CHAPTER 5

CYCLIC CHANGES IN THE HISTOCHEMICAL LOCALIZATION OF FAT IN THE GONADS

In most animals there are seasonal variations in the size of the gonads and in the relative quantity of its constituent elements. Certain histological and histochemical changes in the crustacean gonads with special reference to spermatogenesis and endocrine function have been extensively studied by several workers (Callan, 1940; Knowles and Callan, 1940; Takewaki and Nakamura, 1944; Charniaux, 1953a, b; Charniaux Cotton, 1954; Nath, 1956; Yasuzumi, 1960; Moses, 1961). Stephens (1952) studied the mechanisms regulating the female reproductive cycle in the cray fish, Cambarus. Parameswaran (1955) found that the ovarian maturation and the ovigerous state are related to the neurosecretory activity in the freshwater crab, Paratelphus hydrodromous. The effect of parasitism on the sex reversal in several crustaceans had been studied by various investigators (Giard, 1886; Reinhard, 1950; Tucker, 1930; Charniaux, 1952; Charniaux Cotton,1953). Smith (1913) studied the effect of parasitism on the storage of fat and glycogen and on the formation of pigments by its host. Veillet (1945) and Frentz and Veillet (1953) also attempted to study the effect of parasites on the fat metabolism in crab, Carcinus meanus. They observed a normal fat

metabolism in male crabs in which the parasite was still inside, even though the crabs exhibited advanced feminization. The seasonal variation in the fat content of the gonads in normal marine and freshwater decapod crustaceans was studied by George and Patel (1956). From their studies they concluded that the fat store is utilised for the development of eggs in females, but as a reserve material in both the sexes. George and Desai (1954), while studying the liver fat in Pila globossa, reported that the fat stored in that organ is utilised for the production of reproductive elements.

The present investigation was undertaken with a view to understand the cyclic changes in the fat content of gonads in the freshwater crab in relation to the formation and development of sperms and eggs.

MATERIALS AND METHODS

The common freshwater crabs, Paratelphusa jacquemontii, were collected from local rivers and were used in the present study. Histochemical observations on the seasonal changes in the fat content of gonads were carried out. Gonads of both sexes were dissected out and fixed in calcium-formol and Ciaccios' fixatives. Fixation in Ciaccios' fluid (formalin, 20 ml; 5% potassium dichromate solution 80 ml; glacial acetic acid 5 ml) with prolonged post chromation as recommended by Chou (1957) for vertebrate adipose tissue gave the most

satisfactory preservation of lipids. The tissues were washed, then embedded in gelatin and sectioned at 10 to 15 μ on a freezing microtome. Baker's improved Sudan Black technique (Baker, 1956) was employed for the demonstration of lipids. Neutral fat was demonstrated in the sections by staining with Nile blue sulphate (Cain, 1947) and Fettrot 7 B (Pearse, 1960). Sections treated with 2:1 Chloroform-Methanol mixture served as controls.

For the demonstration of phospholipids, the Acid-Haematin method (Pearse, 1960) was employed. Weak Bouin's fixed tissue after hot pyridine extraction was used as the control.

Sections were also subjected to Schultz's test for the detection of cholesterol-positive lipids.

OBSERVATIONS

These crabs aestivate in May/June, which are the dry months of the year, and start breeding with the onset of rain in July. After the breeding season gonads of both the sexes appeared to be in a regressed state; very few spermatozoa were found in the seminiferous tubules and the ovary contained only immature developing oocytes. During this period both the seminiferous tubules and the interstitial connective tissue of the testis contained very little fat which stained with Sudan Black B (Fig. 1). It showed no

affinity to Fettrot 7B. Afterwards when active feeding started, a gradual increase in the fat content in the seminiferous tubules as well as in the interstitium was noted. The spermatogenic cells and the few spermatozoa left in the tubules showed the presence of acidic fat stained blue with Nile blue sulphate and black with Sudan Black B, while the interstitial connective tissue contained a fairly high amount of neutral fat staining red with Fettrot 7B and pink with Nile blue sulphate. However in August, before the starting of the gonadal development and sperm formation a high level of interstitial neutral fat and tubular acidic fat was seen (Fig. 2). At the same time this fat in the interstitium and tubule showed a strong positive reaction to Schultz's cholesterol test. A gradual depletion of the interstitial cholesterol-positive lipid was noted with the initiation of spermatogenesis. However, towards the later stage of spermatogenesis a decrease in the interstitial neutral fat and a considerable increase in the tubular fat was observed. At this stage the spermatophores were seen to be accumulated in the centre of the seminiferous tubules and these spermatophores contained neutral fat. Gradually an increase in the neutral fat store was observed in the spermatophores and the central portion of the tubule appeared to be the site of neutral fat storage (Fig. 3). The germinal cells at the periphery as well as the tunic of the seminiferous

