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Christian Fauvel

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## The quality of great scallop (*Pecten maximus*) sperm after thawing

Suquet Marc <sup>1,\*</sup>, Gourtay Clémence <sup>1</sup>, Donval Anne <sup>2</sup>, Le Goïc Nelly <sup>2</sup>, Quere Claudie <sup>3</sup>, Malo Florent <sup>1</sup>,  
Le Grand Jacqueline <sup>1</sup>, Ratiskol Dominique <sup>1</sup>, Mingant Christian <sup>1</sup>, Fauvel Christian <sup>4</sup>

<sup>1</sup> Ifremer, UMR 6539, PFOM Department, Station Expérimentale d'Argenton, Argenton, France

<sup>2</sup> IUEM, UMR 6539, Lemar, Technopole Brest Iroise, Plouzané, France

<sup>3</sup> Ifremer, UMR 6539, PFOM Department, Technopole Brest-Iroise, Plouzané, France

<sup>4</sup> Ifremer, UMR 248, Marbec, Station Ifremer, Palavas, France

\* Corresponding author : Marc Suquet, email address : [msuquet@ifremer.fr](mailto:msuquet@ifremer.fr)

### Abstract :

Most publications devoted to the cryopreservation of mollusc sperm have focused on the definition of technical protocols, avoiding the description of sperm quality after thawing. The present study investigated the effects of cryopreservation on sperm quality in the great scallop. Wild scallop were fished during the natural spawning period and conditioned in the hatchery before use. Sperm samples were obtained after intragonadal injection of serotonin and cryopreserved using a previously published protocol. Sperm quality was assessed using a panel of four parameters: sperm motility characteristics, using a computer assisted sperm analysis plugin with Image J, intracellular ATP content using an ATP-Lite kit, sperm integrity, using flow cytometry and sperm morphology, using transmission electron microscopy. For each parameter, fresh (control) and thawed spermatozoa were compared. A significant decrease of both the percentage of motile spermatozoa (reduction: 75%) and sperm swimming speed (86%) were observed for thawed sperm compared with fresh sperm. The percentage of living spermatozoa, as assessed using flow cytometry, was significantly lower for thawed sperm ( $72.4 \pm 2.5\%$ ) compared with fresh sperm ( $86.4 \pm 1.1$ ). However, no significant difference of intracellular sperm ATP content was observed between fresh and thawed sperm. Post thawing, while some spermatozoa showed little or no morphological differences compared with fresh sperm, others had undergone drastic changes, including swelling of the plasma membrane, structural alterations of the chromatin and damage to mitochondria. In conclusion, the descriptive parameters studied in the present work showed that the quality of thawed great scallop sperm was lower than that of fresh cells but was still sufficient for use in aquaculture programs and sperm cryobanking for this species.

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## Highlights

► Post thawing, scallop sperm swimming characteristics were decreased. ► The percentage of living spermatozoa was significantly lower for thawed sperm. ► Various morphological changes were observed using electron microscopy. ► Although decreased, the quality of thawed scallop sperm was sufficient for cryobanking.

**Keywords :** Pecten maximus, Reproduction, Gamete, Sperm, Cryopreservation

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## 1. Introduction

Compared with fish species, sperm cryopreservation research has only been conducted on a limited number of molluscs. This can be explained by the frequent use of traditional rearing techniques with spat mainly collected from the wild rather than obtained from hatchery rearing. However, recent trends in mollusc farming, including breeder selection, creation of special lines and induction of triploids, have boosted the development of hatcheries. The research effort on sperm cryopreservation was first focused on Pacific oyster (*Crassostrea gigas*) because of its high commercial value. Since the first study on this species (Lannan, 1971), more than 50 papers have been published on cryopreservation of Pacific oyster sperm (for review see Hassan et al., 2015).

