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REPRODUCTIVE CHARACTERISTICS OF THE ARCHAEOGASTROPOD *MEGATHURA CRENULATA*

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ABSTRACT A histological and histochemical study was performed on individuals of the archaeogastropod *Megathura crenulata* sampled in the field, in order to ascertain the fundamental features of reproductive biology in this species. Basic aspects addressed were gonad and gamete structure, nature of vitelline reserves, and composition of oocyte coat. Stereological counts and oocyte measurements were performed to obtain a quantitative assessment of the reproductive cycle from June 1999 to June 2000. No simultaneous hermaphrodites were observed. The gonad structure of *M. crenulata* consisted of traversing trabeculae from which gametes developed centrifugally. The gonads of both males and females were homogeneous, allowing reliable data to be obtained from a single histological sample of each individual. Mature gametes greatly dominated the profile throughout the study period; coated oocyte diameters were also very stable. These techniques, routinely applied to the study of reproductive cycles, did not allow the identification of spawning preparedness in this species. Vitelline reserves were dominated by non-staining (putatively lipid) vacuoles; no appreciable quantities of glycogen were observed. The oocyte coat was chiefly composed of acid mucopolysaccharides, conferring both mechanical and antimicrobial protection, as well as limiting egg and larval dispersal.

KEY WORDS: *Megathura crenulata*, Gastropoda, gonads, gametes, reproductive cycle, histochemistry

INTRODUCTION

Many marine natural products present biological activity in humans, and are used in medical testing or in pharmaceuticals (Munro et al. 1987, Ireland et al. 1989, Kobayashi et al. 1989, Suffness et al. 1989, Faulkner 2000). The archaeogastropod *Megathura crenulata* is a keyhole limpet native to the California Pacific Coast, with a reported range from Mendocino County (40°N; 124°W) to Isla Asuncio (27°N; 115°W). Keyhole limpet hemocyanin (KLH) of this organism is used in the treatment of certain forms of bladder cancer (Harris & Markl 1999, 2000). It is thus of considerable medical and economic interest to rear this organism for extraction and purification of the active compound. This goal requires knowledge concerning essential aspects of *M. crenulata* biology, notably feeding, growth, and reproduction.

The sparse studies on reproduction in *M. crenulata* chiefly concern modes of fertilization, which is external and involves several hormonal agents (Tyler 1939, Webber 1977). The lack of data on the reproductive biology of *M. crenulata* may be contrasted with the relatively abundant information concerning the highly-prized abalones (*Haliotis spp*), which are commercially important, edible archaeogastropods (Newman 1967, Purchon 1968, Fretter 1984, Hahn 1989). The present study documents the gonad structure, oocyte histochemistry and reproductive cycle of *M.crenulata* in its natural habitat, with a view toward the long-term objective of cultivation.

MATERIAL AND METHODS

Specimen Collection and Dissection

The animals used in the present study were obtained from June 1999 to June 2000 from the subtidal zone off Long Beach, California (33°45′N, 118°10′W). The specimens were sent by air to France, where they were dissected and fixed in the SMEL (Syndicat Mixte pour l'Equipement du Littoral) laboratory in Blainville, Normandy. Five individuals were dissected each month, except in October 1999, when no sampling was possible. In order to verify the structural homogeneity of the gonad, the entire organ was removed in certain individuals of certain months, and several regions were examined (Table 1): a median region (M), a distal extremity (DE) and a proximal extremity (PE). For samples from February, May, and June 2000, tissue pieces were obtained from labelled L, M and R regions, allowing the distinction of left (L), median (M), and right (R) regions of the gonad. The types of histological sample are summarized in Table 1 for each individual and each sampling date.

Histological Techniques

In most cases, entire gonads were fixed in aqueous Bouin's solution. In an effort to improve fixation, small pieces of the gonad were fixed in March and April; however, the resulting sections showed that this provoked extensive leakage of gametes, such that the sections could not be used for males in either month, and for females in March. From February, May, and June 2000, in which the dissections allowed the distinction of three different regions of the gonad, histological samples were directly fixed. After rinsing for a minimum of 10 h and dehydration in an ascending ethanol–Bioclear® series, the biopsies were embedded in paraffin and sectioned at 7 μm.

Several staining protocols were developed for this study, based on Martoja and Martoja-Pierson (1967), Gabe (1968) and Vacca (1985), as indicated in Table 2. A modified Masson's trichrome protocol using fast green, trioxyhematein, and acid fuschin allowed the topological study of the gonads, distinguishing connective tissue, oocyte coat, cytoplasm, nucleus, chromatin, and nucleolus. Periodic acid–Schiff reagents with positive (oyster digestive gland) and negative (amylase-digested sections of gonad) controls were used to determine the eventual presence of glycogen in the oocyte cytoplasm, as well as neutral mucopolysaccharides (NMPS) in the oocyte coat. Alcian blue was used to determine the presence of acid mucopolysaccharides (AMPS) in the oocyte coat.

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TABLE 1.

Megathura crenulata. Summary of histological sample types for males and females examined.

Date	Males	Male gonad regions	Females	Female gonad regions
06/99	–	–	1	I
	–	–	2	M,P, D
	–	–	3	I
07/99	1	I	1	I
	2	M,P, D	2	I
	3	I	–	–
08/99	1	M,P, D	1	M,P, D
	2	M,P, D	2	I
	3	I	–	–
09/99	1	I	1	M,P, D
	2	I	2	I
	–	–	3	M,P, D
10/99	–	–	–	–
11/99	1	I	1	I
	2	I	2	I
	–	–	3	M,P, D
12/99	1	I	1	I
	2	I	2	I
	–	–	3	M,P, D
01/00	1	M,P, D	1	I
	2	I	2	M,P, D
	3	I	–	–
02/00	1	R, L,M	1	R, L,M
	2	R, L,M	2	R, L,M
	–	–	3	R, L,M
03/00	–	–	–	–
04/00	–	–	1	R, L,M
05/00	1	R, L,M	1	R, L,M
	2	R, L,M	2	R, L,M
	3	R, L,M	–	–
06/00	–	–	1	R, L,M
	–	–	2	I
	–	–	3	R, L,M
	–	–	4	I

I: undetermined gonad region; M: median gonad region; P: proximal region of non-oriented gonad (organ previously removed from individual); D: distal region of non-oriented gonad; R: right region of oriented gonad; L: left region of oriented gonad.

Stereology

Stereological counts were performed in order to quantify the proportions of the different tissue types in the histological sections; variations in the proportions of the different tissue categories thus reflected variations in the different phases of reproductive activity (Weibel et al. 1966, Briarty 1975, Beninger 1987, Morvan & Ansell 1988, Pazos et al. 1996, Mayhew 2000); counts were performed on surfaces of measured area, using a 9 × 9 point matrix on a microscope projector.

