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1 **Standardization of European eel (*Anguilla anguilla*) sperm motility
2 evaluation by CASA software**
3

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29

30 **Abstract**

31 The development of powerful computerized-assisted sperm analysis software (CASA)
32 has made kinetic studies of spermatozoa possible. This system has been used and
33 validated for several species, but some technical questions have emerged regarding fish
34 sample evaluations (i.e., frame rates, sperm dilution, chamber models, time of analysis,
35 magnification lens, etc). In the present study, we have evaluated the effects of different
36 procedural and biological settings with the aim to measure sperm quality parameters on
37 the European eel correctly.
38 The use of different chambers did not affect the sperm motility parameters. However,
39 regarding lens magnification, 10x was the most accurate lens, showing the least
40 variation in the acquired data. Similarly, the frame rate setting resulted in a dramatic
41 effect in some sperm kinetic parameters, primarily in terms of curvilinear velocity, we
42 therefore recommend using the camera's high frame rate setting available. Finally, the
43 reduction in sperm motility over post-activation times suggests that sperm analysis
44 should be performed within the first 60 s after activation of the European eel sperm. In
45 conclusion, some protocol variables of sperm analysis by CASA software can affect the
46 measurement of eel sperm quality parameters, and should be considered before directly
47 comparing results obtained by different laboratories. Moreover, as marine fish species
48 show relatively similar features to sperm kinetic parameters, these results could be
49 considered in the evaluation of the motility of sperm from other fish species.

50

51 **Keywords**

52 ISAS® v1, Spermatozoa, Motility, Frame, Chamber

53

54 # 1. Introduction

55 The economic importance and high commercial demand of the European eel, *Anguilla*
56 *anguilla*, primarily from European and Japanese markets, is well known [1-3].
57 However, the population of the European eel has declined to such a degree that major
58 concerns have been raised for its long-term survival [4,5]. Efforts have been made to
59 understand the life cycle and reproductive biology of this species [6,7] and we already
60 know that in order to overcome the lack of normal spawning stimuli in captivity, it is
61 necessary to use hormones to induce both ovulation and spermiation the use of
62 hormones to induce both ovulation and spermiation is necessary [8,9]. It is particularly
63 advantageous to stimulate the spermiation of male eels so that sperm is available both in
64 a short time and in high volume [10]. In this respect, knowledge of how to manipulate
65 and preserve eel sperm is essential [11-14] and a reliable and standardized methodology
66 to analyze its quality is needed.

67 The evaluation of sperm motility and other kinetic parameters like curvilinear, straight
68 line and average path velocities, as well as morphology, is an essential tool in the
69 examination of sperm quality in many fish species [15-21], including the European eel
70 [10,22-23]. Despite the fact that for many years optic microscopes have conventionally
71 been used to carry out analysis/evaluations, it is considered a subjective method and
72 great variations have been reported [24]. According to Verstegen et al. [25], when
73 subjective optical microscopic evaluation is used in humans and animals, variations of
74 30 to 60% have been reported in the estimation of the motility parameters of the same
75 ejaculates. The computer assisted sperm analysis, or CASA, has been used by an
76 increasing number of researchers worldwide and provides an objective, rapid and
77 multiple-parameter assessment of sperm quality.

78 In order to make it possible to compare the results obtained by different laboratories, all
79 studies that use CASA must describe its methodology very clearly, particularly
80 concerning image acquisition rate, track sampling time, number of cells sampled, type
81 and depth of the chamber used, software name, microscope optic and magnification, etc.
82 [26,27]. Unfortunately, in the majority of publications details of these parameters are
83 not provided, thus reducing the possibility of comparing the results of different
84 laboratories. Furthermore, as there are many different configurations/ways of using
85 CASA, it is important to establish standard methods of enhancing the reliability,
86 comparability and applicability of data produced by different research groups [28-30].

87 As CASA are not ready-to-use devices, they depend largely on the technical settings
88 and standardizing procedures. Thus, the aim of this study was to evaluate different
89 procedural and biological settings such as chamber models, lens magnification, frame
90 rate acquisition, ejaculate portion and post activation times in order to define a standard
91 method to assess the quality of the European eel semen using a CASA system (ISAS®
92 v1).
93

94 2. Material and Methods

95 2.1 Fish handling

96 Sixty adult eel males from the fish farm Valenciana de Acuicultura, S.A. (Puzol,
97 Valencia; East coast of Spain) were moved to our facilities, in the Aquaculture
98 Laboratory of the Universitat Politècnica de València, Spain. The fish were distributed
99 in three 200-L aquaria (approximately 20 males per aquarium) equipped with separate
100 recirculation systems, thermostats/coolers and covered to maintain constant darkness.
101 The eels were gradually acclimatized to sea water (salinity 37 ± 0.3 g/l) and once a
102 week they were anaesthetized with benzocaine (60 ppm) and weighed before receiving
103 the administration of hormones (hCG; 1.5 IU g^{-1} fish) by intraperitoneal injection. The
104 fish were fasted throughout the experiment and were handled in accordance with the
105 European Union regulations regarding the protection of experimental animals (Dir
106 86/609/EEC).
107

