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Dear Editors

I am submitting the revised version of the manuscript entitled “Paternal contribution to development: sperm genetic damage and repair in fish” after addressing the changes suggested by the reviewers.

All the changes required by reviewer 1 have been done. According to the reviewer 3, Figure 3 was modified to include a table of the lesions promoted in DNA by the different genotoxic agents. A Table showing the active DNA repairing pathways associated to specific lesions which have been reported in fish has also been added. All references and scientific names were checked and harmonized.

The more confusing paragraphs (about evolutionary aspects of chromatin organization and about paramutations) were rewritten.

Addressing the editorial comments we have included the Gordon Dixon references.

The review focusses on the contribution of the sperm to the development of the progeny. The current knowledge about chromatin organization, epigenetic patterning, and presence of different RNAs in the sperm is provided, highlighting their important role in sperm quality and the potential use of specific molecules/sequences as biomarkers of quality.

Recent discoveries about the effect of different agents on the stability of the sperm genome, epigenome and transcriptome are reported, with particular attention to environmental factors and factors related to reproductive management in fish farming.

Moreover we summarize the reports linking the changes in some of these sperm components to the ability to fertilize and to progress with the development of the progeny, emphasizing the importance of using high quality sperm to fertilize and the ability of the zygote to repair some of the damages.

*Statement of Relevance

Role of the sperm on achieving high rates of healthy fries

Paternal contribution to development: sperm genetic damage and repair in fish

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Abstract

In this review we provide an overview of the components of the spermatozoa playing an important role in reproductive success beyond fertilization, showing the relationship between the integrity of the diverse elements and the development of a healthy offspring. The present knowledge about fish sperm chromatin organization, epigenetic modifications of DNA and histones and sperm-borne RNAs, essential in controlling embryo development, is summarized, pointing out the possibility of using specific genes or transcripts as biomarkers of sperm quality. Data about commercial species are reported when available and more detailed information about zebrafish sperm is presented.

Considering the implications that the integrity of sperm genome and epigenome has on the preservation of a proper genotype and phenotype in the progeny, the methods applied for the study of chromatin damage and for the study of transcriptome are described. Moreover we discuss some injuring agents affecting paternal information, from the presence of contaminants in the aquatic environment, to the reproductive

practices applied in fish farming. The consequences of fertilizing with damaged spermatozoa, as well as the zygotic ability to repair damage are also reviewed.

Key words

Sperm chromatin, sperm nuclear basic proteins (SNBPs), sperm epigenome, sperm transcriptome, sperm RNAs, early embryo development, sperm quality.

1. Introduction

Sperm quality is defined as the ability to fertilize the oocyte progressing with embryo development. Nevertheless most of the parameters used to evaluate sperm quality are related to the first part of the sentence: the ability to reach the oocyte, fuse the membranes and activate the oocyte metabolism. During the last years it has been clearly established that the contribution of the spermatozoa to the offspring goes beyond the delivery of the haploid genome to conform the genomic information of the zygote. During spermatogenesis chromatin is processed and packaged, contacts between DNA and nuclear matrix are reorganized, epigenetic pattern is totally remodeled and transcription is stopped, a set of RNAs remaining in the cytoplasm of the mature sperm. All these events have a specific contribution to the control of embryo development from the earlier stages. The importance of paternal contribution for the proper development of the progeny is at present one of the most challenging topics in reproduction. Nevertheless, the subject has not been reviewed in fish in spite of their particularly interesting characteristics: fish display a very diversified sperm chromatin organization which involves a variety of sperm nuclear basic proteins (SNBPs); sperm chromatin stability can be affected by a range of different factors, from the presence of genotoxicants in the aquatic environment to some farming practices; and the

mechanisms of sperm selection before fertilization are weaker than in other vertebrates, increasing the probability of fertilization with DNA damaged sperm. Moreover, fish are very good models for the study of the progeny outcome and therefore, of the basic aspects underlying the relationship between sperm genomic integrity and embryo fitness.

The study of the sperm components providing relevant paternal information has been mainly accomplished in zebrafish and is still fragmentary in commercial species. The analysis of chromatin organization has been often accomplished from an evolutionary point of view, but less information exists on epigenetic remodeling or spermatid transcriptome. Nevertheless, the available data reveal a quite similar scenario to that reported in mammalian sperm, with genes packaged in blocks of structurally diversified chromatin, cytoplasmic RNAs whose profile seems to correlate with reproductive success and epigenetic marks delimiting the timing of gene expression. Factors such as changes in the environmental conditions or the use of technologies of reproduction can promote genotoxic and epigenotoxic effects in the sperm, reducing the reproductive efficiency and affecting the genotype and phenotype of the progeny.

This review is aimed to give an overview of the discoveries in this field, encouraging further analysis of paternal contribution to the development given the relevance of this topic for both wild and farmed fish populations.

2. Structural organization of the sperm nucleus

2.1. Nuclear reorganization during spermatogenesis

In most eukaryotic cells, DNA is found to be associated with chromosomal proteins, mainly histones, forming a nucleoprotein complex called chromatin. The basic unit of chromatin organization is the nucleosome, consisting of a core particle plus a

linker DNA region. The nucleosome core consists of a histone octamer (containing two copies of the highly evolutionarily conserved core histones H2A, H2B, H3, H4) around which a segment of 146 pb wraps (Luger et al. 1997). Linker histones (the more variable H1 family) sit at the base of the nucleosome, near the DNA entry and exit, binding to the linker region of the DNA; they are involved in the compaction of the chromatin fiber through the assembly of higher order chromatin structures (Li and Reinberg 2011; Li and Reinberg 2011). This organization not only allows for compaction of the genome within the limited space provided by the nucleus, but also enables the elaborate regulation of gene expression.

An extreme case of compacted chromatin is found in the sperm cell nucleus. During spermatogenesis, a series of morphological and physiological changes occur that will transform the initial diploid spermatogonia into a streamlined, motile, haploid cell that can efficiently transfer the male genetic complement to the egg, the spermatozoon. Through mitotic and meiotic divisions, spermatogonia render spermatocytes and spermatids; these finally transform during the process of spermiogenesis, and become spermatozoa. It is during this last stage that the most conspicuous modifications are observed.

One of the hallmark characteristics of spermiogenesis is the extensive remodeling of the sperm chromatin, which represents one of the most dramatic changes in metazoan chromatin organization. During the process, chromatin becomes highly condensed which allows for an important reduction of the nuclear volume. In most cases, this involves the substitution of somatic histones for other sperm-specific proteins, called generically sperm nuclear basic proteins (SNBPs). SNBPs present an increased basicity (especially in the case of protamines; see below) that allows for a higher packaging ratio, leading to the highly electron-dense chromatin observed in the

spermatozoon. The progressive condensation of the spermiogenic chromatin should not be considered a neutral or passive process, as in many cases it participates, directly or indirectly, in the determination of the final form of the sperm nucleus (Ribes et al. 2001; Ribes et al. 2001).

In addition to packing the DNA into the limited space of the nucleus, this chromatin remodeling brings about the almost complete shut off of gene expression, the erasing of the somatic histone epigenetic component (Ausió 1995), and probably provides some protection against external damaging agents during the journey of the sperm from the male gonad all the way to the egg (Caron et al. 2005).

Fish spermatozoa show more morphologic diversity than those of other vertebrates, and their ultrastructure provides parameters for phylogenetic analysis (Jamieson 1991). Mirroring this morphological diversity of the mature sperm cell, the variability found in the SNBPs is also higher in fishes (see 2.2 below). The protein transitions occurring during spermiogenesis are similarly diverse.

An ancestral model of nuclear protein transitions during spermiogenesis has been proposed by Kurtz and colleagues (Kurtz et al. 2009). According to this model, histones are partially acetylated during early spermiogenesis, which leads to a homogeneous chromatin organized in 20 nm granules. These 20 nm granules containing acetylated H3 and H4 can be considered the most evolutionarily ancestral chromatin conformation preceding condensation in animal spermiogenesis. From this point on, in the most primitive spermiogenesis, histones are deacetylated, and sperm chromatin condenses; meanwhile, in more advanced evolutionary stages, acetylated histones are replaced by other SNBPs that will compact the chromatin. This protein exchange can be direct, or histones can be transiently replaced by transition proteins that will be in turn replaced by the final SNBP. Examples of all these types of transitions can be found in

fishes (see 2.3). An exhaustive characterization of the biochemical and molecular mechanisms involved in this transition was carried out in trout by Gordon Dixon's group (Christensen and Dixon 1982; Marushige and Dixon 1969; Marushige and Dixon 1971; Ling et al. 1969). Although direct exchange is observed in most fishes, transition proteins are found in Chondrichthyes. Thus, in the dogfish *Scyliorhinus canicula*, two new proteins appear at the beginning of nuclear elongation in spermatids: S1 and S2. They will progressively replace somatic histones and will in turn be replaced by the final SNBPs (proteins Z1, Z2, Z3 and S4; see fig. 1A) (Gusse and Chevaillier 1978). The progression in the formation of the different chromatin structures which can be observed during spermiogenesis (granules, fibers, lamellae, etc.) correlates to that of the nuclear protein transitions, and has to be considered as a well-ordered sequence of molecular structure remodeling processes (Chiva et al. 2011). In each remodeling event, a structure type can disassemble and reorganize into the next structure of the remodeling process (Harrison et al. 2005; Martens et al. 2009).

