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Additional Information

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3 1 **Sperm quality parameters of Iberian toothcarp (*Aphanius iberus*) and**
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5 2 **Valencia toothcarp (*Valencia hispanica*): new conservation tools from a**
6
7 3 **gamete perspective**
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61
62 **Abstract**
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64
65 27 The sensitive state of conservation of several endemic fish species such as
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67 28 Iberian toothcarp (*Aphanius iberus*) and Valencia toothcarp (*Valencia hispanica*)
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69 29 has led governments to consider the implementation of conservation measures
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71 30 to preserve their populations. However, limited knowledge about the reproductive
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73 31 biology of these species makes it necessary to investigate different aspects of
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75 32 their reproductive cycle. In this sense, the main objectives of this work were *i*) to
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77 33 advance knowledge of the breeding biology of both species, and *ii*) to develop
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79 34 protocols for the conservation of gametes for the future management and
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81 35 conservation.

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84 36 During the spring of 2019 a temporal series of samplings were carried out in
85
86 37 different places in the Comunitat Valenciana. Sperm samples were collected and
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88 38 sperm motion parameters were assessed for the first time in both species. Kinetic
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90 39 patterns were similar showing high motility and velocity values during the first 30
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92 40 s, and a rapid decrease from that point. At the same time, an in-depth
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94 41 morphometric analysis was carried out using computer-assisted sperm analysis
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96 42 software. Spermatozoa from *A. iberus* and *V. hispanica* showed similar sizes and
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98 43 shapes to other external fertilizers belonging to Cyprinodontiformes, with small
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100 44 spherical heads, unflagellated and without acrosomes.

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103 45 In addition, a new cryopreservation protocol was designed for cryobanking the
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105 46 sperm of these threatened species. Cryopreserved samples showed lower
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107 47 motility than fresh samples but reaching acceptable percentages of motile cells
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109 48 after thawing of around 20 and 25% (*A. iberus* and *V. hispanica*, respectively).
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111 49 This study is the first of its kind to successfully achieve gamete cryopreservation
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113 50 of these two endemic and endangered species from the Iberian Peninsula,
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51 providing new and useful tools to complement the management and conservation

52 programs that are being developed for both species.

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54 **Keywords**

55 killifish; endangered species, motility; cryopreservation; fish; breeding season

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180 **57 1. Introduction**
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182 58 Fish populations of the Mediterranean coast have been declining since the mid-
183
184 59 20th century as a result of several factors such as habitat loss (degradation,
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186 60 fragmentation and destruction), water pollution, dredging and draining processes,
187
188 61 etc. Among these factors, the presence of invasive species is probably the main
189
190 62 cause of this dramatic decline, and nowadays this factor is widely regarded to be
191
192 63 one of the top threats to global biodiversity (Courchamp et al., 2017). In the case
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194 64 of the Iberian toothcarp (*Aphanius iberus*) and Valencia toothcarp (*Valencia*
195
196 65 *hispanica*), the introduction of the Eastern mosquitofish (*Gambusia holbrooki*) to
197
198 66 eradicate malaria in the early 20th century triggered the beginning of the decrease
199
200 67 of both local species. Despite the constant effort made by the government
201
202 68 administrations, both species still have a fragile conservation status. In fact, they
203
204 69 are included in the IUCN Red List of Threatened Species, classified as
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206 70 “Endangered” (*A. iberus*; Crivelli, 2006a) and “Critically endangered” (*V.*
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208 71 *hispanica*; Crivelli, 2006b).

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212 72 The *in situ* measurements carried out during the last few decades (monitoring
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214 73 programs, wetlands restoration, etc.) have been successfully supplemented with
215
216 74 the *ex situ* conservation actions. In this sense, the launch of successful captive
217
218 75 breeding programs carried out at the Center for the Conservation of Freshwater
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220 76 Species of the Valencian Community (CCEDCV, El Palmar, Valencia) has
221
222 77 allowed periodic reinforcements of specific populations and the creation of new
223
224 78 population nucleus (Risueño and Hernández, 2000). However, due to the limited
225
226 79 knowledge on the reproductive biology of these species (García-Alonso et al.,
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228 80 2009), it is necessary to investigate different aspects of their reproductive cycle
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230 81 such as when is the breeding season of these species or when their gamete
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239 82 quality is optimal, in order to carry out artificial insemination to improve the
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241 83 population management of these species (Comizzoli, 2015).
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243 84 On the other hand, to complement the *in situ* and *ex situ* conservation tasks, one
244
245 85 conservation strategy currently applied in fish management is the creation of
246
247 86 genetic resources banks (GRBs) (Martínez-Páramo et al., 2017). The use of
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249 87 GRBs for captive breeding programs make several tasks possible, including *i*) the
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251 88 preservation of the genetic material of endangered species, *ii*) the conservation
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253 89 of certain genetic lines or haplotypes, or *iii*) the recovery of lost genetic
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255 90 characteristics in some populations or individuals (becoming a wild-phenotypic
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257 91 backup). In this sense, one of the important objectives of developing fish sperm
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259 92 cryopreservation protocols is their application in restocking and conservation
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261 93 programs.
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264 94 However, it is important to note that the development of GRBs combines
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266 95 knowledge of reproductive biology and cryobiology techniques. In this regard,
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268 96 knowledge of the breeding cycle of species is essential in order to be able to
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270 97 apply preservation techniques when the best quality genetic material is available.
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273 98 The timing of gamete collection is an essential step in order to obtain high quality
274
275 99 gametes, since best gamete quality is imperative to the success of the fertilization
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277 100 process (Comizzoli, 2015). On the other hand, gamete handling can also affect
278
279 101 (and reduce) gamete quality: when sperm is collected by stripping, several fluids
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281 102 (urine, feces, etc.) can spontaneously activate the sperm due to osmotic changes
282
283 103 in the medium (Beirão et al., 2019). Finally, the choice of the right species-specific
284
285 104 extender for diluting the fresh sperm samples can maintain gamete quality over
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287 105 time (Gallego and Asturiano, 2019; Muchlisin, 2014). Therefore, fish diversity
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298 106 makes it necessary to develop specific cryopreservation protocols according to
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300 107 the species and the material to be preserved (Asturiano et al., 2017).
301
302 108 Summing up, because of the limited knowledge of the reproductive biology of
303
304 109 these endangered species, the aim of this study was *i*) to increase our knowledge
305
306 110 of different aspects of their reproductive process during their breeding season, *ii*)
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308 111 to describe sperm kinetic and morphometric parameters of both species, and *iii*)
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310 112 to develop a gamete cryopreservation protocol for future management and
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312 113 conservation programs.
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317 115 **2. Methods**

319 116 *2.1. Fish handling*

321 117 For the capture of the exemplars, it was used basket fish traps. The traps had a
322
323 118 tubular shape and a structure that difficult the escape of the fishes. On the one
324
325 119 hand, Iberian toothcarps (*A. iberus*) were caught from a captive population in the
326
327 120 CCEDCV facilities. Two fish traps with food inside were placed in the ponds and
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329 121 they were collected after 2 h. The captive population was originally from
330
331 122 Albuixech (Valencia), and it was maintained in the facilities with natural
332
333 123 photoperiod, temperature, and autochthonous vegetation. On the other hand,
334
335 124 Valencia toothcarps (*V. hispanica*) were caught from three different spots of the
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337 125 Albufera Natural Park. It was used 10 fish traps in every case, that were placed
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339 126 among the vegetation. After 2 h, the fish traps were collected and the captured
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341 127 fishes were counted and sexed. A selection of the fishes was carried out to the
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343 128 CCEDCV for the experiment and the ex situ conservation programs that are being
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345 129 developed in the centre.
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357 130 *2.2. Sperm collection*
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359 131 Before handling, breeders (*A. iberus*, n=10-24; *V. hispanica*, n=9-21) were
360
361 132 anaesthetised using clove oil (80 ppm; Kroon, 2015). Due to the small size of
362
363 133 both species, a sponge with a small cut was used to fit the fish into, in order to
364
365 134 minimise fish manipulation during sperm extraction. Sperm samples (1-2 µl) were
366
367 135 collected by the application of abdominal pressure using a microcapillary tube (40
368
369 136 x 1 mm for *A. iberus*; 75 x 1 mm for *V. hispanica*) after cleaning the genital area
370
371 137 with NaCl 0.9% (pH 8.0, 303 mOsm/kg) to avoid contamination by feces, urine or
372
373 138 freshwater. The sperm was then diluted 1:20 for *A. iberus* and 1:50 for *V.*
374
375 139 *hispanica* (sperm:extender) in a PBS medium (pH 8.0, 309 mOsm/kg) and kept
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377 140 in 500 ml microtubes. Microtubes were kept in a portable fridge (4 °C) up to 2
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379 141 hours, until the sperm analyses were carried out at the facilities of the Universitat
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381 142 Politècnica de València.
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387 144 *2.3. Evaluation of sperm motility and kinetic parameters*
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389 145 Samples were activated by mixing water from fish breeding tanks (0 mOsm/kg)
390
391 146 with 2% BSA (w/v) and adjusting the pH to 8.0. The mix was examined using a
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393 147 SpermTrack-10 chamber (Proiser R+D, S.L., Paterna, Spain). Video sequences
394
395 148 of 0.5 s were recorded (at 50 fps) using a video camera (Nikon Digital Sight DS-
396
397 149 5M) mounted on a phase contrast microscope (Nikon Eclipse 80i) with a 10x
398
399 150 objective lens. All the motility analyses were performed in triplicate using the
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401 151 CASA-Mot software (Computer Assisted Semen Analysis; Proiser R+D, S.L.;
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403 152 Paterna, Spain). The parameters considered in this study were: total motility
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405 153 (MOT, %), defined as the percentage of motile cells; progressive motility (pMOT,
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407 154 %), defined as the percentage of spermatozoa which swim in what is essentially
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416 155 a straight line; curvilinear velocity (VCL, $\mu\text{m/s}$), defined as the average velocity of
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418 156 a spermatozoa head along its curvilinear trajectory; straight line velocity (VSL,
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420 157 $\mu\text{m/s}$), defined as the time/average velocity of a spermatozoa head along the
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422 158 straight line between its first detected position and its last position; and the
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424 159 percentage of fast (VAP $>100 \mu\text{m/s}$), medium (VAP = $50\text{-}100 \mu\text{m/s}$) and slow
425
426 160 (VAP = $10\text{-}50 \mu\text{m/s}$) spermatozoa, defining VAP (average path velocity) as the
427
428 161 time/average velocity of a sperm head along its spacial average trajectory. Sperm
429
430 162 samples were considered motile if their total motility was over 10%.
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435 164 *2.4. Spermatozoa morphometric analysis*

