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Gallego Albiach, V.; Peñaranda, D.; Marco Jiménez, F.; Mazzeo, I.; Pérez Igualada, LM.; Asturiano Nemesio, JF. (2012). Comparison of two techniques for the morphometry study on gilthead seabream (Sparus aurata) spermatozoa and evaluation of changes induced by cryopreservation. Theriogenology. 77(6):1078-1087. doi:10.1016/j.theriogenology.2011.10.010,.

The final publication is available at

https://dx.doi.org/10.1016/j.theriogenology.2011.10.010

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REVISED

1 **Comparison of two techniques for the morphometry study on gilthead**
2 **seabream (*Sparus aurata*) spermatozoa and evaluation of changes**
3 **induced by cryopreservation.**
4
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31 ## Abstract

32 The development of powerful software has made possible spermatozoa morphology
33 studies. However, some problems have emerged in relation to protocol standardization
34 to compare results from different laboratories. This study was carried out to compare
35 two techniques commonly used (staining vs phase contrast technique) for the
36 morphometry study of gilthead seabream spermatozoa using an integrated sperm
37 analysis system (ISAS®).
38 Spermatozoa morphometry values were significantly affected by the technique used,
39 and phase contrast technique was found to be the best method, showing lower
40 coefficients of variation on spermatozoa morphometry parameters measurements.
41 Moreover, it has been shown that cryopreservation process produces damage in gilthead
42 seabream spermatozoa, causing negative effects in sperm parameters as spermatozoa
43 morphometry (a decrease in cell volume), motility (from 95 to 68% motile cells) and
44 viability (from 95 to 87% of live cells), being the addition of freezing medium
45 containing cryoprotectant (DMSO) the most important factor that caused the
46 morphometry changes.

47

48 **Keywords**

49 Spermatozoa; Morphometry; Cryopreservation; *Sparus aurata*; ASMA; CASA

50 # 1. Introduction

51 Gilthead seabream (*Sparus aurata*) represents one of the most important cultured
52 species in Mediterranean Sea, being the most produced teleost in the European Union.
53 Despite raised production, little attention has been paid to sperm studies [1,2,3].
54 Spermatozoa morphology studies have been possible by the development of several
55 potent software (known as computer assisted semen analysis, CASA) that has been
56 shown as accurate, objective and repeatable technique [4]. These techniques have been
57 validated for several species with different applications. In mammals, determination of
58 spermatozoa head morphometry has been correlated with fertilization rates [5,6]; in fish,
59 has been used in the development of cryopreservation methods [7].
60 However, despite specific software provide parameters that have improved the
61 morphological spermatozoa assessment, some problems have emerged in relation to
62 previous sample preparation. In one hand, there are methods based on different stains
63 where a number of variables in the protocols as fixation techniques [8,9] or use of
64 different stains [10,11] can influence in subsequent morphometry and, therefore, need to
65 be standardized. On the other hand, there are methods based on phase contrast technique
66 (PC) in which non-stained spermatozoa are analyzed under microscopes with phase
67 contrast lens [12]. In previous studies, PC showed reliable results in teleost fish [7,12],
68 and have as advantage no presenting as many protocol variables to standardize as
69 staining methods, before morphometry sperm analysis. In this sense, to evaluate and
70 find an optimal technique that produces the minimal variation in morphometry is an
71 important requirement to allow comparisons between results from different research
72 groups [13].
73 Moreover, among sperm management techniques, sperm cryopreservation has several
74 applications in aquaculture such as synchronization of gamete availability, preservation
75 of genetic variability or improvement of broodstock management [14]. However, it is
76 known that cryopreservation causes lethal damage in fish spermatozoa and also
77 produces important loss of membrane functions in live cells [15]. In fact, many reports
78 in teleost fish have shown negative effects on morphology, motility and viability
79 [16,17], but actually there are scarce reports on gilthead seabream sperm
80 cryopreservation [18,19].
81 The main objectives of the present study were: first, the assessment of a good technique
82 for morphometry analysis of gilthead seabream spermatozoa using a morphometry

83 software (ISAS®); and second, to know the influence that cryopreservation process has
84 on the sperm quality of the gilthead seabream evaluating changes in spermatozoa
85 morphometry, motility and viability.
86

