ASPECTS OF OVARIAN MATURATION IN THE MOLLY, MOLLIESIA LATIPINNA

By
Robert W. H. Tseng

INTRODUCTION

The molly, Molliesia latipinna, a common brackish water fish on Oahu, Hawaii, is ovoviviparous. This fish has approximately one-month cycles of gestation, parturition and oocyte growth during late spring and summer. The young attain sexual maturity in about four months. These facts make them an interesting species for studies on the maturation of the ovary.

Many papers and reviews regarding the relation between hormones or gonadotropins and ovarian development in fishes have been reported (Pickford and Atz, 1957; Ball, 1960; Ramaswami, 1962; Hoar, 1965, 1969). All experiments confirm that pituitary and chorionic gonadotropins promote ovulation, but few reports deal with the ovarian maturation of ovoviviparous fish. Ovarian maturation and gonadotropin production of the guppy, Poecilia reticulata, show similar cycles (Stolk, 1951a, b; Sokol, 1961). M. latipinna is closely related to P. reticulata and also has similar cycles (Ball and Baker, 1969).

Gonadal development is dependent upon the pituitary gland in all bony fishes. Ball (1962) hypophysectomized the female M. latipinna and Liley (1968) and Pendi (1969a, b) have done the same with the female and male P. latipinna, respectively and agree that the pituitary gland is required for gonadal maturation. After hypophysectomy, vitellogenesis is suppressed by atresia of the larger developing oocytes, and steroidogenesis does not occur in the gonadal tissues.

Few experimental studies exist on gonadotropic effects on ovarian development in fish. In vitro experiments, despite their useful application to endocrine studies, have similarly not been extensively utilized. These studies are important in clarifying the mechanism of ovarian maturation; a time mapping during the process of ovarian development, determination of the target organ, etc. The basic organ culture method for the ovary of M. latipinna is useful for further study of hormonal effects on ovarian maturation. The study of ovarian and pituitary cycles will lead to a better understanding of the relationship between the pituitary gland and ovary interactions.

MATERIALS AND METHODS

The experimental fish, M. latipinna, were netted in Kuliouou Bay on the southeastern coast of Oahu, Hawaii, and maintained in the sea water room (Edmondson Hall, University of Hawaii) in continuously running sea water.
For the ovarian cycle studies 10 to 40 young mollies from the same brood were kept in separate aquaria and on three to five day intervals, three-five female fish were sacrificed for histological preparation through maturation. The whole fish, if two weeks old or younger, was fixed with Helly's fixative; with fish older than two weeks just the ovary was fixed. By using the paraffin method, the sections were 5 to 10 μ in thickness and stained with Mayer's hematoxylin and eosin, but a few were stained with Heidenhain's hematoxylin and eosin. For detecting the presence of fatty material, the crystal technique was employed and the section stained with Sudan Black B.

Ovarian studies and pituitary gland studies were conducted on the same fish. The whole brain was removed from the ventral skull and fixed with Helly's fixative. The brain was trimmed before paraffin embedding for orientation of the pituitary gland during sectioning. Both Azan (Mallory Heidenhain's) and Cameron and Steele (Paraldehyde-fuchsin) stains were utilized, the latter method being the most successful.

The organ culture method employed in this study was the floating-lens paper-technique for liquid media (Chen, 1954). This method involved the use of aseptic technique. It must be mentioned that the detailed procedures developed by Oyama (1968) were used with little modification.

All glassware and other instruments employed in the actual cultivation of ovarian tissues were soaked in Alconox detergent solution, washed, and then boiled for 1.5 hour in about 5 liters of distilled water containing 7 gm sodium carbonate as a buffer. After boiling, the glassware and other instruments were rinsed four times in running tap water and six times in distilled water. These were then dried in a dry-heat oven.

