*Rev. peru. biol.* **6(1)**: 104-109 (1999)

ISSN 1561-0837

# CHARACTERIZATION OF SOME TECHNIQUES OF IMMUNOFLUORESCENCE AND FLUORESCENCE IN CHILEAN MUSSELS *Perumytilus purpuratus* AND *Semimytilus algosus*

# CARACTERIZACIÓN DE ALGUNAS TÉCNICAS DE INMUNOFLUORESCENCIA Y FLUORESCENCIA EN MEJILLONES CHILENOS *Perumytilus purpuratus* Y *Semimytilus algosus*

Orlando Garrido[1] y José Pino[2]

## RESUMEN

Gametos y estadios larvales de los mejillones *P. purpuratus* y *S. algosus* fueron tratados *in vitro* con técnicas de fluorescencia y no fluorescencia a fin de detectar microfilamentos, DNA y gránulos corticales involucrados con estadios de la reproducción como fertilización y clivaje. En *P. purpuratus* se detectó tubulina a nivel de los cilios y el velum; asimismo, la actina fue detectada desde el estadio de fertilización a los estadios de desarrollo tanto en *P. purpuratus* como en *S. algosus*, lo que sugiere que no son descartados durante el proceso de fertilización. Los microfilamentos detectados en ambos mejillones sugieren que ellos juegan un rol importante como integrante del citoesqueleto durante el desarrollo.

**Palabras claves**: Mejillones, Inmunofluorescencia, DAPI, tubulina, *Perumytilus, Semimytilus*, microfilamentos, gránulos corticales, Chile.

## ABSTRACT

Gametes and larval stages of the mussels *P. purpuratus* and *S. algosus* were treated *in vitro* with fluorescent and non-fluorescent techniques in order to detect microfilaments, DNA and cortical granules involved with reproductive status as fertilization and cleaveage stages. In *P. purpuratus* tubulin was detected in cilius and velum in D larval stages, also actin was detected from the fertilization to development stages in both *P. purpuratus* and *S. algosus*. Cortical granule-like structures were observed in larval stages of *P. purpuratus* suggesting they are not discharged during the fertilization process. Microfilaments detected in both mussels suggest they play an important role in the cytoskeleton during development.

**Key words**: Mussels, Immunofluorescence, DAPI, tubulin, *Perumytilus, Semimytilus*, microfilaments, cortical granules, Chile

## INTRODUCTION

Microfilaments, 7-nm cytoskeletal elements, are ubiquitous in eukaryotic cells and participate in motility and changes in cell shape (Schatten *et al.* 1986). Cleveage is the most conspicuous change that reveals the development of an embryo, since eggs from many animals have dark cytoplams, living observations about the behavior of microtubules have been restricted and information about the microtubule system is still meager (Sawada & Schatten 1988). In general, the significance of the microtubules and microfilaments in fertilization and embryogenesis is due the spatial distribution of cytoplasmic components (ooplasmic segregation); after mitosis the chromosomes remain near the cleavage plane and form daughter nuclei just slightly past the site of the cleavage furrow. The center of the interphase aster is separated from the daughter nucleus when the egg is cleaved. The nuclei

[1] Embryology Institute, Biological Science Faculty, Universidad Austral de Chile, Valdivia, Chile.
[2] Reproduction and Development Biology Laboratory, Biological Sciences Faculty. Universidad Nacional Mayor de San Marcos, Lima, Perú.

are moved to the blastomer center after cleavage is completed (Sawada & Schatten 1988).

In order to explore the living cell as it changes during reproduction and development, we used immunocytochemistry and cytochemistry techniques with fluorescent and non-flurorecent labels for examining DNA, microfilament, cortical granules and microtubules (Epel 1980; Schatten 1982; Shroeder 1981; Barack *et al.* 1981). This work describes some aspects of fertilization and embryonic development in two small Chilean mussels (*Perumytilus purpuratus* and *Semimytilus algosus*) by means of light microscopy.

