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# Male germ cells of the Pacific oyster *Crassostrea gigas*: flow cytometry analysis, cell sorting and molecular expression

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**Abstract –** A technique was developed for dissection and isolation of male germ cells in the oyster *Crassostrea gigas.* This procedure can provide cells for the exploration of processes involved in the reproductive physiology of bivalves. Spermatogonia were chosen because of their essential role in spermatogenesis and the impact of gonia proliferation on reproductive effort. A non lethal method for determining sex and reproductive cycle stage was first validated in oysters. This first step was essential in order to constitute a homogeneous pool of oysters at the same stages of gametogenesis. Germ cell fractions were then obtained from a density gradient, and enrichment of each fraction was ratified by electron microscopy and by means of a 2-parameter flow cytometry procedure (DNA and mitochondrial staining). A significant enrichment in spermatogonia and spermatocytes was confirmed by the increased expression of markers of proliferative cells (proliferative cell nuclear antigen, PCNA) and early germ cells (oyster vasa-like gene). A preliminary cell sorting procedure is also reported, which was applied to fractions enriched in spermatogonia.

**Key words:** gametogenesis / germ line / mitochondrial staining / DNA content / Vasa / proliferative cell nuclear antigen / Mollusca / *Crassostrea gigas*

## 1 Introduction

The Pacific oyster, *Crassostrea gigas*, is a successive and irregular protandrous hermaphrodite mollusc. Reproduction in this species is seasonal and timing of each step of germ lineage development is strongly affected by environmental conditions (Mann 1979; Ruiz et al. 1992). The gonad consists of gonadal tubules invaginated in a connective storage tissue where haemolymphatic vessels are numerous (Franco et al. 2008). The volume of the gonadal tubules is directly related to gametogenetic stage from autumn (resting stage followed by spermatogonial mitosis) to summer (ripe gonad) (Heude-Berthelin et al. 2001). The initiation of gametogenesis in males is marked by numerous gonial mitosis directly related to the quality and the quantity of gametes produced at the end of the sexual cycle. This cellular proliferation of spermatogonia is continuous throughout the gametogenetic cycle in Pacific oyster (Franco et al. 2010), although the mechanisms involved in the reinitiation of spermatogenesis and regulation of gonial mitosis are still unknown in this species. These steps of the reproductive cycle depend on the quality and the quantity of gametes produced. Furthermore, reproductive events were reported in triploid oysters considered to be sterile. An analysis of different patterns of the reproductive cycle in these oysters has been described by Jouaux et al. (2010). New knowledge on the first stages of the reproductive cycle in diploid oysters could serve to help us understand this gametogenesis disturbance in their triploid counterparts. In order to characterize specific markers of early gametogenetic events and also to obtain cellular tools to explore the function of these markers, we developed a strategy for early germ cell isolation in male oysters.

The first difficulty encountered in the study of oyster spermatogenesis is the impossibility to determine the sex of animals based on external anatomical criteria. In addition, the seasonal reproductive cycle led to variation in the proportions of each germ cell type in the tubules over the year. Oysters are not perfectly synchronous and the development of protocols for spermatogonia enrichment first requires preliminary strategies to obtain a homogeneous sample of male oysters in stage I and II of the gametogenetic cycle. At these stages, the sex of oysters is discernible from histological slides, and spermatogonia are the majority cell type in the gonadal tubules (Franco et al. 2008, 2010).

In rat, seminiferous tubules containing germinal epithelium and Sertoli cells can be separated from the interstitial tissue using a protocol based on trypsin and collagenase

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dissociation (Bellve et al. 1977). This strategy is not easily transferable to oyster because a large part of gonadal area is made up of connective storage tissue. In trout, additional dissociation steps were developed in order to remove the conjunctive tissue (Loir 1994). Our strategy for oysters was derived from these approaches. Molecular characterization of the different cell populations present in gonadal tubules in oyster, as in seminiferous tubules in mammals, represents a challenge, due to the multitude of germinal cell types and somatic cells present in cell suspensions issued from gonad dissection. Numerous strategies have been developed in vertebrates to isolate these cell populations, among which a bovine serum albumin (BSA) density gradient allowed suspensions of spermatogonia (Com et al. 2003), pachytene spermatocytes and round spermatids (Silandre et al. 2007) to be obtained in rat.

To make a cell composition analysis of complex cell populations and further enrichment of certain cell types, flow cytometry analysis has already been employed by several groups for the study of testicular cell types in mouse and rat (Petit et al. 1995, Suter et al. 1997). These studies are based on the classification of cells according to their DNA content. Additional cell markers, measurable by flow cytometry, have been considered for enriching the sensitivity and specificity of this method. In the rat, mitochondrial staining with the fluochrome nonyl acridine orange (NAO) allowed several germ cell subpopulations to be distinguished within each ploidy group (Suter et al. 1997). Vital mitochondrial staining with Mitotracker green FM® was used to visualize the structural integrity of bovine spermatozoa after flash freezing (Celeghini et al. 2007), and the incorporation of spermatozoa mitochondria during fertilization in the zebra mussel (Misamore et al. 2006). In the Pacific oyster, male germ cells have been characterized by transmission electronic microscopy. Number, size and cytoplasmic localisation of mitochondria change during spermatogenesis. Mitochondria are small and numerous in the spermatogonia, whereas they are large and fewer in spermatocytes and spermatids (Franco et al. 2008). Mitochondria would make an excellent subject of analysis to identify male germ cell populations in oyster.