In males, four tissue categories were identified for stereological purposes: trabecular tissue, developing gametes, mature gametes, and unoccupied space. In females, three tissue categories were identified: coated oocytes, non-coated oocytes, and trabecular tissue. Due to the loose nature of the female gonad tissue, it was impossible to determine whether observed unoccupied spaces were real or artefacts of gonad dissection. Stereolological counts were therefore performed only on areas without visible unoccupied space.

Nine counts were performed for each individual and region of the gonad, and the means (with 95% confidence intervals) of all male and female counts were plotted.

Oocyte Diameters

Oocyte diameters were measured for 30 coated oocytes for each female and for each gonad region, using a calibrated optical micrometer. In order to standardize the measurements, only oocytes in which the nucleolus was visible (approximately the center of the cell) were selected. The evolution of oocyte diameters could then be recorded throughout the year.

RESULTS

Males

Gonad Structure

The general structure of the male gonad is presented in Figure 1 area 1. The gonad was composed of connective tissue trabeculae, from which arise centrifugally the germ cells, first visible as developing aflagellate gametes, and then the mature flagellate spermatozoa. The mature spermatozoa occupied the majority of the sectional area (Fig. 1 area 1, Fig. 2).

Stereology

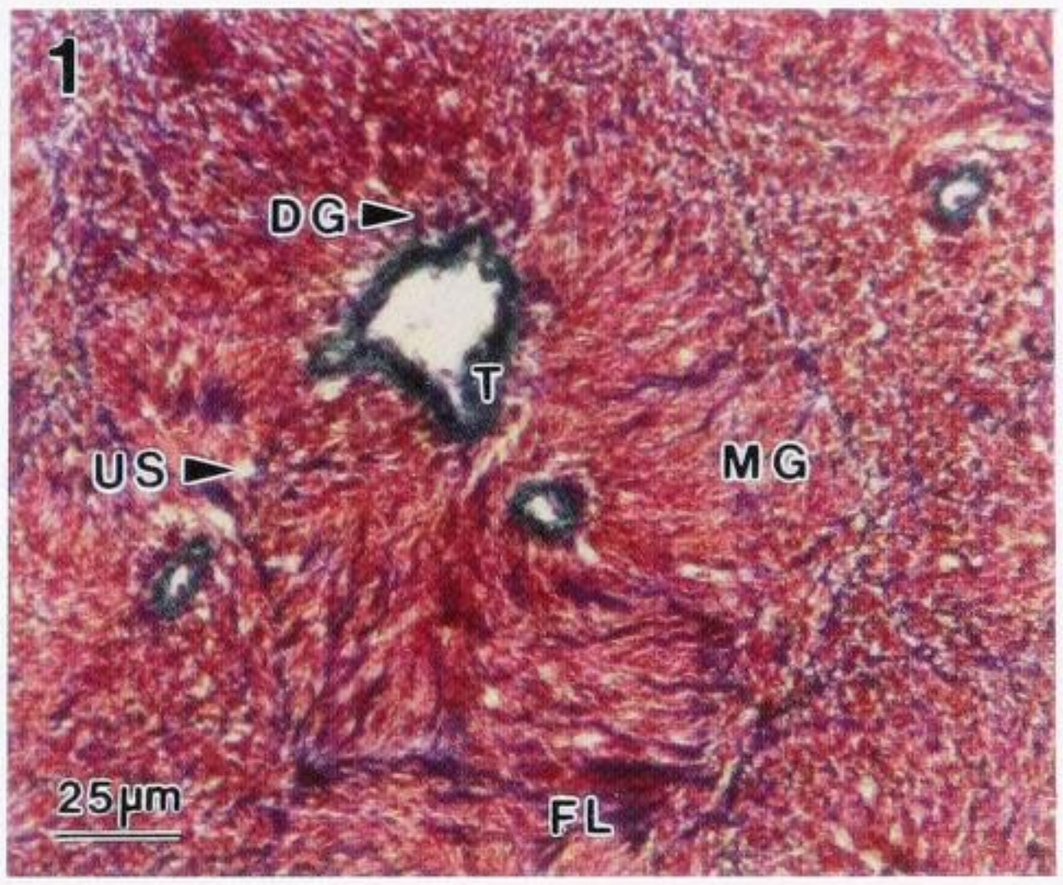
Testicle homogeneity was verified in 1 to 3 individuals from July, August 1999 and January, February and May 2000, using stereological counts. The results shown in Fig. 2 indicate that the male gonad was structurally homogeneous and gametogenetically synchronous. Reliable data for histological study may therefore be obtained from only one histological sample per individual.

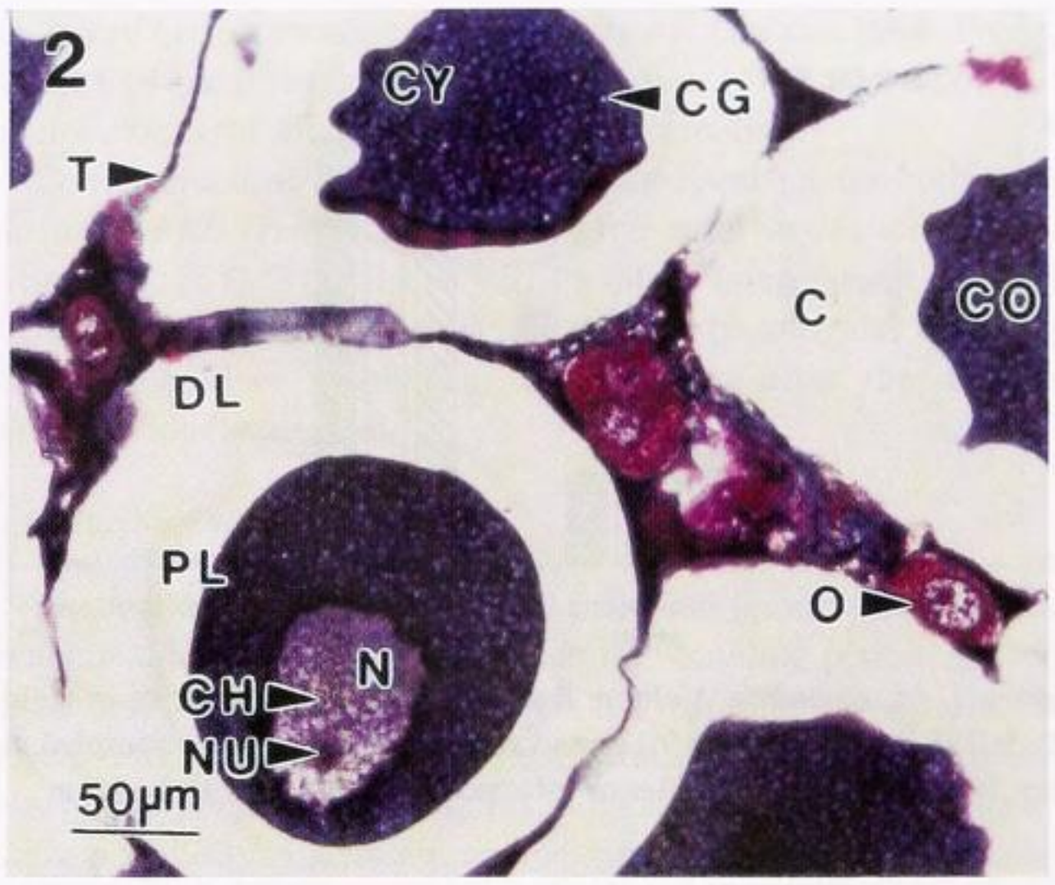
The male gonad presented a stable histological profile throughout the study period from July 1999 to June 2000. Mature spermatozoa occupied almost the entire gonad, with mean volume fractions of 0.77 (May 2000) to 0.88 (November 1999). In comparison, developing gametes occupied a low proportion of the gonad, from 0.095 (November 1999) to 0.16 (July 1999). Trabec-

