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Additional Information

Characterisation of European eel (*Anguilla anguilla*) spermatozoa morphometry using Trumorph tool in fixed and non-fixed samples

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Abstract

Currently, the techniques used to analyse fish spermatozoa head morphology are not only subjective and time-consuming, but also, variable due to alteration of real spermatozoa size during sample manipulation. Thereby, it is important to develop a new method to obtain accurate and objective results. So far, computer-assisted systems for morphometry analysis were developed and validated for mammals, although they could also be adapted to fish spermatozoa. The present study aimed to characterise the European eel spermatozoa morphometry, comparing the measurements (area and perimeter) obtained by computer-assisted spermatozoa between pre-treated or non-treated eel sperm samples (n = 5) with fixation solution. In both protocols, the TruMorph® tool was used to apply a constant force to extend the cells in a thin layer. Images of spermatozoa were captured using a 40x negative phase contrast objective and analysed with the ISASv1 CASA-Morph system. Sperm head morphometry showed significant differences in area and perimeter comparing both protocols. Besides, the head size analysis using TruMorph® without fixation along time showed that the sperm membrane remains intact with the use of this technique, preserving the semipermeable condition. Considering the fact that fixation solution produces dehydration/hydration that could affect the real spermatozoa size, the simple use of TruMorph® without fixation combined with CASA-Morph analysis could allow more realistic measurements of eel spermatozoa head.

Keywords: CASA-Morph system; Sperm morphometry characterisation; Fixation solution; TruMorph® tool; Pressure; Realistic measurements

1. Introduction

Spermatozoa are individual motile cells exposed to different environments from the time produced in the testis to the time of fertilisation (Fauvel et al., 2010). They can be vulnerable not only to xenobiotics, genetic mutation or ageing, but also to cryopreservation protocols, which could result in morphometric damage of the head or flagellum, functional impairment of the energy-producing mitochondria, or development of abnormalities in

sperm structure (Asturiano et al., 2007; Fauvel et al., 2010; Kime et al., 2001). Modifications in sperm structure are evaluated by the examination of sperm cell morphology using different microscopic techniques, including light microscopy with bright field (Tuset et al., 2008) and negative-phase contrast (Gage et al., 1998; Marco-Jiménez et al., 2006; Van-Look and Kime, 2003), scanning and transmission electron microscopy (Křišťan et al., 2014; Medina et al., 2003; Okamura et al., 2005). The protocol for sperm morphology analysis varies according to the technique used, although the most common procedures consist in sample preparation, like sample dilution in fixative solution or smear / air-drying techniques, and in some cases is followed by staining protocols (Fauvel et al., 2010; Sancho et al., 1998; Yeung et al., 1997). However, all these techniques easily produce artefacts that may change spermatozoa morphology (Haidl and Schill, 1993; Soler and Cooper, 2016; Soler et al., 2015, 2016).

Sperm morphology analysis obtained after sample preparation and staining techniques could be time-consuming (Marco-Jiménez et al., 2006; Van-Look and Kime, 2003) and the evaluation could be subjective, even if it follows a standard protocol (e.g. human WHO; WHO, 2010), generating a high variability of results. In this sense, many studies aimed to search for objective, accurate and repeatable methods for sperm assessment (Gallego and Asturiano, 2018a,b). One solution was the development of a computer-assisted system based on image analysis of spermatozoa head (Fauvel et al., 2010; Marco-Jiménez et al., 2006; Tuset et al., 2008), reducing the subjective nature and the technical variation inherent to the morphology and morphometric analysis (Marco-Jiménez et al., 2006; Sancho et al., 1998; Van-Look and Kime, 2003). This kind of automatic system was validated for mammals (Rijsselaere et al., 2004; Yániz et al., 2015), avian (García-Herreros, 2015; Santiago-Moreno et al., 2016) and fish species (Van-Look and Kime, 2003).