437 165 Sperm samples (total motility over 70%) were fixed by adding 1% glutaraldehyde
438
439 166 diluted in PBS buffer and were deposited in microtubes (Eppendorf). An aliquot
440
441 167 of sperm dilution (approximately $5 \mu\text{l}$) was put on a slide and covered with a cover
442
443 168 glass. The sperm samples were examined using a phase contrast microscope
444
445 169 with a 100x contrast phase lens.
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448 170 Microphotographs of the spermatozoa were taken using an ISAS 782M camera
449
450 171 (Proiser R + D, S.L.), and the morphometric analyses of the sperm samples were
451
452 172 performed using the morphometry module of the ISAS software (ASMA;
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454 173 Automated Sperm Morphometry Analysis). The spermatozoa head
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456 174 measurements, including size variables such as length (L, μm), width (W, μm),
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458 175 area (A, μm^2), and perimeter (P, μm) and shape variables such as ellipticity (L/W),
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460 176 rugosity ($4\pi A/P^2$), elongation $(L-W)/(L+W)$, and regularity ($\pi LW/4A$), were
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462 177 calculated automatically by capturing 100 digitized spermatozoa for each sample.
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475 179 *2.5. Scanning electron microscopy (SEM)*
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477 180 Sperm cells were fixed with 2.5% glutaraldehyde diluted in PBS buffer and were
478
479 181 deposited in Eppendorf tubes until the scanning electron microscope analysis.
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481 182 The samples were washed in triplicate with a PBS buffer over a micropore filter
482
483 183 (0.1 μm) and after that, they were dehydrated with different concentrations of
484
485 184 ethanol (30, 50, 70, 80 and 90%, plus three times in 100%), and left 10 min in
486
487 185 each concentration. Samples were fixed using a critical point (LEICA EM
488
489 186 CPD300), assembled over a specific support and were then platinum coated by
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491 187 sputtering. Finally, head and flagellum measurements were taken using
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493 188 SmartSEM software (ZEISS, Germany).
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499 190 *2.6. Cryopreservation*
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501 191 Seven fresh sperm samples from *A. iberus* and eight fresh sperm samples from
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503 192 *V. hispanica* (total motility > 60%) were used for the cryopreservation trials.
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505 193 Samples were diluted 1:8 (sperm:extender) in a PBS medium and 10% (v/v) of
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507 194 methanol, after the predilution made during the sperm collection (view section
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509 195 2.2). Samples were immediately packed in straws of 250 μL . Between two and
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511 196 three straws from each specimen were used, according to sperm available
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513 197 volume. Then, the diluted samples were then incubated for 5 min at 4 °C to ensure
514
515 198 a stable penetration of the cryoprotectant into the cells. The freezing condition
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517 199 was created by placing the straws on a floating structure 6.5 cm over the liquid
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519 200 nitrogen (LN) for 15 min, and then by dropping them into the LN. For the thawing
520
521 201 process, the frozen sperm samples were submerged in water at 40 °C for 13 s
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523 202 (Herranz-Jusdado, 2019). All samples were analysed in triplicate immediately
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525 203 after thawing with the CASA-Mot system previously mentioned (section 2.3).
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2.7. Statistical analysis

The mean \pm standard error was calculated for all sperm parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. Univariate General Linear Model (GLM) and Student-Newman-Keuls (SNK) tests were used to analyze the sperm kinetic parameters along post-activation times. One-way ANOVA was used to analyze the morphometric parameters. Differences between the control samples and the cryopreserved samples were analyzed using a paired sample T-test. Significant differences were detected when $p\text{-value}<0.05$. All statistical analyses were performed using the statistical package SPSS version 24.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Census and status of populations

In the samplings carried out in the field, we caught individuals of *V. hispanica* in two of the three spots prospected (Fig. 1). *V. hispanica* was present in the "Dosser" wetland and the "Enebro" wetland. The "Dosser" wetland had a sex ratio of 2 females:1 male, while "Enebro" wetland had a sex ratio of 1 female:2 males. Finally, in the "Dunas" wetland no *V. hispanica* was caught, but a all the individuals registered were of Eastern mosquitofish (*G. holbrooki*).

3.2. Evolution of reproductive parameters

Regarding sperm production during the breeding season, *A. iberus* showed a higher percentage of spermiating males (with motile cells) in May than in April

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592
593 229 (Fig. 2A). On the other hand, no difference between April and May was found in
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595 230 *V. hispanica*, and the percentage of spermiating males (approximately 50%) was
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597 231 constant during these months (Fig. 2B).
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601 602 233 3.3. Kinetic sperm parameters

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604 234 Sperm motility patterns were similar in *A. iberus* (n=7) and *V. hispanica* (n=13)
605
606 235 (Fig. 3A and 3C). Both species showed high motility values until 30 s post-
607
608 236 activation, reaching maximum MOT values of 71.2 and 74.8%, and maximum
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610 237 pMOT values of 39.5 and 26.9% (*A. iberus* and *V. hispanica*, respectively). After
611
612 238 1 min post-activation, the motility values decreased progressively until reaching
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614 239 values of 10%. The most notable difference in the sperm motion parameters was
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616 240 in the swimming time, with *A. iberus* exhibiting longer values than *V. hispanica*.
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618 241 In this sense, some samples of *A. iberus* spermatozoa were able to move for up
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620 242 to 30 min, whereas the longest that samples of *V. hispanica* spermatozoa were
621
622 243 able to move was for around 10 min.

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624 244 Regarding the kinetic values, the velocity patterns were also similar both in *A.*
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626 245 *iberus* and *V. hispanica* (Fig. 3B and 3D). Both species displayed high VCL
627
628 246 values at the beginning of the activation process, reaching the highest values of
629
630 247 169.5 and 198.3 $\mu\text{m/s}$ (*A. iberus* and *V. hispanica*, respectively). From that point,
631
632 248 a continuous and marked decrease was observed until movement ceased.
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634 249 Regarding fast, medium and slow spermatozoa, *A. iberus* and *V. hispanica*
635
636 250 showed similar trends in the relative percentages. Both species showed an
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638 251 elevated number of fast spermatozoa (>60%) during the 30 s post-activation.
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640 252 However, 1 min after post-activation the levels of fast spermatozoa decreased to
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642 253 less than 40% of the total spermatozoa in both species.
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652 254 **3.4. Morphometric spermatozoa parameters**
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654 255 The morphometric analyses using ASMA software yielded a small spherical head
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656 256 with a long flagellum for *A. iberus* and *V. hispanica*. In addition, no significant
657
658 257 differences were found in the different size parameters including area, perimeter,
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660
661 258 length and width (Table 1). Regarding the shape parameters, some significant
662
663 259 differences were observed. In this sense, *V. hispanica* showed higher values in
664
665 260 ellipticity and elongation than *A. iberus*, while *A. iberus* spermatozoa showed
666
667 261 higher values in rugosity than *V. hispanica*. Images provided by SEM confirm the
668
669 262 similar size and shape obtained by ASMA technique in spermatozoa of both
670
671 263 species (Fig. 5).
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673 264
674
675 265 **3.5. Cryopreservation protocols**
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677 266 Results from the cryopreservation trials showed that the cryopreservation
678
679 267 process caused a reduction of sperm motion parameters (MOT, pMOT, VCL and
680
681 268 VSL) in both species (Fig. 6 and 7). In this sense, sperm samples from *A. iberus*
682
683 269 reached post-thawed MOT values of 21.3%, while in *V. hispanica* the motility
684
685 270 values after cryopreservation process were a little higher, reaching 24.7% of
686
687 271 motile cells. Regarding pMOT, the values of the fresh sperm samples
688
689 272 (approximately 20%) were also significantly higher than those of the
690
691 273 cryopreserved samples (around 10%), but significant differences were only found
692
693 274 at certain post-activation times. In the case of *A. iberus*, differences were found
694
695 275 at 10 and 30 s post-activation, whereas in *V. hispanica*, differences were found
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697 276 at 30 s post-activation.
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699 277 Regarding the kinetic parameters, in both species lower values of fast
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701 278 spermatozoa were shown in the cryopreserved samples than in fresh samples at
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711 279 every post-activation time (10, 20 and 30 s; Fig. 8). While the percentage of fast
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713 280 spermatozoa of the fresh sperm samples was always higher than 50% of the total
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715 281 motile spermatozoa, the percentage of fast spermatozoa of the cryopreserved
716
717 282 samples was always lower than 60% of all motile spermatozoa in both species
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719 283 and at all post-activation times.
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722 284

