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Short Contribution

Sexual differentiation of reproductive tissue in bivalve molluscs: identification of male associated polypeptide in the mantle of *Mytilus galloprovincialis* Lmk

ALEXANDER T. MIKHAILOV[1]*, MARIO TORRADO and JOSEFINA MÉNDEZ

Institute of Sciences of the Health and Department of Cell and Molecular Biology, University of La Coruña, La Coruña, Spain

ABSTRACT We have addressed the question of sexual reproductive tissue dimorphism in bivalve molluscs, *Mytilus galloprovincialis* Lmk, which is a stable gonochoric species although with no apparent differences in gonad morphology of both sexes. At all periods of the annual cycle the proteins specific of male/female gonads were identified. One of these proteins, "male-associated polypeptide" with apparent MW 39 kDa (MAP-39), has been biochemically and immunochemically characterized. MAP-39 concentration in male mature gonads achieved up to 10% of the total soluble protein while in female ones only traces of this protein could be detected. In male mantle, MAP-39 expression was associated with the process of gonad development and maturation as well as gamete spawning, although this polypeptide has been localized in fibroblast-like cells, membrane of follicles and connective tissue matrix of the mantle but not in germinal cells.

KEY WORDS: *bivalve molluscs, mantle tissue, sex-associated polypeptide, sexual dimorphism*

The mantle is the main site of gonad development in the bivalve mollusc *Mytilus*. The mantle tissue consists of storage cells (adipogranular and vesicular cells) which form a connective tissue matrix supporting the gonadal acini (follicles) where the differentiation and maturation of the gametes occur. The proportion of germinal (follicle) to connective tissue is variable and depends upon the stages of the annual reproductive cycle (Lowe *et al.*, 1982; Gabbott, 1983; Pipe, 1987). In *M. galloprovincialis* mussels collected in Galicia (Spain), the development of the gonad tissue begins in autumn and gametogenesis then proceeds throughout the winter, culminating in spawning in spring and early summer. Spawning may occur throughout the summer until late August or September; then mussels enter into the quiescent phase when the germinal (follicle) tissue is almost absent in the mantle (Crespo and Espinosa, 1990; Mancebo *et al.*, 1992).

The aim of this research was to identify the proteins specific of male or female mantle tissue of *M. galloprovincialis* Lmk. A low incidence of hermaphroditism (see Gosling, 1992) suggests that *M. galloprovincialis* is a stable gonochoric species, although we could not detect the sex chromosomes (Méndez *et al.*, 1990) or any external sex-specific characteristics.

It was sometimes possible to tell the sex of individuals by the color of their gonads (frequently female gonads are orange while male are creamy-white; Fig. 1a). However, we found that the color of the gonads could not be used for correct sex discrimination in *M. galloprovincialis* because pink- and orange-colored mantles have been detected not only in females but also in about 30% of males (n 61). On the other hand, about 30% of females (n 59) displayed white- and yellow-colored mantles.

The sex specific proteins/genes in *Mytilus* have not been found yet with the exception of sex difference in the mitochondrial DNA transmission (Skibinski *et al.*, 1994; Zouros *et al.*, 1994) in *M. edulis*.

We have compared the protein patterns of male/female mantle tissue during the annual cycle. The stage of the cycle and gonad conditions of each mantle sample used for electrophoretical and immunochemical analysis was assessed by stereological analyses of the corresponding second mantle lobe and calculating the percentage of the sections occupied by genital (follicle) tissue. During the process of gonad development and maturation, the follicle "fraction" was gradually increased until as much as 95% of the mantle was occupied by fully mature follicles. During the gonad involution, the area of mantle occupied by genital tissue decreased and at the stage of sexual rest the gonad was rudimentary. However, we have found that the proportion of follicle to total mantle tissue could not be used as an absolute criterion for functional maturity of the gonad. In particular, we observed that only 30-40% of mussel mantles were occupied by genital tissue

Abbrevations used in this paper: MAP, male-associated polypeptide; PEB, protein of ejaculatory bulb.

***Address for reprints:** Department of Cell and Molecular Biology, University of La Coruña, Campus de Zapateira s/n, E-15071 La Coruña, Spain. Fax: 34.81.104129.

[1]Permanent address: Koltzov' Institute of Developmental Biology, Russian Academy of Sciences, Vavilova Str. 26, Moscow 117808, Russia. FAX: 7.095.1358012.