108 2.2 Sperm collection and sampling

109 Sperm samples were collected 24 h after the administration of the hormone because
110 previous studies [31] have demonstrated that this is moment when the highest sperm
111 quality is found. In preparation for sperm collection the fish were anesthetized, and after
112 cleaning the genital area with fresh water to avoid the contamination of the samples
113 with faeces, urine and sea water, and thoroughly drying the fish, the sperm were
114 collected by abdominal pressure. A small aquarium air pump was modified to obtain a
115 vacuum breathing force and the sperm was collected in a tube. A new tube was used for
116 every male and distilled water was used to clean the collecting pipette between each
117 male.
118 Sperm samples were collected between the 6th and the 13th week and kept in plastic
119 tubes under refrigeration (4 ^{o}C) during 1-2 hours prior to the analyses.

120

121 **2.3 Sperm motility evaluation, CASA settings and the analyzed parameters**

122 Sperm was activated by mixing 1 µl of sperm with 200 µl of artificial sea water (Aqua
123 Medic Meersalz, 37 g/l, with 2% BSA (w/v), pH was adjusted to 8.2; [13]). All the
124 motility analyses were performed by triplicate by the motility module of ISAS®v1
125 (Proiser R+D, S.L.; Paterna, Spain) using an ISAS® 782M camera recorder capturing 60
126 frames per second (fps). At least 400-700 spermatozoa were captured in each field
127 adjusting the brightness and contrast in the CASA settings in relation to the microscope
128 light with the aim to reach spermatozoa clearly defined. Range size particle were
129 defined between 2 and 20 µm in the CASA settings. The counting chamber used in all
130 experiments was the ISAS D4C20 chamber, with the exception of the "chamber trial",
131 in which ISAS and Makler chambers were compared.

132 The parameters considered in this study were total motility (MOT, %); progressive
133 motility (PM, %), defined as the percentage of spermatozoa which swim forward in an
134 essentially straight line; the percentage of fast (FA; VAP > 100 µm/s), medium (ME;
135 VAP = 50-100 µm/s) and slow (SL; VAP = 10-50 µm/s) spermatozoa; curvilinear
136 velocity (VCL, in µm/s), defined as the time/average velocity of a sperm head along its
137 actual curvilinear trajectory; straight line velocity (VSL, µm/s), defined as the
138 time/average velocity of a sperm head along the straight line between its first detected
139 position and its last position; average path velocity (VAP, µm/s), defined as the
140 time/average of sperm head along its spatial average trajectory; straightness (STR, %),
141 defined as the linearity of the spatial average path, VSL/VAP; and beat cross
142 frequency (BCF, in beats/s), defined as the average rate at which the curvilinear sperm
143 trajectory crosses its average path trajectory. Spermatozoa were considered immotile if
144 their VCL was lower than 10 µm/s.

145 In order to perform an in-depth analysis, sperm samples were classified into three
146 classes based on the percentage of motile spermatozoa: Class I (C-I)= 0-25% of motile
147 cells; Class II (C-II)= 25-50% of motile cells; and Class III (C-III) >50% of motile cells.
148 All trials were carried out using each one of these motility classes (except the ejaculate
149 portion trial, in which only C-III class was used).

150

151 **2.4 Effect of chambers and magnification lens.**

152 Different tools can be used for sperm motility evaluation by CASA systems. In this trial
153 two chamber models commercially available: the ISAS D4C20 disposable chamber (20

154 µm deep; Proiser R+D, S.L.; Paterna, Spain) versus the Makler reusable chamber (10
155 µm deep; Sefi Medical Instruments, Haifa, Israel) and two magnification lenses (10x
156 versus 20x in a Nikon E400 microscope, negative phase contrast) were tested.
157

158 **2.5 Effect of frame rate**

159 To assess the effect of frame rate upon the system's ability to describe sperm motion,
160 sperm quality parameters at 20, 30 and 60 frames per second (Hz) were compared. With
161 the aim of avoiding variations between replicates within the same sample, the original
162 file, captured at 60 fps, was manually modified using video-analysis software removing
163 1 or 2 frames from every 3 original ones within each video file, as such obtaining files
164 of 30 or 20 fps, respectively.
165

166 **2.6 Effect of ejaculate portion and post activation time.**

167 Sperm samples were collected in two portions: the first collectable millilitre (1st mL)
168 was retrieved in a test tube, while the rest of the sperm (Rest) was collected in another
169 test tube. At the same time, sperm quality parameters of C-III class samples were also
170 measured at different post-activation times (30, 60 and 90 s) with the aim of assessing
171 the effects of the differences in time from the sperm activation event.
172