In this paper, we present an enrichment protocol for oyster early germ cells, coupled with a staining technique for oyster male cell suspensions. This procedure enables the identification of germ cell types based on DNA content and mitochondrial staining. To complement the microscope observations and flow cytometry analysis, expression of the Oyster vasa-like gene (Oyvlg), a vasa ortholog gene that is a marker of early germ cells (Fabioux et al. 2004), and proliferating cell nuclear antigen (PCNA), a marker of proliferative cells (Franco et al. 2010), were measured in the different cell fractions obtained. The spermatogonia and spermatocyte enrichment achieved in this way led to a preliminary trial in cell sorting.

## 2 Material and methods

### 2.1 Animals

Specimens of the Pacific oyster, *Crassostrea gigas* (approximately 3 years old), were purchased from a commercial oyster farm (Manche, France).

### 2.2 Determination of sex and gametogenetic stage by a non lethal method

For each isolation procedure, 75 oysters were used. In order to perform a biopsy of the gonadal area, the shell of each oyster was notched using a circular saw, oysters were maintained in an experimental structure from this point onwards. Biopsies were treated for histological analysis as described previously (Franco et al. 2008), the sex and the gametogenetic stage of each animal were determined. Male oysters in stage I and II were selected for the subsequent experiment.

### 2.3 Cell isolation

The gonadal area of thirteen selected oysters was dissected. Tissues were rapidly rinsed in sterile sea water (SSW), cut into small pieces with scissors, and incubated in Hank's 199 medium; (aCl 250 mM, KCl 10 mM, MgSO 25 mM, CaCl 2.5 mM, Hepes 10 mM, pH 7.4, 1100 mOsm) containing 0.1% collagenase and 0.05% deoxyribonuclease (DNase) and supplemented with penicillin (60 mg $L^{-1}$) and streptomycin (100 mg $L^{-1}$), for 3 h at room temperature, in a rotating shaker. The enzymatic digestion was stopped by transfer of the tissues to fresh medium containing 1% bovine serum albumin (BSA) and an antibiotic supplement. After one night at 17 °C, the pieces of tissues were decanted and crushed in fresh medium with a Dounce piston. Cell suspensions were filtered successively through 100 $\mu$m and 30 $\mu$m nylon meshes to remove any remaining tissues. The cell suspension was incubated for one hour at 17 °C in culture flasks. The supernatant was centrifuged (500 g, 15 min, 15 °C) and the pellet re-suspended in SSW containing 0.2% BSA. The cell population issued from this dissociation procedure formed the initial population then put through a BSA gradient. The adherent cell population retained on the culture flasks was treated for electron microscopy.

Separation of the different cell types was realised by unit gravity sedimentation through a continuous BSA gradient (0.5 to 2%) in a Sta-Put apparatus (Bellvé et al. 1977). After 4 h of sedimentation, 40 cell fractions were collected and centrifuged (500 g, 15 min, 15 °C).

### 2.4 Electron microscopy

Cells from the initial population or issued from the BSA gradient were fixed for 15 min in 3.2% glutaraldehyde in 0.31 M cacodylate buffer (pH 7.4) supplemented with 0.25 M sucrose. They were then post-fixed for 2 h at 4 °C using 1% osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) containing 0.36 M sucrose, rinsed (2 × 5 min) and incorporated into 1.5% agar. They were dehydrated in increasing concentrations of ethanol (2 × 5 min 70% ethanol, 3 × 15 min 95% ethanol, and 4 × 15 min 100% ethanol) and impregnated for 20 min in 50% ethanol/50% Epon (v/v), and, finally, 20 min in Epon 100%. Ultrathin sections (80 nm) were stained with uranyle acetate followed by lead citrate and examined with a JEOL 10–11 transmission electron microscope.

**Table 1.** Analysis of cell fractions issued from the sedimentation procedure on the basis of DNA content. Different categories of ploidy considered: haploid (C, spermatids, spermatozoa), 2C ($G_0$-$G_1$ phase, spermatogonia, spermatocytes I and II, and intragonadal somatic cells), S phase (spermatogonia, spermatocytes I), 4C ($G_2$-M, spermatogonia, spermatocytes I). Results are expressed in percentage of events by category of ploidy (10 000 events analysed by fraction).

