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Sperm motility parameters and spermatozoa morphometric characterization in marine species: a study of swimmer and sessile species

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Abstract

The biodiversity of marine ecosystems is diverse and a high number of species coexist side by side. However, despite the fact that most of these species share a common fertilization strategy, a high variability in terms of the size, shape and motion of spermatozoa can be found. In this study, we have analysed both the sperm motion parameters and the spermatozoa morphometric features of two swimmer (pufferfish and European eel) and two sessile (sea urchin and ascidian) marine species.

The most important differences in the sperm motion parameters were registered in the swimming-time period. Sessile species sperm displayed notably higher values than swimmer species sperm. In addition, the sperm motilities and velocities of the swimmer species decreased sharply once the sperm was activated, whereas the sessile species were able to maintain their initial values for a long time. These results are linked directly to the species-specific lifestyles. While sessile organisms, which demonstrate limited or no movement, need sperm with a capacity to swim for long distances in order to find the oocytes, swimmer organisms can move towards the female and release gametes near it, and therefore the spermatozoa does not need to be able to swim for such a long time period.

At the same time, sperm morphology is related to sperm motion parameters and in this study an in depth morphometric analysis of ascidian, sea urchin and pufferfish spermatozoa, using CASA software, has been carried out for the first time. A huge variability in shapes, sizes, and structures of the studied species was found using electron microscopy.

Keywords

Spermatozoa; ASMA; European eel; Sea urchin; Ascidian; Pufferfish

1. Introduction

A wide diversity of reproductive strategies and fertilization methods can be found in marine fauna [1]. External fertilization (broadcast spawning) is the most common reproductive strategy in aquatic environments, and is generally thought to be ancestral to internal methods of reproduction. However, several factors are involved in this kind of reproductive strategy, including the evolution of both male and female gametes in the aquatic environments. Regarding eggs, features such as cell size, the size and shape of accessory structures, and the chemoattractants of some marine species have already been documented [2]. With regards to male gametes, external fertilization involves a fundamental process known as sperm competition [3]. This mechanism, defined as when sperm from two or more males compete for the fertilization of eggs, can lead to a wide range of morphological and physiological adaptations/variants and, over time, reproductive traits such as spermatozoa morphology, swimming-time period or swimming speed [4] have been filtered by natural selection. Therefore, it seems reasonable to suppose that species sharing similar reproductive strategies might also share similar spermatozoa features, whereas species with radically different reproductive strategies, might display differences both in morphological as well as in kinetic sperm parameters.

In terms of sessile species, which show limited or even no movement over the substrate, one factor in particular is fundamental in the fertilization of their gametes: the proximity of the congeners usually ensures reproductive success, whereas isolation can lead to reproductive failure. With the aim of reducing this issue, the eggs of some marine species are able to release specific substances called chemoattractants. These can be detected by the spermatozoa who orient their swimming direction up the concentration gradient towards the oocyte [5,6,7].

On the other hand, marine fish can swim and both males and females are able to come together to reproduce and, for this reason, fish sperm does not need to be able to swim for a long time period in order to find the female gametes. In addition, in some fish species the egg micropyle closes quickly after contact with seawater, and the spermatozoa must find the micropyle within a short time period, ranging from several seconds to a few minutes [8]. This means that fish spermatozoa exhibit high motility at the beginning of sperm activation, and the kinetic traits of marine fish sperm are defined by/ based on these biological and behavioural premises.

Thus, the main goal of this study was to use a computer-assisted sperm analysis (CASA) system and assisted-sperm morphology analysis (ASMA) software, to evaluate the kinetic and morphometric sperm parameters of some marine species belonging to different taxa. The data obtained were used to compare the main differences between the species, and to inquire into some theories to explain the biological reasoning for the size, shape and motion of the spermatozoa. Scanning electron microscope pictures were captured in order to evaluate the shape of the sperm cells.

2. Materials and methods

2.1 Animal handling, sperm collection and sampling

All the trials were carried out in accordance with the animal guidelines of the University of Tokyo on Animal Care and, the eels specifically, were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

The Pufferfish (*Takifugu niphobles*) displays an unusual spawning behavior at Arai Beach near the Misaki Marine Biological Station (MMBS, Japan). Large schools of fish arrive to the beach with the spring tide, around the time of the new moon, and spawning takes place repeatedly from 2 hours before sunset until sunset. During that time, pufferfish males were caught and moved to the MMBS facilities. To collect the sperm, the genital area was cleaned with freshwater to avoid contamination of the samples by faeces, urine or seawater. The sperm was collected by applying gentle pressure and then diluted (1:50) in a seminal plasma-like solution (SLS; [9]).

Eel males (*Anguilla anguilla*) were moved from the Valenciana de Acuicultura, S.A. fish farm (Spain) to the aquaculture facilities at the Universitat Politècnica de València (UPV, Spain). The fish were gradually acclimatized to artificial seawater and maturation was induced through weekly intraperitoneal hormone injections over the course of 11 weeks (hCG; 1.5 IU g^{-1} fish). The sperm samples were collected by abdominal pressure 24 h after the hormone injection [10] and after the genital area had been cleaned in order to avoid contamination by faeces, urine or seawater. The sperm was collected by applying gentle pressure and then diluted (1:50) in a P1 medium ([9]).

Ascidians (*Ciona intestinalis*) were collected in Aburatsubo Bay (Kanagawa, Japan) and kept in constant light to prevent spontaneous spawning. Having removed the tunic and opened the body with scissors, the oviduct was punctured with forceps to remove the

eggs. The sperm was obtained by puncturing the sperm duct with forceps and extracting the sperm using a Pasteur pipette. The sperm was then diluted (1:50) in seawater (SW, see section 2.2).

Sea urchins (*Anthocidaris crassispina*) were also collected in Aburatsubo Bay (Kanagawa, Japan) and the sperm was obtained by the administration of an intracoelomic injection of 1 ml of acetylcholine solution (0.5 M) into the soft tissue of the oral surface of the animal. The animal was gently shaken after the injection to distribute the acetylcholine to all the gonads. The sperm was then collected using a pipette and diluted (1:50) in a seminal plasma-like solution (SLS).

All the sperm samples (n= 8; for each species) were kept in their diluted form at 4 ºC until both the motility and morphometric analyses were carried out.

2.2 Assessment of sperm motility parameters

The diluted sperm was activated by mixing 0.5 µl with 4 µl of artificial seawater (SW) consisting of 460 mM NaCl, 10 mM KCl, 36 mM $MgCl_2$, 17 mM $MgSO_4$, 9 mM $CaCl_2$, and 10 mM HEPES, with 1% BSA (w/v) and a pH adjusted to 8.2. In the case of the ascidian, sperm motility was initiated by SW containing SAAF, prepared from the unfertilized eggs as per the method described previously by Yoshida et al. [12].