87 ## 2. Materials and methods

88

89 ### 2.1 Fish sampling and sperm collection

90 In September 2009, 21 gilthead seabream male breeders from a hatchery were moved to
91 the facilities of the Universidad Politécnica de Valencia (Spain). Males (1911 ± 79 g)
92 were kept joined in a 1750 L fiberglass tank in a recirculation seawater system with
93 compressed air supply. Photoperiod was natural and fish were handly fed using
94 commercial fish feed once a day to apparent satiation. The study was carried out during
95 the middle of the reproductive season (December-January).
96 For sperm collection fish were anesthetized with benzocaine (60 mg/L) and after
97 cleaning the genital area with freshwater and thoroughly drying to avoid contamination
98 of samples with faeces, urine and sea water, milt was collected by gentle abdominal
99 pressure. A small aquarium air pump was modified to obtain a vacuum breathing force
100 and to collect the sperm. A new tube was used for every male and distilled water was
101 used to clean the collecting pipette between different males. Samples were maintained
102 at 4 ºC until analysis and were evaluated before 60 min after extraction.
103

104 ### 2.2 Evaluation of motility

105 Immediately after collection, the motility of sperm samples was assessed by mixing 1µl
106 of sperm with 10 µl of artificial sea water (in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9,
107 Na_2SO_4 28.2, KCl 9.4; 2% BSA (w/v), pH 8.2) adjusted to 1000 mOsm/kg as activation
108 media [20]. All the motility analyses were performed in triplicate by the same trained
109 observer to avoid subjective differences at 30s post-activation. Sperm samples from
110 three males having over 90% of total motile spermatozoa were pooled to avoid
111 individual male differences.
112

113 ### 2.3 Freezing and thawing protocol

114 Gilthead seabream sperm was cryopreserved using the extender proposed by Fabbrocini
115 *et al*. [18] (1% NaCl, 300 mOsmol/kg plus 5% DMSO), optimized by Cabrita *el al*. [19]

116 adding 10 mg/ml BSA to protect the plasma membrane and avoid sperm aggregation.
117 One sperm dilution 1:6 (sperm: extender) was used.
118 Sperm was packaged in 0.25 ml straws (IMV® Technologies, Láigle, Cedex, France),
119 sealed with modeling paste and frozen in liquid nitrogen vapour, 1 cm above the liquid
120 nitrogen level for 10 min, before being plunged into the liquid nitrogen for storage.
121 Thawing took place in a 60 ºC water bath for 5 s.
122
123 **2.4 Fluorescence stain analysis**
124 Fluorescence stain analysis Live/Dead Sperm Viability Kit [SYBR/Propidium Iodide
125 (PI) of Invitrogen (Barcelona, Spain)] was used to evaluate the viability of spermatozoa.
126 The sperm diluted in P1 medium [21] was mixed with SYBR Green and PI, and kept for
127 20 min in dark incubation at room temperature. The final SYBR Green concentration
128 was 104 times diluted from the original stock (2 µM) and PI 103 times from original
129 stock (24 µM). At least 100 spermatozoa per sample were assessed in an
130 epifluorescence microscope, using UV-2A (EX: 450-490 nm, DM: 505, BA: 520) filter.
131 Spermatozoa were classified as dead when nuclei showed red fluorescence over sperm
132 head and alive when they showed green fluorescence.
133
134 **2.5 Techniques for morphometry measurements**
135 For the both techniques sperm samples were diluted 1:50 (v/v) in P1 medium (NaCl
136 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4; 2% BSA (w/v), pH 8).
137
138 a) Staining technique (HH): Smears were prepared by carefully dragging a 20 mL drop
139 of the diluted sperm across a slide and air-drying it during 2 h. Harris's Hematoxylin
140 (Panreac Química S.A.) staining was carried out during 30 min after fixing samples 10
141 min in methanol. Slides were then exposed to dry air and kept permanently mounted
142 with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany) and viewed using a 1000x
143 non-phase lens.
144
145 b) Phase contrast technique (PC): Sperm pooled samples were fixed with 2.5%
146 glutaraldehyde and were deposited in eppendorf tubes. An aliquot of sperm dilution
147 (approximately 10 µl) was introduced in an Improved Neubauer hemocytometer to
148 sperm analysis). Sperm samples were viewed using a 1000x phase contrast lens.
149

150

151 **2.6 Spermatozoa morphometry analysis**

152 Morphometry analyses of sperm pools were performed using the morphometry module
153 of the ISAS® software (Proiser R+D, S.L.; Buñol, Spain) using a ISAS® 782M camera.
154 Spermatozoa head measurements calculated automatically by ISAS® after selecting the
155 appropriate cells included the size variables: length (L), width (W), area (A), and
156 perimeter (P); and shape variables: ellipticity (L/W), rugosity ($4\pi A/P^2$), elongation (L-
157 W)/(L+W) and regularity ($\pi LW/4A$).