173 **2.7 Statistical analysis**

174 The mean and standard error (SE) were calculated for all the sperm quality parameters.
175 Shapiro-Wilk and Levene tests were used to check the normality of data distribution and
176 variance homogeneity, respectively. The one-way analysis of variance (ANOVA) and
177 Student's *t*-test were used to analyze data with normal distribution. Significant
178 differences between post-activation times were detected using the Tukey multiple range
179 test ($P<0.05$). For non-normally distributed populations, Kruskal-Wallis one-way
180 ANOVA on ranks and Mann-Whitney *U*-test were used. All statistical analyses were
181 performed using the statistical package SPSS version 19.0 for Windows software (SPSS
182 Inc., Chicago, IL, USA).
183

184 # 3. Results

185 The sperm cell detection parameters used in this study were suitable for fish sperm
186 evaluation. Quality control analysis performed using the playback facility showed that

187 all spermatozoa observable in the field were detected and recorded.

188

189 **3.1 Effect of chambers and magnification lens.**

190 The different chambers used in this trial did not significantly affect the sperm quality
191 parameters in any motility class (Table 1). However, samples analyzed by the ISAS®
192 disposable chamber showed slightly higher values in almost all the sperm motility
193 parameters (although no significance differences were found). The coefficients of
194 variation (CV) of samples within the same motility class obtained with both chambers
195 were quite similar (Figure 1), with much higher CV's in C-I than in C-II and C-III
196 classes.
197 On the contrary, the different magnification lenses used in this study significantly
198 affected some of the sperm quality parameters in the different sperm classes (Table 2).
199 Samples analyzed using the 20x lens showed lower values than those analyzed using the
200 10x lens, with more significant differences in C-II and C-III. In addition, the
201 coefficients of variation within the same motility class (Figure 1) with the 20x lens were
202 much higher than with the 10x lens, with much higher CV's in C-I than in C-II and C-
203 III.

204

205 **3.2 Effect of frame rate setting**

206 The frame rate setting (FR) had no effect neither on the total and progressive motile
207 cells nor on the proportion of fast, medium and slow spermatozoa (data not shown).
208 However, other kinetic values were deeply affected by FR (Figure 2). VCL and BFC
209 showed a progressive increase with significant differences as the FR increased while
210 STR showed a reverse trend, decreasing as the FR increased. VSL did not show
211 significant differences in any motility class whereas VAP only showed statistical
212 differences in C-II motility class.

213

214 **3.3 Effect of ejaculate portion and post activation time.**

215 Sperm quality parameters obtained by the first collectable millilitre (1st mL) and the rest
216 of the sperm (Rest) were similar and no significant differences were evident (Table 3).
217 Regarding changes in sperm parameters after sperm activation, significant differences
218 were found on MOT, FA, and SL (Figure 3). The most affected parameter was MOT,
219 showing a progressive decrease in motile cells after the activation time with significant
220 differences in the different classes, in which motility value recorded at 90 s was lower

221 than motility obtained at 30 s. The percentage of fast spermatozoa showed a similar
222 trend, but without significant differences in C-II. The percentage of slow spermatozoa
223 only showed significant differences in the highest motility class (C-III).
224

225 4. Discussion

226 The subjective sperm quality evaluation, widely used in many laboratories working with
227 male gametes, depends on the skill, perception and training of the researcher who
228 evaluates the sperm samples [15,17,32]. In the last few years, several CASA software
229 have been developed with the aim of achieving an objective evaluation of sperm quality
230 parameters [33]. However, although these systems provide the most accurate and
231 repeatable technique currently available, they need to be standardized before their use.
232 Despite the beneficial effects of this standardization process in human andrology [34],
233 there is little data about domestic animals [35,36] and to this day there are no studies
234 about the standardization of procedures in fish species. In this study, we have assessed
235 different technical and biological settings in order to standardize the sperm quality
236 evaluation of European eel to be used as a sperm model of for teleost fish.
237 Several different chambers can be used for the analysis of spermatozoa using CASA
238 systems. The choice of chamber depends on several factors and in this trial two chamber
239 models have been evaluated. The Makler chamber is a round reusable sperm counting
240 chamber (10 µm depth) loaded by drop displacement, while the ISAS DC420 chamber
241 is a rectangular disposable sperm counting chamber (20 µm depth) loaded by capillarity.
242 All these factors (shape, loading method, depth, etc.) can affect the sperm parameters, as
243 occurs in other species like humans [27,37] or bull [29,38]. However, in our study, the
244 different chambers used did not affect fish sperm quality parameters in any motility
245 class. This result suggests that in the case of eel sperm and, in fish sperm with similar
246 sperm features in general, it is possible to evaluate the sperm quality parameters with
247 different kind of chambers without compromising the final result.
248 On the other hand, the different magnification lenses used in this study significantly
249 affected many of the sperm quality parameters. In this case, the result could be related
250 to the sample size, which can affect the results of sperm analysis. If an insufficient
251 number of spermatozoa are analyzed during the video capture a non-accurate
252 measurement of sperm parameters will be obtained due to a higher data
253 variation/dispersion [21]. In this case, the number of spermatozoa captured by the 20x

