environmental conditions. Reproductive studies of haliotids from around the world have shown a wide range of reproductive patterns both between and within species (Hahn, 1992). Reviews of spawning seasons by Boolootian *et al.* (1962), Shepherd & Laws (1974) and Hahn (1989) show great variability of spawning season. The temperate abalone *Haliotis rufescens* Swainson, was found to be filled with mature gametes and capable of spawning throughout the year in the southern part of its geographical range, while spawning only occurred in the warmest summer months in the northern part of its range (Boolootian *et al.*, 1962; Young & DeMartini, 1970; Giorgi & DeMartini, 1977). *Haliotis cracherodii* Leach around Pacific Grove, California, had a spawning period from June to October (Boolootian *et al.*, 1962), whereas in *Haliotis discus hannai* Ino in Japan, spawning period took place around August to late September (Tomita, 1967, 1968; Mugiya *et al*, 1980; Yahata & Takano, 1970). *Haliotis iris* Gmelin in New Zealand exhibits spatial variability in its reproductive cycle. Studies by Poore (1973), Sainsbury (1982) and Wilson & Schiel (1995) in the South Island demonstrated variability between localities and years, but with a general pattern of spawning in late summer to autumn (February or March to April). Hooker & Creese (1995) reported two major spawnings in *H. iris* in the North Island, June-July and September-October, with a minor spawning in February-March. In *Haliotis diversicolor diversicolor* Reeve from Taiwan, the spawning period occurred during September to October (Takashima *et al.*, 1978).

Among abalone species found in Thailand, a preliminary study of *Haliotis varia* Linnaeus around Bon Island, Phuket Province showed that spawning occurred at several intervals throughout the year during January-February, April-May, June-July and November-December (Bussarawit *et al.*, 1990). The gametogenic cycle was also studied in another species, *Haliotis ovina* Gmelin, at Khangkao Island, Chonburi Province (Jarayabhand *et al.*, 1994), in which the spawning period occurred between June and November. Singhagraiwan & Doi (1992) studied the spawning pattern of *Haliotis asinina* Linnaeus in captivity. Based on observations of the monthly spawning frequency, the major spawning period of wild broodstock, *H. asinina* occurred in October, while the pond-reared broodstock could spawn from September to November and January. However, they did not study the gametogenic processes or reproductive cycle. Therefore one of the aims of the present study was to investigate the gametogenic processes and their possible cyclical pattern during different months of the year in *H. asinina* from their natural habitat at Samed Island, Rayong Province and from a land-based culture system. In addition, the development of the gonad was also studied. The knowledge gained from the present study could be applied in the induction

of spawning and seed production for the possible improvement of the aquaculture system of this abalone species.

MATERIALS AND METHODS

Collection of abalone

Adult *H. asinina* were collected by SCUBA diving from the coral reefs at Tien Bay, Samed Island, Rayong Province. The abalone from a land-based culture system were provided by the Coastal Aquacultrue Development Center, Prachuap Khiri Khan Province and by the Marine Biological Station, Chulalongkorn University, Chonburi Province. They were reared in raceways or concrete tanks which were well flushed with mechanically circulated water and an air delivery system to maintain a stable and controlled environment. Sea water was pumped directly from nearby bays and filtered before use. The optimum level of salinity was about 22.5-32.5 ppt. They were fed with a diet of macroalgae, *Gracilaria fisheri* (Xia et Abbot) Abbott, Zhang et Xia, and supplemented with artificial food for abalone.

The abalone were collected monthly for a period of one year (January - December 1997). Shell length, total live weight, and sex of these abalone were recorded individually. In order to determine the reproductive stages of the gonad, two techniques were used : determination of gonad index and histological study.

Determination of gonad index

The gonad index was based on the following equation (Poore, 1973).

$$GI = (GA \times 100) / TA$$

where GI was gonad index; GA was gonad area measured by a planimeter; and TA was the total area of the section (also measured by planimeter).

Histological study

Abalone were anesthetized in 5% magnesium chloride for one hour before the gonads were cut and fixed in Bouin's fluid overnight. They were washed in 70% ethanol and dehydrated in a graded series of ethanol. Then they were cleared with dioxane, infiltrated and embedded in paraffin. Specimens were sectioned at 5 μm in thickness, and stained with hematoxylin-eosin and Periodic Acid Schiff (PAS) and hematoxylin. They were observed under an Olympus Vanox light microscope.

Developmental study

Juvenile abalone aged 1-13 months were obtained from the land-based culture system. At least ten abalone (five males and five females) from each age group were used. They were anasthesized and the tissues of hepatopancreas and gonads were separated and processed for light microscopy as previously described.

RESULTS

Size range of male and female

A total of 173 *H. asinina* were collected from their natural habitat. There were 100 males and 73 females (Table 1). Among males, the size range in shell length was 58.1 ± 18.6 - 85.0 ± 7.6 mm, and that in total weight was 78.1 ± 35.4 - 147.5 ± 33.0 g (Table 1). Among females, the size range in shell length was 60.4 ± 17.1 - 82.6 ± 6.1 mm, and that in total weight was 64.4 ± 32.4-134.9 ± 25.3g (Table 1).

From the land-based culture system, a total of 189 *H. asinina* were collected. There were 116 males and 73 females (Table 2). Among males, the size range in shell length was 49.3 ± 4.4 - 61.4 ± 8.3 mm (Table 2), and that in total weight was 53.4 ± 1.0 - 61.6 ± 2.9g (Table 2). Among females, the size range in shell length was 51.2 ± 8.6 - 61.3 ± 12.0 mm, and that in total weight was 53.2 ± 1.0 - 61.4 ± 3.6 g (Table 2).

Gonad index

Monthly means (± S.D.) of the gonad index of *H. asinina* collected from their natural habitat are shown in Figure 1. In males, the lowest gonad indices were in May (28.5 ± 10.3) and October (26.5 ± 2.9). In females, the lowest gonad index was in October (15.2 ± 6.8). However, in May and September, the animals also showed a relatively poor gonad condition (30.4 ± 10.0 in May, 28.3 ± 8.8 in September).

Monthly means (± S.D.) of the gonad index of *H. asinina* from the land-based culture system are shown in Figure 2. In males, the lowest gonad index was in September (22.1 ± 4.0). In the females, the lowest gonad index was in October (18.5 ± 3.1).

Histology of gonad and reproductive stages

Our findings confirmed the description of cells in oogenesis and spermatogenesis by Apisawetakan *et* al. (1997). The cells in the oogenetic process can be classified into six stages : oogonium (Og), and five stages of oocytes, *i.e.*, with light basophilia (Oc_1), with intense basophilia and oil droplets (Oc_2), with primay yolk granules (Oc_3), with secondary yolk

granules and thin jelly coat (Oc_4), and mature ovum with fully formed jelly coat (Oc_5). The cells in the spermatogenetic process can be classified into 13 stages : spermatogonium (Sg), five stages of primary spermatocytes (PrSc), secondary spermatocyte (Ssc), four stages of spermatids (St) and two stages of spermatozoa (Sz).

The reproductive stages of *H. asmina* collected from their natural habitat and the land-based culture system were assessed by observing changes in gonad histology, especially the characteristics of cellular association. The stages of gonad maturation during one reproductive cycle can be classified into five distinct phases: proliferative, premature, mature, spawning and spent.

1. *Proliferative phase.* In this phase, the gamete cells begin to reappear to commence a new reproductive cycle. At the initiation of this phase, the gonads contain mainly early-stage cells, and all of them are attached to the trabeculae (connective tissue in the gonads). The ovary contains primarily Og, which usually form clusters near the capsule (Fig.3A). Oc_1 and Oc_2 are rapidly increased in number (Fig.3C). In the testis, there are mostly newly produced Sg and PrSc, but no Sz (Figs.3B,3D). The clusters of these early-stage cells are located around the short and dilated trabeculae. The hepatopancreas is large and occupies most of the cross sectional profile of the conical organ when compared to the total gonad area.

2. *Premature phase.* In this phase, gametogenesis proceeds with a rapid increase in numbers and sizes of various gamete cells. The gonads become enlarged in volume, and the trabeculae become thinner. The ovary contains Og, Oc_1, Oc_2 and predominantly Oc_3 (Figs.4A,4C). At the beginning, there are abundant teardrop shaped Oc_3,which are still attached to the trabeculae, and Oc_4 and Oc_5 cells occur later (Fig.4C). The testis contains mainly Sg, PrSc and a few of St and Sz, all of which aggregate around the trabeculae (Figs.4B,4D).

3. *Mature phase.* This phase is a period of rapid growth of gonads which are reflected by striking differences in color between the two sexes (yellow in male and dark green in female). The rates of cell proliferation start to diminish, and the gonads contain primarily late-stage germ cells, while the few of early-stage cells are still present and are restricted to area immediately around the trabeculae, which become thinner. In the ovary, there are abundant Oc_5, but there are only few remaining and widely scattered Oc_1 (Figs.5A,5C). All of the Oc_5 appear fully mature and are liberated into the lumen of the oogenetic compartment. In the testis, there are mostly late-stage male germ cells, *viz.*, St and Sz (Figs.5B,5D). The most noticeable characteristic of the testis in this phase is the vast number of Sz_2 that lie in rows that surround the earlier cell stages which are still

closely attached to the trabeculae. As a result the testis appears to have a maximum density of late-stage cells. Prior to spawning, all of Sz_2 are dispersed into the gonad lumen and intermingled with other late-stage cells. The thin band of Sg and PrSc surrounding the trabeculae is still evident.

4. *Spawning phase.* This spawning phase is the period when abalone are ready for breeding, during which the completely mature and viable eggs or sperm are released from the gonads. The gonads are significantly decreased in size during spawning, and the gonadal wall becomes wrinkle when compared with the former phase (Figs.6A,6B). Only mature sperm or eggs are discharged and the earlier stages of gamete cells are still attached to the dilated trabeculae (Figs, 6C,6D). The spawning phase occurs at least twice during the one year period of observation; partial spawning could be observed during the year in most males. The recovery of gametogenesis is initiated immediately after spawning.

5. *Spent phase.* This phase is the period after spawning when fully mature gamete cells are completely discharged (Figs.7A,7B). This phase is also indicative of complete spawning, during which the gonads exhibit the breaking down of connective tissue stroma, and gametogenic activity momentarily ceases (Figs.7C,7D). The gonads are greatly decreased in size and become creamy in color in both sexes. The quiescent gonads show small cross sectional profiles in contrast to that of the hepatopancreas.

Reproductive cycle

The reproductive cycles of adult *H. asinina* from the natural habitat and land-based culture system are shown in Figures 8 and 9, respectively. *H. asinina* in both populations spawn twice during the year. In the natural habitat, the first spawning occurs from April to May in both males and females. In a land-based culture, the first spawning occurs from February to April in males and from March to April in females. Hence, spawning occurs later in *H. asinina* from the natural habitat. The second spawning begins in August in both males and females *H. asinina* from both the natural habitat and in the culture. But, *H. asinina* reared in the culture appears to spawn longer (August to November in males and August to October in females) than those from the natural habitat (August to October in males and August to September in females).

Each gonadal cycle of *H. asinina* both from the natural habitat and the land-based culture requires 5-8 months (Table 3). In *H. asinina* from the natural habitat, the gonadal cycles start from June to October and November to June in males, and from June to November and December to

June in females (Table 3). In *H. asinina* from the land-based culture, the gonadal cycles start from April to November and October to April in males, and from April to November and November to April in females (Table 3).

Development of the gonad

It was found that there was no gonad development in *H. asinina* from one month up to the age of four months (Fig.10A). In the three- to four-month-old abalone, the gonad capsule was observed around the hepatopancreas (Fig.10B). The gonads first appeared around the hepatopancreas when the abalone reached the age of five months (Figs.10C, 10D) and became fully mature in the nine-month-old abalone (Figs.10E,10F).

DISCUSSION

There have been a number of studies on the course of reproductive cycle in various abalone species by many investigators. The two methods that are frequently used for determining an entire reproductive cycle of a population are : 1) measurement of the relative size of the gonads or gonad indices (GI), and 2) assessment of histological changes in the gonads (Hahn, 1989; Webber & Giese, 1969; Giese, 1959; Grant & Tyler, 1983; Ault, 1985). The gonad index (GI) is simple and a well-defined method for characterizing the reproductive cycle in many marine invertebrates (Giese, 1959). GI involves the comparison between gonad size and body size, and spawning is assumed to occur when GI drops rapidly. However, GI is not always a valid index for reproductive organ development in all species of abalone because gonad size is not actually proportional to animal size, especially in very large or small animals (Shepherd & Laws, 1974). In addition, GI only relates gonad area to a constant parameter (e.g. shell length) of the animal (Young & DeMartini, 1970; Hahn, 1981).

A more precise index that can define the reproductive cycle better is the use of histological examination of gonadal sections, which can give considerable details of cellular association and the time interval between successive spawning (Webber & Giese, 1969). Tomita (1967, 1968), Giorgi & DeMartini (1977) and Ault (1985) classified the reproductive stages in various temperate species of *Haliotis* into 5 - 6 distinct phases that are most clearly defined in females (activation, gametogenesis, proliferation of gametes, ripeness, spawning and resting). Generally, the names of these phases differ depending on the researchers, but they usually refer to the same phenomena (Hahn, 1989). In the present study, histological examination of monthly samplings of *H. asinina* both from the

natural habitat and from a land-based culture system revealed five distinctive reproductive stages during the year, *viz.*, proliferative, premature, mature, spawning and spent phases.

The proliferative phase is characterized by the regeneration of gametes for a new cycle. The gonads contain mostly early-stage germ cells in both sexes. Giorgi & DeMartini (1977) and Ault (1985) in studying *H. rufescens*, found that the ovary contained primarily small oocytes usually less than 50 μm in diameter, while Tomita (1967), in studying *H. discus hannai* reported that there were mainly oogonium, and yolkless and oil drop-oocytes in this stage.

