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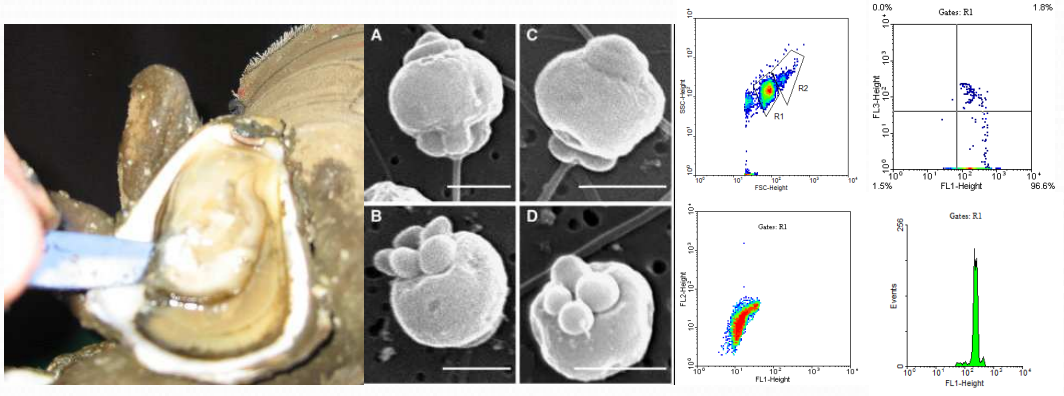
Development of experimental methods for the determination of sperm quality in Pacific oyster (*Crassostrea gigas*)

Mise au point d'outils de détermination de la qualité des spermatozoïdes chez l'huître creuse (*Crassostrea gigas*)



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This work is for my fellow citizens and countrymen who were dead in the earthquake of 12 may 2008, for those who have lost their children and for those who have lost their parents. I pray for the dead to live happily in the Heaven; I wish for the survivors to be courageous to battle against the disaster and to be stronger to face their future life.

Table of contents

Introduction…….....5

1. ***Crassostrea gigas*……..……5**
   1.1 History and distribution
   1.2 General description of reproduction processes

2. **Objectives…….....5**

Material and methods ……..……..7

1. **General procedures…….7**
   1.1 Collection and maintenance of animals
   1.2 Gamete preparation

2.Optimisation of fertilisation methods ……...……...8
2.1 Effect of oocyte aging
2.2 Effect of oocyte concentration
2.3 Effect of sea water volume
2.4 Effect of spermatozoa : oocyte ratio
2.5 Effect of gamete contact time
2.6 Measurement of fertilisation success

3. **Flow cytometry analysis of spermatozoa……...9**
3.1 Instrument
3.2 Chemicals
3.3 Sample preparation for mitochondrial membrane potential (MMP) analysis
3.4 Validation of the assay using MMP inhibitor (CCCP)
3.5 Stability of JC-1 staining
3.6 Effect of Store Gigas solution on MMP
3.7 Application to sperm quality assessment

4. **Data analysis……..…….10**

Results and discussion……..……11

1. Fertilisation experiment……...……...…11
1.1 Effect of oocyte ageing
1.2 Effect of oocyte concentration
1.3 Effect of seawater volume
1.4 Effect of spermatozoa : oocyte ratio
1.5 Effect of the gamete contact time

2. Flow cytometry analysis of spermatozoa……14
2.1 Effect of CCCP on JC-1 staining
2.2 The kinetics of probe accumulation at RT and on ice
2.3 Comparison of MMP of spermatozoa prepared in Store Gigas solution and FSW

2.4 Conservation of sperm mitochondrial function in the fridge
2.5 The relationship between fertilization success and MMP

Conclusion and perspective…….18

References……..……...19

Acknowledgments……..…22

Lists of figures

1. Effect of oocyte aging on D-larval yield…….……..11
2. Effect of oocyte concentration on D-larval yield……..12
3. Effect of fertilisation volume on D-larval yield……12
4. Effect of sperm : oocyte ratio on D-larval yield……...13
5. Effect of incubation time on D-larval yield……..13
6. Representative example of flow cytometric analysis of spermatozoa……..14
7. Representative cytofluorometric analysis of MMP in isolated spermatozoa……15
8. FL2 : FL1 ratio after JC-1 staining for isolated spermatozoa maintained at room temperature and on ice with and without CCCP……...16
9. The comparison of F2 : FL1 ratio for isolated spermatozoa diluted in FSW and in Store Gigas solution treated with and without CCCP……...16
10. FL2 : FL1 ratio in isolated spermatozoa according to conservation duration……...17
11. The comparison of fertilization success and FL2 : FL1 ratio (0 hour) for three sperms……...18

Introduction

1. The oyster, *Crassostrea gigas*

1.1 History and distribution

The evidence of oyster consumption goes back into prehistory, as evidenced by dead oyster shells found worldwide. Oysters were an important food source in all coastal areas where they could be found, and oyster fisheries were an important industry where they were plentiful. Oysters are a favourite among exotic foods and research now shows this shellfish to be a rich source of zinc and iron. The Pacific oyster, *Crassosotrea gigas*, is the native oyster of the Pacific coast of Korea, Japan and China. It has been introduced to the west coast of North America and to the south of Australia. It was introduced to France between 1966 and 1974 (Laing et al., 2006).

1.2 General description of reproduction processes

Oyster consume algae and other water-born nutrients, by filtering up to five litres of water per hour. Oyster usually mature by one year of age and they mature within 3 months in some conditions. They are protandric, during their first year they spawn as males. Over the next two or three years, they release eggs. Bay oysters are usually prepared to spawn by the end of June in France. An increase in water temperature prompts a few initial oysters to spawn. This triggers a spawning "chain reaction", which clouds the water with millions of eggs and sperm. A single female oyster can produce up to 100 million eggs annually. Spawned oocytes and many ovarian oocytes undergo spontaneous germinal vesicle breakdown (GVBD) and remain blocked in metaphase 1 until they are fertilized (Osanai, 1985). Oocytes can be fertilized whether or not GVBD has occurred prior to insemination. After fertilization, eggs develop into larvae, which eventually settle on a hard substrate becoming then a so-called "spat". Oyster spat are 25 mm or less in length. Settlement of many species of bivalve, including oysters, seems to be stimulated by the proximity of adults of their species.

2. Objectives

It is widely admitted that reproductive success of *C. gigas* is highly variable and that this great variability is partially due to inconsistence gamete quality (Boudry et al., 2002). The quality of the spermatozoa can be characterized by their fertilization capacity and second by embryo development yield. Sperm quality results from multiple cellular and subcellular parameters of the gametes. The quality of spermatozoa has been extensively studied in many freshwater and sea water fishes (Billard et al., 1995) but it is little known about molluscs and especially bivalves. However, because shellfish aquaculture is currently evolving to the strict control of larval and spat production, there is need for a better assessment of sperm quality in commercial hatcheries. To achieve this, objective and reproducible experimental methods to study oyster sperm quality are needed.