tubules showed lesser amounts of fat. The interstitium contained numerous individual neutral fat globules. Later a decrease in the interstitial fat globules together with a change in the position of the tubular fat from the centre to the periphery of the tubule was noted (Fig. 4). This shift in the localization of fat was as a result of the removal or transport of the mature spermatozoa from the tubules to the vas deferens. A gradual depletion of the fat in the tubules as well as in the interstitium was noted towards the aestivation period in summer (Fig. 5). The very little stored fat seen in the tubules and in the interstitium during that period was negative to the Schultz's test. When Baker's acid haematin test was carried out, during the early stages of development the spermatophores showed moderate activity(Fig.6). The spermatogenic cells and the interstitium were completely negative to the reaction. The dark blue colour was more intense in the fully mature sperms (Fig. 7). Pyridine extraction, however, did not prevent them from maintaining an equally intense bluish colour.

In the immature ovary very small amount of fat was seen which stained only with Sudan Black B. Small oocytes did not show any fat at all. As the development advanced small fat globules, started appearing at the periphery of the developing oocyte cytoplasm (Fig. 8). The ovarian

connective tissue stained moderately with Sudan Black B. In certain stages of the oocyte development, numerous fat granules around the nucleus and comparatively large fat globules at the periphery of the ova were noticed (Fig. 9). A gradual increase in the connective tissue acidic fat was also seen. Towards the ripening of the ova the fat globules increased considerably in number and size and finally the whole cytoplasm of the mature ova was filled with large fat globules (Fig. 10). The nucleus was completely negative. The fat globules in the cytoplasm stained intensely with Fettrot 7B thereby showing the presence of neutral fat. But in the early stages the oocytes were completely devoid of any neutral fat. In addition to the large neutral fat globules in the cytoplasm of the fully mature ova, small neutral fat globules were abundant in the ovarian stroma (Fig. 11). The vitelline membrane also showed the presence of numerous minute neutral fat granules. Towards summer, nearing the aestivation period numerous small oocytes were seen among the mature eggs (Fig. 12). These developing oocytes contained only acidic fat, whereas the mature ova showed the presence of both acidic and neutral lipids. A gradual decrease in the connective tissue neutral fat was noticed during that period.

During the initial stages of the ovarian development the connective tissue showed the presence of cholesterol-