Another remarkable feature during this phase is the reciprocal relationship between the sizes of gonads and hepatopancreas (Boolootian *et al.*, 1962; Webber & Giese, 1969). Hepatopancreas is relatively large when compared to the total area of conical organ. Boolootian *et al.* (1962) reported that, in *H. cracherodii* and *H. rufescens*, the size of hepatopancreas exhibited an inverse relation to that of the gonad. During this phase, the hepatopancreas attains a maximum growth while the gonad activity is relatively quiescent. The precipitous drop in the size of hepatopancreas will occur during the rapid growth period of gonads. Boolootian *et al.* (1962) suggested that materials were mobilized from the hepatopancreas for the growth of the gonad.

The premature phase is the period of rapid increases in numbers and sizes of gamete cells. The ovary contains predominantly Og, Oc_1, Oc_2, and Oc_3 which is similar to those reported in the premature stage of *H. discus hannai* (Tomita, 1967; Mugiya *et al.*, 1980); while Sg, Sc and a few of Sz are evident in the testis during this phase. Ault (1985), in studying *H. rufescens*, also indicated that there were numerous developing germ cells in this stage. Hence the major events of development in this phase involve the rapid growth of gonads and notably the initiation of vitellogenesis in the ovary.

The mature phase is characterized by a notable enlargement of the gonads which exhibit striking differences of color between both sexes. The ovary contains mostly late-stage germ cells (*i.e.*, Oc_5) with widely scatterred Oc_1, and the testis is mostly filled with Sz. Before spawning occurs, Oc_5 are detached from trabeculae and released into the ovarian lumen, while spermatogenic cells differentiate rapidly into Sz, which are concentrated around the partially degraded and thinner trabeculae. Finally, the cells of the testis are dispersed and become completely displaced by fully mature Sz.

The spawning phase is the time when sexually mature abalone start to release their mature gametes. The period of spawning is the most important criterion for success of reproduction of various abalone species

reared in close aquaculture systems (Boolootian *et al.*, 1962; Webber & Geise, 1969; Young & DeMartini, 1970; Giorgi & DeMartini, 1977; Tomita, 1967, 1968; Mugiya *et al.*, 1980; Poore, 1973; Newman, 1967; Harrison & Grant, 1971; Shepherd & Laws, 1974). From many previous studies, spawning periods have been found to vary considerably among various species of abalone, and from year to year according to geographical locations, and such local conditions as food supply, temperature and day length (Cox, 1962; Webber & Giese, 1969; Mugiya *et al.*, 1980; Uki & Kikuchi, 1984; Kinne, 1970; Shepherd, 1973). Thus, some investigators (Boolootian *et al.*, 1962; Shepherd & Laws, 1974) have classified various *Haliotis* spp. into three groups according to their spawning season : those which spawn during summer, those which spawn during seasons other than summer, and those that exhibit year-round spawning.

In Thailand, Jarayabhand *et al.* (1994) reported that there were two peaks of spawning in *H. ovina* (June and November). Singhagraiwan & Doi (1992) reported the spawning period of wild broodstock of *H. asinina* to be around October, while the pond-reared broodstock could spawn from September to November and January. In contrast, the spawning period of *H. asinina* from the natural habitat and from the land-based culture system in the present study occurs twice a year. While this is the general pattern of spawning for most members of the population during the spawning periods, individuals with different stages of the gonadal cycle can be observed most frequently in males. Some are in mature phase, some are ready to spawn, and some are already spent. This is the extended breeding season which occurs in many marine invertebrates (Giese, 1959).

The spent phase is characterized by the lack of gamete cells and the breakdown of trabeculae or connective tissue in the gonads, which is similar to that previously found in *H. rufescens* (Giorgi & DeMartini, 1979). According to Shepherd & Laws (1974), the spent phase is expressed when there is a complete discharge of gamete cells. Giese (1959) defined the spent phase in marine invertebrates as a post-spawning quiescent stage that is indistinguishable between male and female. In the present study, during the spent period the gonads of *H. asinina* are greatly reduced in size and become creamy in color and the sexes of animals cannot be distinguished from each other.

From the data collected from a one year period, it could be concluded that spawning of *H. asinina* both from the natural habitat and those reared in the closed culture system can occur at least twice yearly provided that environmental condition and food supply are optimal. Each gonadal cycle consisting of five phases of development needs 5-8 months.

ACKNOWLEDGEMENTS

This study received financial support from the Thailand Research Fund #BRG 408004 and PG 2/015/2539.

Table 1 Sample size and gonad index of male and female *H. asinina* collected monthly from a natural habitat (January - December, 1997).

Month sampled	Sample size		Size (mean ± S.D.)			
			Male		Female	
	Male	Female	Shell length (mm)	Total weight (g)	Shell length (mm)	Total weight (g)
Jan.	5	5	72.2 ± 13.1	99.3± 46.6	78.7 ± 14.5	124.1 ± 49.6
Feb.	6	4	61.3 ± 29.3	106.0 ± 65.5	77.8 ± 12.6	119.3 ± 37.2
Mar.	7	5	65.4 ± 15.4	88.4 ± 29.4	82.6 ± 6.1	134.9 ± 25.3
Apr.	12	9	58.1 ± 18.6	78.1 ± 35.4	79.1 ± 13.7	125.8 ± 39.6
May	11	9	67.5 ± 17.0	94.5 ± 44.2	72.2 ± 12.1	101.4 ± 39.2
June	12	6	70.9 ± 17.8	105.3 ± 51.4	60.4 ± 17.1	79.5 ± 43.8
July	14	7	62.5 ± 17.3	83.5 ± 26.9	61.2 ± 18.7	83.7 ± 39.2
Aug.	9	8	85.0 ± 7.6	147.5 ± 33.0	61.2 ± 13.5	73.1 ± 41.6
Sept.	8	5	66.7 ± 18.6	91.3 ± 50.0	55.3 ± 15.7	64.4 ± 32.4
Oct.	5	5	65.5 ± 24.0	93.2 ± 68.3	68.3 ± 25.0	96.7 ± 66.7
Nov.	6	5	63.1 ± 16.1	81.0 ± 33.1	65.5 ± 19.8	89.0 ± 54.4
Dec.	5	5	60.4 ± 25.4	91.3 ± 63.9	67.8 ± 11.0	71.6 ± 47.6

Table 2 Sample size and gonad index of male and female *H. asinina* collected monthly from a land-based culture system (January - December, 1997).

Month sampled	Sample size		Size (mean ± S.D.)			
			Male		Female	
	Male	Female	Shell length (mm)	Total weight (g)	Shell length (mm)	Total weight (g)
Jan.	10	6	57.8 ± 9.1	60.3± 3.1	51.2 ± 8.6	57.8 ± 2.0
Feb.	9	6	55.5 ± 5.7	59.5 ± 1.9	59.1 ± 5.3	60.8 ± 3.0
Mar.	10	5	49.3 ± 4.4	61.6 ± 2.9	47.6 ± 7.8	56.5 ± 2.8
Apr.	9	7	61.4 ± 8.3	53.4 ± 1.0	39.5 ± 5.0	53.2 ± 1.0
May	8	8	41.9 ± 4.2	54.2 ± 1.8	44.4 ± 8.7	55.3 ± 2.5
June	9	7	52.6 ± 9.3	58.3 ± 3.9	59.8 ± 7.6	61.0 ± 3.3
July	11	5	55.8 ± 6.5	59.6 ± 2.4	57.5 ± 2.8	60.2 ± 3.3
Aug.	10	6	53.0 ± 3.1	58.7 ± 1.1	54.6 ± 6.9	59.3 ± 1.8
Sept.	11	6	58.6 ± 6.4	60.6 ± 2.2	53.5 ± 11.0	58.8 ± 3.3
Oct.	8	6	56.0 ± 4.2	59.7 ± 1.5	61.3 ± 12.0	61.4 ± 3.6
Nov.	10	5	60.8 ± 8.4	61.3 ± 2.8	51.3 ± 9.0	57.9 ± 2.6
Dec.	11	6	58.6 ± 5.4	60.6 ± 1.9	53.2 ± 4.6	58.6 ± 3.7

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Maleeya KRUATRACHUE[1], Somjai APISAWETAKAN[2], E.Suchart UPATHAM[1], Prasert SOBHON[2], Saowapa SAWATPEERA[3], Vicharn INGSRISAWANG[4] and Tanin SINGHAGRAIWAN

[1]*Department of Biology and* [2]*Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[3]*Institute of Marine Science, Burapha University, Chonburi 20130, Thailand*
[4]*Eastern Marine Fisheries Development Center, Rayong 21160, Thailand*

EXPLANATION OF FIGURES

FIG 1 Seasonal changes in the mean gonad indices of male and female *H. asinina* from a natural habitat.

FIG 2 Seasonal changes in the gonad indices of male and female *H. asinina* from a land-based culture system.

FIG 3 Photomicrographs of paraffin sections of the proliferative phase showing regeneration of gamete cells after the spawning and spent phases. The ovary (A, C) contains mainly stage I and II oocytes ($Oc_{I,II}$), which are rapidly increased in number. The testis (B, D) contains mostly spermatogonia and primary spermatocytes (PrSc). Trabeculae (tr), which are depleted of cells and breaking down in spent phase, start to regenerate and appear short and dilated. ca, capillary; cp, gonad capsule; HP, hepatopancreas; Sc, spermatocyte.

FIG 4 Paraffin sections of premature phase showing rapid increases in the number and size of various cells. The ovary (A, C) contains mostly early-stage oocytes (Oc_{I-II}), and late-stage oocytes (Oc_{I-V}) start to appear and gradually increase in number. The testis (B, D) contains various stages of primary spermatocytes (Sc) and spermatids (St) together with a few spermatozoa (Sz), all of which are located close to the trabeculae (tr). je, jelly coat; HP, hepatopancreas.

FIG 5 Paraffin sections of the mature phase showing rapid growth of gonads. The ovary (A, C) contains primarily fully mature Oc_V with only a few widely scattered early-stage cells (Oc_I and Oc_{II}). The testis (B, D) contains mostly late spermatids (St) and spermatozoa (Sz), which lie in rows. Finally, they become dispersed and displaced into the luminal area of the testis. HP, hepatopancreas; Sc, spermatocyte; tr, trabeculae.

FIG 6 Paraffin sections of the spawning phase showing the period when abalone release viable sperm or eggs from the gonads. The ovary (A, C) contains only earlier stage oocytes (Oc_I) which are still attached to dilated trabeculae (tr). The testis (B, D) contains only early-stage male germ cells with a few spermatozoa (Sz). cp, gonad capsule; HP, hepatopancreas.

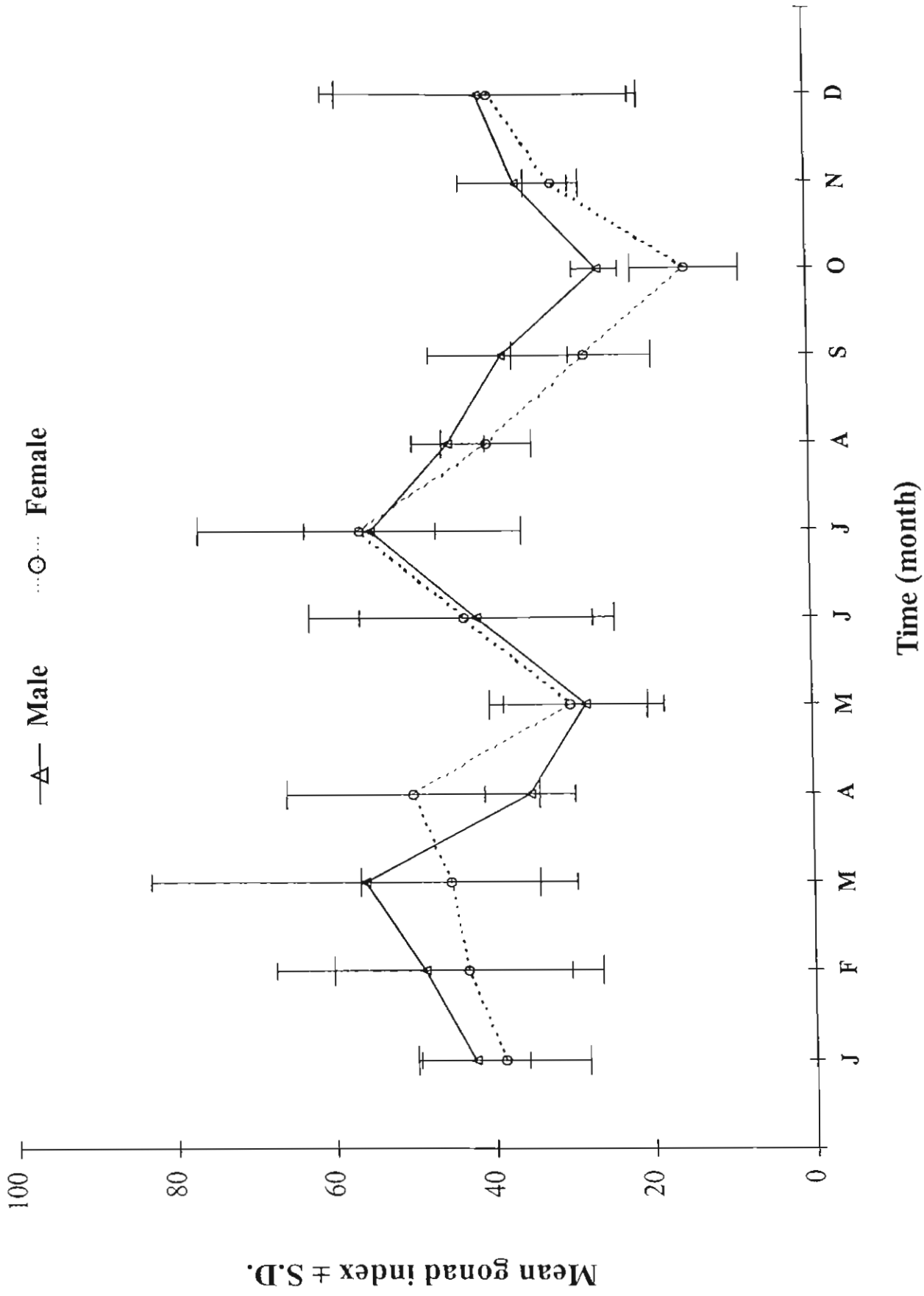
FIG.7 Paraffin sections of the spent phase showing the complete discharge of gamete cells, and the breaking down of trabeculae (tr) and associated connective tissues in both sexes (female in A and C, male in B and D). ca, capillary; cp, gonad capsule; HP, hepatopancreas; Oc_1, oocyte stage 1; Sz, spermatozoa.

