HOKKAIDO UNIVERSITY

Title	ENDOCRINOLOGICAL STUDIES ON THE REPRODUCTION OF THE FEMALE GOLDFISH, CARASSIUS AURATUS L., WITH SPECIAL REFERENCE TO THE FUNCTION OF THE PITUITARY GLAND
Author(s)	YAMAZAKI, FUMIO
Citation	MEMOIRS OF THE FACULTY OF FISHERIES HOKKAIDO UNIVERSITY, 13(1), 1-64
Issue Date	1965-05
Doc URL	https://hdl.handle.net/2115/21842
Type	departmental bulletin paper
File Information	13(1)_P1-64.pdf

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ENDOCRINOLOGICAL STUDIES ON THE REPRODUCTION OF THE FEMALE GOLDFISH, *CARASSIUS AURATUS* L., WITH SPECIAL REFERENCE TO THE FUNCTION OF THE PITUITARY GLAND

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INTRODUCTION

As in other vertebrates, the pituitary gland of the fish exerts decisive control over reproduction by secreting trophic hormones and mediates between external environment and reproductive organs. Therefore it is thought to be the most important subject for the investigation of the reproduction of fish to analyze the functions of the pituitary gland connected with various reproductive processes.

Studies on the morphology of the gonads of fish were begun more than a century and a half ago by Cavolini (1792). Since that time, numerous investigators have engaged in the study of teleostean gonads and a great deal of information on their morphological changes was obtained. Especially the formation of eggs in teleosts has been examined morphologically and cytochemically in detail by numerous embryologists and cytologists.

However, little is known of endocrinological mechanisms concerning the egg formation and other events associated with reproduction in teleosts. The existence of gonadotrophic hormones in fish pituitary glands was first indicated by Houssay (1931) who observed that the injection of pituitary extracts of *Micropogon opercularis* and *Luciopimelodus pati* into *Cnesterodon decemmaculatus* induced ovulation half a month before the normal breeding season. Cardoso (1934), Percira and Cardoso (1934), Hasler, Meyer and Field (1939), Nishino (1948, 1949), Suzuki (1957), Kobayasi and Yamabayashi (1957) and Chaudhuri (1960) also observed that the injection of saline suspensions or extracts of fish pituitary glands into the same or other kinds of fish resulted in the enlargement of the gonads or acceleration of ovulation. The same results were obtained by many workers using HCG or other mammalian gonadotrophins (Damas 1933, Ichikawa & Kawakami 1948, Kawajiri, Shimadate, Koyama & Miyajima 1948, Ramaswami & Sundararaj 1957,

Ramaswami & Lakshman 1958, Sneed & Clemens 1959, Hibiya & Kasahara 1962, Suzuki & Mitsuya 1964). On the other hand, negative results were obtained by Koch and Scheuring (1936), Johnson and Riddle (1939), Matthews (1939), Combs and Burrows (1959) and others using mammalian pituitary glands or gonadotrophins.

These investigations were needed by fish culturists who were anxious for the methods of artificial control of spawning. In these investigations, however, it appears that technical experimentation has far exceeded academic studies as Sneed and Clemens (1960) have pointed out.

Relatively few experiments aimed at pure biological or endocrinological information, hitherto, have been conducted. Vivien (1939, 1941) found that after the removal of the pituitary gland from *Gobius*, marked regressive changes occurred in the ovaries and large oocytes underwent atresia while small ones remained intact. The same results were obtained by Yamazaki (1961) in *Carassius auratus* and by Barr (1963) in *Pleuronectes platessa*. Matthews (1940), Rasquin (1951) and Stoll (1957) succeeded in inducing yolk formation in the oocytes of immature fish by means of injection or implantation of pituitary glands. The presence of both FSH and LH was demonstrated in the pituitary glands of *Lepisosteus* and salmon by Witschi (1955). Maturation and ovulation in loach were studied by Kawamura and Motonaga (1950) after the treatment with the acetone-dried pituitary glands of frogs, and Nadamitsu (1961) observed also *in vitro* ovulation in the loach using frog pituitaries.

An excellent review on the relation of the pituitary gland to reproduction of fish was given by Pickford and Atz (1957), and Ball (1960) stated suggestive opinions about the reproduction of female fish.

At present, however, the information on the mechanisms of various events of reproduction in fish is still very fragmentary and inconclusive. Therefore, further experimental investigations are indispensable. Especially, in order to elucidate the control of the pituitary gland over reproduction, it is most important, at first, to examine both effects of hypophysectomy on the various events associated with reproduction and of the preparations injected into hypophysectomized fish. This is the reason why the present writer undertook a study on the reproduction of fish from the endocrinological viewpoint centering around the function of the pituitary gland.

The goldfish was employed as an experimental animal because the material has a very convenient size, and can be easily obtained and handled.

Before going further, the author wishes to express his hearty thanks to Professor Kiichiro Yamamoto, Faculty of Fisheries, Hokkaido University for his kind guidance during the course of the present study with important advice, helpful encouragement and valuable criticisms and for his kind reading of the present

manuscript. The author is also very grateful to Professors Saburo Saito and Shinjiro Kobayashi, Faculty of Fisheries, Hokkaido University, who have been interested in the present study and kindly offered much advice. A debt of gratitude is also owed to Assistant Professor Tatsuro Kubo and Mr. Kazunori Takano for their kind help and advice in the course of the present study. Thanks are also offered to Messrs. Kaoru Shirai, Hiroshi Yoshioka, Hiroshi Onozato and Isao Oota, for their friendly help in various ways in the course of the present study. For the collection of pituitary glands of dog salmon, the author is greatly indebted to Mr. Kazuhiko Nishino and Dr. Tetsuo Kobayashi of the Hokkaido Salmon Hatchery. The present study was supported, in part, by a Grant in Aid for Fundamental Scientific Research from the Ministry of Education, donated to Professor Kiichiro Yamamoto.

MATERIAL AND METHODS

The goldfish used in the present studies were restricted to a variety known as "Wakin". The fish, 4–15.5 cm in length from the tip of the snout to the base of the tail, were stocked either in a large outdoor aquarium (about 5 m×3.5 m× 0.9 m in depth) or in an aquarium (1.3 m×3.5 m×0.3 m) set in the greenhouse of the Fisheries School of Hokkaido University, which was supplied with underground water of about 13°C. They were cultured with a boiled mixture of dry shrimp and wheat meal until needed.

Hypophysectomy was carried out by the method of opercular approach recommended by Abramowitz (1937) and Pickford and Atz (1957) in *Fundulus*. This method gave also good results in goldfish. No fish died, thanks to the direct effects of the operation. The fish operated on fed well and sometimes appeared to compete with one another for their food. They were fed with earthworms or bloodworms as well as the food mentioned above.

All experiments except those on oviposition, which were done in an outdoor aquarium, were carried out in an indoor aquarium. In order to examine the seasonal changes of ovaries, sampling of the fish was performed monthly from the stock kept in the outdoor aquarium. At fixation, records were made of body length, body weight and ovary weight in all fish used in the present study. For histological observations, ovaries were fixed with Bouin's solution. They were cut at 7–10 micra in thickness by the usual paraffin method and stained with Delafield's haematoxylin-eosin, Heidenhain's haematoxylin-light green and Heidenhain's azan modification of Mallory's triple stain. Hypophysectomy was checked by either dissection of pituitary regions or sectioning of that region.

The detailed methods employed in each experiment will be described fully in each section. The stage of ovarian eggs is represented according to Yamamoto and Yamazaki's (1961).

RESULTS

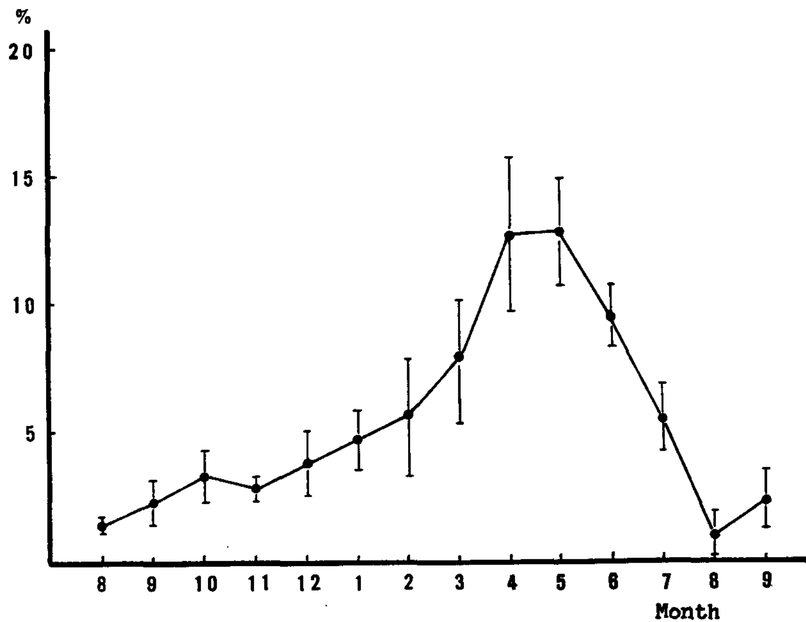
Part I. Cyclic changes in the ovary

In most of higher vertebrates, the estrus is repeated cyclically. This cycle is composed of follicular growth, ovulation and formation of corpus luteum. The hormonal control of these phenomena is well understood by the presence of two kinds of gonadotrophins in the pituitary gland; follicle stimulating hormone (FSH) which causes the follicular growth and lutenizing hormone (LH) or interstitial cell stimulating hormone (ICSH) by which the secretion of ovarian hormones and formation of corpus luteum are induced. Ovulation is caused mainly by LH.

To study the reproduction of goldfish endocrinologically, the ovarian change must be examined at first.

1. Changes in ovary weight

The spawning period of goldfish covers about one and a half months from May to early July. The rest of the year is spent in building up a new stock of



Text-figure 1. Seasonal change in ovary weight, represented by the gonad index

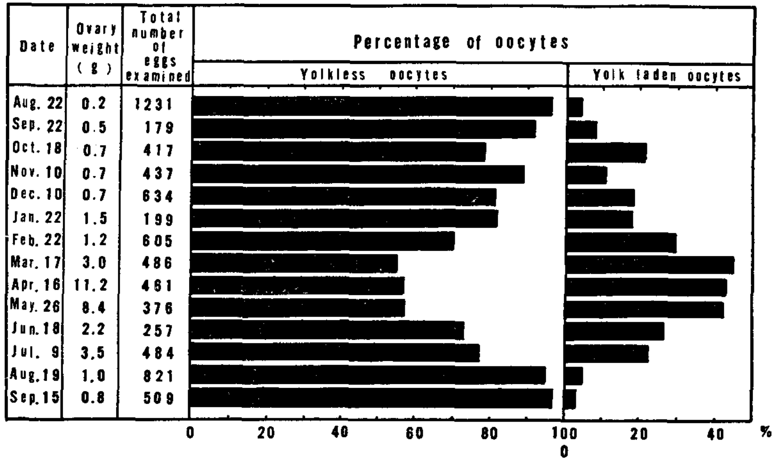
eggs. Therefore, the ovary changes in weight cyclically with one year interval. This change in ovary weight is shown in Text-figure 1 in which ovary weight is expressed as the percentage of body weight. The number of fish examined was 120 in total. The vertical line in the figure represents 95 per cent confidence limits.

In August the mean percentage of ovary weight per body weight was the smallest value showing only 1.4 per cent. From this point on, ovary weight showed a gradual increase from month to month throughout autumn and winter. In March and April, ovaries grew quickly and were filled with large eggs with much yolk. The mean value in April amounted to 12.7% which was about 3 times those in winter. In May the value became largest, being 12.8 per cent. Eggs within these ovaries were seen from outside as large, yellow, spherical spots. Henceforth, the ovary gradually decreased in weight as a result of the spawning. The slow decreasing in ovary weight along with spawning may be attributed to the asynchronous development of oocytes which causes the fish to spawn several times in a season. In late July and August, ovaries were characterized by the degeneration of yolk-laden eggs and they were reduced in size. Ovary weight fell off to 1.0 per cent by the end of August. The above mentioned cyclic change was repeated again during the next season.

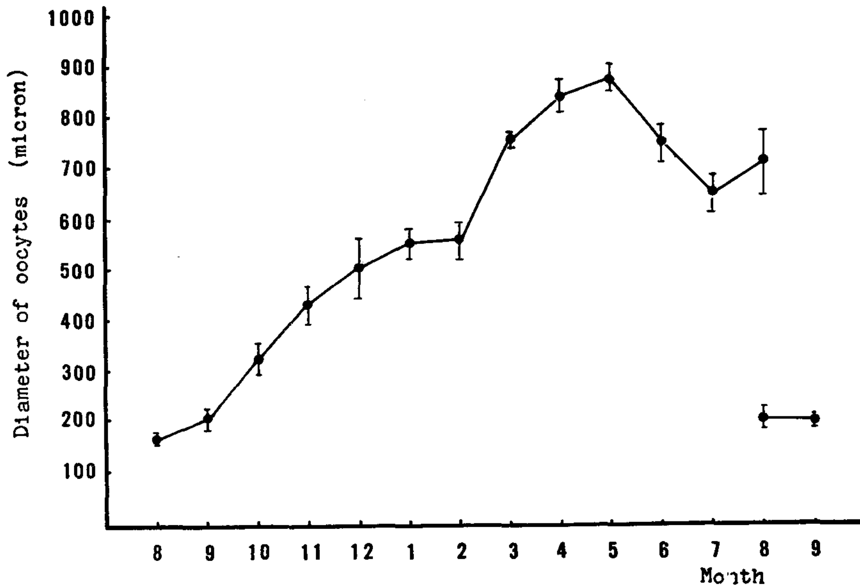
2. Annual changes in the occurrence and growth of yolk-laden eggs

An annual change in the occurrence of yolk-laden eggs is shown in the Text-figure 2. All eggs larger than 20 micra in diameter were enumerated under a microscope using serial sections of ovaries, and the percentage of yolk-laden eggs was counted. From the Text-figure, it is clear that yolk-laden eggs increase gradually in number from September to March, the value in March being 45 per cent. No difference is seen among the values of March, April and of May. Owing to spawning, the percentage of the yolk-laden eggs begins to decrease in June. The minimum percentage in yolk-laden eggs is found in August and September, the values being less than 5 per cent. During this period the ovaries are nearly filled with yolkless eggs.

In Text-figure 3, an annual change in the growth of yolk-laden eggs is presented. The largest twenty eggs in each ovary were chosen and measured. Serial sections prepared from two or three ovaries in each month were used for this measurement. The mean egg diameter of each month is plotted in the figure. The vertical line represents 95 per cent confidence limits. In August, the mean egg diameter is 170 micra. As the month proceeds, the diameter rapidly becomes large until winter. In winter the growth of eggs is retarded and an inflection point in the growth curve is found in February. Then the eggs recover their rapid growth and they reach 760 micra on the average by March. The maximum



Text-figure 2. Seasonal change in the occurrence of yolk-laden eggs



Text-figure 3. Seasonal change in the growth of oocytes

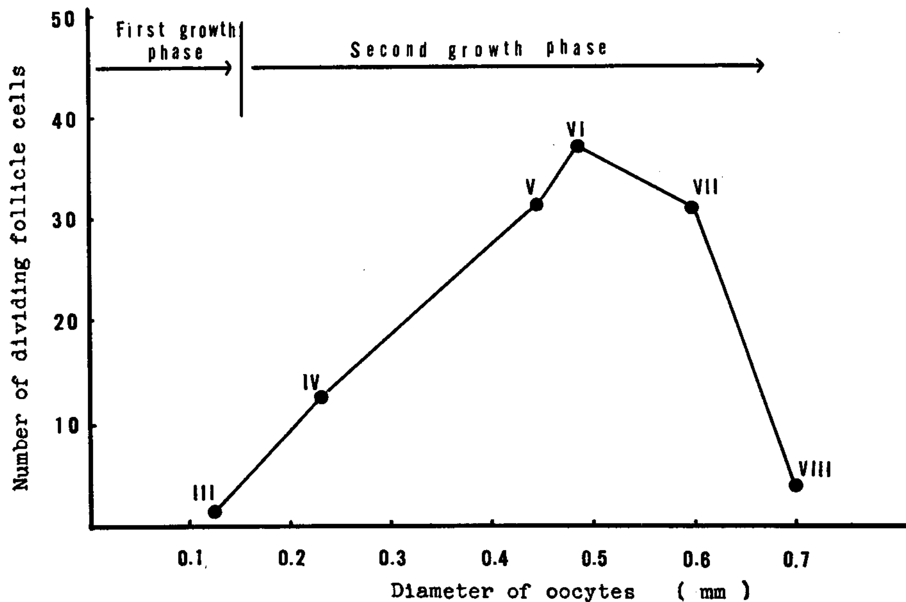
size is seen in May, being 880 micra. As a result of the spawning of the most advanced eggs, the value for June is found to be smaller than that for May. A conspicuous decrease in egg size is seen in July. In August two distinct groups of eggs were observed in the ovaries, that is, one of a few eggs of about 712 micra with much yolk and the other of a large number of eggs of about 205 micra. All eggs of the former group disappeared in September and the mean egg diameter decreased to 201 micra.

