Aquaculture xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Factors enhancing fish sperm quality and emerging tools for sperm analysis

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ARTICLE INFO

Article history:
Received 30 October 2013
Received in revised form 9 March 2014
Accepted 12 April 2014
Available online xxxx

Keywords:
Sperm quality assessment
Sperm quality predictors
Cryopreservation
Oxidative stress
Fish sperm

ABSTRACT

With this review we try to give a comprehensive overview of the current methods used in research to assess sperm quality. In addition, we identify some of the most important factors for enhancing sperm production and quality, including, broodstock nutrition, epigenetics and sperm management (cryopreservation). Sperm quality can be assessed by analyzing different parameters from simple methods to very sophisticated approaches involving molecular tools. Parameters related with sperm composition or function (e.g. spermatozoa plasma membrane lipids, seminal plasma composition, motility activation) have successfully characterized a sperm sample but could not respond to the causes behind sperm defects. Reactive oxygen species (ROS) are one of the causes of the impairment of sperm traits. High contents of ROS are capable of producing cell apoptosis, DNA strand breakages, mitochondria function impairment, and changes in membrane composition due to sugars, lipids, and amino acid oxidation, affecting at later times sperm fertilization ability. Recently, the importance of spermatozoa RNAs in the fertilization and early embryo development has been clearly demonstrated in different species, including fish. Spermatozoa delivers more than the paternal genome into the oocyte, carrying also remnant mRNA from spermatogenesis. These RNAs have been found in sperm from human, rodent, bovine, and recently in several fish species, demonstrating the important predictive value of spermatozoa transcripts present only in those samples with high motility or from males with higher reproductive performance. The content of those transcripts can be changed during gametogenesis process influencing their content in spermatozoa.
We will focus this review on sperm quality markers, in new trends on sperm analysis, and in the use of these tools for the identification of factors enhancing gamete quality. Basic research in this field is helping to develop appropriate quality evaluation methodologies and early biomarkers of reproductive success, with potential future industrial applications.

1. Introduction

Control of sperm quality is a major issue for the aquaculture industry, both for the production of well-established commercial species and for the introduction of new ones with high commercial interest. The identification of predictive estimators or markers of sperm quality would also have major applications in other research fields and in the development of biotechnological companies. However it has been extremely difficult to accurately estimate the quality of sperm and to correlate these quality estimators with the capacity of these cells, not only to reach the oocyte and fertilize the egg, but as it will be demonstrated, to contribute to a successful early embryonic development. This quality evaluation may be relevant in order to better understand the mechanisms by which sperm is affected and to control some of the factors influencing overall gamete quality. Most of these factors are related with male breeder performance, life history, social context, or to husbandry conditions such as broodstock nutrition, environmental manipulation and spawning induction protocols or procedures for sperm handling and management.

This review gives an overview of the new trends on sperm analysis and emerging tools for sperm quality determination, with a focus on the most relevant factors affecting sperm production and quality, including, epigenetics, broodstock nutrition, and sperm management (cryopreservation). The range of optimal indicators should be defined according to the different species, sperm fate or reproductive strategy such as artificial fertilization, cryopreservation, gene banking, or mass

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http://dx.doi.org/10.1016/j.aquaculture.2014.04.034
0044-8486/

production. Basic research in this field is helping develop appropriate quality evaluation methodologies and early biomarkers of reproductive success, with potential future industrial applications for early prediction of gamete quality.

2. Factors enhancing sperm quality

There are several extensive reviews on sperm quality highlighting the potential factors that may contribute to an improvement of sperm quality or that produce detrimental effects. Most reviews deal with aspects related with broodstock improvement (Cabrita et al., 2011b) while others directly identify factors capable of affecting sperm quality traits in several fish species (Bobe and Labbé, 2010; Cabrita et al., 2009; Fauvel et al., 2010; Rurangwa et al., 2004) or relate broodstock management procedures with gamete features (Migaud et al., 2013). In this sense, this review will particularly be focused on the principal aspects for enhancing sperm quality trying to give updated information on the latest highlights in research.

2.1. The role of breeders' nutrition, epigenetics and cryopreservation on sperm quality enhancement

Although it may seem strange these three subjects are related when we speak about factors enhancing sperm quality. Nutrition enhancement has proved to ameliorate the quality of sperm by improving several spermatozoa traits. In fact not only seminal or plasma membrane composition may be positively modulated through the incorporation of certain compounds in the feeds, although most of the reports deal with an increase of DHA and ARA in plasma membrane of fish feed on polyunsaturated fatty acids (PUFA) enriched diets in species such as European seabass, *Dicentrarchus labrax* (Asturiano et al., 2001), and carp, *Catla catla* (Nandi et al., 2007). Plasma membrane fluidity is determined by the phospholipids (PL) and cholesterol (CHO) composition, and CHO regulates the lipid chain order and molecular organization of the membranes (Muller et al., 2008; Wassall and Stillwell, 2009). Therefore their modulation has a direct impact on sperm physiology and functionality. Several authors have investigated the PL, CHO and fatty acids contents in sperm and their interaction to understand how its composition affects sperm quality and influence its ability to successfully fertilize the eggs (Beirão et al., 2012; Henrotte et al., 2010; Lahnsteiner et al., 2009; Muller et al., 2008). These compounds are also inducers of sex steroid and eicosanoid production, particularly prostaglandins, and thereby their involvement in gonad development may also potentiate sperm quality (Alavi et al., 2009; Izquierdo et al., 2001). In Senegalese sole (*Solea senegalensis*), the supplementation of PUFA on diet increased the percentage of progressive spermatozoa and sperm velocity as a consequence of DHA, ARA and EPA increase in sperm membranes, particularly if this supplementation was complemented by an addition of antioxidants (Beirão, 2011). However, modulation of fish nutritional requirements may affect other parts of the fish spermatozoa. Although there are no reports on this subject in fish species, several recent reviews, exploring the promising field of nutrigenomics, discussed on the important role that epigenetic mechanisms play at the nexus between nutrition and the genome (Farhud et al., 2010). Nutrient–gene interactions enable various nutrients to transiently influence the expression of specific subsets of genes. It is becoming increasingly evident that by interacting with epigenetic mechanisms, that regulate chromatin conformation, transient nutritional stimuli at critical ontogenic stages can yield lasting influences on the expression of genes or in the methylation process of certain genes during gametogenesis. If such epigenetic changes occur in the gametes, and they are transmittable to the next generation, the quality of sperm not only in our breeders but also on the breeders of future generations in captivity can be affected. Therefore, it seems clear the positive concomitant effects of nutrition and epigenetics on sperm quality enhancement.

Breeder's nutrition can play an important role as an enhancement factor of sperm quality. There are several reports on the incorporation of antioxidant substances into feed proving to have a favorable effect on sperm quality, especially when it needs to be reinforced to sustain manipulations such as cryopreservation. It has been widely assumed that cryopreserved sperm quality is influenced by the quality of initial fresh samples (Cabrita et al., 2009), and therefore once more the cascade events of enhanced nutritional factors may also interfere in this process. It is assumed that high quality samples enhanced by nutritional factors are more prone to resist to cryodamage and therefore will better sustain the cryopreservation process.

The incorporation of probiotics in fish feeds has been one of the new areas of research for the improvement of gamete quality. There are some studies that demonstrate the importance of *Lactobacillus rhamnosus* in improving fish reproductive performance. Most of the studies have been performed in model species like the zebrafish (*Danio rerio*) where it was shown that oral administration of probiotics (10^6 CFU) stimulated reproduction by increasing *gnrh3* expression (Gioacchini et al., 2010), anticipated sex differentiation, and influenced sex ratio, probably through modulation of *sox9*, an autosomic gene also involved in chromosomal control of testis differentiation (Avella et al., 2012; Carnevali et al., 2013). Regarding the improvement of sperm quality, another study in zebrafish demonstrated the relevance of *L. rhamnosus* in target genes such as *fhsb* and *lepa* expressed in testicular cells (Riesco et al., 2013) that were considered in other species as markers of sperm quality and fertility.

