Document downloaded from:

http://hdl.handle.net/10251/182572

This paper must be cited as:

Blanes-García, M.; Risueño, P.; Pérez Igualada, LM.; Asturiano, JF.; Gallego Albiach, V. (2021). Sperm quality parameters of Iberian toothcarp (Aphanius iberus) and Valencia toothcarp (Valencia hispanica): new conservation tools from a gamete perspective. Aquaculture. 530:1-9. https://doi.org/10.1016/j.aquaculture.2020.735819

The final publication is available at

https://doi.org/10.1016/j.aquaculture.2020.735819

Copyright Elsevier

Additional Information

Sperm quality parameters of Iberian toothcarp (*Aphanius iberus*) and Valencia toothcarp (*Valencia hispanica*): new conservation tools from a gamete perspective

M. Blanes-García[a], P. Risueño[b], L. Pérez[a], J.F. Asturiano[a], V. Gallego[a,*]

[a] *Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n. Edificio 7G 46022, Valencia, Spain.*

[b] *Centro de Conservación de Especies Dulceacuícolas de la Comunitat Valenciana (CCEDCV). Plaza 04, El Palmar, Valencia, Spain.*

*Corresponding author:

Dr. Victor Gallego

Grupo de Acuicultura y Biodiversidad

Instituto de Ciencia y Tecnología Animal

Universitat Politècnica de València

Camino de Vera s/n 46022 Valencia (Spain)

email: vicgalal@upvnet.upv.es

Phone: +34 645 515 527

Abstract

The sensitive state of conservation of several endemic fish species such as Iberian toothcarp (*Aphanius iberus*) and Valencia toothcarp (*Valencia hispanica*) has led governments to consider the implementation of conservation measures to preserve their populations. However, limited knowledge about the reproductive biology of these species makes it necessary to investigate different aspects of their reproductive cycle. In this sense, the main objectives of this work were *i*) to advance knowledge of the breeding biology of both species, and *ii*) to develop protocols for the conservation of gametes for the future management and conservation.

During the spring of 2019 a temporal series of samplings were carried out in different places in the Comunitat Valenciana. Sperm samples were collected and sperm motion parameters were assessed for the first time in both species. Kinetic patterns were similar showing high motility and velocity values during the first 30 s, and a rapid decrease from that point. At the same time, an in-depth morphometric analysis was carried out using computer-assisted sperm analysis software. Spermatozoa from *A. iberus* and *V. hispanica* showed similar sizes and shapes to other external fertilizers belonging to Cyprinodontiformes, with small spherical heads, uniflagellated and without acrosomes.

In addition, a new cryopreservation protocol was designed for cryobanking the sperm of these threatened species. Cryopreserved samples showed lower motility than fresh samples but reaching acceptable percentages of motile cells after thawing of around 20 and 25% (*A. iberus* and *V. hispanica*, respectively). This study is the first of its kind to successfully achieve gamete cryopreservation of these two endemic and endangered species from the Iberian Peninsula,

providing new and useful tools to complement the management and conservation programs that are being developed for both species.

Keywords

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

1. Introduction

Fish populations of the Mediterranean coast have been declining since the mid-20th century as a result of several factors such as habitat loss (degradation, fragmentation and destruction), water pollution, dredging and draining processes, etc. Among these factors, the presence of invasive species is probably the main cause of this dramatic decline, and nowadays this factor is widely regarded to be one of the top threats to global biodiversity (Courchamp et al., 2017). In the case of the Iberian toothcarp (*Aphanius iberus*) and Valencia toothcarp (*Valencia hispanica*), the introduction of the Eastern mosquitofish (*Gambusia holbrooki*) to eradicate malaria in the early 20th century triggered the beginning of the decrease of both local species. Despite the constant effort made by the government administrations, both species still have a fragile conservation status. In fact, they are included in the IUCN Red List of Threatened Species, classified as "*Endangered*" (*A. iberus*; Crivelli, 2006a) and "*Critically endangered*" (*V. hispanica*; Crivelli, 2006b).

The *in situ* measurements carried out during the last few decades (monitoring programs, wetlands restoration, etc.) have been successfully supplemented with the *ex situ* conservation actions. In this sense, the launch of successful captive breeding programs carried out at the Center for the Conservation of Freshwater Species of the Valencian Community (CCEDCV, El Palmar, Valencia) has allowed periodic reinforcements of specific populations and the creation of new population nucleus (Risueño and Hernández, 2000). However, due to the limited knowledge on the reproductive biology of these species (García-Alonso et al., 2009), it is necessary to investigate different aspects of their reproductive cycle such as when is the breeding season of these species or when their gamete

quality is optimal, in order to carry out artificial insemination to improve the population management of these species (Comizzoli, 2015).

On the other hand, to complement the *in situ* and *ex situ* conservation tasks, one conservation strategy currently applied in fish management is the creation of genetic resources banks (GRBs) (Martínez-Páramo et al., 2017). The use of GRBs for captive breeding programs make several tasks possible, including *i*) the preservation of the genetic material of endangered species, *ii*) the conservation of certain genetic lines or haplotypes, or *iii*) the recovery of lost genetic characteristics in some populations or individuals (becoming a wild-phenotypic backup). In this sense, one of the important objectives of developing fish sperm cryopreservation protocols is their application in restocking and conservation programs.

However, it is important to note that the development of GRBs combines knowledge of reproductive biology and cryobiology techniques. In this regard, knowledge of the breeding cycle of species is essential in order to be able to apply preservation techniques when the best quality genetic material is available. The timing of gamete collection is an essential step in order to obtain high quality gametes, since best gamete quality is imperative to the success of the fertilization process (Comizzoli, 2015). On the other hand, gamete handling can also affect (and reduce) gamete quality: when sperm is collected by stripping, several fluids (urine, feces, etc.) can spontaneously activate the sperm due to osmotic changes in the medium (Beirão et al., 2019). Finally, the choice of the right species-specific extender for diluting the fresh sperm samples can maintain gamete quality over time (Gallego and Asturiano, 2019; Muchlisin, 2014). Therefore, fish diversity

makes it necessary to develop specific cryopreservation protocols according to the species and the material to be preserved (Asturiano et al., 2017).

Summing up, because of the limited knowledge of the reproductive biology of these endangered species, the aim of this study was *i*) to increase our knowledge of different aspects of their reproductive process during their breeding season, *ii*) to describe sperm kinetic and morphometric parameters of both species, and *iii*) to develop a gamete cryopreservation protocol for future management and conservation programs.

2. Methods

2.1. Fish handling

For the capture of the exemplars, it was used basket fish traps. The traps had a tubular shape and a structure that difficult the escape of the fishes. On the one hand, Iberian toothcarps (*A. iberus*) were caught from a captive population in the CCEDCV facilities. Two fish traps with food inside were placed in the ponds and they were collected after 2 h. The captive population was originally from Albuixech (Valencia), and it was maintained in the facilities with natural photoperiod, temperature, and autochthonous vegetation. On the other hand, Valencia toothcarps (*V. hispanica*) were caught from three differents spots of the Albufera Natural Park. It was used 10 fish traps in every case, that were placed among the vegetation. After 2 h, the fish traps were collected and the captured fishes were counted and sexed. A selection of the fishes was carried out to the CCEDCV for the experiment and the ex situ conservation programs that are being developed in the centre.

2.2. Sperm collection

Before handling, breeders (*A. iberus,* n=10-24*; V. hispanica,* n=9-21) were anaesthetised using clove oil (80 ppm; Kroon, 2015). Due to the small size of both species, a sponge with a small cut was used to fit the fish into, in order to minimise fish manipulation during sperm extraction. Sperm samples (1-2 µl) were collected by the application of abdominal pressure using a microcapillary tube (40 x 1 mm for *A. iberus*; 75 x 1 mm for *V. hispanica*) after cleaning the genital area with NaCl 0.9% (pH 8.0, 303 mOsm/kg) to avoid contamination by feces, urine or freshwater. The sperm was then diluted 1:20 for *A. iberus* and 1:50 for *V. hispanica* (sperm:extender) in a PBS medium (pH 8.0, 309 mOsm/kg) and kept in 500 ml microtubes. Microtubes were kept in a portable fridge (4 ºC) up to 2 hours, until the sperm analyses were carried out at the facilities of the Universitat Politècnica de València.

2.3. Evaluation of sperm motility and kinetic parameters

Samples were activated by mixing water from fish breeding tanks (0 mOsm/kg) with 2% BSA (w/v) and adjusting the pH to 8.0. The mix was examined using a SpermTrack-10 chamber (Proiser R+D, S.L., Paterna, Spain). Video sequences of 0.5 s were recorded (at 50 fps) using a video camera (Nikon Digital Sight DS-5M) mounted on a phase contrast microscope (Nikon Eclipse 80i) with a 10x objective lens. All the motility analyses were performed in triplicate using the CASA-Mot software (Computer Assisted Semen Analysis; Proiser R+D, S.L.; Paterna, Spain). The parameters considered in this study were: total motility (MOT, %), defined as the percentage of motile cells; progressive motility (pMOT, %), defined as the percentage of spermatozoa which swim in what is essentially

a straight line; curvilinear velocity (VCL, μm/s), defined as the average velocity of a spermatozoa head along its curvilinear trajectory; straight line velocity (VSL, μm/s), defined as the time/average velocity of a spermatozoa head along the straight line between its first detected position and its last position; and the percentage of fast (VAP >100 μm/s), medium (VAP = 50-100 μm/s) and slow (VAP = 10-50 μm/s) spermatozoa, defining VAP (average path velocity) as the time/average velocity of a sperm head along its spacial average trajectory. Sperm samples were considered motile if their total motility was over 10%.