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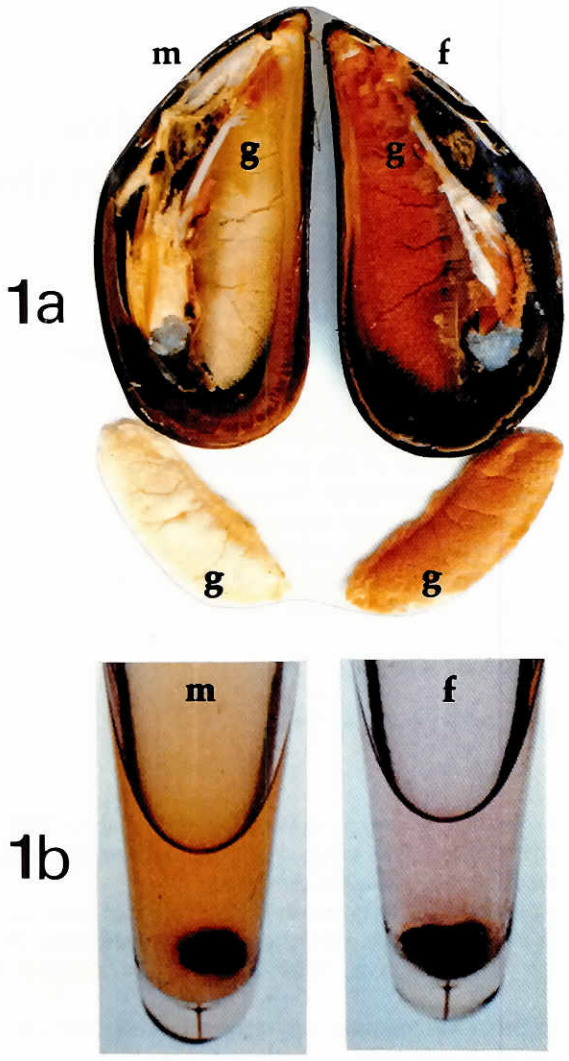

Fig. 1. Sex-dependent differences in the coloration of the mantle (gonad) tissue. (a) *"Opened" sexually matured male (m) and female (f) mussels (each valve contains the gonad and other tissues; the gills covering the gonads were removed) and isolated creamy-white and orange gonads (g).* **(b)** *Colorimetric testing: small pieces (about 20 mg) of male and female gonads* **(a)** *were treated (100°C, 20 min) with the mixture of thiobarbituric and trichloroacetic acids (Jabbar and Davies, 1987); as a result, a yellow (male) or pink (female) coloration developed.*

whereas the corresponding follicles had been filled by mature gametes. To avoid such problems, we have also determined the gonad conditions (see Seed, 1969) of each mantle sample utilized for biochemical or immunochemical analysis, by observing the gonad histology and cytological characterization of the gametes.

At each period of the annual cycle the proteins specific of male or female gonads were identified. One of these proteins with an apparent 39 kDa MW designated as "male-associated polypeptide" (MAP-39) has been studied. In male mature gonads, MAP-39 concentration could achieve up to 10% of the total soluble protein, whereas in the females its concentration either did not exceed 0.5%, or the corresponding fraction could not be electrophoretically detected (Fig. 2).

In males, the expression of MAP-39 is seasonally regulated and also depends on the stage of gonad development. The highest concentrations occurred during the reproductive period, subsequently declined to a minimum level at the stage of sexual rest (Fig. 3) and then increased again during the next round of gonadal development. In females, the same seasonal changes of MAP-39 expression were observed but its concentrations were significantly lower during all periods of the annual cycle (up to an undetectable level at the stage of sexual rest). As an electrophoretical reference, we have used the fraction with about 43 kDa MW (see Fig. 2) because its concentration was insignificantly changed in the mantle tissue during all periods of the annual cycle.

The expression of MAP-39 also depends on the presence of the mature spermatozoa in the follicles: the maximum expression took place prior to spawning and declined significantly after sperm emission. It is notable that the severe reduction of MAP-39 expression after spawning ("spent" gonads) could be observed in the mantles characterized by different "concentration" of mature germinal tissue (Fig. 4).