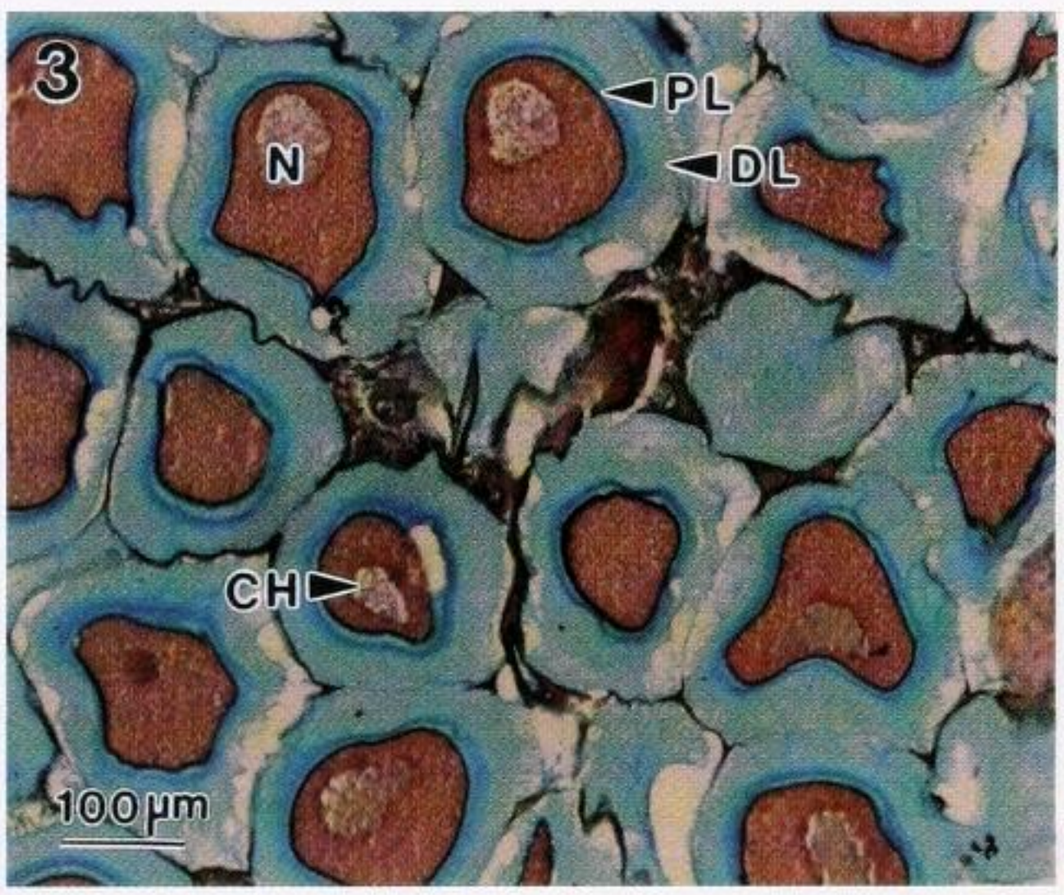
TABLE 2.

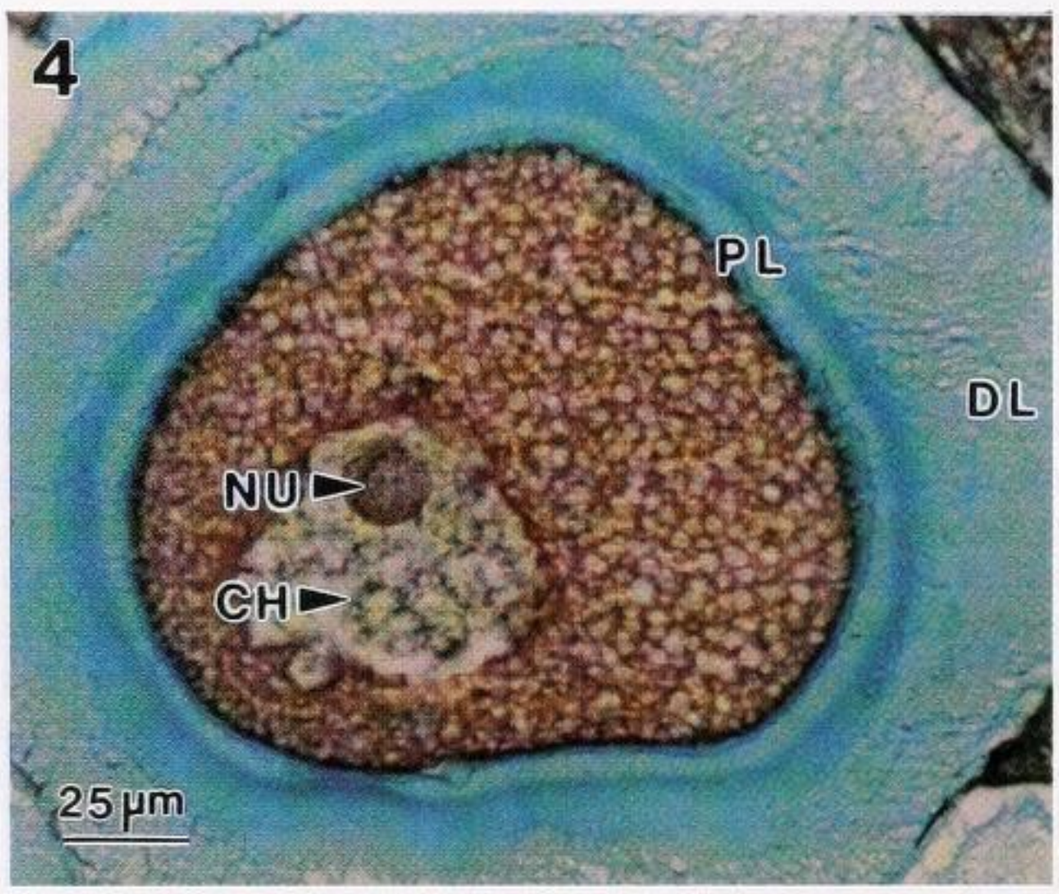
Summary of stains used, and celullar and molecular components targeted.

