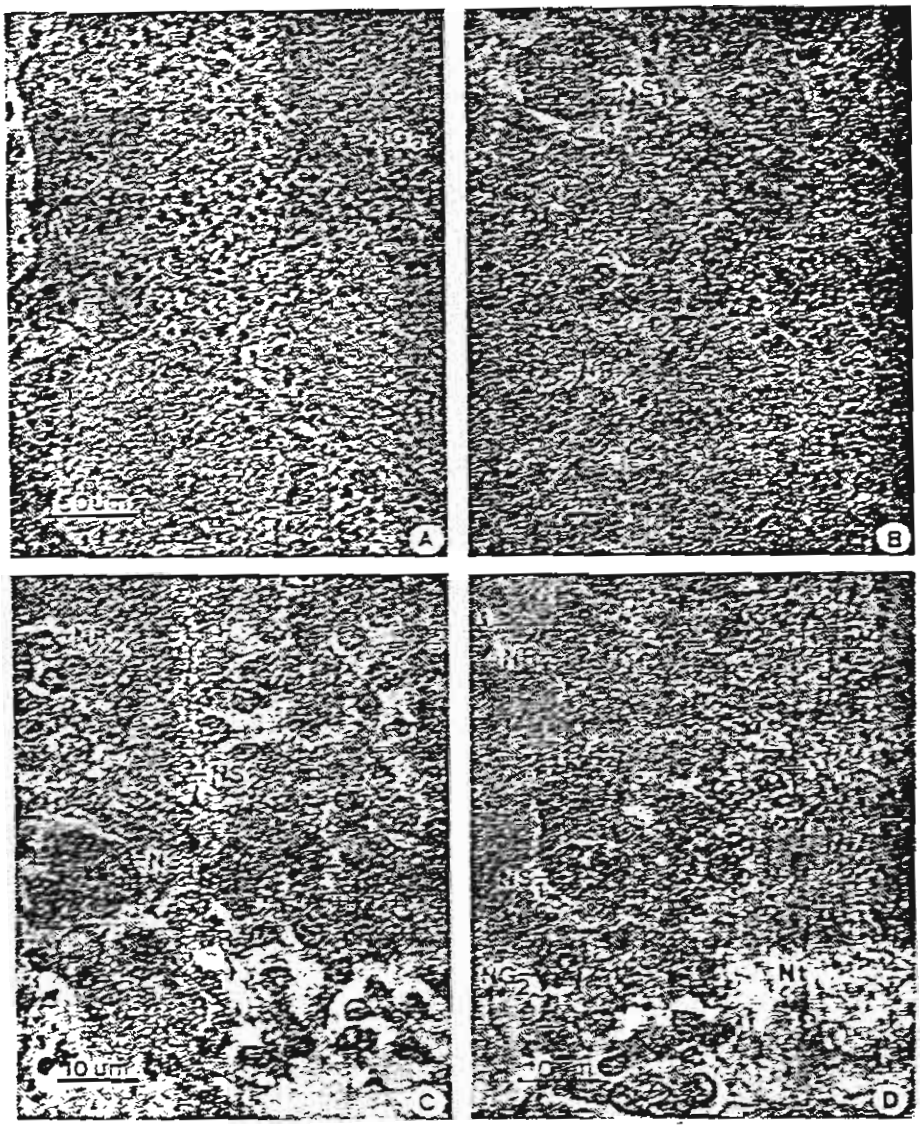


Figure 4. Medium-power (A) and high-power (B–D) micrographs showing various types of nerve cells in the cortex stained with H&E. NS_1, Type 1 neurosecretory cell; NS_2, Type 2 neurosecretory cell; NR_1, Type 1 neuron; NR_2, Type 2 neuron; NG_1, Type 1 neuroglia; NG_2, Type 2 neuroglia; NG_3, Type 3 neuroglia; Nt, nerve tract.

and contain round nuclei (4–6 µm in diameter) with patchy heterochromatin. The cytoplasm is extremely thin and does not contain neurosecretory granules (Figs. 3D and 4B and C).

Type 3 Neuron (NR_3)

These cells are a little smaller than NR_2, about 4 µm in diameter. They occur in the innermost cell layer of the cortex (Figs. 3D and 6). The nucleus is elliptical (3 µm in diameter) and contains completely dense heterochromatin (Fig. 3D). There are no neurosecretory granules in the cytoplasm.

Type 1 Neuroglia (NG_1)

These cells are scattered throughout the cortical region of the ganglion (Figs. 4B and 6). They are small (3–6 µm in diameter) and contain a spindle-shaped nucleus (Fig. 4B). The nuclear membrane is a little crenated, with a thin rim of heterochromatin attached to its inner surface, whereas most of the remaining chromatin is euchromatic (Fig. 4B).

Type 2 Neuroglia (NG_2)

The cell body and nuclear size of these cells are similar to those of NG_1, but they show completely dense chromatin (Figs. 3C and 4D). NG_2 lie in a single row on the basement membrane (Figs. 4D and 6).

Type 3 Neuroglia (NG_3)

These are small cells with spindle-shaped nuclei (2–3 µm) that contain completely dense heterochromatin. They are scattered among nerve bundles of the medulla (Figs. 3B, 4A, 5D, and 6).

DISCUSSION

The anatomy of the nervous system of the tropical abalone, *H. asinina*, is similar to those of primitive prosobranchs and other species of abalone described by Crofts (1929) and Fretter and Graham (1962). Crofts (1929) reported that in *H. tuberculata*, *H. lamellosa*, and *H. cracherodii*, the cerebral ganglia sent the nerves to supply the epipodia, tentacles, eyes, and statocysts. Through

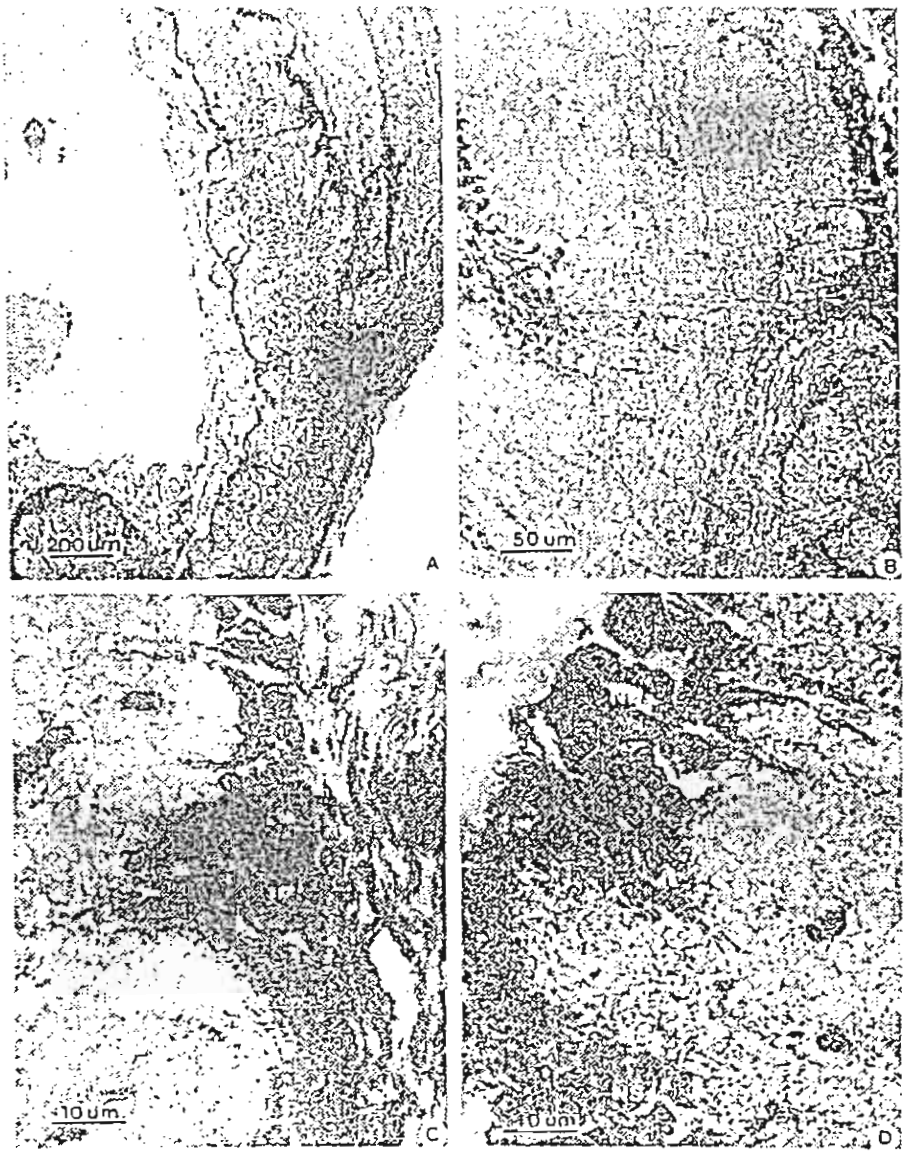


Figure 5. A low-power micrograph showing longitudinal section of cerebral ganglion stained with CH-P. Do, dorsal; La, lateral; Ve, ventral. (B–D) Medium-power (B) and high-power (C and D) micrographs showing longitudinal sections of cerebral ganglion stained with CH-P. Co, cortex; Np, neuropil; NG_3, Type 3 neuroglia; NS_1, Type 1 neurosecretory cell; NS_2, Type 2 neurosecretory cell; NR_1, Type 1 neuron; NR_2, Type 2 neuron; NG_3, Type 3 neuroglia.

these nerves, the animals receive chemosensory, mechanosensory, and visual input from their environment. Hence, the cerebral ganglia are probably the most important center for nervous integration, comparable to the brain in higher animals. The cerebral ganglia in *H. asinina* are also connected with the buccal ganglion and pleuropedal ganglion mass. It was, therefore, suggested that the cerebral ganglia could serve as a center for coordinating and modulating various functions mediated by the rest of the nervous system (Jahan-Parwar and Fredman 1976).

The cerebral ganglia of *H. asinina* are surrounded by a loose connective tissue that is rich in collagen-like fibers, threaded with capillary plexuses. This connective tissue sheath is quite different from that of *Helix aspersa* Muller, which is composed of two layers, the outer layer being packed with globuli cells and the inner being dense and lamellated (Fernandez 1966). The histological study presented here of the cerebral ganglia of *H. asinina* revealed that they contain eight cell types: two types of neurosecretory cells, three types of neurons, and three types of neuroglia. Yahata (1971) and Hahn (1994a) described four types of neurons in *N. discus* and *H. discus hannai*. They are called Type A, Type B, Type C, and Type D cells. Type A and Type B cells are neurosecretory cells. On the basis of the similarities in size and shape and their nuclear characteristics, distribution, and staining affinity, the neurosecretory cells Type 1 (NS_1) and Type 2 (NS_2) in this study should correspond to Type A and Type B cells, respectively, as reported by Yahata (1971) and Hahn (1994a). In *N. discus*, Type A cells were further divided into Type A-I cells, which contain neurosecretory granules stained with PF and CH-P, and Type A-II cells, the secretory granules of which stained with phloxine but not CH-P. Type A-II cells of *H. discus hannai* appear to be larger (20–32 μm) than the NS_1 of *H. asinina* (20 μm). However, the cell bodies of both Type A cells (Hahn 1994a) and NS_1 stained with PF.

Neurosecretory cells are found in large quantities and variety in molluscan ganglia, which are the principal source of hormones. The functions of neurosecretory cells in the cerebral ganglia are

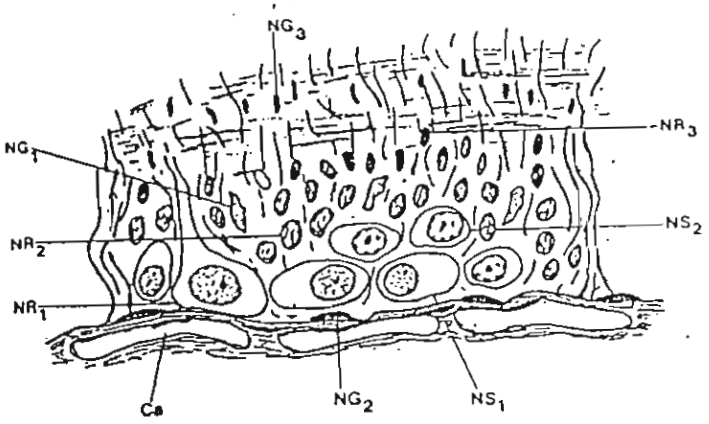


Figure 6. A diagram illustrating structure and cellular composition of the cortex and medulla of cerebral ganglion. NS_1, Type 1 neurosecretory cell; NS_2, Type 2 neurosecretory cell; NR_1, Type 1 neuron; NR_2, Type 2 neuron; NR_3, Type 3 neuron; NG_1, Type 1 neuroglia; NG_2, Type 2 neuroglia; NG_3, Type 3 neuroglia; Ca, capillary.

thought to be related to reproduction (Yahata 1971, Yahata 1973, Hahn 1994a, Hahn 1994b). Yahata (1971) found that Type A and Type B cells in the cerebral ganglia of *N. discus* showed seasonal changes in the staining intensity of PF. These cells began to accumulate PF granules in June, when the gonads started to mature, and the PF stain intensity continued to increase until it reached a maximum in September, which was the month of spawning. Hence, the rise and fall of the neurosecretory material coincided with gonadal maturation (Yahata 1971). However, the injection of crude homogenate of the cerebral ganglia into ripe females, *N. discus*, did not induce spawning, but there was a considerable gain in the mean body weight from the increase in water uptake (Yahata 1973).

Hahn (1994a) reported that the neurosecretory activities in Type A and Type B cells in the cerebral ganglia of *H. discus hannai*, as reflected by the staining intensity of cytoplasmic material, varied with the reproductive cycles. The neurosecretory activity of Type A cells was correlated with vitellogenesis in the ovaries of females, but not with gonad maturation and spermatogenesis in males. The neurosecretory activity in Type B cells in both sexes did not show any correlation with gametogenesis, vitellogenesis, or spawning. Further studies are clearly needed on the neuroendocrine activities and functions of neurosecretory cells of cerebral ganglia in abalone, including NS_1 and NS_2 cells in *H. asinina*.

There are three types of neurons in the cerebral ganglia of *H. asinina*, whereas only two types of neurons (Type C and Type D cells) were described in *N. discus* and *H. discus hannai* (Yahata 1971, Hahn 1994a). On the basis of the size and shape of cells and their nuclei, NR_2 are quite similar to Type C cells, whereas NR_3 probably correspond to Type D cells. These cells did not have any neurosecretory granules in their cytoplasm. NR_1 cells have not been reported in *N. discus* or *H. discus hannai* (Yahata 1971, Hahn 1994a). These cells are the largest neurons in the cerebral ganglia of *H. asinina*. They are pyramidal and multipolar in shape with a round nucleus, and no neurosecretory granules are observed in the cytoplasm. Compared with the classification of neurons in the nervous systems of higher vertebrates, it is possible that NR_1 may be concerned with motor activities because of their large size and multipolarity, whereas NR_2 and NR_3 are most likely to be associated neurons.

Three types of neuroglia were observed in the cerebral ganglia of *H. asinina*. To the best of our knowledge, there has not yet been any classification of neuroglia in any species of abalone. NG_1 are probably the general glia cells of the cortex because of their uniform distribution in all cell layers of the cortex. NG_2, because of their unique lining of the basement membrane, could be a part of the blood–nerve barrier that gates out the undesirable factors from the blood supplied by the capillaries. NG_3, on the other hand, are glia cells of the neuropil of the medullary region. It remains to be proved whether they are involved in the synthesis of the myelin-like structure surrounding the nerve fibers in the neuropil.

ACKNOWLEDGMENT

This study received financial support from the Thailand Research Fund. TF BRG 4080004 and PG 2/015/2539

BRG 4080004 and PG 2/015/2539

LITERATURE CITED

Bullock, T. H. 1965. Mollusca: Gastropoda. pp. 1283–1386. *In:* T. H. Bullock and G. A. Horridge (eds.). Structure and Function in the Nervous System of Invertebrates. Freeman, San Francisco.

Crofts, D. R. 1929. Haliotis. *Liverpool Mar. Biol. Committee Memoirs* 29:1–174.

Dorsett, D. A. 1986. Brains to cells: the neuroanatomy of selected gastropod species. pp. 101–187. *In:* The Mollusca. A. O. D. Willows (ed.). vol. 9. Academic Press, New York.

Fernandez, J. 1966. Nervous system of the snail *Helix aspersa* I. Structure and histochemistry of ganglionic sheath and neuroglia. *J. Comp. Neurol.* 127:157–181.

Fretter, V. & A. Graham. 1962. British Prosobranch Molluscs. Bartholomew Press, Dorking. pp. 299–320.

Gomori, G. 1941. Observations with differential stains on human Islets of Langerhans. *Am. J. Pathol.* 17:395–406.

Gomori, G. 1950. Aldehyde-fuchsin: a new stain for elastic tissue. *Am. J. Clin. Pathol.* 20:665–666.

Hahn, K. O. 1994a. The neurosecretory staining in the cerebral ganglia of the Japanese abalone (ezoawabi), *Haliotis discus hannai*, and its relationship to reproduction. *Gen. Comp. Endocrinol.* 93:295–303.

Hahn, K. O. 1994b. Gametogenic cycle of the Japanese abalone (ezoawabi), *Haliotis discus hannai*, during conditioning with effective accumulative temperature. *Aquaculture.* 122:227–236.

Jahan-Parwar, B. & S. Fredman. 1976. Cerebral ganglion of *Aplysia*—cellular organization and origin of nerves. *Comp. Biochem. Physiol.* 54A:347–357.