Recent studies have demonstrated the effect of probiotics in European eel spermatogenesis using different concentrations of *L. rhamnosus* IMC 501® (10^3, 10^5, 10^6 CFU/ml) (Sinbyotec, Italy) added daily to the rearing water from the 6th week of hCG treatment for inducing maturation (Santangeli et al., 2013). These authors recorded significant higher sperm volume, and an improvement in the percentage of motile cells and straight swimming velocity compared to controls and to the lowest dose. These changes were also associated with the increasing levels of *Activin*, *arα*, *arβ*, *pr1* and *fshr* expression during the first 2 weeks of treatment, proving some evidences of the involvement of these molecular markers in physiological spermatozoa processes.

Although with some controversies, the replacement of fish meal by plant proteins in some cases seems to improve the quality of sperm. In a recent study, Nyina-Wamwiza et al. (2012) demonstrated that total replacement of fish meal by agriculture plants meal produced an increase in sperm volume, spermatocrit, spermatozoa integrity and motility parameters of sperm in African catfish (*Clarias gariepinus*). Although the positive effects could be associated with a different source of plant protein, some effects could be also associated with the modified proportion of fatty acids in each tested meal, since an increase in the quantity of PUFAs due to the replacement of fish meal by a high amount of plant ingredients (including oils) had a positive impact on sperm motility. Previously, we have debated that PUFAs are also implicated in physiological functions linked to sperm activity.

Sperm cryopreservation is a safe method to store and preserve the male genetic material. Its use should benefit the fish farming industry at different levels, from management of reproduction and genetic selection of males with high reproductive value to the use of high quality selected samples. Protocols for fish sperm cryopreservation have been successfully developed for several teleost species (Cabrita et al., 2009). Despite the identification of some cell damage, there are several ways to enhance the quality of samples. One approach was previously mentioned by nutritional incorporation of certain compounds that will benefit sperm traits. Another approach is by the incorporation of certain substances in the extender media of samples. This effect increases post-thaw sperm survival rates. Martínez-Páramo et al. (2012a,b) found higher motility rates and a decrease in DNA fragmentation in European seabass sperm cryopreserved with the addition of antioxidants such as vitamins (α-tocoferol and ascorbic acid) or sulfur-containing amino acids like taurine and hypotaurine. These amino

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acids were also shown to promote a decrease in DNA fragmentation in gilthead seabream, *Sparus aurata* cryopreserved sperm (Cabrita et al., 2011a).

Cryopreservation may also ensure sperm quality by the selection of disease-free material being a safety method for seed supply.

3. Emerging tools for sperm analysis

Although most of the time the requests for sperm quality analysis are coming from the industry, the new demands on research nowadays imply new techniques for sperm analysis, that probably, in a not so far future, will be used by fish farming companies. In this sense there are several factors that in our opinion may have contributed to this new generation of tools that in various ways will improve the knowledge on sperm quality assessment. Some of these advancements can be related with the development of hundreds of protocols for the cryopreservation of sperm from different species or to the use of sperm in toxicological assays.

In the last decade the number of publications in sperm cryopreservation doubled, corresponding to the introduction of new cultivated species, and therefore to an increased need to apply this tool in new reproductive management strategies. There has been also a huge concern in the conservation of threatened species due to an increase in their number, being cryopreservation an excellent way to store this valuable material. Although cryopreservation can be a useful tool it can also induce certain types of species-specific damage, as mentioned before. Consequently, an exhaustive quality analysis of samples is important to guarantee the benefits of the cryopreservation technique. In this regard, research has associated oxidative stress to certain types of damage such as peroxidation of lipids, induction of oxidative DNA damage, formation of protein adducts, ROS production involving electron leakage from the sperm mitochondria, and electron transport chain impairment. The net result of mitochondrial ROS generation is the damage of these organelles and the initiation of an intrinsic apoptotic cascade, and as consequence spermatozoa lose motility, DNA integrity and vitality (Aitken et al., 2012). The mammalian background research on oxidative stress performed since the early 40s and the awareness of the importance of oxidative stress in the etiology of male infertility (last 25 years, Alvarez et al., 1987; Aitken and Clarkson, 1987) helped to understand these concepts and their importance in sperm quality. As in mammalian research, the analysis of fish sperm quality does not come close to evaluating the full range of properties that spermatozoa need to express in order to search for predictable markers of sperm quality. The latter will be only possible when we have a complete understanding of the molecular mechanisms regulating sperm function and appreciate how this process can be influencing sperm functionality. The discovery of new-born mRNA in mammalian species, and more recently in fish, may open a hole in this window, conducting to new advancements and to new trends in sperm quality research (see more details in next section).

Another point that raised the interest of sperm analysis has been the application of sperm as a biomarker in toxicity studies. Current strategies in monitoring programs for marine–coastal areas usually require the integration of chemical analyses and biological testing in order to better evaluate the bioavailable fraction of toxicant interacting with living organisms (Coulaud et al., 2011; Macova et al., 2010). Therefore test batteries need to include organisms representing different phyla and different trophic levels (Macken et al., 2009). In recent years ecotoxicological tests have been standardized for gametes and embryos from a range of aquatic species. These tests have gained more relevance due to EU regulations on the use of animals in experimentation, because in some species this material can be easily collected without sacrificing the animal, can be stored until the tests are carried out and sperm from commercial species can be used for this purpose (Fabbrocini et al., 2012). Therefore, sperm quality tests proposed as a bioassay are a promising starting point for the development of toxicity tests that are increasingly tailored to the needs of ecotoxicology and environmental quality evaluation strategies (Fabbrocini et al., 2012).

3.1. Oxidative stress markers

In living organisms, the production of reactive oxygen species (ROS) occurs as consequence of the normal metabolisms of the cells. Thus, free radicals generation can be from the mitochondrial respiratory chain or from intracellular enzyme systems as xanthine- and NADH-oxidases (Baker and Aitken, 2004). Within the most common ROS known are the hydroxyl radical ($-OH$), the hydrogen peroxide (H_2O_2), the superoxide radical (O_2^-) and some derivates such as the hydroperoxyl radical ($OOH\cdot$). These reactive species play an important role in many cellular pathways regulated by redox events. However, ROS are highly reactive molecules that can interact with proteins, lipids, DNA and RNA, promoting cell injury at several levels. Because of this injuring potential, the level of ROS is strictly controlled by cell-specific antioxidant systems (electron-scavengers and redox-sensitive enzymes) to avoid an "oxidative stress situation" as a result of an imbalance in the production and detoxification of ROS. In aquatic species, environmental factors such as changes in temperature, oxygen levels and salinity, besides exposure to transition metals (i.e. iron, copper, chromium, mercury) or pesticides (i.e. insecticides, herbicides, fungicides and oil and related pollutants) can induce oxidative stress (reviewed by Lushchak, 2011). The exposure to all of these factors in their natural environment makes aquatic organisms good models for the study of oxidative stress damage.

The fish spermatozoa characteristics make this type of cells very prone to suffer oxidative stress-related damage, mainly due to the high content of PUFA that makes the membrane a good target for ROS, but DNA (inducing single- and double-strand DNA breaks) and proteins could be also affected. Therefore in recent years, several oxidative markers have been developed in fish sperm by analyzing different structures in the spermatozoa (Fig. 2).

In fish sperm, seminal plasma provides the major defense against ROS, due to the low content of cytoplasm in spermatozoa (Shiva et al., 2011). Several authors have analyzed the antioxidants and oxidant defensive enzymes present in spermatozoa and seminal plasma of different fish species. Ascorbic acid and uric acid are considered important antioxidants in teleost fish, being the metabolites occurring in highest concentration in sperm of several species such as burbot (*Lota lota*), perch (*Perca fluviatilis*), bleak (*Alburnus alburnus*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) (Ciereszko and Dabrowski, 1995; Lahnsteiner and Mansour, 2010; Lahnsteiner et al., 2010). Besides metabolites scavengers of ROS, there are several enzymatic systems that can be used as markers of oxidative stress. The main enzymes responsible for the detoxification of reactive oxygen species in fish sperm are: catalase, superoxide dismutase (SOD), glutathione reductase (GSR) and peroxidase (GPX) (Lahnsteiner and Mansour, 2010; Mansour et al., 2006).