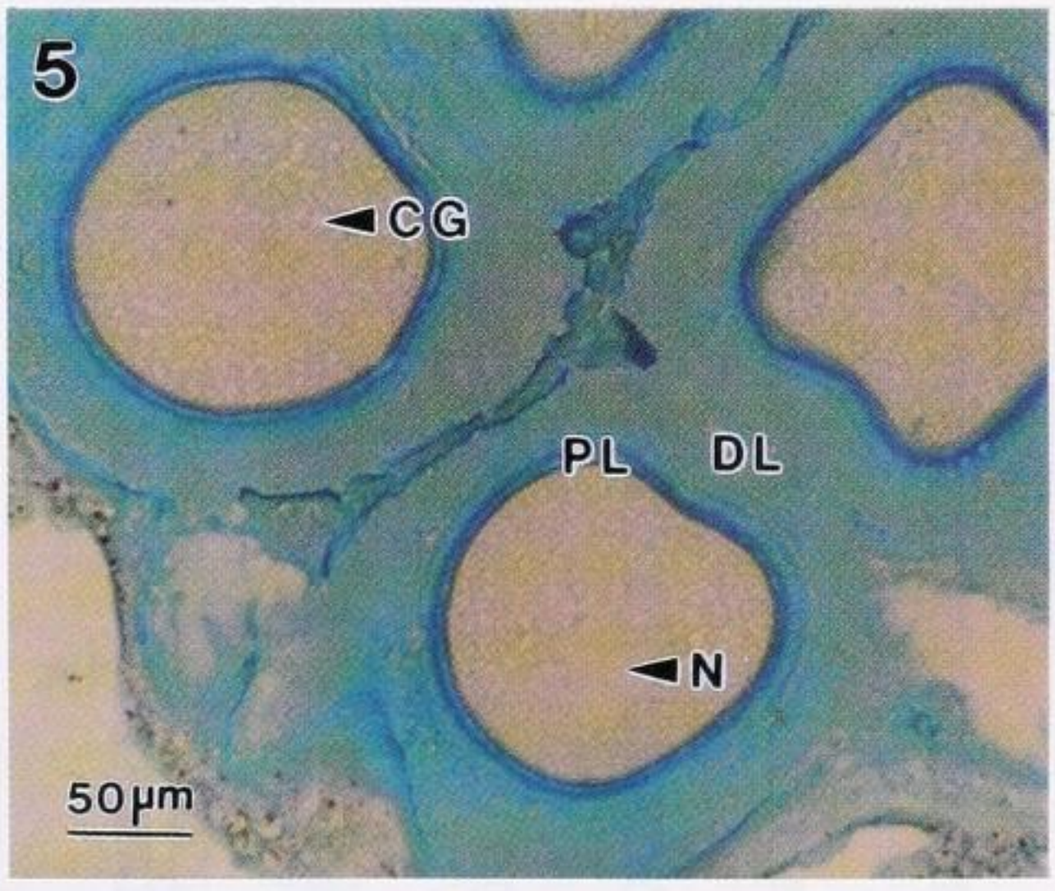
Stain used	Structures and molecules targeted	Cellular and tissue constituents
Acid fuschin	Cytoplasmic granules	Cytoplasmic granules
Fast green	Reticulate fibres, collagen	Connective tissue
Trioxyhematein	Nucleic acids	Nucleus, nucleolus and chromatin
PAS-alcian blue	Neutral and acid mucopolysaccharides; glycogen	Oocyte coat Possible glycogen