Working on the quality of gametes of oyster would thus permit:

1) Accompanying new developments of spat production.

For example, the production of triploid oysters (breeding diploid oocytes and tetraploid spermatozoa), represents about 15% of *C. gigas* produced in French hatcheries. Such genetically controlled production needs a reliable control of gamete quality and a better management of it, because of the high price of tetraploid males (1000-1500 euros per one oyster).

2) Improving gamete management.

Aquaculture production in controlled conditions requires gamete management methods such as short term conservation, cryoconservation, artificial fertilization and incubation. The recent works on oyster sperm cryopreservation underlined the importance of the gamete initial quality for sperm survival after thawing (Dong et al., 2005)

3) Providing methods for other research domains (ecotoxicology).

The gamete has been used as a bio-indicator of modification and perturbation of environment (Kime et Nash, 1999).

From a series of laboratory experiments, it appears that fertilisation success in several sessile and sedentary broadcast spawners depends on gamete concentration, gamete age, contact time between gametes, distance between spawning individuals and hydrodynamic conditions (Levitan et al., 1991; Alan et al., 2006; Hodgson et al., 2007). It needs a protocol which should make a minimal variation of fertilization success among replicates for small gamete lots.

Fertilisation ability is the ultimate indicator of the functional status of sperm, but fertilisation assays are often time-consuming, costly and impractical (Jenkins, 2000). In addition, female factors such as egg quality may influence the results between assays. Sperm motility evaluation has been a standard method of assessing sperm quality for over 50 years (Ammann, 1988). However the estimation of sperm motility by visual, subjective assessment is burdened with a large component of variability (Varner et al.,1991). Because of the inherent variability in motility assessments, Jaskso et al. (1991) suggested that stallion sperm motility evaluation alone was not an adequate estimation of fertility. Hence, alternative methods of objectively assessment sperm function are useful.

The **flow cytometer** (*cyto*: cell, *meter*: measure) is a unique instrument used in both health care and research laboratories. *Cytometry* refers to the measurement of the physical and chemical characteristics of cells. *Flow cytometry* is the process in which measurements are made while cells in a liquid suspension are forced to flow one at a time through a measuring device. It has been used to assay cellular constituents in fundamental biology, immunology, pharmacology, toxicology and clinical research. The adaptation of flow cytometry to sperm structural and functional assessments began with measurement of sperm DNA content (Evinson et al., 1980). Its application to semen analysis has gradually increased over the last 10-15 years. It is now applied to semen evaluation of traits such as cell viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity and DNA status.

Flow cytometry has the capacity to detect labelling by multiple fluorochromes associated with individual spermatozoa, meaning that more than one semen attributes can be assessed simultaneously. Flow cytometry has a high level of experimental repeatability and has the advantage of being able to analyse large number of spermatozoa and work with small

sample sizes. This feature has an added benefit for semen analysis, as few single sperm parameters show significant correlation with fertility in vivo for semen within the acceptable range of normality (Larsson et al., 2000) and the more sperm parameters that can be tested, the more accurate the fertility prediction becomes (Amman et al., 1993). This makes the flow cytometer a very sensitive method for the detection of subtle differences among spermatozoa that may not be apparent using other techniques.

One of the most frequently assay applied to spermatozoa is the measurement of cell membrane integrity as an estimation of cell viability. Dual-staining techniques using SYBR 14 or SYBRgreen I coupled to propidium iodide (PI) allow to distinguish membrane damaged cells from membrane intact cells (Garner et al., 1995; Paniagua et al., 1998; Suzuki et al., 2003; Paniagua et al., 2006). SYBR 14 or SYBR Green I are cell permeant dyes which stain the double-stranded DNA of viable and dead cells emitting a green fluorescence. PI, cell impermeant dye, can only penetrate the cells with damaged membrane and emits red fluorescence. Moribund spermatozoa are stained green-orange. Although these assays are good indicators of cell integrity, they are little informative on functional capacity of spermatozoa.

Energy for spermatozoa motility is provided by the mitochondria, which are localized on the midpiece. Hence, any changes in the mitochondrial function may be reflected in sperm motility (Ericsson et al., 1993). Recently Rhodamine 123 (Rh 123) has been used to assessed the sperm mitochondrial membrane potential (MMP) or $\Delta\psi_m$ (Adams et al., 2003; Paniagua et al., 1998, 2006). Although Rh 123 can distinguish living cells based on mitochondrial membrane potential, it can not distinguish between mitochondria with high or low membrane potential because of several energy-independent Rh123-binding sites (Lopez-Mediavilla et al., 1989). The lipophilic, cationic compound, JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl carbocyanin iodide) is a ratiometric probe, which differentially label mitochondria with high and low membrane potential (Cossarizza et al., 1993). Under high membrane potential, JC-1 forms multimers known as J-aggregate after accumulation in mitochondria (emitting an orange fluorescence at 590 nm) while it forms monomers (M-band) in mitochondria with low membrane potential (emitting a green fluorescence at 530 nm). The excitation for JC-1 is 488nm.

The objective of the training experience is (1) to establish an experimental fertilisation protocol in oyster, the first critical step in efficient hatchery culture; (2) to evaluate the oyster sperm quality by measuring mitochondrial function and state with flow cytometry.

Material and methods

1. General procedures

1.1 Collection and maintenance of animals

C. gigas were collected in Charentes (France) and transfered in the experimental hatchery of Ifremer (Argenton, France). They were fed two microalga (*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 200L aquarium with running sea water at 19°C in order to obtain gonad maturation.

1.2 Gamete preparation

Gametes were collected from the oyster by the dry stripping method (Allen et al., 1992). Briefly gonads were dissected out whole and placed in a small glass bowl with 5ml stock solution (Store Gigas) (Brizard et al., 2001) for sperms or with 5ml filtered sea water (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 chunk of gonads. After diluted between 1/1000 and 1/10000, sperm concentration was determined by Coulter Counter in all the experiments. The concentration of oocytes was determined by microscopic count. The concentration of spermatozoa used for flow cytometric analysis was $1*10^7$ ml^{-1}.

2. Optimisation of fertilisation methods

All the experiments were carried out with triplicate trials at 19°C. After oyster stripping, female gametes were conserved in 2 litters containing FSW while male gametes were stored in "Store Gigas" solution at 4°C prior fertilisation. As all experimental conditions of fertilization could not be tested at once, five separated experiments have been conducted. Thus, the results of previous experiment could be considered in optimising the following one.

2.1 Effect of oocyte aging

After 1, 2, 3, 4 hours of maintenance in FSW at 19°C, 50000 ovocytes from 3 females were transferred into a beaker to obtain a sperm : oocyte ratio of 150 : 1 in an adjusted volume of 50ml.

