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Effects of Different Stages of Cryopreservation of Red Tilapia (*Oreochromis niloticus*) Sperm and the Variability between Three Individual Fish in Response to Cryopreservation

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ABSTRACT

Cryopreservation is a technique that makes long term storage of fish semen possible. The objectives of the present study were to determine the effects of different stages of cryopreservation and individual variability of fish in the cryopreservation process. This study involved five stages of cryopreservation (fresh sperm, sperm + extender, after equilibration time, after vapor phase and after freezing) and three different fish. Sperm movement and velocity distribution for fresh and after frozen-thawed semen were evaluated using an automated semen analyzer. The motility of red tilapia fish sperm decreased gradually in the freezing process. The highest value of motility was obtained at fresh sperm (96.93 ± 0.53%), followed by sperm with extender (92.64 ± 1.05%), after equilibration time (84.86 ± 2.26%) and after vapor phase (67.82 ± 3.28%), whilst the lowest value of motility was obtained for sperm after freezing (61.39 ± 3.62%). For individual fish, Fish 1 significantly showed the highest total motility (77.50 ± 1.86%).

Keywords: Cryopreservation, red tilapia sperm, sperm motility

ABSTRAK

Kriopengawetan semen ikan adalah salah satu teknik yang membolehkan penyimpanan semen ikan. Tujuan kajian ini dijalankan adalah untuk menentukan kesan peringkat yang berbeza dalam kriopengawetan dan perbezaan individu ikan dalam proses kriopengawetan. Kajian ini melibatkan lima peringkat kriopengawetan (sperma asal, sperma + ekstender, selepas masa pengimbangan, selepas fasa wap, dan selepas penyejukbekuan) dan ikan-ikan yang berbeza. Motiliti sperma dan halaju penyebaran untuk sperma asal dan selepas sejukbeku dinilai menggunakan penganalisa sperma automatik. Motiliti sperma ikan tilapia merah menurun secara beransur-ansur dalam proses penyejukbekuan. Nilai motiliti yang paling tinggi adalah pada sperma asal (96.93 ± 0.53%), diikuti dengan sperma + ekstender (92.64 ± 1.05%), selepas masa pengimbangan (84.86 ± 2.26%) dan selepas fasa wap (67.82 ± 3.28%), sementara itu, nilai motiliti sperma yang paling rendah adalah selepas penyejukbekuan (61.39 ± 3.62%). Bagi individu ikan, Ikan 1 menunjukkan motiliti yang paling tinggi (77.50 ± 1.86%) secara bererti.

Kata kunci: Kriopengawetan, sperma tilapia merah, motiliti sperma

INTRODUCTION

Sperm cryopreservation for fish is essentially an unexplored field although many species of fish are threatened with extinction. Cryopreservation of sperm from genetically superior brood fish can preserve genetic resources and provide greater availability of improved material. Cryopreservation is a technique that makes long-term storage of fish sperm possible. The cryopreservation of sperm process involves the following steps of temperature reduction, cellular dehydration, freezing and thawing (Medeiros *et al.*, 2002). Generally, sperm cells are not adapted to endure extreme low temperatures; therefore, cryopreservation of these cells happens at the expense of cellular viability and normal function.

Every stage of cryopreservation affects the sperm. During cryopreservation, there are varying degrees of severity of cellular damage induced by distinct mechanisms at each of the cryopreservation phases, and the functional state of the frozen-thawed cells is the result of the injuries accumulated throughout the freezing process (Medeiros *et al.*, 2002). In the past few years, cryobiological studies focusing on the adaptation of cooling rates to biophysical properties of sperm, changes of sperm packaging systems as well as the accurate and consistent freezing of large numbers of samples have led to the improvement of cryopreservation protocols (Roca *et al.*, 2006).

