REPRODUCTIVE MECHANISMS IN CRUSTACEA

First Edition

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REPRODUCTIVE MECHANISMS IN CRUSTACEA

First Edition

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REPRODUCTIVE MECHANISMS IN CRUSTACEA

The Crustacea constitute an important group in the fisheries and aquaculture industry; in spite of this, much remains unknown about the physiological mechanisms controlling their reproduction. However, in order to achieve a stable and reliable means of aquaculture of crustacean species without dependence on the use of natural spawners and seed, it is necessary to achieve the control of reproduction in captivity. Therefore, it is essential to gain full understanding of the reproductive mechanisms of species of economic importance and Crustacea in general. We have been employing mainly the freshwater prawn, *Macrobrachium nipponense*, the giant freshwater prawn *M. resenbergii* and the kuruma prawn, *Penaeus japonicus* as experimental material to study gametogenesis and reproductive rhythms, and have obtained extensive profiles for molting hormone levels and yolk protein titers in these and other species. In this review, the results of this laboratory are presented as part of a larger discussion concerning the general knowledge of reproductive mechanisms in Crustacea which have been obtained thus far.

1. The processes of oogenesis and spermatogenesis

During the process of gametogenesis, primordial germ cells differentiate and ultimately become sperm or egg cells. This process is essentially the same in a variety of species. The basics will be elaborated here (Fig. 1－31), in reference to the literature on *M. nipponense* (Han, 1988) and on *P. japonicus* (Hong, 1977; Yano, 1990a).

1.1 Oogenesis

In *P. japonicus*, the ovaries exhibit a sac-like lobular appearance and are positioned on the left and right sides, extending from the top of the carapace over the hepatopancreas. As ovaries develop, they enlarge, reaching the head portion and down towards the tail. Oviducts extend from both lobes of the ovaries, and culminate in ovipores positioned at the third pair of pereiopods (Fig. 1).

During oogenesis, oogonia undergo proliferation mitosis and increase in number; at a certain point, oogonia commence the early phases of meiosis and become primary oocytes. Here they become arrested in Prophase I, during which time they accumulate yolk protein, and mature. With the resumption of meiosis, this process culminates in ovulation. *In M. nipponense*, this progression has been observed histologically, and is divided into 7 stages (Fig. 2). These are described as follows.

1) Oogonia stage

Oogonia possess relatively large nuclei and very little cytoplasm. Oogonia are seen in the central portion of the ovary; they repeatedly undergo mitosis and increase rapidly in number.

2) Meiotic stage

Oogonia which have just undergone proliferation mitosis become primary oocytes and enter meiosis. At this early stage, oocytes possess a large nucleus and little cytoplasm. Many chromosomes can be seen within the nucleus.

3) Previtellogenic stage

Primary oocytes which have become arrested in Prophase I begin a phase of growth. Oocyte diameter increases, and one to two large nucleoli can be seen within the nucleus. The cytoplasm becomes basophilic, indicating the accumulation of ribonucleic acids.

4) Endogenous vitellogenesis

The cytoplasm of oocytes of this stage are replete with granules which stain PAS positive. These are recognized as endogenous glycoprotein granules. From this time, follicle cells begin to develop around the perimeter of the oocyte membrane and eventually surround the oocytes.

5) Exogenous vitellogenesis

Lipid droplets and yolk globules accumulate in oocytes, and oocytes increase dramatically and rapidly in size. Yolk globules are formed from vitellogeinin (yolk protein precursor, lipoprotein) incorporated from the hemolymph. The origin of the vitellogenin is considered to be the hepatopancreas or fat body. The vitellogenin is secreted into the hemolymph and taken up actively by the oocytes.

6) Maturation

An extremely thin membrane appears between the oocytes and the follicle cells which surround them. With the completion of vitellogenesis, the oocytes which until now have been arrested in Prophase I, recommence meiosis and the germinal vesicle seen at the cell center begins to disintegrate (germinal vesicle breakdown: GVBD), and subsequently migrate to the animal pole.

7) Ovulation

After GVBD, yolk-laden primary oocytes are ovulated. Soon after ovulation, spawning and fertilization occur. Thereafter, the primary and secondary meiotic divisions occur accompanied by the extrusion of the first and second polar bodies.

In the kuruma prawn, a similar process is seen as in *M. nipponense*. In addition, the formation of a fertilization envelope via what is referred to as "cortical reaction" has been studied in several crabs and penaeid prawn species (see Meusy and Payen, 1988 for review). In crabs, the fertilization envelope arises from granular material accumulated within the vitelline envelope and ring-shaped elements arising from egg cortical vesicles. The cortical reaction of penaeids is of particular interest; cortical specializations are comparatively large and rod-shaped (their size is 40 μm while egg diameter is 270 μm). These rods are positioned perpendicular to the oolemma in a radial fashion, and are separated from the external environment by a membrane surrounding the entire egg. At spawning, rods are expelled rapidly, and later form a homogeneous jelly which envelops the eggs.

1.2 Spermatogenesis

The testes are comprised from a number of small lobules and positioned on the left and right flanks. Additionally on the left and right are the coil-shaped vas deferens which are connected to the testes, and culminate in spermatophores opening at the fifith pair of pereiopods. The processes of spermatogenesis and spermiation is divided into five stages.

1) Spermatogonia stage

Spermatogonia contained in the seminiferous tubules divide mitotically and increase in number.

2) Primary spermatocyte stage

Some spermatogonia, after dividing by mitosis undergo additional growth and become primary spermatocytes. In these cells, the nucleus is spherical in shape and stains strongly by hematoxylin.

3) Secondary spermatocyte stage

Primary spermatocytes undergo the first meiotic division, and each gives rise to two secondary spermatocytes. Secondary spermatocytes show the same morphology as that of primary spermatocytes, but their size is halved.

4) Spermatid stage

Secondary spermatocytes undergo the second meiotic division with each spermatocyte giving rise to two spermatids (i.e. four spermatids from the original primary spermatocyte). The nucleus exhibits a distorted sickle shape.

5) Spermatozoan stage

Without further division, the spermatids undergo conversion into spermatozoan cells, a conversion process which includes the shrinkage of the nucleus and the loss of cytoplasm. The fully formed sperm cells show a pinhead shape.

2. Reproductive rhythms and environmental factors

Most animal species harbor a specific breeding season, which is a function of their ecology. Such patterns, in which a number of reproductive changes occur seasonally, are referred to as "reproductive rhythms." Reproductive rhythms are thought to be controlled in part by daylength, water temperature, feed availability, and other environmental factors of the like. In Crustacea, a number of species harbor such seasonal patterns, and much has been reported concerning the influences of environmental factors (Sastry, 1985; Meusy and Payen, 1988). Here, we describe our results on the freshwater prawn *M. nipponense* in Lake Kasumigaura as the focal point of this discussion.

2.1 Annual rhythms

Annual rhythms vary with species (Table 1); furthermore, within species, rhythms often vary among strain in accordance with habitat. In general, in species which inhabit the mid to upper latitudes, breeding extends from spring to fall; however, those of the lower latitudes exhibit a relatively long breeding season which does not have much apparent connection to season. In deep-sea species, it is often seen that breeding takes place during winter.

In Lake Kasumigaura, the freshwater prawn exhibit a life span of nearly three years, and breed during the summers of their second and third years of life. The gonads of the females and how they develop during a one-year period have been examined in this laboratory by Han (1988); this data is presented in Fig. 32. Gonadosomatic index (GSI: gonad weight × 100/body weight) increases rapidly from April to June, during which period, ovaries are replete with oocytes which are undergoing vitellogenesis (exogenous). From June to August, high GSI values continue, and almost all prawns are obsereved to spawn eggs.

During this season, individuals spawn continually. Nearly all individuals that possess oocytes which have completed vitellogenesis also harbor ovulated follicles. In individuals which possess oocytes still undergoing vitellogenesis, a range of reproductive stages is seen among these oocyes. In September, individuals brooding spawned eggs are rarely seen, and GSI values become low. At the periphery of the ovaries are seen atretic eggs, and in the center portions are seen oogonia and immature oocytes ranging from the pre-meiotic stages to the pre-vitellogenic stages. From October to March, GSI values remain low. In October and November, oogonia undergo proliferation mitosis at the ovaries, and numbers of immature oocytes increase; however, these degenerate before entering into the early vitellogenic stages, as this is a period of ovarian rest. From December to March, immature oocytes once again attain the early vitellogenic stages and continue to develop in accordance with reproductive rhythm and in preparation for the onset of the breeding season.

The above can be summarized as four main stages as follows (Fig. 33):

1) April－June: vitellogenesis advances, with the approach of breeding; yolk accumulation in the oocytes occurs rapidly.

2) June－August: Spawning season. Spawning is repeated continually; one individual may spawn several times.

3) September－November: Breeding recession. Reproduction and spawning terminate; mature oocytes which remain in the ovary unspawned degenerate.

4) December－March: Pre-vitellogenic stage. Oocyte proliferation renews, but development does not proceed into early vitellogenesis.

In male freshwater prawn as well, GSI increases from April to June (Fig. 32). In histological observation of the testes, spermatogonia undergo proliferation mitosis from April and spermiation occurs in May. From June to August, GSI values are high, and in the seminiferous tubules of the testes, are numerous sperm, and spermiation is carried out actively. From September to March, GSI gradually decreases. Some spermatogonia still remain within the seminiferous tubules, but without undergoing further proliferation, they gradually disintegrate. In summary, similarly to females, male reproduction becomes quite active from April, culminating in spermiation in the June–August breeding season. Subsequently, sperm production ceases from September to March (Fig. 33).

2.2 Environmental factors

Annual reproductive rhythms are much decided by a variety of environmental parameters. This is due to the necessity of species-specific adaptation to environment in context of condition of habitat, for example, when food prey is most abundant, or temperature is most suitable for breeding. Generally, in the mid-latitudes, water temperature and daylength vary greatly with season; thus species which are found in these regions are greatly influenced by these parameters (Table 2). In tropical species, the parameters of importance are considered to be the timing of the rainy and dry seasons and/or feed availability, since daylength and temperature are almost constant.

The freshwater prawn undergoes oogenesis and spermatogenesis from April to June, heading toward the breeding season. During this time, water temperature on the average increases from 10℃ to 18℃ and daylength goes from 13L (time which is light) to 14L. On the other hand, when the breeding season ends in September, water temperature decreases from 26℃ to 22℃, and daylength decreases from 14L to 13L. In our laboratory, it was considered that the commencement and termination of breeding season may be dependent on water temperature and daylength, and therefore, several experimental investigations concerning the influences of these parameters were carried out (Han, 1988).