2.4. Spermatozoa morphometric analysis

Sperm samples (total motility over 70%) were fixed by adding 1% glutaraldehyde diluted in PBS buffer and were deposited in microtubes (Eppendorf). An aliquot of sperm dilution (approximately 5 μl) was put on a slide and covered with a cover glass. The sperm samples were examined using a phase contrast microscope with a 100x contrast phase lens.

Microphotographs of the spermatozoa were taken using an ISAS 782M camera (Proiser R + D, S.L.), and the morphometric analyses of the sperm samples were performed using the morphometry module of the ISAS software (ASMA; Automated Sperm Morphometry Analysis). The spermatozoa head measurements, including size variables such as length (L, μm), width (W, μm), area (A, μm^2), and perimeter (P, μm) and shape variables such as ellipticity (L/W), rugosity ($4\pi A/P^2$), elongation (L-W)/(L+W), and regularity ($\pi LW/4A$), were calculated automatically by capturing 100 digitized spermatozoa for each sample.

2.5. Scanning electron microscopy (SEM)

Sperm cells were fixed with 2.5% glutaraldehyde diluted in PBS buffer and were deposited in Eppendorf tubes until the scanning electron microscope analysis. The samples were washed in triplicate with a PBS buffer over a micropore filter (0.1 µm) and after that, they were dehydrated with different concentrations of ethanol (30, 50, 70, 80 and 90%, plus three times in 100%), and left 10 min in each concentration. Samples were fixed using a critical point (LEICA EM CPD300), assembled over a specific support and were then platinum coated by sputtering. Finally, head and flagellum measurements were taken using SmartSEM software (ZEISS, Germany).

2.6. Cryopreservation

Seven fresh sperm samples from *A. iberus* and eight fresh sperm samples from *V. hispanica* (total motility > 60%) were used for the cryopreservation trials. Samples were diluted 1:8 (sperm:extender) in a PBS medium and 10% (v/v) of methanol, after the predilution made during the sperm collection (view section 2.2). Samples were immediately packed in straws of 250 µL. Between two and three straws from each specimen were used, according to sperm available volume. Then, the diluted samples were then incubated for 5 min at 4 °C to ensure a stable penetration of the cryoprotectant into the cells. The freezing condition was created by placing the straws on a floating structure 6.5 cm over the liquid nitrogen (LN) for 15 min, and then by dropping them into the LN. For the thawing process, the frozen sperm samples were submerged in water at 40 °C for 13 s (Herranz-Jusdado, 2019). All samples were analysed in triplicate immediately after thawing with the CASA-Mot system previously mentioned (section 2.3).

2.7. Statistical analysis

The mean ± 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

(Fig. 2A). On the other hand, no difference between April and May was found in *V. hispanica*, and the percentage of spermiating males (approximately 50%) was constant during these months (Fig. 2B).

3.3. Kinetic sperm parameters

Sperm motility patterns were similar in *A. iberus* (n=7) and *V. hispanica* (n=13) (Fig. 3A and 3C). Both species showed high motility values until 30 s post-activation, reaching maximum MOT values of 71.2 and 74.8%, and maximum pMOT values of 39.5 and 26.9% (*A. iberus* and *V. hispanica*, respectively). After 1 min post-activation, the motility values decreased progressively until reaching values of 10%. The most notable difference in the sperm motion parameters was in the swimming time, with *A. iberus* exhibiting longer values than *V. hispanica*. In this sense, some samples of *A. iberus* spermatozoa were able to move for up to 30 min, whereas the longest that samples of *V. hispanica* spermatozoa were able to move was for around 10 min.

Regarding the kinetic values, the velocity patterns were also similar both in *A. iberus* and *V. hispanica* (Fig. 3B and 3D). Both species displayed high VCL values at the beginning of the activation process, reaching the highest values of 169.5 and 198.3 μm/s (*A. iberus* and *V. hispanica*, respectively). From that point, a continuous and marked decrease was observed until movement ceased. Regarding fast, medium and slow spermatozoa, *A. iberus* and *V. hispanica* showed similar trends in the relative percentages. Both species showed an elevated number of fast spermatozoa (>60%) during the 30 s post-activation. However, 1 min after post-activation the levels of fast spermatozoa decreased to less than 40% of the total spermatozoa in both species.

3.4. Morphometric spermatozoa parameters

The morphometric analyses using ASMA software yielded a small spherical head with a long flagellum for *A. iberus* and *V. hispanica.* In addition, no significant differences were found in the different size parameters including area, perimeter, length and width (Table 1). Regarding the shape parameters, some significant differences were observed. In this sense, *V. hispanica* showed higher values in ellipticity and elongation than *A. iberus,* while *A. iberus* spermatozoa showed higher values in rugosity than *V. hispanica*. Images provided by SEM confirm the similar size and shape obtained by ASMA technique in spermatozoa of both species (Fig. 5).

3.5. Cryopreservation protocols

Results from the cryopreservation trials showed that the cryopreservation process caused a reduction of sperm motion parameters (MOT, pMOT, VCL and VSL) in both species (Fig. 6 and 7). In this sense, sperm samples from *A. iberus* reached post-thawed MOT values of 21.3%, while in *V. hispanica* the motility values after cryopreservation process were a little higher, reaching 24.7% of motile cells. Regarding pMOT, the values of the fresh sperm samples (approximately 20%) were also significantly higher than those of the cryopreserved samples (around 10%), but significant differences were only found at certain post-activation times. In the case of *A. iberus*, differences were found at 10 and 30 s post-activation, whereas in *V. hispanica*, differences were found at 30 s post-activation.

Regarding the kinetic parameters, in both species lower values of fast spermatozoa were shown in the cryopreserved samples than in fresh samples at

every post-activation time (10, 20 and 30 s; Fig. 8). While the percentage of fast spermatozoa of the fresh sperm samples was always higher than 50% of the total motile spermatozoa, the percentage of fast spermatozoa of the cryopreserved samples was always lower than 60% of all motile spermatozoa in both species and at all post-activation times.

4. Discussion

4.1. Census and status of populations

A. iberus and V. hispanica are two endemic species of the Iberian Peninsula whose current populations are in a fragile status of conservation. Even though populations of both species have increased in the last few years thanks to the reintroduction and reinforcement programs of the local government (Activity report CCEDCV, 2018), the random appearance of exotic species can negate prior conservation efforts (Silva et al., 2019). For example, a population growth was seen after the initial reintroduction of the species in the “Dunas” wetland in late 90s (sampled during this study); however, nowadays, no endemic fish have been detected in this wetland, and all the fishes caught during the samplings were Eastern mosquitofish (*G. holbrooki*). In this sense, it has been demonstrated that the existence of Eastern mosquitofish annihilates the populations of *A. iberus and V. hispanica* in a short space of time (Caiola and de Sostoa, 2005; Rincón et al., 2002). Considering that the eradication of invasive species is an utopian solution (Haubrock et al., 2018), the only reasonable solution for long-term conservation of endemic species would be to improve their competitiveness against invasive species. In this sense, it has been demonstrated that the optimal salinity range of *G. holbrooki* is between 15 and 25 g/l (Alcaraz et al., 2008), but *A. iberus* is able

to tolerate a larger range of salinity (between 5 and 60 g/l; Oltra and Todolí, 2000). Therefore, the reuse or the construction of artificial bodies of water with high salinity could benefit this endemic species.

A similar trade-off happens with the temperature. In this regard, the Eastern mosquitofish is a North American species adapted to live in temperate water (from 15 to 35 ºC; Riehl and Baensch, 1996), but usually prefers warmer waters (31-35 ºC; Pyke, 2005). When temperatures are low, it is less voracious and dominant, a fact that favours the *V. hispanica* population (Carmona-Catot et al., 2013; Rincón et al., 2002). Our results support this theory because in the “Dosser” and "Enebro" wetlands (showing temperatures lower than 20 ºC), no Eastern mosquitofish was caught during the samplings. However, in the “Dunas” wetland (where temperatures higher than 20 ºC were recorded), Eastern mosquitofish was the prevalent species. Taking into consideration the above, the choice of optimal places for carrying out population reinforcements of *V. hispanica* is essential to success of any recovery programmes. In this respect, the Albufera Natural Park contains specific areas of upwellings (named locally as "ullals"), where the temperature remains around 18 ºC throughout the whole year, thus these would be optimal places to carry out population reinforcements and the creation of new populations areas (Technical report GVA, 2015).

4.2. Reproductive parameters

Breeding in captivity programs for threatened species require profound knowledge of the reproductive biology of the species in question. Unfortunately, scientific reports concerning Cyprinodontiformes (to which *A. iberus and V. hispanica* belong) are notably scarce (Gonzalez et al., 2018; Rubio-Gracia et al.,

2019; Verdiell-Cubello et al., 2014). The data obtained in this study show that spermiating males from both species were found in most of the samplings carried out in April. These data corroborates those of several other authors, who have established that the breeding period of these species is between March and September, with reproduction peaks in summer months (Caiola et al., 2001; Oliva-Paterna et al., 2009). Nevertheless, in the *A. iberus* population the number of spermiating fish was higher in May than in April, while no difference in the percentage of spermiating males was found in the case of *V. hispanica*.