## MATERIALS AND METHODS

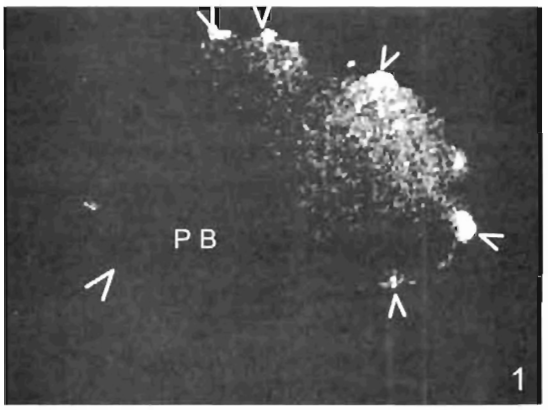
Gametes from the Chilean mussels *P. purpuratus* and *S. algosus* were obtained by raising the temperature of sea water, and the fertilization was made *in vitro* condition. Male and female were placed at 4 °C over the night, followed by an increase in temperature to 20 °C (Loosanoff & Davis 1963). Ripe mussels of *P. purpuratus* released large number of gametes. This method of spawning failed in *S. algosus*, the gametes in this specie where obtained by squashing the gonad of ripe specimens. In *S. algosus* autofertilization was obtained because mature ovocites and sperms of the same individual were utilized. These fertilized eggs were cultured in 1M urea pH 8,1 for at least 15 minutes, examined and if the fertilization envelopes remained the incubation was repeated; next the fertilized eggs were gently rinsed three times with calcium-free sea water containing 10 mM Tris; 2,5 mM EGTA (N,N´-Tetraacetic acid), pH 8,1, until the appropriate stage was obtained. Stages of development were fixed onto poly-L-Lysine-coated coverslips (1mgr/ml polylysine) with absolute methanol during 5 minutes. Stained with 50 ug/ml rhodamine-phalloidin in Phosphate Buffer Saline (PBS) (1:40) (Barak, *et al.* 1981; Cline and Schatten 1986) for F-Actin (Oligomeric and polymeric) for 30 minutes at room temperature, rinsed with PBS.

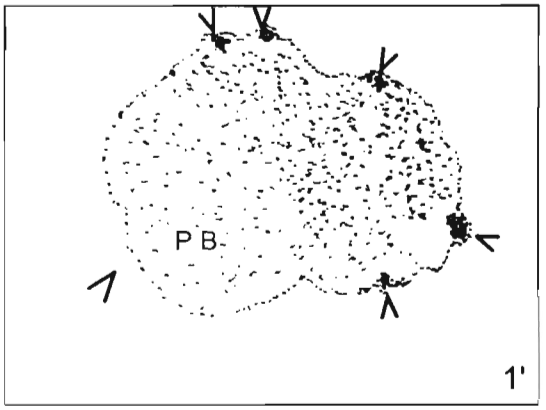
DNA was visualized with specific compound: 4,6 diamino-2-phenylindole (DAPI) (Thombes *et al.* 1992); the coverslip was mounted on the top of a small drop of glycerol and examined with fluorescence microscopy epifluorescense rhodamine filters. For microtubules and DNA detection, about one hundred eggs without envelopes were gently pipeted onto polylysine coated-coverslips, treated with extraction buffer composed of 25% glycerol, 25mM Hepes, 0,5 mM MgCl, 10% Methanol, pH 6,9 with 1% Triton X-100 (Schatten *et al.* 1985) during 20 minutes at room temperature. The microtubules were recognized by indirect immunofluorescence (IFI) using an FITC antimouse IgG antibody (Sigma). The mouse monoclonal antibody was detected by a secondary antibody conjugated to Rhodamine and/or fluorescein, for introducing this stain into the egg without disrupting the cell surface structure, the membranes were gently permeabilized with lysolecithin (Miller *et al.* 1978). Dithiothreitol (DTT) was used to remove the viteline layer from unfertilized eggs (Cline and Schatten 1986). Additionally the DNA was labeled with DAPI. The cortical granules were observed in unfertilized eggs. The eggs were dejellied by several passages through 100um bolting silk, attached onto a monolayer by polylysine-coated slides and then their tops were sheared off by squirt of a calcium-free medium containing 10 mM DTT (Cline & Schatten 1986) and tripsine pH 8,0. The isolated eggs surfaces were examined with phase microscopy.