Joosse, J. 1979. Evolutionary aspects of the endocrine system and of the hormonal control of reproduction of molluscs. pp. 119–157. *In:* E. J. W. Barrington (ed.). Hormones and Evolution. vol. 1. Academic Press, New York.

Miller, W., R. S. Nishioka & H. A. Bern 1973. The "juxtaganglionic" tissue and the brain of the abalone, *Haliotis rufescens* Swainson. *Veliger.* 16:125–129.

Yahata, T. 1971. Demonstration of neurosecretory cells in the cerebral ganglion of the abalone, *Nordotis discus* Reeve. *Bull. Fac. Fish. Hokkaido Univ.* 22:207–214.

Yahata, T. 1973. Demonstration of neurosecretory cells in the cerebral ganglion of the abalone, *Nordotis discus* Reeve infected with ganglional suspensions. *Bull. Jpn. Soc. Sci. Fish.* 39:1117–1122.

HISTOLOGICAL STUDIES OF THE PLEURO-PEDAL GANGLION, VISCERAL GANGLION AND PEDAL CORD GANGLIA OF *HALIOTIS ASININA* LINNAEUS

A. Thongkukiatkul, E.S. Upatham, P. Sobhon, M. Kruatrachue, C. Wanichanon, T. Pumthong and J. Nugranad

ABSTRACT

The pleuro-pedal, visceral and pedal cord ganglia of *Haliotis asinina* were studied by light microscopy using hematoxylin-eosin, chrome-hematoxylin-phloxine and paradehyde-fuchsin stains. These ganglia contain ten types of cells : three types of neurosecretory cells (NS_{1-3}), four types of neurons (NR_{1-4}) and three types of neuroglia (NG_{1-3}). NS_{1-3} contain round nuclei with three different patterns of heterochromatization and neurosecretory granules in the cytoplasm. NR_{1-4} do not have neurosecretory granules. NR_1 are giant neurons with oval or pyramidal shape. NR_{2-4} are small neurons with oval bodies and nuclei, and they are the majority of neuronal cells in the cortex of the ganglia. NG_{1-3} are spindle-shaped cells containing similar shaped nuclei. NG_1 and NG_3 are the main neuroglia of the cortex and medulla of the ganglia, respectively, while NG_2 are a part of the blood-nerve barrier that borders the connective tissue capsule of the ganglia.

KEY WORDS *Haliotis asinina*, histological studies, pleuro-pedal ganglion, visceral ganglion, pedal cord ganglia

INTRODUCTION

The nervous system of *Haliotis asinina* Linnaeus consists of a pair of cerebral ganglia, a pleuro-pedal ganglion mass, and a visceral ganglion and a pair of pedal cord ganglia (Upatham *et al.*, 1998). Arising from the pleuro-pedal ganglion are two loops of nerve cords : the visceral cord and the paired pedal nerve cords. The visceral cord twists into a figure 8 around the visceral mass. At its posterior end is a single visceral ganglion that gives off many nerves going to digestive and reproductive organs (Upatham *et al.*, 1998). The paired pedal cords run parallel along the midline of the foot muscle and send off many nerves to innervate the foot muscles (Upatham *et al.*, 1998).

Hahn (1992) described several cell types in the cerebral and pleuro-pedal ganglia of *Haliotis discus hannai* Ino. Two types of neurosecretory cells had been described in these ganglia (Hahn, 1992, 1994). Yahata (1971) also demonstrated two types of neurosecretory cells in the cerebral ganglia of *Nordotis discus* Reeve. Upatham *et al.* (1998) reported on the classification

and histology of cells in the cerebral ganglia of *H. asinina*. There were eight types of nerve cells in the cerebral ganglia : two types of neurosecretory cells, three types of neurons, and three types of neuroglia (Upatham *et al.*, 1998). However, there is virtually no information on the histology of nerve cells in the pleuro-pedal, visceral, and pedal cord ganglia of *H. asinina*. Hence, the objective of the present study is to study the histology and classification of cell types, and their characteristics in the pleuro-pedal, visceral, and pedal cord ganglia of *H. asinina*.

MATERIALS AND METHODS

Mature 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. These animals were reared in a land-based aquaculture system with well circulated and aerated sea water. They were given appropriate algal food *ad libitum*, and kept under normal daylight. Abalone were anesthetized with 5% MgCl, after which their shells were removed. The pleuro-pedal, visceral, and pedal cord ganglia were dissected out and fixed in Bouin's fluid in 0.14 M NaCl for 12 hours. Tissues were dehydrated through a graded series of ethanol, infiltrated with dioxane, and embedded in paraffin. Sections were cut at 5-6 μm thickness and stained with hematoxylin eosin (H&E), chrome-hematoxylin-phloxine (CH-P) (Gomori, 1941) and paraldehyde-fuchsin (PF) (Gomori, 1950). Neurons and cells in the pleuro-pedal, visceral and pedal cord ganglia were observed and evaluated for their cell size and shape, nuclear size and shape, and staining affinity under an Olympus Vanox light microscope.

RESULTS

Histological study

Pleuro-pedal ganglion This is a large ganglion. Each ganglion is surrounded by a loose connective tissue rich in collagen-like fibers and capillaries in close contact with the ganglion capsule. The ganglion is composed of two parts : the outer cortex and the inner medulla. In the cortex, there are numerous neurosecretory cells, neurons and neuroglia. The dorsal and lateral edges of the ganglion's cortex are relatively thick, and contains 4-6 cell layers. In contrast, the ventral edges of the cortex of the ganglion are thin and contain only 2-3 layers of cells. There are many layers of cells where the nerve branches out. There are many bundles of nerve tracts in the medullary region

Visceral ganglion. This ganglion appears like a dumb-bell, with both sides of the ganglion form a bulb-like structure. Most of the lateral and ventro-medial parts of the ganglion have thick cortex that contains 2-3 layers of cells. In contrast, the remaining parts are relatively thin, and contain only a single layer of ganglionic cells. The neuropil of the ganglion consists of both longitudinal and cross sectional nerve tracts.

Pedal cord ganglia. These are the enlarged portions of the two parallel pedal cords, which are paired along the elongated strands of the cords; the pairs are linked by irregular commissures. Each ganglion is composed of two parts : the outer cortex and inner medulla. The ganglion is surrounded by a loose connective tissue and capillaries. In the cortex, there are numerous neurosecretory cells, neurons and neuroglia. The ventral part of the ganglion's cortex is relatively thick, and contains 4-5 layers of cells. In contrast, the dorsal and lateral parts of the cortex of the ganglion are thin and contain only 2-3 layers of cells. There are many bundles of nerves intersecting in the medullary region of the ganglion. The ganglionic cells in the pedal cord ganglia are generally more numerous than those in the visceral ganglion.

Classification of cell types

The cells in the pleuro-pedal, visceral and pedal cord ganglia can be classified into ten types based on their histological characteristics and staining affinities to H&E, CH-P and PF. These include three types of neurosecretory cells (NS_1, NS_2, NS_3), four types of neurons (NR_1, NR_2, NR_3 and NR_4) and three types of neuroglia (NG_1, NG_2 and NG_3).

Type 1 neurosecretory cell (NS_1). These cells are the largest neurosecretory cells. Most cells occur along the periphery of the cortex, resting on the basement membrane of the ganglion capsule. The cell body is round or oval (10x20 μm). The nucleus is round (7 μm in diameter), which contains mostly pale-stained euchromatin with only a thin rim of heterochromatin binding to the internal surface of the nuclear envelope. The nucleolus is small but very distinct. The cytoplasm shows a clear boundary. It is stained reddish pink with H&E, and pinkish purple with CH-P. There are numerous neurosecretory granules which are stained deep violet with PF and fill the entire cytoplasm.

Type 2 neurosecretory cell (NS_2). These cells are smaller than NS_1. They are located in the inner cell layer. The cell body is round or oval and of medium size (10x12 μm). The nucleus is round (7 μm in diameter) with most blocks of heterochromatin attached to its periphery with some in the center. Together, they resemble a clock-face pattern. The nucleolus is denser than that of NS_1. The stainings of NS_2 are similar to those of NS_1. The cytoplasm

contains fewer neurosecretory granules than does NS_1, and they stained deep violet with PF.

Type 3 neurosecretory cell (NS_3). These cells are the smallest neurosecretory cells. They are medium in size (8x10 μm). The nucleus is round (6 μm in diameter) and located on one side of the cell. The nucleus contains a thick rim of heterochromatin attached to the periphery, and some thick heterochromatin cords in the center. The cytoplasm does not show a clear boundary but contains abundant granular material.

Type 1 neuron (NR_1). These cells are the largest neurons and have a pyramidal shape (25x40 μm). Their long axons extend inwardly into the medulla of the ganglion. The nucleus is round (13 μm in diameter), and contains almost entirely euchromatin, with large and eccentrically located nucleolus. The cytoplasm stained homogeneously pink with H&E and CH-P. There are no neurosecretory granules in the cytoplasm.

Type 2 neuron (NR_2). These cells are the most numerous among neuronal cells. They are concentrated mostly in the middle cell layer of the cortex. They have a round to oval shape (4-6 μm in diameter), and contain oval nuclei (4 μm in diameter) with patchy heterochromatin. The cytoplasm is extremely thin and does not contain neurosecretory granules.

Type 3 neuron (NR_3). These cells are slightly smaller than NR_2 (4 μm in diameter), while having similar shape. They occur in the innermost cell layer of the cortex. The nucleus is elliptical (3 μm in diameter) and contains completely dense heterochromatin. There are no neurosecretory granules in the cytoplasm.

Type 4 neuron (NR_4). These cells occur in small number. The nucleus is round or oval (4-6 μm in diameter) with a thick rim of heterochromatin attached to the periphery, while the central area is clear. The nucleolus, with round shape, is very distinct. There are no neurosecretory granules in the cytoplasm. These cells are fewest in number among neurons.

Type 1 neuroglia (NG_1). These cells are scattered throughout the cortical region of the ganglion. They are small spindle-shaped cells (3x6 μm) that contain similar shaped nuclei. The nuclear membrane is a little crenated, with a thin rim of heterochromatin attached to its inner surface, whereas most of the remaining chromatin is euchromatic. There is only a thin rim of cytoplasm around the nucleus .

Type 2 neuroglia (NG_2). The cell body and nuclear size of these cells are similar to those of NG_1 but they show completely dense chromatin. NG_2 lie in a single row on the basement membrane.

Type 3 neuroglia (NG_3). These are the smallest cells with spindle-shaped nuclei (2-3 μm) that contain completely dense heterochromatin. They are scattered among nerve bundles of the medulla.

DISCUSSION

Histological studies of the pleuro-pedal, visceral and pedal cord ganglia of *H. asinina* revealed that they contain ten cell types: three types of neurosecretory cells, four types of neurons and three types of neuroglia.

Neurosecretory cells are found in the pleural ganglion of many gastropods such as *Bithynia tentaculata* (Linnaeus)(Andrews, 1968), *Lymnaea stagnalis* Linnaeus (Wendelaar Bonga, 1970), *Bulinus truncatus* (Audouin) (Boer *et al.*, 1977) and *Haliotis* spp. (Hahn 1992). Hahn (1992) described several cell types found in *H. discus hannai*, but only two cell types, #1 -and #7-cells are thought to be neurosecretory cells. Cell type #1 has oblong shape about 8-20 μm in diameter and contains a large nucleus (5-10 μm in diameter). Cells type #7 is large (16 μm) with a small nucleus (4 μm). Their secretory granules were stained with PF. Based on the similarities in size and shape, and staining affinity, the neurosecretory cells type 1 (NS_1) and type 2 (NS_2) in the present study should correspond to cells type #1 and #7 as reported by Hahn (1992). The neurosecretory activities of cells type #1 were correlated with gametogenesis, while cells type #7 show a strong correlation with the induction of spawning (Hahn, 1992). The histological characteristics of NS_1 and NS_2 in the pleuro-pedal ganglion are quite similar to those in the cerebral ganglia (Upatham *et al.*, 1998).

There are also three types of neurosecretory cells in the visceral ganglia of other mollusks such as *L. stagnalis* (Wendelaar Bonga, 1970) and *Australorbis glabratus* Pilsbry (Lever, 1965). Lever (1965) described only one type of neurosecretory cells in *A. glabratus*. These cells are large, ranging between 50-70 μm, with a nucleus of 13-48 μm. The cytoplasm contains large violet-black droplets. It is different from those reported in *H. asinina*. The functions of neurosecretory cells in the visceral ganglion are thought to be related to reproduction (Yahata, 1973), inducing spawning (Hahn, 1992) and stimulating diuresis (Wendelaar Bonga, 1972).

The pedal cord ganglia consist of ganglionic cells, arranged peripherally in a thick layer, which is similar to the situation reported in *Haliotis tuberculata* Linnaeus (Crofts, 1929). The large cells tend to be situated on the outside of the ganglion, while the smaller cells towards the center. The histological study of the pedal cord ganglia of *H. asinina* revealed that they also contains three types of neurosecretory cells. Boer *et*

al. (1977) described three types of neurosecretory cells in the pedal ganglion of *B. truncatus*. They are called dark green cells, yellow green cells and yellow cells. Based on similarity in size, the NS_1 in the present study should correspond to the dark green cells of *B. truncatus*. However, the functions of these neurosecretory cells of pedal ganglia have not yet been demonstrated.

There are four types of neurons in the pleuro-pedal, visceral and pedal cord ganglia of *H. asinina* whereas only one type of neurons is described in *B. tentaculata*. It is noticeable that the giant neurons (NR_1) are more abundant in the pleuro-pedal ganglia than in the cerebral ganglia (Upatham *et al.*, 1998). These cells are multipolar and possess characteristics similar to large motor cells such as ventral horn motor cells of spinal cord and Pukinje cells of cerebellum in vertebrates. As such, they may be involved in controlling and co-ordinating motor activities, especially that of pedal muscle. Other neurons (NR_{2-4}) are small and appear like ordinary neurons and globuli cells described in *Aplysia californica* (Cooper), which could be association neurons (Bullock & Horridge, 1965). However, there is a paucity of giant neurons (NR_1) in the visceral ganglion in comparison to those in the pleuro-pedal and pedal cord ganglia. Thus rather than having a control over substantial motor activities, the neurons of the visceral ganglion are thought to be related to the control of functions of the visceral organs (Crofts, 1929).

The present study also demonstrated the presence of three types of neuroglia in the pleuro-pedal, visceral and pedal cord ganglia of *H. asinina*. Upatham *et al.* (1998) reported that the cerebral ganglia of *H. asinina* also contained three types of neuroglia. These neuroglia in the pleuro-pedal, visceral and pedal cord ganglia have similar characteristics to the NG_{1-3} described in the cerebral ganglia of *H. asinina* (Upatham *et al.*, 1998).

ACKNOWLEGEMENTS

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

LITERATURE CITED

ANDREWS, E.B. 1968. The fine structure of the nervous system of *Bithynia tentaculata* (Prosobranchia) in relation to possible neurosecretory activity. *The Veliger*, 14: 13-20.

BOER, H.H., ROUBOS, E.W., VAN DALEN, H. & GROESBEEK, J.R.F. TH. 1977. Neurosecretion in the basommatophoran snail *Bulinus truncatus* (Gastropoda, Pulmonata). *Cell and Tissue Research*,176: 57-67.

BULLOCK, T.H. & HORRIDGE, G.A. 1965. *Structure and Function in the Nervous System of Invertebrate*. Vol.2. Freeman, San Francisco.

CROFTS, D.R. 1929. *Haliotis*. In *Liverpool Marine Biology Committee Memoirs on Typical British Marine Plants and Animals*. (Edited by Johnstone, J.). The University Press of Liverpool. 174 pp.

GOMORI, G. 1941. Observations with differential stains on human Islets of Langerhans. *American Journal of Pathology*, 17: 395-406.

GOMORI, G. 1950. Aldehyde-funchsin : A new stain for elastic tissue. *American Journal of Clinical Pathology*, 20: 665-666.

HAHN, K.O. 1992. Review of endocrine regulation of reproduction in abalone *Haliotis* spp. In *Abalone of the World : Biology, Fisheries and Culture*. (Edited by Shepherd, S.A., Tagner, M.J. & Guzman Del Proo, S.A.), pp. 49-58. Fishing News Books, London.

HAHN, K.O. 1994. The neurosecretory staining in the cerebral ganglia of the Japanese abalone (ezoawabi), *Haliotis discus hannai* and its relationship to reproduction. *General and Comparative Endocrinology*, 93: 295-303.

LEVER, J., DE VRIES, C.M., & JAGER, J.C. 1965. On the anatomy of the central nervous system and on the location of neurosecretory cells in *Australorbis glabratus*. *Malacologia*, 2: 219-230.

UPATHAM, E.S., THONGKUKIATKUL, A., KRUATRACHUE, M., WANICHANON, C., CHITRAMVONG, Y.P., SAHAVACHARIN, S. & SOBHON, P. 1998. Classification of neurosecretory cells, neurons and neuroglia in the cerebral ganglia of *Haliotis asinina* Linnaeus by light microscopy. *Journal of Shellfish Research*, 17. (In press).

WENDELAAR BONGA, S.E. 1970. Ultrastructure and histochemistry of neurosecretory cells and neurohaemal areas in the pond snail *Lymnaea stagnalis*. *Zeitschriff fuer Zellforschung und Mikroskopische Anatomie*, 108: 190-224.

WENDELAAR BONGA, S.E. 1972. Neuroendocrine involvement in osmoregulation in freshwater snail *Lymnaea stagnalis*. *General Comparative Endocrinology Supplement*, 3: 308-316.

YAHATA, T. 1973. Induced spawning of abalone (*Nordotis discus* Reeve) injected with ganglional suspensions. *Bulletin of the Japanese Society of Science and Fisheries*, 39: 1117-1122.