The sex ratio of the wild populations of *V. hispanica* sampled in this study, ranged from 2:1 (female:male) in the “Dosser” wetland to 1:2 in the “Enebro” wetland. The inequality in the sex ratio could be due to several factors in the wild such as *i*) differences in the mortality rate of the two sexes, this tending to be higher in males because of predation due to their colouration during the reproduction period (Labbaci et al., 2019); *ii*) differences in life expectancy, similar to what occurs in the *V. hispanica* population studied in Catalonia, where the maximum age of females was 4 years, whereas that of males was only 3 years (Caiola et al., 2001), or *iii*) the environmental sex determination, where some factors (such as temperature) determine the gender as occurs in others Cyprinodontiformes (Barón et al., 2002; Yamamoto et al., 2014). In this study, the sex ratio found in the different places where sampling took place in the Albufera Natural Park could be explained by a combination of the previously described factors. In practical terms, the knowledge of sex ratio parameters could be a useful tool for optimising the breeding captive programs carried out in the CCEDCV, where different populations from different wetlands are maintained separately in their facilities.

On the other hand, a key factor in order to successfully carry out *ex situ* conservation programs is the knowledge of gamete quality of the breeding animals. Regarding males, fish sperm motility is nowadays considered the best biomarker for the quality of fish spermatozoa, including certain aspects such as *i*) the number of motile spermatozoa, *ii*) how they move, and *iii*) the duration of movement (Gallego and Asturiano, 2018). Ours is the first study to use the CASA-Mot system to report, the sperm motion parameters of *A. iberus and V. hispanica*. The motility and velocity values of both species were high when activated by freshwater and showed a gradual decrease over time. These data agree suggest similarities with other Cyprinodontiformes species such as *Fundulus grandis*, whose sperm kinetic pattern was very similar to that of *A. iberus and V. hispanica*, with a motility peak at 30 s post-activation, that decreased gradually over the next 10 min (Tiersch and Yang, 2012).

The main difference between these species was the post-activation swimming time: while several samples of *A. iberus* spermatozoa moved for more than 15 min (including a sampling that reached 30 min), samples of *V. hispanica* spermatozoa moved for less than 10 min. These differences could be due to the different fertilization strategies used for the two species (Gallego et al., 2014). While *V. hispanica,* males and females swim close to the water´s surface and use macrophytes to release gametes (eggs and spermatozoa), *A. iberus* swim in a lower layer of the water column and prefer to release the gametes in deeper vegetation (Rincón et al., 2002). In addition, *A. iberus* males push the females against the vegetation, and competition between males to fertilize the eggs may exist (Vargas and de Sostoa, 1997). In this regard, the differences in the distribution along the water column (known as vertical segregation) and the

mating strategies could explain the variance in the spermatozoa motion values (Rincón et al., 2002).

Furthermore, when we compare our results (from oviparous species) with ovoviviparous species belonging to the same order (Cyprinodontifomes), the hypothesis of “fertilization strategies and sperm motion” gains strength. In this regard, sperm from ovoviviparous species such as *Jenynsia multidentata* (Roggio et al., 2014) and *Poecilia reticulata* (Gasparini et al., 2014), show lower velocities than sperm form oviparous species (*A. iberus* and *V. hispanica)*. This can be explained by the sperm competition theory: while spermatozoa from live-bearing species do not have to battle with other spermatozoa in the water column (the sperm is directly released into the female); spermatozoa from external fertilizers must compete to reach the oocyte in the external medium (Simpson et al., 2013).

Regarding morphology, ASMA analyses plus SEM revealed that *A. iberus and V. hispanica* spermatozoa showed a similar size and shape to the sperm of other Cyprinodontiformes: small head-rounded heads, uniflagellated and without acrosomes (Mattei, 1991). However, the head size of *A. iberus and V. hispanica* spermatozoa was bigger than that of species from a closely related order: *Odontesthes* (Gárriz and Miranda, 2013). Finally, despite the sperm cells of both species (*A. iberus* and *V. hispanica*) showing a spherical shape, significant differences were found between some parameters such as ellipticity and elongation.

4.3. Sperm cryopreservation

The creation of genetic resources banks as a tool for biodiversity conservation is fundamental for the conservation and management of threatened species (Holt

et al., 1996). Regarding *A. iberus* and *V. hispanica*, long-term gamete preservation could allow for the creation of a genetic stock, preserving the different haplotypes that both species present. For example, an haplotype of *V. hispanica* became extinct in its original location (Albuixech, Valencia), and nowadays it is only maintained in captivity in the CCEDCV facilities (Technical report GVA, 2015). Therefore, cryobanking can complement *in situ* and *ex situ* conservation programs by preserving genotypes of certain populations that for stochastic reasons could be extinguished in the wild.

This study has been the first to obtain positive results in sperm cryopreservation for these two threatened species, signaling an advance in complementing the current *in situ* and *ex situ* conservation programs (Technical report GVA, 2015). Although the sperm motility values of the cryopreserved-thawed samples which were reached in the trials were not very high (20 and 25% on *A. iberus* and *V. hispanica*, respectively), the establishment of the foundations for the first protocol will become the basis for further improvement over the next few years.

The first handicap we find when freezing sperm from small species (such as *A. iberus* and *V. hispanica*) is the tiny volume that they produce (usually less than 2 µL). Although gametes are usually collected by stripping, in small fish (i.e. zebrafish, medaka, etc.) the collection of gametes could be done by surgical extraction in order to try to optimize sperm extraction (Viveiros and Godinho, 2009). In this study, the application of this method was not viable due to the fragile status of conservation of both species. In addition, the low volume collected during the gamete extraction made it impossible to carry out a complete battery of cryopreservation experiments and compare different experimental variables (type of cryoprotectant, sperm:extender ratio, cooling and thawing rates, etc.).

Therefore, sperm samples of *A. iberus* and *V. hispanica* were cryopreserved based on a cryopreservation protocol previously developed by our group (Herranz-Jusdado et al., 2019).

In other fish species with similar technical limitations (hard management, tiny sperm volume collected, etc.), several authors have reported similar motility results in cryopreservation trials to those we obtained in this study (about 30-40% decrease compared to the fresh samples). Regarding zebra fish (*Danio rerio*), Diogo et al. (2018) reported that MOT, VCL and VSL values decreased by approximately 50% compared to fresh samples. In *Odontesthes bonariensis*, only the use of DMSO and egg yolk as cryoprotectants provided positive results in the cryopreservation process, although motility values decreased 40% compared to fresh samples (Lichtenstein et al., 2010; Lichtenstein and Miranda, 2007). In medaka (*Oryzias latipes*), Yang et al. (2010) reported that the motility of cryopreserved samples (using methanol 10% v/v) decreased 30% compared to fresh samples.

To sum up, this study improves our knowledge of the reproductive biology of *A. iberus* and *V. hispanica* by reporting sperm motion parameters and spermatozoa morphometric features. In addition, this study is the first of its kind to achieve gamete cryopreservation of these species. These are all new tools which can be used to complement the management and conservation programs that are being developed.

Declaration of interest

The authors declare no conflict of interests.

Ethics statement

This study was carried out in strict accordance with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV) and final permission was given by the local government for managing endangered fish species (Generalitat Valenciana).

Acknowledgements

This project has received funding from the Generalitat Valenciana under the "*Subvenciones para la realización de proyectos de I+D+I desarrollados por grupos de investigación emergentes (GV/2019/130)*". VG has a postdoc grant from the MICIU (IJCI-2017-34200). We would like to thank all the technicians of the CCEDCV for their work and effort during the samplings, specially to J. Velázquez, J. Hernández and A. Pradillo.

References

Alcaraz, C., Bisazza, A., García-Berthou, E., 2008. Salinity mediates the competitive interactions between invasive mosquitofish and an endangered fish. Oecologia 155, 205–213. https://doi.org/10.1007/s00442-007-0899-4

Asturiano, J.F., Cabrita, E., Horvath, A., 2017. Progress, challenges and perspectives on fish gamete cryopreservation: A mini-review. Gen. Comp. Endocrinol. 245, 69–76. https://doi.org/10.1016/j.ygcen.2016.06.019

Barón, B., Bückle, F., Espina, S., 2002. Environmental factors and sexual differentiation in *Poecilia sphenops* Valenciennes (Pisces: Poeciliidae). Aquac.

Res. 33, 615–619.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., Cabrita, E., 2019. Sperm handling in aquatic animals for artificial reproduction. Theriogenology 133, 161–178. https://doi.org/10.1016/j.theriogenology.2019.05.004

Caiola, N., de Sostoa, A., 2005. Possible reasons for the decline of two native toothcarps in the Iberian Peninsula: Evidence of competition with the introduced Eastern mosquitofish. J. Appl. Ichthyol. 21, 358–363. https://doi.org/10.1111/j.1439-0426.2005.00684.x

Caiola, N.A., Vargas, M.J., de Sostoa, A., 2001. Life history pattern of the endangered Valencia toothcarp, *Valencia hispanica* (Actinopterygii: Valenciidae) and its implications for conservation. Arch. für Hydrobiol. 150, 473–489.

Carmona-Catot, G., Magellan, K., García-Berthou, E., 2013. Temperature-specific competition between invasive mosquitofish and an endangered cyprinodontid fish. PLoS One 8. https://doi.org/10.1371/journal.pone.0054734

CCEDCV, 2018. *Activity report.* Valencia, Spain: Direcció General de Medi Natural i d'Avaluació Ambiental.

Comizzoli, P., 2015. Biobanking efforts and new advances in male fertility preservation for rare and endangered species. Asian J. Androl. 17, 640–645. https://doi.org/10.4103/1008-682X.153849

Courchamp, F., Fournier, A., Bellard, C., Bertelsmeier, C., Bonnaud, E., Jeschke, J.M., Russell, J.C., 2017. Invasion biology: specific problems and possible solutions. Trends Ecol. Evol. 32, 13–22.