| Fraction | Haploid (C) | $G_0$-$G_1$ (2C) | S phase | $G_2$-M (4C) |
|---|---|---|---|---|
| Initial population | 29.5 | 13 | 16.1 | 19.8 |
| 8 | 41.2 | 10.7 | 12.7 | 14.3 |
| 12 | 33.8 | 12.1 | 14.7 | 18.4 |
| 13 | 33.9 | 12.1 | 13.5 | 19.6 |
| 14 | 30.5 | 12.8 | 15.8 | 18.6 |
| 16 | 48.7 | 9.8 | 12.0 | 10.4 |
| 17 | 37.2 | 11.1 | 13.6 | 15.3 |
| 20 | 25.8 | 11.2 | 14.1 | 16.7 |
| 24 | 17.1 | 10.8 | 16.5 | 20.3 |
| 26 | 17.3 | 11.0 | 18.3 | 21.6 |
| 27 | 18.1 | 11.8 | 20.0 | 17.7 |
| 29 | 20.5 | 13.3 | 20.7 | 12.4 |
| 30 | 30.6 | 14.3 | 17.8 | 7.9 |
| 32 | 40.0 | 12.1 | 12.3 | 4.5 |
| 33 | 53.2 | 12 | 10.2 | 3.5 |

$G_0$: Resting phase, S: Synthesis phase (DNA synthesis), M: Mitotic phase (nuclear division).

### 2.5 Cell staining for flow cytometry analysis

Cell fractions issued from the BSA gradient were fixed in 70% ethanol and stored at –20 °C until fluorescent staining (Table 1). These cells were then centrifuged and washed twice with PBS to remove the fixative. Mitochondrial staining was carried out by addition of PBS (1 ml) containing 200 nM MitoTracker Green FM (Invitrogen®) and incubation was realised in the dark at room temperature for 10 min. Cells were centrifuged and rinsed twice with PBS, suspended in 50 $\mu$l solvent containing 500 $\mu$l propidium ioide (PI) solution (Coulter DNA Prep Reagents kit, Beckman Coulter®), and incubated in the dark at room temperature for 20 min.

The fluorescent emission of stained cells was measured on an Epics XL cytometer (Beckman coulter) connected to a computer running Expo 32 ADC software. The excitation source consisted of one argon-ion laser operated at 488 nm (15 mW). PI and Mitotracker green FM fluorescent emissions were monitored using 620 and 525 nm band-pass filters respectively. At least 10 000 events were evaluated for each sample.

### 2.6 First attempts at cell sorting

Cell fractions issued from the BSA gradient were fixed in paraformaldehyde (PFA) 3% and stored at –20 °C until fluorescent staining. Cells were centrifuged and washed twice with phosphate buffered saline (PBS) to remove the fixative. Mitochondrial staining was carried out by addition of PBS (1 ml) containing 200 nM MitoTracker Green FM (Invitrogen) and incubating in the dark at room temperature for 10 min. Cells were centrifuged and rinsed twice with PBS.

Analysis and sorting were realised on a FACS Epics Altra flow cytometer (Beckman Coulter) connected to a computer running Expo 32 MultiComp software. The excitation source consisted of one argon-ion laser, operated at the 488 nm (15 mW). Mitotracker green FM fluorescence was collected with a 505–545 nm band-pass filter. At least 10 000 events were evaluated for each sample.

Sorted cells were examined under an epifluorescence microscope with filters for detection of red and green fluorescence (Nikon Eclipse 80i), equipped with a Nikon DXM12 camera.

### 2.7 Molecular expression of cell fractions

Total RNA was isolated from fractions 11, 15, 19, 21, 25, 28, 31, 34, 38 using a microRNeasy kit (Quiagen®) following the manufacturer's instructions. Samples were pooled after ultrastructure observations and flow cytometry analysis. To prevent genomic DNA contamination, a 15 min DNase I treatment was carried out at room temperature. For cDNA synthesis, reverse transcription (RT) was carried out with 20 U RNasin (Promega), 0.5 mM deoxynucleotide triphosphates (dNTPs), 200 U reverse transcriptase from Moloney murine leukemia virus (Promega), and 100 ng random primer, at 37 °C for 90 min.

Real-time polymerase chain reaction (PCR) analysis was performed using an iCycler apparatus (BioRad®) with the iQ™ SYBR Green Supermix PCR kit (Biorad) for real time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95 °C/15 s, 60 °C/15 s) with the specific primers. Accurate amplification of the target amplicon was verified by performing a melting curve. Amplification of cDNA was performed using PCNA specific primers (forward, 5′GCTGCTCGATTTAGACTGTGA 3′; reverse, 5′ CTTGACTCCTTCCTTTGTACA 3′) and Oyvlg specific primers (forward, 5′ TCCCCGAGGAGATTCAGA 3′; reverse, 5′ ACGTCAGTGCAAGCACCA 3′). A parallel amplification of the oyster actin (actin, GenBank accession no. AF026063) reference transcript was also performed (primers: forward, 5′ GCCCTGGACTTCGAACAA 3′; reverse, 5′ CGTTGCCAATGGTGATGA 3′). PCR analysis was