2.2. Sperm nuclear basic proteins (SNBPs)

The SNBPs of fish can be classified into three main groups: Histones (H-type), protamines (P-type), and protamine like (PL-type) (Ausió et al. 2011). The histones correspond to a group of chromosomal proteins rich in basic amino acids (10-30% lysine- 5-14% arginine) and with a very similar structural organization (in many instances identical) to that of somatic canonical core histones. Core histones consist of a typical dimerizing histone fold domain (HFD) (Arents and Moudrianakis 1995), flanked by N- and C-terminal intrinsically disordered domains usually referred to as histone tails. Protamines are a group of highly diverse arginine (and sometimes cysteine)-rich proteins (> 30% arginine) with a small size that can range from 30-100 amino acids

(Kasinsky et al. 2011). Structurally these are intrinsically disordered proteins (IDPs) (Dunker et al. 2001) that do not exhibit any structural features in solution, but can adopt highly organized structures upon interaction with DNA (Roque et al. 2011). These proteins displace and completely replace the somatic histone counterpart over the course of spermiogenesis. Protamine-like proteins are a highly heterogeneous group of chromosomal proteins with a composition rich in both lysine and arginine residues – which are all evolutionarily related to linker histones (Eirín-López and Ausió 2009). As protamines, they can replace the somatic histones, albeit to a different extent, during spermiogenesis. Structurally, these proteins often retain the characteristic winged helix fold (WHD) of linker histones (Kasinsky et al. 2001), a reminder of their evolutionary origin. Hence, from a biochemical point of view, the PL-type represents an intermediate between the H- and P-types.

Fig. 1A shows an acetic acid-urea polyacrylamide gel electrophoretic analysis of the SNBPs of several representative fish species, encompassing the three main SNBP types described above. The H-type is represented by the gilthead seabream *Sparus aurata*. Electrophoretically, these proteins exhibit an identical pattern to that of the histone composition of any eukaryotic somatic cell – but with an increasing amount of histone H1 (Ausió et al. 2014). A similar composition has been described for other well-known fishes such as the zebrafish (*Danio rerio*) (Ausió et al. 2014) and the goldfish *Carassius auratus* (Muñoz-Guerra et al. 1982), amongst others. The PL-type shown in Fig. 1A corresponds to another species of commercial interest, the red mullet *Mullus surmuletus* (Saperas et al. 2006). In this species, an arginine-rich PL-I protein containing the WHD typical of histones of the H1 family also replaces most of the somatic histones in the mature sperm (Saperas et al. 2006). The PL-type has also been described in the winter flounder (*Pseudopleuronectes americanus*), though in this case

the PL components appear to coexist with the histone complement in the sperm (Watson and Davies 1998). The most widespread SNBP type in fish, however, is – by far – that of protamines. Protamines are found in both Osteichthyes and Chondrichthyes, like sea bass (*Dicentrarchus labrax*) and salmon (*Oncorhynchus sp.*) and the dogfish *S. canicula* (Fig. 1A). In Chondrichthyes, in addition to arginine, the protamines also contain cysteine – as in the S4, Z1 and Z2 components of *S. canicula* protamines (Fig. 1A) (Kasinsky et al. 2011).

2.3. Diversity of chromatin condensation patterns in fish sperm

Regardless of the particular type of SNBP compositions described above, all fish exhibit a very similar type of structural chromatin organization at their early spermatid stages of differentiation, at the onset of spermiogenesis. Such organization is characterized by a fibro-granular arrangement, with granules of approximately 20 nm in diameter, consisting exclusively of histones (Fig. 1B, small circles). Although the exact number of nucleosomes in each of these granules is not known, it is tempting to speculate that they correspond to the tetra-nucleosome structures that represent the basic intermediate state in the folding of the chromatin fiber (Ausió 2015).

As spermiogenesis proceeds, depending on the SNBP type expressed (Fig. 1A), four main groups of chromatin compaction can be distinguished which can be epitomized by each one of the representative species shown in Fig. 1A. In the first group, represented here by *S. aurata* (H-type SNBP), the fibro-granular structures observed at the early stages of spermiogenesis coalesce together as the differentiation proceeds (Kurtz et al. 2009) (Fig. 1B-1). In the second group, represented by *M. surmuletus* (PL-type SNBP), the PL proteins displace the histones, and at the molecular level lead to the formation of highly intertwined nucleoprotein complexes (Saperas et al.

2006). This eventually leads to the formation of thicker fibrillar structures with a diameter of approximately 50 nm in more advanced spermatids (Saperas et al. 1993) (Fig. 1B-2). These coarse fibers are very reminiscent of the similar structures observed during spermiogenesis of invertebrates containing this type of SNBPs (Casas et al. 1993). A third group encompasses the fish with arginine-rich protamines (P-type SNBP), such as *D. labrax*. The replacement of histones by protamines leads, in this case, to the formation of highly condensed nucleoprotamine complexes (Balhorn 2007) that develop into the glomerular structures of 150 ± 50 nm observed in the late stages of spermiogenesis (Saperas et al. 1993) (Fig. 1B-3). An interesting example of this has been recently described in the Myctophidae fish *Lampanyctus crocodilus* (jewel lanternfish) (Ribes et al. 2015). Finally, group four is the one typically found in those Chondrichthyes species that, in addition to arginine, also contain cysteine in their protamine composition (P-type SNBP); *S. canicula* is such a one. Although the first step in the histone-to-protamine transition is similar to that of the previous group, in this case, this results in the formation of lamellar structures of approximately 15 nm of thickness, like those shown in Fig. 1B-4. The next chromatin transition in all these groups is the convergence of the chromatin structures into a highly compact electron-dense nuclear organization, a result of thermodynamically-driven processes of either nucleation and growth or spinodal decomposition (Fig.1B). These processes usually involve highly dynamic reversible transitions in the post-translational modifications (i.e. acetylation and phosphorylation) of the SNBPs involved (Kasinsky et al. 2011).

An important remaining question has to deal with the reason for the compositional and structural variability of sperm chromatin in fish. The answer is not straightforward, as a similar heterogeneous pattern of SNBP distribution is also observed in many different groups of protostome and deuterostomes (Eirín-López and

Ausió 2009). In vertebrates, only fish and amphibians retain this heterogeneity, as reptiles, birds, and mammals almost exclusively contain protamines in their sperm (Ausió et al. 2007) – and thus protamines are always present in those groups at the tips of the phylogenetic tree (Ausió et al. 2007). The sporadic presence of the three main SNBP types over the course of evolution can be better explained by an evolutionary model such as that of punctuated equilibrium (Lieberman and Eldredge 2014), in which the evolutionary molecular mechanism of death-and-birth evolution plays a very important role in the evolution of the individual SNBP types (Eirín-López and Ausió 2009).

It had been proposed that the sporadic distribution of SNBPs in fish (Figure 2) could be explained by a model of horizontal evolutionary transfer of retroviral arginine-rich encoding sequences (Jankowski et al. 1986). In other words, protamines could have appeared in fish through a repeated and independent acquisition of the protamine gene via retroviral infection. However, a subsequent more extensive analysis showed that protamine distribution in fish was not random as initially believed and that the heterogeneous presence of histones and protamines could be better explained by a repeated and independent loss of the expression of the protamine gene – or the loss of the gene itself (see Saperas et al. 1994, for a more detailed discussion).

3. Non genomic information provided by the spermatozoa

The idea that spermatozoon is more than a mere carrier of genomic information has become ingrained recently. In mammalian spermatozoa it is accepted that genome packaging has the purpose of protecting the DNA during the journey to the oocyte, but recent findings have suggested other implications for further embryogenesis, the

paternal epigenome playing a relevant role during early embryo development (Brykczynska et al. 2010; Hammoud et al. 2009; van der Heijden et al. 2006). Yamauchi et al. (2011) (Yamauchi et al. 2011) categorized at least six different kinds of epigenetic information in mammalian sperm: sperm chromatin-associated proteins, perinuclear theca proteins, DNA loop configuration by the sperm nuclear matrix, DNA methylation, histone modifications, and sperm RNAs. These recent findings reveal the complexity of paternal information delivered by the sperm, which requires a correct protamination, sufficient histone retention and a specific pattern of DNA methylation and histone modifications to achieve a successful fertilization (Gannon et al. 2014).