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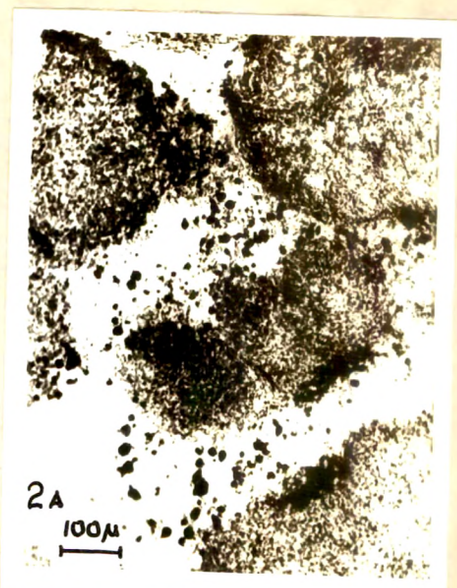
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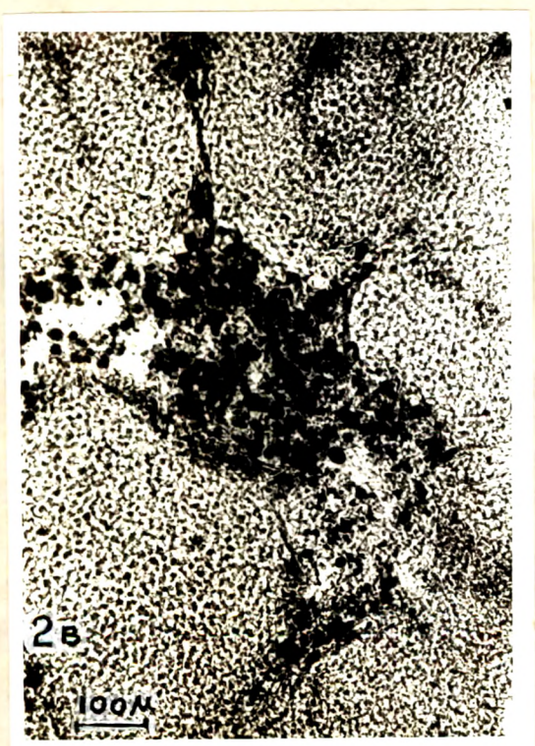
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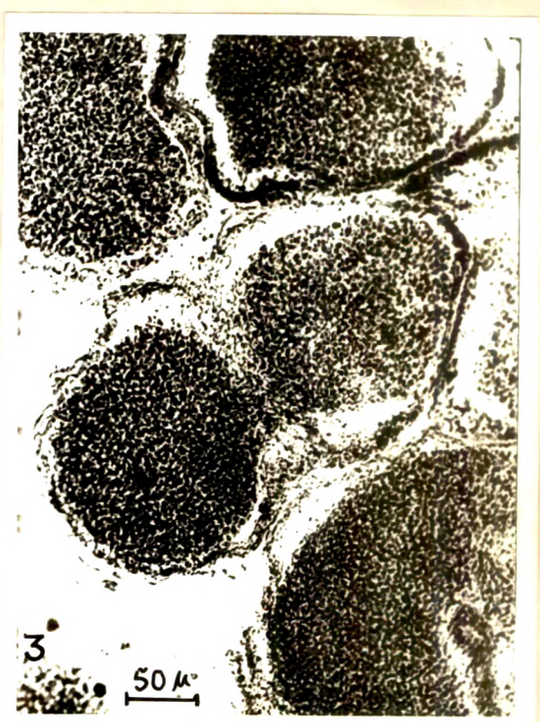
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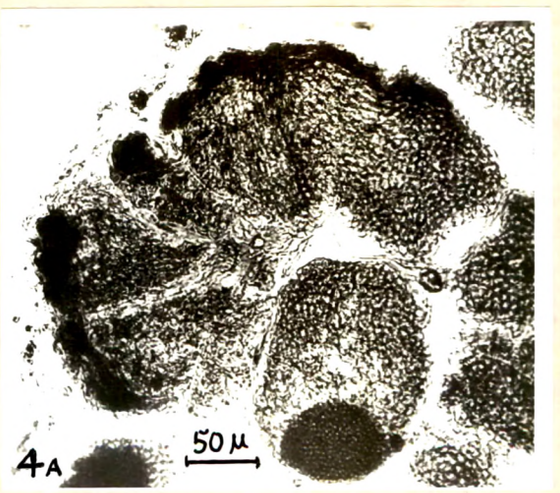
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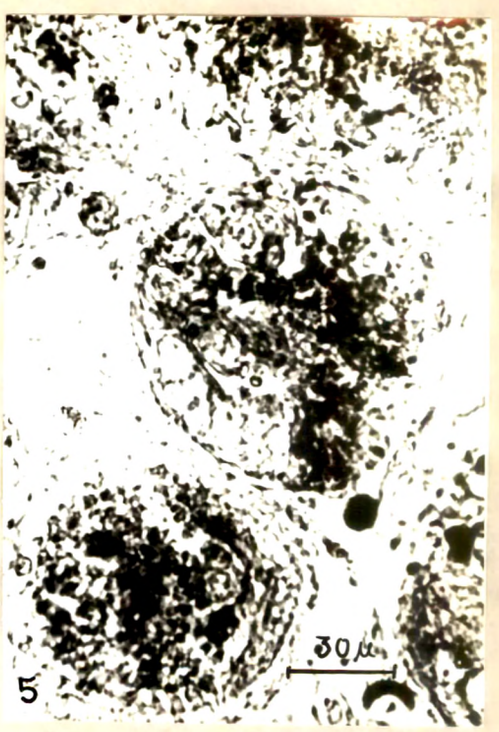