724 285 **4. Discussion**

726 286 *4.1. Census and status of populations*

728 287 *A. iberus* and *V. hispanica* are two endemic species of the Iberian Peninsula
729
730 288 whose current populations are in a fragile status of conservation. Even though
731
732 289 populations of both species have increased in the last few years thanks to the
733
734 290 reintroduction and reinforcement programs of the local government (Activity
735
736 291 report CCEDCV, 2018), the random appearance of exotic species can negate
737
738 292 prior conservation efforts (Silva et al., 2019). For example, a population growth
739
740 293 was seen after the initial reintroduction of the species in the “Dunas” wetland in
741
742 294 late 90s (sampled during this study); however, nowadays, no endemic fish have
743
744 295 been detected in this wetland, and all the fishes caught during the samplings were
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746 296 Eastern mosquitofish (*G. holbrooki*). In this sense, it has been demonstrated that
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748 297 the existence of Eastern mosquitofish annihilates the populations of *A. iberus* and
749
750 298 *V. hispanica* in a short space of time (Caiola and de Sostoa, 2005; Rincón et al.,
751
752 299 2002). Considering that the eradication of invasive species is an utopian solution
753
754 300 (Haubrock et al., 2018), the only reasonable solution for long-term conservation
755
756 301 of endemic species would be to improve their competitiveness against invasive
757
758 302 species. In this sense, it has been demonstrated that the optimal salinity range of
759
760 303 *G. holbrooki* is between 15 and 25 g/l (Alcaraz et al., 2008), but *A. iberus* is able
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770 304 to tolerate a larger range of salinity (between 5 and 60 g/l; Oltra and Todolí, 2000).
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772 305 Therefore, the reuse or the construction of artificial bodies of water with high
773
774 306 salinity could benefit this endemic species.
775
776 307 A similar trade-off happens with the temperature. In this regard, the Eastern
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778 308 mosquitofish is a North American species adapted to live in temperate water
780
781 309 (from 15 to 35 °C; Riehl and Baensch, 1996), but usually prefers warmer waters
782
783 310 (31-35 °C; Pyke, 2005). When temperatures are low, it is less voracious and
784
785 311 dominant, a fact that favours the *V. hispanica* population (Carmona-Catot et al.,
786
787 312 2013; Rincón et al., 2002). Our results support this theory because in the “Dosser”
788
789 313 and "Enebro" wetlands (showing temperatures lower than 20 °C), no Eastern
790
791 314 mosquitofish was caught during the samplings. However, in the “Dunas” wetland
792
793 315 (where temperatures higher than 20 °C were recorded), Eastern mosquitofish
794
795 316 was the prevalent species. Taking into consideration the above, the choice of
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797 317 optimal places for carrying out population reinforcements of *V. hispanica* is
798
799 318 essential to success of any recovery programmes. In this respect, the Albufera
800
801 319 Natural Park contains specific areas of upwellings (named locally as "ullals"),
802
803 320 where the temperature remains around 18 °C throughout the whole year, thus
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805 321 these would be optimal places to carry out population reinforcements and the
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807 322 creation of new populations areas (Technical report GVA, 2015).
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812 324 *4.2. Reproductive parameters*

813 325 Breeding in captivity programs for threatened species require profound
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815 326 knowledge of the reproductive biology of the species in question. Unfortunately,
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817 327 scientific reports concerning Cyprinodontiformes (to which *A. iberus* and *V.*
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819 328 *hispanica* belong) are notably scarce (Gonzalez et al., 2018; Rubio-Gracia et al.,
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829 329 2019; Verdiell-Cubello et al., 2014). The data obtained in this study show that
830
831 330 spermiating males from both species were found in most of the samplings carried
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833 331 out in April. These data corroborates those of several other authors, who have
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835 332 established that the breeding period of these species is between March and
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837 333 September, with reproduction peaks in summer months (Caiola et al., 2001;
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839 334 Oliva-Paterna et al., 2009). Nevertheless, in the *A. iberus* population the number
840
841 335 of spermiating fish was higher in May than in April, while no difference in the
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843 336 percentage of spermiating males was found in the case of *V. hispanica*.
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846 337 The sex ratio of the wild populations of *V. hispanica* sampled in this study, ranged
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848 338 from 2:1 (female:male) in the “Dosser” wetland to 1:2 in the “Enebro” wetland.
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850 339 The inequality in the sex ratio could be due to several factors in the wild such as
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852 340 *i*) differences in the mortality rate of the two sexes, this tending to be higher in
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854 341 males because of predation due to their colouration during the reproduction
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856 342 period (Labbaci et al., 2019); *ii*) differences in life expectancy, similar to what
857
858 343 occurs in the *V. hispanica* population studied in Catalonia, where the maximum
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860 344 age of females was 4 years, whereas that of males was only 3 years (Caiola et
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862 345 al., 2001), or *iii*) the environmental sex determination, where some factors (such
863
864 346 as temperature) determine the gender as occurs in others Cyprinodontiformes
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866 347 (Barón et al., 2002; Yamamoto et al., 2014). In this study, the sex ratio found in
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868 348 the different places where sampling took place in the Albufera Natural Park could
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870 349 be explained by a combination of the previously described factors. In practical
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872 350 terms, the knowledge of sex ratio parameters could be a useful tool for optimising
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874 351 the breeding captive programs carried out in the CCEDCV, where different
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876 352 populations from different wetlands are maintained separately in their facilities.
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353 On the other hand, a key factor in order to successfully carry out *ex situ*
354 conservation programs is the knowledge of gamete quality of the breeding
355 animals. Regarding males, fish sperm motility is nowadays considered the best
356 biomarker for the quality of fish spermatozoa, including certain aspects such as
357 *i)* the number of motile spermatozoa, *ii)* how they move, and *iii)* the duration of
358 movement (Gallego and Asturiano, 2018). Ours is the first study to use the CASA-
359 Mot system to report, the sperm motion parameters of *A. iberus* and *V. hispanica*.
360 The motility and velocity values of both species were high when activated by
361 freshwater and showed a gradual decrease over time. These data agree suggest
362 similarities with other Cyprinodontiformes species such as *Fundulus grandis*,
363 whose sperm kinetic pattern was very similar to that of *A. iberus* and *V. hispanica*,
364 with a motility peak at 30 s post-activation, that decreased gradually over the next
365 10 min (Tiersch and Yang, 2012).
366 The main difference between these species was the post-activation swimming
367 time: while several samples of *A. iberus* spermatozoa moved for more than 15
368 min (including a sampling that reached 30 min), samples of *V. hispanica*
369 spermatozoa moved for less than 10 min. These differences could be due to the
370 different fertilization strategies used for the two species (Gallego et al., 2014).
371 While *V. hispanica*, males and females swim close to the water's surface and use
372 macrophytes to release gametes (eggs and spermatozoa), *A. iberus* swim in a
373 lower layer of the water column and prefer to release the gametes in deeper
374 vegetation (Rincón et al., 2002). In addition, *A. iberus* males push the females
375 against the vegetation, and competition between males to fertilize the eggs may
376 exist (Vargas and de Sostoa, 1997). In this regard, the differences in the
377 distribution along the water column (known as vertical segregation) and the