The sperm-seawater mix was examined using a SpermTrack-10® chamber (Proiser R+D, S.L.; Spain). Video sequences were recorded (at 50 fps) using a high-sensitivity video camera mounted on a phase contrast microscope with a 10x objective lens. All the motility analyses were performed in triplicate using the motility module of ISAS® (Proiser R+D, S.L.; Spain). All the sperm samples were evaluated in the two hours that followed extraction.

The parameters examined in this study were total motility (TM, %), defined as the percentage of motile cells; progressive motility (PM, %), defined as the percentage of spermatozoa which swim in an essentially straight line; curvilinear velocity (VCL, µm/s), defined as the time/average velocity of a spermatozoa head along its actual 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; average path velocity (VAP, µm/s), defined as the time/average of a sperm head along its spatial average trajectory; and finally, the percentage of fast (VAP > 100 µm/s), medium (VAP = 50-100 µm/s) and slow (VAP = 10-50 µm/s)

spermatozoa; Spermatozoa were considered immotile if their VAP was lower than 10 µm/s.

2.3 Spermatozoa morphometric analysis

All the sperm samples were diluted 1:50 (v/v) in their own diluent (see section 2.1). The spermatozoa were fixed with 2.5% glutaraldehyde and were deposited in Eppendorf tubes. An aliquot of sperm dilution (approximately 10 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 and the morphometric analyses of sperm samples were performed using the morphometry module of the ISAS® software (Figure 1). The spermatozoa head measurements, including size variables such as length (L), width (W), area (A) and perimeter (P); and shape variables such as elipticity (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.

2.4 Scanning electron microscopy

Sperm cells were fixed with 4% glutaraldehyde and were deposited in Eppendorf tubes until the scanning electron microscopy analysis. The samples were frozen in slush N_2 and attached to the specimen holder of a CT-1000C Cryo-transfer system (Oxford Instruments, Oxford, UK) interfaced with a JEOL JSM-5410 scanning electron microscope (SEM). The samples were then fractured and transferred from the cryostage to the microscope sample stage, where the condensed surface water was sublimed by controlled warming to -90 ºC. Finally, the samples were gold coated by sputtering and viewed at an accelerating voltage of 15 KeV.

2.5 Statistical analysis

The mean ± standard error was calculated for all the sperm motility parameters. Shapiro-Wilk and Levene tests were used to check the normality of data distribution and variance homogeneity, respectively. One-way analysis of variance (ANOVA) was used to analyze data with normal distribution. Significant differences were detected using the Tukey multiple range test ($P \leq 0.05$). For non-normally distributed populations, Kruskal-Wallis one-way ANOVA on ranks was used. All the statistical analyses were performed

using the statistical package SPSS version 19.0 for Windows software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Sperm motility parameters

The most notable difference in the sperm motion parameters was registered in the swimming-time period (defined as the length of time the spermatozoa are able to move in seawater), with the sessile species exhibiting higher values than the swimmer species. The Pufferfish and European eel spermatozoa were able to move for 1.5 and 15 min respectively (Figure 2A,B); while both sea urchin and ascidian spermatozoa were able to move for longer, around 45 min (Figure 2C,D).

Moreover, different sperm motility patterns were found in the analyzed species (Figure 2). The swimmer species displayed higher TM values (≥77%) than the sessile species (≤59%) at the start of sperm activation. However, the spermatozoa of the sessile species were able to maintain their initial TM values for a long time, while the spermatozoa of the swimmer species exhibited a sharp decrease in TM after the sperm was activated. Regarding PM, the swimmer species displayed higher values (55 and 46%, in pufferfish and European eel, respectively) than the sessile species, which displayed low values over time (≤10%).

Different sperm velocity patterns were also found (Figure 3). The sessile species displayed higher VCL values (241 and 296 μm/s, in sea urchin and ascidian, respectively) than the swimmer species (≤183 μm/s). In addition, the highest VCL values were registered at different moments: in the sessile species they were recorded in the middle of the swimming-time period while in the swimmer species they were recorded just after sperm activation. Regarding VSL, similar patterns to VCL were found, but with noticeably lower values.

On the other hand, pufferfish showed a dramatic decrease in terms of the percentage of fast spermatozoa, displaying values of less than 50% 30 s post-activation (Figure 4A). In contrast, the European eels had more than 50% fast spermatozoa until 10 min after activation (Figure 4B). On the other hand, the sessile species displayed more fast spermatozoa than the swimmer species, and they were able to maintain those high values throughout most of the swimming-time period (Figure 4C,D).

3.2 Assisted spermatozoa morphometric analysis (ASMA) and scanning electron microscopy (SEM).

Different sizes and shapes of spermatozoa were found in the analyzed species. The smallest spermatozoa analyzed were those of the Pufferfish, with significantly lower values than the other species in all the size parameters (Figure 5). The spermatozoon consisted of a small cylindrical head without an acrosome (Figure 6A). No mitochondria were externally evident, due to the fact that these organelles are located behind the nucleus occupying the anterior region of cytoplasmic sleeve [13]. The flagellum was exceptionally long, 12-13 times longer than the head (Table 1).

The European eel spermatozoa displayed the highest values in all of the size parameters (Figure 5). The head was asymmetrical along the longitudinal axis, and gently curved and elongated with a hook-shaped superior end facing inwards towards the crescent (Fjigure 6B). A single spherical mitochondrion was located at the anterior portion of the head, exactly opposite the flagellum, which was 5-6 times longer than the head (Table 1).

The sea urchin spermatozoa displayed the second highest values in many of the size parameters, including area, perimeter and length (Figure 5). The head had a conical shape with a small acrosome located in the apical zone (Figure 6C). A single spherical mitochondrion was located at the posterior side of the head. The flagellum was 4-5 times longer than the head (Table 1) and had a balloon-like structure at the end of it.

Finally, the ascidian spermatozoa, like the pufferfish spermatozoa, were small in size, with the shortest head length (Figure 5). The spermatozoon was comprised of a cylindrical and thin head, which was partly surrounded by an elongated and relatively big mitochondrion (Figure 6D). The flagellum was 10-11 times longer than the head (Table 1).