254 magnification lens was much less than those assessed with the 10x lens, therefore the
255 coefficients of variation obtained by the 20x lens were much higher than those obtained
256 with the 10x lens within the same motility class. Thus, the results obtained by the 10x
257 lens should be *a priori* more accurate and precise than the results obtained by the 20 x
258 lens.
259 The number of frames acquired per second (fps) can influence the quality of the
260 acquisition and the sperm quality parameters [26]. It has been demonstrated in literature
261 that low frame rates can underestimate the real value of kinetic traits [28,29]. The higher
262 the quantity of track information available during the sperm capture (increasing fps), the
263 more accurate the reconstruction of the sperm trajectories obtained, more closely
264 resembling the real trajectory. Thus, the reduction in the fps could generate significant
265 variations in several kinetic parameters. In our trial, the frame rate setting had no effect,
266 neither on total motility nor on progressive motility. However, other sperm quality
267 parameters like VCL, STR or BFC were deeply affected by frame rate. Our results
268 corroborate previous studies [26,29], in which it has been suggested that increases in
269 frame rate drastically increase the measured VCL without substantial impact on VAP,
270 resulting in a decrease in STR. In this respect, it seems reasonable to think that the
271 higher number of fps we use will generate the more “real” the spermatozoa trajectory.
272 However, what is the limit of fps?. This limit depends on several factors, from the
273 kinetic features of the sperm with which we are working (it is quite different to work
274 with low and linear than with fast and non-linear spermatozoa movements) to the
275 laboratory´s ability to invest in the best camera available in the market. In this respect,
276 most of the papers about mammal sperm carried out with CASA software use an
277 acquisition rate of 50-60 Hz [39-42]. However, this rate seems to be chosen due to
278 hardware/software facilities and not due to theoretical considerations. Regarding fish
279 sperm, the problem is bigger because fish spermatozoa are considered one of the fastest
280 describing non-linear trajectories. Toth et al. [43] suggested that frame rates >60 fps
281 should be used when analysing fish sperm. In this sense, Wilson et al. [28] reported that
282 97 fps is the lower limit to obtain acceptable trajectories in zebra fish, and Castellini et
283 al. [26] reported that fish sperm require a frequency of 290 fps to fully trace the
284 movement path. Thus and to sum up, it is important to take into account that the
285 comparison of results between different laboratories/research groups which use a
286 different number of frames acquired per second (fps) may not be valid.

287 On the other hand, it is well known that marine fish spermatozoa are quiescent in the
288 seminal plasma, and the hyperosmolality of the sea water is the trigger that initiates the
289 motility [44]. However, the ability of spermatozoa to swim is eventually dependent on
290 their previous maturation in the sperm ducts, where some essential processes in
291 acquiring movement capability take place, such as changes in pH and ionic composition
292 of seminal plasma, as well as the action of the progestin DHP [45,46]. While in some
293 mammals [47] it has been demonstrated that the portion of ejaculate evaluated can
294 affect the sperm quality parameters, scarce studies have been developed in fish. For
295 example, in rainbow trout, the spermatozoa collected from the distal portions of the
296 sperm duct display better motility than the spermatozoa collected from the proximal
297 portions [48]. Peñaranda et al. [49] suggested that the high concentration of lipoproteins
298 (HDL-proteins) present in the seminal fluid can interact with the spermatozoa plasma
299 membrane to maintain its lipid composition during storage in the sperm duct. In our
300 trial, significant differences in sperm quality parameters between the first collectable
301 millilitre and the rest of the sperm were not evident. In the case of European eel, an
302 endangered marine species able to produce a high volume of sperm (1–4 mL 100 g^{-1}
303 fish; [8,10,31]), this result confirms the possibility of using sperm produced by males
304 under hormonal treatment in artificial fertilizations. This result increases the economical
305 profitability of the relatively expensive hormonal treatment necessary to obtain the
306 sperm [10] and enhances the need for good cryopreservation techniques to reduce the
307 male broodstocks and the hormones required to produce enough amounts of sperm.
308 Moreover, regarding change in movement parameters after sperm activation, total
309 motility was the most affected factor, displaying a progressive decrease in the
310 percentage of motile cells after the activation time. Usually in marine and freshwater
311 species, most of the sperm traits used to characterize motility decline within tens of
312 seconds to a few minutes, depending on the species, and this general decrease leads to
313 an eventual full arrest of spermatozoa by ATP consume [50]. In the case of European
314 eel, in addition to the reduction in motility, a decrease in the percentage of fast
315 spermatozoa was also evident over time. As such our results suggest that sperm analysis
316 in European eel sperm should be performed within the first 60 s after activation.
317 To summarize, CASA systems are useful tools for carrying out studies about
318 spermatozoa kinetic parameters in fish species. However, some questions have emerged
319 regarding sperm sample evaluations, and as such several procedural and technical
320 settings should be standardized and validated before comparing results obtained by

