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Effect of Cryopreservation on Bio-Chemical Parameters, DNA Integrity, Protein Profile and Phosphorylation State of Proteins of Seawater Fish Spermatozoa

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1. Introduction

Fish sperm cryopreservation is considered as a valuable technique for artificial reproduction and genetic improvement (Chao & Liao, 2001; Kopeika et al., 2007; Rana, 1995; Suquet et al., 2000). Semen quality must be monitored when attempts are made to increase the efficiency of artificial fertilization, to cryopreserve only sperm of high quality, and to evaluate frozen-thawed sperm. Cryopreserved sperm usually shows, with respect to fresh sperm, a lower quality, since the freezing–thawing procedure affects DNA and protein integrity (Labbe et al., 2001; Zilli et al., 2003, 2005), membrane lipids (Maldjian et al., 2005; Müller et al., 2008), sperm motility (Linhart et al., 2000; Ritar, 1999; Rodina et al., 2007; Zilli et al., 2005), fertilization ability (Gwo & Arnold, 1992; Rana, 1995), and also larval survival (Suquet et al., 1998). Spermatozoa genome alteration due to cryopreservation may affect only late embryonic development and larval survival (Kopeika et al., 2003a, 2003b, 2004; Suquet et al., 1998), but not the early events in embryonic development, because these are controlled by maternally inherited information (Braude et al., 1988). On the contrary, defects in sperm proteins (degradation and/or change of the phosphorylation state) may compromise sperm motility, fertilization ability, and the early events after fertilization (Cao et al., 2003; Huang et al., 1999; Lessard et al., 2000).

The most common parameters used to evaluate sperm quality are fertilization ability, motility (rate and duration) and cellular (chemical and/or biochemical) parameters. Fertilizing capacity is the most conclusive test of sperm quality but the use of this marker is laborious and requires the availability of eggs (McNiven et al., 1992). Motility is normally evaluated as percentage and duration, but some authors also use velocity, flagellum beat frequency, or other parameters measured by computer-assisted sperm analysis (Ciereszko et al., 1996; Cosson et al., 2000; Rurangwa et al., 2001). Cellular bio markers has been used to evaluate spermatozoa quality of different fish species such as Atlantic salmon (Aas et al., 1991; Hwang & Idler, 1969), rainbow trout (Ciereszko & Dabrowski, 1994; Lahnsteiner et al.,1996a, 1998) and sea bass (Zilli et al., 2004). All these

parameters have been also used to evaluate the effect of cryopreservation on spermatozoa quality.

Here we reviewed data obtained by our group, on the effect of freezing-thawing procedures on sea bass and sea bream sperm. In particular, data concerning the effect of cryopreservation on bio-chemical parameters, DNA integrity, protein profile and phosphorylation state, are reported.

2. Effect of freezing-thawing procedure on sea bass spermatozoa bio-chemical parameters. Use of intracellular ATP concentration and seminal plasma ß-D-glucuronidase activity as quality marker of fresh and frozen-thawed semen

2.1 Effect of cryopreservation on sea bass semen

The cryopreservation of spermatozoa is known to result in considerable damage to cellular structures such as plasma membrane, nucleus, mitochondria, and flagellum (Conget et al., 1996; Drokin et al., 1998; Lahnsteiner et al., 1992; 1996b; Zhang et al., 2003). Spermatozoa plasma membrane is the cellular structure most susceptible to damage during cryopreservation (Baynes & Scott, 1987). It is well known that the activity of intracellular enzymes, the seminal plasma protein concentrations and the seminal plasma enzyme activities can be used to evaluate spermatozoan plasma membrane integrity (Babiak & Glogowski, 1997; Lahnsteiner et al., 1998; McNiven et al., 1992). Our group has evaluated (Zilli et al., 2004) the effect of cryopreservation on sea bass semen quality by measuring chemical and biochemical parameters (reported in Table 1), before and after sperm cryopreservation. Results obtained demonstrated that, the used cryopreservation protocol did not cause significant injuries to spermatozoan plasma membranes, since the activity of the intracellular enzymes as well as the seminal plasma protein concentrations and ß-D-glucuronidase activity were not affected by the freezing-thawing procedure. The absence of injuries at the plasma membrane level was also supported by the observation that eosin uptake was similar in fresh and frozen-thawed spermatozoa (75-80%). Only malate dehydrogenase activity and intracellular ATP concentration resulted significantly higher in cryopreserved than in fresh samples, while the pH of seminal plasma resulted significantly lower after freezing

Since respiration rate, malate dehydrogenase activity, and intracellular ATP concentration did not decrease in frozen/thawed spermatozoa we concluded that spermatozoan mitochondria were intact and active after cryopreservation procedure. The increases in both intracellular ATP concentration and malate dehydrogenase activity following the freezing-thawing procedure has been also reported in *Silurus glanis* (Ogier de Baulnyet al., 1999). The increase of intracellular ATP concentration occurs during early freezing of sperm (from +20°C to -10°C) (Baynes & Scott, 1987) and is most probably attributable to dimethyl sulfoxide, used as cryoprotectant, which interferes with cellular metabolism (McConnell et al., 1999). No data is available concerning the kinetics and structure of mitochondrial malate dehydrogenase in fish. In other vertebrates, mitochondrial malate dehydrogenase shows a complex dependence on the ionic environment, which influences both kinetics and structure (Birktoft et al., 1989; Bleile et al., 1977; Harada & Wolf, 1968;

Ruggia et al., 2001; Wood et al., 1981). The increase of malate dehydrogenase activity after cryopreservation could be a consequence of the oxidative stress that occurs during the freezing phase, as previously suggested (Lahnsteiner et al., 1998), or could be due to the presence of anions that increase the activity of the enzyme by stabilizing the dimeric form (Ruggia et al., 2001).

Parameters	Fresh sperm	Cryopreserved sperm
Respiration rate (µg O_2/min × ml seminal fluid)	6.71±0.76a (N=22)	7.09±1.22a (N=18)
Aspartate aminotransferase (U/mg protein)	0.010±0.008a (N=62)	0.012±0.009a (N=45)
Malate dehydrogenase (U/mg protein)	0.054±0.03a (N=65)	0.079±0.046b (N=61)
Isocitrate dehydrogenase (U/mg protein)	0.11±0.05a (N=45)	0.18±0.10a (N=40)
Intracellular ATP (µmoli/protein)	1.22±0.65a (N=72)	1.92±1.11b (N=66)
Intracellular triglycerides (µmoli/protein)	0.33±0.24a (N=48)	0.25±0.14a (N=38)
Intracellular glycerol (µmoli/protein)	0.28±0.18a (N=45)	0.21±0.15a (N=27)
Seminal plasma osmolality (mOsm/Kg)	352.1±19.7a (N=22)	354.9±20.7a (N=18)
Seminal plasma pH	8.21±0.45a (N=62)	7.65±0.61b (N=45)
Seminal plasma protein (mg/l)	815.3±174.5a (N=45)	837.1±180.0a (N=40)
Seminal plasma triglycerides (µmoli/l)	226.2±107.2a (N=72)	181.8±103.0a (N=66)
Seminal plasma ß-D-glucuronidase (U/l)	0.0083±0.0066a (N=48)	0.0093±0.0049a (N=38)

Table 1. Chemical and biochemical parameters measured in sea bass spermatozoa and seminal plasma before and after cryopreservation. Values (± SD) in a row with the same letter are not significantly different (P>0.01). N=Number of sperm samples from different males. (This table was originally published in Zilli et al., Biol. Reprod 2004)

2.2 Relationship of sperm and seminal plasma parameters and fertilization rate in fresh and cryopreserved semen samples

The most common parameters used to evaluate sperm quality are fertilization ability, motility (rate and duration) and cellular (chemical and/or biochemical) parameters. In sea bass we identified simple and cost-effective markers of sperm quality that would replace conventional motility and fertility evaluation assays, using both fresh and frozen-thawed sperm. Parameters of sperm metabolism and seminal plasma were tested by evaluating correlations with the fertilization rate using simple regression analysis and square relationship analysis.