Most publications devoted to the cryopreservation of mollusc sperm have focused on the definition of technical protocols, including specific aspects such as the nature and the concentration of the cryoprotectant, the composition of the extender, and the definition of the cooling and thawing rates (Chao and Liao, 2001; Gwo, 2000). After thawing, sperm quality is generally described using only a small number of parameters, most often confined to the percentage of motile spermatozoa and their fertilization capacity (Kawamoto et al., 2007; Vitiello et al., 2011). However, a wide range of descriptive parameters is available, including the assessment of fine movement parameters using Computer Assisted Sperm Analysis (CASA) devices, cell integrity using ultrastructure or flow cytometry, sperm functioning with intracellular ATP content or fertilization capacity and DNA

damage using Tunel or SCSA (Sperm Chromatin Structure Assay), as suggested for fish sperm (Cabrita et al., 2010; Fauvel et al., 2010). The effects of sperm cryopreservation on sperm quality were investigated in Greenshell mussel (*Perna canaliculus*), using a panel of complementary parameters describing the different types of damage to sperm function: DNA damage, sperm viability, motility and fertility, showing that freezing and thawing had adverse effects on all these parameters (Smith et al., 2012).

Great scallop is a functional hermaphrodite, both mature ovaries and testes being present during the spawning period and spermatozoa and oocytes being released by a single animal (Devauchelle and Mingant, 1991). Investigations on scallop sperm biology remain limited: the mature spermatozoon has a bullet-shaped head, a short acrosome and can be considered as being of the primitive type (Dorange and Le Pennec, 1989). A sperm “maturation process” along the genital ducts has been suggested. Sperm can be collected after intragonadal serotonin injection or thermal shock. Sperm movement can be activated by dilution in seawater and movement duration is long, lasting 4 to 10 h (Faure et al., 1994). Recently, a sperm cryopreservation protocol was published (Suquet et al., 2014), in which the percentage of motile spermatozoa decreased from fresh ( $80 \pm 4\%$ ) to thawed sperm ( $11 \pm 2\%$ ). However, the effects of cryopreservation on thawed sperm quality were not investigated. The present work aims to describe the quality of great scallop sperm after thawing and to compare it with fresh sperm, using a panel of descriptive parameters including sperm motility, viability, intracellular ATP content and morphology.

## 2. Material and methods

### 2.1. Animals

Adult great scallops (mean weight  $\pm$  SEM:  $91.2 \pm 2.9$  g) were captured by divers in the bay of Brest (Finistère, France) during the natural spawning period (May to October; Paulet et al., 1988). The scallops were then transferred to the Argenton experimental hatchery (Ifremer) and maintained in a 600 L raceway (17°C, salinity 34.0) where they were fed daily on a mixture of microalgae (*Isochrysis*

*galbana* and *Chaetoceros calcitrans*) for one month. The sperm collection method and laboratory analysis are given briefly below as these were previously described in detail in Suquet et al. (2013 and 2014).

## 2.2 Sperm collection

The scallops were treated with 100  $\mu$ l of a 10 mM serotonin solution (dilution in seawater) and injected in the testes. They were then transferred to 2 L beakers, filled with 0.5 L seawater (19°C, filtered at 1  $\mu$ m). Sperm shedding was observed at 10 to 30 min post injection and samples were pipetted directly from the sperm plume, to ensure a high concentration. Sperm samples were then stored at 4°C until use.

## 2.3 Sperm cryopreservation

Sperm samples were diluted v/v with a solution containing the saline solution DCSB4, as an extender (Paniagua-Chavez et al., 1998) and polyethylene glycol (PEG: 20%), as a cryoprotectant. Straws (500  $\mu$ l) were cryopreserved on a tray floating 5.5 cm above liquid nitrogen (LN) for 10 min and then plunged into LN. The straws were then thawed in a water bath (25°C, 15s).