321 different laboratories. In this study, we have assessed different technical and biological
322 settings in order to standardize the evaluation of sperm quality in the European eel and
323 use it as a sperm model for teleost fish. We have discovered that some protocol
324 variables in sperm analysis by CASA software (ISAS® v1) can affect the measurement
325 of eel (fish) sperm quality parameters. Notably, neither the type of chamber nor the
326 ejaculate portion affected the sperm quality parameters, suggesting that either type can
327 be used for sperm evaluation in European eels. Finally, in order to carry out a suitable
328 analysis on sperm quality parameters in European eel, we would suggest a few
329 recommendations regarding its application: i) use the lowest available magnification
330 lens, with the aim of avoiding ~~a~~ big spread in data; ii) use the highest available frame
331 rate, with the aim of obtaining the most "real trajectory" of the spermatozoa and iii)
332 perform the analysis within the first 60 s after activation.
333

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342

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481 ## Table legends

482

483 **Table 1.** Mean ± SE of sperm quality parameters for different chamber models on
484 different sperm classes (C-I, C-II and C-III) at 30 s post-activation time.
485 No significant differences were found between chamber models.

486

487 **Table 2.** Mean ± SE of sperm quality parameters for different microscopy
488 magnifications on different sperm classes (C-I, C-II and C-III) at 30 s post-activation
489 time. Asterisks indicate significant differences between microscopy magnifications.

490

491 **Table 3.** Mean ± SE of sperm quality parameters in the first collectable millilitre (1st
492 mL) and the rest of the sperm (Rest) in high quality sperm samples (C-III) at 30 s post-
493 activation time. No significant differences were found between different ejaculate
494 portions.

495

496 ## Figure legends

497

498 **Figure 1.** Coefficients of variation (CV´s) for each chamber model (Makler and ISAS)
499 and each microscopy magnification (10x and 20x) on different sperm classes (C-I, C-II
500 and C-III).

501

502 **Figure 2.** Kinetic parameters at different frame rates (20, 30 and 60 fps) on different
503 sperm classes (C-I, C-II and C-III). Data are expressed as mean ± SE and different
504 letters indicate significant differences between frame rates.

505

506 **Figure 3.** Sperm quality motility parameters at different post-activation times (30, 60
507 and 90 s) on different sperm classes (C-I, C-II and C-III). Data are expressed as mean ±
508 SE and different letters indicate significant differences between times.

509 **Table 1.**

510

		C-I		C-II		C-III	
		Makler	**ISAS**	**Makler**	**ISAS**	**Makler**	**ISAS**
MOT	%	16.0 ± 1.6	20.8 ± 2.4	38.6 ± 3.2	42.7 ± 3.7	57.6 ± 2.3	62.8 ± 3.6
PM	%	4.3 ± 0.8	5.5 ± 1.0	19.6 ± 2.4	21.1 ± 2.5	22.1 ± 2.2	26.1 ± 2.2
FA	%	7.3 ± 1.2	9.0 ± 1.3	27.0 ± 2.8	30.0 ± 3.4	38.6 ± 2.9	45.3 ± 3.5
ME	%	4.4 ± 0.5	5.9 ± 0.9	6.1 ± 0.8	6.8 ± 0.7	12.7 ± 0.9	11.2 ± 0.9
SL	%	4.4 ± 0.5	5.8 ± 0.9	5.5 ± 0.8	5.9 ± 0.8	6.3 ± 0.6	6.3 ± 0.5
VCL	µm/s	96.1 ± 5.4	94.3 ± 6.3	140.4 ± 7.5	143.0 ± 7.8	136.1 ± 6.3	145.6 ± 6.0
VSL	µm/s	37.0 ± 2.9	38.5 ± 3.7	69.8 ± 5.4	69.6 ± 4.8	62.5 ± 4.2	69.0 ± 4.1
VAP	µm/s	54.3 ± 3.2	55.6 ± 4.2	87.8 ± 5.3	88.7 ± 5.2	85.0 ± 4.6	92.3 ± 4.6
STR	%	66.7 ± 1.5	67.0 ± 1.9	77.4 ± 2.4	77.4 ± 1.5	72.3 ± 1.6	73.8 ± 1.3
BFC	beats/s	21.6 ± 2.2	24.3 ± 2.0	31.9 ± 1.6	34.9 ± 1.3	30.8 ± 1.2	31.1 ± 1.2