In order to investigate the parameters which induce breeding, six groups of *M. nipponense* were reared under combinations of daylength (12L and 15L) and water temperature (10℃, 16℃, 22℃), and ovarian development was observed from April to June (Fig. 34). At the beginning of rearing, GSI values were low, and yolk accumulation in the oocytes had not yet commenced. In females reared at 22℃, GSI began to increase, and egg-brooding individuals began to appear within one month, without connection to daylength. Within two months, almost all individuals were brooding. In the 16℃ group, after one month of rearing, GSI increased somewhat, but egg-holding individuals did not appear; this had no relationship to daylength. There was however, some advancement of yolk protein uptake at the ovaries, and oocytes were observed that were in the stages of secondary vitellogenesis. Egg-brooding individuals appeared after two months of rearing. In contrast, in the 10℃ group, even after two months of rearing, increases in GSI and the occurrence of spawning were not observed. From the above results, it can be inferred that the factor which induces the start of breeding is not change in daylength, but rather, increase in water temperature. Nextly, actual coastal temperatures were examined in context of breeding rhythms, revealing that vitellogenesis commences in *M. nipponense* when temperatures reach above 12℃. Thus in April, females respond to such temperature changes, and undergo vitellogenesis more actively as temperature continues to rise; spawning commences in June (Fig. 33).

Subsequently, we investigated the termination factors of the breeding season, and to do so, female *M. nipponense* were reared from August to November under combinations of daylength (12L and 15L) and water temperature (28℃ and 22℃) in four groups. The frequency of the cessation of spawning was examined (Fig. 35). In the 28℃-15L group, all individuals continued to spawn until the end of September, but in the 28℃-12L group, 90% of the individuals ceased spawning. Similarly, in the 22℃-15L group, 80% of the individuals continued to spawn, but in the 22℃-12L group, all individuals ceased spawning. After September, all prawns reared under 12L ceased spawning without connection to water temperature, but in the 15L group, all those reared at 28℃ continued to spawn, and of those which were reared

at 22℃, 70% continued to spawn. Fom these results, it was elucidated that the environmental factor which controls the termination of spawning in September is decreasing daylength to a much greater extent than is decreasing water temperature (Fig. 33).

In contrast, in other *Macrobrachium* species, for example, the tropical *Macrobrachium rosenbergii* (giant freshwater prawn), water temperature and daylength are not extremely important. When the giant freshwater prawn is raised under a variety of water temperature-daylength combinations, the division line between spawning and non-spawning is not clear-cut, and only a insignificant difference in spawning frequency is seen (Chavez Justo et al., 1991). In the Hoogly river in India, the *M. rosenbergii* breeds between December and July. The climax of the breeding season coincides with the occurrence of the North monsoons between March and May. In *M. rosenbergii* inhabiting the southern parts of India, the breeding season occurs from August or September, and breeding is most active during the Southwestern monsoon season from October to November. Monsoon season implies wet season, and therefore water quality and level are expected to change and affect feed availability (to increase). This is thought to be the stimulus for breeding in this species.

3. Spawning rhythms

In numerous crustacean species, during a singular spawning season, spawning occurs not just once, but is repeated multiple times. Each individual species appears to habor its own specific, regulated pattern, according to when spawning occurs. Here we introduce two examples: *M. nipponense* in which spawning is synchronized with the molt cycle, and the red-armed crab (*Sesarma haematocheir*) in which larval release is governed by tidal rhythms.

3.1 Molting and spawning rhythms

With the exception of the kuruma prawn, and only a few other species, most crustaceans spawn an egg mass and brood it until hatchout via attachment to the reproductive setae. If molting were to occur during brooding, the egg mass would be shed and lost. Therefore, mechanisms exist such that during brooding, molting does not occur. The synchronization of molting and spawning takes on a number of regulatory patterns according to species (Fig. 36: Adiyodi, 1985). (1) in *M. nipponense* and a number of isopods, during an intermediate point in the mold cycle, the ovaries begin to develop, and immediately following the next molt, females mate, spawn, and brood. (2) In crabs, the molt cycle is of a long duration, and between each molt, females may undergo ovarian development and carry out spawning and brooding one or more times. (3) In barnacles, molt intervals are short, and ovarian development is completed not over one molt cycle, but is extended over several. When ovarian development is completed the female spawns and broods immediately after the next molt. The egg mass hatches before the next molt.

In *M. nipponense*, ovaries develop within one molt cycle, and spawning occurs after ecdysis; therefore, the molt cycle and egg development cycle are intricately linked (Fig. 2: Han, 1988). Post-spawning ovaries are observed to contain oogonia and oocytes ranging from the pre-meiotic stages to the previtellogenic stages, in addition to mature unspawned oocytes and ovulatory follicles. At this point immediately following spawning, the next cycle begins and oogonia once again undergo proliferation mitosis, and become oocytes. GSI values are low, ranging around 0.6−0.7% (Fig. 37). Prior to yolk accumulation in the ovaries, hemolymph yolk protein (vitellogenin) levels begin to increase (Fig. 37).

At an intermediate time in the molt cycle, yolk uptake commences, and oocytes in the stages of exogenous vitellogenesis appear. GSI values begin to become high. In the latter intermediate stages of the molt cycle, yolk protein accumulation becomes very active, and oocyte diameter enlarges greately. GSI values increase dramatically, and hemolymph vitellogenin levels reach peak levels. Finally, molting occurs, which is followed by mating, and spawning. During the spawning season, this cycle occurs repeatedly. Under rearing conditions of 28℃, the duration of this is about 14 days, while at 22℃, this is 25 days. The length of the spawning cycle is determined by water temperature.

3.2 Tidal rhythms

In certain species, tidal rhythms are an integral part of the reproductive physiology. The red-armed crab *S. haematocheir* inhabits land areas close to the shore, but when females are ready to hatch their brooded egg masses, they move to the sea. Larval release coincides with the full moon or new moon, which is when high tide occurs. The female proceeds toward the sea at sundown, usually between 19 and 21 hrs to spawn. After larval release, the female soon mates with the male, and spawns another egg mass, thus beginning a new brooding cycle(Hashimoto, 1972).

4. Endocrinological mechanisms

As was touched upon in Section 1, the process of gonadal maturation is under hormonal control, but many details still remain unclear. Up until present, the existence of hormonal factors and their significance have been demonstrated experimentally. While several hormonal substances have been purified (adiyodi, 1985; Charniaux-Cotton and Payen, 1988), the identities of many crustancean reproductive hormones remain unknown. Recently, amino acid sequences of crustacean hyperglycemic hormone (CHH) family peptides in the eyestalk including molt-inhibiting hormone (MIH) and vitellogenesis-inhibiting hormone (VIH) were determined in several species (Fig. 38: Chang et al. 1990; Huberman et al. 1993; Kegel et al. 1989, 1991; Keller 1992; Martin et al. 1993; Soyez et al. 1991; Tensen et al. 1991; Webster 1991; Yang 1994; Yasuda 1994). Here we will introduce separately for males and females those regulatory factors which are considered to be involved in gonadal matuation.

4.1 Females

1) Mitosis of oogonia and previtellogenesis of oocytes

During sexual differentiation, primordial germ cells differentiate into oogonia. Experimentally, if ovaries alone are cultured *in vitro*, this processes proceeds automatically, thus it is considered that this process is not under hormonal control. If immature crab (*Eriocheir sinensis*) are eyestalk-ablated, DNA synthesis in the oogonia becomes very active; therefore, the proliferation mitosis of the oogonia is thought to be under the control of an inhibitory factor in the eyestalks. In the same species, even if the Y-organ, the site of ecdysteroid production, is removed surgically, oogonia still undergo proliferation, and enter the previtellogenic phases; therefore, it appears that ecdysteroids do not play a significant role in gonadogenesis. However, if both the eyestalks and the Y-organ, are removed, previtellogenic oocytes actually degenerate. In order to maintain normal oocyte function in ablated crabs, it is necessary to apply continuous dosage of ecdysteroids. However, it is still unclear exactly how eyestalks factors and ecdysteroids are involved in this phenomenon.

2) Vitellogenesis

The site of vitellogenin (yolk protein precursor) synthesis is the fat body in isopods and amphipods, and the hepatopancreas or ovaries in crabs and prawns. In *M. nipponense*, the hepatopancreas and subepidermal tissue are considered to be the site of vitellogenin synthesis (Han et al. 1994, Fig. 39, 40 and 41). Vitellogenin produced at these sites is secreted into the hemolymph and is accumulated into the oocytes as yolk globules. This is a dramatic physiological change, and hormonal mechanisms related to this process have been investigated to a relatively large extent. Here, five major hormones and their functionings are presented (Fig. 42).

A. Vitellogenesis-inhibiting hormone (VIH)

It has been long-established that in a variety of crustacean species that via removal of the eyestalks, ovarian maturation occurs automatically, and therefore it was considered that an inhibitory factor exists in the eyestalks. In 1987, this hormone was purified from the American lobster, as a neurosecretory peptide having a molecular weight of between 7000 to 8000 kDa. Since, its amino acid sequence (77 residues) has been elucidated by Soyez et al. (1991) via microsequencing and mass spectrometric techniques.

VIH is synthesized at the X-organ of the eyestalk; the X-organ is comprised of neurosecretory cells. The X-organ contains perikarya whose axons end in the sinus gland. VIH is secreted from here into the hemolymph. In the crayfish, *Crangon crangon* and related species, eyestalk VIH activity is at ultimate low levels just before and during spawning, and increases towards the end of the spawning season. Two possibilities have been given consideration concerning the manner in which VIH inhibits vitellogensis: 1) inhibition of vitellogenin synthesis at the fat body, hepatopancreas or ovaries, and its release, 2) inhibition of the uptake of hemolymph vitellogenin into the oocytes. Of the two possibilities, there appears to be more support for the latter.

B. Vitellogenesis-stimulating hormone (VSH)

It has been observed in a number of species that by transplantation of brain tissue or thoracic ganglion from mature females into immature females, the ovaries of the recipients can be induced to mature. Therefore, it is considered that these tissues harbor a factor which promotes vitellogenesis. However, while there are many experimental investigations that support such a hypothesis, VSH itself as not been isolated and purified, and therefore its existence remains a postulation. The mechanism by which VSH may stimulate vitellogenesis also remains unclear.

C. Vitellogenesis-stimulating ovarian hormone (VSOH)

It does appear that continued production of vitellogenin requires a factor from the ovaries. In the amphipod, *Orchestia gamarella* and the isopod, *Porcellio dilatatus* the following experiments have been performed. 1) In males which are androgenic gland-ectomized, spermiation stops, but vitellogenesis does not automatically begin. However, if ovaries are transplanted into these males, vitellogenin synthesis occurs at the fat body. 2) Females which are undergoing vitellogenesis are ovariectomized: vitellogenin synthesis at the fat body ceases. From the above results, it can be considered that there exist hormone (s) at the ovary which stimulate further vitellogenin synthesis at the ovary; this factor is referred to as vitellogenesis-stimulating ovarian hormone (VSOH). However, in the isopod, *Armadillium vulgare*, opposite results have been obtained demonstrating that the ovary is not necessary for continued vitellogenin synthesis, and therefore the existence of this hormone may not be common to all crustaceans. It will be necessary to clarify the roles and identity of VSOH in subsequent studies.

D. Ecdysteroids

Ecdysone and 20-hydroxyecdysone (Fig. 43) have been identified as the molting hormones in Crustacea (Skinner, 1985; Chang and O'Connor, 1988). The precursor form, ecdysone, is produced at the Y-organ from exogenous cholesterol, passed into the hemolymph, and oxidated to 20-hydroxyecdysone in the peripheral tissues (Nakamura et al. 1991). Molting activity has been confirmed by detailed experimentation in which ecdysteroid administration causes accelerated molting. In Crustacea in general, hemolymph titers increase dramatically immediately preceeding ecdysis, which is the actual shedding of the cuticle, (Okumura, et al. 1989 and 1992: Fig. 44). Hemolymph titers are regulated by the secretion of molt-inhibiting hormone (MIH) from the X-organ/sinus gland complex. In addition to ecdysone and 20-hydroxyecdysone, numerous other ecdysteroids exist, which exhibit structural variations at the side chain or steroid nucleus. In some insect species, varying ecdysteroids are thought to play complimentary, additional roles in the stimulation of vitellogenesis (Sakurai, 1986). The same can be considered for the Crustacea as well.