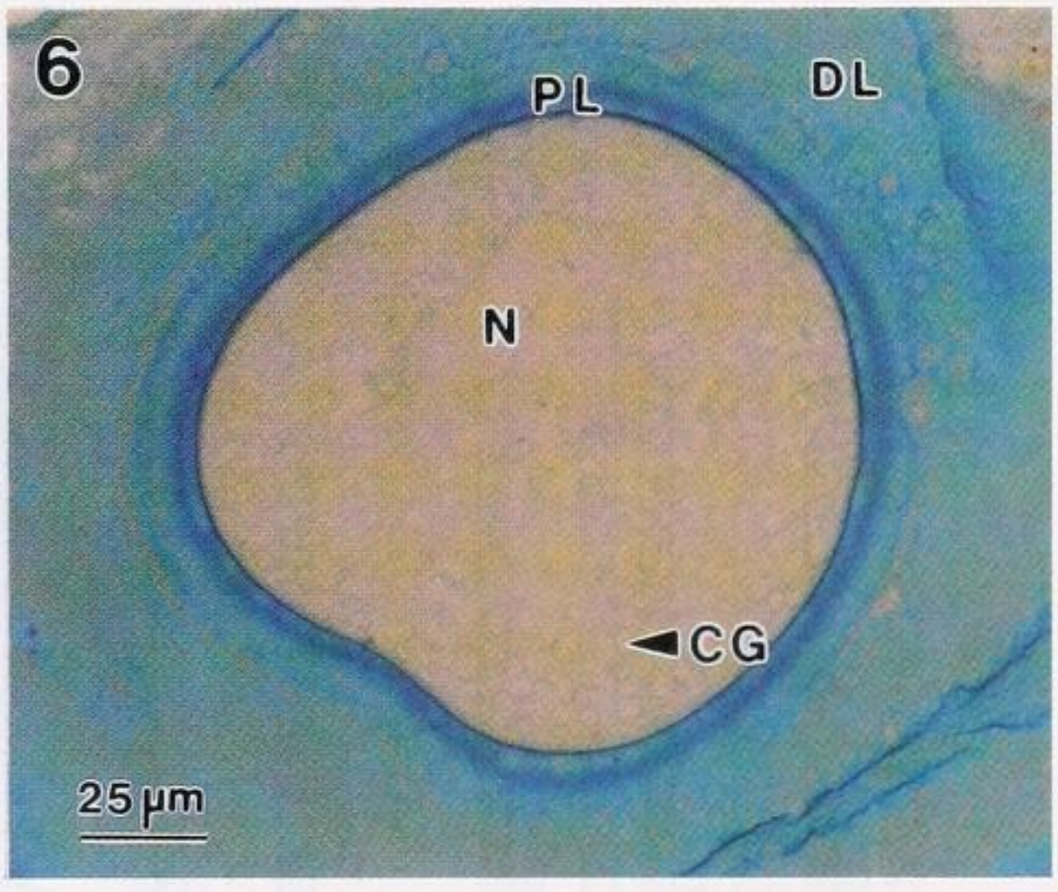


Figure 1. *M. crenulata* **gonad. Photomicrographs of paraffin-embedded sections. Area (1) Histological section of male gonad. Modified Masson's trichrome protocol. T: trabecular tissue; DG: developing gametes; MG: mature gametes; FL: flagella; US: unoccupied space. Area (2) Histological section of female gonad. Modified Masson's trichrome protocol. N: nucleus; T: trabecular tissue; CO: coated oocyte; O: non-coated oocyte; C: coat; NU: nucleolus; PL: proximal layer of oocyte coat; DL: distal layer of oocyte coat; CY: cytoplasm; CH: heterochromatin; CG: unstained cytoplasmic globule. Area (3, 4) Female gonad. Trioxyhematein and alcian blue stains. DL: distal layer of oocyte coat; PL: proximal layer of oocyte coat; N: nucleus; NU: nucleolus; CH: heterochromatin. Area (5, 6) Female gonad. PAS-alcian blue protocol. N: unstained nucleus; DL: distal layer of oocyte coat; PL: proximal layer of oocyte coat; CG: cytoplasmic globule.**

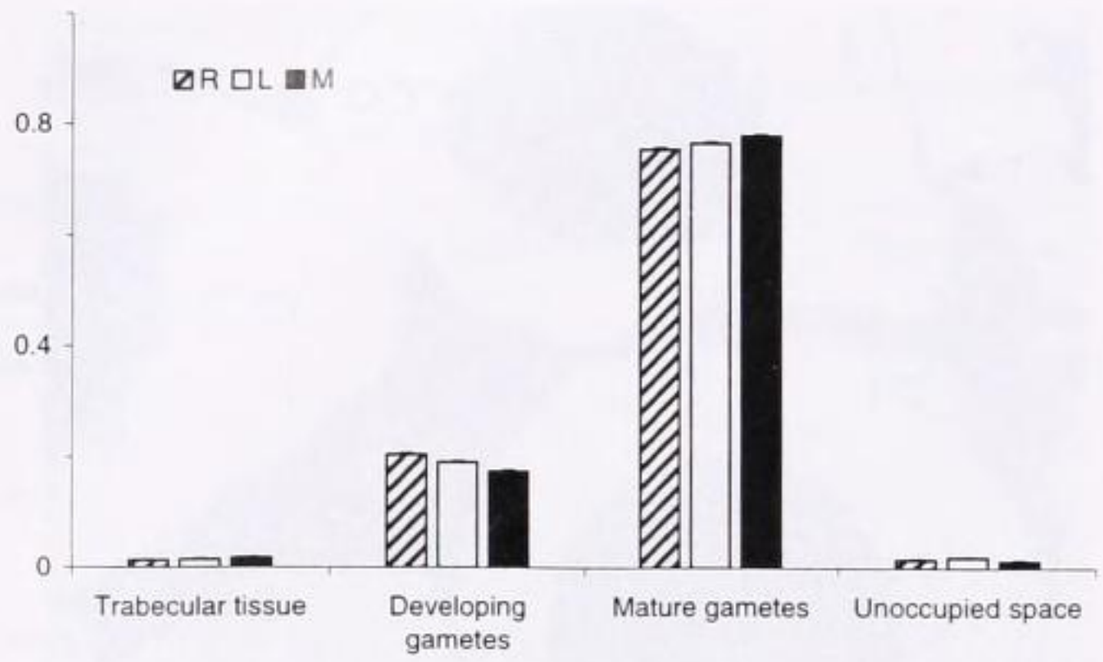


Figure 2. ***M. crenulata.*** **Volume fractions of tissue categories in right (R), left (L), and median (M) gonad regions of three males sampled in May 2000. The 95% confidence intervals are too small to be seen.**

ular tissue occupied a low and stable proportion of the testicle (approximately 0.03). Unoccupied space was rare (Fig. 3).

Females

Gonad Structure and Oocyte Histochemistry

The structure of the female gonad is shown in Figure 1, area 2. Trabeculae consisted of connective tissue (Fast Green positive) and were often masked by the acid fuschin. Uncoated oocytes adhered to the trabecular tissue and were small (approximately 25 μm) their cytoplasm was less intensely stained compared to coated oocytes. Unstained regions of trichrome-stained sections corresponded to the oocyte coat, which like the oocytes themselves appeared to be of constant dimension when the sectional plane passed through the nucleus. The cytoplasm of coated oocytes stained intensely with both trioxyhematein and acid fuschin, indicating the presence of numerous cytoplasmic globules. Many unstained globules (poorly visible in photographs due to the great staining heterogeneity of the sections) were present in the cytoplasm of the coated oocytes (Fig. 1 areas 2, 5, 6). Of the major biochemical tissue constituents, Masson's trichrome does not stain lipids (which are extracted during section preparation) or AMPS; as the globules were not alcian-blue positive (Fig. 1 areas 3–6), they were very probably lipid in nature.

The intensity of the cytoplasmic staining obscured the nucleus of those oocytes for which the sectional plane did not pass through the nucleus. When visible, the large nucleus (approximately 75 μm) presented dispersed heterochromatin and a single nucleolus (Fig. 1 area 2).

The oocyte coat appeared to be composed of two layers: a high-density proximal layer and a lower-density distal layer (Fig. 1 area 2). Staining with alcian blue confirmed this structure, and identified the principal coat constituent as AMPS (Fig. 1 areas 3–6). Counterstaining with trioxyhematein was extensive in the cytoplasm, indicating the presence of large quantities of nucleic acids, suggesting considerable anabolic activity (Fig. 1 areas 3, 4). The negative PAS reaction indicated both an absence of appreciable quantities of glycogen in the oocytes, and an absence of NMPS in the oocyte coat (Fig. 1 areas 5, 6).