511

512 **Table 2.**

513

		C-I		C-II		C-III	
		10x	**20x**	**10x**	**20x**	**10x**	**20x**
MOT	%	18.5 ± 1.8	18.3 ± 2.3	42.2 ± 3.1	39.1 ± 3.8	63.8 ± 2.1	56.6 ± 3.6
PM	%	4.8 ± 0.9	5.0 ± 0.9	22.1 ± 2.3	18.6 ± 2.6	27.8 ± 2.0*	20.4 ± 2.3
FA	%	8.4 ± 1.2	7.9 ± 1.3	32.0 ± 2.8	25.1 ± 3.2	47.8 ± 2.5*	36.1 ± 3.6
ME	%	5.7 ± 0.7	4.6 ± 0.6	5.6 ± 0.6	7.3 ± 0.9	10.5 ± 0.8*	13.3 ± 1.0
SL	%	4.4 ± 0.5	5.9 ± 0.9	4.6 ± 0.5	6.8 ± 1.0	5.5 ± 0.4*	7.1 ± 0.7
VCL	µm/s	101.2 ± 4.6	89.2 ± 6.7	153.6 ± 5.1*	129.8 ± 8.6	152.3 ± 5.0*	129.4 ± 6.5
VSL	µm/s	39.3 ± 2.8	36.2 ± 3.8	74.9 ± 4.1	64.6 ± 5.7	72.2 ± 3.8*	59.2 ± 4.2
VAP	µm/s	58.3 ± 2.9	51.6 ± 4.3	94.6 ± 3.9	82.0 ± 5.9	95.4 ± 4.0*	81.9 ± 4.8
STR	%	66.4 ± 1.6	67.4 ± 1.8	78.4 ± 1.4	76.4 ± 2.4	74.8 ± 1.1	71.3 ± 1.7
BFC	beats/s	26.2 ± 1.2*	19.7 ± 2.6	35.7 ± 1.1*	31.1 ± 1.6	33.0 ± 1.1*	28.9 ± 1.2