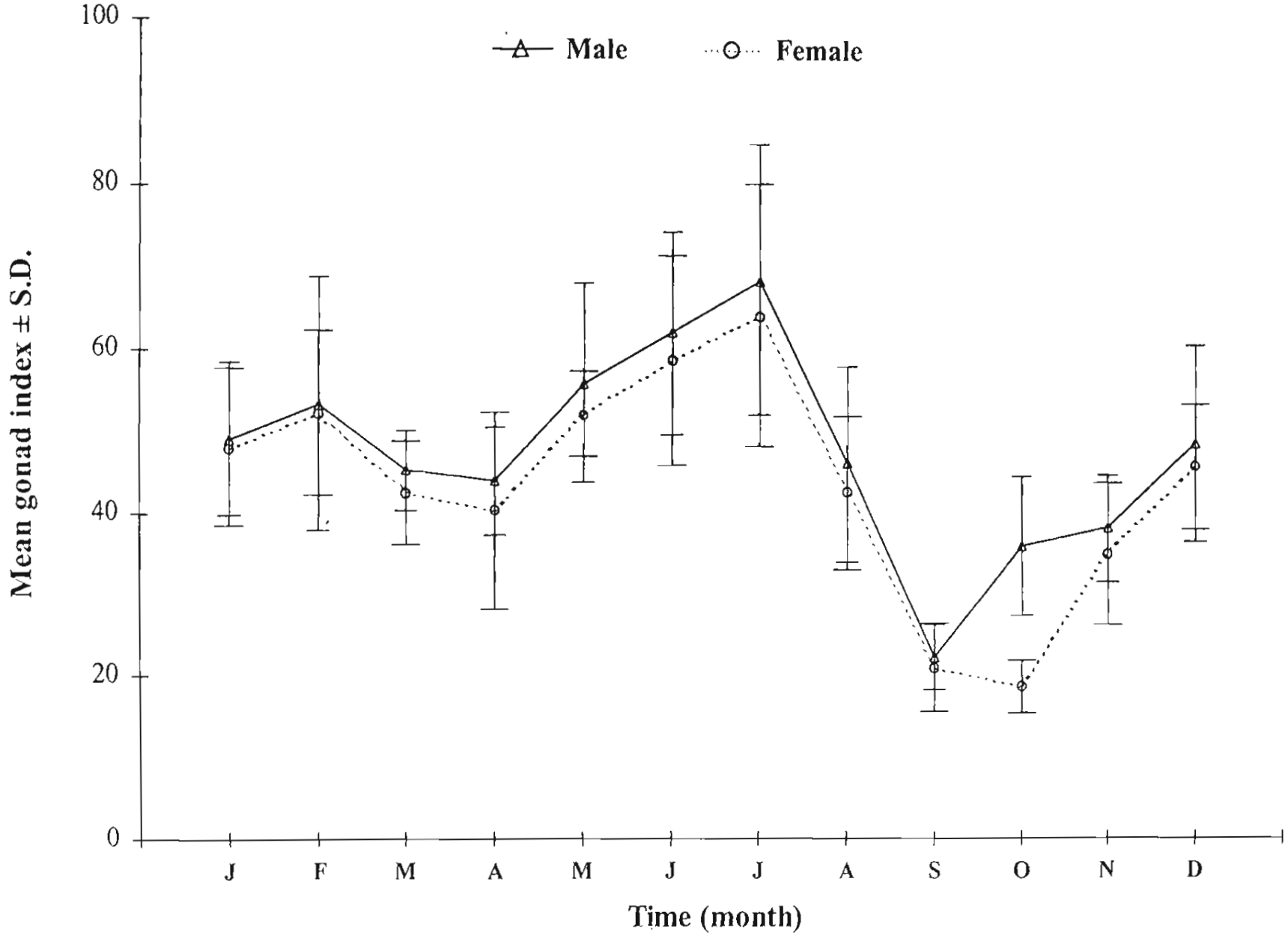
FIG.8 The reproductive cycle of adult *H. asinina* from a natural habitat during a one-year period : (1) the proliferative phase occurs around June to July and November to December in males, and around June to July and December to January in females. (2) The premature phase occurs around June to July and December to January in males, and around June to July and February to March in females, (3) The mature phase occurs around August to September and February to March in males, and around July to August and April to May in females, (4) the spawning phase occurs around August to October and April to May in males, and around August to September and April to May in females, (5) the spent phase occurs around September to October and May to June in males, and around October to November and May to June in females.

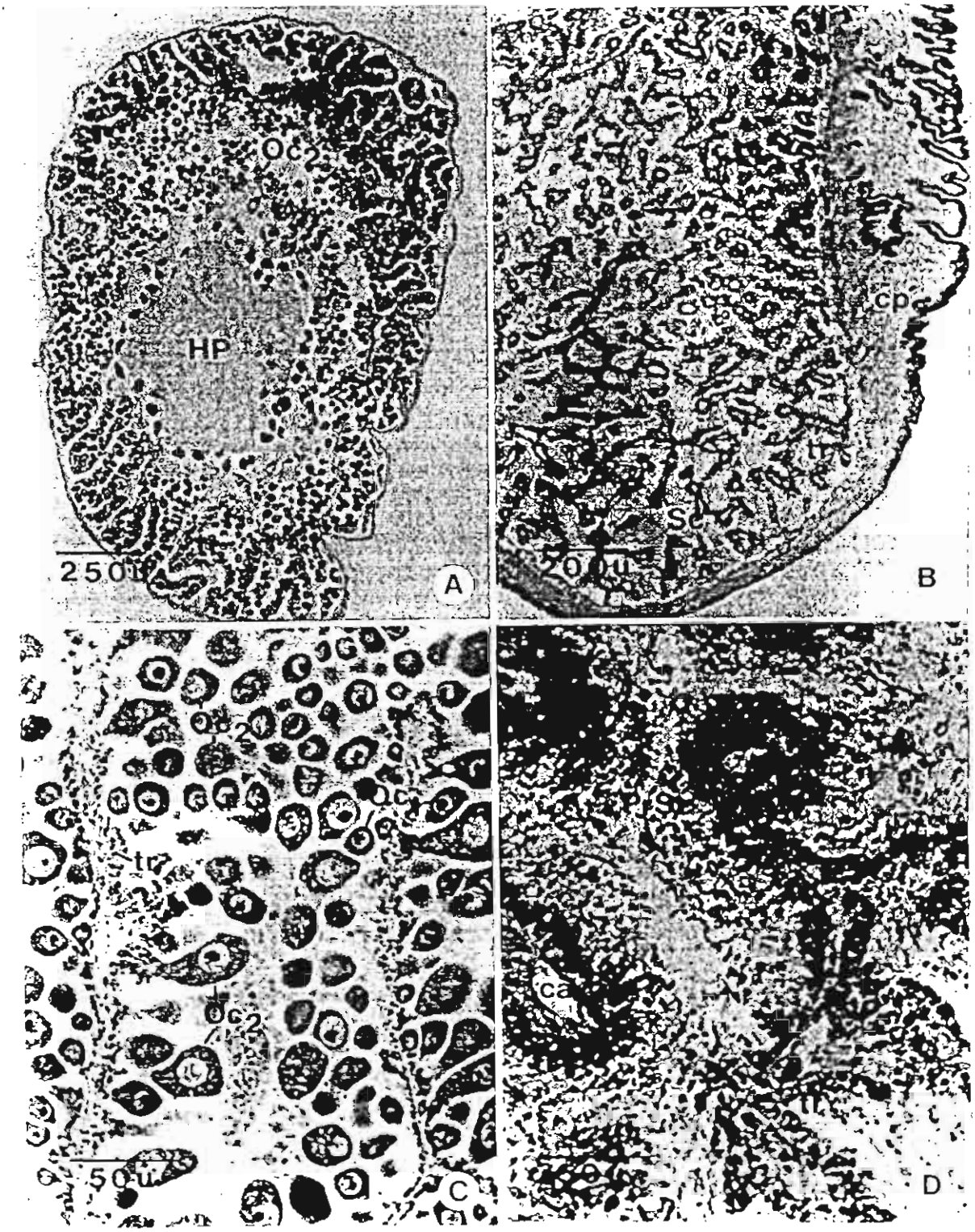
FIG.9 Reproductive cycle of adult *H. asinina* reared in a land-based culture system during a one-year period : (1) the proliferative phase occurs around April to May and October to November in males, and around April to May and November to December in females, (2) the premature phase occurs around April to May and December to January in males, and around May to June and January to February in females, (3) the mature phase occurs around June to August and February to April in both sexes, (4) the spawning phase occurs around August to November and February to April in males, and around August to October and March to April in females, (5) the spent phase occurs around September to November and March to April in both sexes.

FIG.10 A. Hepatopancreas (HP) of one- to four-month-old abalone. Gonad development was not observed. B. Higher magnification of the hepatopancreas (HP) capsule. Notice the formation of gonad capsule (arrow) and trabeculae (tr). C. The ovary of a five-month-old female abalone around hepatopancreas (HP). Early-stage oocytes (Oc) could be observed. D. The testis of a five-month-old male abalone around the hepatopancreas (HP). Various stages of spermatogenesis, spermatocytes (Sc) and spermatozoa (Sz) could be observed. E-F. Ovary (E) and testis (F) of a nine-month-old abalone. The gonads had become fully mature. cp, gonad capsule; HP, hepatopancreas; Oc, oocyte; Sz, spermatozoa.