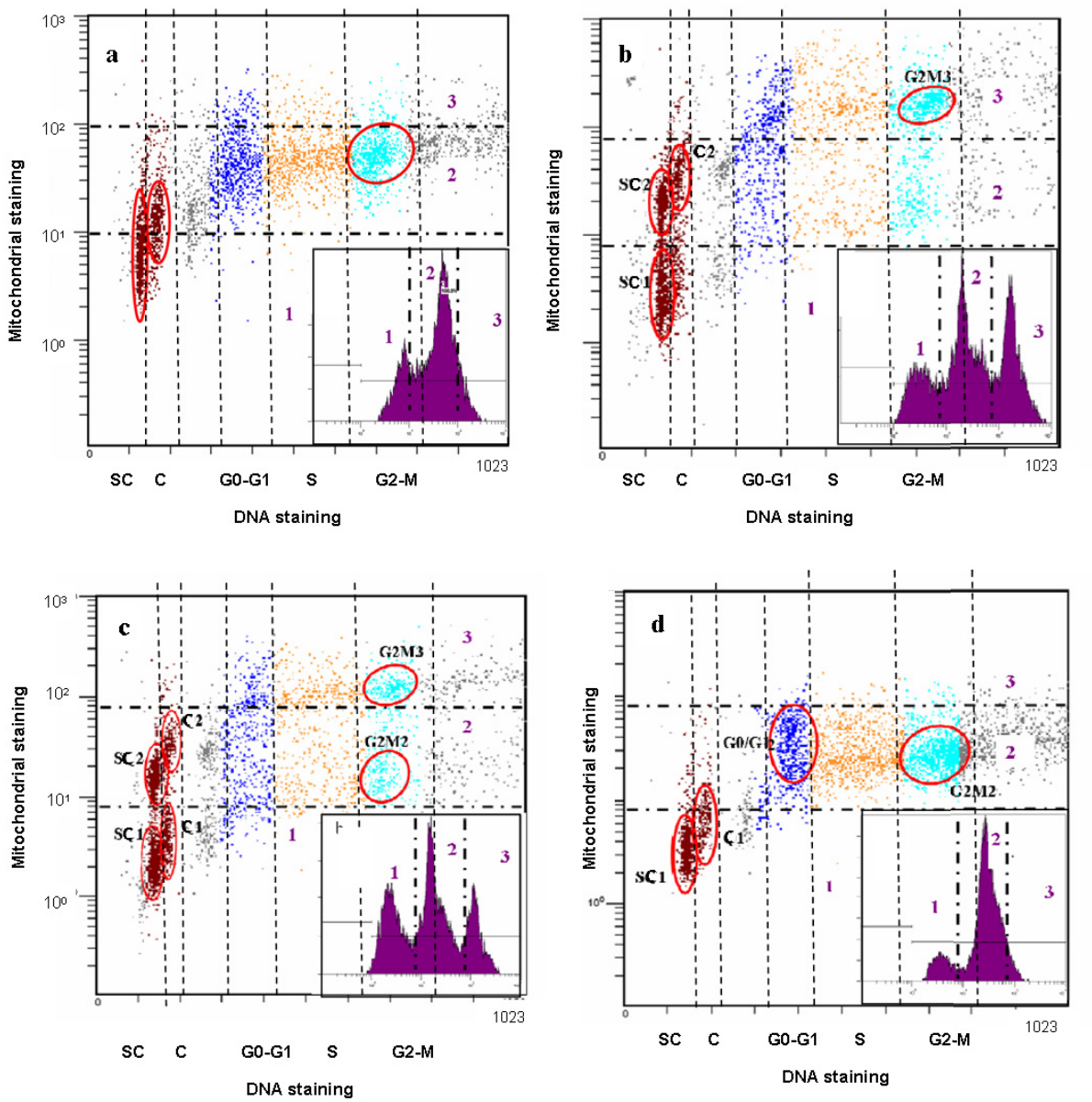


**Fig. 1.** Flow cytometry analysis of the initial population, subfractions 8, 16 and 24 on the basis of DNA content and mitochondrial staining. Testicular cells of the initial population (**a**), (inset) three levels of mitochondrial staining considered for analysis: low (1), medium (2) and high (3) of subfraction 8 (**b**), subfraction 16 (**c**), subfraction 24 (**d**). SC: subhaploid population. C: haploid population. $G_0$-$G_1$: 2C population. S: S phase (DNA synthesis phase). $G_2$-M: 4C population.

performed in triplicate for one sample. The relative level of gene expression was calculated for one copy of the actin reference using the following formula: $N = 2^{(\mathrm{Ct\,actin} - \mathrm{Ct\,Cg-gene})}$.

## 3 Results

### 3.1 Characterisation of the initial population

Identification of cell subpopulations according to DNA content and mitochondrial staining was carried out on the initial population (suspension free of haemocytes and conjunctive cells). PI staining led to the identification of 3 main populations: two 1C populations, one haploid (C) and the other subhaploid (SC), made up of spermatids and spermatozoa and representing 29% of the initial population; and a 4C population made up of $G_2$-M ($G_2$ phase and mitotic phase of the cell cycle), spermatogonia and spermatocytes I and representing 19.8% of the total initial population (Fig. 1a and Table 1). For mitochondriome characteristics based on mitochrondial staining, three levels of staining were distinguished for the subsequent analysis: low (1), medium (2) and high (3) (Fig. 1a, inset). In the initial population, the majority of cells were stained at low and medium levels.