## 2.4. Sperm quality assessment

For each parameter, both fresh (control) and thawed spermatozoa were observed (n = 6 scallops). Sperm motility was assessed after 1 : 625 dilution in seawater (19°C, 5 gL<sup>-1</sup> BSA, Tris 20 mM, adjusted to pH 8.1). Sperm samples of 7  $\mu$ l were transferred to Fast-Read 102 cells (Biosigma®, Cona, Italy). Sperm motility was observed under a phase contrast microscope (Olympus BX51,  $\times$  10 magnification), immediately and 1 h post sperm activation. The percentage of motile spermatozoa was visually estimated by counting 6  $\times$  30 cells. Sperm movement was then recorded (Sony camera, 60

frames  $s^{-1}$ , 10 s film duration). Sperm Velocity of the Average Path (VAP) was determined using a CASA plug-in developed for Image J (Wilson-Leedy and Ingermann, 2007) and adapted to scallop sperm (number of frames: 240, minimum VAP for motile sperm:  $3 \mu m s^{-1}$ , minimum number of spermatozoa observed: 30).

Intracellular sperm ATP content was assessed, immediately post activation, by bioluminescence (ATP lite kit, Perkin Elmer, triplicates). Sperm concentration was determined by counting in a Thomas-type hemocytometer, to express ATP content as  $nmole \cdot 10^{-9}$  spermatozoa (dilution 100  $\mu l$  sperm : 500  $\mu l$  seawater).

Sperm viability was studied by flow cytometry (Guava EasyCyte Plus, Millipore), according to Le Goïc et al. (2013), using dual staining (Live/Dead® Sperm Viability kit, Molecular probes). Briefly, an aliquot of 200  $\mu l$  sperm (concentration:  $10^6$  spermatozoa  $ml^{-1}$ ) was stained with both SYBR-14, which penetrates cells with intact membranes, and Propidium iodide (PI), which stains cells with membrane damage. The results were expressed as the percentage of living spermatozoa. Furthermore, Side Scatter (SSC: relative sperm complexity) was also provided, to estimate sperm morphological changes.

Then, changes in sperm morphology following cryopreservation were assessed using transmission electron microscopy. Sperm samples of four scallops were fixed for 1 h in 3% glutaraldehyde, buffered to pH 7.2 with sodium cacodylate (0.1 M, adjusted to 1200 mOsmol  $L^{-1}$  by adding NaCl). Samples were then centrifuged (3000 rpm, 10 min) and post-fixed for 1 h in 1% osmium tetroxide. After dehydration through an ascending ethanol series, samples were embedded in Epon resin. Ultrathin sections were stained with 7% uranyl acetate lead citrate (Reynolds, 1963) and examined using a Jeol TEM JM 1400.

## 2.5. Statistical analysis

Results are presented as mean  $\pm$  SEM. Percentages of motile sperm were arcsin square root transformed. Means were then compared (Statistica 6 software) using Student t-tests or one-way

ANOVA. For ANOVA and when significant differences were observed ( $P < 0.05$ ), a Fisher a posteriori test was used to compare the means.

### 3. Results

A significant decrease in the percentage of motile sperm ( $P < 0.001$ ) and VAP ( $P < 0.001$ ) was observed for thawed sperm compared with fresh, both immediately and 1 h post sperm activation (Fig. 1a and b). For each treatment (fresh or thawed sperm), no significant differences were observed between values assessed immediately and 1 h post sperm activation, for either the percentage of motile sperm or VAP.

No significant difference of intracellular sperm ATP content was observed between fresh ( $203.1 \pm 42.0 \text{ nmole} \cdot 10^{-9} \text{ spermatozoa}$ ) and thawed sperm ( $148.1 \pm 18.2 \text{ nmole} \cdot 10^{-9} \text{ spermatozoa}$ ).

When assessed by flow cytometry, the percentages of living sperm were significantly higher ( $P < 0.001$ ) for fresh sperm compared with thawed (Fig. 2). Furthermore, significantly higher SSC values ( $P < 0.001$ ) were observed for fresh sperm ( $60.9 \pm 1.4$ ) compared with thawed cells ( $55.4 \pm 2.0$ ).