Amporn THONGKUKIATKUL[1], E.Suchart UPATHAM[2], Prasert SOBHON[3], Maleeya KRUATRACHUE[2], Chaitip WANICHANON[3], Tanate PUMTHONG[4] and Jintana NUGRANAD[4]

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

[2]*Departments of Biology and* [3]*Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

[4]*Coastal Aquaculture Development Center, Prachuap Khiri Khan 77000, Thailand*

FIG. 1

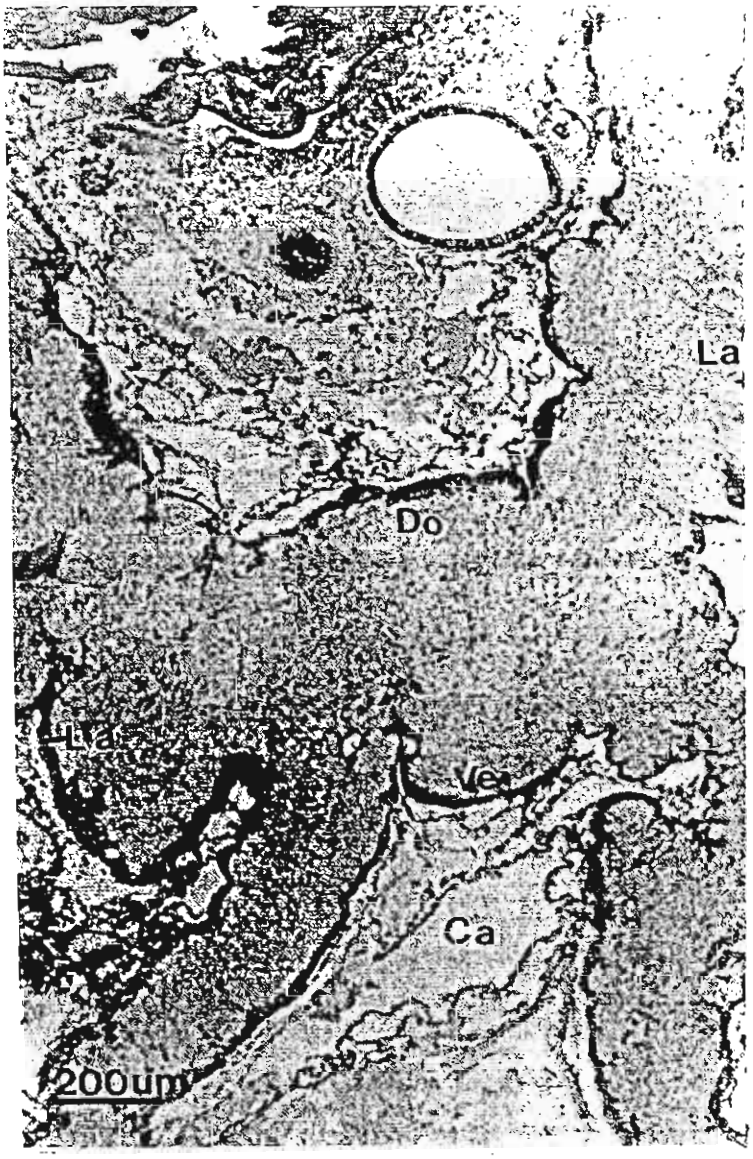


FIG.

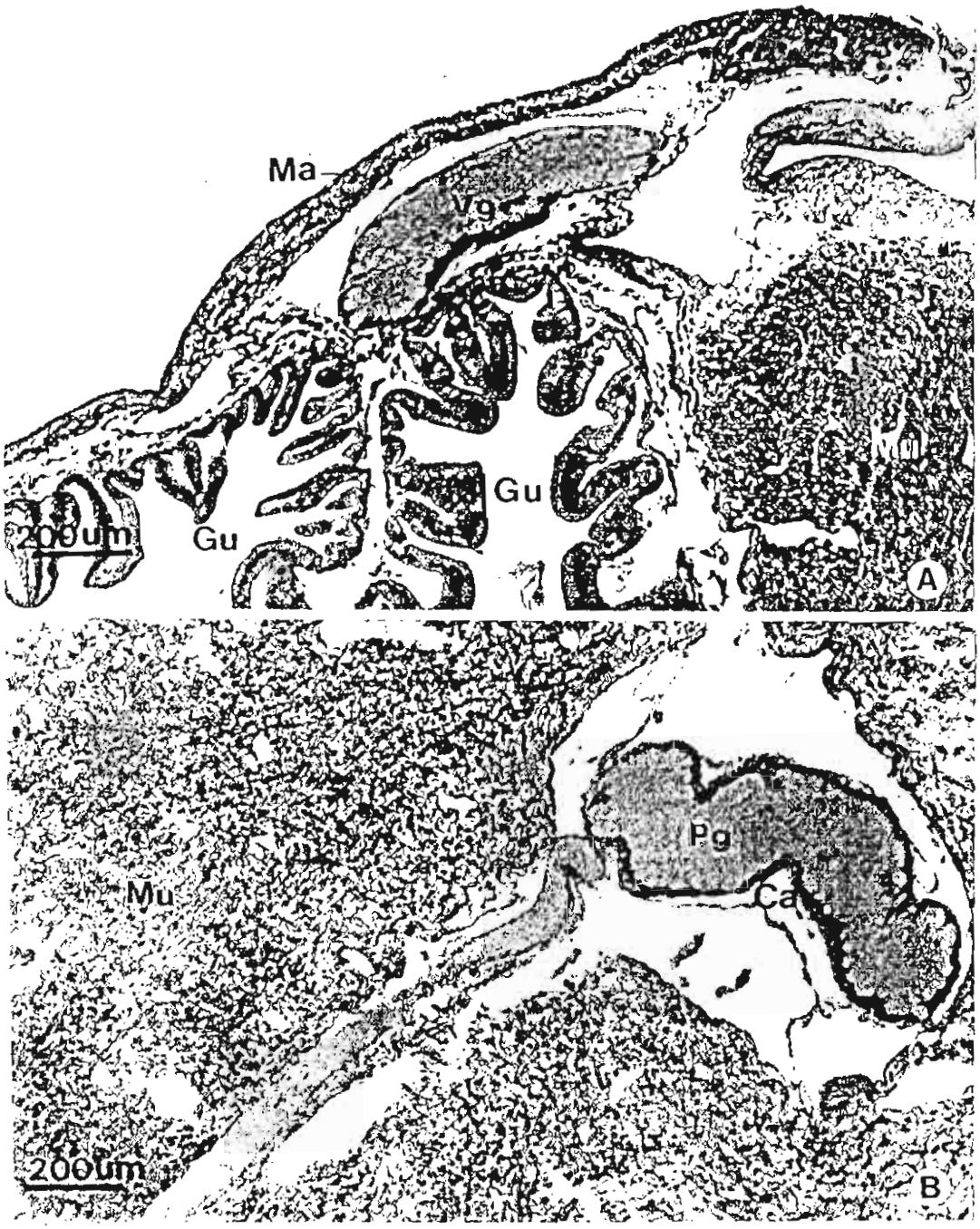
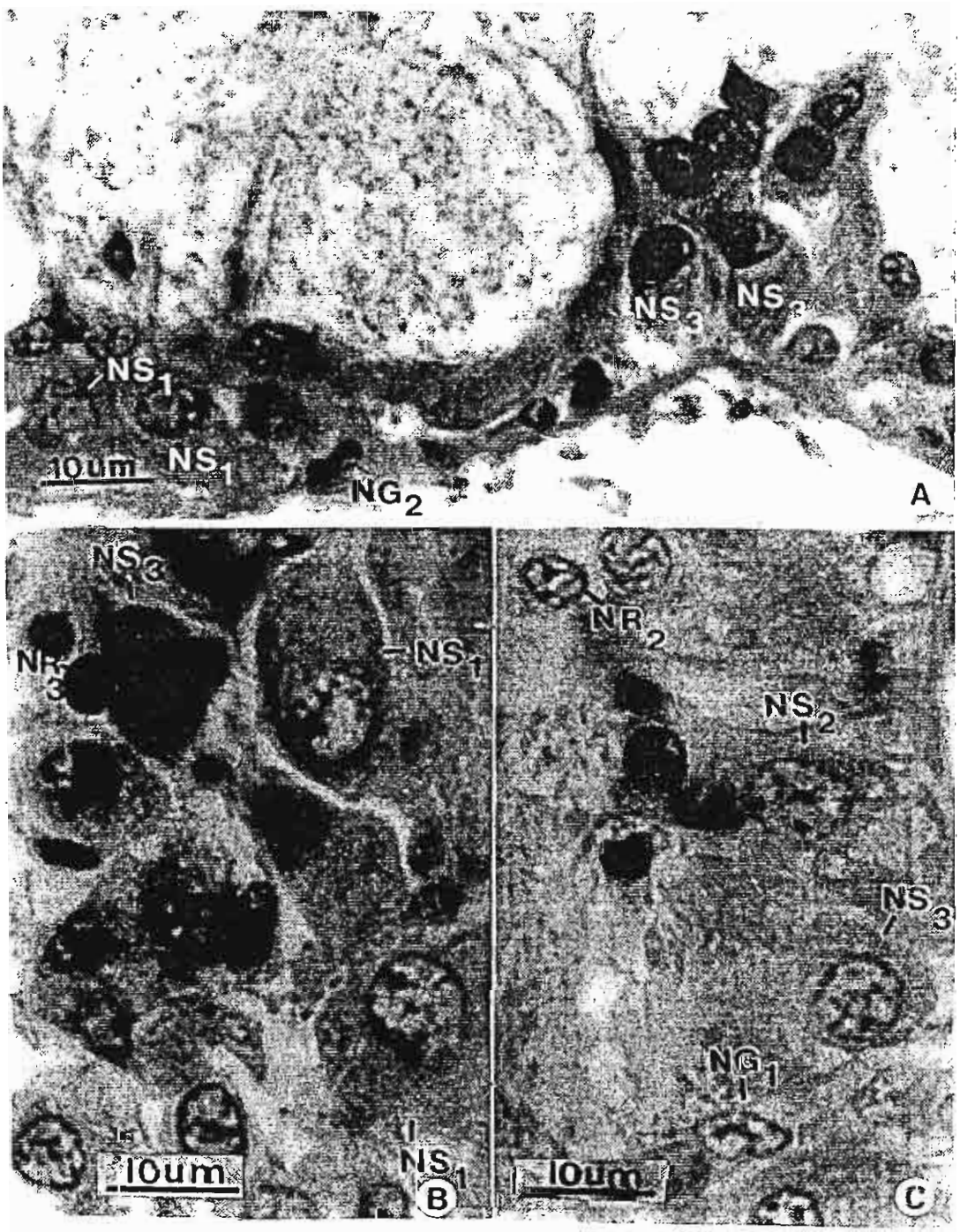
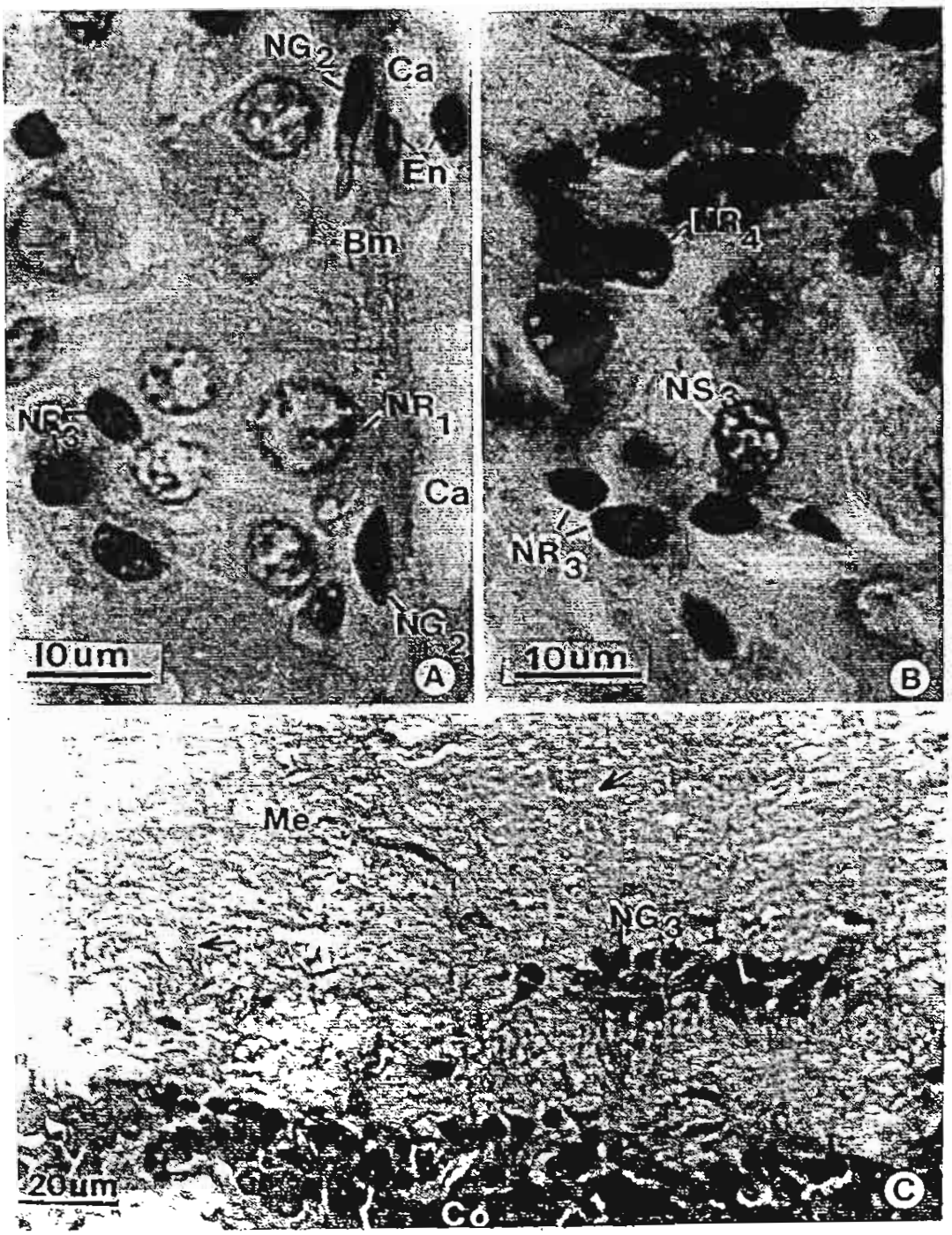


FIG. 3


NS3
NS2
NS1
10um
NS1
NG2
A
NS3
NR3
NS1
NS1
10um
B
NR2
NS2
NS3
NG1
10um
C


NG2
Ca
En
Bm
NR1
NR3
Ca
NG2
10um
A
NR4
NS3
NR3
10um
B
Me
NG3
20um
Co
C

ScienceAsia
Journal of the Science Society of Thailand
วารสารของสมาคมวิทยาศาสตร์แห่งประเทศไทย

March 16 , 1999

Dr.Prasert Sobhon
Department of Anatomy
Faculty of Science
Mahidol University

Dear Dr.Prasert

Thank you very much for submitting the manuscript entitled Classification of Germ Cells, Reproductive Cycle and Maturation of Gonads in *Haliotis Asinina* Linnaeus (Code 9901-005, received 29 January 1999) for consideration for publication in ScienceAsia.

The manuscript has been read by two independent referees, whose reports are enclosed for your information. Although the referees have recommended acceptance of your manuscript, there are a number of queries and comments which require clarification from you. In addition, the manuscript needs to be revised in light of their comments. Please reply to every point of the referees' comments or queries, and send 3 copies of the revised manuscript, together with the diskette, back to me as soon as possible, preferably within 3 months.

Looking forward to receiving the revised manuscript and reply to the referees from you soon. Thank you again for your interest in contributing to our journal.

Sincerely

Prof.Dr.Yongyuth Yuthavong
Editor
ScienceAsia

EDITORIAL OFFICE : Public Information Department, Thailand's National Science and Technology Development Agency, 73/1 Rama VI Road, Rajdhevee, Bangkok 10400, Thailand.
Tel: +66-2-644 8150-4 ext. 101, 107 Fax: +66-2-644 8215

CLASSIFICATION OF GERM CELLS, REPRODUCTIVE CYCLE AND MATURATION OF GONADS IN HALIOTIS ASININA LINNAEUS

PRASERT SOBHON[a], SOMJAI APISAWETAKAN[a], MALEE CHANPOO[a], CHAITIP WANICHANON[a], VICHAI LINTHONG[a], AMPORN THONGKUKIATKUL[c], PADERMSAK JARAYABHAND[d], MALEEYA KRUATRACHUE[b], SUCHART UPATHAM[c] AND TANATE POOMTHONG[e].

[a] *Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*
[b] *Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*
[c] *Department of Biology, Faculty of Science, Burapha University, Chonburi,20131.*
[d] *Department of Marine Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.*
[e] *Coastal Aquaculture Development Center, Department of Fishery, Ministry of Agriculture and Cooperatives, Klong Wan, Prachuabkirikhu, 77000.*

ABSTRACT

Germ cells in the gonads of Haliotis asinina, a species of abalone found along the coast of Thailand, were classified by light and electron microscopies. Germ cells in oogenetic units could be classified into six stages according to their histological and ultrastructural characteristics: oogonium and five stages of oocytes, i.e., Oc_1 with light to intense basophilia and abundant polyribosomes, with some in large aggregates; Oc_2 with intense basophilia, oil droplets, numerous well developed Golgi complexes and rough endoplasmic reticulum, but little secretory granules; Oc_3 with a few yolk granules and 2 types of cortical granules; Oc_4 with increasing number of yolk granules, numerous cortical granules and thin jelly coat; and Oc_5 is the mature ovum with 2 types of yolk granules, numerous cortical granules and fully formed jelly coat. The cells in spermatogenetic units could be classified according to the pattern of chromatin condensation into thirteen stages: spermatogonium, five stages of primary spermatocytes, secondary spermatocyte, four stages of spermatids and two stages of spermatozoa.