There are various factors that can affect the initial quality of sperm. Variability between individual fish is one of the main factors that can affect fish sperm cryopreservation. Individual fish could give differences or variations in sperm quality. This could be due to the differences in lipid composition of the sperm plasma membrane, where it has been suggested that it could be a factor in variable freezability of sperm (Parks and Graham, 1992). Furthermore, the variation in the success rate of cryopreservation can also be caused by variation in the response of sperm of individual males to the cryopreservation process (Christensen and Tiersch, 2005). Thus, to overcome this problem selective breeding could be used to improve those factors in male fish, by using parameters such as motility or possibly even cryopreservation performance.

For marine and freshwater fish sperm, there are also differences in survivability due to cryopreservation. Sperm of marine fish species are easier to cryopreserve than those of freshwater species (Billard *et al.*, 1995). This fact could be explained by the specific composition of membranes especially by the cholesterol: phospholipid ratio. Cholesterol modulates bilayer fluidity through interaction with membrane phospholipids (Parks and Lynch, 1992). The cholesterol:phospholipid ratio is 2 to 3 times higher in marine than in freshwater fish (Drokin, 1993). As phosphatidylcholine protects cells from osmotic and cold stresses, the high level of phosphatidylcholine found in marine sperm could also explain the higher capacity of cryoresistance (Drokin, 1993). Therefore, cryopreservation of freshwater fish sperm is more challenging compared to marine fish sperm.

There is limited information and research on fish sperm cryopreservation. This study was done to improve success rate and increase information on fish sperm cryopreservation. The objectives of the present study were to determine the effects of different stages of cryopreservation, variability between three individual fish and to develop a suitable cryopreservation protocol for red tilapia (*Oreochromis niloticus*) fish sperm.

MATERIALS AND METHODS

Collection of Semen

Semen collection was carried out with the gentle squeezing technique of the male fish abdomen. The semen was transferred to 100 mL capillary tubes. This technique should be done carefully to avoid any mixing of semen with the urine, blood, feces or waste matter which may reduce the sperm motility. Sometimes, training of fish during squeezing is important to avoid contamination.

Extender

The extender used was tris citric acid yolk extender (TCAYE) as described by Asmad (2005).

Freezing and Thawing

Semen was processed immediately (within 10-15 minutes after collection) upon arrival at the laboratory to avoid degradation in quality.

- **Semen dilution**

The semen was diluted at a ratio of 1:9 (semen: TCAYE extender). The eppendorf tube was then shaken slowly to mix the extender and semen thoroughly.

- **Semen enveloping**

The diluted semen was placed in a French straw (0.5 mL). Each end of the straw was sealed with an electric sealer. These straws were then arranged on a special rack for further processing.

- **Equilibration**

The rack of straws filled with semen was placed in the refrigerator at 4 °C for 60 minutes as equilibration time.

- **Freezing**

This two stage process involved exposure to liquid nitrogen vapor at a temperature of -80 °C for 5 minutes and complete submersion of straw into the liquid nitrogen (-196 °C) for 10 minutes. Some straws were placed in the liquid nitrogen tank for long-term sperm storage.

- **Thawing**

This process was carried out by placing the straws in the water bath at 30 °C for 30 seconds. Then, the straws were wiped dry and both sealed ends were cut off to extract the sperm.

Analysis of Sperm

Fresh and post-thawed sperms were analyzed using the automated semen analyzer (IVOS) to evaluate sperm movement characteristics and velocity distribution.

Statistical Analysis

All data were analyzed by using the Statistical Package for Social Science (SPSS) software package. The effect of factors and parameters measured was demonstrated by using the Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT).

RESULTS

Effects of Different Stages of Freezing Process on Fresh and Frozen-Thawed Sperm of Red Tilapia (*Oreochromis niloticus*)

The results from this study determined the effects of different stages in the freezing process on fresh and frozen-thawed sperm in red tilapia (concentration, total motility, progressive motility and velocity distribution; rapid, medium, slow and static).

Table 1 shows the concentration, total motility, progressive motility and velocity distribution at five different stages in freezing. Among the five stages, the fresh sperm stage showed the highest values of concentration, total motility and progressive motility; whereas, the sperm after freezing stage gave the lowest values, respectively. The results obtained demonstrated that there were significant differences among fresh sperm to fresh sperm with extender, after vapor phase and after freezing (for concentration), fresh sperm to sperm after equilibration time, vapor phase and freezing stage (for total motility), and fresh sperm to sperm after vapor phase and after freezing stage (for progressive motility).