Crivelli, A.J., 2006a. *Aphanius iberus*. The IUCN Red List of Threatened Species [WWW Document]. URL https://www.iucnredlist.org/species/1846/8299534

Crivelli, A.J., 2006b. *Valencia hispanica*. The IUCN Red List of Threatened Species. [WWW Document]. URL https://www.iucnredlist.org/species/22829/9392487

Diogo, P., Martins, G., Quinzico, I., Nogueira, R., Gavaia, P.J., Cabrita, E., 2018.

Electric ultrafreezer (−150°C) as an alternative for zebrafish sperm cryopreservation and storage. Fish Physiol. Biochem. 44, 1443–1455.

Gallego, V., Asturiano, J.F., 2019. Fish sperm motility assessment as a tool for aquaculture research: a historical approach. Rev. Aquac. 1–28. https://doi.org/10.1111/raq.12253

Gallego, V., Asturiano, J.F., 2018. Sperm motility in fish: Technical applications and perspectives through CASA-Mot systems. Reprod. Fertil. Dev. 30, 820–832. https://doi.org/10.1071/RD17460

Gallego, V., Pérez, L., Asturiano, J.F., Yoshida, M., 2014. Sperm motility parameters and spermatozoa morphometric characterization in marine species: A study of swimmer and sessile species. Theriogenology 82, 668–676. https://doi.org/10.1016/j.theriogenology.2014.05.026

García-Alonso, J., Ruiz-Navarro, A., Chaves-Pozo, E., Torralva, M., García-Ayala, A., 2009. Gonad plasticity and gametogenesis in the endangered Spanish toothcarp *Aphanius iberus* (Teleostei: Cyprinodontidae). Tissue Cell 41, 206–213.

Gárriz, Á., Miranda, L.A., 2013. Ultrastructure of fresh and post thawed sperm of pejerrey *Odontesthes bonariensis* (Atheriniformes). Neotrop. Ichthyol. 11, 831–836. https://doi.org/10.1590/S1679-62252013000400011

Gasparini, C., Kelley, J.L., Evans, J.P., 2014. Male sperm storage compromises sperm motility in guppies. Biol. Lett. 10. https://doi.org/10.1098/rsbl.2014.0681

Gonzalez, E., Cunha, C., Ghanavi, H., Oliva-Paterna, F., Torralva, M., Doadrio, I., 2018. Phylogeography and population genetic analyses in the Iberian toothcarp (Aphanius iberus Valenciennes, 1846) at different time scales. J. Hered. 109, 253–263. https://doi.org/10.1093/jhered/esx076

GVA, 2015. *Technical report. Conservación de peces marismeños en la Comunitat Valenciana. Balance de 25 años de trabajo*. Valencia, Spain: Direcció General de Medi Natural i d'Avaluació Ambiental.

Haubrock, P.J., Criado, A., Monteoliva, A.P., Monteoliva, J.A., Santiago, T., Inghilesi,

A.F., Tricarico, E., 2018. Control and eradication efforts of aquatic alien fish species in Lake Caicedo Yuso-Arreo. Manag. Biol. Invasions 9, 267–278.

Herranz-Jusdado, J.G., 2019. Improvement of techniques for sperm evaluation and cryobanking in European eel. Universitat Politècnica de València.

Herranz-Jusdado, J.G., Gallego, V., Morini, M., Rozenfeld, C., Pérez, L., Müller, T., Horváth, Á., Ohta, H., Asturiano, J.F., 2019. Eel sperm cryopreservation: An overview. Theriogenology 133, 210–215. https://doi.org/10.1016/j.theriogenology.2019.03.033

Holt, W. V., Bennett, P.M., Volobouev, V., Watson, P.F., 1996. Genetic resource banks in wildlife conservation. J. Zool. 238, 531–544. https://doi.org/10.1111/j.1469-7998.1996.tb05411.x

Labbaci, A., Chaoui, L., Kara, M.H., 2019. Age, growth and reproduction of the Mediterranean killifish *Aphanius fasciatus* Nardo, 1827 in Mellah Lagoon (Eastern Algeria). Environ. Biol. Fishes 102, 663–674.

Lichtenstein, G., Elisio, M., Miranda, L.A., 2010. Development of sperm cryopreservation techniques in pejerrey *Odontesthes bonariensis*. Aquaculture 306, 357–361. https://doi.org/10.1016/j.aquaculture.2010.05.016

Lichtenstein, G., Miranda, L.A., 2007. Desarrollo de diluyentes y evaluación de métodos para la criopreservación de esperma de pejerrey bonaerense (Odontesthes bonariensis), in: I Reunión Conjunta de Sociedades de Biología de La República Argentina. Huerta Grande, Córdoba, Argentina.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., Cabrita, E., 2017. Cryobanking of aquatic species. Aquaculture 472, 156–177. https://doi.org/10.1016/j.aquaculture.2016.05.042

Mattei, X., 1991. Spermatozoon ultrastructure and its systematic implications in fishes. Can. J. Zool. 69, 3038–3055. https://doi.org/10.1139/z91-428

Muchlisin, Z.A., 2014. Current status of extenders and cryoprotectants on fish

spermatozoa cryopreservation. Biodiversitas, J. Biol. Divers. 6, 66–69. https://doi.org/10.13057/biodiv/d060114

Oliva-Paterna, F.J., Ruiz-Navarro, A., Torralva, M., Fernández-Delgado, C., 2009. Biology of the endangered cyprinodontid *Aphanius iberus* in a saline wetland (SE Iberian Peninsula). Ital. J. Zool. 76, 316–329. https://doi.org/10.1080/11250000802488159

Oltra, R., Todolí, R., 2000. Reproduction of the endangered killifish *Aphanius iberus* at different salinities. Environ. Biol. Fishes 57, 113–115. https://doi.org/10.1023/A:1007579527064

Pyke, G.H., 2005. A review of the biology of *Gambusia affinis* and *G. holbrooki*. Rev. Fish Biol. Fish. 15, 339–365. https://doi.org/10.1007/s11160-006-6394-x

Riehl, R., Baensch, H.A., 1996. Aquarien Atlas, Band 1, 10th ed. Mergus Verlag GmBH, Melle, Germany.

Rincón, P.A., Correas, A.M., Morcillo, F., Risueño, P., Lobón-Cerviá, J., 2002. Interaction between the introduced eastern mosquitofish and two autochthonous Spanish toothcarps. J. Fish Biol. 61, 1560–1585. https://doi.org/10.1006/jfbi.2002.2175

Risueño, P., Hernández, J., 2000. Planes de recuperación en peces de la Comunidad Valenciana: el fartet y el samaruc. Publicaciones Biol. la Univ. Navarra Serie Zool, 17–30.

Roggio, M.A., Hued, A.C., Bistoni, M.A., Teves, M.E., Giojalas, L.C., 2014. Spermatozoa characterization in the one-sided livebearing *Jenynsia multidentata* (Cyprinodontiformes: Anablepidae). Rev. Biol. Trop. 62, 997–1006. https://doi.org/10.15517/rbt.v62i3.7988

Rubio-Gracia, F., García-Berthou, E., Latorre, D., Moreno-Amich, R., Srean, P., Luo, Y., Vila-Gispert, A., 2019. Differences in swimming performance and energetic costs between an endangered native toothcarp (*Aphanius iberus*) and an invasive mosquitofish (*Gambusia holbrooki*). Ecol. Freshw. Fish 29.

https://doi.org/10.1111/eff.12509

Silva, G., Weber, V., Green, A.J., Hoffmann, P., Silva, V., Volcan, M., Lanés, L.E., Stenert, C., Reichard, M., Maltchik, L., 2019. Killifish eggs can disperse via gut passage through waterfowl. Ecology 0, 55–58. https://doi.org/10.1002/ecy.2774

Simpson, J.L., Humphries, S., Evans, J.P., Simmons, L.W., Fitzpatrick, J.L., 2013. Relationships between sperm length and speed differ amont three internally and three externally fertilizing species. Evolution (N. Y). 68, 92–104. https://doi.org/10.1111/evo.12199

Tiersch, T.R., Yang, H., 2012. Environmental salinity-induced shifts in sperm motility activation in *Fundulus grandis*. Aquaculture 324–325, 145–150. https://doi.org/10.1016/j.aquaculture.2011.10.023

Vargas, M.J., de Sostoa, A., 1997. Life-history pattern of the Iberian toothcarp Aphanius iberus (Pisces, Cyprinodontidae) from a Mediterranean estuary, the Ebro Delta (Spain). Netherlands J. Zool. 47, 143–160.

Verdiell-Cubello, D., Ruiz-Navarro, A., Torralva, M., Moreno-Valcárcel, R., Oliva-Paterna, F., 2014. Habitat use of an endangered cyprinodontid fish in a saline wetland of the Iberian Peninsula (SW Mediterranean Sea). Mediterr. Mar. Sci. 15, 27–36. https://doi.org//10.12681/mms.432

Viveiros, A.T.M., Godinho, H.P., 2009. Sperm quality and cryopreservation of Brazilian freshwater fish species: A review. Fish Physiol. Biochem. 35, 137–150. https://doi.org/10.1007/s10695-008-9240-3

Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R.S., Strüssmann, C.A., 2014. Coexistence of genotypic and temperature-dependent sex determination in pejerrey Odontesthes bonariensis. PLoS One 9, 1–8. https://doi.org/10.1371/journal.pone.0102574

Yang, H., Norris, M., Winn, R., Tiersch, T.R., 2010. Evaluation of cryoprotectant and cooling rate for sperm cryopreservation in the euryhaline fish medaka *Oryzias latipes*. Cryobiology 61, 211–219. https://doi.org/10.1016/j.cryobiol.2010.07.006

Tables

Table 1. Morphometric parameters of sperm head of *Aphanius iberus* (n=9) and *Valencia hispanica* (n=8) measured with ASMA software using optic microscopy. Dates are expressed as the mean ± SEM (standard error of the mean). Different letters mean significant differences between species (p-value ≤ 0.05).