Cellular composition of the initial population was investigated by ultrastructural observation. The population contained numerous cell types. Cell identification was based on

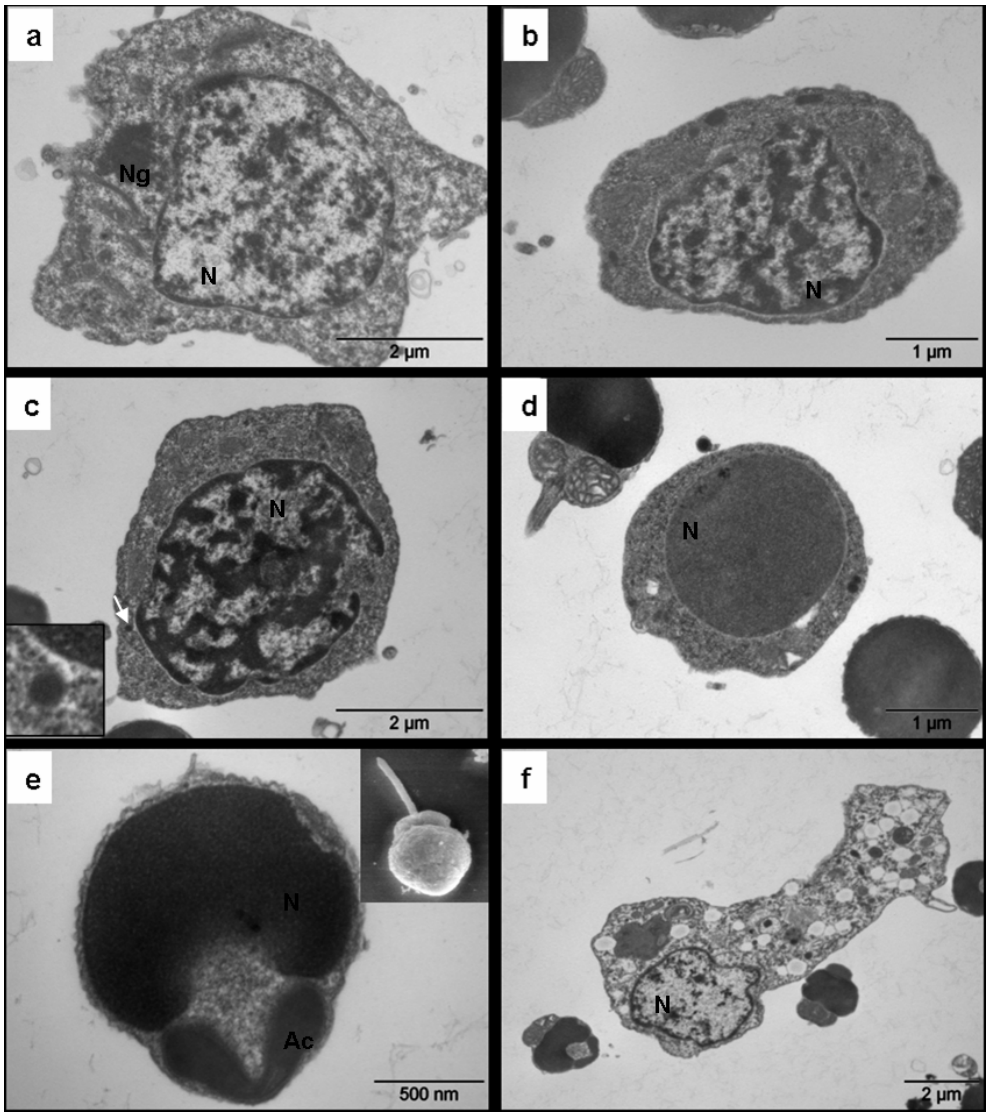


**Fig. 2.** Ultrastructure of cell types in the initial population. Spermatogonia type I (**a**), primary spermatocyte (**b**), secondary spermatocyte with proacrosomal vesicle (arrow and inset) (**c**), spermatid (**d**), spermatozoa (SEM, inset) (**e**), intratubular somatic cell (ISCs) (**f**). N: nucleus, Ng: nuage, Ac: acrosome.

the ultrastructural characteristics of male germ cells and associated cells described in Franco et al. (2008). Spermatogonia type I, spermatocytes I, spermatocytes II, spermatids, spermatozoa and intragonadal somatic cells (ISCs) were subsequently characterized in the initial population (Fig. 2).

### 3.2 Characterization of separated subfractions (after BSA gradient)

Different fractions distributed along the BSA gradient were qualified by electron microscope examination. All fractions contained spermatids and spermatozoa. In fractions <20 (lower part of the gradient) numerous cell residues (Fig. 3a) and groups of intragonadal somatic cells (ISCs) were observed (Fig. 3b). No early germ cells were identified in this part of the gradient. In fractions 20 to 30 (middle of the gradient), type II spermatogonia with numerous small mitochondria and prominent nucleoli were identified (Fig. 3c). Spermatocytes I, characterized by elongated mitochondria, were also present (Fig. 3d). The fractions >30 were enriched in spermatocytes II (Fig. 3e) and spermatozoa, but also presented numerous cell residues (Fig. 3f).

