilization ability and the number of total motile cells or the number of cells belonging to SP3 per egg, demonstrated that the latter has a higher correlation. However, both the percentage of motile cells and the

Table 4 – Percentage of S. senegalensis sperm cells in each subpopulation (SP1, SP2 and SP3) after cryopreservation in straws.

	Time/				Total				Total				Total				Total
	Subpopulation		15 sec		15 sec		30 sec		30 sec		45 sec		45 sec		60 sec		60 sec
Cryoprotectant	distance from LN_2	SP1	SP2	SP3		SP1	SP2	SP3		SP1	SP2	SP3		SP1	SP2	SP3	
Control		17.35	13.85	29.43	60.63	16.57	16.40	17.04	50.01	15.03	14.38	5.80	35.21	13.17	9.90	1.62	24.69
	1 cm^{abc}	10.75	8.08	11.83	30.65	8.73	6.97	6.03	21.74	7.76	5.49	1.89	15.14	6.01	3.10	0.35	9.46
	2 cm^{abc}	11.00	7.69	10.76	29.45	9.86	6.06	4.22	20.15	8.14	3.84	1.10	13.09	6.78	2.33	0.36	9.47
	3 cm^{abcdef}	9.16	8.59	10.23	27.98	7.30	6.28	3.67	17.24	5.17	4.01	0.73	9.91	4.16	1.86	0.14	6.16
	4 cm^{abc}	9.52	8.03	13.43	30.98	8.48	7.63	4.33	20.44	7.40	5.05	0.81	13.27	5.42	2.20	0.10	7.72
10% Me_2SO	8 cm^{bcdefg}	5.78	4.82	7.69	18.29	4.95	4.41	2.76	12.13	4.07	2.32	0.54	6.93	2.52	0.87	0.10	3.49
	1 cm^{abc}	11.38	9.37	12.71	33.46	8.66	7.56	5.74	21.96	5.62	4.83	1.77	12.22	3.67	2.61	0.19	6.47
	2 cm^{a}	**7.69**	**7.69**	**14.53**	**29.90**	**5.43**	**6.26**	**7.75**	**19.44**	**4.64**	**4.23**	**2.34**	**11.21**	**3.83**	**2.69**	**0.51**	**7.03**
	3 cm^{abcde}	6.75	7.71	9.46	23.92	4.65	6.13	5.11	15.89	4.04	3.78	1.63	9.45	3.23	1.94	0.36	5.54
	4 cm^{bcdefg}	5.64	6.23	8.30	20.17	3.10	4.40	2.63	10.13	2.29	2.17	0.54	5.00	1.32	0.53	0.01	1.86
5% Me_2SO	8 cm^{cdefg}	6.29	6.26	5.87	18.42	3.75	3.82	2.33	9.90	2.61	2.21	0.55	5.37	1.78	0.78	0.13	2.69
	1 cm^{fg}	4.23	5.28	2.74	12.25	3.19	2.37	2.28	7.83	1.50	0.85	0.20	2.56	0.57	0.44	0.00	1.01
	2 cm^{ab}	**7.00**	**7.21**	**11.62**	**25.83**	**6.31**	**6.44**	**8.12**	**20.88**	**5.13**	**4.29**	**3.38**	**12.79**	**4.21**	**3.44**	**0.91**	**8.55**
	3 cm^{bcdefg}	7.85	7.56	6.13	21.54	5.67	5.75	3.72	15.14	4.46	3.57	2.04	10.07	2.86	3.13	0.61	6.60
	4 cm^{abcdefg}	5.49	5.49	7.39	18.37	4.13	4.38	4.81	13.32	3.37	4.12	1.70	9.19	3.04	2.54	0.46	6.04
10% EG	8 cm^{cdefg}	4.52	4.63	5.83	14.98	3.40	3.22	2.47	9.09	2.77	1.89	0.51	5.17	1.84	0.63	0.13	2.60
	1 cm^{g}	3.50	2.88	1.03	7.40	2.14	1.67	0.61	4.41	1.73	1.03	0.21	2.97	0.85	0.59	0.02	1.47
	2 cm^{bcdefg}	6.91	6.93	6.25	20.08	4.52	5.81	3.31	13.64	3.55	3.41	1.01	7.97	2.57	1.89	0.31	4.76
	3 cm^{defg}	6.54	6.69	5.83	19.06	4.28	4.48	2.32	11.08	3.70	2.85	0.78	7.33	2.72	1.79	0.26	4.76
	4 cm^{bcdefg}	8.18	7.74	7.61	23.52	5.85	6.42	4.27	16.54	4.22	3.56	1.43	9.21	2.97	2.55	0.30	5.82
15% EG	8 cm^{efg}	5.57	4.98	4.34	14.89	3.32	3.26	2.89	9.47	1.86	2.33	0.73	4.92	1.65	1.44	0.16	3.26
	1 cm^{bcdefg}	7.04	6.53	8.79	22.36	4.93	3.72	2.69	11.34	3.46	3.48	0.58	7.51	3.01	1.36	0.12	4.48
	2 cm^{abcdef}	8.32	6.89	12.60	27.81	7.25	5.77	4.53	17.55	6.50	4.14	0.57	11.23	4.88	1.46	0.16	6.50
	3 cm^{bcdefg}	8.31	7.59	9.92	25.82	7.00	4.66	2.55	14.21	4.50	2.32	0.27	7.10	2.61	0.81	0.03	3.46
	4 cm^{abcd}	12.08	8.02	12.21	32.32	10.42	7.22	5.02	22.66	8.66	4.88	0.81	14.36	6.94	2.17	0.16	9.27
10% PG	8 cm^{defg}	6.84	5.61	6.46	18.90	5.32	3.03	1.34	9.69	3.49	1.63	0.23	5.35	2.61	0.66	0.04	3.32
	1 cm^{abcdef}	12.46	7.73	7.38	27.56	9.56	5.25	3.27	18.08	7.89	3.97	1.01	12.88	6.84	2.60	0.19	9.63
	2 cm^{abcdefg}	10.37	7.80	7.13	25.30	7.42	4.59	4.10	16.11	6.20	3.33	1.19	10.73	5.91	2.18	0.41	8.50
	3 cm^{ab}	9.29	7.86	14.43	31.58	7.54	6.94	6.80	21.28	6.95	6.07	2.26	15.28	5.83	3.92	0.33	10.08
	4 cm^{abcdefg}	7.71	6.15	11.29	25.15	4.84	5.06	5.40	15.29	4.18	4.19	1.24	9.62	3.97	2.75	0.20	6.92
15% PG	8 cm^{abcdefg}	9.05	6.59	9.45	25.09	7.31	4.92	4.11	16.34	6.22	4.16	0.74	11.12	5.25	1.85	0.14	7.23

Different letters indicate significant differences ($p<0.05$) between the different treatments for the SP3 obtained with Univariate General Linear Model (n=5). Values closer to the control are marked in bolt.

subpopulation size have a relatively low correlation index (r = 0.77 and r = 0.82 for average motility and SP3, respectively), once other factors affect spermatozoa fitness to fertilize.

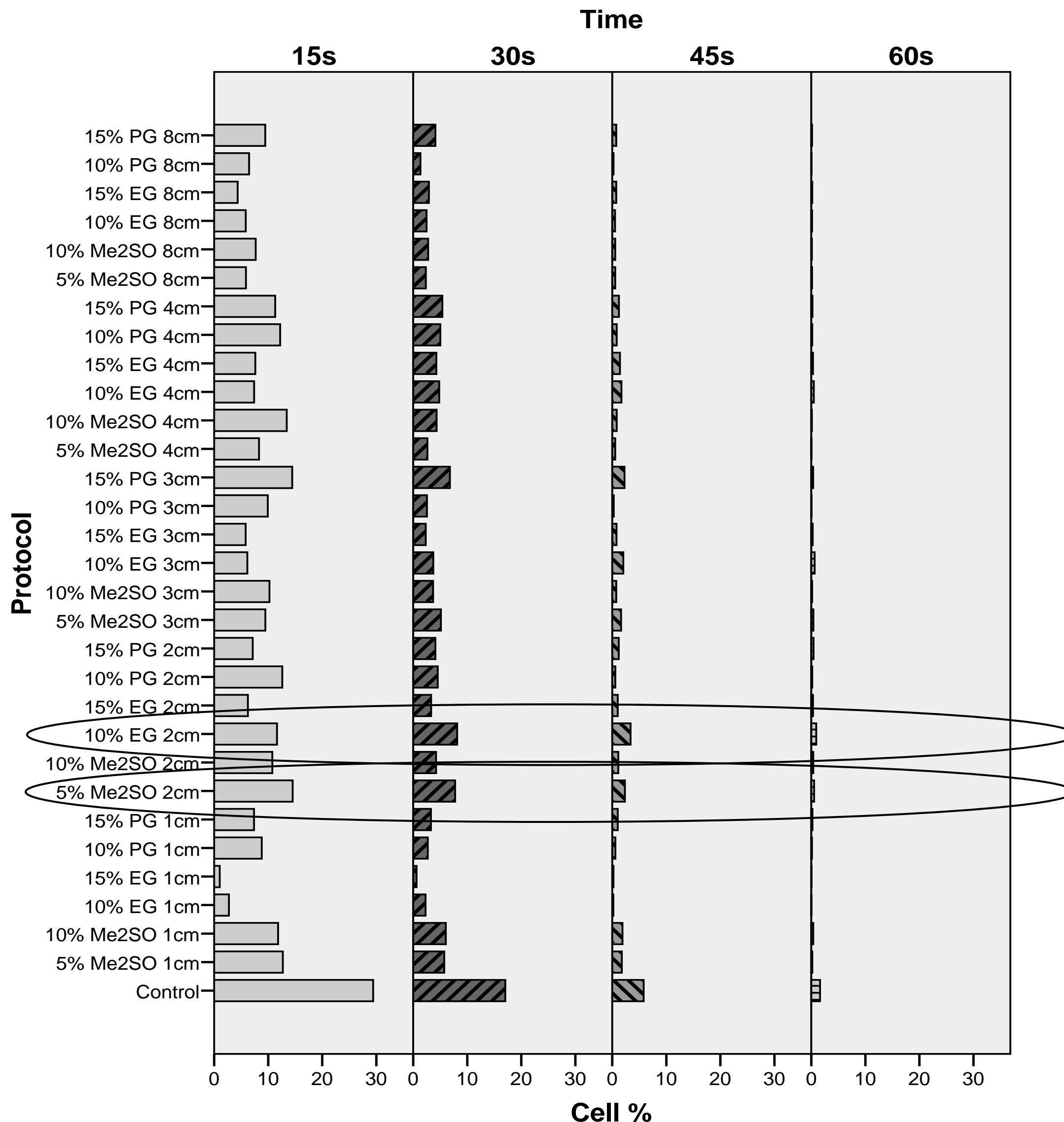


Fig 4 - Cell percentage of SP3 through time for each protocol for straws. Circles represent the cryopreservation protocol with closest results to the control. Data and statistics are detailed in Table 3.

It is also important to notice that, if only average motility had been analyzed, we would have considered freezing in straws with 10% PG at 4 cm as the best cryopreservation method (with 32%, 23%, 14% and 9% motility at 15, 30, 45 and 60 s, respectively). Nevertheless, when SP3 is considered as the key subpopulation, the best protocol is the one using 5% Me_2SO at 2 cm (with 15%, 8%, 2% and 0.5% of cells in SP3 at 15, 30, 45 and 60 s, respectively). With regard to the cryovials, the results considering the overall sperm motile population and only SP3, in terms of

results closer to the control are in agreement (10% Me_2SO at 1 cm). Therefore, we should bear in mind the importance of the information that the use of subpopulations contributes to this type of studies, helping us to understand that the motile population is not homogeneous and its composition varies temporarily and according to the preservation method applied (freezing rates, cryoprotectants etc.).

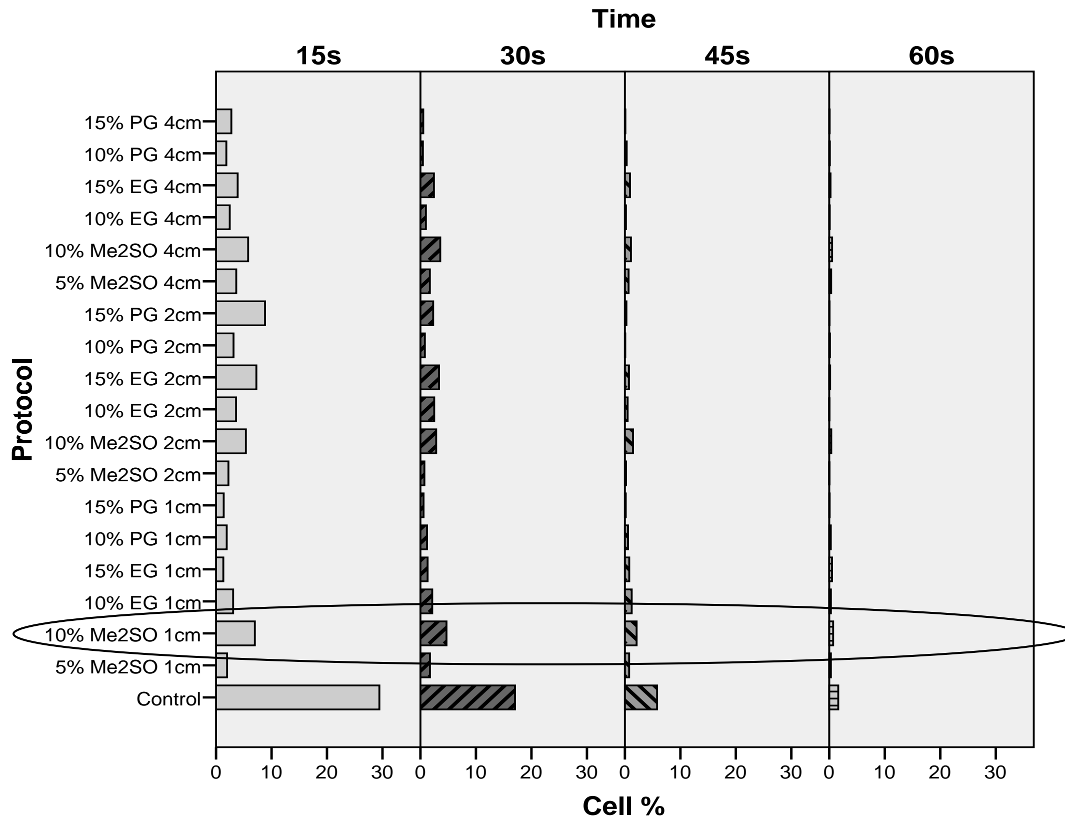


Fig 5 - Cell percentage of SP3 through time for each protocol for cryovials. Circle represent the cryopreservation protocol with closest results to the control. Data and statistics are detailed in Table 4.

In the present work, we used two different packaging volumes. The advantages and disadvantages of using large cryopreservation volumes in fish sperm have already been discussed by different authors [5,6,9,14,17,26,33]. Cryovials have the advantage over straws of being easier to use for routine practice in hatcheries, allowing large volumes to be cryopreserved. Most authors obtained lower fertilization rates using this packaging system, but consider that the decrease was mostly compensated by the benefits of using large volumes [6]. In this study, a significant decrease in SP3 subpopulations in samples frozen in cryovials when compared to the same samples frozen in straws was observed. These differences in the evolution of the subpopulations were related to the thickness of the two containers, causing

lower temperature diffusion in the cryovials (freezing and thawing curves presented a lower slope in the cryovials when compared with the straws) and also to the different volume to surface area ratio as discussed by Cabrita et al. [5]. This implied greater dehydration during freezing and more chance of the cellular structure being damaged. Moreover, during thawing there was a higher probability of recrystalization. Additionally, Horvath et al. [14] attributed the lower efficiency of large cryopreservation volumes to slower freezing rates, which expose spermatozoa to the toxic effects of the cryoprotectants for longer. Under the tested conditions, the best freezing rate for straws was 21.6 ± 3.3 ºC/min from 4 to -20 ºC and 82.0 ± 17.7 ºC/min from -20 to -100 ºC and for cryovials 6.6 ± 1.2 ºC/min from 4 to -20 ºC and 19.8 ± 1.8 ºC/min from -20 to -100 ºC. These values are difficult to compare with those reported by other authors since not always the same rates are analyzed. The optimum freezing/thawing rate is highly variable between species [8,35] and its efficacy is related with the dehydration process and the prevention of intracellular ice formation during freezing and with recrystalization during thawing [8,34]. It is interesting to note that in cryovials ice nucleation occurs later than in straws, and this will cause greater cell dehydration and longer exposure to supercooling, which is responsible for cold shock damage, as explained by Wolfe et al. [34]. Hence, the lower mean motility values in the cryovials and the fact that SP1 is the main population at 15 s in the cryovials, might be explained by damaged cell structure as well as lethal ion concentration due to excessive dehydration [34].