The gonads of adult H. asinina reared in land-based culture system exhibit five phases of reproductive cycle during the year: these are proliferative, premature, mature, spawning and spent phases. Gonads in proliferative and premature phases contain primarily gonial cells, early oocytes$_{1-3}$ and spermatocytes, while mature phase contains mainly late stage cells, i.e., oocytes$_{4-5}$ in ovary and spermatids and spermatozoa in testis. The spawning phase occurs at least twice during each year: from March to April and August to October in females, and with similar intervals but slightly prolonged duration in males. Spent phase, occurring after the period of spawning, is characterized by a complete discharge of gamete cells and the breakdown of connective tissue stroma. It takes approximately 5 to 6 months for gonads to regenerate their connective tissue stroma and germ cell population, and finally become repleted with mature cells again.

In developing H. asinina definitive gonads appear to be clearly separated from hepatopancreas at 2 months. Histologically, gonial cells appear at 2 months, early spermatocytes and spermatids at 4 months; early oocytes (Oc_{1-2}) at 6 to 7 months. While completely mature spermatozoa could arise in the gonads as early as 6 to 7 months, mature oocytes (Oc_{4-5}) occur much later at 10 to 11 months. The male animals tend to reach full sexual maturity and start normal reproductive cycle as early as 7 to 8 months, while female animals reach sexual maturity and start reproductive cycle around 11 to 12 months.

KEYWORDS: *Haliotis asinina*, gametogenesis, germ cells, reproductive cycle, gonad development

INTRODUCTION

There are three species of abalone along the Thai coasts, namely, *H. asinina*, *H. ovina*, *H. varia*[1-3], which are also distributed generally over the Indo-western Pacific area, especially in coastal reef zones of Southeast Asia[4-5]. These abalone species are found along the Thai Gulf and Andaman Sea, usually in the crevices on coral and rocky reefs, at the depth of 1 to 7 m of water[1-3,6]. Among the three species, *H. asinina* has the largest size and the most economic potential because of their maximum proportion of flesh[7] and good taste. *H.asinina* is primarily found off the eastern coast of the Gulf of Thailand around Chonburi, Rayong and Trad provinces[6,8]. Since collection from natural habitat could not keep pace with market demand, an efficient aquaculture system for this abalone is required. However, there are still lack of certain aspects of knowledge that could aid the large scale production of larvae for aquaculture. These are: 1) the probable spawning periods and the frequencies of spawning of land-cultured broodstocks during the year; 2) the age when the abalone reach full sexual maturity and can be used as broodstocks; and 3) the possibility of using artificial means to induce spawning when the gonads are fully developed, so that mature gamete cells from both sexes could be obtained simultaneously.

Among abalone species found in Thailand, preliminary study in *H. varia* around Bon Island, Phuket, showed that spawning occurred at several intervals throughout the year during January-February, April-May, June-July and November-December[9]. Gametogenic cycle was also studied in another species, *H. ovina*, at Khangkao Island, Chonburi province[10], in which the spawning period occurred between June and November. So far there has not yet been any studies of the gametogenic cycle as well as the development and structure of reproductive organs in *H. asinina*. Therefore, the aims of the present study are to investigate the reproduction of *H. asinina* that have been reared in land-based culture system with respect to 1) the gonadal histology and the gametogenic processes, especially the classification of various stages of germ cells in the testis and ovary of this abalone based on light and electron microscopic observations; 2) possible cyclical pattern of gonadal histology during different months of the year; and 3) the development of the gonads and the ages that abalone of both sexes reach full sexual maturity. The knowledge gained could be applied in determining the appropriate time for induction of spawning, and to increase gamete production, for the improvement of aquaculture system of this abalone species.

MATERIALS AND METHODS

Collection of abalone 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 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[7]. They are fed with a diet of macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food for abalone.

For studies of the gonadal histology, ultrastructure and the cyclical changes during the year, adult abalone, aged at least 24 months, were collected monthly for a period of one year. The fixed gonads were prepared for light and electron microscopic observations by the paraffin, semithin, and conventional TEM methods.

For development of the gonads, samples of juvenile abalone reared in the closed-culture system as mentioned above were collected monthly from the age of 1 to 12 months, and the gonads were processed for light microscopic observations.

Light Microscopy

Abalone were anesthetized in 5% magnesium chloride ($MgCl_2$) for one hour, for paraffin sections the gonads were cut and fixed in either Bouin's solution, or 3% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4, at 4°C, for overnight. The tissue blocks were then washed in 70% ethyl alcohol for removal of the Bouin's fixative, and glutaraldehyde fixative was removed by washing with phosphate buffer three times. Then, the specimens were dehydrated in graded series of ethyl alcohol (70-100%) for 30 minutes each, cleared with dioxane, infiltrated and embedded in paraffin wax. Blocks of specimens were sectioned at 5-micron thick, and finally stained with heamatoxylin-eosin, or PAS-heamatoxylin, and observed in an Olympus Vanox light microscope.

Transmission Electron Microscopy

For semithin sections and TEM studies, gonads were cut into very small pieces and fixed in a solution of 3% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4, at 4°C, for overnight. The specimens were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, at 4^0C, for 2 hours. Then, they were dehydrated in graded series of ethanol (50-100%) for 30 minutes each, cleared in two changes of propylene oxide, infiltrated in a mixture of propylene oxide and Araldite 502 resin at the ratios of 3:1 for 1 hour, 2:1 for 2 hours and 1:2 for overnight, then embedded in pure Araldite 502 resin for at least 6 hours, and finally polymerized at 30^0C, 45^0C and 60^0C for 24, 48 and 48 hours, respectively. Blocks of specimens were sectioned at 1-micron thickness by ultramicrotome and stained with Methylene blue for light microscopic observations, and ultrathin sections were cut and stained with lead citrate-uranyl acetate and viewed under a Hitachi TEM H-300 at 75 kV.

RESULTS

1. Gonadal Histology

The conical organ consists of the hepatopancreas surrounded by the testis or ovary (Fig.1C,D). At the base of the organ, the hepatopancreas appears large and occupies most of the cross-sectional profile (Fig.1C); while it becomes smaller towards the tapered end of the organ where most of the tissue belongs to the gonads (Fig.1D). Both testis and ovary are surrounded by a capsule which is composed of the outer single layer of epithelial cells, and the inner layer of dense collagenous fibers mixed with smooth muscle cells (Fig.1K,2D). The thickness of this capsule varies according to the gonadal cycle during the year.

The connective tissue from the capsule extends perpendicularly into the interior of the gonad to form septa or trabeculae that are branched, and connected at the innermost ends with the thin loose capsule of hepatopancreas. As a result the gonads are partitioned into small compartments, each containing various stages of maturing germ cells (Fig.1E,1J). Within the connective tissue of each trabecula, there are small vessels running through its whole course (Fig.1F,1L,M), which may be capillaries that branch out from the larger subcapsular vessels. Around the capillaries, parallel to the long axis of the trabeculae, there are packs of smooth muscle cells and collagen fibers that are intermingled with small cells exhibiting dense ellipsoid nuclei (Fig.1K,1M). Some of the latter may be fibroblasts, while others may be follicular or supporting cells that surround oogonia and developing oocytes. Some small cells contain granules that show similar characteristics as endocrine cells.

Each trabecula acts as the axis on which growing germ cells are attached (Fig.1E,F,1J,M). Early stage cells, such as spermatogonia, initial stages of primary spermatocytes and oogonia, are closely adhered to the trabeculae. Middle stage germ cells, such as secondary spermatocytes and developing oocytes, are more detached and appear further away from the trabeculae; while late stage cells, such as spermatids, spermatozoa and mature oocytes, are completely detached and move to the outermost region from the axis. Such an appearance gives rise to a discrete group of germ cells surrounding each trabecula, which is termed spermatogenic or oogenic unit.

2. Classification of Germ Cells

Germ cells appearing in the gonads could be classified, according to their structural features as observed under the light and transmission electron microscopes, as follows:

2.1 Spermatogenic cells Based on the nuclear characteristics and the cell sizes, the male germ cells of *H. asinina* can be classified into 13 stages.

Spermatogonium (Sg) (Fig.1G) Sg is a spherical or oval-shaped cell with diameter about 8-10 μm. Its nucleus is round or slightly indented with diameter about 6-7 μm. The nucleus contains mostly euchromatin with only small chromatin blocks attached to the inner surface of nuclear envelope. The nucleolus is prominent and stands out from the rather transparent nucleoplasm. Sg are bounded to trabeculae.

Primary spermatocytes (PrSc) (Fig.1G-H,4A-C) PrSc consists of 5 stages, *i.e.*, leptotene (LSc), zygotene (ZSc), pachytene (PSc), diplotene (DSc), and diakinetic or metaphase (MSc) stages. The early cells (from LSc to PSc) are round and become increasingly larger, then they (from DSc to MSc) are gradually decreased in size. Another distinctive differences among various stages of PrSc is the pattern of chromatin condensation and the relative amount of euchromatin versus heterochromatin.

Leptotene spermatocyte (LSc) (Fig.1G,H,4A) These round-shaped cells are larger than Sg with diameter about 10-12 μm and also contain large round nuclei, each with diameter about 8 μm. There is a thin rim of heterochromatin along the nuclear envelope and small blocks of heterochromatin scattered evenly throughout the nucleus. The nucleolus is still present but not as prominent as in Sg.

Zygotene spermatocyte (ZSc) (Fig.1G,H,4A) ZSc has approximately the same size as LSc. The distinguishing features of ZSc is the heterochromatin blocks which are increasing in size and density, and they are coupled at many points by synaptonemal complexes. The nucleolus disappears completely.

Pachytene spermatocyte (PSc) PSc still shows round shape with slightly smaller size than those of LSc (about 8 μm in size and 5 μm in nuclear diameter). Under LM (Fig.1G,H) it is characterized by the heterochromatin which appears as long threads or thick fibers that are entwined into "bouquet pattern", and becoming visible throughout the nucleus. Under TEM (Fig.4A-C) these chromatin "threads" are actually thick blocks consisting of tightly packed 30 nm fundamental chromatin fibers.

Diplotene spermatocyte (DSc) (Fig.1G,H,4A-C) This cell resembles PSc, except the nucleus becomes smaller (about 4 μm), and the chromatin blocks become increasingly thicker and packed closer together in the denser nucleoplasm than in earlier stages.

Diakinetic and Metaphase spermatocytes (MSc) (Fig.1H,4B,C) These stages exhibit thick chromosomes that move to the equatorial region, while the nuclear membrane disintegrates and completely disappears in MSc.

Secondary spermatocyte (SSc) (Fig.4B,C) SSc is a small round cell about 7 μm in diameter with the nucleus about 4 μm. They show thick chromatin blocks that are crisscrossing one another, thus appearing as checker-board or XY figures. The individual chromatin fibers in the block are loosened up, and each still maintains the size of 30 nm.

Spermatids (St) (Fig.1F-H,4B,C) There are 4 stages of spermatids, *i.e.*, spermatid I (St_1), spermatid II (St_2), spermatid III (St_3) and spermatid IV (St_4) depending on the size, chromatin granulation and condensation. All stages are round or oval, and ranging in size from 6 μm in St_1 to 3 μm in St_4.

Spermatid I (St_1) (Fig.1G) St_1 can be distinguished by their chromatin which appears as fine granules under LM, that are uniformly spread throughout the nucleus. As a result the whole nuclei appear moderately dense without any intervening transparent areas of nucleoplasm. Under TEM the 30 nm chromatin fibers becomes loosely packed and uniformly distributed throughout the nucleus.

Spermatid II (St_2) (Fig.1G,H) The general features of St_2 are similar to those of St_1 but the nucleus, which remains round, decreases in size and is located eccentrically within the cell. As a result the chromatin fibers become more closely packed, and the nucleus appears denser but still uniform.

Spermatid III (St_3) (Fig.1G,H,4B,C) The cell becomes smaller and assumes more oval shape with eccentrically-located and elongated nucleus. The chromatin begins to condense into dark blocks with intervening light area of nucleoplasm, individual chromatin fiber is enlarged to 40 nm.

Spermatid IV (St_4) (Fig.1H) The cell becomes smallest but still appears oval. Its chromatin becomes completely condensed, thus the nucleus appears rather opaque; however, the outlines of individual chromatin fibers could still be observed, and each is enlarged to 60 nm.

Spermatozoa (Sz) (Fig.1F-I,4D) There are 2 stages of spermatozoa: Sz_1 is the immature spermatozoon that begins to show highly elongated nucleus with completely dense chromatin, thus the outlines of chromatin granules are barely discernible. There is a cap-like structure apposing on one side of the ellipsoid nucleus, which is the maturing acrosome. The tail is short with a pair of centrioles moving to the neck region, from which the axonemal microtubules start to form.

In mature spermatozoa (Sz_2) (Fig.1I,4D) the nucleus is fully elongated and slightly tapered at the anterior end, with the size about 1x3 μm. The chromatin is completely dense and the anterior portion of the head is covered by acrosome with central core element (Fig.4D). Five globular mitochondria surround the centrioles in the neck region. Zig-zag microtubules link mitochondria to the plasma membrane covering the distal half of the nucleus. The tail is lengthened, and consists of 9+2 axonemal microtubule doublets surrounded by plasma membrane. Both immature and mature sperm are completely detached from the germinal epithelium and come to lie in the space between adjacent spermatogenic units (Fig.1I,4B,D).

2.2 Oogenetic cells There are 6 stages of female germ cells of *H. asinina*, including oogonium and five stages of growing oocytes.

Oogonium (Og) (Fig.1K,L) Og is a round or oval-shaped cell, whose size is about 10-12 μm. Its nucleus is round and about 7 μm in diameter. It contains small blocks of heterochromatin attached to the inner surface of nuclear envelope, with the remaining majority appearing as euchromatin. The nucleolus is present but may not be as prominent as in Sg. The cytoplasm is stained light blue by heamatoxylin-eosin and methylene blue, which implies its basophilic property due to the presence of moderate amount of ribosomes. Og are attached to the capsular side of trabeculae and usually are concentrated in groups (Fig.1K,L). Each Og is surrounded by flat, squamous-shaped follicular cells.

Stage I Oocyte (Oc_1) (Fig.1K,L,5A-C) Oc_1 is a round or scallop-shaped cell that is closely adhered to the trabecula. It is about 15-24 μm in size, with a round nucleus about 12 μm in diameter. The nucleus exhibits densely packed chromatin in the form of numerous lampbrush chromosomes. The nucleolus is present but tends to be obscured by the rather dense chromatin and nucleoplasm. The cytoplasm is stained deep blue with heamatoxylin-eosin and methylene blue, which indicates its intense basophilic property, reflecting the presence of numerous polysomes, newly developed rough endoplasmic reticulum (RER) and Golgi complexes (Gc) as observed in TEM (Fig.5C). Newly released ribosomes are packed into large mass around nuclear envelope (Fig.5B). There is very few secretory granules. Due to its enlarged size each Oc_1 is surrounded by few follicular cells.

Stage II Oocyte (Oc_2) (Fig.1K,L,5D,6A) Oc_2 becomes larger and transforms into columnar shape, with the cell size around 30x55 μm, and nuclear size about 22 μm. It is still attached to the connective tissue of trabecula by the narrow part, and each Oc_2 is surrounded by several follicular cells. The nucleus exhibits increasingly decondensed chromatin and nucleolus. Thus the nucleolus and nuclear membrane are clearly distinct due to the more transparent nucleoplasm and the presence of mostly euchromatin. The cytoplasm is stained light blue similar to Og, and contains cluster of clear lipid droplets (Fig.5D). At TEM level it was observed to contain numerous well-developed Gc, RER and still abundant ribosomes. There are 2 types of secretory granules: SG_1 and SG_2 (~330 and 450 nm in diameter) with electron lucent and electron dense matrix, respectively (Fig.6A,B).

Stage III Oocyte (Oc_3) (Fig.1M,6B) This cell becomes increasingly larger and assumes flask or pear shape, with the narrow side or base still attached to the connective tissue of trabecula. The cell size is about 35-70 μm, with the nuclear size about 20 μm. The nucleus contains mostly euchromatin, as most of the lampbrush chromosomes become almost completely unraveled, and the nucleoplasm is

quite transparent. The nucleolus is distinct and becomes enlarged due to the uncoiling of nucleolar chromatin. In addition to increasing number of clear lipid droplets, the cytoplasm begins to show reddish yolk platelets (Fig.1M) which are electron dense under TEM. Fine blue granules representing SG_1 and SG_2 are evenly distributed between lipid droplets and yolk platelets. At TEM these granules are seen concentrated around Gc (Fig.6B). Follicular cells surround both the cell body and its base near trabecula.

Stage IV Oocyte (Oc_4) (Fig.2A,C,6C) This cell is large and assumes a pear or polygonal shape, but still attached to trabecula by slender cytoplasmic process. The cell size is about 60-80 μm, with nuclear size about 35 μm. The nucleus contains mostly euchromatin and completely transparent nucleoplasm (Fig.2A,C,6C). Hence the nucleolus is clearly visible, and it also becomes enlarged due to the complete uncoiling of its chromatin. The cytoplasm is filled with reddish and electron dense yolk platelets (each about 1500-2500 nm in diameter) mixed with numerous lipid droplets (each about 1500-3000 nm in diameter) (Fig.6C). Fine blue-stained granules which represent SG_1 and SG_2 are decreased in central area of the cytoplasm, since most are probably translocated to the area underneath the plasma membrane. A thin layer of jelly coat begins to form on the outer surface of the cell membrane (Fig.2C). This coat is PAS positive and may be formed by the released content of SG_1, which were seen exocytosed at the oocyte's plasma membrane (Fig.6D). The coat is in turn surrounded by follicular cells.