In fresh sperm, among the measured cellular metabolites and enzymes, only ATP concentration and aspartate aminotransferase activity showed significant linear correlations ($P<0.0001$) with fertilization rate (Fig. 1) and the calculation of the partial correlation coefficient revealed that these two parameters were not correlated ($Pr=-0.323$). Malate dehydrogenase activity and sperm triglyceride concentration had a quadratic relation with fertilization rate: $R^2=0.31$, $P<0.001$ for malate dehydrogenase; $R^2=0.28$, $P<0.01$ for triglyceride concentration (see Zilli et al. 2004 for details).

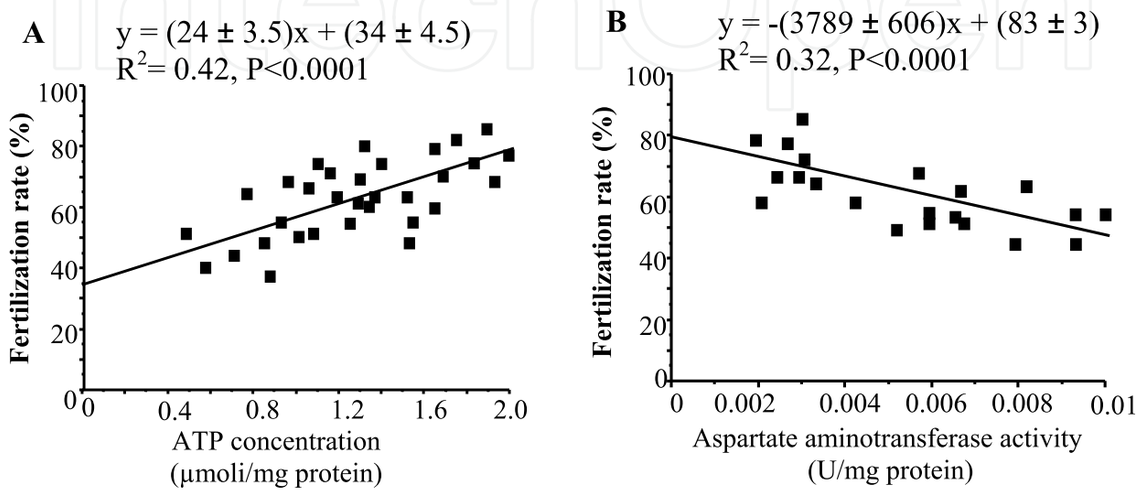


Fig. 1. Relationship between fertilization rate and ATP concentration (A, N=32) or aspartate aminotransferase activity (B, N=21) using sea bass fresh sperm samples. Samples obtained from different males were used to measure the ATP concentration and the aspartate aminotransferase activity and to perform the fertilization trials. N=Number of sperm samples from different males. (This figure was originally published in Zilli et al., Biol. Reprod 2004).

ATP concentrations of >1.8 mmol/mg protein characterized sperm with fertilization rates ≥75%. The relationship between ATP concentration and fertilization rate is due to the fact that the flagellar beat frequency of spermatozoa depends on ATP concentration and dynein ATPase activity (Christen et al., 1987; Lahnsteiner et al., 1998). Thus, intracellular ATP concentration could be used instead of sperm motility as a predictor of fertilization ability. Determination of ATP concentration has some advantages over motility assessment: it is not subjective as is motility determination based on microscopic observation (McNiven et al., 1992) and it is faster and less expensive with respect to the Computer Assisted Sperm Analysis system. Aspartate aminotransferase activity activities of 0.3 mU/100 mg protein characterized sperm with fertilization rates of 75%. A correlation between the activity of this enzyme and fertilization rate was also found in lake whitefish (*Coregonus clupeaformis*) (Ciereszko & Dabrowski, 1994) and rainbow trout (Lahnsteiner et al., 1998). The physiological meaning of this relationship is uncertain.

Among the seminal plasma (tested) parameters, only β-D-glucuronidase activity and potassium concentration had a significant linear relation ($P<0.01$) with fertilization rate (Fig.

2) and also in this case the calculation of the partial correlation coefficient revealed that these two parameters were not correlated.

β-D-Glucuronidase activity is negatively correlated with fertilization rate. This enzyme is involved in hydrolysis of β-glucuronides to glucuronic acid and is located most frequently in lysosomes (Rawn, 1983). It is located in the spermatic duct epithelium, usually in areas where lytic processes occur, and is also secreted into the seminal fluid (Lahnsteiner et al., 1994). A correlation between this activity of this enzyme and fertilization rate was also found in rainbow trout (Lahnsteiner et al., 1998). An increase of seminal plasma β-D-glucuronidase activity indicates degeneration or aging processes in the semen (Lahnsteiner et al., 1998). Seminal plasma potassium concentration concentrations of 17 mM characterized sperm with fertilization rates of 75%. Quadratic functions were used to described the relationship between fertilization rate and potassium concentration in other fish species, i.e., bleak (*Alburnus alburnus*), Atlantic salmon, and rainbow trout (Aas et al., 1991; Lahnsteiner et al., 1998).

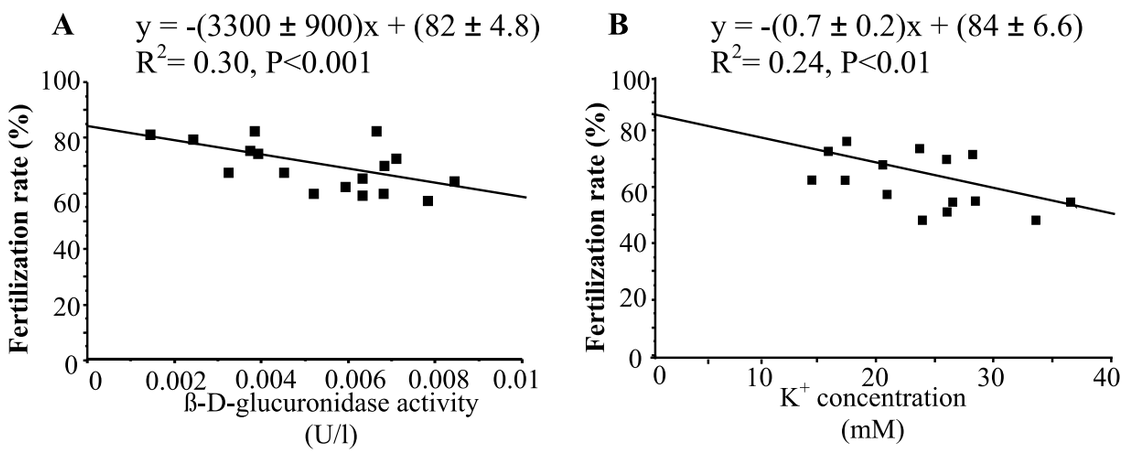


Fig. 2. Relationship between fertilization rate and seminal plasma β-D-glucuronidase activity (A, N=17) or seminal plasma potassium concentration (B, N=15) using fresh sea bass sperm samples. Samples obtained from different males were used to measure β-D-glucuronidase activity and seminal plasma potassium concentration and to perform the fertilization trials. N=Number of sperm samples from different males. (This figure was originally published in Zilli et al, Biol Reprod 2004)

Sperm and seminal plasma parameters of fresh semen that showed linear correlation with the fertilization rate have been also used in multiple regression models to predict the fertilization ability. Three models were tested: the first model included sperm ATP concentration and aspartate aminotransferase activity, the second model included the seminal plasma β-D-glucuronidase activity and potassium concentration while the third model included ATP, aspartate aminotransferase and β-D-glucuronidase (potassium concentration was exclude due to its linear relathionshep with ATP). Results indicated that sea bass fresh semen fertilization rate was well predicted by the first multiple regression model, which included cellular parameters (see Zilli et al 2004 for details).