4. Discussion

Marine ecosystems display a huge biodiversity and a large number of species belonging to different taxa coexist side by side. Most of these species share a common fertilization strategy, broadcast spawning, in which gametes both from males and females are released into the seawater. However, despite this common method of reproduction, a high variability in terms of spermatozoa traits can be found between the different taxa, genus, species, even at an intra-specific level [14]. In this study, we have analyzed both

256 the sperm motion parameters and the spermatozoa morphometric features of four
257 species belonging to two marine groups which display fundamentally different
258 lifestyles: the swimmer and sessile species.
259 The most important difference in the sperm motion parameters was registered in the
260 swimming-time period. The sessile species (sea urchin and ascidian) exhibited the
261 longest values, with total motility values remaining at nearly 20% up until at least 40-45
262 min after activation. In reference to this, it has been reported that the sperm of the
263 majority of sessile marine invertebrates is able to remain motile and viable for a long
264 period of time, from a few minutes in some echinoderms [15] to many hours and even
265 as long as a day in some species of ascidian [16]. However, in addition to understanding
266 how long the sperm are able to move for, finding out how they move would also seem
267 essential. The increasing availability of CASA systems has allowed the estimation of
268 new parameters regarding sperm motion [17]. Progressive motility and spermatozoa
269 velocities are currently recognized as essential sperm traits because they are linked to
270 fertilization success [18,19]. However, in this study, and in terms of progressive
271 motility, the sea urchin and ascidian sperm displayed negligible values over time (≤5
272 and ≤10%, respectively), and once activated most of their spermatozoa swam in
273 continuous concentric circles. So, how are the spermatozoa able to find the oocyte
274 swimming that way? In fact, for sea urchin or ascidian spermatozoa, finding an egg to
275 fertilize in a vast ocean might seem as impossible a task as looking for a needle in a
276 haystack. However, some external fertilizers have devised an effective strategy in order
277 to overcome this problem: the eggs are able to release chemical elements that guide the
278 sperm towards them. This mechanism, known as chemotaxis, has been researched in
279 some marine species, from benthic invertebrates [20] to pelagic fish [21,22]. During this
280 process, the chemoattractant interacts with the flagellum, generating asymmetric
281 flagellar movements and therefore, causing the reorientation of the spermatozoa [23]. In
282 ascidian (*Ciona intestinallis*) it has been reported that the chemoattractant, called
283 SAAF, has no effect on sperm kinetic parameters such as curvilinear velocity. However,
284 SAAF is able to modulate the swimming direction of the spermatozoa so they swim up
285 the concentration gradient towards the oocyte [22], increasing the number of
286 spermatozoa which swim in an essentially straight line. In terms of the spermatozoa
287 velocities of sessile species, notably higher curvilinear velocity (VCL) values were
288 registered than straight-line velocity (VSL) values, probably because VSL is directly
289 related to progressive motility. In this respect, scarce data about marine invertebrates

290 can be found in literature. Yoshida et al. [23,24] reported some velocity values for the
291 ascidian species, but these data were not obtained by the CASA system, so it was
292 impossible to discriminate between the different types of velocities (VCL, VSL and
293 VAP). In terms of sea urchin species, VCL values of about 150 μm/s have been reported
294 in *Heliocidaris erythrogramma* [25], and slightly higher in *Anthocidaris crassispina*
295 [26]. However, this study describes the performance of the sperm velocities over time,
296 and has shown that sessile species are able to maintain high VCL values for a long time
297 (20 and 40 min, in the case of sea urchin and ascidian, respectively).
298 On the other hand, the swimmer species (pufferfish and European eel) exhibited shorter
299 swimming-time periods than the sessile species. Motility in marine fish species has been
300 widely studied [8], and sperm motility can last from a few seconds (bogue [27]) to up to
301 20 min (conger [28]). In the present study, the swimming-time period of the two
302 swimmer species analysed was quite different. In the case of the pufferfish (*Takifugu*
303 *niphobles*), the sperm swimming-time period was just 90 s, and the motility values and
304 spermatozoa velocities decreased drastically after sperm activation. This sperm motion
305 pattern could be explained by the peculiar spawning behavior of this species, where the
306 males and females (ratio 20:1) leave the seawater and release the gametes on the shore´s
307 pebbles [29]. Therefore, pufferfish males must race amongst themselves and release the
308 sperm in the right location in order for the spermatozoa to reach and fertilize the oocyte
309 as fast as possible. As such, the high values found for total and progressive motility and
310 spermatozoa velocities over the first 20 seconds after activation support this hypothesis
311 and are what allow the pufferfish spermatozoa to reach the eggs quickly. In contrast, the
312 European eel sperm exhibited a longer swimming-time period of around 15 min, and the
313 motility values and spermatozoa velocities decreased gradually over time. Similar
314 results for this species were reported by Gibbons et al. [30], who estimated that eel
315 spermatozoa can swim for more than 20 min with little change in their kinetic
316 parameters. In the case of the European eel, little is known about its spawning
317 behaviour in the wild, so relating the motility parameters to spawning ethology is a
318 difficult task. However, it is assumed that the spawning ground is located in the waters
319 of the Sargasso Sea [31], and detailed descriptions of the spawning behaviour under
320 controlled lab conditions have been given in the last few years [32]. In this respect, the
321 spawning of the European eel seems to be collective, large scale, and possibly triggered
322 by pheromones. In this way, the pelagic eggs released from the female will float in the
323 water column for a long-time, thus the spermatozoa which are able to remain in motion

for longer will have the greatest chances of fertilizing the oocytes. However, in some fish species the egg micropyle closes quickly after contact with seawater, after several seconds to a few minutes, so the spermatozoa must achieve their objective as soon as possible after activation.

Moreover, in addition to motility parameters, spermatozoa morphology has been a widely analysed feature of marine fish [33-35]. Fish spermatozoa differ greatly from one another and a widespread range of shapes, sizes, and structures can be found [36]. In this respect, morphometric features such as cryopreservation [37,38], environmental pollution [39] and sperm quality [40,41] have successfully been used in different field studies,. In the present study a morphometric analysis of ascidian, sea urchin and pufferfish spermatozoa using CASA software has been carried out for the first time. In the case of the European eel, previous studies have already been published using this technique [42-44]. Sperm motion parameters can be directly influenced by spermatozoa morphology and the morphometry of sperm and somestudies have shown interesting relationships and trade-offs between these parameters. In the case of sea urchin, a high correlation between flagellum length and spermatozoa velocities has been observed at an intra-male level [25]; and in bluegill (*Lepomis macrochirus*), a positive relationship was found between sperm head length and swimming speed [45]. However, other authors have not found any relationship between sperm morphology and motility parameters [46,47], probably because a combination of several traits such as i) the head size, ii) the number and relative size of mitochondria, iii) the flagellum length or iv) the availability of ATP, defines the sperm motion parameters. In this study, the pufferfish spermatozoa were the smallest spermatozoa analyzed, but showed the highest flagellum/head ratio. Some studies have shown that flagellum length is linked to faster velocities and shorter swimming-time periods [48,49], and the pufferfish spermatozoa data appears to corroborate this. In terms of the sessile species, a big mitochondrion was located at the posterior end of the sea urchin head and surrounded part of the head of the ascidian. It is well known that the mitochondrion provides energy used to propel the cell, determining “how” and “for how long” the flagellum moves [50]. Therefore, the mitochondrion/head volume ratio could define the sperm motion patterns, as it does in mammal species [51], with high ratios resulting in spermatozoa which are able to swim for longer. Our data agree with this hypothesis, with the sessile species spermatozoa exhibiting the biggest mitochondria in relation to their heads, having the longest swimming-time periods. However, making comparisons and hypothesis about the

relationship between spermatozoa morphometry and motility at an interspecific level is a complicated task, because sperm evolution has occurred at an intraspecific level. Therefore, further studies about these relationships and trade-offs within species should be carried out to reveal the functional link between sperm structure and motion.