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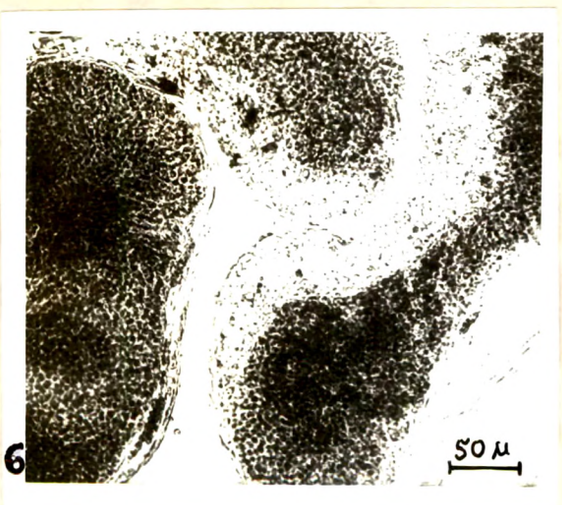
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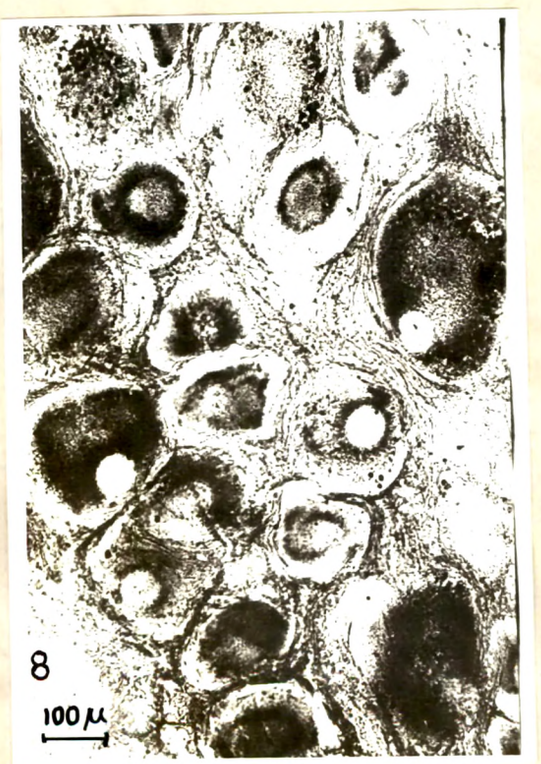
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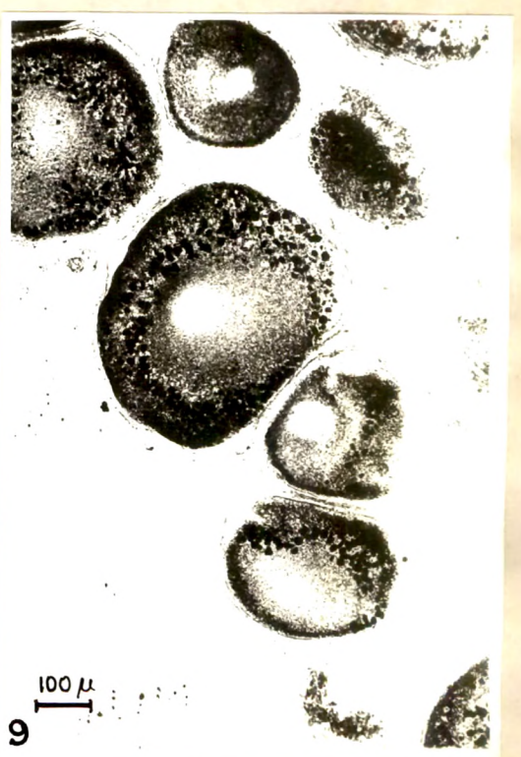
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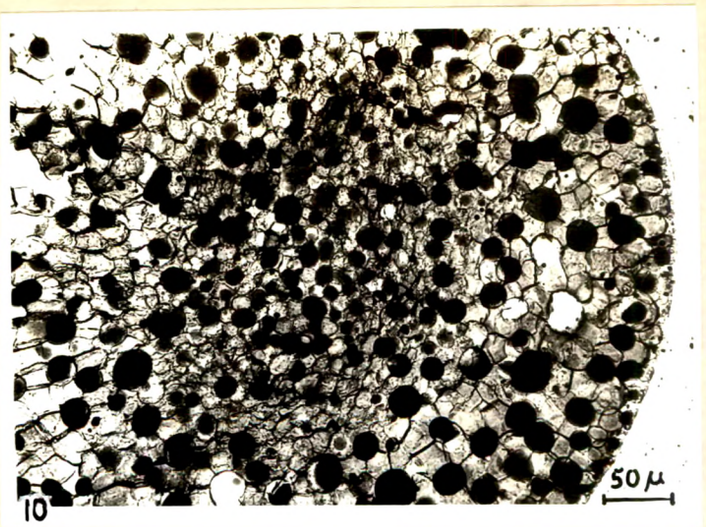