Stage V Oocyte (Oc_5) (Fig.2B-D) This is the fully mature oocyte before being released from the adult female. Oc_5 is the largest cells with polygonal or round shape, with the cell size about 80-140 μm and the nuclear size about 40 μm. The nucleus exhibits similar characteristics as that of Oc_4, but with completely enlarged and clear nucleolus. Oc_5 could be divided into 2 subgroups based on the characteristics of yolk platelets observed under LM (Fig.2D). The first subgroup contains small and similar size yolk platelets that are scattered evenly throughout the cytoplasm. In the second subgroup, the yolk platelets are variable in size, and most are large bodies that could be formed by the coalescence of the smaller yolk platelets. Stripe of fine blue granules are also located underneath the cell membrane as in Oc_4 (Fig.2C,D). The thick PAS positive jelly coat attains its maximum thickness and is uniform around the outer surface of the cell membrane, but without the surrounding layer of follicular cells. Under TEM jelly coat appears fibrous in comparison to the amorphous appearance in Oc_4 (Fig.6D). All Oc_5 are completely detached from the connective tissue of trabeculae.

3. Reproductive Cycle

The reproductive cycle of *H. asinina* was assessed by observing the changes in the gonad histology, especially the characteristics of cellular association during one year period. The stages of gonad maturation during one reproductive cycle of the abalone cultured in a closed land-based system could be classified into 5 distinct phases as follows.

Proliferative phase (Fig.2E-I) This is a period in which gamete cells begin to regenerate to commence a new reproductive cycle. At the initiation of this phase, the gonads contain mainly early stage cells, and all of them are closely attached to the trabeculae. The ovary (Fig.2E,F) contains primarily Og, which usually form clusters near the capsular side, and Oc_1 and Oc_2 which are rapidly increased in number. In the testis (Fig.2G-I) there are mostly Sg and PrSc, but neither St nor Sz are present. The clusters of these early stage cells are located around the short and dilated trabeculae. The hepatopancreas is quite large in size and occupies most of the cross sectional profile of the conical organ when compared to the total gonad area. This phase usually occurs immediately after the spawning, and lasts for 2 months around April to May and October to November.

Premature phase (Fig.2J-M) This phase is the period when gametogenesis proceeds at full speed with rapid increase in numbers and sizes of various cells, while hepatopancreas is slowly reduced in its relative size; the gonads become enlarged in volume and trabeculae become thinner. At the beginning, the ovary (Fig.2J,K) contains Og, Oc_1, Oc_2 and predominantly Oc_3, most of which are still attached to the trabeculae; and later Oc_4 and Oc_5 cells occur. The testis (Fig.2L,M) contains mainly Sg, PrSc, increasing number of St and a few of Sz, all of which aggregate around the trabeculae. This

phase lasts about 1 months following the proliferative phase, usually around May to June and January to February in female; and it takes place around April to May and December to January in male.

Mature phase (Fig.3A-E) This phase is a period of rapid growth of gonads which are reflected by striking differences in color between the two sexes. The rates of cells proliferation start to diminish, and the gonads contain primarily late stage germ cells, while only a few of the early stage cells are still present and restricted to area immediately around trabeculae. Hepatopancreas is further decreased in size, and trabeculae become slimmer. In the ovary (Fig.3A,B) there are abundant Oc_5, but only few remaining and widely scattered Oc_1. All of Oc_5 appear fully mature and are liberated into the lumen of oogenetic compartment. In the testis (Fig.3C-E) there are mostly late stage male germ cells, i.e., St and Sz. The most noticeable characteristics of the testis in this phase is the vast number of Sz_2 which lie in rows that in turn surround the earlier cell stages which are still closely attached to the trabeculae (Fig.3D). As a result the testis appears to have maximum density of late stage cells. Prior to spawning, all of Sz_2 are dispersed into gonadal lumen and intermingled with other late stage cells (Fig.3E). Thin bands of Sg and PrSc surrounding the trabeculae are still evident. This phase lasts for 2 months usually from June to July and February to March in both sexes.

Spawning phase (Fig.3F-J) This 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, and the gonadal wall becomes wrinkle when compared with the former phase (Fig.3H). Mostly ripen sperm or eggs are discharged while the earlier stages of gamete cells are still attached to the dilated trabeculae. After spawning, the yellowish granular substances (Fig.3G) remain in the lumen of gonadal compartments in both sexes. Spawning phase occurs at least twice during the one year period of observation, usually from August to October and March to April in female, and around August to November and February to April in male. In addition, partial spawning could be observed throughout the year in some males.

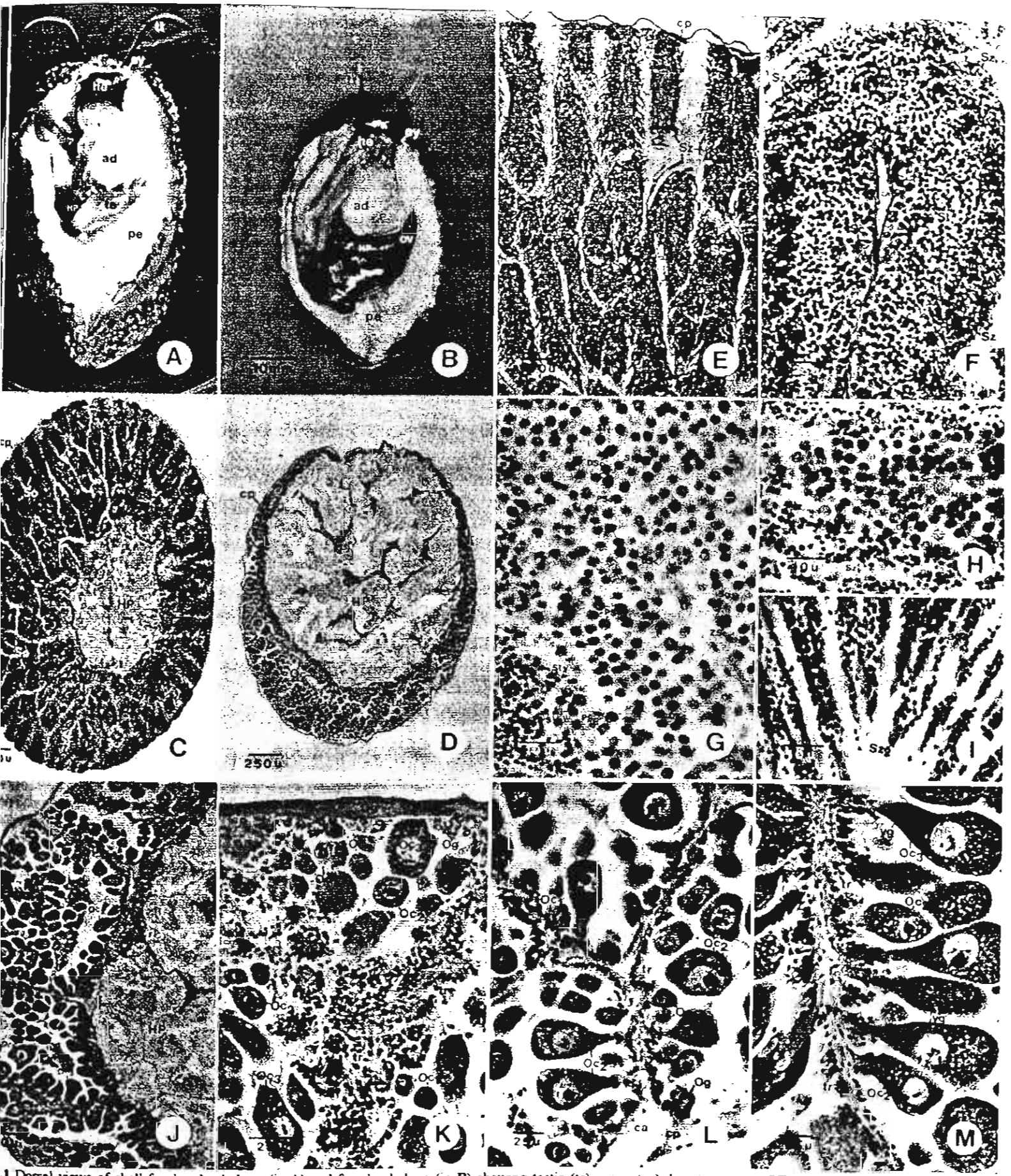
Spent phase (Fig.3K-N) This is the period after spawning when fully mature gamete cells are completely discharged. The gonads exhibit the breaking down of connective tissue stroma, and gametogenic activity momentarily cease. However, there are still clusters of gonial cells remain attached to parts of the gonads' capsule. As a result the gonads are greatly decreased in size and become creamy in color in both sexes. These quiescence gonads show small cross-sectional profiles in contrast to those of the hepatopancreas, which becomes very large in relative size (Fig.3M). This phase occurs after spawning around September to November and February to April in both sexes.

4. Maturation of Gonads

In developing *H.asinina*, definitive gonads appear during 2 months. The initial sign is the separation of hepatopancreatic capsule into 2 separate layers with clusters of gonial cells start to appear in the space between the two layers of capsules. Early spermatocytes (PrSc, SSc) and spermatids (St_{1-4}) could be detected at 4 months, while the ovary could be distinguished from the testis by the presence of few Og in contrast to fairly numerous primary spermatocytes. Spermatocytes, spermatids and mature spermatozoa are increasing in number during 6 to 7 months. While testis are rapidly enlarging and surrounding almost half of the circumference of the conical organ, ovary is much less developed and contains only oogonia and early oocytes ($Oc_{1,2}$). By 8 to 9 month the testis becomes enlarged to almost completely surround the hepatopancreas, and it already contains fully mature spermatozoa; while the ovary tends to be delayed in development and contains only early oocytes (Oc_{1-3}). By 10 to 11 month the testis appears fully developed, while the ovary starts to enlarge substantially and mature oocytes (Oc_{4-5}) begin to appear. Thus the male animals tend to reach full sexual maturity and start normal reproductive cycle as early as 7 to 8 months, while female animals reach sexual maturity and start reproductive cycle around 11 to 12 months (see Table I, Fig.7).

Table I Summary of the Key Features During the Course of Development of Gonads in ***Haliotis asinina***

Age (months)	General Structure	Gametogenic Unit	Cell Types	Phases of Cycle
2	-Separation of gonadal capsule from hepato-pancreatic (HP) capsule. -Development of few muscle cells in gonadal capsule. -sexually indistinguishable.	none	only few undifferentiated gonial cells attached to capsules	none
4	-**male**: testicular tissue covering a quarter of HP capsule. -**female**: ovary shows no further development. -sexually distinguishable.	incomplete none	Sg, PrSc, SSc, St_{1-4} Og	proliferative none
6	-**male**: testis covering half of HP capsule. -**female**: ovary still small and not well developed.	complete none	Sg, PrSc, SSc, St_{1-4}, Sz_1 Og, Oc_1	premature none
7	-**male**: testis covering half of HP capsule. -**female**: ovary still small.	complete begin to develop from sprouting trabeculae	Sg, PrSc, SSc, St_{1-4}, Sz_{1-2} Og, Oc_1, Oc_2	mature very early proliferative
8	-**male**: testis covering slightly over half of HP capsule. -**female**: ovary covering about a quarter of HP capsule.	complete incomplete	Sg, PrSc, SSc, St_{1-4}, Sz_{1-2} Og, Oc_1, Oc_2, Oc_3	mature early proliferative
9	-**male**: testis covering all HP capsule. -**female**: ovary covering half of HP capsule.	complete incomplete	Sg, PrSc, SSc, St_{1-4}, Sz_{1-2}, Og, Oc_1, Oc_2, Oc_3	mature proliferative /premature
11	-**male**: testis covering all HP capsule and much thickened. -**female**: ovary covering slightly over half of HP capsule.	complete and numerous complete and increasing in number	Sg, PrSc, SSc, St_{1-4}, Sz_{1-2} Og, Oc_1, Oc_2, Oc_3, Oc_4, Oc_5	mature mature



.1 Dorsal views of shell-freed male abalone (in **A**) and female abalone (in **B**) showing testis (te), ovary (ov), hepatopancreas (HP), adductor muscle (ad), pedal muscle (pe), head (he), eyes (ey), and tentacle (tt). **C)** A cross-section of the testis showing hepatopancreas (HP) surrounded by testicular tissue which is, in turn, surrounded by a thin connective tissue capsule (cp). **D)** a cross-section of the ovary showing hepatopancreas (HP) surrounded by ovarian tissue and fibrous capsule; **E,F)** a spermatogenic unit consists of a central trabeculae (tr) arising from capsule (cp), surrounded by various stages of germ cells; in **F** a capillary (ca) is present inside each trabeculae, and successive maturing stages of germ cells lie at different distance from the connective trabecula (Sc-spermatocyte, St-spermatid and Sz-spermatozoa); **G-I)** sections showing various stages of male germ cells surrounding each trabecula, they are spermatogonia (Sg), primary spermatocytes (LSc-leptotene; ZSc-zygotene, PSc-pachytene, DSc-diplotene; MSc-metaphase stage), spermatid ($St_{1,2}$), and spermatozoa ($Sz_{1,2}$), in **I** there are rows of fully mature spermatozoa (Sz_1), which are the most typical characteristic in mature phase of male abalone; **J)** an oogenic unit also consists of an axis of trabecula (tr) with closely attached early stage oocytes ($Oc_{1,2}$). The fully mature oocytes (Oc_3) are released into the central area of the compartment partitioned off by adjacent trabeculae; **K-M)** sections showing stage I, II and III oocytes ($Oc_{1,3}$) which exhibit intensely basophilic cytoplasm , in **M** there are stage III oocytes (Oc_3) showing the presence of eosinophilic yolk granules (arrows) in the cytoplasm when compared with the former stage oocytes

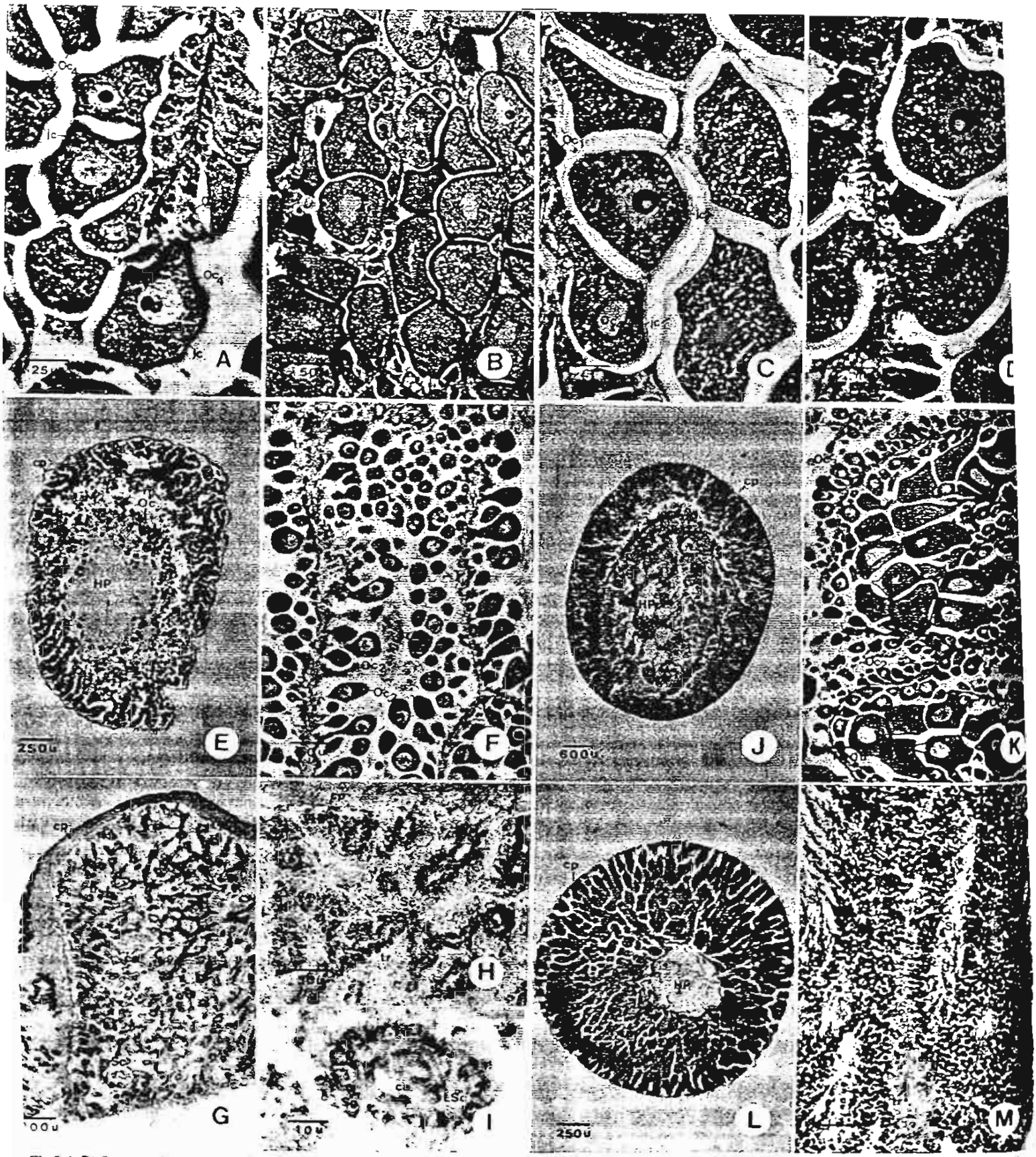


Fig.2 A-D) Sections showing stage IV and V ($Oc_{4,5}$), notice the first appearance of a thin jelly coat (jc), which is PAS positive, and increasing number of eosinophilic volk granules (vg) in $Oc_{4,5}$. The increasing amount of euchromatin, which is pale stained, and the enlargement and vesiculation of nucleolus are also noticeable. Blue stripe underneath the oocyte's plasma membrane (arrow) is present in $Oc_{4,5}$. In D there are two subtypes of stage V cells: the upper cell (1) shows small and evenly distributed eosinophilic yolk granules, and the lower cell (2) shows large platelet of yolks. E-I) Sections of "proliferative phase", showing the regeneration of gamete cells after spawning and spent phases. The ovary (E,F) contains only $Oc_{1,2}$, which are rapidly increased in numbers. The testis (G-I) contains mostly Sg and LSc. Trabeculae, which are depleted of cells and breaking down in spent phase, start to regenerate and appear short and dilated. J-M) Sections of "premature phase", showing rapid increase in numbers and sizes of various cells. The ovary (J,K) contains mostly early stage oocytes ($Oc_{1,2}$) and late stage oocytes ($Oc_{4,5}$) start to appear and gradually increase in numbers. The testis (L,M) contains various stage of primary spermatocytes (PrSc), spermatid (St) together and a few spermatozoa (Sz), all of which are located close to the trabeculae.

