UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

EVALUATION OF DIFFERENT EXTENDERS FOR COLD STORAGE OF MEAGRE (*ARGYROSOMUS REGIUS*) SEMEN

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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ABSTRACT

"EVALUATION OF DIFFERENT EXTENDERS FOR COLD STORAGE OF MEAGRE (*ARGYROSOMUS REGIUS*) SEMEN"

Semen refrigeration is usually recommended as a cheap and simple procedure that facilitates artificial reproduction techniques. The main objective of this experiment was to develop a semen refrigeration protocol for meagre that is considered a potential candidate for aquaculture diversification in Southern Europe. This thesis also contributes for the understanding of the causes of the fish sperm quality degradation during refrigeration.

Three extenders (non-activating medium (Fauvel et al., 1998); NaCl 0.9% and; NaCl 0.9% with glycine and glucose) and three different dilutions (1:4, 1:9 and 1:19, sperm:extender) were tested in a full factorial design. The following quality parameters were assessed along the storage time: sperm motility parameters; percentage of viable sperm; adenosine triphosphate (ATP); lipid peroxidation in the form of malondialdehyde (MDA); and bacteriology.

The 0.9% NaCl and the 0.9% NaCl with glycine and glucose extenders and the dilutions 1:4 and 1:9 kept a higher percentage of motile cells for longer, as well as higher sperm velocity. Sperm viability and ATP had better results with 0.9% NaCl and the 0.9% NaCl with glycine and glucose extenders. The MDA values were lower in treatments with dilution 1:4 when compared to those with dilution 1:9. In the CFU/ml values, no differences were found between extenders and dilutions. Motility parameters were strongly correlated with viability, whereas no or weak correlations existed with the remaining parameters. Thus, motility and viability seem to have the most impact in the loss of semen quality. According to the results, meagre semen could be kept refrigerated using 0.9% NaCl, in dilution 1:4, for up to 10 days.

Key-words: Semen; Fish; Argyrosomus regius; Cold storage; Extenders

RESUMO

"AVALIAÇÃO DE DIFERENTES DILUIDORES PARA A REFRIGERAÇÃO DE SÉMEN DE CORVINA (*ARGYROSOMUS REGIUS*)"

A refrigeração de sémen é tipicamente recomendada como um procedimento barato e simples que facilita as técnicas de reprodução artificial. O principal objectivo desta experiência foi desenvolver um protocolo de refrigeração de sémen para a corvina, que é considerada uma potencial candidata para a diversificação de aquacultura no sul da Europa. Esta tese contribui também para a compreensão das causas da degradação da qualidade de sémen de peixe durante a refrigeração.

Três diluidores (*non-activating medium* (Fauvel et al., 1998); NaCl 0,9%; e NaCl 0,9% com glicina e glucose) em três diferentes diluições (1:4, 1:9 e 1:19, sémen:diluidor) foram testados num plano factorial completo. Os seguintes parâmetros de qualidade espermática foram avaliados ao longo do tempo de armazenamento: parâmetros de mobilidade do sémen; percentagem de espermatozoides viáveis; adenosina trifosfato (ATP); peroxidação lipídica na forma de malondialdeído (MDA); e bacteriologia.

Os diluidores NaCl 0,9% e NaCl 0,9% com glicina e glucose e as diluições 1:4 e 1:9 mantiveram uma percentagem de células móveis mais elevada por mais tempo, bem como maior velocidade dos espermatozóides. A viabilidade e o ATP tiveram melhores resultados com NaCl 0,9% e NaCl 0,9% com glicina e glucose. Os valores de MDA foram mais baixos em tratamentos com as diluições 1:4, quando comparados com aqueles com as diluições 1:9. Nos valores de bacteriologia não foram encontradas diferenças entre diluidores e diluições testadas. Os parâmetros de mobilidade correlacionaram-se fortemente com a viabilidade, enquanto inexistentes ou fracas correlações foram encontradas entre os restantes parâmetros. Por conseguinte, mobilidade e viabilidade parecem ter o maior impacto na perda de qualidade do sémen. De acordo com os resultados, o sémen de corvina pode ser mantido refrigerado usando o diluidor NaCl 0,9% na diluição 1:4 até 10 dias.

Palavras-chave: Sémen; Peixe; Argyrosomus regius; Refrigeração; Diluidores

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AEC	Adenylate energy charge
ATP	Adenosine triphosphate
BSA	Bovine serum albumen
C	Control/undiluted
CASA	Computer-assisted sperm analysis
CFU	Colony-forming units
FAO	Food and Agriculture Organization
GnRHa	Gonadotropin-releasing hormone agonist
GzLM	Generalized Linear Model
HBSS	Hanks' balanced salt solution
HPLC	High-performance liquid chromatography
IPMA	Instituto Português do Mar e Atmosfera (Portuguese Institute of Sea and Atmosphere)
LIN	Linearity
MDA	Malondialdehyde
MOT	Percentage of motile cells
NAM	Extender NAM
NAM4	Treatment with extender NAM in dilution 1:4
NAM9	Treatment with extender NAM in dilution 1:9
NAM19	Treatment with extender NAM in dilution 1:19
NC	Extender NC
NC4	Treatment with extender NC in dilution 1:4
NC9	Treatment with extender NC in dilution 1:9
NC19	Treatment with extender NC in dilution 1:19
NCG	Extender NCG
NCG4	Treatment with extender NCG in dilution 1:4
NCG9	Treatment with extender NCG in dilution 1:9
NCG19	Treatment with extender NCG in dilution 1:19
PI	Propidium iodide
PROG	Percentage of progressive spermatozoa
PS	Pseudomonas CN Agar
STR	Straightness
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TSA	Trypticase soya agar
VAP	Average path velocity
VCL	Curvilinear velocity
VIAB	Viability
VSL	Straight line velocity

ACTIVITIES DEVELOPED IN THE SCOPE OF THE MASTER'S DEGREE

1. Internship

Faculty of Veterinary Medicine – University of Lisbon (FMV-UL)

For three weeks, in July of 2014, some work was done in the facilities of the Faculty of Veterinary Medicine dedicated to research concerning Fish and Aquaculture. The work consisted in a preliminary approach to a possible research concerning the behavior of a species of shrimp, *Palaemonetes varians*. The preferences of this species regarding color of the substrate was assessed. During this period, aquaria were prepared for the individuals, and their behavior was observed and registered. A visit to an aquaculture was also done.

During these three weeks it was possible to acquire some experience in the planning and implementation of a preliminary project.

Oceanário de Lisboa

The first segment of the internship took place from January 5th to March 27th of 2015, in Oceanário de Lisboa (Lisbon Oceanarium). The team of veterinarians was accompanied during the entirety of the internship. The species that were observed and handled during the internship consisted of: mostly fish, both bony and cartilaginous, from different ecosystems; some species of amphibians; aquatic birds; a species of reptile (*Caretta caretta*); and one species of mammal (*Enhydra lutris*). Some of the activities which I accompanied included observation and diagnosis of individuals, routine analysis, reproductive ecography and necropsies. Due to an agreement with the institution, this section won't be further elaborated.

This internship allowed to obtain knowledge and a better understanding of aquatic animal medicine, mostly of fish, including some experience in reproduction of different species.

Aquaculture Research Station of the Portuguese Institute for the Ocean and Atmosphere (EPPO – IPMA)

The second segment of the internship took place from the 7th of April to the 26th of June of 2015, with two weeks of additional work in September, in the Aquaculture Research Station of the Portuguese Institute for the Ocean and Atmosphere, in Olhão. A portion of work took place in the facilities of the Center of Marine Sciences of the University of Algarve, in Faro.

During this internship took place the research project addressed by this thesis. The activities done in the scope of this project were done mostly by myself and my supervisor, but a team of biologists, as well as aquaculture and research technicians provided us with valuable support.

Experience was obtained in induction of handling of fish, as well as induction of anesthesia and collection of semen by stripping. I prepared the extenders that were tested and used them to dilute the semen. The semen quality parameters that were evaluated also represent areas of acquired expertise. I learned to use a computer-assisted sperm analysis in order to evaluate concentration, motility and viability. Nonetheless, the latter was mostly evaluated by myself through observation and counting in a fluorescence microscope. I prepared solutions and did ATP extraction and quantification, which included manipulation of a microplate reader. The MDA assay procedure was observed by myself. For bacteriology, I prepared growth medium plates and performed inoculations, as well as counting of colonies. I also obtained experience in the isolation, propagation and identification of genera of bacteria with biochemical tests. Lastly, in general, time planning and teamwork skills were developed.

2. Formation and Publications

A poster was displayed at the Aquaculture Europe 2014 congress, organized by the European Aquaculture Society: "Studies on behavior and activity of the shrimp *Palaemonetes varians*", by Santos, M. and Afonso, F. (2014).

In July of 2015 I attended the seminar "Meagre production – science and practice", organized by the EPPO-IPMA. During this seminar the results of the several works of the AQUACOR project were presented, regarding different aspects of the production of meagre, such as: performance of different production systems; feed; reproduction; larvae and fry production.

In October of 2015 I attended the XV National Spanish Congress and I Iberian Congress of Aquaculture in Huelva (Spain), organized by the Spanish Aquaculture Society. The communications I attended were related to different subjects such as: reproduction in captivity of Senegalese sole (*Solea senegalensis*) and other species; diversification of aquaculture with species such as meagre (*Argyrosomus regius*) and common octopus (*Octopus vulgaris*); production and sanitary quality of mollusks and crustaceans; sustainability and environmental impact in the scope of aquaculture. A poster was displayed: "Refrigeração do sémen de corvina (*Argyrosomus regius*): desenvolvimento de um protocolo e avaliação de parâmetros de qualidade" (Refrigeration of meagre semen (*Argyrosomus regius*): development of a protocol and evaluation of quality parameters), by Santos, M., Soares, F., Moreira, M., Cabrita, E. and Beirão, J. (2015).

BIBLIOGRAPHIC REVISION

1. Aquaculture in the world, and in Portugal

The human population is expected to increase to more than 11 billion by the end of the century (Lutz, Sanderson & Scherbov, 2001), leading to a bigger consumption of resources such as food (Pimentel, Huang, Cordova & Pimentel, 1997). This will result in an increase in fish consumption, a protein-rich food recommended as part of the human diet (Sargent & Tacon, 1999). Under these circumstances, and since the vast majority of the wild fish stocks are already overexploited or have reached their maximum productive capacity, further demand of fish should be met by aquaculture instead of capture (Grafton, Kompas & Hilborn, 2007).

The first written records regarding aquaculture date back to 2 500 years ago in China (Rabanal, 1988). Nowadays, it is the fastest growing animal production industry (Gjedrem, Robinson & Rye, 2012). The portion of global fish production corresponding to aquaculture, in 2015, was forecast to be 46% (Food and Agriculture Organization of the United Nations [FAO], 2015), while in 2003 it was only 31% (FAO, 2015). Quoting the words of the inventor of modern management and business visionary Peter Drucker, "Aquaculture, not the Internet, represents the most promising investment opportunity of the 21st century".

The fish consumption per capita in Portugal is the highest in the European Union, and it is estimated to remain so, with a predicted consumption of 58 kg per capita per year, in 2020 (Failler, 2007). To support this high demand the country relies on a large volume of fish commodity imports, which in 2009 represented more than half of the national supply (Teixeira, Rodrigues, Cavadas & Neto, 2013). At the same time, only 5% of fish produced in Portugal comes from aquaculture, whereas the remaining 95% comes from fisheries (FAO, 2014). Thus, the development of aquaculture would decrease Portugal's dependence on imports.

There are several factors in favour of the development of aquaculture in Portugal. As previously stated, the consumption of fish is very high. There is funding from the European Commission towards the development of aquaculture (Associação Portuguesa de Aquacultura [APA], 2014). Furthermore, it is one of the warmest European countries, due to the Mediterranean climate (Kalkan & Canuyrt, 2012). Finally, it has a vast coastline and a large area covered with lagoons, which can be used for aquaculture (Kalkan & Canuyrt, 2012). Nonetheless, the lack of economic power and the bureaucracy involved (licensing procedures take several years) have hindered the investment in aquaculture (APA, 2014).

Aquaculture production in Portugal, over the last 30 years, has fluctuated between 5 and 11 thousand tonnes, approximately (FAO, 2014). Of the total volume produced, 93% refers to brackish or marine aquaculture, and fish comprises 55% of its volume (Instituto Nacional de Estatística [INE], 2016). The main species produced in 2014 were grooved carpet shell (*Ruditapes decussatus*), turbot (*Scophthalmus maxima*), mussels (*Mytilus sp*.) and gilthead seabream (*Sparus aurata*) (INE, 2016). When it comes to marine fishes, the markets are currently saturated (Couto et al., 2016), especially because of competition with countries like Spain or Greece. An alternative to prevent this saturation is the development of technology for the production of new species. This is the aim of projects such as the AQUACOR project of IPMA, regarding meagre (*Argyrosomus regius*) and its production.

2. Biology of the meagre

The meagre, *Argyrosomus regius* (Asso, 1801), is part of the *Sciaenidae* family, which belongs to the Perciformes order. A common characteristic of this family, that gave its members the name of croakers and drums, are the sounds they produce by beating specific muscles against the swim bladder, making it resonate (Ramcharitar, Gannon & Popper, 2006). The *Argyrosomus* name comes from the Greek: *argyros*, meaning silver, and *soma*, body. Indeed, this species has a silver to bronze coloured elongated body, with a fairly large head and a yellow mouth cavity (Stipa & Angelini, 2005). In the wild, they reach up to 2 m in length and 50 kg in weight (Stipa & Angelini, 2005). The meagre's geographic distribution includes the Eastern Atlantic coast and the Mediterranean and Black Seas (Haffray et al., 2012; Lagardère & Mariani, 2006). They are carnivorous and, although the diet varies with their size, they feed mostly on small crustaceans when juveniles (Jiménez et al., 2005), and fish when adults (Quéro & Vayne, 1985; Stipa & Angelini, 2005).

Meagre are gonochorists (Gil, Grau, Basilone, Ferreri & Palmer, 2013; Schiavone, Zilli, Storelli & Vilella, 2012), meaning each individual has one constant gender. The reproductive season occurs from April to June (Gil et al., 2013), with aggregation near estuaries and salt marshes (Quéro, 1989; Stipa & Angelini, 2005). During this season the males emit the characteristic *croaks* (Lagardère & Mariani, 2006), whilst spawning occurs. The females lay eggs that are fertilized externally and hatch in 48 hours (Haffray et al., 2012). The larvae leave the nursing area as juveniles, when the summer ends, and migrate back to spawning areas upon reaching maturity (González-Quirós et al., 2011). During the winter, adults migrate to deeper waters, and reduce feeding activity. Water temperature seems to be a

determining factor in this species' biology, affecting namely its feeding requirements, trophic migration and reproduction (Stipa & Angelini, 2005).

Meagre appears to be a resilient species, due to its high fecundity (Haffray et al., 2012) and growth rate (Monfort, 2010). However, their aggregation and emission of spawning sounds makes meagre a species vulnerable to capture (Catalán, Jiménez, Alconchel, Prieto & Muñoz, 2006; Sadovy & Cheung, 2003).

3. Meagre as an advantageous species for aquaculture

Meagre has been farmed in Europe since the late nineties, first in France and then Italy. Since then, other countries like Spain, Portugal and Greece have started to show some interest in this species. In 2000, the total global production was of only 33 tonnes, but the number has been growing and in 2010 over 10 000 tonnes were produced in aquaculture (Monfort, 2010).

This species has several strengths that support its production. It has a fast growth rate, reaching about 1 kg in the first year and 2.5 kg in the second (Duncan et al., 2013) and a low feed conversion rate, between 0.9 and 1.2 (Monfort, 2010). Meagre responds well to the hormonal induction of spawning and the eggs and larvae have high quality (Mylonas et al., 2013a; Mylonas, Mitrizakis, Sigelaki & Papadaki, 2011). A study by Roo, Hernández-Cruz, Borrero, Schuchardt & Fernández-Palacios (2010) obtained promising results for larval production at an industrial rate. As a euryhaline species, meagre tolerates diverse environments, such as land based cultivation with brackish water (Jiménez et al., 2005; Ribeiro, Soares, Quental-Ferreira, Gonçalves & Pousão-Ferreira, 2013), while also withstanding tank captivity fairly well (Pastor, Grau, Massutí & Sánchez-Madrid, 2002). It is also resistant to handling and there are few publications reporting disease problems, indicating that meagre might be somewhat resistant to infectious diseases (Stipa & Angelini, 2005). Additionally, it has a good filleting yield of 42% (Grigorakis, Fountoulaki, Vasilaki, Mittakos & Nathanailides, 2011) and its fillets received high sensory acceptability scores (Giogios, Grigorakis & Kalogeropoulos, 2013). The intramuscular fat content is low, around 1% (Grigorakis et al., 2013), when compared to species such as gilthead seabream (Özogul, Özogul & Alagoz, 2007) and European sea bass (*Dicentrarchus labrax*) (Lanari et al., 1999). At the same time, the fat has a high nutritional value (Giogios, Grigorakis & Kalogeropoulos, 2013) and its low content allows refrigeration for a longer period of time (Poli, et al., 2003). Altogether, these characteristics make meagre a very attractive species for the aquaculture diversification (Monfort, 2010; Poli et al., 2003), particularly in southern Europe.

As a new species adapted to aquaculture, important advances are expected to occur in meagre production, which should lead to a reduction of the juveniles' price (Stipa & Angelini, 2005). In this context, most of the research is focused on the improvement of meagre production techniques and the increase of its efficiency and profitability (Duncan et al., 2013). As an example, research in the field of reproduction will certainly result in regular production of high quality spawns.

4. Overview of meagre reproduction

Reproduction of meagre, as in fish in general, depends on the brain-hypothalamus-pituitary-gonad axis. Environmental stimuli like photoperiod and temperature are responsible for the control of this axis and have a crucial role in the control of gamete maturation and release (Cardinaletti et al., 2010). In the end of the axis lie the gonads, which are the reproductive organs in which gametes' production occurs, termed gametogenesis (Wooton & Smith, 2014). Gametes originated from germ cells after several mitotic divisions and meiosis (Okutsu, Suzuki, Takeuchi, Takeuchi & Yoshizaki, 2006; Stickney, 2000). In males, the gametogenesis occurs in the testes and is called spermatogenesis, producing spermatozoa. In females, the gametogenesis occurs in the ovaries and is called oogenesis, producing oocytes (Patiño & Sullivan, 2002). During oogenesis takes place the vitellogenesis that consists in the accumulation of yolk proteins within the oocyte (Patiño & Sullivan, 2002), essential for the formation of eggs. Mylonas, Mitrizakis, Papadaki & Sigelaki (2013) evaluated female and male gamete development of meagre over the reproductive period, in captivity, and observed that both spermatogenesis and vitellogenesis took place between April and June, in accordance with their natural reproductive season.

Meagre reach maturity at the age of 2.7 years for males and 3.5 years for females (Gil et al., 2013) and the length at maturity ranged 26.8-61.6 cm for males and 35.8-110 cm for females (González-Quirós et al., 2011; Schiavone et al., 2012; Soares et al., 2015). Additionally, Gil et al. (2013) concluded that the annual fecundity increases with length, weight and age, ranging from 0.9 to 4.2 million oocytes in reared females.

The reproduction of meagre in captivity is one of the bottlenecks of its production, similar to most species new to aquaculture (Schiavone et al., 2012). One example is the fry cost, which is still very high (Schiavone et al., 2012), in part because of unpredictability of its production. Nonetheless, industrial fish farming requires broodstocks that provide an adequate supply of fertilized eggs (Duncan et al., 2012) and juveniles (Stickney, 2000). However, some advances

in meagre reproduction are taking place. As an example, different research groups recently reported spontaneous spawning of meagre in captivity (Mylonas et al., 2013b; Pastor et al., 2013; Soares et al., 2015). Nowadays, fertilized eggs can either be obtained with wild-caught fish acclimated to captivity (Duncan et al., 2012; Soares et al., 2015) or with first-generation fish born in captivity (Soares et al., 2015). Indeed, when adequate nutrition is provided, both types of broodstocks can spawn good eggs and produce high larval quality, suitable for a hatchery production (Soares et al., 2015). Oviparous females in particular, like the meagre, have a higher and more diverse nutritional demand due to oogenesis (Stickney, 2000). A commonly observed problem in meagre reproduction is the failure to undergo oocyte formation and spawning. This also occurs in other species, due to a deficiency in the feed (Cabrita, Robles & Herráez, 2008).

Additionally, there have been some developments in hormonal induction protocols for meagre. These consist in the usage of a gonadotropin-releasing hormone agonist (GnRHa), given via injections or slow-release implants, to both males and females (Duncan et al., 2008; Duncan et al., 2012; Mylonas et al., 2013a; Pastor et al., 2013). Biglino (2015) observed a reduction in semen volume and sperm concentration when using GnRHa implants in males, but no apparent effect over motility or viability. At the same time, Duncan et al. (2008) and Pastor et al. (2013) obtained high egg fecundity after the administration of GnRHa to the females. Indeed, a single injection or a slow-release implant of GnRHa led to the production of fertilized eggs in sufficient number and quality for a commercially viable hatchery production (Duncan et al., 2012).

The current and future research should lead to new knowledge on meagre reproductive biology, which will be crucial to improve the broodstocks' reproductive performance (Mylonas et al., 2013b).

5. Artificial reproduction

Adequate management of broodstock and gametes requires certain environmental conditions and procedures, including artificial reproduction techniques. Artificial reproduction is the reproduction in a non-natural way, with the interference and manipulation of humans. It ensures the availability of fertilized eggs over time or when necessary, and maximizes the number of larvae produced (Suquet, Dreanno, Fauvel, Cosson & Billard, 2000). Furthermore, it grants some protection from the environment and pathogens, leading to a bigger survival rate and growth. In addition, species that cannot spawn naturally or reproduce in captivity can be propagated through these methods, using for example *in vitro* fertilization. Some

technologies require artificial reproduction. Polyploidy production, already done in other sciaenid species, is one example (Ballarin et al., 2004). It leads to the formation of fish that, being sterile, have less incidence of diseases or higher growth rate (Piferrer et al., 2009). Lastly, these techniques allow selective breeding, which consists in crossing the genetic material of specific individuals (Dupont-Nivet, Vandeputte, Haffray & Chevassus, 2006).

Meagre gametes may be collected for artificial reproduction (Poli et al, 2003), particularly when spawning is not occurring naturally (Marino et al., 2000). Semen can be collected by stripping, which might not be effective in every individual (Mylonas et al., 2013b), whereas eggs can also be collected by stripping after hormonal treatment, as seen in Figure 1 (DIVERSIFY, n.d.).

Figure 1: The process of stripping eggs from a female meagre (DIVERSIFY, n.d.).

Sperm and eggs are usually mixed in a certain ratio that maximizes the gamete usage. In general, to ensure genetic variability when doing artificial reproduction, a pool of semen from several males is used (Mylonas, Fostier & Zanuy, 2010). Nevertheless, for selective breeding, the use of only one specific male is also possible.

6. Gamete storage

When conducting artificial reproduction male and female gametes are not always available at the same time. In these cases gametes have to be stored in advance. In this context, gamete storage is an essential tool for artificial fertilization and breeding programs for several reasons. Fertilization can be synchronized according to gametes' availability (Cabrita et al., 2010; Suquet, 2000). Furthermore, it also allows for the gametes' transportation between locations such as collection site and hatchery, making easier and cheaper genetic exchanges (Cabrita et al., 2010; Kopeika et al., 2007; Suquet, 2000). The total volume of the gametes can

be efficiently used, which is particularly important when low volumes are available (Cabrita et al., 2010). Finally, in the case of semen cryopreservation, genetic material of valuable males can be theoretically preserved indefinitely (Cabrita et al., 2010; Suquet, 2000).

After collection, gametes start to suffer degradation processes. Their quality decreases, causing a reduced fertilizing ability. In some species, gametes may be stored unaltered from hours to days, at room temperature (Mylonas et al., 2010). Alternatively, the storage conditions can be manipulated to delay the degradation processes (Mishra, Patra, Dash, Verma & Routray, 2016; Yasui et al., 2015). Some examples of parameters that can be manipulated are temperature, oxygen supply, gas exchange, the storage medium (extender) and its dilution ratio, as well as the replenishment of the extender during storage (Kowalski et al., 2014; Mishra et al., 2016). For both female and male gametes of several fish species there are different storage protocols, which involve the manipulation of one or more of these factors.

Regarding storage temperatures, refrigeration is done above 0^{o}C (Yasui et al., 2015). This can be achieved in a simple way, with the use of a refrigerator or a cooler with ice packs for both oocytes and semen, either undiluted or diluted (Komrakova & Holtz, 2009; Wayman, Tiersch & Thomas, 1998). Depending on the species, the gametes may be refrigerated for several days while keeping some quality.

Alternatively, gametes can be stored at temperatures below 0^{o}C. In fishes, only semen can be stored at subzero temperatures (Asturiano, Cabrita & Horváth, 2016). The larger size of the oocytes, the yolk content and the different permeability of the oocytes' membranes causes some difficulties in their storage, namely the penetration of cryoprotectants and lack of uniformity in the cooling process (Asturiano et al., 2016; Chao & Liao, 2001). On the other hand, it is relatively easier to preserve semen of most fish species, either through short term cooling or long term cryopreservation in liquid nitrogen, at -196^{o}C (Agarwal, 2011; Gwo, Chen & Cheng, 2002; Stoss, 1983). Although cryopreservation allows the cells' preservation for a longer period of time, and several cryopreservation protocols have been developed for different fish species, there are not many cases of practical applications outside of the research environment (Cabrita et al., 2010). Indeed, from the aquaculture perspective semen refrigeration remains the best alternative (Viveiros, Isaú, Figueiredo, Leite & Maria, 2010a).

Extenders are the substances in which gametes are diluted and preserved. Oocytes are generally stored in ovarian fluid (Bellard, 1988), since the usage of dilution media has shown no advantages (Stoss, 1983). In the case of semen storage the usage of extenders is more common. Although it is only essential for cryopreservation, for the incorporation of

cryoprotectants (Agarwal, 2011; Kopeika, Kopeika & Zhang, 2007), they are an important complement for refrigeration as well, extending the spermatozoa's viability (Lahnsteiner, Berger, Weismann & Patzner, 1996; Maria, 2014). An ideal extender, according to Mann (1964), should not activate semen motility, should be isotonic and have a good buffering capacity and should include nutrients, antioxidants, antibacterial substances and stabilizing colloids. Keeping these characteristics in consideration, the extender's composition may vary. A simple and effective extender is a solution of NaCl (Fabbrocini et al., 2000; Maria, Viveiros, Freitas & Oliveira, 2006; Oliveira, Viveiros, Maria, Freitas, & Izaú, 2007). The addition of other substances to this extender may be proven beneficial, such as glucose (Viveiros, Orfão, Maria & Allaman, 2009). He and Woods III (2003) also proved that the addition of some aminoacids to a NaCl solution, such as glycine, improved the quality of stored semen. There are also extenders with more complex compositions, usually mimicking the seminal plasma composition. As an example, for the European sea bass it is usually recommended a non-activating medium, with different salts, glucose and BSA (Fauvel, Suquet, Dreanno, Zonno, & Menu, 1998). Additionally, there are commercially available extenders that may be used, such as BTS™ and ACP® (Nascimento, Maria, Pessoa, Carvalho, & Viveiros, 2010; Viveiros, Maria, Orfão, Carvalho, & Nunes, 2008).

The atmosphere with which the gametes are stored, excluding cryopreservation, affects their quality. Generally, normal air is used, but oxygen can be added (Billard, 1981). During storage, gametes consume oxygen (Robitaille, Mumford & Brown, 1987), and thus aerobic conditions should be provided. For this reason, addition of oxygen to the containers results in a longer storage time (Bellard, 1988; Komrakova & Holtz, 2011) for both oocytes (Billard, 1981) and semen (Bellard, 1988). Nevertheless, the presence of oxygen can also be the cause of lipids' peroxidation, resulting in damage to the cells with deterioration of quality (Chen et al., 2010). Some factors must also be taken in consideration, such as frequency of renewal of atmosphere (Marques & Godinho, 2004), which depends on the amount of oxygen consumed. This varies with the species, semen dilution and ratio of volume of semen to atmosphere. Diluting semen or stocking it with more air allows a reduction in this frequency (Bellard, 1988).

Other aspect to take into consideration in the refrigeration protocols is, in the case of semen, the sedimentation over time, which can be avoided by slightly shaking the containers periodically (Stoss, Büyükhatipoglu & Holtz, 1978). In the case of oocytes their storage is affected by the weight of eggs and the number of layers (Komrakova & Holtz, 2009). In most

cases, these protocols' optimization is done empirically, testing different parameters and evaluating the gametes' quality over time.

