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Setting of a procedure for experimental fertilisation of Pacific oyster (*Crassostrea gigas*) oocytes

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ABSTRACT

The quality of oyster spermatozoa has become an issue for modern aquaculture. However, reliable protocols used to assess gamete quality are lacking. The aim of this work is to define a standardised experimental fertilisation protocol for Pacific oyster oocytes.
Six experiments have been carried out in this study. The optimal conditions for Pacific oyster experimental fertilisation have been defined: (1) oocytes can be conserved in seawater for at least 4 h before fertilisation, (2) oocyte concentration: 100–1000 ml^{-1} seawater, (3) fertilisation volume: 10–100 ml, (4) spermatozoa: oocyte ratio: 400 to maintain discriminating conditions, (5) gamete contact time: longer than 10 min and (6) fertilisation and incubation temperatures: both 19 °C. The effect of individual male significantly influenced the fertilisation success. The results of this study will be exploited for spermatozoal quality analysis in combination with other techniques.

1. Introduction

It is widely admitted that reproductive success of *Crassostrea gigas* is highly variable and that this variability is partially due to inconsistent gamete quality (Boudry et al., 2002). Gamete quality can be characterized by fertilisation rates and by embryo development yields. It results from multiple cellular and subcellular parameters of the gametes. The quality of gametes has been extensively studied in many freshwater and seawater fishes (Billard et al., 1995) but it is little known about molluscs and especially bivalves. Shellfish aquaculture requires a strict control of the reproduction process including a better assessment of gamete quality. In other domains, like ecotoxicology, the gametes have been used as a bio-indicator of modification and perturbation of environment (Kime and Nash, 1999). For these purposes, an objective and reproducible experimental fertilisation procedure is indispensable.

From a series of laboratory experiments, it appears that fertilisation success in several sessile and sedentary mollusc broadcast spawners depends on gamete concentration, gamete age, contact time between gametes, distance between spawning individuals and hydrodynamic conditions (Levitan, 1991; Hodgson et al., 2007; Adams et al., 2008). The experimental techniques can greatly influence the fertilisation success. An experimental incubation for Pacific oyster embryos has been established in previous work (Suquet et al., 2007). The aim of this work is to establish an experimental fertilisation protocol for Pacific oyster oocytes.

2. Materials and methods

2.1. Oyster conditioning and gamete stripping

Pacific oysters were collected in Charentes (France) and transferred to the experimental hatchery of Ifremer (Argenton, France). They were fed two microalgae (*Isochrysis galbana* clone Tahitian (T-Iso) and *Chaetoceros gracilis*) at a ratio of dry mass of algae to oyster of 2:100 and maintained in 200 L tanks with running seawater at 19 °C in order to obtain gonad maturation (Chavez-Villalba et al., 2002).

Gametes were collected from the oyster by the dry stripping method (Allen and Bushek, 1992). Briefly, gonads were dissected out and placed in a small glass bowl with 5 ml salt stock solution "Store Gigas" (Brizard et al., unpublished) for sperms or with 5 ml filtered at 1 µm seawater (FSW) for oocytes. The gonads were cut up into small chunks and the gonad material was agitated in the solution to release the gametes. The sperm suspension was filtered at 20 µm to remove the large chunks of gonad material. The oocyte suspension was successively filtered at 110 µm and 60 µm to remove the large and small chunks of gonads. After dilution to 1/1000 or 1/10000 in seawater, sperm concentration was determined by Coulter Counter in all the experiments. The concentration of oocytes was determined by microscopic count (3*50 µl). After oyster stripping, female gametes were conserved in a 2 L container with FSW and at 19 °C, while male gametes were stored in "Store Gigas" solution at 4 °C prior to fertilisation. Fertilisation occurred in a 1.8 L beaker.

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As all experimental conditions of fertilisation could not be tested at once, six separate experiments have been conducted. Thus, the results of previous experiments could be considered in optimising the following one. All the experiments were carried out with triplicate trials. When not stated, fertilisation standard procedure was as follows: after 30 min of contact between spermatozoa and oocytes, beakers were filled up to 1.8 L with FSW at 19 °C for incubation. The D-larval yield (48 h) was used to estimate the fertilisation success (number of D-larvae.total number of embryos^{-1}). Non viable larvae (translucent or non D shaped) were not included.

2.2. Experimental design

2.2.1. Effects of oocyte conservation duration in FSW

After 1, 2, 3, and 4 h of maintenance in FSW prior to fertilisation, 50,000 oocytes from three females were transferred into a beaker to obtain a spermatozoa:oocyte ratio of 150:1 in an adjusted volume of 50 ml. Spermatozoa were pooled from three males.

2.2.2. Effect of oocyte concentration

Five oocyte concentrations were tested (100 ml^{-1}, 500 ml^{-1}, 1000 ml^{-1}, 5000 ml^{-1} and 10,000 ml^{-1}) using three individual females. The fertilisation was conducted in 40 ml FSW at a sperm: oocyte ratio of 200:1. Spermatozoa were pooled from three males.