Several investigations have shown that ecdysteroids have involvement in egg development. 1) In *O. gammarella*, and *P. dilatatus*, if the Y-organ is removed, vitellogenin synthesis ceases. 2) In female Y-organ-ectomized *P. dilatatus* in which vitellogenin synthesis has therefore ceased, if ecdysteroids are administered, vitellogenin synthesis recommences. 3) In female crab, *Carcinus maenus*, if the Y-organ is removed, ovarian development stops. 4) On the contrary, in post-molt shrimp *Palaemon serratus* and *O. gammarella*, 20-hydroxy-

ecdysone administration causes vitellogenin production to stop. These results suggest that ecdysteroids stimulate vitellogenesis in some cases, and inhibit it in others, and thus appear to indicate contradictory roles for ecdysteroids. Yet, ecdysteroid action may be a function of concentration and particular molt stage of the organism; thus what seems contradictory may actually have connection to threshold levels.

In the giant freshwater prawn, ecdysteroids are accumulated in the oocytes during vitellogenesis (Wilder et al., 1991). All ecdysteroids in the embryo decrease gradually during the nauplius phase and with the formation of the compound eye and the appearance of the carapace and other body-like structures, marking morphogenesis to the zoeal stage, embryos show the beginning of a continuous and dramatic increase in ecdysteroid concentrations sustained until larval hatchout (Wilder et al., 1990). It is be postulated that the presence of ecdysteroids in ovaries and eggs represents a reserve of maternal ecdysteroids which are necessary at the commencement of embryonic development and with the differentiation of embryonic tissue capable of ecdysteroid synthesis, ecdysteroids increase rapidly to play a role in later embryonic development.

E. Juvenile hormone (JH)

In insects, JH is involved in metamorphosis and was first discovered via this functioning (Yamashita, 1986). More recently, in addition, it has been ascertained that JH additionally controls vitellogenesis (Sakurai, 1986). Thereafter, it had been postulated that JH is similarly involved in metamorphosis and vitellogenesis in Crustacea as well, and many investigations thus followed. In 1987, not JH, but its unepoxidated precursor form, methyl farnesoate (MF) was first detected in a crustacean species by Laufer and Borst, 1988 (Fig. 45). It is now known that MF is synthesized and secreted at the mandibular organs (MO). In several crab species, it has been ascertained that MF secretion by the MO's is six times higher in females actively undergoing vitellogenesis than in previtellogenic females. The administration of MF has been shown to stimulate oocyte growth *in vitro* in the shrimp, *Penaeus vannamei* (Tsukimura and Kamemoto, 1991). Such research has suggested that in Crustacea as well, juvenoid compounds play a role in stimulating vitellogenin production. On the other hand, in *Artemia*, it has been demonstrated that MF stimulates Na/K-ATPase activity, and therefore may be involved in osmoregulation and the mediation of ecdysis (molting) (Ahl et al., 1991). In the giant freshwater prawn, fluctuations of hemolymph MF were the same both in the reproductive molt and common molt cycles: a peak was observed at the early premolt stage. However, vitellogenin production was not observed to be accelerated by MF administration in eyestalk-ablated prawns (Wilder et al. 1994). As of yet, the exact functionings of MF remain unclear.

F. Others

In a limited number of crustacean species, the presence of several vertebrate sex hormones, such as testosterone, estradiol 17β, etc. have been observed. Thus far, the involvement of progesterone and 17α-hydroxyprogesterone in the stimulation of vitellogenin synthesis in the kuruma prawn has been reported, but the actual mechanisms by which this stimulation is achieved have not been elucidated.

It has also been reported that the administration of human chorionic gonadotropin (HCG) to kuruma prawn (Yano, 1990b) and to *O. gammarella* results in increased ovarian development. Therefore, it appears that HCG functions as a VSH; however, there exists the possibility that the action of HCG here is a pharmacological. HCG may possess a molecular structure similar to that of the yet uncharacterized VSH.

3) Ovulation

As mature oocytes enter the ovulatory process, meiotic arrest is broken, and the germinal vesicle collapses and migrates from the center of the oocyte to the oocyte periphery. This culminates in ovulation. In the grass shrimp (*P. serratus*) (Lanot and Cledon, 1989), hemolymph ecdysteroid titers become elevated during this interval. Experimentally, if ovaries

at this stage are dissected out and incubated in the presence of ecdysteroid, ovulation of the oocytes is seen to occur. However, if ovaries are incubated in the absence of ecdysteroid, ovulation does not occur (Fig. 46). These results indicate the strong possibility that ecdysteroid is the ovulation-inducing hormone.

4.2 Males

Testes development and its endocrine mechanisms in males have not been a focal point of investigation to the extent that female reproductive mechanisms have been. The most followed male endocrine factor is produced by the androgenic gland (androgenic gland hormone: AH) and is involved in sexual differentiation and spermiation. Additionally, factors which are thought to be involved in spermatogenesis include eyestalk and thoracic ganglion factors, but knowledge beyond the indication that these exist is completely lacking.

A. Androgenic gland hormone (AH)

Ovaries transplanted into male *O. gammarella* are transformed into testes; the existence of AH was first confirmed in this manner. AH is synthesized and released by the androgenic gland, an organ attached laterally to the vas deferens. The androgenic gland exists only in males, and its presence seems common to most crustacean species. The wide involvement of AH in male reproduction is described below.

i. Sexual differentiation: Ovaries transplanted to males are transformed into testes; if the androgenic gland is removed, spermatogenesis ceases and oocytes begin to appear. Androgenic gland implants into females undergoing vitellogenesis cause vitellogenin synthesis to cease; if androgenic gland is implanted into immature females, ovaries are transformed into testes. Such experimental work has been carried out in isopods, amphipods and in the giant freshwater prawn (*M. rosenbergii*) and in various crab and crayfish species, confirming these phenomena.

ii. Secondary sex characteristics: If immature females are implanted with androgenic gland, appendages take on male morphology, but if males are androgenic gland-ectomized, these take on female characteristics; this indicates that the androgenic gland secretes a factor which induces masculine secondary sex characterisitics.

iii. Spermatogenesis: In males, mitosis of the spermatogonia ceases, when the androgenic gland is removed. In organ culture, mitosis does not occur in the absence of AH; therefore, it can be inferred that AH is necessary to the process of spermatogenesis. However, in the crab, *C. maenus*, spermatogonia are maintained even in the absence of AH. Yet, in general, there exists a correlation between the extent of spermatogenic activity and the size of the androgenic gland.

Two classes of AH have been confirmed to exist. In *A. vulgare*, two forms of AH have been purified and identified as 17,000 and 18,300 Da proteinaceous material. On the other hand, in the *C. maenus*, two AH's have been demonstrated as farnesyplacetone and hydrohexafarnsylacetone, which are 18-carbon skeleton terpenoid molecules (Fig. 47). Whether this implies that different AH's play varying roles in male reproduction is unknown. However, AH can also act in a pheromonal capacity; both proteinaceous AH and isoprenoid AH inhibit vitellogenin synthesis in respective species, indicating the universality of these substances. Of interest, farnesylacetone has been shown to inhibit vitellogenesis in the ovaries of the crab, *C. maenus* in a seasonal manner (Berreur-Bonnefant and Lawrence, 1984). Farnesylacetone appears to act through a mechanism in which protein synthesis is inhibited via modification of RNA transcription, as demonstrated by organ culture experiments.

B. Brain factors

In *Palaemon* shrimp species, and in the crayfish, *Crangon crangon*, if the foreportion of the brain is destroyed, the proliferation mitosis of the spermatogonia in the testes ceases,

and the vas deferens and androgenic gland atrophy. However, if brain from untreated males is transplanted into surgically-treated males, these organs are maintained. In organ culture, if *O. gammarella* testes are incubated alone, the spermatogonia degenerate; but if male brain tissue is added to the culture medium, spermatogonia can be maintained. Female brain tissue can not maintain the spermatogonia in culture. From these results, it can be inferred that a number of factors which stimulate spermiation exist in the brain. These may be male equivalent of the female VSH.

C. Eyestalk factors

If male kuruma pawn are eyestalk-ablated, and are then injected with eyestalk extract, testes development can be stimulated. Therefore, the existence of an eyestalk factor which targets the testes has been postulated. Alternatively, removal of the eyestalks can lead to a hypertrophy of the androgenic gland. The above results are contradictory, but it is suggested that differing eyestalk factors are present and act in varying processes.

5. Artificial propagation/fertilization

In order to increase the efficiency of seed production, improved rates of fertilization are very much necessitated; however, there are many sources of failure: mating may be successful, but the sperm receptacle could be lost to shocks, such as molting. Additionally, for rearing purposes, it is often necessary to hybridize new strains, but in actuality, males and females of different species often do not mate. For such reasons, improved artificial insemination technology is desirable and necessary to establish. A number of methods have been attempted, which fall into three main categories.

(1) The sperm receptacle is obtained from the male, and is implanted into the thelycum of the female, or is attached to the female abdomen.

(2) Females which are unmated and therefore do not possess attached sperm receptacles are reared in aquaria; when these females spawn, advancely-prepared spermatic fluid is introduced into and dispersed in the tanks, achieving fertilization,

(3) Matured ovaries are dissected out from the female and fragmentated; sperm fluid is administered to fertilize the eggs.

Of the above, method (1) has been mainly employed and tested in the giant freshwater prawn (Sandifier and Smith, 1979), lobster (Criddle and Chang, 1985), crayfish (Berill, 1985), and kuruma prawn (Bray et al., 1982; Lin and Tei, 1984; Lin and Tin, 1986; Lin, 1989). Here, we will introduce the techniques of sperm receptacle implantation of Lin (1989) for kuruma prawn.

In penaeid species, mating in, for example, the kuruma prawn, black tiger prawn, and redtail prawn occurs immediately after the female molts. In the white shrimp *P. vannamei*, mating is carried out between the male and the intermolt female. During mating, the male deposits a sperm receptacle in the thelycum of the female. After mating, if the abdomen of the female is viewed, the sperm receptacle can be seen deposited in the thelycum. Next, the sperm receptacle is dissolved by fluid secreted by the female thelycum, and a uniform sperm fluid results. When spawning occurs, eggs are released through the thelycum, and are thus fertilized by this semen. Thus, fertilization occurs externally.

To carry out sperm receptacle implantation, it is first necessary to obtain the sperm receptacle from the male. The following methods are available. 1) Remove by anatomical dissection. 2) Squeeze out by exertion of pressure. 3) Pipette out by suction. 4) Remove by pincette without extensive dissection. 5) Release by running a direct current (2–4 V) longitudinally for 20 seconds between the fifth walking leg and the first abdominal segment. After obtaining the sperm receptacle by one of the above methods, the stopper portion is removed and the receptacle can be implanted in the female thelycum.