Fig. 1



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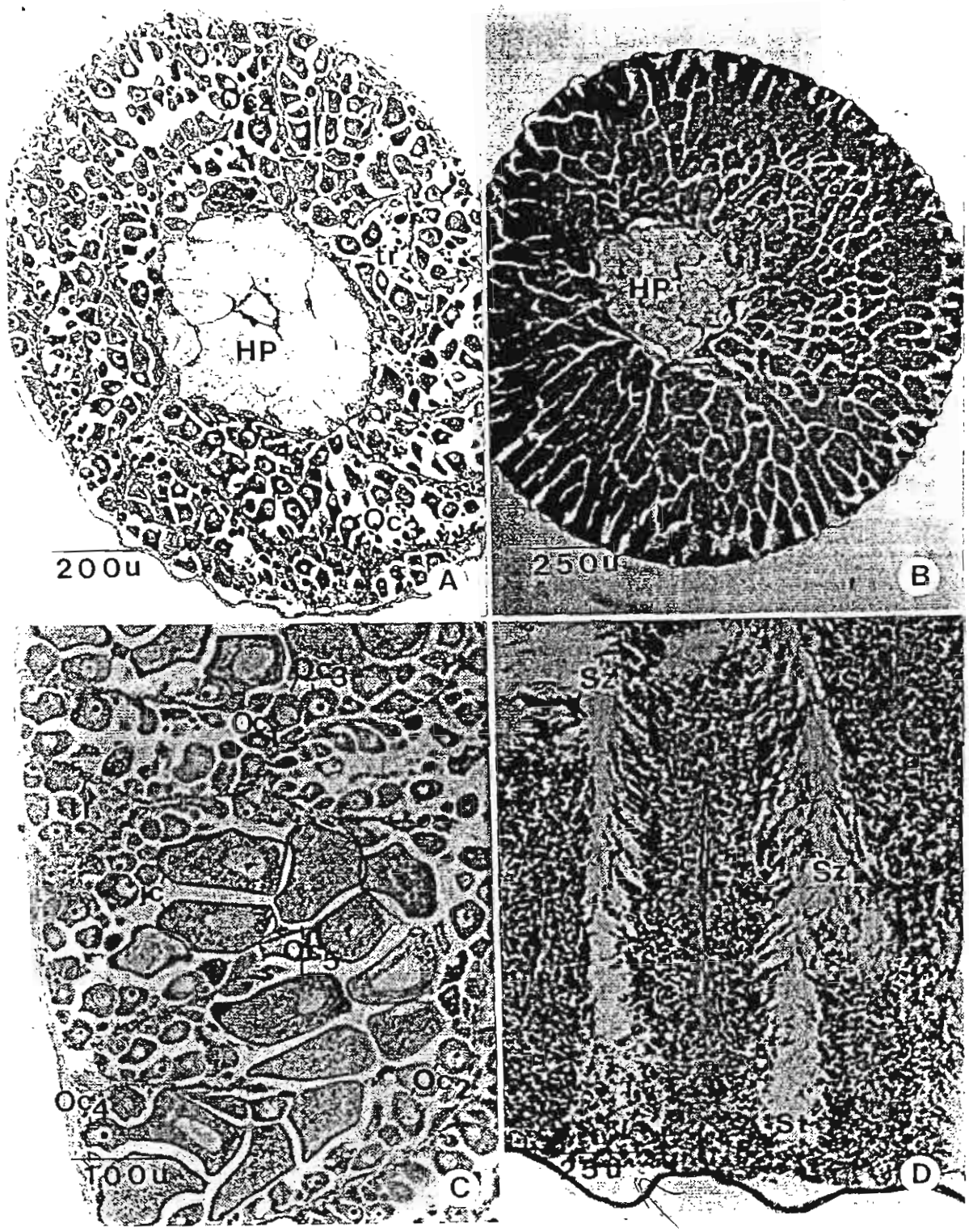
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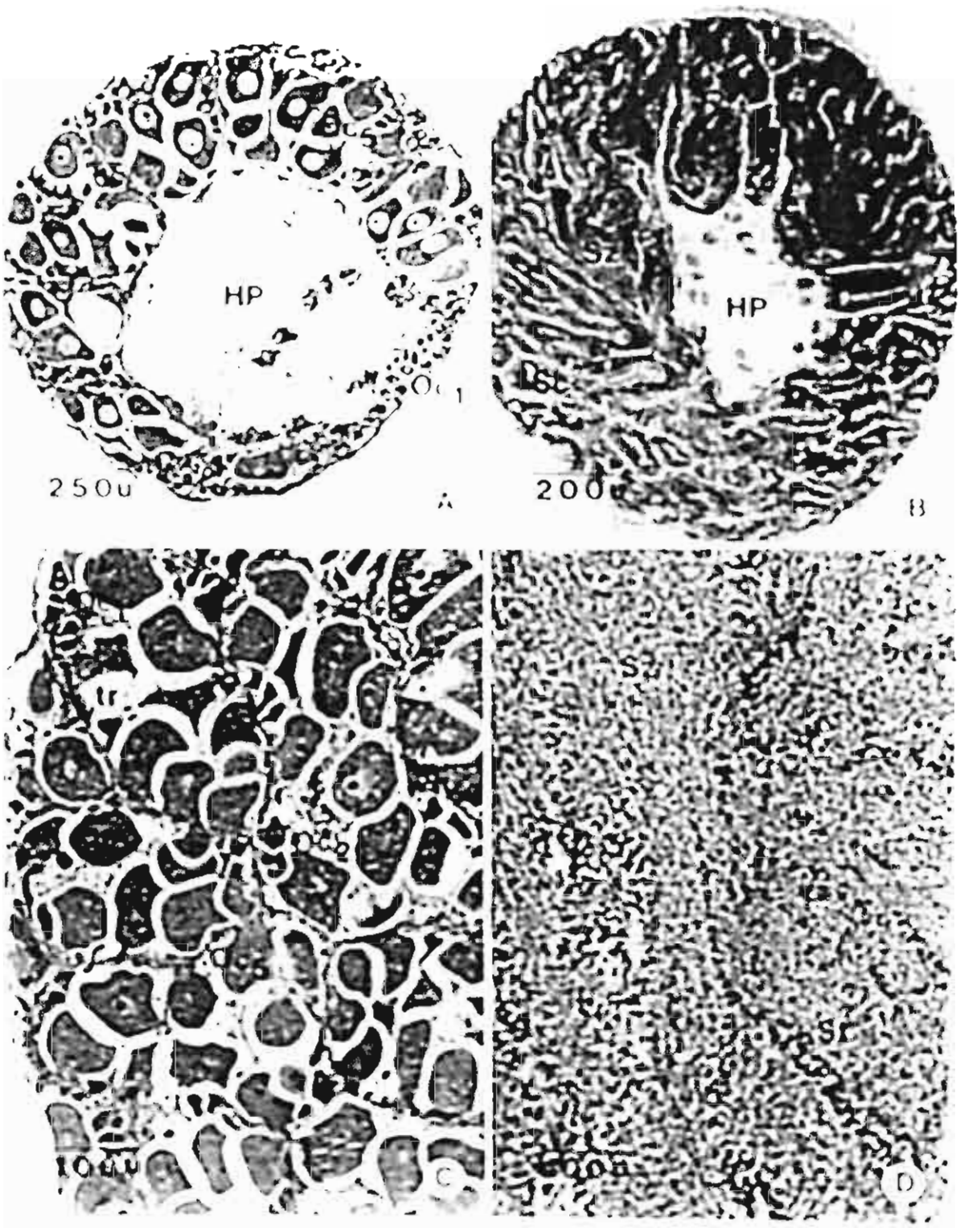
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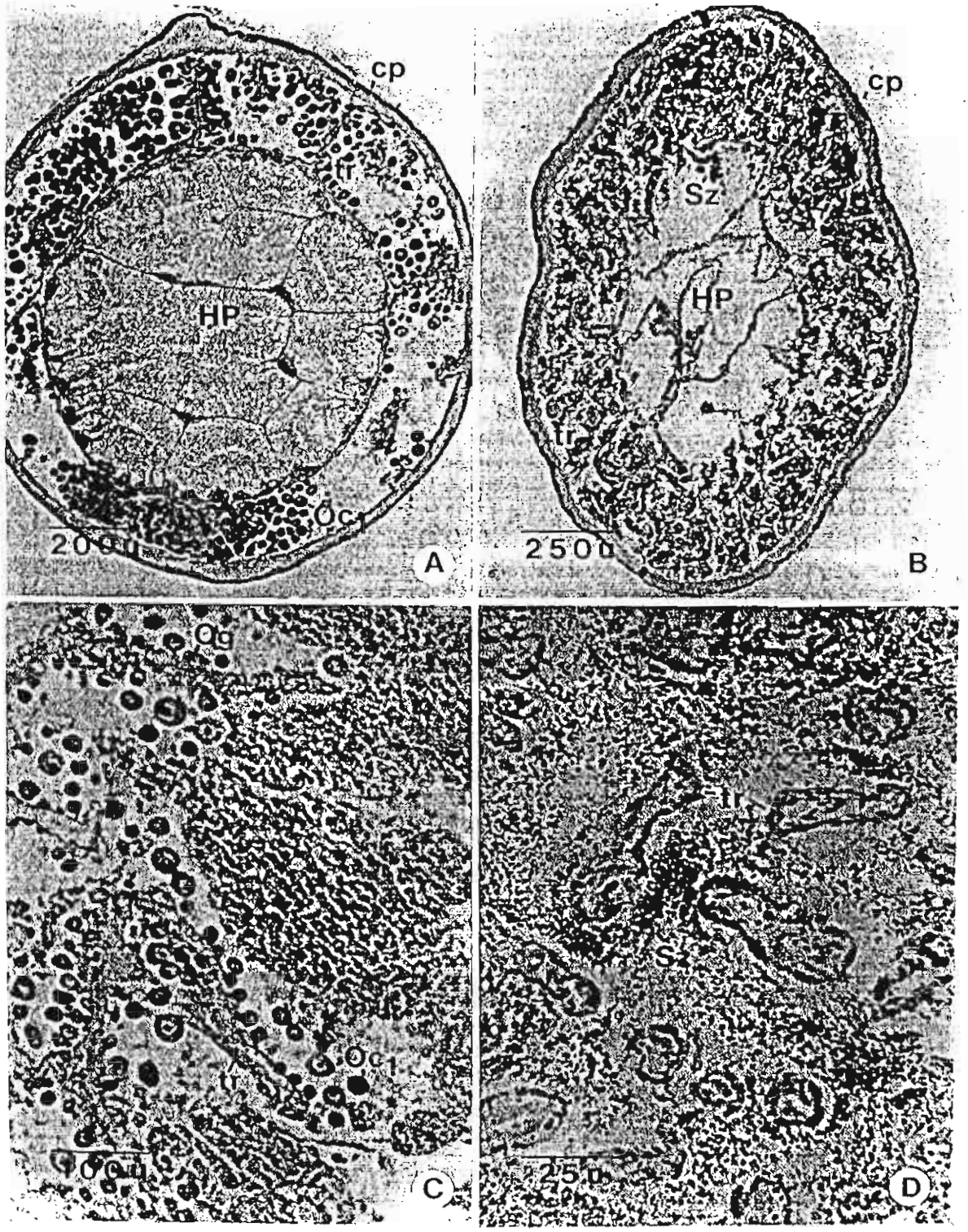
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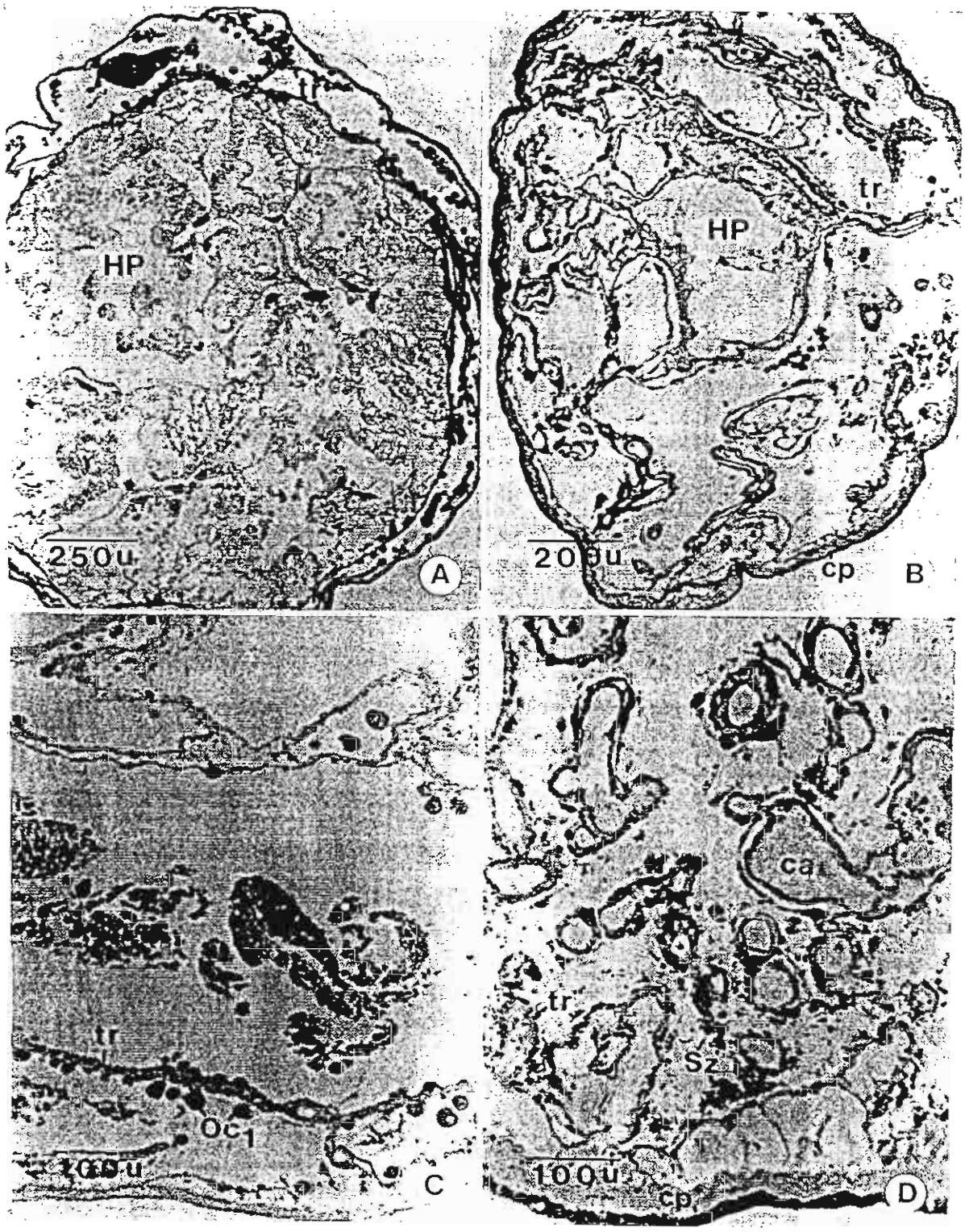
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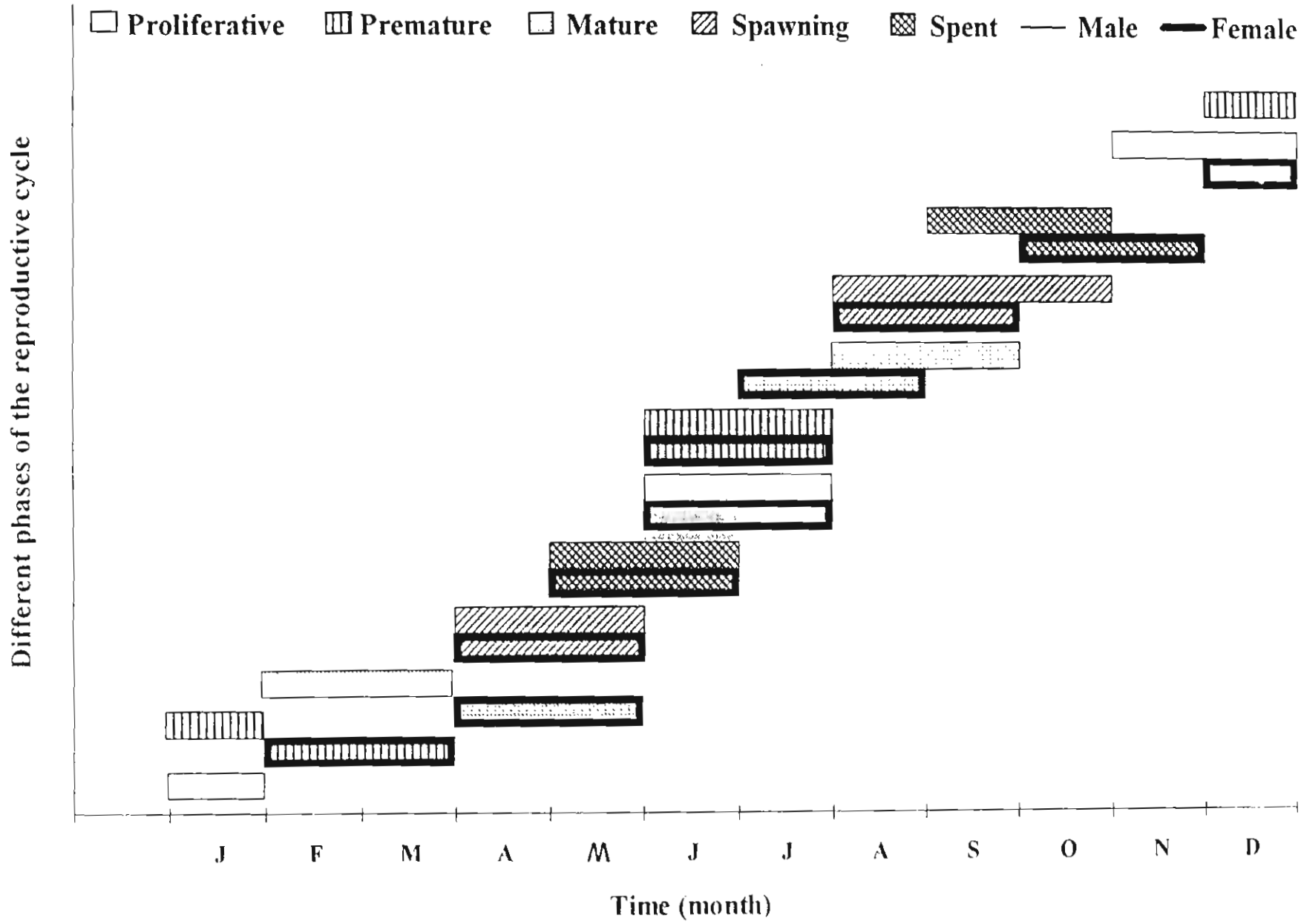
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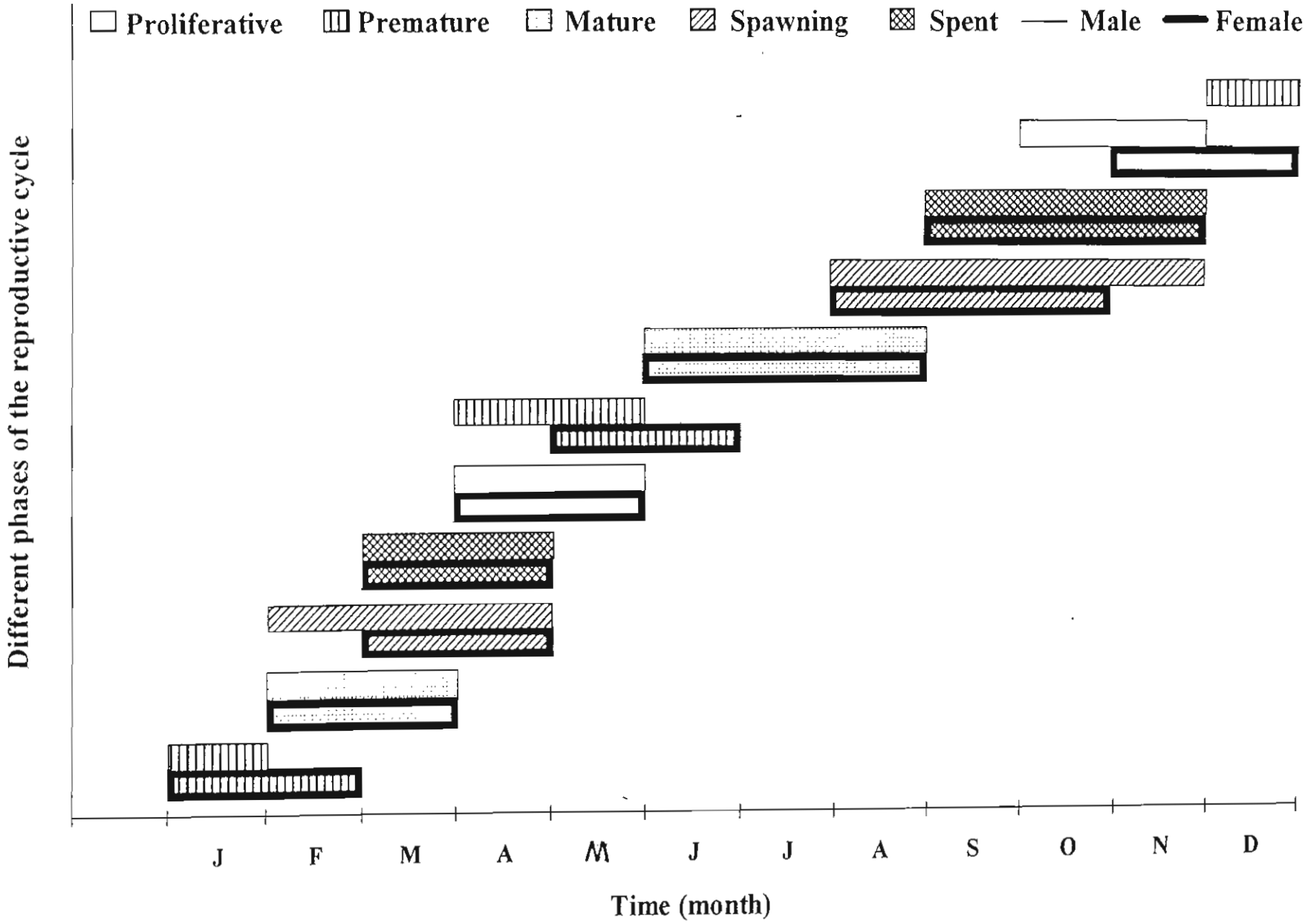
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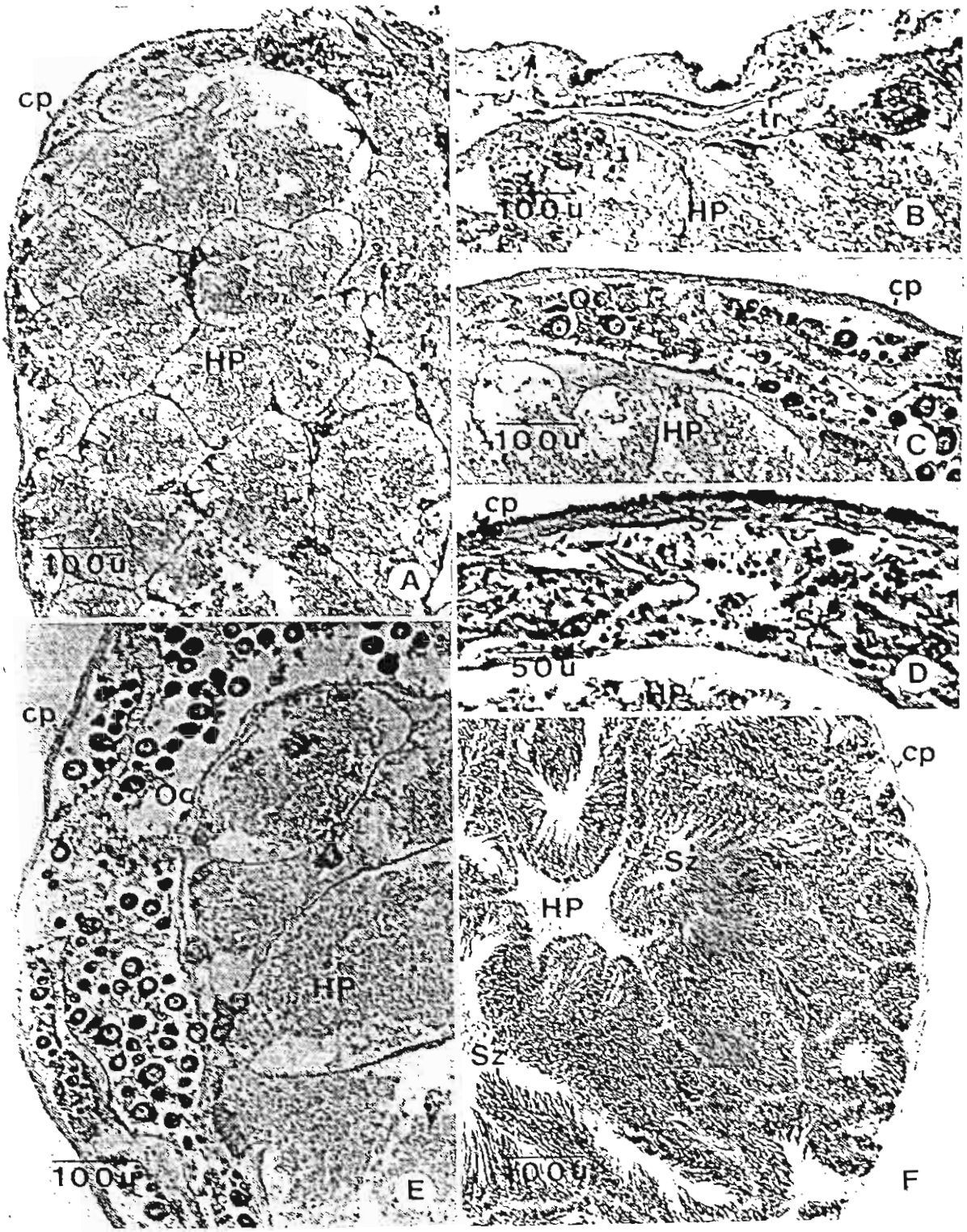
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THE ULTRASTRUCTURE OF NEURONS AND NEUROGLIA IN THE CEREBRAL AND PLEURO-PEDAL GANGLIA OF *HALIOTIS ASININA* LINNAEUS