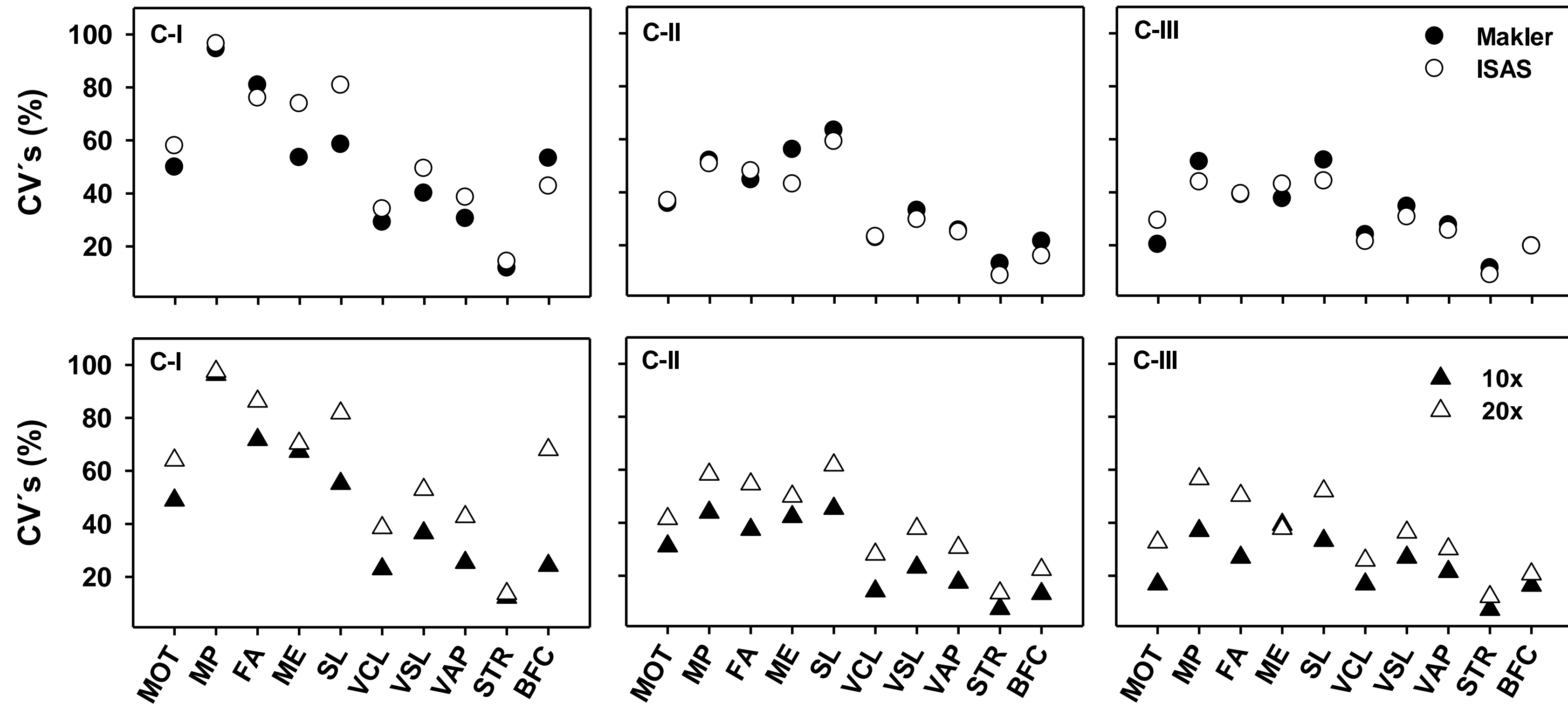
514

515 **Table 3.**

516

		1st mL	Rest
MOT	%	74.75 ± 3.19	73.29 ± 3.78
PM	%	39.36 ± 3.36	36.65 ± 5.49
FA	%	61.16 ± 5.09	58.80 ± 5.26
ME	%	10.39 ± 2.56	10.60 ± 1.93
SL	%	3.20 ± 0.33	3.89 ± 0.60
VCL	µm/s	162.88 ± 7.28	156.95 ± 6.34
VSL	µm/s	87.03 ± 5.77	81.76 ± 6.37
VAP	µm/s	110.58 ± 6.33	104.16 ± 6.03
STR	%	78.41 ± 1.12	77.83 ± 1.87
BFC	beats/s	30.70 ± 1.12	30.44 ± 0.75