158

159 **2.7 Influence of number of spermatozoa in the morphometry analysis**

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

164

165 **2.8 Experimental design**

166 *Experiment 1. Evaluation of two techniques for the morphometry spermatozoa analysis.*
167 To determine the technique effect on spermatozoa morphometry 15 pools of fresh sperm
168 were analyzed. At least 100 spermatozoa from each pool were digitized and analyzed
169 using both techniques: HH and PC. Moreover, to evaluate the accuracy of the PC
170 technique, subsets of 25, 50, 75 and 100 randomly selected spermatozoa were compared
171 to determine the minimum sample size needed to characterize the whole population.

172

173 *Experiment 2. Influence of cryopreservation on sperm morphometry.*
174 To determine the cryopreservation effect on spermatozoa morphometry during
175 cryopreservation process, differing live and dead spermatozoa, 15 pools were analyzed.
176 At least 100 live, 100 dead and 100 randomly selected spermatozoa were analyzed for
177 each frozen–thawed pool.

178

179 *Experiment 3. Influence of cryopreservation on sperm motility and viability.*
180 Motility (percentage of motile cells) and viability (percentage of live cells) values of 15
181 pools were recorded for fresh sperm and frozen-thawed sperm.

182

183 **2.9 Statistical analysis**

184 The mean and standard error of the mean were calculated for spermatozoa morphometry
185 parameters (area, perimeter, length, width, ellipticity, elongation, rugosity and
186 regularity). Shapiro-Wilk and Levene tests were used to check the normality of data
187 distribution and variance homogeneity, respectively. One-way analysis of variance
188 (ANOVA) and *t*-student test were used to analyze data with normal distribution.
189 Significant differences between treatments were detected using the Student-Newman-
190 Keuls (SNK) multiple range test ($P<0.05$). For non-normally distributed populations,
191 Kruskal-Wallis one-way ANOVA on ranks and Mann-Whitney *U*-test were used.
192 Moreover, "within-pools" coefficient of variation (CV_{wp}) represents the mean of CV
193 obtained for each pool, and the "between-pools" coefficient of variation ($CV_{bp)}$ was
194 estimated as the CV of all data pools. All statistical analyses were performed using the
195 statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL,
196 USA).
197

198 **3. Results**

199 In Experiment 1, PC technique offered better images of spermatozoa head in terms of
200 intensity, contrast and image definition, in comparison with those obtained by HH
201 technique (Figure 1, A1 vs B1). However, whatever that was the origin of the images,
202 once digitalized and converted to gray scale by ISAS® software, they had similar
203 aspects (Figure 1, A2/3 vs B2/3, for PC and HH, respectively) but showing the
204 differences caused by the technique used.
205 Spermatozoa displayed larger size when were stained with hematoxylin (HH) and
206 significant differences with non-stained spermatozoa (PC) were obtained on all size and
207 shape parameters, indicating the handling procedure affected the final results (Table 1).
208 Coefficients of variation both within pools and between pools obtained in the study
209 showed that HH technique presented higher variability than PC technique on all
210 morphometry parameters analyzed (Table 2), so PC technique was showed as the most
211 accurate method for the morphometry analysis.
212 No significant differences in morphometry values were recorded within each pool
213 counting 100, 75, 50 or 25 cells with the exception of pool 1 (Table 3), in which area
214 obtained from 25 spermatozoa measurement was significantly lower to those obtained
215 with 50, 75 or 100. However, it is important to note that when comparing between

216 different pools, significative differences become more evident with the increase of
217 analyzed spermatozoa from 25 to 100.
218 In Experiment 2, only significant differences in size parameters were found in width,
219 where fresh sperm cells showed higher values than diluted and frozen-thawed
220 spermatozoa (Figure 2). In shape parameters, diluted and frozen-thawed sperm cells
221 showed similar values between them, with significant higher values in ellipticity and
222 elongation than fresh spermatozoa. With the exception of area and perimeter, live
223 spermatozoa showed significant differences with dead spermatozoa on the rest of size
224 and shape parameters (Figure 3.). Dead cells showed higher length and lower width,
225 showing results a more elliptical and elongated shape than live spermatozoa. Also, dead
226 spermatozoa showed higher rugosity and lower regularity than live cells.
227 In experiment 3, motility and viability cells showed significant differences between
228 fresh and frozen-thawed sperm (Figure 4). Fresh spermatozoa showed higher
229 percentages of motility (95%) than frozen-thawed sperm (68%). Viability of cells
230 showed a decrease after the cryopreservation process, with final values around 87% of
231 live spermatozoa.
232