MALEEYA KRUATRACHUE[a], AMPORN THONGKUKIATKUL[b], PRASERT SOBHON[c], E.SUCHART UPATHAM[a], CHAITIP WANICHANON[c], PRAPEE SRETARUGSA[c] AND VICHAI LINTHONG[c]

[a]*Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*

[b]*Department of Biology, Faculty of Science, Burapha University, Chonburi 20131, Thailand.*

[c]*Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*

ABSTRACT

The ultrastructure of neurons and neuroglia in the cerebral and pleuro-pedal ganglia of Haliotis asinina *are described. There are four types of neurons (NR_{1-4}) and three types of neuroglia. (NG_{1-3}). The NR_1, which is the largest nerve cell, has a round nucleus with a thin rim of heterochromatin attached to the nuclear envelope. The cytoplasm contains numerous rough endoplasmic*

reticulum, mitochondria, but only a few small elementary granules. The nuclei of NR_2 , NR_3 and NR_4 have increasingly condensed heterochromatin. The cytoplasm of these cells contain relatively few organelles such as rough endoplasmic reticulum, mitochondria and elementary granules. The NG_1 is spindle-shaped with little perinuclear cytoplasm. The NG_2 and NG_3 are highly ellipsoid in shape. The cytoplasm of NG_1 and NG_3 is very thin and contains ribosomes, a few rough endoplasmic reticulum and mitochondria. The cytoplasm of NG_2 is extremely thin and contains only ribosomes. There are no elementary granules in the neuroglia.

KEY WORDS *Haliotis asinina*, neuron, neuroglia, ultrastructure

INTRODUCTION

Bullock and Horridge[1] classified the neurons in the ganglia of gastropods on the basis of morphology (size and nuclear - cytoplasmic ratio of the cell body and perikaryon). In the pulmonate snails such as *Achatina fulica* Bowdich, *Helix pomatia* Linnaeus, *Arion ater* Linnaeus and *Limax maximus* Linnaeus, the classification of neurons is based on cell size[2-4]. They are giant neurons, ordinary neurons and globuli cells. The giant neurons are characterized by their large size and irregularly-shaped nucleus[4]. The nuclei are often flattened or lobated[5]. The nucleoli are usually large and annular[6]. The surface of the giant neuron is often deeply indented by processes of glial cells[7-8]. The ordinary neurons are divided into large, medium and small cells[1-4]. They are similar to the giant neurons in having large clear nuclei with one or several nucleoli, abundant cytoplasm and thick processes[1]. The ultrastructure of ordinary neurons has been described in many gastropod species such as *Aplysia californica* Cooper[7], *Lymnaea stagnalis* Lamarck[8]. *Archachatina marginata* Lamarck[9], *Helix aspersa* Muller[10], *A. fulica*[11], *Bithynia tentaculata* (Linnaeus)[12] and *Haliotis rufescens* Swainson[13]. The cytoplasm contains a large number of small mitochondria, innumerable cisternae of rough endoplasmic reticulum, free ribosomes, polyribosomes, lysosomes, numerous Golgi complexes and elementary granules. The free ribosomes and cisternae of the rough endoplasmic reticulum are especially concentrated near the nucleus and in the region of the axon hillock[1]. The globuli

cells contain chromatin-rich nuclei. The cytoplasm is scant and contains organelles similar to those found in the ordinary neurons[11].

Glial cells are distributed between the neurons and their sheath cells and along the outer surfaces of blood vessels[7,14,15]. The nuclei are round or oval. There are two types of glial cells in *L. stagnalis*[8,14]. The first type of neuroglia indents into the perikaryon and into the axon of the large neurons. Cell organelles are scarce in the cytoplasm of those glial cells except for mitochondria. Usually some glycogen is present. A second type of glial cells (filamentous glial cells) is characterized by a large number of thin filaments (50 A°) which are comparable to tonofilaments. Moreover, these cells contain abundant mitochondria, a rather extensive rough endoplasmic reticulum, numerous Golgi complexes, and lysosome-like structures[8]. The ultrastructure of glial cells of *L. stagnalis* is similar to those of *A. marginata*[9] and *A. californica*[7].

From the previous reports on the ultrastructure of neurons and neuroglia in the ganglia of various species of gastropods, it is apparent that the knowledge of ultrastructure of neurons and neuroglia is still lacking in *Haliotis asinina* Linnaeus. Therefore, the present study reports on the fine structure of neurons and neuroglia in the cerebral and pleuro-pedal ganglia of *H. asinina*.

MATERIALS AND METHODS

The adult abalone *H. asinina*, with a shell length of 4-5 cm, were obtained from the Coastal Aquaculture Development Center, Klong Wan, Prachuap Khiri Khan Province, Thailand. Abalone were anesthesized with 5% MgCl, after which their shells were removed. The cerebral and pleuro-pedal ganglia were dissected out and fixed in a mixture of 4% glutaraldehyde and 2% paraformaldehyde in 0.1M Millonig buffer (pH 7.8) at 4°C for 24 hours. Specimens were washed six times with 0.1 M Millonig buffer. They were postifixed in 1% OsO_4 in 0.1 M Millonig buffer for 2 hours, then dehydrated through a graded series of ethanol. They were embedded in Spurr's resin. The sections were stained with uranyl acetate in 70% ethanol and lead citrate and examined with a Hitachi H-300 transmission electron microscope operating at 75 KV.