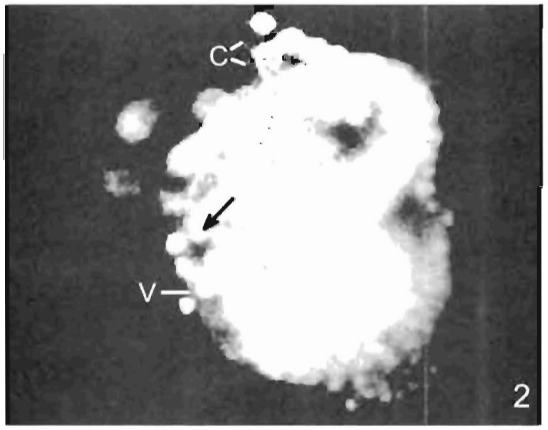
## RESULTS

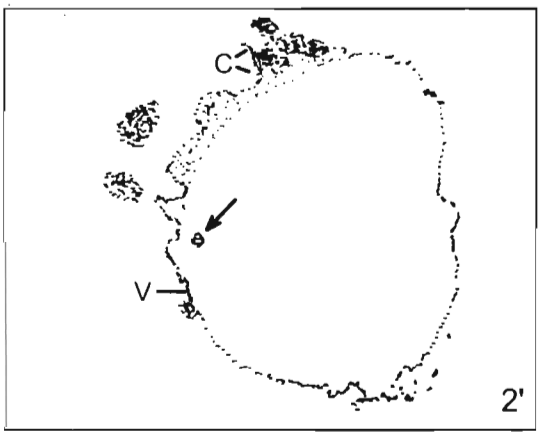
### Polymeric and oligomeric actin detection with Rhodamine-Phalloidin

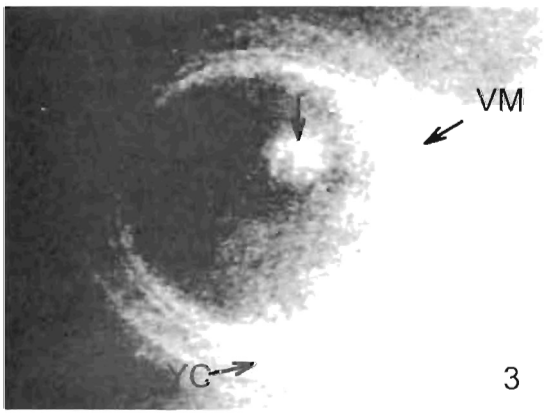
Microfilaments were detected throughout the cortex of fertilized eggs and dividing cells