Stereology

Ovarian homogeneity was verified in June, August, September, November and December 1999, and in February, April, May and June 2000. The near-identical volume fractions for the three identified ovary regions demonstrated the histological homogeneity of this organ (Fig. 4). These data confirm that the female gonad was structurally homogenous and gametogenetically synchronous. As was the case for the male gonad, representative histological data may thus be obtained from a single histological sample per individual. Similarly, the female gonad showed a stable tissue profile throughout the sampling period. Coated oocytes represented the great majority of the mean volume fraction (Fig. 5), from 0.83 (June 1999) to 0.94 (November 1999). Uncoated oocytes represented a small mean volume fraction, from 0.01 (November 1999) to 0.04 (June 1999). The mean volume fraction of trabecular tissue was also small: 0.035 (May 2000) to 0.12 (June 1999).

Oocyte Diameters

Given the homogeneity of the ovary, oocyte diameters were pooled for all females of a given sampling date. Mean diameters varied only slightly, from 125 to 135 μm without the coat (Fig. 6).

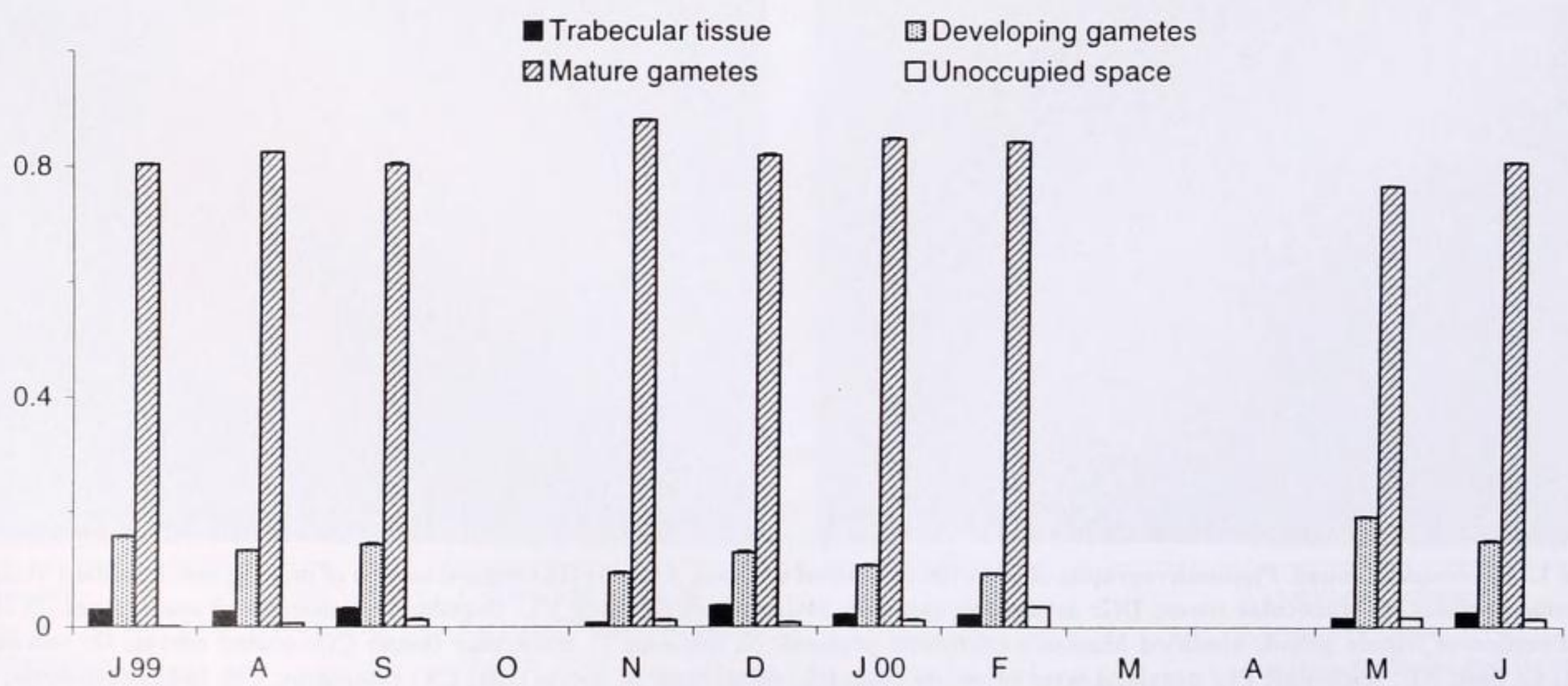


Figure 3. ***M. crenulata.*** **Evolution of tissue volume fractions in males, July 1999 to June 2000. The 95% confidence intervals are too small to be seen. Data unavailable in October, March, and April due to sampling difficulties.**

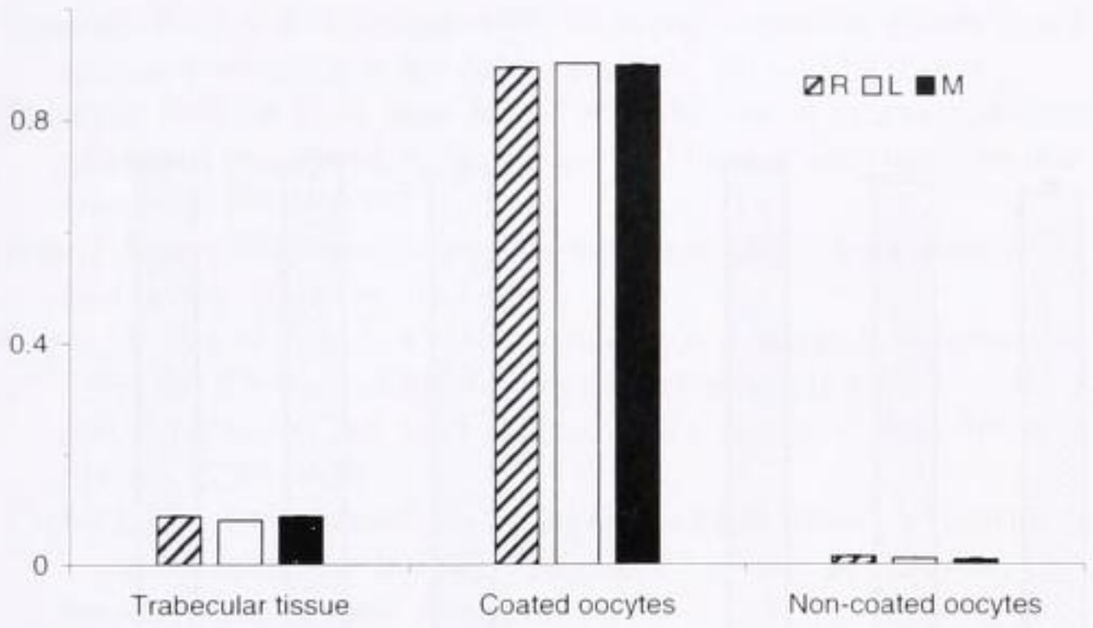


Figure 4. ***M. crenulata.*** **Volume fractions of tissue categories in right (R), left (L), and median (M) gonad regions of three females sampled in February 2000. The 95% confidence intervals are too small to be seen.**

This difference was not statistically significant (parametric ANOVA, normality and heteroscedasticity verified, $P \leq 0.05$).