2.2 Effect of oocyte concentration

Five oocyte concentrations were tested (100/ml, 500/ml, 1000ml, 5000/ml and 10000/ml) using 3 individual females. The fertilisation was conducted in 40 ml FSW at a sperm : oocyte ratio of 200 : 1.

2.3 Effect of sea water volume for fertilisation

Three fertilisation volumes (10ml, 50ml and 100ml) were tested. Oocytes from three females were pooled and adjusted at a concentration of 500 ml^{-1} at a sperm : oocyte ratio 200 : 1.

2.4 Effect of spermatozoa : oocyte ratio

Six sperm : oocyte ratios were tested 10 : 1, 100 :1, 500 :1, 2000 :1. Ooocytes were pooled from three oysters and the concentration was adjusted at 500 ml^{-1} for fertilisation. Three male were tested in a fertilisation volume of 50ml.

2.5 Effect of gamete contact time

Six incubation durations were tested :10 seconds, 10, 20, 30, 60 and 90 minutes. The oocytes from three females were mixed together and the concentration of oocytes used for

fertilization was 500 ml^{-1}. Three males were tested. The fertilisation was performed in 50 ml FSW at a sperm : oocyte ratio of 400. At the indicated contact time, the samples were filtered with a 20 µm mesh and then proceed as below

2.6 Measurement of fertilisation success

After 30 minutes (except the last experiment) of contact between spermatozoa and oocytes, beakers were completed up to 1.8L with FSW to monitor embryo and larval development. Because difficulties to distinguish the four embryo stages, the D-larval yield (48 hours after fertilization) was used to estimate the fertilization success.

3.Flow cytometry analysis of spermatozoa

3.1 Instrument

Samples were analysed on a FACScalibur flow cytometer **(**Becton Dickinson, San Jose, CA, USA), equipped with standard optics and an Argon laser tuned at 488 nm, and running at 200 mW. Calibration was carried out using standard beads (Fluoresbrite plain YG 1.0 µM, Polysciencies Inc., Warrington, PA, USA). The filter in front of the fluorescence 1 (green) photo-multiplier (PMT) transmits at 530 nm and has a bandwidth of 30 nm; the filter used in the FL2 (orange) channel transmits at 585 nm and has a bandwidth of 42 nm; the filter used in the FL3 (red) channel transmits at 650 nm and has a bandwidth of 50 nm. Flow rate was 10-12 $\mu l.minute^{-1}$. At least 10,000 events were analysed from each sample.

3.2 Chemicals

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Sigma (UK); JC1 was purchased from Fluoroprobes (UK).

3.3 Sample preparation for mitochondrial membrane potential (MMP) analysis

The spermatozoa from three individual oysters were used for flow cytometric analysis of their mitochondrial membrane potential (MMP). Prior incubation with JC-1 probe, spermatozoa concentrations were adjusted to approximatively 10^7 in 1 mL of FSW.

According to the experiments, cells were incubated with or without MMP activators or inhibitors for 10 min at room temperature. Then, all samples were proceeded as follows: 10 µL of JC-1 at 0.5 mM (in DMSO) was prediluted in 190 µL FSW in a separated tube, and combined with sperm samples. After an incubation of 10 min at room temperature (RT), samples were diluted 10 min in FSW to block JC-1 interaction with other cell membrane and were considered ready for flow cytometry analysis.

3.4 Validation of the assay using MMP inhibitor (CCCP)

A MMP inhibitor was used to validate MMP measurement in oyster sperm using JC-1. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was chosen because it is a protonophore that uncouples oxidation from phosphorylation by dissipating the chemiosmotic gradient and induces dissipation of $\Delta\psi_m$. 4 µL of CCCP (100 µM in DMSO) was added to three 10^7 ml^{-1} spermatozoa suspension (from individual oysters) for comparison with the same sperm

samples without CCCP. After 10 minutes of incubation at RT with and without CCCP, samples were proceeded as described in 3.3.

3.5 Stability of JC-1 staining

3 sperm samples (from individual oysters) were prepared for JC-1 measurement as described in 3.3. After the 1/10 dilution, samples were divided in two sets. One set was maintained at room temperature and the other one was maintained on ice. Samples were then analysed after 10, 25, 80 and 160 min. of incubation.

3.6 Effect of Store Gigas solution on MMP

Two aliquots of 3 spermatozoa suspensions (from individual oysters) were diluted and adjusted to 10^7 in 1 mL of Store Gigas solution and FSW, respectively. JC-1 assay were performed as described above.

3.7 Application to sperm quality assessment

The aim of the experiment was (1) to test the efficiency of spermatozoa conservation condition (in the Store Gigas solution at 4°C) to maintain mitochondrial functionality; (2) to establish a relationship between fertilization success and MMP.

Sperms from three males used in this experiment were prepared in the hatchery of IFREMER (Argenton) in the morning and were transported to LEMAR for flow cytometric analysis in the afternoon.

To assess fertilisation success, oocyte concentration was adjusted to 500 mL^{-1}, in a fertilisation volume of 50ml and incubated with sperm at a ratio of 400 spermatozoa/oocyte. After 30 minutes of contact time, samples were filtered at 20 µm and were incubated in a 1.8L beaker fully filled with FSW forn larval development monitoring.

During the transport from the hatchery to the laboratory, sperms were conserved on ice and transferred at arrival in the fridge (T=0 hour). After 0, 18, 22, 46 hours of conservation in the fridge, three samples were prepared and analysed by flow cytometry.

Each spermatozoa sample was diluted in FSW, FSW+CCCP, FSW+caffeine (10%). After 10 minutes of incubation at RT, JC-1 was added and samples processed as before.

4. Data analysis

Flow cytometric data are processed using winMDI 2.8 software. All processed data were analysed by statistica (http://www.statsoft.com/). The percentage data were arcsin square-root transformed to achieve homogeneity of variances prior to analysis. The difference in D-larval yield was examined using one or two ways of analysis of variance (ANOVA). When the D-larval yield was significantly different, Tukey' multiple rank comparison was used. The statistical significance was set at $p<0.05$.

Result and discussion

1.Fertilisation optimisation

1.1 Effect of oocyte ageing

During the first four hours, the maintenance of oocytes in sea water had no significant effect to D-larvae yield. This is rather long period over which oyster oocytes can still be fertilized after release by female (figure 1). In other molluscs, fertilisation success for oocyte from *Patella ulyssiponensis* and *Patella vulgata* remains high for up to 12 hours old and 6 hours old, respectively (Alan et al., 2006).This reflects as an adaptive response to sedentary life and external fertilization 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 and fertilization can not occur before the gametes have died (Levitan et al., 1991; André and Lindegarth 1995). Others have suggested that increased gamete longevity could enhance fertilization success when sperm availability varies (Williams and Bentley 2002; Yund and Meidel 2003).

