Comparison of two techniques for the morphometry study on gilthead seabream (*Sparus aurata*) spermatozoa and evaluation of changes induced by cryopreservation.

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Abstract
The development of powerful software has made possible spermatozoa morphology studies. However, some problems have emerged in relation to protocol standardization to compare results from different laboratories. This study was carried out to compare two techniques commonly used (staining vs phase contrast technique) for the morphometry study of gilthead seabream spermatozoa using an integrated sperm analysis system (ISAS®).

Spermatozoa morphometry values were significantly affected by the technique used, and phase contrast technique was found to be the best method, showing lower coefficients of variation on spermatozoa morphometry parameters measurements. Moreover, it has been shown that cryopreservation process produces damage in gilthead seabream spermatozoa, causing negative effects in sperm parameters as spermatozoa morphometry (a decrease in cell volume), motility (from 95 to 68% motile cells) and viability (from 95 to 87% of live cells), being the addition of freezing medium containing cryoprotectant (DMSO) the most important factor that caused the morphometry changes.

Keywords
Spermatozoa; Morphometry; Cryopreservation; Sparus aurata; ASMA; CASA
1. Introduction

Gilthead seabream (*Sparus aurata*) represents one of the most important cultured species in Mediterranean Sea, being the most produced teleost in the European Union. Despite raised production, little attention has been paid to sperm studies [1,2,3]. Spermatozoa morphology studies have been possible by the development of several potent software (known as computer assisted semen analysis, CASA) that has been shown as accurate, objective and repeatable technique [4]. These techniques have been validated for several species with different applications. In mammals, determination of spermatozoa head morphometry has been correlated with fertilization rates [5,6]; in fish, has been used in the development of cryopreservation methods [7].

However, despite specific software provide parameters that have improved the morphological spermatozoa assessment, some problems have emerged in relation to previous sample preparation. In one hand, there are methods based on different stains where a number of variables in the protocols as fixation techniques [8,9] or use of different stains [10,11] can influence in subsequent morphometry and, therefore, need to be standardized. On the other hand, there are methods based on phase contrast technique (PC) in which non-stained spermatozoa are analyzed under microscopes with phase contrast lens [12]. In previous studies, PC showed reliable results in teleost fish [7,12], and have as advantage no presenting as many protocol variables to standardize as staining methods, before morphometry sperm analysis. In this sense, to evaluate and find an optimal technique that produces the minimal variation in morphometry is an important requirement to allow comparisons between results from different research groups [13].

Moreover, among sperm management techniques, sperm cryopreservation has several applications in aquaculture such as synchronization of gamete availability, preservation of genetic variability or improvement of broodstock management [14]. However, it is known that cryopreservation causes lethal damage in fish spermatozoa and also produces important loss of membrane functions in live cells [15]. In fact, many reports in teleost fish have shown negative effects on morphology, motility and viability [16,17], but actually there are scarce reports on gilthead seabream sperm cryopreservation [18,19].

The main objectives of the present study were: first, the assessment of a good technique for morphometry analysis of gilthead seabream spermatozoa using a morphometry
software (ISAS®); and second, to know the influence that cryopreservation process has on the sperm quality of the gilthead seabream evaluating changes in spermatozoa morphometry, motility and viability.

2. Materials and methods

2.1 Fish sampling and sperm collection
In September 2009, 21 gilthead seabream male breeders from a hatchery were moved to the facilities of the Universidad Politécnica de Valencia (Spain). Males (1911 ± 79 g) were kept joined in a 1750 L fiberglass tank in a recirculation seawater system with compressed air supply. Photoperiod was natural and fish were handly fed using commercial fish feed once a day to apparent satiation. The study was carried out during the middle of the reproductive season (December-January).

For sperm collection fish were anesthetized with benzocaine (60 mg/L) and after cleaning the genital area with freshwater and thoroughly drying to avoid contamination of samples with faeces, urine and sea water, milt was collected by gentle abdominal pressure. A small aquarium air pump was modified to obtain a vacuum breathing force and to collect the sperm. A new tube was used for every male and distilled water was used to clean the collecting pipette between different males. Samples were maintained at 4 ºC until analysis and were evaluated before 60 min after extraction.