Semen storage protocols are far more diverse and more frequently used than oocytes' storage protocols. Artificial reproduction techniques normally involve semen storage until collection of oocytes (Rurangwa, Kime, Ollevier & Nash, 2004; Stoss, 1983). In the case of the Sciaenidae family, there are already semen storage protocols for different species, testing different extenders, osmolalities and dilution ratios (Leclercq et al., 2014; Wayman et al. 1998; Wayman, Thomas & Tiersch, 1997). More recently, research done under the DIVERSIFY-EU project, in 2014, found that in meagre sperm quality decreases rapidly after collection, if not diluted in an extender.

Meagre is a species in the early phases of adaptation to aquaculture, which will benefit from the improvement of artificial reproduction techniques that could assist in the management of broodstocks. Preliminary results regarding the refrigeration of meagre semen, done under the DIVERSIFY-EU project, gave promising results for the development of artificial reproduction. The usage of European sea bass extenders efficiently sustained the spermatozoa's motility for more than 24 hours. However, there are no protocols for semen storage in this species.

7. General considerations on semen and spermatozoa

The ejaculate semen is composed of spermatozoa and seminal plasma. Spermatozoa are the cells responsible to deliver the male genetic information to the egg, and have the capacity to become motile (Miller, Brinkworth & Iles, 2010). The seminal plasma is an enriched medium responsible for maintaining spermatozoa in a quiescent state until ejaculation and providing energy resources (Alavi & Cosson, 2006).

In teleost fish, the spermatozoa structure is divided in head, mid-piece and flagellum (Ginzburg, 1968; Islam & Akhter, 2011). The head is occupied in the most part by the nucleus, containing the genetic material (Ginzburg, 1968; Islam & Akhter, 2011). In the case of the teleosts, the head does not contain an acrosome (Dzyuba & Cosson, 2014), and in meagre it is oval-shaped (Schiavone et al., 2012). The mid-piece is linked to the head (Ginzburg, 1968; Islam & Akhter, 2011) and contains the mitochondria (Berois et al., 2011; Ginzburg, 1968). The flagellum is the apparatus responsible for the movement (Islam & Akhter, 2011), and originates from the posterior part of the nucleus (Cosson et al., 2008). It produces waves that propagate from proximal to distal tip (Boryshpolets, 2011), resulting in cell motility. ATP is the major source of energy for the flagellar beating, produced by the

mitochondria present in the mid-piece (Boryshpolets, 2011; Dzyuba et al., 2016). Nevertheless, during the fast motility period there is a very high ATP consumption that is not compensated by the mitochondria production (Ingermann, 2008). As a result, the energy stores are depleted during this time leading to the spermatozoa immobilization in a short period (Cosson, 2010; Dreanno, Seguin, Cosson, Suquet & Billard, 1999b). This means that motility depends on the endogenous ATP and other energy molecules' stores present before activation (Cabrita et al., 2010). In most external fertilizing fishes spermatozoa are immotile when inside the testes (Cosson et al., 2008; Ginzburg, 1968). After ejaculation motility is triggered by changes in the osmotic or ionic environment, which in the case of most marine species is caused by seawater (Cosson et al., 2008).

8. Sperm quality and its evaluation

Sperm quality can be defined as the ability to successfully fertilize an egg and develop into a normal embryo (Bobe & Labbé, 2010; Rurangwa et al., 2004). The measurement of different sperm quality parameters has several applications in aquaculture. They can be used as predictors of the fertilizing ability (Bobe & Labbé, 2010; Rurangwa et al., 2004), and thus their evaluation is important to increase artificial reproduction efficiency (Zilli, Schiavone, Zonno, Storelli & Vilella, 2004). When there is a reduced amount of eggs (Rurangwa et al., 2004), the risk of a low fertilization rate and loss of egg batches is prevented (Bobe & Labbé, 2010). Furthermore, cryopreservation should be only done to sperm of proven high quality (Zilli et al., 2004). Sperm quality evaluation also helps to determine the optimal time for semen collection, when its quality is variable along the season (Babiak, Ottesen, Rudolfsen & Johnsen, 2006; Fabbrocini et al., 2000). The occurrence of contamination during collection (Dreanno et al., 1998) and the effect of repeated stripping (Aas, Refstie & Gierde, 1991) may also be assessed. Finally, it helps to improve handling and storage protocols (Fabbrocini et al., 2000; Linhart et al., 2004), as is the case of the present thesis.

Fertilization success is the most integrative parameter and the best indicator of sperm quality (Bobe & Labbé, 2010). Nonetheless, for practical reasons, there is the need to use other parameters, which do not require the usage of eggs. The most commonly used are motility parameters, such as percentage of motile cells and motility duration or spermatozoa's swimming speed (Beirão et al., 2015, 2011b; Schiavone et al., 2012). Other parameters less frequently used are sperm concentration, sperm cell membrane integrity or viability (Beirão, Pérez-Cerezales, Martínez-Páramo & Herráez, 2010; Cabrita et al., 2011), ATP content (Montgomery, Brown, Gendelman, Ota & Clotfelter, 2014), lipid peroxidation (Martínez-

Páramo, Diogo, Beirão, Dinis & Cabrita, 2012) or bacteriology (Jenkins & Tiersch, 1997; Viveiros et al., 2010b), to mention a few.

In most cases it is difficult to correlate one specific parameter with fertilization rate, since they evaluate specific cellular functions, and fertilization depends on many (Bobe & Labbé, 2010). Furthermore, they show the mean value for the whole spermatozoa population, whereas only a small number of spermatozoa is needed to ensure fertilization (Bobe & Labbé, 2010). Sperm quality parameters are, then, partial descriptors of fertilization ability (Bobe & Labbé, 2010). In this context, sperm quality schemes should be built based on several parameters at the same time. These parameters should be investigated and improved, to increase the number of spermatozoa which meet the requirements for fertilization to occur (Bobe & Labbé, 2010).

According to Bobe & Labbé (2010) there are several factors that can influence the sperm quality, most of them related to broodstock management (Billard, Cosson, Perchec & Linhart, 1995). One example is stress, which can be caused by fish handling or environmental conditions. Nutrition is another factor with impact in sperm quality, as feed should have an adequate formula, especially regarding lipid composition and vitamins (Asturiano et al., 2001; Dabrowski & Ciereszko, 1996). Sperm handling during and after collection can also affect its quality, such as adequate storage conditions as temperature, medium and duration (Babiak et al., 2006; Peñaranda, Pérez, Fakriadis, Mylonas & Asturiano, 2008; Suquet et al., 1998).

In the case of meagre, the sperm quality has already been evaluated for concentration, for aspects of motility such as percentage of progressive motile cells and duration of progressive forward movement and morphology (Mylonas et al., 2013b; Mylonas et al., 2016; Schiavone et al., 2012). While Mylonas et al. (2013b) state that sperm quality does not significantly change during the reproductive season., Schiavone et al. (2012) verified a variation during the reproductive season, which goes in accordance to what occurs in other species, and may be due to the aging of spermatozoa (Babiak et al., 2006; Peñaranda et al., 2008).

9. Sperm quality parameters

9.1. Concentration

Sperm concentration is usually presented as the number of cells per milliliter (Junior et al., 2008; Wirtz & Steinmann, 2006). In teleost semen the cell concentration is highly variable, due to factors such as species, individual and time in the reproductive season (Butts, Litvak & Trippel, 2010; Piironen & Hyvärinen, 1983). In several species studied by Piironen and

Hyvärinen (1983) and Poole and Diilane (1998), the concentration ranged from 2.2 to 127.4 x 10^9 ml^{-1}. Results obtained by Mylonas et al. (2013b) in meagre semen obtained a mean cell concentration between 19 and 32 x 10^9 ml^{-1}. This parameter can be correlated, in some instances, with other parameters such as fertilization rate and motility (Ciereszko & Dabrowski, 1994; Hwang & Idler, 1969). Nevertheless, its main importance involves the optimization of the usage of semen when doing artificial reproduction, and the sperm to egg ratio needs to be adjusted (Bombardelli, Mörschbächer, Campagnolo & Syperreck, 2006; Sanches et al., 2011).

Several methods can be used to assess sperm concentration of fish semen. The most common involves counting the number of spermatozoa with a microscope and a cell counting chamber, such as a Neubauer chamber (Junior et al., 2008; Wirtz & Steinmann, 2006). These methods, despite cheaper, are time consuming (Fauvel, Suquet & Cosson, 2010). Spermatocrit determination, with hematocrit capillary tubes, is faster and simpler, giving a result in percentage equivalent to sperm concentration (Ciereszko & Dabrowski, 1993). However, this technique is not as effective in marine fish because spermatozoa do not sediment efficiently, which may be due to their density being similar to that of seminal plasma (Fauvel et al., 2010). Alternatively, the value of absorbance, obtained by spectrophotometry, can be used if a correlation with concentration has been previously established (Ciereszko & Dabrowski, 1993) Furthermore, some authors also suggest the use of flow cytometry or coulter counter, which are highly precise but expensive methods (Fauvel et al., 2010). Lastly, a modern image analysis software connected to a microscope is also frequently used to determine concentration fast and efficiently (Fauvel et al., 2010). Depending on the species, the correlation coefficient between these three methods may be high (Ciereszko & Dabrowski, 1993).

9.2. Motility

Sperm motility is an integrative parameter that combines different cellular structures (Bobe & Labbé, 2010). It is the most commonly used parameter to compare different experimental conditions such as sperm collection procedures, semen extenders, or sperm storage conditions (Bobe & Labbé, 2010).

In general, the initiation, duration and the patterns of motility differ between species (Rurangwa et al., 2004). In teleosts with external fertilization, such as meagre, the motility tends to be brief (Coward, Bromage, Hibbitt & Parrington, 2002) and spermatozoa usually swim at high speed and frequency, in rectilinear motion (Cosson et al., 2008; Cosson, 2010).

In meagre the percentage of motile spermatozoa varies between 53% and 74%, with a duration of 34 to 80 seconds (Schiavone et al., 2012). In most species, fertilization usually occurs between 5 and 20 seconds after spermatozoa's activation, thus the most useful motility data is obtained during this period (Kime et al., 2001).

In several species a correlation between motility and ability to fertilize the eggs has been found (e.g. Cosson et al., 2008; Ottesen, Babiak & Dahle, 2009), including refrigerated and cryopreserved sperm (e.g. Beirão et al., 2011a; Ciereszko & Dabrowski, 1994). Some examples are the correlations between sperm motility parameters and fertilization or hatching rate, in cryopreserved semen in African catfish (*Clarias glariepinus*) (Rurangwa et al. 2001), gilthead seabream (Beirão et al. 2011a), common carp (*Cyprinus carpio*) (Leveroni, Calvi, Zoccarato, Gasco & Andrione, 1993; Leveroni, Calvi, Zoccarato, Gasco & Andrione, 1994) and *Alburnus alburnus* (Lahnsteiner et al., 1996), or in refrigerated rainbow trout (*Oncorhynchus mykiss*) semen (Ciereszko & Dabrowski, 1994).

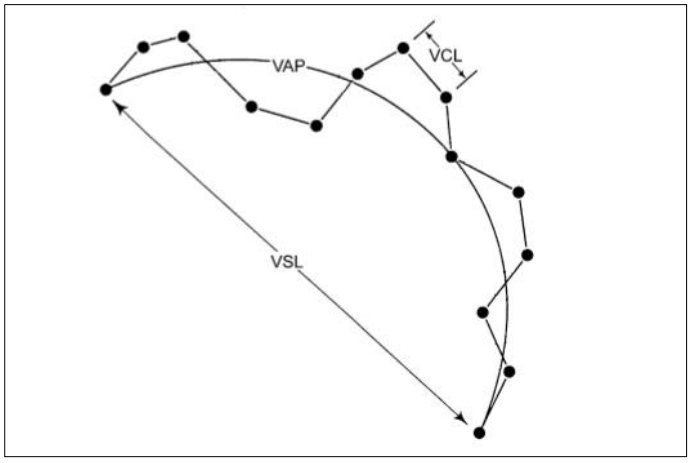
Motility can be assessed subjectively, by direct observation on a microscope (Kime et al., 2001). In these cases, the researcher measures the motility duration and estimates the percentage of motile sperm (Fauvel et al., 2010). To increase the accuracy of these measurements, sperm motility may be videotaped and posteriorly analyzed by different researchers (Kime et al., 2001). Alternatively, nowadays, more laboratories are using computer-assisted sperm analysis (CASA) systems (Fauvel et al., 2010; Kime et al., 2001) to objectively assess motility. The CASA systems analyze sperm movement of every single spermatozoa in a short video file, which is recorded with a camera attached to a microscope and connected to a computer (Rurangwa et al., 2004). This system measures several motility and velocity parameters (Fauvel et al., 2010). The parameters most commonly used for fish sperm analysis are described in Table 1. The differences between the three velocity parameters are represented in Figure 2. The large data sets obtained can be used to compare different sperm samples and thus reveal differences in experimental protocols, individuals and species, to mention a few (Linhart, Rodina, Gela, Kocour & Vandeputte, 2005; Rurangwa et al., 2001).

Table 1: Sperm motility parameters, their abbreviation, unit, and description (Rurangwa et al., 2004).

PARAMETER (unit)	DESCRIPTION
Duration of Motility (s)	time interval in which the cells remain motile
MOT • Percentage of Motile Cells (%)	percentage of cells that exhibit some motility

PROG • Percentage of Progressive Spermatozoa		
	(%)	percentage of cells with forward progressive movement
MOC • Concentration of Motile Cells		
	(spz/ml)	concentration of cells that exhibit some motility
VCL • Curvilinear Velocity		
	(µm/s)	considering the entire distance travelled
VSL • Straight Line Velocity		
	(µm/s)	considering a straight line between initial and final point
VAP • Average Path Velocity		
	(µm/s)	considering the distance travelled as a smoothed path
LIN • Linearity		
	(%)	obtained by VSL/VAP • a measure of path curvature
STR • Straightness		
	(%)	obtained by VAP/VCL • a measure of side to side movement

Figure 2: Schematic representation of some motility patterns measured by a CASA system. VCL, curvilinear velocity, in the entire distance travelled; VSL, velocity in a straight line; VAP, velocity of an average path. Black circles represent the position of the cell in each frame. Adapted from Rurangwa et al. (2004).



9.3. Viability

Sperm viability refers to the percentage of live spermatozoa (Garner & Johnson, 1995). Although viability should be attributed to the capacity of sperm to fertilize an egg, this parameter evaluates the percentage of spermatozoa with an integral plasma membrane (Fauvel et al., 2010; Rurangwa et al., 2004).

Different authors have found a correlation between sperm viability and other semen quality parameters, such as sperm velocity parameters in common carp semen and hatching rate in African catfish (Linhart et al., 2005; Rurangwa et al., 2001).

Viability tests are based on staining protocols, which confer different coloration to viable and dead cells (Rurangwa et al., 2004). Each stain has a different cell permeation ability,

depending on the plasma membrane's integrity (Fauvel et al., 2010). Protocols may only use one dye, such as tryptan blue (Lubzens et al., 1997; Rurangwa, Biegniewska, Slominska, Skorkowski & Ollevier, 2002), or a combination of two, such as eosin and nigrosine (Maria et al., 2006). Alternatively, fluorescent dyes can also be used, to facilitate cell distinction. A commonly used combination of these is propidium iodide (PI) with SYBR-14, for dead and viable cells respectively (Rurangwa et al., 2004), but other combinations have been proposed, such as PI with YO-PRO 1 (Beirão et al., 2010). These stains usually target nucleic acids of the spermatozoa. In the case of the PI/SYBR-14 combination, PI cannot cross the intact plasma membrane, whereas SYBR-14 leaks out when the plasma membrane is deteriorated (Nagy, Jansen, Topper & Gadella, 2003; Rurangwa et al., 2004). The percentage of viable cells can be assessed under a microscope, by direct counting or using an image analysis system (De Baulny, Labbé & Maisse, 1999). Other ways to do so are through flow cytometry (Beirão et al., 2010), with an automatic counter (Fauvel et al., 2010) or a fluorometer (McNiven, Gallant & Richardson, 1993).

9.4. ATP

ATP, together with adenosine diphosphate (ADP) and adenosine monophosphate (AMP), compose the Adenylate Energy Charge (AEC). The AEC can be considered the intracellular content of available energy, for it is composed of high energy nucleotides. Since the values of ATP and AEC are highly correlated, it is reasonable to evaluate just one of these parameters (Fauvel et al., 2010), such as ATP, which has simple and precise protocols (Perchec, Jeulin, Cosson, Andre & Billard, 1995). As previously mentioned, the ATP present in the spermatozoa is the major energy source for flagellar beating and, as demonstrated by Dreanno et al. (1999b) and Cabrita et al. (2005), it can be used as a sperm quality estimator. Indeed, a positive correlation between ATP content and percentage of motile cells was found in some species such as turbot (De Baulny et al., 1996) and steelhead trout (*Oncorhynchus mykiss*) (Bencic, Krisfalusi, Cloud & Ingermann, 1999b). Furthermore, Bencic et al. (1999b) also observed a positive correlation between ATP and fertility rate in steelhead trout.

ATP determination can be done with a bioluminescence technique that uses firefly luciferin-luciferase, to which there are commercial assay kits (Fauvel et al., 2010; Perchec et al., 2005). The reaction between ATP, luciferin and luciferase leads to the production of luminescence, which can be detected with a luminometer or scintillation counter (Rieger, 1997; Yang, Ho, Chen & Hu, 2002). There are also methods which enable the quantification of not only ATP but also the other components of AEC, such as high pressure liquid chromatography

(Dziewulska, Rzemieniecki & Domagala, 2010; Mukai & Okuno, 2004) and nuclear magnetic resonance spectroscopy (van den Thillart, van Waarde, Muller, Erkelens & Lugtenburg, 1990).

9.5. Lipid Peroxidation

During the aerobic metabolic processes, cells produce reactive oxygen species (ROS). These have cytotoxic properties, causing damages to the desoxyribonucleic acid (Cabrita et al., 2011; Potts, Notarianni & Jefferies, 2000), ATP depletion (Aramli, Kalbassi, Nazari & Aramli, 2013), abnormalities in morphology (Ball, 2008), and lipid peroxidation (Zhou et al., 2006), which leads to a decrease in motility and cell viability (Aramli et al., 2013; Ball, 2008), and a decrease in sperm quality (Apel & Hirt, 2004). The cell membrane's high content in polyunsaturated fatty acids (PUFAs) makes fish spermatozoa quite susceptible to lipid peroxidation (Labbe et al., 1995; Zhou et al., 2006). To counteract the ROS effects semen has antioxidant mechanisms (Lahnsteiner & Mansour, 2010), which include different antioxidant substances present in the seminal plasma (Cabrita et al., 2011; Potts et al., 2000). However, *in vitro* storage of the semen increases ROS formation (Ball, 2008; Zhou et al., 2006) namely because dilution in the extenders reduces the antioxidants' concentration of seminal plasma (Cabrita et al., 2011; Martínez-Páramo, Martínez-Pastor, Martínez-Rodríguez, Herráez & Cabrita, 2009). Nevertheless, antioxidants could be added to stored semen to prevent cellular injuries caused by ROS (Aramli et al., 2013).

During lipid peroxidation, several by-products are formed. Their measure is frequently used to quantify lipid peroxidation (Devasagayam, Boloor & Ramasama, 2003). The thiobarbituric acid (TBA) assay is the most used to evaluate lipid peroxidation *in vitro* (Buege & Aust, 1978; Devasagayam et al., 2003). It measures the by-products which react with thiobarbituric acid – thiobarbituric acid reactive substances (TBARS) -, such as malondialdehyde (MDA) (Potts et al., 2000; Devasagayam et al., 2003; Yahyavi, Kaykhaii & Hashemi, 2016). This assay is sensitive but non-specific (Devasagayam et al., 2003; Zhou et al., 2006), since TBA might react with other organic components. Alternatively, MDA can be measured using commercially available kits (Beirão et al., 2015; Martínez-Páramo et al., 2012).

9.6. Bacteriology

Bacterial growth can be the cause of sperm quality decrease especially when semen is stored in refrigeration. Bacteria may be present in semen as part of the normal flora, due to contamination or with origin in the extender (Bartha, 2009). They can reduce sperm quality

due to the oxygen consumption, and the production of extracellular enzymes and metabolic by-products that affect spermatozoa (Jenkins & Tiersch, 1997). The growth of bacteria has been associated to decreased fertility (Saad, Billard, Theron & Hollebecq, 1988; Stoss & Refstie, 1983), low motility (Christensen & Tiersch, 1996; Isaú, 2014) and viability (Stoss et al., 1978), as well as increased morphological changes (Jenkins & Tiersch, 1997). Indeed, Jenkins & Tiersch (1997) observed that the usage of a sterile extender, as opposed to a non-sterile one, extended the number of days in which there was sperm motility. Moreover, some authors have suggested the use of antibiotics to inhibit bacterial growth and improve sperm survival and fertility (Billard et al., 2004; Viveiros et al., 2010b).

Bacteriological evaluation can be done quantitatively or qualitatively. The quantitative analysis is the estimation of the number of bacteria per unit of volume by counting the number of colony-forming units (CFUs) (Elain et al., 2015; Jenkins & Tiersch, 1997). The qualitative analysis involves determining the presence of bacteria and their identification (Plusquellec, Beucher, Le Lay, Le Gal & Cleret, 1991) by means of various techniques that evaluate different phenotypical characteristics of the bacteria. Examples of these are observation of morphology and motility, Gram staining, catalase or oxidase test, and glucose fermentation (Salanitro, Blake & Muirhed, 1977).

9.7. Fertilization success

Fertilization success is the most integrative estimator of sperm quality, since it requires the interaction of all sperm cellular functions, rather than one or some of them (Bobe & Labbé, 2010). In fact, in numerous occasions there is no correlation between other sperm quality parameters, such as motility parameters or viability, and fertilization (Bobe & Labbé, 2010; Cabrita et al., 2010). It should also be kept in mind that only a small fraction of spermatozoa are needed to fertilize the eggs, whereas most of the sperm quality parameters take into account the average of overall sperm population (Bobe & Labbé, 2010).

In general, to evaluate sperm fertilization success, sperm and eggs are mixed at a certain ratio to observe the percentage of ova fertilized per amount of sperm cells (Beirão et al. 2011a). Usually, only the amount of fertilized eggs is evaluated, without contemplating further developmental success (Shields, Brown & Bromage, 1997). This is easy to observe in species with transparent eggs, such as meagre (Gamsiz & Neke, 2008). Nonetheless, the hatching rate might also be assessed, incubating the eggs (Cabrita et al., 2010). It should be taken in consideration that there may be significant differences between fertility and hatching rate

(Cabrita et al., 2009; Pérez-Cerezales et al., 2010). Furthermore, the impact of egg quality must also be taken in consideration when evaluating the results (Bobe & Labbé, 2010).

EXPERIMENTAL STUDY

1. Objectives

The primary objective of this study was to develop a protocol for the refrigeration of meagre semen that facilitates artificial reproduction techniques both for research and for meagre production. The secondary objective was to contribute for the understanding of the causes of the fish sperm quality degradation during refrigeration. For this, meagre sperm was refrigerated using three extenders in three different dilutions, taking in consideration the differences in the semen quality parameters along the time. Semen quality was analyzed in terms of sperm motility, viability, ATP content, lipid peroxidation and bacteriology.

2. Material and methods

2.1. Experimental animals

The meagre broodstock used in this experiment was kept in two 18 m^3 outdoor in the Aquaculture Research Station of the Portuguese Institute for the Ocean and Atmosphere (EPPO-IPMA). Each tank had 9 individuals born in the research station, with a mean weight of 7 315 ± 750 g and length of 91.8 ± 4.4 cm. Most of the individuals were not previously sexed, but an initial screening showed that the ratio was of two males per female. The tanks were covered with shade nets and exposed to natural photoperiod and temperature conditions. Tanks were supplied with filtered seawater from the nearby estuary, pumped at a continuous rate of 1 L/s. The water salinity changed according to the natural tidal cycles. The mean water temperature was 21.9^{o}C (maximum of 26.9^{o}C and minimum of 15.6^{o}C), and the mean dissolved oxygen concentration was 6.3 mg/L (maximum of 9.3 and minimum of 2.2). Fish were fed *ad libitum* with commercial pellets twice a week.

2.2. Semen collection

Semen was collected in seven different occasions during meagre reproductive season, between April and June. Sampling took place between 10 am and 12 pm, once every 6 to 8 days. One tank was used in each day of sampling, alternating between them. This way fish in each tank had at least 12 days to recover from sampling.

In the sampling days, the water level of the tank was lowered to about 30 cm. Before semen collection, fish were mildly sedated with 50 ppm of 2-phenoxyethanol directly added to the tank and a net was placed to separate fish already sampled, as shown in Figure 3.

Figure 3: Image of one tank during semen collection, showing the lowered water level and the net separating fish already sampled.

Fish were caught individually and placed onto a 200 liters tank shown in Figure 4, with 300 ppm of 2-phenoxyethanol (Neiffer and Stamper, 2009). Meagre were kept there until they showed signs of anesthesia.

Figure 4: Two meagre on the 200 liters tank with anesthesia, showing loss of equilibrium, by being upside down.

Besides the loss of equilibrium, which causes fish to turn upside down (Fig. 4), fish were also tested for a lack of response to stimuli, grasping the base of the tail (Neiffer and Stamper, 2009). Before removing the fish from the anesthesia tank, they were gently pressed near the

genital papilla to observe whether they were spermiating or not. Males that were not spermiating were returned to the main tank. Spermiating males were enveloped in a wet towel, and placed in dorsal decubitus in an acrylic cylinder half with some water on its bottom (Fig. 5).

Figure 5: Moment of semen collection, with the meagre upside down and enveloped in a wet towel, in the acrylic cylinder half. Abdominal pressure was being applied and semen was collected with a syringe.

Since the sperm sampling procedure took less than 2 minutes, it could be performed out of the water, without the need of an artificial ventilation system (Harms and Bakal, 1995). The semen was obtained by applying pressure abdominally in a craniocaudal motion. Semen was collected with a 5 ml sterile syringe and placed in 1.5 ml microtubes kept in a styrofoam box with ice packs. The genital papilla was frequently wiped with tissue paper to avoid contamination, particularly before applying abdominal pressure and after the discharge of urine or feces. In the event of contamination the syringe and its contents were discarded.

2.3. Refrigeration and samples' processing

Samples with no signs of contamination and a percentage of motile spermatozoa over 70% were pooled in a sterilized 15 ml falcon. Each pool had semen of 3 to 6 males. A total of 7 pools were used in this study.

Subsamples of 1.5 to 2.5 ml of each pool were placed in sterilized and labelled 15 ml falcons, and combined with one of three different extenders, in one of three different dilutions, in a full factorial design. A control sample, with 1.5 to 2.5 ml of undiluted sperm, was also kept. A total of 10 treatments were tested (3 extenders × 3 dilutions + 1 control). Dilutions were

selected after a preliminary test. The dilutions used were (in ratio of volume semen:extender) 1:4, 1:9 and 1:19.

The extenders' composition was the following:

- Extender NAM, Non-activating medium: NaCl 7.5 mg/mL, KCl 0.11 mg/mL, $MgCl_2$ 1.23 mg/mL, $CaCl_2$ 0.39 mg/mL, $NaHCO_3$ 1.68 mg/mL, Glucose 0.08 mg/mL, BSA 10 mg/mL, diluted in distilled water (Fauvel et al., 1998);
- Extender NC: 0.9% NaCl;
- Extender NCG: 0.9% NaCl, Glycine 0.0038 mg/mL, Glucose 0.001 mg/mL (He and Woods III, 2003).

The osmolality was adjusted to 300 mOsm, according to the observed value of meagre's seminal plasma in a preliminary experiment. The pH of the extenders was adjusted according to the pH of the NAM, which was 7.7 (Fauvel et al., 1998). NAM was chosen because it is the extender used for European sea bass sperm (Fauvel et al., 1998), and there are some resemblances between European sea bass and meagre sperm. NC was designed to be a simple and inexpensive extender. Finally, the extender NCG was based on NC, with the addition of glycine that, according to the results obtained by He and Woods III (2003), improves the quality of striped bass' (*Morone saxatilis*) stored semen. All the extenders were autoclaved before mixing with the semen samples. In the case of NAM, BSA was only added after this procedure, to avoid protein denaturation.

The falcons were kept in a refrigerator at 4^{o}C (Fig. 6). They were opened daily for gas exchanges and gently shaken to avoid formation of cell deposit. There was a temperature sensor in the refrigerator to control the daily maximum and minimum temperature it reached (Annex A, Table 13).

Figure 6: The refrigerator shelf. In the front, the falcons with the various samples from three separated pools. On the left, bottles with the extenders and reagents for the ATP protocol. On the right, a falcon filled with water and with the temperature sensor.