Petri dishes and other small items were then prepared for sterilization. The small items were packaged and sealed, while the Petri dishes were prepared in the following manner: five sheets of 9 cm Whatman number 2 filter paper rings were layered in the dishes. In each dish a watch glass was placed in the cut out portion of the paper. The covers were replaced, and dishes and packaged items were sterilized for 20 minutes at 121°C in an autoclave set at 15 psi.

 Pipettes were soaked in distilled water after use and washed in a solution composed of 120 gm KOH, 120 ml distilled water, and 900 ml of 95% ethyl alcohol. After rinsing, first in running tap water for at least eight hours and then in distilled water for two hours, the pipettes were dried, the mouth-parts plugged with cotton, and placed in canisters and sterilized for four hours in a dry-heat oven set at 100°C.

Ovarian explants were performed in a "sterile" hood where air movement was minimal. Ten ml of double distilled sterile water was pipetted on the filter paper in the bottom of the dish. Care was taken not to wet the watch glass. One ml of chemically defined medium was put in the watch glass, and a piece of lens paper floated on the surface of the medium. The fish was rinsed with 70% ethyl alcohol and then killed; the ovary was removed and washed in separate medium before placement in the Petri dish. Finally, the ovary explants were placed on the floating lens paper, thereby forming a small depression (Fig. 1).
The lens paper was cut into pieces 1.5 cm sq. They were then placed into Petri dishes and washed three times each in ether and 95% ethyl alcohol for 30 minutes for each wash and five times in distilled water for 15 minutes per wash. The cleaned pieces of lens paper were treated for five minutes with a silicone solution consisting of one part Siliclad (Clay-Adams, Inc.) and 100 parts distilled water. After five minutes immersion, the pieces of lens paper were rinsed five times with distilled water, each rinse for 15 minutes. The pieces of lens paper were dried in the dry-heat oven for two hours in open Petri dishes.

Medium 199 (Baltimore Biological Laboratories) with Hank's base without serum, Eagle's MEM with Earle's base and 20% serum (Grand Island Biological Company) and combinations of above two media were used for the chemically defined medium. Fifteen mg Streptomycin, 3000 units Penicillin G potassium, and 0.07 M NaCl were added to 100 ml medium as a germicide.

 Cultures were incubated for seven days in a temperature controlled chamber equipped with a day-night light regulator set for 12 hours photoperiods. The temperature was maintained at about 25°C. On the third and fifth day during incubation period, the culture medium was removed with a sterile hypodermic syringe and needle and replaced with fresh medium. On the seventh day the ovary explants were removed from the culture medium and immediately fixed in Helly's formula.

OBSERVATIONS AND RESULTS

A. Ovary Development

The mature male mollies can be distinguished from mature female fish by possession of a specialized anal fin, the gonopodium (Fig. 2). The ovary of *M. latipinnia* a small unpaired body, occupies the posterior dorsal part of the peritoneal cavity and is enveloped by a thin pigmented peritoneal membrane. The follicle is composed of a single layer of cells supported by a thin theca. There is funnel-like invagination of the ovarian lining to each ovum. At the posterior end of the ovary is a short oviduct which leads to the genital opening. Each ovary usually has up to forty ova. In the early stages the small ova are white in color. After maturation, the ova contain large amounts of yellowish yolk, and the ovary becomes very large in comparison to the early stages.

In the natural environment and under suitable laboratory Conditions, females give birth to broods of from one to forty young at intervals of about thirty days during late spring and summer, and in somewhat longer intervals during other seasons. The fully developed oocytes are fertilized in the ovarian follicles by spermatozoa which are stored for long periods in the ovarian epithelium. The fertilized ova are not ovulated but are retained in the follicle which acts as a brood chamber for the developing embryos. The embryos develop synchronously, and when the fry are fully developed, the follicles rupture and the brood is expelled via the oviduct over a few hours. After a brood has been born, the partially developed oocytes (juvenile oocytes) with a maximal diameter of about 0.2 mm grow rapidly to maturity and reach a diameter of 2 mm in about five to eight days (Fig. 3). Fertilization occurs over a period of less than four days and begins about five to eight days after
the voiding of the last brood (Table 1).