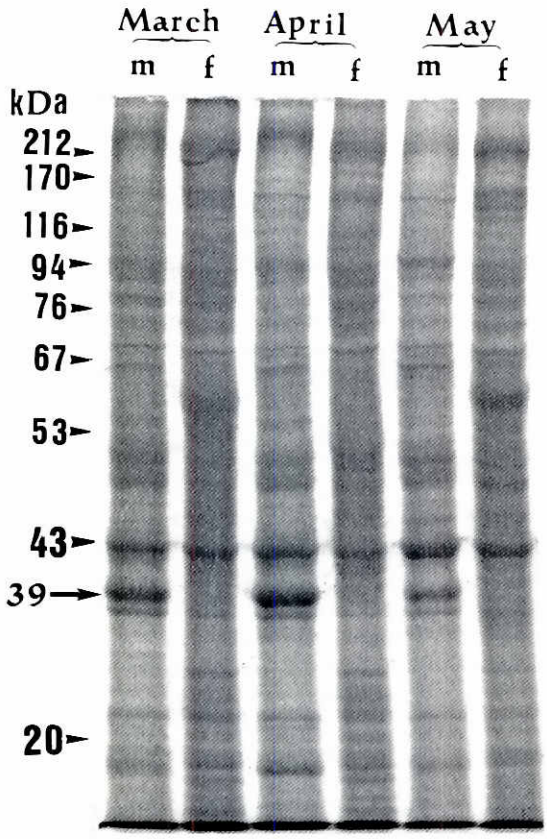

Fig. 2. SDS-PAGE showing water-soluble protein patterns of male (m) and female (f) mantle tissues. *The gonads were dissected from the animals collected in March, April and May, 1993 (period of reproduction). Follicle "fraction", % (m/f): March: 80/85; April: 90/85; May: 70/75. Arrow indicates the position of the MAP-39 fraction. In males, the variations in MAP-39 fraction intensity are connected with the differences (70-90%) in the follicle "concentration" of the mantles (see Fig. 3 and text). In females, the MAP-39 fraction is either not detected (May) or present in trace (March, April).*

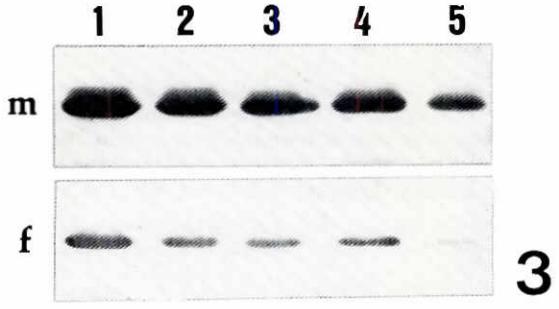

Fig. 3. Immunoblot analysis of MAP-39 expression in male (m) and female (f) mantles characterized by different concentration of genital (follicle) tissue. *Follicle "fraction", % (m/f):* **(1)** *90/85 (May, 1993);* **(2)** *70/75 (July, 1993);* **(3)** *50/45 (August, 1993);* **(4)** *40/40 (September, 1993),* **(5)** *30/30 (October, 1993). Samples of male and female mantles (5 mg of the total soluble protein per run) were subjected to SDS-PAGE using "Mini-Protean II" electrophoretic cell (Bio-Rad); after electrophoretic transfer, MAP-39 fraction was immunoassayed. In both sexes MAP-39 expression depends on the "concentration" of the genital (follicle) tissue in the mantle. However, there are significant differences in the levels of MAP-39 expression in each male/female mantle samples (higher in males) containing the same "concentration" of follicle tissue.*

Using immunofluorescence, MAP-39 has been detected in membrane of follicles, connective tissue matrix and fibroblast-like cells of the mantle but not in germinal cells (Fig. 5). We have not observed the positive immunofluorescence specific of MAP-39 in mantle adipogranular and vesicular connective tissue cells. In female gonads MAP-39 has also been found in membranes of follicles and mantle connective tissue matrix only but the corresponding immunofluorescent reactions were very weak.

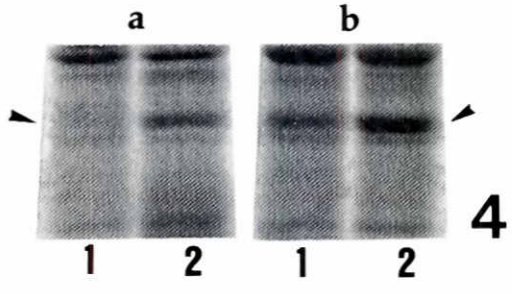

Fig. 4. Fragment of the electroproteinogram showing MAP-39 expression in male gonads after (1) and prior to (2) spawning. *Follicle "fraction", %:* **(a)** *50-60 (period of general reduction of the mantle area occupied by "mature" follicles; September, 1993);* **(b)** *80-90 (period of reproduction; the mantles are full of follicles; July, 1993). Arrows indicate the position of the MAP-39 fraction. Samples (30 μg of the total soluble protein per run) extracted from the mantles having different "concentration" of genital (follicle) tissue were subjected to SDS-PAGE using "Protean II xi" electrophoretic cell (Bio-Rad). In both variants* **(a,b)**, *the intensity of the MAP-39 fraction decreased after spawning; this effect was very strong in the sample a-1 because MAP-39 expression also depends on the "concentration" of genital (follicle) tissue in the mantle (see Fig. 3).*

It could be speculated that MAP-39 may be involved in a process of sperm release from gonadal acini. This speculation is in agreement with the fact that MAP-39 detected in *M. galloprovincialis* is characterized by immunochemical similarity with the protein of ejaculatory bulb (PEB) of *Drosophila melanogaster* (see below).