In velocity distribution, fresh sperm with extender stage showed the highest value of rapid and medium velocity compared to the other stages. On the other hand, sperm after freezing gave the lowest value of rapid and slow velocities. The highest value of static velocity was the sperm after the freezing stage. Fresh sperm gave the lowest value. There were significant differences among fresh sperm to sperm after vapor phase and freezing (for rapid velocity distribution), fresh sperm to sperm

for all stages (for medium velocity distribution) and fresh sperm to sperm after vapor phase and freezing (for static velocity distribution). However, there was no significant difference within the freezing process for slow velocity distribution.

Table 1. Concentration, total motility, progressive motility and velocity distribution (mean ± S.E.) of red tilapia at different stages of the freezing process

Stages in freezing process	N	Concentration (million/mL)	Total motility (%)	Progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
Fresh sperm	28	181.44 ± 6.04^{b}	96.93 ± 0.53^{c}	42.21 ± 3.17^{c}	50.57 ± 3.99^{b}	16.86 ± 1.62^{c}	25.50 ± 1.72^{a}	3.14 ± 0.52^{a}
Fresh sperm + extender	28	216.44 ± 9.55^{c}	92.64 ± 1.05^{c}	40.57 ± 2.51^{ab}	52.93 ± 2.92^{b}	10.71 ± 1.09^{b}	28.79 ± 1.94^{a}	7.36 ± 1.05^{a}
After equilibration time	28	168.92 ± 11.82^{b}	84.86 ± 2.26^{b}	40.00 ± 1.84^{ab}	48.18 ± 2.39^{b}	7.36 ± 0.46^{a}	29.29 ± 1.89^{a}	15.18 ± 2.28^{b}
After vapor phase	28	108.83 ± 9.93^{a}	67.82 ± 3.28^{a}	33.50 ± 2.92^{b}	38.46 ± 3.37^{a}	6.14 ± 0.55^{a}	23.21 ± 1.98^{a}	32.18 ± 3.28^{c}
After freezing	28	98.49 ± 10.83^{a}	61.39 ± 3.62^{a}	26.04 ± 2.53^{a}	30.21 ± 3.00^{a}	7.36 ± 0.51^{a}	23.18 ± 2.58^{a}	38.61 ± 3.62^{c}

Note: ^{a,b,c}Means with different superscripts within a column were significantly different ($P < 0.05$)

Effects of Different Stages in the Freezing Process for Three Individual Fish on Fresh and Frozen-Thawed Sperm in Red Tilapia (*Oreochromis niloticus*)

Fresh semen

Table 2 demonstrates the concentration, total motility, progressive motility and velocity distribution of fresh sperm among the three fish. There was no significant difference in sperm characteristics of concentration and progressive motility. However, in total motility, there were significant differences among Fish 1 to Fish 2 and 3, in where Fish 1 showed the highest total motility. There was no significant difference among the three fish for rapid, medium and slow velocity distribution, respectively. However, in static velocity there were significant differences between Fish 1 and Fish 3, in where Fish 3 showed the highest static velocity.

Table 2. Concentration, total motility, progressive motility and velocity distribution (mean ± S. E.) of red tilapia for fresh sperm.

Fish	N	Concentration (million/mL)	Total motility (%)	Progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	12	168.26 ± 8.57^{a}	98.67 ± 0.14^{b}	37.00 ± 4.76^{a}	51.33 ± 5.01^{a}	19.17 ± 3.13^{a}	28.50 ± 2.31^{a}	1.50 ± 0.15^{a}
2	8	195.25 ± 30.78^{a}	96.25 ± 0.86^{a}	49.00 ± 5.12^{a}	46.00 ± 10.45^{a}	14.00 ± 1.71^{a}	21.50 ± 3.77^{a}	3.75 ± 0.86^{ab}
3	8	187.40 ± 11.48^{a}	95.00 ± 1.34^{a}	43.25 ± 6.59^{a}	54.00 ± 6.44^{a}	16.25 ± 2.69^{a}	25.00 ± 2.96^{a}	5.00 ± 1.34^{b}

