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Research Article

Biological characteristics of sperm in European flat oyster (*Ostrea edulis*)

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Abstract – In brooding mollusc species that incubate larvae before their release in seawater, the biology of gametes remains poorly explored. The present study describes some biological characteristics of sperm of the European flat oyster, a native species that has been over-exploited in the past and is nowadays classified as an endangered species in Europe. Flat oysters were collected by divers in the Rade de Brest (Finistère, France), during its natural reproduction period. Gonadal pH is acidic (6.31 ± 0.10). Spermatozoa are clustered in spermatozeugmata, an acellular structure in which the sperm heads are embedded. After their transfer in seawater, spermatozeugmata have a mean diameter of 64 ± 3 µm and they release free spermatozoa for a mean duration of 21 ± 3 min. Immediately after their release, the mean percentage of motile spermatozoa was 48.5 ± 12.6%. At 10 min after dilution in seawater, movement of spermatozoa was no more observed. Biological characteristics of European flat oyster sperm are compared to those observed in the Pacific oyster, regarding the unique reproductive behaviour of the former species and the role of spermatozoa transfer played by spermatozeugmata. The present results aim to improve the knowledge of reproduction and natural recruitment processes, support conservation and restoration measures and favour the establishment of management protocols of gametes and larvae in this endangered species.

Keywords: Oyster / *Ostrea edulis* / brooding species / sperm / spermatozeugmata / endangered species

1 Introduction

Molluscs can be classified into two groups regarding their reproductive strategy: the broadcasting species releasing their gametes in seawater where fertilization and larval development take place which is the case for the Pacific oyster (*Crassostrea gigas*) and, the brooding species incubating their larvae in the inhalant chamber before their release into seawater which occurs for half of all living oyster species (O'Foighil and Taylor, 2000). The brooding behaviour is frequent in molluscs, more than 100 clam species adopting a parental care of their larvae (Sellmer, 1967).

In brooding oyster species of the genus *Ostrea*, males produce spermatozeugmata (singular: spermatozeugma), a cluster composed of a central core where spermatozoa are attached by their heads, and sperm flagella radiate freely. After their transfer in seawater, spermatozeugma dissociation is observed, releasing free swimming spermatozoa. Oocytes released in the inhalant chamber are fertilized after sperm uptake by females. The knowledge of sperm biology recently expanded in broadcasting oysters such as the Pacific oyster, showing a long movement duration of spermatozoa fueled by a de novo ATP synthesis (Boulais et al., 2015a) and a fertilization capacity both driven by sperm energy stores and spermatozoon viability (Boulais et al., 2017). On the contrary, sperm biological characteristics of brooding oyster species remain little explored.

The presence of spermatozeugmata was confirmed in five flat oyster species including the Argentine one (*Ostrea puelchana*; Morriconi and Calvo, 1979) and the Chilean oyster (*Ostrea chilensis*; Jeffs, 1998). In the Olympia oyster (*Ostrea lurida*), the dissociation of spermatozeugmata was observed shortly after sperm transfer in seawater (Hori, 1933). In the Australian flat oyster (*Ostrea angasi*), the pattern of spermatozeugma development during gametogenesis and its morphology were reported (Hassan et al., 2016). The fine morphology of the European flat oyster (*Ostrea edulis*) spermatozeugma and the structural changes associated with its dissociation were described (O'Foighil, 1989).

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Bivalve sperm can be collected by gonad scarification, but also after serotonin injection as previously observed in six bivalve species (Gibbons and Castagna, 1984). Male clams (*Nutricola confusa* and *N. tantilla*) released spermatozeugmata when exposed to fluvoxamine, a serotonin reuptake inhibitor (Falese et al., 2011). In the Pacific oyster, gonad pH was acidic and the motility of spermatozoa was triggered by sudden alkalinization of sperm environment when released in seawater (Boulais et al., 2018).