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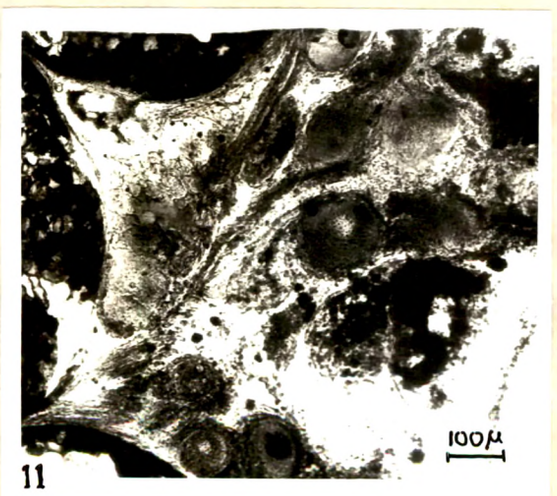

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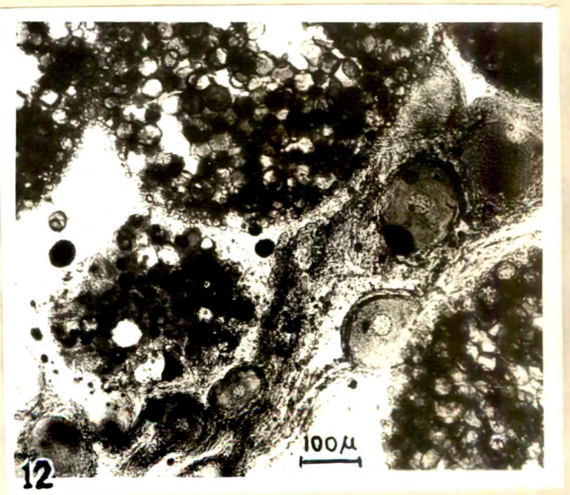

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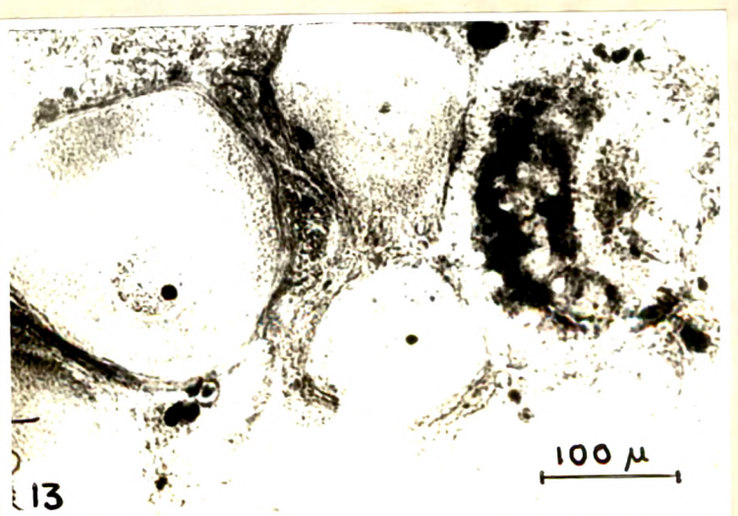

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EXPLANATION TO THE FIGURES

Fig. 1. T. S. of testis (July) stained with Sudan Black B showing very little amount of fat in the tubules and connective tissue.