Taken together these observations suggest that the mantle tissue of *M. galloprovincialis*, in spite of its identical structure in both sexes, is sexually dimorphic at the level of protein tissue expression. We could not detect any references dealing with sex differences in the protein composition (expression) of the mantle tissue in *Mytilus*. Sex-dependent activity of the enzymes observed in the mature gonads of *M. edulis* (see Gabbot, 1983; Churchill and Livingstone, 1989) and *M. galloprovincialis* (Suteau *et al.*, 1985), with higher levels in females, may be connected with the presence of the oocytes in the gonads rather than reflect the influence of sex on mantle protein expression. MAP-39 identified in the mantle of *M. galloprovincialis* revealed the positive immunoblot reaction with antibodies against PEB of *Drosophila*

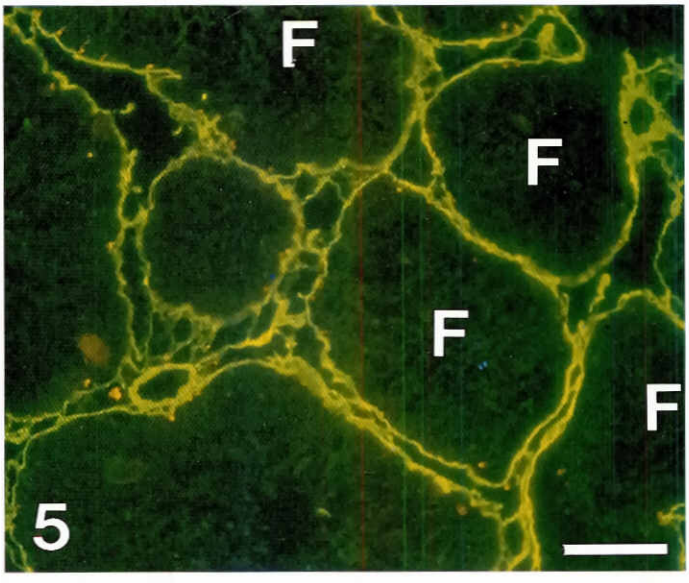

Fig. 5. Immunofluorescent localization of MAP-39 in male mantle tissue. *Follicle "fraction", %- about 90 (mature gonad, reproductive period). MAP-39 is expressed in membrane of follicles, connective tissue fibers and around the duct-like structures. There is no positive antibody staining of germinal cells into follicles (F). Bar, 100 μm.*

males (A.T. Mikhailov, L.I. Korochkin, M. Torrado, M. Kopantzeva, unpublished results). Whether PEB is structurally related to MAP-39 is not known yet. Several possible functions exist for PEB in *Drosophila* males (Ludwig *et al.*, 1993). Further research will therefore be directed towards studying the biochemical and molecular similarity between PEB and MAP-39.

Experimental Procedures

Animals were obtained from commercial suppliers of La Coruña (Galicia, Spain) and monthly determinations of the protein expression in the mantle tissue were made over one year (1993-94). One mantle lobe of each animal was used for histological examination and the other for biochemical and immunochemical analysis.

Three methods were used for sex determination: (1) microscopic examination of mantle smears; (2) colorimetric mantle test elaborated for *M. edulis* (Jabbar and Davies, 1987) which we applied to tell the sex of *M. galloprovincialis* (see Fig. 1b); and (3) histological examination of the mantle tissue.

The mantle lobes were homogenized in 100 mM Tris-EDTA buffer (pH 7.0) containing the protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml leupeptin; Sigma) and 0.01% sodium azide. After 1 h incubation on ice, debris was sedimented (20000g, 2°C, 1 h) and the resulting supernatants were used for analysis.

SDS-PAGE was performed in accordance with Laemmli (1970) using 8-15% gels which were stained with Coomassie blue. The gels were scanned by a DU-70 spectrophotometer (Beckman) and calculations including peak pick, area integration and MW determination were made. For immunoblotting, the fractions (after SDS-PAGE) were transferred onto nitrocellulose membrane (Schleicher and Schuell) and immunoreactivity in blot-strips was assessed (Mikhailov and Simirskyi, 1991) using anti-mantle sera and peroxidase labeled goat anti-rabbit immunoglobulins (Sigma) as a secondary reagent. Anti-mantle sera were raised by immunization of rabbits with 39 kDa bands cut from gels in which male mantle extracts were separated by SDS-PAGE (Mikhailov and Simirskyi, 1991). Indirect immunofluorescence of Carnoy-fixed, paraffin-embedded tissue specimens was used (Mikhailov and Simirskyi, 1991). FITC-conjugated goat anti-rabbit immunoglobulins (Sigma) were used as secondary antibodies. For histological analysis, tissues were fixed in Bouin's solution, dehydrated in ethanol, washed in xylol and embedded in paraffin. The sections (5-8 μm) were stained with hematoxylin-eosin. The area occupied by follicles (follicle "fraction") was measured using standard stereological methods (Lowe *et al.*, 1982) and expressed as a percentage of the total area of the transverse mantle sections (see Seed, 1969).

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