

Classification of Germ Cells, Reproductive Cycle and Maturation of Gonads in *Haliotis asinina* Linnaeus

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ABSTRACT Germ cells in the gonads of *Haliotis asinina*, a species of abalone found along the coast of Thailand, were classified basing on 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, ie, Oc₁ with light to intense basophilia and abundant polyribosomes, with some in large aggregates; Oc₂ with intense basophilia, oil droplets, numerous well developed Golgi complexes and rough endoplasmic reticulum, but little secretory granules; Oc₃ with a few yolk granules and 2 types of cortical granules; Oc₄ with increasing number of yolk granules, numerous cortical granules and thin jelly coat; and Oc₅ 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, ie, 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 They are also distributed generally over the Indo-western Pacific area, especially in the 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.^{1-3,6} Among the three species, *H. asinina* has the largest size and the most economic potential because of their maximum proportion of flesh⁷ 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, certain aspects

of knowledge that could aid the large scale production of larvae for aquaculture are still lacking. These are: 1) the probable spawning periods and the frequencies of spawning of land-culture broodstocks during the year; 2) the age when the abalone reaches full sexual maturity and could be used as broodstocks; and 3) the possibility of using artificial means to induce spawning when the gonads are fully developed in order that mature gamete cells from both sexes could be obtained simultaneously.

Among abalone species found in Thailand, preliminary study of *H varia* around Bon Island, Phuket, revealed 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 occurred between June and November. So far there has not yet been any studies of the gametogenic cycle as well as the development 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 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 age of full sexual maturity in both sexes. The findings could be applied in determining the appropriate time for induction of spawning, and to increase gamete production leading to 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, Prachaukirkhun province, and Marine Biological Station, Chulalongkorn University, Angsila, Chonburi province. They are kept in concrete tanks housed in the shade and well flushed with mechanically circulated filtered sea water as well as 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 macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food for abalone.

For the study of the gonadal histology, ultra-structure 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°C, 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°C, 45°C and 60°C 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 are branched out from the larger subcapsular vessels. Around the capillaries and 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 show similar characteristics as endocrine cells by containing granules.

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, *ie*, leptotene (LSc), zygotene (ZSc), pachytene (PSc), diplotene (DSc), and diakinetik 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. The distinctive differences among various stages of PrSc are 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, *ie*, 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 nucleus appears 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 $1 \times 3 \mu\text{m}$. 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 hematoxylin-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 hematoxylin-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 $30 \times 55 \mu\text{m}$, and nuclear size about $22 \mu\text{m}$. 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 $35\text{-}70 \mu\text{m}$, with the nuclear size about $20 \mu\text{m}$. 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 $60\text{-}80 \mu\text{m}$, with nuclear size about $35 \mu\text{m}$. 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 $1500\text{-}2500$ nm in diameter) mixed with numerous lipid droplets (each about $1500\text{-}3000$ 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 $80\text{-}140 \mu\text{m}$ and the nuclear size about $40 \mu\text{m}$. 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