Sperm can be exposed to oxidative stress from spermatogenesis, because of the high rates of mitochondrial oxygen consumption inherent to the extremely active replicative process (Aitken and Roman, 2008). Although during gametogenesis ROS can play an important role in oxidative damage, compared to other germ cells, such as spermatogonia type A, B or even spermatocytes, the resistance of spermatozoa to this attack is lower, being more easily attacked by oxygen species than other germ cells (Fig. 1). This fact is associated with the high protection given by scavengers to spermatogonia and to the fact that the cytoplasm where these enzymes act is reduced in spermatozoa (Miura and Miura, 2011).

Mature spermatozoa can suffer the attack of free radicals when is preserved to be used in artificial fertilization practices (Shaliutina et al., 2013a). For instance, during cryopreservation, the antioxidant barrier provided by seminal plasma is extremely weakened, mainly due to sperm dilution in the extender that reduces the concentration of seminal plasma compounds (Cabrita et al., 2011a).

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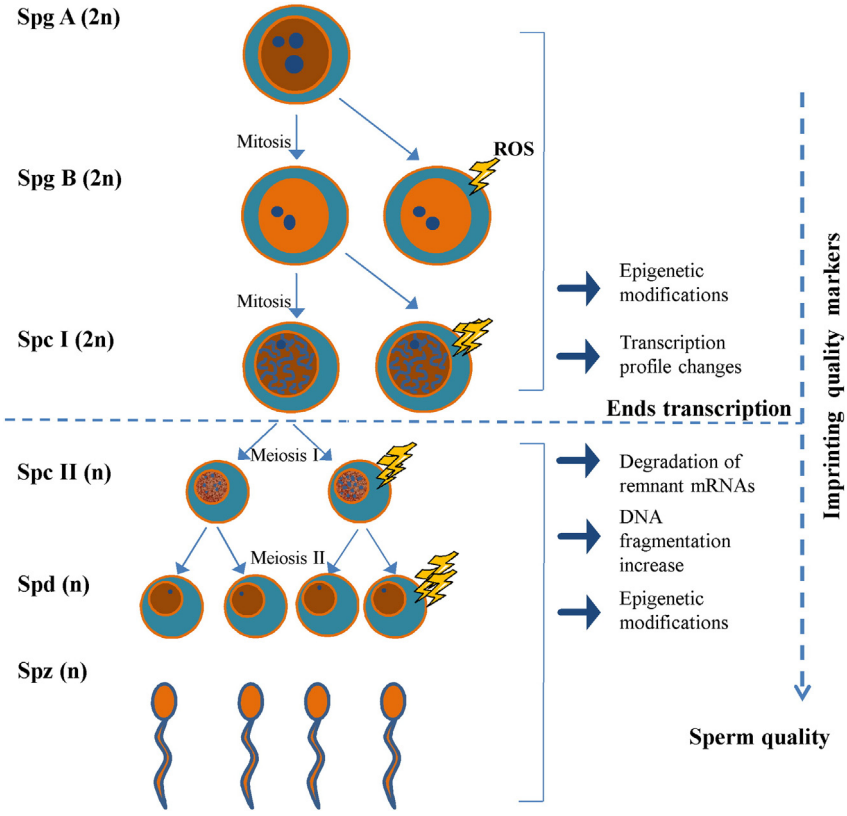

Fig. 1. Steps during fish spermatogenesis targeting points of damage that could be transmitted to spermatozoa, imprinting a quality marker in each cell.

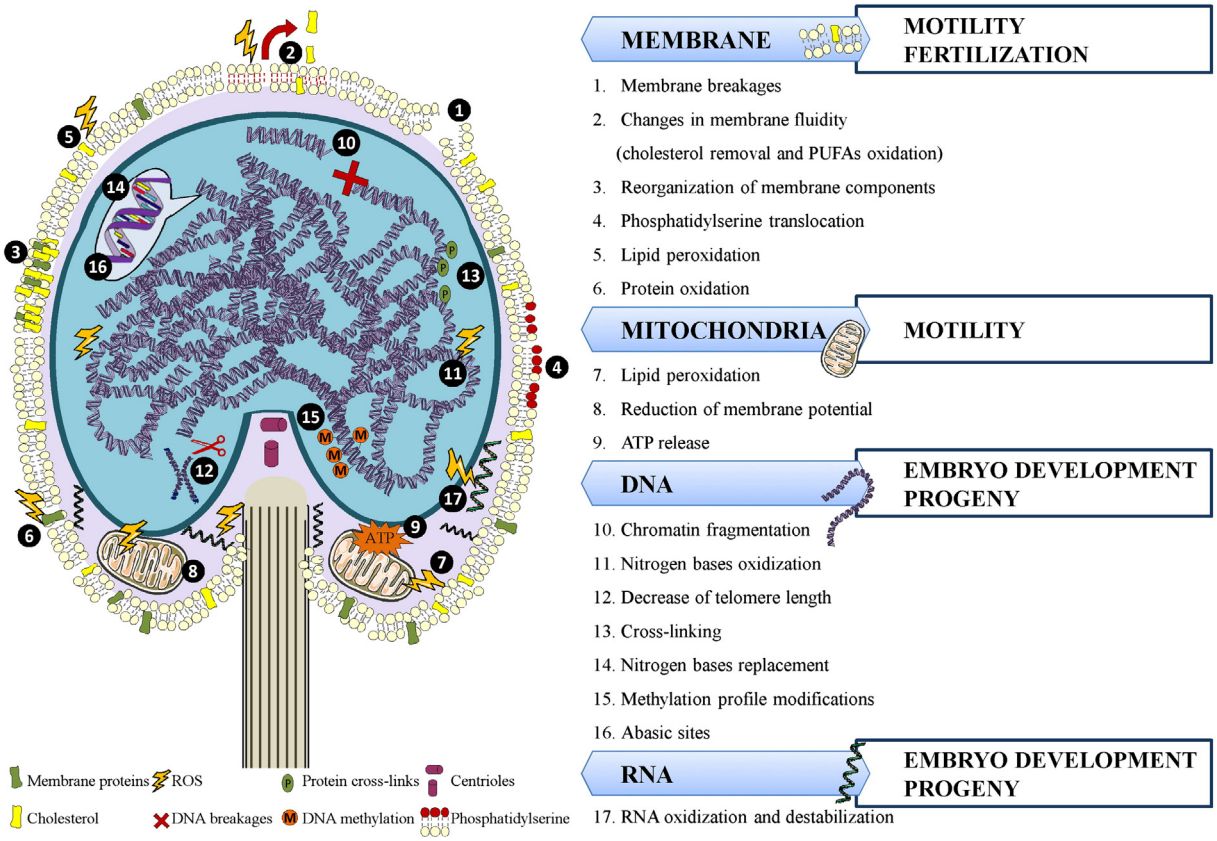

Fig. 2. Fish spermatozoa illustration showing the principal types of damage occurring in plasma membrane, mitochondria, DNA and RNA that could be used as sperm quality markers. These markers can identify changes in sperm motility, fertilization ability, embryonic development and progeny fitness.