233 ## 4. Discussion

234 The present study shows the importance of the choice of an accurate and standardized
235 method for the morphometry characterization of fish spermatozoa, in this case of
236 gilthead seabream. Despite digitalized images obtained by ISAS® software using both
237 techniques had similar contrast and intensity, the best initial images were obtained by
238 PC technique. In this sense, initial images with a high quality are necessary to obtain
239 reliable results by CASA analysis [11], therefore phase contrast method seems to be the
240 best technique for the sperm morphometry analysis.
241 In relation to staining method, different stains as Diff-Quick, Hemacolor, Hematoxylin
242 or Spermac have been widely used in other fish species as Atlantic cod [22], rainbow
243 trout [23] or perch [24], but there are no previous studies analyzing their effects on the
244 spermatozoa morphometry in gilthead seabream. However, despite HH technique has
245 also been used with great results in other fish spermatozoa [25], coefficients of variation
246 on size and shape parameters were significantly lower with PC technique. These
247 differences may be caused because staining methods have several analytical variables
248 such as different staining products and fixation protocols [25,26,27], that may influence

249 on morphometry parameters and must be standardized previously and properly. Also, it
250 should be considered that staining protocol used in this study is not still optimized for
251 this specie. The development of a staining technique is not simple and should
252 incorporate many tests with different extenders and times to dry, fix and color. So, more
253 studies should be developed to improve and to know the real possibilities of this
254 technique on the spermatozoa morphometry in gilthead seabream and similar species.
255 On the contrary, sample preparation in phase contrast technique is direct and simple,
256 which minimizes the possibility that sperm head dimensions would be influenced along
257 protocol stages [25]. In this sense, good results were obtained using PC technique in
258 some species of marine teleosts as sharpsnout sea bream (*Diplodus puntazzo*), gilthead
259 seabream (*Sparus aurata*) or European eel (*Anguilla anguilla*) [12,26]. The use of
260 techniques that decrease coefficients of variation should be a prerequisite for any large-
261 scale scientific application in commercial aquaculture [13]. Therefore, PC technique is
262 showed as a useful and simple method for measuring head spermatozoa, avoiding
263 variability on morphometry parameters.
264 Moreover, biological variation is another variable that can affect the results of the
265 morphometry sperm analysis: if an insufficient number of spermatozoa are analyzed, the
266 variation in a sample population may make confuse the interpretation of acquired data
267 [27]. The present study showed that no significant differences were found in a same
268 pool (except in one pool) when was analyzed considering different numbers of cells.
269 However, this result can be read from two viewpoints. In one hand, to analyze fewer
270 spermatozoa can reduce substantially the laboratory workload but, on the other hand, if
271 an insufficient number of spermatozoa is analyzed, the coefficient of variation in each
272 sperm sample will increase, making more difficult to detect differences between pools,
273 males, treatments, etc. [11,27]. In this sense, differences between pools became evident
274 by increasing the number of analyzed cells. Our results proved the importance of
275 choosing an accurate, reliable and standardized method for spermatozoa morphometry
276 analysis with the aim of identifying variations in gilthead seabream sperm samples.
277 In relation to morphometry changes induced by cryopreservation process, to our best
278 knowledge it is the first report on the morphometric analysis of cryopreserved gilthead
279 seabream spermatozoa. During cryopreservation process, diluted sperm and frozen-
280 thawed spermatozoa showed a little decrease of head area, as occur in other species
281 [25,28]. It has been suggested that these changes on head size are due to a cell water
282 flux from inside spermatozoa to the external medium as a result of a high osmolality of