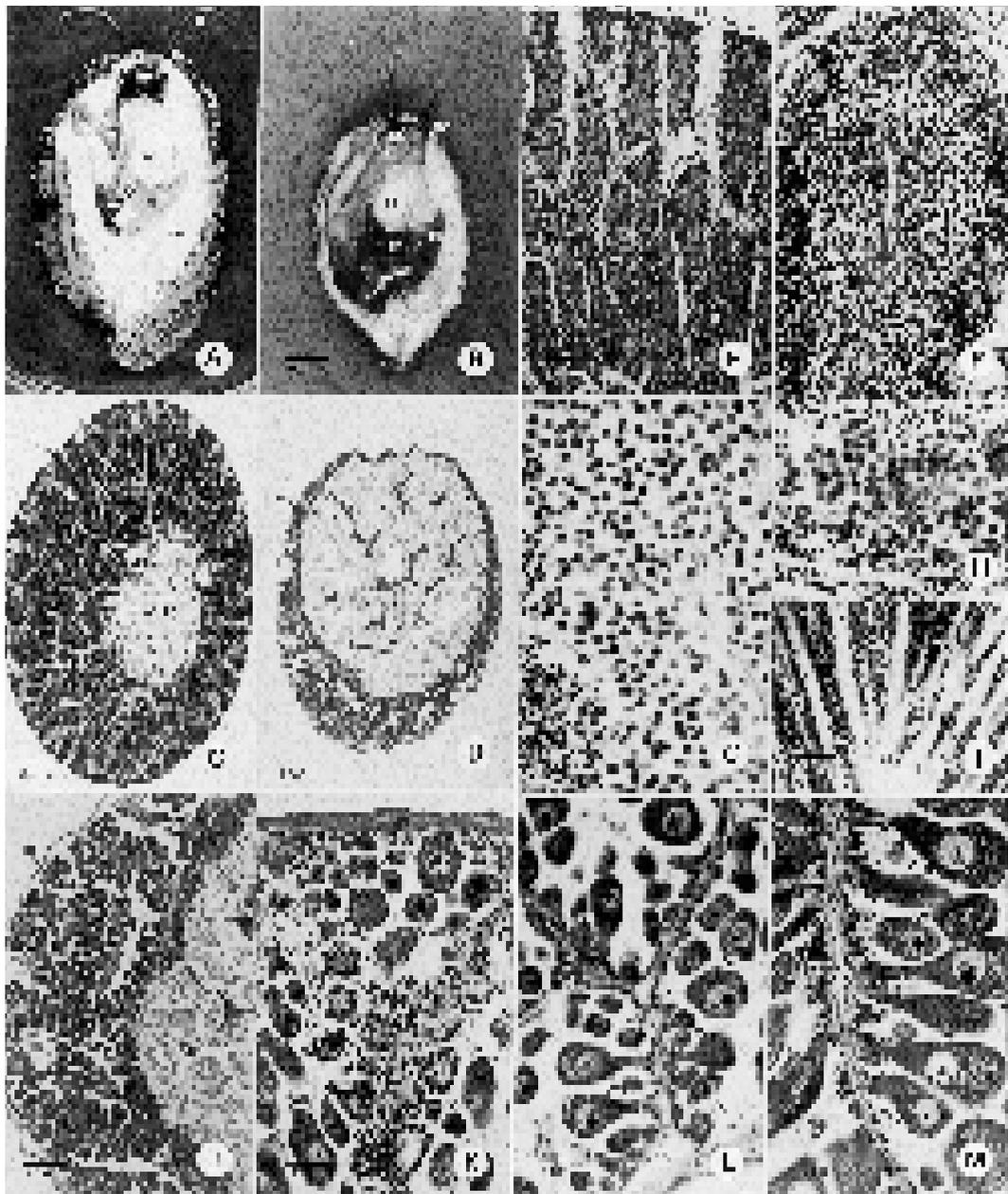


Fig 1. Dorsal views of shell-free male abalone (in A) and female abalone (in B) showing testis (te), ovary (ov), hepatopancreas (HP), adductor muscle (ad), pedal muscle (pe), head (he), eyes (ey), and tentacle (tt). C) A cross-section of the testis showing hepatopancreas (HP) surrounded by testicular 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 (St₁₋₄), and spermatozoa (Sz₁₋₂); in I there are rows of fully mature spermatozoa (Sz₂), 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_{1,3}). The fully mature oocytes (Oc₃) 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 (Oc₃) showing the presence of eosinophilic yolk granules (arrows) in the cytoplasm when compared with the former stage oocytes.