Sperm production of a studied species may be described using different parameters including the number of spermatozoa collected, the characteristics of their movement and, in brooding species, the kinetic of spermatozeugma dissociation. Improving the description of sperm biological characteristics sustains the knowledge of the reproduction behaviour of the studied species and the comparison between different specific reproductive strategies. This knowledge is especially required for the European flat oyster, whose world production collapsed from 30 000t in 1961 to 3 000 in 2014 (FAO, 2017). Native populations of European flat oyster suffered a drastic decline because of overfishing, habitat destruction, environmental pollution and diseases, mainly caused by two protozoans *Marteilia refringens* and *Bonamia ostrea* (Gercken and Schmidt, 2014). The European flat oyster is now considered as a threatened species by OSPAR commission whose protection must be increased by several approaches including the study of its reproduction success (Haelters and Kerckhof, 2009).

The present paper describes some biological characteristics of sperm output in European flat oyster, including (i) the number of spermatozoa released in relation with sperm collection technique used, (ii) the kinetic of spermatozeugma dissociation, and (iii) the movement characteristics of spermatozoa after their release from spermatozeugmata.

2 Material and methods

2.1 General procedure

Flat oysters (mean weight ± SEM: 95 ± 4 g) were collected by divers within a wild population, in the Rade de Brest (Finistère, France; annual water temperature range: 6.8–20.6 °C; Pouvreau et al., 2017), during the natural reproductive period of this species (10th May to 30th September 2016 and 2017). Oysters were transferred to the Argenton experimental hatchery where they were maintained in a 600-L raceway (seawater: 17 °C, salinity 34 ‰) and continuously fed with a mixture of micro-algae (*Isochrysis galbana* and *Chaetoceros neogracile*: 10^9 cells of each species/day/animal; Gonzalez-Araya et al., 2013).

To determine their internal pH, the gonad and the digestive tract were incised and a microelectrode was directly inserted. Triplicate values of pH were recorded (IQ150, IQ Scientific instrument).

Sperm was sampled directly in the gonad using an automatic pipette fitted with a 200 µL tip. Then, sperm samples were dispersed in an activating solution (1 µm filtered seawater at 19 °C, Tris 20 mM adjusted to pH 8.1 adding HCl, BSA: 2 g/L): depending on individual sperm concentration, 20–50 µL were diluted into 500 µL activating solution. Sperm samples of 12 µL were transferred to Fast-Read 102 cells (Biosigma®, Cona, Italy). Spermatozeugma morphology and their dissociation were observed under a phase contrast microscope (Olympus BX51, × 10 to 40 magnification). The movement of spermatozoa was recorded (video camera Qicam fast, 60 frames/s, 4 s recording duration) and sperm movement characteristics (percentage of motile spermatozoa, VCL: curvilinear velocity, VAP: velocity of the average path) were assessed using a computer assisted sperm analyser (CASA) plug-in developed for the Image J software (Wilson-Leedy and Ingermann 2007) and calibrated to oyster sperm according to Boulais et al. (2015a). The size of spermatozeugmata was measured using Image J.

Sperm concentration was assessed on a Malassez cell, after a 1:2 to 1:10 dilution (sperm:seawater, one drop of formalin added to prevent the movement of spermatozoa).

2.2 Experimental procedure

The size of spermatozeugmata was measured 2 min after sperm transfer in activating solution ($n = 13$ oysters, 15–30 spermatozeugmata/oyster). The kinetic of spermatozeugma dissociation was described using four different parameters: firstly, the percentage of active spermatozeugmata: 2 min after transfer in activating solution, spermatozeugmata were considered as active when they release free spermatozoa [percentage of active spermatozeugmata = (number of active spermatozeugmata/total number of spermatozeugmata) × 100; $n = 10$ oysters], secondly, the duration of spermatozeugma activation: the dissociation of spermatozeugmata was observed up to the end of the process. Spermatozeugmata were considered as inactive when no spermatozoa were released from the cluster during a 1 min duration ($n = 8$ oysters, 3 spermatozeugmata/oyster), thirdly, the maximum rhythm of spermatozoa release: 2 min after the transfer of sperm in activating solution, the release of spermatozoa from spermatozeugmata was recorded during 1 min and the number of spermatozoa released was counted ($n = 15$ oysters, 3 spermatozeugmata/oyster; because the number of spermatozoa released from spermatozeugmata decreased at the end of spermatozeugma activation period, this result can be considered as the higher rhythm of spermatozoa release), and fourthly, the decrease of spermatozeugma diameter: changes in spermatozeugma diameter as a function of time was measured between 2 and 150 min after dilution in activating solution ($n = 6$ oysters, 15–30 spermatozeugmata/oyster).