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947 378 mating strategies could explain the variance in the spermatozoa motion values
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949 379 (Rincón et al., 2002).
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951 380 Furthermore, when we compare our results (from oviparous species) with
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953 381 ovoviviparous species belonging to the same order (Cyprinodontiformes), the
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955 382 hypothesis of “fertilization strategies and sperm motion” gains strength. In this
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957 383 regard, sperm from ovoviviparous species such as *Jenynsia multidentata* (Roggio
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959 384 et al., 2014) and *Poecilia reticulata* (Gasparini et al., 2014), show lower velocities
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961 385 than sperm from oviparous species (*A. iberus* and *V. hispanica*). This can be
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963 386 explained by the sperm competition theory: while spermatozoa from live-bearing
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965 387 species do not have to battle with other spermatozoa in the water column (the
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967 388 sperm is directly released into the female); spermatozoa from external fertilizers
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969 389 must compete to reach the oocyte in the external medium (Simpson et al., 2013).
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971 390 Regarding morphology, ASMA analyses plus SEM revealed that *A. iberus* and *V.*
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973 391 *hispanica* spermatozoa showed a similar size and shape to the sperm of other
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975 392 Cyprinodontiformes: small head-rounded heads, unflagellated and without
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977 393 acrosomes (Mattei, 1991). However, the head size of *A. iberus* and *V. hispanica*
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979 394 spermatozoa was bigger than that of species from a closely related order:
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981 395 *Odontesthes* (Gárriz and Miranda, 2013). Finally, despite the sperm cells of both
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983 396 species (*A. iberus* and *V. hispanica*) showing a spherical shape, significant
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985 397 differences were found between some parameters such as ellipticity and
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987 398 elongation.
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993 400 4.3. Sperm cryopreservation

994 401 The creation of genetic resources banks as a tool for biodiversity conservation is
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996 402 fundamental for the conservation and management of threatened species (Holt
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1006 403 et al., 1996). Regarding *A. iberus* and *V. hispanica*, long-term gamete
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1008 404 preservation could allow for the creation of a genetic stock, preserving the
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1010 405 different haplotypes that both species present. For example, an haplotype of *V.*
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1012 406 *hispanica* became extinct in its original location (Albuixech, Valencia), and
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1015 407 nowadays it is only maintained in captivity in the CCEDCV facilities (Technical
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1017 408 report GVA, 2015). Therefore, cryobanking can complement *in situ* and *ex situ*
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1019 409 conservation programs by preserving genotypes of certain populations that for
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1021 410 stochastic reasons could be extinguished in the wild.
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1023 411 This study has been the first to obtain positive results in sperm cryopreservation
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1025 412 for these two threatened species, signaling an advance in complementing the
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1027 413 current *in situ* and *ex situ* conservation programs (Technical report GVA, 2015).
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1029 414 Although the sperm motility values of the cryopreserved-thawed samples which
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1031 415 were reached in the trials were not very high (20 and 25% on *A. iberus* and *V.*
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1033 416 *hispanica*, respectively), the establishment of the foundations for the first protocol
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1035 417 will become the basis for further improvement over the next few years.
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1037 418 The first handicap we find when freezing sperm from small species (such as *A.*
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1039 419 *iberus* and *V. hispanica*) is the tiny volume that they produce (usually less than 2
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1041 420 μL). Although gametes are usually collected by stripping, in small fish (i.e.
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1043 421 zebrafish, medaka, etc.) the collection of gametes could be done by surgical
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1045 422 extraction in order to try to optimize sperm extraction (Viveiros and Godinho,
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1047 423 2009). In this study, the application of this method was not viable due to the fragile
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1049 424 status of conservation of both species. In addition, the low volume collected
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1051 425 during the gamete extraction made it impossible to carry out a complete battery
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1053 426 of cryopreservation experiments and compare different experimental variables
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1055 427 (type of cryoprotectant, sperm:extender ratio, cooling and thawing rates, etc.).
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1065 428 Therefore, sperm samples of *A. iberus* and *V. hispanica* were cryopreserved
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1067 429 based on a cryopreservation protocol previously developed by our group
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1069 430 (Herranz-Jusdado et al., 2019).

1071 431 In other fish species with similar technical limitations (hard management, tiny
1072 432 sperm volume collected, etc.), several authors have reported similar motility
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1074 433 results in cryopreservation trials to those we obtained in this study (about 30-40%
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1076 434 decrease compared to the fresh samples). Regarding zebra fish (*Danio rerio*),
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1078 435 Diogo et al. (2018) reported that MOT, VCL and VSL values decreased by
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1080 436 approximately 50% compared to fresh samples. In *Odontesthes bonariensis*, only
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1082 437 the use of DMSO and egg yolk as cryoprotectants provided positive results in the
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1084 438 cryopreservation process, although motility values decreased 40% compared to
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1086 439 fresh samples (Lichtenstein et al., 2010; Lichtenstein and Miranda, 2007). In
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1088 440 medaka (*Oryzias latipes*), Yang et al. (2010) reported that the motility of
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1090 441 cryopreserved samples (using methanol 10% v/v) decreased 30% compared to
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1092 442 fresh samples.

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1095 443 To sum up, this study improves our knowledge of the reproductive biology of *A.*
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1097 444 *iberus* and *V. hispanica* by reporting sperm motion parameters and spermatozoa
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1099 445 morphometric features. In addition, this study is the first of its kind to achieve
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1101 446 gamete cryopreservation of these species. These are all new tools which can be
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1103 447 used to complement the management and conservation programs that are being
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1111 450 **Declaration of interest**

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1114 451 The authors declare no conflict of interests.

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1124 453 **Ethics statement**
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1126 454 This study was carried out in strict accordance with the recommendations given
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1128 455 in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal
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1130 456 Decree 53/2013 regarding the protection of animals used for scientific purposes
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1132 457 (BOE 2013). The protocol was approved by the Experimental Animal Ethics
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1134 458 Committee from the Universitat Politècnica de València (UPV) and final
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1136 459 permission was given by the local government for managing endangered fish
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1138 460 species (Generalitat Valenciana).
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1160 470 **References**
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1537 621 **Tables**
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1541 623 **Table 1.** Morphometric parameters of sperm head of *Aphanius iberus* (n=9) and
1542
1543 *Valencia hispanica* (n=8) measured with ASMA software using optic microscopy.
1544 624
1545 Dates are expressed as the mean \pm SEM (standard error of the mean). Different
1546 625
1547 letters mean significant differences between species (p-value \leq 0.05).
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| | <i>A. iberus</i> | <i>V. hispanica</i> |
|---|--------------------|---------------------|
| Area (μm^2) | 4.84 \pm 0.06 | 4.81 \pm 0.04 |
| Perimeter (μm) | 8.04 \pm 0.05 | 8.02 \pm 0.04 |
| Lenght (μm) | 2.55 \pm 0.01 | 2.55 \pm 0.01 |
| Width (μm) | 2.35 \pm 0.01 | 2.32 \pm 0.01 |
| Ellipticity | 1.09 \pm 0.01 b | 1.10 \pm 0.004 a |
| Elongation | 0.04 \pm 0.001 b | 0.05 \pm 0.002 a |
| Rugosity | 0.95 \pm 0.001 a | 0.94 \pm 0.001 b |
| Regularity | 0.97 \pm 0.003 | 0.97 \pm 0.004 |

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1596 629 **Figure legends**
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1600 631 **Figure 1.** Percentage of *Valencia hispanica* (males, females, fries) and Eastern
1601
1602 mosquitofish captured with fish traps in different wetlands of Albufera Park (El
1603 632
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1605 633 Dosser, Enebro and Dunas).
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1607 634

1609 635 **Figure 2.** Percentage of spermiating males with motile cells (Sperm+),
1610
1611 spermiating males without motile cells (Sperm) and no spermiating males, (No
1612 636
1613 spermiating males, (No sperm) during the breeding season in *Aphanius iberus* (A; n=10-24) and *Valencia*
1614 637
1615 638 *hispanica* (B; n=9-21).
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1617 639

1619 640 **Figure 3.** Sperm kinetic parameters in *Aphanius iberus* (A and B; n=7) and
1620
1621 *Valencia hispanica* (C and D; n=13) at different post-activation times. Data are
1622 641
1623 expressed as the mean \pm SEM (standard error of the mean). Different letters
1624 642
1625 mean significant differences over time (p -value \leq 0.05).
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1628 644 Graphs show total motility (MOT), progressive motility (pMOT), curvilinear
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1630 645 velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP).
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1632 646