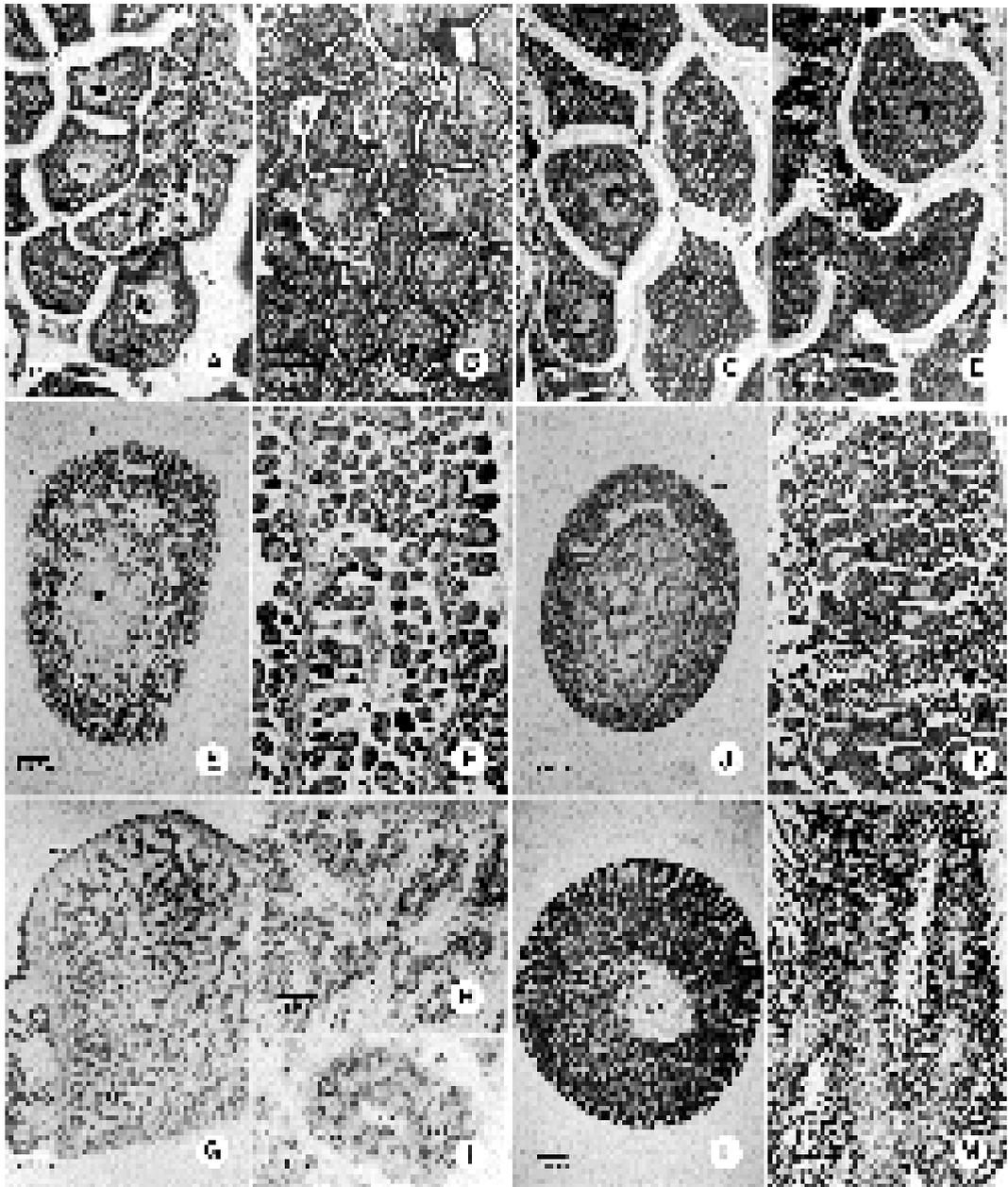


Fig 2. A-D) Sections showing stage IV and V ($O_{c_{4,5}}$): notice the first appearance of a thin jelly coat (jc), which is PAS positive, and increasing number of eosinophilic yolk granules (yg) in $O_{c_{4,5}}$. The increasing amount of euchromatin, which is pale stained, and the enlargement and vesiculation of nucleolus are also noticeable. Blue stripe underneath the oocyte's plasma membrane (arrow) is present in $O_{c_{4,5}}$. 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 $O_{c_{1,2}}$, 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 ($O_{c_{1,3}}$) and late stage oocytes ($O_{c_{4,5}}$) 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.