When sperm was transferred in seawater, free spermatozoa were continuously delivered from spermatozeugmata, so preventing the assessment of changes of sperm movement characteristics in relation with time. Thus, sperm movement characteristics were assessed using two different techniques: first, assessment of immediate movement characteristics: the movement characteristics of spermatozoa were recorded immediately after their release from spermatozeugmata ($n = 19$ oysters, >30 spermatozoa/film), and second, changes of movement characteristics as a function of time after transfer in activating solution: in order to avoid a continuous delivery of fresh spermatozoa from spermatozeugmata, sperm suspension was filtered at 20 µm to discard spermatozeugmata, 1 min after sperm transfer in activating solution. Then, the movement of free spermatozoa was recorded each min, from 2 min after

sperm transfer in activating solution up to the end of their movement ($n = 11$ oysters, >30 spermatozoa/film).

Finally, the number of spermatozoa collected in different oysters was evaluated using two different release techniques: firstly, gonad stripping in seawater ($n = 8$ oysters) using a protocol previously developed in Pacific oyster (Boulais et al., 2015b), and secondly, chemical induction: oysters were treated with 150 µL of a 10 or 50 mM serotonin solution diluted in seawater. Serotonin was injected in the gonad and oysters were transferred in 2 L beakers filled with 0.6 L seawater or, for some oysters treated with 50 mM serotonin, filled with a 10^{-3} mM solution of fluvoxamine diluted in 0.6 L seawater (control: injection of 150 µL SW, $n = 8$). For both collection techniques and three hours after oyster treatment when spermaozeugmata were dissociated, spermatozoa concentration was evaluated ($n = 8$ oysters, except for fluvoxamine $n = 4$) and the total number of spermatozoa released was calculated for each oyster (spermatozoa concentration × seawater volume in each beaker).

2.3 Data analysis

Data were presented as mean ± SEM. Percentages were arcsin square-root transformed to normalize variances and homogeneity of variances was verified (Levene's test). Data were compared using one or two way analysis of variance. When significant differences were observed ($P < 0.05$), a Fisher a posteriori test was used. The decrease of the percentage of motile spermatozoa in relation with time was evaluated by linear regression.

3 Results

The pH values of gonad (6.31 ± 0.10) and digestive tract (6.13 ± 0.08) were acidic and not significantly different.

Spermatozeugmata were observed within sperm samples (Fig. 1A). Their shape is variable, from round to no geometrical form. Spermatozeugmata are composed of two parts: the core where sperm heads are deeply embedded and the periphery where flagella radiate freely (Fig. 1B). A gentle flagellar beating is often observed, sometimes leading to a slow and regular movement of spermatozeugmata. The size of spermatozeugmata was significantly different ($P < 0.001$; Fig. 2) between individuals. The mean value observed in $n = 13$ oysters was 64 ± 3 µm.

When non-diluted in activating solution, spermatozeugmata were inactive, i.e. free spermatozoa were not released. A few seconds after dilution in activating solution, the dissociation of spermatozeugmata was observed and spermatozoa swam off the cluster (Fig. 1C). The percentage of active spermatozeugmata showed a high variability ($P < 0.05$) between oysters (Fig. 3A). The duration of spermatozeugma activation depended on oysters ($P < 0.05$), ranging from 11 to 34 min (mean: 21 ± 3 min; Fig. 3B). Among the oysters observed, a significantly different number of free spermatozoa was released by spermatozeugmata in 1 min, ranging from 1 to 130 ($P < 0.01$; Fig. 3C). A significant decrease of spermatozeugma size in relation with time after dilution in activating solution was observed between 2 and 60 min ($P < 0.01$). Then, a plateau was observed up to 150 min (Fig. 3D). At the end of their dissociation, a broken membrane was observed at the periphery of spermatozeugmata. Free spermatozoa were present outside the cluster while residual spermatozoa are still observed inside spermatozeugmata (Fig. 1D).