Both phenomena described above are correlated well with the change in ovary weight.

3. Changes in the follicular epithelium

Accompanying the growth of the oocyte, the cyclical change in the follicular epithelium must be taken into consideration, because this epithelium takes important roles in the nutrition of oocytes or yolk accumulation (Narain 1956, Raven 1961, Yamazaki 1963). The most conspicuous changes in the follicular epithelium are the increase in number of cells and in thickness of the epithelium. For the evidence of the former fact, changes in number of mitotic divisions of follicle cells were examined with fifty-two oocytes ranging from 100 to 750 micra in diameter obtained from three female fish. The fish were kept in an aquarium (30 cm × 45 cm × 30 cm) in February for three weeks. The water temperature of the aquarium was maintained at 17°–19°C.

The number of dividing follicle cells was summed up at each stage of the oocyte growth (Text-figure 4). The divisions of follicle cells were first seen in the oocytes of about 100 micra and the number of mitotic figures became numerous in oocytes larger than 150 micra in diameter. The largest number of divisions in follicle cells was found in the medium-sized oocytes which were at the primary



Text-figure 4. Mean number of dividing follicle cells at each stage of oocyte growth. III: late peri-nucleolus stage; IV: early yolk vesicle stage; V: late yolk vesicle stage; VI: primary yolk stage; VII: secondary yolk stage; VIII: tertiary yolk stage

yolk stage, the number being an average of 37 per one oocyte. As the oocytes grew further, the dividing cells rapidly decreased in number and they were rarely detected in the oocytes of 750 micra in diameter. The eggs just before ovulation had no dividing follicle cell.

This cyclical change in the number of dividing follicle cells is not concerned directly with the changes in ovary weight but seems to be closely connected with the growth of oocytes.

4. Discussion

The ovary of most fish changes cyclically in weight in accordance with its reproductive cycle. In goldfish, the gonad index begins to increase gradually in September from about 1% and arrives at 10–20% by the spawning season. Then it falls to the minimum after spawning. More than 45% yolkless oocytes are present in the ovaries through the year. Especially in August and September, they are most abundant and occupy more than 90% of the total eggs. Hickling (1930) called these oocytes reserve fund eggs and Vladykov (1956) called them recruitment stock eggs. These yolkless oocytes have great significance in supplying the eggs to be spawned in the following spawning season. The origin of these yolkless oocytes has long been discussed by many workers (Franchi, Mandl & Zuckerman 1962). Naturally this may be concerned with the problems of when and where oogonial multiplication occurs. However, the problems are still not settled in most fish.

The growth of these yolkless oocytes is generally called first growth or primary growth of oocytes (Hann 1927, Craig-Bennett 1930, Bullough 1939, Yamamoto 1955, Ball 1960, Yamazaki 1961). This phase of growth seems not to bring any large influence on ovary weight.

On the other hand, in fish the increase in ovary weight depends mainly on the occurrence and growth of yolk-laden oocytes. The growth of these yolk-laden oocytes is distinguished as the second growth or the secondary growth of oocytes by many investigators as described above.

In goldfish, the oocytes with yolk begin to appear in the ovaries in September and gradually increase in number as the time passes, and spawning occurs several times during May and early July. During this time oocytes in various stages of development are found in the ovaries. At the end of spawning many atretic oocytes with yolks are found. These changes in ovaries show clearly that goldfish belong to the type of asynchronism in oocyte development as already pointed out by Yamamoto and Yamazaki (1961). The other species of fish belonging to this type are sardines (Clark 1934, Ishida *et al.* 1959), bitterling (Bretschneider & de Wit 1947, Shirai 1962), Medaka (Yamamoto & Yoshioka 1964) and so on.

In the fish belonging to this type, the ovary decreases in weight owing not

only to ovulation or oviposition but also to the degeneration of oocytes with yolk (Yamamoto & Yamazaki 1961).

Therefore it may be said that the cyclic change in the ovary of goldfish is composed of oogonial multiplication, the first and the second growth of oocytes, ovulation, oviposition and degeneration of oocytes.

Part II. Multiplication of oogonia

Franchi *et al.* (1962) classified the origin of oocytes in fishes according to the following four categories: 1) oocytes are derived from proliferation of the germinal epithelium, 2) they are derived from mitotic division of the follicular cells, 3) some oocytes are formed from pre-existing oogonia, while others are formed from epithelial cells, and 4) all definitive germ cells arise from pre-existing oogonia which have remained in the ovary from the time of their arrival in the genital ridges during embryonic development. However, it is unknown to what category goldfish belong. Recently Yamamoto (1962) and Yamamoto & Shirai (1962) found clear oogonial division in the ovaries of adult *Oryzias latipes* and *Rhodeus ocellatus* respectively. As to the time of appearance of oogonial division in adult fish the following results have been obtained; namely, the division appears shortly after spawning (Craig-Bennett 1930, Matthews 1938, Bullough 1942, Barr 1963), throughout the year (Yamamoto & Shirai 1962) or during the spawning season (Yamamoto 1962).

It is uncertain whether these contradictory results on the origin of oocytes are due to species specificity, and further, whether these multiplications of oogonia are under some endocrine controls as suggested by Bullough (1942).

1. Morphology of oogonia and their mitotic division

Numerous oogonial cysts were observed in spent ovaries (Figs. 1, 2, 3, 4) which included many atretic eggs (Figs. 1, 3). The cysts ranging 60–150 micra in size were found embedded in the wall of ovigerous folds (Figs. 3, 5, 7, 9). No limitation was found in the distribution of the cysts, but throughout the ovaries the cysts were detected. Each cyst contained several to several hundred oogonia with a large nucleus of 12–14 micra in diameter in which one large nucleolus stained deeply with haematoxylin and scattered chromatin materials of strand form were observed. The nucleus was enclosed with the very thin cytoplasm.

Dividing oogonia were observed in the ovaries obtained in May, June, July, August, October and February. Especially after spawning, many dividing oogonia were detected. They had rod-like chromosomes and showed a typical mitotic figure as already known in other fish (Yamamoto 1962, Yamamoto & Shirai 1962). The size of the oogonia at the metaphase of mitosis was 14 micra in diameter. Some of these oogonia appear to develop immediately into the oocytes of meiotic prophase.

2. Pituitary gland and oogonial multiplication

Ninety-six adult fish were hypophysectomized extending all the year around and their ovaries were examined to ascertain whether the dividing oogonia were present or not. The results showed that no dividing oogonia were found in these ovaries except a few. All cell components of the ovaries were shrunk and the degenerating oocytes became numerous as the day after the operation elapsed. Therefore it may be said that hypophysectomy inhibits not only the growth of ovaries, but also the multiplication of oogonia.

On the other hand, seven hypophysectomized fish were implanted or injected with pituitary glands of goldfish or dog salmon three or four weeks after hypophysectomy. In all these fish, some cysts composed of a few oogonia were found embedded in the wall of ovigerous folds (Figs. 5, 7, 9). The oogonia were normal in size and appearance, and many oogonia were found in the process of division (Figs. 6, 8, 10). Especially two of these fish had numerous cysts in which dividing oogonia were included. The ovaries of two fish out of five treated with goldfish pituitary glands had many atretic oocytes which showed frequently dividing figures of invading granulosa cells. The ovaries of two fish treated with dog salmon pituitary glands had only a few atretic oocytes (Fig. 9). Both pituitary glauds of goldfish and dog salmon proved to have the same effects on the multiplication of oogonia.

3. Discussion

The possibility that the division of oogonia ceases during the young stage of life and that the eggs are formed from the stock of small oocytes already present in the ovaries has been supported by a number of investigators (Goodrich *et al.* 1934, Hickling 1935, Yamamoto 1956), because they failed to find any sign of mitotic figures of oogonia in ovaries of adult fish. In goldfish, however, distinct oogonial division was observed in adult fish which had once experienced spawning. Especially after the spawning season, many dividing oogonia were found in the oogonial cysts which were situated in the wall of ovigerous folds. Some of these divided oogonia soon gave rise to the oocytes of the meiotic prophase. Therefore it may be definitely concluded that in goldfish the new crop of oocytes in the adult fish are produced by the proliferation of resident oogonia as described by Hann (1927) on *Cottus bairdii*, Matthews (1938) on *Fundulus heteroclitus*, Craig-Bennett (1930) on *Gasterosteus aculeatus*, Yamamoto (1962) on *Oryzias latipes* and Yamamoto and Shirai (1962) on *Rhodeus ocellatus*. Thus the goldfish may be grouped in the fourth category of Franchi *et al.*'s classification, *i.e.*, all definitive germ cells arise from pre-existing oogonia.

However, the multiplication of oogonia was not found throughout the year but limited to some period. This suggests that some factors concerned with oogonial

division are connected with season. The present investigation on the goldfish failed to establish any of the factors, but obtained suggestive results. Oogonial division was inhibited by hypophysectomy, while numerous oogonial divisions appeared in the ovaries, when the pituitary glands were injected or implanted into the peritoneal cavity of the hypophysectomized fish. These facts show that pituitary hormone may be concerned with oogonial division.

Bullough (1942) found that in *Phoxinus*, the injection of oestrone stimulates the oogonial division and an abnormally large number of young oogonia are produced in the post-spawning ovaries. This raises the question of whether the pituitary causes the oogonial division directly or indirectly through the ovarian hormone. Further experimental works are needed to elucidate the problem.

Part III. The first growth phase

1. Morphological characteristics

The first growth phase of oocytes corresponds to the prophase of meiosis. During the phase germ cells show the so-called premeiotic phenomena and grow from 14 micra to 150 micra in diameter. This phase may be divided into three stages in conformity with the previous paper of Yamamoto and Yamazaki (1961), namely, 1) the chromatin nucleolus stage, 2) the early peri-nucleolus stage, and 3) the late peri-nucleolus stage.

1) Chromatin nucleolus stage

After the last oogonial division, the oogonia of 12–14 micra in diameter, transform into oocytes (Fig. 11). The youngest oocytes are nearly the same in figure as the oogonia and are found embedded in nests (Fig. 12). The nucleus has a conspicuous nucleolus which is connected with chromatin threads. Then the oocyte nuclei pass through a sequence of characteristic stages in which the chromatin undergoes a regular series of successive changes. The typical chromatin nucleolus which is stained deeply with haematoxylin is found to appear in one side of the nucleus (Fig. 13). Chromatin threads connected with the chromatin nucleolus are also observed within the nucleus. The oocytes having these characteristic nuclei of the synaptic stage are found forming cysts (Fig. 14). As the oocytes grow, the chromatin threads make arrangement near the periphery of the nucleus. A large nucleolus showing a complicated form is found in the center of the nucleus. Some oocytes of this stage are present within cysts (Fig. 16) while others are outside of cysts (Fig. 15). This deeply stained nucleolus becomes triangular or crescent in shape and attaches to the nuclear membrane with its base (Fig. 17). On the opposite side of the nucleolus, there are found chromatin threads. The crescent-shaped nucleolus becomes thin (Figs. 18, 19) and finally disappears from sight along with the growth of oocytes. Simultaneously with these nuclear changes, the oocytes

detach themselves from the cyst and become located singly in the wall of ovigerous lamellae.

The oocytes pertaining to this stage have the size ranging from 12 micra to 20 micra in diameter. During this stage the cytoplasm shows little increase.

2) Early peri-nucleolus stage

The nucleus increases in size and the cytoplasm becomes thicker (Figs. 20, 21). Then the nucleoli increase greatly in number and size and the cytoplasm becomes stained deeply with haematoxylin (Fig. 22). The yolk nucleus is seen in the cytoplasm, although it is not so distinct as in the following stage. The yolk nucleus lies at first close to the nuclear membrane and then moves to the periphery of the cytoplasm with the growth of the oocyte. The oocytes become surrounded with a very thin follicular layer. The oocytes in this stage range from 20 micra to 150 micra in diameter.

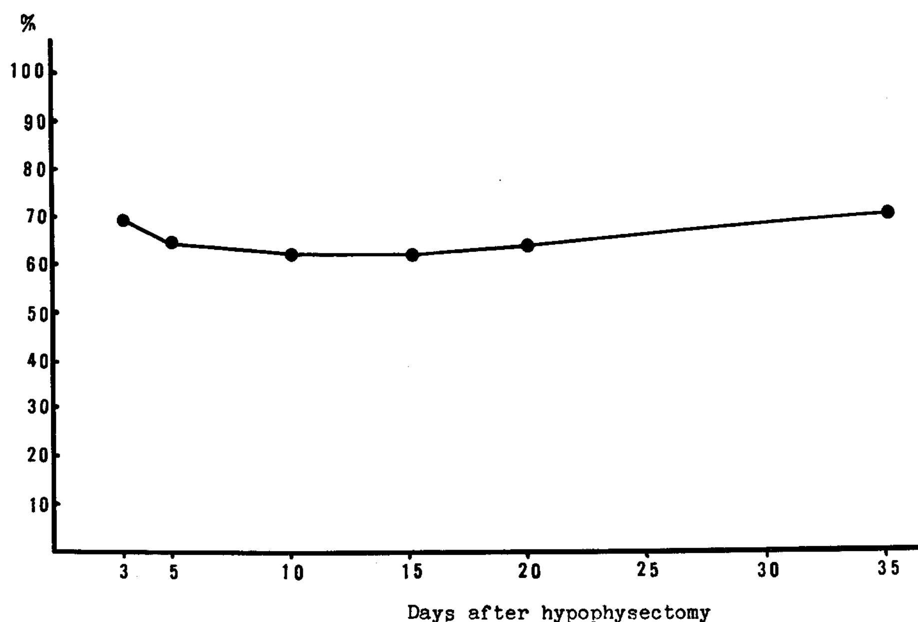
3) Late peri-nucleolus stage

This stage is only distinguished from the previous one by the large size of the oocytes and weak affinity of their cytoplasm to haematoxylin. The yolk nucleus which has a dotted form and dimensions of 10–15 micra in diameter is clearly found situated in the outer part of the cytoplasm. The follicular layer around the oocyte becomes clear and sometimes a few dividing cells are found in this follicular layer. The diameter of the oocytes in this stage varies from 110 micra to 160 micra (Fig. 23).

2. Effects of hypophysectomy

Thirty-three fish were hypophysectomized during the period from December, 1962 to January, 1963. They were reared in aquaria whose water temperature ranged from 10°C to 25°C. Three, 5, 10, 15, 16, 20, 25 and 36 days after hypophysectomy, they were killed. Pieces from the ovaries were fixed with Bouin's solution and cut 7 micra in thickness by the usual paraffin method. Serial sections were stained with Delafield's haematoxylin. Five hundred eggs were examined more or less in each fish to obtain the percentage of yolkless eggs to total eggs. In four controls which were kept at 20°C and killed 3, 7, and 21 days after, yolkless eggs were counted at 60–75 per cent. Nearly the same percentage of yolkless eggs were obtained in hypophysectomized fish throughout the experimental period (Text-figure 5). In the hypophysectomized fish, the cytoplasm of the yolkless eggs was stained deeply with haematoxylin and it was very difficult to find the yolk nucleus in the cytoplasm. In some fish late peri-nucleolus stage eggs seemed to decrease in number.