All the samples were analyzed for concentration. Due to the limited samples' volume, not all analysis were conducted in all pools. The number of pools evaluated, as well as the frequency of evaluation of each quality parameter, is shown in table 2. The first 5 pools were analyzed for sperm motility and viability within one hour after collection (considered 0 h data point) in the control sample. Motility and viability were evaluated in all the treatments at 12, 24, 36, 48 h, and every 24 h after that. The motility was measured until the percentage of motile cells was zero, or until 15 days. The viability was measured until 7 days or after observing zero percent of motility. Pools 1 to 4 were also analyzed for ATP within one hour after collection (considered 0 h data point) in the control sample, and in all treatments at 12 and 24 h, and every 48 h, for as long as viability was evaluated. MDA was evaluated in the last two pools (6 and 7). It was measured on day 0 in the control and at days 3, 6 and 9 in all the treatments. Bacteriology was analyzed in 3 pools (5, 6 and 7). In day 0 only the control was inoculated, whereas the control, the dilutions 1:4 from each extender, and the three extenders as negative control, were inoculated at days 5 and 10. It was not logistically feasible to inoculate the plates for the evaluation of all the 10 treatments. Therefore, only the 1:4 dilution treatments were assayed for bacteriology, as representatives of each extender, these had the highest semen concentration, and thus the highest concentration of bacteria from semen. To allow for comparisons, sperm motility and viability was measured on the same days as MDA and inoculations.

Table 2: Number of pools and frequency of evaluation of each sperm quality parameter.

	MOTILITY	VIABILITY	ATP	MDA	BACTERIOLOGY
NUMBER OF POOLS EVALUATED	5	5	5	2	3
FREQUENCY OF EVALUATION	Daily	Daily	Days 0, 0.5, 1, 3, 5	Days 0, 3, 6, 9	Days 0, 5, 10

2.4. Concentration and motility

Sperm concentration and motility were evaluated using the CASA software ISAS® by PROiSER R+D and a UOP UB202i microscope by Prymus© with a 10x lens, attached to a Basler© camera connected to a laptop with the software.

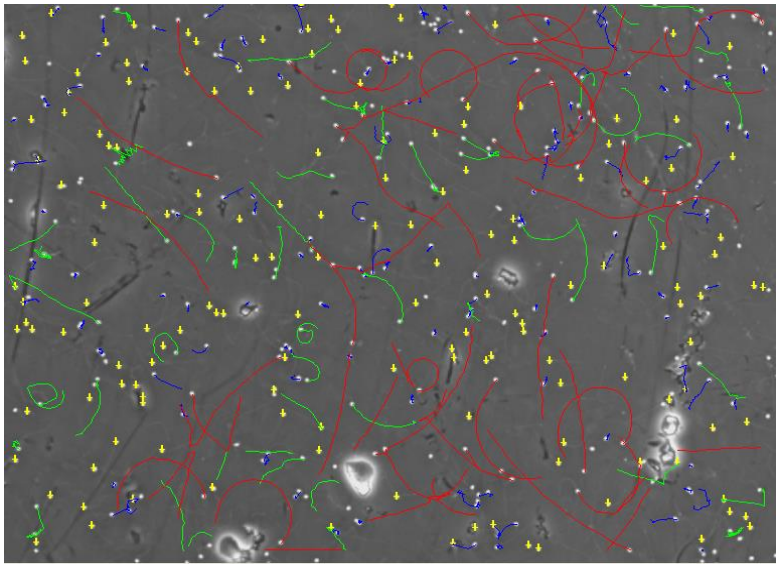
For concentration, the samples were diluted between 10 to 100 times in 0.9% NaCl dependent on the treatment dilution. This was repeated six times for each treatment of each pool.

For motility evaluation, the sperm was activated by adding 1.5 µl of the semen (volume adjusted according to treatment dilution) in a Makler chamber with 20 µl of the research station seawater. Motility was recorded 15 seconds after activation.

The following parameters were analyzed: MOT, PROG, VCL, VAP, VSL, LIN and STR. The software settings were adjusted to meagre sperm: 50 frames captured per second; scale was calibrated at 10x; particle area (μm^2) between 1 and 70; connectivity, 14; Progressivity: 80% of the STR and; minimum number of images to calculate ALH: 10. An example of an image obtained during this evaluation is in Figure 7.

Each reading was repeated three times.

Figure 7: Image obtained with the CASA software. Different colours represent spermatozoa with different motility speeds. Yellow dots represent immotile spermatozoa, whereas, blue, green and red represent slow, medium and fast spermatozoa respectively, and their paths.



2.6. Viability

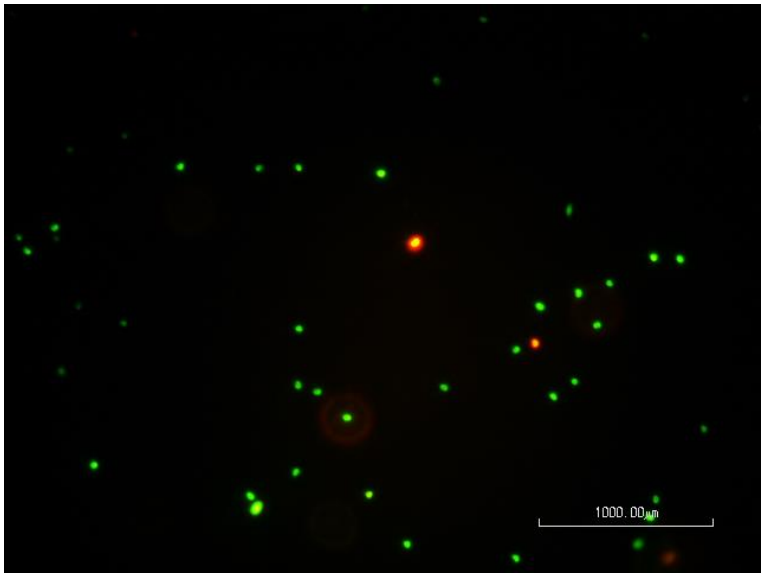
Sperm viability was measured with the double stain PI and SYBR-14 from the kit Live/Dead® Sperm Viability Kit from Invitrogen™. The cells were observed under a Nikon® Eclipse Ci fluorescence microscope with 10x magnification lens with a Nikon® LH-M100C-1 100w Mercury Lamphouse.

One hundred µl of 0.9% NaCl were added to different volumes of sperm according to the different treatments dilution (control and 1:4 dilutions, 0.5 µl of sperm; 1:9 dilutions, 1 µl of sperm and; 1:19 dilutions, 1.5 µl of sperm). The cell suspensions were then stained with 0.5 µl pre-diluted SYBR-14 (1:9 in 0.9% NaCl) and 0.75 µl of PI (stock solution). After 10 minutes of incubation in the dark and on ice, the suspension was slightly mixed, and a droplet

placed between a slide and cover slip. The preparation was immediately observed under the fluorescence microscope in a dimly lit room. Different fields were observed and at least 200 cells were counted. Cells were differentiated between viable cells (marked green with SYBR-14) and damaged or dead cells (marked red with PI) as observed in Figure 8, and the percentage of viable cells was recorded.

This process was repeated three times per sample.

Figure 8: Image taken in the fluorescence microscope of a sample observed after incubation, showing the difference between cells coloured green (viable cells) and red (dead cells).



2.7. ATP

The following solutions were prepared in advance of the ATP extractions: Sorensen stock solutions A and B, and ethylenediaminetetraacetic acid (EDTA) solution (composition in Annex B, Table 14). The three solutions were autoclaved and stored refrigerated for 1 month maximum. Sorensen buffer and the 2% TCA solution (composition in Annex B, Table 14) were freshly prepared every day.

For the ATP extraction, 10 µl of each treatment were mixed with 150 µl of 2% TCA solution in microtubes kept on ice, and left for 15 min. Subsequently, the microtubes were vortexed for some seconds, and centrifuged at 4^{o}C, 10 000 g, for 10 min. One hundred µl of the resulting supernatant was diluted in 500 µl of Sorensen buffer and stored at -80^{o}C until further analysis. ATP extracts were analyzed with the Adenosine 5′-triphosphate (ATP) Bioluminescent Assay Kit from Sigma-Aldrich®, following the manufacturer's instructions with some adjustments to sperm samples. The kit ATP mix, buffer and ATP standard were divided in aliquots and frozen at -80^{o}C, in advance. The ATP mix was kept from the light, wrapped in aluminum foil. These aliquots were thawed before usage and discarded afterwards.

ATP extracts and reactives were kept on ice during all the procedure. For the ATP curve, serial dilutions of 1 µmol/ml ATP standard were done with HPLC grade H_2O (Table 3).
The curve was kept for a maximum of 8 hours. A working ATP mix solution was prepared by mixing 1.2 ml of ATP buffer with 50 µl of ATP mix.

Table 3: Curve points used in the ATP protocol.

CURVE POINTS	1	2	3	4	5	6	7
ATP (nmol/ml)	25	6.25	1.25	0.25	0.0625	0.0125	0.0025

The luminescence was measured in a BioTek's Synergy 4™ multi-mode microplate reader, using black 96-well microwell plates. For each plate reading a new curve and two blanks (HPLC grade H_2O) were prepared. One hundred µl of the working ATP mix solution was added to the plate wells and left to rest for 5 minutes at room temperature in the dark. After this time, 100 µl of the curve points, blanks and extracts were added to the respective wells. The plate was automatically shaken for 3 minutes and read for luminescence. Because strong luminescence can affect the results in adjacent wells, one well of interval was left between all wells with a sample. Each sample was analyzed in duplicate in different plates.

2.8. MDA

To measure the MDA a spectrophotometric assay kit, BIOXYTECH® MDA-586™ (*Oxis*Research™), was used following the protocol developed for fish sperm by Martínez-Páramo et al. (2012). From each treatment 50 µl were used, that were kept in ice all the time. In the case of the control, this volume was diluted 1:1 with 0.9% NaCl. For the cell suspension from treatments NAM and NCG the extender was replaced by 0.9% NaCl after centrifugation at 13 000 g and 4^{o}C for 10 min. All the cell suspensions were briefly vortexed, to resuspend the sperm cells, after which 450 µl of 0.9% NaCl were added to all of them, followed by 5 µl of a solution with 200 µM of sodium ascorbate and 40 µM of iron sulfide. This step fragments the existing lipid peroxides and generates MDA, which will be measured (Annerén, & Epstein, 1987). Then, the cell suspensions were slightly shaken and incubated in a dry bath for 30 minutes at 37^{o}C, in the dark. Meanwhile, a calibration curve was prepared (Table 4), by diluting MDA standard solution (20µM) with bidistilled water.

Table 4: Curve points used in the MDA protocol.

CURVE POINTS	1	2	3	4	5	6
MDA (μM)	10	8	4	2	1	0

After the incubation time, 100 µl of each cell suspension and curve points were transferred to another microtube, each with 320 µl of R1 solution (N-methyl-2-phenylindole, in acetonitrile). In addition, a control of turbidity was prepared with 100 µl from a previously prepared cell suspension. To prevent further lipid peroxidation in this control 320 µl of acetonitrile solution were added (Botsoglou, 1994). Five µl of Probucol were then added to all the microtubes (cell suspensions, control and curve points). This minimizes the reaction with 4-hydroxyalkenals, although they do not produce significant colour under the conditions of the assay (*Oxis*Research™, 2001). Subsequently, all microtubes were briefly vortexed and 75 µl of R2 reagent (hydrochloric acid) were added in each one, and then incubated at 45^{o}C in a dry bath, in the dark, for 60 minutes. Afterwards, the microtubes were centrifuged at 10 000 g and 4^{o}C for 10 minutes. Two hundred µl of supernatant from each microtube were placed in well of a 96-well microplate, in duplicate for each cell suspension, curve point, blank and control. Absorbance was read at 586nm in a BioTek's Synergy 4™ multi-mode microplate reader immediately, as acetonitrile may damage the bottom of the multiwell plate.

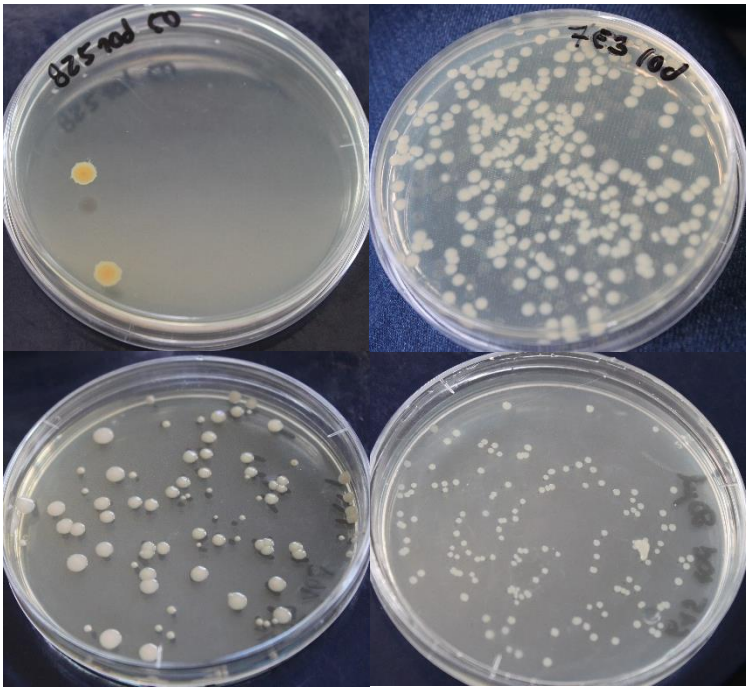
2.9. Bacteriology

For each sample, three plates with Trypticase Soya Agar (TSA), from Sigma-Aldrich®, and three with Pseudomonas CN Agar (PS), from Merck Millipore®, were inoculated by spread plate technique. TSA was chosen as a general nutrient medium for the growth of bacteria, also used by Viveiros et al. (2010s) and Jenkins and Tiersch (1997). PS, a medium selective for *Pseudomonas aeruginosa*, was chosen because *Pseudomonas* sp. are prevalent not only in semen but also in the gastrointestinal tract, water and soil (Boonthai et al., 2016; Jenkins & Tiersch, 1997; Nayak, 2010; Sader & Jones, 2005). Fifty µl of the corresponding treatment were used per plate. In the case of the control, 100 µl were used per plate after pre-dilution 1:9 in sterilized 0.9% NaCl. The incubations were conducted at 24^{o}C. Incubators were weekly disinfected with 70% ethanol to prevent cross contamination.

A potential fungal presence suspected by macroscopic observation was confirmed microscopically by wet mount of a portion of colony. The number of colony forming units (CFUs) of bacteria (Fig. 9) were counted on the 2nd and 7th days of incubation. The last count was the one used for data analysis.

Representative colonies of plates from the same medium, pool, treatment and day of inoculation, were selected based on phenotypical differences. They were photographed when observed and isolated on the 7th day of incubation of their respective plate. Isolation was done with an inoculating loop, touching the colony (preferably isolated from others, and not old) and streaking onto a plate with the TSA medium, which was incubated again for 2 to 7 days at 24^{o}C, in order to purify the colonies.

Figure 9: Plates with apparent bacterial growth after 7 days of incubation.



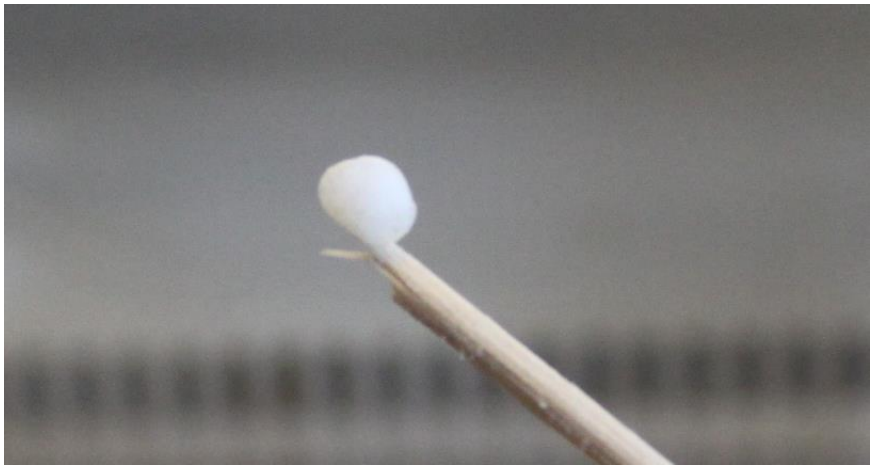
So as to identify the genus of the bacteria, several biochemical identification tests were done in fresh colonies, with 2 days, as described by Holt (1994) and Buller (2004). The following tests were made:

- Morphology and mobility assessment (Bryant and Small, 1956): a portion of colony was mixed with 0.9 ml of sterilized 0.9% NaCl. A drop of this wet mount was placed between slide and cover slip, and observed in a Nikon® Eclipse Ci microscope with a 100x oil-immersion lens. Bacteria were evaluated as to morphology and motility.
- Gram test with potassium hydroxide (KOH) (Buck, 1982): A drop of 3% KOH was placed on a sterile surface. The colony was touched with a sterile toothpick, and dipped repeatedly in 3% KOH. If a viscous string was formed between the drop and toothpick, the bacteria was Gram negative. If not, the bacteria was Gram positive.
- Oxidase test (Ishikawa, Nakajima, Yanagi, Yamamoto & Yamasato, 2003): A portion of the colony was touched with a sterile toothpick and streaked in an oxidase test strip

from bioMérieux ©. A color change to purple or deep blue indicated that the colony was oxidase positive.

- Catalase test (Chang, Kim & Shin, 1997): A portion of the colony was touched with a sterile toothpick and in it was placed a drop of 3% hydrogen peroxide solution. The formation of gas bubbles (Fig. 10) indicated a positive result, meaning the colony had the enzyme catalase.

Figure 10: Positive result of a catalase test, with the formation of gas bubbles.



- Oxidative/fermentation glucose test (Hugh and Leifson, 1953): The colony was mixed in two tubes with a liquid medium of Oxidative Fermentative Test Nutrient Agar of Sigma-Aldrich®. One tube was covered by paraffin, to differentiate growth on anaerobic and aerobic conditions. Both tubes were incubated for 2 days. If the anaerobic or both tubes turned yellow, the result was fermentation of glucose. If only the aerobic tube turned yellow, the result was oxidation of glucose. If the aerobic tube turned blue, then it did not metabolize glucose, and oxidized peptones.
- Growth in mediums with different percentages of salinity: Each bacteria was incubated in non-selective mediums with 0, 3, 5 and 10% of salinity. After 5 days it was determined if the bacteria grew or not in each percentage of salinity.

2.10. Data analysis

The statistical analysis was conducted with the IBM SPSS® Statistics 20 for the correlation analysis, generalized linear models (GzLM) and multiple comparisons and with the R software (version 3.3.1) and the packages Rcmdr (Fox, 2005) and RcmdrPlugin.survival (Fox & Sá Carvalho, 2012) for the survival analysis.

The concentration values of each pool were determined from control sample. The mean and standard error of the mean were calculated for the total number of pools (n = 7).

The values of the triplicates done for sperm motility were averaged by pool, treatment and day. In time zero only the control was tested, and the value obtained was assumed to be the time zero value of the other treatments from the same pool. No value of VCL, VAP, VSL, LIN or STR was attributed when motility was 0%. The values of each parameter (MOT, PROG, VCL, VAP, VSL, LIN and STR) were tested for normality with a Shapiro-Wilk test (Annex C, Table 15). Since the data was not normally distributed, they were tested for correlations with a Spearman's rank correlation coefficient. Some parameters were discarded from further analysis when they had similar biological meaning and were significantly correlated. Significant differences between treatments for MOT, VCL and LIN, from the five initial pools for the first seven days were detected with a GzLM because the data was not normally distributed (Annex C, Table 16). Both extender and dilution were considered as factors. MOT, VCL and LIN were tested for multiple comparisons with a Mann-Whitney U, performed for the different combinations of pairs of each of the factors. The average time it takes for the percentage of motile cells to reach zero was determined with two survival analysis. One to compare extenders and another to compare dilutions.

Regarding viability, the triplicates done for each day, treatment and pool were averaged. In time zero only the control was tested, and assumed to be the value at time zero for the other treatments. The Shapiro-Wilk tests showed that for some treatments the values were not normally distributed (Annex C, Table 17). Therefore, significant differences between treatments were investigated with a GzLM, followed by a Mann-Whitney U test for the pairs of values of the different extenders. The average time it takes for a population to present only 50% of viable cells was detected with survival analysis. Due to the fluctuation verified in viability values over time, the 50% value was obtained with linear regression equations (Annex C, Table 18) on each of the five initial pools. Two different survival analysis were done. One to compare extenders and another to compare dilutions.

In the ATP luminescence readings the mean of the two blanks was subtracted to all readings in the respective plate. The ATP value in μmol/ml of each extract was calculated from the curve equation. The values which gave less or equal to zero were considered to be zero. After this, the values of ATP were converted from μmol/ml to μmol/10^6 cells, dividing each value

of ATP for the respective concentration value. For samples with a repeated ATP analysis, the two values obtained for the same sample were averaged. When the highest value for a data point was more than 15 times superior to the second highest value, it was considered to be procedural error and it was removed. In time zero only the control was tested. This was assumed to be the value of the other treatments in time zero. To study the significant differences between treatments only the values obtained at 12 h and days 1 and 3 were considered since after that the ATP values were below the detection limit. The Shapiro-Wilk test showed that the data was not normally distributed (Annex C, Table 18), and so significant differences were tested with a GzLM, with extender and dilution as factors.

For MDA, the values were corrected with respective cell concentration and dilution, in order to obtain MDA in $\mu M/10^6$ cells. In time zero only the control was tested. This was assumed to be the initial value for the other treatments. The Shapiro-Wilk test showed that the data was not normaly distributed (Annex C, Table 19). A GzLM was performed to detect significant differences between the values, with extender and dilution as factors, followed by a Mann-Whitney U test for the pairs of values of the different dilutions.

Finally, for the bacteriology results, the number of colonies counted in each plate at the seventh day of incubation was divided by the inoculation volume, to obtain the CFU/ml value of that inoculation. Counts under 30 were considered to be 0. When the highest value of a triplicate was above one hundred and more than 3 times superior to the second highest value, it was removed. The CFU/ml values of the three plates for each pool, treatment, day and medium were averaged. According to the Shapiro-Wilk test of normality the values were not normally distributed (Annex C, Table 21). Thus, significant differences between treatments were tested with a Kruskall-Wallis, performed for the values of day 5, and another for the values of day 10.

Correlations between the results obtained in each different assay were tested with the Spearman's coefficient test, since the data was not normally distributted (Annex C, Tables 22, 22, 23 and 24). Pairs of values with zeros were discarded. When the sample size was three or less the correlation was not performed.

3. Results

3.1. Concentration

The sperm concentration values obtained for the control sample of each pool ranged from 26.30 to 62.47 x 10^9 spz/ml (Annex D, Table 23). The average concentration was 43.45 ± 18.17 x 10^9 spz/ml.

3.2. Motility

Most sperm motility parameters were highly correlated (Table 5) with each other. MOT was selected for further analysis because is the most used motility parameter, whereas progressive movement, PROG, was discarded as it was correlated ($\rho = 0.942$, $n = 379$, $p < 0.01$) with MOT. VCL was also selected for analysis, this is the velocity parameter that corresponds to the spermatozoa's actual path and had a high and significant correlation with the other two velocity parameters, VSL ($\rho = 0.931$, $n = 379$, $p < 0.01$) and VAP ($\rho = 0.992$, $n = 379$, $p < 0.01$). Lastly, LIN also correlated to STR ($\rho = 0.804$, $n = 379$, $p > 0.01$), and was selected to be further used because it is more widely reported than STR.

Table 5: Spearman's rho test of correlation between the values obtained for all the sperm motility parameters measured with CASA.

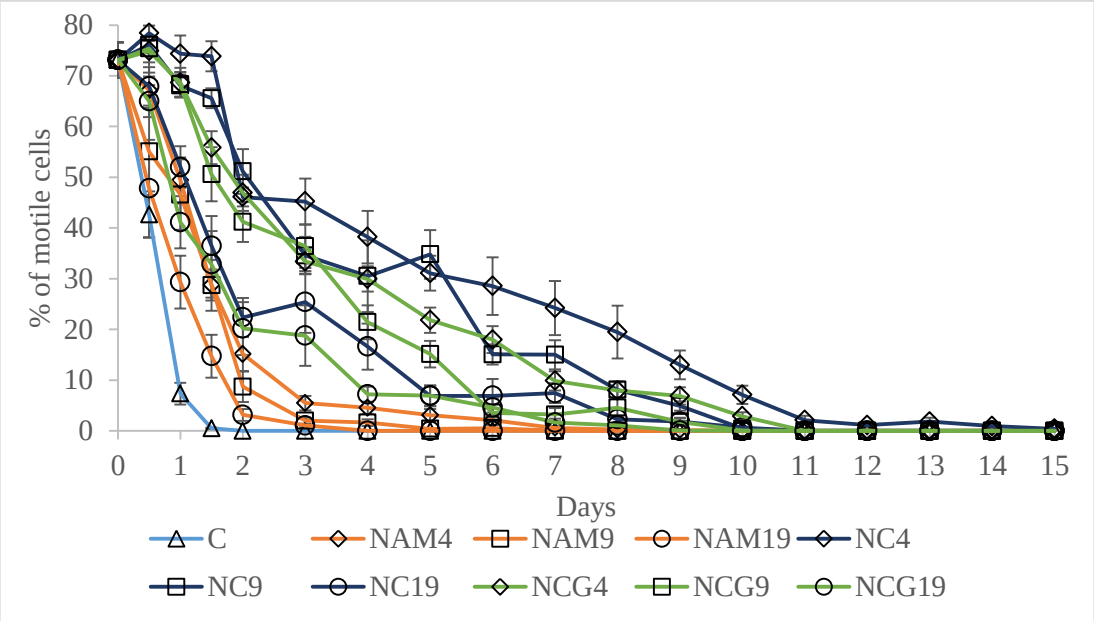
	PROG	VCL	VSL	VAP	LIN	STR
MOT	0.942*	0.794*	0.646*	0.758*	0.017	-0.203*
PROG		0.827*	0.756*	0.804*	0.213*	-0.042
VCL			0.931*	0.992*	0.220*	-0.136*
VSL				0.953*	0.485*	0.108
VAP					0.279*	-0.114
LIN						0.804*

Note: MOT, percentage of motile cells; PROG, percentage of progressive motile cells; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; and STR, straightness. The sample size refers to the seven pools, and all the days during which motility was assessed. N = 379. *Correlation is significant at the 0.01 level (2-tailed).

The mean percentage of motility was 73.12 ± 3.48% at day 0 (Annex E, Table 27). This value decreased along the time for all treatments. All the treatments had a higher percentage of motile cells than the control at all times (Graph 1; Annex E, Table 27). The control reached 0% motility on average at one and a half days of storage (Graph 2), which was less than half of the average of the extenders and dilutions tested.

There were significant differences between the extenders for the MOT parameter (Wald χ^2 (2) = 67.186, p < 0.001). The post-hoc analysis (Annex E, Table 28 for a more complete overview of the Mann-Whitney U test) revealed significant differences between all the pairs of extenders. The treatments with the extenders NC and NCG, when compared with those with NAM, kept higher percentage of motile cells during a longer period.

Graph 1: Mean percentage of motile spermatozoa (MOT) of each treatment for each day of assessment.

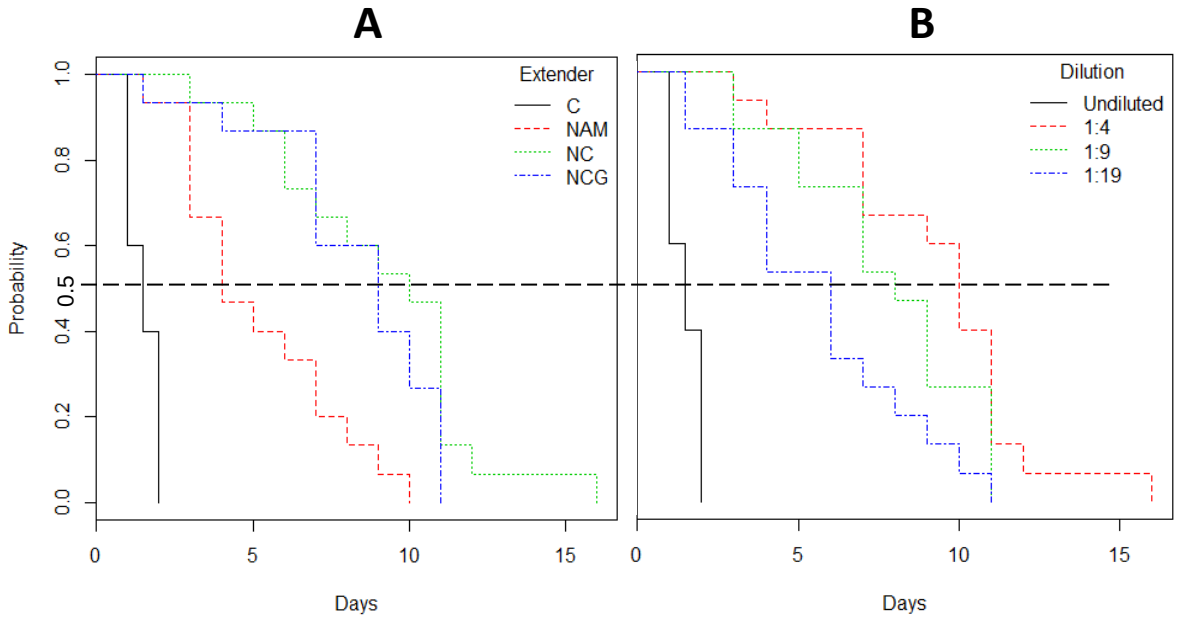


Note: Control in light blue, NAM in orange, NC in dark blue and NCG in green. 1:4 dilution is represented with ◆, 1:9 dilution with ■ and 1:19 dilution with ●. The value at day zero, for all the treatments, was considered to be equal to that of the control. Motility was assessed every 12 h on the first two days and every 24 h after that. Mean values ± standard error of the mean. The sample size refers to the first five pools in which sperm motility was assessed, with CASA. N = 5.