283 DMSO [7,29,30]. In this sense, gilthead seabream seminal plasma shows an osmolality
284 between 360-390 mOsmol/kg [18,19,31] and the addition of cryoprotector (DMSO) on
285 the freezing medium can increase osmolality up to 1000 mOsmol/kg [14], causing these
286 changes in cell volume.
287 However, besides variations in spermatozoa head area, width was the morphometry
288 parameter most influenced by cryopreservation process, where diluted and frozen-
289 thawed spermatozoa showed lower values than fresh cells. These width changes caused
290 also significant differences in other related shape parameters, and cells submitted to
291 cryopreservation process showed higher values on ellipticity and elongation. These
292 shape changes can be due to multiple factors related to cryopreservation process, as
293 progressive dehydration of the spermatozoa and/or a loss of sperm membrane integrity
294 and functionality [32,33]. Moreover, no significant differences in any shape parameters
295 (except roughness) were found between cells on diluted sperm and frozen-thawed
296 sperm, suggesting that, in addition to the known effect generated by the freezing rates,
297 the morphometry changes induced in the cryopreservation process were also due to the
298 dilution of sperm with the freezing medium, in addition to freezing and thawing
299 processes itself. The differences found in roughness between cells on diluted sperm and
300 frozen-thawed sperm are probably due to membrane damage caused by the formation of
301 ice crystals on the freezing process.
302 Also, differences in head size and shape between live and dead spermatozoa were
303 detected after cryopreservation process, surely because the dead spermatozoa have
304 modified the membrane function and do not have the ability to maintain osmotic
305 equilibrium [7,25]. Thus, a different proportion of dead spermatozoa in a frozen-thawed
306 sample could underestimate or overestimate the real values of cryopreserved
307 spermatozoa morphometry. Therefore, it is important to know the proportion of live and
308 dead spermatozoa present in samples to can compare cryopreservation results between
309 different laboratories, animal species or cryopreservation protocols.
310 On the other hand, it is known that cryopreservation has a negative effect on
311 spermatozoa motility and viability, which affect both marine and freshwater fish species
312 [38,39,40]. Some important features to establish cryopreservation protocols are the
313 appropriate choice of the extender, the cryoprotectant as well as the cooling and thawing
314 conditions [14]. In the present work, we have used the protocol proposed by Fabbrocini
315 *et al.* [18] optimized through the addition of BSA by Cabrita *et al.* [19]. In this study,
316 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

This work was financed by the Spanish Ministry of Science and Innovation (MICINN; AGL2007-64040-C03-00, Project SELECTBREAM). V. Gallego and I. Mazzeo were supported by predoctoral scholarships financed by the Spanish MICINN and Generalitat Valenciana, respectively. D.S. Peñaranda had a postdoctoral grant from UPV.

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474 ## Table legends

475

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

479

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

483

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

489

490 ## Figure legends

491

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

496

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

501

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

504

505 **Figure 4.** Percentage of motile cells and spermatozoa viability in fresh sperm (FRESH)
506 and frozen-thawed sperm (CRYO) in gilthead seabream. Data are expressed as mean ±

507 SEM and different letters indicate significant differences.

508 **Table 1.**

	Technique	
	PC	**HH**
Area (μm^2)	4.89 ± 0.051*	6.98 ± 0.126
Perimeter (µm)	8.08 ± 0.044*	10.31 ± 0.090
Length (µm)	2.57 ± 0.014*	3.14 ± 0.016
Width (µm)	2.34 ± 0.014*	2.75 ± 0.032
Ellipticity	1.10 ± 0.004*	1.16 ± 0.009
Elongation	0.05 ± 0.002*	0.07 ± 0.003
Rugosity	0.94 ± 0.001*	0.82 ± 0.003
Regularity	0.97 ± 0.001*	0.98 ± 0.004

509 **Table 2.**

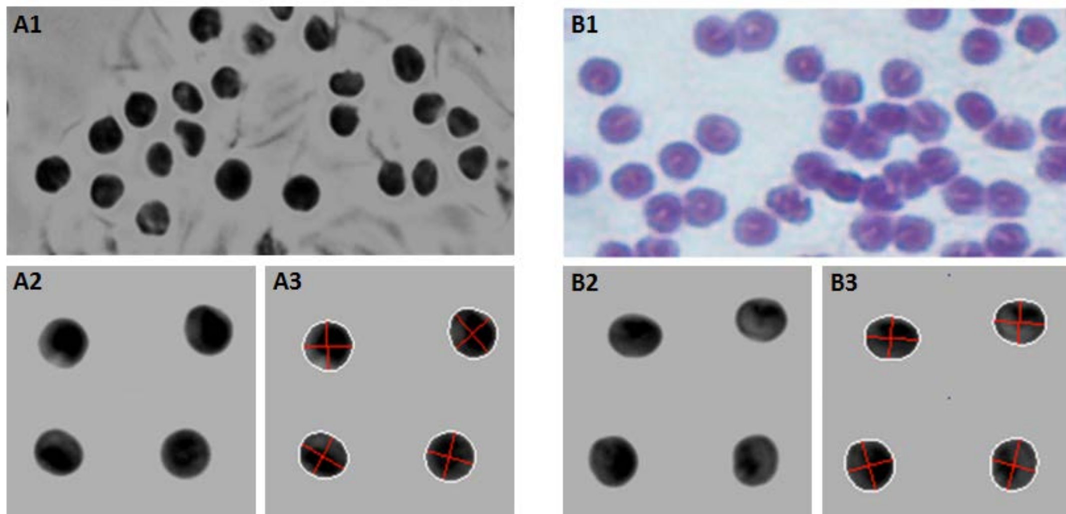
	CV_{wp}		CV_{bp}	
	PC	**HH**	**PC**	**HH**
Area (µm^2)	6.01	12.43	4.02	7.00
Perimeter (µm)	3.09	6.95	2.13	3.37
Length (µm)	3.65	7.73	2.09	1.95
Width (µm)	5.16	8.19	2.29	4.57