1634 647 **Figure 4.** Percentage of fast (FA), medium (ME) and slow (SL) spermatozoa at
1635
1636 different post-activation times in *Aphanius iberus* (A; n=7) and *Valencia hispanica*
1637 648
1638 (B; n=13).
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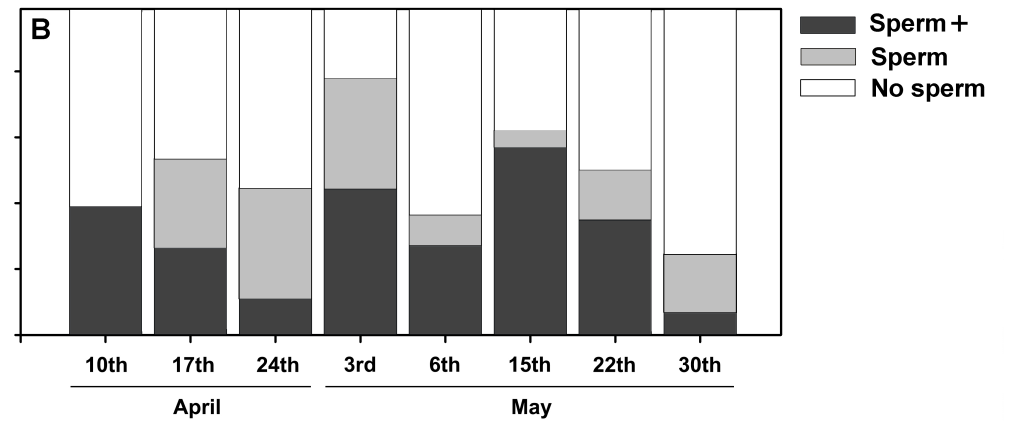
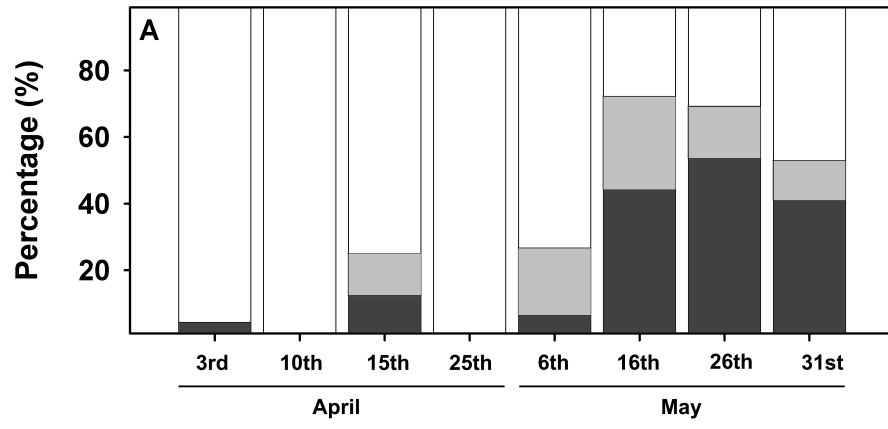
1643 651 **Figure 5.** Scanning electron microscopy of spermatozoa of *Aphanius iberus* (A
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1645 and B) and *Valencia hispanica* (C and D). Scale bar is showed in the figure.
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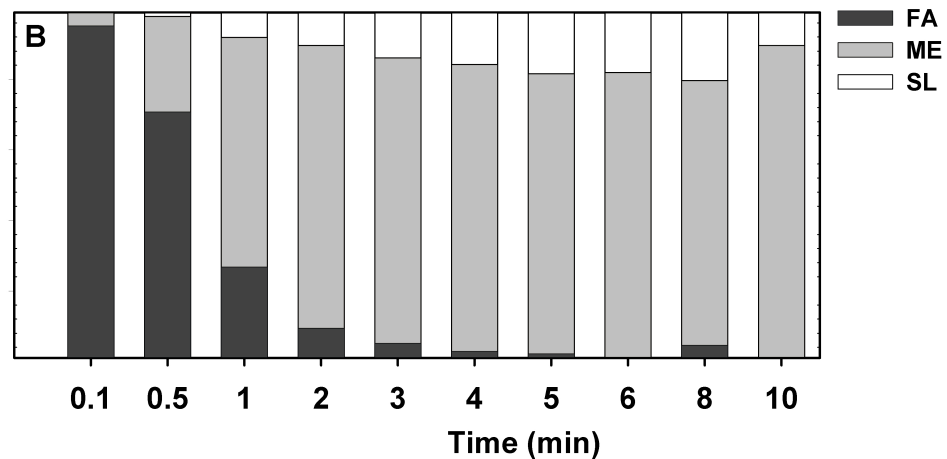
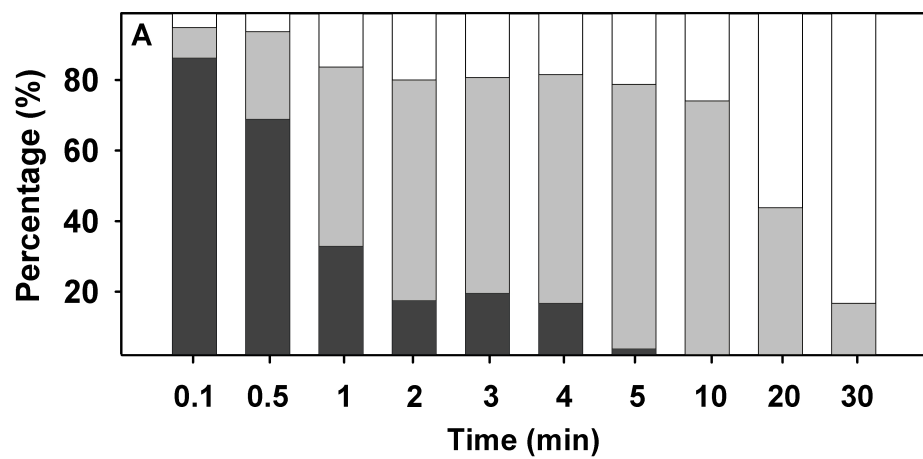
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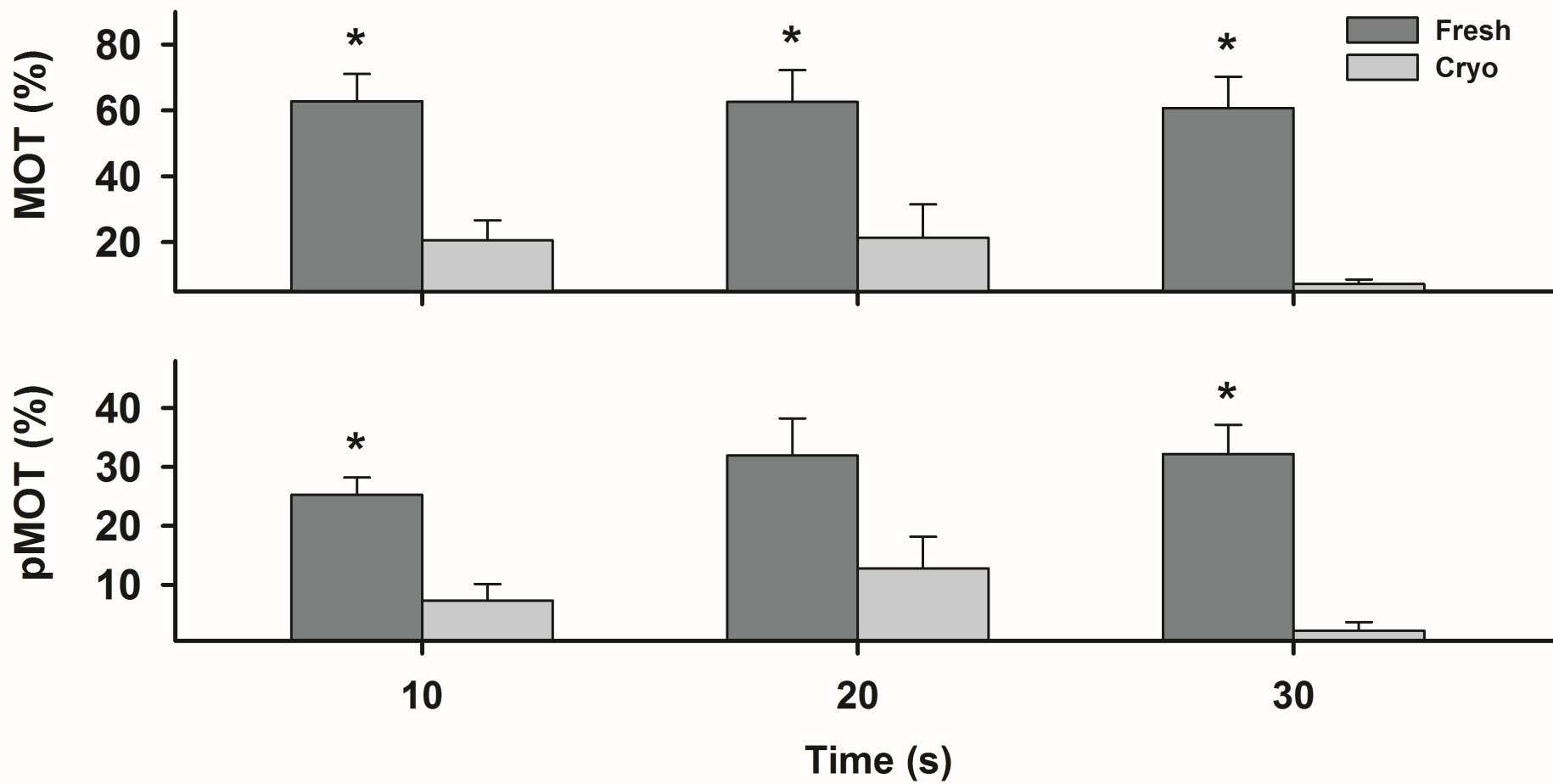
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1655 654 **Figure 6.** Total motility (MOT) and progressive motility (pMOT) of fresh and
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1657 655 thawed sperm samples (Cryo) at different post-activation times in *Aphanius*
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1659 656 *iberus*. Data are expressed as the mean \pm SEM (n=7). Asterisk means significant
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1661 657 differences (p-value \leq 0.05).