Because sperm ATP concentration and seminal plasma β-D-glucuronidase activity among the tested parameters produced the highest correlation coefficients, we also investigated their relationship with fertilization rate in frozen-thawed samples. These parameters showed a linear relationship with fertilization rate also after the freezing-thawing procedure (Fig. 3) similar to what happens for fresh semen.

For pratical application the measuraments of ATP concentration and seminal plasma β-D-glucuronidase activity represents an alternative simple and cost-effective tests for evaluating sea bass sperm fertilization ability before and after cryopreservation.

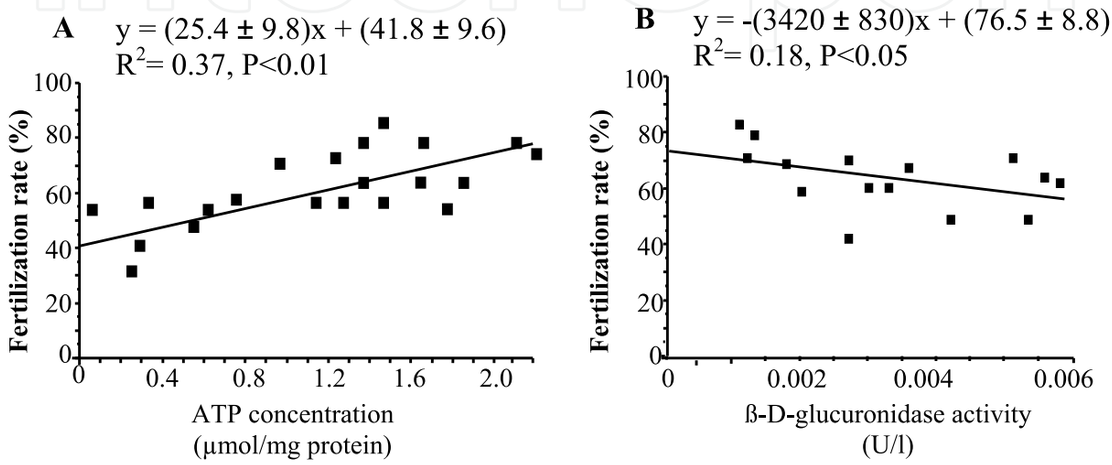


Fig. 3. Relationship between fertilization rate and ATP concentration (A, N=21) or seminal plasma β-D-glucuronidase activity (B, N=15) using cryopreserved sea bass sperm samples. Samples obtained from different males were used to measure the ATP concentration and β-D-glucuronidase activity and to perform the fertilization trials. N=Number of sperm samples from different males. (This figure was originally published in Zilli et al., Biol Reprod 2004).

3. Effect of cryopreservation on DNA integrity on sea bass spermatozoa

Sperm DNA fragmentation could be a consequence of the freezing-thawing process and the resulting genome alterations could affect late embryonic development and survival of larvae (Suquet et al., 1998). There are different methods to determine the DNA fragmentation, among these an effective tool is single-cell gel electrophoresis (SCGE). Introduced by Ostling & Johanson in the 1984 it has become a recognized method for detecting DNA damage in a variety of vertebrate cell types, including sperm (Fairbairn et al., 1995; Hughes et al., 1997; Steele et al., 2000). In this assay, the fragmented DNA migrates toward the anode, giving the appearance of a ''comet tail '' while the undamaged DNA appears as intact comet heads (lacking tail). These comets can be easily visualized when stained with DAPI. By using this technique we have demonstrated (Zilli et al, 2003) that the cryopreservation protocol (Fauvel et al 1998) used to cryopreserve the sea bass sperm cause significantly damage at DNA level (Figure 4). Results, expressed in terms of the ''percent tail DNA'' (% DNA_T) and ''tail moment'' (MT) (Ashby et al., 1995; Helma & Uhl, 2000; Johnson & Ferris, 2002; Piperakis et al., 1999) were reported in table 2.

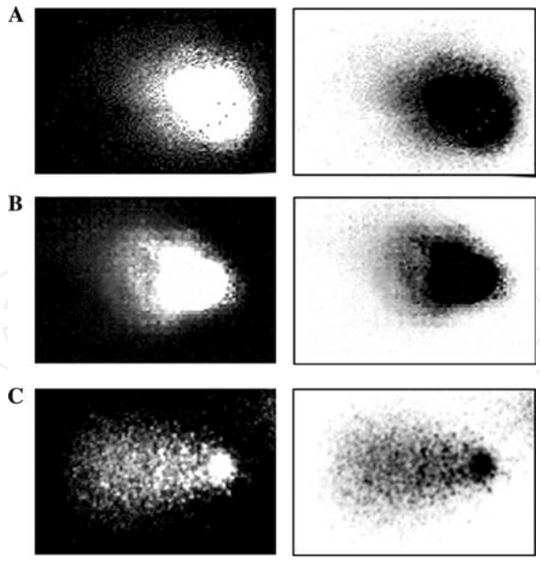
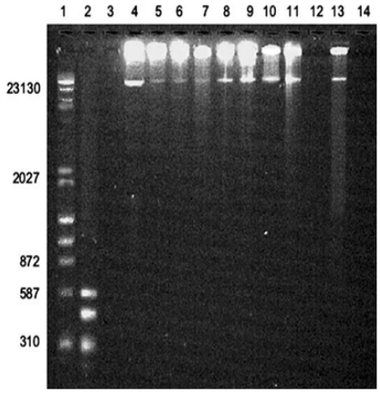


Fig. 4. The appearance of fresh (A), frozen-thawed (B) and unprotected frozen-thawed (C) sea bass sperm following preparation by the SCGE assay. On the right are shown the negative images of the same preparation used to perform the analysis. (This figure was originally published in Zilli et al., Cryobiology 2003)

Parameter analysed	Fresh sperm	Frozen-thawed sperm	Unprotected frozen-thawed sperm
Motile sperm (%)	75±15a	67±18a	n.d.
Fertilization rate (%)	74±15a	70±12a	n.d.
Motility duration (sec)	129±46a	28±8b	n.d.
Percent Tail DNA	32.7±11.1a	38.2±11.2b	65.2±10.2c
Tail Moment	375.2±190.7a	498.9±166.4b	2345.1±725.2c

Table 2. Effect of cryopreservation on DNA integrity, sperm motility and fertilizing ability determined on fresh and frozen-thawed in the presence or absence of cryoprotectant (Me_2SO). Values in a row with the same letter are not significantly different ($P>0.01$). n.d.: not detectable. (This table was originally published in Zilli et al., Cryobiology 2003).