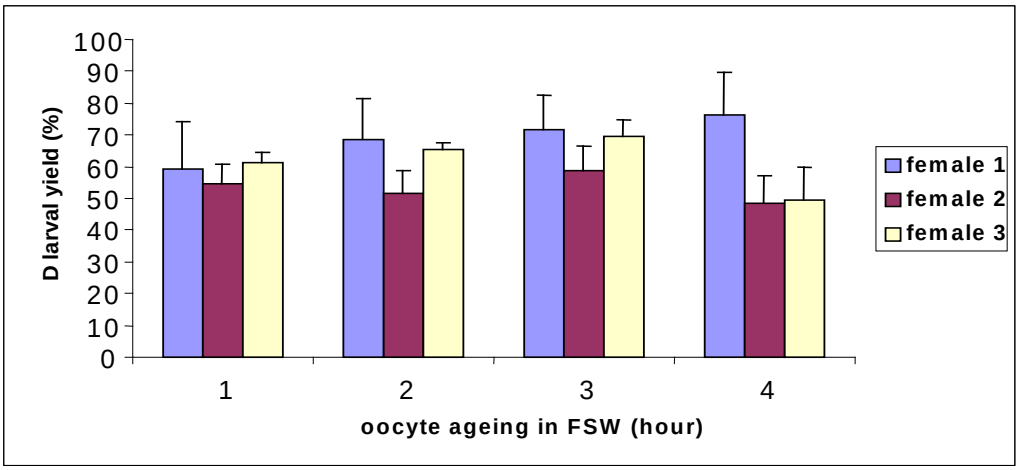

Figure 1. Effect of oocyte aging on D-larval yield (mean ± SD, n=3).

1.2 Effect of oocyte concentration

When the oocyte concentration increased from 1000 ml^{-1} to 5000 ml^{-1}, there was a significant decrease of D-larvae yield. A second decrease was observed between 5000 ml^{-1} to 10000 ml^{-1} (figure 2). It has been suggested that the deleterious effect of high oocyte density is cause but a water-soluble substance, originating from the zygote rather than oxygen depletion nor the physical crowding of eggs (Rampersad et al., 1994).

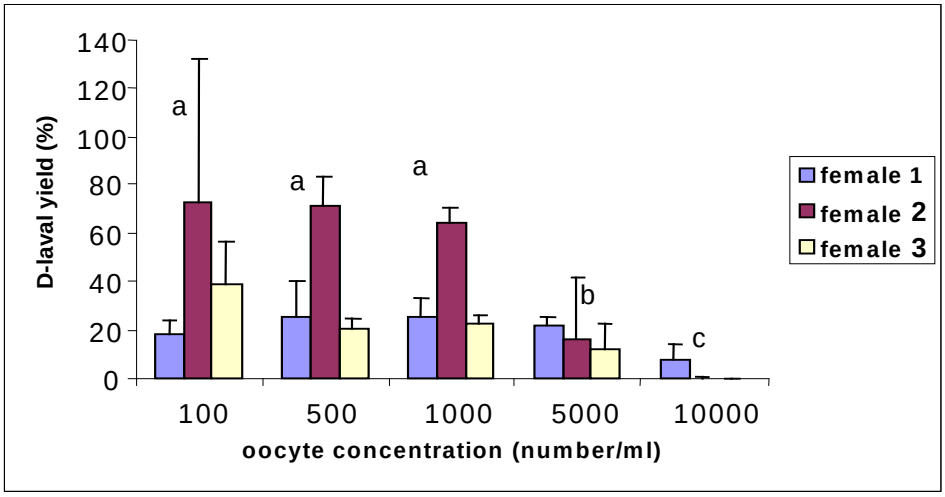

Figure 2. Effect of oocyte concentration on D-larval yield (mean ± SD, n=3, different letters refer to significantly different results).

1.3 Effect of seawater volume for fertilisation

Fertilization volume had no significant effect to D-larval yield (Fig. 3). Suquet et al. (2007) have shown that when the density of oyster oocyte rises from 100/ml to 500/ml, D-larval yield significantly diminishes. In the present, after 30 minutes of incubation, the 1.8 litre baker was fully filled with FSW. Thus the initial density of oocyte in 100ml fertilization volume was 28/ml which was inferior to 100/ml.

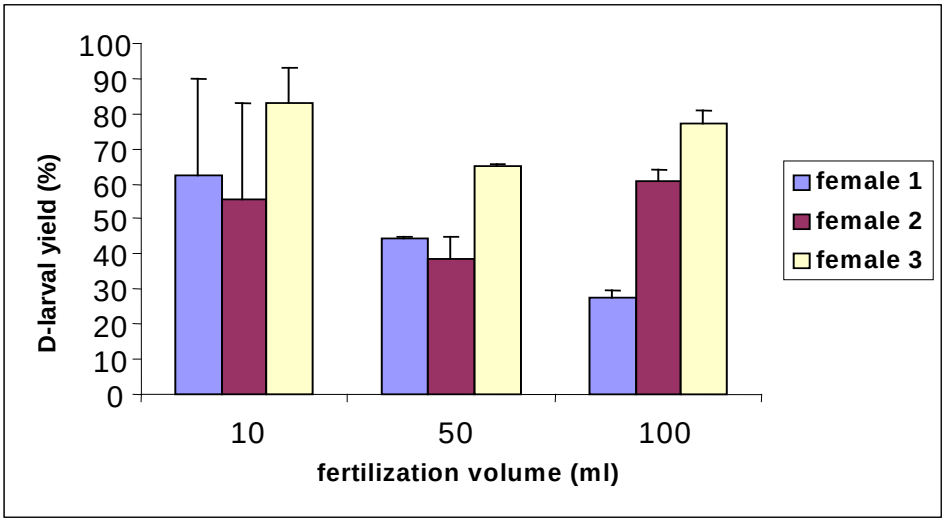

Figure 3. Effect of fertilisation volume on D-larval yield (mean ± SD, n=3).

1.4 Effect of spermatozoa : oocyte ratio

A significantly increase of D larval yield was recorded when the spermatozoa : oocyte ratio increased from 100 to 500. The effect of male was significant on fertilization success (Fig. 4). This confirms that gamete concentration is a crucial factor for the production of larvae. Santos and Nascimento (1985) reported that fertilisation in *Crassostrea rhizophorea* is optimum with a sperm : oocyte ratio ranging from 100:1 to 5000: 1, resulting thus in more than 50% normal larvae. Excessive amounts of sperm during fertilization process were shown to decrease the percentage of viable larvae, likely because of polyspermy (Stephano and

Gould., 1988). Marine invertebrates develop different mechanisms to prevent polyspermy (Gould and Stephano, 2003). Mechanisms allowing to block polyspermy in *Crassostrea* oocytes consist of two steps: a sodium-dependent fast block and a slow permanent block at the oocyte plasma membrane (Togo and Morisawa, 1999).