However, most eggs in the first growth phase remained intact without any morphological changes and the fish which were reared for twenty-five days at 20°C had the ovaries occupied with only yolkless eggs, atretic eggs and fibrous con-



Text-figure 5. Change in percentage of the oocytes in the first growth phase after hypophysectomy

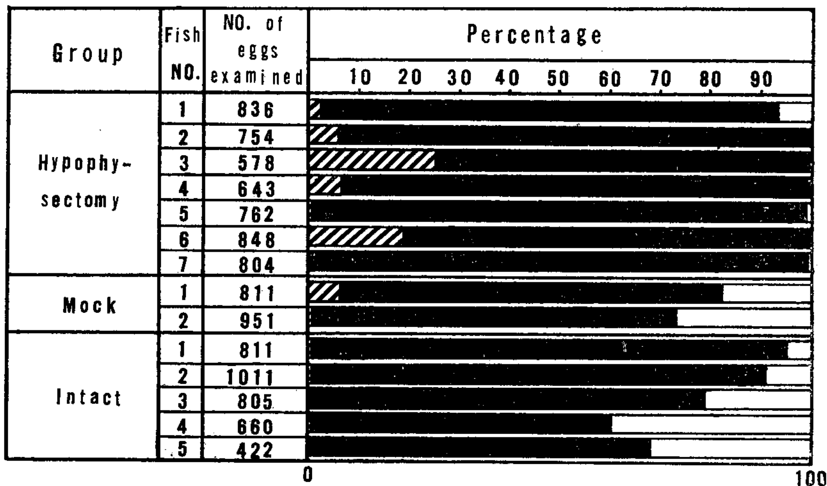
Table 1. Gonad weight of hypophysectomized and control fish

Group	Fish No.	Date of operation	Date killed	Days lived after operation	B. L. (cm)	G.W. (g)
H. sectomy	1	Sept. 3	Sept. 30	27	7.8	0.3
	2	Sept. 3	Sept. 30	27	7.8	0.3
	3	Sept. 3	Sept. 30	27	7.8	0.4
	4	Sept. 9	Oct. 14	35	6.8	0.3
	5	Sept. 9	Oct. 28	49	8.5	0.2
	6	Sept. 9	Dec. 16	98	10.2	1.1
	7	Sept. 9	Dec. 16	98	6.7	0.1
Mock	1	Sept. 9	Oct. 14	35	9.0	0.8
	2	Sept. 9	Oct. 28	49	9.3	0.7
Intact	1		Sept. 13		7.8	0.3
	2		Oct. 2		8.8	0.6
	3		Oct. 27		8.9	0.8
	4		Oct. 27		9.0	1.7
	5		Dec. 26		9.4	3.4

nective tissue (Fig. 29).

Seven fish were hypophysectomized in September when new yolk formation began to start. Sampling was made 27, 35, 49 and 98 days after hypophysectomy. Two fish were subjected to a mock operation on September 9 and killed 35 and 49 days later. Intact fish were sacrificed on September 13, October 2 and 27, and December 26 as a control (Table 1).

The percentages of atretic and normal eggs were obtained by counting 422–1011 eggs from the serial sections of ovaries of all the fish. The results are figured in Text-figure 6. Intact fish killed on September 13 contained 95.1 per cent peri-nucleolus



Text-figure 6. Percentage of atretic and normal oocytes found in the ovaries of the fish shown in Table 1. oblique line: atretic oocytes; black: yolkless oocytes; empty: yolk-laden oocytes

stage eggs. In the control group ovaries gained weight, and many oocytes with yolk appeared within them as time passed. Two fish subjected to the mock operation had also many yolk-laden eggs. On the contrary, all hypophysectomized fish showed no increase in ovary weight. Three fish had only a few yolk-laden eggs in their ovaries, but the other had no oocytes with yolk. All the fish except two produced 91.3–99.8 per cent eggs of the peri-nucleolus stage. The two exceptional fish had many atretic eggs in addition to 74.6 and 81.1 per cent yolkless oocytes. Thus the ovaries of hypophysectomized fish were proved to remain in an immature state. The results obtained in the present study show clearly that hypophysectomy does not affect the oocytes in the first growth phase.

3. Discussion

The oocytes in the first growth phase are characterized by the absence of yolk. The follicular epithelium is formed in this phase, but the cells in the epi-

thelium are very thin and inactive in multiplication. During this phase, oocytes do not show any conspicuous morphological changes except those in the nucleus. These oocytes serve only as a reserve supply and occupy more than 45 per cent of all ovarian eggs all the year around. The same facts are known in many other species of fish. Hickling (1930, 1935) stated that a stock of small eggs is built up during the first ten years of life of the hake, and Bullough (1939) stated the same opinion concerning the minnow. These facts may indicate that the growth of this phase is more monotonous and is slow in tempo as Yamamoto (1955) has suggested in *Oryzias latipes*.

In goldfish, after the end of the spawning season, numerous oocytes in the chromatin nucleolus stage grow into the oocytes in the early peri-nucleolus stage and after the absorption of atretic eggs the ovaries are filled only with many yolkless oocytes. On the other hand, at this time, the gonadotrophic potency of the pituitary gland decreases greatly in goldfish (Scruggs 1951) and also in other fish (Gerbilsky 1940). Therefore it is reasonable to assume that the growth of the yolkless eggs may occur without pituitary gonadotrophin.

This supposition is supported by the results of the present study, that is, eggs in this phase always remained intact in the ovaries of all hypophysectomized fish. In the fish hypophysectomized in January and kept for about three weeks at 20°C the ovaries became filled with only the oocytes of this phase and atretic oocytes. This condition of ovaries in hypophysectomized fish remained unchanged even after a month or more. The fish hypophysectomized in September when the ovaries are in pre-vitellogenesis yielded no or only a few (0.2%) yolk-laden oocytes even 3 months after the operation and the ovaries were full of the oocytes of this phase alone. The same results were obtained by Vivien (1939, 1941) in *Gobius paganellus* and Barr (1963) in *Pleuronectes platessa*. They reported that there is a critical size below which oocytes are unaffected by hypophysectomy. Further, Barr (1963) concluded that early meiotic prophase activity can continue in the absence of the pituitary gland and that some production of new primary oocytes can take place even a year after hypophysectomy.

In general, growth is always brought about by metabolism. Therefore the hormones relating to metabolism such as corticosteroid or thyroid hormone may be concerned with the growth of the eggs and it is well known that the pituitary gland controls the secretion of these metabolic hormones. From the present study, however, it was shown clearly that the eggs in the first growth phase can grow without the pituitary gland. Therefore it may be reasonably concluded that the pituitary gonadotrophin is not directly concerned with the growth of the oocytes in the first growth phase as Bullough (1951) and Ball (1960) have suggested.

Part IV. The second growth phase

1. Morphological characteristics

This phase is characterized by the accumulation of yolk. Two kinds of yolks, namely, yolk vesicles and yolk globules are recognized in oocytes. They are easily distinguished from each other by their different mode of appearance and chemical nature. Oocytes grow from 150 micra to about 900 micra in diameter and increase very much in volume during this phase. Therefore, this phase holds the most important part in the growth of the oocytes. Two stages may be distinguishable according to the process of yolk accumulation, namely, the yolk vesicle stage and the yolk stage.

1) Yolk vesicle stage

This is the stage in which yolk vesicles are formed in the ooplasm. The oocytes in this stage range from 150 micra to 400 micra in diameter. The yolk vesicles appear as pale vesicles when they are fixed with Bouin's solution, while if they are fixed with fixatives containing potassium bichromate such as Zenker's, Regaud's and Champy's solutions, they are stained black or brown with Delafield's haematoxylin. They are PAS-positive and contain mucopolysaccharides. The yolk vesicle stage may be subdivided into two stages, namely, the early yolk vesicle stage and the late yolk vesicle stage.

The early yolk vesicle stage represents the stage in which the yolk vesicles come into sight as minute bodies and make a single layer situated in the peripheral region of the ooplasm (Fig. 24). The zona radiata is very thin and is not stained red with eosin. Follicle cells begin to increase in number mitotically. In the late yolk vesicle stage, the yolk vesicles are accumulated centripetally accompanied with enlargement in their size. The zona radiata becomes thick and is stained red with eosin or azocarmine. The nucleus becomes irregular in outline and the peripheral nucleoli become elliptical, bar-shaped or amoeboid in form (Fig. 25).

2) Yolk stage

This is the stage in which yolk globules are accumulated in the ooplasm. When the yolk vesicles have occupied an outer half part of the cytoplasm, the yolk globules appear at first in the peripheral cytoplasm as minute granules. Then they are accumulated in the inner part of the oocyte. The globules are spherical in form and gradually increase in size. They are nearly PAS-negative and stained black with Heidenhain's haematoxylin. The zona radiata with clear radial striation becomes thicker and measures about 10 micra in thickness. The nucleus becomes more irregular in form and the peripheral nucleoli are, at this time, not spherical but often irregular in form. The oocytes pertaining to this stage have the dimensions ranging from 350 micra to 900 micra in diameter. This stage may be subdivided into three stages, namely, the primary, secondary

and tertiary yolk stage.

The primary yolk stage represents the time when the yolk globules begin to appear in the oocytes. The follicular layer increases in thickness and in cell number. The yolk vesicles also grow in size and number and occupy the outer two thirds of the cytoplasm (Fig. 26).

The secondary yolk stage is characterized by the rapid accumulation of yolk globules in the inner part of the cytoplasm. This accumulation results in the rapid growth of oocytes. The yolk vesicles are gradually shifted outerwards to take arrangement in a few rows in the periphery of the oocytes. Both the nucleus and the nucleoli are also irregular in form as in the previous stage. Follicle cells are large in size and many mitotic figures are found among them, though somewhat fewer than in the previous stage (Text-figure 4, Fig. 27).

The tertiary yolk stage is the final stage of yolk formation. The oocytes are filled with yolk globules. The yolk vesicles are distributed on the peripheral region of the oocyte, making one or two rows. The nucleus shows a spherical form and fairly smooth contour. A few nucleoli showing a spherical form are located in the interior of the nucleus free from its membrane. The micropyle having a large micropylar cell is detected at one pole where yolk vesicles are scattered sparsely or they disappear completely. The follicle cells are somewhat flat compared with the previous stage and the dividing follicle cells are nearly invisible in the follicular epithelium. During this stage, the growth of oocytes is not so conspicuous as in the last stage (Fig. 28).

2. Effects of hypophysectomy

a. General effects on the oocytes of the second growth phase

The fish which gave more than 10 per cent of the second growth phase oocytes are usually found in the period from October to the following July (Text-fig. 2). At the present experiment, therefore, hypophysectomy was carried out during this period. Thirty-two females were hypophysectomized in 1959 and 1960. At the same time, a mock operation which exposed the pituitary gland but left it intact was done on nineteen fish (Table 2). All the fish operated on were reared in the aquaria of 70 cm×103 cm×30 cm or of 160 cm×130 cm×45 cm in dimension. They were fed with earthworms and a boiled mixture of chopped shrimp and wheat meal.

In general, the oocytes in this phase suffer much atresia with the passing of time after hypophysectomy and become corpora atresia. Thus, the ovaries are occupied by only the oocytes in the first growth phase and corpora atresia (Fig. 29). All the cells found in the ovary such as stroma cells, germ cells and follicle cells make shrinkage. The ovaries become soft, decrease in size and weight, and gradually change their colour into yellow brown. But these decreases in the size

Table 2. Seasonal changes of the atretic index in hypophysectomized and in mock operated fish

Mock control				Hypophysectomized fish			
Date of operation	Days lived after operation	Atretic index	B. L. (cm)	Date of operation	Days lived after operation	Atretic index	B. L. (cm)
Jan. 12	159	−	8.9	Apr. 15	14	−	10.1
Apr. 15	14	−	11.5	Apr. 15	28	+	10.7
Apr. 15	28	−	9.0	Apr. 15	42	−	10.0
Apr. 15	42	+	13.5	Apr. 22	21	+	9.4
May 6	28	−	11.7	May 6	7	−	9.1
May 6	56	−	10.4	May 6	14	−	9.3
				May 8	14	−	10.3
				May 6	21	+	9.5
				May 6	42	+++	9.2
				May 6	56	++++	11.1
June 6	28	−	9.8	June 27	7	+	15.5
June 6	59	+	10.6	June 6	14	+	10.1
				June 6	28	+++	11.1
				June 6	35	++	10.8
July 7	70	−	10.7	July 7	28	++	10.4
July 23	36	−	7.1	July 7	43	++++	10.3
July 23	36	−	7.1	July 7	44	++	10.5
July 23	36	−	7.2	July 7	70	+++	11.0
Aug. 5	9	−	8.8	Oct. 2	7	−	8.7
Aug. 5	16	−	6.8	Oct. 2	28	−	9.3
Aug. 5	36	−	9.8	Oct. 2	28	+	8.9
Aug. 2	64	−	7.2	Oct. 30	49	+	8.3
				Oct. 15	71	−	7.2
				Oct. 3	77	−	6.8
				Oct. 3	77	+	7.8
				Oct. 3	77	−	7.0
				Oct. 8	78	−	7.9
				Oct. 8	78	−	10.9
				Oct. 8	78	+	8.0
				Oct. 8	78	+	9.2
				Oct. 8	78	++	11.5
				Nov. 9	118	++	11.4

The mark − represents ovaries containing 0 to 5% of atretic oocytes; +: 5 to 15%; ++: 15 to 25%; +++: 25 to 35%; ++++: more than 35%.

and weight of ovaries are not so rapid as the change of the oocytes themselves. The corpora atresia are recognized as yellow spots from the outside of the ovaries. All oocytes do not change simultaneously, but they change asynchronously in each stage. The oocytes in the late yolk vesicle stage, the primary and the secondary yolk stage are more sensitive to hypophysectomy and tend to degenerate at first. On the other hand, the oocytes in the early yolk vesicle stage and some of the oocytes in the tertiary yolk stage remained unchanged for a long time, although they also showed atresia finally. Then these corpora atresia are gradually absorbed.

Atretic changes after hypophysectomy as well as spontaneous atresia which is found mainly in the oocytes in spent ovaries, begin to occur in the peripheral region of the oocytes. At first, yolk vesicles are melted and sometimes they make a colloidal mass. This colloidal mass is not stained with haematoxylin but stained in blue with Mallory's azan stain. The nucleus disappears from sight, granulosa cells are hypertrophied, and the zona radiata is broken into fragments. At the next step, yolk globules are also melted from the peripheral region and they seem gradually absorbed by the granulosa cells invading the oocytes. The oocytes become weak in stainability and irregular in shape. Finally these atretic oocytes are composed almost entirely of granulosa cells and become small in size. In hypophysectomized fish kept in low water temperature, the nuclei of some oocytes are found intact for a long time even after the peripheral region of oocytes has been degenerated and the follicle cells have already been hypertrophied.

There is some difference in the velocity of atretic changes according to season. In Table 2, the approximate percentage of atretic oocytes to the total eggs in the ovaries of hypophysectomized fish is shown. From this table it may be recognized that for the occurrence of more than 10 per cent atretic eggs, more than 3 weeks elapsed in April and May, and in July it was two weeks, while in October or November the changes took more than eleven weeks. Therefore, it may be said that hypophysectomy exerts influence on the ovaries of operated fish more quickly in June and July than in October and November. This seasonal change in the velocity of degeneration of oocytes seems to be intimately connected with both the state of yolk accumulation and water temperature. The water temperature in July was at about 17°–21°C and in November it was at about 5°–8°C.

b. Influence of water temperature on the velocity of oocyte degeneration

Many workers said that in fish the effects of hypophysectomy develop slowly and atresia takes several weeks or even months (Vivien 1941, Pickford & Atz 1957, Ball 1960), whereas in mammals the final stage of the atresia in the ovaries is reached in one or two weeks. This may be mainly due to the difference in body temperature. In the present section, the relation between velocity of degeneration and temperature has been studied.

Procedure

This experiment was carried out in the period from the middle of December to early February. During this season only a few oocytes in the tertiary yolk stage were visible and the ovaries were almost occupied with the eggs below the secondary yolk stage. Fifty-three fish were chosen and hypophysectomized. They ranged from 6.3 cm to 11.2 cm in body length, mostly 7–9 cm. No fish died from the operation. The fish operated on were divided into four groups which were kept respectively in each aquarium set in the greenhouse. Water temperature in

each aquarium was controlled by a regulator and heater (100 or 200 W) at just 10°C, 15°C, 20°C and 25°C, though the water temperature of 10°C aquarium varied from 9.8° to 10.7°C during experimental period. The size of the aquaria was 30 cm ×45 cm×30 cm whose water was circulated by the air lift filteration method. Thirteen fish proved to be males at the time of autopsy and five fish were found to have been operated on unsuccessfully, so that thirty-six females served as useful material. They were killed according to the following schedules shown in Tables 3, 4, 5, and 6. Then the ovaries were fixed with Bouin's solution and the serial sections of the ovaries were made.