### TABLE I.

THE OVARY CYCLE SHOWING THE TIMING AND OVERLAPPING OF DEVELOPING OOCYTES

<table>
<thead>
<tr>
<th>Day</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growing</td>
<td>----→ Fertilization ----→ Embryos ----→ Birth</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>Juvenile</td>
<td>Growing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oocytes</td>
<td>----------</td>
<td>oocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oocytes</td>
<td>---→ oocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**B. OVARY HISTOLOGY STUDIES**

Oocytes are generated from numerous nests of cells scattered within the ovary. These oogonial nests contain oocytes of very early stages (Fig. 4). As the ovary develops oocytes are arranged in such a way that the more mature ones are at the periphery of the ovary while young ones are in the middle (Fig. 5). The oocyte developmental stages were classified according to Yamamoto and Yamazaki (1961) and Yamamoto and Yoshioka (1964) with little modification. Using this classification, the following observations were made:

1. **Chromatin-nucleolus stage**

   Oocytes smaller than 20 μ are distributed sparsely around the oogonial nests and a very thin layer of cytoplasm surrounds a large nucleus. Within the nucleus there are many long, thin chromatin threads in a network appearance intermingled with one another. The cytoplasm is quite strongly basophilic (Fig. 6).

2. **Peri-nucleolus stage**

   Oocytes between 20 to 75 μ in diameter have a large nucleus with many nucleoli of peripheral arrangement. The cytoplasm increases relative to volume. Initially, the cytoplasm is stained deeply with hematoxylin; later, it decreases its affinity to hematoxylin and stains faintly. The only strongly basophilic portion are the nucleoli (Fig. 7).

3. **Yolk vesicle stage**

   Yolk vesicles are formed in oocytes of about 75 μ. These minute vesicles are deposited initially in the peripheral region of the cytoplasm and then accumulate and enlarge in size. Staining in Sudan Black B indicates the presence of fatty material in these vesicles. The cytoplasm decreases its affinity to hematoxylin and the follicle epithelium begins to thicken. During this lengthy stage the oocytes grow to 200 μ in diameter (Fig. 8).

4. **Yolk stage**

   Oocytes grow to between 200 to 500 μ. Yolk globules begin to form between the vesicles, and subsequently move to the inner part.
of the cytoplasm. These globules increase in number and size, accumulating rapidly in the inner cytoplasm. The follicle layer increases in thickness, and the granulosa cells grow larger in size (Fig. 9, 10).

5. Ripe egg stage

Oocytes are larger than 500 μm in diameter. The yolk mass grows further and occupies all the interior of the oocyte. The yolk is stained red with eosin.

6. Pregnant stage

If the eggs are fertilized in the ovary, they remain in follicles and develop into embryos. As the embryos grow, the yolk becomes less abundant until at maturity no yolk remains and parturition ensues. The spent follicles then collapse and hypertrophy occurs forming a thick theca (Fig. 11, 12).

C. PITUITARY GLAND STUDIES

1. General histology

The pituitary gland is located at the antero-ventral portion of the brain, it is oval in shape and 1 to 2 mm in diameter in mature female *M. latipinnata* (Fig. 13). It consists of two parts, the neurohypophysis and the adenohypophysis. The adenohypophysis is divided into the pars distalis and the pars intermedia. The pars distalis consists of a rostral and a proximal area in the anterior and middle, respectively. The neurohypophysis is interdigitated into the adenohypophysis (Fig. 14).

2. Gonadotropins

The presence of gonadotropin secreting cells was determined histologically by the Cameron-Steele method. The gonadotropin secreting cells of *M. latipinnata* are located in the ventral part of the proximal pars distalis. The appearance of the secreting cells varies in relation to the cyclic development of the ovary. In the immature fish the ventral part of proximal pars distalis contains no gonadotropin secreting cells as determined by the absence of positive staining. During mid-pregnancy, the cells are small in size (Fig. 15). Beginning at late pregnancy, the gonadotropin cells start to enlarge until fertilization takes place at the peak of the cycle (Fig. 16). This gonadotropin cells cycle is correlated with the ovarian cycle. When oocytes begin the vitellogenesis process at the yolk vesicle stage, the oocytes begin to enlarge and secrete fatty matters. The gonadotropin secreting cells also show the same type of beginning, following the oocyte maturation cycle. Gonadotropin development also shows the cyclic change (Table II).