In post-molt black tiger prawn (*P. monodon*) in which ovaries are not sufficiently mature, if sperm receptacle implantation is carried out, ovaries mature within 4 − 5 days and a high spawning rate of 69 − 85% can be obtained. In female *P. penicillatus* at post-molt, the carapace is still soft; if sperm receptacle implantation is done on individuals with immature ovaries, ovaries mature within 3−8 days and a hatching rate of 89−94% is observed. Furthermore, in individuals 3−6 days after molting in which the carapace becomes hard and ovaries are already maturing, spawning can be obtained within 2−4 days after transplant with hatch-out rates of 80−91%. In those prawns after 8−19 days of molting which possess a hard carapace and mature ovary, if spawning occurs the night of receptacle implantation, hatchout rates are low (0−38%). This discrepancy might be attributable to the possibility that with the elapse of time after molting, the copulatal fluid (fluid secreted by the female thelycum which dissolves the sperm case and releases it as sperm) activity may become decreased. Additionally, as the time from implantation to spawning becomes shortened, the transformation from sperm receptacle to sperm fluid may not be sufficient. To solve such problems, a technique in which trypsin is introduced into the copulatal fluid to facilitate the transformation into semen, has been attempted. In *P. penicillatus*, in which the carapace is hardening and the ovaries are developed, the following four treatments have been tested and compared: Ⅰ) Sperm receptacle is soaked in artificial copulatal fluid and transplanted; additional artificial copulatal fluid is introduced. Ⅱ) The sperm receptacle is crushed; sperm bundles are obtained and implanted, with the introduction of artificial copulatal fluid. Ⅲ) Only sperm is introduced/implanted. Ⅳ) As a control, untreated sperm receptacles are implanted. In comparison of all four methods, the order of efficacy regarding hatching rates is Ⅱ > Ⅰ > Ⅲ > Ⅳ. Therefore, it was ascertained that artificial copulatal fluid can be used to improve hatching rates.

In this manner, by using sperm receptacle implantation, hybridization has been carried out between black tiger prawn and *P. penicillatus* (Lin et al., 1988), between the giant freshwater prawn and several varieties, between the crayfish *Orconectes rusticus*, and *O. propiniques*) (Berill, 1985), and between American lobster and European lobster (Criddle and Chang, 1985).

Perspective

Here, we have introduced a representation of recent knowledge on reproductive mechanisms in Crustacea. However, investigations up until present have covered only a portion of the many crustacean species that exist. The Crustacea are among the most highly diversified groups of aquatic animals, and their reproductive ecology is varied and wide-ranging. It will be necessary to continue basic research encompassing many more species. It will be necessary to examine these in detail, putting their reproductive strategies in context of the environmental conditions of their habitats. Recent techniques in analytical chemistry and molecular biology should be used to purify and identify crustacean hormones; their basis for action and their molecular mechanisms must be clarified. In comparison to the vertebrates, research on invertebrates has not advanced as much. Further research would contribute not only to the fishries industry, but also to the realm of basic science. Much can be expected in future studies.

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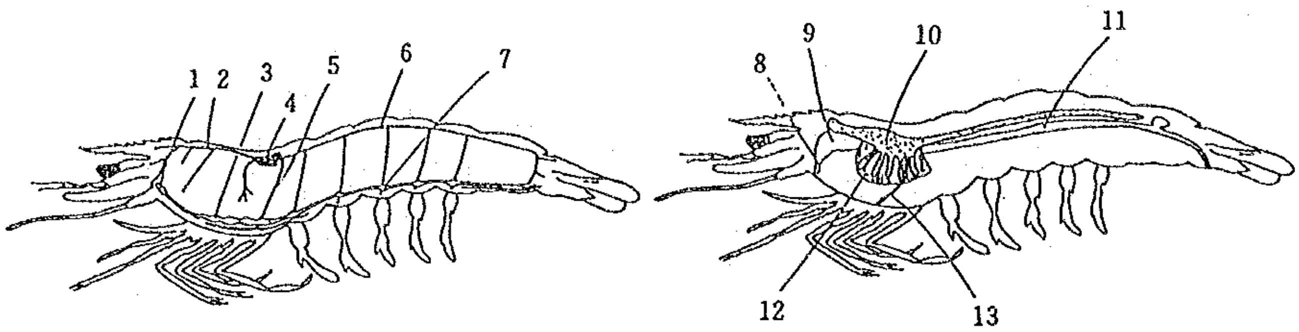


A. Circulatory and nervous system B. Digestive and reproductive system

Fig. 1 Anatomy of the female kuruma prawn, and related species. 1, brain (supraesophageal ganglion); 2, anterior aorta; 3, thoracic ganglion; 4, heart; 5, sternal artery; 6, posterior aorta; 7, ventral nerve cord; 8, esophagus; 9, stomach; 10, ovaries; 11, intestine; 12, hepatopancreas; 13, oviduct.

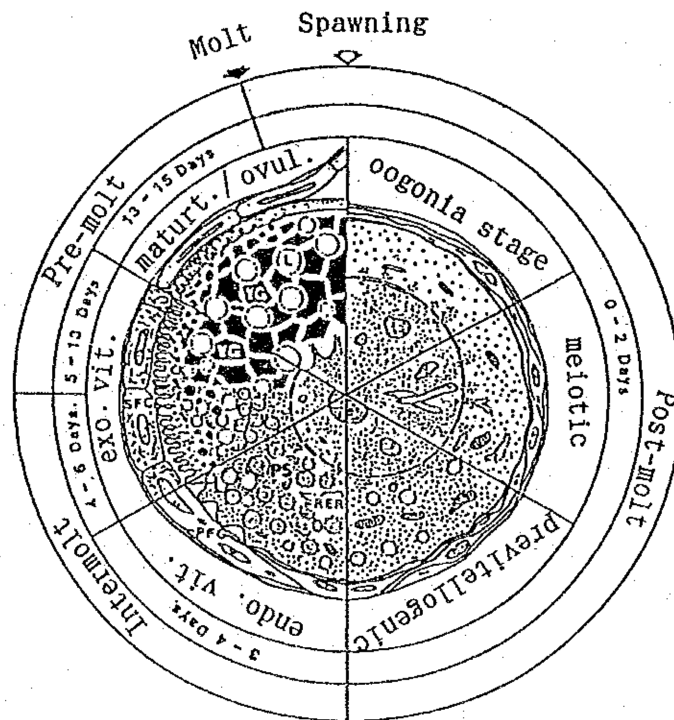


Fig. 2 Relationship between oocyte development and molt stage in *M. nipponense*. L: Lipid globules, YG: yolk globules, RER: rough endoplasmic reticulum, PFC: primary follicle cells, SFC: secondary follicle cells, PS: PAS positive granules, endo. vit: endogenous vitellogenesis, exo. vit: exogenous vitellogenesis, maturt: maturation, ovul: ovulation (han, 1988).

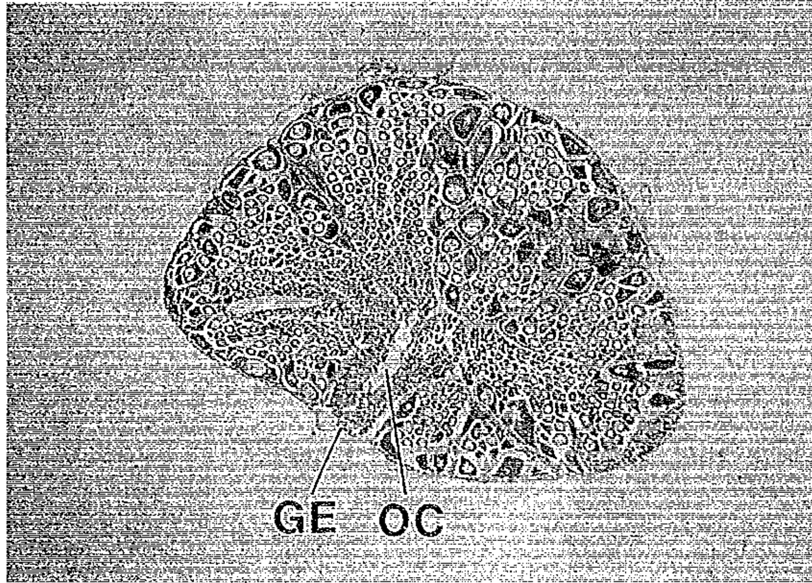


Fig. 3 Ovarian cavity (OC) and germinal epithelium (GE) in immature ovary of the kuruma prawn, *Penaeus japonicus*. × 350

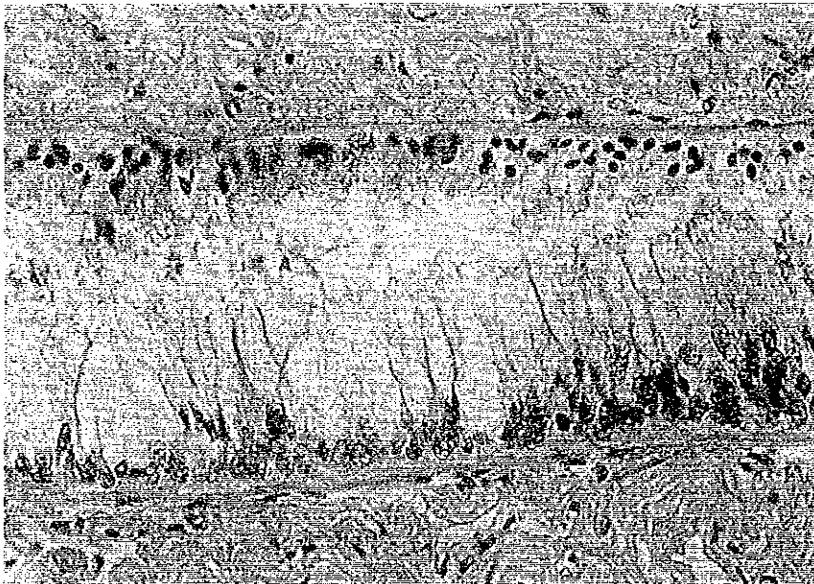


Fig. 4 Longitudinal section of the oviduct, showing inner villi. × 350 (*P. japonicus*)

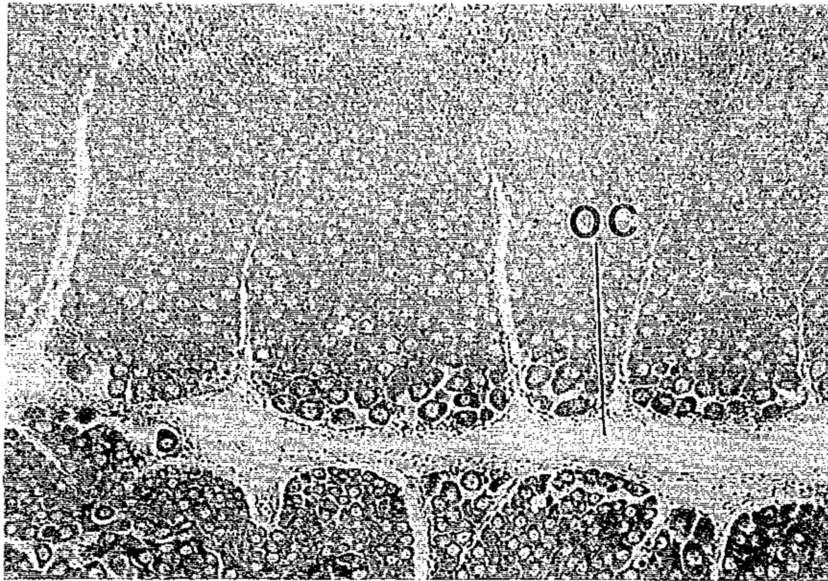


Fig. 5 Longitudinal section of immature ovary in *P. japonicus*. OC, ovarian cavity. × 90