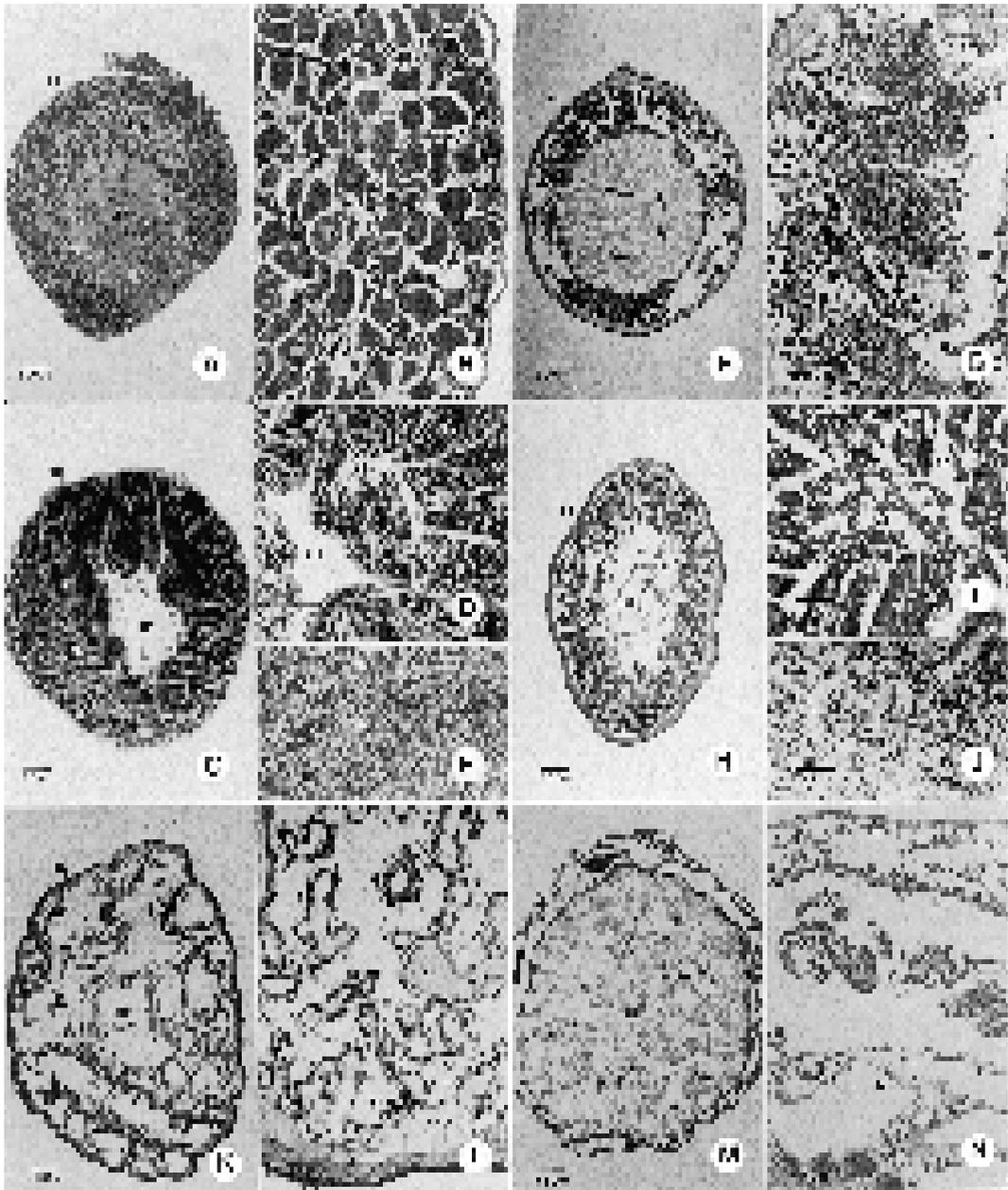


Fig 3. A-E) Sections of "mature phase", showing rapid growth of the gonads. The ovary (A,B) contains primarily fully mature Oc_5 with only a few widely scattered early stage cells ($Oc_{1,2}$). The testis (C-E) contains mostly late spermatids (St) and spermatozoa (Sz), which lie in rows and at low power 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.