### Table II

<table>
<thead>
<tr>
<th>Gonadotropin secreting cells</th>
<th>Non-differentiated</th>
<th>Medium</th>
<th>Large</th>
<th>Small</th>
<th>Medium to small</th>
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</thead>
<tbody>
<tr>
<td>Ovarian cycle</td>
<td>Chromatin— Yolk</td>
<td>Yolk</td>
<td>Mid—</td>
<td>Late—</td>
<td>Ripe-egg preg.</td>
</tr>
<tr>
<td>oocytes</td>
<td>Vesicle</td>
<td>Ripe</td>
<td>preg.</td>
<td>stages</td>
<td>stage stage</td>
</tr>
<tr>
<td>cycle</td>
<td>Peri-nucleolus</td>
<td>stages</td>
<td>stages</td>
<td>stage</td>
<td>stage stage</td>
</tr>
</tbody>
</table>
D. OVARY ORGAN CULTURE

1. Medium

Among three media, i.e., medium 199, Eagle's MEM and combination of the two media, the ovary explants culture in Medium 199 had the highest percentage of survival (Table III).

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of ovary explants cultured</th>
<th>No. of ovary explants survived</th>
<th>Survival percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 199</td>
<td>20</td>
<td>13</td>
<td>65%</td>
</tr>
<tr>
<td>MEM</td>
<td>10</td>
<td>2</td>
<td>20%</td>
</tr>
<tr>
<td>Combination</td>
<td>10</td>
<td>4</td>
<td>40%</td>
</tr>
</tbody>
</table>

All slides of in vitro ovary explants show that the connective tissue surrounding the oocytes proliferated quite well in comparison with those in vivo ovaries slides, but some of the ova may die after seven days culture (Fig. 17).

2. Oocytes culture stage

By using Medium 199 for cultivation of six oocytes development stages, the oocytes at yolk vesicle stage produced the best results. The oocytes at the stages of chromatin-nucleolus and peri-nucleolus stage were too small to be handled, while the stages after the yolk stage, the oocytes contained much yolk which caused difficult histological problems. Furthermore, the oocytes at that stage were almost mature. The pregnant embryo culture did not show anything concerned with ovary maturation though good results after seven days cultivation were obtained; the embryos were still alive and active.

DISCUSSION

The Molly, *M. latipinna*, is a livebearing fish. It is generally considered to be ovoviviparous as the yolk content of the oocytes is consumed during the parturition of the young. According the Scrimshaw (1945) they are not truly ovoviviparous. They not only develop within the ovary but also draw some nourishment from the ovary. He observed very little change in dry weight between fertilized oocytes and embryos at parturition, meaning that the mother has given some nourishment to the young for the metabolic costs of respiration in the developing tissues so that the total weight of the young at birth is almost the same when it is fertilized.

When a brood of embryos is in the ovary, the growth of oocytes for the next brood stops. As soon as the brood of young are given birth, the juvenile oocytes then begin to grow rapidly and mature as a new crop of ova within five to eight days of the birth of the previous brood.
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This suggests that the embryos may exert some inhibitory influence on oocyte growth. This inhibition also shows quantitative effects depending on the size of the brood. The more embryos developing within a brood, the smaller the size of the juvenile oocytes. This inhibitory effect may be induced by certain materials secreted by the gonadotropins.