The fractions issued from the density gradient were analysed on the basis of DNA content (Table 1). For fractions 1 to 7 and fractions 33 to 40, corresponding to the lower and upper ends of gradient (high and low BSA concentration), respectively, the number of cells was not sufficient to allow cytometry analysis. Haploid and subhaploid cells were found in all fractions. Fractions 8 to 17 and 30 to 33 were enriched in haploid cells, fractions 24 to 30 were enriched in cells in S phase, fractions 18 to 23 showed no enrichment, and fractions 24 to 26 showed enrichment in $G_2$-M phase cells (4C).

The complete cytometry analysis based on DNA content and mitochondrial staining (with three levels: low –1, medium

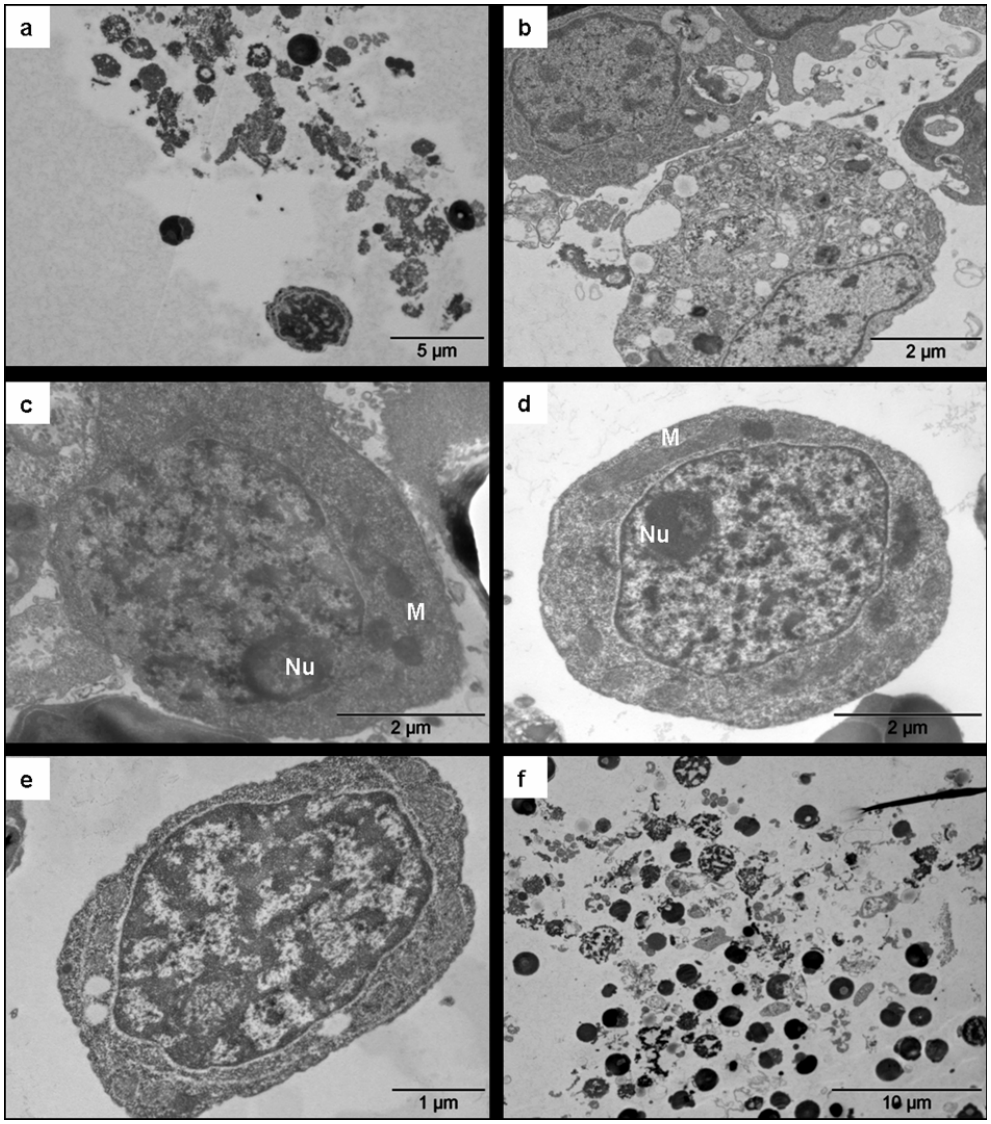


**Fig. 3.** Ultrastructure of different cell fractions along the BSA gradient. Fractions <20 (lower part of the gradient) (**a** and **b**): cell remains (**a**) and groups of intracellular somatic cells (**b**). Fractions 20–30 (**c** and **d**): spermatogonia type II (**c**) and primary spermatocyte (**d**). Fractions <30 (top of the gradient) (**e** and **f**): secondary spermatocyte (**e**) and spermatozoa and cell remains (**f**). Nu: nucleolus, M: mitochondria.