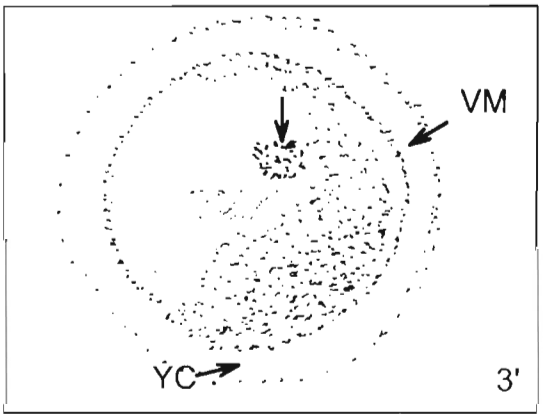


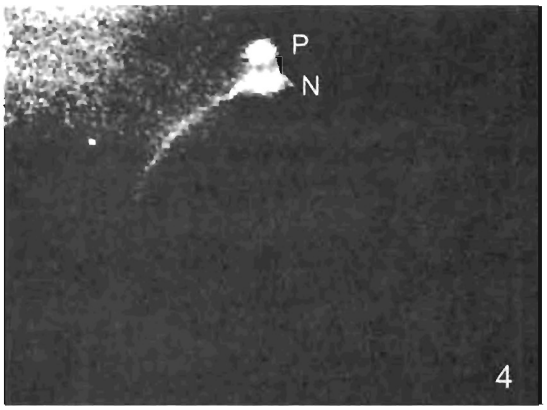



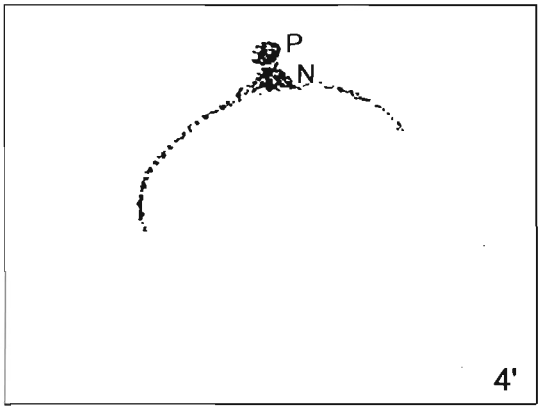



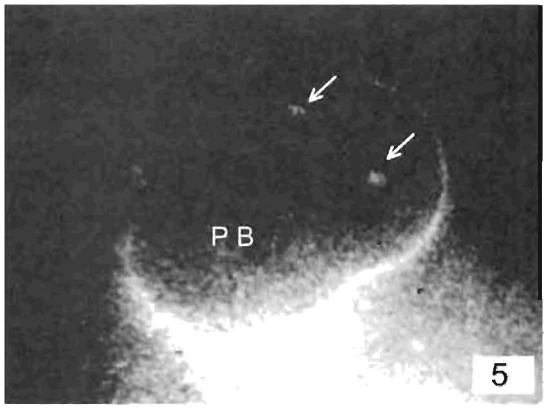



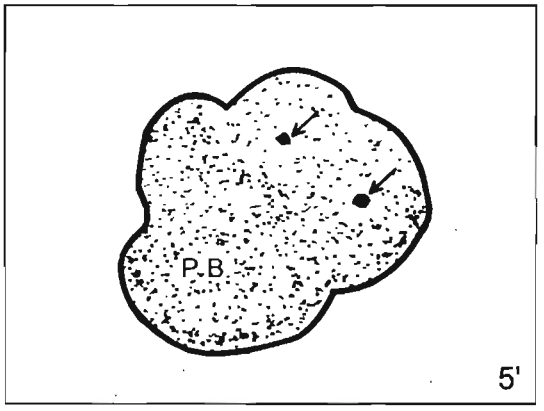



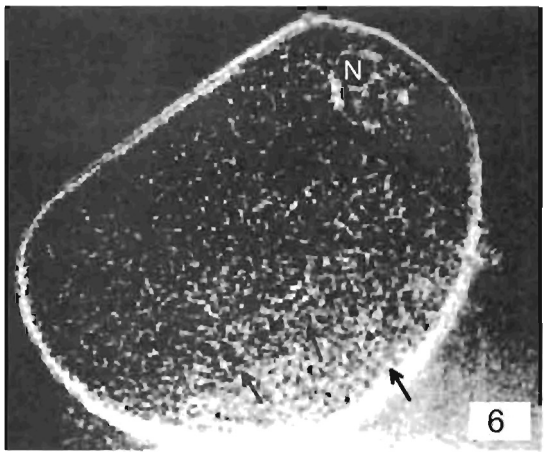

**Figures 1.** Rhodamine-Phalloidin staining of microfilament in dividing *P. purpuratus* eggs. Arrow head, fluorescent foci; arrow, difuse fluorescence; PB, polar body (X400);
**Figure 2.** Staining of the same preparation with antitubulin monoclonal antibody and with DAPI dye. C, cilius; V, vellum; arrow, nucleus (DAPI) (X400).
**Figure 3.** Unfertilized *P. purpuratus* egg stained with DAPI. VM, vitelline membrane; YC, jelly coat; arrow, nucleus (X400).
**Figures 1'-3':** Schematic drawings to facilitate the comprehension of the legends of the illustrations.



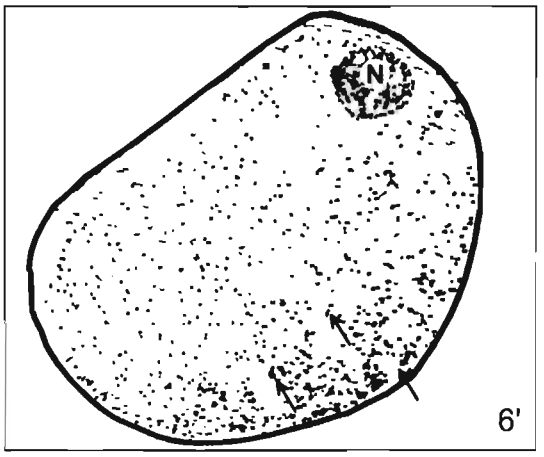




**Figure 4.** Fertilized *P. purpuratus* egg stained with DAPI. PB, polar body; arrow, blastomeres nucleus (X400).

**Figure 5.** *S. algosus* larval stage stained with DAPI. PB, Polar body; arrow, blastomeres nucleus (X400).

**Figure 6.** Isolated unfertilized egg surface of *P. purpuratus*. Arrows, cortical granule-like structure; N, nucleus (X400).