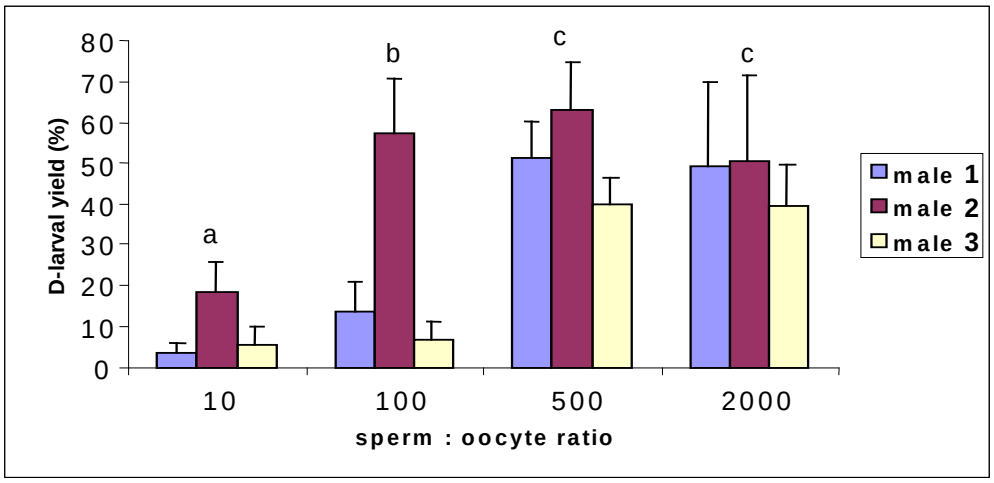

Figure 4. Effect of sperm : oocyte ratio on D-larval yield (mean ± SD, n=3, different letters refer to significantly different results).

1.5 Effect of the gamete contact time

A significant increase of D larval yield was observed between 10s and 10 min. contact duration between spermatozoa and oocytes. There was no significant difference between 10 to 90 minutes of contact between gametes. The effect of male was significant on fertilization success (Fig. 5). So most of fertilization occurred within the first 10 minutes of contact for the oyster, *C. gigas*. Similarly, most of fertilization occurred within 5 minutes in sea urchin and asteroid (Levitan et al., 1991 and Babcock et al., 1999). However, it has been shown that fertilization success is highly variable (5-70%) in the first few minutes in limpet (Alan et al., 2006). Authors claim that this variability may be due to the inexact sperm concentration and inadequacies in experimental techniques.

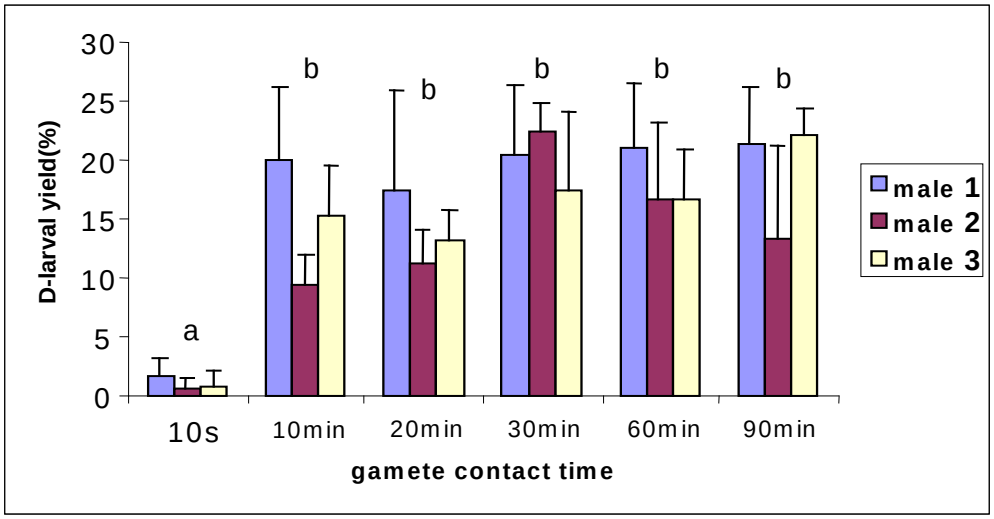

Figure 5. Effect of incubation time on D-larval yield (mean ± SD, n=3, different letters refer to significantly different results).

2. Flow cytometry analysis of spermatozoa

A representative flow cytometric analysis is shown in figure 6. In the density plot of FSC versus SSC (A), two regions R1 and R2 correspond to single cell spermatozoa and aggregated spermatozoa respectively. In B, only the results of analysis for R1 are shown and they correspond to JC-1 staining of spermatozoa in FL1 and FL2. The values of FL1, FL2 are expressed in arbitrary unit of fluorescence intensity and events correspond to the number of analysed spermatozoa.