As reviewed by Ward (Ward 2010) the association DNA-proteins defines three major structural domains in mammalian sperm chromatin: i) the toroids in which DNA is wrapped by protamines; ii) the nucleosomal regions with about 1-10 % of the histone-bound chromatin (Brykczynska et al. 2010) and iii) the matrix attachment regions (MARs) where the DNA is attached to the sperm nuclear matrix. The special nucleosome retention is not a random process, rather is a programmatic event to retain histones at key loci for embryo development with a contribution to totipotency, developmental decisions and imprinting patterns (Brykczynska et al. 2010; Hammoud et al. 2009; Arpanahi et al. 2009). The special nuclear configuration of the mature gamete is reached thanks to ordered epigenetic changes taking place during the spermatogenic process and is considered crucial for a suitable sperm function (Gannon et al. 2014; O'Doherty and McGettigan 2014; Schagdarsurengin et al. 2012). Anomalous retention of histones has been noticed at promoters of developmental-related genes in many infertile men (Hammoud et al. 2011), reflecting the major function that this impaired situation could have in infertility. There is no data about such a regionalization in fish spermatozoa in which the different chromatin compaction models should provide

different scenarios. The absence of remnants of histones in some specific genes has been confirmed in rainbow trout sperm (*Oncorhynchus mykiss*) (González-Rojo et al. 2014) and keta salmon (*Oncorhynchus keta*) (Frehlick et al. 2006), whose chromatin is fully compacted with protamines. Other species such as zebrafish (*D. rerio*) (Wu et al. 2011) and seabream (*S. aurata*) (Kurtz et al. 2009) also show a homogeneous association with SNBPs, being in these species histones. Nevertheless, differential DNA-protein associations (including nuclear matrix proteins) could also be expected in genes related to early development, the different epigenetic patterning contributing to establish a non-homogeneous landscape.

The potential role of DNA loop organization was highlighted by Yamauchi and coworkers (Yamauchi et al. 2011) who proposed a model for the inheritance of the DNA loop domain organization of the paternal genome and who stated that DNA organization by the nuclear matrix is a key factor for a proper DNA replication in the zygote, considering the sperm nuclear matrix as a possible checkpoint for chromatin stability evaluation.

3.1. The importance of chromatin methylation

One of the common epigenetic marks in sperm chromatin is DNA methylation, which occurs at cytosine residues of the CpG dinucleotides (Portela and Esteller 2010) and is considered a powerful mark to silence genes (Carrell 2012). A correct pattern of DNA methylation is a requirement for fertility, embryo development and viability of the offspring (O'Doherty and McGettigan 2014; Jenkins and Carrell 2012b; Jenkins and Carrell 2012a). With the description of the human sperm methylome, a methylation status at 70 % of genomic CpGs has been considered as “normal” in human sperm (Molaro et al. 2011), lower to that observed in zebrafish, with 95% of methylated CpGs

(Potok et al. 2013). Sperm DNA methylation pattern is close to that observed in embryonic stem cells, showing hypomethylation at developmental promoters (Hammoud et al. 2009; Schagdarsurengin et al. 2012). After fertilization, sperm DNA is quickly demethylated in mammals except in some specific loci (O'Doherty and McGettigan 2014; Miller et al. 2010; Okada et al. 2010) which are supposed to be necessary for a proper progress in early embryogenesis. However in zebrafish, whose sperm chromatin, as was explained before, is fully compacted with histones (Wu et al. 2011), DNA reprogramming after fertilization follows a different mechanism, early embryos inheriting sperm DNA methylome (Jiang et al. 2013). Maintenance of a proper methylation at specific gene promoters is required for an appropriate sperm function and aberrant methylation patterns have been associated with infertility and poor sperm quality in men (Jenkins and Carrell 2012b; Jenkins and Carrell 2012a; Houshdaran et al. 2007). Moreover inappropriate methylation status has been suggested to be related with recurrent pregnancy loss (El Hajj et al. 2011).

Regarding histone modifications, a different pattern may be expected in mammalian and fish sperm. In the mammalian sperm, nucleosomal regions compacted with histones are those packaging specific coding regions expressed soon after fertilization; e.g. HOX genes, morphogenesis regulators, developmental related sequences or promoters of ncRNAs and miRNAs (Brykczynska et al. 2010; Hammoud et al. 2009; Arpanahi et al. 2009). In contrast, in many fish species, histones are packaging the entire genome. Further expression of those genes involved in early development could be regulated by the particular pattern of histone modifications. Histones are able to lead epigenetic changes through tail modification at lysine and serine residues, comprising methylation, acetylation, ubiquitination and phosphorylation (Jenkins and Carrell 2012b). An active transcriptional state is created when H3K4

methylation, H3 and H4 acetylation and H2B ubiquitination occur; while H3K9 and H3K27 methylation, H3 and H4 deacetylation or H2A ubiquitination confer a gene silencing status (Schagdarsurengin et al. 2012; Carrell 2012; Jenkins and Carrell 2012b). In mammalian and zebrafish sperm, repressive or active marks at gene promoters have been described in genes that are silenced or activated in the early embryo, respectively (Brykczynska et al. 2010; Hammoud et al. 2009; Wu et al. 2011). Moreover, bivalent marks with H3K4me3 and H3K27me3, specific from sperm and embryonic stem cells, are found in development-related genes (Bernstein and Hake 2006), these regions showing a hypomethylated DNA (Brykczynska et al. 2010; Hammoud et al. 2009). These special marks are believed to maintain genes in a repressed state in the spermatozoa, but ready to be activated after fertilization. The scenario seems to be similar in human and zebrafish, suggesting that despite the different chromatin organization, epigenetic marks drive the sequential activation of the paternal genes required for the early stages of development.

3.2. The sperm transcriptome. mRNAs in the sperm cytoplasm

Spermatozoa possess a considerable store of RNA particles in spite of its transcriptional inactivity and the lack of rRNA (Johnson et al. 2011; Miller et al. 2005) that excludes the translation process in the sperm cell (Miller et al. 2005; Jodar et al. 2013). During spermatogenesis, a constant transcription activity exists until the spermatid formation; then, cytoplasm and RNA content are reduced gradually to the residual or chromatoid body. The original explanation for the presence of RNA in the spermatozoa was attributed to an incomplete removal of cytoplasmic RNA during spermiogenesis but recent data suggest a selective retention of particular transcripts (Johnson et al. 2011; Miller et al. 2005; Hamatani 2012; Hosken and Hodgson 2014).

Spermatic RNAs are coded by the diploid genome, being equivalent in all the spermatozoa from the ejaculate, independently of the particular haplotype of each spermatozoon (Hosken and Hodgson 2014). The diverse set of RNAs in mature spermatozoa is a varied source of coding and non-coding RNAs which include messenger RNA (mRNA), small-interfering RNA (siRNA), micro RNA (miRNA), Piwi-interacting RNA (piRNA) and long non-coding RNA (lncRNA) (Schagdarsurengin et al. 2012; Jodar et al. 2013; Hosken and Hodgson 2014).

Human transcripts are the best described amongst mammals, mainly due to the availability of genomic information and next generation tools. Since the first attempts to decipher the RNA profile of human spermatozoa (Miller et al. 2010; Kumar et al. 1993; Miller et al. 1999; Pessot et al. 1989), technologies have evolved to high throughput ones which have facilitated to a large extent the analysis of spermatozoa RNA profiles. The first published study of the transcriptome revealed that approximately 3000-7000 different coding transcripts were present in human spermatozoa (Ostermeier et al. 2002). This study confirmed a sperm-specific mRNA profile which included transcripts with a potential role in fertilization and development. Several reports suggest that the retention of mRNAs in mature sperm is species specific, revealing that they are released into the oocyte and maintained in the zygote (Johnson et al. 2011; Hamatani 2012; Avendano et al. 2009; Ostermeier et al. 2004). Transcriptome of zebrafish was analyzed by Wu and colleagues (Wu et al. 2011), who identified that the most enriched transcripts are from 1731 genes (encoding ribosomal proteins, actin, tubulin, etc), corresponding to genes showing histone activating marks and DNA hypomethylation in sperm chromatin. Even if the specific role of these spermatic RNAs is not clear, different hypothesis and suggestions have been made supposing a remarkable role in zygotic and early embryo development (Hamatani et al. 2004). Hosken and Hodgson

(Hosken and Hodgson 2014) hypothesized four different potential roles for sperm RNAs that are: i) markers of relatedness of brother sperms, mainly when sperm populations are mixtures from several males, as occurs in many teleosts, ii) a police force to keep some paternal control in spite of the individual differences provided by the haploid genome of each spermatozoon, iii) “nuptial gifts” that sperm deliver to the oocyte to be directly used for translation or iv) “Trojan horses” able to change the female reproductive capacity.