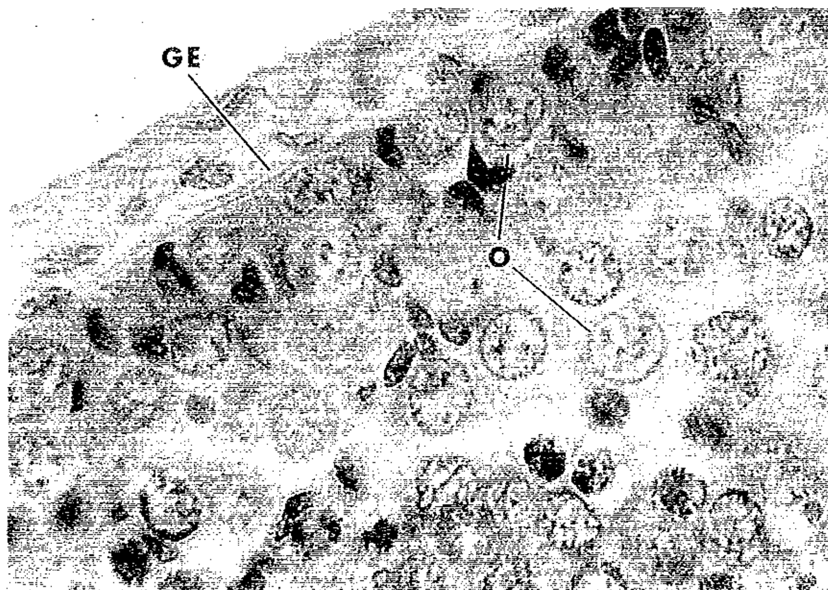


Fig. 6 Oogonia (O) in the germinal epithelium (GE). × 900 (*P. japonicus*)

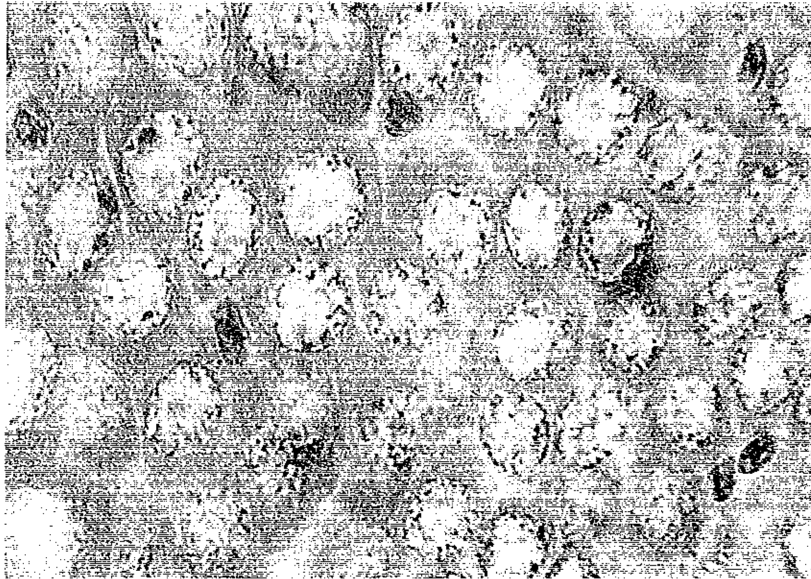


Fig. 7 Oocytes at the previtellogenic stage. × 900 (*P. japonicus*)

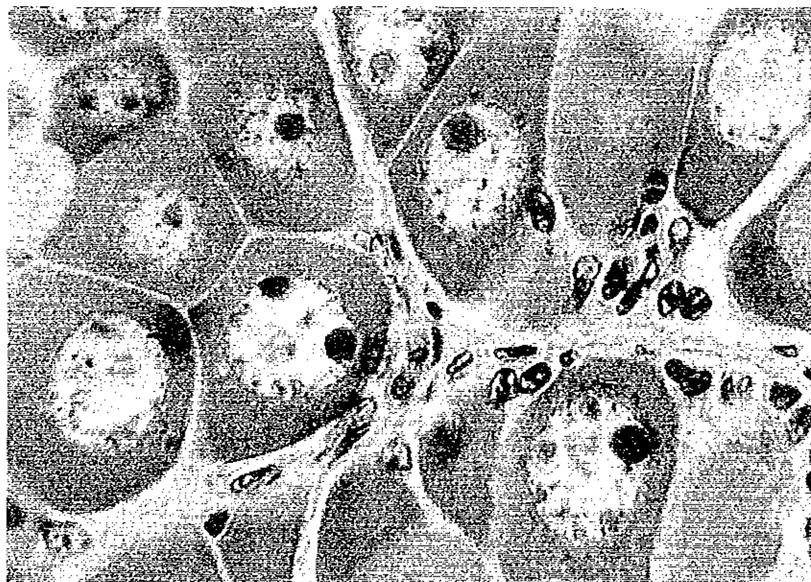


Fig. 8 Oocyte at the previellogenic stage. × 900 (*P. japonicus*)

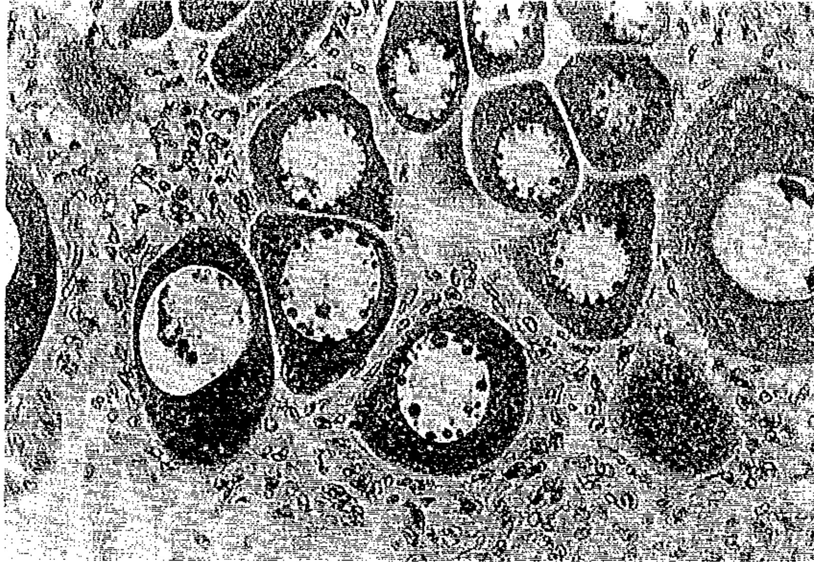


Fig. 9 Oocyte at the previtellogenic stage (perinucleolus stage). × 900 (*P. japonicus*)

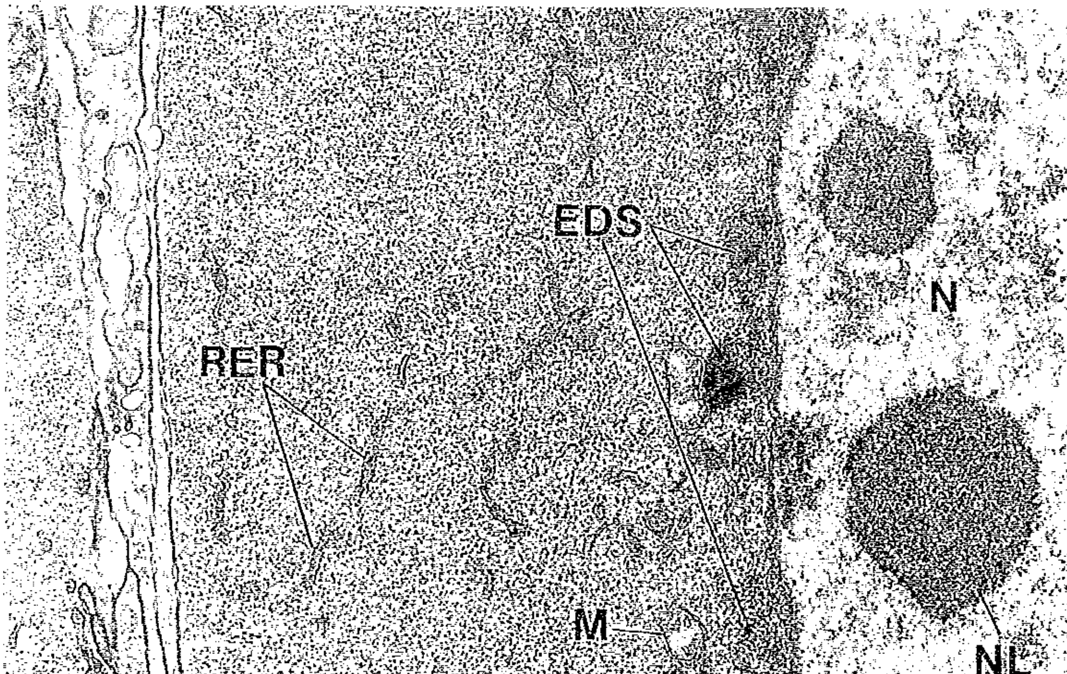


Fig. 10 Electron micrograph of an immature ovarian oocyte in the previtellogenic stage (perinucleolus stage), showing electron−dense substances. N, nucleus; NL, nucleolus; M, Mitochondria; RER, rough endoplasmic reticulum. × 15,000 (*P. japonicus*)

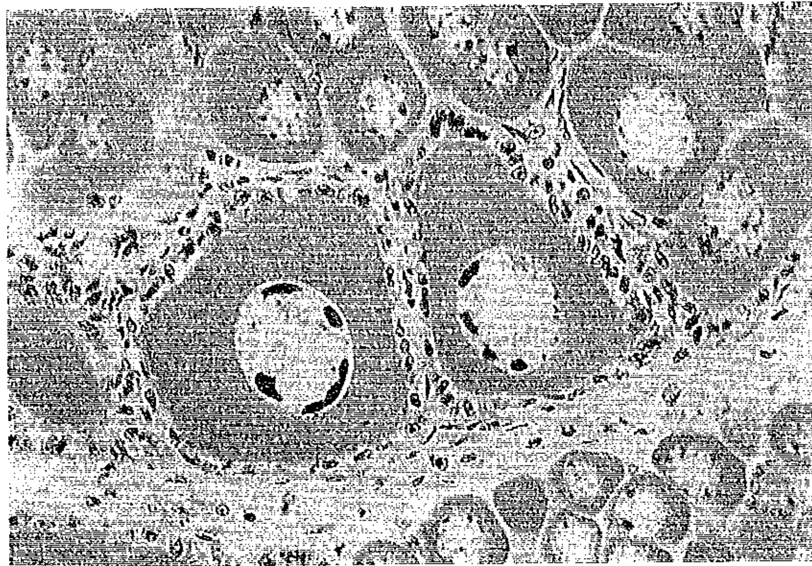


Fig. 11 Oocyte at the previtellogenic stage (fused nucleolus stage). × 350 (*P. japonicus*)