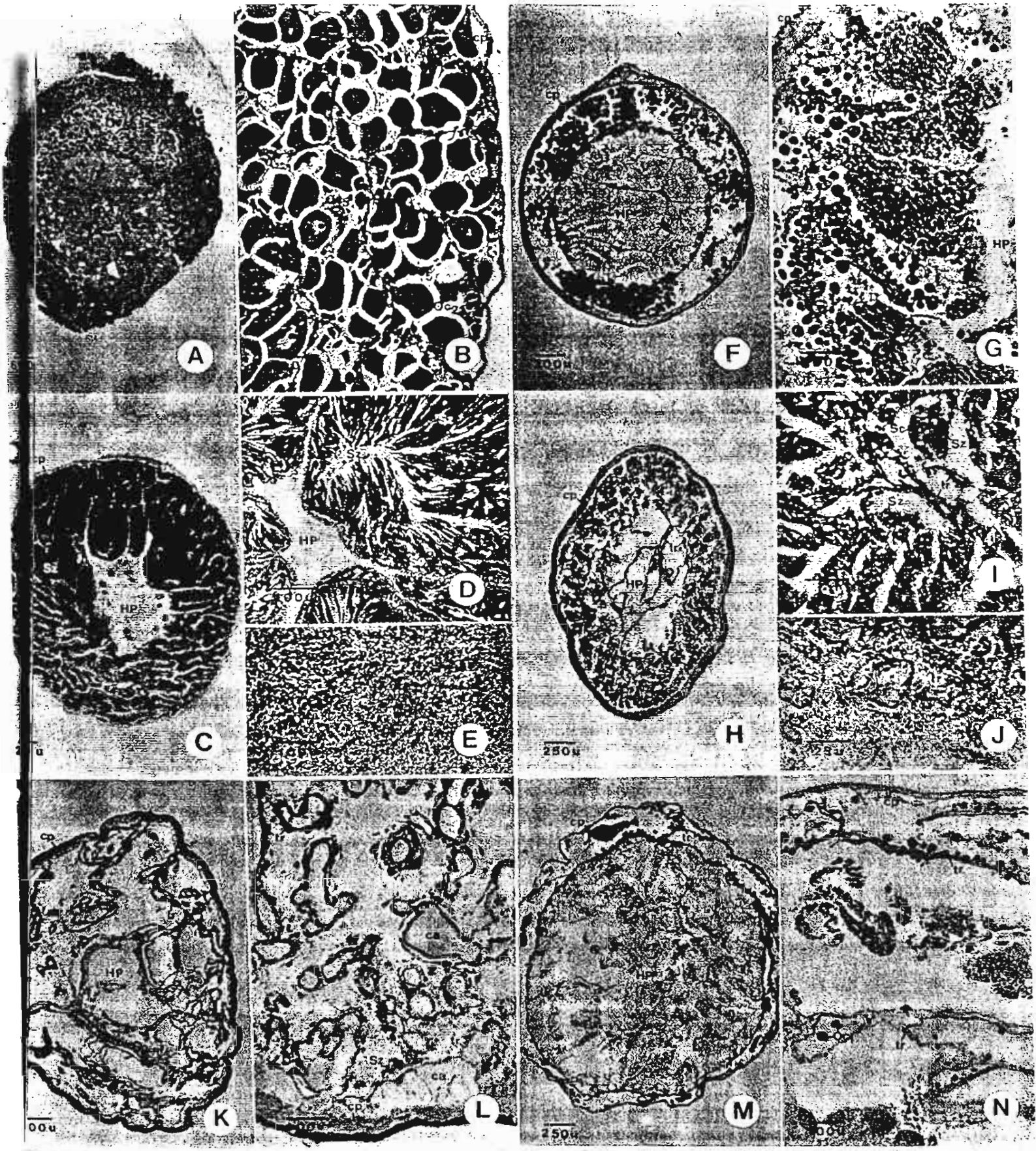


Fig.3 A-E) Sections of "mature phase", showing rapid growth of the gonads. The ovary **(A,B)** contains primarily fully mature Oc_5 with only a few widely scattered early stage cells (Oc_{1-2}). The testis **(C-E)** contains mostly late spermatids (St) and spermatozoa (Sz), which lie in rows and at low power appear streaky **(D)**. Finally they become dispersed and released into luminal area of the testis. **F-J)** Sections of "spawning phase", showing the period when abalone release the viable sperm or eggs from the gonads. The ovary **(F,G)** contains only the earlier stage oocytes which are still attached to the dilated trabeculae. Some yellowish granular substances (arrow) is present in the ovarian lumen. The testis **(H-J)** contains only early stage of male germ cells with a few of spermatozoa (Sz). **K-N)** Sections of "spent phase", showing the complete discharge of gamete cells, and the breaking down of trabeculae and associated connective tissues in both sexes. Notice the hepatopancreas which becomes larger in relative size.

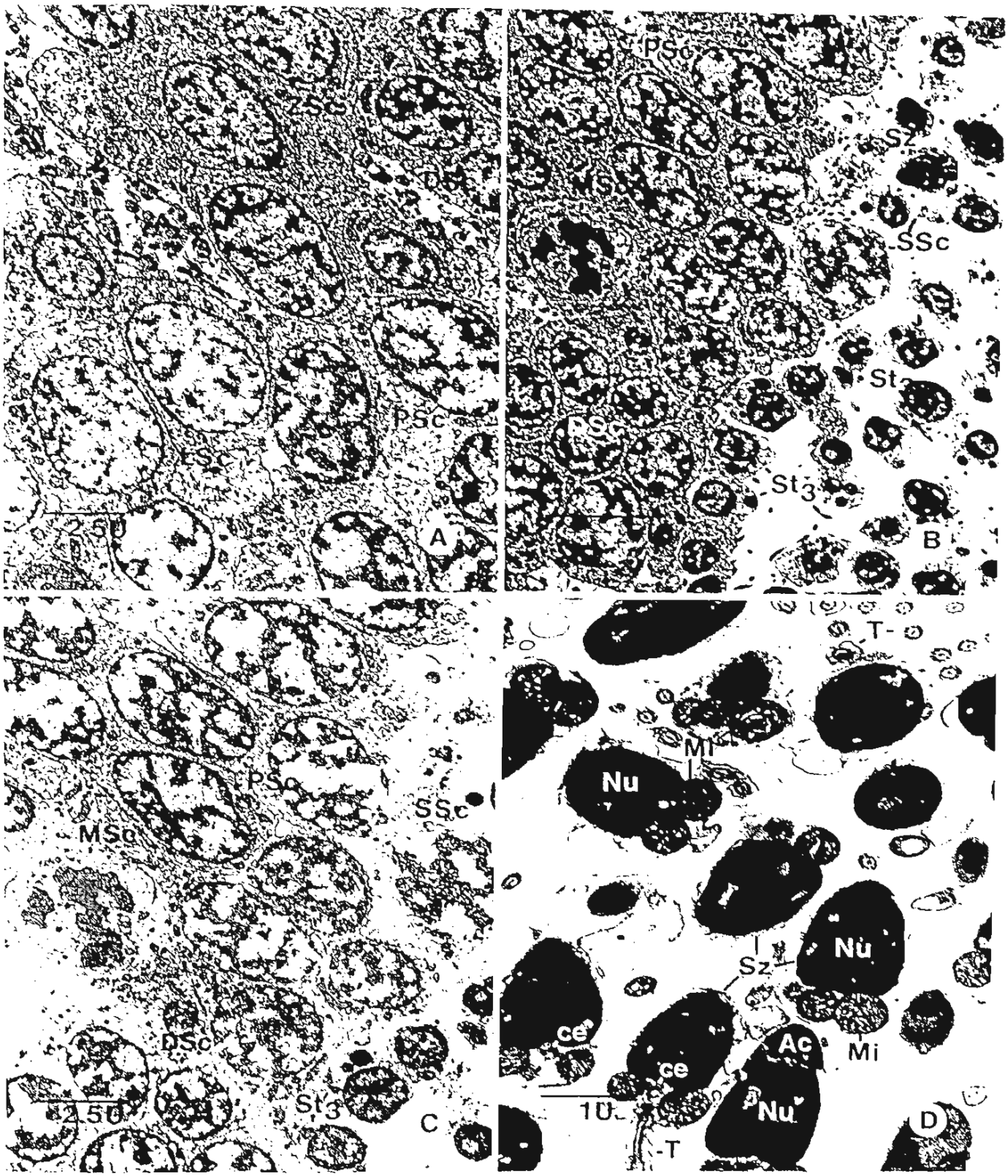


Fig.4 A-C) Electron micrographs showing various stages of male germ cells, including leptotene (LSc), zygotene (ZSc), pachytene (PSc), diplotene (DSc), secondary spermatocyte (SSc), spermarids (St). **D)** Spermatozoa exhibiting dense nucleus (Nu), acrosome (Ac), globular mitochondria (Mi), centriole (ce), and tails (T) with axonemal complexes.

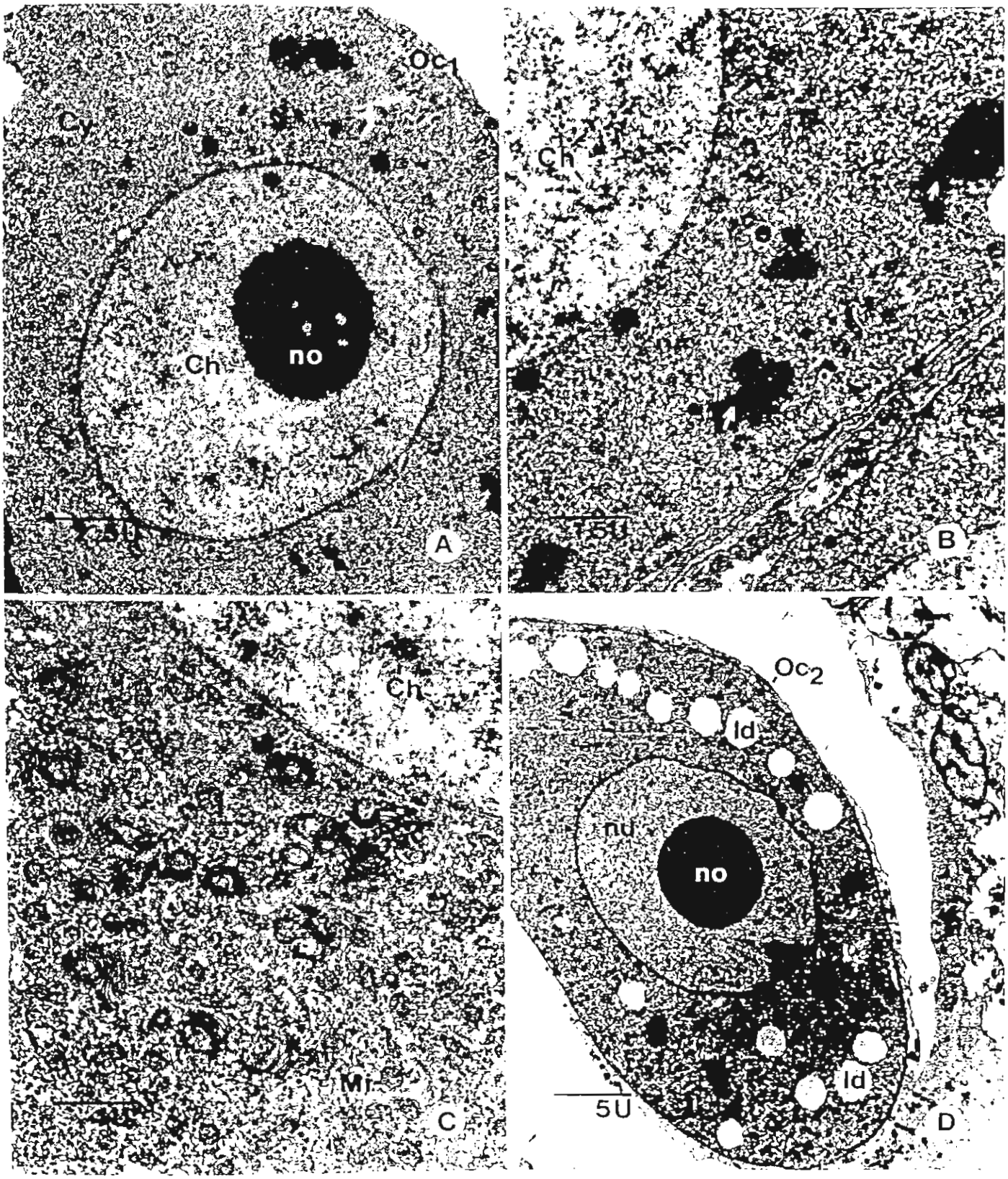


Fig.5 A,B) Early stage I oocyte (Oc_1), exhibiting nucleus with lampbrush chromosomes (Ch), dense nucleolus (no), and cytoplasm (Cy) with abundant ribosomes, some of which are aggregated in crystal-like bodies (arrows) **C)** Late Oc_1, exhibiting the extensive development of Golgi complexes (Gc) and mitochondria (Mi). **D)** Stage II oocyte (Oc_2), exhibiting lipid droplets (ld), nucleus with uncoiled and clear chromatin and nucleolus (no)

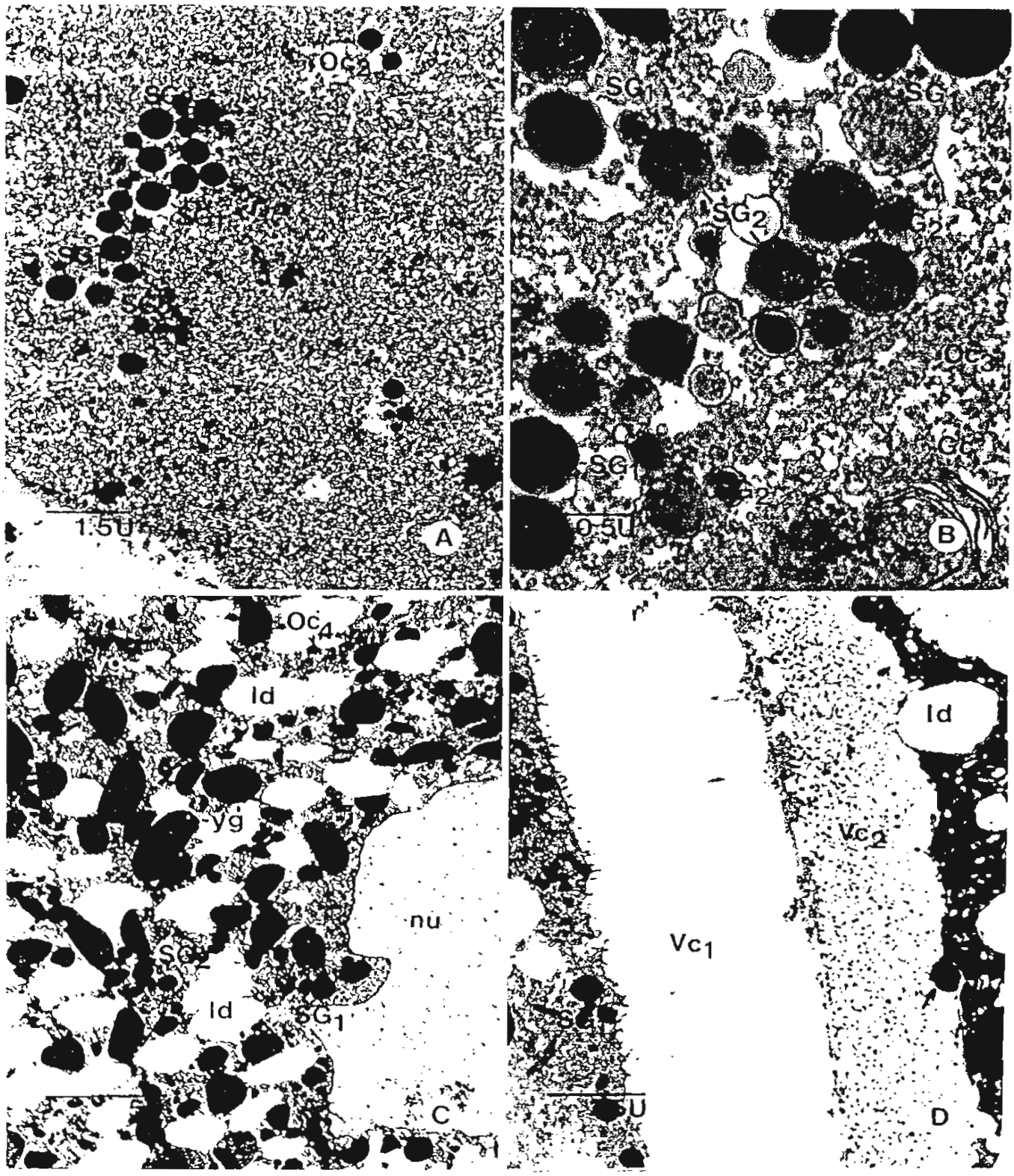


Fig.6 A,B) The cytoplasm of Oc_2 (A) and Oc_3 (B) exhibiting high concentration of dense jelly coat granules (SG_1) and lighter cortical granules (SG_2) around Golgi complexes (Gc). **C)** Fourth stage oocyte (Oc_4) exhibiting very light nucleus (nu) due to completely uncoiled chromatin. The cytoplasm contains numerous large yolk granules (yg), small SG_1 and SG_2 granules. **D)** The homogeneous jelly coat of Oc_4 (Vc_1) and fibrous jelly coat of Oc_5 (Vc_2). Notice the exocytosis of SG_1 into jelly coat Vc_1 (arrow).