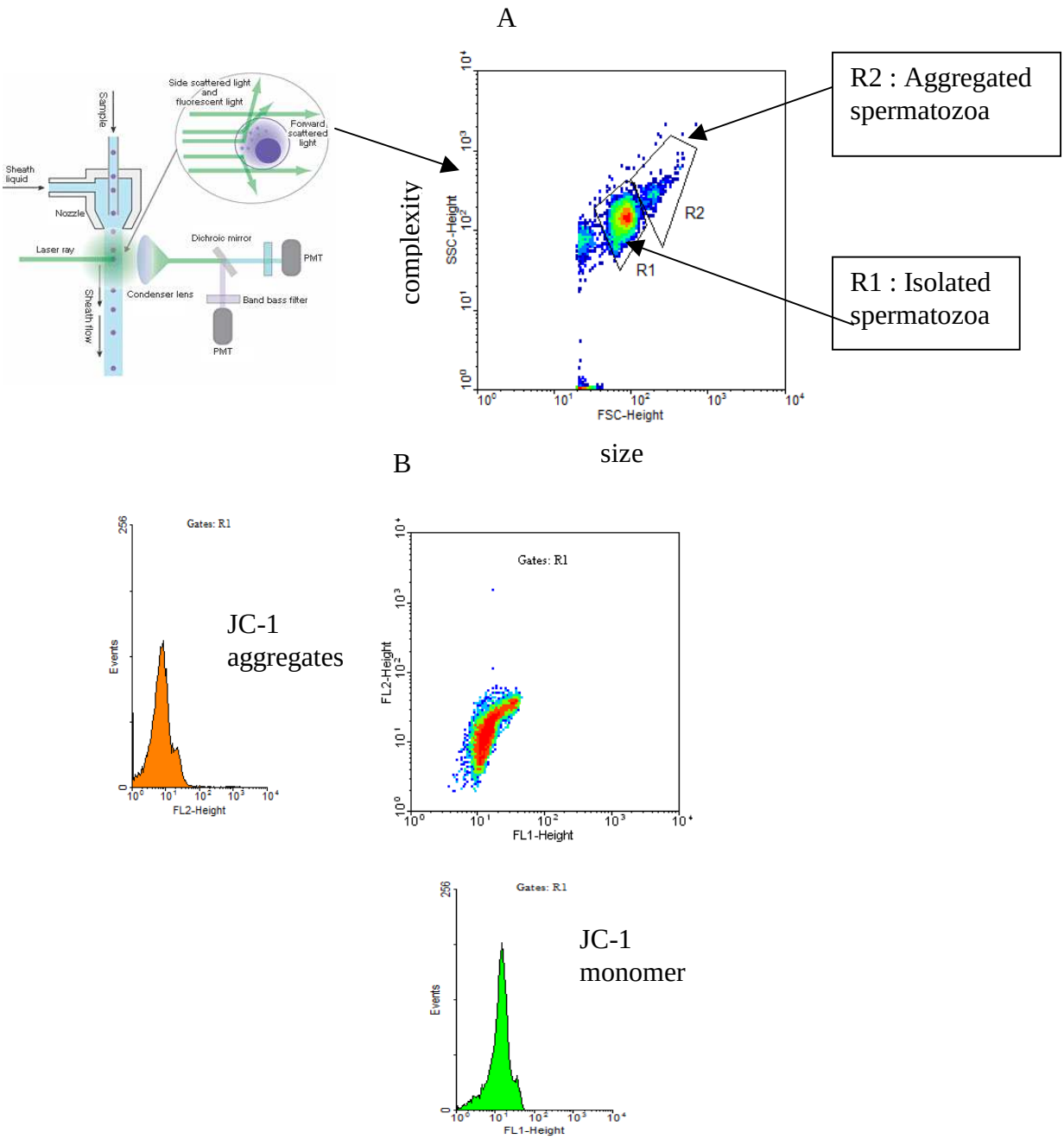

Figure 6. Representative example of flow cytometric analysis of spermatozoa. (A) a density plot of FSC versus SSC (B) a FL1 vs FL2 density plot and corresponding histograms of isolated spermatozoa stained with JC-1.

2.1 Effect of CCCP on JC-1 staining

When sperms were treated with CCCP (an inhibitor of mitochondrial membrane potential), orange fluorescence of sperm drastically reduced while green fluorescence remained stable (Fig. 7 A and B). The observed inhibitory effect of CCCP confirms that JC-1 can be used to measure mitochondrial membrane potential (MMP) in oyster spermatozoa. Typically, it is described that upon lowering the $\Delta\psi_m$, the JC-1 aggregates dissipate into monomers and lead to a shift from red to green fluorescence. However, in our study, the intensity of the green fluorescence from the JC-1 monomer form seems to be insensitive to $\Delta\psi_m$ changes as previously observed by Cossarizza et al. (1996). It has been shown that there exists a strict correlation between ratio (FL2/FL1) and the TPP^+-measured membrane potential values with a regression coefficient of 0.992 and $P<0.0001$ (Cossarizza et al., 1996). Some investigators have used JC-1 as a ratiometric probe (Bowser et al.,1998). Thus, FL2/FL1 ratio appeared thus to provide a good estimation of MMP and we will use hereafter it as a functional parameter of sperm activity. This type of analysis has the advantage of measuring MMP independently of mitochondrial mass or volume. Based on the same premise, JC-1 monomer has been instead used to monitor changes in mitochondrial mass (Mancini et al., 1997).

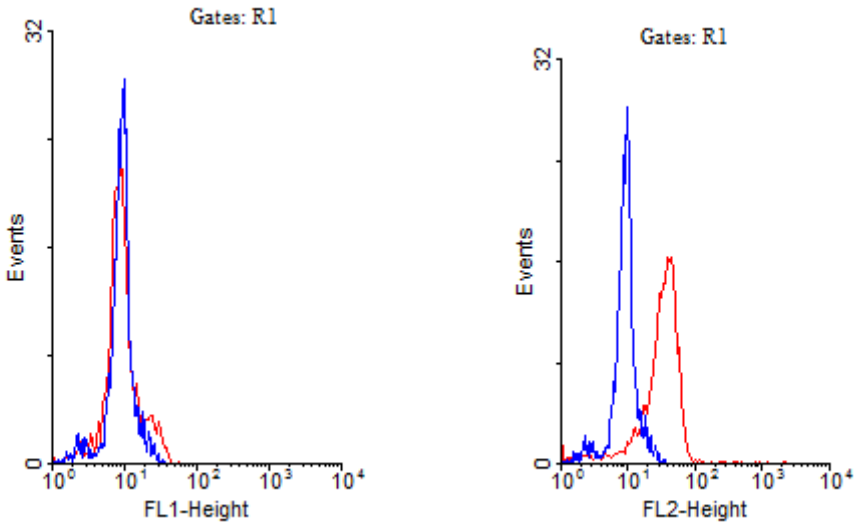

Figure 7. Representative cytofluorometric analysis of MMP in isolated spermatozoa (R1) treated without (red) and with (blue) CCCP.

2.2 The kinetics of probe accumulation at RT and on ice

To develop objective measurement of MMP in semen samples, it is fundamental to achieve standardized protocols. We compared two conditions of spermatozoa maintenance (at RT or on ice) prior flow cytometric analysis. Our results (Fig. 8) showed that the FL2 : FL1 ratio was more stable when samples are maintained on ice than that at RT. So if the sample could not be analysis immediately, it should be conserved on ice. FL2 : FL1 ratio was fairly stable for samples treated with CCCP both at RT and on ice.

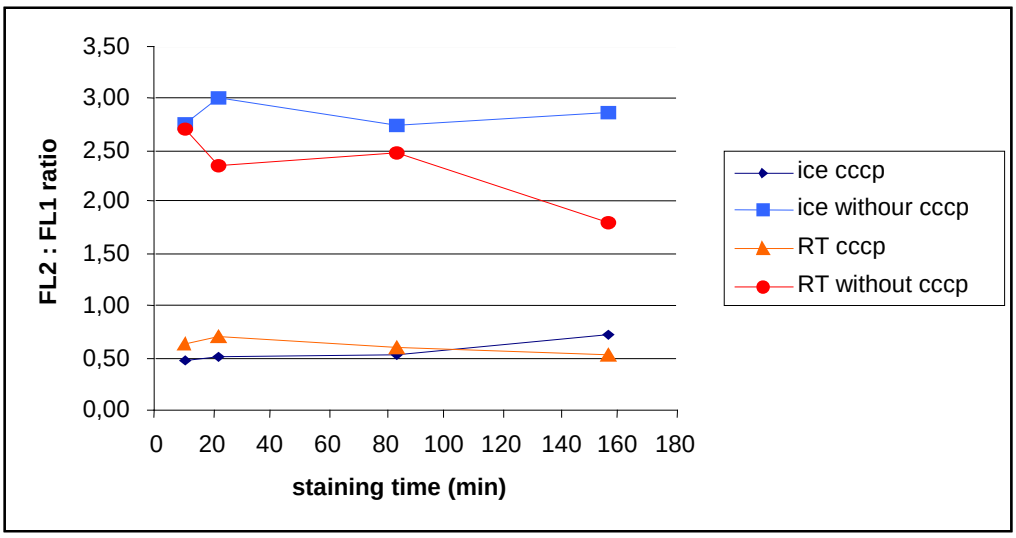

Figure 8. FL2 : FL1 ratio after JC-1 staining for isolated spermatozoa maintained at room temperature and on ice with and without CCCP. At the indicated time, spermatozoa were analysed by flow cytometry.