DISCUSSION

To our knowledge, the results of the present study constitute the first report on the gonad structure, reproductive cycle and oocyte histochemistry in *Megathura crenulata.* A much more abundant literature exists for the commercially exploited archaeogastropods of the family Haliotidae, to which frequent reference will be made.

Gonad Structure

The gonad structure of *M. crenulata*, with gametes developing centrifugally from traversing trabeculae, resembles the well-known example of the Haliotidae (Newman 1967, Young & DeMartini 1970, Cochard 1980). In all of these cases, no ciliated evacuating ducts were observed; gametes are presumably expulsed via contractions of the gonad tegument, as is in *H. midae* (Newman 1967).

No simultaneous hermaphrodites were observed in any of the specimens studied; the dominant possible sexual modes for *M. crenulata* are therefore either gonochoric or successive hermaphrodite. Most prosobranchs are gonochoric, but there are a small number of hermaphroditic species (Fretter & Graham 1964, Fretter 1984). Complete resolution of this question in *M. crenulata* will require extensive sampling and long-term rearing.

Gonad structure was shown to be homogeneous for both male and female *M. crenulata*, as is also the case for *Haliotis midae* (Newman 1967). This result will facilitate future studies on the gonad of this species. By standardizing the histological sampling zone, it should be possible to reduce even further the residual inter-individual variation.

Reproductive Cycle

The marked stability of both the male and female *M. crenulata* gonad histological profile throughout the sampling period, as well as the uniform oocyte size, preclude the use of either criterion in determining gamete maturity, or even the state of spawning readiness of the gonad. The stable histological profile raises an interesting possibility: spawning readiness may depend on fine-tuning oocyte reserves rather than on synchronizing protracted periods of vitellogenesis. This is in contrast to the situation in the Haliotidae. A bimodal oocyte size distribution was observed in *H. midae* (Newman 1967); in *H. roei*, three oocyte maturation stages, characterized by different diameters, were observed (Shepherd & Laws 1974). Several oocyte sizes co-exist in *H. tuberculata*, with progressive growth from January to mid-July (Cochard 1980). Similarly, marked changes in histological profile characterize the reproductive cycle of *H. midae* males (Newman 1967). Whereas mature spermatozoa were found in *H. rufescens*, this appeared to correspond to a lack of variation in gonad indices and therefore year-round dribble spawning (Young & DeMartini 1970). Similarly, *H. asinina* synchronously spawns every two weeks on the Southern Great Barrier Reef (Jebreen et al. 2000). In the present case, the gonad appears ready to spawn at any moment in the reproductive cycle. The true state of gamete maturity must therefore be ascertained by other means, as suggested below.

A crude indicator of spawning readiness could involve determination of a gonosomatic index (DeVlaming et al. 1982); indeed, despite the uniform histological profile, considerable variations in gonad volume were observed over the sampling period in the few individuals dissected for histological processing. However, such a

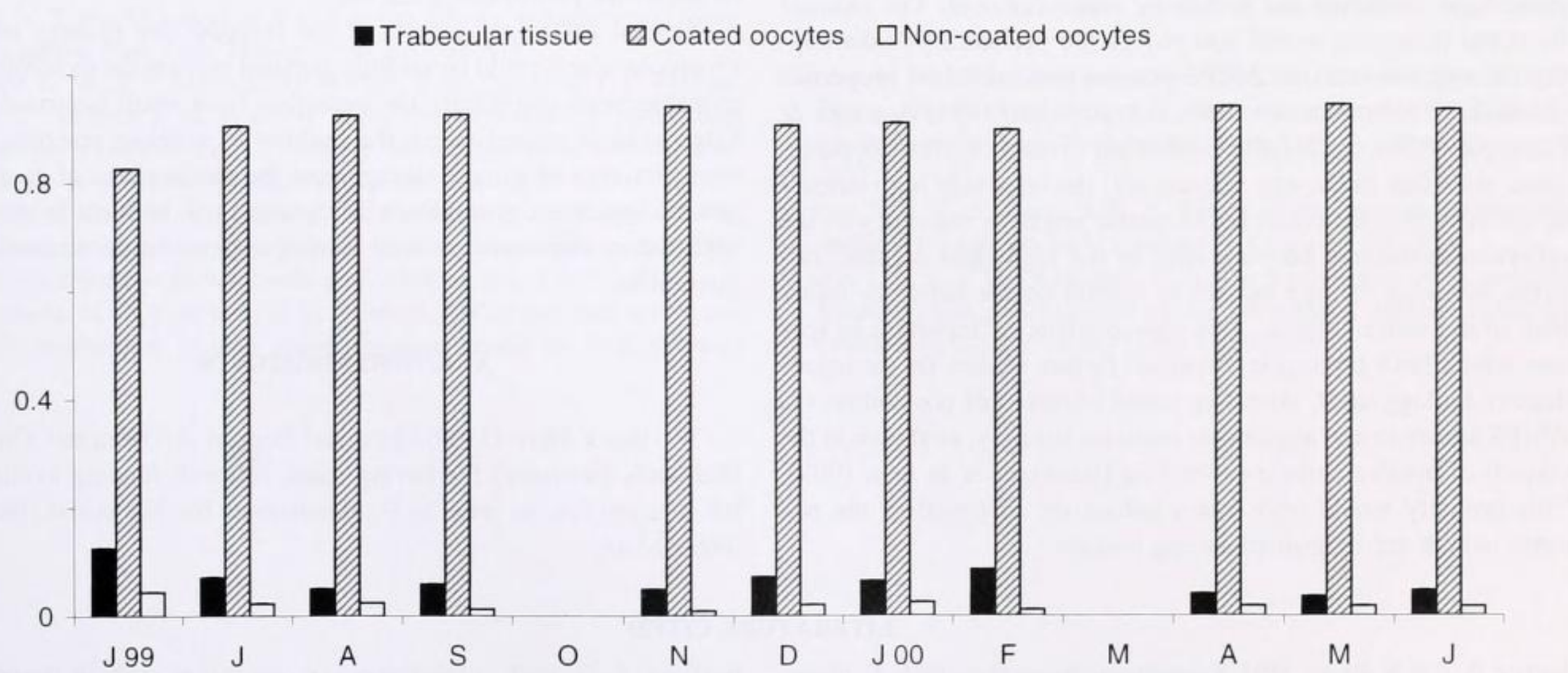


Figure 5. ***M. crenulata.*** **Evolution of tissue volume fractions in females, June 1999 to June 2000. The 95% confidence intervals are too small to be seen. Data unavailable in October and March due to sampling difficulties.**