Note: ^{a,b}Means with different superscripts within a column were significantly different ($P < 0.05$)

Fresh sperm with extender

Table 3 shows the concentration, total motility, progressive motility and velocity distribution of fresh sperm with extender for Fish 1, 2 and 3. There was no significant difference in sperm characteristics of concentration and progressive motility. In total motility, there were significant differences among the three fish in which Fish 1 showed the highest total motility. There was no significant difference among the three fish for rapid and slow velocity distribution. However, there were significant differences between Fish 1 and Fish 3 (for medium velocity distribution) and Fish 1 and 3 to Fish 2 (for static velocity distribution), in which Fish 1 and Fish 2 showed the highest medium and static velocity, respectively.

Table 3. Concentration, total motility, progressive motility and velocity distribution (mean ± S. E.) of red tilapia for fresh sperm with extender.

Fish	N	Concentration (million/mL)	Total motility (%)	Progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	12	198.16 ± 10.72^{a}	95.50 ± 0.71^{b}	40.17 ± 3.90^{a}	54.33 ± 4.35^{a}	14.00 ± 1.91^{b}	27.50 ± 2.10^{a}	4.50 ± 0.71^{a}
2	8	241.82 ± 27.75^{a}	87.75 ± 2.38^{a}	39.00 ± 5.60^{a}	48.25 ± 6.43^{a}	9.75 ± 1.42^{ab}	29.25 ± 5.54^{a}	12.25 ± 2.38^{b}
3	8	218.47 ± 5.28^{a}	93.25 ± 1.66^{b}	42.75 ± 4.10^{a}	55.50 ± 4.95^{a}	6.75 ± 0.86^{a}	30.25 ± 3.02^{a}	6.75 ± 1.66^{a}

Note: ^{a,b}Means with different superscripts within a column were significantly different ($P < 0.05$)

Sperm after equilibration time

Table 4 indicates the concentration, total motility, progressive motility and velocity distribution of sperm after equilibration time for Fish 1, 2 and 3, respectively. There were significant differences in sperm characteristics of concentration and total motility among Fish 1 and 3 to Fish 2, in which Fish 1 showed a significantly higher concentration and total motility. However, in progressive motility, there was no significant difference among all fish. There was no significant difference among the three fish for rapid, medium and slow velocity distribution. However, there were significant differences between Fish 1 and 3 to Fish 2 for static velocity distribution, in which Fish 2 showed the highest static velocity.

Table 4. Concentration, total motility, progressive motility and velocity distribution (mean ± S. E.) of red tilapia sperm after equilibration time.

Fish	N	Concentration (million/mL)	Total motility (%)	Progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	12	188.92 ± 13.14^{b}	91.67 ± 1.17^{b}	42.92 ± 3.73^{a}	52.67 ± 4.69^{a}	7.08 ± 0.72^{a}	31.83 ± 3.50^{a}	8.33 ± 1.17^{a}
2	8	121.80 ± 28.63^{a}	73.38 ± 5.72^{a}	38.00 ± 2.20^{a}	42.38 ± 3.11^{a}	6.50 ± 0.85^{a}	24.63 ± 3.62^{a}	26.75 ± 5.77^{b}
3	8	186.03 ± 14.71^{b}	86.13 ± 2.07^{b}	37.63 ± 2.19^{a}	47.25 ± 2.47^{a}	8.63 ± 0.75^{a}	30.13 ± 1.22^{a}	13.88 ± 2.07^{a}

Note: ^{a,b}Means with different superscripts within a column were significantly different ($P < 0.05$)

Sperm after vapor phase

Table 5 demonstrates the concentration, total motility, progressive motility and velocity distribution of sperm after the vapor phase among the three fish. There were significant differences in sperm characteristics of concentration (between Fish 1 and 3 to Fish 2), total motility (among all fish) and progressive motility (between Fish 1 to Fish 2 and 3), in where Fish 1 showed the highest concentration, total motility and progressive motility. There was no significant difference in rapid and medium (between Fish 1 to Fish 2 and 3), slow (within Fish 1 and 2 to Fish 3), and static velocity distribution (among all fish).