2.3 Comparison of MMP of spermatozoa prepared in Store Gigas solution and FSW

The FL2 : FL1 ratio in FSW was higher than that in Store Gigas (Fig. 9). When the spermatozoa were observed with microscope, there was no movement in Store Gigas while a majority of sperm moved in FSW. Since the oxidative phosphorylation needs a high MMP, this may suggest that the mitochondria could play an important role for ATP supplementation in oyster sperm flagella movement. It has been shown that the glycolysis is the major ATP supplier in mouse sperm flagella movement (Mukai and Okuno, 2004)

Figure 9. The comparison of F2 : FL1 ratio for isolated spermatozoa diluted in FSW and in Store Gigas solution treated with and without CCCP (mean ± SD, n=3).

2.4 Conservation of sperm mitochondrial function in the fridge

At time 0, caffeine increased the FL2 : FL1 ratio by 1.5 times while the FL2 : FL1 ratio was higher than 3, caffein had little effect to MMP (Fig. 10A). Caffeine can increase sperm motility (Levin at al., 1980 and Jiang at al., 1984). The effect has been attributed to

their potent phosphodiesterase (FDE) inhibitory action, to elevation of adenosine 3'5-cyclic monophosphate (cAMP) levels within the sperm and to the stimulation of glycolysis (Tash and Means, 1983). Wheather caffeine has direct effect on MMP or on glycolysis that arises MMP needs further confirmation. Sperm MMP in sample 1 was different from that in sample 2 or 3 (figure 10D). The reason was unknown and it needs further investigation.

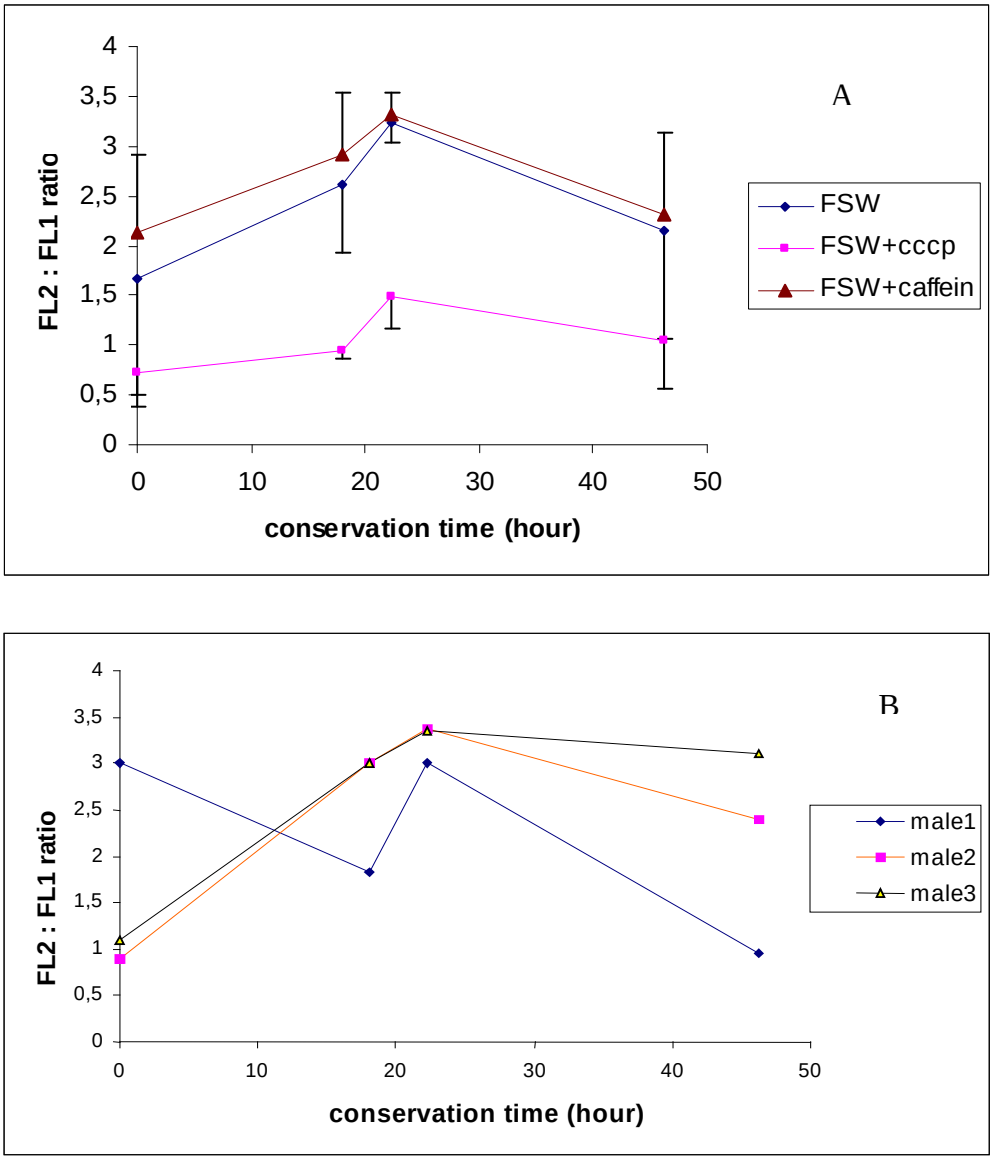

Figure 10. FL2 : FL1 ratio in isolated spermatozoa according to conservation duration. The spermatozoa were diluted in FSW, FSW+CCCP, and FSW+ caffeine solution and analysed by flow cytometry (A) (mean ± SD, n=3). Comparison of the evolution of FL2 : FL1 ratio in three individual spermatozoa samples diluted in FSW(B).

2.5 The relationship between fertilization success and MMP

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In comparison with 1 and 2 or 1 and 3, it seems that a relationship exists between fertilization success and MMP (Fig. 11). To further confirm this observation, it is needed to increase the number of sperm samples.