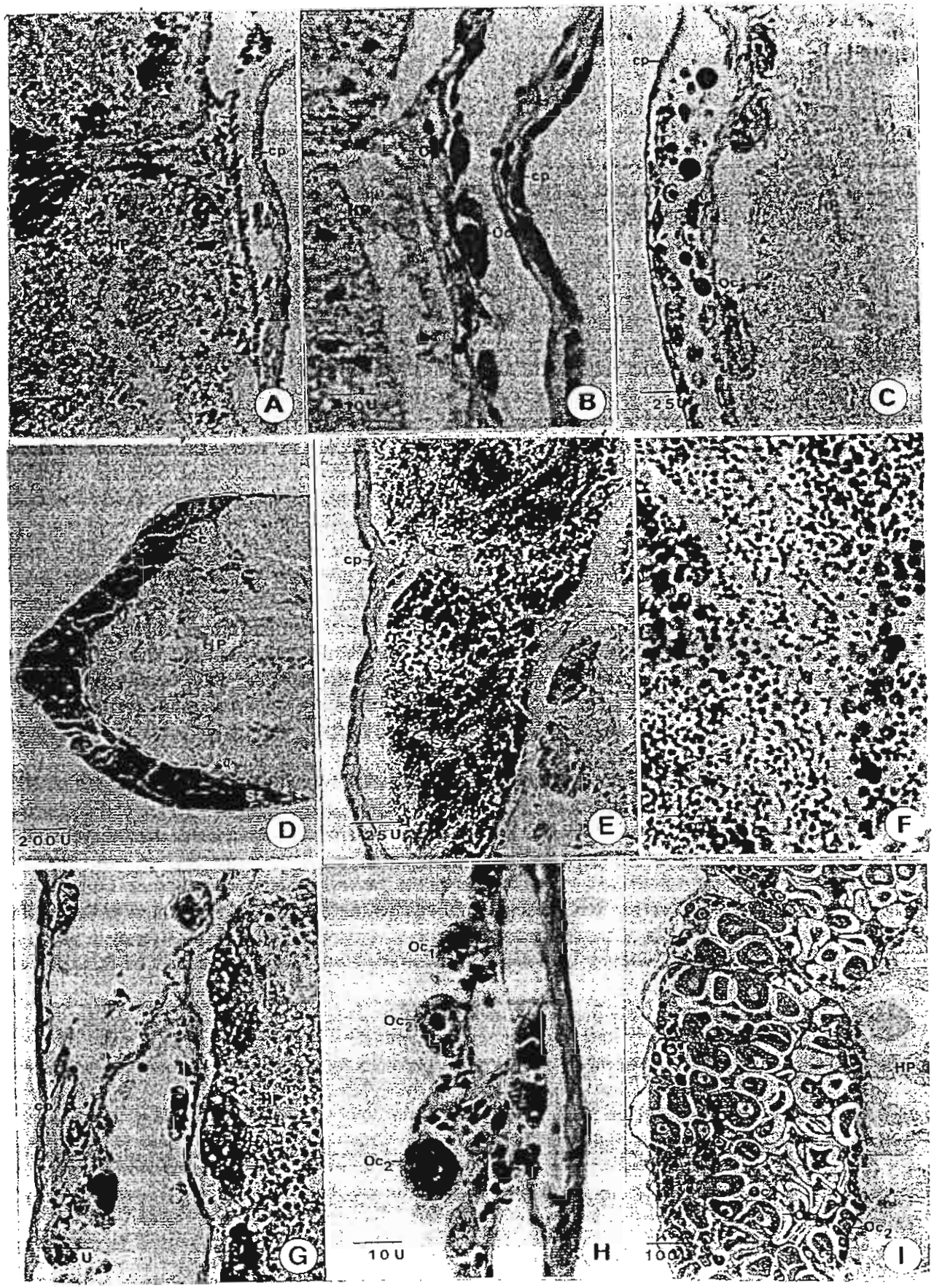


Fig.7 The development of gonads: **A)** the separation of gonadal capsule (cp) from hepatopancreas (HP) at 2 months; **B)** the presence of oogonia (Og) and possibly Oc_1 on the gonadal capsule bordering the hepatopancreas (HP); **C)** the ovary at 6 months showing the presence of early oocytes, mostly Oc_1; **D,E,F)** the testis at 4 months (in **D**) and 6 months (in **E**) showing full range of spermatocytes (Sc), spermatids (St) and some spermatozoa (Sz); **G,H)** ovary at 7 months showing the formation of trabecula (tr) and the presence of early oocytes, mostly Oc_1 and Oc_2; **I)** ovary at 11 months in mature phase, containing mostly $Oc_{4,5}$ and a few $Oc_{1,2}$.

DISCUSSION

Gonadal Structure and Classification of Cells in Gametogenesis

The first accounts of reproductive biology on an abalone species, *H. tuberculata*, was published by Stephenson[11] since 1924, and Croft[12] in 1929, who showed that the basic framework of the gonads is composed of fibrous capsular and trabecular supports, from which germ cells appear to generate. Similar histological studies in other species were later performed by many investigators[13-22]. More recently, a fine structural study of the ovarian cells in the red abalone, *H. rufescens*, was also undertaken by Martin *et al.*[23]. All these studies confirmed similar pattern of structural organization of the gonads; however, there are some disagreements on the classification of the stages of germ cells in the oogenetic and spermatogenic processes[15,16,18,20]. Utilizing a high resolution TEM to study the relative abundance of various organelles, particularly ribosomes and the development of rough endoplasmic reticulum and Golgi complexes in the cells, Martin *et al.*[23] suggested that there are 5 stages of female germ cells in *H. rufescens*, which they termed oogonium, presynthetic oocyte, synthetic oocyte, early postsynthetic oocyte and fully developed postsynthetic oocyte. We feel that the classification based on size alone, as adopted by many investigators, is not a good criterion for dividing cells in a single line of differentiation into various stages, because in reality these cells are undergoing continuous development. A better criterion would be to divide the cells according to the changes in histological and ultrastructural features which reflect the beginning of different synthetic activities in various developmental stages. In our study of *H.asinina*, we have used the following light and electron microscopic characteristics for dividing the stages of female germ cells: 1) the appearance of nucleus and nucleolus especially with regard to the uncoiling of chromatin, as reflected by the clarity of the two structures; 2) the clarity of nuclear membrane which is the result of the density difference between the condensed chromatin in the nucleus and the surrounding cytoplasm; 3) the basophilia or the bluishness imparted to the cytoplasm of the cells by basophilic dyes which reflect the abundance of ribosomes in the cytoplasm; 4) the presence of lipid droplets; 5) the development of secretory organelles particularly rough endoplasmic reticulum and Golgi complexes; 6) the occurrence of basophilic secretory granules including cortical granules, and eosinophilic yolk granules, and their relative abundance; and 7) the presence of jelly coat surrounding the egg cells. By using these rather stringent morphological criteria, we have identified 5 stages of egg cells, starting from oogonia (Og) which are the smallest cells closely attached to the connective tissue trabecula. These cells could maintain a constant pool of early stem cells, particularly those that are clustered towards the capsular side of trabeculae. During the spent period when most mature oocytes are released from the ovary and the connective tissues of trabeculae are breaking down, these cells are the only remaining group of germ cells. The restoration of gonadal structure during proliferative phase is carried out by the regeneration of connective tissues of trabeculae and the proliferation of this pool of oogonia.

The first stage of oocytes (Oc_1) including cells of different sizes ranging from 20-24 μm. The most pronounced characteristics that they exhibit is the increasing basophilia or bluishness of their cytoplasm. And because of the similar degree of density between the cytoplasm on one hand, and the partially condensed chromatin and dense nucleoplasm on the other, the outline of nuclear membrane could not be easily discerned under LM. The nucleolus, while present, is not outstanding. All Oc_1 are surrounded by a single layer of flat follicular cells. Under TEM we found that there is increasing amount of ribosomes which reflects the intense cytoplasmic basophilia. While ribosomes are rapidly synthesized during the early stage of Oc_1, definite surge in the number and degree of development of Golgi complexes and RER are observed only in late Oc_1. These two subgroups of Oc_1 do not yet exhibit any secretory granules. Thus they may correspond to the presynthetic oocytes as described by Martin *et al.*[23], when cells are preparing themselves for the onset of synthetic activities.

Oc_2 is the stage that first shows the presence of lipid droplets in the less intense basophilic cytoplasm. Due to the decondensation of most chromatin, and the increased translucence of the nucleoplasm, the nuclear boundary could be clearly observed under LM. For similar reasons the

nucleolus also becomes more distinct; and because of its enlargement the nucleolar activities for ribosomal synthesis is believed to be on the increase[24]. Under TEM, a few definite SG_1 and SG_2 granules start to appear in this stage, by clustering around Golgi complexes. Thus Oc_2 could represent the initial phase of synthetic activities when jelly coat (SG_1) and cortical granules (SG_2) are first synthesized.

Oc_3 is the stage which eosinophilic yolk granules first appear, and later is increasing in number; hence rendering the cytoplasm of Oc_3 more reddish in contrast to that of Oc_2, while the basophilic or bluish SG granules are seen scattered evenly between yolk granules and lipid droplets. We believed, therefore, that this is the stage where there is intense synthetic activities, since under TEM numerous SG_1 and SG_2 as well as yolk granules appear in large numbers; particularly SG_1 and SG_2 were seen concentrating around Golgi complexes. Oc_3 is still surrounded by a single layer of follicular cells, which by this time consists of several cells because of the increase in size of the cell. In addition, Oc_3 is further detached from the connectives of trabeculae and assumes a pear or even tear-drop shape. The chromatin becomes completely euchromatic and the nucleolus is enlarged further as its chromatin are almost completely uncoiled; this implies the active transcriptional as well as translational activities.

Oc_4 is the stage where a thin jelly coat is first detectable, and it is sandwiched inbetween the egg's cell membrane and the surrounding layer of follicular cells. Under LM the cytoplasm of Oc_4 becomes increasingly eosinophilic and appears more reddish due to the staining of numerous yolk granules by eosin. While the jelly coat is intensely PAS positive, the yolk granules are completely PAS negative. The contrasting feature implies that there may be very little or no carbohydrate moieties in the yolk granules, while these are the major constituent of the jelly coat. Under TEM the cytoplasm of Oc_4 is filled with SG_1, SG_2 and yolk granules, which reflect the near saturation of synthetic activities. The chromatin of Oc_4, like that of Oc_3, is completely in euchromatic state and the nucleolus is fully enlarged due to the complete uncoiling of its chromatin, and under LM it even appears eosinophilic. These indicate still high levels of both nuclear and nucleolar transcriptional activities. Another remarkable feature of Oc_4 under LM is the appearance of a narrow bluish stripe in the cytoplasm just underneath the cell membrane, while the bluishness of the remaining mass of cytoplasm is much decreased in comparison to Oc_2 and Oc_3. This could be due to the high concentration of basophilic SG_1 and SG_2 granules which are translocated to this area as observed under TEM. Some of the more electron SG_1 granules are also seen exocytosed to the cell's periphery, and thus is believed to contribute material to the formation of the jelly coat. In contrast, SG_2 contains more electron lucent material than SG_1. They may be the actual cortical granules that are concentrated in the narrow cytoplasmic zone underneath the plasma membrane, and thus are kept in reserve for cortical reaction upon fertilization of the egg by the sperm.

Oc_5 is the stage where the jelly coat becomes uniformly thick and deprived of surrounding layer of follicular cells. Under TEM the jelly coat is transformed from homogeneous in Oc_4 to fibrous structure in Oc_5. There is no division of this cell coat into jelly and vitelline layers as reported in other species[25]. Thus Oc_5 appears completely mature and is fully detached from the trabeculae. The absence of follicular cells might allow the detachment of Oc_5 into space between trabeculae and ready them for release from the ovary. From this appearance it could be speculated that the major roles of follicular cells are protective and helping to maintain the adherence between oocytes and trabecula connective tissue, while the former are undergoing maturation. In addition, follicular cells could be involved in nutritive function for oocytes, and their roles in synthesizing the jelly coat could not yet be ruled out. Under LM the cytoplasm of Oc_5 is laden with reddish yolk granules. Based on the size of these yolk granules there could be 2 subgroups of Oc_5: one containing small granules of uniform size while the other contains very large granules, both of which appear very electron opaque under TEM. It is still not possible to confirm whether these are two separate stages of Oc_5, or that the latter merely represent the final stage in which small yolk granules are coalesced to form larger ones. In any cases these two subgroups of Oc_5 should represent fully mature cells. In comparison to the work of Martin *et al.*[23], Oc_4 could represented the early postsynthetic cells and Oc_5 late postsynthetic cells; even though, judging from ultrastructural features certain degree of synthetic activities must still be carried out in these cells.

Up to now most studies have not rigorously categorize various spermatogenic cells of *Haliotis*, apart from suggesting broadly that there are 4 stages, *i.e.*, spermatogonia, spermatocytes, spermatids and spermatozoa[16,18,20]. In the present study, the male germ cells in *H. asinina* could be classified into 13 specific stages according to the size, shape, appearance of chromatin and the presence or absence of nucleolus. Spermatogonium is the earliest cell whose nucleus contains almost all euchromatin which results in the nucleus being very clear and nucleolus is prominent. Spermatogonia divided mitotically to give rise to primary spermatocytes, which pass through 5 stages as in the first meiotic division of vertebrates' germ cells[26]. These prophase cells exhibit different forms of chromatin condensation, beginning with small to larger blocks of heterochromatin that are evenly scattered throughout the nucleus in LSc and ZSc. Heterochromatin blocks transform to thread-like pattern that are increasing in thickness and length, and become more entwined in PSc and DSc. Finally in diakinetic and MSc stages chromatin appears as pairs of chromatids that are translocated to the equatorial region. Secondary spermatocytes are quite numerous in comparison to those in vertebrates and they have heterochromatin that exhibit checker-board or XY-figure pattern.

Four stages of spermatids could be identified in *H.asinina* based on the nuclear size, shape and chromatin condensation. Under LM the first two stages exhibit finely granulated chromatin that appears homogeneous and evenly stained throughout the nuclei. Thus St_1 and St_2 could be distinguished by the difference in size (St_1 about 6 μm versus St_2 about 4 μm), and by the denser nuclear material in St_2. The latter is due to the reduction of nuclear volume which results in the closer packing of chromatin fibers, even though each fibers still maintain their width of 30 nm. In the third stage (St_3) the chromatin fibers begins to be tightly wound together into large dense blocks, particularly along the nuclear envelope, leaving clear areas between the blocks. At this stage individual fiber increases in size to 40 nm. Eventually, the decrease in volume of nucleus and its more ellipsoid shape results in the total condensation of chromatin mass in St_4, and individual chromatin fiber is enlarged to 60 nm.

The two stages of spermatozoa are distinguished by their ellipsoid nuclei. Sz_1 also shows the initial formation of acrosome as a clear cap-like structure on one end of the nucleus, while exhibiting only short tail. Under TEM, there is the formation of axonemal complexes from centriolar pair that move to the neck area just distal to the nucleus. Later, three to five globular mitochondria become localized around the centrioles. In Sz_2 the nucleus is elongated further and chromatin appears completely dense with the outline of 60 nm fibers (or granules) barely discernible. Sz_2 exhibits a completely formed tail that is long and point outwards from each trabecula.

Reproductive Cycle

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 a reproductive cycle of a population are: 1) the measuring of the relative size of gonads with respect to the size of conical organ which is termed gonad indices (GI); and 2) the assessing of histological changes in the gonads[17,27-30]. GI is not always a valid index for development of the gonads because GI only relates gonad area to constant parameters (e.g. the size of conical organ) of the animal, and it does not take variation in hepatopancreas size into account[18,31]. The more precise index that can define of reproductive cycle better is the use of histological examination of gonad sections, which can give considerable details of cellular association and the time interval between successive phases[17]. Many investigators, including Tomita[15-16], Lee[32], Giorgi & DeMartini[33], Ault[30], classified the reproductive stages in various temperate species of *Haliotis* into 5 to 6 distinct phases which are more clearly defined in females. In the present study, these various phases were also observed in *H. asinina*. Histological examination of monthly samplings of the brooding stocks cultured in the land-based culture system reveal 5 distinctive gonadal patterns during the year, *i.e.*, proliferative, premature, mature, spawning and spent phase.