In the first day of refrigerated storage the percentage of motile cells of the control treatment dropped to less than 10% and to 0% in the second day. In the treatments with NC the percentage of motile cells was kept above 50% in the first day. Whereas, in the treatments with NCG and NAM it was kept above 40% and 25% respectively. After one and half days, the percentage of motile cells in all treatments with the extender NAM were below the values of the treatments with NC and NCG. At the fourth day, the treatments with NAM had less than 5% motile cells, whereas, the treatments with extenders NC and NCG had more than 15% and 5% motile cells respectively. As seen in Graph 2, the samples with the extenders NC or NCG took longer, on average, to reach 0% motile cells, when compared to the samples with the extender NAM (11, 9 and 4 days, respectively).

There were also significant differences between the dilutions for the MOT parameter (Wald χ^2 (2) = 32.811, p < 0.001) (Table 6; Annex E, Table 28 for a more complete overview of the Mann-Whitney U test). The post-hoc analysis revealed that there were significant differences between all the pairs with the exception of 1:4 with 1:9.

Graph 2: Survival analysis until the percentage of motility reaches zero, comparing the values obtained in five pools for: A, the different combinations of extenders (NAM, NC, NCG, C); B, the different combinations of dilutions (1:4, 1:9, 1:19, Control).



Note: Probability 0.5 is indicated with a dashed line. $N_{NAM, NC, NCG, 1:4, 1:9, 1:19} = 15$. $N_C = 5$.

Table 6: Mann-Whitney U test for the values of MOT, VCL and LIN in pairs of extenders and pairs of dilutions.

	Pair		N	Significantly higher element		
	1	2		MOT	VCL	LIN
Extender	C	NAM	166	2	2	2
	C	NC	180	2	2	2
	C	NCG	180	2	2	2
	NAM	NC	256	2	2	2
	NAM	NCG	256	2	2	2
	NC	NCG	270	1	none	none
Dilution	C	1:4	180	2	2	2
	C	1:9	173	2	2	2
	C	1:19	170	2	2	2
	1:4	1:9	263	none	none	none
	1:4	1:19	260	1	1	1

	1:9	1:19	253	1	1	1

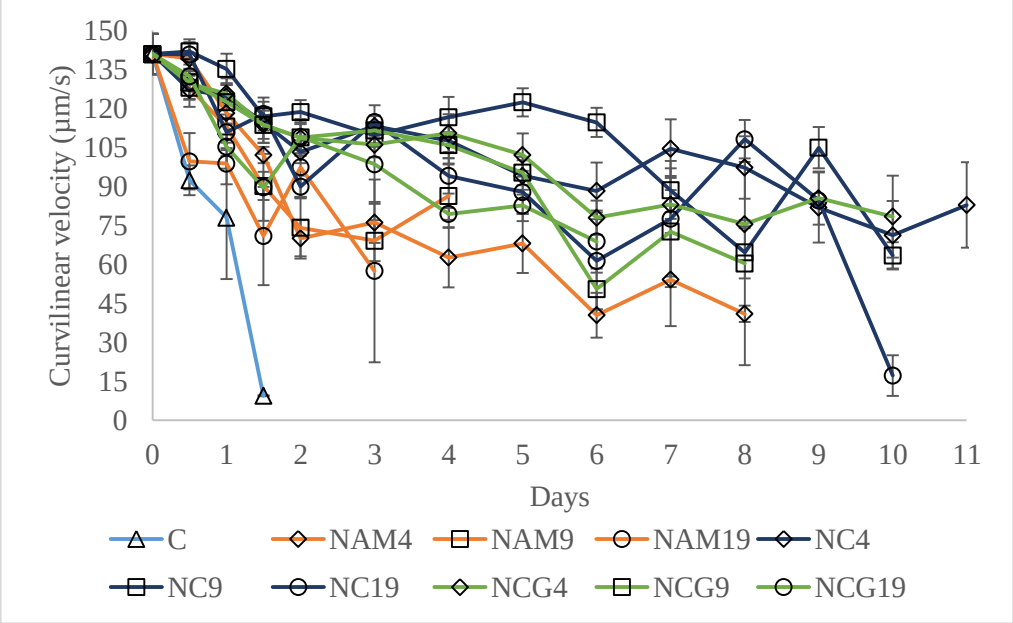
Note: The "Significantly higher element" indicates: if there was no significant difference between the elements of the pair (none); or that there was significant difference between elements of the pair (1 or 2 corresponding to the element with the significantly higher value). Values obtained from the seven pools, from all the days in which motility was assessed for the pair of treatments. The N column corresponds to the sample size value.

Although the dilutions 1:4 and 1:9 did not differ, in the survival analysis (Graph 2) the dilution 1:4 retained the percentage of motile cells above 0% for the longer time, on average ten days. The dilution 1:9 had an average of 8 days, and 1:19 had an average of 6 days. Along the days of evaluation the control had always lower MOT values. When comparing the three dilutions of the same extender, the 1:19 dilution always had lower MOT values, whereas the values of dilution 1:4 were generally higher.

The last recorded day of refrigerated storage was the fifteenth day, for the treatment NC dilution 1:4, with values barely above the 0% of motile cells. This treatment was the only one with a percentage of motile cells above zero after day 11.

The VCL was on average 140.90 ± 7.75 µm/s at day 0 (Annex E, Table 29). This value decreased along the time for all treatments and this decrease was faster in the control treatment (Graph 3). There were significant differences between the extenders (Wald χ^2 (2) = 82.962, $p < 0.001$).

Graph 3: Mean curvilinear velocity (VCL) of each treatment for each day of assessment and treatment.



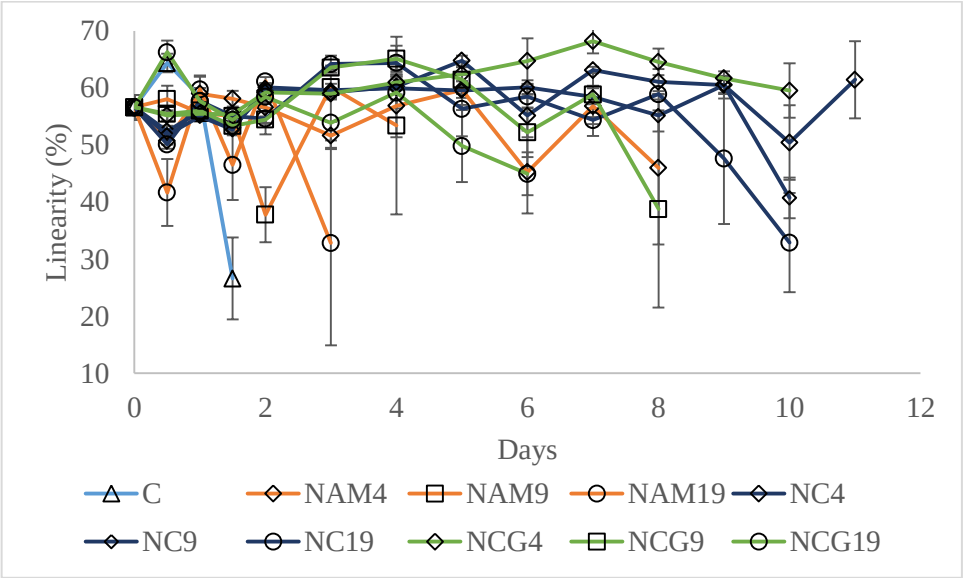
Note: Control in light blue, NAM in orange, NC in dark blue and NCG in green. 1:4 dilution is represented with ◊, 1:9 dilution with □ and 1:19 dilution with ○. The value at day zero, for all the treatments, was considered to be equal to that of the control. Motility was assessed every 12 h on the first two days and every 24 h after that. Mean values ± standard error of the mean. The sample size refers to the first five pools in which motility was assessed, with CASA. N = 5.

The post-hoc analysis (Annex E, Table 30 for a more complete overview of the Mann-Whitney U test) revealed significant differences between all the pairs of extenders, with the exception of NC with NCG. All the extenders had significantly higher values than the control. Indeed, in every time point the VCL of the control was lower than that of the other treatments tested. In the first day of refrigerated storage the VCL of the control was below 80 µm/s, while it was above 95 µm/s in all the samples tested. The VCL values of the extender NAM were significantly lower than those of the extenders NC and NCG. After two days of refrigerated storage, VCL of the treatments with NAM was between 70 and 97 µm/s, while it was between 90 and 119 µm/s in those with NC and NCG. At the fourth day of refrigerated storage, the treatments with NAM had an average VCL of less than 86 µm/s, nonetheless this value only refers to motile cells and for this treatment means less of 5% of the cells. In treatments with NCG and NC the VCL was above 79 µm/s and 94 µm/s respectively.
Also for VCL, there were significant differences between the dilutions (Wald χ^2 (2) = 37.054, $p < 0.001$). The post-hoc analysis (Table 6; Annex E, Table 30 for a more complete overview of the Mann-Whitney U test) revealed that there were significant differences between all the pairs with the exception of 1:4 with 1:9. The dilution 1:19 had the lowest VCL. In fact, the general trend for the different dilutions from the same extender (Annex E, Table 29), at the same time point, was that the dilution 1:19 had the lowest value. Indeed, at the fourth day of storage VCL of the dilution 1:19 of the extender NAM there were no motile cells. Whereas, at day four, the dilution 1:19 of the extender NC had a VCL of 75 µm/s, while it was above 100 µm/s in the other two dilutions. In day 7 the dilution 1:19 had an average VCL below 20 µm/s, while the dilutions 1:4 and 1:9 had values above 70 µm/s. A similar trend was observed for the extender NCG.

The mean LIN was 56.61 ± 2.16 % at day 0 (Annex E, Table 31). For the control LIN was the second highest at 12 h of storage, but from then on had the lowest values. There were significant differences between the extenders (Wald χ^2 (2) = 71.353, $p < 0.001$). The results of the post-hoc analysis (Annex E, Table 32 for a more complete overview of the Mann-Whitney U test) are similar to those from the VCL parameter. The control had lower values than the

extenders tested. From these three, NAM had the lowest percentage of LIN. There were no differences between the values from the extenders NC and NCG. At the sixth day of storage, samples from the extender NAM had less than 45% LIN, while those from NC and NCG had more than 45% LIN (Annex E, Table 31).

Graph 4: Mean percentage of linearity (LIN) of each treatment for each day of assessment and treatment.



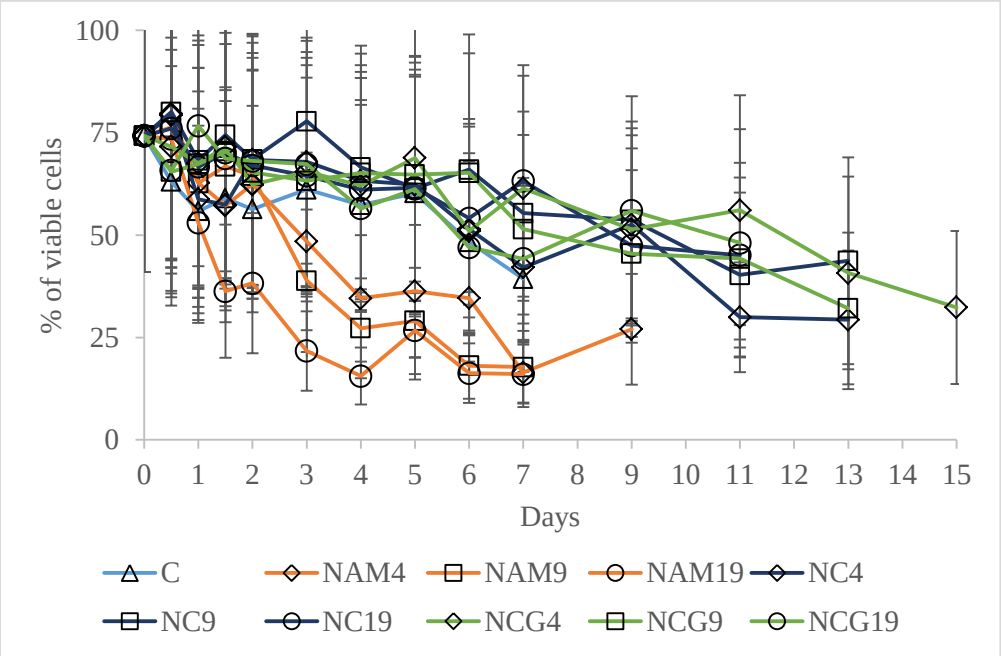
Note: Control in light blue, NAM in orange, NC in dark blue and NCG in green. 1:4 dilution is represented with ◆, 1:9 dilution with ■ and 1:19 dilution with ●. The value at day zero, for all the treatments, was considered to be equal to that of the control. Motility was assessed every 12 h on the first two days and every 24 h after that. Mean values ± standard error of the mean. The sample size refers to the first five pools in which motility was assessed, with CASA. N = 5.

There were also significant differences between the dilutions used (Wald χ^2 (2) = 47.142, p < 0.001). The post-hoc analysis (Table 6; Annex E, Table 32 for a more complete overview of the Mann-Whitney U test) revealed that, similar to what was verified for MOT and VCL, there were differences between the values of all the pairs with the exception of 1:4 with 1:9. The control presented the lowest values, and of the three dilutions tested, 1:19 had the lowest LIN. For the different dilutions of the same extender (Annex E, Table 31), at the same time point, in general the dilution 1:19 had the lowest LIN. For example, at the fourth day, the LIN of the dilution 1:19 of NCG was 36%, while it was 61% and 65% in dilutions 1:4 and 1:9, respectively. The difference was smaller in NC, for which dilutions 1:4 and 1:9 had 60% LIN, and dilution 1:19 had 51%. At the seventh day of storage the LIN of the dilution 1:19 was less than half than that of 1:4 and 1:9, in the case of the extenders NC and NCG.

3.3. Viability

The average percentage of viability was 74.12 ± 33.20% at day 0 (Annex F, Table 33). The values decreased along the storage time in all the samples. There were significant differences between the extenders used (Wald χ^2 (2) = 114.887, p < 0.001), but not between the dilutions (Wald χ^2 (2) = 6.285, p = 0.099). The post-hoc analysis (Table 7; Annex F, Table 34 for a more complete overview of the Mann-Whitney U test) revealed significant differences between most pairs of extenders.

Graph 5: Mean values obtained for each treatment, along the days of evaluation.



Note: Control in light blue, NAM in orange, NC in dark blue and NCG in green. 1:4 dilution is represented with ◊, 1:9 dilution with ◘ and 1:19 dilution with ○. The value at day zero, for all the treatments, was considered to be equal to that of the control. Error bars with the standard error of the mean. Samples were only measured for viability when the MOT value was higher than 0.Therefore in some instances the date points were obtained with less than five pools. No mean value was attributed when it had values from less than three pools. N value ranged from 5 to 3.

Table 7: Mann-Whitney U test done to the pairs of extenders.

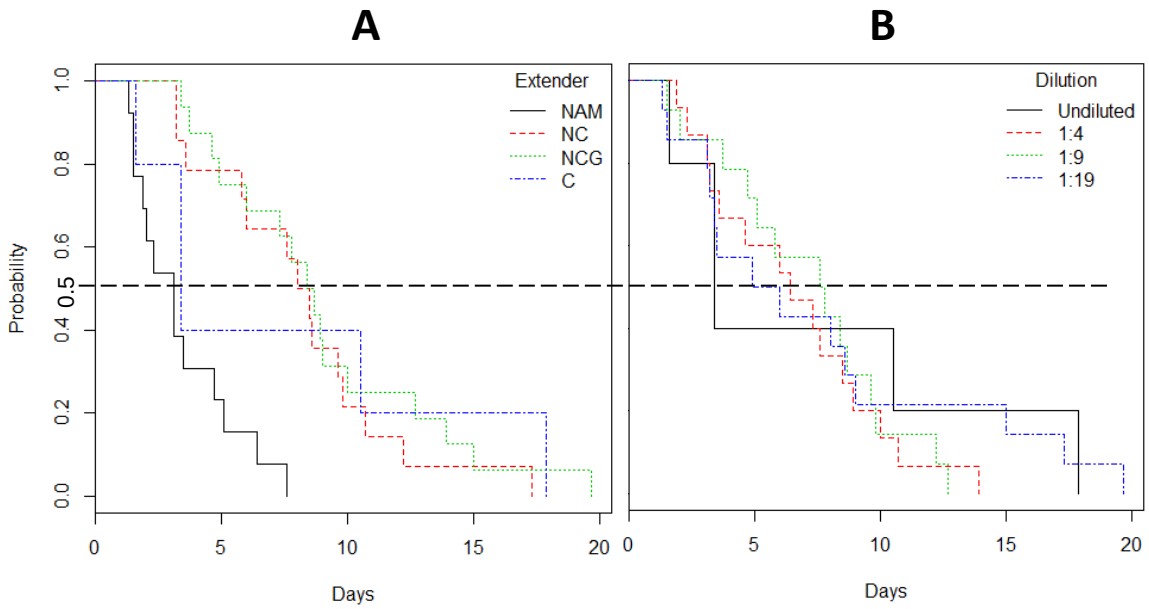
	Pair		Significantly higher element
	1	2	
Extender	C	NAM	1
	C	NC	2
	C	NCG	2
	NAM	NC	2
	NAM	NCG	2

	NC	NCG	none

Note: The "Significantly higher element" indicates: if there was no significant difference between the elements of the pair, with "none"; or that there was significant difference ($p < 0.05$) between elements of the pair, indicating the number (previously attributed in column "Pair") corresponding to the element with the significantly higher VIAB. N corresponds to the values obtained from the seven pools, from all the days in which viability was assessed. $N_C = 42$, $N_{NAM} = 133$, $N_{NC} = 130$ and $N_{NCG} = 127$.

From the third day on, the extenders NC, NCG, and the control had viability values higher than the treatments with the extender NAM. At the fourth day of storage the viability of the samples with the extender NAM ranged 16 and 35%, while those from extender NC, NCG and control ranged from 57 to 67%. At the seventh day of storage the viability of extender NAM was lower than 18%, while the control was 39% and the values from the extenders NC and NCG were above 42%.

Graph 6: Survival analysis until the viability reaches 50%, comparing the values obtained in five pools for: A, the different combinations of extenders (NAM, NC, NCG, C); B, the different combinations of dilutions (1:4, 1:9, 1:19, Control).



Note: Probability 0.5 is indicated with a dashed line. $N_{NAM, NC, NCG, 1:4, 1:9, 1:19} = 15$. $N_C = 5$.

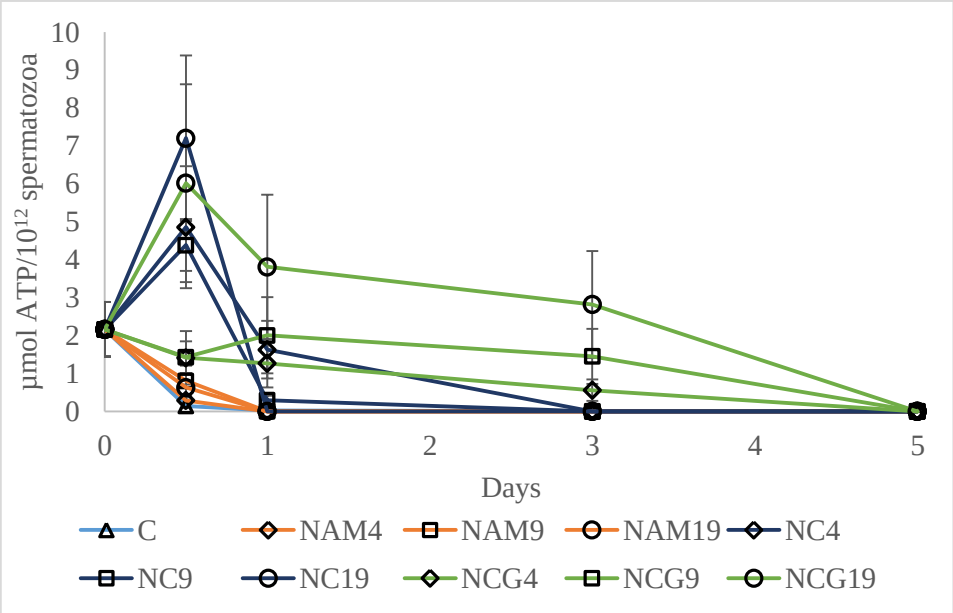
Samples incubated in the extenders NC and NCG had in most cases higher viability than the control. The values from these two extenders did not differ between themselves.

As seen in Graph 5, which represents the average time to reach 50% viability, the samples with the extenders NC or NCG took, on average, eight and a half days to reach 50% viability, while the control and the samples with the extender NAM took on average less than four days. Although there were no significant differences between dilutions, the samples from dilution 1:19 reached 50% viability on average at around 6 days of storage, the dilution 1:4 reached the same percentage on average at six and a half days, and the dilution 1:9 at around 8 days (Graph 6).

3.4. ATP

The average value of ATP was 2.16 ± 0.72 µmol ATP per 10^{12} spermatozoa at day zero (Annex G, Table 35) and at the fifth day of storage these values were below the detection limit for all the treatments (Graph 7).

Graph 7: Mean ATP values obtained for each treatment, from five pools, along the days of viability evaluation.



Note: Control in light blue, NAM in orange, NC in dark blue and NCG in green. 1:4 dilution is represented with ◆, 1:9 dilution with ■ and 1:19 dilution with ●. Mean value at time zero was the same for all the samples, based on the mean from the control. Error bars with the standard error of the mean. N = 4.

Table 8: Mann-Whitney U test done to the pairs of extenders.

	Pair		Significantly higher element
	1	2	
Extender	C	NAM	none
	C	NC	none
	C	NCG	none
	NAM	NC	2
	NAM	NCG	2
	NC	NCG	none

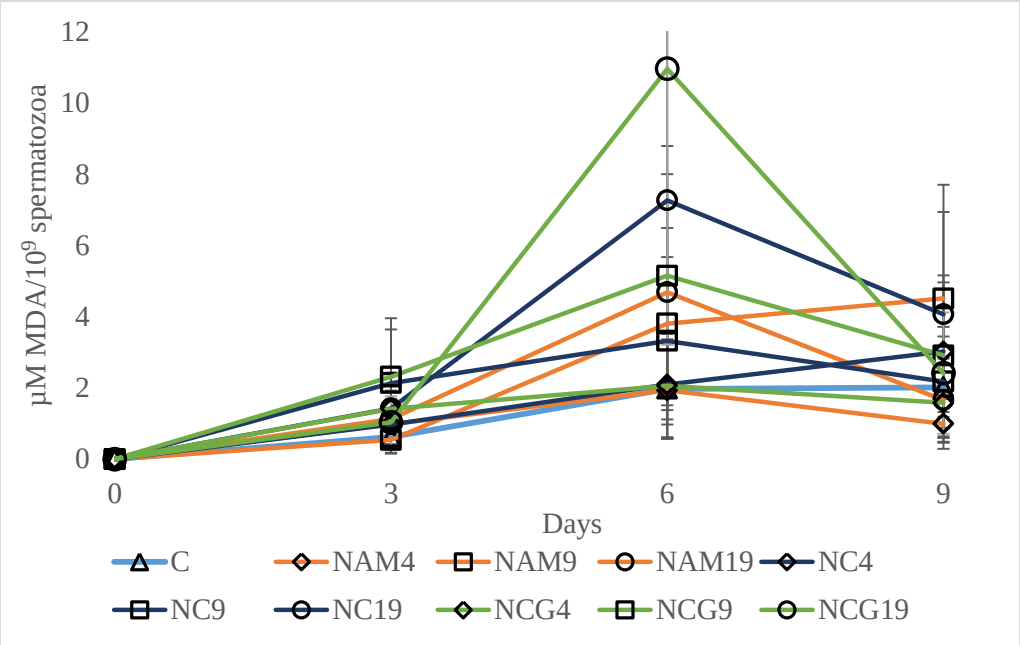
Note: The "Significantly higher element" indicates: if there was no significant difference between the elements of the pair, with "none"; or that there was significant difference ($p < 0.05$) between elements of the pair, indicating the number (previously attributed in column "Pair") corresponding to the element with the significantly higher ATP. N corresponds to the values obtained from the seven pools, from all the days in which viability was assessed. $N_C = 12$, $N_{NAM} = 34$, $N_{NC} = N_{NCG} = 35$.

There were significant differences between the extenders (Wald $\chi 2$ (2) = 6.645, $p < 0.05$), but not between the dilutions (Wald $\chi 2$ (2) = 4.364, $p = 0.113$). The post-hoc analysis (Table 8; Annex G, Table 36 for a more complete overview of the Mann-Whitney U test) revealed significant differences between the pairs NAM and NC, and NAM and NCG, but none with the control treatment. The extender NAM had lower values than NC and NCG. In fact, the ATP values of the samples with the extender NAM were lower than the other two extenders at all times. Samples kept in the extender NAM only kept the ATP value above the detection limit until the first day of storage. At 12 hours of refrigerated storage all the treatments with the extenders NC or NCG had more than 1.40 µmol ATP per 10^{12} spermatozoa, while the ATP content of the samples with NAM was below 0.85 µmol ATP per 10^{12} spermatozoa.

3.5. MDA

The value of MDA on time zero was 0 µM MDA per 10^6 spermatozoa (Annex H, Table 37). The values increased in all the treatments until the sixth day of storage, and decreased between days 6 and 9 in most treatments (Graph 8). Furthermore, the values from day 9 were all higher than those at day 3, from the corresponding treatment.

Graph 8: Mean and standard error of the mean of the values of MDA at days 0, 3, 6 and 9, in which it was quantified.



Note: Control in light blue, NAM in orange, NC in dark blue and NCG in green. 1:4 dilution is represented with ◆, 1:9 dilution with ■ and 1:19 dilution with ●. Mean value of the treatments at time zero was considered to be equal to the mean of the control. Pools 6 and 7 were used in this evaluation. N = 2.

There were no significant differences in the values between the groups of extenders (Wald χ^2 (2) = 1.765, p = 0.414), but there were significant differences between dilutions (Wald χ^2 (2) = 7.216, p < 0.05). The post-hoc analysis (Table 9; Annex H, Table 38 for a more complete overview of the Mann-Whitney U test) revealed significant differences between only one pair of dilutions. The MDA content of dilution 1:9 was higher than that of dilution 1:4. As seen in Table 37, from Annex H, the mean values from dilution 1:9 were higher than those from dilution 1:4 from the same extender, with a couple of exceptions.

Table 9: Mann-Whitney U test done to the MDA values in pairs of different dilutions.

	Pair (1 x 2)		Significantly higher element
Dilution	C	1:4	none
	C	1:9	none
	C	1:19	none

	1:4	1:9	2
	1:4	1:19	none
	1:9	1:19	none

Note: The "Significantly higher element" indicates: if there was no significant difference between the elements of the pair, with "none"; or that there was significant difference ($p < 0.05$) between elements of the pair, indicating the number (previously attributed in column "Pair") corresponding to the element with the significantly higher MDA. N corresponds to the values obtained from the two pools, from all the days in which MDA was assessed. $N_{C \times 1:4} = N_{C \times 1:9} = N_{C \times 1:19} = 24$. $N_{1:4 \times 1:9} = N_{1:4 \times 1:19} = N_{1:9 \times 1:19} = 36$.

3.6. Bacteriology

The CFU/ml obtained from the extenders was discarded as a contamination source, since its values were mostly zero.

Table 10: Mean ± standard error of the mean of the CFU/ml obtained for each treatment (C, NAM, NC and NCG) and day, in TSA medium. Values from three pools. N = 3.