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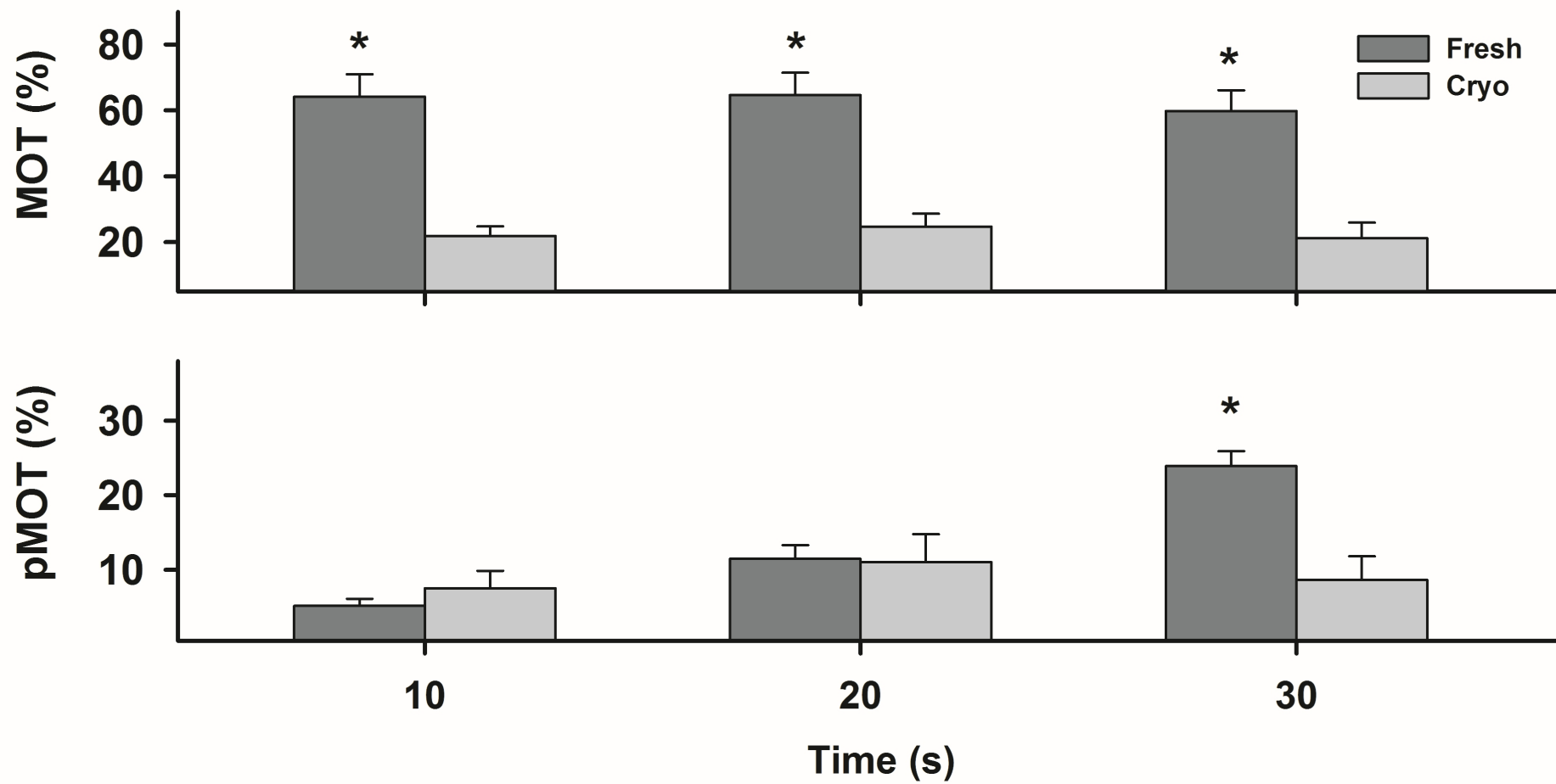
1665
1666 659 **Figure 7.** Total motility (MOT) and progressive motility (pMOT) of fresh and
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1668 660 thawed sperm samples (Cryo) at different post-activation times in *Valencia*
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1670 661 *hispanica*. Data are expressed as the mean \pm SEM (n=8). Asterisk means
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1672 662 significant differences (p-value \leq 0.05).
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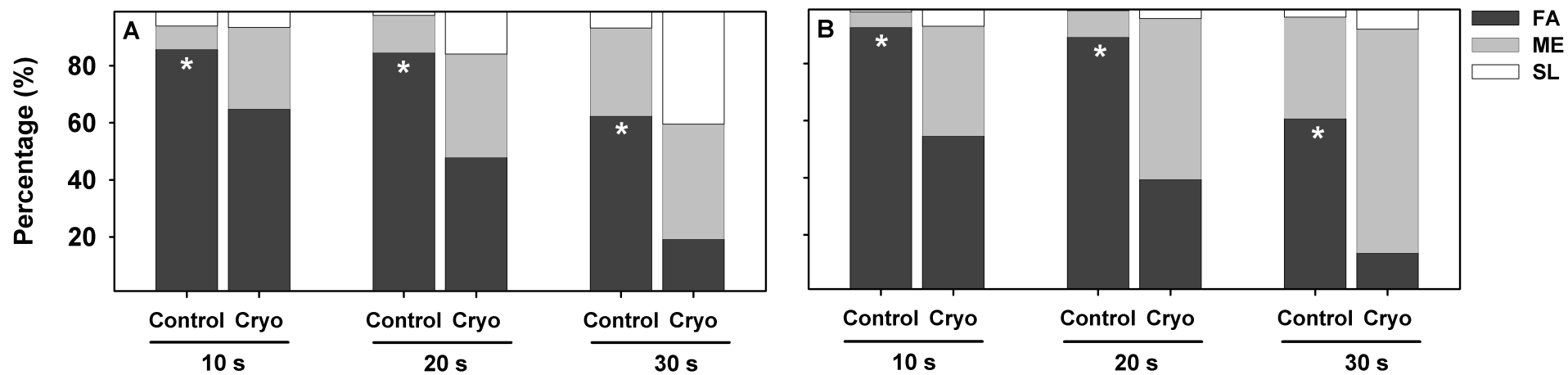
1676 664 **Figure 8.** Percentage of fast (FA), medium (ME) and slow (SL) spermatozoa of
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1678 665 fresh and thawed sperm samples (Cryo) at different post-activation times in
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1680 666 *Aphanius iberus* (A; n=7) and *Valencia hispanica* (B; n=8). Data are expressed
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1682 667 as the mean \pm SEM (standard error of the mean). Asterisk means significant
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1684 668 differences (p-value \leq 0.05).
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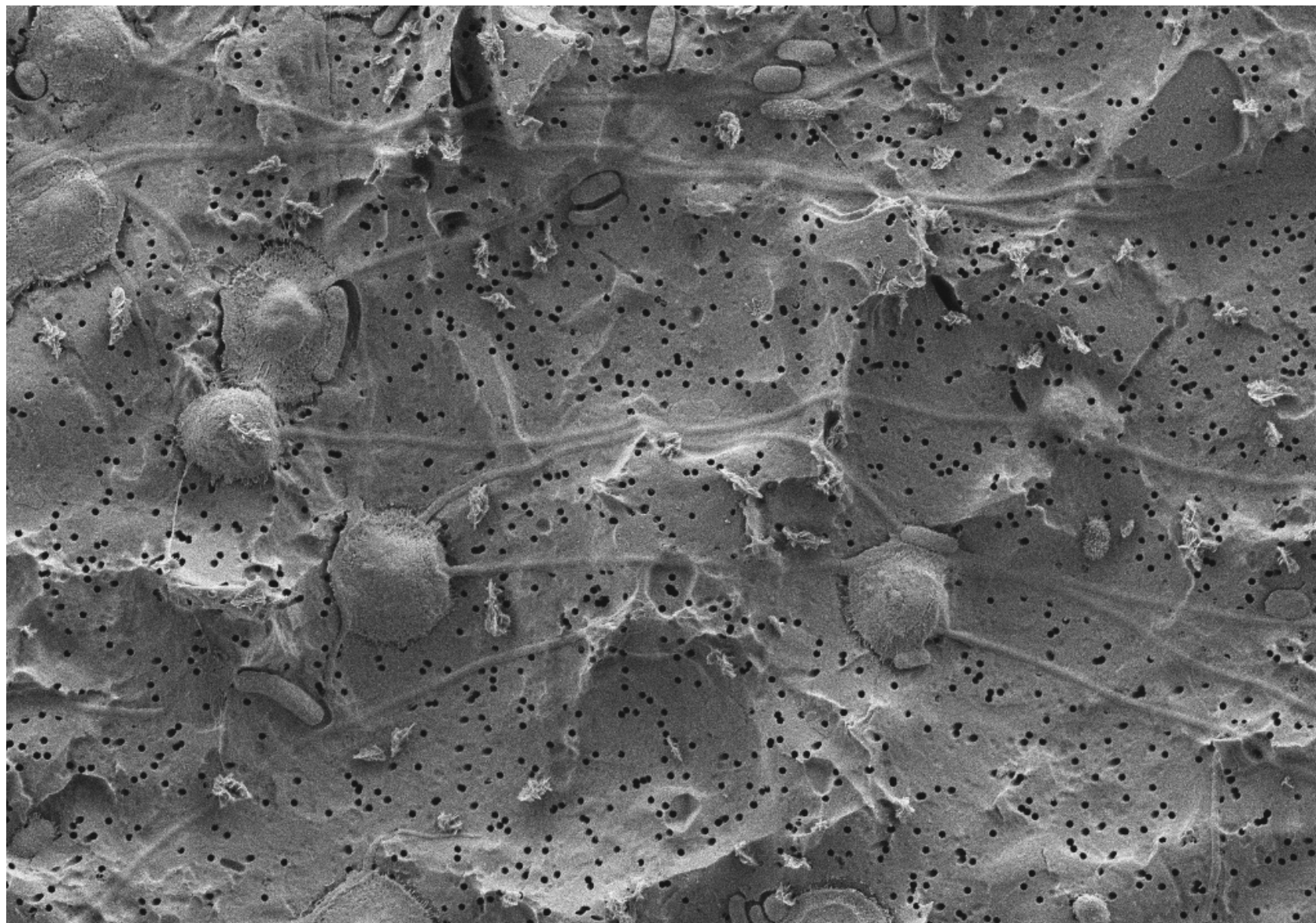