To enumerate the number of the oocytes, it is very convenient and useful to count the nuclei of the oocytes, because the size of the nucleus changes only a little with the growth of the oocytes. But atretic oocytes have no nucleus and are very complicated in form, so that it is very difficult to count them exactly. Therefore, the percentage of atretic oocytes and the normal ones of both growth phases were obtained by the following procedures:

At first, the percentage of normal oocytes in both growth phases was obtained by counting the nuclei of the two kinds of oocytes. In the next, in order to know the percentage of normal oocytes in the second phase and atretic oocytes, all normal and atretic oocytes in the second growth phase which appeared in the microscopical sight were counted without regard to the presence of the nucleus by making sure whether they were normal or atretic. As only the oocytes of the second growth phase underwent atresia and the size of both kinds of oocytes was about the same, the rate of normal oocytes to atretic ones could be estimated properly from the number obtained. Then the percentage of atretic oocytes and normal ones in both growth phases was calculated from both groups of values obtained above.

Results

The results obtained were shown in Table 3, 4, 5, and 6 together with the gonad index (gonad weight×100/body weight). No significant difference could be found in the gonad index between the groups of different temperatures because of the high fluctuation in individual values. Therefore, the gonad index was not useful to check the degree of degeneration of ovaries in this experiment. However, the dependence of ovary degeneration on water temperature is clearly shown in the changes in oocyte composition. At 25°C, two fish killed three days after hypophysectomy had 12 per cent and 15 per cent atretic oocytes. The number of the atretic oocytes increased further five days after, being 35 per cent and 24 per cent respectively. Four fish were killed ten days after. In the ovary of one fish out of these four, nearly all oocytes in the second growth phase were atretic and it was impossible to count them exactly. The other three fish had 33–36 per cent atretic oocytes. Sixteen days after, no oocyte in the second growth phase was

Table 3. Changes in oocyte composition in the ovaries of hypophysectomized fish kept at 25°C

Fish No.	B. L. (cm)	$\frac{GW \times 100}{BW}$	Days after operation	No. of oocytes counted	Percentage of oocytes		
					First growth	Second growth	Atretic
1	7.7	3.5	3	387	73	15	12
2	7.2	5.5	3	440	50	35	15
3	10.9	17.4	5	134	40	25	35
4	7.2	6.3	5	532	62	14	24
5	8.4	22.4	10	63	52	15	33
6	7.9	3.5	10	288	55	10	35
7	7.8	1.5	10	443	62	2	36
8	6.3	0.8	10	357	94	6	impossible to count
9	8.8	2.0	16			0	

Table 4. Changes in oocyte composition in the ovaries of hypophysectomized fish kept at 20°C

Fish No.	B. L. (cm)	$\frac{GW \times 100}{BW}$	Days after operation	No. of oocytes counted	Percentage of oocytes		
					First growth	Second growth	Atretic
1	7.7	7.4	3	581	59	39	2
2	8.9	6.0	5	659	62	36	2
3	8.7	22.0	10	344	42	38	20
4	6.7	2.5	10	563	50	16	34
5	8.2	3.7	10	524	44	15	41
6	9.7	2.4	25			0	
7	7.4	1.6	25			0	

Table 5. Changes in oocyte composition in the ovaries of hypophysectomized fish kept at 15°C

Fish No.	B. L. (cm)	$\frac{GW \times 100}{BW}$	Days after operation	No. of oocytes counted	Percentage of oocytes		
					First growth	Second growth	Atretic
1	8.8	1.8	3	577	84	16	0
2	8.1	5.3	3	500	63	37	0
3	8.0	1.8	5	499	71.9	28	0.1
4	8.2	6.3	10	547	59	33	8
5	7.2	9.1	10	638	67	29	4
6	9.9	10.1	15	399	32	59	9
7	6.6	2.4	15	463	79	20	1
8	8.8	1.7	20	561	36	35	29
9	9.4	2.2	36	600	70	14	16

observed in the ovary (Table 3).

At 20°C, two fish killed three days after hypophysectomy had only 2 per cent atretic oocytes. In this case atretic oocytes also increased with the passage of postoperative days and the final stage was arrived at 25 days after (Table 4). Therefore, the velocity of degeneration was not so rapid as that seen at 25°C.

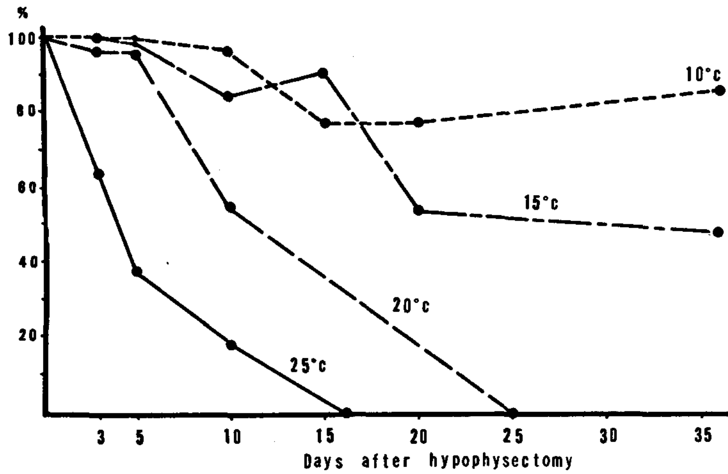
At 15°C, no atretic oocyte was found in the ovaries of fish killed three days after hypophysectomy (Table 5). The velocity of degeneration was slow and the final stage was not detected during the experimental period. At the end of the present experiment (36 days after hypophysectomy), 14 per cent of normal oocytes in the second growth phase were still observed.

At 10°C, few oocytes which appeared to be in the process of degeneration were found in the ovaries of the fish killed ten days after, while many intact oocytes with yolk were also observed in the ovaries. Even 36 days after, 4 per cent of atretic oocytes and 19 per cent of normal oocytes in the second growth phase were found in the ovary (Table 6). Therefore degeneration proceeded much slowly when compared with that of the group reared at 15°C.

In Text-figure 7, the changes in the percentage of normal to total second growth phase oocytes seen in the fish kept at various temperatures are shown. From this figure, it is also clear that the decrease in normal oocytes after hypophysectomy depends on water temperature. At 25°C the final stage is seen in about two weeks and below 15°C, 50 to 80 per cent of the eggs in the second growth phase remained in the ovaries even thirty-six days after so that the final stage may be, perhaps, reached in several months.

Table 6. Changes in oocyte composition in the ovaries of hypophysectomized fish kept at 10°C

Fish No.	B. L. (cm)	$\frac{GW \times 100}{BW}$	Days after operation	No. of oocytes counted	Percentage of oocytes		
					First growth	Second growth	Atretic
1	9.3	0.9	3	764	86	14	0
2	8.4	5.1	3	455	72	28	0
3	8.9	2.6	5	458	74.9	25	0.1
4	9.4	2.9	5	658	76.9	23	0.1
5	8.8	1.2	10	692	83	17	0
6	10.2	5.4	10	431	69	31	0
7	8.1	11.4	10	470	73.8	26	0.2
8	12.0	14.4	15	313	76	18	6
9	8.7	3.7	20	746	74	17	9
10	10.0	4.0	20	432	83	15	2
11	8.8	2.1	36	891	77	19	4



Text-figure 7. Changes in the percentage of normal oocytes in the second growth phase after hypophysectomy

c. The effects of hypophysectomy on the follicle cells

Follicle cells take the most important roles in the formation of yolks. Therefore, it is indispensable to examine the effects of hypophysectomy on the follicle cells. Fish used in this study ranged from 7.2 cm to 8.6 cm in body length. They had been cultured in an outdoor aquarium of 5 m×3.5 m×0.9 m until needed. The experiments were carried out from January to early March. During this period the water temperature of the aquarium varied from 0.5°C to 4.0°C. Fish were taken from this aquarium and operated on. Then they were kept in an indoor aquarium (30 cm×45 cm×30 cm) in which water temperature was controlled by a regulator and heater at 19°–21°C. Two fish kept in the outdoor aquarium were sampled as initial control. The number of materials used and the postoperative days are shown in Table 7. The ovaries were fixed with Bouin's solution and cut serially at 7 micra in thickness with the usual paraffin method. The sections were stained with Delafield's haematoxylin-eosin and Heidenhain's haematoxylin-light green.

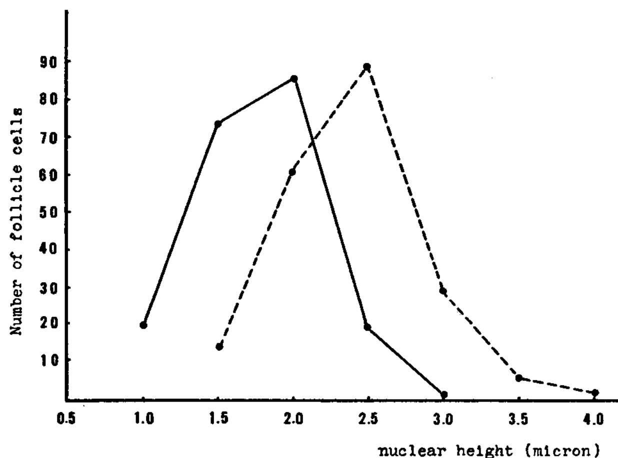
Table 7. The number of materials used for the observations on the effects of hypophysectomy on follicle cells

	Initial control	3 days	One week	Two weeks	Three weeks
H. sectomy		2	2	1	1
Mock		2		1	2
Intact	2	2	1	3	1
Water temp. (C)	2.5	19—21			

The follicular epithelium lying on the outer surface of the oocyte membrane consists of a single layer of flat cells with elliptical nuclei whose long axes are horizontal to the oocyte membrane. The thickness and frequency of the mitotic division of these cells change with the growth of the oocyte (Yamazaki 1963). In this study, therefore, the oocytes of the late yolk vesicle stage were taken as index oocytes to avoid the fluctuation owing to the stages of the oocytes. The effects of hypophysectomy on the follicle cells were examined from three points, namely, changes in the thickness of the cell nuclei, changes in the number of dividing cells and the degeneration of the cells.

1) *Dimension of the nuclei of follicle cells*

The index oocytes of normal appearance were chosen from the ovaries of fish one week after hypophysectomy. Intact fish were taken as a control. The nuclei of two hundred follicle cells were measured in each group. Frequency curves

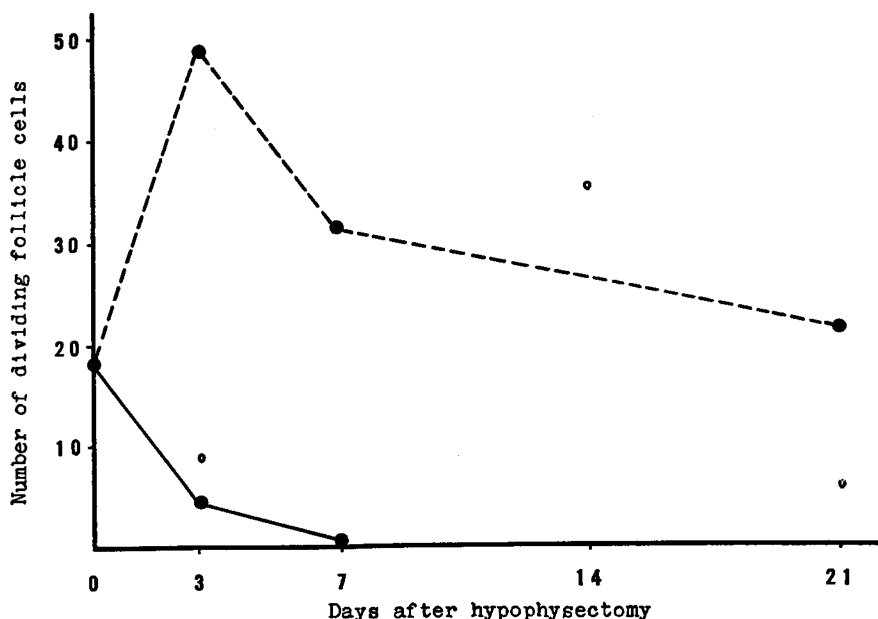


Text-figure 8. Frequency curve of the nuclear height of follicle cells.
broken line: intact fish; solid line: hypophysectomized fish

were made from this data (Text-figure 8). In the intact fish, nuclear height ranges from 1.5 micra to 4.0 micra and the frequency curve has its peak at the point of 2.5 micra, while in the hypophysectomized fish the height extends from 1.0 to 3.0 micra and the peak of the curve is found at 2.0 micra. The mean values of the two groups were 2.2 micra and 1.5 micra respectively. The difference in thickness of the follicle cells between the fish killed three days after hypophysectomy and intact fish is shown in Figs. 30, 31 and 32. This data shows that the follicle cells become small soon after hypophysectomy. But the follicle cells become thick when the oocytes begin to degenerate. In the ovaries of hypophysectomized fish after two weeks had elapsed, the index oocytes were all atretic, the follicle cells were all hypertrophied and they seemed to take part in the absorption of the oocytes.

2) The number of dividing follicle cells

Ten index oocytes were chosen at random from each ovary and the total number of dividing follicle cells was counted in each oocyte. The results are shown in Text-figure 9. The water temperature of the aquarium was at 2.5°C when the initial controls were taken out. The dividing follicle cells in this initial control were 18 in mean number. The number of mitotic figures greatly increased in intact controls after the fish were transferred into the indoor aquarium. Three days after the transfer, the largest number of dividing cells was 75 per one oocyte and the mean was 49. This may be due to the change in water temperature. One week later, the number decreased to some extent and was 32 on the average. Three weeks later, the number was 21.7 per one oocyte.



Text-figure 9. Changes in the number of dividing follicle cells. broken line: intact fish; solid line: hypophysectomized fish; empty circle: mock operated fish

On the other hand, the dividing follicle cells decreased rapidly in number in the hypophysectomized group. Three days after hypophysectomy, clear figures in the metaphase or telophase of mitosis were seldom observed and only the figures in prophase were found in the follicular epithelium. The mean number of them was 4.2 per one oocyte. One week after, the dividing follicle cells were rerely observed even in the eggs showing normal appearance. While in the mock operated controls the number of the dividing follicle cells was very changeable, namely, the mean number was eight per one egg in the fish three days after operation,

35 per one egg two weeks after, and six per one egg three weeks after. This data indicates that the dividing activity of follicle cells depends mainly on the pituitary gland, and partly on some physiological factors.

3) *The degeneration of follicle cells after hypophysectomy*

The other morphological change in the follicular epithelium is the degeneration of follicle cells. This degeneration is characterized by the disappearance of the nuclear membrane and the presence of granules stained deeply with haematoxylin (Figs. 33, 34). Some granules were large and others small. Sometimes, the granules appeared as one large mass. The mean number of degenerating cells was eight per one egg in the fish three days after the operation and three per one egg in those one week after the operation. The degenerating cells were not observed in the intact goldfish.

3. The implantation and injection of pituitary materials into hypophysectomized fish

Goldfish used in this study measured 6.9 cm to 10.7 cm in body length. They were hypophysectomized and cultured for more than three weeks. The water temperature of the aquarium varied from 17°C to 21°C. The fresh pituitary glands used for implantation and injection were obtained from goldfish whose body length ranged from 6.9 cm to 14.2 cm, being 8.7 cm on the average. The implantation was made into the peritoneal cavity of one hypophysectomized fish. This fish received the implantation seven times every other day 4 to 6 glands at one time. The total number implanted was 34 glands (Table 8). The fish was killed on the day following the last implantation. The ovary was fixed with Bouin's solution and sectioned with the usual paraffin method to 7 micra in thickness. This experiment was carried out during July.

Four hypophysectomized fish were injected with suspension of fresh pituitary glands. At one injection, they received about 0.2 cc of 0.6% NaCl solution in which 3–4 glands were suspended. Eight injections were made in each fish during the period from February 22 to May 9 and then the fish were killed on May 10.

Two fish were injected with salmon pituitary suspension made of two or three acetone-dried glands in 0.6% NaCl solution. Injections were made 6 times every other day. The total number of the glands was 14 (Table 8). An autopsy was made on the day following the last injection. This treatment was performed during December.