To sum up, our study reports, for the first time, the sperm motion parameters and spermatozoa morphometric features of some marine species belonging to different taxa of the animal kingdom, showing an example of sperm diversity of swimmer and sessile marine species**.**

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References

[1] Serrao EA, Havenhand J. Fertilization strategies. In: Wahl M, editor. Marine Hard Bottom Communities. Ecological Studies, Berlin: Springer; 2009, p. 149-64.

[2] Marshall DJ, Keough MJ. The evolutionary ecology of offspring size in marine invertebrates. Adv. Mar. Biol. 2007;53:1-60.

[3] Levitan DR. Sperm limitation, gamete competition, and sexual selection in external fertilizers. In: Birkhead TR, Møller AP, editors. Sperm Competition and Sexual Selection. San Diego: Academic Press; 1998, p. 175-217.

[4] Parker GA, Pizzari T. Sperm competition and ejaculate economics. Biological Reviews 2010;85(4):897-934.

[5] Yoshida M, Kawano N, Yoshida K. Control of sperm motility and fertility: Diverse factors and common mechanisms. Cell. Mol. Life Sci. 2008;65:3446-3457.

[6] Yoshida M, Yoshida K. Sperm chemotaxis and regulation of flagellar movement by Ca^{2+}. Mol. Hum. Reprod. 2011;17:457-465.

[7] Yoshida M, Hiradate Y, Sensui N, Cosson J, Morisawa M. Species specificity of sperm motility activation and chemotaxis: A study on ascidian species. Biol. Bull. 2013; *in press*.

391 [8] Cosson J, Groison A, Suquet M, Fauvel C, Dreanno C, Billard R. Studying sperm
392 motility in marine fish: An overview on the state of the art. J. Appl. Ichthyol.
393 2008;24(4):460-86.
394 [9] Gallego V, Pérez L, Asturiano JF, Yoshida M. Study of pufferfish (*Takifugu*
395 *niphobles*) sperm: development of methods for short-term storage, effects of different
396 activation media and role of intracellular changes in Ca^{2+} and K^{+} in the initiation of
397 sperm motility. Aqualculture 2013; *in press* (doi: 10.1016/j.aquaculture.2013.07.046).
398 [10] Gallego V, Mazzeo I, Vílchez MC, Peñaranda DS, Carneiro PCF, Pérez L, et al.
399 Study of the effects of thermal regime and alternative hormonal treatments on the
400 reproductive performance of European eel males (*Anguilla anguilla*) during induced
401 sexual maturation. Aquaculture 2012;354–355(0):7-16.
402 [11] Peñaranda DS, Pérez L, Gallego V, Barrera R, Jover M, Asturiano JF. European
403 eel sperm diluent for short-term storage. Reprod. Domest. Anim. 2010;45(3):407-15.
404 [12] Yoshida M, Inaba K, Morisawa M. Sperm chemotaxis during the process of
405 fertilization in the ascidians *Ciona savignyi* and *Ciona intestinalis*. Dev. Biol.
406 1993;157(2):497-506.
407 [13] Morisawa S. Ultrastructural studies of late-stage spermatids and mature
408 spermatozoa of the puffer fish, *Takifugu niphobles* and the effects of osmolality on
409 spermatozoan structure. Tissue and Cell 2001;33(1):78-85.
410 [14] Haszprunar G. Fish evolution and systematics: Evidence from spermatozoa. J.
411 Evol. Biol. 1991;5(4):721-3.
412 [15] Rahman MS, Tsuchiya M, Uehara T. Effects of temperature on gamete longevity
413 and fertilization success in two sea urchin species, *Echinometra mathaei* and
414 *Tripneustes gratilla*. Zool. Sci. 2009;26(1):1-8.
415 [16] Johnson SL, Yund PO. Remarkable longevity of dilute sperm in a free-spawning
416 colonial ascidian. Biol. Bull. 2004;206(3):144-51.
417 [17] Gallego V, Carneiro PCF, Mazzeo I, Vílchez MC, Peñaranda DS, Soler C, et al.
418 Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by
419 CASA software. Theriogenology 2013;79(7):1034-40.
420 [18] Kime DE, Van Look KJW, McAllister BG, Huyskens G, Rurangwa E, Ollevier F.
421 Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in
422 fish. Comp. Biochem. Physiol., C: Comp. Pharmacol. Toxicol. 2001;130(4):425-33.

[19] Gasparini C, Simmons LW, Beveridge M, Evans JP. Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. Plos One 2010;5(8):e12146.

[20] Miller RL. Sperm chemo-orientation in metazoa. In: Metz CB, Monroy A, editors. Biology of Fertilization, New York: Academic Press; 1985, p. 275-337.

[21] Hart NH. Fertilization in teleost fishes: Mechanisms of sperm-egg interactions. Int. Rev. Cytol. 1990;121:1-66.

[22] Yanagimachi R, Cherr GN, Pillai MC, Baldwin JD. Factors controlling sperm entry into the micropyles of salmonid and herring eggs. Dev. Growth Differ. 1992;34:447-61.

[23] Yoshida M, Ishikawa M, Izumi H, De Santis R, Morisawa M. Store-operated calcium channel regulates the chemotactic behavior of ascidian sperm. Proc. Natl. Acad. Sci. 2003;100(1):149-54.

[24] Yoshida M, Murata M, Inaba K, Morisawa M. A chemoattractant for ascidian spermatozoa is a sulfated steroid. Proc. Natl. Acad. Sci. 2002;99(23):14831-6.

[25] Fitzpatrick JL, Garcia-Gonzalez F, Evans JP. Linking sperm length and velocity: the importance of intramale variation. Biol. Lett. 2010;6(6):797-9

[26] Au DWT, Chiang MWL, Tang JYM, Yuen BBH, Wang YL, Wu RSS. Impairment of sea urchin sperm quality by UV-B radiation: Predicting fertilization success from sperm motility. Mar. Pollut. Bull. 2002;44(7):583-9.