Evidences of the role of spermiatic RNAs in the control of development are provided by paramutations. A paramutation is a change in the inherited phenotype generated by an epigenetic state (Jodar et al. 2013; Hamatani 2012). In mice this phenomenon was observed in the *Kit* locus, responsible for the skin pigmentation: *Kit*+/+ heterozygote mice are characterized by the presence of white tail and feet whereas homozygote *Kit*+/+ mice show a normal pigmentation. Nevertheless homozygotes originated from heterozygote males maintain and transfer the white spotted phenotype to their own offspring independently of the genotype. Rassoulzadegan and colleagues (Rassoulzadegan et al. 2006) demonstrated that this paramutated phenotype was associated with the zygotic transfer of RNA molecules. An additional role has been proposed for mRNA related to their potential ability to support the interaction between the nuclear membrane and histone-bound DNA (Miller et al. 2005; Hamatani 2012). Global analysis of spermatozoa mRNAs have pointed out the correlation between specific human mRNA profiles and different disorders (male infertility, teratozoospermia, smoking habits, etc.) (Jodar et al. 2013; Hamatani 2012), that support their use as male infertility markers.

The knowledge of fish sperm transcriptome is still in a preliminary stage. Different reports show the modification of the mRNA profiles after sperm

cryopreservation in gilthead seabream (*S. aurata*) (Guerra et al. 2013), after exposure of adult zebrafish (*D. rerio*) males to different contaminants (Lombó et al. 2015) or after treatment of mummichog (*Fundulus heteroclitus*) with 5 α -dihydrotestosterone (Feswick et al. 2014). The sensitivity of specific transcripts to these agents makes them good candidates for biomonitoring.

3.3. Controlling gene expression with miRNA

Hundreds of small non-coding miRNAs have been identified in animals and plants and have been recently described in spermatozoa, mainly human (Krawetz et al. 2011; Ostermeier et al. 2005). Their ability to regulate gene expression post-transcriptionally is considered a key mechanism during cell differentiation (Hamatani 2012). miRNA are set in intragenic or in intergenic areas in the whole genome (Kotaja 2014). They are initially processed by two endonucleases: Drosha and Dicer, resulting in a 21-22 nucleotides RNA. Then a RNA-induced complex (RISC) is formed with one strand of the miRNA, guiding to specific sequences to degrade it, repressing its translation (Meister 2013; Wang and Xu 2015). Some evidences point to a great variety of biological processes in which miRNAs are involved, including among others embryo development, cell proliferation or apoptosis (Wang and Xu 2015). Dicer1 or Drosha knockout mouse models, in which miRNA processing is compromised, resulted also in impaired spermatogenesis and fertility after birth due to faulty germ cell proliferation (Maatouk et al. 2008; Wu et al. 2012). The essential and multiple functions played by miRNAs during spermatogenesis have been largely evaluated (Jodar et al. 2013; Kotaja 2014; Luo et al. 2015). In addition, an implication of paternal miRNAs in embryo development has been hypothesized because the potential capacity for repressing translation of zygotic transcripts, or regulating transcription by interaction with specific

promoters (Jodar et al. 2013). Their mechanism of action is similar among vertebrates, so findings in mammals could be the basis for their study in fish. One of the most abundant miRNAs in mammalian spermatozoa is miR-34c (Jodar et al. 2013; Krawetz et al. 2011), which has been described as a crucial element for the first cleavage in mouse embryos. Several targets for this miRNA have been described as key for early embryo development (Jodar et al. 2013), supporting the proposal that miRNA profile could be used as marker of sperm quality and male infertility (Kotaja 2014), for clinical or ecotoxicological diagnosis in reproductive medicine or animal production.

Moreover, miRNA precursors (pi-miRNAs), which could be processed by zygotic enzymes after fertilization, are also present in mammalian spermatozoa (Jodar et al. 2013).

4. Analysis of the integrity of the paternal information

4.1 Methods applied for the study of genetic integrity

Since some years ago, when the sperm DNA integrity was correlated with its ability to coalesce with the oocyte (Evenson et al. 1999), the study of sperm genomic stability has been considered as a principal feature to determine the sperm quality (Agarwal and Allamaneni 2005).

There are a number of methods for the evaluation of sperm chromatin integrity: the comet assay (being the most commonly applied), the Sperm Chromatin Structure Assay (SCSA), the Terminal transferase dUTP Nick End Labelling (TUNEL) and the Sperm Chromatin Dispersion test (SCD or halo test). The comet assay or SCGE (Single Cell Gel Electrophoresis), initially described by Ostling and Johanson (Ostling and Johanson 1984) is extensively used for the determination of DNA fragmentation. Spermatozoa cells embedded into an agarose gel are lysed using detergents, high salt

concentration and eventually DTT and are subsequently exposed to an alkaline or neutral electrophoresis to detect both single and double-strand breaks or only double-strand breaks, respectively (Cortés-Gutiérrez et al. 2014; Olive and Banath 2006). Different stains can be used to reveal the DNA spots in the gel (DAPI, propidium iodide or ethidium bromide), spermatozoa nuclei displaying after electrophoresis a comet-like shape where the fragmented DNA migrates faster creating the comet tail. DNA fragmentation can be quantified using specific software, the more fragmented the DNA, the higher the proportion of DNA in the comet tail. A recent methodology of two-tailed comet assay has been reported by Cortés-Gutiérrez and colleagues (Cortés-Gutiérrez et al. 2014; Enciso et al. 2009). After a non-denaturant electrophoresis to first separate double-strand damaged DNA, cells are subjected to an alkaline electrophoresis, perpendicularly to the first one, the results showing two dimensional tails that allow distinguishing single and double-strand breaks in the same sperm cell. As other techniques, the comet assay have some limitations (Olive and Banath 2006; Ribas-Maynou et al. 2013) but it is considered as very sensitive, accurate and able to reveal a wide range of sperm DNA damage (Aitken et al. 2013; Aitken and De Iuliis 2007). In the field of aquaculture and of aquatic ecotoxicology, it has been applied to several species such as rainbow trout (*O. mykiss*) (Cabrita et al. 2005; Pérez-Cerezales et al. 2009; Pérez-Cerezales et al. 2010; Pérez-Cerezales et al. 2011), zebrafish (*D. rerio*) (Riesco et al. 2012), seabream (*S. aurata*) (Cabrita et al. 2011), seabass (*D. labrax*) (Martínez-Páramo et al. 2013), sturgeon (*Acipester gueldenstaedtii*, and *Acipester baerii*) sperm. (Shaliutina et al. 2013) or sole (*Solea senegalensis*) (Beirao et al. 2009), being considered a reliable tool for the analysis of genomic integrity and for the evaluation of the sensitivity of the spermatozoa to different genotoxic agents.

SCSA, developed by Dr. Evenson and colleagues (Evenson et al. 1999), is a referent and repeatable technique employing the metachromatic dye acridine orange to discern, under low pH conditions, between the denatured DNA (red fluorescence) or the native form (green fluorescence) using flow cytometry (Evenson et al. 1999; Evenson and Jost 2000). The DNA fragmentation index (DFI) (ratio between red fluorescence and total fluorescence) is used to express the results. This technique is widely used in mammals and it is considered a useful approach for the clinical diagnosis of human fertility, mainly for assisted reproductive technologies (ART) (Evenson et al. 1999; Ribas-Maynou et al. 2013; Bungum et al. 2011; Farah et al. 2013; Palermo et al. 2014). However the application of SCSA for the analysis of teleost spermatozoa does not provide reliable results, probably due to the requirement of modifications in the denaturation process, in accordance to the specific chromatin packaging.

The TUNEL assay (firstly described by Gavrieli and colleagues (Gavrieli et al. 1992)) specifically detects single and double-strand breaks (Gorczyca et al. 1993). The method detects free 3'-OH ends in DNA strands, binding to them FITC-labeled nucleotides catalyzed by deoxynucleotidyl transferase. Subsequently, sperm cells are analyzed either by microscopy or flow cytometry (Lewis et al. 2013; Loo 2011). This is a rather expensive procedure mainly used for the analysis of human sperm (Aitken and De Iuliis 2007; Evenson et al. 2007), but it has also been employed successfully for seabream and seabass spermatozoa (Cabrita et al. 2011) and for tuna germ cells analysis (Corriero et al. 2009).

The SCD test was developed recently as the commercial kit Halosperm® (Fernández et al. 2005), with the aim to provide a simple, accurate and easy-to-use tool for the determination of DNA fragmentation. The sperm is treated with an acidic solution that promotes a relaxation of fragmented chromatin loops, forming halos

around the non-damaged part of the nuclear structure (Coughlan et al. 2015). Then sperm chromatin is revealed using a DNA-binding stain and images are acquired by microscopy. This technique has been applied to fish species such as tench sperm (López-Fernández et al. 2009) and is also used for clinical purposes. However, critical reports stated that low correlation exist between halo measurements and artificial reproductive technologies (ART) outcomes (Lewis et al. 2013).