The great scallop sperm head has a bullet shape and a reduced middle piece (Fig. 3a). A conical acrosome is observed and the subacrosomal space is filled with a fine fibrillar material (Fig. 3b). The large nucleus contains dense granular chromatin, showing two depressions: an anterior one, in the subacrosomal space and a posterior one, located at the basal pole, where the proximal centriole is inserted (Fig. 3a). In the middle piece, four mitochondria can be identified (Fig. 3c). The distal centriole is located in the middle piece, near the base of the flagellum (Fig. 3d). The axoneme contains the typical '9+2' pattern of tubules (Fig. 3h). The condition of the thawed spermatozoa was found to be variable. For some spermatozoa, little or no morphological changes were noted, for the plasma membrane, nucleus, mitochondria (Fig. 3e) or acrosome (Fig. 3f). On the other hand, drastic changes could be distinguished in some spermatozoa: the plasma membrane could appear swollen, a wide space appearing between the membrane and the nuclear surface (Fig. 3g). The flagellar plasma membrane could also become swollen (Fig. 3h). Mitochondrial damage was observed: Fig. 3i and 3j show enlarge intracristal spaces and plasma membrane disruption. Structural alterations of the

chromatin also appeared, showing the presence of invaginations (Fig. 3i) and even sperm head degeneration (Fig. 3j).

#### 4. Discussion

The sharp decrease in thawed sperm survival observed in the great scallop agrees with the decrease in sperm quality biomarkers assessed in the present study: cryopreservation had a significant effect on sperm movement characteristics, sperm morphology and sperm viability, but not on sperm intracellular ATP content.

A high recovery of thawed sperm (assessed as the percentage of motile sperm relative to a fresh control) is commonly observed in molluscs (Fig. 4), ranging from 65% in Pacific oyster (Dong et al., 2005) to 23% in flat oyster (Horvath et al., 2012). Among the molluscs that have been studied, the limited survival of great scallop sperm after thawing (75% reduction in the percentage of motile sperm) may be explained by the fact that only one other study to date has been devoted to sperm cryopreservation in this species (Suquet et al., 2014), compared with more than 50 studies on oyster species (for review see Hassan et al., 2015). Furthermore, sperm survival after thawing may vary between species, even close ones, since 51% survival was reported in another pectinid species, the Chilean scallop, *Argopecten purpuratus* (Espinoza et al., 2010).

Complementary characteristics of sperm motility, such as movement velocity, have rarely been studied in molluscs and, when assessed, often rely on subjective estimates with an arbitrary scale (Dupré and Espinoza, 2004). The use of a CASA device enables objective estimation of sperm movement speed: a 27% reduction in thawed sperm VAP was reported in Greenshell mussels (Smith et al., 2012), which is limited compared with the decrease of 86% assessed for great scallop sperm in the present study.

The mean intracellular ATP content of fresh scallop sperm ( $203 \pm 14$  nmole  $10^{-9}$  sperm) was found to be close to that previously estimated in the same species ( $212 \pm 133$  nmole  $10^{-9}$  sperm; Suquet et al., 2013) and to values reported in Pacific oyster ( $186 \pm 16$  nmole  $10^{-9}$  sperm; Boulais et al., 2015). The marked reduction of thawed scallop sperm movement characteristics observed in the



present study cannot be explained by a decrease in sperm intracellular ATP content, because no significant difference in this parameter was found between fresh and frozen cells. The intracellular ATP content of frozen sperm had not been previously assessed in mollusc species. In marine fish, the decrease of ATP content in thawed seabream (*Sparus aurata*) sperm samples compared with fresh ones ranged from 50 to 95%, affecting sperm movement characteristics (Cabrita et al., 2005). In turbot (*Scophthalmus maximus*), a 40% decrease in sperm ATP content was measured post thawing (Dreanno et al., 1997). Sperm movement lasts a long time in scallop (8 to 10 h), compared to values recorded in marine fish species (a few minutes). This long movement duration suggests that scallop sperm could partially restore the energy catabolized during the swimming phase. It appears that the capacity for ATP regeneration is maintained in thawed scallop sperm because the percentage of motile cells was not significantly different between measurements taken immediately post thawing and those taken one hour later (Fig. 1a). The maintenance of ATP regeneration of thawed sperm is also sustained by the absence of damage to mitochondria in some thawed spermatozoa, as observed by electron microscopy.