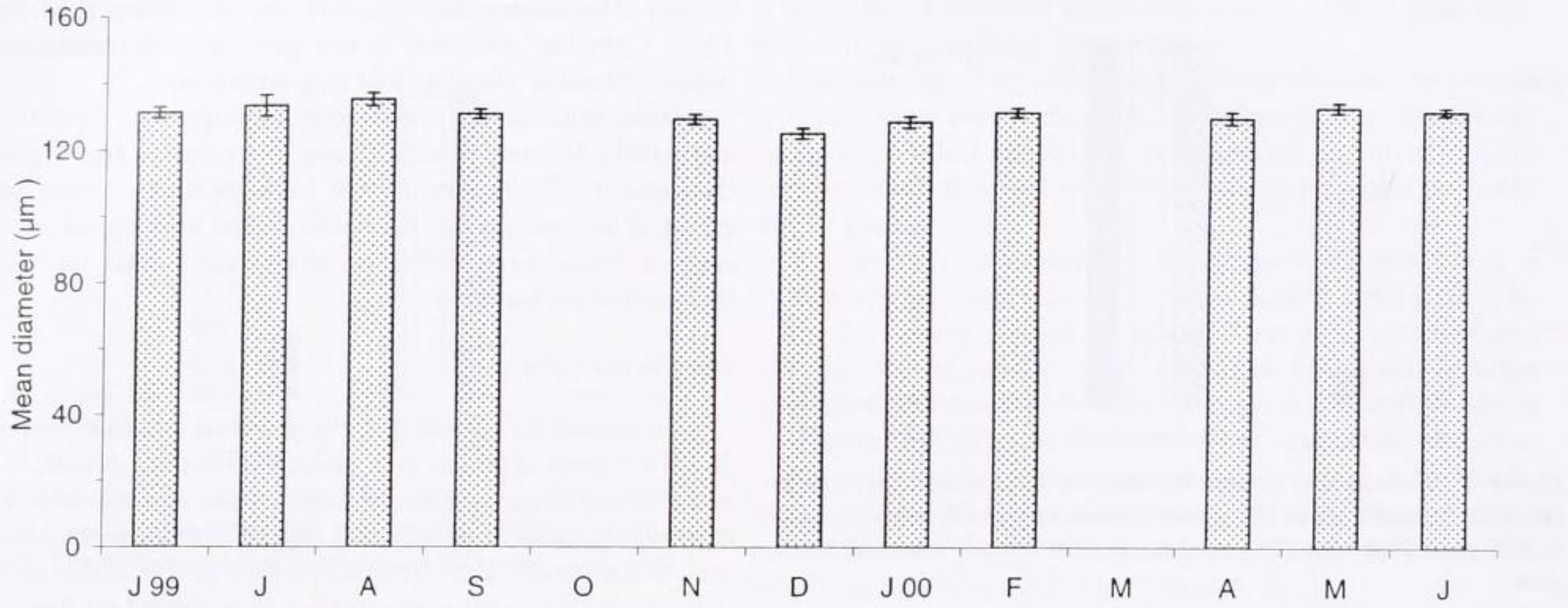


Figure 6. Evolution of *M. crenulata* coated-oocyte mean diameters (measured without their coats) from June 1999 to June 2000. Vertical bars are 95% confidence intervals. Data unavailable in October and March due to sampling difficulties.

technique requires the sacrifice of relatively large numbers of animals and is therefore not feasible in the context of the commercial exploitation of this species reared in captivity for repeated extraction of valuable hemolymph.

Oocyte Histochemistry

The presence of an oocyte coat, observed in mature oocytes of *M. crenulata*, is typical of archaeogastropods (Newman 1967, Cochard 1980). In gastropods with coated oocytes, gamones intervene in the modification of the coat to permit fertilization (Fretter & Graham 1964, Webber 1977, Fretter 1984). The results of the present study establish AMPS as a dominant component of the oocyte coat in *M. crenulata*. AMPS possess several chemical and mechanical properties which may confer important advantages to the oocytes: (i) Due to the high viscosity of AMPS (Beninger & St-Jean 1997, Davies & Hawkins 1998), they provide mechanical protection to the oocytes; (ii) AMPS reduce frictional resistance, thus allowing better water movement (Hoyt 1975, Daniel 1981, Davies & Hawkins 1998) over the egg masses, and hence improved gas exchange and metabolic waste removal. The reduced frictional resistance would also reduce the probability of dislodging the egg masses; (iii) AMPS possess anti-microbial properties (Sasikala & Subramoniam 1987, Subramoniam 1991, Beninger & Larocque 1998), potentially conferring protection from opportunistic microbes in the egg masses; (iv) the relatively high density of the AMPS coats could act to confer negative buoyancy to the otherwise positively buoyant (due to the high lipid content) oocytes, allowing the egg masses to remain on the substrate rather than in the water column. This characteristic is important in species which limit propagule dispersal; further studies on the reproductive biology of *M. crenulata* could address this possibility; (v) AMPS adhere to and agglutinate particles strongly, as shown in the context of bivalve particle processing (Beninger & St-Jean 1997). This property would once again reduce the dispersal of the oocytes, which are oviposited as egg masses.

The lack of positive PAS staining in the oocyte cytoplasm eliminates the possibility of glycogen as a reserve in the oocytes of this species. The chief oocyte reserve appears to be lipid (visible as clear globules in the trichrome-stained sections), as is the rule in the Mollusca (Gallager & Mann 1986, Lucas et al. 1986, Caers et al. 1999, Lu et al. 1999). Large lipid reserves have been reported in the ovaries of both *Haliotis* and *Megathura* genera (Webber 1977).

Although histological examination is a well-established technique for the detailed documentation of reproductive cycles (Webber 1977, Beninger 1987, Barber & Blake 1991), the results of the present study show that this approach, while very useful for elucidating other aspects of the reproductive biology of *M. crenulata*, cannot be used to follow and pinpoint spawning preparedness in this species. However, the eventual rearing of *M. crenulata* will require this information; even more desirable would be a non-destructive technique of monitoring the reproductive status of broodstock. Such biological monitoring of broodstock could lead to increased fertilization success, and hence increased production of adults for pharmacological use.

Several additional aspects of the reproductive biology of *M. crenulata* which could be usefully pursued include the dynamics of gametogenesis (especially the transition from small uncoated oocytes to large coated ones), the buildup of vitteline reserves, the characteristics of gamete storage, and the mechanisms of gametogenetic synchronization. Such information will be most helpful in both the management of wild stocks, and in future aquaculture operations.

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