These methodologies offer information about global DNA damage, but do not provide a detailed picture of injuries at the level of single genes or specific DNA sequences. Gene-specific alternative techniques have been developed that inform about the differential susceptibility of particular genes or nuclear territories. The combination of the comet assay with Fluorescence In Situ Hybridization (FISH) has made possible to simultaneously detect global DNA fragmentation and damage in specific genomic sequences (Santos et al. 1997). There are a variety of available probes but the most widely used are repetitive sequences such as centromere, telomere or ribosomal DNA repeats. A similar technique used to determine DNA breaks (both single and double-strands breaks) in the whole genome or in a specific DNA sequence is the DBD-FISH (DNA Breakage Detection-Fluorescence In Situ Hybridization) that can be used to recognize DNA regions sensitive to denaturation (Fernández et al. 2011; Gleib and Schlormann 2014). Quantifying the number of lesions in specific DNA sequences is also possible with the use of a qPCR based methodology. This assay is based on the delay of the polymerase in its progression on the template DNA at the injured spots (Rothfuss et al. 2010). This method was applied for the evaluation of seabream and rainbow trout sperm integrity (González-Rojo et al. 2014; Cartón-García et al. 2013) and to assess damage in germinal cells of zebrafish (Riesco and Robles 2013).

The application of immunocytochemistry to the detection of DNA damage is a different approach allowing the location of specific modified nucleotides in the spermatozoa. Using this technique, González-Rojo and collaborators (González-Rojo et al. 2014) have recently examined the distribution of oxidized guanosine (8-OHdG) in the sperm nucleus of rainbow trout subjected to different treatments, showing that different genotoxicants promote a different distribution of oxidative damage.

Considering all the methodologies available to assess the sperm DNA damage, each one provides partial and specific information. In fact, criticisms have been made about the results from these tests because they are not equivalent (Lewis et al. 2013). Aitken and colleagues (Aitken et al. 2013) reported that lesions detected by SCSA or the comet assay are not the same as the one revealed by TUNEL assay. Ribas-Maynou and colleagues (Ribas-Maynou et al. 2013) proposed that the best method to assess chromatin lesions in sperm is the alkaline comet assay, followed by TUNEL, SCD and SCSA. So in conclusion we can determine that the rational choice of a method should rely on the particular objective and that a combination of different methodologies would provide a more informative diagnostic about sperm quality or about the damages promoted by a particular agent.

4.2. Prospective markers of sperm quality and genotoxicity in fish

After the identification of the paternal molecules providing essential information to fertilize and to progress with the development of a healthy offspring, it is rational to select those with the potential to be used as biomarkers of sperm quality. Assessing the chromatin integrity with any of the available methods is useful for the diagnosis of samples to be used for artificial fertilization or subjected to further storage procedures. The limits of “tolerable damage level” are related to the ability of the zygote to repair

the spermatid DNA, that in trout have been established in a 10% of fragmented DNA (Pérez-Cereales et al. 2010). Nevertheless, this information could be more precise if chromatin regions or DNA sequences particularly susceptible to damage i.e. with a relevant role in embryo development, were specifically analyzed. In mammalian sperm genes at nucleosomal regions are considered good candidates, given their lower compaction degree and their close relationship with developmental decisions. The more relaxed packaging allows an easier access of the transcription machinery, but is considered to render them more susceptible to genotoxic damage (Noblanc et al. 2013). The identification of genes particularly susceptible to damaging is in progress in rainbow trout sperm –exclusively compacted with protamines-. The available data reveal that developmental genes located at the peripheral nuclear area, are more prone to oxidative stress generated by treatment with H₂O₂, but not to UV irradiation which produces a homogeneous damage (González-Rojo et al. 2014). These results confirm that chromatin blocks showing higher accessibility are more subject to specific kinds of genotoxic damage, but reveals that this point is not dependent on the nuclear basic proteins associated to DNA, establishing that differences in susceptibility to damage are specific for genotoxic mechanisms. From the analyzed genes, the authors suggested *HoxB5bi* as a good candidate biomarker of genotoxic injury in trout sperm, allowing early and sensitive detection of damage. Considering the broad patterning of chromatin organization among teleosts, identifying candidate genes could be a challenging task to develop on a species specific basis.

The presence of specific RNAs could also be a useful biomarker as it has been stated above. The available information in fish sperm is still scarce in this respect. Nevertheless, the study of eleven transcripts in testicular cells of good and bad zebrafish breeders revealed differences in the abundance of 9 transcripts, 7 being more abundant

and 2 with a decreasing occurrence in good breeders. Moreover, two of the more abundant transcripts in zebrafish good breeders, *bdnf* and *kita*, were also correlated with sperm quality in seabream: samples with high motility carrying more of those transcripts than samples with lower quality (Guerra et al. 2013). These results point to *bdnf* and *kita* as good candidates for biomarkers of sperm quality, because they have been correlated not only with motility, but also with success in the reproductive process in terms of a healthy progeny. Additional studies performed in a larger number of species could confirm the most suitable transcripts to choose for this purpose.

A different approach is related to the use of zygotic indicators of repair activity after fertilization: the more damaged the sperm DNA, the more intense repairing effort in the zygote. This approach is particularly useful for environmental biomonitoring of genotoxicants. The repair ability of male germ cells is high during mitotic and meiotic phases within spermatogenesis process, but disappears at later phases. Post-meiotic spermatids have a highly compacted nucleus, limiting the access to the proteins of the DNA repair machinery (Baarends et al. 2001; Marchetti et al. 2015). In those late steps, sperm DNA is very prone to suffer genetic lesions which can accumulate and can be transmitted to the zygote, making maternal DNA repair mechanisms the responsible to correct the failures after fertilization (Aitken et al. 2014; Fernández-Díez et al. 2015). As reviewed by Kienzler and coworkers (Kienzler et al. 2013) the repairing activity is lower in fish than in mammalian cell lines, mainly for the nucleotide excision repair pathway (NER), but is more efficient in embryo and larval stages than in adults in vivo. Nevertheless this approach is informative of both, the paternal and maternal gamete status. Fernández-Díez and collaborators (Fernández-Díez et al. 2015) showed that in trout embryos, the expression of BER and NER pathway genes is more affected by the

quality of the fertilized eggs than by paternal DNA defect (UV irradiated sperm used in the fertilization process).

5. Factors affecting the paternal contribution to development

Sperm DNA damage can be promoted by intrinsic and extrinsic factors. The former are consequence of failures in the recombination process during meiosis, in the cell renewal by apoptosis induction during spermatogenesis (Shaha et al. 2010) or during chromatin remodeling at spermiogenesis (Hendrich and Bickmore 2001; Li et al. 2014; Rathke et al. 2014). Moreover, membranes from immature spermatozoa are very susceptible to suffer lipid peroxidation, whose products are genotoxic and mutagenic and can affect to the mature ones (Aitken et al. 2014). Extrinsic factors such as environmental factors (Sakkas and Alvarez 2010) or sperm cryostorage (Pérez-Cerezales et al. 2011) could be responsible for generating DNA damage and for increasing lesions promoted during spermatogenesis. Moreover, epigenetic information can also be affected by factors such as nutrition, the presence of pollutants in the aquatic environment (Feil and Fraga 2012) and some others related to fish production practices (Fig. 3).

5.1. Environmental factors

Anthropogenic sources of contaminants are numerous and aquatic ecosystems are the ultimate receiving compartment of millions of chemical compounds stemming from agriculture, industry, urban activities and transport. So a large array of environmental contaminants (ECs) including heavy metals, pesticides, urban or industrial sewage effluents and pharmaceuticals can be found in surface waters located

in watersheds under human pressure and can deeply affect aquatic organism fitness (mainly survival, growth and reproduction) through different mechanisms of toxicity.

During the last two decades, a large part of the studies dealing with the impact of ECs on fish reproduction has been devoted to the endocrine disruption of reproduction (Arukwe 2001; Jobling S et al. 1998; Matthiessen and Sumpter 1998). In particular, the ability of some compounds to interfere with endocrine functions in fish testes has been shown to diminished reproductive parameters such as sperm production, motility, velocity and fertilizing ability both in laboratory conditions and in the wild (Hatef et al. 2013; Kime and Nash 1999).