In the fish implanted with fresh pituitary glands, the gross appearance and weight of the ovary were about the same as those of the controls. The ovary was yellow-brown and somewhat compact. However, histological observations revealed that in the implanted fish many oocytes with yolk vesicles were found in the ovary in addition to atretic oocytes and a comparatively small number of

Table 8. Effects of the pituitary glands and various mammalian gonadotrophins on the yolk formation in hypophysectomized fish

	Dosage units	Interval of treatment in day	Number of treatment	Total units	Body weight (g)	Ovary weight (g)	Maximum egg diameter (micron)	Stage
Gonatropin (HCG)	25	0	10	250	48.4	1.1	176	1
	25	0	10	250	38.5	0.4	150	0
	50	0	10	500	16.5	0.5	215	1
	100	0	10	1000	23.9	0.9	193	1
	50	2	10	500	39.1	0.5	164	0
	100	2	10	1000	18.5	0.3	200	1
Gonatropin (HCG) +Anteron (PMS)	10+25	0	10	100+250	19.7	0.5	140	0
Serotropin (PMS)	50	0	10	500	36.2	0.5	196	1
	200	0	10	2000	25.3	0.6	200	1
	100	2	7	700	33.5	1.0	160	0
Synahorin (HCG+APE)	20	0	10	200	59.2	0.9	156	1
	10	0	10	100	36.2	0.9	176	1
	10	0	10	100	20.4	0.25	204	1
	10	2	7	70	20.5	0.4	155	0
	10	2	7	70	19.9	0.3	215	1
	21	2	7	147	22.2	0.4	215	1
Prae-hormone (APE)	50	0	7	350	29.1	2.8	156	0
	50	0	8	400	28.4	0.7	150	0
	100	2	10	1000	30.5	1.0	160	0
Hypohorin (APE)	5	0	10	50	12.2	0.25	154	0
	10	0	10	100	14.9	0.2	160	0
	5	2	7	35	18.9	0.2	182	1
	10	2	7	70	20.5	0.6	157	0
Implantation of pituitary glands of goldfish	4-6 glands	1	7	34 glands	37.9	1.0	380	2
Injection with suspension of pituitary glands of goldfish	3-4 glands	1	8	25 glands (mean)	42.4	1.9	680	5
					38.8	1.3	450	3
					22.9	0.8	420	3
					23.3	0.4	273	1
Acetone-dried pituitaries of dog salmon	2-3 glands	1	6	14 glands	87.4	3.1	320	1
					25.0	0.7	230	1
Control					26.6	0.6	137	0
					16.1	0.25	107	0
					35.5	0.7	156	1
					24.9	0.4	140	0
					23.9	0.2	154	0
					27.0	0.4	196	1
					21.3	0.1	143	0
					15.8	0.1	171	1

0: yolkless; 1: early yolk vesicle; 2: late yolk vesicle; 3: primary yolk; 4: secondary yolk; 5: tertiary yolk.

yolkless oocytes (Fig. 39). The largest oocytes were 380 micra in diameter and had the thick zona radiata with clear striation. The follicular layer was also stimulated and had numerous dividing cells (Fig. 40). On the contrary, some ovaries of the controls contained only yolkless oocytes and others included a very few early yolk vesicle stage oocytes and corpora atresia, besides many yolkless oocytes (Figs. 36, 37). All oocytes in these ovaries were below 196 micra in diameter (Table 8). The fish injected with fresh pituitary suspension showed the same results. In three fish out of four, yolk globules were accumulated in the oocytes. One of them especially had the oocytes with many yolk globules, those being in the tertiary yolk stage (Fig. 38). The size of the largest oocyte was 608 micra in diameter (Table 8). The one other fish had a less stimulated ovary in which only early yolk vesicle stage oocytes were found.

Suspension of acetone-dried dog salmon pituitary glands showed also the same effects on the yolk formation of goldfish. The ovaries of the experimental fish were milky white. One of them had many oocytes in the yolk vesicle stage, the largest ones measured 320 micra in diameter. Dividing cells were also seen in the follicular epithelium of some oocytes (Fig. 41). In the other fish, the largest oocytes were 230 micra in diameter and they corresponded to the early yolk vesicle stage (Table 8).

All pituitary materials used proved to be effective for the induction of yolk formation of the goldfish, although there were some differences in efficiency between fresh goldfish pituitaries and the acetone-dried salmon pituitaries.

From these results it may be reasonable to conclude that the pituitary glands have the substance which stimulates the follicular epithelium and induces the oocytes to yolk accumulation.

4. Injection of mammalian pituitary gonadotrophin, human chorionic gonadotrophin and pregnant mare serum into hypophysectomized fish

As mentioned in the previous section, the pituitary gland of the goldfish has a substance which exerts an influence on the yolkless oocytes and stimulates follicle cells to form yolks. This study was carried out in order to determine the relation between this substance and FSH of mammals. Thirty-one females were hypophysectomized and reared for more than three weeks at 17°C to 23°C as in the previous section. Twenty-three of them were treated with the following gonadotrophins: 1) Gonatropin (HCG, Teikokuzoki), 2) Gonatropin+Anteron (PMS, Schering), 3) Serotropin (PMS, Teikokuzoki), 4) Synahorin (HCG+APE: anterior pituitary extracts of mammals, Teikokuzoki), 5) Prae-hormone (APE, Shionogi), 6) Hypohorin (APE, Teikokuzoki). These gonadotrophins were injected into the peritoneal cavity once a day. The dosage and number of injections were shown in Table 8. The other eight hypophysectomized fish served as controls.

1) Gonatropin (HCG)

Four hypophysectomized fish were injected once a day for 10 days continuously and two were injected every three days with 25, 50 or 100 units of Gonatropin. The experiments were made during July, November and December. The fish treated were killed on the day following the last injection. Their ovaries were very small, weighing 0.3 g to 1.1 g and yellowish in colour. They contained many corpora atresia (Figs. 42, 43, 44). In two of these fish, all oocytes remained in the yolkless stage and no oocyte with yolks was found in the ovaries (Figs. 42, 43). They were under 164 micra in diameter. In the other fish, very few oocytes with yolk vesicles were found in the ovaries but most oocytes were also in the yolkless stage (Fig. 44). The largest oocyte measured 215 micra in diameter.

2) Gonatropin (HCG)+Anteron (PMS)

A mixture of HCG (10 u) and PMS (25 u) was injected once a day into a hypophysectomized fish for ten successive days. The injected fish was killed on the day following the last injection. The ovary was yellowish in colour and very small, weighing 0.5 g. No oocyte with yolk was found in the ovary and all oocytes were under 150 micra in diameter (Fig. 45).

3) Serotropin (PMS)

Two fish were injected with 50 units and 200 units of Serotropin for ten successive days and one fish was injected seven times with 100 units every three days. The injection was made during November and December. An autopsy was made on the day following the last injection. Their ovaries were small, weighing 0.5–1.0 g and yellowish in colour. One of them had no oocytes with yolks but many corpora atresia and all normal oocytes remained in yolkless stage. The diameter of these oocytes was under 160 micra. In other fish, a few oocytes with yolk vesicles were found in the ovaries besides a large number of corpora atresia and oocytes in the yolkless stage (Figs. 46, 47). The largest oocyte was 200 micra in diameter.

4) Synahorin (HCG+APE)

Three fish were injected intraperitoneally with Synahorin for ten successive days. One fish was given 20 units per injection and the others 10 units per injection. Treatment was made in November and December. Ovary weights were 0.25–0.9 g. Many atretic oocytes were found in the ovaries. Yolk vesicles were not formed in the oocytes except in a few oocytes (Figs. 48, 49, 50). The cells in the follicular epithelium did not show division and all the oocytes were under 204 micra in diameter.

Three other fish were injected seven times with twenty-one or ten units of Synahorin every three days. One fish was done during November and the other

two fish were done during February. The ovaries of these fish were very small and weighing 0.3 g or 0.4 g. Histological observations revealed that the ovary of one fish contained no oocyte with yolk but the others contained somewhat large oocytes. The size of the largest oocytes was 215 micra and larger than those of the controls (Table 8). However, they were still in the early yolk vesicle stage which was also observed in the three control fish (Table 8). Therefore this gonadotrophin may have had a weak potency to induce the formation of yolks.

5) *Prae-hormone (APE)*

Three fish were injected with Prae-hormone. The injections of 50 units or 100 units per fish were made for seven or eight successive days during July in two fish, the other one was injected every three days during November and December. One fish which was injected 7 times continuously had a large ovary (2.8 g). Histological examinations showed that the ovary contained no normal yolk-laden oocyte and was filled with many atretic ones (Fig. 51). The largest oocytes were 156 micra in diameter. The other fish had a small ovary of 0.7 g or 1.0 g. All the oocytes remained in the yolkless stage (Fig. 52). The other histological characteristics were the same as those seen in the control fish (Figs. 36, 37). Therefore it may be said that this gonadotrophin had no effect on the yolk formation of goldfish.

6) *Hypohorin (APE)*

Four fish were injected with five or ten units of Hypohorin, two of them were done for ten successive days, the others every three days. The ovaries were very small and contained no oocyte with yolks except one (Table 8, Figs. 53, 54).

These results in comparison with controls showed that the gonadotrophins used in the present experiment had very little effect on the yolk formation of goldfish. It is well known that the gonadotrophins used, except HCG, are effective for the enlargement of follicles in mammals. Therefore, the present data suggests that the hormone which stimulates the follicles and induces yolk formation in the oocytes of goldfish may be of a different quality than the FSH of mammals.

5. Relationship between yolk formation and water temperature

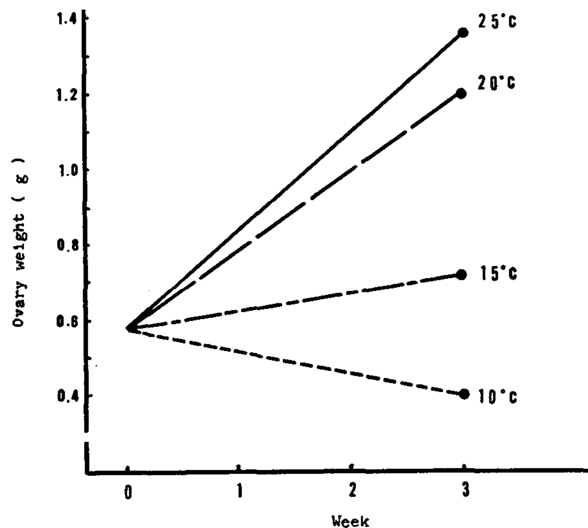
As shown in the previous section, the yolk formation of oocytes in the goldfish depends on the hormone secreted from the pituitary gland. It may be reasonably assumed that the effects of external factors must be translated first into quantitative changes in the secretion of hormone of pituitary glands.

Temperature may be the most important environmental factor affecting the physiology of poikilothermic animals. There is no excellent method to analyze directly the amount of pituitary hormone concerned with yolk formation.

In order to get some information about the secretion of this hormone at various water temperature, therefore, the degree of yolk accumulation was examined

by measuring the diameter of the oocytes in the fish cultured at various water temperatures.

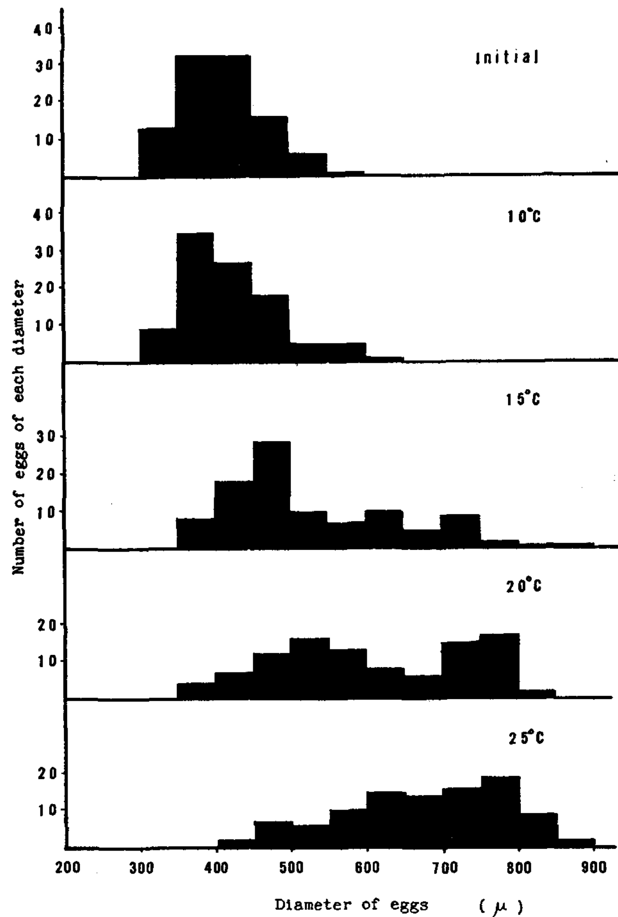
Forty fish were purchased from a commercial dealer and reared in an aquarium in the greenhouse for about two weeks at 13°C to 14°C. They were small in size and ranged from 4.4 cm to 5.6 cm in body length. These fish were divided into four groups, each consisting of ten fish. Each group was kept at 10°C, 15°C, 20°C and 25°C respectively. The lowest temperature (10°C) was not regulated completely but changed a little (9.7°C–11°C). The experiments were carried out from January 31 to February 20. After the fish had been kept in each temperature for three weeks, three females were picked up at random and killed. The body weight and gonad weight of the fish were recorded and pieces of ovaries were fixed with Bouin's solution. All ovaries in each group were almost the same in gross appearance. Therefore the pieces from the two fish were sectioned serially in each group and the diameter of the fifty largest oocytes was measured in each fish.



Text-figure 10. Graph showing the changes in ovary weight of goldfish subjected to various water temperatures

The change of ovary weight is shown in Text-figure 10. Mean ovary weight was 0.57 g in initial controls, while three weeks after, it was 0.4 g in the 10°C fish, 0.75 g in the 15°C fish, 12.0 g in the 20°C fish and 13.5 g in the 25°C fish. All the fish at 10°C showed no increase in ovary weight but the fish kept at above 15°C had markedly enlarged ovaries.

The histogram of oocyte diameter in each group is shown in Text-figure 11. Mean diameter was 556 micra in initial controls, 603 micra in the fish at 10°C,



Text-figure 11. Histogram showing the size of the most advanced oocytes in the ovaries of goldfish subjected to various water temperatures

873 micra at 15°C, 825 micra at 20°C and 889 micra at 25°C. This figure shows that yolk formation may be accelerated a great deal in the fish kept at above 15°C. In the fish kept at 20°C and 25°C for three weeks, yolk formation was nearly completed in a large number of oocytes. But in the fish kept at 25°C, some atretic oocytes were observed. Therefore, critically high water temperature for yolk formation is judged to be 25°C and the most suitable temperature for yolk formation may be 15°C to 20°C.

The difference in yolk formation between the fish at various water temperatures may be attributed in part to the quantitative changes in the secretion of pituitary hormone concerned with yolk formation. However, further studies must be done to reach a conclusion concerning this problem.

6. Discussion

The second growth phase of the oocytes is characterized by the formation of yolks. In goldfish the yolk formation begins in the oocytes of 150 micra in diameter. In most fish, this phase begins when the oocytes arrive at 100 to 200 micra in diameter (140 micra in *Asteanax mexcanus* (Bullough 1939), 150 micra in *Oryzias latipes* (T. S. Yamamoto 1955), 170 micra in *Liopsetta obscura* (Yamamoto 1956), 115 to 120 micra in *Scomber scomber* (Bara 1960), 190 micra in *Clupea harengus* (Polder 1961), 160 micra in *Rhodeus ocellatus* (Shirai 1962), 150 micra in *Plecoglossus altivelis* (Honma & Tamura 1962)). During this growth phase, two kinds of yolks are accumulated in goldfish; the first kind is the yolk vesicles, the second is the yolk globules. The formation of the yolk globules is dominant in the latter half of this phase. During this phase the oocyte diameter increases from 150 micra to approximately 900 micra, and the volume of the oocytes becomes about two hundred fold. This phase, therefore, occupies the main part of the oocyte growth. A further morphological characteristic of this phase lies in that the follicle cells increase much in number by mitotic division. The most intensive multiplication of the follicle cells is observed in the oocytes of 400 to 600 micra in diameter. In the oocytes above 750 micra and under 100 micra in diameter, division of the follicle cells is rarely found.

The oocytes showing such characteristics were found to be much effected by hypophysectomy. The oocytes of 350 to 700 micra in diameter were most sensitive to the hypophysectomy. The division of the cells in the follicular epithelium ceased and the thickness of the epithelium decreased immediately after hypophysectomy. Some follicle cells disintegrated after the operation. Then the oocytes with yolks were degenerated and finally absorbed.