–2 and high –3) was realised on all of the fractions. Results of this analysis are presented for three fractions (8, 16 and 24) representative of the different patterns of germ cell subpopulations (Fig. 1). Fraction 8 (Fig. 1b) was composed of more than 40% haploid cells. Using the combination of DNA content and mitochondrial staining, we were able to distinguish four subpopulations in this part of the gradient: two subhaploid populations (named SC-1 and SC-2) with different mitochondrial staining (low and medium, respectively), a haploid population (C-2) with medium mitochondrial staining, and a $G_2$-M population with a high mitochondrial staining ($G_2$-M -3). In fraction 16 (Fig. 1c), subhaploid (SC-1 and SC-2), haploid (C-2) and $G_2$-M ($G_2$-M -3) populations were present. A fourth haploid population (C-1) with a low mitochondrial staining and a second $G_2$-M population with medium mitochondrial staining ($G_2$-M -2) were also qualified. In fraction 24 (Fig. 1d), populations SC-1, C-1 and $G_2$-M -2 were identified. In this fraction, a homogenous $G_0$-$G_1$ population was observed with medium mitochondrial staining ($G_0$-$G_1$ -2).

### 3.3 Molecular expression of cell fractions

Expressions of PCNA and Oyvlg (Vasa homologous) were measured in the initial population and in the different fractions issued from the BSA gradient. Expression of these two markers was greater in the all fractions in the 20 to 30 range compared with the other fractions issued from the gradient (Fig. 4).

### 3.4 First attempts at cell sorting

First attempts at cell sorting after mitochondrial staining were performed in the region of interest of the BSA gradient

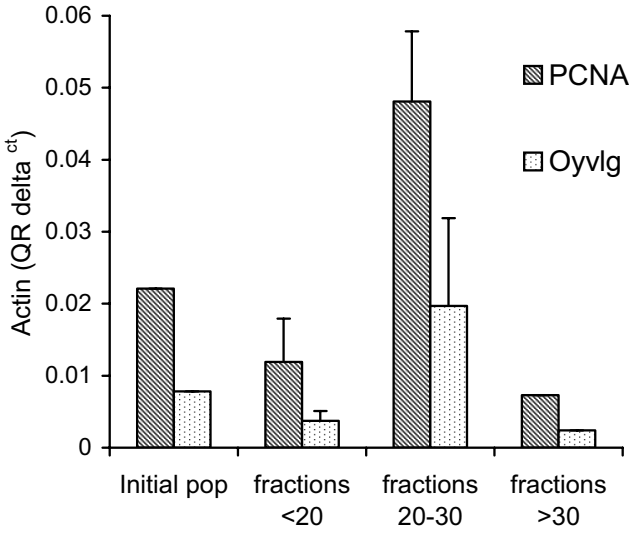


**Fig. 4.** Expression of proliferating cell nuclear antigen (PCNA) and Oyvlg (Vasa homologous) in the initial population and in the different cell fractions issued from the BSA gradient. Results are expressed in relative quantity of actin expression (QR $\Delta^{ct}$).

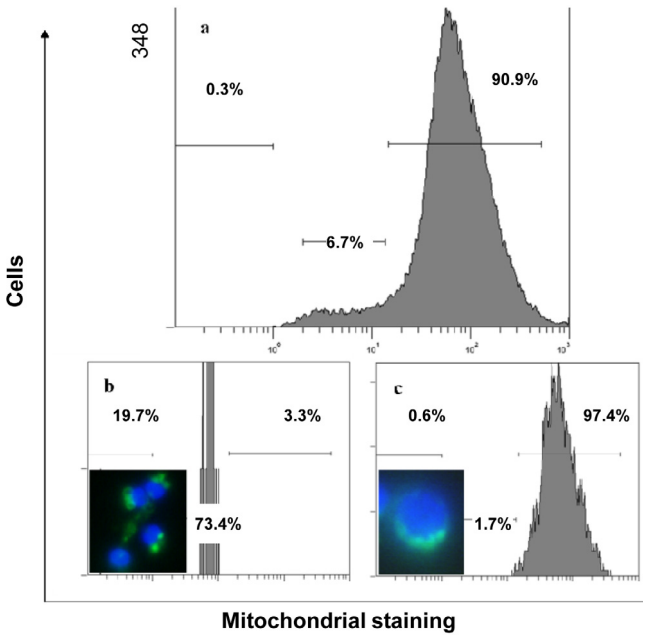


**Fig. 5.** Cell sorting performed on the region of interest in the BSA gradient (fractions 20–30) on the basis of mitochondrial staining. Cytometry analysis (**a**). Cell sorting led to the separation of two populations, which were observed under an epifluorescence microscope (**b** and **c**).

(fractions 20–30) (Fig. 5). The cell sorting procedure permitted two populations of cells to be separated. Due to the low number of purified cells, we opted for morphological description of isolated cells under an epifluorescence microscope (Fig. 5b, c).