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In European seabass post-thawed sperm, it was observed that there is a significant increase in the activity of the enzymatic antioxidant system, probably to counteract the destabilization of the antioxidant barrier after dilution in the extender (Martínez-Páramo et al., 2012b, 2013a). This reaction seemed to be enough to protect lipids and proteins from oxidative damage, though DNA remained exposed to ROS attack showing levels of chromatin fragmentation around 55%, which were reduced by addition of antioxidants into the freezing media (Martínez-Páramo et al., 2013a). DNA damage induced by oxidative stress, is not limited to direct effect on chromatin fragmentation, but may alter gene expression or induce epigenetic deregulation by way of posttranslational histone modifications after hypo- or hypermethylation (Chervona and Costa, 2012; Ziech et al., 2011). In recent studies, genes related with apoptosis (pro-apoptotic-*bax and bad*; anti-apoptotic-*bcl-2*, *bcl-xl*) induced by oxidative stress revealed to be good biomarkers not only in fish germ cells exposed to xenobiotics or contaminants, but also in the assay of sperm cryopreservation (Jeong et al., 2009; Nadzialek et al., 2010). These markers revealed to be good indicators of the effect of antioxidants (Zn, Se, Vit. E and C) supplementation in fish diets on germ cell quality (Cabrita et al., 2013).

Several techniques have been used to quantify oxidative damage in fish sperm. However, the evaluation of oxidative stress markers is a key question in the investigation of oxidative stress because of the impossibility to monitor the level of ROS *in vivo* in the biological processes (Lushchak, 2011). Therefore, the most common approach to evaluate oxidative stress, is quantifying the products of cellular constituents modified by ROS. We summarize some of the markers and techniques used for the evaluation of oxidative stress in fish (Table 1).

3.1.1. Quantification of ROS

The use of fluorescent probes is a very useful technique to quantify the level of ROS in the cell. There are several fluorophores that after being incorporated into the cell can be modified by oxidative reaction, emitting fluorescence. The intensity of the fluorescence emitted is supposed to be proportional to the levels of ROS, and can be quantified by several methods such as fluorescence microscopy, flow cytometry or spectrophotometry. The dihydroethidium (DHE) is a reagent used to quantify superoxide anion (Hagedorn et al., 2012). This molecule exhibits blue-fluorescence in the cytoplasm until oxidized, where it intercalates within the DNA, labeling the nucleus with red fluorescence. Similarly, the acetate ether of dichlorofluorescein penetrates inside the cell where it is cleaved by esterases to dichlorofluorescein (DCF) that is easily oxidized to the green fluorescent form (Pérez-Cerezales et al., 2010a,b). These probes were used in zebrafish (Hagedorn et al., 2012) and rainbow trout (Pérez-Cerezales et al., 2010a,b) to compare the levels of ROS in fresh and cryopreserved sperm, showing a significant increment of these reactive species in post-thaw samples.

3.1.2. Total antioxidant status

The analyses of the antioxidant status in the sperm samples must include the analyses of low and high molecular mass antioxidants. Among the low molecular mass antioxidants are metabolites such as tocopherol, ascorbic acid, uric acid, reduced glutathione, selenium, and zinc, among others. The analyses of the high molecular mass antioxidants include the enzymatic activity of GPX, GSR, SOD and catalase. There are commercial kits that provide an overall indication of antioxidant status, including metabolites and antioxidant enzymes but they can also be measured by routine enzymatic assays (Lahnsteiner and Mansour, 2010; Li et al., 2010a,b; Mansour et al., 2006; Shaliutina-Kolesova et al., 2013). This methodology has been used to characterize the level of total antioxidants in seminal and blood plasma of European seabass and gilthead seabream (Martínez-Páramo et al., 2013b) and in Atlantic cod (*Gadus morhua*), where it was demonstrated that the antioxidant capacity was positively related to motility and velocity of frozen-thawed sperm (Butts et al., 2011). Markers of the sperm antioxidant system were also determined in burbot, perch, bleak, brown trout (Lahnsteiner and Mansour, 2010), common carp (*Cyprinus carpio*) (Li et al., 2010a,b), Arctic char (*Salvelinus alpinus*) (Mansour et al., 2006), brook trout (*Salvelinus fontinalis*), Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon

Table 1
Techniques used for the analysis of sperm quality in fish species.

Assay	Structure	Reference
Cell viability		
Probes: Hoechst, PI/SYBR-14, acridine orange, DAPI, eosine, trypan blue, YO-PROl	Plasma membrane	Cabrita et al. (2009)
Lytic enzymes: acid phosphatase, alkaline phosphatase (LD-glucuronidase	Sperm	Lahnsteiner et al. (1998)
Metabolic enzymes: malate dehydrogenase, lactate dehydrogenase, aspartate aminotransferase, adenosine triphosphatase	Seminal plasma	Lahnsteiner et al. (1998)
HOS-test		
Hyper or hyperosmotic test	Plasma membrane	Cabrita et al. (2009)
Spermatozoa motility		
CASA, analysis of images captured with stroboscopic light, subjective scoring	Sperm	Rurangwa et al. (2004); Cosson et al. (2008)
ATP metabolism		
ATP and ADP levels, creatine phosphate, ATPase, adenylate kinase	Sperm	Lahnsteiner et al. (1998)
Mitochondria functionality		
Probes: JC1, Rhodamine 123; Mitotracker	Mitochondria	Cabrita et al. (2005a,b); Liu et al. (2007)
MTT assay	Mitochondria	Aziz (2006)
Spermatozoa morphology		
Electron microscopy, ASMA	Spermatozoa	Marco-Jiménez et al. (2006)
Oxidative stress		
Anti-oxidative enzymes: catalase, glutathione peroxidase, superoxide dismutase	Seminal plasma	Martínez-Páramo et al. (2012a,b)
Anti-oxidative potential	Seminal plasma	Martínez-Páramo et al. (2013a,b)
Lipid peroxidation		
Free radicals: dihydroethidium-DHE assay, dichlorofluorescein-DCF assay)	Seminal plasma	Pérez-Cerezales et al. (2010a,b); Hagedorn et al. (2012)
MDA determination (TBARS assay), 8-isoprostane level	Spermatozoa	Martínez-Páramo et al. (2012a,b); Khosrowbeygi and Zarghami (2008)
Fluorescent dyes: Bodipy-Cll	Spermatozoa	Hagedorn et al. (2012)
Protein oxidation		
Carbonyl group (DNPH-2,4-dinitrophenilhidrazine reaction)	Spermatozoa	Martínez-Páramo et al. (2012a,b)
DNA fragmentation and oxidation		
Comet assay (with or without FISH or endonucleases)	Chromatine	Cabrita et al.(2009); Pérez-Cerezales et al. (2009)
TUNEL assay	Chromatine	Cabrita et al. (2011a,b)
SCSA	Chromatine	Evenson et al. (1999)
OxiDNA assay (8-oxoguanine assay)	Chromatine	Cambi et al. (2013)
Fertility assay	Sperm	Cabrita et al. (2009)

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(*Acipenser baerii*) and sterlet (*Acipenser ruthenus*) (Shaliutina-Kolesova et al., 2013).

Besides these techniques, new techniques characterizing the antioxidant system are being developed in mammals. In humans, it has been demonstrated that *gpx1* and *gpx4* are differentially expressed in different donors, suggesting a role of these enzymes in male infertility (Garrido et al., 2004). Moreover, low expression of the *gpx* family in sperm has been related with asymmetric divisions in embryos (Meseguer et al., 2006). Paralogs of mammalian *gpx1* and *gpx4* were found to be present in teleosts such as zebrafish (Thisse et al., 2003), common carp (Hermesz and Ferencz, 2009), Southern bluefin tuna (*Thunnus maccoyii*) (Thompson et al., 2010), goldfish (*Carassius auratus*) (Choi et al., 2007), amberjack (*Seriola lalandi*) (Bain and Schuller, 2012) and olive flounder (*Paralichthys olivaceus*) (Choi et al., 2008). These markers of oxidative stress were used in brown trout (Hansen et al., 2006) and Atlantic salmon (Olsvik et al., 2005) exposed to different environmental conditions or to pollutants in the water. In fish, studies related to the expression of antioxidant enzyme genes have been focused in the detection of these enzymes in different tissues (i.e. gill, intestine and liver) and not specifically in sperm. However, considering the previous results obtained in mammals, the correlation of the mRNA expression of the antioxidant enzymes in sperm could be promising to understand the molecular causes of fertilization failure related to oxidative stress damage.