517 **Figure 1.**



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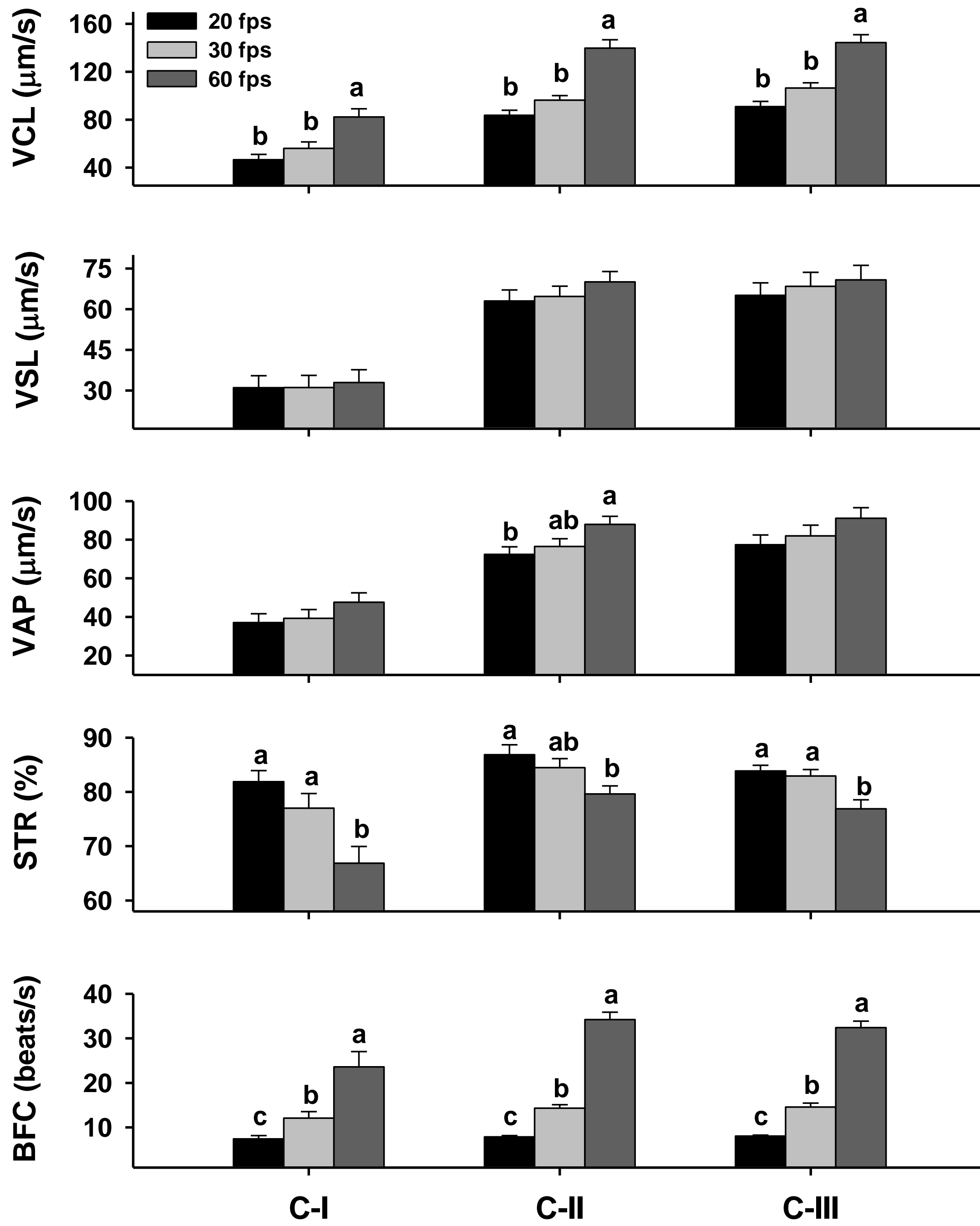
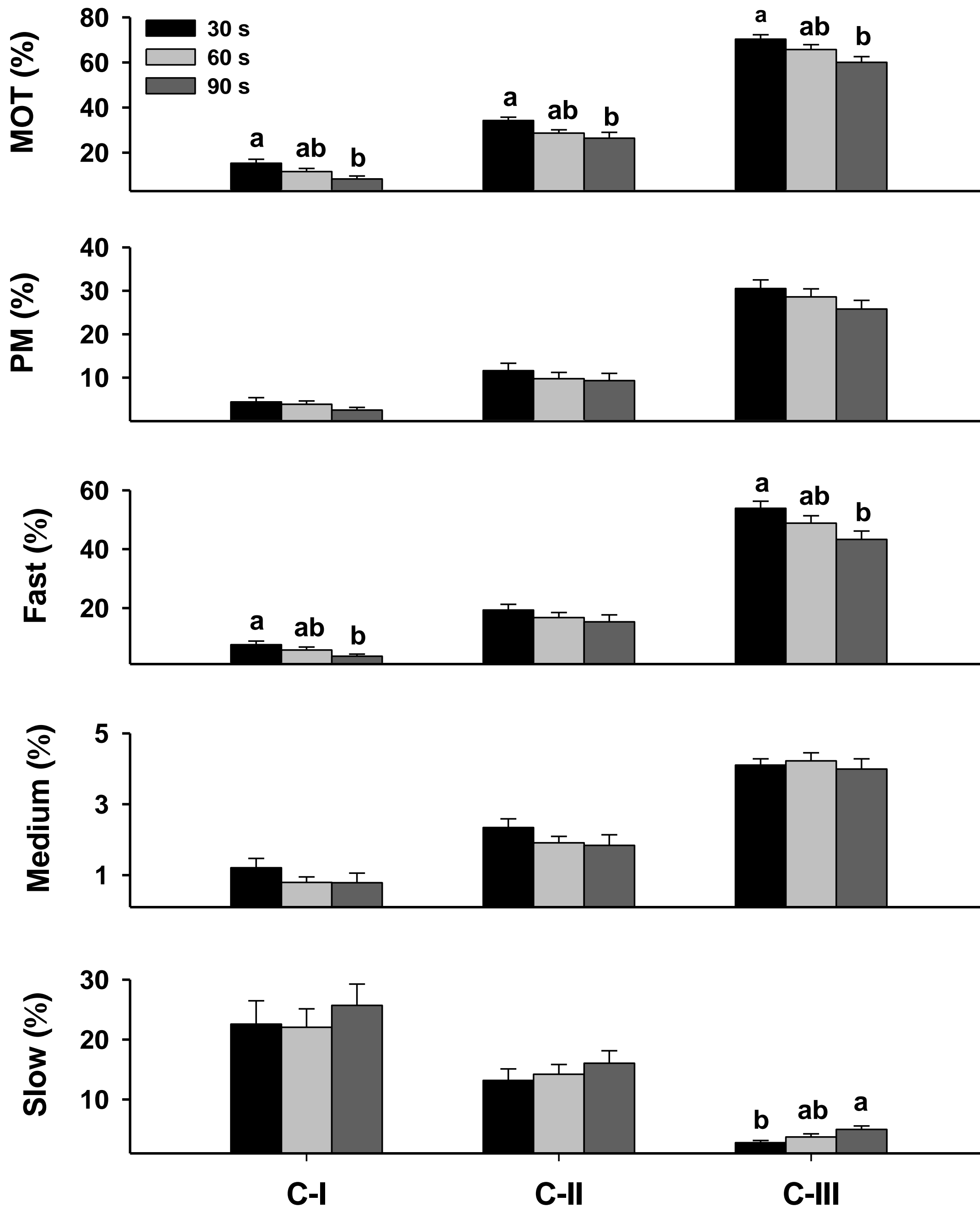
519 **Figure 2.**



520

521 **Figure 3.**



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