2.2 Evaluation of motility
Immediately after collection, the motility of sperm samples was assessed by mixing 1µl of sperm with 10 µl of artificial sea water (in mM: NaCl 354.7, MgCl₂ 52.4, CaCl₂ 9.9, Na₂SO₄ 28.2, KCl 9.4; 2% BSA (w/v), pH 8.2) adjusted to 1000 mOsm/kg as activation media [20]. All the motility analyses were performed in triplicate by the same trained observer to avoid subjective differences at 30s post-activation. Sperm samples from three males having over 90% of total motile spermatozoa were pooled to avoid individual male differences.

2.3 Freezing and thawing protocol
Gilthead seabream sperm was cryopreserved using the extender proposed by Fabbrocini et al. [18] (1% NaCl, 300 mOsmol/kg plus 5% DMSO), optimized by Cabrita et al. [19]
adding 10 mg/ml BSA to protect the plasma membrane and avoid sperm aggregation. One sperm dilution 1:6 (sperm: extender) was used. Sperm was packaged in 0.25 ml straws (IMV® Technologies, Láigle, Cedex, France), sealed with modeling paste and frozen in liquid nitrogen vapour, 1 cm above the liquid nitrogen level for 10 min, before being plunged into the liquid nitrogen for storage. Thawing took place in a 60 ºC water bath for 5 s.

2.4 Fluorescence stain analysis
Fluorescence stain analysis Live/Dead Sperm Viability Kit [SYBR/Propidium Iodide (PI) of Invitrogen (Barcelona, Spain)] was used to evaluate the viability of spermatozoa. The sperm diluted in P1 medium [21] was mixed with SYBR Green and PI, and kept for 20 min in dark incubation at room temperature. The final SYBR Green concentration was 104 times diluted from the original stock (2 µM) and PI 103 times from original stock (24 µM). At least 100 spermatozoa per sample were assessed in an epifluorescence microscope, using UV-2A (EX: 450-490 nm, DM: 505, BA: 520) filter. Spermatozoa were classified as dead when nuclei showed red fluorescence over sperm head and alive when they showed green fluorescence.

2.5 Techniques for morphometry measurements
For the both techniques sperm samples were diluted 1:50 (v/v) in P1 medium (NaCl 354.7, MgCl2 52.4, CaCl2 9.9, Na2SO4 28.2, KCl 9.4; 2% BSA (w/v), pH 8).

a) Staining technique (HH): Smears were prepared by carefully dragging a 20 mL drop of the diluted sperm across a slide and air-drying it during 2 h. Harris’s Hematoxylin (Panreac Química S.A.) staining was carried out during 30 min after fixing samples 10 min in methanol. Slides were then exposed to dry air and kept permanently mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany) and viewed using a 1000x non-phase lens.

b) Phase contrast technique (PC): Sperm pooled samples were fixed with 2.5% glutaraldehyde and were deposited in eppendorf tubes. An aliquot of sperm dilution (approximately 10 µl) was introduced in an Improved Neubauer hemocytometer to sperm analysis). Sperm samples were viewed using a 1000x phase contrast lens.
2.6 Spermatozoa morphometry analysis

Morphometry analyses of sperm pools were performed using the morphometry module of the ISAS® software (Proiser R+D, S.L.; Buñol, Spain) using a ISAS® 782M camera. Spermatozoa head measurements calculated automatically by ISAS® after selecting the appropriate cells included the size variables: length (L), width (W), area (A), and perimeter (P); and shape variables: ellipticity (L/W), rugosity (4πA/P²), elongation (L-W)/(L+W) and regularity (πLW/4A).

2.7 Influence of number of spermatozoa in the morphometry analysis

One hundred properly digitized spermatozoa were analyzed from five pooled samples. Subgroups of 25, 50, 75 and 100 randomly selected spermatozoa were compared both between pools and within pools to determine the minimum sample size needed to characterize the sperm population.

2.8 Experimental design

Experiment 1. Evaluation of two techniques for the morphometry spermatozoa analysis.

To determine the technique effect on spermatozoa morphometry 15 pools of fresh sperm were analyzed. At least 100 spermatozoa from each pool were digitized and analyzed using both techniques: HH and PC. Moreover, to evaluate the accuracy of the PC technique, subsets of 25, 50, 75 and 100 randomly selected spermatozoa were compared to determine the minimum sample size needed to characterize the whole population.