In the cryopreservation trial we also tested different cryoprotectants. A good CPA needs to enable cells to cool and recover from the ultra-low temperature (below -100 ºC) without changes in metabolisms and structure [11] and to do this it needs to present low toxicity for the cells, high permeability and water solubility. The effect observed for the different CPAs is related with an equilibration point between cryoprotectant toxicity and its efficacy [8,35]. The fact that Me_2SO was the CPA providing better results in this study as well as in previous studies on *S. aurata* [10], *Pagrus major* [17] and *Acanthopagrus latus* [13] sperm cryopreservation, seems to indicate that it is the best cryoprotectant for Sparidae spermatozoa.

The use of a clustering analysis has allowed the detection of sperm subpopulations according to motility parameters in *S. aurata* semen, and their variation through time. This statistical procedure has not just pointed out the existence of different fish sperm subpopulations apparently with different fertilizing ability, but has also shown that the subpopulation structure of samples was differentially affected by the cryopreservation protocol depending on the type of cryoprotectant, freezing rates and packaging systems used. When we consider SP3 evolution through time, the treatments giving results closer to the control were, 5% Me_2SO at 2 cm from the LN_2 for straws and 10% Me_2SO at 1 cm from the LN_2 for cryovials. The existence of different sperm subpopulations should be taken into account when optimizing fish sperm cryopreservation protocols. Further studies should try to investigate how these sperm motility

based subpopulations might be related with sublethal damage on spermatozoa after cryopreservation and integrate this data with other sperm quality parameters to better predict its fertilization ability.

Acknowledgments

This work was supported by a PhD grant (SFRH/BD/31990/ 2006) and by the project Petri (PTR 95.1026.OP.01). Samples were provided by the Maresa Sa fish farm, Huelva, Spain. The authors would like to thank Vânia Barradas for her invaluable help in data analysis.

References

[1] T. Abaigar, W.V. Holt, R.A.P. Harrison, G. del Barrio, Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments, Biol. Reprod. 60 (1999) 32–41.

[2] J. Beirão, F. Soares, M.P. Herráez, M.T. Dinis, E. Cabrita, Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level, Theriogenology 72 (2009) 1251–1261.

[3] E. Cabrita, R. Alvarez, L. Anel, K.J. Rana, M.P. Herráez, Sublethal damage during cryopreservation of rainbow trout sperm, Cryobiology 37 (1998) 245–253.

[4] E. Cabrita, R. Alvarez, E. Anel, M.P. Herráez, The hypoosmotic swelling test performed with coulter counter: a method to assay functional integrity of sperm membrane in rainbow trout, Anim. Reprod. Sci. 55 (1999) 279–287.

[5] E. Cabrita, V. Robles, R. Alvarez, M.P. Herráez, Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization, Aquaculture 201 (2001) 301–314.

[6] E. Cabrita, V. Robles, S. Cunado, J.C. Wallace, C. Sarasquete, M.P. Herráez, Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5ml macrotubes, Cryobiology 50 (2005) 273–284.

[7] S.J. Casselman, A.I. Schulte-Hostedde, R. Montgomerie, Sperm quality influences male fertilization success in walleye (*Sander vitreus*), Can. J. Fish. Aquat. Sci. 63 (2006) 2119–2125.

[8] N. Chao, C.I. Liao, Cryopreservation of finfish and shellfish gametes and embryo, Aquaculture 197 (2001) 161–189.

[9] S. Chen, X. Ji, G. Yu, Y. Tian, Z. Sha, Cryopreservation of sperm from turbot (*Scophthalmus maximus*) and application to large-scale fertilization, Aquaculture 236 (2004) 547–556.

[10] A. Fabbrocini, S.L. Lavadera, S. Rispoli, G. Sansone, Cryopreservation of seabream (*Sparus aurata*) spermatozoa, Cryobiology 40 (2000) 46–53.

[11] B.J. Fuller, Cryoprotectants: the essential antifreezes to protect life in the frozen state, Cryoletters 25 (2004) 375–388.

[12] M.J.G. Gage, C.P. Macfarlane, S. Yeates, R.G. Ward, J.B. Searle, G.A. Parker, Spermatozoal Traits and Sperm Competition in Atlantic Salmon: Relative Sperm Velocity Is the Primary Determinant of Fertilization Success, Curr. Biol. 14 (2004) 44–47.

[13] J.C. Gwo, Cryopreservation of yellowfin seabream (*Acanthopagrus latus*) spermatozoa (Teleost, Perciformes, Sparidae), Theriogenology 41 (1994) 989– 1004.

[14] A. Horvath, E. Miskolui, S. Mihalffy, K. Osz, K. Szabo, B. Urbanyi, Cryopreservation of common carp (*Cyprinus carpio*) sperm in 1.2 and 5 ml straws and occurrence of haploids among larvae produced with cryopreserved sperm, Cryobiology 54 (2007) 251–257.

[15] F. Lahnsteiner, B. Berger, T. Weismann, R. Patzner, Changes in morphology, physiology, metabolism, and fertilization capacity of rainbow trout semen following cryopreservation, Prog. Fish-Cult. 58 (1996) 149–159.

[16] S.C. Le Comber, C.G. Faulkes, K.J.W. Van Look, W.V. Holt, C. Smith, Recovery of sperm activity after osmotic shock in the three-spined stickleback: Implications for pre-oviposition ejaculation, Behaviour 141 (2004) 1555– 1569.

[17] Q.H. Liu, J. Li, S.C. Zhang, F.H. Ding, X.Z. Xu, Z.Z. Xiao, S.H. Xu, An efficient methodology for cryopreservation of spermatozoa of red seabream, *Pagrus major*, with 2-mL cryovials, J. World. Aquacult. Soc. 37 (2006) 289–297.

[18] Q.H. Liu, J. Li, Z.Z. Xiao, F.H. Ding, D.D. Yu, X.Z. Xu, Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus major*), Aquaculture 263 (2007) 20–25.

[19] F. Martinez-Pastor, V. Garcia-Macias, M. Alvarez, P. Herráez, L. Anel, P. de Paz, Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process, Biol. Reprod. 72 (2005) 316–327.

[20] F. Martinez-Pastor, A.R. Diaz-Corujo, E. Anel, P. Herráez, L. Anel, P, De Paz, Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm, Theriogenology 64 (2005) 958–974.

[21] F. Martinez-Pastor, E. Cabrita, F. Soares, L. Anel, M.T. Dinis, Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts, Reproduction 135 (2008) 449–459.

[22] R. Muiño, C. Tamargo, C.O. Hidalgo, A.I. Pena, Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation, Anim. Reprod. Sci. 109 (2008) 27–39.

[23] C. Ortega-Ferrusola, B.M. Garcia, V.S. Rama, J.M. Gallardo-Bolanos, L. Gonzalez- Fernandez, J.A. Tapia, H. Rodriguez-Martinez, F.J. Pena, Identification of Sperm Subpopulations in Stallion Ejaculates: Changes after Cryopreservation and Comparison with Traditional Statistics, Reprod. Domest. Anim. 44 (2009) 419– 423.

[24] A. Quintero-Moreno, J. Miro, A.T. Rigau, J.E. Rodriguez-Gil, Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates, Theriogenology 59 (2003) 1973–1990.

[25] A. Quintero-Moreno, T. Rigau, J.E. Rodriguez-Gil, Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis, Theriogenology 61 (2004) 673–690.

[26] M. Rodina, D. Gela, M. Kocour, S.M.H. Alavi, M. Hulak, O. Linhart, Cryopreservation of tench, *Tinca tinca*, sperm: Sperm motility and hatching success of embryos, Theriogenology 67 (2007) 931–940.

[27] G. Rudolfsen, L. Figenschou, I. Folstad, O. Kleven, Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua* L.), Aquacult. Res. 39 (2008) 212– 216.

[28] E. Rurangwa, F.A.M. Volckaert, G. Huyskens, D.E. Kime, F. Ollevier, Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*), Theriogenology 55 (2001) 751–769.

[29] E. Rurangwa, D.E. Kime, F. Ollevier, J.P. Nash, The measurement of sperm motility and factors affecting sperm quality in cultured fish, Aquaculture 234 (2004) 1–28.

[30] M. Suquet, C. Dreanno, C. Fauvel, J. Cosson, R. Billard, Cryopreservation of sperm in marine fish, Aquacult. Res. 31 (2000) 231–243.

[31] A.R. Taddei, F. Barbato, L. Abelli, S. Canese, F. Moretti, K.J. Rana, A.M. Fausto, M. Mazzini, Is cryopreservation a homogeneous process? Ultrastructure and motility of untreated, prefreezing, and postthawed spermatozoa of *Diplodus puntazzo* (Cetti), Cryobiology 42 (2001) 244–255.

[32] L.M. Thurston, P.F. Watson, A.J. Mileham, W.V. Holt, Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation, J. Androl. 22 (2001) 382–394.

[33] Y.M. Velasco-Santamaria, V.M. Medina-Robles, P.E. Cruz-Casallas, Cryopreservation of yamu (*Brycon amazonicus*) sperm for large scale fertilization, Aquaculture 256 (2006) 264–271.

[34] J. Wolfe, G. Bryant, Cellular cryobiology: Thermodynamic and mechanical effects, Int. J. Refrigeration. 24 (2001) 438–450.

[35] E. Woods, J. Benson, Y. Agca, J. Critser, Fundamental cryobiology of reproductive cells and tissues, Cryobiology 48 (2004) 146–156.

Chapter 2.2.

(Short communication)

Detection of early damage of sperm cell membrane in Gilthead seabream (*Sparus aurata*) with the nuclear stain YO-PRO 1

J. Beirão, S. Pérez-Cerezales, S. Martínez-Páramo,
M. P. Herráez

Department of Molecular Biology, Faculty of Biology, University of León,
León, Spain

Journal of Applied Ichthyology 26 (2010) 794–796

doi: 10.1111/j.1439-0426.2010.01560

Summary

For an accurate evaluation of spermatozoa membrane state, both in fresh and cryopreserved sperm, it is important to have an assay capable of identifying small changes in membrane permeability. In this study we examined for the first time in fishes the feasibility of the YO-PRO 1 nucleic acid stain to detect early membrane changes in samples frozen using different sperm cryopreservation protocols. Gilthead seabream (*Sparus aurata*) sperm were cryopreserved using 1% NaCl as extender and three different cryoprotectant mixtures: 5% DMSO, 5% DMSO plus 10 mg.ml^{-1} BSA and 5% DMSO plus 10% egg yolk. Fresh and frozen ⁄ thawed sperm motility were evaluated with CASA software and sperm membrane integrity was evaluated by flow cytometry with two assays: PI⁄SYBR 14 and PI ⁄ YO-PRO 1. The PI ⁄ YO-PRO 1 assay allowed the identification of a subpopulation of cells, comprised of disturbed cell membrane, which was not noticed when using the common PI ⁄ SYBR 14 assay. This subpopulation increased in abundance after sperm cryopreservation and, as was perceived by flow cytometry, these changes in membrane permeability and ⁄ or integrity were also accompanied by morphometric modifications.

Introduction

Fish sperm membrane is the key structure responsible for the environment reception of stimulus and triggering responses as motility activation, its structure is highly sensitive to environmental stress and alterations on it can lead to spermatozoa malfunction (Cabrita et al., 2008). Hence, the detection of early changes in the sperm cell membrane is of utmost importance for the evaluation of spermatozoa viability after freezing ⁄ thawing procedures. Research on mammalian sperm has reported the use of a new nucleic acid stain (YO-PRO 1) that permeate cells just after membrane destabilization, in conditions where the commonly used PI (propidium iodide) normally used with SYBR 14 does not permeate. Despite good results obtained with mammalian cells (Kumaresan et al., 2009) this nuclear dye has never been tested in fish spermatozoa. The aim of the present research was to test the applicability of this new nucleic acid stain to assist in optimizing fish sperm cryopreservation protocols. For this purpose we used sperm of gilthead seabream (*Sparus aurata*).

Material and methods

Gilthead seabream semen was obtained from a commercial fish farm and transported refrigerated (4ºC) to our facilities at the University of León. Semen cryopreservation and analysis of fresh samples were performed within 3 h after sampling. Sperm was cryopreserved in 0.5 ml French straws (IMV) with 5% DMSO (Dimethyl sulfoxide) in 1% NaCl and two different membrane stabilizers were tested: 10 $mg.ml^{-1}$ BSA (bovine serum albumin) and 10% egg yolk (v⁄v). Dilution rate was 1:6 (sperm:extender). An equilibration time of 4 min (room temperature) plus 10 min at 2 cm from LN_2 was applied, after which straws were plugged directly into LN_2 (liquid nitrogen). Semen was thawed in a water bath at 25ºC for 30 s.

Sperm motility was measured with CASA software, ISAS (Proiser, Valencia, Spain) and an optical phase contrast microscope (Nikon Eclipse E-200, Tokyo, Japan) with a 10× phase contrast objective, attached to a digital camera Basler 302fs (Basler Vision Technologies, Ahrensburg). The software settings were adjusted to fish spermatozoa: 25 images per second; 1 to 90 μm^2 for head area and VCL (curvilinear velocity) > 10 $\mu m.s^{-1}$ to classify a spermatozoon as motile. For motility activation immediately after thawing 10 µl of artificial seawater at room temperature was added to 1 µl of pre-diluted semen (1 µl semen: 25 µl sucrose 300 $mOsm.kg^{-1}$). Motility was recorded 15, 30, 45 and 60 s after activation. From the parameters rendered by the software we used the percentage of motile cells and VCL ($\mu m.s^{-1}$). Significant differences between treatments were detected with a linear mixed model using the Sidak correction ($P < 0.05$).

Membrane viability of fresh and thawed semen was measured with PI ⁄ SYBR 14 and PI ⁄ YO-PRO 1 in a flow cytometer (FACSort Plus Analyzer, Becton–Dickinson, USA) equipped with standard optics and an Argon ion laser (Innova 90, Coherent, USA), with CellQuest software for Macintosh (version 3.0, Becton–Dickinson, USA). Semen samples were pre-diluted, 5 µl fresh samples and 30 µl cryopreserved samples, in 1% NaCl to a final volume of 500 µl. To evaluate membrane viability with the PI ⁄ SYBR 14 assay, Live ⁄ Dead Sperm Viability Kit (Invitrogen, Leiden, The Netherlands) was used as described by Cabrita et al. (2005). For the PI⁄ YO-PRO 1 assay, YO-PRO 1 (Invitrogen, Leiden, The Netherlands) was added to the sperm to a final concentration of 150 nM and incubated 10 min in the dark at 4ºC. Then PI (Invitrogen, Leiden, The Netherlands) was added to a final concentration of 2 $\mu g.ml^{-1}$ and left to incubate for 5 min in the dark at 4ºC. After the incubation time the samples were analyzed on the cytometer, acquiring 10 000 cells per sample. The red fluorescence emitted by PI was detected using a 610 nm filter and the green fluorescence emitted by SYBR 14 and YO-PRO 1 with a 516 nm filter. Samples were analyzed thrice. Percentage data were normalized by arcsine transformation and analyzed with a one-way ANOVA. Significant differences between the PI ⁄ SYBR 14 assay and the PI ⁄ YO-PRO 1 assay, and between each treatment for the PI ⁄ YO-PRO 1 assay were detected with a SNK (Student- Newman-Kleus) multiple range test ($P < 0.05$). All statistical analyses were conducted with SPSS software (version 15.0).

Results and Discussion

Our results showed the existence of two sperm subpopulations in the PI⁄ SYBR 14 assay, one permeable to PI (non viable cells) stained red and other stained green (viable cells) only permeable to SYBR 14, whereas three populations were detected with the PI ⁄ YO-PRO 1 assay, both in fresh and cryopreserved sperm (Fig. 1). In the PI⁄YO -PRO 1 assay the first sperm subpopulation was composed of non-stained spermatozoa (viable cells), the second subpopulation comprised of cells permeable to PI and the third by cells permeable to YO-PRO 1 but not to PI. There were no significant differences ($P < 0.05$) between the percentage of cells stained with PI in both assays, as well as, between the percentage of cells stained with SYBR 14 detected with the classic assay and the percentage of non-stained cells plus YO-PRO 1 stained cells in the PI⁄ YO -PRO 1 assay (subpopulations 1 and 3). The PI ⁄ YO-PRO 1 has allowed us to detect a third subpopulation (R2) (Fig. 1) of cells characterized by a disturbed membrane which is non-permeable to PI, but whose permeability is already modified. This subpopulation is not detected when viability is evaluated with other viability probes. Previous studies by our group have revealed the existence of a membrane disturbed subpopulation in Senegal sole (*Solea senegalensis*) sperm with the apoptotic marker Annexin V (Beirão et al., 2008).