The results obtained indicate that the cryopreservation protocol used for sea bass sperm (Fauvel et al 1998): (1) is without effect on both sperm rate motility and fertilizing ability; (2) significantly reduced the duration of motility, (3) is associated with DNA damage that, although significant, is of low magnitude and (4) demonstrated the fundamental role played by cryoprotectant (Me_2SO) in reducing fish sperm DNA fragmentation. The role played by Me_2SO was also demonstrated by using DNA laddering (Fig. 5A). When the analysis was performed on fresh semen samples no smearing was detectable (lanes 4 and 5). In some frozen-thawed semen samples (lanes 7 and 11) but not in all (see lanes 6, 8, 9, and 10) a small degree of laddering seems to be present. On the contrary, in unprotected frozen-thawed semen DNA laddering was clearly evident (lane 13).



A

Sample	% DNA_T	M_T
Sample 4	32.8±9.9 a	371.7±189.8 c
Sample 5	32.3±11.8 a	391.2±180.1 c
Sample 6	38.3±11.2 b	486.7±154.2 d
Sample 7	39.9±6.6 b	508.2±146.6 d
Sample 8	38.6±11.1 b	498.1±184.1 d
Sample 9	38.4±12.3 b	491.3±165.3 d
Sample 10	38.8±10.7 b	496.9±161.2 d
Sample 11	40.1±7.7 b	514.3±178.0 d

B

Fig. 5. DNA fragmentation of sea bass sperm samples. (A) Agarose gel electrophoresis of DNA isolated from sea bass sperm. Lanes 1 and 2: DNA molecular weight markers (pb); lanes 4 and 5: 2 lg of DNA isolated from fresh sperm; lanes 6, 7, 8, 9, 10, and 11: 2 lg of DNA isolated from frozen-thawed sperm; lane 13: 2 lg of DNA isolated from unprotected frozen-thawed sperm; (B) SCGE assay in fresh samples (4 and 5) and frozen-thawed samples (6-11) of sea bass sperm. Values are given as mean ± SD. Values within a column followed by the same letter are not significantly different (P>0.01). (Modified from Zilli et al., Cryobiology 2003)

Since in some frozen-thawed semen samples (lanes 7 and 11 of Fig. 5A) analyzed by DNA laddering analysis, but not in all (lanes 6, 8, 9, and 10), a small degree of laddering seems to occur, we analyzed the same samples with the SCGE method. The results reported in Fig 5B confirmed the presence of DNA fragmentation in the samples 7 and 11; in addition it revealed a significant degree of DNA fragmentation in the samples 6, 8, 9, and 10 with respect to fresh samples (4 and 5). In any case it must be underlined that within the frozen/thawed samples (6, 7, 8, 9, 10, and 11) no statistically significant differences in the DNA fragmentation was revealed by the SCGE method (Fig 5B).

DNA laddering has been used in many studies to obtain a qualitative analysis of DNA fragmentation (Duke & Cohen, 1986; Homma-Takeda et al., 2001; Sun et al., 1999). It is a very simple method, but the most critical problem with DNA electrophoretical analysis are its inability to provide quantitative measurement and its low sensitivity. In fact, random double-stranded or rare single-stranded DNA fragmentation in cells, cannot be detected by this technique. On the contrary, the SCGE or Comet assay has been recognized as one of the most sensitive techniques for measuring DNA strand breaks (Collins et al., 1997). For human sperm, comet assay has been shown to have a significant relationship both to the SCSA (Sperm Chromatin Structure Assay) (Larson et al., 2001) and the TUNEL assay (Terminal Deoxynucleotidyl Transferasemediated Nick End Labelling), another technique for detecting the incidence of DNA fragmentation (Sakkas et al., 1999). The use of the Comet assay in alkaline conditions is a usefull tool to carry out a quantitative analysis of DNA fragmentation. Previous works (Collins et al., 1997; McKelvey-Martin et al., 1993) have reported that the assay resolves break frequencies up to a few hundred per cell, definitely well beyond the range of fragment size for which conventional electrophoresis is suitable. Since introduction of the Comet assay protocol (Ostling & Johanson, 1984), there have been modifications of it for use with various cell types, including sperm (Fairbairn et al., 1995; Hughes et al., 1997; Steele et al., 2000). We have adapted to fish sperm the method developed by Steele et al. (2000) and we have evaluated the effect of cryopreservation on sea bass sperm DNA.

A small but significant effect of cryopreservation on DNA integrity has been demonstrated in studies carried out by Labbe et al. (2001) using sperm trout. They tested how the sperm cryopreservation affected the nuclear DNA stability and whether the progeny development was modified when eggs were fertilised with cryopreserved spermatozoa. They concluded that cryopreservation of trout sperm only slightly affected sperm DNA stability and that the use of cryopreserved sperm did not impair offspring survival and quality. Analogous studies carried out on sperm of other fish species have not revealed DNA damage after cryopreservation. The freeze-thaw process did not cause genome alterations in turbot sperm since the fertilisation rate, the hatching rate, the larval survival rate (up to ten days) and the larval weight, were similar with both fresh and frozen-thawed sperm (Suquet et al., 1998). Similarly, no effect of the freeze-thaw process on the nucleus of Atlantic croaker spermatozoa was reported (Gwo et al., 2003). Moreover, the growth of tilapias (up to 800 g) and channel catfish (up to 130 g) were not altered using thawed spermatozoa (Chao et al., 1987; Tiersch et al., 1994). The DNA damage that we observed in the cryopreserved sea bass sperm did not affect fertilization capacity and motility. Different authors have reported that the DNA fragmentation is associated with a decrease of fertilization ability, abnormal embryo cleavage and decreased embryo survival (Gwo et al., 2003; Kopeika et al., 2003a, 2003b, 2004; Sun et al., 2000). Fauvel et al. (1998b) found a lower hatching rate for eggs inseminated with frozen-thawed sea bass sperm (69%) when compared with those obtained with fresh sperm (81%), although the fertilisation rates were similar. The presence of the significant degree of DNA fragmentation that we measured after cryopreservation of sea bass sperm could explain, at least partially, this observation. Since the establishment of fish sperm cryobanks could play a crucial role in the genetic management and conservation of aquatic resources the advancement of cryopreservation protocols that avoid DNA fragmentation/aberration are necessary and the SCGE technique is a useful tool to rich this goal.

4. Effect of cryopreservation on sea bass protein profile

Defects in sperm proteins may compromise sperm motility, fertilization ability, and the early events after fertilization (Cao et al., 2003; Huanget al., 1999; Lessard et al., 2000). Protein screening has become an excellent approach with which to evaluate changes in expression due to different stresses. Using this method it has been demonstrated that the reduction in motility observed in boar and human spermatozoa following cryopreservation was associated with a decrease in heat shock protein 90 during cooling (Cao et al., 2003; Huanget al., 1999). Similarly, the loss of P25b (a protein associated with the plasma membrane covering the acrosome) may be responsible, at least in part, for the decrease in fertility following the freezing/thawing procedure of bull semen (Lessard et al., 2000). Cryoinjuries due to cryopreservation have been reported for thawed spermatozoa of many freshwater (Rana, 1995) and marine fish species (Gwo et al., 1992; Lahnsteiner et al., 2000). Shrinkage of the plasma membrane of the midpiece, breakage of mitochondria, and coiling of the axoneme have been observed.Cryopreserved sea bass sperm showed similar fertilization rates and class motility compared with fresh sperm, but also showed a decline in motility duration (Fauvel et al., 1998a), changes in metabolism (Zilli et al., 2004), and lower hatching rates (Fauvel et al., 1998b). For these reason we used (Zilli et al., 2005) the 2-DE to verify whether the cryopreservation procedure, applied to sea bass milt, affected the expression of proteins involved in the control of sperm functions and, in addition, matrix-associated laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify some of these proteins.