2.2.3. Effect of fertilisation volume

Three fertilisation volumes (10 ml, 50 ml and 100 ml) were tested. Oocytes collected from three females were fertilised using a sperm pool from three males. Oocyte concentration was adjusted at 500 ml^{-1} and spermatozoa:oocyte ratio was fixed at 200:1.

2.2.4. Effect of spermatozoa:oocyte ratio

Four sperm:oocyte ratios were tested 10:1, 100:1, 500:1, and 2000:1. Oocytes were pooled from three oysters and the concentration was adjusted to 500 ml^{-1} for fertilisation. Three males were individually tested in a fertilisation volume of 50 ml.

2.2.5. Effect of gamete contact time

Six contact times between spermatozoa and oocytes were tested: 10 s, 10, 20, 30, 60 and 90 min. The oocytes from three females were pooled and the concentration of oocytes used for fertilisation was 500 ml^{-1}. Three males were individually tested. The fertilisation was performed in 50 ml FSW at a sperm:oocyte ratio of 400. At the indicated contact time, the samples were respectively filtered with a 20 µm mesh in order to remove spermatozoa.

2.2.6. Effect of temperature

Four combinations of temperature were tested for fertilisation and incubation respectively: (1) 19 °C–19 °C, (2) 15 °C–19 °C, (3) 19 °C–15 °C and (4) 15 °C–15 °C. The fertilisation occurred in 50 ml FSW; spermatozoa:oocyte ratio was fixed at 400; the oocyte concentration was adjusted to 500 ml^{-1}; oocytes were pooled from three females and spermatozoa were collected from three males. After 20 min fertilisation, the beaker was fully filled.

2.3. Data analysis

The percentage data were arcsin square-root transformed to achieve homogeneity of variance prior to two way analysis of variance (ANOVA). When the D-larval yield was significantly different, Tukey's multiple rank comparison was used.

3. Results and discussion

3.1. Effects of oocyte conservation duration in FSW

During the first 4 h, the maintenance of oocytes in seawater had no significant effect on D-larval yield (Fig. 1A). As a consequence, the fertilisation experiment may last a minimum of 4 h. In other molluscs, fertilisation success for oocytes from *Patella ulyssiponensis* and *Patella vulgata* remains higher than 40% for up to 12 h old and 6 h old in seawater, respectively (Hodgson et al., 2007).This reflects an adaptive response to sedentary life and to external fertilisation of these species. The ecological significance of gamete longevity of free spawning invertebrates has been debated by a number of authors. Some have argued that gamete longevity is not important because hydrodynamic processes will rapidly dilute gametes (Levitan, 1991; André and Lindegarth, 1995). Others have suggested that increased gamete longevity could enhance fertilisation success when sperm availability varies (William and Bentley, 2002; Yund and Meidel, 2003).

3.2. Effect of oocyte concentration

When the oocyte concentration increased from 1000 ml^{-1} to 5000 ml^{-1}, there was a significant decrease of D-larval yield ($P<0.05$). A second decrease was observed between 5000 ml^{-1} to 10,000 ml^{-1} (Fig. 1B). It has been suggested that the deleterious effect of a high oocyte density in *Crassostrea rhizophorae* is caused by a water-soluble substance, originating from the zygote rather than oxygen depletion nor the physical crowding of eggs (Rampersad et al., 1994).

3.3. Effect of fertilisation volume

Between 10 and 100 ml, fertilisation volume had no significant effect on D-larval yield (Fig. 2A). However, a significant effect of females was observed ($P<0.05$).

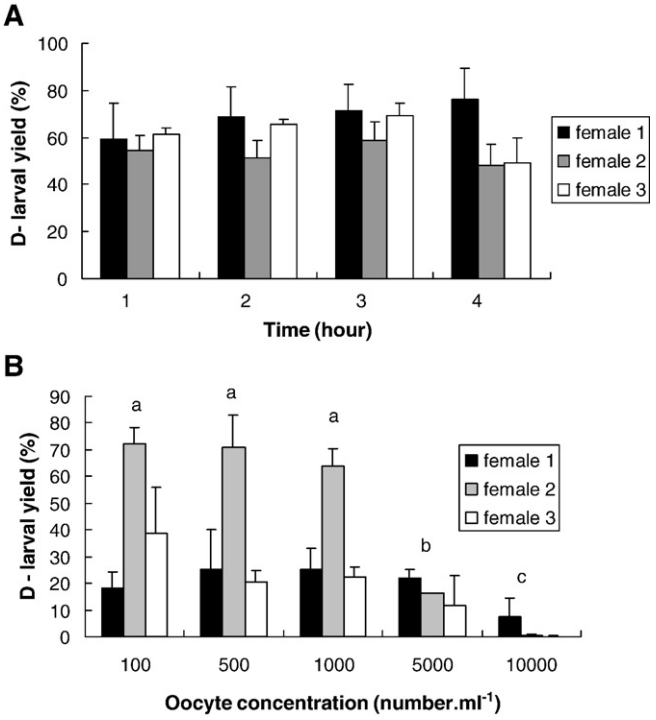

Fig. 1. A: Effect of oocyte conservation duration in FSW on D-larval yield, B: Effect of oocyte concentration on D-larval yield (mean±SD, $n=3$; different letters refer to significantly different results).