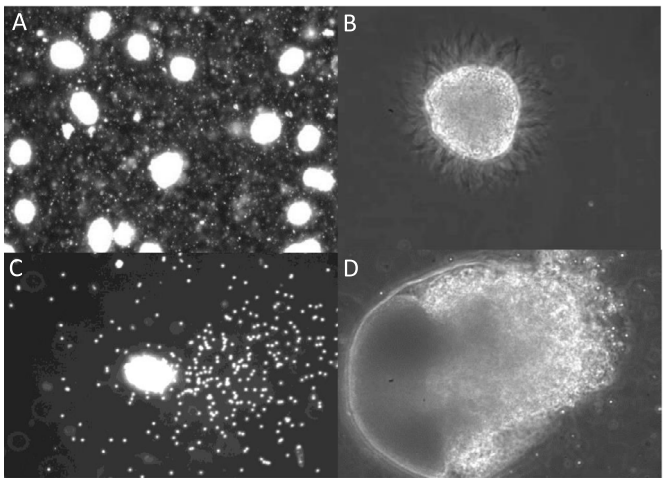

Fig. 1. Spermatozeugmata observed by phase contrast microscopy: (A) general view of several spermatozeugmata (magnification ×10), (B) view of a spermatozeugma showing the core and the flagella at the core periphery (magnification ×40), (C) active spermatozeugma releasing free spermatozoa, 2 min after transfer in seawater (magnification ×20) and (D) spermatozeugma at the end of the dissociation phase, one hour after transfer in seawater (magnification ×40).

Just after their release from spermatozeugmata, movement characteristics of free spermatzoa were the following ones: percentage of motile spermatozoa: 48.5 ± 12.6%, VCL: 131.1 ± 12.8 µm/s and VAP: 50.9 ± 5.2 µm/s. After spermatozeugma filtration, a significantly linear decrease of the percentage of motile spermatozoa in relation with time was assessed ($P < 0.01$, $R^2 = -0.97$, $y = -2.06x + 18.28$) and after 10 min, no movement was recorded (Fig. 4). The mean VCL and VAP were 68.5 ± 8.7 and 25.3 ± 3.1 µm/s, respectively. No significant decrease of both VCL and VAP was observed in relation with time after transfer in seawater (data not shown).

After gonad stripping, a mean of $2.27 \pm 0.98 \times 10^8$ spermatozoa was collected per oyster. The total number of spermatozoa individually shed after chemical treatments ranged from $2.63 \pm 1.40 \times 10^8$ to $6.37 \pm 1.88 \times 10^8$ spermatozoa, without any significant difference between treatments (injection of 10 or 50 mM serotonin, injection of serotonin and incubation in a solution of fluvoxamine or seawater injection as a control).

4 Discussion

Spermatozeugmata occur in many freshwater and marine fish, mollusc and annelidae species (Tab. 1). Spermatozeugmata were also reported in three species of Xenurobrycon (Characidae; Burns et al., 2008), in several species belonging to the tubicifids (Tubicifidae and Naididae; Ferraguti et al., 1989) and in many gastropods (Robertson, 2007).

In the scientific literature, different roles have been reported for spermatozeugmata: either maintain spermatozoa

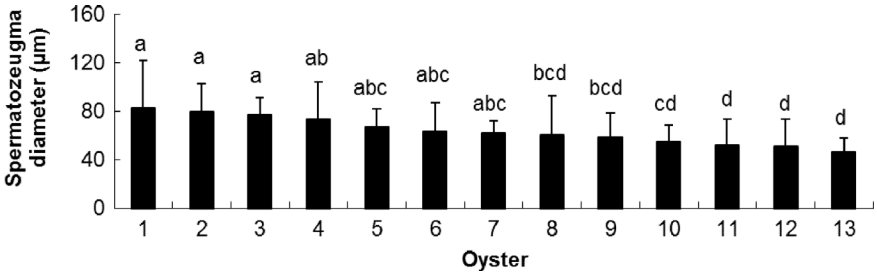

Fig. 2. Mean individual diameter of spermatozeugmata assessed 2 min after transfer in seawater (different letters refer to significantly different values).