Computer-assisted sperm analysis for morphology (CASA-Morph) have been used on fish to study the effect of contaminants (Van-Look and Kime, 2003), hormonal stimulation of sperm maturation (hCG; Asturiano et al., 2006) and cryopreservation (Asturiano et al., 2007; Billard, 1983; Billard et al., 2001; Peñaranda et al., 2008) on the sperm morphological and morphometric characteristics. This technique was also used to study the possible correlation between swimming performance and sperm morphology (Tuset et al., 2008). Nevertheless, the use of CASA-Morph to evaluate spermatozoa morphology has not

solved the problem related to the artefacts produced by the different sample preparation protocols, which lead to a diversity of morphometric results (Hidalgo et al., 2018; Soler et al., 2003, 2016) and a lack of standardised morphology software settings for each species (Boersma et al., 1999, 2001; Davis and Gravance, 1993; Gravance and Davis, 1995). Therefore, the optimisation of morphological sperm evaluation is crucial and must include techniques focused on the analysis of raw semen preparations to keep the natural seminal fluid that could allow the appearance of cells with native shape (Cooper, 2012). Spermatozoa morphology assessment in natural environment conditions means that the cells should be alive, although they still need to be immobilised in the absence of chemical fixatives (Abraham-Peskir et al., 2002; Cooper et al., 2004; Fetic et al., 2006). TruMorph® system is a new tool that was developed to produce physical constraints of the cells preventing spermatozoa movement without damaging them. A uniform and constant force of 5.0 ± 0.1 kp applied to the coverslip (20×20 mm) surface for five seconds spreads the fluid (3 μL of semen) in a depth of approximately 6.2 μm. Under these conditions, the spermatozoa must be positioned laterally providing a better view of the cell, which permits a more reliable analysis of head and midpiece details (Soler et al., 2015, 2016).

The applicability of methodologies for morphological sperm evaluation is limited in fish due to the biological characteristics of the sperm and spermatozoa head shape. Therefore, the main aim of this study was to describe for the first time a specific protocol for the assessment of fish spermatozoa morphometry in living cells using a new technique that was validated for mammalian spermatozoa. We have used the European eel spermatozoa as a model. The results were obtained by a CASA-Morph system and compared the TruMorph® tool in unfixed samples and a fixative protocol that were previously reported. Finally, the changes in the spermatozoa head morphometry were evaluated over time after using the TruMorph® protocol without fixation.

2. Material and Methods

The care and use of experimental animals complied with the Universitat Politècnica de València animal welfare laws, guidelines and policies as approved by the Committee of Ethics of Animal Experimentation (2015/VSC/PEA/00064).

2.1. Fish handling and sampling

European eel males (body weight around 100-120 g) were maintained in tanks with seawater and recirculation system at constant temperature (20 °C) and weekly treated with intraperitoneal injections of recombinant human chorionic gonadotropin (hCG; Ovitrelle, Merck S.L., Madrid; 1.5 IU/g of fish body weight) during 15 weeks (Gallego et al., 2012a). During the spermiation period, sperm samples were weekly collected by abdominal pressure 24 h after the administration of the hormone according to Pérez et al. (2005). Sperm was sampled taking special care to avoid contamination with faeces, urine, and seawater. Samples were diluted 1:50 (sperm:extender) in P1 medium (in mM: 125 NaCl, 20 $NaHCO_3$, 2.5 $MgCl_2$6H2O, 1 $CaCl_2$2H_2O, 30 KCl in distilled water) with pH (8.5) similar to seminal plasma and supplemented with BSA (2%, w:v; Peñaranda et al., 2010) and kept in plastic tubes at 4 °C until sperm kinetic analyses, which were carried out during the next hour (Gallego et al., 2013).

2.2. Sperm motility assessment

Sperm motility was assessed activating each P1-diluted sperm sample with artificial water (in mM: 354.7 NaCl, 52.4 $MgCl_2$, 9.9 $CaCl_2$, 28.2 Na_2SO_4, 9.4 KCl, in distilled water) with 2% (w:v) bovine serum albumin (BSA), pH adjusted to 8.2. The activation was performed in a Spermtrack® 10 counting chamber (PROISER R+D, S.L., Paterna, Spain) mixing 0.5 µL of P1-diluted sperm sample with 4.5 µL of artificial seawater. Sperm motility was analysed by using the CASA-Mot module of the Integrated Semen Analysis System (ISAS®v1; PROISER) that included a phase-contrast microscope with ×10 magnification connected to an ISAS 782M video camera (PROISER), with a frame rate of 60 frames per second (fps). All samples were analysed in triplicate 15 s after activation. Total sperm motility (MOT; %) were recorded for the evaluation of sperm quality, being values higher than 70% considered to define the best sperm quality. For the experiment, an ejaculate from five individuals with high sperm quality (motile cells > 70%) was used.