Table 5. Concentration, total motility progressive motility and velocity distribution (mean ± S. E.) of red tilapia sperm after vapor phase

Fish	N	Concentration (million/mL)	Total motility (%)	Progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	12	129.83 ± 10.36^{b}	83.92 ± 1.47^{c}	45.58 ± 1.69^{b}	53.58 ± 1.87^{b}	7.83 ± 0.55^{b}	22.58 ± 2.19^{a}	16.08 ± 1.47^{a}
2	8	57.82 ± 13.25^{a}	49.00 ± 2.82^{a}	26.88 ± 4.70^{a}	28.38 ± 4.45^{a}	4.75 ± 1.25^{a}	15.75 ± 4.19^{a}	51.00 ± 2.82^{c}
3	8	128.36 ± 19.58^{b}	62.50 ± 4.72^{b}	22.00 ± 5.44^{a}	25.88 ± 6.25^{a}	5.00 ± 0.85^{a}	31.63 ± 2.42^{b}	37.50 ± 3.28^{b}

Note: ^{a,b,c}Means with different superscripts within a column were significantly different ($P < 0.05$)

Sperm after freezing

Table 6 shows the concentration, total motility and progressive motility of sperm after freezing for Fish 1, 2 and 3. There were significant differences in sperm characteristics of concentration (between Fish 1 and 3 to Fish 2), total motility (among all fish), and progressive motility (between Fish 1 and Fish 2), in which Fish 3 showed the highest concentration, whereas, Fish 1 showed the highest total motility and progressive motility. There were significant differences for rapid and slow velocity (between Fish 1 and Fish 2), and static velocity distribution (among the 3 fish), where Fish 1 showed the highest rapid and slow velocity, whereas, Fish 2 showed significantly the highest static velocity. However, in medium velocity distribution, there was no significant difference among all fish.

Table 6. Concentration, total motility, progressive motility and velocity distribution (mean ± S. E.) of red tilapia sperm after freezing

Fish	N	Concentration (million/mL)	Total motility (%)	Progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
1	12	108.98 ± 16.53^{b}	77.50 ± 1.86^{c}	33.83 ± 3.86^{b}	39.67 ± 4.51^{b}	8.00 ± 0.90^{a}	29.50 ± 4.86^{b}	22.50 ± 1.86^{a}
2	8	42.23 ± 4.85^{a}	38.75 ± 5.19^{a}	16.38 ± 4.35^{a}	17.88 ± 4.98^{a}	5.75 ± 0.86^{a}	15.25 ± 2.60^{a}	61.25 ± 5.19^{c}
3	8	139.01 ± 14.35^{b}	59.88 ± 3.50^{b}	24.00 ± 2.37^{ab}	28.38 ± 2.72^{ab}	8.00 ± 0.57^{a}	21.63 ± 3.06^{ab}	40.13 ± 3.50^{b}

Note: ^{a,b}Means with different superscripts within a column were significantly different ($P < 0.05$)