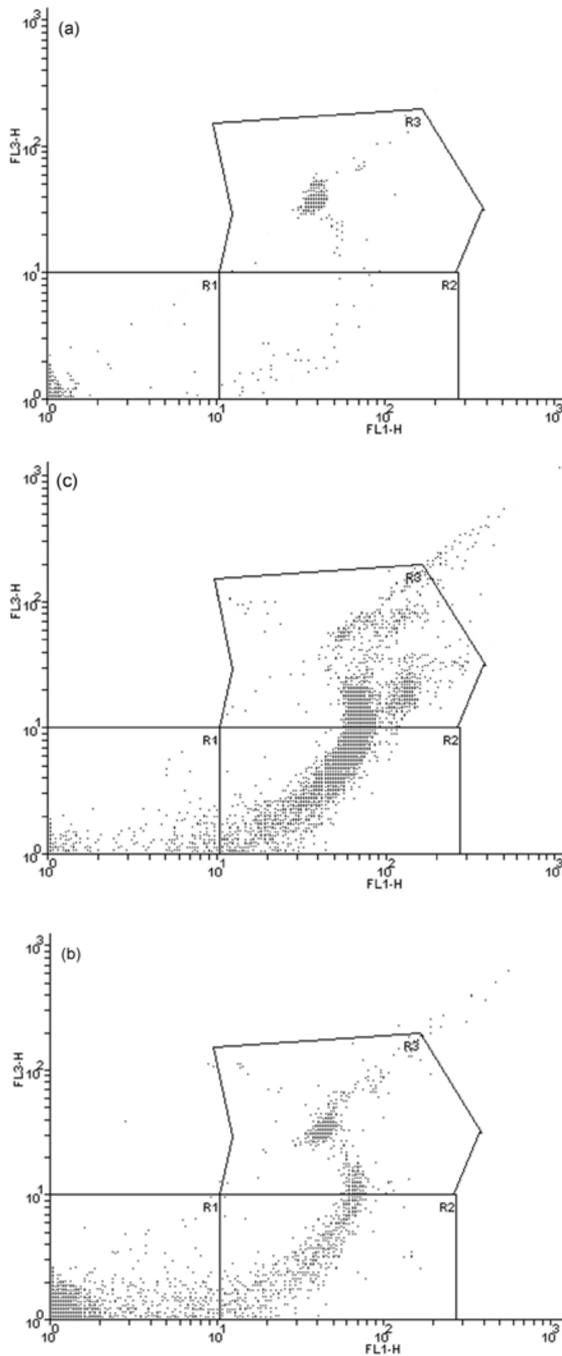


Fig 1 - Example of flow cytometry dot plot for (a) fresh sperm; (b) cryopreserved sperm with 5% DMSO plus BSA; and (c) 5% DMSO plus egg yolk 10%. Green (FL1) and red (FL3) was collected for 10 000 events. Events in (R1) region are negative for both PI and YOPRO 1, in region (R2) are positive for YO-PRO 1 and region (R3) positive for PI.

There was a significant increase (P < 0.05) of the YO-PRO1 and PI positive cells after freezing ⁄ thawing (Fig. 2). This increase was slightly higher in samples frozen with DMSO than in those frozen with DMSO plus BSA but the difference was not significant (P < 0.05). Sperm frozen with DMSO plus egg yolk presented the highest percentages of cells stained with YO-PRO 1 and PI. According to the CASA data there was a significant decrease (P < 0.05) in percentage of motile cells after thawing, for all tested cryopreservation treatments (Fig. 3). Moreover, as detected by flow cytometry, the FSC signal (forward scatter) that correlated with cell volume and the SSC signal (right scatter) that correlated with cell complexity, were significantly different (P < 0.05) between the non-stained (viable), the YO-PRO 1 and PI subpopulations (Fig. 4).

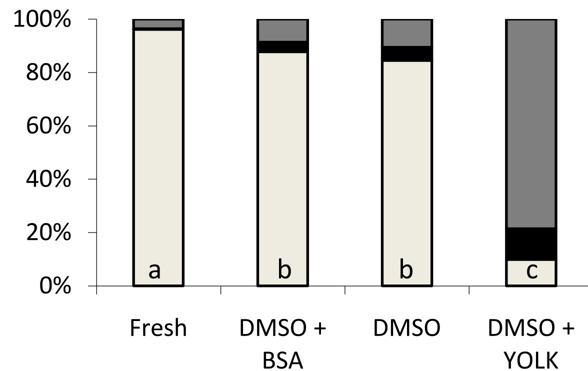


Fig 2 - Percentage of cells non-stained (white), YO-PRO 1 stained (black) and PI stained (grey) for each treatment. Different letters indicate significant differences in the viable population, between treatments (P < 0.05).

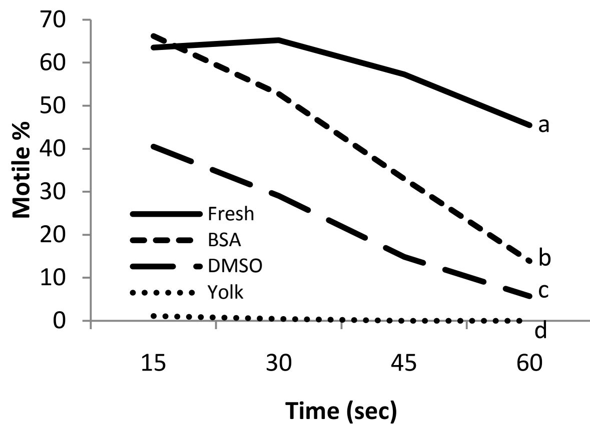


Fig 3 - Mean percentage of motile cells for each treatment 15, 30, 45 and 60 s after activation. Different letters at the end of each line indicate significant differences between treatments (P<0.05).

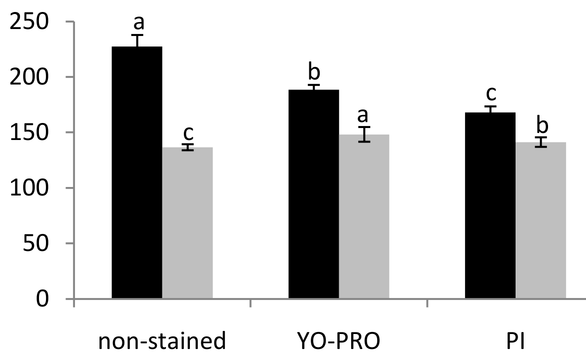


Fig 4 - FSC (black) and SSC (grey) mean values ± SD for each cell subpopulation detected by flow cytometry. Different letters indicates significant differences between subpopulations for each parameter (P<0.05).

In this work we have for the first time applied the YO-PRO 1 stain to fish spermatozoa in order to detect permeability changes after sperm cryopreservation. Most of authors relate these modifications with apoptosis (Martínez-Pastor et al., 2008; Kumaresan et al., 2009). The application of this nucleic acid stain allowed us to detect a membrane disturbed sperm subpopulation that is not detected with PI, which is usually used in combination with SYBR-14 for the analysis of cell viability. The addition of membrane stabilizers did not improve sperm viability, however sperm motility was improved with the addition of BSA. In the case of egg yolk, an important decrease both in cell viability and motility was observed. According to the obtained flow cytometry data, spermatozoa belonging to the different observed subpopulations (viable cells, non-viable cells and membrane disturbed cells) demonstrated by the PI⁄ YO-PRO 1 assay, present significant different sizes (FSC) and complexity (SSC). This fact indicates that changes in membrane permeability or integrity are also accompanied by morphometric changes.

Acknowledgements

This work was supported by a PhD grant (SFRH ⁄ BD ⁄ 31990 ⁄ 2006) and the project PTR95-1026.OP.01 from the Spanish Ministry of Science and Innovation.

References

Beirão, J.; Cabrita, E.; Soares, F.; Herráez, M. P.; Dinis, M. T., 2008: Cellular damage in spermatozoa from wild-captured *Solea senegalensis* as detected by two different assays: comet analysis and Annexin V–Fluorescein staining. J. Appl. Ichthyol. 24, 508– 513.

Cabrita, E.; Robles, V.; Cunado, S.; Wallace, J. C.; Sarasquete, C.; Herráez, M. P., 2005: Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. Cryobiology 50, 273–284.

Cabrita, E.; Robles, V.; Herráez, P., 2008: Sperm quality assessment. In: Methods in Reproductive Aquaculture: Marine and Freshwater Species. E. Cabrita, V. Robles and P. Herráez (Eds), Taylor & Francis, Boca Raton, FL, pp. 93–147.

Kumaresan, A.; Kadirvel, G.; Bujarbaruah, K. M.; Bardoloi, R. K.; Das, A.; Kumar, S.; Naskar, S., 2009: Preservation of boar semen at 1.8 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. Anim. Reprod. Sci. 110, 162–171.

Martínez-Pastor, F.; Fernández-Santos, M. R.; del Olmo, E.; Domínguez-Rebolledo, A. E.; Esteso, M. C.; Montoro, V.; Garde, J. J., 2008: Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod. Fert. Develop. 20, 547–556.

Chapter 2.3.

Improving sperm cryopreservation with antifreeze proteins: effect on seabream plasma membrane lipids

J. Beirão[a], L. Zilli[b], S. Vilella[b], E. Cabrita[c], R. Schiavone[b], M.P. Herráez[a]

[a]Dept. of Molecular Biology, Faculty of Biology, University of León, 24071 León, Spain
[b]Dept. of Biological and Envir. Sciences and Tech., Lab. of Comparative Physiology, University of Salento, Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy
[c]ICMAN-Institute of Marine Science of Andalusia, Spanish National Research Council, Avda. República Saharaui 2, 11510 Puerto Real, Cádiz, Spain

Under review

Abstract

Changes in the plasma membrane lipid composition have been related to a decrease in sperm quality during cryopreservation. Antifreeze proteins (AFPs) have been tested because of their ability to depress the freezing point and their potential interaction with membranes, but controversial effects were reported. In the present study we analyzed the lipid composition of both sperm head plasma membrane (HM) and flagella separately, after cryopreservation with an extender containing 5% DMSO either alone or with AFPI and AFPIII (1µg/mL). We used sperm from a teleost, *Sparus aurata*, because its structure is simpler than that of mammalian sperm, to avoid changes in lipid profiles due to acrosomal losses. Compared with the control (cryopreservation with 5% DMSO alone) the addition of AFPIII increased the straight line velocity (VSL) and percentage of viable cells. In addition, freezing with DMSO alone increased the phosphatidylserine (PS) content, increased the saturated fatty acids (palmitic and stearic) and decreased the polyunsaturated fatty acids (mainly PUFA) in both HM and flagella. HM had a higher amount of saturated fatty acids than flagella and was more affected by cryopreservation without AFPs, suffering decreases in C20:3n3, C20:5n3 (EPA) and C22:5n3. The amount of unsaturated fatty acids in HM was positively correlated with the quality parameters whereas the saturated ones correlated negatively. AFPs, especially AFPIII, seem to have interacted with unsaturated fatty acids avoiding changes in lipidic profiles during cryopreservation and stabilizing the plasma membrane organization both in head and flagella. This effect contributes to improving sperm quality after cryopreservation.

Keywords: Antifreeze protein, sperm, flagella, head plasma membrane, *Sparus aurata*

1. Introduction

Sperm plasma membrane is characterized by a particular lipid composition when compared with most cell membranes, their content in polyunsaturated lipid species (PUFA) being hundreds of times higher and including large amounts of diPUFA phospholipids, very unusual except in sperm, retina and some brain areas (Ladha 1998; Wassall & Stillwell 2008). This particular composition has been related to their specific functions, considering that it promotes the creation of microdomains with different fluidity, fusogenicity and permeability characteristics, required to reach the ova and fuse with it (Wassall and Stillwell 2008). The lipid content varies in mammals during maturation and capacitation (Ladha 1998), defining the characteristic five membrane domains (acrosome, equatorial region, postacrosome, mid-piece and principle piece) over the specialized spermatozoa regions (Ladha 1998). Several studies performed during the last years confirm the influence of membrane molecular species of lipids on sperm motility and viability (Miller *et al*. 2004; Am-in *et al*. 2011). Differences in plasma membrane lipid profiles (cholesterol/phospholipids ratio, saturated/unsaturated fatty acids ratio or the presence/abundance of particular molecular species) have been noticed in boar and men between groups of individuals with different sperm quality (Aksoy *et al*. 2006; Watherhouse *et al*. 2006; Am-in *et al*. 2011), showing the importance that the lipidic profile of plasma membrane has on sperm function.

Sperm membrane composition is, in turn, affected by changes in feeding regime (Pustowka *et al*. 2000; Bongalhardo *et al*. 2009), thermal regime (Labbé & Maisse 1996), or storage procedures and different modifications have been noticed as a consequence of cryopreservation (Chakrabarty *et al*. 2007). Changes in the sperm plasma membrane during cryopreservation include lipid phase transition resulting in a spatial redistribution of the membrane components, modification of membrane domains and microdomains and consequently re-aggregation of membrane proteins (Muller *et al*. 2008).

The evaluation of the effects of cryopreservation on the molecular species of sperm membrane lipids in mammals could be impaired by the fact that freezing/thawing also promotes processes of capacitation, leakage of acrosomal content, induction of acrosomal reaction, etc, linked to significant modifications in the lipidic profiles (Visconti *et al*. 1999). The overall differences in sperm characteristics of the thawed sample could be reduced using a simpler cell model such as teleost fish spermatozoa. Teleost fish spermatozoa are structurally simple, lack acrosome and possess a short or absent mid-piece. After ejaculation survival lasts for no longer than some minutes and neither capacitation nor acrosomal reaction takes place, suggesting that changes in lipidic profiles, if any, must be very subtle. Studies on the lipidic composition of some fish species, mainly salmonids, have been developed by Labbé & Maisse (1996) and Bell *et al*. (1997), but no reports exist on the changes promoted during cryopreservation. Different

membrane domains have not been described in detail, but some differences in the plasma membrane recovering the head and tail should be expected according to their different functional requirements. The head plasma membrane (HM) plays a major role in detecting the transition from seminal plasma environment to freshwater/seawater environment, sensing the ionic changes that lead to motility activation, and it is also responsible for gamete fusion (Bobe & Labbé 2010). On the other hand, flagella have a determinant importance as the mechanism responsible for driving the spermatozoa towards the oocytes.

Arctic fish species have developed specific proteins with antifreeze capacity (AFPs), to resist freezing temperatures. AFPs act by depressing the freezing point, modifying the ice-crystal formation process, preventing recrystallization and interacting with plasma membrane at low temperatures (Tomczak *et al*. 2002a; Venketesh & Dayananda 2008), thus allowing these species to survive in waters colder than the equilibrium freezing point of their internal fluids (Inglis *et al*. 2006). It seems that AFPs bind to the lipid bilayer increasing the phase transition temperature of the membranes and stabilizing them. Although there is a consensus that its action mechanism is dependent on the membrane composition, a predictive model on how AFPs interact with lipid membranes has not been defined and this issue is the focus of several research studies (Tomzack *et al*. 2002; Inglis *et al*. 2006). Although the natural expression of AFPs in polar fishes have been shown to increase fish sperm resistance to cryopreservation (Le François *et al*. 2008), to our knowledge only the addition of antifreeze glycoproteins (AFGP) has been tested in fish sperm cryopreservation, improving the fertilization ability after the freezing/thawing process (Karanova & Tsvetkova 1994; Karanova *et al*. 1997). Reports in mammals sperm of the use of antifreeze proteins (AFPs) in the extender composition (Younis *et al*. 1998; Prathalingam *et al*. 2006), showed improved sperm quality after cryopreservation but no data about their potential effects preventing changes on lipidic profiles of the membrane have been provided.

The aim of this study is to evaluate in Gilthead seabream (*Sparus aurata*) spermatozoa the effects of cryopreservation on sperm plasma membrane lipids and evaluate the potential protective effect of AFPs addition on plasma membrane from head and flagella separately. Gilthead seabream will be used as a representative model, since cryopreservation protocols are well established (Fabbrocini *et al*. 2000; Cabrita *et al*. 2005; Beirão *et al*. 2010, 2011) but some losses in sperm quality are still reported when cryopreserved spermatozoa resistance to hypoosmotic shock was analyzed, revealing changes in membrane functionality (Cabrita *et al*. 2005).

2. Material and Methods

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (Spain/Italy) and were reagent grade or higher. Solvents were purchased from Romil (Spain).

2.1. Sampling and cryopreservation procedure

Sperm samples were obtained from the Aquaculture Research Center of Acquatina (University of Salento, Italy) between November and January. Sperm was collected by applying gentle abdominal pressure and collecting the milt from the urogenital pore with a 2 mL plastic Pasteur pipette. Care was taken to avoid contamination with urine or seawater. Samples were kept at 4ºC until transportation to the laboratory facilities in University of Salento and processing (± 60 min). Semen from individual males was pooled in order to have enough volume for the different experimental treatments and the subsequent analytical procedures. During the experiment, 8 sperm pools (8 to 10 males each pool) of 7 mL were used.