4.1 Proteins expression in two-dimensional electrophoresis gels: Differences between fresh and frozen-thawed sea bass sperm samples

To perform two-dimensional analysis sperm samples with similar fertilization rates (70%–90%) and percentage of motility (80%–100%), before and after cryopreservation, were used to extract proteins. All the sperm samples used showed lower motility duration after the cryopreservation procedure. 163 spots were detected in all gels prepared from fresh samples (with molecular masses ranging between 190 and 10 kDa and isoelectric points between 3.5 and 8.0) and were used for comparative analysis. Results of a typical experiment performed on sperm samples before and after cryopreservation are showed in figure 6 (A and B).

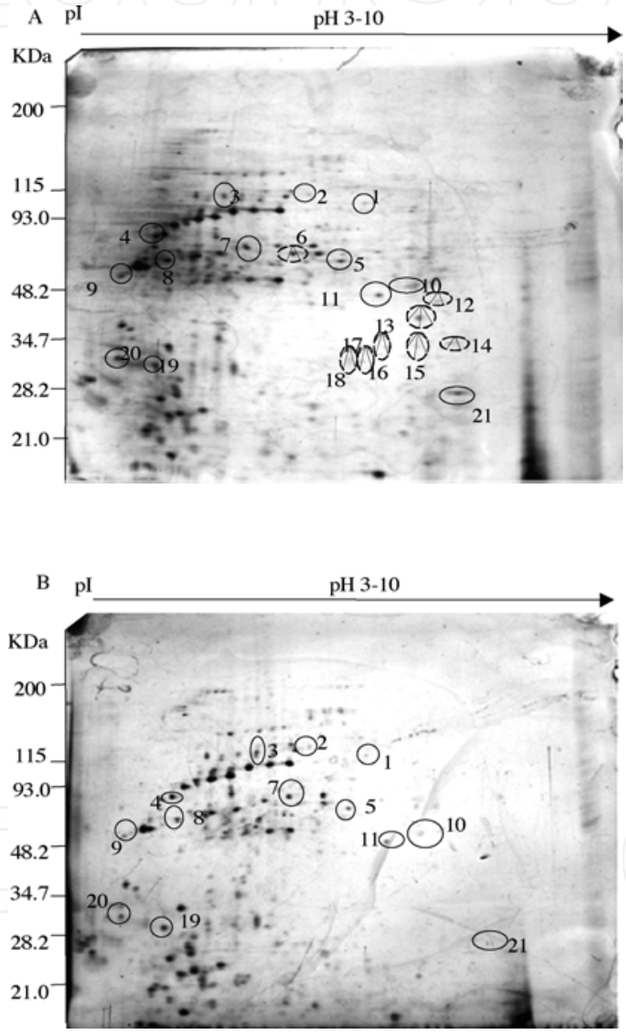


Fig. 6. Two-dimensional Electrophoresis (2-DE) maps of fresh (A) and cryopreserved (B) sea bass sperm proteins. 2-DE was performed on an immobilized pH 3–10 strip, followed by the second-dimensional separation on 12.5% polyacrylamide gels. The separated proteins were stained with silver staining. Spots that are less expressed after cryopreservation are highlighted with a continuous line; spots that are entirely absent after cryopreservation are marked with a dotted line. (This figure was originally published in Zilli et al., Biol Reprod 2005).

Differences were observed (by visual inspection and by using image analysis software) in the protein profiles of fresh and cryopreserved sperm samples. In fact, in the cryopreserved sperm samples, among the 163 spots considered, 13 were significantly (P<0.05) less expressed, and 8 completely disappeared. These 21 spots are highlighted in Figure 6 and the normalized spot volumes listed in Table 3. A decrease in protein abundance or spot disappearance in sperm after the cryopreservation procedure may be due to either leakage of proteins from spermatozoa to the extracellular medium or to degradation following freezing-thawing stress. The leakage of proteins is ruled out because we have previously demonstrated that the intracellular protein concentration and the seminal plasma protein concentrations do not change after cryopreservation (Zilli et al., 2004). Consequently, protein degradation seems to be responsible for the reduction in spot abundance (and disappearance). Similar results have been also reported in human and boar semen (Cao et al., 2003; Huanget al., 1999) and bull sperm (Lessard et al., 2000).

Spot Number	Normalised spot volume in fresh sperm (N=6)	Normalised spot volume in cryopreserved sperm (N=6)
SPOT 1	17.8±2.1	7.0±1.1
SPOT 2	10.7±1.1	5.9±1.5
SPOT 3	48.0±9.1	20.2±5.1
SPOT 4	40.8±5.2	29.3±4.1
SPOT 5	54.9±11.3	20.6±3.0
SPOT 6	42.9±2.2	-
SPOT 7	36.8±3.3	25.5±5.2
SPOT 8	110.0±23.0	33.4±6.3
SPOT 9	52.7±12.0	38.7±9.0
SPOT 10	31.9±8.0	4.7±2.0
SPOT 11	30.6±7.0	7.4±5.0
SPOT 12	34.9±15.2	-
SPOT 13	44.3±13.	-
SPOT 14	34.3±11.3	-
SPOT 15	11.4±3.2	-
SPOT 16	8.4±2.8	-
SPOT 17	6.2±3.5	-
SPOT 18	11.8±4.2	-
SPOT 19	30.6±7.2	10.6±4.2
SPOT 20	102.8±3.6	42.9±3.3
SPOT 21	46.3±6.6	29.2±7.3

Table 3. Differences in abundance of spots in fresh and cryopreserved sea bass sperm. Spot adundance is expressed as mean ± SD of normalized spot volume measured in six different gels. (This table was originally published in Zilli et al., Biol Reprod 2005).

4.2 Identification of protein spots by MALDI-TOF

Five of the protein spots shown in Table 3 were analyzed by MALDI-TOF for protein identification. Three were selected among the spots that significantly decreased after cryopreservation (5, 8, and 20) and two (6 and 13) were taken from among those that were absent in the gel obtained with frozen-thawed sperm (Fig. 6 and Table 3). Protein identification was performed by three search programs (PeptIdent, Mascot, and MS-Fit).

Three out of five sea bass proteins processed were found to have homologies with existing sequences in the databases used (Table 4). These proteins were identified from protein sequences already described in other teleost species and amphibians. In particular, two were from *Brachidanio rerio* (spots 5 and 20) and one was from *Xenopus laevis* (spot 13). Table 4 summarizes the data of the bio-informatics analysis for these proteins. For spot 5, the search engine PeptIdent found a homology with a protein of *Brachidanio rerio* (similar to SKB1 of human and mouse). This is a highly conserved cytoplasmic protein with methyltransferase activity that interacts with the members of the Janus family tyrosine kinases (JAK) (Pollack et al., 1999). Genome activation is one of the first critical events in the life of a new organism. Both the timing of genome activation and the array of genes activated must be controlled correctly, and these events depend on changes in chromatin structure and availability of transcription factors (Latham & Schultz, 2001).