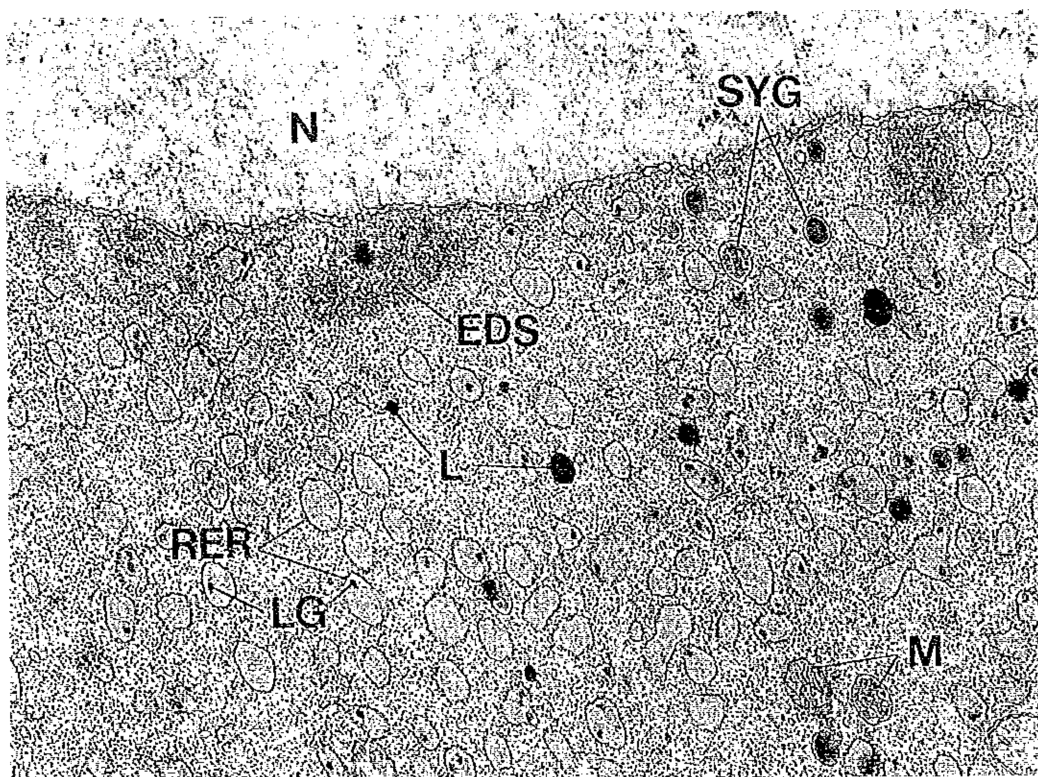


Fig. 12 Electron micrograph of an immature ovarian oocyte at the endogenous vitellogenesis stage. × 16,000 (*P. japonicus*)

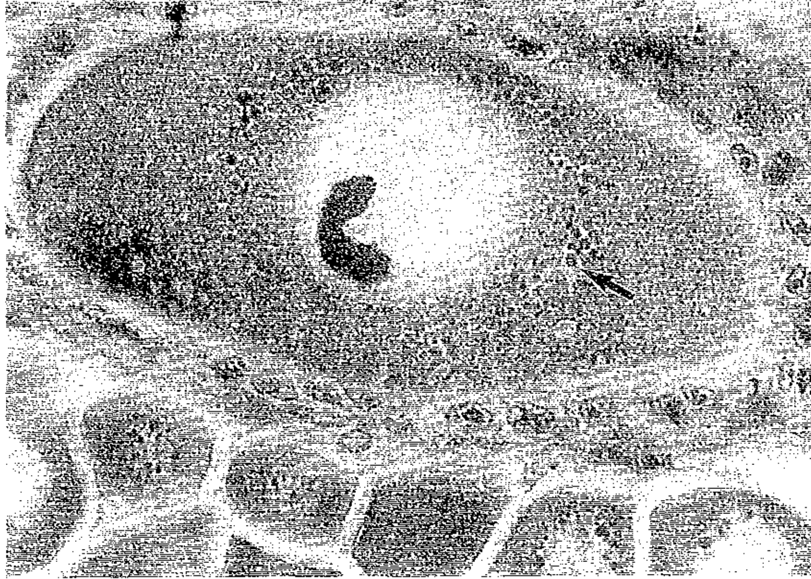


Fig. 13 Oocyte at the exogenous vitellogenesis stage (early yolk globule stage). × 900
Arrow, yolk globule. (*P. japonicus*)

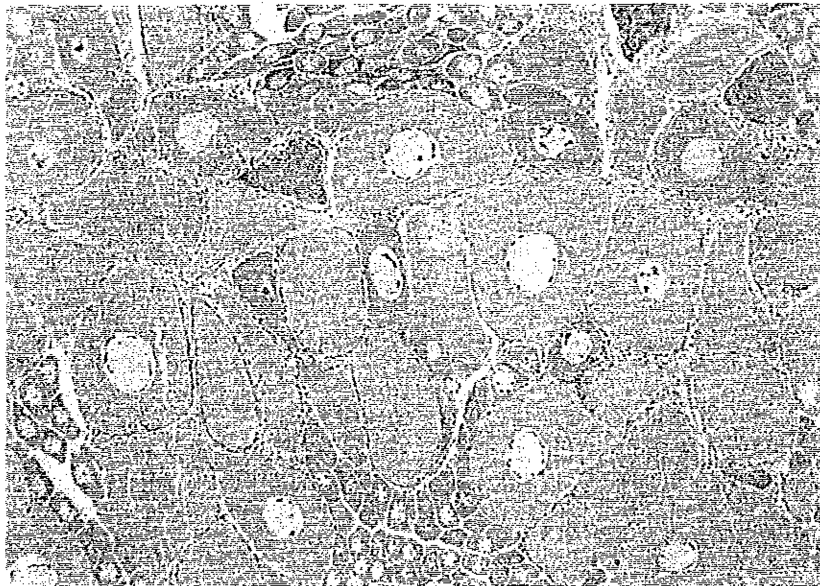
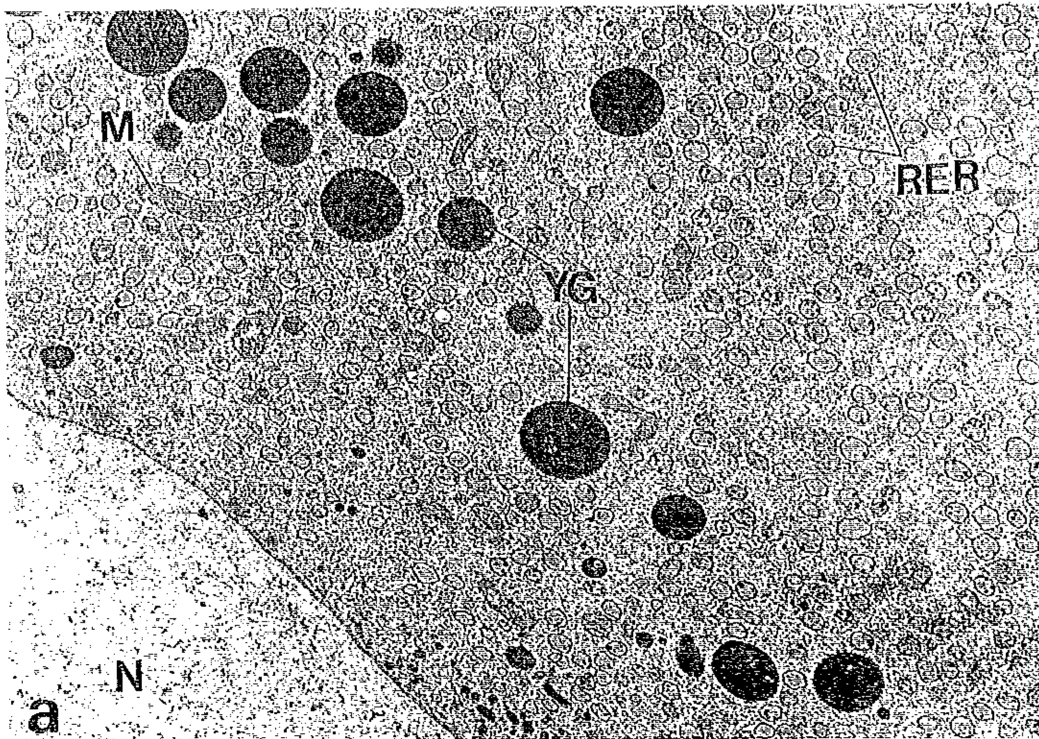


Fig. 14 Oocyte at the exogenous vitellogenesis stage (late yolk globule stage). × 900
(*P. japonicus*)