[27] Lahnsteiner F, Patzner RA. Sperm motility of the marine teleosts *Boops boops*, *Diplodus sargus*, *Mullus barbatus* and *Trachurus mediterraneus*. J. Fish Biol. 1998;52(4):726-42.

[28] Cosson J, Groison A-, Suquet M, Fauvel C, Dreanno C, Billard R. Marine fish spermatozoa: Racing ephemeral swimmers. Reproduction 2008;136(3):277-94.

[29] Yamahira K. The role of intertidal egg deposition on survival of the puffer, *Takifugu niphobles*, embryos. J. Exp. Mar. Biol. Ecol. 1996;198(2):291-306.

[30] Gibbons BH, Baccetti B, Gibbons IR. Live and reactivated motility in the 9+0 flagellum of anguilla sperm. Cell Motil. 1985;5(4):333-50.

[31] Aarestrup K, Økland F, Hansen MM, Righton D, Gargan P, Castonguay M, et al. Oceanic spawning migration of the European eel (*Anguilla anguilla*). Science 2009;325(5948):1660.

[32] van Ginneken VJT, Maes GE. The European eel (*Anguilla anguilla*), its lifecycle, evolution and reproduction: A literature review. Rev. Fish Biol. Fish. 2005;15(4):367-98.

457 [33] Asturiano JF, Marco-Jiménez F, Peñaranda DS, Garzón DL, Pérez L, Vicente JS, et
458 al. Effect of sperm cryopreservation on the European eel sperm viability and
459 spermatozoa morphology. Reprod. Domest. Anim. 2007;42(2):162-6.
460 [34] Lahnsteiner F, Patzner RA. Sperm morphology and ultrastructure in fish. Fish
461 Spermatology 2008:1-61.
462 [35] Marco-Jiménez F, Peñaranda DS, Pérez L, Viudes-De-Castro MP, Mylonas CC,
463 Jover M, et al. Morphometric characterization of sharpsnout sea bream (*Diplodus*
464 *puntazzo*) and gilthead sea bream (*Sparus aurata*) spermatozoa using computer-assisted
465 spermatozoa analysis (ASMA). J. Appl. Ichthyol. 2008;24(4):382-5.
466 [36] Baccetti B. Evolutionary trends in sperm structure. Comparative Biochemistry and
467 Physiology Part A: Physiology 1986;85(1):29-36.
468 [37] Peñaranda DS, Pérez L, Fakriadis G, Mylonas CC, Asturiano JF. Effects of
469 extenders and cryoprotectant combinations on motility and morphometry of sea bass
470 (*Dicentrarchus labrax*) spermatozoa. J. Appl. Ichthyol. 2008;24(4):450-5.
471 [38] Gallego V, Peñaranda DS, Marco-Jiménez F, Mazzeo I, Pérez L, Asturiano JF.
472 Comparison of two techniques for the morphometry study on gilthead seabream (*Sparus*
473 *aurata*) spermatozoa and evaluation of changes induced by cryopreservation.
474 Theriogenology 2012;77(6):1078-87.
475 [39] van Look KJW, Kime DE. Automated sperm morphology analysis in fishes: The
476 effect of mercury on goldfish sperm. J. Fish Biol. 2003;63(4):1020-33.
477 [40] Asturiano JF, Marco-Jiménez F, Pérez L, Balasch S, Garzón DL, Peñaranda DS, et
478 al. Effects of hCG as spermiation inducer on European eel semen quality.
479 Theriogenology 2006;66(4):1012-20.
480 [41] Tuset VM, Trippel EA, De Monserrat J. Sperm morphology and its influence on
481 swimming speed in Atlantic cod. J. Appl. Ichthyol. 2008;24(4):398-405.
482 [42] Marco-Jiménez F, Pérez L, Castro MPVD, Garzón DL, Peñaranda DS, Vicente JS,
483 et al. Morphometry characterization of European eel spermatozoa with computer-
484 assisted spermatozoa analysis and scanning electron microscopy. Theriogenology
485 2006;65(7):1302-10.
486 [43] Marco-Jiménez F, Garzón DL, Peñaranda DS, Pérez L, Viudes-de-Castro MP,
487 Vicente JS, et al. Cryopreservation of European eel (*Anguilla anguilla*) spermatozoa:
488 Effect of dilution ratio, fetal bovine serum supplementation, and cryoprotectants.
489 Cryobiology 2006;53(1):51-7.

490 [44] Peñaranda DS, Marco-Jiménez F, Pérez L, Gallego V, Mazzeo I, Jover M, et al.
491 Protein profile study in European eel (*Anguilla anguilla*) seminal plasma and its
492 correlation with sperm quality. J. Appl. Ichthyol. 2010;26(5):746-52.
493 [45] Burness G, Casselman SJ, Schulte-Hostedde AI, Moyes CD, Montgomerie R.
494 Sperm swimming speed and energetics vary with sperm competition risk in bluegill
495 (*Lepomis macrochirus*). Behav. Ecol. Sociobiol. 2004;56(1):65-70.
496 [46] Casselman SJ, Schulte-Hostedde AI, Montgomerie R. Sperm quality influences
497 male fertilization success in walleye (*Sander vitreus*). Can. J. Fish. Aquat. Sci.
498 2006;63(9):2119-25.
499 [47] Gage MJG, Macfarlane C, Yeates S, Shackleton R, Parker GA. Relationships
500 between sperm morphometry and sperm motility in the Atlantic salmon. J. Fish Biol.
501 2002;61(6):1528-39.
502 [48] Stockley P, Gage MJG, Parker GA, Møller AP. Sperm competition in fishes: The
503 evolution of testis size and ejaculate characteristics. Am. Nat. 1997;149(5):933-54.
504 [49] Levitan DR. Sperm velocity and longevity trade off each other and influence
505 fertilization in the sea urchin *Lytechinus variegatus*. Proc. Biol. Sci.
506 2000;267(1443):531-4.
507 [50] Cosson J, Billard R, Cibert C, Dreanno C, Linhart O, Suquet M. Movements of fish
508 sperm flagella studied by high speed videomicroscopy coupled to computer assisted
509 image analysis. Polskie Archiwum Hydrobiologii 1997;44(1-2):103-13.
510 [51] Ciftci HB and Zulkadir U. The correlation between bull sperm head dimensions
511 and mitochondrial helix length. J Anim Vet Adv 2010;9(7):1169-1172.
512

Table legends

Table 1. Head (H) and flagellum (F) length of spermatozoa of swimmer (pufferfish and European eel) and sessile (sea urchin and ascidian) species. Data are expressed as mean ± SEM (n=15). Different letters mean significant differences between the different species.