On the other hand, the implantation or injection of the fresh pituitary gland of the goldfish or the injection of the suspension of acetone-dried pituitary glands of dog salmon into hypophysectomized fish more than three weeks after the operation the division of follicle cells resumed and stimulated the yolkless oocytes to the formation of yolks.

Therefore, it may be said with certainty that the pituitary gland of fish has follicle-stimulating hormone (FSH) which produces a multiplication of follicle cells and induces the second growth of oocytes through the accumulation of yolks.

It is said that the hormones do not produce a new biochemical reaction but rather influence the rate and intensity of the existing reaction (Williams 1962). In the present study, it could not be determined what biochemical reactions occurred in the oocytes. However, many investigators (Yamamoto T. S. 1955, Yamamoto K. 1958) using the histochemical method suggested that when yolk vesicles began to appear in the oocytes, conspicuous biochemical changes occurred in the oocytes.

Mitochondria and Golgi bodies increased in number with the beginning of yolk formation and extruded nucleoli were observed frequently at this time (Raven 1961). These bodies were thought to be very closely connected with the synthesis of the egg materials and metabolism (Raven 1961). These facts show that the intense biochemical reactions occur in the oocytes with the beginning of yolk formation. This intense reaction is postulated to be connected with FSH of the pituitary glands.

It is well known that pituitary glands of mammals also contain FSH which induces the enlargement of ovarian follicles through the secretion of liquor folliculi, the mitotic proliferation of follicle cells and so on (Chester Jones & Ball 1962).

In the present study, however, various gonadotrophins which have FSH activity of mammals were almost ineffective or very weak in activity for the stimulation of follicle cells and for the yolk formation in the oocytes of goldfish. Reviewing the literature relative to this point, von Ihering & Azevedo (1937), Hansen (1939), Matthews (1939), Landgrebe (1941) got negative results after the administration of mammalian pituitary glands or their extracts into various kinds of fish. Any fish to which they were administered did not mature. Johnson & Riddle (1939) injected FSH of mammals into rainbow trout but they obtained negative results for yolk formation. Pickford (1959) stated that hypophysectomized *Fundulus* are completely unaffected by mammalian follicle stimulating hormone. Witschi (1955) observed that the pituitary gland of fish has very little FSH activity for mammals. These facts may clearly show that the FSH of fish is very different qualitatively from the FSH of mammals.

The FSH of fish stimulates vitellogenesis in oocytes. This is, however, different from Kazanskii's hormone of vitellogenesis because the acetone-dried pituitary gland of dog salmon (*Oncorhynchus keta*) was effective for the formation of yolks in the hypophysectomized fish as shown in the present experiment. According to him, stickleback's pituitary gland contained a special hormone concerned with vitellogenesis which was destroyed by the treatment with acetone (cited from Pickford & Atz 1957).

At present, nothing is known with certainty about the mechanisms of the secretion of FSH in fish. But from the literature concerning this, it is very clear that various environmental factors affect the maturation of fishes. The present study demonstrated that at 20° and 25°C water temperatures yolk formation was completed in fish in three weeks, about four months before normal spawning season, while at 10°C, little changes in the gonadal condition were discerned in three weeks. Regarding the retarding effects of lower water temperature, it is uncertain whether the secretion of FSH from the pituitary gland is inhibited or not. However, if

exogenous gonadotrophin is administrated to fish, the fish become ripe at temperature below the critical ones (Pickford & Atz 1957). Therefore, there is a possibility that the retardation of yolk formation under lower water temperature may result from a deficiency of FSH secretion from the pituitary gland.

Part V. Ovulation

The first experiment concerning the artificial ovulation of fish was performed in 1931 by Houssay. He injected pituitaries from other teleost fish into the species of small size, *Cnesterodon decemmaculatus*. One or three days after the injection, most of the fish ovulated. Since then numerous experiments concerning the acceleration of ovulation were performed in fish by many workers using pituitary glands or their extracts taken from fish or other vertebrates. Further, the fact that the ovulation of fish is under the control of pituitary glands has been clearly demonstrated by Vivien (1941) and Yamazaki (1962). They removed the pituitary gland from female gobies and goldfish just before spawning and thereby ovulation was prevented from occurring normally. As for the hormonal control of ovulation in fish, however, many problems still remain unsolved. This study aims to get some information on the problems using goldfish as material. All the experiments concerned with this part were carried out during the spawning seasons from 1961 to 1963.

1. Morphological changes of ovarian eggs associated with ovulation

In the natural condition, the ovulation in goldfish takes place during the night, so that from 8 to 11 p.m., ovaries were obtained from six fish which had been expected to ovulate, and then they were fixed with Bouin's solution. Serial sections were made in each ovary by the usual paraffin methods and stained with Delafield's haematoxylin and eosin. From the observations of these ovaries, it was found that the characteristic stages of ovarian eggs associated with ovulation in goldfish were the migratory nucleus stage and the pre-maturation stage. Discharge of ovarian eggs from follicles into ovarian lumen appeared to take place in only a few minutes.

1) Migratory nucleus stage

The oocytes in this stage have about the same size as those in the tertiary yolk stage and ranged from 650 to 1000 micra in diameter. The nucleus of the egg is moving toward the animal pole where a clear, wedge-shaped micropyle is situated and yolk vesicles are absent. Around the nucleus there is frequently found a viscid substance. Nucleoli are few in number and round in shape. They are situated in the inner part of the nucleus or close to the nuclear membrane. The yolk globules become enlarged in size, large ones being 26 micra in diameter. The zona radiata of about 7 to 8 micra in thickness has clear radial striations (Fig. 55). Divisions of follicle cells are never seen in the epithelium.

2) Pre-maturation stage

This stage is characterized by the nuclear change in the eggs. The eggs have the same size as those in the previous stage. After the nucleus has arrived at the animal pole, the nuclear membrane disappears suddenly and no boundary can be seen between the nucleoplasm and cytoplasm (Figs. 56, 58). The nucleoli take a complicated form and then lose their affinity to stain and finally fade away. Chromatin elements are found distributed in the cyto-nucleoplasmic mass of the animal pole where a large micropylar cell having a nucleus with a few distinct nucleoli is found plugging the micropyle canal (Fig. 58). Numerous eggs in this stage are found in the ovaries of fish obtained just before ovulation. During this stage living eggs change in nature from opaque to translucent. The ovulated eggs present in the ovarian lumen are ripe in condition.

3) Ripe eggs

Ripe eggs are easily extruded from the genital pore by only a slight pressure against the belly. These eggs are adhesive in living condition. This adhesive nature of the eggs appears to be attributed to the very thin membrane lying on the outermost part of the eggs. In a fixed condition, the yolk globules of large size are enclosed by the cortical cytoplasm which is thick at the animal pole and becomes thin toward the opposite pole. The cortical alveoli are found embedded in the cortical cytoplasm. Between yolk globules a network of cytoplasm connected with the cortical layer may be discernible. At the animal pole the micropyle is seen running through the zona radiata of 13 micra in thickness (Fig. 57).

2. Effects of mammalian pituitary gonadotrophin, human chorionic gonadotrophin and pregnant mare serum on ovulation

The present experiment was carried out during May and June. Fish used were 7.2 cm to 13.4 cm in body length. They were injected intraperitoneally with the following four preparations of gonadotrophin, 1) Gonatropin (HCG), 2) Synahorin (HCG+APE), 3) Hypohorin (APE), 4) Serotropin (PMS). All these preparations are of Teikokuzoki Co.. The experimental fish had been kept in the indoor aquarium at about 14°C water temperature and after injection they were transferred into the outdoor aquarium whose water temperature was maintained at 17°C to 19°C. Neither males nor aquatic plants were placed together with the injected fish in the aquarium in order to prevent the occurrence of spawning.

1) Gonatropin

Seven fish were injected with 20 unit or 30 unit HCG per 10 g of body weight. They had a very large belly and their mean ponderal index (BW/BL^3) was 0.0572. The results are presented in Table 9. Six fish out of seven ovulated and ripe eggs were held in the ovarian lumen on the day following the injection. Ovulated eggs weighed 0.3 g–12.0 g per fish. The weight of the ovulated eggs was approximately

Table 9. Effects of Gonatropin (HCG) and Synahorin (HCG+APE) on the ovulation of goldfish

	Fish No.	B. L. (cm)	B.W. (g)	Ponderal index	U. per 10 g B.W.	Total unit	Ovulation	Weight of ovulated eggs (g)	Days from injection to ovulation
Gonatropin	1	12.0	93.0	0.0538	30	279.0	+	12.0	1
	2	10.1	58.0	0.0563	30	180.0	+	3.5	1
	3	9.0	37.2	0.0510	30	120.0	+	5.3	1
	4	6.9	26.7	0.0813	30	90.0	+	2.6	1
	5	9.3	34.5	0.0429	20	69.0	+	0.3	1
	6	8.9	40.7	0.0577	20	80.0	+	4.1	1
	7	10.3	62.3	0.0570	20	120.0	−		
Synahorin	1	8.9	40.0	0.0567	10	40.0	+	4.5	1
	2	11.0	59.9	0.0450	5	29.9	+	1.9	1
	3	10.0	55.0	0.0534	5	27.5	+	4.7	1
	4	9.3	38.0	0.0472	5	19.0	+	1.4	1
	5	8.5	34.8	0.0567	5	17.4	+	2.8	1
	6	10.9	74.8	0.0578	5	37.4	−		

in proportion to body weight. One fish had not ovulated even two days after injection. From this data it may be said that HCG injections of more than 20 units per 10 g of body weight are very effective for the ovulation of goldfish.

2) Synahorin

Six fish were injected with 5 units or 10 units of Synahorin per 10 g of body weight. Their ponderal indices were 0.0528 on the average. The results obtained are presented in Table 9. Five fish out of six responded to the injection and ovulated on the day following the injection. The weight of ovulated eggs varied from 1.4 g to 4.7 g per fish. The difference in ovulated egg weights between individual fish appears to depend mainly on the difference in the ponderal index. The results obtained show that Synahorin injections more than 5 units per 10 g of body weight are effective for ovulation and thereby ovulation of goldfish takes place on the day following the injection.

3) Hypohorin

Five fish were used for the test of Hypohorin. Their bellies were large due to the enlargement of ovaries and the mean ponderal index of the fish was 0.0576. Five units and 7.5 units of Hypohorin per 10 g of body weight were administrated to these fish. The results are presented in Table 10. One fish out of five ovulated on the next day and two fish ovulated two days after the injection. The ovulated eggs were 2.8 g to 4.5 g. The ovulated fish were only those injected with 5 units per 10 g of body weight. Those with 7.5 units per 10 g of body weight made no response to the injections. This data may indicate that this preparation has

lower potency for ovulation than the previous two preparations.

4) Serotropin

Seven fish were used for the experiment of Serotropin. They were also gravid and their ponderal index was 0.0543 on the average. They were injected with 10 units, 30 units, 50 units or 100 units of Serotropin per 10 g of body weight. The results obtained are presented in Table 10. Only two fish out of seven ovulated

Table 10. Effects of Hypohorin (APE) and Serotropin (PMS) on the ovulation of goldfish

	Fish No.	B. L. (cm)	B.W. (g)	Ponderal index	U. per 10 g B.W.	Total units	Ovulation	Weight of ovulated eggs (g)	Days from injection to ovulation
Hypohorin	1	8.0	28.8	0.0563	7.5	21.0	−		
	2	7.7	28.5	0.0614	7.5	18.0	−		
	3	8.9	39.8	0.0565	5	20.0	+	4.5	2
	4	7.1	19.3	0.0539	5	9.7	+	2.8	2
	5	8.5	36.8	0.0599	5	18.2	+	3.4	1
Serotropin	1	8.9	40.6	0.0576	100	406.0	−		
	2	7.2	18.0	0.0482	100	180.0	−		
	3	7.7	26.3	0.0576	50	145.0	+	0.3	1
	4	11.1	70.3	0.0514	50	365.0	−		
	5	8.6	40.3	0.0634	30	129.0	−		
	6	13.4	117.3	0.0488	30	352.0	+	10.3	2
	7	8.1	28.2	0.0531	10	28.2	+	2.8	1

on the next day, and one fish of 30 unit group ovulated two days after the injection. No fish of the 100 unit group ovulated even two days after the injection. This data may indicate that this preparation is also less effective for ovulation than Gonatropin and Synahorin.

3. Effects of Gonatropin (HCG) on the ovulation of the fish once-spawned

The experiments were carried out during the period from July 1 to 7. Twenty-three fish which seemed to have finished spawning were chosen and injected intraperitoneally with Gonatropin once a day. Whether ovulation had taken place in the fish or not was checked every morning before the injection. At the time of the first injection, the body length and body weight of the fish were also recorded.

Eight fish were treated with 50 units of the preparation per injection. The results are presented in Table 11. The mean ponderal index of this group was 0.0472. These fish were not so conspicuous in abdominal enlargement. Three fish ovulated on the day following the first injection and one ovulated after two injections. The others showed no ovulation even after three or five injections.

Eight fish were used for the test with 25 units of Gonatropin. The results

Table 11. Effects of the HCG injection on the ovulation of intact once-spawned goldfish

Fish No.	B. L. (cm)	No. of injection (each 50 u.)	Total units injected	Ovulation	Days to ovulation from first injection
1	8.9	1	50	+	1
2	8.2	1	50	+	1
3	8.1	1	50	+	1
4	9.6	2	100	+	2
5	9.7	3	150	Partial	3
6	9.7	3	150	−	
7	8.1	5	250	−	
8	9.0	5	250	−	

Table 12. Effects of the HCG injection on the ovulation of intact once-spawned goldfish

Fish No.	B. L. (cm)	No. of injection (each 25 u.)	Total units injected	Ovulation	Days to ovulation from first injection
1	10.2	1	25	+	1
2	7.9	1	25	+	1
3	8.7	1	25	+	1
4	7.9	2	50	+	2
5	8.3	2	50	Partial	2
6	9.1	2	50	−	
7	10.2	3	75	+	4
8	9.3	3	75	+	4

Table 13. Effects of the HCG injection on the ovulation of intact once-spawned goldfish

Fish No.	B. L. (cm)	No. of injection (each 10 u.)	Total units injected	Ovulation	Days to ovulation from first injection
1	8.6	2	20	Partial	2
2	8.8	2	20	−	
3	8.9	2	20	−	
4	8.4	2	20	−	
5	9.2	5	50	−	
6	8.5	5	50	−	
7	9.2	6	60	−	

obtained were presented in Table 12. The mean ponderal index of this group was 0.0518 which was larger than that of the previous group. In this group, three fish ovulated on the day following the first injection, one ovulated after two injections and two others after 3 injections. The other two fish did not respond to the second injection.

Seven fish were treated with 10 units of HCG. The results obtained are presented in Table 13. The mean ponderal index of this group was 0.0519. No fish treated made response to the injection.

4. Effects of hypophysectomy on ovulation

Nine fish void of ripe eggs in their ovarian lumen but were being pursued by males were chosen from the indoor aquarium. Five fish out of nine were hypophysectomized and the other had a mock operation. The operations were performed in the early morning. After the operation they were put into 0.2 per cent cooled saline water (about 6°C) and kept in it for 3 hours. During this time the water temperature in the aquarium was raised gradually to 12.0°C. Then the fish were transferred into an outdoor aquarium of 130 cm×60 cm×45 cm, which was divided into two compartments with a wire-netting partition and set with a bunch of aquatic plants and active males in each compartment. The water temperature of the outdoor aquarium ranged from 17°C to 19.1°C. The fish were observed for five days to determine whether or not they ovulated. Then they were killed. The ovulated fish were killed on the day that ovulation was observed. The body length, body weight and gonad weight were recorded. The ovaries were fixed with Bouin's solution and cut ten micra in thickness, then stained with Delafield's haematoxylin and eosin. The ponderal index was measured in each fish.