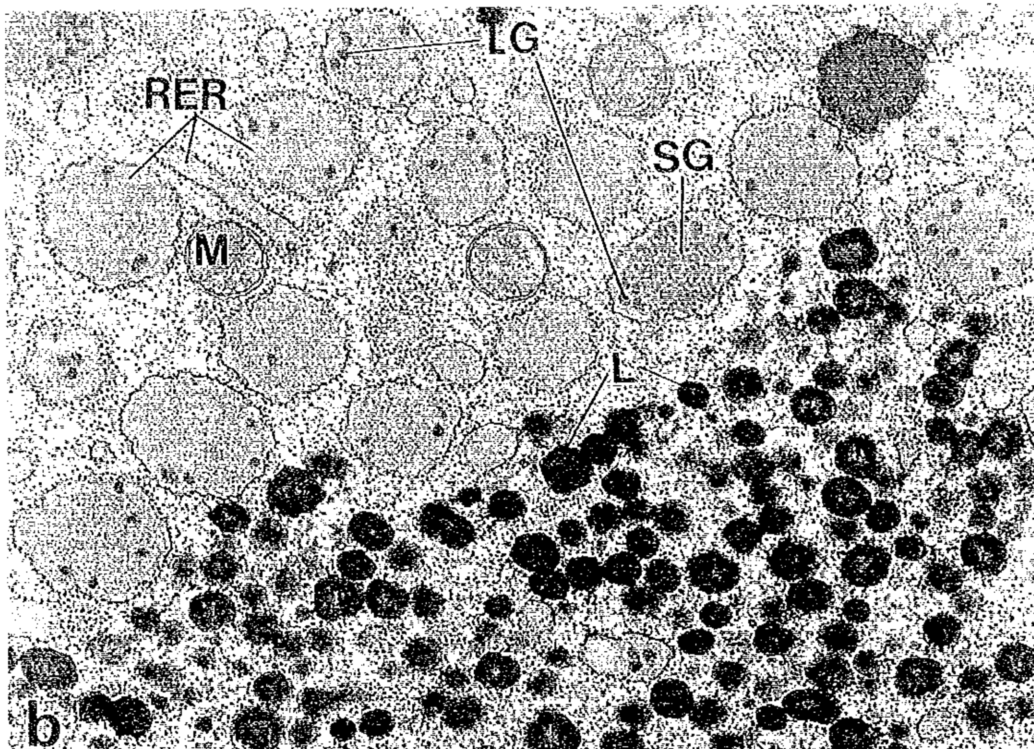
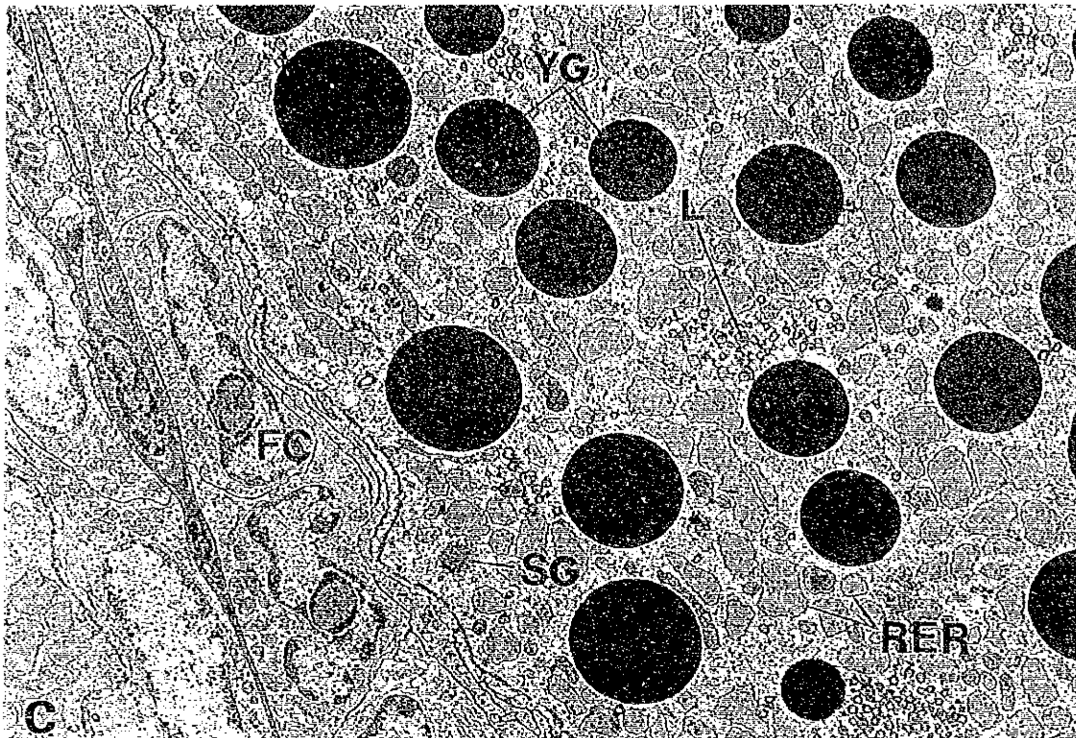
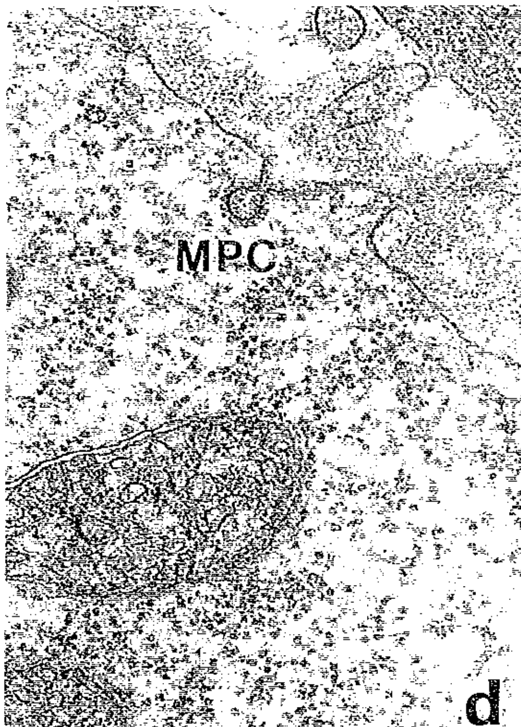


Fig. 15 Electron micrographs of mature ovarian oocyte at the exogenous vitellogenesis stage. a) photograph showing many RER in the cytoplasm and large yolk globules (YG) around the nucleus. × 7,000 b) Expanded RER containing two types of granules, large (LG) and small (SG) granules. × 12,000 (*P. japonicus*)





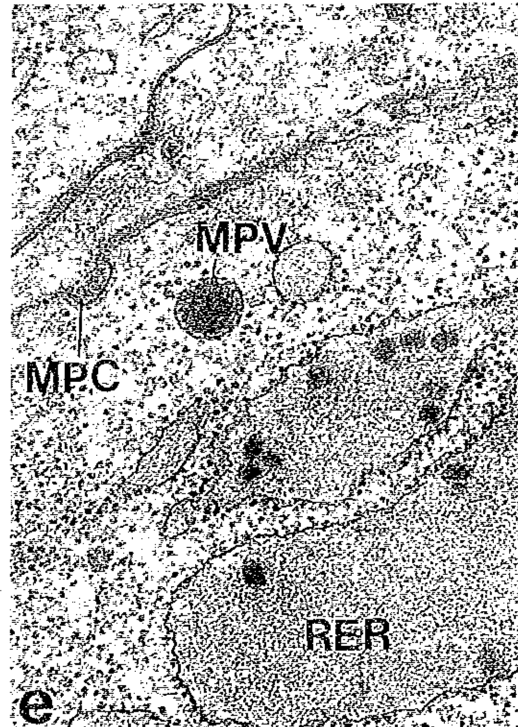
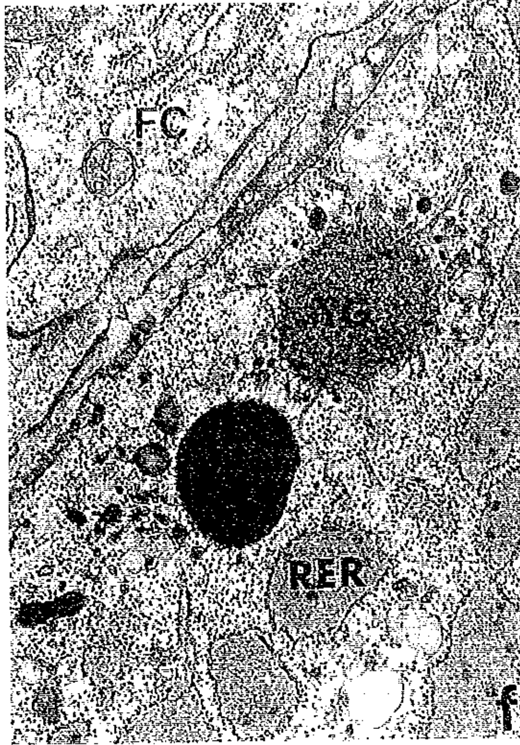
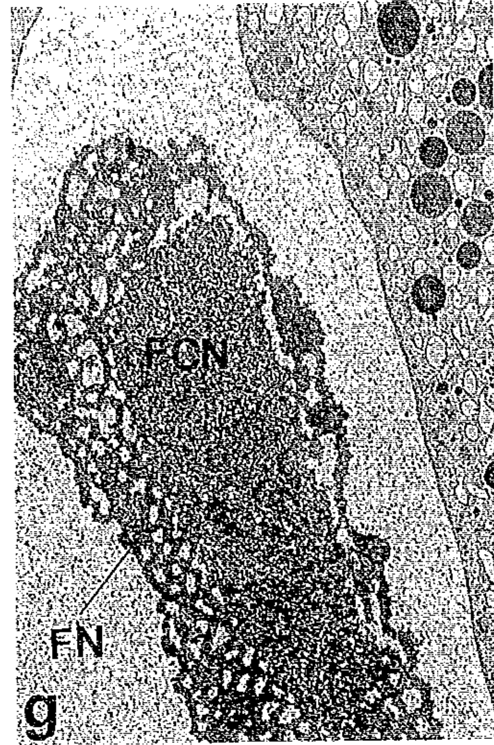


Fig. 15 c) Many large yolk globules and well developed RER containing large and small granules are present. × 5,000 d, e and f) Electron micrographs showing micropinocytosis (MPC) and micropinocytotic vesicle (MPV) on the cytoplasmic membrane. d) × 50,000; e) × 50,000; f) × 20,000. N, nucleus; M, Mitochondria; RER, rough surfaced endoplasmic reticulum; FC, follicular cell. g) Ultrastructure of nucleolus. Note the fibrillar center (FCN) and fibrillar nucleolonema (FN), × 6,000





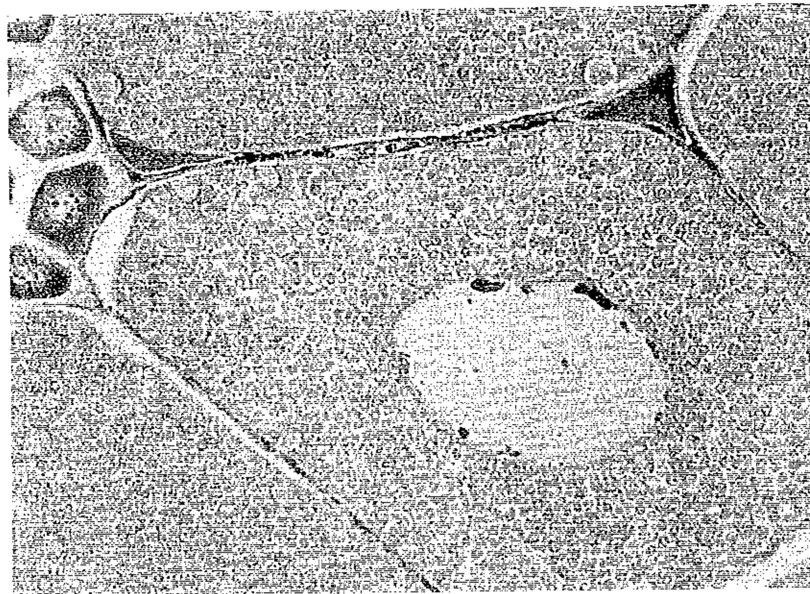


Fig. 16 Oocyte at the maturation stage (early cortical alveoli stage)showing small cortical alveoli at the periphery. × 450 (*P. japonicus*)

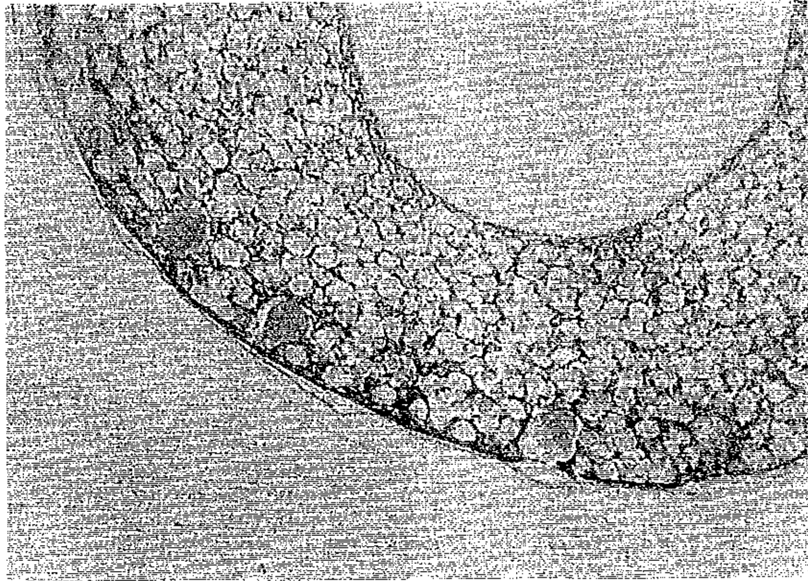


Fig. 17 Oocyte at the maturation stage (early cortical alveoli stage) stained with PAS. × 900 (*P. japonicus*)