	Day		
Treatment	0	5	10
C	0	0	242200.00 ± 39984.00
NAM		15689.00 ± 2643.34	18513.00 ± 2860.36
NC		7528.90 ± 1271.05	84.44 ± 28.15
NCG		0	23255.56 ± 3516.67

Table 11: Mean ± standard error of the mean of the CFU/ml obtained for each treatment (C, NAM, NC and NCG) and day, in PS medium. Values from three pools. N = 3.

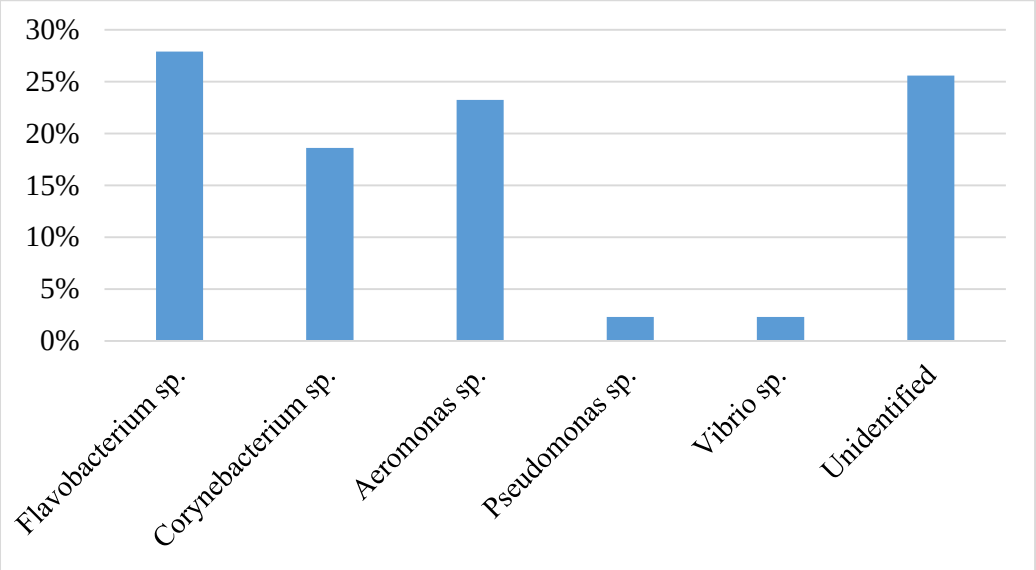
	Day		
Treatment	0	5	10
C	333.33 ± 111.11	1222.20 ± 407.41	0
NAM		120.00 ± 40.00	2268.89 ± 296.61
NC		0	0
NCG		0	413.33 ± 74.77

The value of CFU/ml on time zero was 0 for TSA (Table 10). At day 5 and at day 10 of incubation there were no significant differences between CFU/ml of the treatments tested (χ^2 (3) = 2.212, p = 0.530 and χ^2 (3) = 1.528, p > 0.676 respectively).

The value of CFU/ml on time zero was 333.33 ± 111.11 for PS mediums (Table 11). The CFU/ml was not significantly different between the treatments tested at day 5 (χ^2 (3) = 4.992, p = 0.172), nor at day 10 (χ^2 (3) = 2.212, p = 0.530).

Of all the isolated colonies, 25% were not possible to identify (Graph 9 and Table 39 from Annex I). The most identified genus was Flavobacterium sp., comprising 28% of all the colonies, followed by Aeromonas sp. and Corynebacterium sp., 23% and 19%, respectively. Lastly, Pseudomonas sp. and Vibrio sp. represent around 2% each.

Graph 9: Relative frequency of the genera of the bacteria that were isolated at the seventh day of incubation in the TSA and PS mediums from the three pools. N = 43.



3.7. Correlations

Viability was positively correlated with MOT (ρ = 0.323, n = 293, p < 0.01) and VCL (ρ = 0.223, n = 293, p < 0.01) and negatively correlated with LIN (ρ = -0.231, n = 293, p < 0.01), as seen in Table 12. ATP content was correlated with MOT (ρ = 0.168, n = 35, p < 0.05) (Table 12).

Table 12: Spearman's rank correlation coefficient between the different parameters tested.

	MOT	VCL	LIN	VIAB
VIAB	0.323**	0.223**	-0.231**	
ATP	0.350*	-0.024	-0.148	0.261
MDA	0.071	0.198	0.266	0.222
CFU/ml TSA	***	***	***	-0.100
CFU/ml PS	***	***	***	0.500

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
*** Correlation not performed due to the sample size (N ≤ 3).

Note: MOT, percentage of motile cells; VCL, curvilinear velocity; LIN, linearity; VIAB, percentage of viable cells; ATP, adenosine triphosphate; MDA, malondialdehyde; TSA, Trypticase Soya Agar medium; PS, CN Pseudomonas Agar medium. Values equal to zero were excluded. For VIAB x MOT, VCL and LIN, N = 293. Values from seven pools and all the days of evaluation. For ATP x MOT, VCL and LIN, N = 35. For ATP x VIAB, N = 32. Values from five pools, obtained between 0 and 3 days of storage. For MDA with MOT, VCL and LIN, N = 33. For MDA x VIAB, N = 57. Values from two pools, obtained between 3 and 9 days of storage. For CFU/ml TSA x VIAB, N = 5. For CFU/ml PS x VIAB, N = 4. Values from three pools, obtained between 0 and 10 of storage.

There was no correlation between the MDA values and the other measured parameters ($\rho \geq 0.266$, $n \geq 32$ and $p > 0.05$) (Table 12). The correlation tests between CFU/ml of the TSA and PS medium and the remaining parameters were considered not biologically meaningful because of the reduced sample size ($n \leq 3$).

4. Discussion

The reproduction of meagre is one of the areas that requires research and improvement, for the production of high quality spawns at a lower price. In this work a protocol for semen refrigeration in meagre was developed that will facilitate the artificial reproduction techniques. To determine the best extender and dilution ratio for the protocol, semen quality was evaluated during several days based on the following quality parameters: sperm motility, sperm viability, energy content (ATP), lipid peroxidation (MDA) and bacteriology.

4.1. Concentration

The cell concentration values obtained in the present thesis were generally higher compared to the values reported for meagre by Mylonas et al. (2013b). The maximum concentration obtained on this experiment was 63 x 10^9 spz/ml versus 32 x 10^9 spz/ml obtained by Mylonas et al. (2013b). Nonetheless, these values are within the range reported for several teleost species, by Piironen and Hyvärinen (1983) and Poole and Diilane (1998).

The differences between cell concentration values in this work and those obtained by Mylonas et al. (2013b) could be due to normal variability between individuals and different experimental conditions, but also caused by different measurement methods. While in this study the concentration was assessed with a CASA system, Mylonas et al. (2013b) used a Neubauer chamber. This last method, hematocytometry, is the gold standard, while CASA systems require the determination of a compensation factor to ensure accuracy (Kuster, 2005).

Nonetheless, different factors could have affect cell concentration such as different feeding regimes, the size of the fish (in this study fish were larger, average of 7.3 kg versus average of 4.6 kg in Mylonas et al. (2013b)), or the rearing conditions (outdoor tanks in this study versus indoor tanks in the study by Mylonas et al. (2013b)).

4.2. Motility

The percentage of motile spermatozoa of fresh semen immediately after collection in this study (73 ± 3.5%) was similar to the one obtained in the other two studies in meagre. Schiavone et al. (2012) obtained between 53 and 74% (depending on the time during the spawning season), and Mylonas et al. (2013b) had values that ranged between 44% and 80%. In all these studies, the motility was evaluated with a CASA system. Samples with this high percentage of motile cells should be able to achieve high fertilization rates, given that the quality of eggs is good and enough volume of sperm is used (e.g. Casselman et al. 2006; Ottesen et al. 2009). In relation to the sperm velocity immediately after collection, the values of 141 ± 8 μm/s observed for meagre in this study were slightly higher than the values described for other Perciformes. As an example, the Lahnsteiner & Patzner (1998) described for the white seabream (*Diplodus sargus*) 127 ± 23 μm/s VCL, whereas, the European sea bass semen, which has some similarities to meagre semen had a curvilinear velocity lower than 80 μm/s at 15 seconds after activation (Fauvel et al., 1998). On the other hand, the linearity of meagre's fresh semen immediately after collection of 57 ± 2% appears to be lower, when compared to other teleosts. While zebrafish (*Danio rerio*) semen have an average linearity of 84% (Wilson-Leedy & Ingermann, 2007) the bluegill (*Lepomis macrochirus*) has an average linearity of 76%.

As expected, during refrigeration, the values of percentage of motility, curvilinear velocity and linearity obtained with the undiluted sample were lower than those from any of the diluted treatments. Also, in another sciaenid, the Atlantic croaker (*Micropogonias undulatus*), it was verified that the undiluted sample reached zero motility earlier than the diluted treatments, in semen refrigerated at 4^{o}C (Leclercq et al., 2012). In effect, as stated by Lahnsteiner et al. (1996) and Maria et al. (2014), diluting semen with an extender contributes to its preservation, which translates in better values of quality parameters throughout time.

The highest percentages of motility along the time were obtained with dilutions 1:4 and 1:9, and with the extenders 0.9% NaCl and 0.9% NaCl with glucose and glycine. No differences were found between the results obtained with dilutions 1:4 and 1:9. This was also verified in Atlantic cod (*Gadus morhua*) (DeGraaf & Berlinsky, 2004) and black sea bass (*Centropristis*

striata) (DeGraaf, King, Benton & Berlinsky, 2004), when comparing refrigerated semen, diluted with 1:4 and 1:9 modified Mounib's extender. Nonetheless, and although not significantly, the dilution 1:4 kept motility for longer in black sea bass semen, as also observed in this experiment.

Comparing with other Sciaenids, the results obtained for motility along storage time appear similar or better. Atlantic croaker semen diluted in 1:2 or 1:9 HBSS retained motility for less than 4 days (Leclercq et al., 2014), whereas in the present work, at 4 days, it had on average 38% and 31% motile cells when diluted 1:4 or 1:9 in 0.9% NaCl, respectively. Semen of black drum (*Pogonias cromis*) diluted in 202 or 286 mOsm/kg HBSS retained 8% of motile cells at the eighth day of refrigerated storage (Wayman et al., 1997), while in this experiment it retained 13% of motility until the ninth day, in semen diluted 1:4 in 0.9% NaCl. In the present experiment the motility was kept for a maximum of 15 days, with the 1:4 dilution of 0.9% NaCl. These good results could be due to the meagre semen being more resistant to refrigeration or because of a positive effect of some of the treatments tested. Additionally, in this thesis only extenders with 300 mOsm/kg were tested, chosen according to the osmolality of meagre's seminal plasma. Whereas, in red drum (*Sciaenops ocellatus*) semen refrigerated at 1^{o}C and diluted in HBSS or NaCl with 200 mOsm/kg kept some motility until the thirteenth day, while the same extenders with 300 or 400 mOsm/kg kept motility for only 11 days (Wayman et al., 1998). The storage temperature of 4^{o}C in this study was selected, for practical reasons of usage in a fish farm facility. In this sense, the Wayman et al. (1998) results could indicate that a lower refrigeration temperature together with a slightly decrease of the extenders' osmolality might extend meagre sperm usage.

From the different semen quality parameters, motility has been shown to be the parameter that more frequently correlates with fertilization rate. See examples in species such as European sea bass (Fauvel, Savoye, Dreanno, Cosson, & Suquet, 1999) and gilthead seabream (Beirão et al., 2011a). Therefore, when selecting for a semen refrigeration protocol special emphasis should be placed in the sperm motility evaluation. According to the sperm motility values, semen should be refrigerated with 0.9% NaCl and 0.9% NaCl with glucose and glycine in dilutions 1:4 and 1:9.

4.3. Viability

The percentage of viability of fresh semen immediately after collection was 74 ± 33%. This value is in a range normally observed in other species. For example, in the literature the values for other euryhaline species vary between 94% in Mangrove red snapper (*Lutjanus*

argentimaculatus) (Vuthiphandchai, Chomphuthawach & Nimrat, 2009) to 83% for brook trout (*Salvelinus fontinalis*) (Nynca & Ciereszko, 2009).

In the present work, while there was no difference between the dilutions tested (1:4, 1:9 or 1:19), the extender had a significant effect in the percentage of viable cells during refrigeration. Whereas the semen viability was not prolonged by NAM, compared with the undiluted sample, both the samples diluted in 0.9% NaCl and 0.9% NaCl with glycine and glucose had more than 50% viability for 10 days. Different authors have already described that the dilution of semen prolonged its viability during refrigeration (e.g. Maria et al. 2006; McNiven et al. 1993). Thus, the negative effects of the extender NAM in the sperm viability were unexpected. Even more so, the undiluted sample had lower values of MOT and VCL when compared to NAM, and these two parameters are correlated with VIAB. This unexpected result may be due to the NAM composition, for example the ionic composition and the addition of the BSA could be unapropriate, as this extender was developed for the cryopreservation of sea bass (Fauvel et al., 1998). However, the cause for the lower viability values with the NAM extender are not clear.

In general, the decrease in the viability with time helped explained the decrease in the sperm motility parameters. In effect, and similar to the observation made by other authors in different teleosts, there was a positive correlation between sperm viability and the percentage of motile cells (Beirão et al. 2011b; Horváth et al. 2008; Lanes et al. 2008), as well as sperm velocity (Linhart, Rodina, Flajshans, Gela & Kocour, 2005). Linearity was also correlated with viability. Despite the absence of publications correlating both parameters, this goes in accordance with the correlation found between the other two motility parameters.

4.4. ATP

The ATP content of fresh meagre semen, assessed after its collection, was relatively low (2.16 µmol of ATP per 10^{12} spermatozoa) compared with other marine teleosts with a similar motility period and velocity. As an example, European sea bass semen had 115 µmol of ATP per 10^{12} spermatozoa (Dreanno et al., 1999a), and turbot semen had between 1450 and 1080 µmol of ATP per 10^{12} spermatozoa (Suquet et al., 1998). Expectedly, the ATP content decreased over time, by more than half between 12 h and day 1, with the exception of the samples stored in the extender NCG. When compared with other species, the decrease verified in the present study was more abrupt. In refrigerated beluga (*Huso huso*) and steelhead trout semen the ATP decreased by half after three to four days (Aramli, 2014; Bencic et al., 1999b).

In the present work the values fell to undetectable levels in all treatments within five days, even though some still had motility values above 30%. Despite the fact that the samples diluted in 0.9% NaCl with glycine and glucose were the only ones with measurable values above the detection limit in the third day of refrigeration, they were not statistically different from the control. In contrast, Dziewulska et al. (2010) reported that the content of ATP in Atlantic salmon semen was significantly higher when it was diluted, when compared to undiluted. Nevertheless, the present work analysis refers to the entire period of refrigeration, whereas the observations by Dziewulska et al. (2010) refers only to one time point (10 h). An analysis focused in specific time points of the meagre sperm refrigeration, is likely to find similar differences betwen undiluted sperm and the two best extenders (0.9% NaCl and 0.9% NaCl with glycine and glucose).

As expected, the ATP content, associated to the energy and thus movement of the spermatozoa, was positively correlated with MOT. A similar correlation has been found also in some freshwater teleosts, such as rainbow trout (De Baulny et al., 1996; Lahnsteiner et al., 1998) and bluegill (*Lepomis macrochirus*) semen (Burness et al., 2005), but not in bleak (*Alburnus alburnus*) semen (Lahnsteiner et al., 1996). Therefore, the depletion of the energy reserves in the form of ATP may partially explain the loss of the motility of the refrigerated sperm. Nonetheless, contrary to the correlation found by Burness et al. (2005) in bluegill, in this work there was no significant correlation between ATP and swimming velocity, neither between ATP and linearity. Characteristics other than ATP content may influence the swimming parameters.

4.5. MDA

As expected, the MDA values increased in all treatments with the refrigeration time, peaking in the 6^{th} day, with the highest value being 109.63 ± 77.52 from the extender 0.9% NaCl with glucose and glycine in the dilution 1:19. Likewise, in another teleost, the Persian sturgeon (*Acipenser persicus*), the levels of TBARS, also indicative of lipid peroxidation, increased significantly with the storage time (Aramli et al., 2013). In meagre, however, a decrease was observed between days 6 and 9, in most treatments. Since this was verified in both pools, assessed in different occasions, it is assumed that this was not due to an error in the thiobarbituric acid assay. The production of MDA requires oxygen and lipids. A depletion of these would lead to a lower production of MDA, which was not the case. The quantity of oxygen did not differ between the several days, as the falcons were opened regularly. Furthermore, as lipids are the main component of the spermatozoa's membranes, it appears

that these would not be a limiting factor. However, there are no publications regarding the half-life of MDA. Thus, the cause for this decrease is not clear.

There was no difference between the extenders used, only between dilutions 1:4 and 1:9, with the latter having higher MDA values. Either the samples are not differently affected by the dilutions and extenders tested, or this is due to the small sample size (n = 2). The MDA values had to be measured in the same day in which the samples were collected and it was not logistically feasible to evaluate it in more than two pools.

Finally, although lipid peroxidation leads to degradation of spermatozoa, there was no correlation between the MDA values and viability or motility parameters. Aramli et al. (2013) and Ball (2008) also did not find correlations between them. Thus, despite not being a parameter with a greater influence, MDA appears to somewhat impact semen quality.

The results evidence that lipid peroxidation occurs during the refrigerated storage of meagre semen. Taking this in consideration, antioxidants should be added to the extenders design to prevent cell injury caused by oxidative stress (Lahnsteiner & Mansour, 2010; Shaliutina-Kolešová et al., 2013).

4.6. Bacteriology

The number of CFU/ml increased with the refrigeration time in most treatments, as expected, and also observed by Viveiros et al. (2010a) in piracanjuba. No differences were found between the CFU/ml of the different treatments at days 5 and 10, in TSA and in the PS mediums. Nonetheless, the mean CFU/ml in the TSA medium was more than ten times superior for the undiluted treatment at the last day of incubation than for the diluted treatments tested. This could indicate that undiluted semen promotes the growth of more bacteria when compared to the diluted sperm. The number of pools tested might not have been sufficient to obtain statistically significant results. Aditionally, and despite care taken with disinfection during the plate's incubation, the possibility of cross contamination can not be totally discarded.

Most authors associate the presence and growth of bacteria with the degradation of semen quality, with negative effects in fertilization, motility and viability (Nimrat & Vuthiphandchai, 2008). The sample size for correlation of CFU/ml from either TSA or PS mediums with the parameters was small, meaning that the results of the statistical test were inconclusive. Statistics with a larger sample size could lead to the verification of additional correlations.

During the qualitative analysis five bacteria genera were identified. The most prevalent genus was Flavobacterium sp. Although it is normally found in the water of freshwater fish aquaculture (Kubilay, Altun & Savas, 2009; Welker, Shoemaker, Arias & Klesius, 2005), it has also been identified in the intestinal tract of marine fish (Nayak, 2010). Aditionally, Boonthai et al. (2016) found it in semen collected without cleaning the urogenital area. Despite the care taken in the present work to clean the urogenital papilla from water, urine and feces, there is the possibility of some contamination during the sampling procedure, as there was no disinfection. Pathogenic species of Flavobacterium sp. have been isolated in fish semen (Kumagai & Nawata, 2011; Madsen & Dalsgaard, 2008), but Oplinger and Wagner (2015) do not consider semen as a relevant infectious medium for this genus. Therefore, the main concern regarding the presence of Flavobacterium sp. is associated to the possible effect in semen degradation.

Concerning Aeromonas sp., it is found in both freshwater and marine fish (Nimrat & Vuthiphandchai, 2008). It has been found in hatchery water of rainbow trout (Kubilay et al., 2009), skin of freshwater fish (Austin, 2006), digestive tract of freshwater fish (Nayak, 2010; Nimrat & Vuthiphandchai, 2008) as well as semen of silver barb (*Barbodes gonionotus*) (Boonthai et al., 2016). In fact, in rainbow trout, Aeromonas sp. was isolated in semen collected with and without cleaning the urogenital area (Boonthai et al., 2016). Thus, its presence in the present experiment could be due to contamination or with origin in the sperm.

Regarding Corynebacterium sp. and Vibrio, these genera have been isolated from the skin (Austin, 2006; Austin & Al- Zahrani, 1988; Gillespie & Macrae, 1975; Gilmour, McCallum & Allan, 1976) and intestinal tract (Austin & Al- Zahrani, 1988; Nayak, 2010; Trust & Sparrow, 1974) of marine and freshwater fish, as well as euryhaline in the case of Corynebacterium sp. This genus is the most prevalent in hatchery water of rainbow trout (Kubilay et al., 2009). The presence of these two genera is most likely related with contamination.

Finally, bacteria from the genus Pseudomonas sp. are commonly found in soil and water (Sader & Jones, 2005), but are also present in fish, as part of the microbiota of the semen (Boonthai et al., 2016; Jenkins & Tiersch, 1997) and gastrointestinal tract (Nayak, 2010) of both marine and freshwater fish. Boonthai et al (2016) isolated it in channel catfish (*Ictalurus punctatus*), even when collecting semen with a catheter and cleaning of urogenital pore, although in a smaller number, meaning that it may be present in the semen of that species. Therefore, the detection of Pseudomonas sp. in the present study could have been isolated due to contamination or presence in the semen. Jenkins and Tiersch (1997) consider Pseudomonas

sp. to be responsible for the shortening of the semen storage period. Since in this experiment the genus was only identified once its effects were not adequately assessed.

The usage of antibiotics such as gentamycin (Viveiros et al., 2010a) is considered essential by some authors (DeGraaf & Berlinsky, 2004; Segovia, Jenkins, Paniagua-Chavez & Tiersch, 2000). However, as observed by Oplinger and Wagner (2015) in production-size trials, the usage of antibiotics in the extenders causes a decrease in egg fertilization rate. In the present work we discarded the hypothesis of using antibiotics, since one of the objectives was to develop a protocol that could be used by fish farmers.

5. Conclusions

The sperm samples in the extenders 0.9% NaCl and 0.9% NaCl with glycine and glucose in the 1:4 and 1:9 (sperm:extender) dilutions kept better semen quality, namely in motility, viability and bacteriology, when compared to the undiluted treatment and the other treatments tested.

Both the decrease in sperm viability, the ATP depletion, the increase in lipid peroxidation and the increase in bacterial counts seem to contribute to the degradation of the sperm quality during refrigerated storage. Thus, the adition of antioxidants to the extender may increase its quality.

Since extenders with different osmolalities gave better motility results in another Sciaenidae, this may also be tested futurely.

Overall, the best option according to the results for the refrigeration of meagre semen was the dilution 1:4 (sperm:extender) in the extender 0.9% NaCl. At the fourth day of storage, it had more than 40% of motile cells, and at the 10^{th} day of storage it had approximately 10%. This means that if enough volume of sperm is available, good fertilization rates could still be obtained even after 10 days of refrigeration, in these conditions.

BIBLIOGRAPHY

Aas, G.H., Refstie, T., & Gjerde, B. (1991). Evaluation of milt quality of Atlantic salmon. *Aquaculture, 95*(1), 125-132.

Agarwal, N.K. (2011). Cryopreservation of fish semen. *Himalayan Aquatic Biodiversity Conservation & New Tools in Biotechnology*, 104-127.

Agriculture and Agri-Food Canada (2013). Inside Portugal: the fish and seafood trade. Retrieved Jun. 16, 2016, from http://www5.agr.gc.ca/resources/prod/Internet-Internet/MISB-DGSIM/ATS-SEA/PDF/6430-eng.pdf

Alavi, S.M.H., & Cosson, J. (2006). Sperm motility in fishes.(II) Effects of ions and osmolality: a review. *Cell biology international, 30*(1), 1-14.

Alavi, S.M.H., Cosson, J., Karami, M., Amiri, B. M., & Akhoundzadeh, M.A. (2004). Spermatozoa motility in the Persian sturgeon, *Acipenser persicus*: effects of pH, dilution rate, ions and osmolality. *Reproduction, 128*(6), 819-828.

Annerén, K.G., & Epstein, C.J. (1987). Lipid peroxidation and superoxide dismutase-1 and glutathione peroxidase activities in trisomy 16 fetal mice and human trisomy 21 fibroblasts. *Pediatric research, 21*(1), 88-92.

Aramli, M.S. (2014). ATP content, oxidative stress and motility of beluga (*Huso huso*) semen: effect of short-term storage. *Reproduction in Domestic Animals, 49*(4), 636-640.

Aramli, M.S., Azarin, H., & Farsi, P. (2015). Motility parameters, adenosine triphosphate content and oxidative stress indices of sterlet, Ac*ipenser ruthenus* sperm after 6 days of storage. *Aquaculture Research*, doi: 10.1111/are.12855

Aramli, M.S., Kalbassi, M.R., Nazari, R.M., & Aramli, S. (2013). Effects of short-term storage on the motility, oxidative stress, and ATP content of Persian sturgeon (*Acipenser persicus*) sperm. *Animal reproduction science, 143*(1), 112-117.

Asso, I.D. (1801). Introduccion á la ichthyologia oriental de España. *Anales de Ciencias Naturales de Madrid, 4*, 28-52.

Associação Portuguesa de Aquacultura (2014). *Aquacultura em Portugal*. Retrieved Jun. 14, 2016, from http://oceano21.inegi.up.pt/userfiles/file/Eventos/2014/FEVEREIRO2014/MissaoChile/APA.pdf

Asturiano, J.F., Cabrita, E., & Horváth, Á. (2016). Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. *General and Comparative Endocrinology.*

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), 173-190.

Austin, B. (2006). The bacterial microflora of fish, revised. *The Scientific World Journal*, *6*, 931-945.

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.

Ball, B.A. (2008). Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Animal reproduction science*, *107*(3), 257-267.

Ballarin, L., Dall'Oro, M., Bertotto, D., Libertini, A., Francescon, A., & Barbaro, A. (2004). Haematological parameters in *Umbrina cirrosa* (Teleostei, Sciaenidae): a comparison between diploid and triploid specimens. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *138*(1), 45-51.

Barrett, I. (1951). Fertility of salmonoid eggs and sperm after storage. *Journal of the Fisheries Board of Canada*, *8*(3), 125-133.

Bartha, M.M.P. (2009). Análise bacteriológica de sêmen de catitus (*Tayassu tajacu*) criados em cativeiro. Master's thesis in Animal Sciences. Belém, PA: Universidade Federal do Pará.

Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S., & Herráez, M. P. (2011a). Effect of cryopreservation on fish sperm subpopulations. *Cryobiology*, *62*(1), 22-31.

Beirão, J., Pérez- Cerezales, S., Martínez- Páramo, S., & Herráez, M.P. (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*(5), 794-796.

Beirão, J., Soares, F., Pousão-Ferreira, P., Diogo, P., Dias, J., Dinis, M.T., Herráez, M.P., & Cabrita, E. (2015). The effect of enriched diets on *Solea senegalensis* sperm quality. *Aquaculture*, *435*, 187-194.

Beirão, J., Soares, F., Herráez, M.P., Dinis, M.T., & Cabrita, E. (2011b). Changes in *Solea senegalensis* sperm quality throughout the year. *Animal reproduction science*, *126*(1), 122-129.

Bellard, R. (1988). Artificial insemination and gamete management in fish. *Marine & Freshwater Behaviour & Phy*, *14*(1), 3-21.

Bencic, D.C., Krisfalusi, M., Cloud, J.G., & Ingermann, R.L. (1999b). Maintenance of steelhead trout (*Oncorhynchus mykiss*) sperm at different *in vitro* oxygen tensions alters ATP levels and cell functional characteristics. *Fish Physiology and Biochemistry*, *21*(3), 193-200.

Bencic, D.C., Krisfalusi, M., Cloud, J.G., & Ingermann, R.L. (1999a). ATP levels of chinook salmon (*Oncorhynchus tshawytscha*) sperm following *in vitro* exposure to various oxygen tensions. *Fish Physiology and Biochemistry*, *20*(4), 389-397.

Berois, N., Arezo, M.J., & Papa, N.G. (2011). Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. *Biological research*, *44*(2), 119-124.

Biglino, T. (2015). *Reproduction of hatchery-produced meagre (*Argyrosomus regius*) in captivity: effects of GnRHa implants on sperm quality*. Master's thesis. Lund: Department of Biology, Faculty of Science, Lunds University.

Billard, R. (1981). Short-term preservation of sperm under oxygen atmosphere in rainbow trout (*Salmo gairdneri*). *Aquaculture*, *23*(1), 287-293.

Billard, R., & Cosson, M.P. (1988). Sperm motility in rainbow trout, *Parasalmo mykiss*; effect of pH and temperature. *Colloques de l'INRA (France), 44.*

Billard, R., Cosson, J., Noveiri, S.B., & Pourkazemi, M. (2004). Cryopreservation and short-term storage of sturgeon sperm, a review. *Aquaculture*, *236*(1), 1-9.