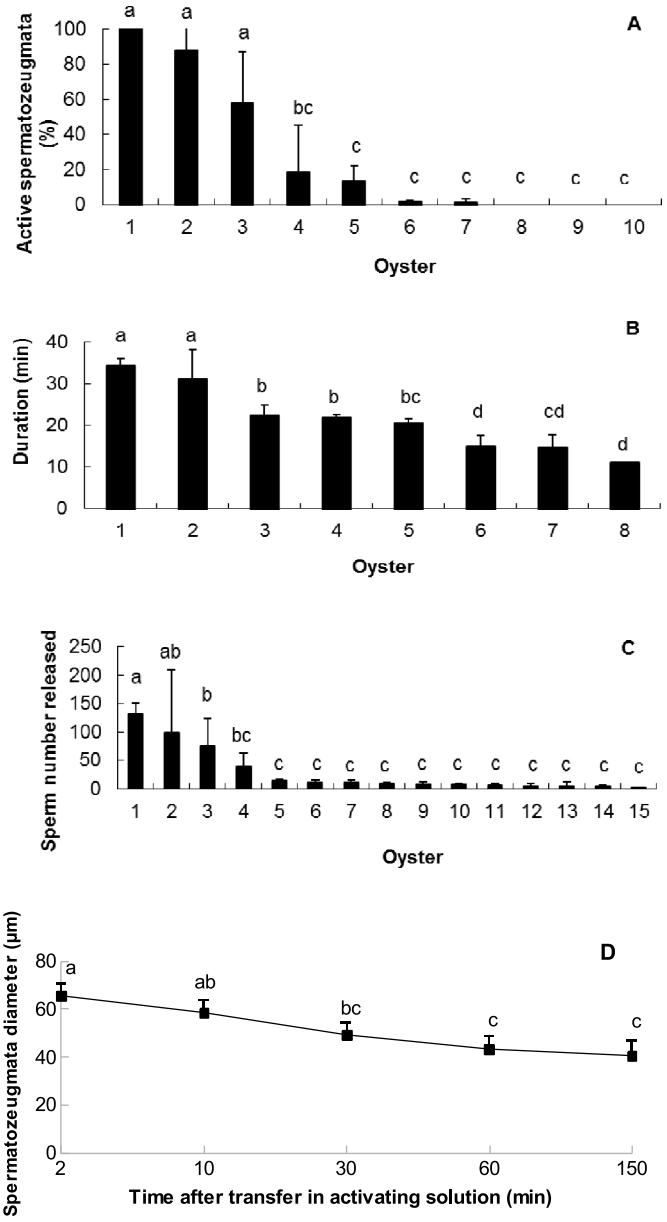

Fig. 3. Spermatozeugmata dissociation: (A) individual percentage of active spermatozeugmata, (B) individual duration of spermatozeugma activation, (C) sperm release during 1 min, and (D) mean changes in spermatozeugma size as a function of time after transfer in activating solution (different letters refer to significantly different values).

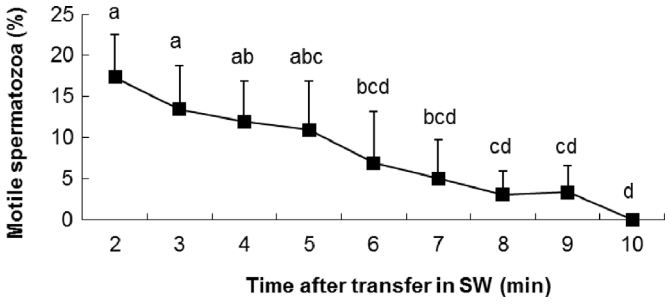

Fig. 4. Changes in the percentage of motile spermatozoa, assessed after spermatozeugma filtration, in relation with time after activation (different letters refer to significantly different values).

immotile so as to preserve intracellular energy stores and cell morphology (Serrao and Havenhand, 2009), or provide complementary energetic sources to spermatozoa such as lipids (Bucklands-Nicks and Chia, 1977), or favour spermatozoa transport up to females (Lynn, 1994), or protect spermatozoa against environmental changes (O'Foighil, 1989), or decrease self-fertilization (Coe, 1931) since spermatozoa still embedded in cluster cannot fertilize oyster eggs (O'Foighil, 1989), or protect spermatozoa against some possible immune reaction of the females (Parreira et al., 2009). In European flat oyster, these different roles played by spermatozeugmata must be studied. However, our observations confirm that this structure maintains spermatozoa immotile.