RESULTS

From the histological observation, , there are four types of neurons (NR_{1-4}) and three types of neuroglia (NG_{1-3}) in the cerebral and pleuro-pedal ganglia[16,17].

Type I neuron (NR_1). The cell body is round or oval with very large size (14x28 μm). The nucelus is round (9 μm in diameter) with a thin rim of heterochromatin attached to the nuclear envelope. Most of the remaining

chromatin is finely dispersed euchromatin that is scattered uniformly throughout the nucleus (Fig.1A). The cytoplasm contains numerous mitochondria and stacks of rough endoplasmic reticulum (Fig.1A). Only few small elementary granules are present.

Type 2 neuron (NR_2). The cell body is oval and about 4-6 μm in diameter. The nucleus also has an oval shape (4-6 μm in diameter), with thick patches of heterochromatin along the periphery and in the central area (Fig.1B). The cytoplasm is very thin and contains a few rough endoplasmic reticulum, mitochondria, polyribosomes and some elementary granules (Fig.1B).

Type 3 neuron (NR_3). The cell body and nuclear size of these cells are similar to those of NR_2. However, in the nucleus, the heterochromatin is increased greatly in comparison to NR_2 (Fig.1C). The cytoplasm is relatively thin comparing to the size of the nucleus, and contains sparing amount of rough endoplasmic reticulum and mitochondria, a few polyribosomes and small elementary granules.

Type 4 neuron (NR_4). These cells are round in shape and about 4-6 μm in diameter. The nucleus contains thin patches of heterochromatin along the nuclear embrane, while most of the nucleoplasm in the central area is clear (Fig.1D). The cytoplasm is thin and contains the usual organelles such as, rough endoplasmic reticulum, mitochondria, ribosomes, along with some elementary

granules (Fig.1D). These cells are rarely observed in the cerebral ganglia, while they are more numerous in the pleuro-pedal ganglia.

Type 1 neuroglia (NG_1). The cell body is spindle-shaped with little perinuclear cytoplasm. The nucleus is also spindle-shaped with patches of heterochromatin attached to the periphery of the nuclear membrane, and few large blocks of heterochromatin occur in the central area (Fig.2A). The cytoplasm contains a few rough endoplasmic reticulum, mitochondria and ribosomes (Fig.2A). No elementary granules were observed. These neuroglia are intermingled with neurons in all layers of the cortex.

Type 2 neuroglia (NG_2). The cell body (3x8 μm in diameter) and nucleus (3x5 μm in diameter) are highly ellipsoid in shape. The nucleus contains a dense heterochromatin strip along the nuclear envelope, continuing into large blocks in the central region (Fig. 2B). The cytoplasm is extremely thin and contains only ribosomes (Fig.2B). These neuroglia form a single sheet of continuous cell layer adjacent to the basement membrane, which is surrounded in turn by ganglionic connective tissue capsule.

Type 3 neuroglia (NG_3). The cell body and nuclear characteristic of these neuroglia are similar to those of NG_2, but they are smaller in size and the nuclear membrane is more indented (Fig.2C). The nucleus contains dense heterochromatin (Fig.2C). The cytoplasm is very thin and contains ribosomes, a

few rough endoplasmic reticulum and mitochondria (Fig.2C). These neuroglia are interspersed amongst the nerve tracts in the neuropil.

DISCUSSION

The neurons in the cerebral and pleuro-pedal ganglia of *H. asinina* can be divided into 4 types (NR_{1-4}) based on the nuclear characteristic. NR_1 is the largest neuron and exhibit the ultrastructural features similar to typical motor neurons of vertebrates such as ventral horn motor cells of the spinal cord and Purkinje cells in the cerebellum. Their chromatin is completely euchromatic with a prominent nucleolus, while the cytoplasm contains abundant rough endoplasmic reticulum and mitochondria, but only a few typical elementary or neurotransmitter granules.

Other types of neurons (NR_2, NR_3, NR_4) may belong to the same group. They are characterized by increasing condensation of heterochromatin. The cytoplasm is small but contains sizable numbers of organelles particularly polyribosomes and mitochondria. These features are similar to association neurons in the vertebrate nervous system, such as small neurons in the molecular layer of cerebellum of mammals.

The ultrastructure of ordinary neurons had been studied extensively in the ganglia of several pulmonates such as *L. stagnalis*[18], *Helisoma tenue* (Phillippi)[19], *A. californica*[7], *H. pomatia*[3], *H. aspersa*[10], *A. marginata*[9] and *A.*

fulica[11]. The studies on the neurons of prosobranchs were reported in *H. rufescens*[13] and *B. tentaculata*[12]. The ultrastructure of the ordinary neurons in *H. asinina* is rather simple compared with those in the pulmonates. They contain the usual cytoplasmic organelles such as rough endoplasmic reticulum, mitochondria, ribosomes, polyribosomes, similar to the neurons of pulmonates but in smaller numbers. Their Golgi bodies and lysosomes are not observed as frequent as those of the pulmonate neurons. In addition, the ordinary neurons of pulmonates usually contain a large number of elementary granules. There are only a few elementary granules in *H. asinina* neurons. These granules are not stained by chrome-hematoxylin and paraldehyde-fuchsin. It is possible that the small vesicles of the neurons might incorporate neurotransmitters, which are non-peptides or glycoprotein[18]. Similar granules, thought to contain neurotransmitters have been described in the neurons of *L. stagnalis*[18] and *A. marginata*[9].

Special types of inclusions and secretory granules have been reported in the neurons of *H. rufescens* and *B. tentaculata*[12,13]. In *H. rufescens*, the cerebral neurons contain large membrane-bound inclusions showing various degrees of organization such as packed membranes or filaments, clumps of strongly osmiophilic material, and homogeneous pale material. Frequently, a single inclusion shows all three features[13]. No such inclusions were observed in *H. asinina* neurons. Andrews[12] reported the presence of lipofuscin spherules in *B.*

tentaculata neurons. We did not observe these spherules in the neurons of *H. asinina*.

The neuroglia of the cerebral and pleuro-pedal ganglia of *H. asinina* which contain spindle-shaped nuclei and little perinuclear cytoplasm are different from those described in *B. tentaculata*[12], *L. stagnalis*[14] and *A. marginata*[9] which have several mitochondria, rough endoplasmic reticulum, Golgi complexes and lysosome-like structures. Furthermore, they are interspersed amongst the neurons, but do not indent into the cytoplasm of the neurons like those described for the neuroglia of pulmonates[1].

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EXPLANATION OF FIGURES

Fig. 1 A. A low power micrograph of type 1 neuron (NR_1) in the cerebral ganglia showing a round nucleus (Nu) with a prominent nucleolus (No). The cytoplasm contains numerous mitochondria (Mt) and stacks of rough endoplasmic reticulum (RER).

B. A medium power micrograph of type 2 neuron (NR_2) in the cerebral ganglia showing an oval nucleus (Nu) with patches of heterochromatin (He) along the nuclear envelope and in the central area. C. A medium power micrograph of type 3 neuron (NR_3) in the cerebral ganglia showing an oval nucleus (Nu) with completely dense heterochromatin (He) and a thin rim of cytoplasm. D. A medium power micrograph of type 4 neuron (NR_4) in the pleuro-pedal ganglia showing a round nucleus (Nu) with thin patches of heterochromatin (He) along the nuclear membrane. The cytoplasm contains a few rough endoplasmic reticulum (RER), mitochondria (Mt) and elementary granules (EG).

Fig.2 A. A medium power micrograph of type 1 neuroglia (NG_1) in the pleuro-pedal ganglia showing a spindle-shaped nucleus (Nu) with patches of heterochromatin (He) in the peripheral and central regions. The cytoplasm contains a few rough endoplasmic reticulum (RER) and mitochondria (Mt). B. A medium power micrograph of type 2 neuroglia (NG_2) in the pleuro-pedal ganglia showing a nucleus (Nu) with very dense heterochromatin (He). The cytoplasm is extremely thin and contains only ribosomes. C. A medium power micrograph of type 3 neuroglia (NG_3) in the pleuro-pedal ganglia showing a nucleus (Nu) with dense heterochromatin (He). The cytoplasm contains a few rough endoplasmic reticulum (RER) and mitochondria (Mt).

Fig 1

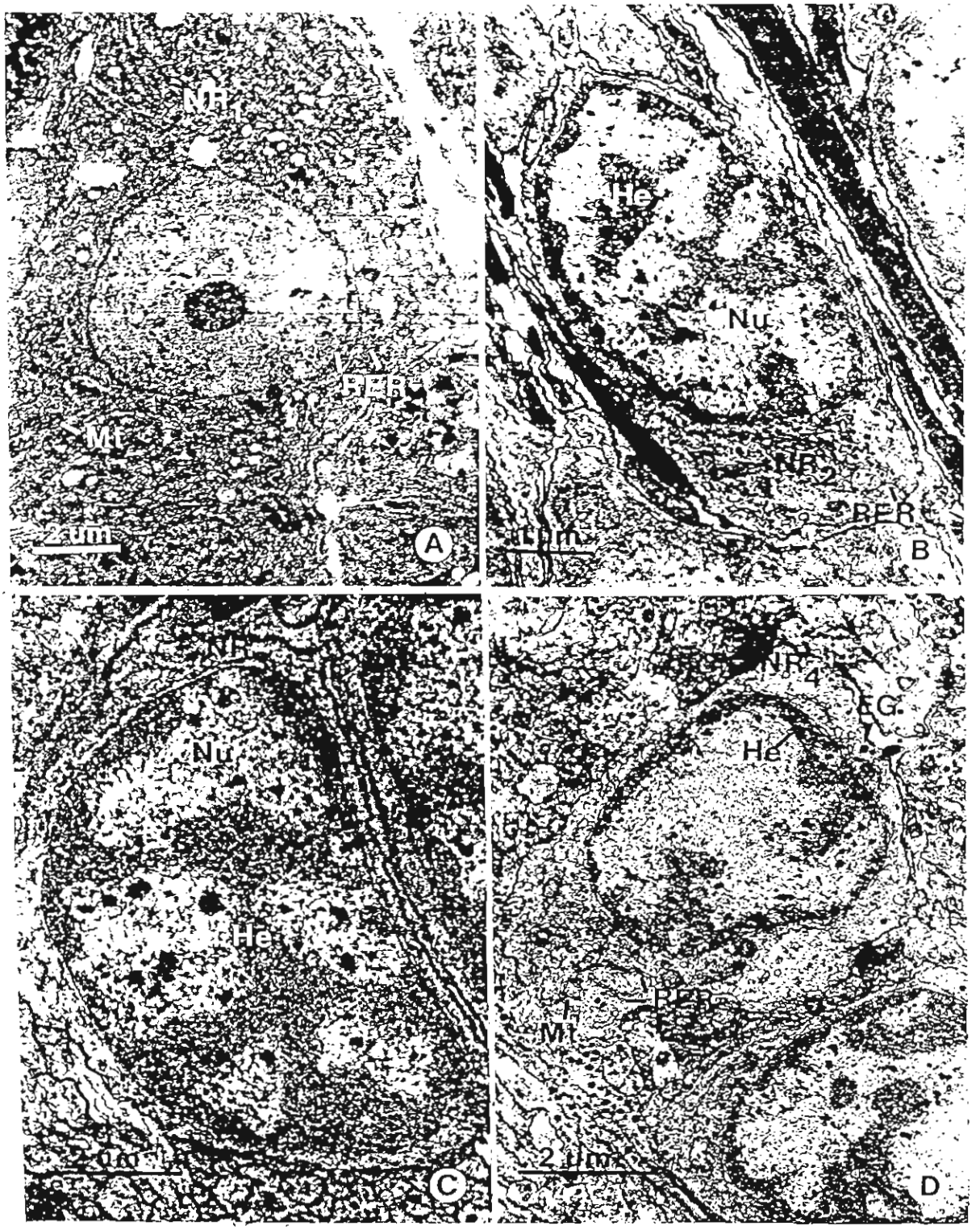
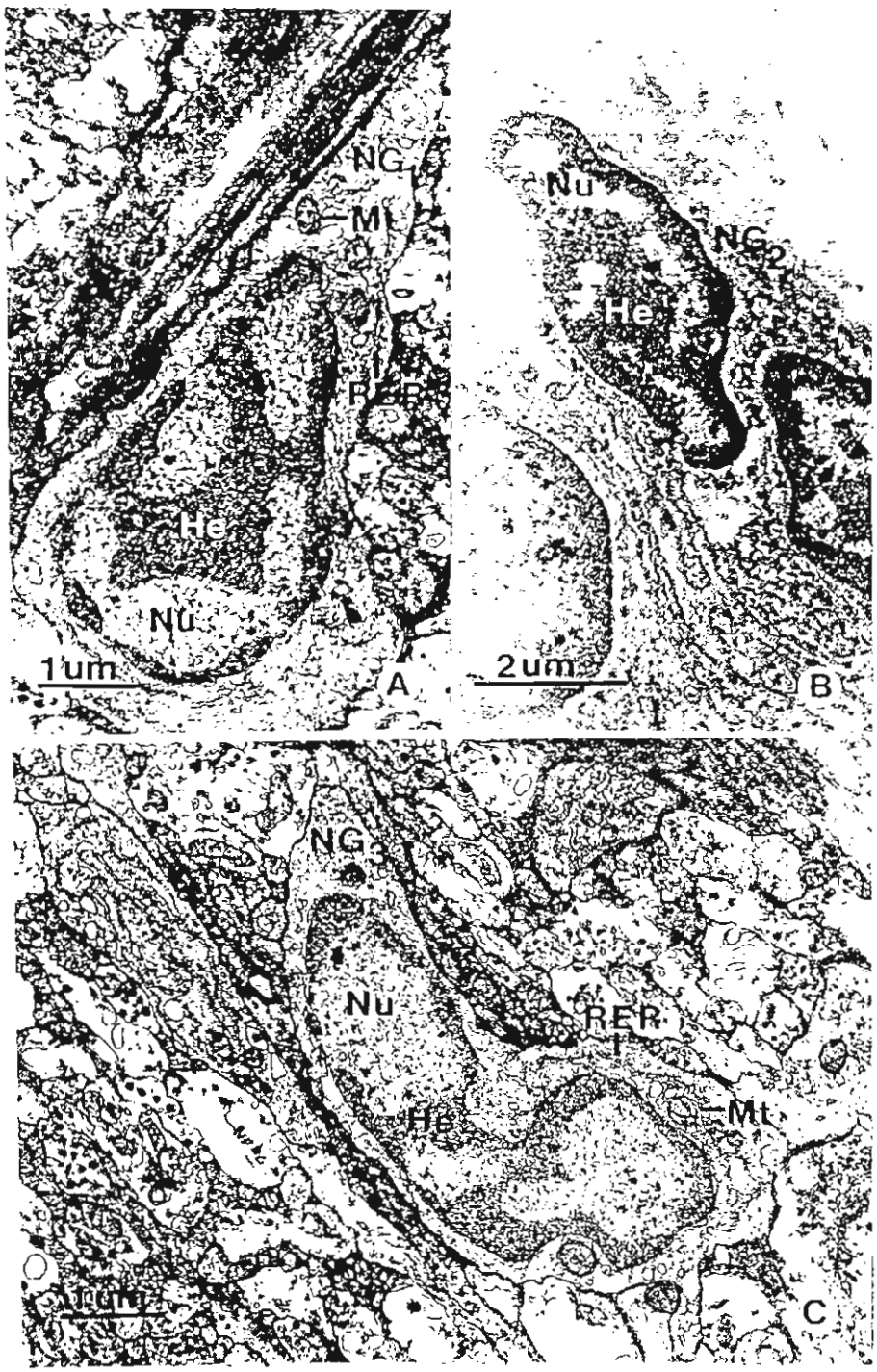