**Figures 4´- 6´:** Schematic drawings to facilitate the comprehension of the legends of the illustrations.

of *P. purpuratus* and S. algous, indicating that microfilaments were found in association with the entire egg surface. In the equatorial region of dividing cells thick spots of cortical staining were detected in relationship with the leading edges of the cleavage furrow (Figure 1).

### Tubulin detection with Antitubulin monoclonal antibody

Microtubules detection was made in D larval stage by first labelling with monoclonal anti-ß-tubulin antibody. The immunofluorescence was observed in the surface of the vellum and in the cilium. DNA was labeled with DAPI in the same specimens (Figure 2).

### DNA detection with DAPI

DNA was clearly visualized using DAPI in unfertilizing eggs, fertilized eggs and blastomeres of larval stages of *P. purpuratus* and *S. algosus*. In unfertilized eggs staining DNA was incorporated to the chromosomes of female nucleus of the oocyte (Figure 3) and male pronucleus of the sperm. During the fertilization process staining DNA was observed in the nucleus of secondary oocyte and polar body (Figure 4). In larval stages DNA was observed in the nucleus of the blastomeres (Figure 2 and 5).

### Cortical Granules detection

The isolated unfertilized eggs surfaces examined with phase microscopy are seen with a uniform arrangement of cortical granule-like structures (Figure 6). Secretion was not observed when *in vitro* adding 1mM $CaCl_2$ onto the isolated eggs surface.

## DISCUSSION

The immunocytochemistry localization of microfilaments with monoclonal anti-actin antibody is very difficult by background staining due to the monomeric actin. Rhodamine-labelled phalloidin impairs this process because of detected oligomeric and polimeric actin. Rhodamin phalloidin detection of egg microfilaments was shown to provide a sensitive and easy alternative to transmission electron microscopy for studies of microfilament patterns during fertilization and early development of marine invertebrates (Bestor & Chatten 1981; Schatten *et al.* 1986; Sawada and Schatten 1988; Perez *et al.* 1991). This method permits a global detection of microfilaments in a large number of cells in different stages of development (Cline and Schatten 1986). The detection of polymerized actin in the fertilized and cleaving eggs of *P. purpuratus* and *S. algosus* suggests that this microfilament plays an important role in the organization of the cytoskeleton of these mussels.

During fertilization and cell division the tubulin images may delineate centrosomal structures. Centrosomes, which organize microtubule configurations, undergo replication and division process of the blastomers (Cline and Schatten 1986). The monoclonal antibody was used in D larval stages of *P. purpuratus* and recognized by indirect immunofluorescence the presence of tubulin molecules. The detection of eggs and embryos microfilaments is shown here to provide a sensitive alternative to the complicated techniques involved in Transmission Electron Microscopy for the analysis of their changing configuration during fertilization and early development, and may well prove valuable in the investigation of other alteration in cell shape (Cline & Longo 1986).

Cortical granules are specialized membrane-bound secretory granules located beneath the plasmatic membrane of most invertebrates and vertebrates eggs (Raven 1970; Guraya 1982). In most organisms that possess cortical granules, the content of the

granules are discharged by exocytosis when the sperm activates the eggs during the fertilization (Schuel 1978). The exocytosis of cortical granules can be obtained by artificially increasing the concentration of $Ca^{+2}$ (Vacquier 1975). Mussels of the genus *Mytilus* possess cortical granules (Humphreys 1962). The eggs of some invertebrates contain non-secretory cortical granules that are not discharged during fertilization and persist during early embryonic development, as in the surfclam *Spisula* (Longo and Anderson 1970) and the mussel *Mytilus* (Humphreys 1967). *P. purpuratus* may have cortical granules similar to *Mytilus*. A more comprehensive study is quite necessary in order to confirm his hypothesis.