3.1.3. Lipid peroxidation

Lipid peroxidation is a marker currently used to evaluate oxidative stress in fish sperm because of the high amount of polyunsaturated fatty acids that constitute the spermatozoa membrane (Li et al., 2010a, b; Mansour et al., 2006; Shaliutina et al., 2013a). Lipid peroxidation is usually measured by the quantification of a final product of the oxidation of lipids, the malondialdehyde (MDA). The TBARS assay is the technique usually employed to evaluate lipid peroxidation, by quantification of the malondialdehyde present in the sample that reacts with the thiobarbituric acid used as reagent and with the final product being measured using a spectrophotometer. Mansour et al. (2006) used this technique to quantify lipid peroxidation in Arctic char sperm demonstrating a reduction of this parameter in those fish fed an antioxidant supplemented diet. Other authors, using the same technique in gilthead seabream, determined that lipid peroxidation was associated with the impairment of the fertilizing capability of sperm after acute exposure to surfactants (Rosety et al., 2007). Other works demonstrated that sperm preservation processes (cryopreservation or short-term storage) induce higher levels of lipid peroxidation in common carp (Li et al., 2010a,b), Russian and Siberian sturgeons (Shaliutina et al., 2013a). This technique was also used to determine lipid peroxidation in brook trout and sterlet sperm (Shaliutina-Kolesova et al., 2013).

The TBARS reaction is relatively nonspecific because thiobarbituric acid, besides MDA, also reacts with many types of compounds, such as aldehydes, amino acids, and carbohydrates. Thus, new commercial kits have been designed to minimize interference from other lipid peroxidation products. This kind of kits (Oxis BIOXYTECH MDA-586) has been used in European seabass, showing that sperm lipid peroxidation is correlated with differences in sperm quality during reproductive season and could be used as a biomarker of peroxidation in this species (Martínez-Páramo et al., 2012a).

The lipophilic probe BODIPY® can be also used to quantify lipid peroxidation. This fluorescent probe is incorporated into the cell membranes exhibiting a change in the spectral emission, shifting from red to green after interaction with peroxyl radicals. This technique usually used in mammals (Aitken et al., 2007; Ortega-Ferrusola et al., 2009) has been recently applied in zebrafish sperm by Hagedorn et al. (2012).

3.1.4. Protein oxidation

Similarly to the analyses of lipid peroxidation, oxidization of proteins is measured by quantification of carbonyl groups, that can be spectrophotometrically quantified through DNPH (2,4-dinitrophenilhidrazine) reaction at 360 nm (Levine et al., 1990).

This method has been used to quantify protein oxidization in several fish species, such as European seabass (Martínez-Páramo et al., 2012b), Russian and Siberian sturgeons (Shaliutina et al., 2013a), sterlet, common carp and brook trout (Shaliutina-Kolesova et al., 2013). In Russian and Siberian sturgeons and common carp, similarly to the result obtained from the lipid peroxidation analyses, the level of protein oxidation increased in the preserved samples (Li et al., 2010a,b; Shaliutina et al., 2013a).

3.1.5. DNA damage

According to Aitken et al. (2012), oxidative stress is one of the main mechanisms responsible for DNA strand fragmentation. In the DNA, the nitrogen bases, particularly guanine, are the main targets of ROS attack, generating 8-hydroxy, 2′-deoxyguanosine (8OHdG). This reaction weakens the bond between the guanine and the adjacent ribose unit, leading to loss of the oxidized base, destabilizing the DNA structure, and resulting in localized strand breakages. Several methods have been used to quantify the effect of ROS on DNA integrity. For instance, the presence of 8 OHdG can be quantified by flow cytometry using assays incorporating labeled avidin, which binds to 8OHdG with great affinity. However, the most widely method used to quantify the percentage of fragmented chromatin in fish sperm is the comet assay (Cabrita et al., 2005b; Martínez-Páramo et al., 2009; Shaliutina et al., 2013a; Zilli et al., 2003). This technique will be explained in detail in the next section.

3.1.6. Mitochondria dysfunction

In the majority of eukaryotic cell types, mitochondrial energy metabolism is the most important source of ROS (Kowaltowski et al., 2009). The complex redox mechanisms occurring in mitochondrial microenvironment are able to control ROS production at the levels required for the normal functionality of this organelle. However, oxidative stress situations leading to damage in the mitochondrial membrane can impair the mitochondrial respiratory efficiency promoting the release of ROS, and creating a vicious circle in which mitochondria might be the generator and the victim of the oxidative damage (Ferramosca et al., 2013). In human sperm, it was reported that oxidative stress negatively affects sperm mitochondrial respiration by an uncoupling between electron transport and ATP synthesis, and as a result it was suggested that this reduced mitochondrial respiratory efficiency decreased the progressive motility of spermatozoa (Ferramosca et al., 2013).

There are several dyes that could be used to evaluate the mitochondria membrane potential and functionality. Through the use of rhodamine 123 in red seabream (*Pagrus major*) and in rainbow trout and catfish it was showed that the percentage of spermatozoa with functional mitochondria was significantly reduced after cryopreservation (Liu et al., 2007; Ogier de Baulny et al., 1997, 1999). In striped bass (*Morone saxatilis*) and gilthead seabream, the JC-1dye was successfully used to evaluate the mitochondrial transmembrane potential of sperm incubated at different osmolarities (Guthrie et al., 2008) or exposed to different dilution ratios in the extender media during cryopreservation (Cabrita et al., 2005a).

3.2. Genome analysis as marker of sperm quality

Traditionally the chromatin damage has been poorly considered in the assessment of sperm quality. Chromatin integrity has been clearly related with the success of fertilization in mammals. Evenson et al. (1999) established the correlation between chromatin fragmentation index in a sperm sample and the ability to fertilize oocytes. Most of the factors potentially damaging for chromatin are also aggressive for other cell structures related to fertilization, resulting in a positive selection of spermatozoa carrying undamaged chromatin by the exclusion of damaged cells, unable to reach or penetrate the egg (Hourcade et al.,

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2010). Nevertheless chromatin modifications could happen in the absence of measurable effects on other sperm characteristics, allowing sperm with damaged DNA to fertilize the oocytes. In rainbow trout, Pérez-Cerezales et al. (2010b) demonstrated that spermatozoa carrying at least 10% of fragmented DNA fertilize the egg and produced larvae. It is now admitted that sperm chromatin damage has higher effects on early embryo development than on the process of fertilization (Speyer et al., 2010), given its role in the control of gene expression from the first steps of embryo development (Carrell and Hammoud, 2010; Delbés et al., 2010; Ward, 2010). Different authors described the consequences of the use of bad quality sperm with low chromatin integrity on the offspring outcome both at embryonic and at later larval stages (Speyer et al., 2010; Zini, 2011). In rainbow trout the increase of abortion rates at different developmental stages promoted by fertilization with DNA-damaged sperm was reported (Pérez-Cerezales et al., 2010b) and a differential expression of 8 genes related to growth and development in the obtained larvae was later identified (Pérez-Cerezales et al., 2011). The importance of sperm chromatin integrity goes beyond the fertilization ability, contributing to the overall progeny development with a wider role than the simple transmission of their genome.

Many different factors could affect fish sperm chromatin stability, from exposure to toxicants or radiations (Dietrich et al., 2005, 2007, 2010), to aging, cold storage (Pérez-Cerezales et al., 2009), cryopreservation (Cabrita et al., 2005b; Labbé et al., 2001; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2009, 2010a; Zilli et al., 2003), or changes in thermal regime during spermiogenesis, etc. The reported results showed a different susceptibility to damage between species that could be related to differences in the chromatin structure. Chromatin compactation follows different patterns during fish spermiogenesis, being the sperm nuclear basic protein (SNBP) composition very heterogeneous. In some species like the gilthead seabream, histones are not substituted by protamines and the DNA remains associated to histone-like proteins in mature spermatozoa (Kurtz et al., 2009), whereas other species, such as chum salmon, have fully replaced somatic histones by protamines (Frehlick et al., 2006). As an example seabream DNA seems to be less cryosensitive than that from brown trout and this one more cryoresistant than that from rainbow trout (Cabrita et al., 2005b; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2009).