Figure legends

Figure 1. Microphotographs of spermatozoa of swimmer (A: Pufferfish; B: European eel) and sessile (C: Sea urchin; D: Ascidian) species. Pictures were taken using PC technique and digitalized by ISAS software.

Figure 2. Total (TM) and progressive (PM) motility in swimmer (A: Pufferfish; B: European eel) and sessile (C: Sea urchin; D: Ascidian) species at different post-activation times. Data are expressed as mean ± SEM (n=8). Different letters mean significant differences over time.

Figure 3. Curvilinear (VCL) and rectilinear (VSL) velocity in swimmer (A: Pufferfish; B: European eel) and sessile (C: Sea urchin; D: Ascidian) species at different post-activation times. Data are expressed as mean ± SEM (n=8). Different letters mean significant differences over time.

Figure 4. Percentage of fast, medium and slow spermatozoa in each post-activation time in swimmer (A: Pufferfish; B: European eel) and sessile (C: Sea urchin; D: Ascidian) species over time (n=8). Fast spermatozoa, VAP > 100 μm/s; medium spermatozoa, VAP = 50-100 μm/s) and slow spermatozoa, VAP = 10-50 μm/s.

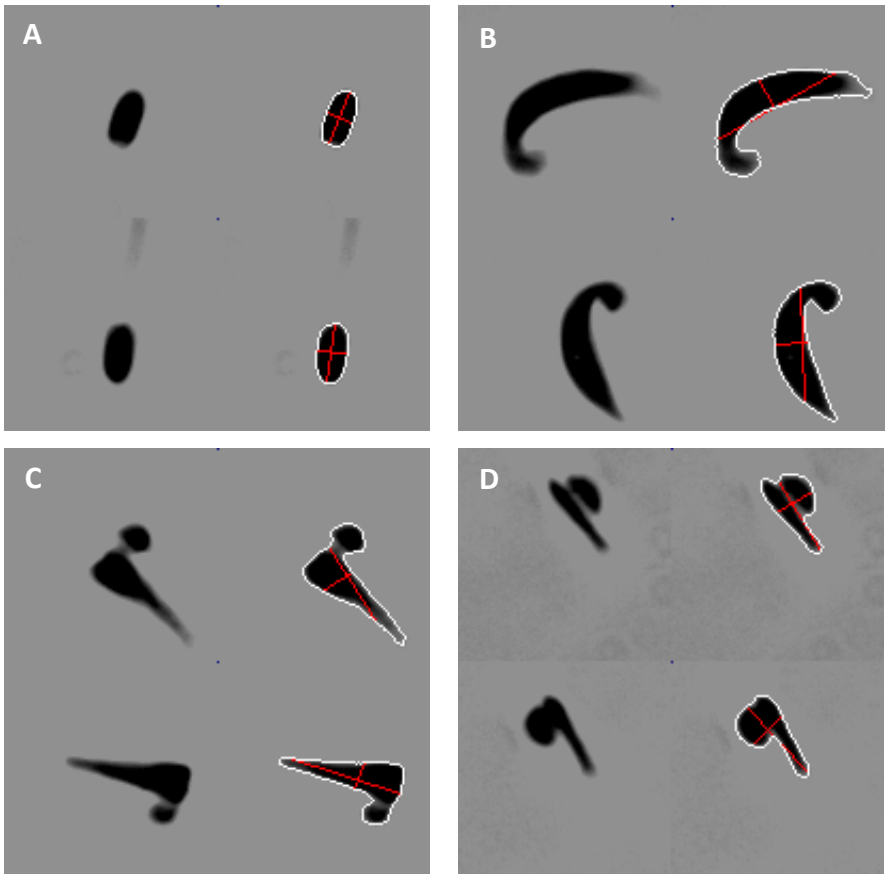
Figure 5. Spermatozoa head size and shape variables in swimmer and sessile species. Data are expressed as mean ± SEM (n=8). Different letters mean significant differences between the different species.

Figure 6. Scanning electron microscopy of spermatozoa of swimmer (A: Pufferfish; B: European eel) and sessile (C: Sea urchin; D: Ascidian) species. Head (h); mitochondrion (m); rootlet (r); flagellum (f); acrosome (a). Scale bar = 5 µm.

Table 1

	Head (µm)	Flagellum (µm)	Ratio F/H
Pufferfish	2.29 ± 0.06 d	28.83 ± 1.12 a	12.6 ± 0.32 a
European eel	5.58 ± 0.11 a	30.31 ± 1.02 a	5.43 ± 0.12 b
Sea urchin	4.56 ± 0.07 b	19.34 ± 0.87 b	4.25 ± 0.09 c
Ascidian	3.04 ± 0.04 c	31.81 ± 1.09 a	10.47 ± 0.15 b