The belly was enlarged in nearly all the fish except one of both groups. In the hypophysectomized group, the ponderal index ranged from 0.0472 to 0.0581,

Table 14. Effects of hypophysectomy on the ovulation of goldfish

Operation	Fish No.	B. L. (cm)	B.W. (g)	Ponderal index	Ovulation	Days to ovulation	G.W. (g)	$\frac{GW \times 100}{BW}$	Atretic oocytes
H. sectomy	1	8.6	36.4	0.0572	−		2.5	20.6	many
	2	8.4	28.0	0.0472	−		4.7	16.8	some
	3	10.0	58.1	0.0581	−		13.6	23.4	some
	4	8.0	26.8	0.0523	−		6.1	22.8	some
	5	7.5	21.9	0.0519	−		3.9	17.8	some
Mock	1	9.3	42.1	0.0535	+	2	7.2	17.1	some
	2	7.5	20.3	0.0481	+	1	2.5	12.3	many
	3	9.8	47.2	0.0501	+	1	4.3	9.1	non
	4	7.7	29.1	0.0637	+	2	1.2	4.1	non

the mean being 0.0535. In the mock control, the value ranged from 0.0481 to 0.0637 and the mean was 0.0536. Therefore no difference in the ponderal indices of both groups was found (Table 14).

Soon after putting the fish into the outdoor aquarium, all of them were pursued actively by males. But the hypophysectomized fish did not ovulate even after five days, and at autopsy, no eggs were found in the ovarian lumen. On the other hand, the mock controls ovulated and spawned naturally after one or two days (Table 14). At the end of the experiment, gonad indices of hypophysectomized fish ranged from 16.8 to 22.8 while those of the mock controls measured from 4.1 to 17.1. This decrease in gonad index of the mock control may be due to spawning. Microscopical observations on the ovaries of hypophysectomized fish revealed that no empty follicles were present in the ovaries while numerous normal eggs in the tertiary yolk stage and a few atretic eggs were found within them (Fig. 59). Exceptionally, one fish had many atretic oocytes in the ovary. On the contrary, many empty follicles were found in the ovaries of the mock control group (Fig. 60). All ovaries except one contained only a few atretic oocytes.

5. Effects of hypophysectomy just before ovulation

In a natural condition, the ovulation takes place at night. Therefore the hypophysectomy was performed during 9 p.m. to 11 p.m. The fish operated on were placed in an aquarium of 20 liter volume by the next morning. The total number of the fish operated on was twenty-eight. The results are summarized in Table 15.

Table 15. Ovulation in hypophysectomized goldfish

Date of operation	No. of fish operated on	No. of ovulated fish
June 15	8	2
June 16	11	3
June 17	9	1
Total	28	6

On June 15, eight fish were hypophysectomized and two fish ovulated the next morning; on June 16 eleven fish were hypophysectomized and three fish ovulated; on June 17 nine fish were operated and one fish ovulated the next morning. In total, six fish out of twenty-eight could ovulate even after hypophysectomy.

These facts may indicate that the pituitary hormone necessary for ovulation has been discharged from the organ several hours before actual ovulation.

6. Effects of HCG on the ovulation of hypophysectomized fish

It was found in the previous experiment that the Gonatropin and Synahorin are very effective for the induction of ovulation in goldfish. However whether

these gonadotrophins exert their effect directly on the ovaries or indirectly the effects are mediated by the pituitary gland of injected fish, is uncertain. Therefore it is necessary to administer HCG to hypophysectomized goldfish in order to settle the problem.

Gonatropin (HCG) was used in the present study. The goldfish were hypophysectomized during the spawning season. Hypophysectomized fish after one to four days following the operation were divided into three groups and the fish of each group were injected intraperitoneally once a day with 50 units, 25 units and 10 units of Gonatropin. The body length and body weight were recorded before the first injection. After the injection they were kept in the aquaria of 30 cm × 45 cm × 30 cm without males but a small bunch of aquatic plants. The water temperature of the aquaria was at about 18°C. Ovulation was checked every day in the morning before injection.

Fifty units of Gonatropin were injected into fourteen hypophysectomized goldfish once a day. The results are presented in Table 16. The mean ponderal index of this group was 0.0537. Seven fish ovulated on the day following the first injection and one fish ovulated two days after the first injection. The other fish did not ovulate even after two to four injections. The percentage of ovulated fish to the total fish was 57.1. The volume of ovulated eggs was nearly the same as those of intact fish and the eggs were fertilized normally.

Nine hypophysectomized fish were injected with 25 units of Gonatropin. The

Table 16. Effects of the HCG injection on the ovulation of hypophysectomized goldfish

Fish No.	B. L. (cm)	Days to first injection from hypophysectomy	No. of injection (each 50 u.)	Ovulation	Days to ovulation from first injection
1	7.3	1	1	+	1
2	7.1	1	1	+	1
3	8.6	2	1	+	1
4	8.8	2	1	+	1
5	10.3	2	1	+	1
6	9.1	2	1	+	1
7	8.9	2	1	+	1
8	8.4	1	2	+	2
9	7.9	1	2	−	
10	8.3	4	2	−	
11	8.4	4	2	−	
12	9.6	2	3	−	
13	9.0	2	3	−	
14	8.4	1	4	−	

Table 17. Effects of the HCG injection on the ovulation of hypophysectomized goldfish

Fish No.	B. L. (cm)	Days to first injection from hypophysectomy	No. of injection (each 25 u.)	Ovulation	Days to ovulation from first injection
1	7.8	2	1	+	1
2	8.4	2	1	+	1
3	9.9	2	1	+	1
4	9.2	4	1	+	1
5	9.1	4	2	−	
6	10.0	2	2	−	
7	10.0	2	3	−	
8	9.0	2	3	−	
9	10.0	2	3	−	

Table 18. Effects of the HCG injection on the ovulation of hypophysectomized goldfish

Fish No.	B. L. (cm)	Days to first injection from hypophysectomy	No. of injection (each 10 u.)	Ovulation	Days to ovulation from first injection
1	8.5	4	4	−	
2	9.9	2	3	−	
3	9.4	2	3	−	
4	6.7	2	3	−	
5	8.6	2	3	−	
6	7.3	2	3	Partial	3
7	8.4	2	5	−	
8	7.9	2	5	−	

mean ponderal index was 0.0548. Four fish ovulated on the day following the first injection. The other fish did not ovulate even after two or three injections. The ovulated fish were 44.4 per cent (Table 17).

Eight hypophysectomized fish were treated with 10 units of Gonatropin. The mean ponderal index was 0.0553. The results are presented in Table 18. In this group, normal ovulation was not observed even after more than two injections. After three injections only one fish ovulated a small number of eggs which were extruded by pressure on the belly.

7. Effects of progesterone on the ovulation

Wright (1961) stated that an important function of hypophyseal hormones at the time of ovulation of frogs is to stimulate the secretion of a steroid which may bring about extrusion of ova, and progesterone is the most active material among various tested steroids. Recently, progesterone has been identified in fish ovaries.

(Chieffi & Lupo 1963, Dean & Chester Jones 1959). However, at present the function of progesterone in relation to fish reproduction is still uncertain. The present experiment was carried out to determine the effects of progesterone on ovulation of goldfish.

Fifteen gravid fish were used for the present study. They were divided into three groups, of five each. Each group of fish was kept in an aquarium of 30 cm ×45 cm×30 cm, whose water temperature was regulated at 20°C. The first group was injected intraperitoneally with 0.25 *iu* of progesterone per 10 g of body weight, the second with 0.5 *iu* and the third one with 0.75 *iu*. The first injection was made on March 22 and the second on March 29. This date was about two months before the normal breeding season. To compare the ovulation inducing potency of progesterone with that of HCG, 30 units of HCG per 10 g of body weight were injected into another group of seven fish. Ovulation was checked every day and ovulated eggs were weighed. Males and aquatic plants were not placed in the experimental aquaria.

In the first group, two fish ovulated on the following injection; one of them responded to the first injection, the other to the second injection (Table 19). The weight of ovulated eggs was 0.05 g and 0.2 g respectively. In the second group, only one fish ovulated on the day following the second injection and the ovulated

Table 19. Effects of progesterone and HCG on the ovulation of goldfish

	Fish No.	Body weight (g)	First injection per 10 g body weight	Days to ovulation						Second injection per 10 g body weight	Days to ovulation				Weight of ovulated eggs (g)
				1	2	3	4	5	6		1	2	3	4	
Progesterone	1	11.5	0.25 iu	−	−	−	−	−	−	0.25 iu	+				0.05
	2	8.6	0.25 iu	−	−	−	−	−	−	0.25 iu	−	−	−	−	
	3	10.5	0.25 iu	−	−	−	−	−	−	0.25 iu	−	−	−	−	
	4	16.3	0.25 iu	+											0.2
	5	8.8	0.25 iu	−	−	−	−	−	−	0.25 iu	−	−	−	−	
	6	7.0	0.5 iu	−	−	−	−	−	−	0.5 iu	−	−	−	−	
	7	9.0	0.5 iu	−	−	−	−	−	−	0.5 iu	−	−	−	−	
	8	8.0	0.5 iu	−	−	−	−	−	−	0.5 iu	−	−	−	−	
	9	11.0	0.5 iu	−	−	−	−	−	−	0.5 iu	−	−	−	−	
	10	13.5	0.5 iu	−	−	−	−	−	−	0.5 iu	+				0.1
	11	9.4	0.75 iu	+											0.2
	12	11.5	0.75 iu	−	−	−	−	−	−	0.75 iu	−	−	−	−	
	13	10.6	0.75 iu	−	−	−	−	−	−	0.75 iu	−	−	−	−	
	14	9.2	0.75 iu	−	−	−	−	−	−	0.75 iu	−	−	−	−	
	15	12.8	0.75 iu	−	−	−	−	+							0.4
HCG	1	9.6	30 u	+											0.3
	2	9.7	30 u	+											0.8
	3	8.8	30 u	+											0.3
	4	14.0	30 u	+											0.1
	5	8.0	30 u	+											0.5
	6	7.7	30 u	+											0.3
	7	6.4	30 u	−											

eggs weighed 0.1 g. In the third group, two fish ovulated. The weight of ovulated eggs was 0.2 g and 0.4 g respectively. In total, the progesterone injection brought about ovulation in five fish out of fifteen and the mean weight of ovulated eggs was 0.19 g. On the other hand, six fish out of seven responded to the injection of HCG and ovulation took place on the day following injection. The weight of ovulated eggs ranged from 0.1 to 0.8 g, being 0.38 g on the average. As no remarkable difference was discerned in body weight between the progesterone group and the HCG group, the results obtained suggest that progesterone has the weaker effect on ovulation.

8. Discussion

In mammals, it is well known that ovulation is induced by the synergic action of FSH and LH. Especially LH acts to produce ovulation. Further it is said that the LH secretion is under the control of the sympathetic and parasympathetic nervous systems and directly of the nucleus of hypothalamus (Takewaki 1953).

The present study showed that the ovulation of fully matured goldfish is completely inhibited by hypophysectomy. The same result was obtained by Vivien (1941) in gobies. On the other hand a great many workers have been able to induce ovulation in various kinds of fish by the administration of fish pituitary materials (Pickford & Atz 1957). These facts clearly indicate that the ovulation of fish is directly dependent on the pituitary gland.

Morphological changes of the oocytes associated with ovulation are characterized by nuclear changes. In goldfish, the migration of the nucleus to the animal pole takes place simultaneously with the disappearance of the nuclear membrane and appearance of prophase chromosomes. Following these nuclear changes, the oocytes are released from the follicular envelope to the ovarian lumen. The same nuclear changes in the oocytes just before ovulation have been described in a loach, by Kawamura & Motonaga (1950). They observed that after the treatment with pituitary materials the migration of the nucleus to the animal pole occurred in three hours and the disappearance of the nuclear membrane in five hours. Therefore it seems a rule that the nuclear changes and ovulation take place in eggs several hours after enough ovulation hormone has been secreted from the pituitary gland.

The present experiment also showed that some hypophysectomized fish operated on a few hours before ovulation could do a normal ovulation. Therefore it may be reasonable to conclude that actual ovulation needs some time after the secretion of pituitary ovulation hormone has been completed. It is said that the time difference between the accomplishment of pituitary secretion and actual ovulation is 9 to 11 hours in the rat and 9 hours in the rabbit (Takewaki 1953). This time difference in goldfish is assumed to be several hours, although it could not be

determined exactly in the present study.

It is known that the ovulation of goldfish is easily stimulated by the active courtship of the male, the presence of aquatic plants, or rapid changes in water temperature or water condition. These facts suggest that the secretion of the ovulation hormone of the pituitary gland is under the control of the nervous system. This secretion may be mediated by hypothalamus as in mammals.

In the present study, the hormone preparations tested may be placed in the following order thanks to their potency for ovulation: PMS<APE<HCG. The similarity of HCG to LH has long been recognized. APE may contain LH. But PMS does not have the action of LH. Therefore the ovulation inducing potency of the preparation could well be ascribed to its LH-like properties. This is supported by Ball's information (1960) that purified mammalian LH will induce ovulation in mature *Misgurnus fossilis* in both intact and hypophysectomized individuals, whereas FSH, prolactin and ACTH is totally ineffective in the same test.

As mentioned previously, the pituitary gland of the goldfish secrets FSH. Therefore it may be concluded that there are two kinds of gonadotrophins secreted from the goldfish pituitary gland, one is FSH and the other is an LH-like ovulation hormone which is responsible for nuclear changes and follicular rupture.

Witschi (1955) identified both FSH and LH from the pituitary glands of *Lepisosteus* and salmon, and reported that LH is relatively rich and FSH is very poor as compared with birds and mammals. Many workers succeeded in inducing artificial ovulation of fish by using mammalian gonadotrophins such as Damas (1933) in *Petromyzon fluviatilis* with ether extracts of pregnant urine and extracts of the anterior pituitary of mammals. Morosova (1936) in *Perca fluviatilis* with pregnant urine and Prolan (HCG), Khan (1938) in *Cirrhina nirigala* with extracts of mammalian pituitary, Ichikawa & Kawakami (1948) in *Cyprinus carpio* with Gonatropin (HCG), Ramaswami & Sundararaj (1957) in *Heteropneustes* with HCG, Ramaswami & Lakshman (1958) in *Heteropneustes* with mammalian FSH and LH, Sneed & Clemens (1959) in goldfish and channal catfish with HCG, and Suzuki & Mitsuya (1964) in *Misgurnus anguillicaudatus* with HCG. These facts are not contradictory to the supposition that the ovulation hormone in fish is an LH-like hormone.

In the present study, hypophysectomized fish responded to the first injection of HCG and ovulation took place on the day following the injection, and only one fish responded to further injections, whereas the intact fish which had finished its first spawning responded to the injection more than the second. This may be due to the fact that HCG does not induce vitellogenesis in oocytes. Therefore the LH-like ovulation hormone is only effective when the fish arrives at a certain stage of maturity. It is uncertain whether the ovulation is induced only by the single

action of the ovulation hormone or by the synergic action of both FSH and the ovulation hormone. But it may be assumed from knowledge of mammals that the two hormones may be synergic.

Wright (1961) has shown that *in vitro* ovulation induced in ovarian fragments of *Rana pipiens* with frog pituitary may be facilitated by simultaneous exposure of the ovary to progesterone. He concluded that the important function of pituitary hormone at the time of ovulation in the frog is to stimulate the secretion of progesterone or of a close derivative which brings about the extrusion of ova. Edgren and Carter (1961) reported that the combination of progesterone 0.1 mg and CG 3 *iu* produced a higher proportion of *in vitro* ovulation than progesterone or CG alone produced. Kirshenblat (1952) found that progesterone and DOCA were the most effective and caused ovulation in all the fish treated. Methyl testosterone caused nuclear maturation but oestradiol benzoate and oestrone were all negative. Ball (1960) who reviewed the effects of steroid on the ovulation stated that gonadal steroid may play an essential part in the ovulatory mechanism. On the other hand, it is shown from the present study that 0.25–0.75 *iu* of progesterone had slight potency to induce ovulation. Therefore, further works are needed to elucidate the significance of progesterone or other sex steroids on the ovulation in fish.

As stated above, the ovulation hormone in fish is thought to resemble the LH of mammals. The LH causes formation of corpus luteum in mammals as well as ovulation. Corpus luteum-like structures are also found in various kinds of fish (Wallace 1904, Matthews 1938, Bretschneider & Duyvene de Wit 1947, Beach 1959, Shirai 1962). Examining the evidence for the presence of the corpora lutea in fish, Pickford and Atz (1957) asserted that no direct evidence has yet been submitted to show that the so-called corpora lutea have a secretory function. Hisaw and Hisaw (1959) stated that in elasmobranch the corpus luteum seems to have not yet adapted itself as an endocrine gland for the production of steroid. Honma *et al.* (1962) also declared the same opinion in *Plecoglossus altivelis*. Many atretic eggs which appeared in hypophysectomized goldfish resemble the pre-ovulation corpora lutea as described by Bretschneider and Duyvene de Wit (1947), Beach (1959) and others. Thus, it is very doubtful whether the formation of the corpus luteum-like structure is under the control of the pituitary gland or not. Therefore, at present, Witschi's (1955) conclusion "induction of ovulation is a much more general and more ancient function of LH than is its role in the formation of corpora lutea throughout the vertebrates" is acceptable.