3.2.1. Methods to assess chromatin integrity

Different methods can be applied to the evaluation of chromatin damage, most of them related to the detection of fragments or packaging failures, whose main characteristics are summarized in Table 2. The comet assay or SCGE (single cell gel electrophoresis) is the technique most commonly used to analyze chromatin fragmentation in fish (Cabrita et al., 2005a,b; Devaux et al., 2011; Dietrich et al., 2007, 2010; Labbé et al., 2001; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2010a,b, 2011; Zilli et al., 2003) and is based on the different electrophoretic migration patterns of DNA fragments according to the size of the strand. Cells immobilized over an agar covered slide are lysed and submitted to electrophoresis, promoting the migration of the small pieces of fragmented DNA. After staining DNA can be observed forming a comet-like tail structure in DNA fragmented cells, preceding the non-fragmented DNA (head of the comet). Specific software allows the analysis of this "comets" for each single cell and thus results can be expressed as mean values for all the analyzed cells in a given sample, or as percentage of cells per range of DNA damage (Beirão et al., 2008; Martínez-Páramo et al., 2009; Pérez-Cerezales et al., 2010b). One of the advantages of this method relies on its versatility since modification of the pH lysis solution allows the differential identification of single or double strand breaks. Detection of lesions other than fragmentation is also possible by combining this technique with a previous digestion with specific endonucleases that will cut the strand in modified bases. In trout sperm digestion with ENDIII or FPG has been applied for the quantification of oxidized cytosines and guanosines, respectively, in non-fragmented positions of the DNA strand (Pérez-Cerezales et al., 2009). Another technique based on the differential migration of chromatin fragments is the sperm chromatin dispersion test (SCD). This method requires species-specific protocols for chromatin decondensation that should be set up for fish, considering the specific differences in the chromatin packaging pattern. This method was used in the evaluation of tench (*Tinta tinca*) sperm reporting a good correlation with the results obtained using the comet assay (López-Fernández et al., 2009).

Fragmentation can also be assessed more specifically using the TUNEL assay (terminal deoxynucleotidyl transferase mediated dUTP-biotin end-labeling), based on the addition of a fluorescent labeled nucleotide to the 3′OH end of the strand. Fragmentation increases the presence of free 3′OH ends, thus the more fragmented the DNA, the higher the fluorescence emitted by the nucleus. Analysis of the results can be made by fluorescence microscopy, but flow cytometry is also compatible with this method, increasing the accuracy with the evaluation of a high number of cells per sample. TUNEL assay is commonly used for the detection of apoptotic cells, but it will effectively reveal DNA fragmentation, being caused by apoptosis or by any other process. In fish sperm TUNEL was used to identify differences in several freezing protocols in gilthead seabream and European seabass sperm (Cabrita et al., 2011a,b). Among the methods compatible with flow cytometry, the sperm chromatin structure assay (SCSA®) developed by Evenson et al. (1980), is one of the most commonly used in the evaluation of human sperm chromatin status, with different commercial kits available for an easy and fast application. It is a simple method based on the metachromatic shift of acridine orange (AO) fluorescence from green, when intercalated into native double-stranded DNA, to red when stacked on single-stranded DNA. This method measures the susceptibility of sperm DNA to acid-induced *in situ* denaturation. Cells preserving DNA integrity emitted in green after treatment, whereas cells with a compromised chromatin emitted red fluorescence. An alternative methacromatic probe is chromomicine A3 (CMA3). Their advantages are the simplicity and the possibility to analyze results by flow cytometry, but there are no reports on their use for sperm fish evaluation, probably because setting up this method to fish also requires facing the different patterns of DNA/proteins packaging between species.

Immunofluorescence methods allow the detection of specific lesions in the DNA strand. Antibodies have been developed against cyclobutane-pyrimidine dimers (CPDs) or 6–4 photoproducts (6-4PPs), typically induced by UV irradiation (Rastogi et al., 2010) used in aquaculture to inactivate the sperm genome in gynogenetic processes. Commercial kits are also available to detect 8-hydroxy,2-deoxyguanosine (8-OHdG), the hallmark of oxidative DNA damage, and are starting to be used for the evaluation of oxidative damage in sperm (Cambi et al., 2013). These methods, still not assayed in fish, allow the evaluation by microscopy or flow cytometry and provide more specific information about the kind of damage present in the DNA.

3.2.2. Analysis of damage in specific regions or genes

All methods previously discussed allow the assessment of the global status of the nuclear genome being good markers of chromatin status. However, some chromosome regions have a differential susceptibility to damage, considering their position on the chromosome, linkage to the nuclear proteins, or other more specific factors. Moreover, some specific paternal genes could have a relevant role in the control of early embryo development (Carrell and Hammoud, 2010; Ward, 2010; Wu et al., 2011). In the sperm of mammals these particular genes seem to be located in chromatin regions more loosely packaged than the rest of the chromatin whereas in zebrafish display a lower DNA methylation degree, as well as specific methylation pattern in their associated histones (Wu et al., 2011). The different epigenetic pattern could render them more susceptible to be injured than other chromatin regions. Moreover, damage in these particular genes could have special relevance, considering that proper embryogenesis relies on their correct expression and that any damage should be immediately repaired after fertilization. Therefore the consideration of these group-

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Table 2
Advantages and disadvantages of different methods used for sperm DNA analysis.

Technique	Analysis	Information provided	Technical requirement	Equipment	Reports in fish	Advantages	Disadvantages
Comet assay	Cell by cell	Overall fragmentation (single and double strand breaks), indirect measurements of specific lesions	Medium	Basic but specific software recommended	Abundant	Cheap and versatile	Time consuming
SCD	Cell by cell	Overall fragmentation	Low	Basic	Scarce	Easy to perform	Species-adaptation required
TUNEL	Cell by cell/average	Fragmentation	High	High	Scarce	Accuracy	Expensive
SCSA CMA3	Average	Chromatin packaging/integrity	Medium	High	None	Simplicity	Species-adaptation required
Immunofluorescence	Cell by cell	Specific lesions in the genome (i.e. presence of 8OHdG)	High	High	None	Accurate identification of lesions	Still not assayed in fish Expensive
qPCR	Average	Total number of lesions in specific regions/genes	High	High	Scarce	Accuracy and analysis of key genes	Expensive, long genomic annotations required

set of genes as markers of sperm quality and early embryogenesis development success could represent a step forward in the assessment of sperm quality.

Telomeres, highly conserved guanine-rich tandem DNA repeats of the chromosomal end, provide chromosomal stability (Agarwal et al., 2010; Meyne et al., 1989; Ocalewicz et al., 2004) and could be regions more prone to suffer damage. Thus, telomere length can be used as a parameter of sperm quality. This tool has been recently applied in fish, showing a decrease in telomere length of rainbow trout spermatozoa after cryopreservation (Pérez-Cerezales et al., 2011). This study revealed that DNA fragmentation promoted by sperm cryopreservation disturbs the telomere region, as it was also reported by some studies on human sperm (Fernandez-Gonzalez et al., 2004). In addition, there were evident changes in telomere length in the larvae obtained with these sperm samples. Telomere length analysis was used to analyze cryodamage in gilthead seabream sperm by Cartón-García et al. (2013) who demonstrated the stability of telomeres from this species under the tested conditions.

The idea of studying DNA damage not in large regions, but in concrete genes or sequences has arrived to the aquaculture field from previous studies performed in other species, mainly in mammals. The application of fluorescence *in situ* hybridization (FISH), using probes for target genes or regions in combination with Comet assay has been used for this purpose in somatic cells. Hybridization with a specific DNA probe of the cells spread over a slide show the position of the target sequence in the tail (fragmented) or the head (unfragmented) of the comet. To our knowledge this technique was never used in fish sperm, although its application in the study of DNA repair mechanisms and genotoxicity in other species has been very useful (Jha, 2008).