	A. iberus	***V. hispanica***
Area (μm²)	4.84 ± 0.06	4.81 ± 0.04
Perimeter (μm)	8.04 ± 0.05	8.02 ± 0.04
Lenght (μm)	2.55 ± 0.01	2.55 ± 0.01
Width (μm)	2.35 ± 0.01	2.32 ± 0.01
Ellipticity	1.09 ± 0.01 b	1.10 ± 0.004 a
Elongation	0.04 ± 0.001 b	0.05 ± 0.002 a
Rugosity	0.95 ± 0.001 a	0.94 ± 0.001 b
Regularity	0.97 ± 0.003	0.97 ± 0.004

Figure legends

Figure 1. Percentage of *Valencia hispanica* (males, females, fries) and Eastern mosquitofish captured with fish traps in different wetlands of Albufera Park (El Dosser, Enebro and Dunas).

Figure 2. Percentage of spermiating males with motile cells (Sperm+), spermiating males without motile cells (Sperm) and no spermiating males, (No sperm) during the breeding season in *Aphanius iberus* (A; n=10-24) and *Valencia hispanica* (B; n=9-21).

Figure 3. Sperm kinetic parameters in *Aphanius iberus* (A and B; n=7) and *Valencia hispanica* (C and D; n=13) at different post-activation times. Data are expressed as the mean ± SEM (standard error of the mean). Different letters mean significant differences over time (p-value ≤ 0.05).
Graphs show total motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP).

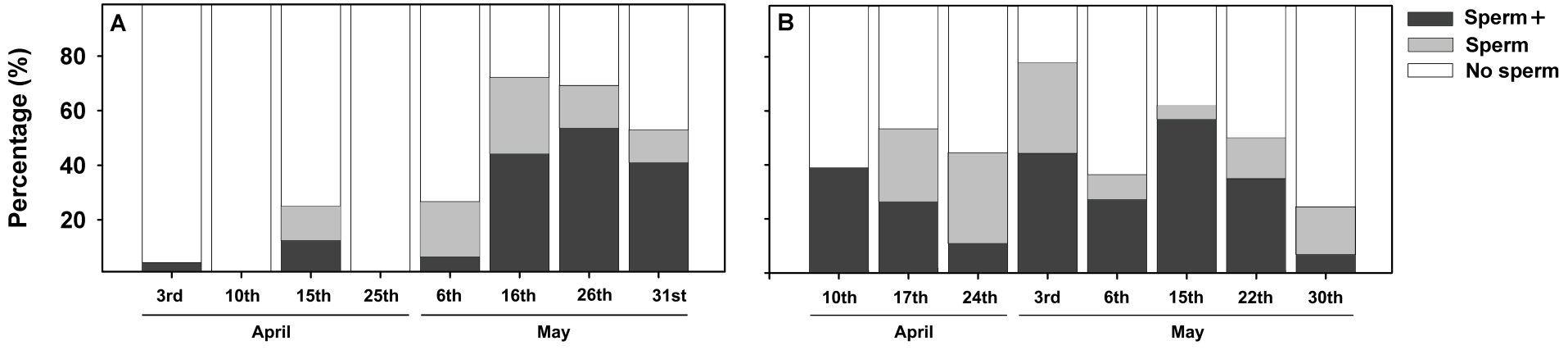
Figure 4. Percentage of fast (FA), medium (ME) and slow (SL) spermatozoa at different post-activation times in *Aphanius iberus* (A; n=7) and *Valencia hispanica* (B; n=13).

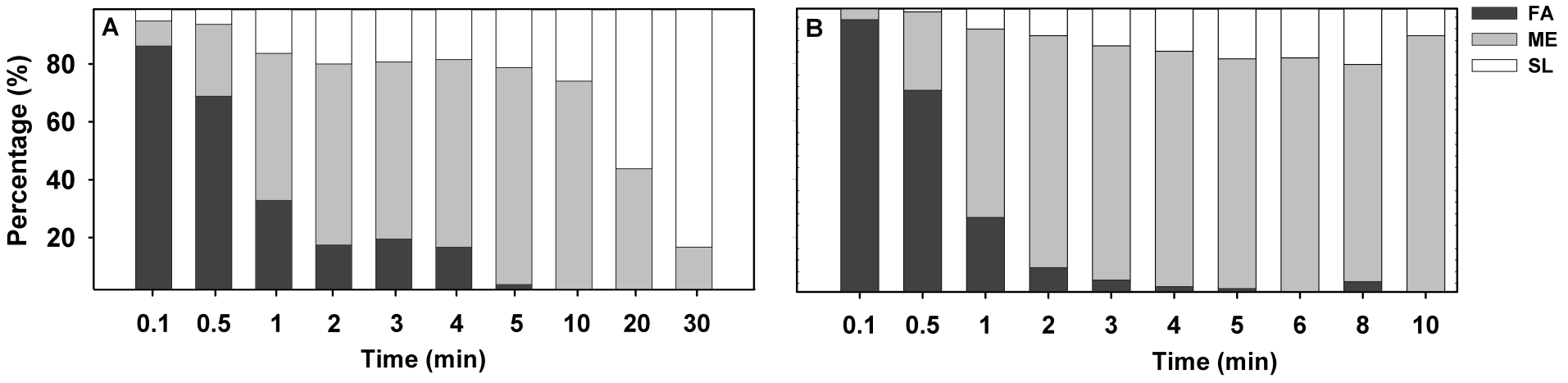
Figure 5. Scanning electron microscopy of spermatozoa of *Aphanius iberus* (A and B) and *Valencia hispanica* (C and D). Scale bar is showed in the figure.

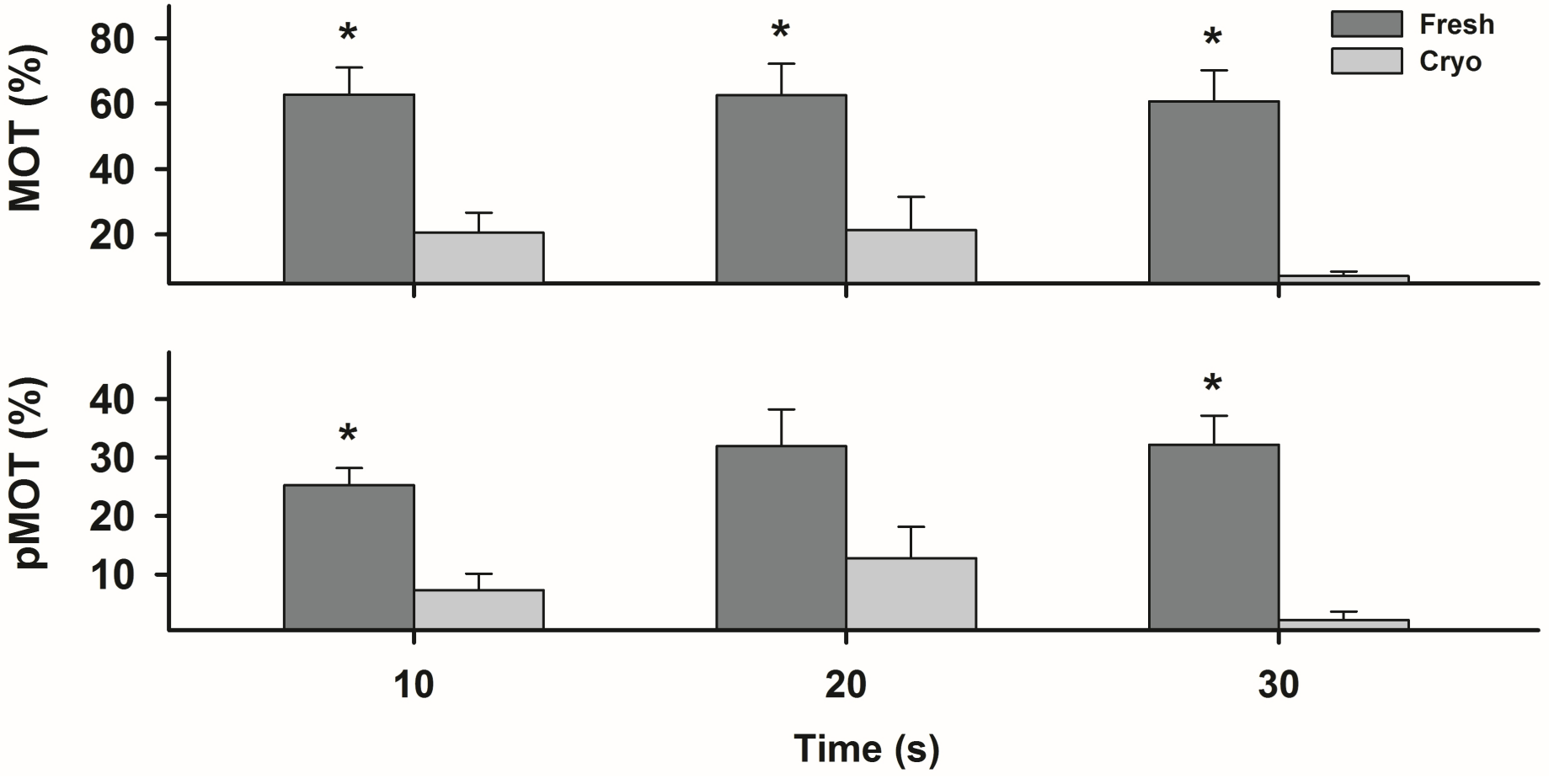
Figure 6. Total motility (MOT) and progressive motility (pMOT) of fresh and thawed sperm samples (Cryo) at different post-activation times in *Aphanius iberus*. Data are expressed as the mean ± SEM (n=7). Asterisk means significant differences (p-value ≤ 0.05).