Sperm motility and viability were analyzed and lipids extracted for four different experimental conditions: fresh (control) and cryopreserved sperm according 3 distinct protocols. Sperm cryopreservation was performed by diluting sperm 1/6 (v/v) in the extender solution (1% NaCl plus 5% DMSO (dimethyl sulfoxide)) with and without AFPI or AFPIII (1µg/mL) and loading it in 0.5 mL French straws (IMV). Previous experiments were conducted to test the best AFPI and AFPIII (A/F Protein Canada Inc., St John's, Canada) concentrations (data not shown).

After loading the straws, an equilibration time of 4 min at room temperature plus 10 min at 2 cm from the liquid nitrogen (LN_2) surface was applied. The straws were then directly plunged into LN_2. Cryopreserved samples were thawed in a water bath at 25ºC for 30 sec.

2.2. Motility and Viability analysis

For motility evaluation fresh and frozen/thawed sperm were prediluted in a non-activating solution of 300 mOsm/kg sucrose (1 µL semen: 25 µL sucrose). One µL of the prediluted sample was then activated with 10µL seawater. Sperm movement was videotaped within 15 sec after motility activation using a Nikon Alphaphot 2 microscope with a 20 × negative phase objective and a Sony CCD black and white video camera (SSC-M188CE). Sperm motility videotapes were analyzed with the CASA system Hobson Sperm Tracker and associated software (Hobson Vision Ltd., Baslow, UK). Settings were optimized for seabream sperm as described by Zilli *et al.* (2008). Percentage of motile cells and straight line velocity (VSL) in µm/sec were recorded. Three videotapes were analyzed for each sample.

Sperm membrane viability was measured with the Live/Dead sperm viability Kit from Molecular Probes (Invitrogen, Italy). Samples were prediluted (1 µL semen: 100 µL in 1% NaCl) and incubated for 5 min with SYBR-14 0.25 µM plus 5 min with propidium iodide 24 µM in the dark at 4ºC. After the incubation time three slides were observed with a fluorescence microscope (Nikon Eclipse E600) with blue excitation (450- 480 nm). At least one hundred cells were counted per slide. Cells stained green were classified as viable and cells stained red were classified as non-viable. Results are presented as percentage of viable cells.

2.3. Head plasma membrane (HM) and flagella isolation

Sperm samples were centrifuged (4 000 × g for 15 min at 4ºC) to eliminate the seminal plasma, and washed with 1% NaCl (addition of five times 1% NaCl volume followed by centrifugation and elimination of the supernatant). For head and flagella separation, samples were resuspended four times their volume in 1% NaCl and passed ten times through a 50 cm × 0.5 mm i.d. capillary attached to a 20 mL syringe. Percentage of separation was controlled by observation under light microscopy.

Eight mL of the samples were carefully layered over 24 mL of a 0.5 – 2 M sucrose gradient, made up in 4 steps (6 mL of 0.5, 1, 1.5 and 2 M) prepared in ultracentrifugation tubes. After centrifugation at 28 000 × g for 45 min at 4ºC, two bands were visible, an upper band (0.5M sucrose) consisting of flagella and a lower band (between 1.5 and 2M sucrose) containing spermatozoid heads. With the help of glass Pasteur pipettes the two bands were individually collected and diluted in 1% NaCl to a total volume of 40 mL. Flagella suspensions were centrifuged at 5 000 × g for 20 min at 4ºC, and the pellets used for lipid extraction. Head suspensions were centrifuged at 3 000 × g for 20 min at 4ºC and 30 mL of distilled water was added to the pellets and the suspensions vortexed for 10 sec. This operation allowed us to lyse the heads. Head suspensions were centrifuged for 25 min at 1 000 × g at 4ºC, to sediment the cellular debris, and the supernatants containing the HM were recovered. To concentrate the HM, supernatants were ultracentrifuged for 20 min at 28 000 × g at 4 ºC. The HM pellets were used for lipid extraction.

2.4. Lipids extraction and analysis

Lipids were extracted from flagella and HM samples according to the procedure by Folch *et al*. (1957) adjusted for seabream sperm and were stored in 300 µL of isopropanol. Cholesterol was measured with a CHOD PAP Kit (Biolabo, France) according to the instructions of the manufacturer, and a calibration curve was prepared with cholesterol (Sigma-Aldrich, Spain) for each measure. Total phospholipids were quantified using the procedure developed by Rouser *et al*. (1970). For phospholipids purification samples were passed through SEP-PAK silica gel

cartridges (Waters, Spain). First, 10 mL chloroform was pushed through the cartridge to elute neutral lipids, then 5 mL acetone was passed through the cartridge to elute glycolipids and finally 10 mL methanol was pushed through to elute phospholipids.

For the detection and quantification of the different phospholipids, samples were analyzed in a High Pressure Liquid Chromatographer (HPLC) (Waters 2695), equipped with an autoinjector and using a UV detector (Waters 996) using a Waters Spherisorb cartridge PSS838521 (Waters, Spain) (5 µm, 250 mm × 3.0 mm i.d.). The following mobile phase was used (acetonitrile/methanol/ phosphoric acid, 130/5/1.5, V/V/V) at a constant flow rate of 1.0 mL/min. Ten µL of each sample were injected and the sample analysis took 20 min. Peaks were identified and measured at 203 nm by comparison with standards. Results are reported as mg/mmol of phospholipids.

For fatty acids analysis samples were first transmethylated following the protocol by Berry *et al*. (1965) and methyl esters were stored in n-Hexane under a nitrogen atmosphere. Fatty acids were analyzed with a gas chromatographer (GC) (Perkin Elmer Autosystem XL) equipped with a flame ionization detector and using a fused silica capillary column Omegawax 250 (Supelco, Spain) (30 m × 0.25 mm i.d. × 0.25 µm film thickness). Helium was used as the carrier gas and the operating conditions were as follows: initial temperature set at 50ºC for 2 min; warming to 200ºC at a rate of 7ºC/min; 2 min at 200ºC; warming to 220ºC at a rate of 0.5ºC and; 5 min at 220ºC. Sample injection volume of 3 µL and total time of the analysis of 70 min. Fatty acid methyl ester standards mixture (C4 – C24:1) was used to identify and quantify the peaks and methyl nonanoate was used as internal standard. Results are presented as a proportion of total fatty acids.

2.5. Statistical analysis

All statistics were conducted using the software SPSS 15.0 for Windows. Significant differences between treatments for the different sperm quality parameters and lipids analysis were detected with a general linear model with Bonferroni's adjustment, considering the sampling months as covariable. Motility, viability and fatty acids data were normalized through arcsine transformation. Differences between fresh HM and fresh flagella were also detected with a general linear model with Bonferroni's adjustment, considering the sampling months as covariable. $P<0.05$ was considered statistically significant. Results are reported as mean values ± SEM (standard error of the mean). Correlations between the distinct parameters were detected with Pearson's parametric correlation ($p<0.05$ or $p<0.01$).

3. Results

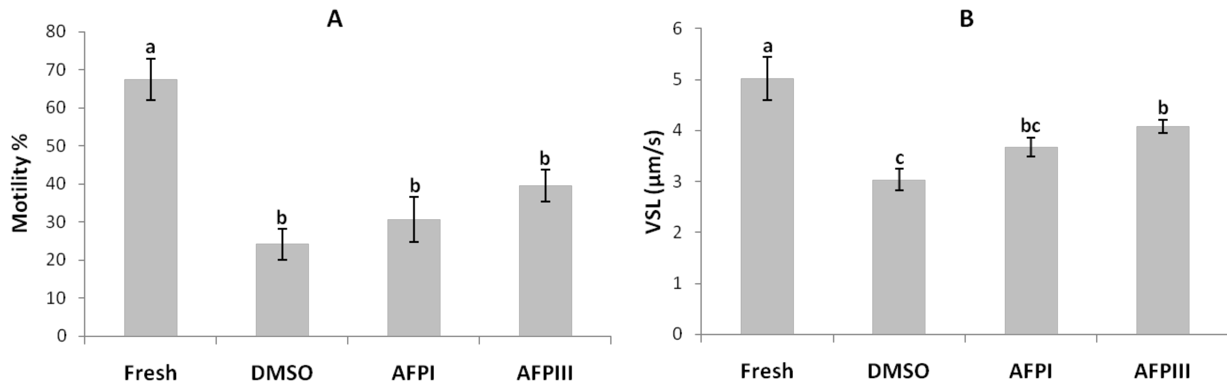


Fig 1 – A) Percentage of motile cells and; B) mean sperm straight line velocity (VSL) obtained for the four experimental conditions (fresh; cryopreserved with 5% DMSO; cryopreserved with 5% DMSO plus 1µg/mL AFPI and; cryopreserved with 5% DMSO plus 1µg/mL AFPIII). Data correspond to mean values ± SEM. Differences letters mean significant differences (p<0.05) (n=8).

Cryopreserved samples showed lower percentages of motile spermatozoa and lower VSL than control fresh sperm. For the percentage of motile sperm there were no significant differences between the different cryopreservation treatments as observed in Fig 1A. On the other hand, VSL values were significantly higher for the samples cryopreserved with AFPIII (4.08 ± 0.13 µm/sec) compared with samples cryopreserved with DMSO (3.03 ± 0.21 µm/sec) (Fig. 1B). Also for the percentage of viable cells there were significant differences between fresh and frozen samples, as well as between samples cryopreserved with AFPIII or DMSO (50.5 ± 2.75 % and 33.43 ± 3.01 %, respectively) (Fig 2).

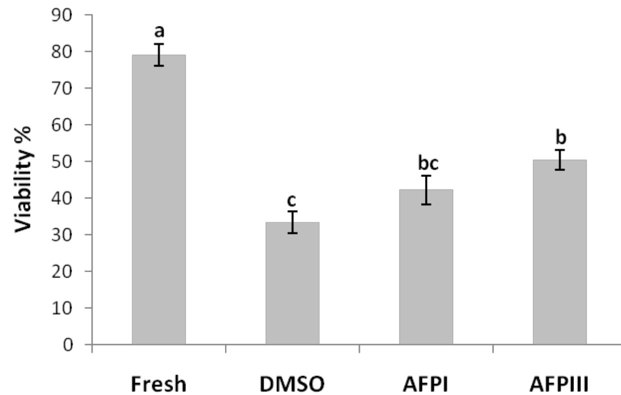


Fig 2 – Percentage of viable cells obtained for the four experimental conditions (fresh; cryopreserved with 5% DMSO; cryopreserved with 5% DMSO plus 1µg/mL AFPI and; cryopreserved with 5% DMSO plus 1µg/mL AFPIII). Data correspond to mean values ± SEM. Differences letters mean significant differences (p<0.05) (n=8).

The cholesterol/phospholipids ratio (Cho/Phos) was higher in the flagella than in the HM, but this difference was not significant, and neither were there any significant differences between the treatments for both flagella and HM (Table

1). For the analyzed phospholipids concentrations, presented in Table 1, the concentrations were different between HM and the flagella for fresh sperm, the concentration of both PE and PS being higher in flagella extracts than in HM extracts. Phosphatidyl-choline (PC) presented the highest values in both HM and flagella, ranging from 755 ± 61 to 539 ± 107 mg/mmol of phosphate, followed by phosphatidyl-ethanolamine (PE). Phosphatidyl-serine (PS) and phosphatidyl-inositol (PI) presented minor concentrations. Additionally, lysophosphatidyl-choline and sphyngomyelin were detected by HPLC, but their values were not considered since a very high variability was noticed. Only the PS from the HM was affected by freezing/thawing showing significantly higher values in DMSO treatment compared with the fresh samples.

Comparing the fresh sperm fatty acids composition between HM and flagella (Table 1), there were significantly higher amounts of saturated fatty acids (Stearic acid and total saturated) and lower amounts of unsaturated fatty acids (oleic acid, DHA (docosahexaenoic acid) and total MUFA (monounsaturated fatty acids)) in the HM than in the flagella membranes. Also the DHA/EPA (eicosapentaenoic acid) ratio was significantly higher in the flagella extracts than in HM extracts. The fatty acids composition of the HM was more affected by the cryopreservation procedures than was that of the flagella. No differences in the molecular species of fatty acids from HM were observed with regard to the fresh samples when AFPI or AFPIII were added to the extender. Nevertheless, there was an increase in the amount of saturated fatty acids when sperm was cryopreserved with DMSO without AFPs, although this increase was only significant for the palmitic acid (C16:0). The opposite effect occurred for several PUFAs, such as EPA (C20:5n3) and C22:5n3, with a decrease in its relative amount after cryopreservation with DMSO. When samples frozen with the different extenders were compared, no significant differences were noticed between samples frozen with DMSO and those including AFPI, but relevant changes were observed between sperm cryopreserved with or without AFPIII: the total saturated fatty acids were at a lower concentration in the sperm cryopreserved with AFPIII than in samples cryopreserved with DMSO, whereas a higher content in either total monounsaturated (Total MUFA), total polyunsaturated (Total PUFA) and n3 fatty acids was detected after cryopreservation with AFPIII comparing with DMSO. Furthermore the ratio of both EPA/AA (arachidonic acid) and DHA/EPA in the HM was also significantly different between the DMSO and AFPIII cryopreservation procedures.

Table 1 (next page) – Cholesterol/phospholipids ratio, phospholipid classes (mg per mmol of phospholipids) and the more abundant fatty acids (%) composition of the head plasma membrane (HM) and flagella of the *S. aurata* for the four experimental conditions (fresh; cryopreserved with 5% DMSO; cryopreserved with 5% DMSO plus 1µg/mL AFPI and; cryopreserved with 5% DMSO plus 1µg/mL AFPIII).

		Fresh	DMSO	AFPI	AFPIII
HM	Cho/Phos	0.74 ± 0.21	1.13 ± 0.30	0.96 ± 0.27	1.02 ± 0.30
Flagella	Cho/Phos	1.22 ± 0.09	1.38± 0.14	1.22 ± 0.09	1.15 ± 0.10
HM	Phospholipids mg /mmol of Phosphate				
	PI	ND	124.14 ± 124.14	ND	130.50 ± 130.50
	PS	**57.79 ± 12.14**^{b}*	**139.95 ± 65.44**^{a}	**41.28 ± 6.59**^{b}	**71.24 ± 17.92**^{ab}
	PE	141.40 ± 42.68*	188.02 ± 32.08	85.72 ± 51.10	148.67 ± 38.17
	PC	708.69 ± 74.12	612.45 ± 88.47	546.97 ± 89.72	539.45 ± 106.73
Flagella	PI	ND	250.55 ± 208.73	61.48 ± 43.01	ND
	PS	155.85 ± 25.61	151.16 ± 25.20	153.18 ± 13.53	164.49 ± 19.21
	PE	426.22 ± 30.18	376.22 ± 57.02	402.00 ± 28.66	421.93 ± 14.07
	PC	652.59 ± 40.30	754.82 ± 61.03	670.90 ± 44.44	699.52 ± 55.10
HM	Fatty acids (%)				
	C16:0 (Palmitic acid)	**18.04 ± 1.69**^{b}	**21.25 ± 1.45**^{a}	**18.18 ± 1.35**^{ab}	**16.45 ± 0.50**^{b}
	C18:0 (stearic acid)	**15.71 ± 3.62**^{ab}*	**21.06 ± 3.65**^{a}	**17.18 ± 4.05**^{ab}	**11.09 ± 1.35**^{b}
	C18:1n9 (oleic acid)	**12.01 ± 1.01**^{ab}*	**10.36 ± 1.37**^{b}	**12.14 ± 1.08**^{ab}	**13.57 ± 0.53**^{a}
	C18:2n6 (linoleic acid)	8.83 ± 0.91	6.74 ± 1.26	6.97 ± 1.36	9.87 ± 0.57
	C20:2n6	1.62 ± 0.27*	2.83 ± 0.78	2.58 ± 0.54	1.74 ± 0.37
	C20:3n3	**2.11 ± 0.21**^{a}	**1.61 ± 0.23**^{b}	**1.95 ± 0.21**^{ab}	**2.25 ± 0.10**^{a}
	C20:4n6 (AA)	0.19 ± 0.01	0.27 ± 0.09	0.15 ± 0.02	0.18 ± 0.01
	C20:5n3 (EPA)	**11.11 ± 1.09**^{a}	**8.02 ± 1.29**^{b}	**10.69 ± 1.27**^{ab}	**12.24 ± 0.54**^{a}
	C22:5n3	**6.45 ± 0.65**^{a}	**5.47 ± 0.63**^{b}	**6.41 ± 0.72**^{ab}	**7.12 ± 0.36**^{a}
	C22:6n3 (DHA)	20.78 ± 0.60*	17.33 ± 0.83	19.18 ± 2.02	21.18 ± 0.52
	Total saturated	**35.82 ± 5.64**^{ab}*	**45.35 ± 5.37**^{a}	**37.47 ± 5.53**^{ab}	**29.20 ± 1.90**^{b}
	Total MUFA	**13.47 ± 1.17**^{ab}*	**11.98 ± 1.38**^{b}	**13.59 ± 1.22**^{ab}	**15.16 ± 0.62**^{a}
	Total PUFA	**49.44 ± 5.38**^{ab}	**40.97 ± 5.18**^{b}	**48.94 ± 4.40**^{ab}	**55.64 ± 1.52**^{a}
	Total n3	**38.19 ± 4.54**^{ab}	**30.57 ± 4.20**^{b}	**38.60 ± 4.19**^{ab}	**43.20 ± 1.32**^{a}
	Total n6	11.25 ± 0.89	10.41 ± 1.10	10.35 ± 1.14	12.44 ± 0.41
	EPA/AA	**65.96 ± 4.20**^{ab}	**48.37 ± 9.94**^{b}	**69.35 ± 4.16**^{a}	**70.80 ± 4.38**^{a}
	DHA/EPA	**1.74 ± 0.06**^{b}*	**2.22 ± 0.35**^{a}	**1.83 ± 0.06**^{ab}	**1.74 ± 0.06**^{b}
Flagella	C16:0 (Palmitic acid)	16.06 ± 0.23	17.95 ± 1.21	17.88 ± 1.14	17.82 ± 1.44
	C18:0 (stearic acid)	**6.50 ± 0.23**^{b}	**10.30 ± 1.71**^{a}	**8.72 ± 0.69**^{ab}	**8.35 ± 1.19**^{ab}
	C18:1n9 (oleic acid)	15.97 ± 0.18	15.07 ± 0.28	15.58 ± 0.53	15.07 ± 0.39
	C18:2n6 (linoleic acid)	**10.80 ± 0.32**^{a}	**9.85 ± 0.48**^{bc}	**10.63 ± 0.26**^{ab}	**9.58 ± 0.54**^{c}
	C20:2n6	0.72 ± 0.07	0.71 ± 0.08	0.65 ± 0.04	0.87 ± 0.13
	C20:3n3	1.91 ± 0.10	1.87 ± 0.13	2.02 ± 0.05	1.79 ± 0.13
	C20:4n6 (AA)	**0.20 ± 0.01**^{ab}	**0.18 ± 0.00**^{b}	**0.19 ± 0.01**^{ab}	**0.22 ± 0.02**^{a}
	C20:5n3 (EPA)	10.75 ± 0.59	10.31 ± 0.66	10.87 ± 0.18	9.93 ± 0.80
	C22:5n3	7.46 ± 0.44	7.06 ± 0.42	6.92 ± 0.59	6.95 ± 0.58
	C22:6n3 (DHA)	22.99 ± 0.38	20.76 ± 1.43	21.57 ± 0.83	20.08 ± 1.53
	Total saturated (%)	**21.73 ± 2.86**^{b}	**31.02 ± 3.24**^{a}	**27.72 ± 1.09**^{a}	**30.64 ± 1.66**^{a}
	Total MUFA (%)	18.05 ± 0.26	17.14 ± 0.37	17.61 ± 0.61	17.08 ± 0.46
	Total PUFA (%)	53.02 ± 4.16	51.67 ± 3.01	48.17 ± 6.31	49.49 ± 2.94
	Total n3 (%)	40.69 ± 3.88	40.39 ± 2.53	37.48 ± 4.88	39.48 ± 2.97
	Total n6 (%)	12.33 ± 0.38	11.28 ± 0.53	10.69 ± 1.45	10.01 ± 1.26
	EPA/AA	**57.30 ± 2.81**^{ab}	**63.51 ± 3.25**^{a}	**57.13 ± 3.50**^{ab}	**50.25 ± 5.78**^{b}
	DHA/EPA	2.05 ± 0.06	2.011 ± 0.05	1.99 ± 0.08	2.04 ± 0.05