More accurate procedures based on quantitative polymerase chain reaction (qPCR) have arisen in the last decade. These methods can be applied to the evaluation of multiple genes or sequences giving a global assessment of sperm DNA damage (Ayala-Torres et al., 2000; San Gabriel et al., 2006; Santos et al., 2006). The analyses are based on the capacity of certain DNA lesions (abasic sites, cross-linking, double lesions, modification of nitrogen bases, strand breakages, DNA fragmentation), to reduce and block polymerase progression in template DNA, which finally results in a reduction of the template amplification and threshold cycle (Ct) delay (Rothfuss et al., 2010; Sikorsky et al., 2004). Nuclear and mitochondrial evaluation of specific DNA damage has been reported independently with success (Santos et al., 2006). Rothfuss et al. (2010) developed a rapid and quantitative method to evaluate the relative levels of damage in mitochondrial DNA by using a semi-long qPCR amplification of mitochondrial DNA fragments of different lengths. The formula proposed by this group is based the correlation of the Ct obtained from two different amplicons (a small and a large one). This analysis provides specific damage as number of lesions per 10 kb in a concrete gene/region. These novel approaches have been applied to aquaculture species by our group (Cartón-García et al., 2013) for the first time. We studied the number of lesions produced by different cryopreservation protocols in specific genes of gilthead seabream spermatozoa. Two nuclear genes with important roles in embryo development (*Igf1* and *Gh*) and two mitochondrial genes (*Cytb* and *CoI*) were studied and DNA fragmentation was analyzed by the comet assay. The number of lesions/10 kb registered in samples that showed very low fragmentation rate with the comet assay was always higher on the mitochondrial genes than on those nuclear ones related to embryo development. More recent studies with trout sperm clearly revealed a differential sensitivity to damage of nuclear genes which is dependent on the source of damage. Comparison between 7 early transcribed genes related to embryo development and 2 late transcribed ones non-required for early embryo development, revealed important differences in the susceptibility to ROS between both groups, being the late transcribed genes more resistant to oxidative damage. Nevertheless, susceptibility to UV irradiation was similar between them (González-Rojo et al., 2013). Studying gene-specific damage can be used as an excellent complement approach for traditional techniques and could help to identify particular genes whose integrity could be used as a biomarker of sperm quality. Nevertheless, this accurate and sensitive qPCR method is expensive and time consuming requiring highly-skilled technologies. Application to most of the commercial species could be hindered by the lack of gene sequence annotations available.

3.3. Transcripts as predictors of sperm quality

It is well known that spermatozoa are transcriptionally inactive cells (Lalancette et al., 2008) and during decades it was assumed that spermatozoa RNA population was non-functional because these molecules are remnant from spermatogenesis. However, nowadays it is totally accepted that spermatozoa provide more than the paternal genome into the oocyte and the residual mRNAs from spermatogenesis can have key roles in early embryonic development and success of fertilization, in humans and other mammalian species (Garcia-Herrero et al., 2011; Johnson et al., 2011; Lalancette et al., 2008; Ostermeier et al., 2002). In studies made in bull (*Bos taurus*), spermatozoa transcriptome profiling has been presented as a potential tool to evaluate semen quality (Bissonnette et al., 2009), and in humans a differential transcriptomic profile has been found in spermatozoa capable of producing pregnancy (Garcia-Herrero et al., 2011). To date, the use of microarrays in aquaculture has not focused on the evaluation of the RNA profile for predicting gamete quality. Most of the transcriptomic studies performed in fish in the field of reproduction have been descriptive and fall within one of these three categories: (1) spermatogenesis and testicular development and (2) evaluation of hormones, drugs or contaminants on these processes, and more recently (3) the study of sperm quality markers.

Within the first group, we can mention some studies carried in rainbow trout where more than 3000 differentially expressed genes were grouped according to their expression profiles in developing testes and isolated germ cells (Schulz et al., 2010). Taking into account that the transcripts in the spermatozoa are relevant for fertilization and early development and are remnants from spermatogenesis, the

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establishment of these gene expression clusters of male gonad development in the rainbow trout could be useful as a tool to potentially predict sperm quality. With this in mind, and assuming spermatozoa quality markers not only as end-points in assays, it is important to consider that molecular markers could reflect the mechanisms through which "sperm quality" is imprinted during spermatogenesis (Fig. 1). In this direction Gardner et al. (2012) described the expression profiles of certain genes in mature gonadal tissues of Atlantic blue fin tuna (*Thunnus thynnus*). Expressed sequence tags (ESTs) potentially related to components significant to spermatogenesis were included such as meiosis, sperm motility and lipid metabolism. Microarray technology has also allowed the discovery of novel markers. Yano et al. (2009) identified *notch1* as a molecular marker for type A spermatogonia by microarray analysis in transgenic rainbow trout. In other studies in the same species, next-generation sequencing was combined with microarray for transcriptome analysis, confirming the expression of known spermatogonial markers to be higher in spermatogonia A than in testicular somatic cells (Hayashi et al., 2012). In this way, these results were extremely useful to understand gametogenesis in freshwater species, opening way for further more detailed characterization of specific transcripts present at key developmental stages of spermatogenesis.

Within the second group, different studies explore the effects of hormones like progestin, androgens and estrogens on testicular gene expression by microarray analysis. It has been found that Sertoli cells change the expression profile of growth factors in the presence of steroids, and that these changes in expression subsequently modulate germ cell proliferation and differentiation (Schulz et al., 2010). In zebrafish, the exposure to 17α-ethinyloestradiol compromised the reproductive health of breeding individuals and, by transcriptomic analysis of gonads, it was confirmed that the expression of 114 genes was altered after the exposure to this molecule. The most affected genes were those related to the regulation of cell cycle progression, the ubiquitin system and glutathione peroxidase were affected by the treatment and associated with the decrease in gamete quality in both genders (Santos et al., 2007). The ubiquitin system has been implicated in the degradation of paternal mitochondria at fertilization, and in degradation of poor quality sperm (Sutovsky, 2003).

However, all these transcriptomic studies are not focused on the use of microarrays as a direct tool to predict sperm quality in fish. Recently, our group has performed a different approach to investigate on the role of mRNAs as quality markers in fish spermatozoa. Using a qPCR approach, we have recently defined a set of transcripts which have a different profile in testicular cells from good and bad zebrafish breeders (Guerra et al., 2013). The transcripts identified as predictors of good breeding performance were *bdnf*, *lhcgr*, *lepa*, *bik*, *dmrt1*, *fshb* and *hsd17b4*, whereas *bik* and *hsd17b4* were more abundant in bad zebrafish breeders. Although these results were obtained with a model species, we were interested on exploring the possibility of transferring these findings to relevant teleost species from a commercial point of view. Spermatozoa transcripts from *S. aurata* were analyzed in males with either good or bad sperm motility, which has been described as one of the most reliable parameters for predicting fertility in some species (Cosson et al., 2008). Males with low sperm motility registered low levels of *bdnf*, *bik* and *kita* comparing to the control group males with high sperm motility. However, changes in sperm transcripts and/or transcriptome profile cannot be only understood as a way to predict fertilization ability of males, it can also be considered as an emerging tool to evaluate sperm analysis after a process such as cryopreservation. As it was mentioned before, cryopreservation can produce several types of damage to the cells, affecting sperm functionality at later stages. Some studies have reported the decrease or even the elimination of some transcripts after cryopreservation (Garcia-Herrero et al., 2011). Our group reported the absence of certain transcripts after human sperm cryopreservation (Riesco and Robles, 2013; Valcarce et al., 2013a,b) and we speculate that this fact could be due to a change caused by cryopreservation in the interaction protein–mRNA that can make the mRNA more susceptible to degradation, although until now difficult to prove in fish sperm. Contrary to these observations, we have reported a different tendency after *S. aurata* sperm cryopreservation. Although cryopreservation has been reported to decrease transcript levels in spermatozoa from other species, the protocol employed for gilthead seabream sperm cryopreservation did not affect mRNA levels. This is a relevant observation since it is known that changes in spermatozoa transcripts could have a serious effect on fertilization or even on the offspring. Therefore, transcripts level analysis in the spermatozoa could also be understood as a tool to select the most efficient and safe cryopreservation protocol. Moreover, it is known that fertilization with cryopreserved spermatozoa with DNA-damage alters gene expression in the surviving embryo and larva (Pérez-Cerezales et al., 2011), indicating that transcriptome analysis in the progeny could be reflecting sperm quality.