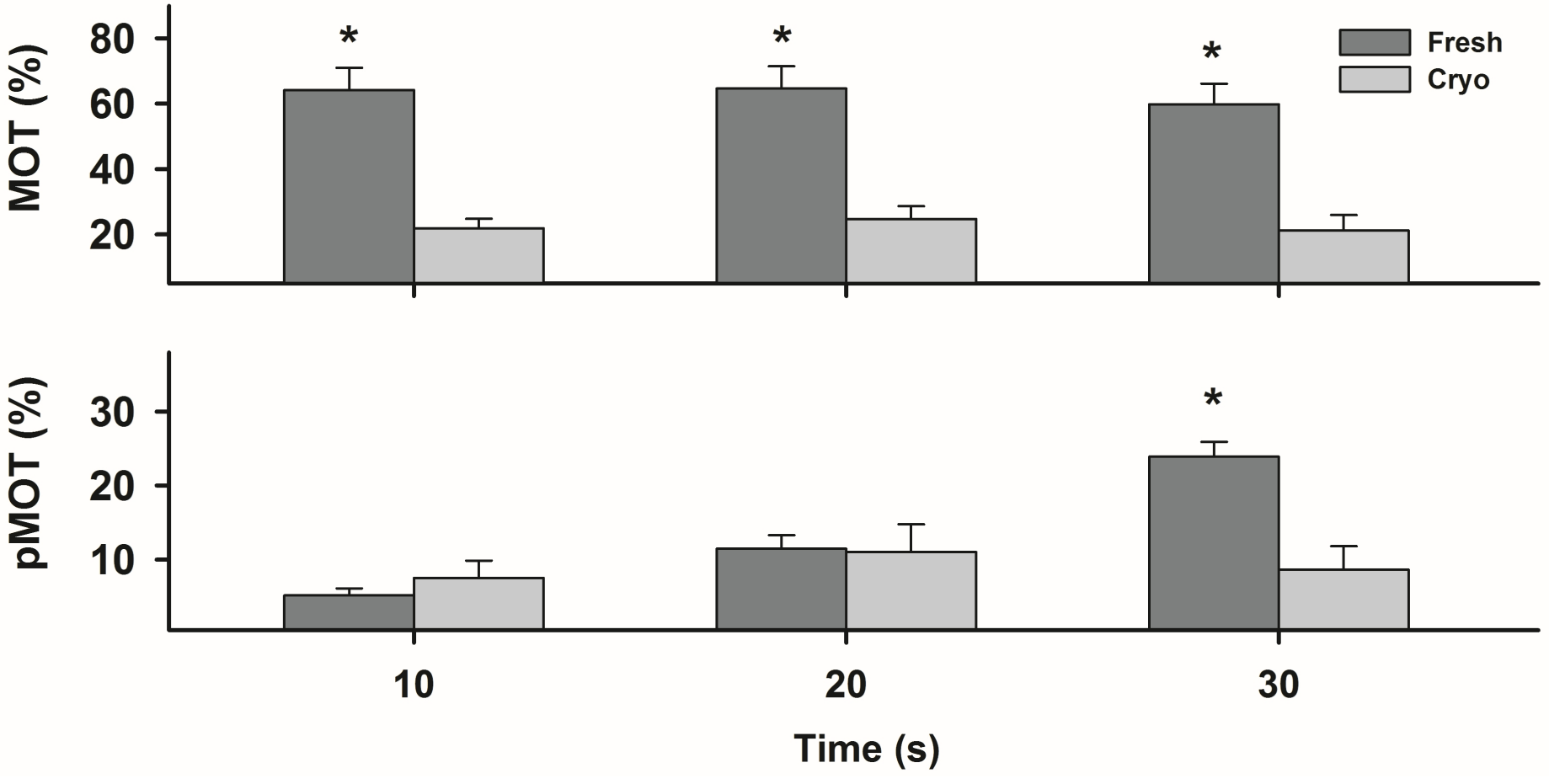
Figure 7. Total motility (MOT) and progressive motility (pMOT) of fresh and thawed sperm samples (Cryo) at different post-activation times in *Valencia hispanica*. Data are expressed as the mean ± SEM (n=8). Asterisk means significant differences (p-value ≤ 0.05).

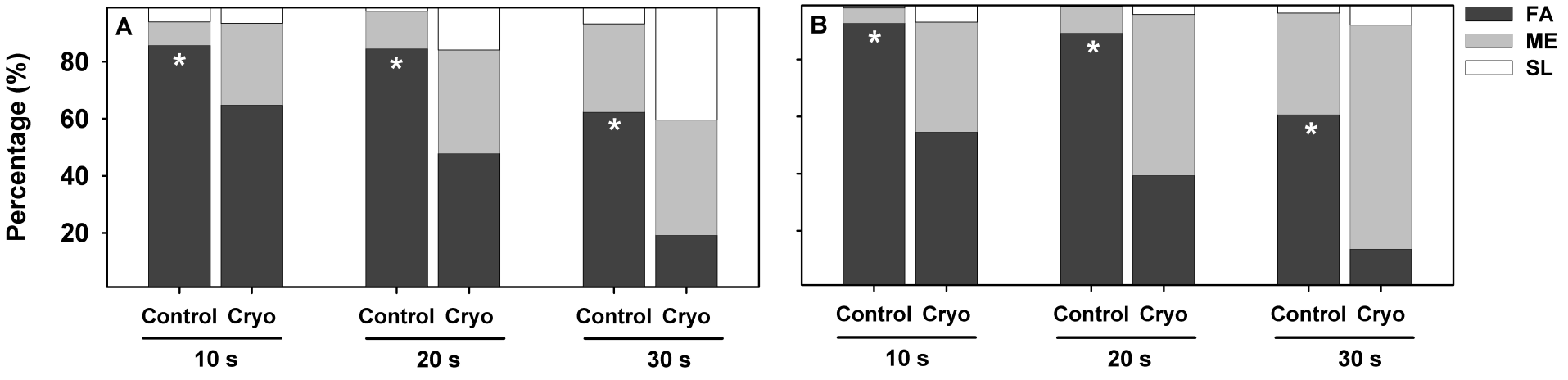
Figure 8. Percentage of fast (FA), medium (ME) and slow (SL) spermatozoa of fresh and thawed sperm samples (Cryo) at different post-activation times in *Aphanius iberus* (A; n=7) and *Valencia hispanica* (B; n=8). Data are expressed as the mean ± SEM (standard error of the mean). Asterisk means significant differences (p-value ≤ 0.05).


A
B
Percentage (%)
80
60
40
20
3rd
10th
15th
25th
6th
16th
26th
31st
April
May
10th
17th
24th
3rd
6th
15th
22nd
30th
April
May
Sperm +
Sperm
No sperm