510 **Table 3.**

511

N	Male	Area (µm^2)	Perimeter (µm)	Length (µm)	Width (µm)	Ellipticity	Elongation	Rugosity	Regularity
25	1	5.03 ± 0.07*	8.20 ± 0.06	2.59 ± 0.02ab	2.38 ± 0.03	1.09 ± 0.01	0.04 ± 0.005	0.94 ± 0.003	0.96 ± 0.006
	2	5.15 ± 0.09	8.32 ± 0.07	2.67 ± 0.02a	2.36 ± 0.03	1.14 ± 0.01	0.06 ± 0.006	0.93 ± 0.004	0.96 ± 0.005
	3	5.06 ± 0.05	8.21 ± 0.05	2.61 ± 0.02ab	2.39 ± 0.03	1.09 ± 0.01	0.04 ± 0.006	0.94 ± 0.003	0.97 ± 0.006
	4	4.80 ± 0.09	8.01 ± 0.08	2.55 ± 0.02b	2.29 ± 0.03	1.12 ± 0.02	0.06 ± 0.007	0.94 ± 0.004	0.96 ± 0.005
	5	5.04 ± 0.05	8.23 ± 0.04	2.62 ± 0.02ab	2.37 ± 0.03	1.11 ± 0.02	0.05 ± 0.007	0.94 ± 0.003	0.97 ± 0.006
50	1	5.20 ± 0.05a	8.33 ± 0.04a	2.62 ± 0.02b	2.43 ± 0.02a	1.08 ± 0.01a	0.04 ± 0.004b	0.94 ± 0.002	0.97 ± 0.005
	2	5.15 ± 0.06a	8.32 ± 0.05a	2.68 ± 0.02a	2.36 ± 0.02b	1.14 ± 0.01b	0.06 ± 0.005a	0.93 ± 0.002	0.98 ± 0.004
	3	5.03 ± 0.04a	8.20 ± 0.03ab	2.61 ± 0.01b	2.37 ± 0.02ab	1.10 ± 0.01ab	0.05 ± 0.004ab	0.94 ± 0.002	0.97 ± 0.004
	4	4.79 ± 0.05b	7.99 ± 0.05b	2.54 ± 0.01c	2.29 ± 0.02b	1.11 ± 0.01ab	0.05 ± 0.005ab	0.94 ± 0.002	0.96 ± 0.004
	5	5.05 ± 0.04a	8.24 ± 0.03a	2.63 ± 0.01ab	2.36 ± 0.02ab	1.11 ± 0.01ab	0.05 ± 0.005ab	0.93 ± 0.002	0.96 ± 0.004
75	1	5.19 ± 0.04a	8.32 ± 0.03a	2.62 ± 0.01b	2.43 ± 0.01a	1.08 ± 0.01b	0.04 ± 0.003b	0.94 ± 0.002a	0.97 ± 0.003
	2	5.15 ± 0.05ab	8.32 ± 0.04ab	2.68 ± 0.01a	2.36 ± 0.02bc	1.14 ± 0.01a	0.06 ± 0.004a	0.93 ± 0.002b	0.97 ± 0.004
	3	5.01 ± 0.04b	8.19 ± 0.03b	2.62 ± 0.01b	2.36 ± 0.02bc	1.11 ± 0.01a	0.05 ± 0.004a	0.94 ± 0.001ab	0.97 ± 0.003
	4	4.77 ± 0.04c	7.98 ± 0.03c	2.54 ± 0.01c	2.29 ± 0.02c	1.12 ± 0.01a	0.05 ± 0.004a	0.94 ± 0.002a	0.96 ± 0.003
	5	5.07 ± 0.03ab	8.25 ± 0.03ab	2.62 ± 0.01b	2.37 ± 0.01b	1.11 ± 0.01ab	0.05 ± 0.004ab	0.94 ± 0.002ab	0.96 ± 0.004
100	1	5.22 ± 0.03a	8.35 ± 0.03a	2.62 ± 0.01b	2.44 ± 0.01a	1.07 ± 0.01b	0.04 ± 0.002b	0.94 ± 0.001a	0.96 ± 0.003
	2	5.12 ± 0.04b	8.29 ± 0.03a	2.67 ± 0.01a	2.36 ± 0.02b	1.13 ± 0.01a	0.06 ± 0.003a	0.93 ± 0.002b	0.97 ± 0.003
	3	4.98 ± 0.03bc	8.17 ± 0.02b	2.61 ± 0.01b	2.34 ± 0.01b	1.12 ± 0.01a	0.06 ± 0.003a	0.94 ± 0.001ab	0.96 ± 0.003
	4	4.83 ± 0.03c	8.02 ± 0.03c	2.56 ± 0.01c	2.31 ± 0.01b	1.11 ± 0.01a	0.05 ± 0.003a	0.94 ± 0.001a	0.96 ± 0.003
	5	5.06 ± 0.03b	8.25 ± 0.02ab	2.62 ± 0.01b	2.37 ± 0.01b	1.11 ± 0.01a	0.05 ± 0.003a	0.94 ± 0.001b	0.96 ± 0.003