Billard, R., Cosson, J., Perchec, G., & Linhart, O. (1995). Biology of sperm and artificial reproduction in carp. *Aquaculture*, *129*(1), 95-112.

Bobe, J., & Labbé, C. (2010). Egg and sperm quality in fish. *General and comparative endocrinology*, *165*(3), 535-548.

Bombardelli, R.A., Mörschbächer, E.F., Campagnolo, R., Sanches, E.A., & Syperreck, M.A. (2006). Dose inseminante para fertilização artificial de ovócitos de jundiá cinza, *Rhamdia quelen* (Quoy & Gaimardm, 1824). *Revista Brasileira de Zootecnia*, *35*(4), 1251-1257.

Boonthai, T., Khaopong, W., Sangsong, J., Sooksawat, T., Nimrat, S., & Vuthiphandchai, V. (2016) Semen collection methods affect the bacterial composition of post-thawed semen of silver barb (*Barbodes gonionotus*). *Animal reproduction science*, *166*, 90-98.

Boonthai, T., Khaopong, W., Sangsong, J., Sooksawat, T., Vuthiphandchai, V., & Nimrat, S. (2014). Evaluation of the potential source of bacterial contamination during cryopreservation process of silver barb (*Barbodes gonionotus*) sperm. *Aquaculture Research*, *47*(7), 2101-2113.

Boryshpolets, S. (2011). Energetic and motility of fish spermatozoa. Ph.D Thesis. Vodňany: Faculty of Fisheries and Protection of Waters - University of South Bohemia.

Boryshpolets, S., Dzyuba, B., Stejskal, V., & Linhart, O. (2009). Dynamics of ATP and movement in Eurasian perch (*Perca fluviatilis L.*) sperm in conditions of decreasing osmolality. *Theriogenology*, *72*(6), 851-859.

Botsoglou, N.A., (1994) Rapid, Sensitive, and Specific Thiobarbituric Acid Method for Measuring Lipid Peroxidation in Animal Tissue, Food and Feedstuff Samples, J. *Agric. Food Chem*. *42*, 1931-1937.

Bouck, G.R., & Jacobson, J. (1976). Estimation of salmonid sperm concentration by microhematocrit technique. *Transactions of the American Fisheries Society*, *105*(4), 534-535.

Bryant, M.P., & Small, N. (1956). The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *Journal of Bacteriology*, *72*(1), 16.

Buck, J.D. (1982). Nonstaining (KOH) method for determination of gram reactions of marine bacteria. *Applied and Environmental Microbiology*, *44*(4), 992-993.

Buege, J.A., & Aust, S.D. (1978). [30] Microsomal lipid peroxidation. *Methods in enzymology*, *52*, 302-310.

Buller, N.B. (2004). Biochemical identification tables. In Buller, N.B., *Bacteria from fish and other aquatic animals: a practical identification manual*. (pp. 137-221). Cambridge, MA: CABI Publishing.

Burness, G., Moyes, C.D., & Montgomerie, R. (2005). Motility, ATP levels and metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *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., Engrola, S., Conceição, L., Lacuisse, M., Herráez, M.P., Pousão-Ferreira, P., & Dinis, M.T. (2007). Preliminary attempts on the cryopreservation of dusky grouper (*Epinephelus marginatus*) sperm. *Aquaculture Europe*, *24*, 27.

Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., & Dinis, M.T. (2011). The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. *Animal reproduction science*, *125*(1), 189-195.

Cabrita, E., Robles, V., & Herráez, P. (Eds.). (2008). *Methods in reproductive aquaculture: marine and freshwater species*. Boca Raton: CRC Press, Taylor & Francis Group.

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

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., & Herráez, M.P. (2010). Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology*, *26*(5), 623-635.

Calvi, S.L., Zoccarato, I., Gasco, L., & Andrione, A. (1994). Effetto dell'aggiunta di trealosio e/o albumina e della concentrazione di metanolo sulla motilità del seme crioconservato di carpa (*Cyprinus carpio* L.). *Rivista Italiana di Acquacoltura*., *29*, 45-51.

Cardinaletti, G., Franzoni, M.F., Palermo, F.A., Cottone, E., Mosconi, G., Guastalla, A., Campantico, E., Tibaldi, E., & Polzonetti-Magni, A.M. (2010). Environmental and neuroendocrine control of fish reproduction. *Recent Advances in Fish Reproduction Biology. Kerala: Research Signpost*, 65-87.

Carpentier, P., & Billard, R. (1978). Conservation à court terme des gamètes de Salmonidés à des températures voisines de 0 C. In *Annales de Biologie Animale Biochimie Biophysique*, *18*(4), 1083-1088.

Casselman, S.J., Schulte-Hostedde, A.I., & Montgomerie, R. (2006). Sperm quality influences male fertilization success in walleye (*Sander vitreus*). *Canadian Journal of Fisheries and Aquatic Sciences*, *63*(9), 2119-2125.

Catalán, I.A., Jiménez, M.T., Alconchel, J.I., Prieto, L., & Muñoz, J.L. (2006). Spatial and temporal changes of coastal demersal assemblages in the Gulf of Cadiz (SW Spain) in relation to environmental conditions. *Deep Sea Research Part II: Topical Studies in Oceanography*, *53*(11), 1402-1419.

Chang, I.S., Kim, B.H., & Shin, P.K. (1997). Use of sulfite and hydrogen peroxide to control bacterial contamination in ethanol fermentation. *Applied and environmental microbiology*, *63*(1), 1-6.

Chao, N.H., & Liao, I.C. (2001). Cryopreservation of finfish and shellfish gametes and embryos. *Aquaculture*, *197*(1), 161-189.

Chen, Y. K., Liu, Q. Li, J., Xiao, Z. Z., Xu, S. H., Shi, X. Ma, D. Y. (2010). Effect of long-term cryopreservation on physiological characteristics, antioxidant activities and lipid peroxidation of red seabream (*Pagrus major*) sperm. *Cryobiology*, *61*(2), 189-193.

Ciereszko, A., & Dabrowski, K. (1993). Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. *Aquaculture*, *109*(3), 367-373.

Ciereszko, A., & Dabrowski, K. (1994). Relationship between biochemical constituents of fish semen and fertility: the effect of short-term storage. *Fish Physiology and Biochemistry*, *12*(5), 357-367.

Cosson, J. (2008). Methods to analyze the movements of fish spermatozoa and their flagella. Alavi, S.M.H., Cosson, J.J., Coward, K., & Rafiee, G. (Eds.), *Fish spermatology*. (pp. 241-266). Oxford, UK: Alpha Science International Ltd.

Cosson, J. (2010). Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. *Journal of Fish Biology*, *76*(1), 240-279.

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

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

Couto, A., Barroso, C., Guerreiro, I., Pousão-Ferreira, P., Matos, E., Peres, H., Oliva-Teles, A., & Enes, P. (2016). Carob seed germ meal in diets for meagre (*Argyrosomus regius*) juveniles: Growth, digestive enzymes, intermediary metabolism, liver and gut histology. *Aquaculture*, *451*, 396-404.

Coward, K., Bromage, N.R., Hibbitt, O., & Parrington, J. (2002). Gamete physiology, fertilization and egg activation in teleost fish. *Reviews in Fish Biology and Fisheries*, *12*(1), 33-58.

Dabrowski, K., & Ciereszko, A. (1996). Ascorbic acid protects against male infertility in a teleost fish. *Experientia*, *52*(2), 97-100.

Dadras, H., Golpour, A., Siddique, M.A.M., & Linhart, O. (2016). Effect of water temperature on the physiology of fish spermatozoon function: a brief review. *Aquaculture Research*.

Dai, T., Zhao, E., Lu, G., Che, K., He, Q., Lu, Y., Fang, Q., Wang, H., Zheng, L., Li, S., Huang, C., & Dong, Q. (2012). Sperm cryopreservation of yellow drum *Nibea albiflora*: A special emphasis on post-thaw sperm quality. *Aquaculture, 368*, 82-88.

De Baulny, B.O., Labbé, C., & Maisse, G. (1999). Membrane integrity, mitochondrial activity, ATP content, and motility of the European catfish (*Silurus glanis*) testicular spermatozoa after freezing with different cryoprotectants. *Cryobiology*, *39*(2), 177-184.

De Baulny, B.O., Le Vern, Y., Kerboeuf, D., Heyrdorff, M., & Meisse, G. (1996). Flow cytometric analysis of plasma membrane damages of rainbow trout and turbot frozen sperm. In Proceedings of the IIR Commission C2, Refrigeration and Production, International Symposium 'Froid et Aquaculture', Bordeaux, France, 20-22 March, pp. 65-72.

DeGraaf, J.D., & Berlinsky, D.L. (2004). Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa. *Aquaculture*, *234*(1), 527-540.

DeGraaf, J. D., King, W., Benton, C., & Berlinsky, D. L. (2004). Production and storage of sperm from the black sea bass Centropristis striata L. *Aquaculture Research*, *35*(15), 1457-1465.

Devasagayam, T.P.A., Boloor, K.K., & Ramasarma, T. (2003). Methods for estimating lipid peroxidation: an analysis of merits and demerits. Indian journal of biochemistry & biophysics, *40*(5), 300-308.

DIVERSIFY (n.d.). *Recent Activities: Assessment of sperm characteristics from DIVERSIFY species.* Retrieved Jun. 16, 2016, from: http://www.diversifyfish.eu/

Dreanno, C. (1998). Régulation de la mobilité des spermatozoïdes de turbot (Psetta maxima) et de bar (*Dicentrarchus labrax*): Etude du métabolisme énergétique, du contrôle ionique, de la morphologie et du pouvoir fécondant. PhD thesis in Vie Agro Santé. Rennes: Université de Rennes.

Dreanno, C., Cosson, J., Suquet, M., Cibert, C., Fauvel, C., Dorange, G., & Billard, R. (1999a). Effects of osmolality, morphology perturbations and intracellular nucleotide content during the movement of sea bass (*Dicentrarchus labrax*) spermatozoa. *Journal of reproduction and fertility*, *116*(1), 113-125.

Dreanno, C., Seguin, F., Cosson, J., Suquet, M., & Billard, R. (1999b). Metabolism of turbot (*Scophthalmus maximus*) spermatozoa: relationship between motility, intracellular nucleotid content and mitochondrial respiration. *Molecular Reproduction and Development, 53*(2), 230-243.

Dreanno, C., Suquet, M., Desbruyères, E., Cosson, J., Le Delliou, H., & Billard, R. (1998). Effect of urine on semen quality in turbot (*Psetta maxima*). *Aquaculture, 169*(3), 247-262.

Dreanno, C., Suquet, M., Fauvel, C., Le Coz, J. R., Dorange, G., Quemener, L., & Billard, R. (1999). Effect of the aging process on the quality of sea bass (*Dicentrarchus labrax*) semen. *Journal of Applied Ichthyology*, *15*(6), 176-180.

Dreanno, C., Suquet, M., Quemener, L., Cosson, J., Fierville, F., Normant, Y., & Billard, R. (1997). Cryopreservation of turbot (*Scophthalmus maximus*) spermatozoa. *Theriogenology*, *48*(4), 589-603.

Duncan, N. J., Estévez, A., Fernández-Palacios, H., Gairin, I., Hernández-Cruz, C.M., Roo, J., Schuchardt, D. & Vallés, R. (2013). Aquaculture production of meagre (*Argyrosomus regius*): hatchery techniques, ongrowing and market. *Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited, Cambridge, UK*, 519-541.

Duncan, N., Estévez, A., Padros, F., Aguilera, C., Esteban, M. F., Norambuena, F., Carazo, I., Carbo, R., & Mylonas, C. C. (2008). Acclimation to captivity and GnRHa-induced spawning of meagre (*Argyrosomus regius*). *Cybium*, *32*(2/Supp), 332-333.

Duncan, N., Estévez, A., Porta, J., Carazo, I., Norambuena, F., Aguilera, C., Gairin, I., Bucci, F., Valles, R., & Mylonas, C.C. (2012). Reproductive development, GnRHa-induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatised to captivity. *Fish physiology and biochemistry*, *38*(5), 1273-1286.

Dupont-Nivet, M., Vandeputte, M., Haffray, P., & Chevassus, B. (2006). Effect of different mating designs on inbreeding, genetic variance and response to selection when applying individual selection in fish breeding programs. *Aquaculture*, *252*(2), 161-170.

Dziewulska, K., Rzemieniecki, A., & Domagała, J. (2010). Motility and energetic status of Atlantic salmon (*Salmo salar* L.) sperm after refrigerated storage. *Journal of Applied Ichthyology*, *26*(5), 668-673.

Dzyuba, B., Bondarenko, O., Fedorov, P., Gazo, I., Prokopchuk, G., & Cosson, J. (2016). Energetics of fish spermatozoa: The proven and the possible. *Aquaculture*.

Dzyuba, V., & Cosson, J. (2014). Motility of fish spermatozoa: from external signaling to flagella response. *Reproductive biology*, *14*(3), 165-175.

Elain, A., Le Fellic, M., Corre, Y. M., Le Grand, A., Le Tilly, V., Audic, J. L., & Bruzaud, S. (2015). Rapid and qualitative fluorescence-based method for the assessment of PHA production in marine bacteria during batch culture. *World Journal of Microbiology and Biotechnology*, *31*(10), 1555-1563.

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

Failler, P. (2007). Future prospects for fish and fishery products. 4. Fish consumption in the European Union in 2015 and 2030. Part 1. European overview. *FAO Fisheries Circular. 972*(4). Retrieved Jun. 16, 2016, from: http://www.fao.org/docrep/010/ah947e/ah947e00.htm

FAO (2014). *Statistics.* Retrieved Jun. 16, 2016 from http://www.fao.org/fishery/statistics/en

FAO (2015). *Food Outlook: Biannual report on global food markets*. Retrieved Jun. 16, 2016, from: http://www.fao.org/3/a-i4581e.pdf

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., & Suquet, M. (1999). Characteristics of sperm of captive seabass in relation to its fertilization potential. *Journal of Fish Biology*, *54*(2), 356-369.

Fauvel, C., Suquet, M., Dreanno, C., Zonno, V., & Menu, B. (1998). Cryopreservation of sea bass (*Dicentrarchus labrax*) spermatozoa in experimental and production simulating conditions. *Aquatic Living Resources*, *11*(6), 387-394.

Fauvel, C., Suquet, M., & Cosson, J. (2010). Evaluation of fish sperm quality. *Journal of Applied Ichthyology*, *26*(5), 636-643.

Fishbase (n. d.). *Order summary for Perciformes.* Retrieved Jun. 16, 2006, from: http://www.fishbase.se/summary/OrdersSummary.php?order=Perciformes

Fox, J. (2005). Getting started with the R commander: a basic-statistics graphical user interface to R. *Journal of statistical software*, *14*(9), 1-42.

Fox, J., & Carvalho, M. S. (2012). The RcmdrPlugin. survival package: Extending the R Commander interface to survival analysis. *Journal of Statistical Software*, *49*(7), 1-32.

Freney, J., Duperron, M.T., Courtier, C., Hansen, W., Allard, F., Boeufgras, J.M., Monget, D., & Fleurette, J. (1991). Evaluation of API Coryne in comparison with conventional methods for identifying coryneform bacteria. *Journal of clinical microbiology*, *29*(1), 38-41.

Gage, M.J., 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. *Current biology*, *14*(1), 44-47.

Gallego, V., Carneiro, P.C.F., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Soler, C., Pérez, L., & Asturiano, J.F. (2013). Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by CASA software. *Theriogenology*, *79*(7), 1034-1040.

Gamsiz, K., & Neke, M. (2008, July). Embryonic development stages of meagre *Argyrososmus regius* Asso 1801 under rearing conditions. Poster session presented at the 8th Larval Biology Symposium, Lisboa, Portugal.

Garner, D.L., & Johnson, L.A. (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biology of reproduction*, *53*(2), 276-284.

Gil, M.D.M., Grau, A., Basilone, G., Ferreri, R., & Palmer, M. (2013). Reproductive strategy and fecundity of meagre *Argyrosomus regius* Asso, 1801 (Pisces: Sciaenidae): implications for restocking programs. *Scientia Marina*, *77*(1), 105-118.

Gillespie, N.C., & Macrae, I.C. (1975). The bacterial flora of some Queensland fish and its ability to cause spoilage. *Journal of Applied Bacteriology*, *39*(2), 91-100.

Gilmour, A., McCallum, M.F., & Allan, M.C. (1976). A study of the bacterial types occurring on the skin and in the intestines of farmed plaice (*Pleuronectes platessa* L.). *Aquaculture*, *7*(2), 161-172.

Ginzburg, A.S. (1968). Fertilization in fishes and the problem of polyspermy. Retrieved Jun. 16, 2016, from: https://archive.org/stream/fertilizationinf00ginz/fertilizationinf00ginz_djvu.txt

Giogios, I., Grigorakis, K., & Kalogeropoulos, N. (2013). Organoleptic and chemical quality of farmed meagre (*Argyrosomus regius*) as affected by size. *Food chemistry*, *141*(3), 3153-3159.

Gjedrem, T., Robinson, N., & Rye, M. (2012). The importance of selective breeding in aquaculture to meet future demands for animal protein: a review. *Aquaculture*, *350*, 117-129.

González-Quirós, R., del Árbol, J., del Mar García-Pacheco, M., Silva-García, A. J., Naranjo, J.M., & Morales-Nin, B. (2011). Life-history of the meagre *Argyrosomus regius* in the Gulf of Cádiz (SW Iberian Peninsula). *Fisheries Research*, *109*(1), 140-149.

Grafton, R. Q., Kompas, T., & Hilborn, R. W. (2007). Economics of overexploitation revisited. *Science*, *318*(5856), 1601-1601.

Grigorakis, K., Fountoulaki, E., Vasilaki, A., Mittakos, I., & Nathanailides, C. (2011). Lipid quality and filleting yield of reared meagre (*Argyrosomus regius*). *International Journal of Food Science & Technology*, *46*(4), 711-716.

Gwo, J.C., Chen, C.W., & Cheng, H.Y. (2002). Semen cryopreservation of small abalone (*Haliotis diversicolor supertexa*). *Theriogenology*, *58*(8), 1563-1578.

Haffray, P., Malha, R., Sidi, M.O.T., Prista, N., Hassan, M., Castelnaud, G., Karahan-Nomm, B., Gamsiz, K., Sadek, S., Bruant, J. S., Balma, P., & Bonhomme, F. (2012). Very high genetic fragmentation in a large marine fish, the meagre *Argyrosomus regius* (Sciaenidae, Perciformes): impact of reproductive migration, oceanographic barriers and ecological factors. *Aquatic Living Resources*, *25*(02), 173-183.

Harms, C.A., & Bakal, R.S. (1994). Techniques in fish anesthesia. In *American Association of Zoo Veterinarians Annual Proceedings*. Pittsburg, USA, pp. 202-210.

Harvey, B. (1993). Cryopreservation of fish spermatozoa. In *Genetic Conservation of Salmonid Fishes* (pp. 175-179). Springer US.

He, S., & Woods III, L.C. (2003). Effects of glycine and alanine on short-term storage and cryopreservation of striped bass (Morone saxatilis) spermatozoa. *Cryobiology*, *46*(1), 17-25.

He, S., & Woods III, L.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.

Holcomb, M., Cloud, J. G., & Ingermann, R. L. (2005). Impact of bacteria on short- term storage of salmonid eggs. *Aquaculture Research*, *36*(15), 1555-1561.

Holt, J.G. (1994). *Bergey's Manual of Determinative Bacteriology*. (9th Ed.). Baltimore: Williams & Wilkins.

Horsley, R.W. (1977). A review of the bacterial flora of teleosts and elasmobranchs, including methods for its analysis. *Journal of Fish biology*, *10*(6), 529-553.

Horváth, Á., 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. *Journal of Applied Ichthyology*, *24*(4), 443-449.

Hoysak, D. J., & Liley, N. R. (2001). Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. *Journal of Fish Biology*, *58*(5), 1286-1300.

Hugh, R., & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *Journal of bacteriology*, *66*(1), 24.

Hwang, P.C., & Idler, D.R. (1969). A Study of Major Cations, Osmotic Pressure, and pH in Seminal Components of Atlantic Salmon. Journal of the Fisheries Board of Canada, *26*(2), 413-419.

Ingermann, R.L. (2008). Energy metabolism and respiration in fish spermatozoa. In Alavi, S.M.H., Cosson, J.J., Coward, K., & Rafiee, G. (Eds.), *Fish spermatology*. (pp. 241-266). Oxford, UK: Alpha Science International Ltd.

Ingermann, R.L., Robinson, M.L., & Cloud, J.G. (2003). Respiration of steelhead trout sperm: sensitivity to pH and carbon dioxide. *Journal of Fish Biology*, *62*(1), 13-23.

Instituto Nacional de Estatística (2016). *Estatísticas da Pesca 2015*. Retrieved Jun. 16, 2016, from: https://www.ine.pt/ngt_server/attachfileu.jsp?look_parentBoui=261842402&att_display=n&att_download=y

Isaú, Z.A. (2006). População bacteriana, motilidade espermática e fertilidade de sêmen de piracanjuba *Brycon orbignyanus* (Valenciennes, 1849) submetido ao resfriamento. Master's thesis in Veterinary Sciences. Lavras: Universidade Federal de Lavras.

Ishikawa, M., Nakajima, K., Yanagi, M., Yamamoto, Y., & Yamasato, K. (2003). *Marinilactibacillus psychrotolerans* gen. nov., sp. nov., a halophilic and alkaliphilic marine lactic acid bacterium isolated from marine organisms in temperate and subtropical areas of Japan. *International journal of systematic and evolutionary microbiology*, *53*(3), 711-720.

Islam, M.S., & Akhter, T. (2011). Tale of fish sperm and factors affecting sperm motility: a review. *Advances in Life Sciences*, *1*(1), 11-19.

Jenkins, J.A., & Tiersch, T.R. (1997). A preliminary bacteriological study of refrigerated channel catfish sperm. *Journal of the World Aquaculture Society, 28*(3), 282-288.

Jiménez, M.T., Pastor, E., Grau, A., Alconchel, J.I., Sánchez, R., & Cárdenas, S. (2005). Revisión del cultivo de esciénidos en el mundo, con especial atención a la corvina *A. regius* (Asso, 1801). *Boletín del Instituto Español de Oceanografía, 21*, 1–4.

Junior, D.P.S., de Moraes, G.V., Ribeiro, R.P., da Costa Caçador, W., Sakaguti, E.S., Povh, J.A., & de Souza, E.D. (2008). Estudo comparativo da indução hormonal da espermiação em piavuçu (*Leporinus macrocephalus*) com extrato de hipófise de frango, coelho e carpa-DOI: 10.4025/actascianimsci. v25i2. 1996. Acta Scientiarum. *Animal Sciences*, *25*(2), 261-266.

Kalkan, S. & Canyurt M. (2012). The fishery potential and sustainable aquaculture in Portugal. *Proceedings of the 3rd International Symposium on Sustainable Development, Sarajevo, Bosnia and Herzegovina, 31 May–01 June*, pp. 84-86. Retrieved Jun. 14, 2016, from: http://eprints.ibu.edu.ba/1230/

Kime, D.E., Van Look, K.J.W., McAllister, B.G., Huyskens, G., Rurangwa, E., & Ollevier, F. (2001). Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *130*(4), 425-433.

Komrakova, M., & Holtz, W. (2009). Factors responsible for successful chilled storage of unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs. *Aquaculture*, *286*(3), 156-163.

Komrakova, M., & Holtz, W. (2011). Storage of unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs in sealed polyethylene (PE) bags. *Aquaculture*, *313*(1), 65-72.

Kopeika, E., Kopeika, J., & Zhang, T. (2007). Cryopreservation of fish sperm. *Cryopreservation and Freeze-Drying Protocols*, 203-217.

Kowalski, R. K., Cejko, B. I., Irnazarow, I., Szczepkowski, M., Dobosz, S., & Glogowski, J. (2014a). Short-Term Storage of Diluted Fish Sperm in Air Versus Oxygen. *Turkish Journal of Fisheries and Aquatic Sciences*, *14*, 831-834.

Kowalski, R. K., Cejko, B. I., Krejszeff, S., Sarosiek, B., Judycka, S., Targońska, K., Kucharczyk, D., & Glogowski, J. (2014b). Effect of albumin and casein supplementation on the common carp Cyprinus carpio L. sperm motility parameters measured by CASA. *Aquaculture international*, *22*(1), 123-129.

Kubilay, A., Altun, S., & Savas, S. (2009). A study on aerobic bacterial flora during incubation of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) eggs in hatchery. *Journal of Fisheries and Aquatic Sciences*, *3*(1), 5.

Kumagai, A., & Nawata, A. (2011). Concentration of *Flavobacterium psychrophilum* in the ovarian fluid and milt of cultured salmonids. *魚病研究*, *46*(4), 116-119.

Kuster, C. (2005). Sperm concentration determination between hemacytometric and CASA systems: Why they can be different. *Theriogenology*, *64*(3), 614-617.

Labbe, C., Maisse, G., Müller, 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.

Lagardère, J. P., & Mariani, A. Spawning sounds in meagre *Argyrosomus regius* recorded in the Gironde estuary, France. *Journal of fish biology*, *69*(6), 1697-1708.

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), 163-181.

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*(1), 130-140.

Lahnsteiner, F., & Patzner, R. A. (1998). Sperm motility of the marine teleosts Boops boops, Diplodus sargus, Mullus barbatus and Trachurus mediterraneus. *Journal of Fish Biology*, *52*(4), 726-742.

Lahnsteiner, F., Weismann, T., & Patzner, R. A. (1997). Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes. *Aquaculture Research*, *28*(6), 471-479.

Lahnsteiner, F., Berger, B., Weismann, T., & Patzner, R.A. (1996). Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. *Fish Physiology and Biochemistry*, *15*(2), 167-179.

Lanari, D., Poli, B.M., Ballestrazzi, R., Lupi, P., D'Agaro, E., & Mecatti, M. (1999). The effects of dietary fat and NFE levels on growing European sea bass (*Dicentrarchus labrax* L.). Growth rate, body and fillet composition, carcass traits and nutrient retention efficiency. *Aquaculture*, *179*(1), 351-364.

Lanes, C.F.C., Okamoto, M., Cavalcanti, P. V., Collares, T., Campos, V. F., Deschamps, J. C., Robaldo, R., Marins, L., & Sampaio, L. A. (2008). Cryopreservation of Brazilian flounder (*Paralichthys orbignyanus*) sperm. *Aquaculture*, *275*(1), 361-365.

Leclercq, E., Antoni, L., Bardon-Albaret, A., Anderson, C.R., Somerset, C.R., & Saillant, E.A. (2014). Spectrophotometric determination of sperm concentration and short-term cold-storage of sperm in Atlantic croaker *Micropogonias undulatus* L. broodstock. *Aquaculture Research*, *45*(8), 1283-1294.

Leveroni Calvi, S., Zoccarato, I., Gasco, L., & Andrione, A. (1993). Effetto del diluitore e delle modalità di congelamento e scongelamento sulla motilità del seme crioconservato di carpa (*Cyprinus carpio* L.). *Rivista Italiana di Acquacoltura*, *28*, 187-195.

Li, Z.H., Li, P., Dzyuba, B., & Randak, T. (2010). Influence of environmental related concentrations of heavy metals on motility parameters and antioxidant responses in sturgeon sperm. *Chemico-biological interactions*, *188*(3), 473-477.

Linhart, O., Gela, D., Rodina, M., & Kocour, M. (2004). Optimization of artificial propagation in European catfish, *Silurus glanis* L. *Aquaculture*, *235*(1), 619-632.

Linhart, O., Rodina, M., Gela, D., Kocour, M., & Vandeputte, M. (2005). Spermatozoal competition in common carp (*Cyprinus carpio*): what is the primary determinant of competition success?. *Reproduction*, *130*(5), 705-711.

Linhart, O., Rodina, M., Flajshans, M., Gela, D., & Kocour, M. (2005). Cryopreservation of European catfish *Silurus glanis* sperm: sperm motility, viability, and hatching success of embryos. *Cryobiology*, *51*(3), 250-261.

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), 13-30.

Luna, S.M. (n. d.). Argyrosomus regius, *Meagre.* Retrieved Jun. 16, 2016, from: http://www.fishbase.se/summary/418

Lutz, W., Sanderson, W., & Scherbov, S. (2001). The end of world population growth. *Nature*, *412*(6846), 543-545.

Madsen, L., & Dalsgaard, I. (2008). Water recirculation and good management: potential methods to avoid disease outbreaks with *Flavobacterium psychrophilum*. *Journal of fish diseases*, *31*(11), 799-810.

Maria, A.N. (2014). *Diluidores e crioprotetores no resfriamento e congelamento do sêmen de piracanjuba (*Brycon orbignyanus*).* Master's thesis in Zootechny. Lavras: Universidade Federal de Lavras.