Proliferative phase is characterized by the regeneration of gamete cells for the new cycle. The gonads contain mostly early stage germ cells in both sexes, such as Og, Oc_1, Oc_2 in the ovary, and

mainly Sg, PrSc without St and Sz in the testis. Giorgi & DeMartini[44] and Ault[30], on studying *H. rufescens*, found that the ovary contained primarily small oocytes usually lesser than 50 μm in diameter; while Tomita[15], on studying *H. discus hannai*, reported that there are mainly oogonia, yolkless and oil drop oocytes in this stage. Another remarkable features during this phase is the reciprocal relationship between the sizes of the gonads to the hepatopancreas, which is similar to that found in other *Haliotid*[13,17]. That is the hepatopancreas is relatively large when compared to the total area of conical organ. Booloottan *et al*.[13] also reported that, in *H. cracherodii* and *H. rufescens*, the size of hepatopancreas exhibits an inverse relationship to gonadal activity. During this phase, the hepatopancreas attains maximum size while the gonad activity is relatively quiescent. The precipitous drop in the size of hepatopancreas will occur during the subsequent phase when there is a rapid growth of the gonads. This implies that hepatopancreas may act as a nutrient storage that is necessary for gamete cells development; it becomes relatively depleted when the proliferation of gonad cells start to surge. Another remarkable histological features observed during this phase is the dilatation of the trabecular vessels which contain large amount of granular materials. This may represent the turgid state of the vessels that are supplying nutrients to the rapidly proliferating and growing gamete cells.

Premature phase is the period of rapid increase in numbers and sizes of gamete cells. The ovary contains predominantly Og, Oc_1, Oc_2, Oc_3 and few Oc_4 which is similar to those reported in the premature stage of *H. discus hannai*[15,34], while Sg, Sc and only few of St and Sz are evident in the testis during this phase. Ault[30], in studying *H. rufescens*, also reported that there were numerous developing early germ cells in this stage. Hence the major events of development in this phase involve the rapid growth of the gonads due to fast proliferation of early germ cells.

Mature phase is characterized by a notable enlargement of the gonads which exhibit striking differences of color between both sexes: greenish in female and yellowish in male. The ovary contains mostly late stage germ cells, *i.e.*, Oc_5 with widely scattered Oc_1; and the testis is mostly filled with St and Sz. Before spawning occurs, Oc_5 are detached from trabeculae and released into the gonadal lumen. During the rapid development of the testis, each trabecula is surrounded successively by a few rows of Sg, PrSc which are closely bound to trabecular connectives, and middle Sc, St appear further away, and Sz are completely detached from trabeculae. In comparison, during the differentiation of Oc_1 to Oc_5 from Og, the cells move along the trabeculae from capsular side towards the hepatopancreas side, until Oc_5 become detached from trabeculae.

Spawning phase is the time when gravid abalone start to release their ripened gametes. The period of spawning is the most important criterion for success of reproduction of various abalone species reared in close aquaculture system[13-19,29,33,35-37]. 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 local environment, such as food supply, temperature and the day length[17,34,38-41]. Thus, some investigators[13,36] have classified various *Haliotid* spp. into 3 groups according to their spawning season: those spawn during summer, those spawn during seasons other than summer, and those that exhibit year-round spawning. Earlier, Singhagraiwan & Doi[42] reported the spawning period of some wild broodstocks of *H. asinina* to peak around October, while the pond-reared broodstocks could spawning throughout the year with several minor peaks during March through September. In contrast, the spawning period of *H. asinina* kept in land-based closed culture system in the present study occurs twice a year: around August to October and March to April in female, and around August to November and February to April in male. While this is the general pattern of spawning for most members of the population, some individual may show irregular periodic spawning throughout the year, especially in males animals.

Spent phase is characterized by the lacking of gamete cells and the breakdown of connective tissue in the gonads, which is similar to that previously finding in *H. rufescens*[33]. According to Shepherd & Laws[36], spent phase is expressed when there is a complete discharge of gamete cells following spawning. Giese[28] defined spent phase in marine invertebrates as a postspawning quiescent stage which is indistinguishable between male and female. In present study, it was observed that during the spent phase the gonads of *H. asinina* are greatly reduced in size and become creamy in

color, and the sexes of animals cannot be distinguished. In contrast, hepatopancreas is relatively increased in size which may be filled up with food reserve.

From the data collected during one year period, it could be concluded that the spawning of *H. asinina* reared in the closed culture system can occur at least twice yearly providing that the culturing condition and food supply are optimal. And that each reproductive cycle, consisting of 5 phases of development needs at least 5 to 6 months to complete itself.

Maturation of Gonads

In previous studies of the gonadal development in *H. asinina*, fecundity was observed in females with the shell length of at least 48 mm for the wild broodstock, and 44 mm of the hatchery-reared broodstock, which was about nine months old[43-44]. On the other hand, the mature gonad of males become obvious in animals with the shell length of at least 31 mm, which was about seven and a half months old[42-44]. The data collected in the present study indicate the same trend. Furthermore, detailed histological study indicated that definitive gonads become clearly separated from the hepatopancreas at 2 month. Testis and ovary could be distinguished by the present of their initial stages of germ cells as early as 4 month. Testis tends to reach maturity quicker than ovary at 7 to 8 months, the time at which St and Sz are found to be abundant. Ovary tends to mature later at 10 to 11 months when it starts to contain mature oocytes (Oc_4 and Oc_5). Thus males tend to reach maturity and assume reproductive cycle much earlier than females.

ACKNOWLEDGEMENTS

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

REFERENCES

1. Nateewathana A, Hylleberge J. (1986) A survey on Thai abalone around Phuket Island and feasibility study of abalone culture in Thailand. *Thai Fish. Gazette* **39**, 177-190.
2. Tookvinart S, Leknim W, Donyadol Y, Preeda-Lampabutra Y, Perng mak P. (1986) Survey on species and distribution of abalone (*Haliotis* spp.) in Surajthani, Nakornsrithammaraj and Songkhla provinces. Technical Paper No.1/1986, National Institute of Coastal Aquaculture, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand. 16p.
3. Nateewathana A, Bussarawit S. (1988) Abundance and distribution of abalones along the Andaman Sea Coast of Thailand. *Kasetsart J. (Nat. Sci.)* **22**, 8-15.
4. Singhagraiwan T, Sasaki M. (1991a) Breeding and early development of the donkey's ear abalone, *Haliotis asinina* Linne. *Thai Mar. Fish. Res. Bull.* **2**, 83-94.
5. Singhagraiwan T, Sasaki M. (1991b) Growth rate of donkey's ear abalone, *Haliotis asinina* Linne, cultured in tank. *Thai Mar. Fish. Res. Bull.* **2**, 95-100.
6. Kakhai N, Petjamrat K. (1992) Survey on species and broodstock collection of abalone (*Haliotis* spp.) in Chon-buri, Rayong and Trad provinces. Technical Paper No.6/1992, Aquaculture Station, Coastal Aquaculture Division, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand.
7. Singhagraiwan T, Doi M. (1993) Seed production and culture of a tropical abalone, *Haliotis asinina* Linne. The Eastern Marine Fisheries Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand. 33p.
8. Sungthong S, Ingsrisawang V, Fujiwara S. (1991) Study on the relative growth of abalone, *Haliotis asinina* Linne, off Samet Island. *Thai Mar. Fish. Res. Bull.* **2**, 120.
9. Bussarawit S, Kawintertwathana P, Nateewathana A. (1990) Preliminary study on reproductive biology of the balone (*Haliotis varia*) at Phuket, Andaman Sea Coast of Thailand. *Kasetsart J. (Nat. Sci.)* **24**, 529-39.
10. Jarayabnand P, Jew N, Manasveta P, Choonhabandit S. (1994) Gametogenic cycle of abalone *Haliotis* ovina Gmelin 1791 at Khangkao Island, Chon-buri province. *Thai J. Aqua. Sci.* **1**, 43-42.
11. Stephenson TA. (1924) Notes on *Haliotis tuberculata*. *J. Marin. Biol. Assoc. UK* **13**, 480-495.

12. Croft DR. (1929) *Haliotis*. L.M.B.C. mem, Liverpool University Press, **29**, 174p.
13. Boolootian RA, Farmanfarmaian A, Giese AC. (1962) On the reproductive cycle and breeding habits of two western species of *Haliotis*. *Biol. Bull.* **122**, 183-93.
14. Newman GG. (1967) Reproduction of the South African abalone *Haliotis midae*. Division of Sea Fisheries, Republic of South Africa, Investigational Report No.64, 24p.
15. Tomita K. (1967) The maturation of the ovaries of the abalone, *Haliotis discus hannai* Ino, in the Rebun Island, Hokkaido, Japan. *Sci. Rep. Hokkaido Fish. Exp. Sta.* **7**, 1-7.
16. Tomita K. (1968) The testis maturation of the abalone, *Haliotis discus hannai* Ino, in the Rebun Island, Hokkaido, Japan. *Sci. Rep. Hokkaido Fish. Exp. Sta.* **9**, 56-61.
17. Webber HH, Giese AC. (1969) Reproductive cycle and gametogenesis in the black abalone *Haliotis cracherodii* (gastropoda: prosobranchiata). *Mar. Biol.* **4**, 152-9.
18. Young JS, DeMartini JD. (1970) The reproductive cycle, gonadal histology and gametogenesis of the red abalone, *Haliotis rufescens* (Swainson). *Calif. Fish and Game* **56**, 298-309.
19. Harrison AJ, Grant JF. (1971) Progress in abalone research. *Tasm. Fish. Res.* **5**, 1-10.
20. Takashima F, Okuno M, Nichimura K, Nomura M. (1978) Gametogenesis and reproductive cycle in *Haliotis diversicolor diversicolor* Reeve. *J. Tokyo Univ. Fish.* **1**, 1-8.
21. Brickey BE. (1979) The histological and cytological aspects of oogenesis in the California abalones. (M.S. Thesis) California State University, Long Beach, California.
22. Tutschultz T, Connell JH. (1981) Reproductive biology of three species of abalone (*Haliotis*) in southern California. *The Veliger* **23**, 195-206.
23. Martin GG, Romero K, Miller-Walker C. (1983) Fine structure of the ovary in the red abalone *Haliotis rufescens* (Mollusca: gastropoda). *Zoomorphology* **103**, 89-102.
24. Schwarzacher HG, Wachtler F. (1993) The nucleolus. *Anat. Embryol.* **188**, 515-36.
25. Monzingo NM, Vacquier VD, Chandler DE. (1995) Structural features of the abalone egg extracellular matrix and its role in gamete interaction during fertilization. *Mol. Reprod. Dev.* **41**, 493-502.
26. Courot M, Hochereau-de Reviers M-T, Ortavant R. (1970) The testis. Johnson AD. (ed.) (1970). Academic Press, New York, 399p.
27 Hahn KO. (1989) Handbook of culture of abalone and other marine gastropods. Hahn KO. (ed.)(1989). CRC Press, Boca Raton, Florida, p.13-39.
28. Giese AC. (1959) Comparative physiology: annual reproductive cycle of marine invertebrates. *Ann. Rev. Physiol.* **21**, 547-76.
29. Grant A, Tyler PA. (1983) The analysis of data in studies of invertebrate reproduction. I. Introduction and statistical analysis of gonad indices and maturity indices. *Int. J. Invertebr. Reprod.* **6**, 259.
30. Ault J. (1985) Some quantitative aspects of reproduction and growth of red abalone, *Haliotis rufescens* Swainson. *J. World Maricult. Soc.* **16**, 398-425.
31. Hahn KO. (1981) The reproductive cycle and gonadal histology of the pinto abalone, *Haliotis kamtschatkana* Jonas, and the flat abalone, *Haliotis walallensis* Stearns. *Adv. Invertebr. Reprod.* **2**, 387.
32. Lee TY. (1974) Histological study on gametogenesis and reproductive cycle of the Korean Coasts. *Bull. Pusan. Fish. Coll.* **14**, 55.
33. Giorgi AE, DeMartini JD. (1977) A study of the reproductive biology of the red abalone, *Haliotis rufescens* Swainson, near Mendocino, California. *Calif. Fish and Game* **63**, 80-94.
34. Mugiya Y, Kobayashi J, Nishikawa N, Motoya S. (1980) Gonadal maturation in the abalone, *Haliotis discus hannai* at Taisei, Hokkaido. *Bull. Fac. Fish. Hokkaido Univ.* **31**, 306-13.
35. Poore GCB. (1973) Ecology of New Zealand abalones, *Haliotis* species (mollusca: gastropoda). *New Zealand J. Mar. Freshwat. Res.* **6**, 534-59.
36. Shepherd SA, Laws HM. (1974) Studies on southern Australian abalone (Genus *Haliotis*). II. Reproduction of five species. *Aust. J. Mar. Freshwat. Res.* **35**, 49-62.
37. Girard A. (1972) La reproduction de l'ormeau Haliotis tuberculata L. *Rev. Trav. Inst. Peches. Marit.* **36**, 163-84.
38. Cox KW. (1962) Review of the abalone of California. *Calif. Fish and Game* **46**, 381-406.
39. Uki N, Kikuchi S. (1984) Regulation of maturation and spawning of an abalone, *Haliotis* (Gastropoda) by external environmental factors. *Aquaculture* **39**, 247-61.
40. Kinne O. (1970) Marine ecology: A comprehensive integrated treatise on life in oceans and coastal waters. Vol.1, Kinne O. (ed.) (1970). Wiley-Interscience, London, p 407-514.

41. Shepherd SA. (1973) Studies on southern Australian abalone (Genus *Haliotis*). I. The ecology of five sympatric species. *Aust. J. Mar. Freshwat. Res.* **24**, 217-57.
42. Singhagraiwan T, Doi M. (1992) Spawning pattern and fecundity of the donkey's ear abalone, *Haliotis asinina* Linne, observed in captivity. *Thai Mar. Fish. Res. Bull.* **3**, 61-9.
43. Singhagraiwan T. (1989a) The experiment on breeding and nursing of Donkey's ear abalone (*Haliotis asinina* Linne). Technical Paper No.21/1989a, The Eastern Marine Fisheries Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand.
44. Singhagraiwan T. (1991) Study on growth rate of donkey's ear abalone (*Haliotis asinina* Linne). Technical Paper No.28/1991, The Eastern Marine Fisheries Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand.

บทคัดย่อ

กระบวนการสร้างเซลล์สืบพันธุ์ในอัณฑะของหอยเป๋าฮื้อ *Haliotis asinina* ประกอบด้วยเซลล์ 13 ขั้น ได้แก่ spermatogonia, primary spermatocytes 5 ขั้น, secondary spermatocyte, spermatids 4 ขั้น, spermatozoa 2 ขั้น ในรังไข่ประกอบด้วยเซลล์ 6 ขั้น คือ oogonia และ primary oocytes 5 ขั้น พ่อแม่พันธุ์ที่เลี้ยงในระบบปิดแสดงวงจรการสืบพันธุ์ในหนึ่งปีที่แบ่งออกได้เป็น 5 ช่วง ตามชนิดและปริมาณของเซลล์สืบพันธุ์ที่ปรากฏในช่วงต่าง ๆ คือ ช่วง proliferative, premature, mature, spawning และ spent ช่วง spawning เป็นช่วงที่มีการปล่อยเซลล์สืบพันธุ์ที่พัฒนาเต็มที่แล้วออกมาจากหอย ซึ่งในหอยตัวเมียมักเกิดขึ้นได้ใน 2 ระยะ คือประมาณเดือนมีนาคมถึงเมษายน และสิงหาคมถึงตุลาคม ส่วนในตัวผู้เกิดได้ 2 ระยะเช่นกัน แต่มักจะกินเวลานานกว่า นอกจากนั้นหอยตัวผู้บางตัวยังสามารถปล่อยเซลล์สืบพันธุ์ได้เกือบตลอดทั้งปี ในหอยวัยเยาว์อัณฑะและรังไข่แยกจากตับเมื่ออายุ 2 เดือน อัณฑะพัฒนาเต็มที่เมื่อ 7-8 เดือน ในขณะที่รังไข่พัฒนาเต็มที่เมื่อ 11-12 เดือน

THE REPRODUCTIVE CYCLE AND THE DEVELOPMENT OF THE GONAD IN THE THAI ABALONE, *HALIOTIS ASININA* LINNAEUS

M. Kruatrachue, S. Apisawetakan, E.S. Upatham, P.Sobhon, S. Sawatpeera, S. Singhakaew, V. Ingsrisawang and T. Singhagraiwan

ABSTRACT

The reproductive cycle and development of the gonad in the Thai abalone, *Haliotis asinina* were studied by light microscopy. Mature *H. asinina* were collected monthly from a natural habitat along Samed Island, Rayong Province, and from a land-based culture system, in order to study the reproductive cycle. The gonads of abalone both from natural habitat and land-based culture exhibit five phases of histological pattern during the year: proliferative, premature, mature, spawning and spent. Spawning occurs at least twice during the year. In the natural habitat, spawning occurs from April to May and August to September in females and from April to May and August to October in males. In the land-based culture system, spawning occurs from March to April and August to October in females and from February to April and August to November in males.

The development of the gonad of *H. asinina* was histologically studied by collecting monthly one- to thirteen-month-old juvenile abalone from the land-based culture system. Gonads appear in six-month-old abalones and become fully mature in nine-month-old animals.

KEY WORDS *Haliotis asinina*, reproductive cycle, development, gonad.

INTRODUCTION

Like most marine invertebrates, abalone are seasonal in their reproduction (Webber & Giese, 1969). The entire reproductive cycle can be divided into a reproductive period, during which the gonads show activity from their first initiation until spawning, and a vegetative period that follows reproduction and corresponds to the acquiescing period of the gonads, during which the reserve stem cells start to proliferate. Gametogenesis is again initiated in preparation for the next reproductive cycle (Giese, 1959).

Seasonal changes of gonad and stages of gametogenesis in temperate abalone species have been studied intensively. According to Cox (1962) and Uki & Kikuchi (1984), the actual spawning period for different abalone species can vary according to geographical location and