In sea bass the observed reduction in SBK1 proteins in cryopreserved sperm could be responsible for abnormal early embryo development, which in turn, could determine the lower hatching rate observed (personal observation). The spot protein 13 matched in Mascot and MS-Fit with a G1/S-specific cyclin E2 protein, which is essential in the control of the cell cycle at the G1/S (start) transition (Moore et al., 2002). Cyclin E is involved in the activation of cyclin-dependent kinase 2 (cdk2). Recently, it has been demonstrated that cdk2 phosphorylates the protein phosphatase, PP1gamma2, a key enzyme in the development and regulation of sperm motility (Huang &Vijayaraghavan, 2004). The observed reduction in sea bass sperm motility duration in frozen-thawed spermatozoa could be a consequence of the cyclin E degradation. The protein spot 20 matched, in MS-Fit, with the hypothetical protein DKFZp566A1524 of unknown function.

Reference Spot	EWM (kDa)	EIP	Identified protein	SWISSPR-OT accession no.	Species ident-ified	TWM (kDa)	TIP	Homology	
								Matched peptides	Coverage %
SPOT 5	80	6.5	Novel protein similar to SKB1 human and mouse (PEPTIDENT)	Q7ZZ07	*Brachidanio rerio*	71.8	5.98	8	22.0
SPOT 6	110	6.0	–	–	–	–	–	–	–
SPOT 8	100	5.2	–	–	–	–	–	–	–
SPOT 13	40	6.8	G1/S-specific cyclin E2 (MASCOT, MS-FIT)	Q91780	*Xenopus laevis*	47.78	6.3	6	20.0
SPOT 20	30	4.5	Similar to hypothetical protein DKFZp566A1524 (MS-FIT)	Q96AZ5	*Brachidanio rerio*	37.13	5.6	4	21.0

Table 4. Results from peptide mass fingerprinting of protein spots excised from 2D gels. EWM: Experimental Weight Mass; EIP: Experimental Isoelectric Point; TWM: Theoretical Weight Mass; TIP: Theoretical Isoelectric Point. (This table was originally published in Zilli et al., Biol Reprod 2005).

The results reported in figure 6 and tables 3 and 4 show that in sea bass spermatozoa the used cryopreservation procedure causes the degradation of 21 sperm proteins, and among these, 2 could be at least partially responsible for the observed decrease in sperm motility duration and the lower hatching rate of eggs fertilized with cryopreserved sperm. In addition, these observations suggest that two-dimensional electrophoresis coupled with MALDI-TOF analysis could be used as a tool to improve cryopreservation procedures.

5. Effect of cryopreservation on proteins phosphorylation state of sea bream sperm

5.1 Molecular mechanisms determining sperm motility initiation in sea bream *sparus aurata*

Most fish spermatozoa are quiescent in the testes, because the osmolality and composition of seminal plasma usually prevent motility in sperm ducts (Billard, 1986). During natural reproduction, fish sperm become motile after discharge into the aqueous environment (in oviparous species) or the female genital tract (in viviparous and ovoviviparous species) (Billard, 1986; Billard & Cosson, 1983; Stoss, 1983). Changes in the ionic and osmotic environment of the sperm cells have been identified as being critical external factors that may be responsible for initiating motility in fish spermatozoa (Morisawa, 1994). Several extracellular factors controlling sperm motility have been reported. In marine (Gwo et al, 1993; Krasznai et al, 2003a, 2003b; Morisawa & Suzuki, 1980; Oda & Morisawa, 1993) and freshwater (Billard, 1986; Morisawa et al., 1983; Stoss, 1983) teleosts, sperm motility is initiated by osmotic shock when sperm are ejaculated. In these species, spermatozoa are quiescent at the osmolality of seminal plasma (referred to as isotonic condition). In freshwater teleost sperm, flagellar motility is initiated by the hypo-osmotic shock, whereas in marine teleost sperm, flagellar motility is initiated by hyperosmotic shock. Furthermore, in medaka (Inoue & Takei, 2003) and tilapia (Linhart et al., 1999), motility regulatory mechanisms of sperm flagella are modulated to suit the spawning environment when they are in freshwater or acclimated to seawater. In herring sperm, motility initiation requires trypsin inhibitor-like sperm-activating peptide from the eggs (HSAPS), and the sperm exhibits chemotaxis when they are close to eggs (Oda et al., 1998; Yanagimachi et al., 1992).The extracellular factors controlling sperm motility (osmolality, ions, sperm-activating peptides, and chemoattractants) act on the flagellar motile apparatus, the axoneme, through signal transduction across the plasma membrane. Second messengers, such as cAMP and Ca, play key roles in the initiation of sperm motility in many animal groups, such as mammals (Lindemann, 1978; Okamura et al., 1985; Tash & Means, 1983), salmonid fish (Morisawa & Okuno, 1982), sea urchin (Cook et al., 1994), mussel (Stephens & Prior, 1992), and tunicate (Opresko & Brokaw, 1983). A cAMP-independent initiation of flagellar motility in sperm was observed in puffer fish (Morisawa, 1994) and striped bass (Shuyang et al., 2004). Second messengers (cAMP and Ca) determine the sperm motility initiation modifying dynein-mediated sliding of the axonemal outer-doublet microtubules through protein phosphorylation/dephosphorylation in different species, such as mammals (Lindemann & Kanous, 1989), rainbow trout, chum salmon, sea urchin (Inaba et al., 1999), and tunicate (Nomura et al., 2000).

In *Sparus aurata* osmolality is the key signal in sperm motility activation and motility initiation depends on a cAMP-dependent protein phosphorylation (Zilli et al., 2008). To elucidate which proteins are involved (phosphorilated/dephosphorilated) in the initiation

of sea bream spermatozoa motility, proteins extracted from spermatozoa before and after motility activation were separated on SDS PAGE, blotted on nitrocellulose membrane, and treated with anti-phosphotyrosine, anti-phosphothreonine, or anti-phosphoserine antibodies.

After motility activation we observed that: 1) two protein bands (76 kDa and 57 kDa) were dephosphorylated and an unspecified number of proteins corresponding to a large band of 9-15 kDa were phosphorylated at tyrosine residues (Fig. 7A); 2) two protein bands (174 kDa and 147 kDa) resulted phosphorilated and an unspecified number of proteins with molecular weights ranging between 15 and 9 kDa were dephophorilated at threonine residues (Fig. 7B); 3) three protein bands (174 kDa, 138 kDa and 70 kDa) and an unspecified number of proteins from 9 to 12 kDa were phosphorylated and only one protein band of 33 kDa was dephosphorylated at serine residues (Fig. 7 C).