Figure 1



553 **Figure 2**

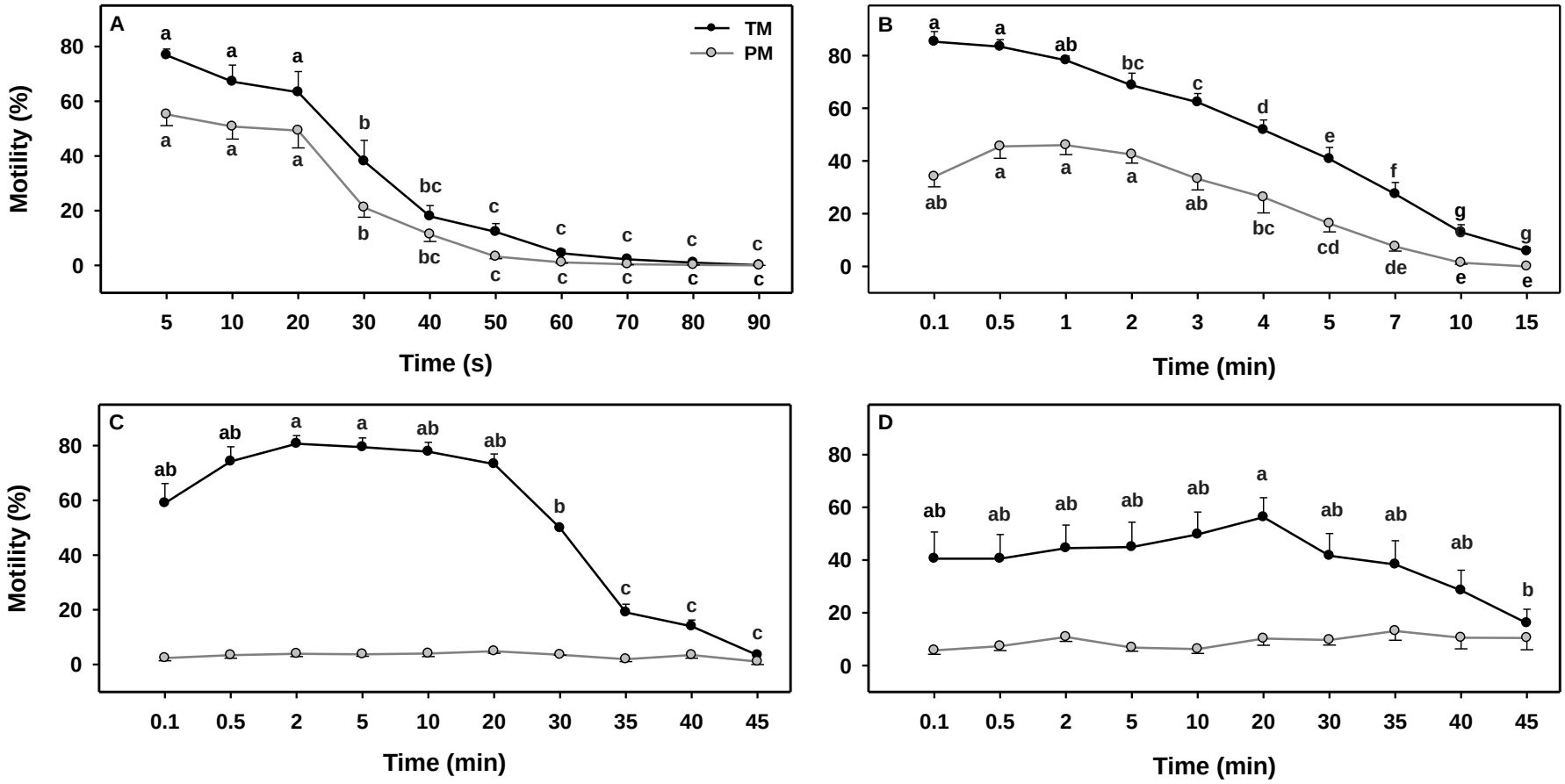


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Figure 3

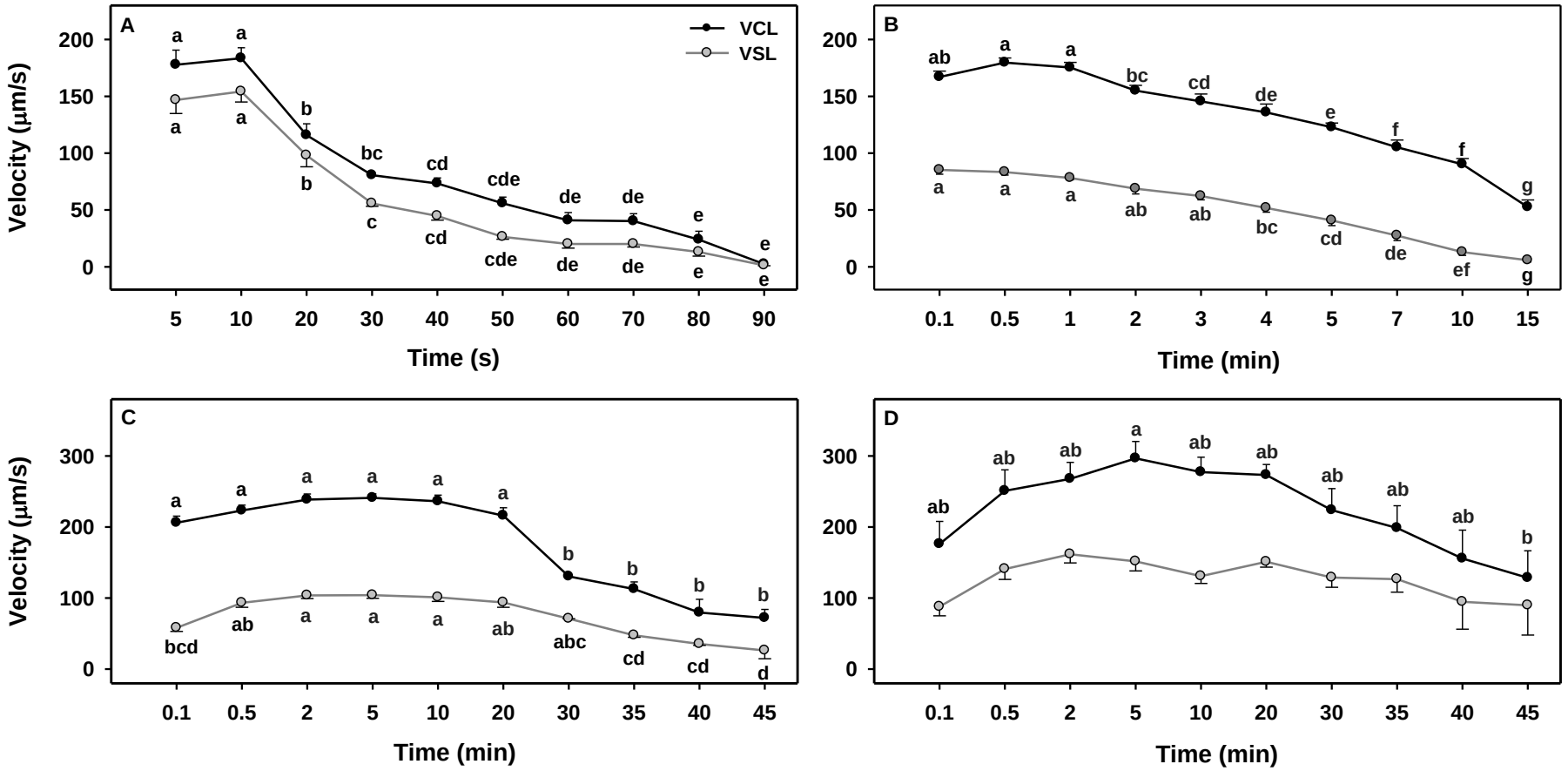
A
VCL
VSL
Velocity (µm/s)
Time (s)
B
Time (min)
C
Velocity (µm/s)
Time (min)
D
Time (min)