## REFERENCES


Barak L. S.; R.R. Yocum and W. W. Webb. 1981. In vivo staining of cytoskeletal actin by autointernalization of nontoxic concentration of nitrobenzoxadiazole Phallacidin. J. Cell. Biol. **89**:24-32.

Bestor, T. and G. Schatten. 1981: Antitubulin Immunofluorescence Microscopy of Microtubules presents during the Pronuclear Movements of Sea Urchin Fertilization. Develop. Biol. **88**:80-91.

Cline, C. A. and G. Schatten. 1986. Microfilament during sea urchin fertilization: Fluorescence detection with Rodaminyl Phalloidin. Gam. Res. **14**:277-291.

Epel, D. 1980. Ionic triggers in the fertilization of sea urchin eggs. Ann. N. Y. Acad. Sci. 339: 74-85.

Guraya, S. S. 1982. Recent advances in the structure, origin, composition and function of cortical granules in animal eggs. Intern. Rev. Cytol. **78**:257-360.

Humphreys, W. J. 1962. Electron microscope studies on eggs of Mylitus edulis. J. Ultrastruct. Res. **7**:467-487.

Humphreys, W. J. 1967. The fine structure of cortical granules in eggs and gastrulae of Mytilus edulis. J. Ultrastruct. Res. **17**:314-326.

Longo, F. J. and E. Anderson. 1970. An ultrastructural analysis of fertilization in the surf clam Spisula solidissima. I. Polar body formation and development of the female pronucleus. J. Ultrastruct. Res. **33**:495-514.

Loosanoff, V. L. and H.C. Davis. 1951. Delayed spawning of lamellibranches by low temperature. J. Mar. Res. **10**: 197-202.

Miller, M. R.; J.J. Castellot and A. B. Pardue. 1978. A permeable animal cell preparation for studying macromolecular synthesis. Biochem. **17**:1073-1080.

Perez, C.; M. Roco; A. Castro; E. Dupre; G. Schatten and C. Barros. 1991. Localization of Microfilaments and a Tubulin-Like Protein in Crustacean (*Rhynchocinetes typus*) Spermatozoon. Molec. Reprod. Develop. **28**: 373-379.

Raven, C.P. 1970. The cortical and subcortical cytoplasm of the Lymnaea egg. Int. Rev. Cytol. 28:1-44.

Sawada, T. and G. Schatten. 1988. Microtubules in Ascidean Eggs during Meiosis, Fertilization, and Mitosis. 1988. Cell Motil. **9**:219-230.

Schatten, G. 1982. Motility during fertilization. Int. Rev. Cytol. **79**:59-163.

Schatten, G.; H. Schatten; I. Spector; C. Cline; N. Paweletz; C. Simerly and C. Petzelt. 1986. Latrunculin inhibits the Microfilaments-mediated Process during fertilization, cleavege and early development in Sea Urchin and Mice. Exp. Cell. Research **166**:191-208.

Schatten, H.; G. Schatten; D. Mazia; R. Balczon and C. Simerly. 1986. Behavior of centrosomes during fetilization and cell division in mouse oocyte and in sea urchin eggs. Proc. Natl. Acad. Sci. USA. **83**:105-109.

Schatten, G.; C. Simmerly and H. Schatten. 1985. Microtubule configurations during fertilization, mitosis and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc. Natl. Acad. Sci. USA. **82**:4152-4156.

Shroeder, T. 1981. Interrelations between the cell surface and the cytoskeleton in cleaving sea urchin eggs. In: Poste, G. and Nicolson, G. L. eds.. Cytoskeletal Elements and Plasma Membrane Organization. Elsevier/North Holland. Pp. 170-216.

Schuel, H. 1978. Secretory functions of eggs cortical granules in fertilization and development. A critical review. Gam. Res. 1: 299-382.

Thombes, R.; C. Simerly; G. Borisy and G. Schatten. 1992. Meiosis, egg activation and nuclear envelope breakdown are differentially reliant on Ca+, whereas germinal vesicle breakdown is Ca+ independent in the mouse oocyte. J. Cell. Biol. **117**:799-811.

Vacquier, V. D. 1975. The isolation of Intact cortical granules from Sea Urchin Eggs: Calcium Ions Trigger Granules Discharge. Dev. Biol. **43**:62-74.