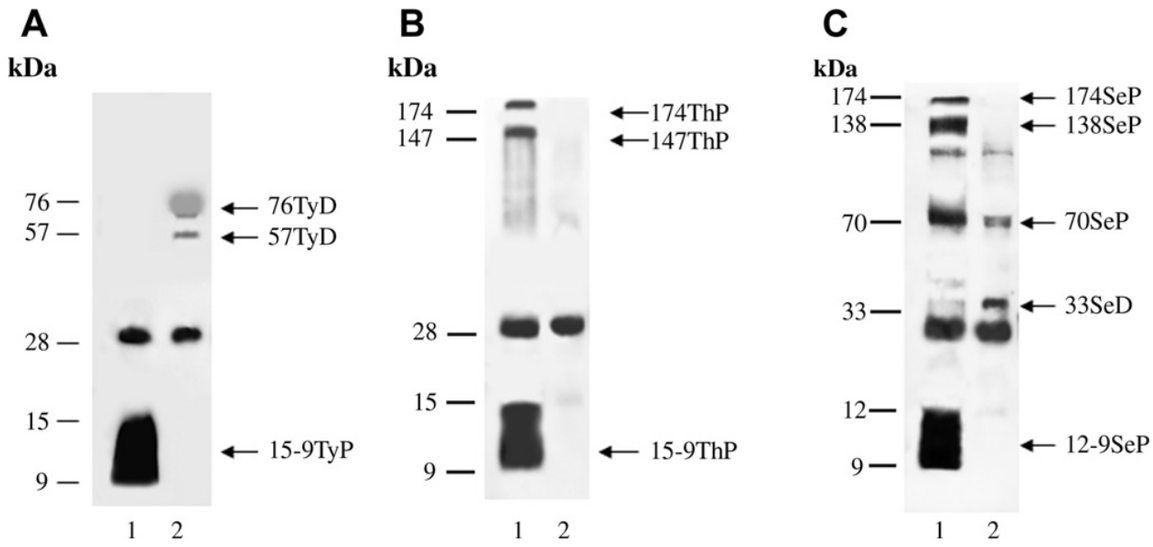
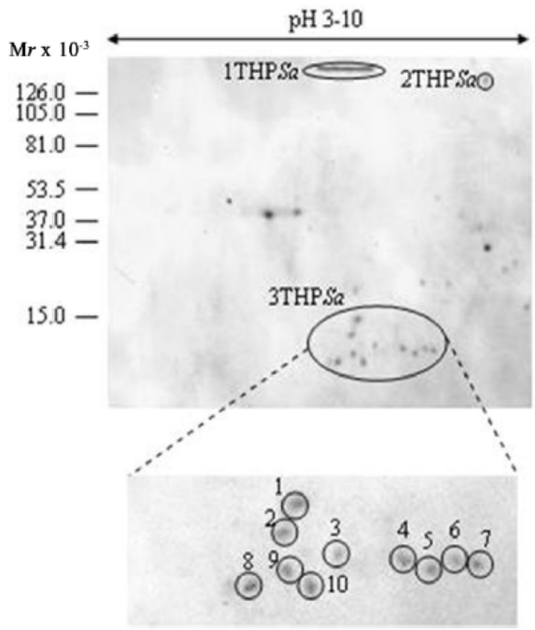


Fig. 7. Motility-dependent phosphorylation/dephosphorylation at tyrosine residues (A), threonine residues, (B) and serine residues (C) in fresh sperm of gilthead sea bream before and after motility activation. Sperm were either activated in seawater (lane 1) or maintained immotile by dilution in non-activating medium (lane 2). Sperm proteins were subjected to Western blotting (30 μg/lane) with anti-phosphotyrosine, anti-phosphothreonine and anti-phosphoserine antibodies. Number on the left indicates the molecular mass of bands. On the right, the names of proteins of interest are indicated. (This figure was originally published in Zilli et al., Cryobiology 2008).

We characterized some of these proteins by using two-dimensional gel electrophoresis (2DE) and the antibody against phosphothreonine. This antibody revealed (figure 8) that: 1) the protein band of 174 kDa (named that 1ThP in figure 7 and identified as 1THPSa in figure 8) was not a single protein but, rather, a cluster of proteins with the same molecular weight (174 kDa) but different pI (5.9–6.29); 2) the protein band of 147 kDa (named 2Thp in figure 7 and identified as 2THPSa in figure 8) was a protein with a pI of 8.7; and 3) the cluster of proteins of 9-15 kDa (named 3ThP in figure 7 and identified as 3THPSa in figure 8) consisted of 10 proteins with pI between 6.1 and 7.6 and molecular weights between 9 and 15 kDa.



Spot	MW (kDa)	pI
1THP*Sa*	174	5.9-6.2
2THP*Sa*	147	8.7
3THP*Sa*		
1	15	6.1
2	13	6.1
3	11	6.2
4	10.5	6.6
5	10	6.8
6	10.5	7.4
7	10	7.6
8	9	6.0
9	10	6.1
10	9	6.1

Notes: 1THP*Sa*, 2THP*Sa*, 3THP*Sa*: spots 1, 2 and 3 Threonine-Phosphorylated in Sparus aurata.

Fig. 8. Western blot analysis with antiphosphothreonine antibody of gilthead sea bream (*Sparus aurata*) sperm proteins separated by 2DE. The 2DE was performed on an immobilized pH 3–10 NL strip, followed by the second-dimensional separation on 13% polyacrylamide gels. The separated proteins were then blotted on nitrocellulose and incubated with antibody. Molecular mass and isoelectric point of proteins of interest are listed. (This figure was originally published in Zilli et al., Biol. Reprod. 2008).

Some of these proteins have been identified by mass spectrometry and results are listed in Table 5. In particular, spots 1 and 2 (belonging to 3THPSa) were identified as acetyl-coenzyme A (CoA) synthetase, spot 5 as A kinase anchor protein (AKAP), and spot 7 as an unnamed protein of *Tetraodon nigroviridis*, which have 70% identity with a novel protein similar to phosphatase and actin regulator 3 of *Danio rerio*. Acetyl-CoA synthetase is well known as an enzyme whose activity is central to the metabolism of prokaryotic and eukaryotic cells. In particular, acetyl-CoA synthetase activates acetate to acetyl-CoA, and it provides the cell with the two-carbon metabolite used in many anabolic and energy generation processes. Therefore, we suppose that this enzyme was activated in motile sperm to increase the level of ATP, which is necessary for flagellar movement. PKA localizes to specific cellular structures and organelles by binding to AKAP molecules via interaction with the regulatory subunits (RI and RII). Therefore, cAMP levels temporally regulate PKA, whereas the spatial regulation within the cell occurs through compartmentalization by binding to AKAP, thus assuring specificity of PKA function. The important role of AKAP as a key regulator of sperm motility is already established (Vijayaraghavan et al., 1997a). In addition, a recent study demonstrated that phosphorylation of AKAP in human sperm results in tail recruitment of PKA and increase of sperm motility, providing evidence for a functional role of phosphorylation of AKAP (Luconi et al., 2004). Regarding the role of phosphatases and kinases in the initiation of sperm motility, many studies have demonstrated that the development and maintenance of motility is regulated by a complex

balance between kinase and phosphatase activities (Tash & Brach, 1994; Vijayaraghavan et al., 1997b).

Reference Spot	EWM (kDa)	EIP	Identified protein	SWISSPROT accession no.	Species identified	TWM (kDa)	TIP	Homology	
								Score	Coverage %
3THPSa									
Spot1/ Spot2	15-13	6.1	LOC568763 similar to Acetyl-coenzyme A synthetase	Q0VG88	*Danio rerio*	15.5	5.3	0.97a	27%
Spot 5	10	6.8	novel protein similar to human A kinase (PRKA) anchor protein 7 (AKAP7)	CAI11962	*Danio rerio*	8.1	6.0	0.79a	26%
Spot 7	10	7.6	Chromosome 11 SCAF14528, whole genome shotgun sequence - Unnamed protein product that have a 70% of identity with novel protein similar to phosphatase and actin regulator 3 (PHACTR3, zgc:109967) [*Danio rerio*]	Q4SQZ9	*Tetraodon nigroviridis*	8.0	7.7	0.28b	21%

Table 5. Results from peptide mass fingerprinting of protein spots excised from 2-D gels of gilthead sea bream sperm.