512 **Figure 1.**



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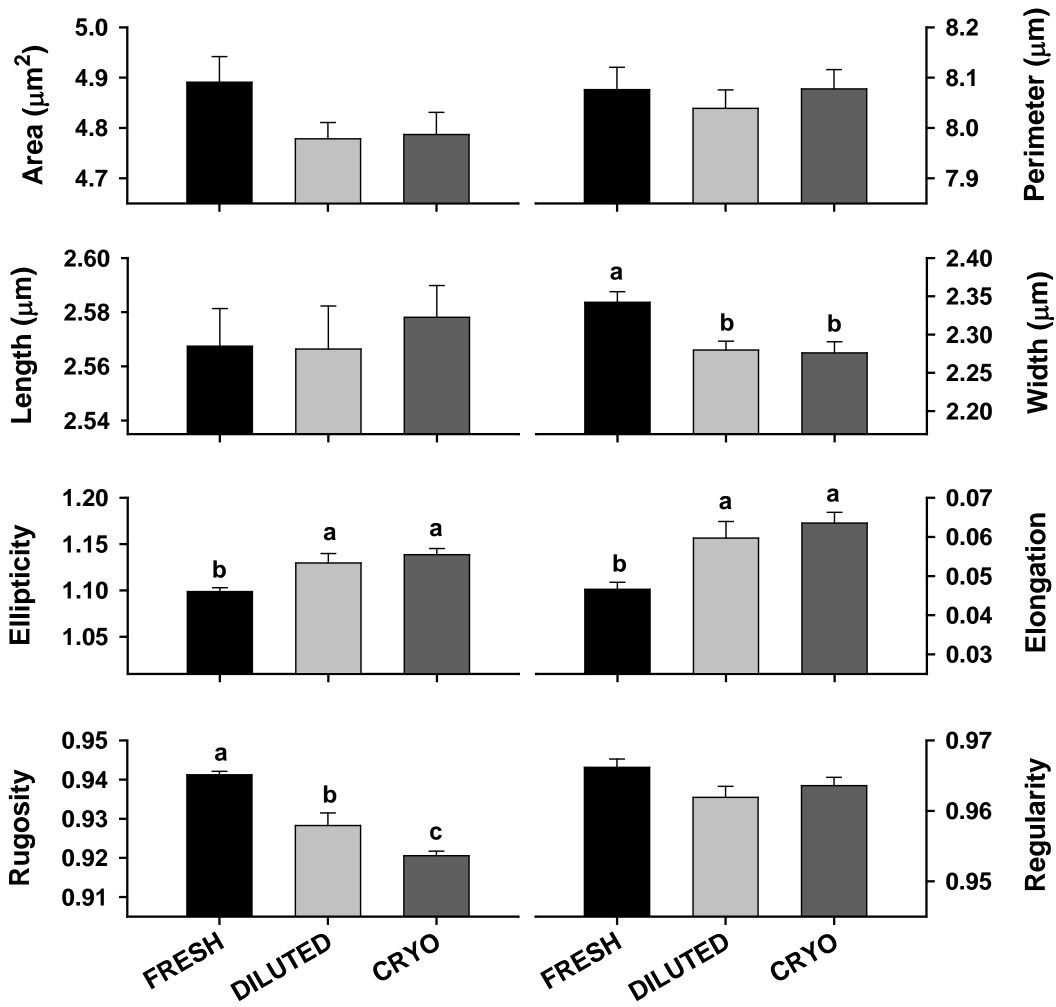