2.3. Experimental design

2.3.1. TruMorph® procedure

A 3 μL-drop of sperm suspension in P1 medium was placed on a cleaned glass slide and covered with a 22×22 mm coverslip. This glass slide was placed into the TruMorph® tool (PROISER) for a light automatic force of 6 kp on all the surface of the coverslip (22×22 mm) during 5 s. Under these conditions, the spermatozoa are constrained in a depth of around 6.2 μm between the glass slide and cover. Sperm samples were observed in a phase-contrast microscope (UOP-UB200i; PROISER) equipped with a ×40 negative phase contrast objective and connected to a video camera (ISASC13-ON; PROISER) with a resolution of 1280×1024 pixels. One field of the visual area with a high number of cells (about 30 well-defined cells) was captured 1 min after the application of pressure on the glass slide. Three replicates were done for each sperm sample. Then, images of individual spermatozoa were selected for morphometric analysis based on the spermatozoa position (frontal side). The parameters (head area, μm^2; head perimeter, μm) were automatically analysed using a CASA-Morph system (ISAS®v1, PROISER). Analysis factor (sensitivity) was adjusted to 0 for the morphometric spermatozoa analysis.

2.3.2. Morphometric analysis after fixation

A fraction of sperm suspension in the P1 medium was fixed using a 2.5% glutaraldehyde solution. After fixing, samples were stored at 4 °C until the morphometric measurements (Marco-Jiménez et al., 2006). For the analysis, the glass slide for each sample was prepared as indicated for the TruMorph® protocol section. A minimum of two hundred spermatozoa was captured in each sample and analysed using the same CASA-Morph system.

2.3.3. Effect of time on morphometric parameters of unfixed spermatozoa

For the five ejaculates, a glass slide was prepared using the TruMorph® procedure. The same field was captured at each 30 s from 1 to 15 min after the application of pressure. This procedure was repeated three times for each ejaculate. The methodology for morphometric analysis followed the same procedure used for TruMorph®.

2.4. Statistical analysis

The data were first assessed for normality and homoscedasticity by using the Shapiro-Wilks and Levene tests. A normal probability plot was used to assess for a normal distribution. The morphometric parameters determined were head perimeter (μm) and head area (μm^2). The mean values were compared by analysis of variance by the generalized linear model (GLM). The GLM model was also used to evaluate the influence fixation methods, on the morphometric variables. For regression analyses, the effects of fixation time with TruMorph® were tested in a polynomial regression model of degree 4, in the form $(y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 + time)$. Results are presented as mean ± standard error of the mean (SEM) and median and first quartile and third quartile. Statistical significance was considered at $P < 0.05$. All data were analysed by using the IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL., USA.).

3. Results

3.1. Fixed versus un-fixed spermatozoa parameters with TruMorph analysis

Individual spermatozoa from each sample were measured by CASA-Morph to compare both methodologies (Figure 1). The total number of spermatozoa analysed for TruMorph® with and without fixation were 1130 and 429, respectively. Mean values for the area were 2.34 ± 0.24 μm^2 (mean ± SEM) and 1.84 ± 0.42 μm^2 for fixation and non-fixation TruMorph® protocols, respectively (Figure 2). For perimeter, spermatozoa head value was also higher for pre-fixed sperm samples (fixation: 7.92 ± 0.77 μm; TruMorph®: 6.70 ± 1.04 μm). Both parameters showed significant differences ($P < 0.05$) between protocols, being the spermatozoa head bigger in sperm samples pre-treated with fixation solution. Moreover, the morphometric parameters showed lower variability for the protocol with fixation technique (coefficient of variation for area: fixed spermatozoa are 10.5% and un-fixed spermatozoa are 22.8%; coefficient of variation for perimeter: fixed spermatozoa are 9.8% and fixed spermatozoa are 15.5%).