DISCUSSION

Effects of Different Stages of Freezing in Red Tilapia Sperm Cryopreservation

Cryopreservation is a technique involving a series of steps including sample collection, sperm extension, cryoprotectant selection, cooling, storage, thawing and viability detection (Tiersch, 2000). Development of protocols for sperm cryopreservation requires suitable choices at each step and consideration of the interactions among the factors. The methodology applied to develop sperm cryopreservation protocols remains mostly empirical because of a lack of conformity between data derived from theoretical work and the observed results, despite the progress of fundamental cryobiology (Gao *et al.*, 1997; Curry *et al.*, 2000). In the cryopreservation process of the present study, every stage showed different results of sperm motility, which meant that every stage had its own percentage of damaging effects especially to sperm motility. The motility of red tilapia fish sperm decreased gradually during the freezing process. The highest value of motility was obtained with fresh sperm (96.93 ± 0.53%), followed by sperm with extender (92.64 ± 1.05%), after equilibration time (84.86 ± 2.26%), and after vapor phase (67.82 ± 3.28%), while the lowest value of motility was obtained for sperm after freezing (61.39 ± 3.62%). This is in agreement with Guruprasad *et al.* (2007), who reported that during cryopreservation, sperm undergo dramatic changes in their intracellular and extracellular environment owing to exposure to cryoprotectant, cooling, freezing and thawing.

From the results, we can observe that the decrease in motility was higher at the vapor phase stage, whereas fresh sperm and fresh sperm with extender stages showed the lowest decreasing of motility compared to other stages. This result shows that the vapor phase plays an important role in the cryopreservation process of red tilapia fish sperm and it is also a critical part that affects the survivability of red tilapia sperm in the cryopreservation process. Previous studies show that cooling and freezing are traumatic events for sperm; the extent of these effects varies with the species (Maldjian *et al.*, 2004). These differences of defects or freezability of sperm are due to the differences in lipid composition of the sperm plasma membrane (Parks and Graham, 1992).

Due to the multiple steps and their interactions, errors at each step can accumulate and lead to considerable losses of variable cells. Thus, careful attention should be given to the numerous details at each step, and care should be taken to reduce or eliminate sources of uncontrolled variation (Leibo, 2000).

Effects of Different Stages of Freezing on Red Tilapia Sperm Cryopreservation for Individual Fish

Variation in the success rate of cryopreservation can be caused by variation in the response of sperm of individual males to the cryopreservation process. The use of selective breeding could improve factors in male catfish such as sperm motility, percent fertilization (dependent on egg quality), or possibly even cryopreservation performance (Christensen and Tiersch, 2005). In the present study, Fish 1 showed the highest value of sperm motility of frozen-thawed sperm compared to Fish 2 and 3. This observation showed that there were significant differences between individual fish in red tilapia sperm motility. This result is in agreement with the study done by Baynes and Scott (1987), which has shown that the decline in motility for fresh, chilled and frozen-thawed sperm was highly variable between individual males. The problem of considerable variation between males in their ability to withstand the freeze-thaw process occurs in other fish species (Baynes and Scott, 1987; Malejac *et al.*, 1990) and in mammals (Watson, 1995). This suggests a need to be selective in the storage and use of semen from individual males to eliminate collections with low frozen-thawed recoveries.

Throughout this study, stages after equilibration time, after vapor phase and after freezing showed significant differences compared to fresh sperm and sperm + extender. Individual fish also affected the percentage of survivability of fish sperm in red tilapia after freezing. From the study, Fish 1 (77.50 ± 1.86%) gave a higher total motility percentage after freezing compared with Fish 2

(38.75 ± 5.19%) and Fish 3 (59.88 ± 3.50%). Sperm are not adapted to survive cryopreservation, and therefore, have variable responses to cooling and re-warming, depending both on individual male and species (Holt, 2000; Thurston *et al*., 2002; Watson, 1990). The temperature related alterations in the organizational structure of the plasma membrane (Holt and North, 1994) may have profound effects on its functional properties. From this study we can suggest that there was variability between individual males of red tilapia fish sperm.

In conclusion, the present study indicates that different stages of fish sperm cryopreservation gave different effects to sperm survivability; therefore, for further study, attention should be given to numerous details at each step to reduce sources of uncontrolled variation. There is also variability between different individual fish, so to reduce the individual's error; a large number of fish samples are needed.

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REFERENCES

Asmad, K. 2005. The effect of equilibration time on cryopreservation of spermatozoa in red tilapia (*Oreochromis niloticus*). BSc. Thesis. Division of Biotechnology, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

Baynes, S. M. & Scott, A. P. 1987. Cryopreservation of rainbow trout spermatozoa: Influence of sperm quality, egg quality and extender composition on post-thawed fertility. *Aquaculture* **66:** 53-67.