However, limiting the study of fish reproduction impairment to endocrine disrupting effects of some ECs would be unrealistic since a huge amount of chemicals can affect reproductive success through other mechanisms. Early studies have shown several detrimental outcomes in organisms exposed to genotoxicants both in aquatic invertebrates and vertebrates such as fish (Anderson and Wild 1994; Belfiore and Anderson 2001; Bickham et al. 2000; Depledge 1998). As an example, these authors have highlighted the weight of gamete loss due to cell death and heritable mutations in lowered genetic diversity population. The fact that defects induced in the germ line can modify the genetic make-up of the population is of primary concern for ecosystems, contrary to somatic alterations that generally remain harmless due to large reproductive surplus (Wurgler and Kramers 1992). Still, it has to be kept in mind that while some great progress has been made in the understanding of genotoxin exposure consequences on mammalian reproduction, a huge gap remains in our comprehension of such effects in fish.

Although exhibiting highly condensed DNA, fish sperm is known to lack efficient defenses such as biotransformation and DNA repair activities against ECs

(Aitken 2004). Moreover, due to limited antioxidant protection and quite high unsaturated fatty acid content, fish sperm has been shown sensitive to oxidative stress involved in DNA damage after exposure to physical or chemical stresses (Li and Reinberg 2011; Cabrita et al. 2011; Hulak et al. 2013; Labbe et al. 1995; Linhartova et al. 2013). Such characteristics make spermatozoa a relevant and sensitive target to assess reproductive impairment through the loss of DNA integrity as demonstrated in several aquatic organisms exposed to environmental stressors (Galloway et al. 2010; Jha AN 2008; Lacaze et al. 2011; Santos et al. 2013a).

Many studies carried out on freshwater or marine fish species have pointed out a loss in sperm DNA integrity and its possible consequences on the quality of the progeny after exposure to physical stressors such as ionizing radiations or hypoxic events (effects of cryopreservation are discussed in another section of this review), or to ECs. The group led by Ciereszko (Ciereszko et al. 2005) showed that after exposure to UV radiation or to hydrogen peroxide used as an oxidative agent, sperm of sea lamprey (*Petromyzon marinus*) was significantly damaged but interestingly oxidative damage was partially repaired by the oocyte after fertilization. A decrease in sperm motility and/or fertilization rate was noted, depending on the compound. Studying the effects of the same stressors on rainbow trout (*O. mykiss*) sperm, Dietrich and colleagues (Dietrich et al. 2005) evidenced a decrease in sperm motility and DNA integrity after a long UV irradiation resulting in the subsequent death of most of the embryos. A dose-dependent decline in sperm motility and fertilizing ability was noted after sperm exposure to hydrogen peroxide. The fungicide vinclozin broadly used in the United States and Europe for crop treatment, bisphenol A used as a carbonate-plastic and epoxy-resin hardener, and duroquinone, a compound used in nanotechnologies, were demonstrated to impair the sperm DNA integrity of the sterlet (*Acipenser ruthenus*),

likely driven by reactive oxygen species (ROS) production (Hulak et al. 2013; Linhartova et al. 2013; Gazo et al. 2013). Results of these studies are in accordance with those of Zhou and coworkers (Zhou et al. 2006) showing that exposure of the common carp (*Cyprinus carpio*) sperm to duroquinone affected sperm motility and increased sperm DNA damage followed by a subsequent decrease in hatching rate. Once again, involvement of oxidative stress through the induction of ROS production in spermatozoa was established. Exposure of rainbow trout (*O. mykiss*) sperm to the trace elements mercury and cadmium led to an increase in DNA damage measured through the comet assay, with deleterious consequences on sperm motility and hatching rate (Dietrich et al. 2010).

Aberrant DNA methylation patterns and modified histone marks have also been reported in many studies as consequence of environmental pollution (Feil and Fraga 2012). The epigenotoxic effects could seriously affect the male gamete. Nevertheless, although several studies using zebrafish as a model organism have described different DNA methylation modifications after exposure of embryos to different toxic compounds (Kamstra et al. 2014), information related to the male gamete is limited. Bhandari and colleagues (Bhandari et al. 2015) gathered the information about the effect of ECs such as bisphenol A (BPA) and 17 α -ethinyl estradiol in aquatic wildlife species, indicating the consequences on the sexual and neural development as well as on the DNA methylation pattern but ignoring the potential effects on the sperm. Recent studies failed to detect changes in DNA global methylation of sperm from males exposed to BPA during adult life, but demonstrated a decrease in certain spermatogenic transcripts, namely insulin receptors *insr α* and *insr β* , that is likely to be related to an increase in the malformation rate in the progeny (Lombó et al. 2015). The effects of this endocrine disruptor on the translation of specific genes during spermatogenesis could be

responsible of the modified transcriptome, with further consequences for the signaling transduction pathways in the embryo, which lead to the development of skeletal and cardiac malformations (Lombó et al. 2015).

Changes in the expression of miRNAs have also been associated in mammals with the exposure to stress conditions or to different toxicants, including tobacco smoke, or environmental contaminants (Brevik et al. 2012; Metzler-Guillemain et al. 2015; Rodgers et al. 2013), that could have further impact in the offspring development.

5.2. Fish production practices

Related to fish production practices, handling and storage of sperm is one of the most relevant factors that could affect the paternal contribution to the offspring. Short-term storage, refrigerating at temperatures above 0 °C, is a very common procedure in species depending on artificial fertilization, whereas long-term storage in liquid nitrogen (cryopreservation) is gaining ground both for artificial breeding and for gene banking in farmed and wild species (Lahnsteiner 2004; Lahnsteiner et al. 2011; Routray et al. 2002; Sarder et al. 2012). Regardless the storage decision, DNA integrity of the spermatozoa must be preserved, especially when gene banking is the goal. However, certain degree of DNA damage is promoted during cold and frozen storage when the protocols used are not perfectly optimized, the selection of samples to be preserved is not strict enough, or the spermatozoa are particularly fragile, as occurs for example in most salmonids (Cabrita et al. 2005; Pérez-Cerezales et al. 2009; Martínez-Páramo et al. 2009; Xu et al. 2014; Zilli et al. 2003). The increase of oxidative stress promoted during storage is considered the most important risk factor. Pérez-Cerezales and coworkers (Pérez-Cerezales et al. 2009) using the comet assay after digestion with endonucleases specific for oxidized bases, revealed that the storage of rainbow trout sperm at 4°C increased the

DNA fragmentation and the base oxidation level as a result of the increase of reactive oxygen species (ROS). Oxidative stress caused by short-term storage was also described in Russian and Siberian sturgeon (*A. gueldenstaedtii*, and *A. baerii*) sperm and was accompanied by an increase in DNA fragmentation after two days of storage in Siberian but not in Russian sturgeon (Shaliutina et al. 2013), pointing out possible specific species differences.

The addition of antioxidants in the storage extenders is a common strategy to reduce oxidative damage, but results are often controversial. The appropriate antioxidant and concentration is species specific as revealed by Cabrita and colleagues (Cabrita et al. 2011), who assayed five amino acids and vitamins at two concentrations each in the cryopreservation extender for gilthead seabream (*S. aurata*) and seabass (*D. labrax*). A decrease in DNA fragmentation post thawing was noticed after the addition of taurine and hypotaurine in gilthead seabream, but any improvement was observed in European seabass, some vitamins even promoting an increase of fragmentation. None or negative effects were observed in the DNA integrity of brook and rainbow (*Salvelinus fontinalis* and *O. mykiss*) trout sperm after the supplementation of extenders for cryostorage with any of ten antioxidants (Lahnsteiner et al. 2011). The analysis of DNA oxidization after short or long-term storage performed in rainbow trout sperm by Pérez-Cerezales and collaborators (Pérez-Cerezales et al. 2009) indicated that oxidization leads the DNA injury in refrigerated sperm, but is not the major factor inducing DNA damage in cryopreserved semen. Sperm cryopreservation entails dramatic physical changes, osmotic stress, cell dehydration, ice crystal formation and other processes playing a role in genotoxicity. The activation of caspases during freezing-thawing or mechanical injuries produced by intracellular ice crystals during the thawing process, are additional mechanisms involved in the DNA cryodamage (Paoli et al. 2014). These

factors can provide an explanation to the inefficiency of antioxidants during sperm freezing in some species.

As showed by Riesco and Robles (Riesco and Robles 2013), using an optimal cryopreservation protocol for zebrafish germinal cells (PGCs), even when cell survival, cell functionality and DNA integrity (analyzed by comet assay) are well preserved, some particular and essential genes can be damaged. In this study *vasa* and *sox2* genes showed a higher susceptibility than other genes (up to 11 lesions per 10kb), that were partially attributed to their position within the nuclei. Considering that *vasa* is a crucial gene for PGCs, involved in germ cell lineage development, and *sox2* is involved in differentiation, damage in these particular genes could have undesirable effects. Guaranteeing the absence of damage after cryopreservation in specific genes could be particularly important, pointing to the need of determining the best candidates for biomarkers.