Significant differences between treatments for the same parameters are marked with different letters and highlighted in bolt ($p<0.05$) (n=8). Significant differences between HM and flagella for fresh sperm are signed by an asterisk and highlighted in grey ($p<0.05$) (n=8).

In the case of the flagella there was a significant increase in the amount of total saturated fatty acids for all three cryopreservation procedures compared with fresh samples, but also in this case more changes in the fatty acids profile were observed when the sperm was frozen without AFPs. The DMSO protocol increases the content in stearic acid and decreases the linoleic concentration and the ratio DHA/EPA in comparison with the fresh sperm. No significant differences were newly observed between this protocol and the one with AFPI. The addition of AFPIII provided significant increases of AA and reduction of the EPA/AA ratio respect to the DMSO.

Percentage of viable cells was found to be negatively correlated with the Cho/Phos ratio of the HM and the amounts of PS in the HM and PC in the flagella (Table 2). The proportion of saturated fatty acids of the HM was negatively correlated with the sperm quality parameters (percentage of viable and motile cells and sperm velocity). On the other hand the proportion of unsaturated fatty acids of the HM was positively correlated with the sperm quality parameters. Moreover the Cho/Phos ratio was also positively correlated with saturated fatty acids and negatively correlated with the unsaturated fatty acids.

Table 2 - Most important obtained correlations between the sperm quality parameters and lipid analysis.

		Viability (%)	VSL	Motility (%)	Membrane Cho/Phos
	Membrane Cho/Phos	-0.403*	-	-0.412*	-
	Membrane PS	-0.604**	-	-0.537**	0.820**
	Flagella PC	-0.472**	-	-0.428*	-
Head Membrane	C16	-	-0.376*	-	-
	C18	-0.369*	-0.371*	-.407*	-
	C18:1n9	0.434*	-	0.481**	-
	C18:2n6	0.443*	0.440*	0.438*	-
	C20:2n6	-0.528**	-	-0.517**	-
	C20:5n3(EPA)	0.457**	0.412*	0.478**	-
	C22:5n3	-	0.384*	0.375*	-
	C22:6n3 (DHA)	0.378*	-	0.405*	-
	Total Saturated	-0.367*	-0.389*	-0.392*	0.686**
	Total MUFA	0.427*	-	0.476**	-0.842**
	Total PUFA	-	0.402*	-	-0.619**
	Total n3	-	0.366*	-	-0.633**
	Total n6	-	0.414*	-	-

Relationships with Pearson parametric correlation (r): significant correlations signed with * for $p < 0.05$; and with ** for $p < 0.01$.

4. Discussion

In this study we have evaluated the effects of sperm cryopreservation on the presence of AFPs with special focus on the lipid composition of the different regions of the sperm (HM and flagella). Clear positive results were observed with the addition of 1µg/mL of AFPIII, promoting an increase in sperm velocity (VSL) and percentage of viable cells after the freezing/thawing process compared to the control freezing/thawing treatment (DMSO). In contrast, the addition of equal concentration of AFPI did not improve the analyzed parameters when compared to the same control treatment. The action mechanism of AFPs on the membranes and on ice crystal growth is believed to be similar for both tested proteins (AFPI and AFPIII): binding to the lipid bilayer, increasing the phase transition temperature of the membranes and stabilizing them (Tomzack *et al*. 2002 BIO; Inglis *et al*. 2006). Additionally, their cryoprotective effect on sperm is clearly species specific, being more notable in some species than others and in some cases even prejudicial, raising the possibility by some authors of possible cytotoxic effects (Payne *et al*. 1994; Younis *et al*. 1998; Prathalingam *et al*. 2006). Comparing our results with those ones obtained by other authors working with mammalian spermatozoa, Younis *et al*. (1998) also observed increased motility after thawing when AFPIII was added to chimpanzee sperm. Nevertheless other studies reported better results with AFPI, such as Prathalingam *et al*. (2006) working with bovine sperm and Payne *et al*. (1994) assaying with ram sperm, whereas Koshimoto & Mazur (2002) noticed a decrease in sperm survival of mouse spermatozoa with both AFPI and AFPIII. The diverse cryoprotective effects of the different AFPs observed in the analyzed species could be related with differences in their interaction to the membrane components, each species having particular arrangements of lipids and characteristic phase transitions temperatures. To our knowledge, there are no reports on the effects of AFPs on sperm membrane composition during the freezing/thawing process. In the present study there was a clear effect of the used cryoprotectant solution (whether used AFPI and AFPIII or not) on the phospholipids and fatty acids composition of the spermatozoa, specially the HM composition.

Membrane fluidity is determinant in the cryopreservation outcome, the higher the membrane fluidity the higher the resistance to the cryopreservation process. The Cho/Phos ratio partly explains membrane fluidity and is usually affected during cryopreservation. Blesbois *et al*. (2005) observed a decrease in the Cho/Phos ratio after cryopreservation of bird sperm. While Chakrabarty *et al*. (2007) observed an increase in the ratio after goat sperm cryopreservation. In the case of rainbow trout this ratio was also negatively correlated with sperm resistance to cryopreservation (Labbé & Maisse 1996). In our case there were no effects of the cryopreservation procedure on the Cho/Phos ratio in either flagella or HM extracts, but the HM Cho/Phos ratio was negatively correlated with the percentage of both viable and motile cells.

While cholesterol increases membrane stability it also has a rigidifying and condensing effect on the membranes and as pointed by Crockett (1998) this, in turn, decreases membrane permeability to ions and polar nonelectrolytes. Furthermore cholesterol variations can also modify the behavior and function of the membranar proteins due to their role in the formation of rafts (Ohvo-Rekila *et al*. 2002; Wassall & Stillwell 2009). Muller *et al*. (2008) consider that the cholesterol changes had to be restricted to a range in order to maintain spermatozoa function. Hence, both motility initiation and fertilization could be compromised by substantial changes in the cholesterol content. Our data did not reveal significant changes in the Cho/Phos ratio, but it is known that cholesterol exerts different effects because of its interaction with proteins and the rest of membrane lipids or the degree of saturation of the fatty acids, which could also define hyperfluidic microdomains as important as rafts for sperm function (Muller *et al*. 2008, Wassall & Sillwell 2009).

We have analyzed the phospholipid and fatty acid composition separately for the flagella and the HM. In mammal, bird and fish, sperm plasma membrane PC and PE usually account for more than 50% of the phospholipids, and PI and PS are usually represented at lower concentrations (Drokin 1993; Buhr *et al*. 1994; Labbé *et al*. 1995; Surai *et al*. 2000). In the present study a significant difference between flagella and HM PE and PS content was observed, since the HM present lower amounts of these phospholipids. Labbé *et al*. (1995) reported in *Oncorhynchus mykiss* that both PE and PS were the most unsaturated phospholipids, and a later study in *Dicentrarchus labrax* and different salmonids by Bell *et al*. (1997) agreed that sperm PS was highly unsaturated whereas PC had the highest amount of saturated fatty acids. Our results showed a higher proportion of unsaturated fatty acids in the flagella (richer in PE and PS), specifically MUFA, what will make this structure more fluid than HM, similar to the observations by Connor *et al*. (1998). Cryopreservation only affected the HM PS amount, being higher in samples cryopreserved only with DMSO, which also display lower values of the sperm quality parameters. Even though several authors have recorded changes in sperm phospholipid composition following cryopreservation, these modifications are usually observed in PC and PE (Chakrabarty *et al*. 2007) that are more susceptible to shed off given their location in the extracellular face of the membrane bilayer (Buhr *et al*. 1994). Therefore the significant increase in PS could be rather the result of other phospholipids decrease such as PC and PE, given that our results refer to phospholipids proportion. Additionally there were some significant negative correlations between both HM and flagella composition in phospholipids (PS and PC respectively) and the percentage of both viable and motile cells. This could be related with their individual fatty acid hydrocarbon chains composition and should be further investigated. Cryopreservation effects on phospholipids composition is highly related with its particular fatty acids content. As already mentioned, the precise effect of AFPs on the membranes is unknown, nevertheless studies with model membranes (liposomes) composed mainly by PC and PE have suggested that AFPs interact with the membranes by the insertion of

a hydrophobic segment of the protein into the membranes, this reaction being highly dependent on the fatty acids composition of the phospholipids (reviewed by Inglis *et al*. 2006). The direct interaction of the hydrophobic domains of AFPs with the hydrophobic fatty acids moieties of membrane lipids could explain their species-specific activity and their positive or negative effects during cryopreservation according to the particular composition of the membrane.

The fact that flagella fatty acids were more unsaturated than those from HM could be related to their higher requirements of fluidity related to movement as suggested Connor *et al*. (1998), and it could also explain the lower rate of modifications of the flagella after the applied cryopreservation treatments: higher unsaturation rates are usually related to higher resistance to freezing/thawing (Waterhouse *et al*. 2006). The evaluation of fatty acids before and after cryopreservation clearly showed a beneficial effect of the addition of AFPs: except for the linoleic acid on the flagella, there were no changes with respect to the fresh samples, but several differences were found between sperm frozen with DMSO and fresh samples or those frozen with AFPs, especially AFPIII. Freezing with DMSO extender without AFPs induced an increase in saturated fatty acids and the loss of unsaturated fatty acids, some of them, such as EPA (C20:5n3) highly unsaturated. Both factors promote losses of fluidity and were negatively correlated to sperm quality parameters (viability, motility and velocity). Similar changes were observed after the cryopreservation of different mammalian spermatozoa by Buhr *et al*. (1994), Cerolini *et al*. (2001) or Chakrabarty *et al*. (2007) who attributed to these changes the decrease in the sperm quality parameters. This issue indicates a direct effect of the AFPs on the stabilization of molecular classes of fatty acids in the membrane, compatible with the theory of an interaction of the protein with the membrane components (Inglis *et al*. 2006). Their different effects on different fatty acids could also indicate that they will probably interact preferentially with certain molecular species. According to our results, AFPIII seem to have avoided the increase in the saturated fatty acids proportion during cryopreservation, and hence would have interacted preferentially with unsaturated fatty acids. This observation is in accordance with results of Tomczak *et al*. (2002b) who observed a stronger association of AFPs with liposomes made with unsaturated fatty acids than with saturated. The differences in the HM and the flagella fresh composition, especially in the amounts of unsaturated fatty acids, may underline their different sensitivity to cryopreservation as well as the different AFPs protection mechanism observed in both structures: while in the HM the EPA/AA ratio increased in the AFPIII and decreased in the DMSO treatment, the converse effect was observed in the flagella. As put forward by Tomczak *et al*. (2002a) the membrane lipid composition dictates the protection conferred by the AFPs during chilling. The observed variations in lipid composition in both HM and flagella cryopreserved with AFPs could have been caused by membrane leakage or free lipids fusion as was observed by Tomczak *et al*. (2001) testing AFPI effects during cryopreservation of liposomes. The fact that sperm quality parameters were positively

correlated with the unsaturated fatty acids, but negatively with the amount of several saturated fatty acids is similar to observations made in boar semen (Am-in *et al*. 2011). This fact was mostly observed in HM, indicating that mainly the HM but not the flagella lipid composition affects both sperm motility and viability. The relevance of highly unsaturated fatty acids in sperm function has also been pointed out by Wassall & Stillwell (2008) who explain its possible role in the organization of hyperfluid microdomains related to sperm-egg fusion. Finally we should stress the fact that not just AFPs protective effect is dependent on specific membrane composition and fatty acids saturation (Wu & Fletcher 2000; Tomczak *et al*. 2002b) but also its toxic effects, which could even be the cause of membrane leakage as was observed in some liposomes (Wu & Fletcher 2000; Tomczak *et al*. 2001). As explained by Inglis *et al*. (2006) it is currently not possible to predict whether a particular AFP will stabilize or destabilize a given lipid system.

Our results have shown that the use of AFPIII in the cryopreservation solution decreases the loss of sperm quality after the freezing/thawing process and helps maintain the lipid composition of the plasma membrane of the analyzed domains (head and flagella) during the freezing/thawing process, exerting a stabilizing effect on gilthead seabream sperm membrane and avoiding at least in part the described sublethal damage caused by sperm membrane disturbance (Cabrita et al. 2005; Beirão et al. 2010). This is the first study analyzing the effects of AFPs on sperm membrane lipids during cryopreservation and helped explain the beneficial effects these proteins could have during the process. Moreover, this is also the first study reporting how the lipidic membrane components of two different sperm domains are affected during freezing/thawing, giving data about fatty acids changes during cryopreservation in fish.

Acknowledgments

The authors are grateful to Manuela Piccinno and Annalea Conte from the University of Salento (Italy) for helping during sampling, to Dr Catherine Labbé from INRA (France) for providing the protocols for lipids analysis and to Francisca Martínez from the ULE-LTI (Spain) for help provided during lipids analysis. This work was supported by the project Petri (PTR 95.1026.OP.01). J. Beirão was supported by a PhD grant from the Portuguese Ministry of Science and Technology (SFRH/BD/31990/2006) co-founded by POPH - QREN - Tipologia 4.1 (FEDER and MCTES).

References

Aksoy Y, Aksoy H, Altinkaynak K, Aydin H & Ozkan A 2006 Sperm fatty acid composition in subfertile men. *Prostaglandins Leukotrienes and Essential Fatty Acids* **75** 75-79.

Am-In N, Kirkwood R, Techakumphu M & Tantasuparuk W 2011 Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. *Theriogenology* **75** 897-903.

Beirão J, Pérez-Cerezales S, Martínez-Páramo S & Herráez M 2010 Detection of early damage of sperm cell membrane in Gilthead seabream (Sparus aurata) with the nuclear stain YO-PRO 1. *Journal of Applied Ichthyology* **26** 794-796.