Billard, R., Cosson, J., Crim, L. W. & Suquet, M. 1995. Sperm physiology and quality. In *Broodstock Management and Egg and Larval Quality*. N. R. Bromage and R. J. Roberts (eds.). Cambridge University Press. p. 53-76.

Christensen, J. M. & Tiersch, T. R. 2005. Cryopreservation of channel catfish sperm: Effects of cryoprotectant, exposure time, cooling rate, thawing conditions, and male-to-male variation. *Theriogenology* **63:** 2103-2112.

Curry, M. R., Kleinhans, F. W. & Watson, P. F. 2000. Measurement of the water permeability of the membranes of boar, ram, and rabbit spermatozoa using concentration-dependent self-quenching of an entrapped fluorophore. *Cryobiology* **41:** 167-173.

Drokin, S. I. 1993. Phospolipid distribution and fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in sperm of some freshwater and marine species of fish. *Aquatic Living Resources* **6:** 49-56.

Gao, D., Mazur, P. & Critser, J. K. 1997. Fundamental cryobiology of mammalian spermatozoa. In *Reproductive Tissues Banking*. A. M. Karow and J. K. Critser (eds.). Academic Press, San Diego. p. 263-327.

Guruprasad, K., Satish, K. A., Dinesh, U., Satish, R. & Pratap, K. 2007. Effect of cryopreservation on sperm DNA integrity in patients with teratospermia. *Fertility and Sterility* Article in press.

Holt, W. V. & North, R. D. 1994. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. *Biology of Reproductive* **51:** 414-424.

Holt, W. V. 2000. Fundamental aspects of sperm cryobiology; the importance of species and individual differences. *Theriogenology* **53:** 47-58.

Leibo, S. P. 2000. Sources of variation in cryopreservation. In *Cryopreservation in Aquatic Species*. T. R. Tiersch and P. M. Mazik (eds.). World Aquaculture Society, Baton Rouge, Louisiana. p. 75-83.

Maldjian, A., Pizzi, F., Gliozzi, T., Cerolini, S., Penny, P. & Noble, R. 2004. Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Theriogenology* **63:** 411-421.

Malejac, M. L., Loir, M. & Maisse, G. 1990. Qualite de la membrane des spermatozoids de truite are-en-ciel *Oncorhynhus mykiss*, relation avec l'aptitude du sperm a la congelation. *Aquatic Living Resources* **3:** 43-54.

Medeiros, C. M. O., Forell, F., Oliveira, A. T. D. & Rodrigues, J. L. 2002. Current status of sperm cryopreservation: Why isn't it better?. *Theriogenology* **57:** 327-344

Parks, J. E. & Graham, J. K. 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenology* **38:** 209-222.

Parks, J. E. & Lynch, D. V. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology* **29:** 255-266.

Roca, J., Rodriguez, M. H., Vazquez, J. M., Bolarin, A., Hernandez, M. & Saravia, F. 2006. To improve the fertility of frozen-thawed boar semen for artificial insemination. In *Control a Pig Production VII.* C. J. Ashworth and R. R. Kracling (*eds.*). Nottingham University Press. p. 261-275.

Tiersch, T. R. 2000. Introduction. In *Cryopreservation in Aquatic Species.* T. R. Tiersch and P. M. Mazik (eds.). World Aquaculture Society, Baton Rouge, LA. p. xix-xxvi.

Thurston, L. M., Siggins, K., Mileham, A. J., Watson, P. F. & Holt, W. V. 2002. Identification of amplified restriction fragment length polymorphism (AFLP) markers linked to genes controlling boar sperm viability following cryopreservation. *Biology of Reproductive* **66:** 545-554.

Watson, P. F. 1990. *Artificial insemination and the preservation of semen.* In *Marshall's Physiology of Reproduction Vol 2.* G. E. Lamming (ed.). Churchill Livingstone, London. p. 747-869.

Watson, P. F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and assessment of their post-thawing function. *Reproductive Fertility and Development* **7:** 871-891.