Oyama (1968) used the chemically defined Medium 199 containing Hank's base without serum and prepared a physiological solution to make a crustacean culture medium. He obtained good results for the marine crab, *Thalamita crenata* ovary culture. Hirose (1971) used three kinds of stock culture media and found Medium 199 containing 0.8% NaCl and 20% calf serum the most suitable medium for Medaka, *Oryzias latipes*, egg culture. The present results support theirs. However, these results are different from Wolf and Quinby's (1969) who suggested that Eagle's MEM with Earle's base is a good medium for fish. They indicate that it has greater buffering capacity although Medium 199 is used more frequently according to the literature.

Ovary explants after seven days cultivation did not produce satisfactory results. The reason may be due to unsuitable pH since the Petri dish could not be sealed tightly to prevent the loss of CO₂ from the sodium bicarbonate on exposure to air. The medium became overly alkaline and thus harmful and even lethal to the explants. But this pH problem did not happen to the ovary culture of Oyama (1968) or Hirose (1971). Whether this is due to species' difference to pH change or the technique used is not clear. Also, it may be due to osmotic stress, nutrient deficiency, oxygen deficiency, or some other factors.

Of the three media used for cultivation, Medium 199 gave the best results, probably because it has the least amount of sodium bicarbonate, thus the pH will not change as much after exposure to air. Sodium bicarbonate functions as a buffer to control the pH and also the glycolytic activity of cells. It is an essential constituent of any medium although it causes the medium to become alkaline on exposure to air when using the watch-glass organ culture method.

The most suitable ovary culture stage of the molly for experiments on hormonal effects is at the time immediately following parturition of the brood. At this time the juvenile oocytes grow rapidly and reach maturity within five to eight days, so the five day cultivation period may be enough to test the effects of hormones on ovary maturation. The problem, however, is the difficulty in controlling the conditions in sea water room aquaria. The changing conditions tend to make the ovarian cycle longer and more irregular.

It is evident that the gonadotropins control the ovarian cycle in teleosts. They stimulate the growing oocytes only after a critical stage—the beginning of vitellogenesis (yolk vesicle stage). After hypophysectomy, small oocytes in the first phase (chromatin nucleolus and perinucleolus stages) continue to grow up to this stage and then undergo atrophic degeneration, while any oocytes past the critical stage and in the second growth phase (after yolk vesicle stage) cease to grow and also atrophy (Ball, 1960).

One very important question to be answered is whether teleosts
possess only one type of gonadotropin or two. Histological technique alone can not solve this question; also, no very definite physiological or biological evidence to prove the presence of one or two gonadotropins in teleosts has been obtained (Pickford and Atz, 1957; Ball, 1960). The mammalian purified fraction of gonadotropin hormones are usually injected into live teleosts or put into in vitro cultures to test the nature of teleost gonadotropin hormones. Of the two mammalian gonadotropins FSH has consistently given negative results, while LH usually elicits both gametogenesis and the steroidogenesis (Sundararam and Goswami, 1966; Ahsan, 1966; Hirose, 1971). Also two chorionic gonadotropins are commonly used for experiments. PMS prepared from the serum of pregnant mares has biological properties similar to a combination of FSH and LH, but more similar to FSH in its action; HCG, prepared from human pregnancy urine, acts more like LH. When injected into fish, HCG has often been found to be quite effective (Sundararam and Nayyar, 1967; Hirose, 1971). But whether the teleosts gonadotropins are chemically related to these mammalian gonadotropins still needs further investigation.

ACKNOWLEDGEMENT

I am gratefully indebted to my advisor, Dr. Fred I. Kamemoto for his patient and ingenious guidance and support to Dr. Pieter B. van Weer for the use of his laboratory facilities and histology technique advice. I would also like to thank Richard E. Tullis and Darrell R. Stokes, their friendship and experimental help is highly appreciated.

LITERATURE CITED


Figure 1. Top view of the culture chamber with all components in place. Watch glass not visible. 0.35X.

Figure 2. The male (top) and female molly, M. latipinnia. The male possesses a specialized anal fin, gonopodium, and the dorsal and caudal fins are considerably larger and more colorful than those of the female. 2X.