3.2. Effect of time on morphometric parameters of un-fixed spermatozoa

A total of 17792 spermatozoa were measured along 900 s (i.e. 15 min) after Trumorph® tool preparation. Between 60 to 240 s after applying pressure, the mean head area showed a quick decrease (from 1.84 ± 0.02 to 1.67 ± 0.01 μm^2), and during the next 225 s, the head area increased to values similar to the initial one (1.83 ± 0.02 μm^2). Then, the head size had a small decrease (1.75 ± 0.01 μm^2) for 30 s followed by slight changes, which correspond to ascending and descending head area up to 900 s (1.85 ± 0.02 μm^2). Analogous results were obtained for head perimeter (Figure 3).

A polynomial regression of degree 4 was used as a model to explain the effect of time on the area and perimeter of the European eel spermatozoa head (Figure 4) during the 15 min of morphology analysis. For both morphometric parameters, the prediction interval included almost all the values and showed in the first minutes a very sharp decline of the head size followed by another decline less abrupt. The overall regression and the degree coefficients are significant ($P < 0.05$) with a multiple correlation coefficient of 0.64 and 0.73 for area and perimeter, respectively.

4. Discussion

A subjective analysis of sperm morphology has been showing a large intra- and inter-laboratory variation (Gallego et al., 2018; Soler et al., 2003). Therefore, to increase the accuracy, repeatability and viability of the results, CASA-Morph systems were developed to perform an objective analysis (Gallego and Asturiano, 2018a,b). However, these techniques still require standardisations of method and variables (Caldeira et al., 2019; Coetzee et al., 1998; Wang et al., 1991).

CASA-Morph systems are based on image analysis techniques and allow the evaluation of a great number of sperm morphometric parameters. In this study, morphometric data from 18082 European eel spermatozoa were measured using one of the marketed CASA-Morph systems that captured individual spermatozoa heads with negative phase-contrast images. Previous studies showed that phase-contrast is the optimal microscopical technique to perform sperm morphometric analysis in fish species. Sample preparation for the phase-contrast technique is simple and direct, avoiding many protocol steps as happens in staining methods, which minimizes the influence of different products on spermatozoa head

dimensions (Gallego et al., 2012b; Marco-Jiménez et al., 2006, 2008; Vladic et al., 2002). Therefore, the variability of size parameters is lower for the phase-contrast technique (Gallego et al., 2012b), which is a prerequisite in the selection of a standard method.

The purpose of the study was to present an accurate and standardised method for the spermatozoa head morphometry characterisation in this species with a new technique, the Trumorph® tool, using two protocols (with and without fixation). However, the researcher should take into account that the number of cells captured in one glass slide is different for both methodologies (with fixation: n =1130; without fixation: n = 429). Hence, at the practical level, it is necessary to prepare more glass slides with Trumorph® protocol without fixation to obtain the same acceptable number of cells (approximately 200 spermatozoa/sample). Beyond that, the area and perimeter were the only parameters considered due to the longitudinal asymmetry and curved and elongated shape of the spermatozoa head with a hook-shaped superior end (Gallego et al., 2014a; Marco-Jiménez et al., 2006). These particular characteristics of European eel spermatozoa are not recognised by the software, which causes an error in the length measurement (Marco-Jiménez et al., 2006). The improvement of the CASA-Morph is a fundamental requirement to detect accurate measurements of all morphometric parameters for different spermatozoa head shapes. Nonetheless, the main finding based on the parameters considered was a higher area and perimeter (2.34 ± 0.01 μm^2 and 7.92 ± 0.02 μm, respectively) of the spermatozoa head evaluated using the protocol with fixation solution. Fixation technique is used to maintain spermatozoa in non-live conditions while morphology is well preserved as close as possible to its natural state for morphometric analysis. On the other hand, the purpose of the Trumorph® tool with raw semen preparations is to keep the cell alive and immobile during the analysis process using pressure. Therefore, the significant differences in head size detected between the two protocols could be related to the loss of integrity and functionality of the spermatozoa membrane in fixed samples and, consequently, the ability of these cells to maintain osmotic equilibrium (Asturiano et al., 2007; Gallego et al., 2012b). Furthermore, both morphometric parameters showed lower variability in samples fixed with 2.5% glutaraldehyde solution, which can be explained by the presence of aldehydes that improve the delineation of the spermatozoa head (Marco-Jiménez et al., 2006; Sancho et al., 1998). The previous study reported high values of area (5.36 ± 0.06