Experiment 2. Influence of cryopreservation on sperm morphometry.

To determine the cryopreservation effect on spermatozoa morphometry during cryopreservation process, differing live and dead spermatozoa, 15 pools were analyzed. At least 100 live, 100 dead and 100 randomly selected spermatozoa were analyzed for each frozen–thawed pool.

Experiment 3. Influence of cryopreservation on sperm motility and viability.

Motility (percentage of motile cells) and viability (percentage of live cells) values of 15 pools were recorded for fresh sperm and frozen-thawed sperm.

2.9 Statistical analysis
The mean and standard error of the mean were calculated for spermatozoa morphometry parameters (area, perimeter, length, width, ellipticity, elongation, rugosity and regularity). Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) and $t$-student test were used to analyze data with normal distribution. Significant differences between treatments were detected using the Student-Newman-Keuls (SNK) multiple range test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks and Mann-Whitney $U$-test were used. Moreover, “within-pools” coefficient of variation ($CV_{wp}$) represents the mean of CV obtained for each pool, and the “between-pools” coefficient of variation ($CV_{bp}$) was estimated as the CV of all data pools. All statistical analyses were performed using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

In Experiment 1, PC technique offered better images of spermatozoa head in terms of intensity, contrast and image definition, in comparison with those obtained by HH technique (Figure 1, A1 vs B1). However, whatever that was the origin of the images, once digitalized and converted to gray scale by ISAS® software, they had similar aspects (Figure 1, A2/3 vs B2/3, for PC and HH, respectively) but showing the differences caused by the technique used. Spermatozoa displayed larger size when were stained with hematoxylin (HH) and significant differences with non-stained spermatozoa (PC) were obtained on all size and shape parameters, indicating the handling procedure affected the final results (Table 1). Coefficients of variation both within pools and between pools obtained in the study showed that HH technique presented higher variability than PC technique on all morphometry parameters analyzed (Table 2), so PC technique was showed as the most accurate method for the morphometry analysis.

No significant differences in morphometry values were recorded within each pool counting 100, 75, 50 or 25 cells with the exception of pool 1 (Table 3), in which area obtained from 25 spermatozoa measurement was significantly lower to those obtained with 50, 75 or 100. However, it is important to note that when comparing between
different pools, significative differences become more evident with the increase of analyzed spermatozoa from 25 to 100.

In Experiment 2, only significant differences in size parameters were found in width, where fresh sperm cells showed higher values than diluted and frozen-thawed spermatozoa (Figure 2). In shape parameters, diluted and frozen-thawed sperm cells showed similar values between them, with significant higher values in ellipticity and elongation than fresh spermatozoa. With the exception of area and perimeter, live spermatozoa showed significant differences with dead spermatozoa on the rest of size and shape parameters (Figure 3.). Dead cells showed higher length and lower width, showing results a more elliptical and elongated shape than live spermatozoa. Also, dead spermatozoa showed higher rugosity and lower regularity than live cells.

In experiment 3, motility and viability cells showed significant differences between fresh and frozen-thawed sperm (Figure 4). Fresh spermatozoa showed higher percentages of motility (95%) than frozen-thawed sperm (68%). Viability of cells showed a decrease after the cryopreservation process, with final values around 87% of live spermatozoa.

4. Discussion

The present study shows the importance of the choice of an accurate and standardized method for the morphometry characterization of fish spermatozoa, in this case of gilthead seabream. Despite digitalized images obtained by ISAS® software using both techniques had similar contrast and intensity, the best initial images were obtained by PC technique. In this sense, initial images with a high quality are necessary to obtain reliable results by CASA analysis [11], therefore phase contrast method seems to be the best technique for the sperm morphometry analysis.