Reproductive aquaculture will undoubtedly obtain higher benefits from transcriptomics in the future, although the lack of genetic tools and annotated sequences in many fish species makes that in many cases microarrays and specific probes can only be designed using limited information from expressed sequence tags (ESTs) or heterologous-species (Yano et al., 2009). Nevertheless the great potential of these technologies in other species, together with the preliminary results that have been reported in fish, let us envision a great applicability of these techniques in the field of aquaculture in the near future.

3.4. Proteome markers

Proteomic analysis of sperm cells in several species including fish has provided valuable information about the proteins involved in sperm physiology and function, in the components of the sperm head, tail and nucleus and in seminal plasma composition (Ciereszko et al., 2012). Specific proteins with key roles on a variety of processes linked to reproductive performance and gamete quality have been reported. Most of the studies made with fish have been designed on species with commercial interest due to de need of improving broodstock management procedures, sperm handling techniques such as cryopreservation or breeding strategies. Proteomic analysis revealed to be a good indicator of cryopreservation success and an indicator of specific cryodamage. In common carp, Li et al. (2010b) found 14 spots corresponding to proteins that were significantly altered after the cryopreservation process. Some of those proteins were identified as being involved in cell metabolism, oxidoreductase activity and signal transduction, membrane trafficking, organization and cell movement. Other authors working in marine species showed, using two dimensional electrophoresis coupled with MALDI-TOF (matrix-associated laser desorption/ionization time-of-flight) analysis, that the use of freezing–thawing procedures caused degradation of 21 sperm proteins in European seabass and few others in gilthead seabream (Zilli et al., 2005, 2008). In gilthead seabream the authors reported a strong effect on the phosphorylation state of proteins responsible for motility activation. Therefore it seems clear that according to the species, some proteins could be more affected than others and that choosing some specific proteins as markers could be interesting not only to study the mechanisms involved in cryodamage but to identify protocols with better success. Defects in sperm proteins may compromise sperm motility, fertilization ability, and the early events after fertilization (Zilli et al., 2005).

Other use of proteomics as a tool for the study of potential markers came from studies on broodstock quality, stripping frequency and changes in quality during the reproductive season. The proteomic profiling of *S. senegalensis* males during spermatogenesis allowed identifying some markers relevant for the different reproductive changes found between F1 and wild males (Forne et al., 2009). In this particular study, the authors identified 49 proteins differentially expressed during the progression of spermatogenesis, sperm maturation and motility, or cytoskeletal remodeling, pointing towards possible causes of differences

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in sperm quality, found previously by other authors between the two types of broodstocks (Cabrita et al., 2006).

Proteome markers were also found in seminal plasma of perch and sterlet related with sperm collection during the reproductive season and consecutive strippings (Shaliutina et al., 2012, 2013b). These authors found specific proteins that were changed in seminal plasma during the course of the reproductive season that could be associated with changes in other sperm traits. In sterlet, some of these proteins were associated with intracellular mechanisms responsible for regulating spermatozoa motility.

Proteomic studies would greatly increase the chance of identifying new biomarkers of male fertility. Such knowledge can contribute significantly to the enhancement of control in reproduction under aquaculture conditions, especially the efficacy of fertilization procedures and semen storage, including cryopreservation (Ciereszko et al., 2012).

4. Future perspective applications to industry: how far are we?

It seems now clear that new biomarkers of sperm quality are needed for a correct evaluation of sperm traits opening new perspectives in research. In addition to this, the commercial availability of those markers seems to be the additional step to follow with potential benefits to the biotechnological companies offering services to the aquaculture industry. Fish sperm selection programs based on certain molecular characteristics could be realistic in a not so far future. Research on biomarkers can allow the design of specific probes for the genes responsible for sperm quality, fertilization success and early indicators of embryonic development. Therefore, once we are able to determine the ideal molecular profile of sperm, selection techniques that enable the enrichment of a sperm sample with spermatozoa with the molecular characteristics considered adequate could increase the reproductive success and give more guarantees of a high quality progeny.

Recently, magnetic-activated cell sorting (MACS) emerged as an alternative technique for sperm selection based on the use of microscopic microbeads that are conjugated to proteins or antibodies to tag cells of interest. Once tagged, these cells are retained in columns and passed through a high gradient magnetic field. Its application as a method for selecting optimal spermatozoa has been extensively described in the literature (Said et al., 2008), particularly in the selection of apoptotic sperm cells via externalization of phosphatidylserine (PS). A similar technique using only PS labeling was already successfully used in characterizing fish sperm (Beirão et al., 2008).

In the case of fish sperm, not only the selection of certain spermatozoa within a sample is important, but most of all, the selection of samples of a certain quality may be more interesting for industry. This may be achieved by using specific mRNA probes labeled with a fluorochrome by fluorescent *in situ* hybridization combined with flow cytometry will allow researchers to easily select samples within a threshold, specific for the species. The same can happen with the selection of certain proteins as biomarkers and the use of flow cytometer immunoassay. All these tests may be available with the integrated use of proteomics, microarray techniques and mass sequencing of sperm samples allowing the design of specific kits for sperm quality analysis.

Nowadays there are several services provided by biotechnological companies to assure the availability of disease-free sperm from several species, such as Atlantic and Pacific salmon, trout, cod and halibut. These companies need to certify the quality of samples not only in terms of specific pathologies, but also by using a set of markers that can rapidly be tested and that can certify sperm quality for progeny control. This will be especially relevant for samples exposed to cryopreservation and commercialized for production purposes.

In summary, several of the techniques mentioned raises the possibility of customized sperm selection in cases where male potential is interesting or where the evaluation of sperm exposed to management procedures such as cryopreservation is needed, or even their use in toxicological tests. This is a step towards a tailored quality assessment depending on the needs, but also ensuring reliable tools that can be employed by the industry to improve the efficiency of reproductive cells and produce high quality progeny.

Acknowledgements

This work was supported by projects CRYOXI-AGL2011-28810 and AGL2011-27787 from MINECO (Spain), GERMFISH (ICMAN-CSIC), by project LE365A11-2 from Junta de Castilla y León (Spain), by the Fundación Ramón Areces (Spain), and by LARVANET (FA0801) and AQUAGAMETE (FA1205) COST actions. V. Robles was supported by Ramon and Cajal program (RYC-2008-02339, MICINN, Spain). S. Martínez-Páramo was supported by FCT — BPD/48520/2008 program (Portugal); M.F. Riesco and D.G. Valcarce were supported by Junta de Castilla y León (ref. E-24-2009-0036681 and ref. EDU/346/2013).

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Please cite this article as: Cabrita, E., et al., Factors enhancing fish sperm quality and emerging tools for sperm analysis, Aquaculture (2014), http://dx.doi.org/10.1016/j.aquaculture.2014.04.034

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Please cite this article as: Cabrita, E., et al., Factors enhancing fish sperm quality and emerging tools for sperm analysis, Aquaculture (2014), http://dx.doi.org/10.1016/j.aquaculture.2014.04.034

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Please cite this article as: Cabrita, E., et al., Factors enhancing fish sperm quality and emerging tools for sperm analysis, Aquaculture (2014), http://dx.doi.org/10.1016/j.aquaculture.2014.04.034

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Please cite this article as: Cabrita, E., et al., Factors enhancing fish sperm quality and emerging tools for sperm analysis, Aquaculture (2014), http://dx.doi.org/10.1016/j.aquaculture.2014.04.034