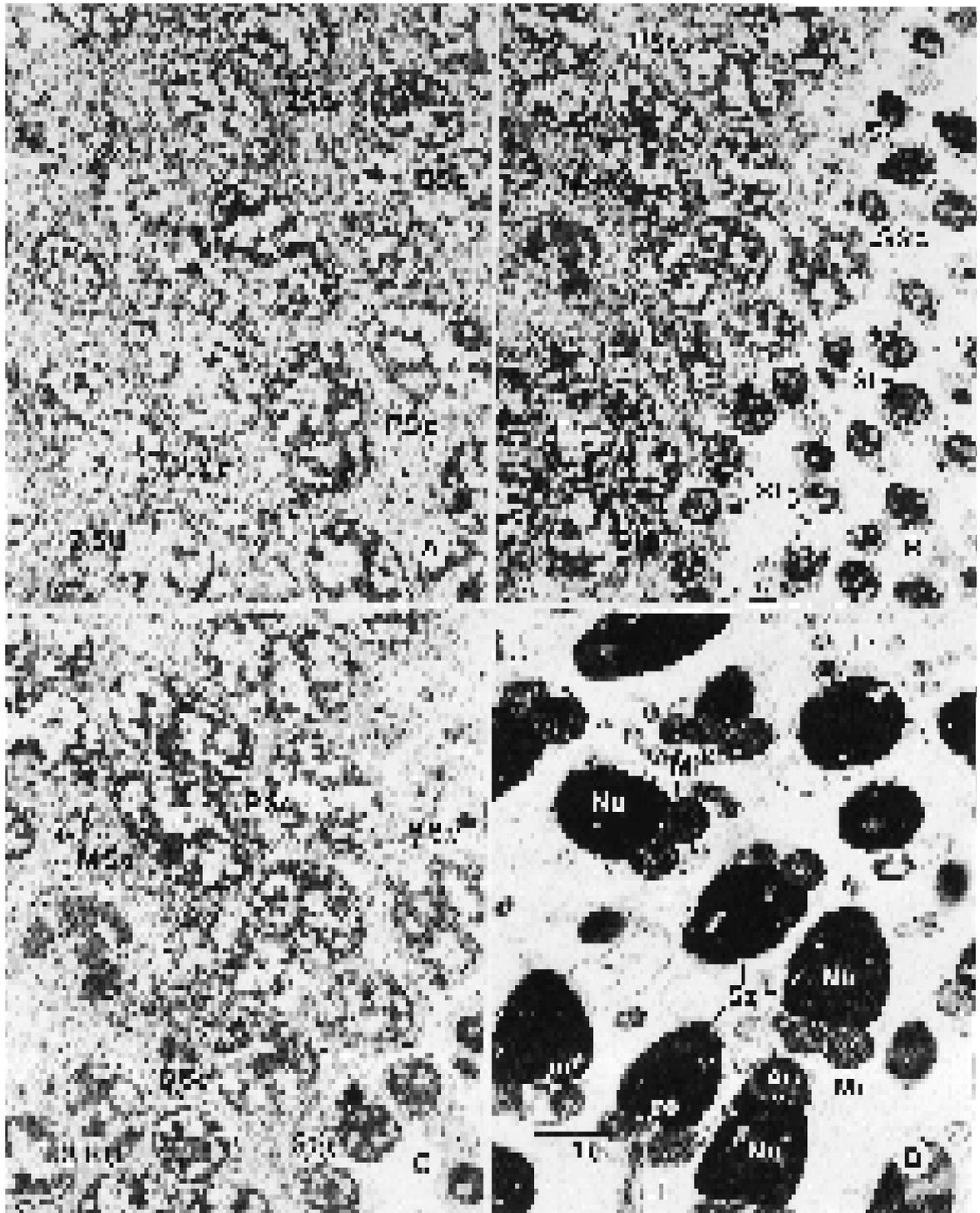


Fig 4. A-C) Electron micrographs showing various stages of male germ cells, including leptotene (LSc), zygotene (ZSc), pachytene (PSc), diplotene (DSc), secondary spermatocyte (SSc), spermarids (St). D) Spermatozoa exhibiting dense nucleus (Nu), acrosome (Ac), globular mitochondria (Mi), centriole (ce),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