In relation to staining method, different stains as Diff-Quick, Hemacolor, Hematoxylin or Spermac have been widely used in other fish species as Atlantic cod [22], rainbow trout [23] or perch [24], but there are no previous studies analyzing their effects on the spermatozoa morphometry in gilthead seabream. However, despite HH technique has also been used with great results in other fish spermatozoa [25], coefficients of variation on size and shape parameters were significantly lower with PC technique. These differences may be caused because staining methods have several analytical variables such as different staining products and fixation protocols [25,26,27], that may influence
on morphometry parameters and must be standardized previously and properly. Also, it should be considered that staining protocol used in this study is not still optimized for this specie. The development of a staining technique is not simple and should incorporate many tests with different extenders and times to dry, fix and color. So, more studies should be developed to improve and to know the real possibilities of this technique on the spermatozoa morphometry in gilthead seabream and similar species.

On the contrary, sample preparation in phase contrast technique is direct and simple, which minimizes the possibility that sperm head dimensions would be influenced along protocol stages [25]. In this sense, good results were obtained using PC technique in some species of marine teleosts as sharpnout sea bream (Diplodus puntazzo), gilthead seabream (Sparus aurata) or European eel (Anguilla anguilla) [12, 26]. The use of techniques that decrease coefficients of variation should be a prerequisite for any large-scale scientific application in commercial aquaculture [13]. Therefore, PC technique is showed as a useful and simple method for measuring head spermatozoa, avoiding variability on morphometry parameters.

Moreover, biological variation is another variable that can affect the results of the morphometry sperm analysis: if an insufficient number of spermatozoa are analyzed, the variation in a sample population may make confuse the interpretation of acquired data [27]. The present study showed that no significant differences were found in a same pool (except in one pool) when was analyzed considering different numbers of cells. However, this result can be read from two viewpoints. In one hand, to analyze fewer spermatozoa can reduce substantially the laboratory workload but, on the other hand, if an insufficient number of spermatozoa is analyzed, the coefficient of variation in each sperm sample will increase, making more difficult to detect differences between pools, males, treatments, etc. [11, 27]. In this sense, differences between pools became evident by increasing the number of analyzed cells. Our results proved the importance of choosing an accurate, reliable and standardized method for spermatozoa morphometry analysis with the aim of identifying variations in gilthead seabream sperm samples.

In relation to morphometry changes induced by cryopreservation process, to our best knowledge it is the first report on the morphometric analysis of cryopreserved gilthead seabream spermatozoa. During cryopreservation process, diluted sperm and frozen-thawed spermatozoa showed a little decrease of head area, as occur in other species [25, 28]. It has been suggested that these changes on head size are due to a cell water flux from inside spermatozoa to the external medium as a result of a high osmolality of
DMSO [7,29,30]. In this sense, gilthead seabream seminal plasma shows an osmolality between 360-390 mOsmol/kg [18,19,31] and the addition of cryoprotector (DMSO) on the freezing medium can increase osmolality up to 1000 mOsmol/kg [14], causing these changes in cell volume.

However, besides variations in spermatozoa head area, width was the morphometry parameter most influenced by cryopreservation process, where diluted and frozen-thawed spermatozoa showed lower values than fresh cells. These width changes caused also significant differences in other related shape parameters, and cells submitted to cryopreservation process showed higher values on ellipticity and elongation. These shape changes can be due to multiple factors related to cryopreservation process, as progressive dehydration of the spermatozoa and/or a loss of sperm membrane integrity and functionality [32,33]. Moreover, no significant differences in any shape parameters (except roughness) were found between cells on diluted sperm and frozen-thawed sperm, suggesting that, in addition to the known effect generated by the freezing rates, the morphometry changes induced in the cryopreservation process were also due to the dilution of sperm with the freezing medium, in addition to freezing and thawing processes itself. The differences found in roughness between cells on diluted sperm and frozen-thawed sperm are probably due to membrane damage caused by the formation of ice crystals on the freezing process.

Also, differences in head size and shape between live and dead spermatozoa were detected after cryopreservation process, surely because the dead spermatozoa have modified the membrane function and do not have the ability to maintain osmotic equilibrium [7,25]. Thus, a different proportion of dead spermatozoa in a frozen-thawed sample could underestimate or overestimate the real values of cryopreserved spermatozoa morphometry. Therefore, it is important to know the proportion of live and dead spermatozoa present in samples to can compare cryopreservation results between different laboratories, animal species or cryopreservation protocols.