Using flow cytometry, morphological damage to great scallop sperm was measured after thawing, showing a 16% decrease of sperm viability compared to fresh cells. Post thaw morphological changes in scallop sperm were confirmed by the significant decrease of SSC values, suggesting changes in cell complexity. Using electron microscopy, damage to the plasma membrane could be observed post thawing. In Chilean scallop, swollen, folded or broken plasma membranes were reported, after thawing (Espinoza et al., 2010). In blue mussel (*Mytilus galloprovincialis*) sperm plasma membrane integrity decreased from 90% in fresh sperm to 21\_25% in thawed cells (Liu et al., 2016), suggesting a similar quality to that found in our work. Modifications of the plasma membrane may affect sperm osmoregulation capacity and its fluidity. In human, sperm survival after thawing depends on the fluidity of the plasma membrane in the fresh spermatozoa (Giraud et al., 2000), suggesting that this membrane plays a key role during cryopreservation.

The fact that little or no damage to the acrosome and mitochondria were observed post thawing means that a high fertilization capacity of great scallop cryopreserved sperm is maintained. In a previous work, we demonstrated that the fertilization ability of thawed sperm was significantly lower

than that of fresh sperm but, fertilization rates similar to those recorded using fresh sperm were observed using a non-limiting sperm to oocyte ratio of 500 (Suquet et al., 2014).

In conclusion, the quality of thawed great scallop sperm was lessened compared with fresh cells, including a decrease of sperm movement characteristics and sperm viability and damage to sperm morphology. Further research is needed to improve sperm survival after thawing, considering the quantity of cryoprotectant used and the incorporation of different components such as proteins and sugars in the extender. However, a 20 to 30% percentage of motile spermatozoa is already sufficient for use in aquaculture programs and sperm cryobanking of the great scallop.

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## Figure captions

**Fig. 1.** Effect of cryopreservation on great scallop sperm motility characteristics, assessed immediately (0 h) and 1 h post sperm activation (n = 6 scallops, mean±SEM, different letters indicate significantly different results), a) percentage of motile spermatozoa, b) Velocity of the Average Path (VAP).

**Fig. 2.** Effect of cryopreservation on great scallop sperm integrity, as assessed by flow cytometry (n = 6 scallops, mean±SEM, different letters indicate significantly different results).

**Fig. 3.** Effect of cryopreservation on great scallop sperm morphology, as assessed by transmission electron microscopy (n = 4 scallops): 3a\_d: fresh sperm, 3e\_j: thawed sperm: a) longitudinal section of spermatozoon, b) longitudinal section of acrosome (arrow: fine fibrillar material), c) cross section of middle piece, d) longitudinal section of middle piece, e) longitudinal section of spermatozoon, f) longitudinal section of acrosome, g) longitudinal section of spermatozoon (arrow: swelling of plasma membrane), h) cross section of flagellum, i) longitudinal section of spermatozoon (arrow: swelling of plasma membrane, double arrow: invagination of nucleus), j) longitudinal section of spermatozoa (double arrow: chromatin disintegration). a: acrosome, dc: distal centriole, f: flagellum, mi: mitochondria, n: nucleus, pc: proximal centriole.

**Fig. 4.** Effect of sperm cryopreservation on the percentage of great scallop motile spermatozoa (percentage of fresh control measurement), in different mollusc species (best survival observed in Pacific oyster, *Crassostrea gigas*: Dong et al., 2005; blue mussel, *Mytilus galloprovincialis*: Di Matteo et al., 2009; Eastern oyster, *Crassostrea virginica*: Yang et al., 2012; Chilean scallop, *Argopecten purpuratus*: Espinoza et al., 2010; pearl oyster, *Pinctada fucata martensii*: Narita et al., 2008; scallop: present study; flat oyster, *Ostrea edulis*: Horvath et al., 2012).