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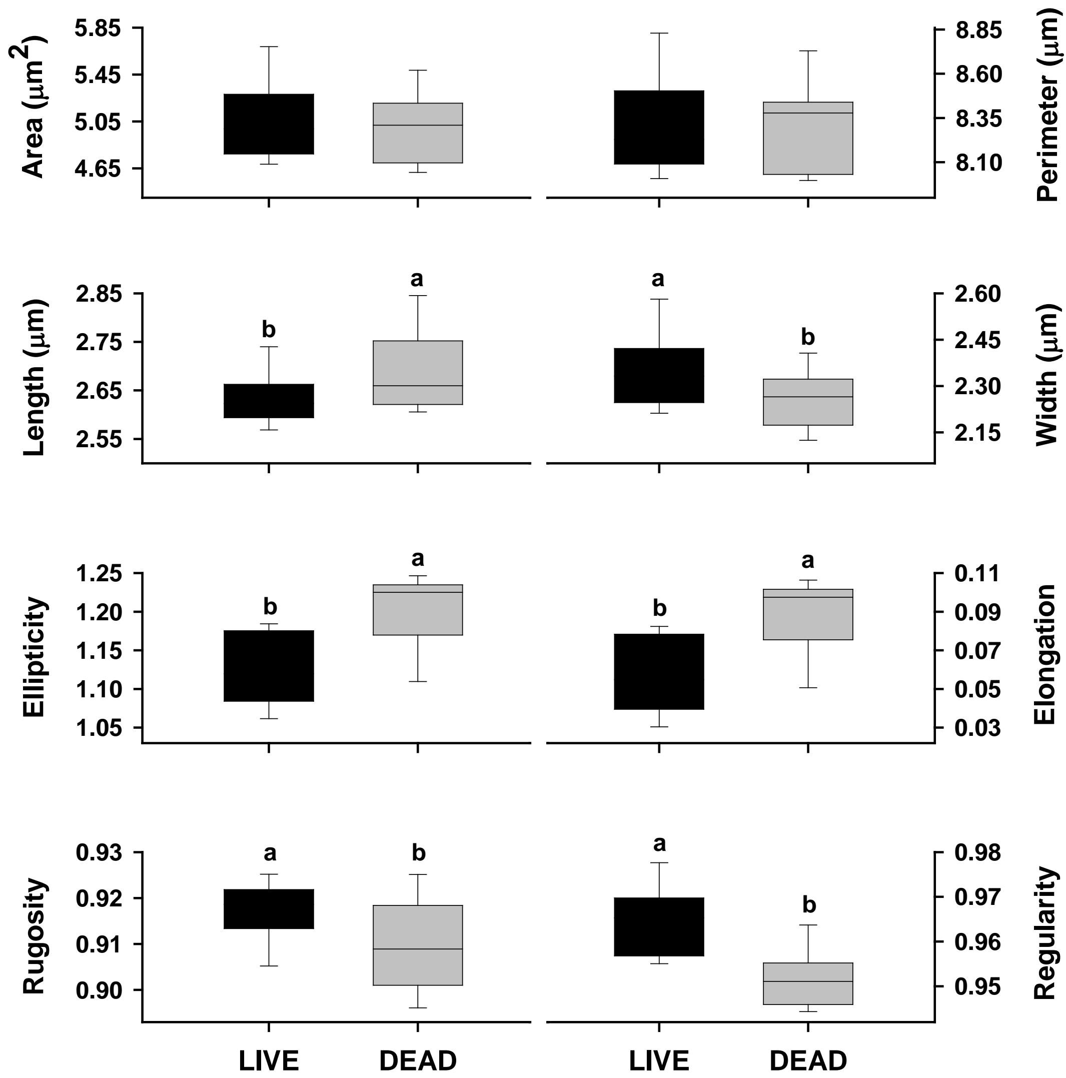
516 **Figure 3.**



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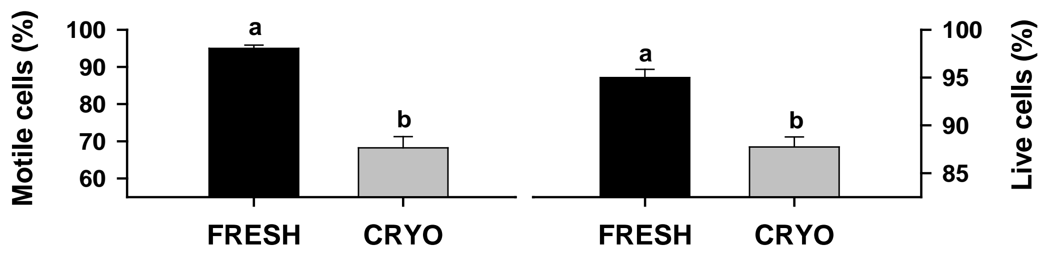
518 **Figure 4.**



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