M. Blanes-García: Validation, Formal analysis, Investigation, Resources, Writing – Original Draft, Visualization. **P. Risueño:** Resources, Review & Editing.

L. Pérez: Writing – Review & Editing. **J .F. Asturiano:** Resources, Writing – Review & Editing, Project Administration; **V. Gallego:** Validation, Investigation, Resources, Writing – Review & Editing, Supervision.



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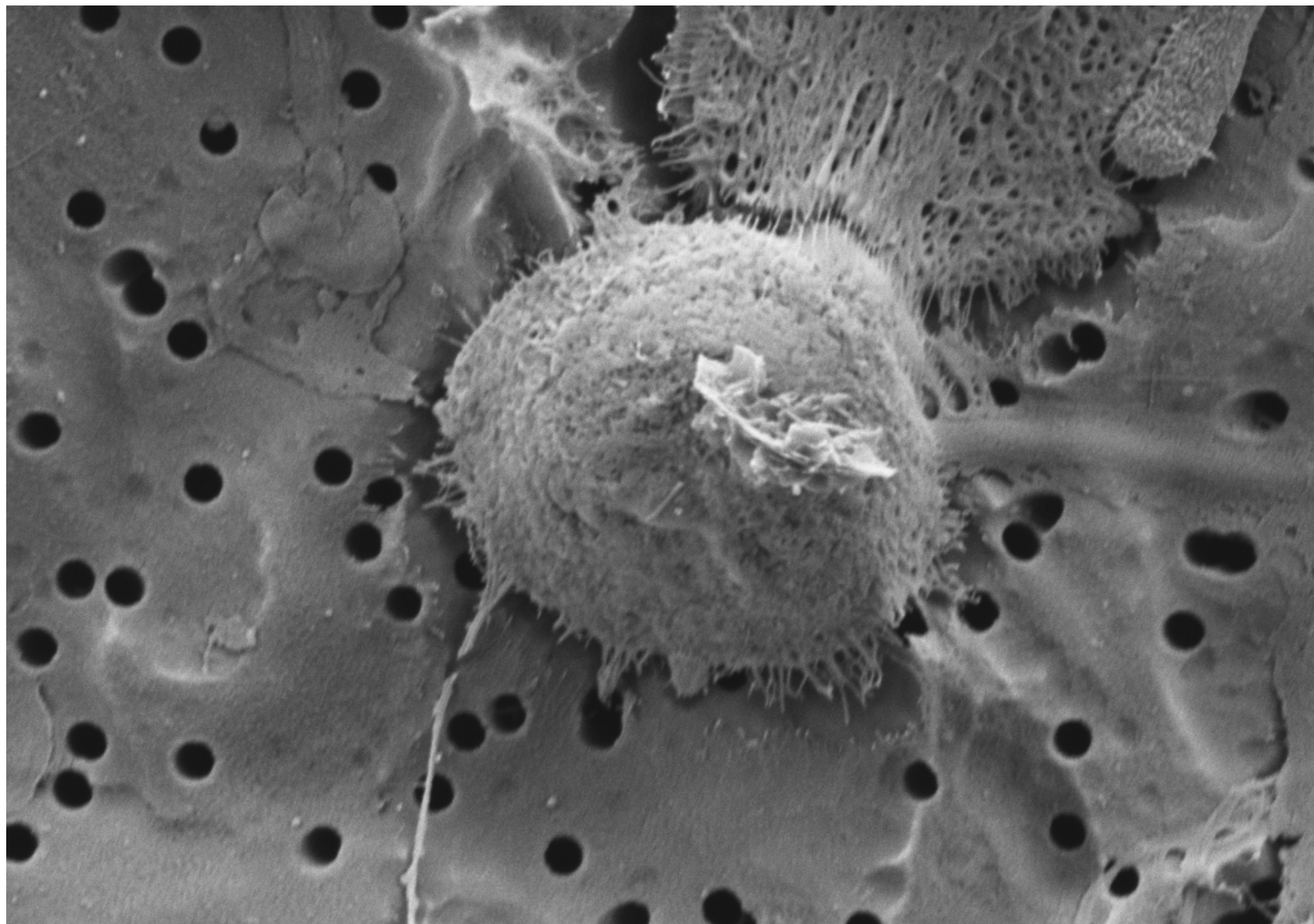
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WD = 4.0 mm

EHT = 1.50 kV
Noise Reduction = Pixel Avg.