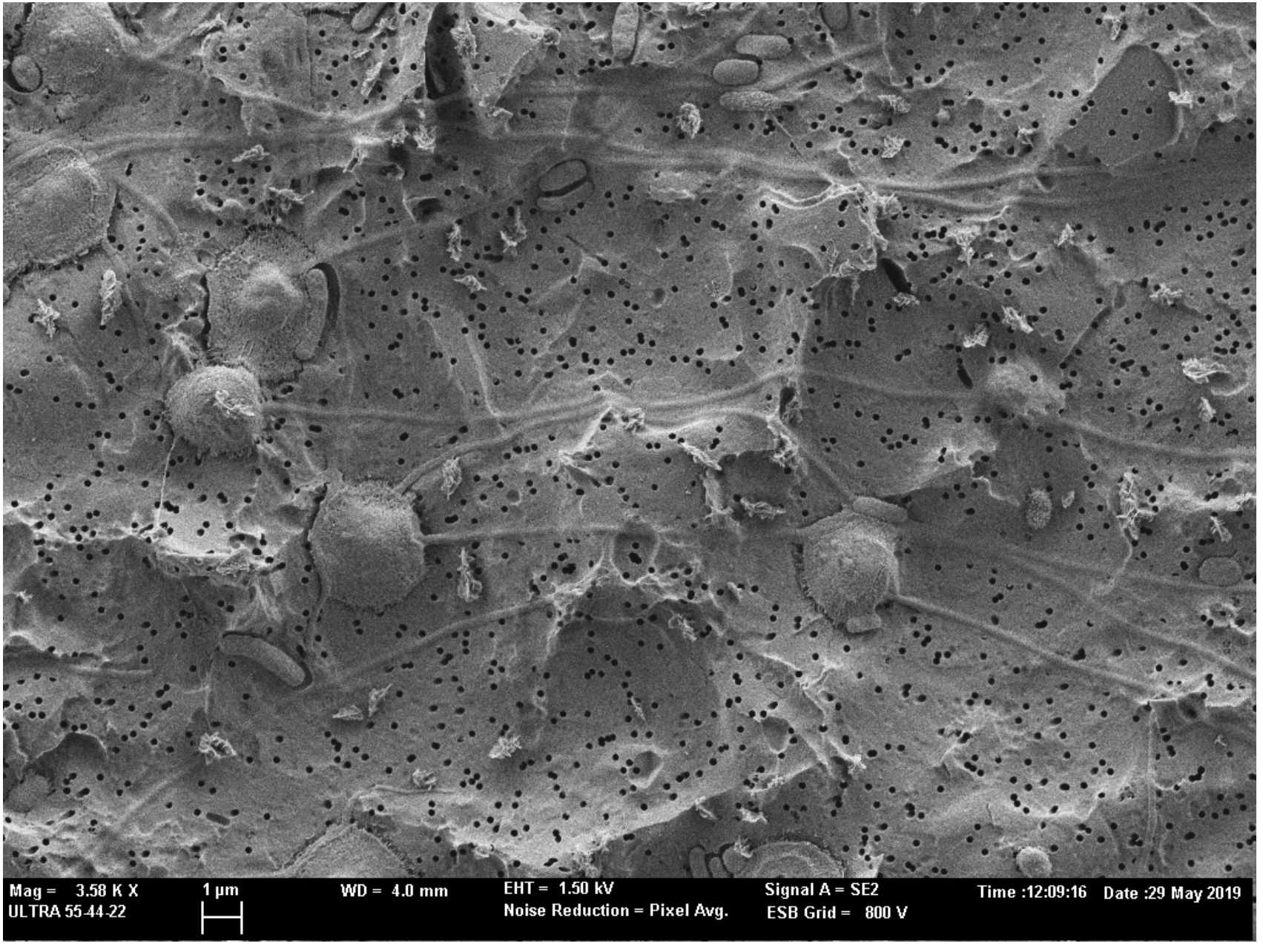

A
B
Percentage (%)
80
60
40
20
0.1
0.5
1
2
3
4
5
10
20
30
6
8
Time (min)
FA
ME
SL

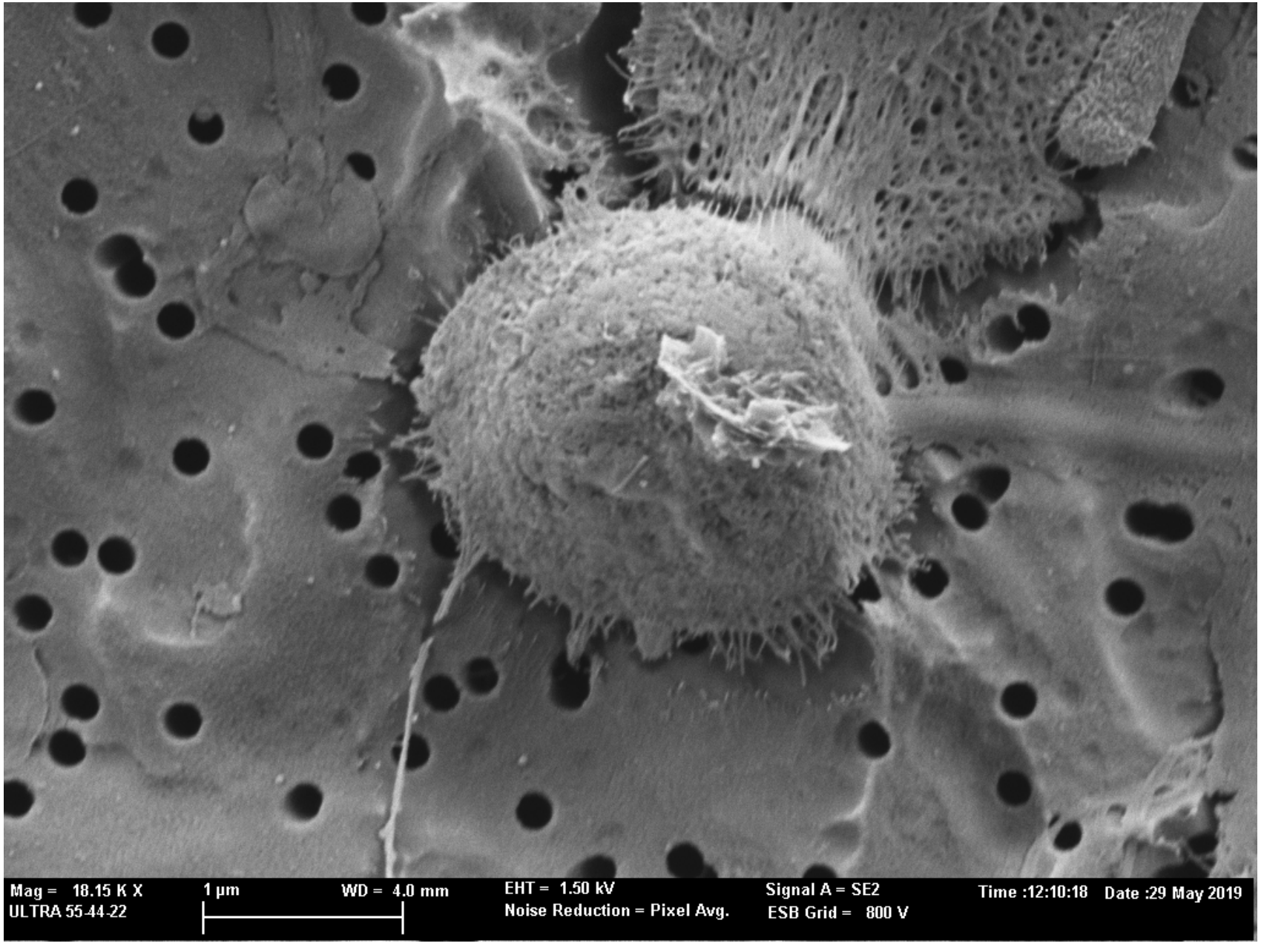

MOT (%)
80
60
40
20
*
*
*
Fresh
Cryo
pMOT (%)
40
30
20
10
*
*
10
20
30
Time (s)

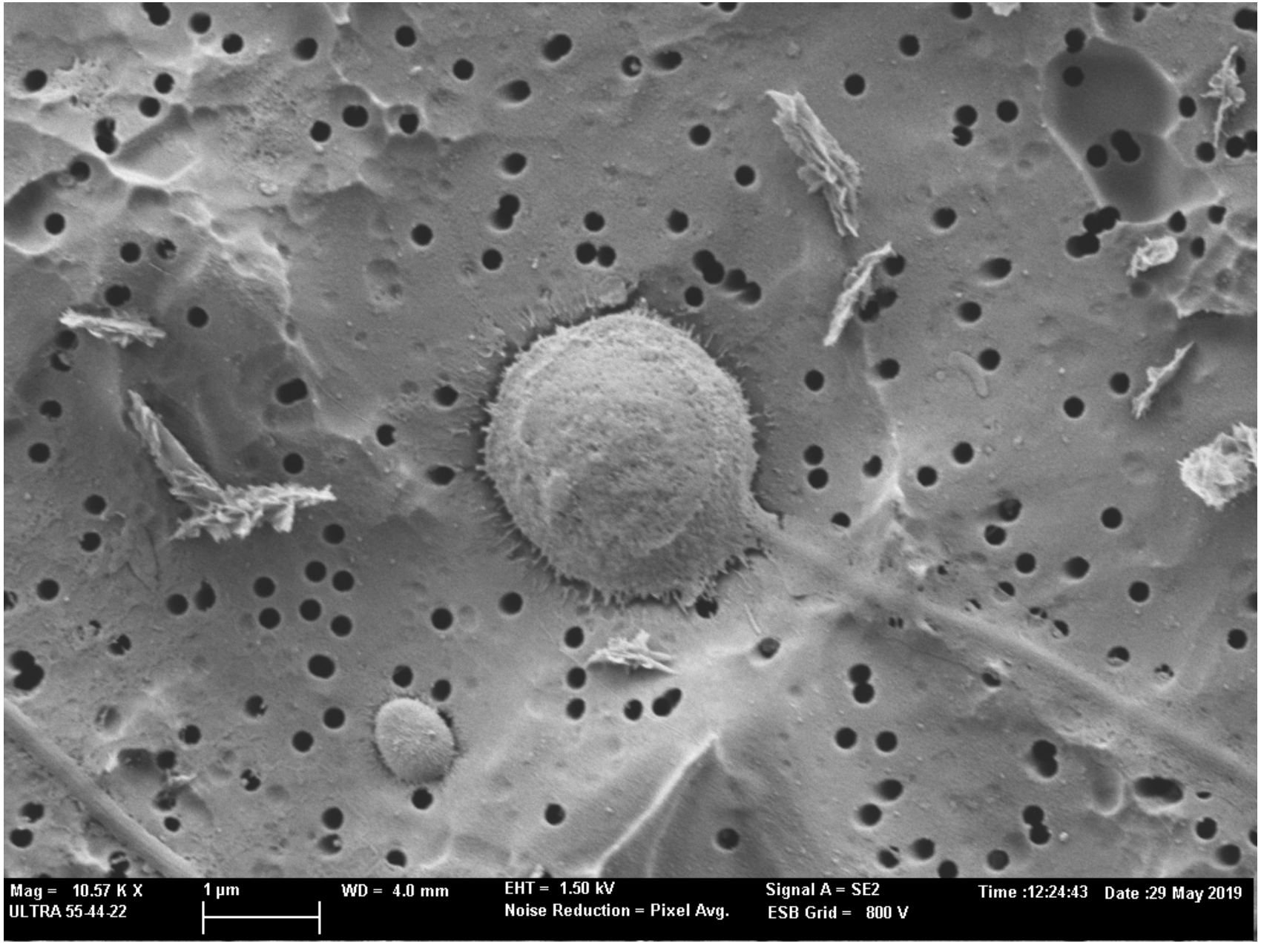

MOT (%)
80
60
40
20
*
*
*
Fresh
Cryo
pMOT (%)
30
20
10
*
10
20
30
Time (s)