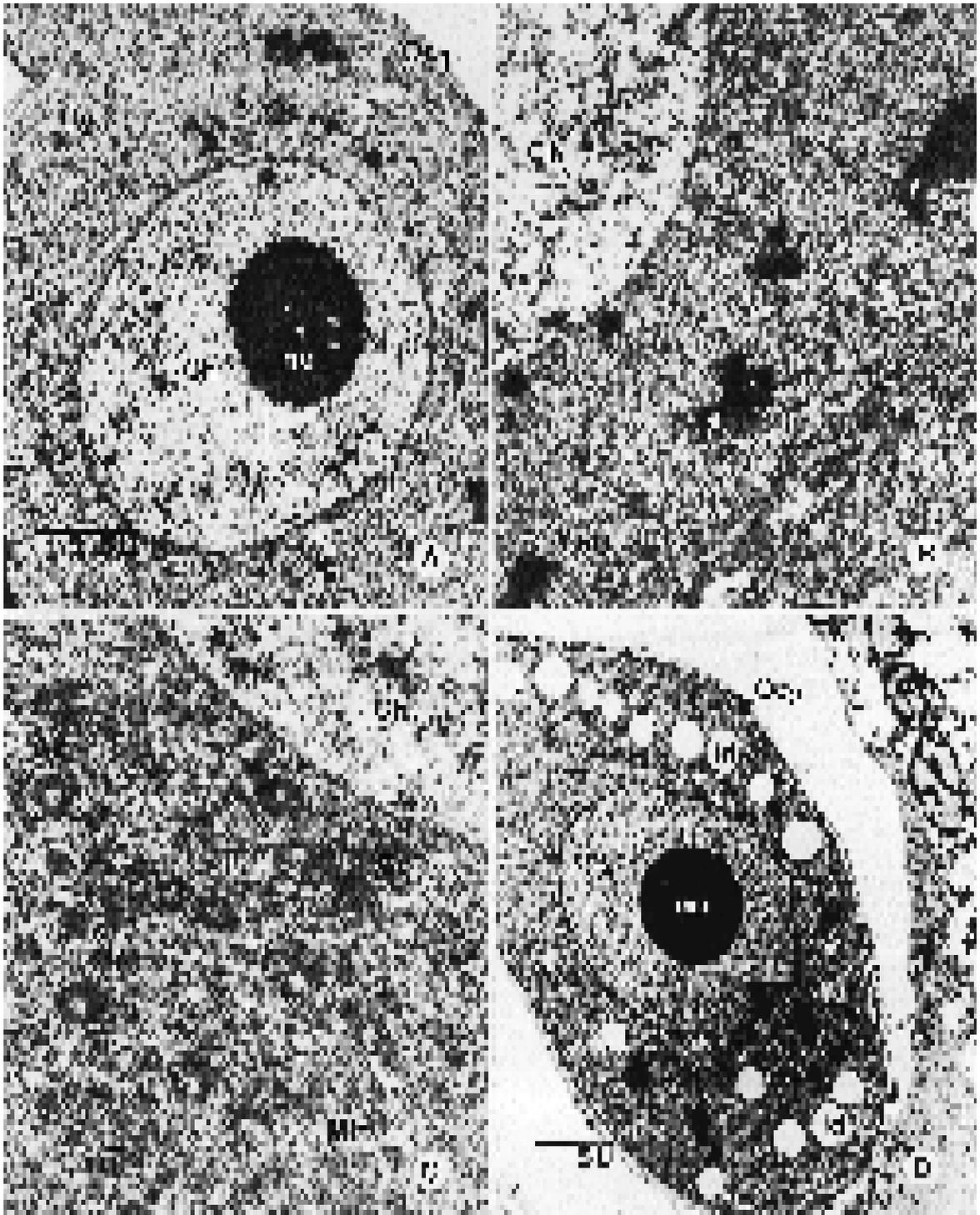


Fig 5. A,B) Early stage I oocyte (Oc_1), exhibiting nucleus with lampbrush chromosomes (Ch), dense nucleolus (no), and cytoplasm (Cy) with abundant ribosomes, some of which are aggregated in crystal-like bodies (arrows). C) Late Oc_1 , exhibiting the extensive development of Golgi complexes (Gc) and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