Figure 3. The pregnant female fish with almost mature embryos and juvenile oocytes. Note some of the white, small oocytes on the dorsal aspect of the abdominal chamber. 4.5X.

Figure 4. Part of an ovary section. Oogonial nests generate numerous, small early developing oocytes. Mayer's hematoxylin and eosin. 1500X.

Figure 5. The arrangement of oocytes in the ovary. The more mature oocytes are located at the periphery of ovary while young ones are in the middle. Mayer's hematoxylin and eosin. 937.5X.

Figure 6. Oocytes at the chromatin-nucleolus stage. A thin layer of cytoplasm surrounds a large nucleus. Many long, chromatin threads are intermingled with one another. Mayer's hematoxylin and eosin. 3750X.
Figure 7. Oocytes at the peri-nucleolus stage. A large nucleus with many strongly basophilic nucleoli of peripheral arrangement. Mayer's hematoxylin and eosin. 1500X.

Figure 8. Oocytes at the yolk vesicle stage. Minute lip droplets begin to deposit in the peripheral region of the cytoplasm. Mayer's hematoxylin and eosin. 1200X.

Figure 9. Oocytes at the yolk stage. Yolk globules deposit between yolk vesicles. The follicle layer increases in thickness. Mayer's hematoxylin and eosin. 480X.

Figure 10. Oocytes at the yolk stage. The follicle layer increases in thickness and the granulosa cells enlarge. Mayer's hematoxylin and eosin. 1500X.

Figure 11. Longitudinal section through abdomen of a pregnant embryo. Note the decrease in yolk volume. Mayer's hematoxylin and eosin. 192X.

Figure 12. The spent oocyte collapses. Hypertrophy of follicle layer. Mayer's hematoxylin and eosin. 937.5X.
Figure 13. Ventral view of the brain. Anterior to the right. Pituitary gland is a small oval structure located at the anterior central portion. 1X.

Figure 14. The mid-sagittal section of the pituitary gland. Anterior to the right. The neurohypophysis (purple stain) is interdigitated into the adenohypophysis. The gonadotropin cells are located at the mid-ventral side. Cameron and Steele stain. 600X.

Figure 15. Proximal pars distalis portion of the pituitary gland at midpregnant stage. Ventral to the right. The gonadotropin cells are located ventrally. Notice they are small and almost unstained during this inactive stage. Cameron and Steele stain. 3750X.

Figure 16. Proximal pars distalis portion of the pituitary gland at the late-pregnant stage. Ventral to the right. The gonadotropin secreting cells are on the ventral portion. Notice they are larger and heavily stained by acidlyc-e-fuchsin showing the initiation of secretion cycle. Cameron and Steele stain. 3750X.

Figure 17. Ovarian explant cultivated in Medium 199. The connective tissue Surrounding the oocyte proliferates well. Mayer's hematoxylin and eosin. 480X.
中文摘要
花鱸魚（Molly）卵巢發育的研究
曾文雄

1. Molly，Molliesia latipinn 價花鱸魚科，為深海之卵胎生小魚，成熟的雌魚約每月生產一次。
2. 雌魚體內受精後，卵子即開始於卵巢上，直至胚胎發育成幼魚，然後排出體外，每胎約產1-40尾幼魚；此時卵巢另有一組初期卵母細胞開始於卵巢上。
3. 幼魚一經排出體外，卵母細胞迅速發育成熟，然後受精，胚胎成長。同時另有新的一組卵母細胞繼
續產生。此種生殖現象，不斷循環。
4. 依組織學的研究，卵巢內卵之發育可分六期：

   (1) 染色體粘核仁期。
   (2) 固原核仁期。
   (3) 染色體期。
   (4) 精囊期。
   (5) 養子期。
   (6) 沐子期。

5. 比較腦下腺及卵巢的腺體切除，顯示二者均有相同的組織變化。
6. 腦下腺的生長促進細胞內激素控制卵之生殖週期。
7. 卵巢的器官培養可獲成功，其方法詳述於本文。