The reduction of some transcripts as a consequence of cryopreservation has also been reported in PGCs from zebrafish (Riesco and Robles 2013). The incubation with cryoprotectants did not induce any variation, revealing that the changes in the stability of the transcripts occur during the process of freezing/thawing. Nevertheless, the level of eleven spermatogenic transcripts from gilthead seabream remained stable after cryopreservation (Guerra et al. 2013). This is a relevant observation showing the suitability of the freezing protocol for gene banking purposes.

Broodstock management can also affect the chromatin stability. Modifying the seasonality of reproduction is a common practice in fish farming and is often accomplished by changes in the environmental factors. These procedures imply that the spermatogenesis takes place during an inappropriate thermal regime that could alter the DNA packaging mechanisms, increase the oxidative stress during this critical period

and also affect the activity of the enzymes implied in the epigenetic remodeling. Increases in DNA fragmentation have been described in rainbow trout sperm at the end of the natural reproductive period (Pérez-Cerezales et al. 2009; Pérez-Cerezales et al. 2010; Pérez-Cerezales et al. 2011; Fernández-Díez et al. 2015) or in sole (*S. senegalensis*) out of the natural spawning seasons (Beirao et al. 2011). In addition, the chromatin of the sperm obtained out of season shows a higher susceptibility to cryodamage or to UV irradiation, increasing significantly the fragmentation rate and the number of lesions in specific genes, that is accompanied by a lower reproductive success (increase in the abortion rates and malformed larvae) (Pérez-Cerezales et al. 2011; Fernández-Díez et al. 2015).

Broodstock nutrition is an additional factor non-yet analyzed in fish sperm that could affect paternal information by different mechanisms. Nutrition has the capacity to modulate the oxidative stress, contributing to generate an appropriate environment, and has a relevant role in the epigenetic patterning. The analysis of its effects deserves further study.

6. Risks associated to fertilization with damaged sperm and potential repair during early embryo development

6.1 Fertilization ability and reproductive outcome

As has been stated above there is a clear link between the alteration of sperm genome or epigenome and the development of fetal disease, the paternal information being a key element during development (Ward 2010; Aitken et al. 2014; Fernández-Díez et al. 2015; Delbes et al. 2010; Wagner et al. 2004). Sperm is a mixture of different subpopulations of cells whose ability to fertilize the oocyte depends on a number of factors including membrane integrity, motility pattern, morphology, etc. (Cosson et al.

1999). Mammalian spermatozoa undergo a strong selection process along the female tract to guarantee fertilization by good quality cells, eliminating defective spermatozoa and diminishing the risks of fertilization with DNA-damaged spermatozoa (Hourcade et al. 2010). In contrast, most external fertilizers such as fish have a different reproductive strategy, requiring weaker selection procedures. Most fish spawn a high number of eggs, energy is not spent on offspring care and only a percentage of the embryos survive. Selective pressure is lower than that undergone by spermatozoa in mammals, increasing the probabilities of fertilization with DNA-damaged spermatozoa (Pérez-Cerezales et al. 2010). Fertilization of rainbow trout ova with sperm carrying different degree of fragmented DNA demonstrated that damaged spermatozoa preserved good fertilization ability, but generated embryos with lower chances of survival (Pérez-Cerezales et al. 2010). Changes in the information contained in the spermatozoa may thus have a relevant impact both in wildlife preservation and aquaculture industry.

Studies regarding ECs in vivo fish exposure or ex vivo fish sperm exposure have focused on the link existing between the genome integrity of sperm and the progeny in terms of development quality, survival and growth efficiency. Devaux and colleagues (Devaux et al. 2011) demonstrated in the salmonids brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*) a clear reproduction impairment after exposure of male during the spawning period to the model genotoxicant MMS (methyl methane sulfonate), representative of direct alkylating compounds frequently recovered in aquatic ecosystems. If paternal exposure to MMS did not influence neither fertilization rate nor survival rate of embryos till the hatching stage, it led to a large array of early developmental abnormalities in embryos and further in hatched larvae. As much as a 20% frequency of skeletal defects was recorded in larvae stemming from MMS-treated trout males. Finally, mortality in progeny of this treated group reached a 3 fold value

after two months of growth compared to the control. The same trend was observed by Santos's group (Santos et al. 2013a; Santos et al. 2013b) in experiments carried out in the three-spined stickleback (*Gasterosteus aculeatus*) exposed to MMS. A significant relationship between abnormal development of progeny and sperm DNA damage level was underlined and progeny defects were clearly demonstrated as being driven by male genome lacking defenses against xenobiotics.

These promising laboratory results stress the needfulness of future studies to investigate such a link between paternal genome integrity and reproduction success in wild fish from the field. If the relevancy of this relationship is demonstrated in actual environmental scenario, it could represent an interesting risk assessment approach for the comprehension of fish population decline due to environmental stressors acting directly or indirectly as genotoxicants. In such a case, a rather simple individual measurement of sperm DNA integrity could be used as a marker to predict a risk at the population level.

Regarding fish farming practices, improving the reproductive outcome is of utmost importance in aquaculture, small differences of survival rates in hatchery being critical for the success of any production. Fertilization with cryopreserved DNA damaged sperm has been related in rainbow trout with an increase of the abortions rate during gastrulation and decreases in hatching rates (Pérez-Cerezales et al. 2010). Similar increases in embryo loss have been observed in carp (Ogretmen et al. 2015) and tench (Rodina et al. 2007) batches fertilized with frozen/thawed sperm carrying fragmented DNA. Other authors have also reported the relationship between sperm DNA quality and embryo survival in fish when suboptimal cryopreservation protocols were applied (Kopeika et al. 2003; Kopeika et al. 2004; Labbe and Maise 2001; Lin and Tsai 2012).

Beyond the embryo death, the presence of sub-lethal damage in the genetic material could persist in the surviving embryos affecting offspring performance, promoting phenotypic changes and compromising their long term survival or health. Pérez-Cerezales and coworkers (Pérez-Cerezales et al. 2011) reported that rainbow trout larvae obtained after fertilization with cryopreserved DNA damaged sperm showed longer telomeres and an over-expression of telomerase, *Tert*, as well as changes in the expression levels of 5 from 7 genes related to growth and differentiation. Abnormal juvenile weight and changes in cortisol response to acute stress were reported by Hayes and colleagues (Hayes et al. 2005) in trout fertilized eggs obtained with frozen sperm. No data were provided about the DNA stability but some cryodamage could be related to these phenotypes.

6.2 *The maternal factor: zygotic repairing ability*

The unfertilized egg of vertebrates contains DNA and RNA polymerases, histone and non-histone chromatin proteins, transcription and translation factors, rRNAs, tRNAs, and maternal mRNAs (reviewed by Sullivan et al. 2015) and (Lubzens et al. 2010)) involved in DNA repair. After fertilization the zygotic mechanisms of DNA repair are activated to correct the failures from paternal origin (Aitken et al. 2014; Fernández-Díez et al. 2015), supporting early embryonic development until the activation of the zygotic transcription during the “mid-blastula transition” stage (MBT). Maternal ability to repair paternal injuries seems to be very effective. It has been estimated that the oocyte is able to repair as much as 8% fragmented DNA in mouse sperm (Ahmadi and Ng 1999) and at least a 10% in trout sperm samples (Pérez-Cerezales et al. 2010). These mechanisms are essential to progress with a proper development, mainly when the paternal gamete has suffered some genotoxic damage,

being deleterious the deficiencies in some of the involved repairing pathways (Pérez-Cerezales et al. 2010; Fernández-Díez et al. 2015).

Different repairing pathways can be activated according to the type of DNA damage, as reviewed by Hakem (Hakem 2008) and as is summarized in Table 1 for fish embryos. Single-strand breaks are repaired by the base excision repair (BER) pathway. Therefore, defective BER activity results in an increase in double-strand breaks after replication (Hilton et al. 2013; Metzger et al. 2013), one of the most detrimental lesions that can lead to genomic instability and cell death (González-Marín et al. 2012). Double strand breaks can, in turn, be repaired by non-homologous recombination end joining (NHEJ) pathway, which increases the genetic instability, or homologues recombination (HR) pathway (Derijck et al. 2008). The inhibition of NEHJ pathway in zebrafish using morpholinos at 1 cell and 6 hpf increased the apoptotic activity and malformations rate (Bladen et al. 2005). The inhibition of the BER pathway in trout zygotes, also induced an important increase of the abortion rates (Pérez-Cerezales et al. 2010) providing a progeny with increased rates of malformed larvae, longer telomeres, higher growth rate and lower apoptotic activity (Fernández-Díez et al. 2015). The inhibition of the apoptotic process represents a selective process boosting survival of individuals more tolerant to DNA damage, with defective DNA checkpoints, that would not survive in optimal conditions. This progeny showed a transcriptomic profile significantly different from that obtained with non-inhibited zygotes and non-damaged sperm, revealing the impact of genotoxic damage and zygotic repair not only for embryo survival, but also for the long term outcome of the siblings (Fernández-Díez et al. 2015). A reduction in DNA repair mechanisms has been described in oocytes from aged mouse (Hamatani et al. 2004), whereas an increase or repairing related transcripts has been noticed in rainbow trout oocytes obtained out of the natural breeding season (Fernández-Díez et al.