Maria, A.N., Viveiros, A.T.M., Freitas, R.T.F., & Oliveira, A.V. (2006). Extenders and cryoprotectants for cooling and freezing of piracanjuba (*Brycon orbignyanus*) semen, an endangered Brazilian teleost fish. *Aquaculture*, *260*(1), 298-306.

Marino, G., Azzurro, E., Finoia, M.G., Messina, M.T., Massari, A., & Mandich, A. (2000). Recent advances in induced breeding of the dusky grouper *Epinephelus marginatus* (Lowe, 1834). *Cahiers Options Méditerranéenes*, *47*, 215-225.

Marques, S., & Godinho, H.P. (2004). Short-term cold storage of sperm from six neotropical characiformes fishes. *Brazilian Archives of Biology and Technology*, *47*(5), 799-804.

Martínez-Páramo, S., Diogo, P., Beirão, J., Dinis, M.T., & Cabrita, E. (2012). Sperm lipid peroxidation is correlated with differences in sperm quality during the reproductive season in precocious European sea bass (*Dicentrarchus labrax*) males. *Aquaculture*, *358*, 246-252.

Martínez-Páramo, S., Martínez-Pastor, F., Martínez-Rodríguez, G., Herráez, M.P., & Cabrita, E. (2009). Antioxidant status in fresh and cryopreserved sperm from gilthead sea bream (*Sparus aurata*). *Second International Workshop on Biology of Fish Gametes, Valencia, Spain, 11-9 September 2009*, pp. 76-77.

McNiven, M.A., Gallant, R.K., & Richardson, G.F. (1993). Fresh storage of rainbow trout (*Oncorhynchus mykiss*) semen using a non-aqueous medium. *Aquaculture*, *109*(1), 71-82.

Merino, O., Risopatrón, J., Sánchez, R., Isachenko, Figueroa, E., Valdebenito, I., & Isachenko, V. (2011). Fish (*Oncorhynchus mykiss*) spermatozoa cryoprotectant-free vitrification: stability of mitochondrion as criterion of effectiveness. *Animal reproduction science*, *124*(1), 125-131.

Miller, D., Brinkworth, M., & Iles, D. (2010). Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction*, *139*(2), 287-301.

Mishra, G., Patra, S., Dash, S. Verma, D. K., & Routray, P. (2016). *In vitro* storage of fish oocytes: effect of storage temperature, media conditions and storage duration on fertilization and larval hatchability of Indian major carp, rohu (*Labeo rohita*). *Aquaculture Research*.

Molecular Probes Inc. (2011). LIVE/DEAD® Sperm Viability Kit (L-7011). Retrieved Oct. 16, 2016, from https://tools.thermofisher.com/content/sfs/manuals/mp07011.pdf

Monfort, M.C. (2010). Present Market Situation and Prospects of Meagre (*Argyrosomus regius*), as an Emerging Species in Mediterranean Aquaculture (Studies and Reviews, No. 89). *Rome, Italy: Food and Agriculture Organization of the United Nations, General Fisheries Commission for the Mediterranean*.

Montgomery, T. M., Brown, A.Gendelman, H. K., Ota, M., & Clotfelter, E. D. (2014). Exposure to 17α- ethinylestradiol decreases motility and ATP in sperm of male fighting fish *Betta splendens*. *Environmental toxicology*, *29*(3), 243-252.

Mukai, C., & Okuno, M. (2004). Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biology of Reproduction*, *71*(2), 540-547.

Mylonas, C.C., Fostier, A., & Zanuy, S. (2010). Broodstock management and hormonal manipulations of fish reproduction. *General and comparative endocrinology*, *165*(3), 516-534.

Mylonas, C.C., Bridges, C., Gordin, H., Ríos, A.B., García, A., De La Gándara, F., Fauvel, C., Suquet, M., Medina, A., Papadaki, M., Heinisch, G., De Metrio, G., Corriero, A., Vassallo-Agius, R., Guzmán, J.-M., Mañanos, E., & Zohar, Y. (2007). Preparation and administration of gonadotropin-releasing hormone agonist (GnRHa) implants for the artificial control of reproductive maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*). *Reviews in Fisheries Science*, *15*(3), 183-210.

Mylonas, C.C., Mitrizakis, N., Castaldo, C.A., Cerviño, C.P., Papadaki, M., & Sigelaki, I. (2013a). Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality. *Aquaculture*, *414*, 318-327.

Mylonas, C.C., Mitrizakis, N., Papadaki, M., & Sigelaki, I. (2013b). Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle. *Aquaculture*, *414*, 309-317.

Mylonas, C.C., Mitrizakis, N., Sigelaki, I., & Papadaki, M. (2011a). Spawning kinetics of individual female meagre (*Argyrosomus regius*) after treatment with GnRHa implants. *Indian Journal of Science and Technology*, *4*(S8), 230-231.

Mylonas, C. C., Salone, S., Biglino, T., de Mello, P. H., Fakriadis, I., Sigelaki, I., & Duncan, N. (2016). Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. *Aquaculture*, *464*, 323-330.

Mylonas, C.C., Zohar, Y., Pankhurst, N., Kagawa, H., & Pavlidis, M.A. (2011b). Reproduction and broodstock management. In Pavlidis, M., & Mylonas, C. (Eds.), *Sparidae: Biology and aquaculture of gilthead sea bream and other species.* (pp. 95-121). Chichester, UK: John Wiley & Sons.

Nagy, S., Jansen, J., Topper, E.K., & Gadella, B.M. (2003). A triple-stain flow cytometric method to assess plasma-and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biology of reproduction*, *68*(5), 1828-1835.

Nair, S.J., Brar, A.S., Ahuja, C.S., Sangha, S.P.S., & Chaudhary, K.C. (2006). A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Animal Reproduction Science*, *96*(1), 21-29.

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*). *Animal reproduction science*, *118*(2), 324-329.

Nayak, S.K. (2010). Role of gastrointestinal microbiota in fish. *Aquaculture Research*, *41*(11), 1553-1573.

Neiffer, D.L., & Stamper, M.A. (2009). Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. *ILAR journal*, *50*(4), 343-360.

Nimrat, S., & Vuthiphandchai, V. (2008). Role of bacteria in the chilled storage and cryopreservation of sperm in aquatic animals: A review. In Schwartz, S. (Ed.), *Aquaculture Research Trends*. (pp. 149-173). New York: Nova Science Publishers, Inc.

Ninhaus-Silveira, A., Foresti, F., Veríssimo-Silveira, R., & Senhorini, J.A. (2006). Seminal analysis, cryogenic preservation, and fertility in matrinxã fish, *Brycon cephalus* (Günther, 1869). *Brazilian Archives of Biology and Technology*, *49*(4), 651-659.

Nynca, J., & Ciereszko, A. (2009). Measurement of concentration and viability of brook trout (Salvelinus fontinalis) spermatozoa using computer-aided fluorescent microscopy. *Aquaculture*, *292*(3), 256-258.

Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., & Yoshizaki, G. (2006). Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. *Proceedings of the national academy of sciences of the United States of America*, *103*(8), 2725-48.

Oplinger, R. W., & Wagner, E. J. (2015). Use of penicillin and streptomycin to reduce spread of bacterial coldwater disease I: antibiotics in sperm extenders. *Journal of aquatic animal health*, *27*(1), 25-31.

Oliveira, A.V., Viveiros, A.T.M., Maria, A.N., Freitas, R.T.F., & Izaú, Z.A. (2007). Sucess of cooling and freezing of pirapitinga (*Brycon nattereri*) semen. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *59*(6), 1509-1515.

Orfão, L.H., Maria, A.N., Nascimento, A.F., Isaú, Z. A., & Viveiros, A.T.M. (2010). Sperm fertility of the subtropical freshwater streaked prochilod *Prochilodus lineatus* (Characiformes) improved after dilution and cold storage. *Aquaculture Research*, *41*(10), e679-e687.

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

*Oxis*Research™ (2001). *Bioxytech MDA-586™: Spectrophotometric Assay for Malondialdehyde.* Retrieved Jul. 20, 2016, from http://www.woongbee.com/StressMarker/StressProtocols/20BIOXYTECH%20MDA-586.pdf

Özogul, Y., Özogul, F., & Alagoz, S. (2007). Fatty acid profiles and fat contents of commercially important seawater and freshwater fish species of Turkey: A comparative study. *Food Chemistry*, *103*(1), 217-223.

Park, C., & Chapman, F. A. (2005). An extender solution for the short-term storage of sturgeon semen. *North American Journal of Aquaculture*, *67*(1), 52-57.

Pastor, E., Grau, A., Massutí, E., & Sánchez-Madrid, A. (2002). Preliminary results on growth of meagre, *A. regius* (Asso, 1801) in sea cages and indoor tanks. EAS Especial Publication, *32*, 422–423.

Pastor, E., Rodríguez-Rúa, A., Grau, A., Jiménez, M.T., Durán, J., del Mar Gil, M., & Cárdenas, S. (2013). Hormonal spawning induction and larval rearing of meagre, *Argyrosomus regius* (Pisces: Sciaenidae). *Bolletí de la Societat d'Història Natural de les Balears*, *56*, 111-127.

Patiño, R., & Sullivan, C.V. (2002). Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry*, *26*(1), 57-70.

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. *Journal of Applied Ichthyology*, *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. *Journal of Cell Science*, *108*(2), 747-753.

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

Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *BMJ*, *316*(7139), 1236-1238.

Piferrer, F., Beaumont, A., Falguière, J. C., Flajšhans, M., Haffray, P., & Colombo, L. (2009). Polyploid fish and shellfish: production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture, *293*(3), 125-156.

Piironen, J., & Hyvärinen, H. (1983). Composition of the milt of some teleost fishes. *Journal of Fish Biology*, *22*(3), 351-361.

Pimentel, D., Huang, X., Cordova, A., & Pimentel, M. (1997). Impact of population growth on food supplies and environment. *Population & Environment*, *19*(1), 9-14.

Plusquellec, A., Beucher, M., Le Lay, C., Le Gal, Y., & Cleret, J.J. (1991). Quantitative and qualitative bacteriology of the marine water surface microlayer in a sewage-polluted area. *Marine Environmental Research*, *31*(3), 227-239.

Poli, B.M., Parisi, G., Zampacavallo, G., Iurzan, F., Mecatti, M., Lupi, P., & Bonelli, A. (2003). Preliminary results on quality and quality changes in reared meagre (*Argyrosomus regius*): body and fillet traits and freshness changes in refrigerated commercial-size fish. *Aquaculture International*, *11*(3), 301-311.

Poole, W.R., & Dillane, M.G. (1998). Estimation of sperm concentration of wild and reconditioned brown trout, *Salmo trutta* L. *Aquaculture research*, *29*(6), 439-445.

Potts, R.J., Notarianni, L.J., & Jefferies, T.M. (2000). Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of DNA strand breaks and lipid peroxidation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *447*(2), 249-256.

Quéméner, L., Suquet, M., Mero, D., & Gaignon, J.L. (2002). Selection method of new candidates for finfish aquaculture: the case of the French Atlantic, the Channel and the North Sea coasts. *Aquatic Living Resources*, *15*(05), 293-302.

Quéro, J.C. (1989). Sur la piste des maigres *Argyrosomus regius* (Pisces, Sciaenidae) du Golfe de Gascogne et de Mauritanie. *Océanis*, *15*(2), 161-170.

Quéro, J.C., & Vayne, J. J. (1985). Le maigre, *Argyrosomus regius* (Asso, 1801)(Pisces, Perciformes, Sciaenidae) du Golfe de Gascogne et des eaux plus septentrionales. *Revue des Travaux de l'Institut des Péches maritimes*, *49*(1-2), 35-66.

Rabanal, H.R. (1988). History of Aquaculture. Retrieved Jun. 14, 2016, from: http://www.fao.org/docrep/field/009/ag158e/AG158E01.htm

Rakitin, A., Ferguson, M.M., & Trippel, E.A. (1999). Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. *Aquaculture*, *170*(3), 349-358.

Ramcharitar, J., Gannon, D.P., & Popper, A.N. (2006). Bioacoustics of fishes of the family Sciaenidae (croakers and drums). *Transactions of the American Fisheries Society*, *135*(5), 1409-1431.

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. *Journal of Fish Biology*, *50*(6), 1309-1328.

Ribeiro, L., Soares, F., Quental-Ferreira, H., Gonçalves, A., & Pousão-Ferreira, P. (2013). Portuguese research studies meagre production in earthen ponds. *Glob Aquac Advocate*, *16*, 38-40. Retrieved Jun. 14, 2016, from: http://pdf.gaalliance.org/pdf/GAA-Ribeiro-May13.pdf

Rideout, R.M., Trippel, E.A., & Litvak, M.K. (2004). Relationship between sperm density, spermatocrit, sperm motility and spawning date in wild and cultured haddock. *Journal of Fish Biology*, *65*(2), 319-332.

Rieger, D. (1997). Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin–luciferase reaction. *Analytical biochemistry*, *246*(1), 67-70.

Robitaille, P.M.L., Mumford, K.G., & Brown, G.G. (1987). 31P nuclear magnetic resonance study of trout spermatozoa at rest, after motility, and during short-term storage. *Biochemistry and Cell Biology*, *65*(5), 474-485.

Roca, J., Martínez, S., Vázquez, J.M., Lucas, X., Parrilla, I., Martínez, E.A. (2000). Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15°C. *Animal Reproduction Science*, *64*(1), 103-112.

Roo, J., Hernández-Cruz, C.M., Borrero, C., Schuchardt, D., & Fernández-Palacios, H. (2010). Effect of larval density and feeding sequence on meagre (*Argyrosomus regius*; Asso, 1801) larval rearing. *Aquaculture*, *302*(1), 82-88.

Rothman, K. J. (1990). No adjustments are needed for multiple comparisons. *Epidemiology*, *1*(1), 43-46.

Rurangwa, E., Biegniewska, A., Slominska, E., Skorkowski, E.F., & Ollevier, F. (2002). Effect of tributyltin on adenylate content and enzyme activities of teleost sperm: a biochemical approach to study the mechanisms of toxicant reduced spermatozoa motility. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *131*(3), 335-344.

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), 1-28.

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.

Saad, A., Billard, R., Theron, M.C., & Hollebecq, M.G. (1988). Short-term preservation of carp (*Cyprinus carpio*) semen. *Aquaculture*, *71*(1-2), 133-150.

Sader, H. S., & Jones, R. N. (2005). Comprehensive *in vitro* evaluation of cefepime combined with aztreonam or ampicillin/sulbactam against multi-drug resistant *Pseudomonas*

aeruginosa and *Acinetobacter* spp. *International journal of antimicrobial agents*, *25*(5), 380-384.

Sadovy, Y., & Cheung, W.L. (2003). Near extinction of a highly fecund fish: the one that nearly got away. *Fish and Fisheries*, *4*(1), 86-99.

Salanitro, J.P., Blake, I.G., & Muirhead, P.A. (1977). Isolation and identification of fecal bacteria from adult swine. *Applied and environmental microbiology*, *33*(1), 79-84.

Sanches, E.A., Marcos, R.M., Baggio, D. M., Tessaro, L., Balen, R. E., & Bombardelli, R. A. (2011). Estimativa da concentração espermática do sêmen de peixe pelo método de espermatócrito. *Revista Brasileira de Zootecnia*, 1163-1167.

Sargent, J.R., & Tacon, A.G. (1999). Development of farmed fish: a nutritionally necessary alternative to meat. *Proceedings of the Nutrition Society*, *58*(02), 377-383.

Sanocka-Maciejewska, D., Ciupińska, M., & Kurpisz, M. (2005). Bacterial infection and semen quality. *Journal of reproductive immunology*, *67*(1), 51-56.

Schiavone, R., Zilli, L., Storelli, C., & Vilella, S. (2012). Changes in hormonal profile, gonads and sperm quality of *Argyrosomus regius* (*Pisces*, *Scianidae*) during the first sexual differentiation and maturation. *Theriogenology*, *77*(5), 888-898.

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.

Shaliutina-Kolešová, A., Gazo, I., Cosson, J., & Linhart, O. (2013). Comparison of oxidant and antioxidant status of seminal plasma and spermatozoa of several fish species. *Czech Journal of Animal Science*, *58*(7), p. 313-320.

Shields, R.J., Brown, N.P., & Bromage, N.R. (1997). Blastomere morphology as a predictive measure of fish egg viability. *Aquaculture*, *155*(1), 1-12.

Smolenski, R.T., Lachno, D.R., Ledingham, S.J.M., & Yacoub, M.H. (1990). Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. *Journal of Chromatography B: Biomedical Sciences and Applications*, *527*, 414-420.

Soares, F., Ribeiro, L., Gamboa, M., Duarte, S., Mendes, A.C., Castanho, S., Barata, M., Lourenço, T.M., & Pousão-Ferreira, P. (2015). Comparative analysis on natural spawning of F1 meagre, *Argyrosomus regius*, with wild broodstock spawns in Portugal. *Fish physiology and biochemistry*, *41*(6), 1509-1514.

Stickney, R. (Ed.). (2000). Encyclopedia of Aquaculture. New York: John Wiley & Sons.

Stipa, P., Angelini, M. (2005). *Cultured Aquatic Species Information Programme: Argyrosomus regius*. Retrieved Jun. 14, 2016, from: http://www.fao.org/fishery/culturedspecies/Argyrosomus_regius/en

Stoss, J. (1983). Fish gamete preservation and spermatozoan physiology. In Hoar, W., Randall, D., & Donaldson, E. (Eds.), *Fish physiology, Volume IX, Part B* (pp. 305-350). London: Academic Press.

Stoss, J., & Refstie, T. (1983). Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture*, *30*(1-4), 229-236.

Stoss, J., Büyükhatipoglu, S., & Holtz, W. (1978). Short-term and cryopreservation of rainbow trout (*Salmo gairdneri Richardson*) sperm. In *Annales de Biologie Animale Biochimie Biophysique* (Vol. 18, No. 4, pp. 1077-1082). EDP Sciences.

Suquet, M., Billard, R., Cosson, J., Dorange, G., Chauvaud, L., Mugnier, C., & Fauvel, C. (1994). Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. *Aquatic Living Resources*, *7*(04), 283-294.

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. *Journal of Fish Biology*, *52*(1), 31-41.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquaculture Research*, *31*(3), 231-243.

Teixeira, A., Rodrigues, S., Cavadas, A., & Neto, B. (2013). Fish and seafood in Portugal - a review of its availability and consumption.

Trippel, E.A., & Neilson, J.D. (1992). Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (*Gadus morhua*) and associated hatching success. Canadian *Journal of Fisheries and Aquatic Sciences*, *49*(10), 2118-2127.

Trust, T.J., & Sparrow, R.A.H. (1974). The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Canadian Journal of Microbiology*, *20*(9), 1219-1228.

van den Thillart, G., van Waarde, A., Muller, H.J., Erkelens, C., & Lugtenburg, J. (1990). Determination of high-energy phosphate compounds in fish muscle: 31P-NMR spectroscopy and enzymatic methods. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *95*(4), 789-795.

Viveiros, A.T.M., Isaú, Z.A., Figueiredo, H.C., Leite, M.A., & Maria, A.N. (2010a). Gentamycin controls bacterial growth during refrigerated storage of piracanjuba, *Brycon orbignyanus*, semen. *Journal of the World Aquaculture Society*, *41*(s1), 57-65.

Viveiros, A.T.M., Maria, A.N., Orfão, L.H., Carvalho, M.A.M., & Nunes, J.F. (2008). Powder coconut water (ACP®) as extender for semen cryopreservation of Brazilian migratory fish species. *Cybium-International Journal of Ichthyology*, *32*, 215.

Viveiros, A. T. M., Nascimento, A. F., Orfão, L. H., & Isaú, Z. A. (2010b). Motility and fertility of the subtropical freshwater fish streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. *Theriogenology*, *74*(4), 551-556.

Viveiros, A.T.M., Orfão, L.H., Maria, A.N., & Allaman, I.B. (2009). A simple, inexpensive and successful freezing method for curimba *Prochilodus lineatus* (Characiformes) semen. Animal Reproduction Science, *112*(3), 293-300.

Vuthiphandchai, V., Chomphuthawach, S., & Nimrat, S. (2009). Cryopreservation of red snapper (*Lutjanus argentimaculatus*) sperm: effect of cryoprotectants and cooling rates on sperm motility, sperm viability, and fertilization capacity. *Theriogenology*, *72*(1), 129-138.

Watson, R. (n.a.). Summary of Biochemical Tests. Retrieved Jun. 14, 2016, from http://www.uwyo.edu/molb2210_lab/info/biochemical_tests.htm

Wayman, W.R., Thomas, R.G., & Tiersch, T.R. (1997). Refrigerated storage and cryopreservation of black drum (*Pogonias cromis*) spermatozoa. *Theriogenology*, *47*(8), 1519-1529.

Wayman, W.R., Tiersch, T.R., & Thomas, R.G. (1998). Refrigerated storage and cryopreservation of sperm of red drum, *Sciaenops ocellatus* L. *Aquaculture Research*, *29*(4), 267-273.

Welker, T. L., Shoemaker, C. A., Arias, C. R., & Klesius, P. H. (2005). Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*. *Diseases of aquatic organisms*, *63*(2-3), 129-138.

Wilson-Leedy, J. G., & 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.

Wirtz, S., & Steinmann, P. (2006). Sperm characteristics in perch *Perca fluviatilis* L. *Journal of Fish Biology*, *68*(6), 1896-1902.

Wootton, R., & Smith, C. (2014). Gametogenesis. In Wootton, R., & Smith, C. (Eds.), *Reproductive Biology of Teleost Fishes*. (pp. 45-80). Chichester, UK: John Wiley & Sons, Ltd.

Yahyavi, H., Kaykhaii, M., & Hashemi, M. (2016). A rapid spectrofluorimetric method for the determination of malondialdehyde in human plasma after its derivatization with thiobarbituric acid and vortex assisted liquid–liquid microextraction. *RSC Advances*, *6*(3), 2361-2367.

Yang, N.C., Ho, W.M., Chen, Y.H., & Hu, M.L. (2002). A convenient one-step extraction of cellular ATP using boiling water for the luciferin–luciferase assay of ATP. *Analytical biochemistry*, *306*(2), 323-327.

Yasui, G. Senhorini, J.A., Shimoda, E., Pereira-Santos, M., Nakaghi, L. S.Fujimoto, T., Arias-Rodriguez, L., & Silva, L. A. (2015). Improvement of gamete quality and its short-term storage: an approach for biotechnology in laboratory fish. *Animal*, *9*(03), 464-470.

Zhou, B., Liu, W., Siu, W.H., O'Toole, D., Lam, P.K., & Wu, R.S. (2006). Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. *Aquatic toxicology*, *77*(2), 136-142.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., & Vilella, S. (2004). Adenosine triphosphate concentration and β-d-glucuronidase activity as indicators of sea bass semen quality. *Biology of Reproduction, 70*(6), 1679-1684.

ANNEX

Annex A: Temperature of refrigeration.

Table 13: Mean maximum and minimum values of temperature of semen storage, as registered daily in the temperature sensor, during the days in which the seven pools were stored..

	Temperature (^{o}C)
Mean Minimum	2.6
Mean Maximum	7.7

Annex B: Composition of solutions used in the ATP protocol.

Table 14: Composition of the solutions that were used during the procedure of extraction and quantification of ATP from meagre semen.

Solution	Composition
Sorensen stock solution A	0.1 M KH_2PO_4 in distilled water
Sorensen stock solution B	0.1 M Na_2HPO_4 □ 2 H_2O in distilled water
Sorensen buffer (pH ≈ 7.8)	10 parts solution A : 95 parts solution B
Ethylenediaminetetraacetic acid (EDTA) solution	2mM $C_{10}H_{16}N_2O_8$ in distilled water (dissolved at pH=8 with the help of Borax)
2% TCA solution	2% trichloroacetic acid in EDTA solution

Annex C: Intermediate tests of the data analysis.

Table 15: Shapiro-Wilk normality test for each motility parameter.

	Statistic
MOT	0.904
PROG	0.924
VCL	0.986
VSL	0.983
VAP	0.989*
LIN	0.898
STR	0.865

Note: MOT, percentage of motile cells; PROG, percentage of cells with progressive movement; VCL, curvilinear velocity; VSL, straight line velocity; The values used were the ones obtained in the seven pools, from all the treatments, in all the days in which motility was assessed. For each test, N = 379. Degrees of freedom = 379. *Normality is statistically significant, $p > 0.05$.

Table 16: Values for the Statistic of the Shapiro-Wilk normality test, done for each combination of treatment.

	MOT	VCL	LIN
C	0.409	0.473	0.524
NAM4	0.727	0.915*	0.796
NAM9	0.658	0.870	0.801
NAM19	0.533	0.650	0.649
NC4	0.949*	0.960*	0.719
NC9	0.936	0.846	0.654
NC19	0.845	0.907	0.799
NCG4	0.953*	0.969*	0.756
NCG9	0.898	0.960*	0.775
NCG19	0.809	0.917	0.789

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. MOT, percentage of motile cells; VCL, curvilinear velocity; and LIN, linearity. The values used were obtained during the initial seven days of evaluation, in five pools. For each test, N = 38. Degrees of freedom = 38. *Normality is statistically significant, $p > 0.05$.

Table 17: Shapiro-Wilk normality test done to the percentage of viability values of each treatment.

	N	Statistic
C	42	0.990*
NAM4	45	0.954*
NAM9	44	0.949*
NAM19	44	0.929
NC4	42	0.983*
NC9	43	0.853
NC19	45	0.912
NCG4	38	0.979*
NCG9	44	0.983*
NCG19	45	0.969*

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values obtained in five pools, in the first seven days, grouped according to combinations of extender with dilution. N corresponds to the sample size. For all the groups, degrees of freedom = 38. *Normality is statistically significant, $p > 0.05$.

Table 18: Linear regression equations, and r^2 value, for the viability values along the time of each treatment from each pool.

	Pool				
	1	2	3	4	5
C	y = -0,2129x + 72,496 r² = 0,8146	y = -0,1533x + 55,904 r² = 0,5621	y = -0,1864x + 65,138 r² = 0,7945	y = -0,0861x + 87,051 r² = 0,7169	y = -0,0976x + 74,632 r² = 0,7688
NAM4	y = -0,3328x + 74,919 r² = 0,8468	y = -0,4319x + 70,177 r² = 0,8684	y = -0,4206x + 72,97 r² = 0,9118	y = -0,1582x + 74,141 r² = 0,7516	y = -0,1604x + 79,141 r² = 0,7686
NAM9	y = -0,2927x + 48,009 r² = 0,7736	y = -0,4126x + 69,586 r² = 0,7743	y = -0,4183x + 65,494 r² = 0,8668	y = -0,3441x + 89,152 r² = 0,9069	y = -0,2965x + 86,559 r² = 0,8793
NAM19	y = -0,3342x + 39,696 r² = 0,8168	y = -0,4314x + 63,377 r² = 0,7294	y = -0,4634x + 66,17 r² = 0,8237	y = -0,3408x + 75,506 r² = 0,7189	y = -0,3821x + 82,486 r² = 0,8373
NC4	y = -0,1297x + 76,569 r² = 0,7044	y = -0,1611x + 62,456 r² = 0,7571	y = -0,2155x + 68,683 r² = 0,7109	y = -0,133x + 84,3 r² = 0,5621	y = -0,2484x + 85,481 r² = 0,7259
NC9	y = -0,1542x + 78,223 r² = 0,8373	y = -0,1729x + 90,042 r² = 0,7181	y = -0,2778x + 88,393 r² = 0,8665	y = -0,1289x + 80,172 r² = 0,7409	y = -0,144x + 92,122 r² = 0,8871
NC19	y = -0,1498x + 78,651 r² = 0,5524	y = -0,1454x + 79,964 r² = 0,811	y = -0,2317x + 67,591 r² = 0,761	y = -0,0612x + 75,346 r² = 0,8332	y = -0,1074x + 88,709 r² = 0,806
NCG4	y = -0,1677x + 85,707 r² = 0,7395	y = -0,1175x + 70,463 r² = 0,6474	y = -0,1943x + 71,371 r² = 0,6895	y = -0,098x + 82,725 r² = 0,6255	y = -0,1017x + 74,289 r² = 0,716
NCG9	y = -0,1103x + 73,096 r² = 0,7754	y = -0,1563x + 79,183 r² = 0,858	y = -0,1952x + 69,946 r² = 0,7588	y = -0,1572x + 81,742 r² = 0,7726	y = -0,1234x + 87,504 r² = 0,6107
NCG19	y = -0,2204x + 68,156 r² = 0,8367	y = -0,1166x + 66,914 r² = 0,6423	y = -0,213x + 74,932 r² = 0,7449	y = -0,0679x + 82,073 r² = 0,6309	y = -0,1734x + 87,275 r² = 0,8177

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19.