In the present study, the size of spermatozeugmata observed in the European flat oyster ranged from 47 to 74 µm diameter, close to that measured in the same species by O'Foighil (25–80 µm; 1989). These values are similar to those assessed in the Olympia flat oyster (40–60 µm; Coe, 1931) and smaller than those observed in the Australian flat oyster (117 µm; Hassan et al., 2016). Because of the decrease of spermatozeugma size in relation with time after dilution in seawater, as observed in the present study, these measurements must be carried out rapidly after sperm transfer in this environment.

When sperm samples of European flat oyster are transferred in seawater, the process of spematozeugma dissociation is immediately initiated and lasts a mean of 21 min. The factors controlling the dissociation of spermatozeugmata and the activation of free spermatozoa are still unknown in flat oyster species. In the lugworm, *Arenicola marina*, a two step mechanism was suggested, the first step

Table 1. Distribution of spermatozeugmata in the sperm of some aquatic species (alphabetically ranked by family).

Phylum	Family	Species	Environnement	Reference
Annelida	Arenicolidae	*Arenicola marina*	Marine	Bentley, 1985
Chordata	Auchenipteridae	*Trachelyopterus galeatus*	Freshwater	Melo et al., 2011
Annelida	Golfingiidae	*Thysanocardia nigra*	Marine	Maiorova and Adianov, 2005
Mollusca	Keliidae	*Pseudopythina tsurumaru*	Marine	Lutzen et al., 2004
Mollusca	Lasaeidae	*Arthritica bifurca*	Marine	Jespersen and Lutzen, 2009
Chordata	Odontaspididae	*Carcharias taurus*	Marine	Lucifora et al., 2002
Mollusca	Ostreidae	*Ostrea angasi*	Marine	Hassan et al., 2016
Mollusca	Ostreidae	*Ostrea chilensis*	Marine	Jeffs, 1998
Mollusca	Ostreidae	*Ostrea edulis*	Marine	O'Foighil, 1989
Mollusca	Ostreidae	*Ostrea lurida*	Marine	Coe, 1931
Mollusca	Ostreidae	*Ostrea puelchana*	Marine	Morriconi and Calvo, 1979
Chordata	Poecilidae	*Poecilia reticulata*	Freshwater	Kinnberg and Toft, 2003
Chordata	Potamotrygonidae	*Potamotrygon magdalenae*	Freshwater	Pedreros-Sierra and Ramirez-Pinilla, 2015
Chordata	Somnosidae	*Centroscymnus coelolepis*	Marine	Girard et al., 2000
Chordata	Triakidae	*Galeorhinus galeus*	Marine	Mc Clusky, 2015
Mollusca	Unionidae	*Anodonta grandis*	Freshwater	Lynn, 1994
Mollusca	Unionidae	*Lampsilis cardium*	Freshwater	Ferguson et al., 2013
Mollusca	Unionidae	*Lampsilis straminea*	Freshwater	Mosley et al., 2014
Mollusca	Unionidae	*Truncila truncata*	Freshwater	Waller and Lasee, 1997
Mollusca	Veneridae	*Nutricola confusa*	Marine	Falese et al., 2011
Mollusca	Veneridae	*Nutricola tantilla*	Marine	Falese et al., 2011

involving the 8, 11, 14-eicosatrienoic acid and inducing the dissociation of spermatozeugmata and the second step triggering the movement of free spermatozoa by a sudden change in extracellular pH during gamete release in seawater (Pacey et al., 1994). The acidic gonadal pH observed in the present study (6.31) suggests an inhibitory role of this factor in European flat oyster, but the whole mechanism controlling spermatozeugma dissociation and spermatozoa activation remains to be described.

In European flat oyster, as in many species having spermatozeugmata, spermatozoa are transported by a process composed of two successive phases: firstly, a passive phase during which spermatozoa are embedded in spermatozeugmata and sperm transportation is mainly due to water currents, and secondly, an active phase beginning when spermatozoa are released from the clusters while their movement is triggered. In contrast, broadcasting oysters as the Pacific oyster only present the active phase of spermatozoa movement.