260 μm^2) and perimeter (14.68 ± 0.13 μm) of European eel spermatozoa head using the phase-
261 contrast technique with the same fixation solution, although, the sperm samples were
262 analysed with a magnification lens of ×100 (Marco-Jiménez et al., 2006). The results of our
263 sperm samples could be compared with this study calculating the theoretical value of the
264 morphometric parameters for the same magnification lens. In the case of sperm head area,
265 our samples present theoretical values at ×100 objective around 5.8 and 4.6 μm^2 for fixed
266 and non-fixed samples, respectively. Regardless of the Trumorph® tool were used in both
267 protocols, the fixed samples showed a theoretical value similar to those published, which
268 means that the pressure did not compromise the cell membrane. Thus, despite the phase-
269 contrast technique with a simple protocol using fixation solution have been recognized as a
270 useful method for sperm morphometry parameters, our study showed that the morphometric
271 results obtained by Trumorph® protocol without fixation could be representative of the real
272 cell size. Furthermore, the definition of a standardised methodology could be also
273 considered a priority, since there is a wide range of sperm morphology protocols used by
274 different laboratories that make it difficult to compare the results with those presented in
275 the literature. Sperm morphology assessment could be influenced by sample preparation
276 with fixation or staining method and the errors associated with the technical factors, such as
277 magnification lens and video camera (Gallego et al., 2012b; Marco-Jiménez et al., 2006;
278 Rijsselaere et al., 2004).
279 Trumorph® tool with raw semen preparations is a new way to analyse sperm morphology
280 and morphometry. This technique was already validated for some mammalian species,
281 including human, boar, bull, dog, fox, goat, mice, rabbit and stallion (Soler et al., 2015,
282 2016). However, the CASA-Morph methodology used for mammals is not directly
283 applicable to fish (Van-Look and Kime, 2003). Fish sperm differs from mammals in two
284 important aspects, such as immotile spermatozoa on ejaculation and sperm activation occur
285 by the contact with the external environment (salt or freshwater). In this way, the study
286 evaluated the accuracy of the Trumorph® for the analysis of fish sperm morphology.
287 European eel spermatozoa were analysed without fixation for 15 min to describe the head
288 size after pressure. The spermatozoa head showed an abrupt reduction of head size
289 followed by a rise to the initial size (around 8 min after pressure) and, afterwards, the cells
290 suffered small oscillations of size. These size changes could be caused by cell water flux

from inside spermatozoa to the external medium (Asturiano et al., 2007; Meyers et al.,2005; Peñaranda et al., 2010), which suggest that the spermatozoa are alive after pressure and did not lose the membrane integrity and functionality. A previous study with Common carp (*Cyprinus carpio*), described the effect of pressure using the Trumorph® in sperm samples diluted in physiological solution and their seminal fluid. The results likewise suggested that the Common carp spermatozoa keep the membrane integrity and functionality after applying pressure to the cells since the samples diluted in seminal fluid showed spermatozoa motility over time (*Personal communication*). In the case of European eel, spermatozoa were not activated by the osmotic pressure since the P1 medium was an isotonic solution and there was no variation in the concentration of ions (Gallego et al., 2014b).

The studies with fish species corroborate previous studies that reported the survival of mammalian spermatozoa after pressure (Soler et al., 2015, 2016). Nonetheless, more studies should be performed to understand how the changes in water flux occur. In the meantime, it is important to define the exact moment of analysis after using the Trumorph® tool with raw sperm samples. However, it is important to take into account that the initial time of analysis could affect the accuracy of the real size of the spermatozoa head since it is necessary to focus the image under the microscope and choose the best field of view after the pressure. For European eel sperm, the capture of images for morphometric analysis of samples diluted in P1 medium must be done between 1′ and 1′30″ after the pressure to get a result as close to the real as possible, although, it may be interesting to observe the model of morphological changes of the spermatozoa head in samples diluted in another medium, such as seminal fluid. Besides, the time of analysis should also be studied for other fish species since the protocols are species-specific.