Beirão J, Cabrita E, Pérez-Cerezales S, Martínez-Páramo S & Herráez M 2011 Effect of cryopreservation on fish sperm subpopulations. *Cryobiology* **62** 22-31.

Bell M, Dick J & Buda C 1997 Molecular speciation of fish sperm phospholipids: Large amounts of dipolyunsaturated phosphatidylserine. *Lipids* **32** 1085-1091.

Berry J, Cevallos W & Wade R 1965 Lipid class and fatty acid composition of intact peripheral nerve and during wallerian degeneration. *Journal of the American Oil Chemists Society* **42** 492-500.

Blesbois E, Grasseau I & Seigneurin F 2005 Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. *Reproduction* **129** 371-378.

Bobe J & Labbé C 2010 Egg and sperm quality in fish. *General and Comparative Endocrinology* **165** 535-548.

Bongalhardo D, Leeson S & Buhr M 2009 Dietary lipids differentially affect membranes from different areas of rooster sperm. *Poultry Science* **88** 1060-1069.

Buhr M, Curtis E & Kakuda N 1994 Composition and behavior of head membrane-lipids of fresh and cryopreserved boar sperm. *Cryobiology* **31** 224-238.

Cabrita E, Robles V, Cuñado S, Wallace J, Sarasquete C & Herráez M 2005 Evaluation of gilthead sea bream, Sparus aurata, sperm quality after cryopreservation in 5ml macrotubes. *Cryobiology* **50** 273-284.

Cerolini S, Maldjian A, Pizzi F & Gliozzi T 2001 Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Reproduction* **121** 395-401.

Chakrabarty J, Banerjee D, Pal D, De J, Ghosh A & Majumder G 2007 Shedding off specific lipid constituents from sperm cell membrane during cryopreservation. *Cryobiology* **54** 27-35.

Connor W, Lin D, Wolf D & Alexander M 1998 Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. *Journal of Lipid Research* **39**

1404-1411.

Crockett E 1998 Cholesterol function in plasma membranes from ectotherms: Membrane-specific roles in adaptation to temperature. *American Zoologist* **38** 291-304.

Drokin S 1993 Phospholipids and fatty-acids of phospholipids of sperm from several fresh-water and marine species of fish. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular* **104** 423-428.

Fabbrocini A, Lavadera S, Rispoli S & Sansone G 2000 Cryopreservation of seabream (Sparus aurata) spermatozoa. *Cryobiology* **40** 46-53.

Folch J, Lees M & Stanley G 1957 A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226** 497-509.

Inglis S, Turner J & Harding M 2006 Applications of type I antifreeze proteins: Studies with model membranes & cryoprotectant properties. *Current Protein & Peptide Science* **7** 509-522.

Karanova M & Tsvetkova L 1994 Cryoprotective properties of antifreeze glycoproteins upon freezing of fish sperm. *Izvestiya Akademii Nauk Seriya Biologicheskaya* **5** 818-827.

Karanova M, Zsvetkova L & Petropavlov N 1997 Effect of antifreeze glycoproteins on quality of cryoconserved carp sperm. *Biofizika* **42** 725-728.

Koshimoto C & Mazur P 2002 Effects of warming rate, temperature, and antifreeze proteins on the survival of mouse spermatozoa frozen at an optimal rate. *Cryobiology* **45** 49-59.

Labbé C & Maisse G 1996 Influence of rainbow trout thermal acclimation on sperm cryopreservation: Relation to change in the lipid composition of the plasma membrane. *Aquaculture* **145** 281-294.

Labbé C, Maisse G, Muller K, Zachowski A, Kaushik S & Loir M 1995 Thermal-acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma-membrane. *Lipids* **30** 23-33.

Ladha S 1998 Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. *Journal of Membrane Biology* **165** 1-10.

Le Francois N, Lamarre S, Tveiten H, Blier P & Bailey J 2008 Sperm cryoconservation in Anarhichas sp., endangered cold-water aquaculture species with internal fertilization. *Aquaculture International* **16** 273-279.

Miller R, Sheffer C, Cornett C, McClean R, MacCallum C & Johnston S 2004 Sperm membrane fatty acid composition in the Eastern grey kangaroo (Macropus giganteus), koala (Phascolarctos cinereus), and common wombat (Vombatus ursinus) and its relationship to cold shock injury and cryopreservation success. *Cryobiology* **49** 137-148.

Muller K, Muller P, Pincemy G, Kurz A & Labbé C 2008 Characterization of sperm plasma membrane properties after cholesterol modification: Consequences for cryopreservation of rainbow trout spermatozoal. *Biology of Reproduction* **78** 390-399.

Ohvo-Rekila H, Ramstedt B, Leppimaki P & Slotte J 2002 Cholesterol interactions with phospholipids in membranes. *Progress in Lipid Research* **41** 66-97.

Payne S, Oliver J & Upreti G 1994 Effect of antifreeze proteins on the motility of ram spermatozoa. *Cryobiology* **31** 180-184.

Prathalingam N, Holt W, Revell S, Mirczuk S, Fleck R & Watson P 2006 Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw. *Theriogenology* **66** 1894-1900.

Pustowka C, McNiven M, Richardson G & Lall S 2000 Source of dietary lipid affects sperm plasma membrane integrity and fertility in rainbow trout Oncorhynchus mykiss (Walbaum) after cryopreservation. *Aquaculture Research* **31** 297-305.

Surai P, Brillard J, Speake B, Blesbois E, Seigneurin F & Sparks N 2000 Phospholipid fatty acid composition, vitamin E content and susceptibility to lipid peroxidation of duck spermatozoa. *Theriogenology* **53** 1025-1039.

Tomczak M, Hincha D, Estrada S, Feeney R & Crowe J 2001 Antifreeze proteins differentially affect model membranes during freezing. *Biochimica et Biophysica Acta-Biomembranes* **1511** 255-263.

Tomczak M, Hincha D, Estrada S, Wolkers W, Crowe L, Feeney R, Tablin F & Crowe J 2002a A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophysical Journal* **82** 874-881.

Tomczak M, Vigh L, Meyer J, Manning M, Hincha D & Crowe J 2002b Lipid unsaturation determines the interaction of AFP type I with model membranes during thermotropic phase transitions. *Cryobiology* **45** 135-142.

Venketesh S & Dayananda C 2008 Properties, potentials, and prospects of antifreeze proteins. *Critical Reviews in Biotechnology* **28** 57-82.

Wassall S & Stillwell W 2009 Polyunsaturated fatty acid-cholesterol interactions: Domain formation in membranes. *Biochimica et Biophysica Acta-Biomembranes* **1788** 24-32.

Waterhouse K, Hofmo P, Tverdal A & Miller R 2006 Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction* **131** 887-894.

Wu Y & Fletcher G 2000 Efficacy of antifreeze protein types in protecting liposome membrane integrity depends on phospholipid class. *Biochimica et Biophysica Acta-General Subjects* **1524**

11-16.

Rouser G, Fleischer S & Yamamoto A 1970 2 dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5** 494-496.

Younis A, Rooks B, Khan S & Gould K 1998 The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (Pan troglodytes) spermatozoa. *Journal of Andrology* **19** 207-214.

Zilli L, Schiavone R, Storelli C & Vilella S 2008 Molecular mechanisms determining sperm motility initiation in two sparids (Sparus aurata and Lithognathus mormyrus). *Biology of Reproduction* **79** 356-366.

Final Discussion

Consensually sperm quality could be defined as the capacity of the sperm to successfully fertilize oocytes and further develop into a normal embryo. Nonetheless, for practical reasons both for aquaculture and research purposes alternative and accurate methodologies are needed to evaluate sperm quality. Specific methodologies for sperm analysis must exist to allow the researcher to study the effects of a particular treatment in fishes (hormonal treatment, testing diets, etc) or in sperm (cryopreservation, ecotoxicology studies) or to understand putative causes for the decrease in sperm fertilizing ability in species showing some kind of reproductive dysfunction.

In this thesis, through the application and development of specific methodologies for fish sperm analysis, we have: 1) evaluated *Solea senegalensis* sperm quality in captivity and searched for possible ways to improve male reproductive performance and; 2) using *Sparus aurata* as a model species for the marine teleost currently farmed, improved sperm cryopreservation protocols and techniques for cryopreservation outcome evaluation at cytophysiological level.

WILD CAPTIVE *SOLEA SENEGALENSIS* SPERM QUALITY ANALYSIS AND IMPROVEMENT

S. senegalensis is a high market value species that has attracted the attention of both aquaculturists and researchers in recent decades, but low spawn quality and availability of F1 broodstocks are impairing its massive production, and sole farming is still dependent on the availability of individuals captured in the wild. Recent studies by Cabrita *et al*. (2006) and Martinez-Pastor *et al*. (2008a) have shown a low sperm quality and volume of stocked animals. In addition some studies showed that these sperm constraints could be partially overpassed by hormonal stimulation (Agulleiro *et al*., 2007; Cabrita *et al*., 2011) and diet control (Anguís *et al*., 2008). Thus an in-depth analysis of sperm quality analysis is mandatory to help solve sole reproductive problems and develop better protocols for broodstock husbandry management.

Bearing in mind the importance of the development of tools for sperm quality analysis of *S. senegalensis*, as a first approach we analyzed the sperm quality of a broodstock during the reproductive season with different approaches: motility analysis, using a CASA system and subpopulation analysis; cell plasma membrane resistance to seawater hyperosmolarity; DNA fragmentation with comet assay and; cell apoptosis with Annexin-V FITC.

For the motility data obtained with CASA we applied a cluster analysis, which allowed us to use the 8 descriptors rendered by the software and group the heterogeneous sperm population in 4 homogeneous subpopulations. Besides the expected decrease in motility observed in sole sperm after activation, the different subpopulations also varied differently through time, and at 60 s the most represented subpopulation was Subpop4 (slow and nonlinear). As demonstrated

by Casselman *et al*. (2006) and Rudolfsen *et al*. (2008) working with other fish species, both sperm progressiveness and velocity affect fertilization success, and thus in *S. senegalensis* fertilization will most likely occur by the Subpop1 (fast and linear), almost absent 1 min after motility activation. As supported by the correlations obtained between the distinct subpopulations at different times, we believe that there was a migration of the sperm from the fast subpopulations to the slow ones and from the linear subpopulations to the nonlinear ones.

The observed sperm viability was very low, in most cases below 50%, caused by the percentage of cells with a damaged membrane (stained with PI) and the presence of apoptotic cells (stained with Annexin-V). The origin of the apoptotic cells is not clear, although their presence has been described in the testes of *Sparus aurata* by Chaves-Pozo *et al*. (2005) at the beginning of spermatogenesis or during the post-spawning period, their presence in the ejaculate indicating some spermiation deregulation. Widely studied in mammals (Paasch *et al*., 2004; Sakkas *et al*., 2010), apoptotic cells are usually eliminated in the testes before ejaculation and their presence in *S. senegalensis* milt could have been caused by environmental stress due to captivity or inappropriate husbandry conditions or be related to a normal mechanism for the elimination of overripe spermatozoa since, in the case of *S. senegalensis* testes, several cell maturation stages coexist at same time in gonad (García-López *et al*., 2006).

A high percentage of spermatozoa was affected by seawater hyperosmolarity after contact with activation solution, required for fertilization (after 45 sec exposure to seawater most samples had less than 10% viable cells). This fact could have been related to poor development of osmoregulatory mechanisms in sperm from this species or to some kind of damage in captive breeders affecting plasma membrane osmoregulation in these cells. Moreover sperm samples presenting higher cell viability were also the ones that better sustained hyperosmotic shock.

Most of the analyzed cells presented some DNA damage. However compared with other marine species (Cabrita *et al*., 2005; Zilli *et al*., 2003), *S. senegalensis* sperm mean DNA fragmentation was lower. DNA fragmentation was negatively correlated with cell resistance to seawater hyperosmotic shock, and positively correlated with the percentage of cells stained with PI before motility activation. Thus seawater hyperosmotic shock could be responsible for the selection of spermatozoa with low DNA fragmentation, also eliminating cells with plasma membrane damage and the non-motile population. Furthermore there were also correlations between the percentage of cells resistant to seawater hyperosmotic shock and the subpopulations at different times, indicating that cell resistance to seawater hyperosmotic conditions modulate population structure after activation. The putative cell lyses observed during the hyperosmotic test can be part of a semen selection process to positively choose cells with better performance, eliminating overripe cells, and preventing them from achieving fertilization.

Following the results obtained during the sole reproductive season, showing very low resistance of the sperm membrane to seawater hyperosmolarity, and the fact that this cell lysis could be a mechanism to eliminate low quality spermatozoon, we addressed a longer term analysis. In *Solea senegalensis*, males spermiate all year-round (Cabrita *et al*., 2006; García-López *et al*., 2006), thus in order to better understand the sperm quality variation and to be able to establish better broodstock manipulation protocols, we evaluated sperm quality throughout the year, applying the analysis used during the reproductive season.

As expected there were males producing sperm during all months. Sperm velocity was significantly higher during the two spawning seasons (spring and autumn) compared with the rest of the year. Both the percentage of motile cells and the progressive sperm were highly variable between males, indicating some asynchrony in the maturation of both sexes, their values decreasing between March and May. Other authors studding fish sperm have shown changes in sperm quality throughout the reproductive season (Babiak *et al*., 2006; Rainis *et al*., 2003).

The mean DNA fragmentation values increased during the spring spawning season and peaked in the summer months following a similar pattern to the water temperature. Banks *et al*. (2005) also observed temperature effect on mammalian sperm DNA integrity. In our case this increase could have been caused by extraction during stripping of already overripe cells presented in the testicular lumen in the later months of the spawning season. The percentage of apoptotic cells was also lower at the beginning of the first spawning (March) and increased until the summer months. Nonetheless these two parameters were not significantly correlated. On the other hand the motility parameters were negatively correlated with the percentage of apoptotic cells, denoting that the membrane destabilization detected with Annexin-V affects the motility mechanism.

The low resistance of cells to seawater hyperosmolarity was a constant throughout the year, however during the summer months these values were significantly even lower, registering values lower than 5% of cells with intact membrane just 15 s after motility activation. This parameter clearly seemed to be a limiting factor, since no matter the time of the year at 60 s almost the entire sperm population was already compromised. Thus fertilization must occur immediately after gamete release. Additionally to the above-mentioned fact that seawater hyperosmolarity could act as a mechanism for the selection of better quality spermatozoa, *S. senegalensis* also presents a reproductive behavior that allows the sperm to achieve oocytes in a short period after ejaculation. As described by Duncan *et al*. (2008) in captive individuals, during spawning the male and female swim in synchrony with the genital ducts held closely together when gametes are released.

Finally there was a high heterogeneity between the sperm quality of different males during the year. Different males were at their peak of semen quality at different moments of the spawning

seasons and even in different periods of the year, and most individuals were desynchronized with females. This fact could have been caused by competitive behavior. In any case this sperm quality variation will result in unequal contribution from males to the offspring.

In accordance with the results obtained in the two previous works we decided to test diets supplemented with fatty acids (DHA) and antioxidants (selenium (Se) and vitamin E (vitE)) aiming to improve *S. senegalensis* sperm quality, especially its membrane resistance to hyperosmotic shock. The results of the diet enrichment were tested during the spawning season, and positive results were observed in the sperm quality even though as initially proposed, cell resistance to seawater hazardous external medium was not improved.

In the males fed on the supplemented diets (DHA plus antioxidants) there was an increase in sperm motility parameters (% progressive sperm and VCL) and, as already explained above, spermatozoa with higher motility would have higher chances of achieving fertilization. Motility values were positively correlated with both PUFAs and DHA proportion in the sperm. Also other authors have observed improved sperm quality after feeding fish with diets with high PUFAs content observing that PUFAs were incorporated in the sperm composition (Alavi *et al*., 2009; Asturiano *et al*., 2001). In the present study cholesterol increased in the males fed on DHA and antioxidants and although it would be expected to affect membrane stability, as observed in *Oncorhynchus mykiss* sperm by Muller *et al*. (2008), such an effect was not detected. This fact could have occurred because this increase was in seminal plasma and not in sperm membrane or be due to the opposite effect by other lipid components. In males fed on DHA and antioxidants, as was expected, there was also an increase in the amount of DHA. As explained by Wassall and Sillwell (2009) DHA has a fluidizing effect on plasma membranes, and at some point this effect is opposed to that of cholesterol. Furthermore there was an increase in MUFAs, specially caused by the increase in oleic acid (C18:1n9) and C20:1n9, and an increase in other unsaturated fatty acids, such as eicosatrienoic acid (C20:3n3) and linoleic acid (C18:2n6). Linoleic acid is the precursor of AA in freshwater fish and evidence of this fact was also found in *Dicentrarchus labrax* (Asturiano *et al*., 2001). In our case there was an increase in this amount, reflected in the EPA/AA ratio (decreasing significantly in the sperm of males fed on supplemented diets). The long chain unsaturated fatty acids (AA, EPA and DHA) are essential for prostaglandin production and the correct testicular maturation and steroidogenesis (Asturiano *et al*., 2000; Cerda *et al*., 1995). Conversely, the amount of saturated fatty acids decreased significantly, perceptible in the amounts of C17, C18 and C24. In mammalian sperm the amount of saturated fatty acids has been negatively correlated with both sperm motility and viability (Aksoy *et al*., 2006; Am-In *et al*., 2011).