During the passive transport phase and for water flow close to 3 cm/s, spermatozeugmata of a freshwater mussel (*Lampsilis straminae*) can be transported up to 8 km in 72 h (Mosley et al., 2014). In another freshwater mussel (*Lampsilis cardium*), the transport of spermatozeugmata on distances as high as 16 km was suggested (Ferguson et al., 2013). The time required for the total dissociation of Australian flat oyster spermatozeugmata ranged from 3.5 to 19.7 h, the fastest dissociation being recorded in female hermaphrodites and the longest one in males (Hassan et al., 2016). In the European flat oyster, sperrmatozeugmata may retain their structural integrity for up to 24 h, but in a limited number of cases only, including spontaneous spawns (O'Foighil, 1989). In the present study, the dissociation of spermatozeugmata was initiated immediately after dilution in seawater. Furthermore, a rapid dissociation of spermatozeugmata was observed since their size was not modified 60 min after dilution in seawater. The mean water current speed measured in the Rade de Brest, where oysters were collected, being close to 7 cm/s (Petton et al., 2016), resulting in a maximum distance covered by spermatozeugmata of 90 m before their total dissociation. However, this passive transport capacity must be further studied using different values of the current speed assessed on various European flat oyster beds.

During the active phase of spermatozoa transportation, the low percentage of free motile spermatozoa assessed in the present study 2 min after their release from spermatozeugmata (17%) was confirmed by previous results (13%; Horvath et al., 2012). This percentage is lower than values observed in the Pacific oyster (70%; Boulais et al., 2015a). The movement duration of free spermatozoa of European flat oyster is short (maximum 10 min) compared to Pacific oyster (24 h; Boulais et al., 2015a). The movement characteristics of free spermatozoa recorded in flat and Pacific oysters result in highly different total distances covered by spermatozoa during their swimming phase: close to 1 cm for the first species and 1 m for the second one (Suquet et al., 2012).

Adding the two transport phases, the total distance covered by spermatozoa of European flat oyster reaches 90 m in our sampling conditions. This distance is, for almost 100% of this value, due to the passive phase of gamete transportation, the active phase providing only 0.001% of the total distance covered. Because of their limited transport capacity, free spermatozoa must be released close to the females during the passive transport phase, to successfully fertilize oocytes stored in the inhalant chamber. In Argentine flat oyster, three reproductive strategies were described: (i) carriage: small epibiotic males being sheltered by a large female on the internal part of its valve, (ii) oyster cluster association: several small size males being attached to a female, and (iii) isolated

oysters being mostly females (Morriconi and Calvo, 1989). The first two strategies reduce the distance to be covered by spermatozoa between males and females so enhancing fertilization success and for the third strategy, oocytes may be fertilized by spermatozoa transported by water currents. In addition, the role of female oysters in sperm incorporation must be considered in the process of fertilization, including their filtration capacity which can be modified by several elements such as prey size (Nielsen et al., 2017), water temperature (Haurea et al., 1998) or salinity and prey density (Hutchinson and Hawkins, 1992). Although limited elements have been previously published in the Argentine flat oyster (Pascual et al., 1989), the hypothesis of possible interactions between females and males, modifying the movement characteristics of free spermatozoa through an internal fluid produced by the female, must be considered in the European flat oyster. Then, the long distance covered by spermatozoa when embedded in clusters, may favour the gene flow among flat oysters. In the wild, paternity analyses of larvae produced by 13 brooding females showed that the number of individuals contributing as males to the progeny was very variable, ranging from 2 to more than 40 oysters (Lallias et al., 2010).

Whatever the collection technique used, stripping or chemical treatment, the total number of free spermatozoa released from the gonads of European flat oyster ranged from 2 to 6×10^8 spermatozoa/oyster. No value was previously published in the different flat oyster species but the present number is 10 times lower than the total number of spermatozoa collected in the Pacific oyster (Suquet et al., 2016).

In conclusion, some biological characteristics of sperm of European flat oyster were described in the present study. Compared to Pacific oyster, the present results highlight the low sperm production observed in this species, assessed in terms of total number of spermatozoa produced and limited movement characteristics of free spermatozoa. However, the passive transport of spermatozeugmata on long distances may compensate the limited movement capacities of free spermatozoa. These preliminary biological characteristics must be completed by further studies including the metabolism of internal energy stores, the factors controlling spermatozeugmata dissociation and spermatozoa activation, and the control of sperm release. This knowledge would sustain the improvement of sperm cryopreservation and spermatozoa cryobanking to protect genetic resources. Furthermore, the understanding of reproductive behaviour of the European flat oyster would provide basic information to improve the management of oyster beds into the wild. All these elements would help further conservation measures of European flat oyster.

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