Fig. 2A. T. S. of the testis (August) stained with Nile blue sulphate showing high amount of neutral fat in the interstitium and comparatively less acidic fat in the seminiferous tubules.

Fig. 2B. Higher magnification of T.S. of testis showing interstitial neutral fat and tubular acidic fat.

Fig. 3. T. S. of testis (late spermatogenesis) stained with Fettrot 7B showing the accumulation of neutral fat containing spermatozoa, in the centre of the seminiferous tubules.

Fig. 4A. T. S. of testis stained with Fettrot 7B showing the increased neutral fat at the periphery of the tubules.

Fig. 4B. Part of the Fig. 4A magnified.

Fig. 5. T. S. of testis (March/April) stained with Fettrot 7B showing less neutral fat in the tubules and interstitium.

Fig. 6. T.S. of testis treated with Baker's acid-haematin for phospholipids in the early stage of spermatogenesis. Note the absence of phospholipids in the interstitium and germinal epethelial cells of the tubules.

Fig. 7. T.S. of testis treated with acid haematin for phospholipids in the late spermatogenesis showing an increase of phospholipid content in the mature spermatozoa, interstitium and basement membrane of the germinal epithelial cells of the tubules.

Fig. 8. T.S. of immature ovary treated with Sudan Black B showing less amount of fat.

Fig. 9. T.S. of developing ovary treated with Fettrot 7B showing small neutral fat granules around the nucleus and comparatively large neutral fat globules at the periphery of the ova.

Fig.10. T.S. of part of a mature egg treated with Fettrot 7B showing numerous neutral fat globules in the egg cytoplasm.

Fig.11. T.S. of ovary stained with Nile blue sulphate showing neutral fat in the connective tissue (March/April).

Fig.12. T.S. of ovary stained with Nile blue sulphate showing the neutral fat containing mature eggs and acidic fat containing small developing oocytes.

Fig. 13. T.S. of ovary treated with acid-haematin for phospholipids. Note the presence of phospholipids in the nucleolus, degenerating cells and to a lesser extent in the connective tissue.

positive lipids. The degenerating cells present in the immature ovary showed strong positive Schultz's test. But the fully mature ova were non-reactive to Schultz's test.

In the immature ovary only the nucleolus was highly positive to the Baker's acid haematin test (Fig. 13). The degenerating cells and the connective tissue of the immature ovary also showed slight staining reaction. An intense dark blue colouration was obtained all over the fully mature ova. The connective tissue of the fully mature ovary was negative to the reaction. Pyridine extracted control sections showed a dark blue colour only at the periphery of the mature ova, the inner medullary portion and the nucleus were completely negative after the pyridine extraction.

DISCUSSION

In the present study a sequence of changes had been observed in the pattern of lipid localization and distribution in the gonads of P. jacquemontii. These changes in the various types of lipids could be correlated with the different stages of gonadal development and other metabolic activities of the animal. The complete absence of neutral fat in the testis and in the ovary in July, immediately after the aestivation indicates the utilization of the stored fat during the non-feeding time. It becomes obvious that almost all the

metabolic requirements of the animal during that period were met by the mobilization and utilization of the organic reserves in the gonads and in the hepatopancreas. Since fat is a convenient form in which energy could be stored, the fat store in these organs becomes the chief metabolic substrate during that period. That accounts for its progressive reduction in the gonads as well as in the hepatopancreas (Chapter 3). Active feeding followed by the increase in the fat content in both testis and ovary is indicative of the fact that in these tissues the reserve materials are stored mainly in the form of fat. But here again a difference in the type of fat present in the interstitium and in the tubule was noted. Accumulation of neutral fat in the interstitium and acidic fat in the tubule before and during the early stages of the spermatogenesis shows that fat is being utilized in the seminiferous tubules for the production of the reproductive elements. The storage of fat in certain animals for utilization in the production of eggs and sperms is well known(George and Desai, 1954). The presence of an increased fat splitting enzyme, lipase was also noted in the tubules during this period (Chapter 6). The interstitium showed no activity of the fat splitting enzyme, thereby indicating that fat utilization takes place only in the tubules.