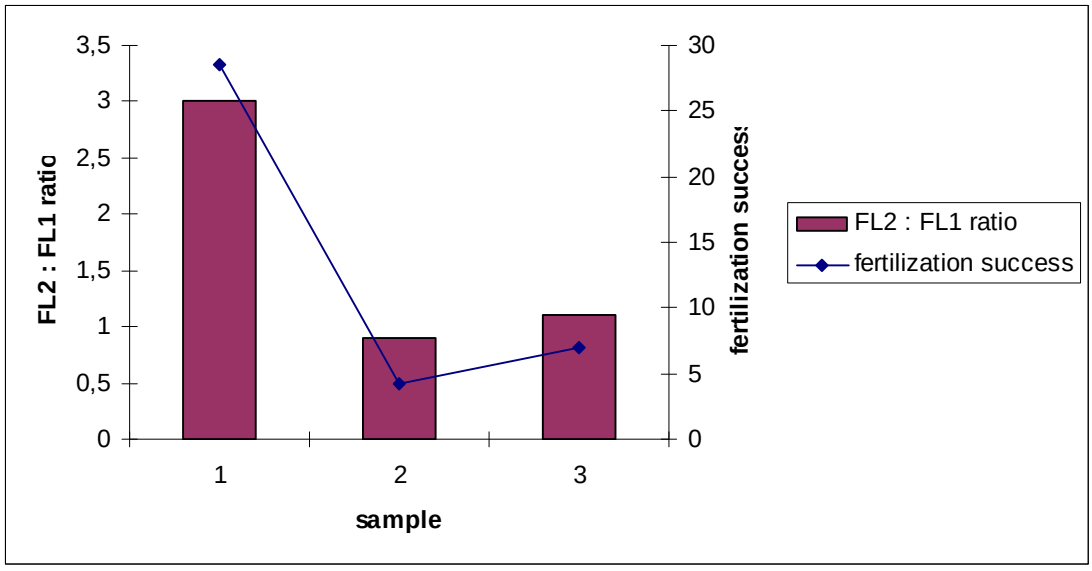

Figure 11. The comparison of fertilization success and FL2 : FL1 ratio (0 hour) for three sperms.

Sperm deterioration can be measured at the membrane and organelle level and an increase of sperm with damaged membranes/organelles will lead to reduced fertility rates. Sperm deterioration at the DNA level may not affect fertilization rates as long as sperm membranes and organelles remain functionally intact. However, the DNA damage leads to a reduced embryo development after the embryonic genome. Thus, to estimate in vitro the oyster spermatozoa quality more precisely, it is suggested to also analyse the DNA quality. (for a review, see Silva-Gadella, 2006).

The goal of semen analysis is to determine the fertilizing potential of the semen sample, using a rapid, inexpensive procedure. It has been established a multiple variable models to predict the fertilization success in human (Morcé and Graham, 2007) and in stallion (Kirk et al. 2005). In a review of the literature, Graham et al. (1980) reported that correlations between fertility and assays for motility, morphology and viability ranged from 0.06 to 0.86, and that no assay provided consistently desirable correlations with fertility. Part of the reason for this lack of consistency is due to the inaccuracy of fertility data (Amann, 1989), part is due to the multi-factorial nature of sperm function, and part is due to inaccuracy of in vitro measurements (Mocé and Graham, 2008). So to evaluate the fertilizing potential of a semen sample, it is suggested to measure several attributes, for a review see Mocé and Graham, (2008).

Conclusion and perspective

Two protocols have been established for Pacific oyster (*C. gigas*) during this study: one for the assessment of sperm fertilization capacity and the other for flow cytometric analysis of sperm mitochondrial membrane potential ($\Delta\psi_m$) with JC-1.

The results of fertilization have shown that the effect of male is significant on fertilization success. Thus it suggests that there exists a variation of sperm quality. In the future, it is needed to test more sperms and establish a multi-variable model (motility, MMP, viability, ATP, integrity of DNA…) to predict the fertilization success in Pacific oyster.

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Abstract

Recently the quality of Pacific oyster (*Crassostrea gigas*) spermatozoa has become a non negligible problem in modern shellfish aquaculture but there are not reliable protocols to determine the quality. Two protocols has been established during this training experience: one for fertilization and the other for flow cytometric analysis of sperm mitochondrial membrane potential (MMP) with JC-1.

Fertilization success was found to be a function of oocyte concentration, spermatozoa : oocyte ratio and contact time between gametes. When the density of oocyte increased from 1000/ml to 5000/ml, the D larval yield decreased; sperm : oocyte ration needed for optimum fertilisation success in vivo ranged between 500 and 2000; the maximal fertilisation success occurred after 10 minutes of incubation; eggs were fertilisable for at most 4 hours when maintained in sea water. The effect of individual male significantly influenced the fertilisation success.

In presence of CCCP, which dissipates the chemiosmotic gradient and induces dissipation of $\Delta\psi_m$, FL2 (aggregates of JC-1) obviously diminished while FL1 (monomer of JC-1) was insensitive to $\Delta\psi_m$. The JC-1 staining in mitochondria was more stable on ice than that at room temperature. Caffeine increased FL2 : FL1 ratio when the ratio was low (inferior to 3) The relationship between fertilisation success and MMP needs further investigations.

The objective of this training experience was to establish reliable experimental protocols to describe the Pacific oyster sperm quality. These protocols will be used to control sperm quality in the future.

Keywords: *Crassostrea gigas*, spermatozoa, fertilization, flow cytometry, JC-1, MMP

Résumé

La qualité du sperme d'huître creuse (*Crassostrea gigas*) est devenu un problème non négligeable pour l'ostréiculture moderne. Cependant, il manque des outils fiables pour déterminer cette qualité. Deux protocoles ont été mis au point pendant ce stage : l'un permet de mesurer la capacité de fécondation d'échantillons de sperme et l'autre analyse le potentiel membranaire mitochondrial du sperme d'huître creuse.

Le taux de fécondation dépend de la concentration en ovocytes, le ratio spermatozoïdes : ovocyte et le temps de contact entre les gamètes. Quand la concentration des ovocytes augmente de 1000/ml à 5000/ml, le taux de larves D diminue. Le ratio optimum spermatozoïdes : ovocyte est compris entre 500 et 2000. Un taux maximum de fécondation est observé après 10 minutes de contact entre les gamètes. Lorsqu'ils sont dilués dans l'eau de mer. les ovocytes sont fécondables pendant 4 heures. Un effet significatif des mâles sur le taux de fécondation est observé.

En présence de CCCP qui diminue le potentiel membranaire mitochondrial, FL2 (agrégat de JC-1) décroît et FL1 (monomère de JC-1) reste stable. L'accumulation de JC-1 dans les mitochondries est plus stable sur la glace qu'à température ambiante. L'ajout de caféine augmente le ratio FL2 : FL1, lorsqu'il est inférieur à 3. Il faudrait établir une relation entre le taux de fécondation et le PMM.

Le travail réalisé lors de ce stage a permis de définir des outils efficaces de description de la qualité des spermatozoïdes chez l'huître creuse. Ces outils seront utilisés à l'avenir pour contrôler la qualité de ces gamètes.

Mots clés : *Crassostrea gigas*, spermatozoïde, fécondation, cytometrie en flux, JC-1, PMM