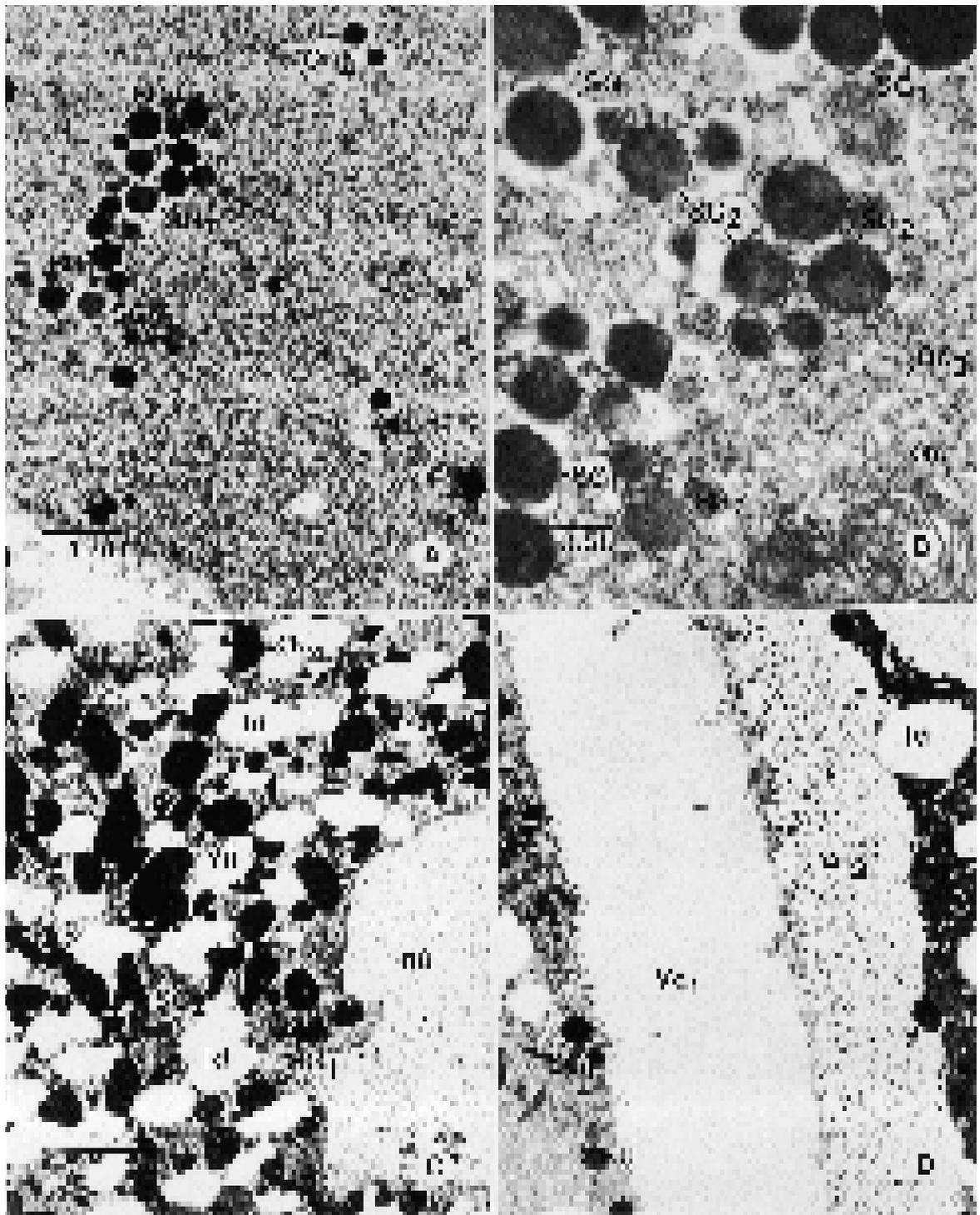


Fig 6. A,B) The cytoplasm of Oc_2 (A) and Oc_3 (B) exhibiting high concentration of dense jelly coat granules (SG_1) and lighter cortical granules (SG_2) around Golgi complexes (Gc). C) Fourth stage oocyte (Oc_4) exhibiting very light nucleus (nu) due to completely uncoiled chromatin. The cytoplasm contains 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).