The gradual depletion of the cholesterol positive lipids from the interstitial connective tissue with the initiation of spermatogenesis suggests the possibility of diverting these materials for the synthesis of certain male sex hormones, which might help in the process of spermiation. The fact that cholesterol is a precursor of testicular androgens in higher animals is well established (Hechter, 1958). Lofts (1962) and John and George (1966) also had shown the presence of cholesterol positive lipids in the interstitial cells and the gradual depletion of it towards spermatogenesis in certain migratory birds. Lofts (1964) had described the cyclical changes in lipid in the Leydig and Sertoli cells of Rana esculenta, and had correlated the appearance of the secretory lipid globules in the Leydig cells with the development of thumb pads. According to him the post-nuptial accumulation of lipid globules as well as cholesterol in the interstitial cells was due to the cessation of male sex hormone production and the appearance of cholesterol positive lipid droplets in the Sertoli cells was found to coincide with the process of spermiation. In the late post-nuptial period he observed that the lipid laden Sertoli cells were getting detached and passing down to the tubular lumen. In the present study, neutral fat containing spermatophores were seen to be accumulated in the tubular lumen during late spermatogenesis. The germinal cells at the periphery of

the tubule and the interstitium showed less fat during that period and that coincided with the increased lipase activity in those regions (Chapter 6). Before the spermatophores were transferred to the vas deferens an increase in the lipase activity as well as a decrease in the tubular fat was observed. From this it may be suggested that at least in the initial stages and in the later stages of spermatogenesis lipids are being utilized in the testis.

In the ovary numerous small immature oocytes were seen in the early stages, i.e. after the aestivation and the subsequent breeding period. These become mature through progressive increase in size, addition of yolk and maturation of nucleus. During the initial stages of the development only sudanophilic material was seen in the oocytes while the cytoplasm of the fully mature ova showed considerably high neutral fat in the form of large globules. This increase in the concentration of the lipid in the ovary side by side with the production and development of the ova may be considered as of general occurrence. An increase in the total as well as neutral lipids, fatty acids and phospholipids was noted towards the maturation of the ova. The disappearance of the neutral fat globules from the connective tissue along with the appearance of small immature oocytes among the fully mature ones can be easily accounted for. It seems that the

neutral fat store of the connective tissue might be transported to the sites of production of the young ova and the same fat store might be utilized for the development of the eggs. George and Patel (1956) had shown the fat store being utilized for the egg development in females.

The presence of cholesterol positive lipids in the ovary is of significance, since it is well known that cholesterol is the precursor of the steroid hormones. Bergman (1949) had stated that cholesterol is a typical sterol of crustaceans. In Orchestia gammarellus, the ovary produces a hormone during yolk deposition which influences the formation of ovigerous hairs (Charniaux-Cotton, 1960). Carlisle and Butler (1956) had suggested that the queen substance of the bees, in certain properties resembles a steroid and the ovarian inhibiting hormone of crustacean is similar to the former compound. In the present investigation, in the early stages of the ovarian development the stroma and the degenerating cells showed remarkably high cholesterol positive lipids. The presence of cholesterol positive lipids in the immature ovary is indicative of its importance as the precursor for the production of certain steroid hormones essential in promoting the growth, maturation and yolk deposition of the ova.

The acid haematin method for phospholipids gave a positive reaction in the spermatozoa. But the inability of hot pyridine to extract the stainable material suggests that they are not to be considered as free phospholipids. Pearse (1960) points out that certain bound lipines are resistent to extraction by hot pyridine. Sinclair (1940) suggested that the testicular phospholipids must have exclusively a non-metabolic function, but at present there is no clue as to what that function is.

In the fully yolk-laden ova only at the cortical region this type of bound phospholipid was seen, whereas the medullary portion showed free phospholipids which was completely extracted with hot pyridine. However, an increase in the phospholipid content, bound or free was noted towards the maturation of the gonads. The presence of phospholipids in the nucleolφus of the immature ova suggests two possibilities:

(1) Phospholipids might be synthesized in the nucleolφus and transported to the nucleus and cytoplasm.

(2) On the other hand it is not unlikely that phospholipids might be synthesized in the nucleus and passed on to the nucleolφus just as the RNA synthesized in the presence of DNA would probably migrate to the nucleolφus to become stored in the ribosomal proteins (Harris,1961). However, the presence of comparatively large amount of phospholipids in the fully mature ova indicates that an increased phospholipid synthesis occurs in the ova towards the maturation.