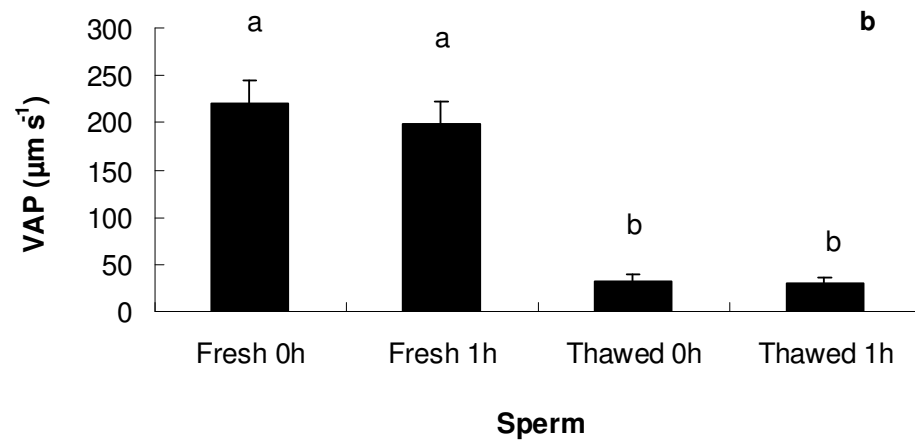
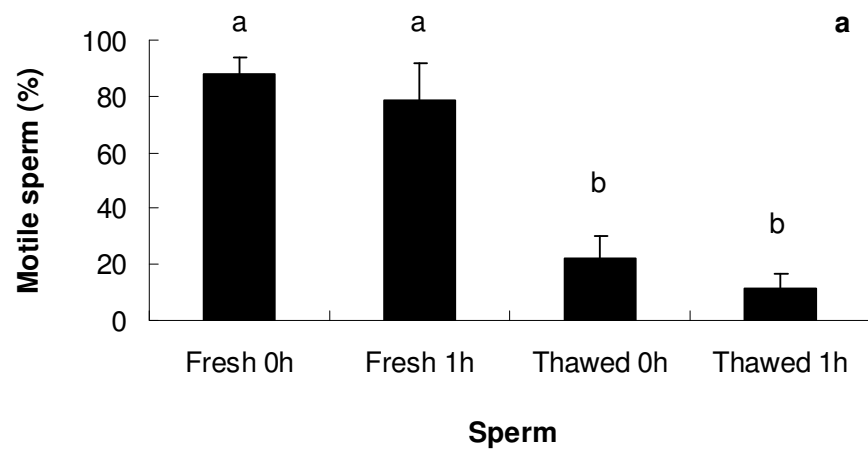