Table 19: Shapiro-Wilk normality test done to the ATP values of each treatment.

	N	Statistic
C	12	0.557
NAM4	12	0.507
NAM9	11	0.345
NAM19	11	0.366
NC4	11	0.644
NC9	12	0.709
NC19	12	0.512
NCG4	12	0.401
NCG9	12	0.702
NCG19	11	0.630

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values obtained at 12 h, 1 and 3 days, in the four pools, grouped according to the different combinations of independent variables (extender and dilution). A significance (Sig.) value below 0.05 indicates the absence of a normal distribution. The N column corresponds to the sample size value. Degrees of freedom = 11. *Normality is statistically significant, $p > 0.05$.

Table 20: Shapiro-Wilk normality test done to the MDA values obtained in each treatment.

	Statistic
C	0.954*
NAM4	0.778
NAM9	0.822*
NAM19	0.856*
NC4	0.931*
NC9	0.948*
NC19	0.837*
NCG4	0.987*
NCG9	0.751
NCG19	0.825*

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values obtained in days 3, 6 and 9, and in both pools. Significance (Sig.) greater than 0.05 indicates the existence of normal distribution. N = 6. Degrees of freedom = 6. *Normality is statistically significant, $p > 0.05$.

Table 21: Shapiro-Wilk normality test done to the CFU/ml values obtained in each treatment, medium and day.

		Day 5	Day 10
		Statistic	
TSA	C	0.750	0.862
	NAM	0.500	0.790
	NC	0.500	0.750
	NCG	0.500	0.750
PS	C	0.750	0.500
	NAM	0.750	0.947*
	NC	0.500	0.500
	NCG	0.500	0.750

Note: C, control; NAM, treatment with extender NAM; NC, treatment with extender NC; NCG, treatment with extender NCG; TSA, Trypticase Soya Agar medium; PS, CN Pseudomonas Agar medium. Values from three pools. The N column corresponds to the sample size value. Degrees of freedom = 3. * Normality is statistically significant, $p > 0.05$.

Table 22: Shapiro-Wilk normality test done to the values obtained in all the pools for VIAB, and the respective values of MOT, VCL and LIN.

	Statistic
VIAB	0.968
MOT	0.931
VCL	0.994*
LIN	0.967

Note: VIAB, percentage of viable cells; MOT, percentage of motile cells; VCL, curvilinear velocity; LIN, linearity. Values of all the treatments and days of evaluation, grouped according to parameter. Values of zero were excluded. N = 293. Degrees of freedom = 293. *Normality is statistically significant, $p > 0.05$.

Table 23: Shapiro-Wilk normality test done between ATP and the respective values of MOT, VCL and LIN, as well as ATP and VIAB.

	ATP x MOT, VCL, LIN	ATP x VIAB
	Statistic	Statistic
ATP	0.836	0.833
MOT	0.874	
VCL	0.462	
LIN	0.716	
VIAB		0.772

Note: ATP, adenosine triphosphate; MOT, percentage of motile cells; VCL, curvilinear velocity; LIN, linearity; VIAB, percentage of viable cells. Values above zero obtained between 0 and 3 days of storage, in five pools. A significance (Sig.) value below 0.05 indicates the absence of a normal distribution. For ATP x MOT, VCL and LIN, N = 35 and degrees of freedom = 35. For ATP x VIAB, N = 32 and degrees of freedom = 32. *Normality is statistically significant, $p > 0.05$.

Table 24: Shapiro-Wilk normality test done between MDA and the respective values of MOT, VCL and LIN, as well as MDA and VIAB.

	MDA x MOT, VCL, LIN	MDA x VIAB
	Statistic	Statistic
MDA	0.707	0.707
MOT	0.809	
VCL	0.957	
LIN	0.891	
VIAB		0.866

Note: MDA, malondialdehyde; MOT, percentage of motile cells; VCL, curvilinear velocity; LIN, linearity; VIAB, percentage of viable cells. For MDA x MOT, VCL, LIN, N = 33 and degrees of freedom = 33. For MDA x VIAB, N = 57 and degrees of freedom = 57. *Normality is statistically significant, $p > 0.05$.

Table 25: Statistic values of the Shapiro-Wilk normality test for the different assessments of correlation, as indicated in the first row.

	CFU/ml TSA x MOT, VCL, LIN	CFU/ml TSA x VIAB	CFU/ml PS x MOT, VCL, LIN	CFU/ml PS x VIAB
TSA	0.999	0.839*		
PS			0.750*	0.820
MOT	0.896		0.790	
VCL	0.997		0.984	
LIN	0.996		0.968	
VIAB		0.861		0.908

Note: TSA, Trypticase Soya Agar medium; PS, CN Pseudomonas Agar medium; MOT, percentage of motile cells; VCL, curvilinear velocity; LIN, linearity; VIAB, percentage of viable cells. The values corresponding to the same days of inoculation and of all the pools, treatments and days were used for each test. For CFU/ml TSA x MOT, VCL and LIN, N = degrees of freedom = 3. For CFU/ml TSA x VIAB, N = degrees of freedom = 10. For CFU/ml PS x MOT, VCL and LIN, N = degrees of freedom = 3. For CFU/ml PS x VIAB, N = degrees of freedom = 6. *Normality is statistically significant, $p < 0.05$.

Annex D: Concentration values and data treatment.

Table 26: Concentration values obtained for the control sample, in each and in all of the pools tested.

Pool	Mean ± S.E.M.
1	26.30 ± 2.54
2	46.40 ± 1.74
3	46.87 ± 4.14
4	23.83 ± 3.68
5	35.87 ± 8.37
6	62.47 ± 5.89
7	62.40 ± 3.29
Total	43.45 ± 2.80

Note: Values shown as mean ± standard error of the mean. Values expressed in 10^9 spz/ml. For each pool, N = 6. Total N = 42.

Annex E: Motility values and data treatment.

Table 27: Mean ± standard error of the mean of the percentage of motile cells for each treatment and day.

	C	NAM 4	NAM 9	NAM 19	NC 4	NC 9	NC 19	NCG 4	NCG 9	NCG 19
0	73.12 ± 3.48									
0.5	42.63 ± 4.58	67.13 ± 3.47	55.05 ± 8.54	47.79 ± 9.60	78.40 ± 1.52	76.07 ± 1.66	67.91 ± 3.78	74.87 ± 2.24	75.37 ± 5.48	64.99 ± 3.16
1	7.34 ± 2.15	49.36 ± 4.55	46.56 ± 6.95	29.31 ± 5.20	74.32 ± 3.62	68.07 ± 2.20	52.00 ± 4.09	68.64 ± 2.92	68.32 ± 2.45	41.12 ± 5.14
1.5	0.48 ± 0.17	28.76 ± 3.05	28.71 ± 5.01	14.71 ± 4.21	73.83 ± 2.96	65.57 ± 1.95	36.48 ± 5.87	55.86 ± 3.25	50.59 ± 5.31	32.83 ± 6.59
2	0	15.14 ± 3.33	8.69 ± 2.97	3.15 ± 1.18	46.11 ± 1.84	51.13 ± 4.44	22.35 ± 3.85	46.92 ± 3.56	41.19 ± 3.93	20.18 ± 5.19
3	0	5.47 ± 1.44	2.00 ± 0.59	1.05 ± 0.36	45.22 ± 4.54	34.56 ± 3.68	25.39 ± 6.09	33.38 ± 2.41	36.39 ± 4.29	18.76 ± 5.96
4	0	4.52 ± 1.24	1.62 ± 0.67	0	38.20 ± 5.18	30.52 ± 7.07	16.61 ± 4.57	29.95 ± 2.47	21.40 ± 3.32	7.19 ± 1.51
5	0	3.09 ± 1.18	0.39 ± 0.19	0	31.09 ± 3.45	34.80 ± 4.81	6.89 ± 2.02	21.82 ± 2.51	15.14 ± 2.62	6.92 ± 2.06
6	0	2.09 ± 0.87	0.54 ± 0.55	0	28.54 ± 5.70	15.06 ± 1.99	6.91 ± 3.29	17.99 ± 2.65	3.57 ± 0.91	4.59 ± 1.83

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

Table 27 (cont.): Mean ± standard error of the mean of the percentage of motile cells for each treatment and day.

	C	NAM 4	NAM 9	NAM 19	NC 4	NC 9	NC 19	NCG 4	NCG 9	NCG 19
7	0	0.60 ± 0.19	0.09 ± 0.04	0	24.22 ± 5.35	15.01 ± 2.87	7.45 ± 1.99	9.86 ± 1.88	3.22 ± 1.29	1.64 ± 0.73
8	0	0.25 ± 0.07	0	0	19.48 ± 5.18	8.11 ± 1.59	2.29 ± 0.64	7.94 ± 1.63	4.57 ± 2.00	1.06 ± 0.47
9	0	0.13 ± 0.06	0	0	13.00 ± 2.85	4.90 ± 0.93	1.81 ± 0.68	6.88 ± 1.72	1.78 ± 0.80	0.05 ± 0.02
10	0	0	0	0	7.10 ± 1.80	0.43 ± 0.09	0.65 ± 0.18	2.86 ± 0.79	0.05 ± 0.02	0
11	0	0	0	0	2.11 ± 0.88	0	0	0	0	0
12	0	0	0	0	1.13 ± 0.51	0	0	0	0	0
13	0	0	0	0	1.81 ± 0.81	0	0	0	0	0
14	0	0	0	0	0.95 ± 0.42	0	0	0	0	0
15	0	0	0	0	0.41 ± 0.18	0	0	0	0	0

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

Table 28: Results of the Mann-Whitney U of all the pairs tested of the various combinations of extenders, and of dilutions, for percentage of motility (MOT) values.

	Pairs (1 x 2)	Mann-Whitney U	Mean rank (1)	Mean rank (2)
Extender	C x NAM	1689.000*	60.53	**92.04**
	C x NC	716.500*	38.92	**107.69**
	C x NCG	873.000*	42.40	**106.53**
	NAM x NC	3802.500*	92.43	**160.83**
	NAM x NCG	4568.500*	98.76	**155.16**
	NC x NCG	7775.500*	**145.40**	125.60
Dilution	C x 1:4	751.000*	39.69	**107.44**
	C x 1:9	907.500*	43.17	**102.41**
	C x 1:19	1620.000*	59.00	**96.84**
	1:4 x 1:9	7890.000	137.56	126.14
	1:4 x 1:19	5330.000*	**156.52**	104.14
	1:9 x 1:19	5815.000*	**147.07**	109.93

Note: The mean rank of each element of the pair is shown, and highlighted in bold when the mean rank corresponds to an element of the pair with statistically significantly higher MOT values. Values from seven pools. $N_C = 45$, $N_{NAM} = 121$, $N_{NC} = 135$, $N_{NCG} = 135$, $N_{1:4} = 135$, $N_{1:9} = 128$ and $N_{1:19} = 128$. * Difference is statistically significant, $p < 0.05$.

Table 29: Mean ± standard error of the mean of the curvilinear velocity for each treatment and day.

	C	NAM4	NAM9	NAM19	NC4	NC9	NC19	NCG4	NCG9	NG19
0	140.90 ± 7.75									
0.5	92.34 ± 5.76	139.17 ± 5.87	127.91 ± 7.31	99.62 ± 10.84	127.06 ± 3.45	141.91 ± 3.54	140.73 ± 5.83	129.56 ± 2.97	130.05 ± 6.80	132.36 ± 1.93
1	77.95 ± 23.63	118.19 ± 3.98	112.98 ± 8.27	98.78 ± 8.04	124.65 ± 5.07	135.15 ± 5.88	110.83 ± 6.85	125.68 ± 5.88	122.41 ± 6.51	105.24 ± 8.39
1.5	9.40 ± 0.13	102.05 ± 3.19	90.13 ± 5.45	70.90 ± 18.94	114.59 ± 7.93	116.95 ± 3.09	117.80 ± 6.33	113.72 ± 5.42	113.68 ± 6.90	89.56 ± 12.89
2		69.98 ± 6.94	74.06 ± 11.92	97.35 ± 11.96	103.21 ± 4.29	118.56 ± 4.67	89.90 ± 4.21	108.60 ± 5.89	108.78 ± 7.10	109.00 ± 6.35
3		76.09 ± 7.90	69.07 ± 7.89	57.44 ± 35.15	113.24 ± 3.79	109.60 ± 4.41	114.68 ± 6.47	106.03 ± 4.87	111.53 ± 5.67	98.48 ± 15.19
4		62.57 ± 11.44	86.33 ± 12.07		107.56 ± 8.83	116.60 ± 7.86	94.00 ± 6.85	110.47 ± 7.30	105.80 ± 5.10	79.35 ± 2.29
5		68.03 ± 11.34			94.10 ± 7.31	122.33 ± 5.40	87.77 ± 8.12	102.10 ± 8.32	95.22 ± 4.85	82.60 ± 6.04
6		40.41 ± 8.63			88.25 ± 10.90	114.58 ± 5.61	61.28 ± 18.61	77.91 ± 6.58	50.48 ± 7.87	68.73 ± 11.99
7		54.12 ± 17.93			104.50 ± 11.27	88.48 ± 8.41	77.52 ± 22.22	83.00 ± 5.53	72.59 ± 21.36	
8		40.93 ± 3.14			97.22 ± 12.04	64.58 ± 10.07	108.12 ± 7.42	75.45 ± 1.55	60.29 ± 39.15	
9					81.84 ± 13.45	104.88 ± 7.94	84.58 ± 0.86	85.47 ± 10.26		

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

Table 29 (cont.): Mean ± standard error of the mean of curvilinear velocity for each treatment and day.

	C	NAM4	NAM9	NAM19	NC4	NC9	NC19	NCG4	NCG9	NG19
10					71.18 ± 13.14	63.40 ± 5.06	17.17 ± 7.85	78.35 ± 15.76		
11					82.82 ± 16.42					

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

Table 30: Results of the Mann-Whitney U of all the pairs tested of the various combinations of extenders, and of dilutions, for curvilinear velocity (VCL) values.

	Pairs (1 x 2)	Mann-Whitney U	Mean rank (1)	Mean rank (2)
Extender	C x NAM	1607.000*	58.71	**92.72**
	C x NC	677.500*	38.06	**107.98**
	C x NCG	789.000*	40.53	**107.16**
	NAM x NC	4250.000*	96.12	**157.52**
	NAM x NCG	4806.000*	100.72	**153.40**
	NC x NCG	8304.000	141.49	129.51
Dilution	C x 1:4	710.000*	38.78	**107.74**
	C x 1:9	835.500*	41.57	**102.97**
	C x 1:19	1528.000*	56.96	**97.56**
	1:4 x 1:9	8402.000	133.76	130.14
	1:4 x 1:19	5975.000*	**151.74**	111.18
	1:9 x 1:19	5985.000*	**145.74**	111.26

Note: The mean rank of each element of the pair is shown, and highlighted in bold when the mean rank corresponds to an element of the pair with statistically significantly higher VCL values. Values from seven pools. $N_C = 45$, $N_{NAM} = 133$, $N_{NC} = 130$, $N_{NCG} = 127$, $N_{1:4} = 135$, $N_{1:9} = 128$, $N_{1:19} = 128$. *Difference is statistically significant, $p < 0.05$.

Table 31: Mean ± standard error of the mean of the percentage of linearity for each treatment and day.

	C	NAM4	NAM9	NAM19	NC4	NC9	NC19	NCG4	NCG9	NCG19
0	56.61 ± 2.16									
0.5	64.37 ± 1.62	50.71 ± 0.62	58.04 ± 2.32	41.67 ± 5.88	52.41 ± 0.97	51.77 ± 0.75	50.10 ± 0.77	55.17 ± 0.92	55.45 ± 1.29	66.25 ± 2.06
1	58.45 ± 3.49	58.99 ± 1.04	55.61 ± 0.78	59.77 ± 2.42	55.86 ± 1.06	55.02 ± 0.91	57.69 ± 0.68	55.47 ± 0.59	56.03 ± 0.79	57.70 ± 0.61
1.5	26.60 ± 7.18	58.11 ± 1.37	55.08 ± 1.23	46.52 ± 6.16	52.99 ± 1.28	52.70 ± 0.64	55.04 ± 1.38	55.39 ± 1.15	53.32 ± 0.90	54.39 ± 0.83
2		56.58 ± 2.85	37.78 ± 4.80	61.15 ± 0.72	59.47 ± 1.00	60.11 ± 0.75	54.62 ± 2.77	59.20 ± 1.64	54.44 ± 0.54	58.45 ± 1.96
3		51.65 ± 2.39	60.16 ± 2.19	32.83 ± 17.94	59.20 ± 0.77	59.63 ± 1.08	64.18 ± 1.48	59.00 ± 0.76	63.56 ± 0.39	53.90 ± 4.41
4		56.88 ± 5.54	53.41 ± 15.57		60.20 ± 1.33	59.96 ± 2.04	64.39 ± 1.49	60.97 ± 1.35	65.10 ± 2.33	59.18 ± 2.76
5		59.66 ± 2.63			64.78 ± 0.87	59.57 ± 0.66	56.34 ± 4.82	62.41 ± 1.23	61.44 ± 3.20	49.83 ± 6.32
6		45.27 ± 7.27			55.15 ± 3.81	60.08 ± 1.27	89.25 ± 17.82	64.71 ± 3.96	52.21 ± 4.35	44.95 ± 3.75
7		56.82 ± 3.14			63.13 ± 0.55	58.44 ± 1.97	54.36 ± 2.77	68.20 ± 2.13	58.86 ± 3.97	
8		45.98 ± 13.42			61.08 ± 2.19	55.16 ± 2.79	58.92 ± 0.03	64.56 ± 2.32	38.79 ± 17.28	
9					60.53 ± 1.61	60.52 ± 2.36	47.64 ± 11.48	61.68 ± 0.28		

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and

NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

Table 31 (cont.): Mean ± standard error of the mean of the percentage of linearity for each treatment and day. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

	C	NAM4	NAM9	NAM19	NC4	NC9	NC19	NCG4	NCG9	NCG19
10					50.42 ± 6.53	40.74 ± 3.55	32.88 ± 8.69	59.54 ± 4.78		
11					61.43 ± 6.78					

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, and all the days of motility assessment. N = 5.

Table 32: Results of the Mann-Whitney U of all the pairs tested of the various combinations of extenders, and of dilutions, for the percentage of linearity (LIN) values.

	Pairs (1 x 2)	Mann-Whitney U	Mean rank (1)	Mean rank (2)
Extender	C x NAM	1745.000*	61.78	**91.58**
	C x NC	1019.500*	45.66	**105.45**
	C x NCG	1068.000*	46.73	**105.09**
	NAM x NC	5061.000*	102.83	**151.51**
	NAM x NCG	5162.000*	103.66	**150.76**
	NC x NCG	9107.000	135.54	135.46
Dilution	C x 1:4	982.000*	44.82	**105.73**
	C x 1:9	1126.500*	48.03	**100.70**
	C x 1:19	1724.000*	61.31	**96.03**
	1:4 x 1:9	7540.000	140.15	123.41
	1:4 x 1:19	5763.000*	**153.31**	109.52
	1:9 x 1:19	6380.000*	**142.66**	114.34

Note: C, control. The mean rank of each element of the pair is shown, and highlighted in bold when the mean rank corresponds to an element of the pair with statistically significantly higher LIN values. Values from seven

pools. $N_C = 45$, $N_{NAM} = 121$, $N_{NC} = 135$, $N_{NCG} = 135$, $N_{1:4} = 135$, $N_{1:9} = 128$, $N_{1:19} = 128$. *Difference is statistically significant, $p < 0.05$.

Annex F: Viability values and data treatment.

Table 33: Mean ± standard error of the mean of the percentage of viability for each treatment and day.

	C	NAM 4	NAM 9	NAM 19	NC 4	NC 9	NC 19	NCG 4	NCG 9	NCG 19
0	74.24 ± 33.20									
0.5	63.06 ± 28.20	79.65 ± 35.62	76.31 ± 34.13	73.46 ± 32.85	79.29 ± 35.46	80.08 ± 35.81	76.04 ± 34.01	71.61 ± 35.80	65.51 ± 32.76	65.80 ± 29.43
1	55.88 ± 24.99	62.81 ± 28.09	62.72 ± 28.05	53.02 ± 23.71	58.78 ± 26.29	68.24 ± 30.52	66.65 ± 29.81	67.56 ± 39.01	67.37 ± 30.13	76.69 ± 34.30
1.5	59.00 ± 26.38	57.20 ± 25.58	66.79 ± 29.87	36.30 ± 16.24	57.40 ± 28.70	74.49 ± 33.31	70.16 ± 31.38	71.51 ± 31.98	70.49 ± 31.53	68.69 ± 30.72
2	56.35 ± 25.20	62.46 ± 27.93	64.49 ± 28.84	38.26 ± 17.11	68.39 ± 30.58	68.51 ± 30.64	67.04 ± 29.98	62.32 ± 27.87	65.31 ± 29.21	68.10 ± 30.45
3	61.14 ±27.34	48.46 ± 21.67	38.87 ± 17.38	21.70 ± 9.70	67.89 ± 30.36	77.79 ± 34.79	64.48 ± 28.83	65.47 ± 29.28	63.26 ± 28.29	67.34 ± 30.11
4	57.36 ± 25.65	34.57 ± 15.46	27.25 ± 12.19	15.59 ± 6.97	63.25 ± 28.29	66.53 ± 29.75	61.08 ± 27.32	62.11 ± 27.77	65.17 ± 29.15	56.51 ± 25.27
5	60.26 ± 30.13	36.28 ± 16.22	29.05 ± 12.99	26.72 ± 11.95	62.35 ± 31.17	61.41 ± 30.70	61.60 ± 27.55	68.86 ± 48.69	64.81 ± 28.98	61.28 ± 27.40
6	48.35 ± 21.62	34.60 ± 15.47	18.10 ± 8.10	16.27 ± 7.28	51.45 ± 25.73	66.00 ± 33.00	54.13 ± 24.21	50.99 ± 25.49	65.24 ± 29.17	47.02 ± 21.03
7	39.33 ± 22.71	16.42 ± 7.34	17.76 ± 8.88	16.06 ± 8.03	42.11 ± 18.83	55.37 ± 24.76	63.23 ± 28.28	61.42 ± 27.47	51.46 ± 23.01	44.21 ± 19.77

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and

NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, in all the days of viability evaluation. N = 5.

Table 33 (cont.): Mean ± standard error of the mean of the percentage of viability for each treatment and day.

	C	NAM 4	NAM 9	NAM 19	NC 4	NC 9	NC 19	NCG 4	NCG 9	NCG 19
9		27.05 ± 13.52			52.58 ± 23.51	53.73 ± 24.03	47.44 ± 23.81	51.39 ± 22.98	45.49 ± 20.34	55.95 ± 28.98
11					29.95 ± 13.39	40.29 ± 20.14	45.09 ± 22.54	56.08 ± 28.04	44.27 ± 19.80	48.10 ± 27.77
13					29.33 ± 16.93	43.73 ± 25.25		40.75 ± 23.53	32.10 ± 18.53	
15								32.34 ± 18.67		

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, ten treatments, in all the days of viability evaluation. N = 5.

Table 34: Results of the Mann-Whitney U of all the pairs tested of the various combinations of extenders, for percentage of viability (VIAB) values.

	Pairs (1 x 2)	Mann-Whitney U	Mean rank (1)	Mean rank (2)
Extender	C x NAM	1873.500*	4615.50	**10784.50**
	C x NC	1908.500*	2811.50	**12066.50**
	C x NCG	1996.000*	2899.00	**11466.00**
	NAM x NC	4263.000*	13174.00	**21542.00**
	NAM x NCG	4425.500*	13336.50	**20593.50**
	NC x NCG	7540.500	17484.50	15668.50

Note: The mean rank of each element of the pair is shown, and highlighted in bold when the mean rank corresponds to an element of the pair with statistically significantly higher VIAB values. The N column

corresponds to the sample size value. $N_C = 42$, $N_{NAM} = 133$, $N_{NC} = 130$ and $N_{NCG} = 127$. *Difference is statistically significant, $p < 0.05$.

Annex G: ATP values and data treatment.

Table 35: Mean ± standard error of the mean of the ATP value for each treatment and day.

	C	NAM 4	NAM 9	NAM 19	NC 4	NC 9	NC 19	NCG 4	NCG 9	NCG 19
0	2.16 ± 0.72									
0.5	0.14 ± 0.05	0.29 ± 0.08	0.81 ± 0.40	0.63 ± 0.34	4.86 ± 1.61	4.38 ± 0.68	7.20 ± 2.18	1.41 ± 0.71	1.43 ± 0.42	6.01 ± 2.61
1	0.02 ± 0.01	0	0	0	1.63 ± 0.76	0.29 ± 0.11	0	1.26 ± 0.63	2.01 ± 1.00	3.81 ± 1.90
3	0.01 ± 0.00	0	0	0	0	0	0	0.56 ± 0.28	1.45 ± 0.73	2.82 ± 1.41

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from five pools, in µmol ATP per 10^{12} spermatozoa. N = 4.

Table 36: Results of the Mann-Whitney U of all the pairs tested of the various combinations of extenders, for ATP values.

	Pairs (1 x 2)	Mann-Whitney U	Mean rank (1)	Mean rank (2)
Extender	C x NAM	173.000	26.08	22.59
	C x NC	186.000	22.00	24.69
	C x NCG	187.000	22.08	24.66
	NAM x NC	464.500*	31.16	**38.73**
	NAM x NCG	469.500*	31.31	38.59
	NC x NCG	601.500	35.81	35.19

Note: The mean rank of each element of the pair is shown, and highlighted in bold when the mean rank corresponds to an element of the pair with statistically significantly higher ATP values. $N_C = 12$, $N_{NAM} = 34$, $N_{NC} = N_{NCG} = 35$. * Difference is statistically significant, $p < 0.05$.

Annex H: MDA values and data treatment.

Table 37: Mean ± standard error of the mean of the MDA value for each treatment and day.

	C	NAM 4	NAM 9	NAM 19	NC 4	NC 9	NC 19	NCG 4	NCG 9	NCG 19
0	0.00 ± 0.00									
3	6.17 ± 4.36	9.83 ± 6.95	5.36 ± 3.79	11.26 ± 7.96	9.74 ± 6.89	21.32 ± 15.08	14.22 ± 10.05	14.15 ± 10.01	23.15 ± 16.37	10.34 ± 7.31
6	19.70 ± 13.93	19.31 ± 13.65	38.06 ± 26.91	46.89 ± 33.16	20.93 ± 14.80	33.21 ± 51.40	72.68 ± 51.40	20.62 ± 14.58	51.49 ± 36.41	109.63 ± 77.52
9	20.18 ± 14.27	9.93 ± 7.02	45.13 ± 91.91	16.56 ± 11.71	30.21 ± 21.36	21.77 ± 28.77	40.69 ± 28.77	15.81 ± 11.18	29.10 ± 20.57	24.14 ± 17.07

Note: C, control; NAM4, treatment NAM in dilution 1:4; NAM9, treatment NAM in dilution 1:9; NAM19, treatment NAM in dilution 1:19; NC4, treatment NC in dilution 1:4; NC9, treatment NC in dilution 1:9; NC19, treatment NC in dilution 1:19; NCG4, treatment NCG in dilution 1:4; NCG9, treatment NCG in dilution 1:9; and NCG19, treatment NCG in dilution 1:19. Values from two pools, in µM MDA/10^{10} cells. N = 2.

Table 38: Results of the Mann-Whitney U of all the pairs tested of the various combinations of dilutions, for MDA values.

	Pairs (1 x 2)	Mann-Whitney U	Mean rank (1)	Mean rank (2)
Dilution	C x 1:4	50.000	11.83	12.72
	C x 1:9	26.000	7.83	14.06
	C x 1:19	34.000	9.17	13.61
	1:4 x 1:9	83.000*	14.11	**22.89**
	1:4 x 1:19	110.000	15.61	21.39
	1:9 x 1:19	159.000	18.67	18.33

Note: The mean rank of each element of the pair is shown, and highlighted in bold when the mean rank corresponds to an element of the pair with statistically significantly higher VCL values. Values from two pools and from days 3, 6 and 9. $N_C = 6$, $N_{1:4} = 18$, $N_{1:9} = 18$, $N_{1:19} = 18$. *Difference is statistically significant, $p < 0.05$.

Annex I: Bacteriology values and data treatment.

Table 39: Results from the bacterial identification procedures, showing the percentage and number of isolates for each of the species that were found, including the ones that were not identified.

Species	Percentage	Number of isolates
Flavobacterium sp.	27.91%	12
Corynebacterium sp.	18.60%	8
Aeromonas sp.	23.26%	10
Pseudomonas sp.	2.33%	1
Vibrio sp.	2.33%	1
Unidentified	25.58%	11

Note: Three pools and TSA and PS plates were used, and isolates done at the seventh day of incubation. N = 43.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