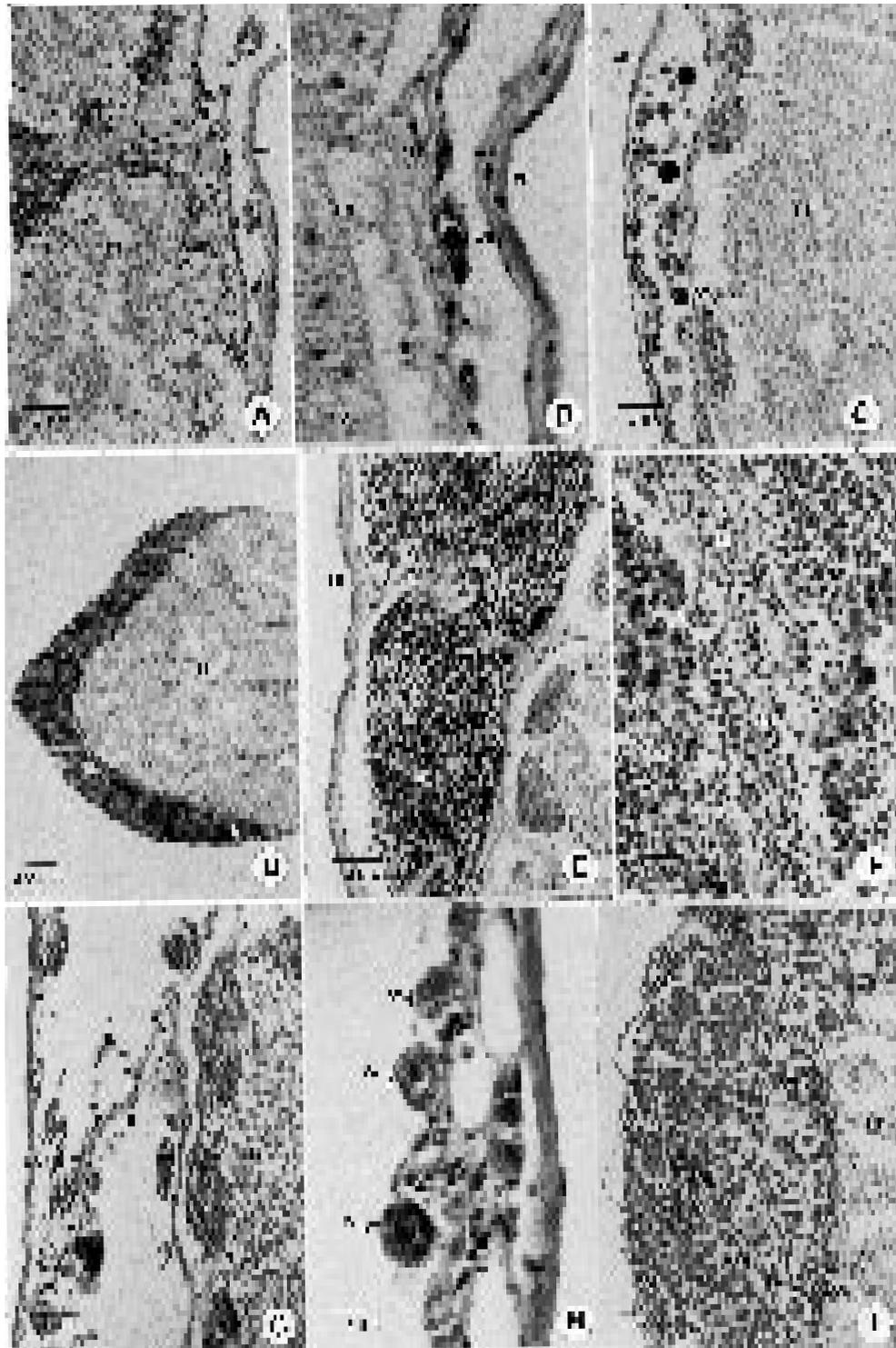


Fig 7. The development of gonads: A) the separation of gonadal capsule (cp) from hepatopancreas (HP) at 2 months; B) the presence of oogonia (Og) and possibly Oc_1 on the gonadal capsule bordering the hepatopancreas (HP); C) the ovary at 6 months showing the presence of early oocytes, mostly Oc_1 ; D,E,F) 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 $Oc_{1,2}$.

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, Oc₁, Oc₂ and predominantly Oc₃, most of which are still attached to the trabeculae; and later Oc₄ and Oc₅ 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 Oc₅, but only few remaining and widely scattered Oc₁. All of Oc₅ 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, *ie*, St and Sz. The most noticeable characteristics of the testis in this phase is the vast number of Sz₂ 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 Sz₂ 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 (St₁₋₄) 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 (Oc₁₋₃). By 10 to 11 month the testis appears fully developed, while the ovary starts to enlarge substantially and mature oocytes (Oc₄₋₅) 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 (Table I, Fig 7).

Table 1. 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 hepatopancreatic (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

DISCUSSION

Gonadal Structure and Classification of Cells in Gametogenesis

The first accounts of reproductive biology on an abalone species, *H tuberculata*, was published by Stephenson¹¹ since 1924, and Croft¹² 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 of 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 were 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*, light and electron microscopic characteristics have been used 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 increase²⁴. 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 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 Oc₅. There is no division of this cell coat into jelly and vitelline layers as reported in other species²⁵. Thus Oc₅ appears completely mature and is fully detached from the trabeculae. The absence of follicular cells might allow the detachment of Oc₅ 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 Oc₅ is laden with reddish yolk granules. Based on the size of these yolk granules there could be 2 subgroups of Oc₅: 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 Oc₅, 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 Oc₅ should represent fully mature cells. In comparison to the work of Martin *et al.*,²³ Oc₄ could represent the early postsynthetic cells and Oc₅ 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, *ie*, 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 diakinetik 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 St₁ and St₂ could be distinguished by the difference in size (St₁ about 6 μm versus St₂ about 4 μm), and by the denser nuclear material in St₂. 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 (St₃) the chromatin fibers begin 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 St₄, and individual chromatin fiber is enlarged to 60 nm.

The two stages of spermatozoa are distinguished by their ellipsoid nuclei. Sz₁ 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 Sz₂ the nucleus is elongated further and chromatin appears completely dense with the outline of 60 nm fibers (or granules) barely discernible. Sz₂ 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 (*eg* 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 phases¹⁷. 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, ie, 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, Oc₁, Oc₂ in the ovary, and mainly Sg, PrSc without St and Sz in the testis. Giorgi & DeMartini³³ 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 were mainly oogonia, yolkless and oil drop oocytes in this stage. Another remarkable feature 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. Boolootian *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 feature 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, Oc₁, Oc₂, Oc₃ and few Oc₄ which is similar to those reported in the pre-

mature 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, ie, Oc₅ with widely scattered Oc₁; and the testis is mostly filled with St and Sz. Before spawning occurs, Oc₅ 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 Oc₁ to Oc₅ from Og, the cells move along the trabeculae from capsular side towards the hepatopancreas side, until Oc₅ 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 & Doi⁴² 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. Giese²⁸ 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 is 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 presence 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 at 10 to 11 months when it starts to contain mature oocytes (Oc₄ and Oc₅). Thus males tend to reach maturity and assume reproductive cycle much earlier than females.

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