Males fed on a diet only supplemented with DHA (without antioxidants) did not show an improvement in sperm quality because of the harmful effects of the generation of reactive

oxygen species (ROS) on spermatozoa, causing the high lipid peroxidation values observed with this diet. This increase in ROS production was caused by increased membrane sensitivity to lipid peroxidation due to the presence of higher amount of PUFAs. The addition of the antioxidants Se and vitE to the diet, has circumvented the higher lipid peroxidation. Furthermore, the increase in lipid peroxidation raised the amount of lysophosphatidyl-choline (LPC) in the sperm samples. LPC increase is the result of phosphatidyl-choline (PC) breakdown as a result of sperm degradation (Glander *et al*., 2002). As a final point the addition of both Se and vitE not only avoided lipid peroxidation, but also contributed to the improvement of the sperm quality traits, as was observed in bovine sperm in the case of Se (Siegel et al., 1980) and in the sperm of other fish species in case of vitE (Canyurt and Akhan, 2008; Rainis *et al*., 2007).

The components supplemented in the diets were clearly incorporated in the semen composition; nonetheless we did not analyze separately seminal plasma and spermatozoon lipid content. Thus, no specific conclusions can be made from putative modifications of the sperm membrane or other specific components of the semen.

EVALUATION AND IMPROVEMENT OF SPERM CRYOPRESERVATION IN MARINE TELEOSTS (*SPARUS AURATA*)

Although several authors developed sperm cryopreservation protocols for different marine teleosts (Cabrita *et al*., 2008; Cabrita *et al*., 2010), there is a lack in standardization and knowledge of the cytophysiological mechanisms that affect sperm quality during the cryopreservation procedure. Most studies only apply rudimentary methodologies to evaluate the tested cryopreservation procedures, such as sperm mean motility values and sperm membrane integrity and thus several established protocols are suboptimal. Moreover, as pointed out by Bobe and Labbé (2010), it is often very difficult to correlate sperm grading in a quality test to the fertilization rate obtained with the same sperm given that fertilizing ability depends upon many cellular parameters.

In the first work we have focus our attention on the effects of cryopreservation on *Sparus aurata* sperm subpopulations. As reviewed by Martínez-Pastor *et al*. (2011) sperm samples are heterogeneous and in any ejaculate spermatozoa with different motility characteristics co-exist. The existence of sperm subpopulations is neglected in most fish sperm studies; however their existence has already been stated by some authors (Cabrita *et al*., 1999; Le Comber *et al*., 2004; Martínez-Pastor *et al*., 2008a).

In this study, similar to what was identified in *S. senegalensis,* three well-defined subpopulations were identified in *S. aurata.* From the identified subpopulations, SP3 presented fast and linear spermatozoa, SP1 slow and non-linear spermatozoa and SP2 slow linear spermatozoa. As reported in different fish species, fertilization success is correlated with spermatozoa swimming speed (Casselman *et al*., 2006; Rudolfsen *et al*., 2008). Similarly, SP3 was the subpopulation best correlated with the hatching rate and thus deserved special focus in the cryopreservation trials. The occurrence of both SP1 and SP2 populations, which represent sperm with lower probability of achieving fertilization, could be the result of semen exhaustion and deterioration after activation as proposed by Quintero-Moreno *et al*. (2003).

The subpopulation structure was clearly affected by the cryopreservation protocols, and there was a significant variation in the subpopulation composition between the different protocols. The different composition in subpopulations obtained between the protocols pointed out which were the less efficient freezing/thawing protocols: the lowest protection conferred to spermatozoa corresponded to the highest probability that spermatozoa belonged to SP2 or SP1 (slow subpopulations) instead of SP3 (fast linear subpopulation). Moreover, similar to the hypothesis proposed by Martinez-Pastor *et al*. (2008a), our results suggested that cells belonging to SP3 at 15 seconds might at later times have moved to SP1 or SP2 due to energy loss, displaying lower velocity and/or linearity. Thus rather than just the sperm subpopulation composition at 15 seconds, we analyzed the overall subpopulation composition throughout time after motility activation. As an example we observed that, using straws, after freezing with 10% EG at 2 cm over the surface of liquid nitrogen, SP3 size was more constant through time than after freezing with 5% DMSO at 2 cm. In the line of the observations by Gage *et al*. (2004), in *Salmo salar*, showing that spermatozoa that maintained straight and rapid motility for longer had a higher probability of achieving fertilization than other sperm cells. So we could state that samples cryopreserved with 10% EG (at 2 cm above LN_2) were more advantageous. Furthermore it was detected that the best results after freezing/thawing observed with the subpopulations analysis did not necessarily correspond to the best results observed with average motility.

In the samples cryopreserved with cryovials a significant decrease in SP3 subpopulations was observed when compared to the same treatments applied with straws. According to the observations by other authors (Cabrita *et al*., 2001 and 2005; Horvath *et al*., 2007), the observed differences were attributed to the difference in thickness between the two containers, causing lower temperature diffusion in the cryovials (freezing and thawing curves presented a lower slope in the cryovials when compared with the straws) and also to the different volume to surface area ratio. Moreover ice nucleation occurred in cryovials later than in straws as shown by our temperature records. All these issues implied a greater dehydration during freezing, longer exposure to supercooling temperatures responsible for cold shock

damage, longer exposure to cryoprotectant toxic effects, higher probability of recrystalization during thawing and hence more chance of damage to the cellular structure.

Finally, DMSO was the tested cryoprotectant that provided better results (higher percentage of SP3). It is interesting to notice that previous studies with other Sparidae also point to DMSO as the best cryoprotectant (Fabbrocini *et al*., 2000; Gwo, 1994; Liu *et al*., 2006).

After better understanding the effects that cryopreservation has on the motile population, we proposed to test a new tool for fish spermatozoa to detect early membrane destabilization for the evaluation of spermatozoa viability after freezing ⁄ thawing procedures. In fish sperm, the membrane is the key structure responsible for the environmental reception of stimulus and for triggering responses such as motility activation. Membrane structure is highly sensitive to environmental stress and alterations can seriously affect spermatozoa functionality (Cabrita *et al*., 2008). Thus the detection of early changes in sperm cell membrane is of utmost importance and therefore we assayed the use of a sensitive probe to subtle changes in membrane permeability: YO-PRO 1.

Our results showed that instead of the two sperm subpopulations detected with the traditional evaluation with PI ⁄ SYBR 14 assay (viable cells and non viable cells) the application of YO-PRO 1 together with PI allowed the detection of a third subpopulation of cells permeable to YO-PRO 1 but not to PI. This subpopulation was composed by cells with a disturbed membrane which were non-permeable to PI, but whose permeability was already modified, similar to the cell population detected in the studies with *S. senegalensis* with Annexin-V. Similar studies in mammalian sperm have attributed these modifications to apoptosis (Kumaresan *et al*., 2009; Martínez-Pastor *et al*., 2008b).

Furthermore after the freezing ⁄ thawing there was a significant increase in both YO-PRO1 and PI stained cells. This fact indicates that cryopreservation in addition to increasing the percentage of membrane-damaged spermatozoa, the percentage of cells with disturbed plasma membrane also increases. The size of the sperm subpopulation stained with YO-PRO 1 was dependent on the applied cryopreservation protocol. Finally the flow cytometry analysis also allowed us to detect that the three different identified subpopulations (YO-PRO 1 and PI stained cells and viable cells) had different size and shape indicating that changes in the sperm membrane permeability or integrity were also accompanied by morphometric changes.

After observing that cryopreservation affects the spermatozoa motile subpopulations differently and that during the cryopreservation process there is an increase in the amount of spermatozoa with destabilized membrane, which could affect the capacity of the sperm to sense the environmental changes that work as a stimulus for triggering the motile machinery,

in our last study we decided to examine the effects that the cryopreservation process has on the head plasma membrane (HM) and flagella lipidic composition. At the same time we attempted to stabilize membrane composition by the addition of AFPs (AFPI and AFPIII) based on the capacity attributed to these proteins to interact with cell membranes (Inglis *et al*., 2006). The best results were obtained with the addition of 1 µg/mL of AFPIII with significantly higher sperm velocity values (VSL) and a significantly higher percentage of viable cells compared with the control samples (cryopreserved only with 5% DMSO as cryoprotectant). The same concentration of AFPI did not produce a significant improvement in sperm quality. AFPs cryoprotective effect is highly species-specific, and it seems to be highly dependent on the membrane composition. For this reason, while some authors reported better results with AFPI (Prathalingam *et al*., 2006), others obtained better results with AFPIII (Younis *et al*., 1998) and in some cases the addition of AFPs had even been cause of a reduction in sperm quality (Koshimoto & Mazur, 2002). According to some hypotheses, AFPs could insert a hydrophobic segment of the protein into the membranes, this reaction being highly dependent on the fatty acids composition of the phospholipids (Tomzack *et al*., 2002; Inglis *et al*., 2006). The interaction of the hydrophobic domains of AFPs with the hydrophobic fatty acids of the membrane lipids could explain their species-specific activity and positive or negative effects during cryopreservation depending on the particular composition of the membrane. For this reason, we have clearly observed different effects depending on the cryoprotectant solution used (whether AFPI and AFPIII are used or not) and on the phospholipids and fatty acids composition of the spermatozoa domains (HM or flagella). The flagella lipidic composition was not so affected by the cryopreservation procedure as the HM. In the case of the HM, cryopreservation with 5% DMSO without AFPs induced an increase in saturated fatty acids and a loss of unsaturated fatty acids, promoting a decrease in fluidity. This unsaturated fatty acid loss was negatively correlated with quality parameters such as viability, motility and velocity.

Lipidic composition differences were noticed between the HM and the flagella, the amount of phosphatidyl-ethanolamine (PE) and phosphatidyl-serine (PS) being significantly higher in the flagella. According to studies performed with other fish species, PE and PS are the most unsaturated phospholipids (Labbé *et al*., 1995; Bell *et al*., 1997). This could also have been the case in our study since not just the PE and PS were significantly in higher concentration in the flagella but also the unsaturated fatty acids, specifically MUFA, which will make the flagella structure more fluid than HM. These higher requirements of fluidity could be related to the flagellar beating has suggested Connor *et al*. (1998).

As a last observation, sperm quality parameters (both motility and viability) were positively correlated with the HM unsaturated fatty acids, but negatively with the amount of several saturated fatty acids. The same correlations were not found for flagella lipids, pointing out that mainly the HM lipid composition affects the tested sperm quality traits.

The addition of AFPs, especially AFPIII, increased sperm quality after the cryopreservation process by stabilizing the lipidic composition of both HM and flagella.

References

Agulleiro M.J., Scott A.P., Duncan N., Mylonas C.C., Cerda J. 2007. Treatment of GnRHa-implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comp. Biochem. Physiol. A-Mol. Integr. Physiol. 147(4): 885-892

Aksoy Y., Aksoy H., Altinkaynak K., Aydin H.R., Ozkan A. 2006. Sperm fatty acid composition in subfertile men. Prostaglandins Leukot. Essent. Fatty Acids 75(2):75-79

Alavi S.M.H., Psenicka M., Policar T., Rodina M., Hamackova J., Kozak P., Linhart O. 2009. Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season. Fish Physiol. Biochem. 35(4):683-693

Am-In N., Kirkwood R.N., Techakumphu M., Tantasuparuk W. 2011. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology 75(5):897-903

Anguís V., Salas-Leiton E., Martín I., Chereguini O. 2008. Influence of the feeding on sperm quality of first generation (f1) *Solea senegalensis* during an annual cycle in two facilities of Spain. Aquaculture Europe 08. Krakow, Poland, September 15-18

Asturiano J.F., Sorbera L.A., Zanuy S., Carrillo M. 2000. Effects of polyunsaturated fatty acids and gonadotropin on prostaglandin series E production in a primary testis cell culture system for the European sea bass. J. Fish Biol. 57(6):1563-1574

Asturiano J.F., Sorbera L.A., Carrillo M., Zanuy S., Ramos J., Navarro J.C., Bromage N. 2001. Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. Aquaculture 194(1-2): 173-190

Banks S., King S.A., Irvine D.S., Saunders P.T.K. 2005. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. Reproduction 129(4):505-514

Babiak I., Ottesen O., Rudolfsen G., Johnsen S. 2006. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 65(8): 1587-1604

Bell M.V., Dick J.R., Buda C. 1997. Molecular speciation of fish sperm phospholipids: Large amounts of dipolyunsaturated phosphatidylserine. Lipids 32(10): 1085-1091

Bobe J. and Labbé C. 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165 (3): 535-548

Cabrita E., Alvarez R., Enel E., Herráez M.P. 1999. The hypoosmotic swelling test performed with coulter counter: a method to assay functional integrity of sperm membrane in rainbow trout. Anim. Reprod. Sci. 55(3-4):279-287

Cabrita E., Robles V., Alvarez R., Herráez M.P. 2001. Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. Aquaculture 201(3-4): 301-314

Cabrita E., Robles V., Rebordinos L., Sarasquete C., Herráez M. P. 2005. Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology 50(2): 144-153

Cabrita E., Soares F., Dinis M. T. 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. Aquaculture 261(3): 967-975

Cabrita E., Robles V., Herráez M.P. 2008. Sperm quality assessment. In: Methods in reproductive aquaculture: Marine and freshwater species. Biology series. Eds: Cabrita E., Robles V., Herráez M.P. CRCPress (Taylor and Francis group), London, UK, 93-147

Cabrita E., Sarasquete C., Martínez-Páramo S., Robles V., Beirão J., Pérez-Cerezales S., Herraéz, M.P. 2010. Cryopreservation of fish sperm: applications and perspectives. J. Appl. Ichtyol. 26(5): 623-635

Cabrita E., Soares F., Beirão J., García-López A., Martínez-Rodríguez G., Dinis M.T. 2011. Endocrine and milt response of Senegalese sole, *Solea senegalensis*, males maintained in captivity. Theriogenology 75(1): 1-9

Canyurt M.A. and Akhan S. 2008. Effect of dietary vitamin E on the sperm quality of rainbow trout (*Onchorynchus mykiss*). Aquac. Res. 39(9): 1014-1018

Casselman S.J., Schulte-Hostedde A.I., Montgomerie R. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci. 63(9):2119-2125

Cerda J., Zanuy S., Carrillo M., Ramos J., Serrano R. 1995. Short-term and long-term dietary-effects on female sea bass (*Dicentrarchus labrax*) - seasonal-changes in plasma profiles of lipids and sex steroids in relation to reproduction. Comp. Biochem. Physiol. C 111(1):83-91

Chaves-Pozo E., Mulero V., Meseguer J., Ayala A.G. 2005. An overview of cell renewal in the testis throughout the reproductive cycle of a seasonal breeding teleost, the gilthead seabream (*Sparus aurata* L.). Biol. Reprod. 72(3): 593-601

Connor W., Lin D., Wolf D., Alexander M. 1998. Uneven distribution of desmosterol and docosahexaenoic acid in the heads and tails of monkey sperm. J. Lipid Res. 39(7): 1404-1411

Duncan N., Carazo I., Chereguini O., Norambuena F., Mañanós E., Estevez A. 2008. Observations of spawning in wild caught and captivity reared broodstock. In: Report of the IV Workshop on the cultivation of Soles, Faro, Portugal, 7-8

Fabbrocini A., Lavadera S.L., Rispoli S., Sansone G. 2000. Cryopreservation of seabream (*Sparus aurata*) spermatozoa. Cryobiology 40(1): 46-53

Gage M.J.G., Macfarlane C.P., Yeates S., Ward R.G., Searle J.B., Parker G.A. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14(1):44-47

García-López A., Fernandez-Pasquier V., Couto E., Canário A. V. M., Sarasquete C., Martínez-Rodríguez, G. 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen. Comp. Endocrinol. 147(3): 343-351

Glander H.J., Schiller J., Suss R., Paasch U., Grunewald S., Arnhold J. 2002. Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines. Andrologia 34(6):360-366

Gwo J.C. 1994. Cryopreservation of yellowfin seabream (*Acanthopagrus latus*) spermatozoa (teleost, perciformes, Sparidae). Theriogenology 41(5):989-1004

Horváth A., Miskolui E., Mihalffy S., Osz K., Szabo K., Urbányi B. 2007. Cryopreservation of common carp (*Cyprinus carpio*) sperm in 1.2 and 5 ml straws and occurrence of haploids among larvae produced with cryopreserved sperm. Cryobiology 54(3): 251-257