5. Conclusion

Morphometric sperm assessment can provide important information about sperm quality. However, this analysis can be affected by several factors, as the sample preparation or technique used, and for that reason need to be standardised. A simple method without staining or fixation techniques could minimise the influence of the protocol in the

spermatozoa head dimensions. Thus, the Trumorph® with raw sperm samples could be a useful tool since the sperm samples are kept in natural environmental conditions and the cells are immobilised only with pressure. In these conditions, the spermatozoa are alive and real measurements of the cells head can be obtained during the analysis process. Nonetheless, it is important to define the initial analysis time to obtain a high accuracy of the characterisation of spermatozoa morphology. In this study, the morphometry assessment of European eel sperm using the Trumorph® tool without fixation suggested that the time of analysis time should be between 1 and 1 min 30″ after pressure.

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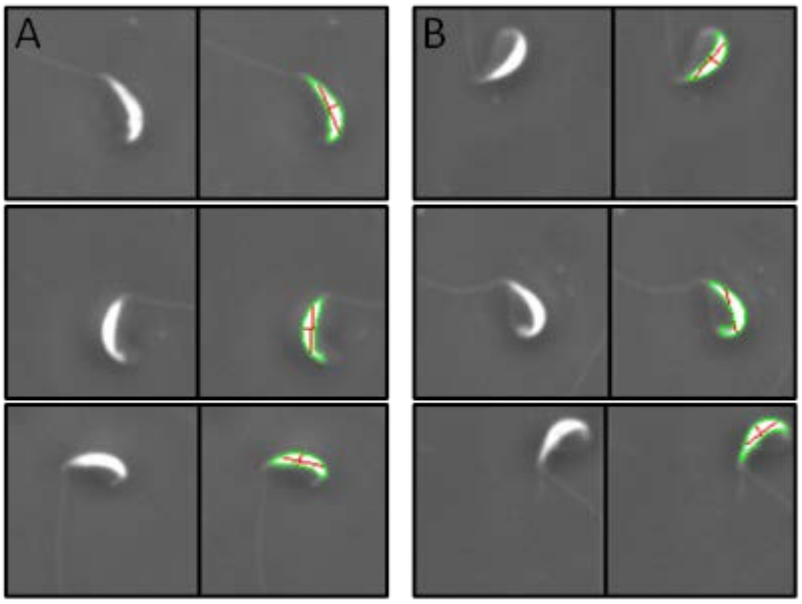
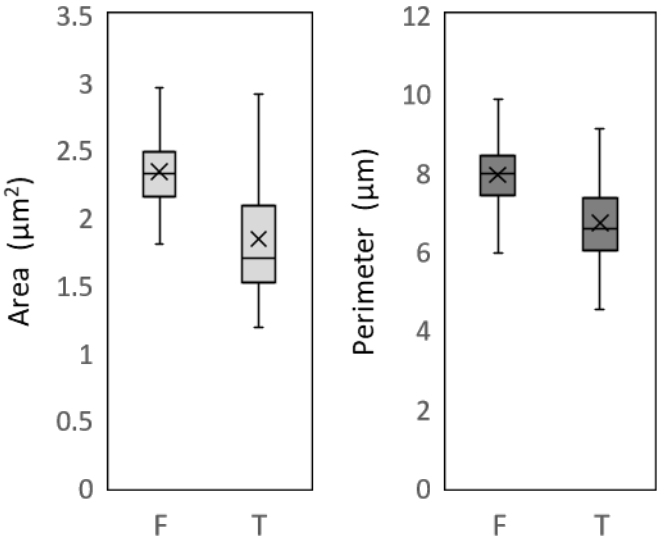
Figure 1: Original (left panel) and false-colour analysed (right panel) paired images from European eel spermatozoa head obtained by phase-contrast microscope at magnification ×100 for both protocols: (A) TruMorph with and (B) without fixation. Green line represents the head perimeter and the red lines represents the major (length) and minor (width) head axes.



514 Figure 2: Head area (µm^2) and perimeter (µm) of European eel spermatozoa from sexual
515 mature males (n = 5) obtained with two protocols: Trumorph® with (F; the total number of
516 spermatozoa = 1130) and without fixation (T; the total number of spermatozoa = 429). Data
517 are presented as median (interquartile range; Q1 and Q3) and minimum and maximum
518 values. The cross represents the mean. Different letters represent significant differences (*P*
519 < 0.05) between techniques.



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522 Figure 3: Effect of time on the head area (µm^2) and perimeter (µm) measurements of
523 European eel spermatozoa after using Trumorph® technique without fixation (*n* = 17792
524 total number of spermatozoa from 5 sperm samples). Data are expressed on mean ± SEM.
525 Asterisk (*) indicate a significant difference between a mean value and the previous one (P
526 < 0.05).