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ESB Grid = 800 V

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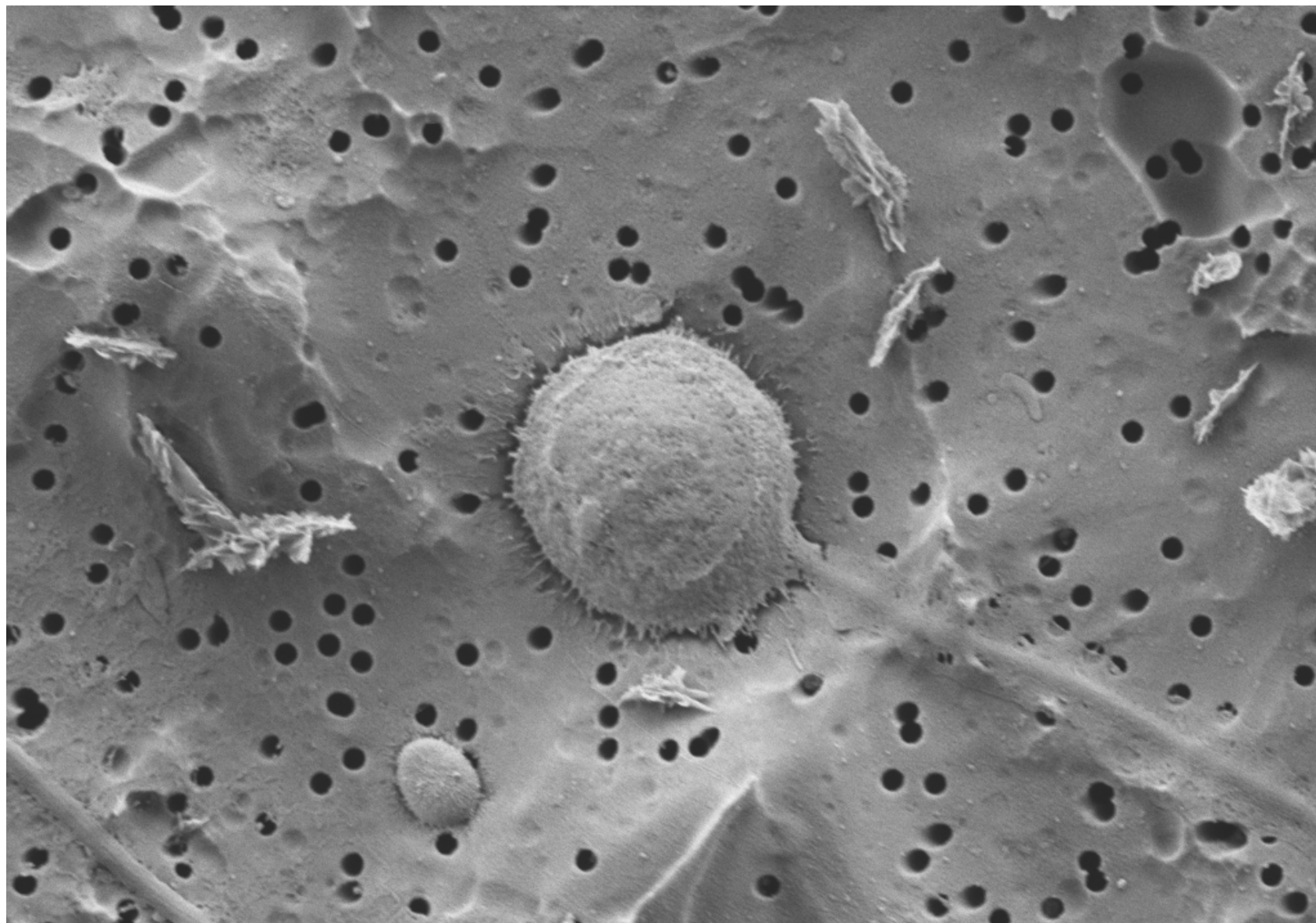
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EHT = 1.50 kV
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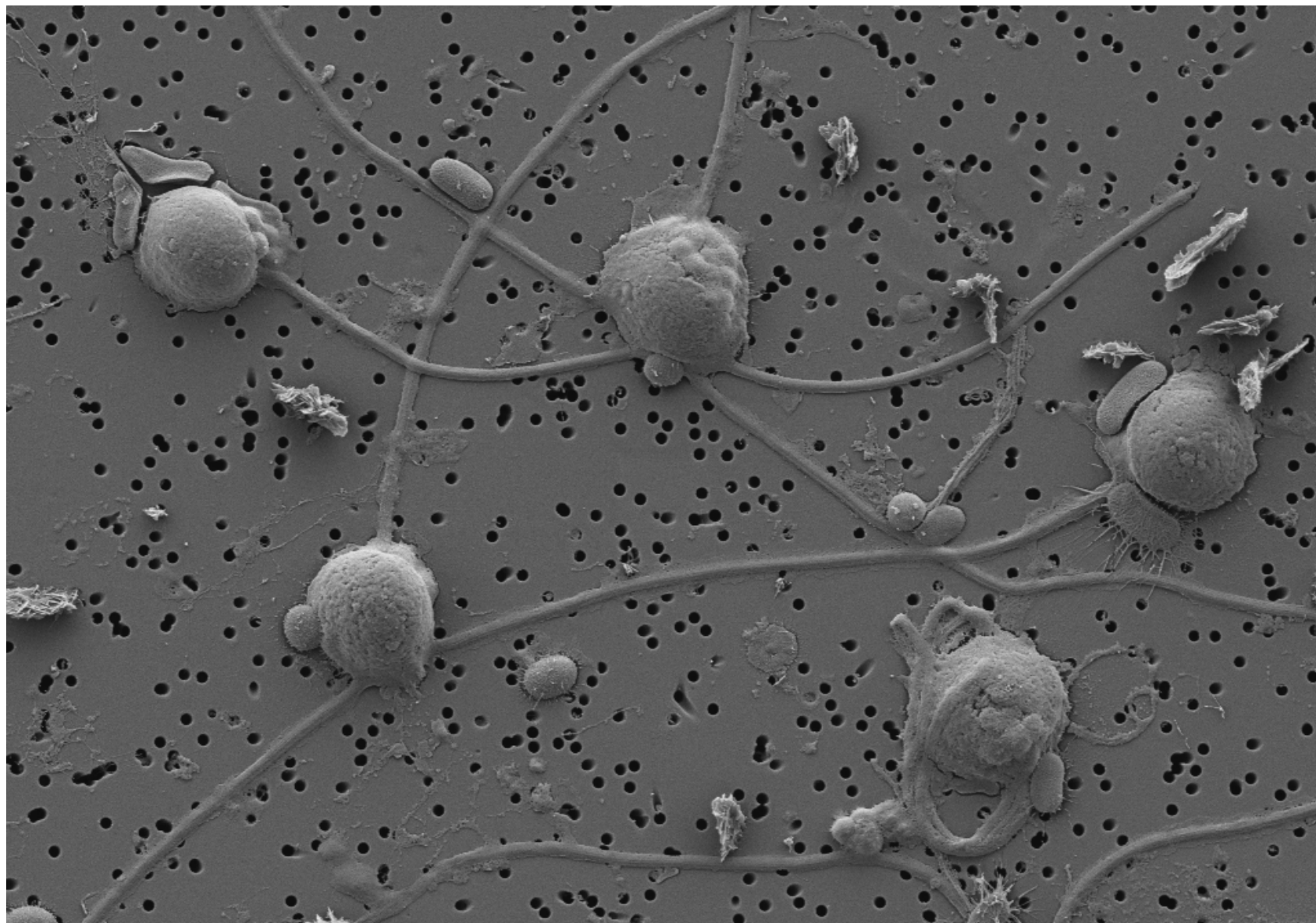
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WD = 4.0 mm

EHT = 1.50 kV
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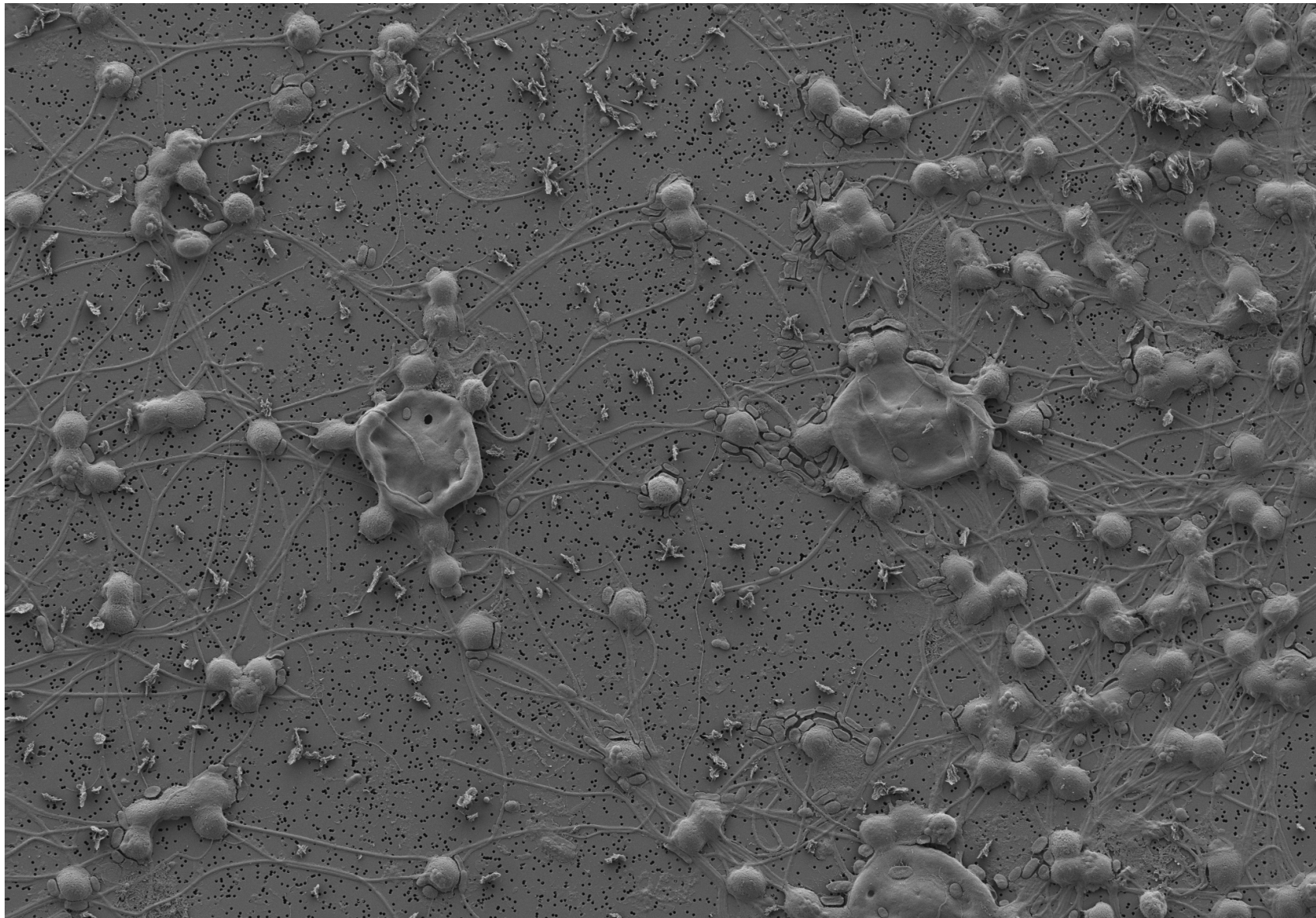
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WD = 5.8 mm

EHT = 1.50 kV
Noise Reduction = Pixel Avg.

Signal A = SE2
ESB Grid = 800 V

Time :10:31:58 Date :29 May 2019



Mag = 1.34 K X
ULTRA 55-44-22

10 μ m

WD = 5.8 mm

EHT = 1.50 kV
Noise Reduction = Pixel Avg.

Signal A = SE2
ESB Grid = 800 V

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