Fig 2



ULTRASTRUCTURE OF SPERMATOZOA IN THE TESTIS OF HALIOTIS ASININA LINNAEUS

Somjai Apisawetakan[1], Prasert Sobhon[1,*], Chaitip Wanichanon[1], Vichai Linthong[1], Maleeya Kruatrachue[2], Suchart Upatham[2,3], Padermsak Jarayabhand[4], Tanate Pumthong[5] and Jintana Nugranard[5]

ABSTRACT

When studied by TEM and SEM, the head of a spermatozoa of *H. asinina* Linnaeus appears as a cone-shaped cell whose size is about 3x1-1.5 μm, with a long tail. The acrosome is an inverted cup-shaped structure, covering the indented anterior end of the head, and containing homogeneously dense matrix. There is an acrosomal core with crystalline structure occupying the concave subacrosomal space with its base resting in the indention of the anterior tip of the nucleus. The nucleus contains chromatin granules about 60 nm in diameter which are densely packed together, and a few nuclear vacuoles. At the posterior end of the nucleus there are 3-5 globular-shaped mitochondria that are adhered to the nucleus by dense plaques of outer membranes, and linked to the posterior half of the sperm membrane by zig-zag filaments. A pair of centriole located in the middle of mitochondria, with the proximal horizontal centriole tightly attached to the nucleus by thickened double plates, and the distal vertical centriole gives rise to a long tail. Axoneme of 9+2 doublets of microtubules makes up the entire core of the tail, whose membrane in the proximal part is crenulated while that in the remaining distal part is tightly-fitted around the axoneme.

KEY WORDS - Spermatozoa, ultrastructure, *Haliotis asinina*

INTRODUCTION

Like in mammalian species mollusc sperm are either cone-shaped or highly elongated with some appearing spiral; all of which conform to the typical architecture of flagellated animal sperm in possessing an acrosome, a head, a mid piece and a tail. In spite of the general similarity, however, there are unique features in the sperm of various mollusc species. By and large, sperm of externally-fertilizing molluscs are considered primitive, by virtue of having cone-shaped heads and long tails that do not have middle pieces. Instead, their mitochondria are located at the base of the nucleus (Franzen, 1955). In contrast, sperm of the

[1] Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
[*] Corresponding author and reprint request.
[2] Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
[3] Department of Biology, Faculty of Science, Burapha University, Chonburi, 20131.
[4] Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[5] Coastal Aquaculture Development Center, Department of Fishery, Ministry of Agriculture and Cooperatives, Klong Wan, Prachuapkhirikun, 77000.

internally-fertilizing molluscs, especially among neogastropods of fresh water, marine and even terrestrial species, possess long slender heads, and elongated middle pieces where helical or long straight mitochondria are concentrically arranged around the axonemes (Walker & MacGregor, 1968; Walker, 1970; Franzen, 1970; Kitajima & Paraense, 1976; Huaquin & Bustos-Obregon, 1981; Healy, 1983; Healy & Willian, 1984; Azevedo & Corral, 1985; Jaramillo *et al.*, 1986; Hodgson, 1986; Gallardo & Garrido, 1989; Sretarugsa *et al.*, 1991; Al-Hajj & Attiga, 1995; Pastisson & Lacorre, 1996). Abalone belong to prosobranch group of gastropods which are considered to be rather primitive, and reproduce by external fertilization. Thus their sperm belong to the first type as described earlier. However, even within their own genus, spermatozoa of abalone exhibit specie-specific characteristics. In the present study, we report the ultrastructure of spermatozoa in *Haliotis asinina*, a species commonly found in the coastal water of tropical region, including Thailand.

MATERIALS AND METHODS

Collection of *H. asinina* specimens

Abalone from land-based culture system are provided by the Coastal Aquaculture Development Center, Prachaubkirikhun Province, and Marine Biological Station, Chulalongkorn University, Angsila, Chonburi Province. They are kept in concrete tanks housed in the shade, which are well flushed with mechanically circulated filtered sea water and aerated by air delivery system to maintain the controlled environment. The optimum level of salinity is about 22.5-32.5 ppt. and the temperature is about 22-26°C (Singhagraiwan & Doi, 1993). They are fed with a diet of macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food for abalone.

Electron Microscopic Study

Small pieces of the testis were prefixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4^0C, for overnight, then prepared for electron microscopic observations by conventional TEM and SEM methods.

For TEM studies, the specimens were post-fixed in 1% osmium tetroxide in the same buffer, at 4^0C, for 2 hours, then dehydrated in a graded series of ethanol, and embedded in Araldite 502 resin. Ultrathin sections were cut and stained with lead citrate-uranyl acetate and viewed under a Hitachi TEM H-300 at 75 kV.

For SEM studies, the specimens were post-fixed, ethanol-dehydrated, and critical-point dried in liquid CO_2, and coated with Platinum-Paladium in an ion-sputtering apparatus. Finally, they were examined with a Hitachi S-2500 scanning electron microscope at 15 kV.

RESULTS

Scanning Electron Microscopy (SEM)

Under SEM, a testicular sperm exhibits cone-shaped head, whose size is about 3 μm in length, 1.5 μm in width at the base of the nucleus and 1 μm at the acrosomal-nuclear junction (Fig.1A-D). Compared to earlier stages of germ cells, the surface of spermatozoa appears smooth (Fig.1B,C). The anterior end is covered by a cup-shaped acrosome, while the posterior end by five spherical mitochondria of similar size. Projecting from the middle of mitochondria is a long, slender, and uniform tail (Fig.1B,C,D).

Transmission Electron Microscopy (TEM)

Under TEM acrosome appears as an inverted cup, whose concavity separating it from the anterior border of the nucleus, which is also indented. This subacrosomal space contains crystalline acrosomal core embedded in a more homogeneous material (Fig.2B,E,F). The acrosomal matrix itself appears homogeneous with varying degree of electron opacity (Fig.2E,F).

The nuclei of most spermatozoa contain completely condensed chromatin which appear electron opaque, except for clear areas of intranuclear vacuoles where there seems to be little chromatin material. Vacuoles are varying in size and distributed randomly throughout the nucleus. In a few spermatozoa, the nuclear chromatin still appear granular with numerous round granules tightly packed together, with each "granule" about 60 nm in diameter (Fig.3B,D). These granules are of similar size and characteristics as those observed in earlier stage spermatids where chromatin is not yet completely condensed.

The posterior border of the nucleus are flanked by spherical mitochondria. In a longitudinal thin section of the nucleus, usually two to three mitochondria could be observed (Fig.2A,B,C). However, in fortuitous cross sections at the level of mitochondria, there appear to be five of these bodies arranged in circle surrounding the centriole (Fig.2A,B). Each mitochondria is tightly apposed to the nucleus at which the nuclear membrane appears thickened (Fig.3B,E,F). Mitochondria contain shelf-like cristae, with some stretching from one side to the opposite side (Fig.3B,E,F).

In the posterior corner of cytoplasm between the nucleus and mitochondria (Fig.3A,B,E,F), there are bundle of thin zig-zag filaments, whose width is about 15-20 nm. These filaments link mitochondria to the adjacent cell membrane, and some filaments appear attached to the latter at specific location (Fig.3B,E,F). These filaments also surround the entire lower half of the nucleus.

The tail piece commences from a pair of centriole whose proximal member is embedded in the socket at the middle region of the slightly indented posterior border of the nucleus (Fig.3A,B,D). The nuclear membrane at this region is also visibly thickened (Fig.3B,D). Long axoneme stretching backwards from the distal centriole that is surrounded by mitochondria (Fig.2C,3A). In cross sections (Fig.3C) axoneme appears as 9+2 doublets of microtubules, and this pattern appears to exist along the whole length of the tail (Fig.2D,3F). Axoneme is covered directly by partially wavy plasma membrane at the proximal end close to the centriole (Fig.2C), while on the rest of the tail it appears fairly smooth and fits snugly around the axoneme (Fig.3F).

DISCUSSION

The shape and general morphology of *H. asinina* sperm is typical of the "primitive type" or "ect-aquasperm" described for mollusc which reproduce by external fertilization, which include chitons, bivalves (Frazen, 1970; Baccetti, 1979) and scaphopoda (Dufresne-Dube *et al.*, 1983). Spermatozoa of these mollusc usually possess short, cone-shaped heads and simple tails that lack mid pieces. In contrast, mollusc whose sperm fertilize the eggs internally tend to have elongated and sometimes also spiraling heads, and the tails that have definitive mid pieces containing mitochondria or their derivertives; and frequently glycogen particles in residual cytoplasmic masses still remaining around parts of the tails (Franzen, 1956; 1983; Anderson & Personne, 1976; Baccetti & Afzelius, 1976; Healy, 1996).

The heads of sperm in *H. asinina* appear to be shorter and more globular in comparison to those of other temperate abalone species, such as *H. rufescens* (Lewis *et al.*, 1980), whose sperm tend to have elongate bullet-shaped heads. There also appear to be more clear areas or intranuclear vacuoles within the nucleus where chromatin mass is lacking. The chromatin in *H. asinina* appear to be "granular type" where large chromatin granules of 50-60 nm are packed tightly side-by-side. During spermiogenesis these large chromatin granules are derived from the periodic thickening of formerly uniform chromatin fibers whose original size in the earliest round spermatid stage is about 20-30 nm (unpublished observation). These granular pattern of chromatin condensation could also be perceived in other primitive gastropods, such as *trochid* genus (Healy 1989; Hodgson *et al.*, 1990), scaphopoda (Dufresne-Dube *et al.*, 1983), and bivalves (Bozzo *et al.*, 1993; Cacas & Subirana, 1994; Johnson *et al.*, 1996). By contrast, in the internally fertilized sperm of most meso- and all neogastropods, opisthobranchs and pulmonates, the chromatin condensation is of "fibrillar-lamellar" type; where the pattern of chromatin condensation goes through three successive phases: granular, fibrillar and lamellar structures, that finally become tightly packed in myelin like whorls (Healy, 1987; 1988; Jaramillo *et al.*, 1986; Gallardo & Garrido, 1989; Amor & Durfort, 1990; Sretarugsa *et al.*, 1991; Caceres *et al.*, 1994). It is possible that these two different patterns of chromatin condensation may be linked to the qualitative difference of basic nuclear proteins, especially protamines, which appear to be more variable among primitive, externally fertilized molluscs and the more modern, internally fertilized molluscs; while histones appear to be more conserved (Subirana *et al.*, 1973; Balhorn *et al.*, 1979; Van Helden *et al.*, 1979; Chiva *et al.*, 1991; Daban *et al.*, 1991a; Caceres *et al*, 1994). Much work remains to be done in mollusc in characterizing and correlating the variation of protamines with the above-mentioned patterns of chromatin condensation.

The acrosome of *H. asinina* has cup-shaped with much less elongation and invagination from nuclear side, in comparison to those of temperate abalone species (Lewis *et al.*, 1980) and other primitive gastropods (Hodgson *et al.*, 1990). The acrosomal material is uniformly homogeneous in contrast to the two clearly separated areas shown in *H. rufescens* (Lewis *et al.*, 1980). The acrosomal core is composed of short thick crystalline-like axis embedded within moderately dense matrix that occupies the whole subacrosomal space. In comparison to other primitive gastropods and temperate abalone species, the core is much shorter. The crystalline material is probably consisted of actin and its associated proteins as reported in other molluscs (Baccetti, 1979; Shiroya *et al.*, 1986; Tilney *et al.*, 1987). This acrosomal core may participate in the extension of acrosomal process during acrosomal reaction and fertilization.

The tail of *H. asinina* sperm consists of an axonemal core of 9+2 doublets of microtubules surrounded directly by plasma membrane. This type of simple tail is also observed in other primitive gastropods (Healy 1989; Hodgson *et al.*, 1990), scaphopoda (Dufresne-Dube *et al.*, 1983) and bivalves (Bozzo *et al.*, 1993; Casas & Subirana, 1994; Johnson *et al.*, 1996), all of which reproduce by external fertilization. In contrast, the mollusc that reproduce by internal fertilization possess tails akin to those of mammalian sperm (Jaramillo *et al.*, 1986; Gallardo & Garrido 1989; Amor & Durfort, 1990; Sretarugsa *et al.*, 1991, Caceres *et al.*, 1994). Such tails usually have mid pieces which contain large cylindrical or helical mitochondria. Fibrous sheathes often surround the axoneme to provide sturdy support that could serve stronger movement than the simple tails found in abalone and other primitive gastropods. Indeed, in the latter there are only five small globular mitochondria located at the posterior end of the nucleus, surrounding proximal and distal centrioles. These mitochondria could probably generate smaller quantity of energy for the less motile sperm of these species.