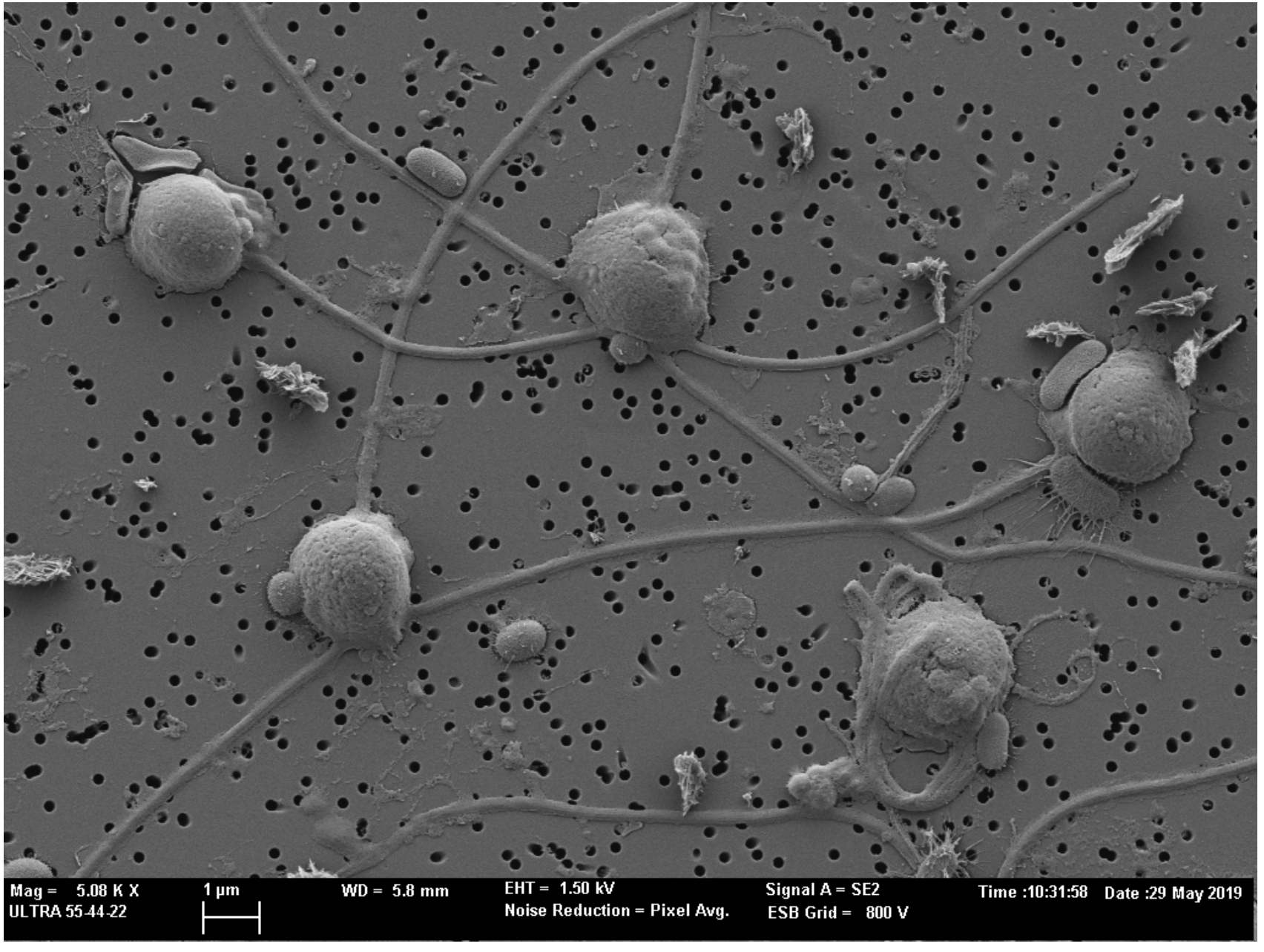

A
B
Percentage (%)
80
60
40
20
*
Control
Cryo
10 s
20 s
30 s
FA
ME
SL

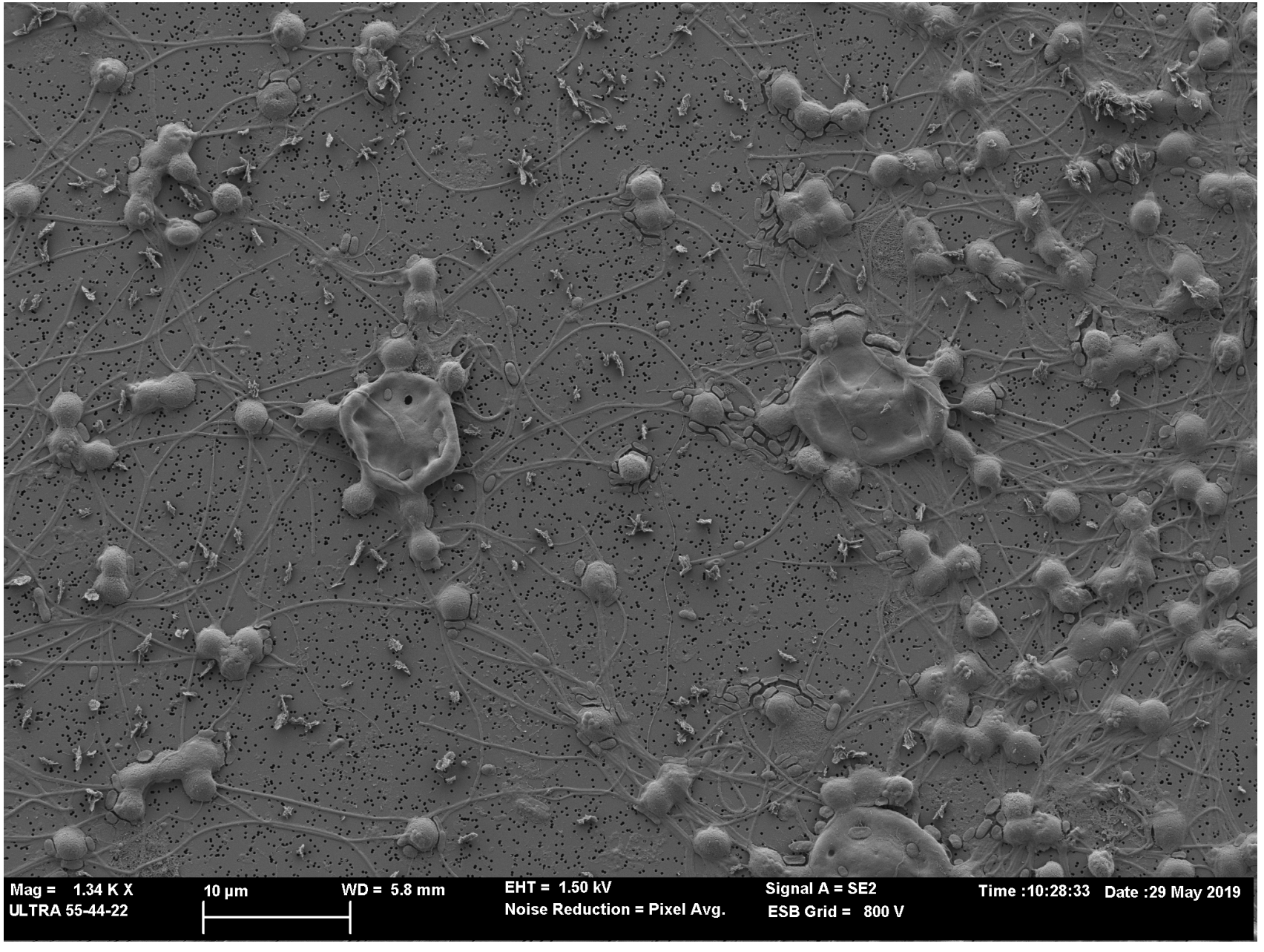
M. Blanes-García: Validation, Formal analysis, Investigation, Resources, Writing – Original Draft, Visualization**. P. Risueño:** Resources, Review & Editing. **L. Pérez:** Writing – Review & Editing. **J .F. Asturiano:** Resources, Writing – Review & Editing, Project Administration; **V. Gallego:** Validation, Investigation, Resources, Writing – Review & Editing, Supervision.


Mag = 3.58 K X
ULTRA 55-44-22
1 µm
WD = 4.0 mm
EHT = 1.50 kV
Noise Reduction = Pixel Avg.
Signal A = SE2
ESB Grid = 800 V
Time :12:09:16
Date :29 May 2019


Mag = 18.15 K X
ULTRA 55-44-22
1 µm
WD = 4.0 mm
EHT = 1.50 kV
Noise Reduction = Pixel Avg.
Signal A = SE2
ESB Grid = 800 V
Time :12:10:18
Date :29 May 2019


Mag = 10.57 K X
ULTRA 55-44-22
1 µm
WD = 4.0 mm
EHT = 1.50 kV
Noise Reduction = Pixel Avg.
Signal A = SE2
ESB Grid = 800 V
Time :12:24:43
Date :29 May 2019


Mag = 5.08 K X
ULTRA 55-44-22
1 µm
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 µm
WD = 5.8 mm
EHT = 1.50 kV
Noise Reduction = Pixel Avg.
Signal A = SE2
ESB Grid = 800 V
Time :10:28:33
Date :29 May 2019