EWM: Experimental Weight Mass; EIP: Experimental Isoelectric Point; TWM: Theoretical Weight Mass; TIP: Theoretical Isoelectric Point. a: Z score of ProFound; b: Normalized score of Aldente. (Modified from Zilli et al., Biol. Reprod. 2008).

5.2 Effect of cryopreservation on phosphorylation state of proteins involved in sperm motility initiation in sea bream *Sparus aurata*

The quality of gilthead sea bream semen was decreased by cryopreservation procedure. Even though the viability (82 ± 5%) of spermatozoa following the freezing–thawing procedure was only slightly (but significant) decreased with respect to that measured in fresh samples (93 ± 4), only the 50% of the thawed spermatozoa could be activated, and showed a motility duration which was one third of that measured in fresh samples. The reduction of sperm motility (percent and duration) is attributable (at least partially) to the effect that the freezing-thawing procedure has on the phosphorylation state of proteins involved in motility initiation (Zilli et al., 2008b).

Phosphorylation/dephosphorylation of tyrosine residues:

Two protein bands (76TyD and 57TyD of figure 7A) which in fresh sperm were completely dephosphorylated after motility initiation, in frozen– thawed remained phosphorylated (Fig. 9A), while the cluster of proteins of 15-9 TyP (Fig. 7A), that were phosphorylated when fresh sperm shifted from the immotile to the motile phase, were much less phosphorylated in frozen–thawed activated sperm (Fig. 9A).

Phosphorylation/dephosphorylation of threonine residues:

Among the proteins that were phosphorylated following motility activation in fresh sperm (Fig. 7B), two bands (named 15-9ThP and 147ThP) were phosphorylated after activation in frozen–thawed spermatozoa (Fig. 9B). However, it must be underlined that within the proteins belonging to the 15-9 ThP band, only one (11 kDa) previously identified as acetyl-coenzyme A synthetase (Zilli et al, 2008a) was phosphorylated after motility activation in cryopreserved sperm (Fig. 9B).

Phosphorylation/dephosphorylation of serine residues:

Among the five previously identified protein bands (Zilli et al, 2008a) that changed their phosphorylation state after motility activation in fresh sperm (Fig. 7C), only two (70SeP and 12-9SeP) were phosphorylated in frozen–thawed sperm after activation (Fig. 9C). The other bands, named 174SeP, 138SeP and 33SeD, did not change their phosphorylation state after activation (Fig. 9C), unlike what happens in fresh sperm (Fig. 7C).

Some proteins (76TyD, 57TyD and 33SeD) that were dephosphorylated after motility activaton in fresh sperm (7A and 7C) but not in cryopreserved spermatozoa (9A and 9C) could not play a key role in sperm motility initiation but could be involved in sperm motility duration and motility characteristics, since the kinematic parameters were significantly reduced by the freezing–thawing procedure.

Our studies also demonstrated that in gilthead sea bream spermatozoa the freezing-thawing procedure increased, independently from the motility activation procedure, protein phosphorylation (mainly at threonine residues), since more phosphorylated proteins were present in non-activated cryopreserved sperm with respect to the fresh sperm. This could be

due, as previously proposed by Perez-Pe et al., (2002), to a membrane modifications that determine conformational changes of these proteins or facilitate calcium influx into the cell (Bailey & Buhr, 1994; McLaughlin & Ford, 1994). This ion could stimulate adenylyl cyclase to initiate cAMP-mediated phosphorylation of sperm protein. Alternatively (or in addition), the cryopreservation procedure could also determine the activation of protein kinases different from PKA (Pommer et al., 2003).

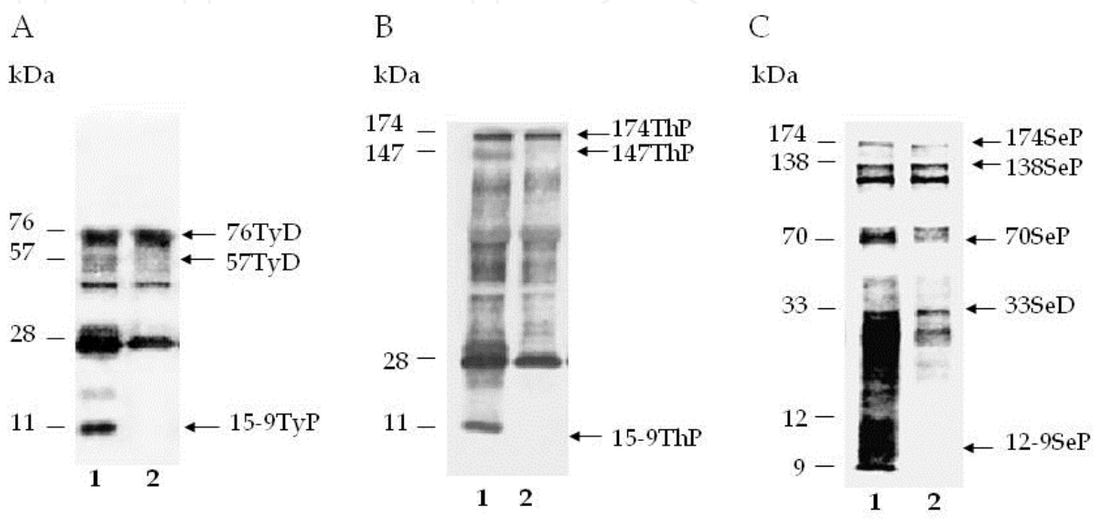


Fig. 9. Motility-dependent phosphorylation/dephosphorylation at tyrosine residues (A), threonine residues (B), and serine residues (C) in frozen–thawed sperm of gilthead sea bream before and after motility activation. Sperm were either activated in seawater (lane 1) or maintained immotile by dilution in non-activating medium (lane 2). Sperm proteins were subjected to Western blotting (30 μg/lane) with antibody. Number on the left indicates molecular mass of bands. On the right, the names of proteins of interest are indicated. (Modified from Zilli et al 2008; Criobiology)

6. Conclusions

Cryopreservation, coupled with insemination and short term storage techniques, will lead to an improvement of gamete management of marine fish species. In particular, sperm cryopreservation is considered as a valuable technique for artificial reproduction and genetic improvement since it allows the selection and the storage of gametes of high quality.

However, although seawater fish spermatozoa of marine fish are more resistant than freshwater species to the dynamic changes in osmotic pressure that occur during the process of cryopreservation (Dzuba & Kopeika, 2002), the freezing-thawing procedure, apart from the experimental protocol used and from the fish species considered, determines: a changes of the kinematic characteristics, damages to proteins and DNA, lipid modification and change of the phosphorylation state of proteins involved in sperm motility initiation. The knowledge of the effects of freezing–thawing procedure on spermatozoa is very important to improve cryopreservation techniques for semen of marine fish for the establishment of sperm cryobanks that could play a crucial role in the genetic management and conservation of aquatic resources.

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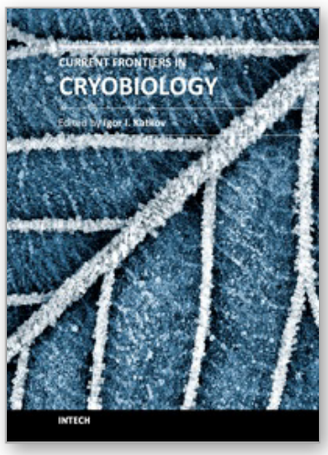

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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