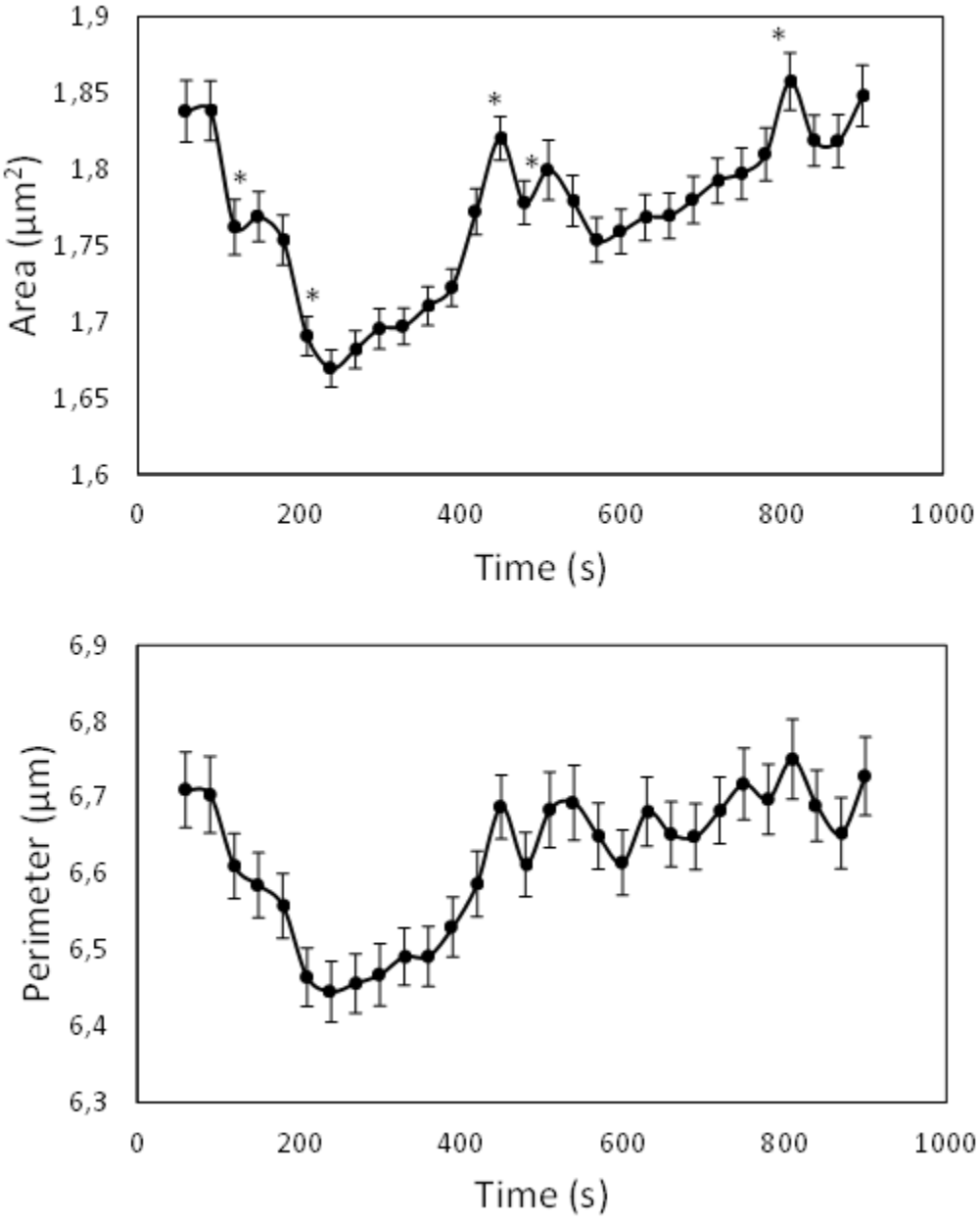

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Figure 4: Prediction interval of the polynomial regression of degree 4 ($y = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4 + time$) for mean values (blue points) of head area (μm^2) and perimeter (μm) of European eel spermatozoa with time (min).