Inglis S.R., Turner J.J., Harding M.M. 2006. Applications of type I antifreeze proteins: Studies with model membranes & cryoprotectant properties. Curr. Protein Pept. Sci. 7(6): 509-522

Koshimoto C. and Mazur P. 2002. Effects of warming rate, temperature, and antifreeze proteins on the survival of mouse spermatozoa frozen at an optimal rate. Cryobiology 45(1):49-59

Kumaresan A., Kadirvel G., Bujarbaruah K.M., Bardoloi R.K., Das A., Kumar S., Naskar S. 2009. Preservation of boar semen at 1.8 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. Anim. Reprod. Sci. 110(1-2):162-171

Labbé C., Maisse G., Muller K., Zachowski A., Kaushik S., Loir M. 1995. Thermal-Acclimation and Dietary Lipids Alter the Composition, but Not Fluidity, of Trout Sperm Plasma-Membrane. Lipids 30(1): 23-33

Le Comber S.C., Faulkes C.G., Van Look K.J.W., Holt W.V., Smith C. 2004. Recovery of sperm activity after osmotic shock in the three-spined stickleback: Implications for pre-oviposition ejaculation. Behaviour 141: 1555-1569

Liu Q.H., Li J., Zhang S.C., Ding F.H., Xu X.Z., Xiao Z.Z., Xu S.H. 2006. An efficient methodology for cryopreservation of spermatozoa of red seabream, *Pagrus major*, with 2-mL cryovials. J. World Aquacult. Soc. 37(3): 289-297

Muller K., Muller P., Pincemy G., Kurz A., Labbé C. 2008. Characterization of sperm plasma membrane properties after cholesterol modification: Consequences for cryopreservation of rainbow trout spermatozoa. Biol. Reprod. 78(3): 390-399

Martínez-Pastor F., Cabrita E., Soares F., Anel L., Dinis M.T. 2008a. Multivariate cluster analysis to study motility activation of *Solea senegalensis* spermatozoa: a model for marine teleosts. Reproduction 135(4): 449-59

Martínez-Pastor F., Fernandez-Santos M. R., del Olmo E., Dominguez-Rebolledo A. E., Esteso M. C., Montoro V., Garde J. J. 2008b. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod. Fertil. Dev. 20(5): 547-556

Martínez-Pastor F., Tizado E.J., Garde J. J., Anel L., de Paz P. 2011. Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. Theriogenology 75(5):783-795

Paasch U., Grunewald S., Agarwal A., Glandera H.J. 2004. Activation pattern of caspases in human spermatozoa. Fertil. Steril. 81:802-809

Prathalingam N.S., Holt W.V., Revell S.G., Mirczuk S., Fleck R.A., Watson P.F. 2006. Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw. Theriogenology 66(8): 1894-1900

Quintero-Moreno A., Miro J., Rigau A.T., Rodriguez-Gil J.E. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. Theriogenology 59(9):1973-1990

Rainis, S., Mylonas C.C., Kyriakou Y, Divanach P. 2003. Enhancement of spermiation in European sea bass (*Dicentrarchus labrax*) at the end of the reproductive season using GnRHa implants. Aquaculture 219(1-4):873-890

Rainis S., Gasco L., Ballestrazzi R., Zoccarato I. 2007. Effects of vitamin E and phosphatidylcholine on qualitative and quantitative parameters of rainbow trout (*Oncorhynchus mykiss*) milt. Ital. J. Anim. Sci. 6(4): 339-350

Rudolfsen G., Figenschou L., Folstad I., Kleven O. 2008. Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua* L.). Aquac. Res. 39(2):212-216

Sakkas D. and Alvarez J.G. 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil. Steril. 93(4): 1027-1036

Tomczak M.M., Hincha D.K., Estrada S.D., Wolkers W.F., Crowe L.M., Feeney R.E., Tablin F., Crowe J.H. 2002. A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. Biophys. J. 82(2):874-881

Wassall S.R. and Stillwell W. 2009. Polyunsaturated fatty acid-cholesterol interactions: Domain formation in membranes. Biochim. Biophys. Acta-Biomembr. 1788(1): 24-32

Younis A.I., Rooks B., Khan S., Gould K.G. 1998. The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa. J. Androl. 19(2): 207-214

Zilli L., Schiavone R., Zonno V., Storelli C., Vilella S. 2003. Evaluation of DNA damage in *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology 47(3): 227-235

Conclusions/Conclusiones

The following conclusions were obtained from the work presented in this thesis:

1. **Wild captive *Solea senegalensis* sperm quality analysis and improvement**

1.1 - The analysis of captive *Solea senegalensis* semen quality through the evaluation of cell viability, resistance to hyperosmotic shock, DNA fragmentation, apoptosis (Annexin-V) and motility parameters provided evidence of a low quality of sperm during the reproductive season, specially related with the low ability of spermatozoa to sustain membrane integrity when exposed to seawater. This parameter correlated with DNA fragmentation, suggesting a selection process to eliminate spermatozoa with high levels of DNA fragmentation before fertilization.

1.2 -The evaluation of the *S. senegalensis* spermatozoa subpopulation structure, based on the motility parameters, demonstrated the presence of four subpopulations within the ejaculate: Subpop1 (fast and linear spermatozoa) was probably the responsible for fertilization. The overall sperm population structure was highly affected during the motility period and correlated with the percentage of cells resistant to seawater osmotic shock, suggesting that changes in this structure were related with sperm exhaustion and osmotic control. Only 1% of cells belonged to the Subpop1 at 60 s, indicating a low probability of achieving fertilization at this time.

1.3 - Sperm production was noticed in captive *S. senegalensis* all year-round, however there was a sperm quality peak within the spring spawning season (March-May) followed by a pronounced decrease. The worse values for most parameters, (percentage of motile and progressive cells, DNA fragmentation, Annexin-V positive spermatozoa and cell resistance to the hyperosmotic shock) were observed during June and July, corresponding to the highest water temperature months. These results indicate a possible effect of this environmental factor, evidencing a sperm ageing during this period. *Solea senegalensis* sperm quality was highly variable between males both within the reproductive seasons and throughout the year.

1.4 – *S. senegalensis* sperm modulation was possible through the addition of DHA (docosahexaenoic acid) accompanied by antioxidants (Se (selenium) and VitE (vitamin E)) to the diet, increasing both velocity and progressiveness and raising the chances for fertilization. Motility values were correlated with the sperm content of several polyunsaturated fatty acids (PUFA), DHA and total PUFA, which increased in sperm from males fed on the experimental diet. The addition of DHA without antioxidant agents did not promote beneficial effects due to the lipid peroxidation increase.

2. **Evaluation and improvement of sperm cryopreservation in marine teleosts (*Sparus aurata*)**

2.1 - The use of a clustering analysis allowed the detection of three spermatozoa subpopulations according to motility parameters rendered by CASA in *Sparus aurata* semen and their variation through time. The fast and linear subpopulation was the best correlated with the hatching rate. The overall population structure was differentially affected by the cryopreservation protocol depending on the type of cryoprotectant, freezing rate and packaging system used. This analysis provided a more accurate method for studying post-thaw sperm quality than the evaluation of average motility parameters.

2.2 - The application of the YO-PRO 1 nucleic acid stain with flow cytometry allowed us to detect a subpopulation of spermatozoa in *S. aurata* with disturbed plasma membrane that was not detected with propidium iodide (PI). Furthermore, it was denoted that changes in membrane permeability or integrity were also accompanied by spermatozoa morphometric changes.

2.3 - *S. aurata* sperm head membrane and flagella presented different lipid composition, the latter being richer in unsaturated fatty acids, which probably confer higher fluidity. Both domains showed different resistance to damage during the cryopreservation with a standard protocol using 5% DMSO as cryoprotectant, the head membrane being more sensitive to the process. Changes included an increase in saturated fatty acids and a decrease in unsaturated fatty acids, mainly the polyunsaturated fatty acids (PUFA).

2.4 - The addition of AFPIII (1µg/mL) to the extender media reduced the damaging effects of the freezing procedure in *S. aurata* sperm, increasing the percentage of viable cells and sperm velocity, as well as avoiding the changes in the membrane composition detected after freezing with 5% DMSO alone. Both sperm motility and viability parameters were correlated with the lipidic composition, suggesting that the stabilizing effect exerted by AFPIII in both analyzed domains (head membrane and flagella) was responsible for the better sperm performance after cryopreservation. Our data support the hypothesis of a direct interaction of AFPs with specific hydrophobic moieties of the membrane lipids.

Los trabajos presentados en esta tesis nos permiten extraer las siguientes conclusiones:

1. Análisis y mejora de la calidad del semen de *Solea senegalensis* en cautividad:

1.1 – El análisis de la calidad del semen de *Solea senegalensis* en cautividad mediante la evaluación de la viabilidad celular, la resistencia al choque hiperosmótico, la fragmentación del DNA, la apoptosis (marcaje con Annexina-V) y diversos parámetros de movilidad, ha puesto en evidencia una baja calidad espermática durante la época reproductiva, especialmente afectada por la baja capacidad del semen para mantener la integridad de la membrana plasmática cuando es expuesto al agua de mar. Este parámetro se ha correlacionado con el nivel de fragmentación del DNA de las células, sugiriendo un proceso de selección para impedir a los espermatozoides con elevado nivel de fragmentación del DNA alcanzar la fertilización.

1.2 – La evaluación de la heterogeneidad del semen de *S. senegalensis* mediante la identificación de subpoblacióнes celulares basadas en los parámetros de movilidad, ha demostrado la presencia de cuatro subpoblaciones en el eyaculado: Subpop1 (espermatozoides rápidos y con trayectoria lineal) probablemente la responsable de la fertilización. La proporción de las distintas subpoblaciones en el eyaculado ha variado a lo largo del tiempo y se correlaciona con el porcentaje de células resistentes al choque hiperosmótico, lo que sugiere que estos cambios están relacionados con el agotamiento de las reservas energéticas, así como con los daños causados por el choque hiperosmótico. A los 60 s, solamente 1% de las células pertenecía a la Subpop1, lo que indica una baja probabilidad de alcanzar la fertilización en este tiempo.

1.3 – La producción seminal se mantiene a lo largo de todo el año, observándose un incremento de calidad durante la época de puesta de primavera (marzo-mayo), seguida por un fuerte descenso en los meses posteriores. Los peores valores para los distintos parámetros analizados (porcentaje de espermatozoides móviles y progresivos, fragmentación del ADN, espermatozoides marcados con Annexina-V y resistencia al choque osmótico) fueron observados en junio y julio, meses en los que las temperaturas del agua eran más elevadas. Los resultados sugieren, por tanto, una influencia de la temperatura en la calidad seminal y evidencian un envejecimiento del semen durante este periodo. La calidad del semen del *S. senegalensis* fue bastante variable entre los machos, tanto en la época de puestas como a lo largo del año.

1.4 – La modulación de la calidad del semen de *S. senegalensis* fue posible con la adición conjunta de DHA (ácido docosahexaenoico) y de antioxidantes (Se (selenio) y VitE (vitamina E)) al pienso. De este modo, se incrementaron la velocidad y la progresividad de los espermatozoides, así como sus posibilidades de fertilizar. Los valores de movilidad

estuvieron correlacionados con el contenido del semen en distintos ácidos grasos poliinsaturados (PUFA), DHA y PUFA total, que se incrementaron en el semen de los peces alimentados con los piensos experimentales. La adición del DHA sin antioxidantes no permitió la mejora de la calidad del semen debido al aumento de la peroxidación lipídica.

2. Evaluación y mejora de la criopreservación seminal de teleósteos marinos (*Sparus aurata*):

2.1 – La aplicación de un análisis de clusters nos ha permitido la detección de tres subpoblaciones de espermatozoides, según los parámetros de movilidad obtenidos con el CASA en *Sparus aurata* y su variabilidad a lo largo del tiempo. La subpoblación rápida y lineal fue la mejor correlacionada con la tasa de eclosión. La composición de las muestras, en términos de subpoblaciones, fue afectada de modo distinto por los protocolos de congelación, según los crioprotectores, la rampa de congelación y el envase utilizado. Este análisis permitió un estudio más preciso de la calidad del semen criopreservado que el que ofrece el uso de los valores medios de los parámetros de movilidad.

2.2 – La aplicación a espermatozoides de *S. aurata* del marcador de ácidos nucleicos YO-PRO 1, conjuntamente con citometría de flujo, nos ha permitido detectar una subpoblación de espermatozoides con la membrana plasmática desestabilizada que no es detectada con el ioduro de propidio (IP). Además, los cambios en la permeabilidad e integridad de la membrana plasmática implican cambios morfométricos del espermatozoide.

2.3 - La membrana plasmática de la cabeza y el flagelo del espermatozoide de *S. aurata* presentaron distinta composición lipídica. El flagelo tuvo una mayor proporción de ácidos grasos insaturados, lo que probablemente le otorga una mayor fluidez. Utilizando un protocolo estándar con DMSO al 5% como crioprotector, las dos estructuras presentaron distinta resistencia al daño durante la criopreservación: la membrana de la cabeza resultó ser más sensible. Estos cambios provocaron un aumento de los ácidos grasos saturados y una disminución de los insaturados, esencialmente de los ácidos grasos poliinsaturados.

2.4 - La adición de AFPIII (1μg/mL) al medio de congelación disminuyó los daños causados por la criopreservación, lo cual incrementó el porcentaje de células viables y la velocidad de los espermatozoides. Además, impidió los cambios en la composición de la membrana, observados tras criopreservar solamente con DMSO 5%. La movilidad y viabilidad se correlacionaron con la composición lipídica, lo que sugiere que la estabilización de las membranas durante la congelación de las dos estructuras (membrana de la cabeza y flagelo) con la adición de AFPIII, fue responsable de la mejora de la calidad del semen

descongelado. Nuestros resultados apoyan la hipótesis de una interacción de las AFPs con regiones hidrofóbicas específicas de los lípidos de membrana.

This is for sure the most difficult part of my thesis, because there are so many people who one way or another have contributed to this work that there is a risk I may forget to mention someone.

I would like to express my gratitude firstly to my supervisors, Paz Herráez and Elsa Cabrita, who have not only taught me but also given me their friendship during these four years. And a reference note to Elsa Cabrita who introduced me to this research area and encouraged me to do this thesis. ¡Muchas gracias, Jefas!

Next, I would like to mention the different places where I have worked:

- The University of León where I spend most of my time and where I have met some great people who, by just making me laugh, have helped me through this year. Thank you to my fellows fish reproduction biologists (Serafín Peréz-Cerezales and Sonia Martínez-Páramo). Thank you to those I have shared the office with, and to all the people in the department who have helped me in the last four years. Thank you to the people in Plant Physiology for not kicking me out of their lab and their nitrogen corner. Thank to the people in LTI who assisted me with lipids analysis, especially Francisca Martínez. ¡Gracias!
- The University of Algarve (Aquagroup) in Portugal, where I performed much of the work presented in this thesis under the supervision of Elsa Cabrita, and in the laboratory of Professor Maria Teresa Dinis. Thank you to the friends I made there and a special mention to those who have participated directly in my work, Florbela Soares, Patricia Diogo and Jorge Dias. Obrigado!
- The University of Lecce in Italy where part of this work was performed in the laboratory of Professor Sebastiano Villela, but where several people also helped and taught me. A special mention to those directly involved in this work, Loredana Zilli, Roberta Schiavone and Annalea Conte. Grazie!
- The IPIMAR CRIP-Sul research station in Portugal, where another part of the work was done in collaboration with Pedro Pousão-Ferreira, and others who contributed by allowing me to perform the studies. Obrigado!

I should also mention the two fish farms that provided samples for this work:

- Tinamenor S.L. and always willing to help, the R+D+i Technical Director Carlos Mazorra
- Mariscos de Esteros S.A (Maresa) and the kind broodstock manager, Lolo

During my time in León, I have not only carried out a research thesis but continued with a live thesis, and grown as a person with the help of friends I have made and situations experienced. To everyone who has made me a better person in one way or another – Thank you.

A special word of gratitude to my wife (D), the one who stands by me day after day, with her amazing, encouraging smile.

Finally, I want to thank the three people who have been at my side since the day I was born, always supporting me and helping me find the way when I get lost (Pá, Ma e Maria).

Thank you all. I mean it.

Appendix

Changes in *Solea senegalensis* sperm quality throughout the year - Letter of aceptance

From: "Nienke de Jong" <anirep@elsevier.com> Subject: ANIREP-D-10-2811R1 Decision
Date: Wed, April 20, 2011 11:11 am To: jbeis@unileon.es

Ms. No. ANIREP-D-10-2811R1
Changes in Solea senegalensis sperm quality throughout the year

Dear Mr Beirão,

I am pleased to be able to inform you that your manuscript has been accepted as
Research Paper for publication in Animal Reproduction Science.

The manuscript will be transferred to our Production Department. Proofs will be sent
to you in due course.

With kind regards,

Nienke de Jong
Editorial Office Manager
Animal Reproduction Science

Chief Editor:
Thank you for your revised paper

Reviewer #1: No further comments