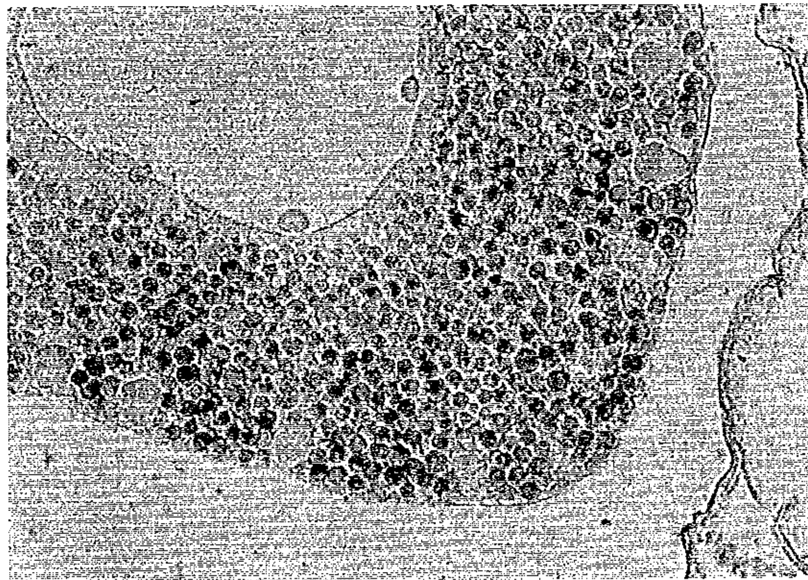


Fig. 18 Oocyte at the maturation stage (early cortical alveoli stage) stained with Sudan Black B (lipid staining). × 900 (*P. japonicus*)

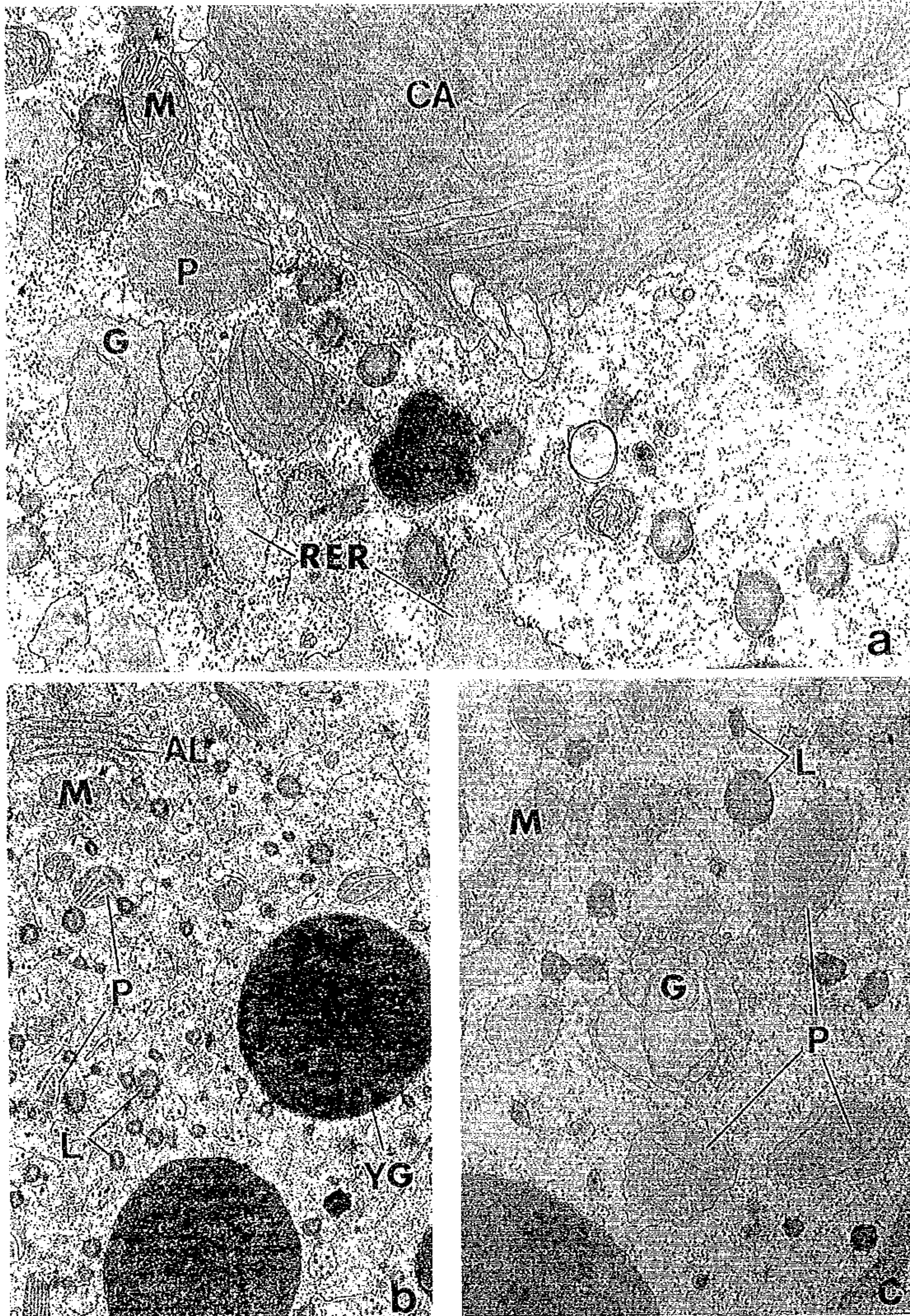


Fig. 19 Electron micrographs of mature oocyte at the maturation stage (early cortical alveloli stage). a) Note cortical alveoli (CA), its precursor (P), and Golgi apparatus (G). × 22,000 b) Precursors of CA. RER reduced in volume. × 12,000 c) Electron micrograph suggesting that CA is produced at Golgi apparatus using RER content. × 20,000 (*P. japonicus*)

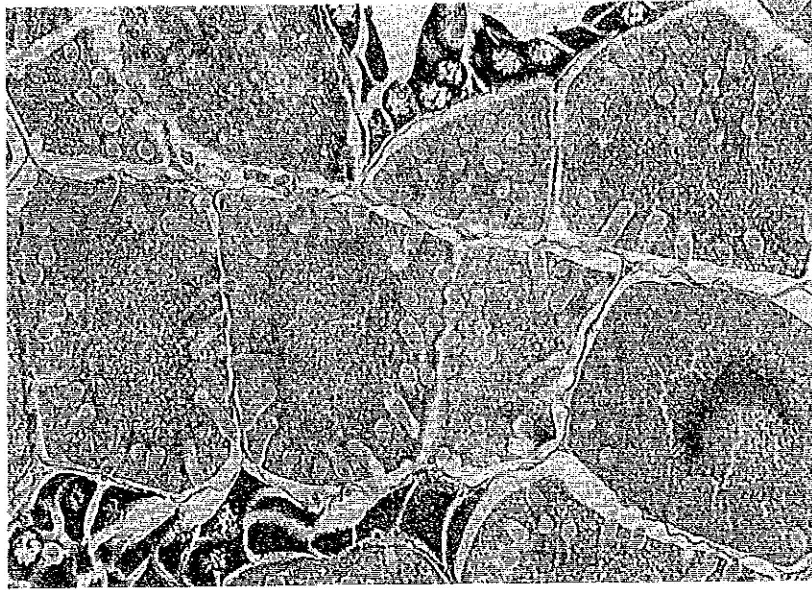


Fig. 20 Oocytes at the maturation stage (late cortical alveoli stage), showing well developed cortical alveoli. × 180 (*P. japonicus*)

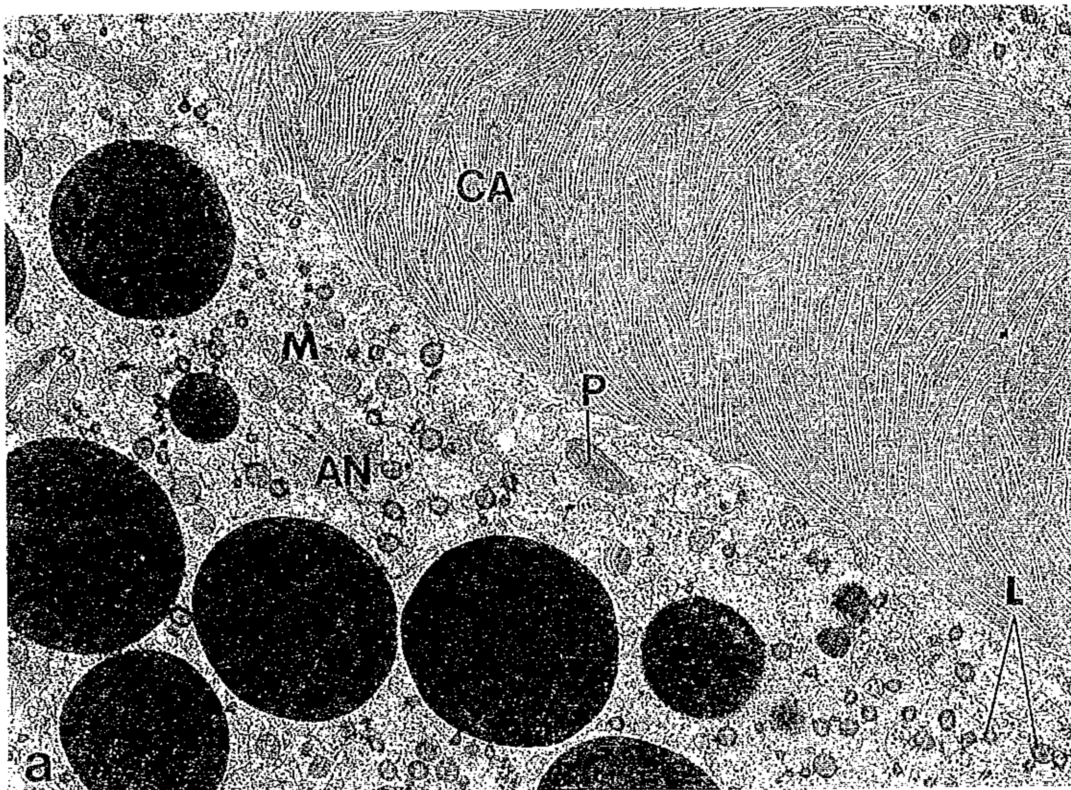


Fig. 21 Electron micrograph of oocyte at the maturation stage (late cortical alveoli stage). a) Well developed cortical alveoli. × 8,000 (*P. japonicus*)