On the other hand, it is known that cryopreservation has a negative effect on spermatozoa motility and viability, which affect both marine and freshwater fish species [38,39,40]. Some important features to establish cryopreservation protocols are the appropriate choice of the extender, the cryoprotectant as well as the cooling and thawing conditions [14]. In the present work, we have used the protocol proposed by Fabbrocini et al. [18] optimized through the addition of BSA by Cabrita et al. [19]. In this study, fresh samples showed values around 95% of motility and viability, but after
cryopreservation there was a significant decrease in both parameters, with values around 68% and 88%, respectively. Usually, in marine fish species the motility of frozen-thawed sperm is quite high [41,42,43] and, in this sense, we obtained also high motility values like other authors in gilthead seabream cryopreserved sperm [18, 19, 44]. This decline in motility and viability could be due to multiple factors; Cabrita et al. [19] reported recently the changes produced after gilthead seabream sperm cryopreservation and they demonstrated that spermatozoa suffer several damages after freezing/thawing process as ATP decrease, loss of membrane functionality and loss of mitochondrial integrity. Nowadays, cryopreservation is a useful tool used on aquaculture production and, despite of sperm of many marine fish species have been cryopreserved, more studies about cellular/molecular damages should be developed to know the limitations of this technique on fertilization.

The main conclusions from this study were that (i) it is possible to use a simple method for measuring head spermatozoa, without staining, which minimizes the possibility that spermatozoa head dimensions would be influenced by procedural protocol; (ii) to get an accurate and reliable spermatozoa morphometry assessment seems necessary to standardize several factors, as the number of analyzed cells, with the aim of detecting small variations between samples; and (iii) cryopreserved process in gilthead seabream caused negative effects in sperm quality parameters as morphometry, motility and viability, being the addition of freezing medium the most important factor causing the morphometry changes.

Acknowledgements

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References


integrity in fresh and cryopreserved rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Cryobiology 1997;34:141-49.


Table legends

**Table 1.** Size and shape morphometry parameters measured on gilthead seabream spermatozoa head using a phase contrast (PC) and staining technique (HH). Data are expressed as mean ± SEM. Asterisk means significant differences between techniques.

**Table 2.** Coefficients of variation (CV, %) within pools (CV\textsubscript{wp}) and between pools (CV\textsubscript{bp}) for morphometry measurements of spermatozoa heads by phase contrast (PC) and staining technique (HH).

**Table 3.** Values of spermatozoa head morphometry parameters of five pools after evaluating 25, 50, 75 or 100 spermatozoa. Different letters indicate significant differences between pools within morphometry parameter and number of counted cells. Asterisk indicates significant differences within same pool when different numbers of counted cells were considered. Data are expressed as mean ± SEM.

Figure legends

**Figure 1.** Morphology of head of gilthead seabream spermatozoa: A1, picture of spermatozoa using PC technique; A2/A3, images of these spermatozoa once digitalized by ISAS\textsuperscript{®} software; B1, picture of stained spermatozoa using HH technique; B2/BA3, pictures of these stained spermatozoa once digitalized by ISAS\textsuperscript{®} software.

**Figure 2.** Size and shape morphometry values of gilthead seabream spermatozoa head on fresh sperm (FRESH), diluted sperm (DILUTED) and frozen-thawed sperm (CRYO). Data are expressed as mean ± SEM and different letters indicate significant differences.

**Figure 3.** Size and shape variables in live and dead gilthead seabream spermatozoa. Data are expressed as mean ± SEM and different letters indicate significant differences.

**Figure 4.** Percentage of motile cells and spermatozoa viability in fresh sperm (FRESH) and frozen-thawed sperm (CRYO) in gilthead seabream. Data are expressed as mean ± SEM.
SEM and different letters indicate significant differences.
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Figure 2.

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Figure 3.

- **Area (µm²)**: LIVE vs. DEAD
- **Length (µm)**: LIVE vs. DEAD
- **Width (µm)**: LIVE vs. DEAD
- **Ellipticity**: LIVE vs. DEAD
- **Elongation**: LIVE vs. DEAD
- **Rugosity**: LIVE vs. DEAD
- **Regularity**: LIVE vs. DEAD
Figure 4.

![Graph showing motile and live cell percentages for FRESH and CRYO samples.](image-url)