The first population contained cells with small nuclei and few and large mitochondria; this should correspond to the spermatids and/or spermatozoa population. The second population contained cells presenting similar characteristics to spermatogonia and/or young spermatocytes I, i.e., large nuclei and small mitochondria grouped near the nucleus.

## 4 Discussion

The development of an isolation procedure for oyster germ cells requires a pool of animals that are homogenous in terms of gametogenetic development. Working with an oyster population of this type, we tested and validated a non lethal method for determining sex and gametogenetic stage. A limitation of this strategy was, however, illustrated by the recurrent presence of spermatids and spermatozoa in BSA-separated fractions, while these cells were absent from the biopsies of oysters chosen for experimentation. This result is probably related to a non homogenous development of gonadal tubules in the oyster gonadal area and to the lack of information on the spatial course of gametogenesis in these tubules. An overall study of the timing of spermatogenesis at the scale of the individual animal would be necessary to target the most adequate areas for biopsies in the oyster model. Injection of BrdU (5′-Bromo-2′-deoxyuridine) could be used, for example, in order to characterize the course of gametogenesis (Parapanov et al. 2008) or the gonial proliferations (Loir 1999; Loppion et al. 2008).

The isolation procedure developed in our study was derived from techniques performed in trout (Loir 1994) and rat (Com et al. 2003; Silandre et al. 2007) to isolate subpopulations of germ cells. One of the final goals of our study is to obtain purified cell suspensions in order to develop homologous functional bioassays in oyster. At present, only haemocytes and storage cells of oysters can be maintained for a few days; these have been used, respectively, in immunological (Labreuche et al. 2006; Lelong et al. 2007) and energetic metabolism (Berthelin et al. 2000; Kellner et al. 2002; Hanquet-Dufour et al. 2006) studies. Until recently, exploration of the functional role of molecules identified in oyster was only based on heterogeneous systems like rabbit chondrocytes to study the role of a chitinase (Badariotti et al. 2006) or zebra fish embryos to explore the function of a receptor of the TGF-$\beta$ family (Le Quéré et al. 2009). Recently, RNA interference was conducted in vivo in oyster to explore the role of Oyvlg in gonad (Fabioux et al. 2009). However, suspensions of germ cells are still essential in work aiming to relate the function of interesting molecules to cell types involved in this function.

At the end of the dissociation procedure, an initial cell population was obtained issued from gonadal tubules and free of haemocytes and conjunctive cells. Cytometry analysis was applied to this initial population using DNA content and mitochondrial staining as parameters. These two parameters have already been employed by several groups for the study of testicular cell types in mouse and rat (Petit et al. 1995; Suter et al. 1997). Subhaploid populations (SC-1 and SC-2) observed in our study should correspond to spermatids advanced in spermiogenesis, and haploid populations (C-1 and C-2) to young spermatids and spermatozoa populations. The $G_0$-$G_1$ population may contain spermatogonia and young spermatocytes I, the $G_2$-M -2 population may contain spermatocytes I (zygotene) and spermatogonia, whereas the $G_2$-M -3 population should correspond to spermatocytes I (pachytene). The

mitochondriome staining of the initial population obtained after the dissociation procedure was not sufficiently selective to allow a direct cell sorting procedure. Subsequently, a further step of cell separation on a density gradient was performed and was found to be efficient in performing an initial cell sorting trial.

The cytometry analysis of the different fractions issued from the BSA gradient displayed a significant enrichment in spermatogonia and spermatocytes I, validated by electron microscopy, in fractions 20 to 30. This enrichment was confirmed by a higher expression of both proliferative and early germ cell markers (Oyvlg, PCNA) in these fractions. In fact, Fabioux et al. (2004) showed, by real time PCR and hybridization in situ, that Oyvlg was expressed in spermatogonia and spermatocytes throughout the Pacific oyster reproductive cycle and developed a RNAi technique to explore the role of Oyvlg in oyster gonad (Fabioux et al. 2009). First attempts at cell sorting were, therefore, conducted on these fractions on the basis of mitochondrial staining. From the morphological characteristics (Franco et al. 2008), the two isolated populations were found to correspond to spermatids/spermatozoa on one hand and to spermatogonia/young spermatocytes on the other. These first results should now be confirmed using a complementary cell sorting procedure based simultaneously on ploidy and the mitochondriome and ratified by electron microscopy identification of cells. However, the number of isolated cells after these multiple steps is always low and may be limiting for a complete characterization procedure including structural, ultrastructural and molecular approaches.

The method described here represents a valuable tool for obtaining fractions enriched in subpopulations of germ cells in the oyster *Crassostrea gigas*. Effective enrichment in spermatogonia and spermatocytes was achieved and confirmed by molecular expression of specific markers of proliferation and stem cells. Regarding the initial cell sorting results obtained in our study, the flow cytometry approach represents a very promising avenue for the study of reproduction and regulative processes of the course of gametogenesis in this seasonally reproducing species.

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