2015). Moreover other endogenous or exogenous factors, could also affect the repairing activity of the zygote compromising the offspring health or characteristics. The oocyte has a high capacity to repair paternal DNA damage, activating more than one single repairing pathway, but long-term consequences for the progeny of any treatment that could affect this repairing activity should be analyzed in depth.

Concluding remarks

The wide variety of sperm nuclear architecture in fish reveals a range of mechanisms controlling the protection of the sperm genome and regulating gene expression. This variability would also imply a differential pattern of association with other nuclear proteins and of epigenetic modifications. Information is still very scarce in wild and farmed species, but the available data confirm that fertilization success, hatching rates and progeny profile highly rely on the conformity of the sperm genome, epigenome and transcriptome. The state of the art should target the study of the involved molecules in different groups of teleosts, driving to the comprehension of the key elements essential for a proper development and to the fundamentals of the transgenerational inheritance via paternal. Considering the importance of the sperm information, as well as of the zygote repairing activity, defining good markers for both factors would be helpful tools in many fields, including fish farming and ecotoxicology.

Figure legends

Fig. 1. A. Acetic-acid urea polyacrylamide gel electrophoretic analysis of the SNBP composition of different representative fish species. The histones (H1, H2A, H2B, H3 and H4), as well as the protamine-like (PL-I) protein, plus the region encompassing the

protamines (P) and the Z1, Z2, Z3 and S4 protamine components of *S. canicula* are shown. **B.** Different types of chromatin folding in fish sperm, resulting from the three major SNBP types (H, PL and P). The small circle represents the putative tetranucleosome structures, and the big circle highlights the 150 ± 50 nm structures. DNA is shown in blue, histone H1 (green), PL (orange), protamine (red), and the nucleosome histone core is represented by gray cylinders.

Fig. 2. Distribution of the different types of SNBPs among fishes. SNBPs are designated as: H, histone type; PL, protamine-like type; and P (protamine type). P* designates queratinous protamines (i.e. protamines containing cysteine). Left, adapted from Ortí and Li (2009). Telostei subdivisions and superorders (right) according to Nelson's classification (Nelson 2006).

Fig. 3. Schematic representation of the spermatoc molecules involved in the transmission of paternal information and the main agents with the potential to alter them, promoting changes affecting the reproductive success. Specific damage promoted in the DNA double strand by different agents is detailed.

Table 1. Pathways involved in the post-fertilization repair of DNA damage which have been identified in fish

DNA lesion in sperm	DNA repair pathways after fertilization	Source	Species
Single strand breaks (SSBs)		(Pei et al. 2011)	1,3,6,13 and 24 hpf zebrafish embryos
Base oxidation Abasic sites	Base Excision Repair (BER)	(Fortier et al. 2009)	Unfertilized eggs and 2,6, 24 and 72 hpf zebrafish embryos
Base deamination		(Pei and Strauss 2013)	12 hpf zebrafish embryos
6-4 photoproducts Cyclobutane pyrimidine dimers (CPDs) Bulky adducts	Nucleotide Excision Repair (NER) Phoenzymatic Repair (PER)	(Kienzler et al. 2013)	Trout cell lines from liver and gills
Base modification	Direct Reversal (DR)	(Pei and Strauss 2013)	12 hpf zebrafish embryos
Base alkylation Base pair mismatches	DNA Mismatch Repair (MMR)	(Feitsma et al. 2008)	3dpf zebrafish
Insertion		(Chen et al. 2016)	6 and 24 hpf zebrafish embryos
Deletion			
DNA double strand breaks (DSBs) Cross-linkage	Homologous Recombination (HR)	(He et al. 2015)	10 and 24 hpf zebrafish embryos
DNA double strand breaks (DSBs)	Non- Homologous End Joining (NHEJ)	(He et al. 2015)	10 and 24 hpf zebrafish embryos

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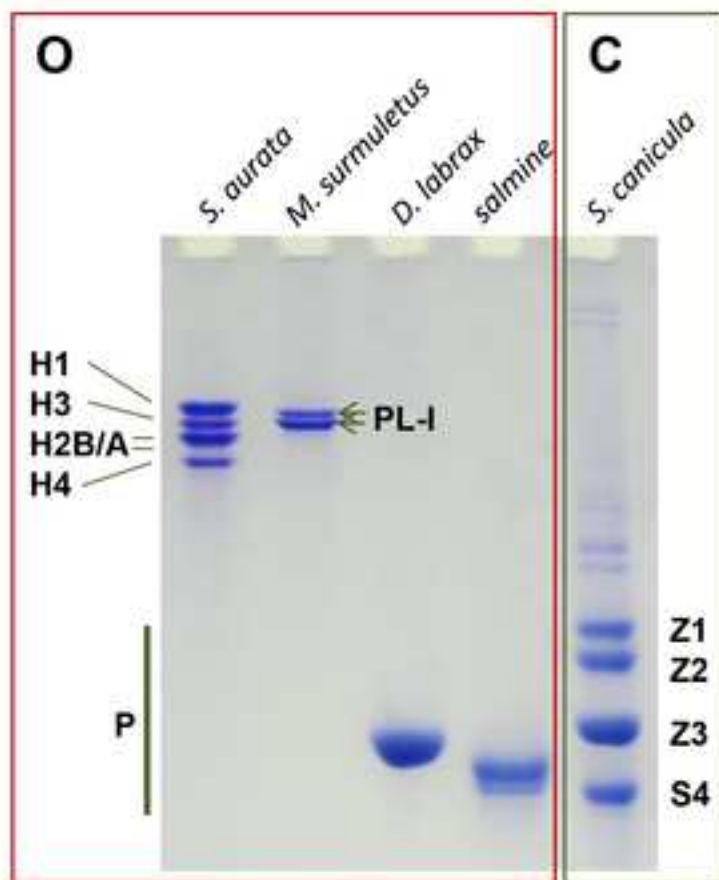
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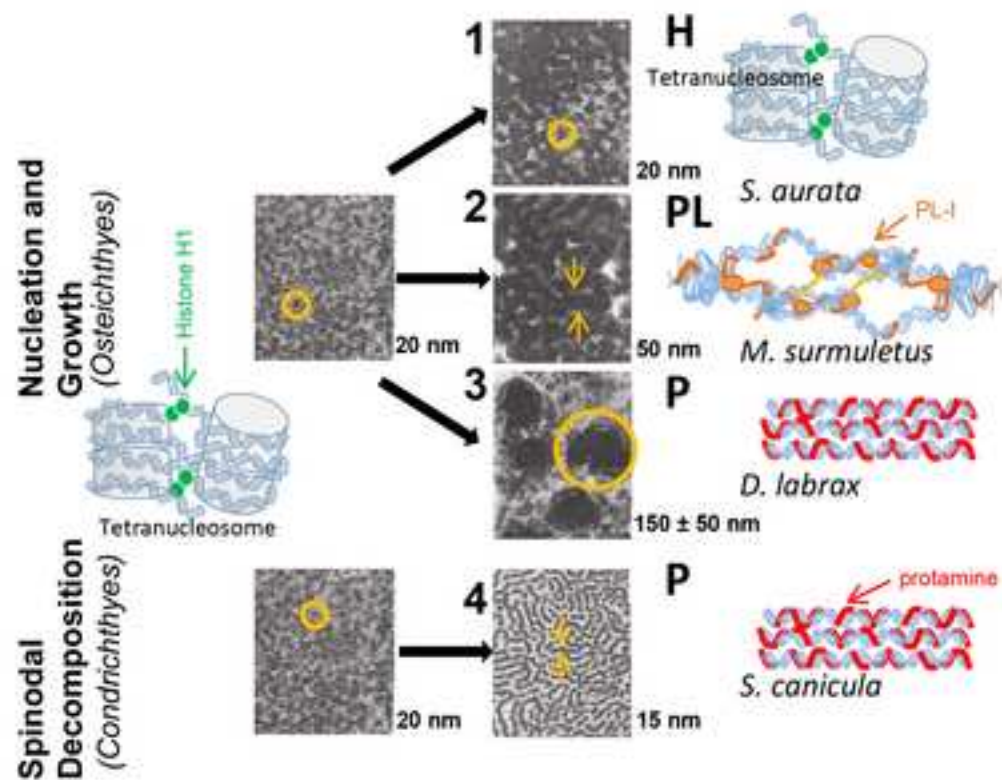
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A



O = Osteichthyes
 C = Chondrichthyes

B



Figure(s)

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