Another remarkable feature in the sperm of *H. asinina*, which have not been reported in sperm of other molluscs, is the presence of zig-zag filaments in the cytoplasm at the posterior corner of the head. It appears that these filaments link mitochondria to the posterior part of the plasma membrane, and some are surrounding the lower half of the nucleus. Because of their zig-zag nature and solid core structure they are probably not microtubules, but their exact component have not yet been identified. We speculate that these filaments could play some roles in controlling the shape of the nucleus as well as the position of mitochondria.

ACKNOWLEDGEMENTS

This investigation was supported by The Thailand Research Fund (Contract BRG4080004 and Senior Research Scholar Fellowship to Prasert Sobhon).

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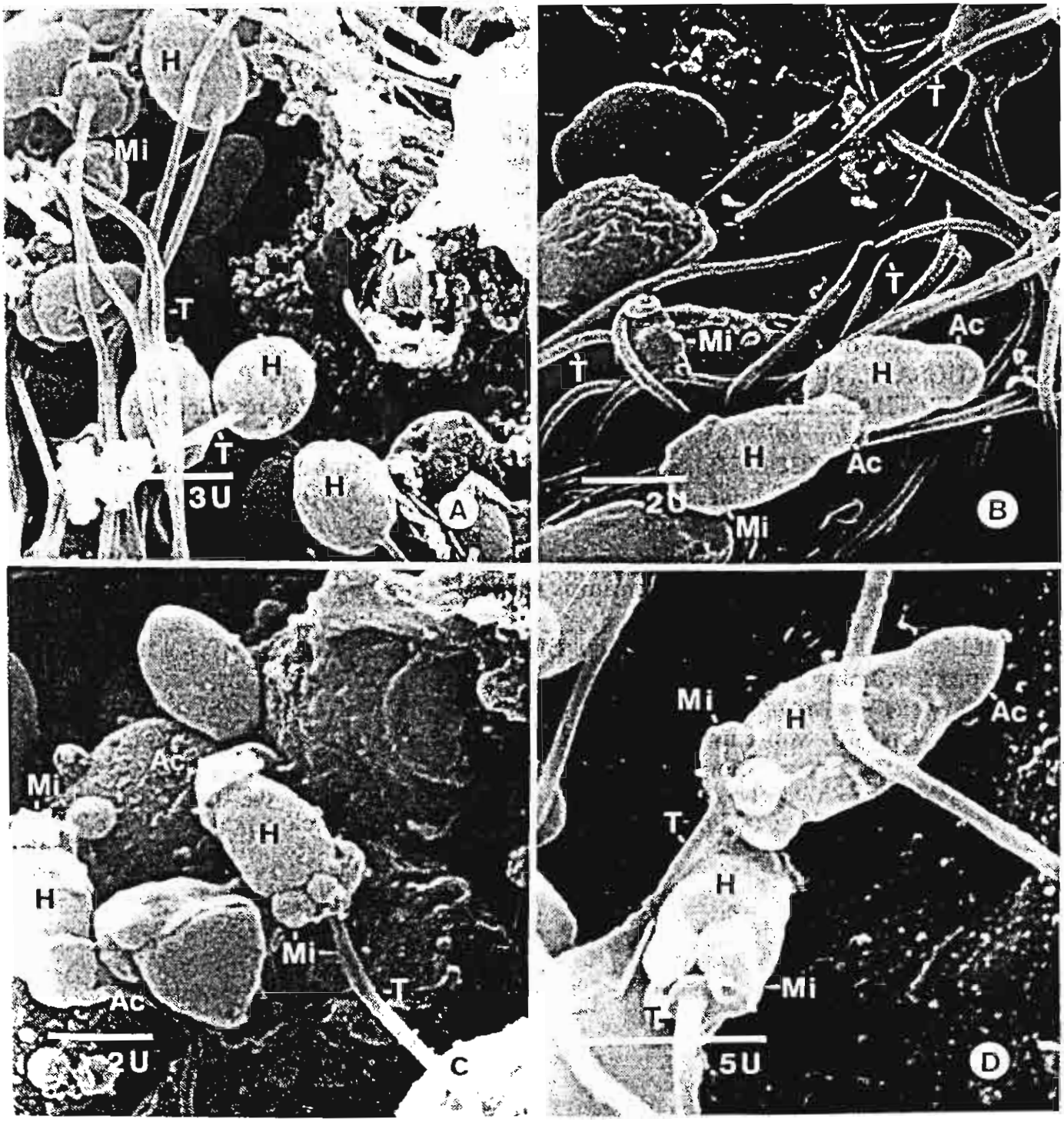

Fig.1 Scanning electron micrographs of spermatozoa in the testis showing cone-shaped heads (H), rod-shaped tails (T) (**A,B**). Five globular mitochondria (Mi)at the base of the head surrounding the proximal region of the tail (**C,D**), and cup-liked acrosome (Ac) covering the anterior end of the head (**C,D**).

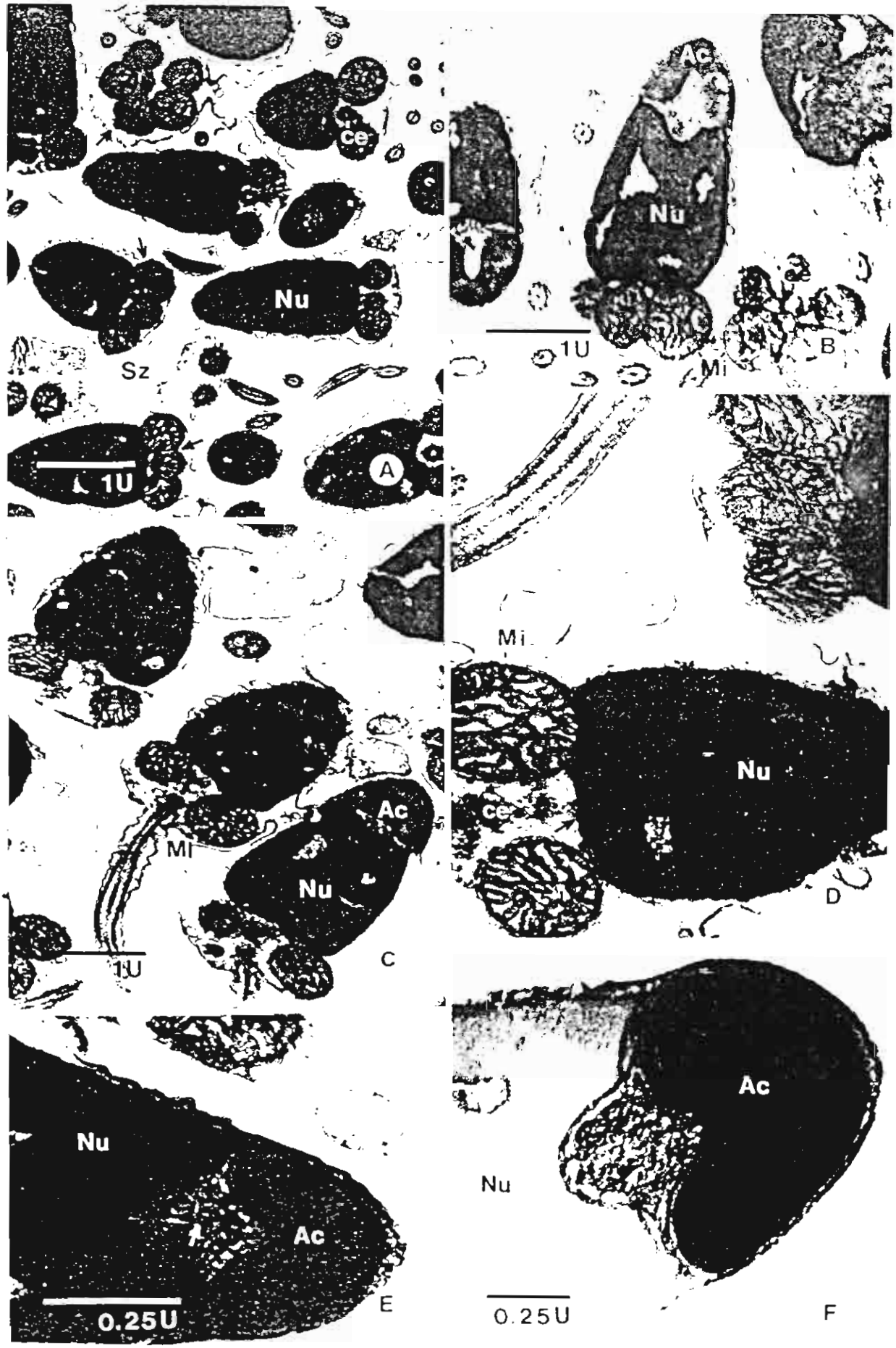

Fig.2 Transmission electron micrographs of mature spermatozoa (Sz) showing nucleus (Nu), acrosome (Ac), plasma membrane (pm) mitochondria (Mi) and vacuoles (va) in the nucleus. Five globular mitochondria (arrows in A,B) surrounding a pair of centriole (ce), which is tightly attached to the nucleus by thickened double plates (arrow in D) at the neck region. An acrosomal core with crystalline structure filling in the concavity of the subacrosomal space at the anterior tip of the nucleus (arrows in E,F)

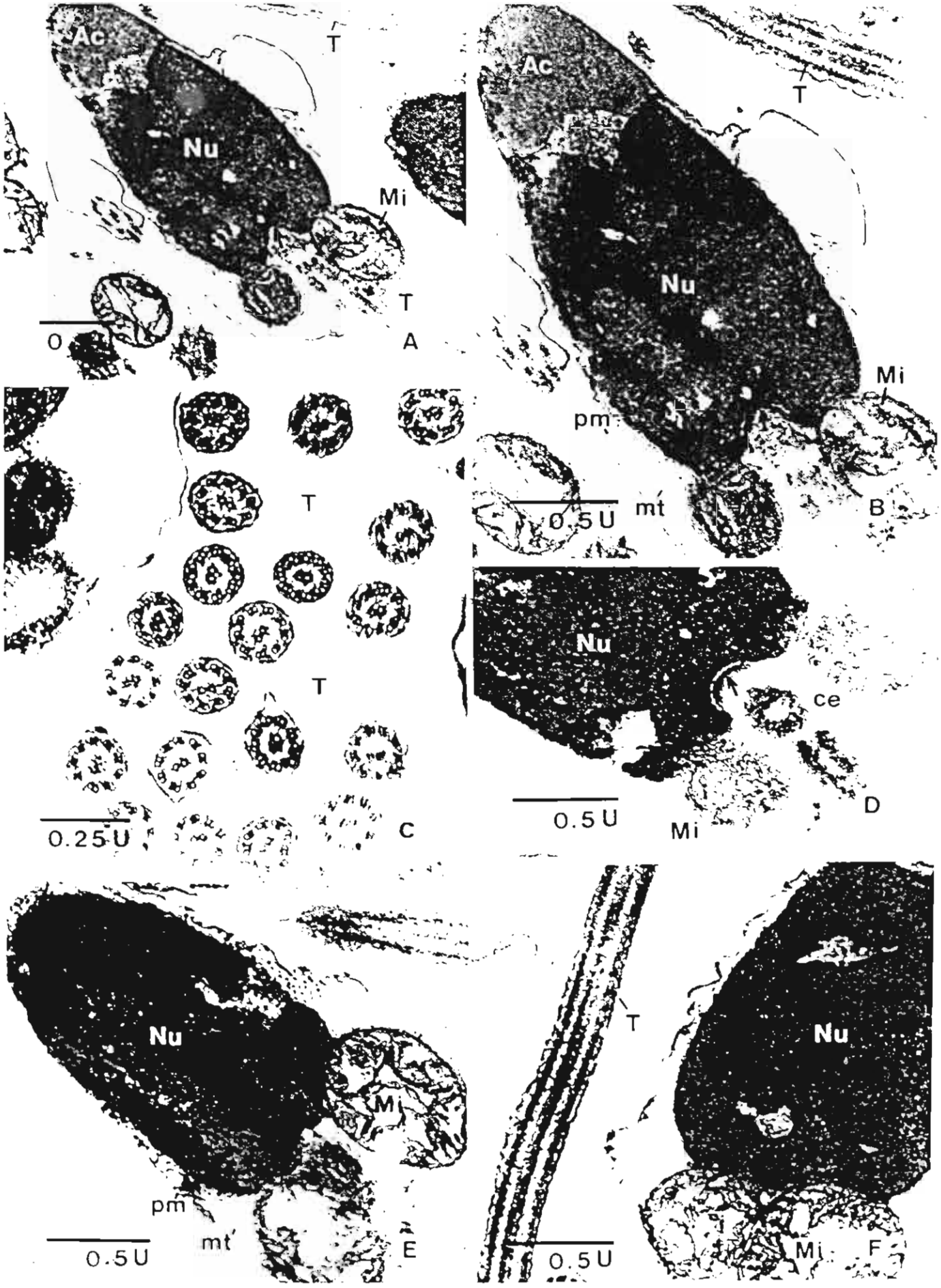

Fig.3 Transmission electron micrographs showing the nuclei (Nu), and tails (T) of spermatozoa consisting of centrioles (ce), the proximal one attached to the nucleus by the thickened plates (arrows in A,B,D). The zig-zag filaments (mt) in the cytoplasm link the nucleus and mitochondria to the posterior sperm membrane (pm) (B,E). The tail consists of axoneme of 9+2 doublets of microtubules surrounded by plasma membrane (cross-section in C, long-section in F). Chromatin in the nuclei of cells in B and D still show incomplete condensation, where individual chromatin granules with 60 nm in diameter are closely packed together, yet the outline of each granules is still visible.