Fig. 1

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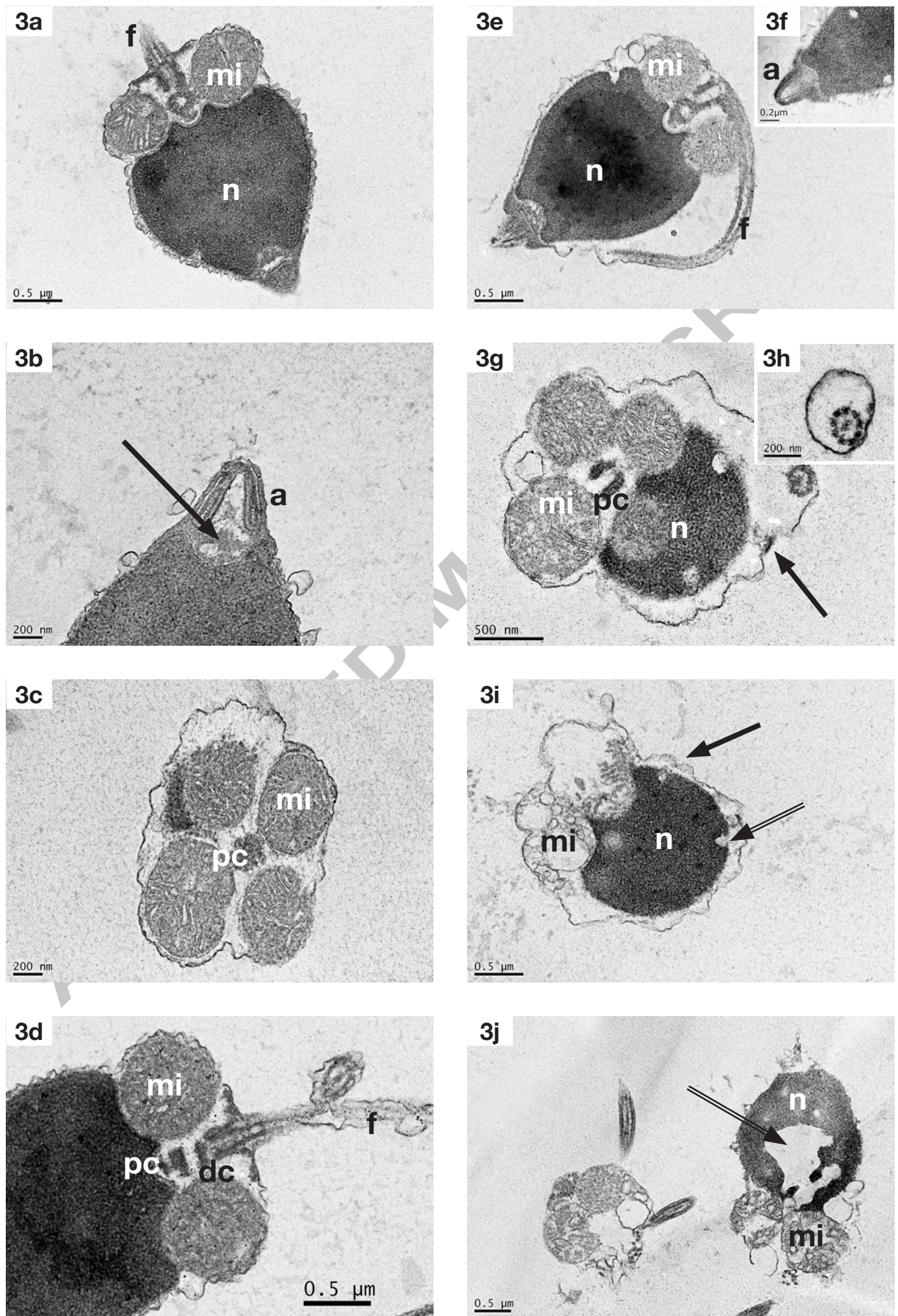
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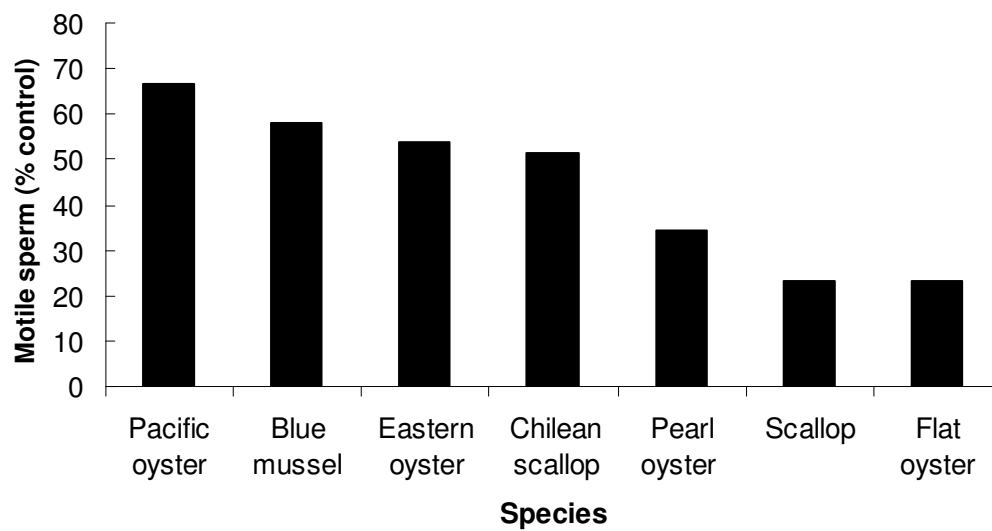
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