Figure 4

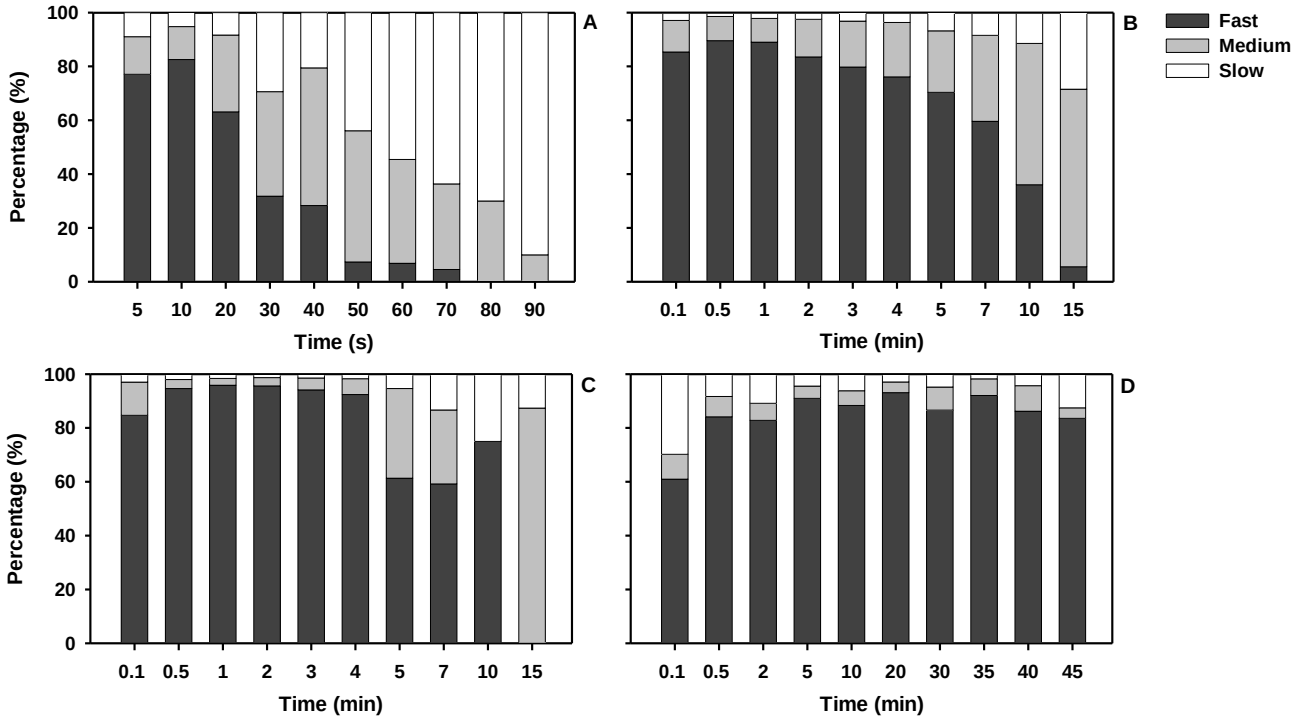
A
B
C
D
Fast
Medium
Slow
Percentage (%)
Time (s)
Time (min)
5 10 20 30 40 50 60 70 80 90
0.1 0.5 1 2 3 4 5 7 10 15
0.1 0.5 2 5 10 20 30 35 40 45

Figure 5

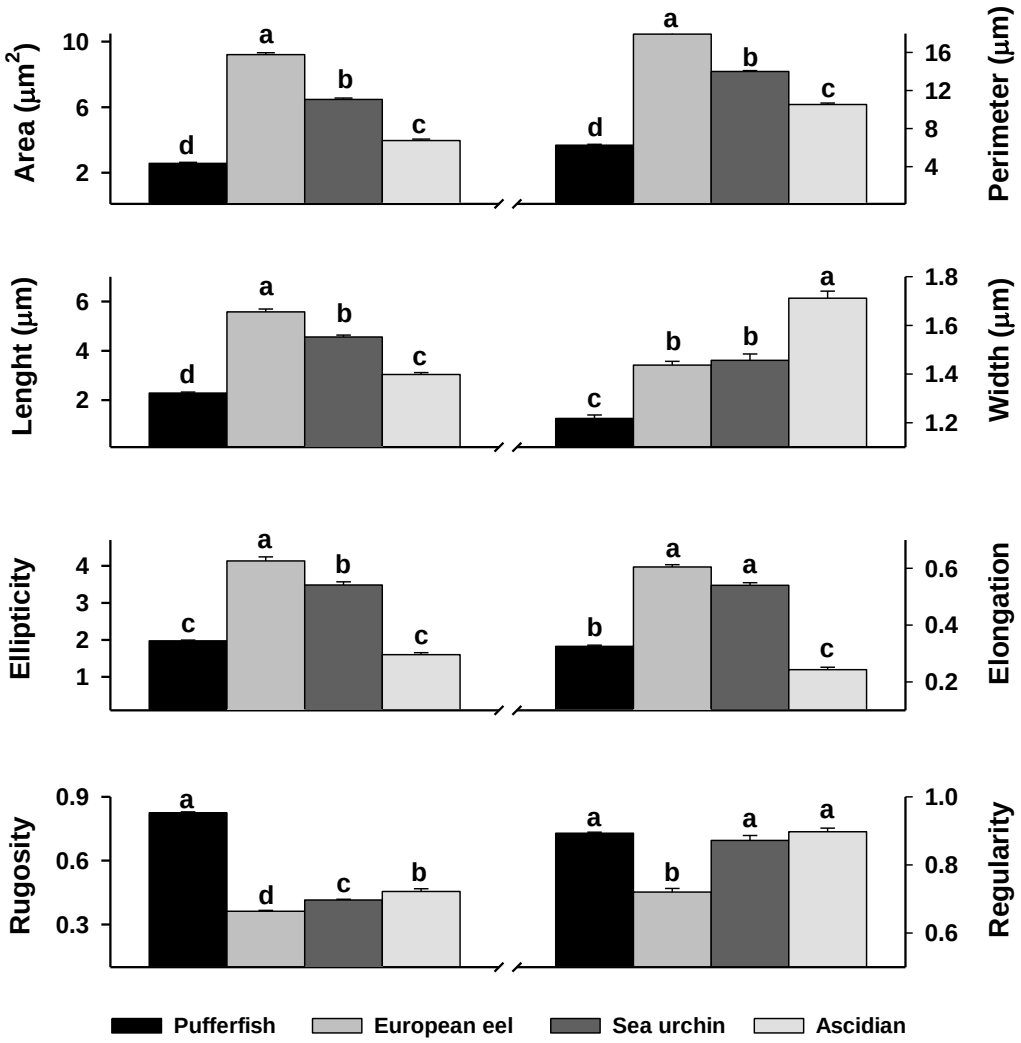
Area (μm²)
Perimeter (μm)
Lenght (μm)
Width (μm)
Ellipticity
Elongation
Rugosity
Regularity
Pufferfish
European eel
Sea urchin
Ascidian

Figure 6