Part VI. Oviposition

As in other vertebrates, the endocrine secretion of gonads in fish is known to affect morphological characteristics such as nuptial or breeding color, gonopodia of

viviparous poeciliids, ovipositor of bitterling and other structures associated with reproduction. Males of stickleback (Ikeda 1933) and Japanese bitterling (Tozawa 1929) lose all spawning and nest building behavior after castration. The evidence that the spawning behavior is dependent upon the secretion of gonad is also supported by the results of the administration of the gonadal steroid (Pickford & Atz 1957). However, some investigators such as Tavolga (1955) reported that the courtship or sexual behavior was not reduced by castration. There are some evidences that sexual or spawning behavior is directly concerned with the secretions of hypophyses. Tavolga (1955) reported that all the pre-spawning behaviors were abolished immediately after hypophysectomy. Wilhelmi *et al.* (1955) demonstrated that neurohypophysial substances induce the rapid occurrence of the spawning reflex, when the substances were injected into *Fundulus heteroclitus*. Moreover, Egami (1959) found that the injection of a mammalian neurohypophysial extract causes not only the spawning reflex in both males and females of *Oryzias latipes*, but also oviposition in females. However, Yamazaki (1962) has reported in goldfish that the spawning behavior was not inhibited by hypophysectomy and the normal oviposition and spawning behavior of males occurred even after hypophysectomy.

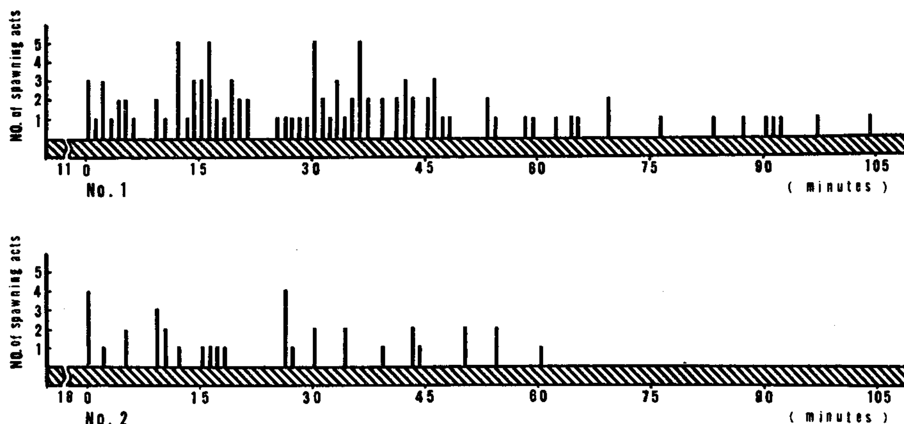
As mentioned above, sexual behavior in fish involves complicated problems connected with the secretions of the pituitary gland and gonads. The present study was carried out to get further light on the problems concerned with spawning behavior, especially on the relationship between the secretions of the pituitary gland and oviposition.

1. General description of spawning behavior and some factors affecting the spawning

Under normal conditions, the spawning activity of females begins after the courtship of males, *i.e.*, the male earnestly follows the female attaching his head to the side of her belly. This activity continues usually for one or two days and induces ovulation in the female. Early on the following morning, when things become visible, males begin to pursue the ovulated female. With approaching light, males become more active in pursuit and the female approaches the aquatic plants with the males. Bending her body rapidly, she oviposites some eggs. Simultaneously the males vibrate their bodies and ejaculate the genital products. By means of the vibration of tails and bodies of both sexes, eggs are scattered around and adhere to aquatic plants or other substitutes. The female quits there with males. These spawning acts are usually repeated from several to several hundred times in a few hours.

In Text-figure 12, the frequency of the spawning acts of two fish is shown. The frequencies were recorded every minute.

The body length of No. 1 fish was 8.9 cm. In this fish, the spawning act be-



Text-figure 12. Graphs showing the frequency of spawning acts of goldfish

gan 11 minutes after pairing with male. The total spawning acts were 102 and the duration of the spawning, which means the time from the first oviposition to the last, was 104 minutes. The highest activity was seen 12, 16, 30 and 36 minutes after the first spawning act (Text-fig. 12). After one hour, the frequency of the spawning was greatly reduced. In general, the frequency of these spawning acts may be changed cyclically.

The body length of the No. 2 fish was 7.1 cm. The total number of spawning acts was 35 and the duration of the spawning was 61 minutes. During this time all the eggs in the lumen were oviposited and no spawning act was observed after one hour. In this fish the frequency of the spawning act was small as compared with that of the previous one. This may be due to the difference in body size which affects the number of ovulated eggs.

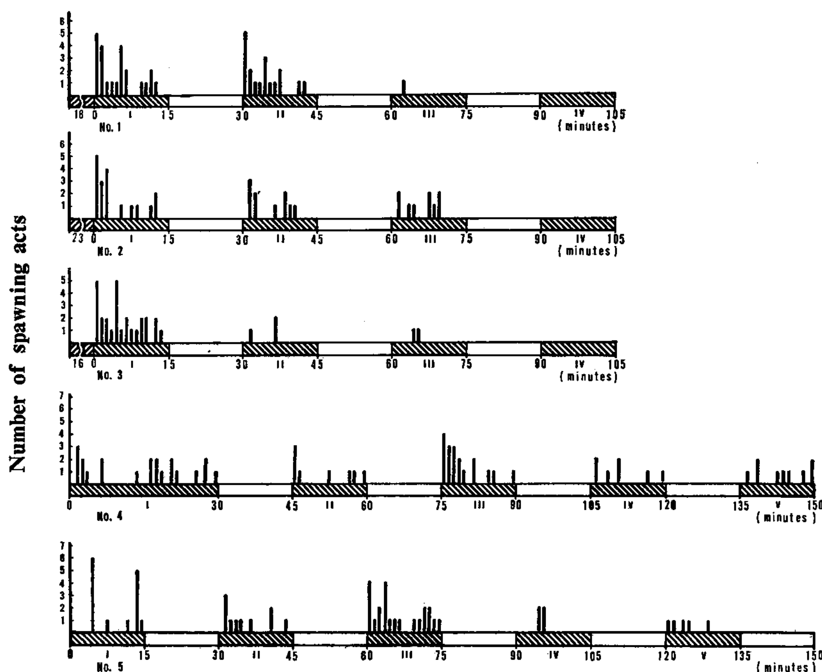
Table 20. Ovulation and oviposition of the female with or without the male

	Fish No.	Ovulation	Days to ovulation	Oviposition
	1	+	2	+
	2	+	3	+
♀ ♂	3	+	4	+
	4	+	2	+
	5	+	3	+
	1	+	2	−
	2	+	2	−
♀	3	+	2	−
	4	+	2	−
	5	+	3	−

The spawning acts of females are generally inhibited if males are not present or unless the males are active. This is shown by the following experiment.

Ten gravid females kept in the aqualium of about 14°C were transferred into the aquarium of 20°C. Then they were divided into two groups, one with five males and the other without males. The fish of both groups ovulated during 2 to 4 days after the fish were transferred into the aquarium of higher water temperature. In the group with males, spawning occurred naturally but in the group without males spawning did not occur (Table 20).

Next, the following experiments were carried out to determine the effects of aquatic plants on the spawning act of females. Five pairs of fish used in the present experiment were shown in Table 21. All females used were induced to ovulate by means of the injection of HCG of 30 units per 10 g of body weight. Experiments were carried out in aquaria of 106 cm×80 cm×45 cm. Each pair of fish from No. 1 to No. 3 was observed independently. But each pair of the fish of both No. 4 and No. 5 was observed simultaneously. One ovulated fish was placed in an aquarium together with an active male and a bunch of aquatic plants. Time of spawning acts and the frequency of the spawning acts were recorded.



Text-figure 13. Graphs showing the frequency of spawning acts in the phases with or without aquatic plants. Oblique line: the phase with aquatic plants; empty: the phase without aquatic plants

The aquatic plants were removed from the experimental aquarium at intervals of 15 minutes. The water temperature during the experiments was at 17°C to 23.5°C. The results obtained are shown in Text-figure 13.

No fish showed any spawning act in the phase without aquatic plants although the male actively pursued the female during this phase. However, all females spawned immediately after introducing aquatic plants. In the No. 1 fish, the total number of spawning acts was 42 in the first phase with aquatic plants. Thirty-seven acts were observed in the first half of the phase and only 5 spawning acts were seen in the last half. In the second phase with aquatic plants, the fish responded immediately to the placement of the aquatic plants and showed the maximum frequency of the acts which was 5 times per minute (Text-fig. 13). The total number of the spawning acts was 18 in this phase. Fourteen acts out of 18 took place in the first half of this phase and in the last half only four spawning acts were recorded. In the third phase the fish did not spawn immediately after introducing the aquatic plants but she spawned only once four minutes later.

The same phenomena were recognized also in the other fish, namely, the highest frequency of the spawning act appeared just immediately after introducing aquatic plants, and then the frequency decreased gradually with the passage of time. These phenomena did not appear in the third or fourth phase with aquatic plants, because of decreasing the number of ripe eggs in the ovarian lumen in each fish. All the fish observed spawned almost all the eggs present in the ovarian lumen during the experiment (Table 21).

Table 21. Materials used in the experiments of oviposition shown in Text-fig. 13

No.	Date	W. t. (C)	Body length (cm)		No. of eggs remained in the ovarian lumen
			Female	Male	
1	May 30	18.3	10.1	8.9	7
2	June 3	17.0	9.3	10.4	0
3	July 1	23.5	13.4	10.3	0
4	July 10	18.0	9.0	8.9	4
5	July 10	18.0	8.9	9.1	0

In the next series of experiments, the effects of ripe eggs present in the ovarian lumen on the spawning act were examined. Four fish with ovulated eggs in the ovarian lumen were used in the experiments. Two of them were induced to ovulation by the injection of HCG, and the others ovulated naturally without any treatment. All the ripe eggs in the ovarian lumen were forced out by mechanical pressure on the belly. The weight of the ripe eggs was recorded. Then they

were transferred into experimental outdoor aquaria. The males showing active courtship behavior were placed with aquatic plants in four aquaria, in which a pair of fish were maintained. Observations were made for about two hours to determine whether they showed spawning acts or not. The results are shown in Table 22. The weight of ripe eggs from the HCG injected fish was about the same as that from the natural ovulating fish. The weight ranged from 1.8 g to 3.1 g. One of four experimental fish showed only one spawning act 77 minutes after the male was placed. Three eggs were found in the lumen of the fish (Table 22).

Table 22. Spawning acts of goldfish whose ripe eggs were forced out by mechanical pressure

Fish No.	B. L. (cm)	Weight of ripe eggs forced out (g)	Time to spawning (minutes)	No. of spawning acts	Duration of spawning (minutes)	No. of eggs remained in the ovarian lumen
1	7.8	2.1	77	1	0	3
2	8.8	1.8		0		0
3	7.9	1.9		0		0
4	8.6	3.1		0		0

The other three fish did not show any spawning act for about 2 hours. During the observation periods males showed active pursuit attaching their heads on the belly of the females and sometimes seemed to beat the females with their heads. However, the females pretended to be indifferent to the males. After the observation had been finished, the female's ovarian lumen was examined, but no eggs were found in it. From these results it is clear that females do not show any spawning acts unless they have ripe eggs in the ovarian lumen.

2. Effects of hypophysectomy on oviposition

As mentioned above, the neurohypophysial hormone is thought to be concerned with the spawning behavior by the S-shaped bending of the body in *Fundulus* and *Oryzias latipes*. Therefore it is very interesting and indispensable to examine whether or not the ovulated fish can show a normal spawning behavior after the removal of the pituitary gland. From this point of view, the following experiments were carried out.

Nine ovulated fish were used. All pituitary glands which contain both adenohypophysis and neurohypophysis, were removed from seven fish. The other two fish had a mock operation. At the time of the operation, some amounts of ripe eggs (0.2 g–0.5 g) in the ovarian lumen were forced out from the genital pore to the operation board by mechanical pressure against the belly. After the removal of the pituitary gland the fish were kept in a small aquarium containing 0.2 per

cent cooled saline water (about 6°C) and kept in it for 3 hours. The water temperature of the aquarium was gradually raised to 9.5°C–12.0°C during this time. Then they were transferred into the outdoor aquarium which water temperature was 17°C to 19.0°C. Both an active male and a bunch of aquatic plants were placed with one operated female. The time to spawning, which is the time which passed before the starting of the spawning act after the operated fish together with a male had been transferred to the outdoor aquarium, duration of the spawning period and the number of spawning acts were recorded in each pair. The results are summarized in Table 23.

Table 23. Effects of hypophysectomy on the spawning acts of goldfish

Group	Fish No.	B. L. (cm)	Initial water temperature (C)	Water temperature of outdoor aquarium (C)	Time to spawning (minutes)	Duration of spawning (minutes)	No. of spawning acts	No. of spawning acts per 10 minutes
Hypophysectomy	1	9.5	9.5	18.5	14	258	196	8
	2	8.8	12.0	17.0	35	20	5	3
	3	10.8	12.0	17.0	55	75	32	4
	4	9.0	11.1	17.0	13	85	53	6
	5	9.3	11.1	19.1	27	143	14	1
	6	7.1	11.1	19.1	43	125	18	1
	7	11.5	9.8	17.0	32	265	30	1
Mock	1	8.7	12.0	17.0	37	133	169	13
	2	8.1	17.0	19.1	12	58	29	5

All the hypophysectomized fish showed normal spawning acts and oviposited naturally. The time to spawning in the hypophysectomized fish was 13 to 55 minutes. The No. 1 fish showed the maximum number of spawning acts, 196, and the duration of the spawning period was 258 minutes. On the contrary the No. 2 fish showed a minimum of spawning acts, 5, and the duration was 20 minutes. All the fish hypophysectomized spawned all eggs in the ovarian lumen except one fish (No. 7) which still retained 0.4 g eggs on the day following spawning.

On the other hand, two ovulated fish which got the mock operation also spawned 12 and 37 minutes respectively after being put into the outdoor aquarium. They showed 169 and 29 spawning acts respectively. The number of spawning acts per 10 minutes seems to be less in hypophysectomized fish than in mock controls. However, no marked difference was observed in the spawning behavior between them.

From these results it may be clearly concluded that ovulated fish show normal spawning acts and they can spawn the ripe eggs present in the ovarian lumen even after the removal of the pituitary gland, if other environmental conditions

are suitable.

The previous experiments were carried out 3 hours after hypophysectomy. Therefore, the following suspicion may arise that the pituitary hormones concerned with the spawning act might be issued out into the blood at hypophysectomy or that the hormone existing in blood before the operation might exert an effect on the spawning act of hypophysectomized fish.

The following experiment was carried out to answer the above suspicion. In the spawning season eight gravid females were hypophysectomized and injected intraperitoneally with HCG. Four fish were given the injection just after hypophysectomy, one fish was done one day after, two fish two days after and the other one four days. They all ovulated on the day following the injection. Thus, their spawning behavior was examined in the same way as in previous experiments. The results are presented in Table 24.

Table 24. Oviposition of hypophysectomized goldfish

Fish No.	B. L. (cm)	Days after operation	Time to spawning (minutes)	Duration of spawning (minutes)	No. of spawning acts	No. of spawning acts per 10 minutes	No. of eggs remained in the ovarian lumen
1	8.8	1	3	173	127	7	0
2	8.1	1	4	165	94	6	2
3	8.7	1	13	63	36	6	4
4	7.3	1	25	90	28	3	3
5	7.1	2	13	52	24	5	5
6	9.9	3	13	116	24	2	63
7	8.8	3	22	97	9	1	3
8	9.2	5	23	82	73	9	2

They showed normal spawning acts for 3 to 25 minutes after pairing with a male. The No. 1 fish showed 127 spawning acts during 173 minutes. Even 5 days after hypophysectomy, the fish was observed to spawn 73 times. The number of spawning acts per ten minutes was 1 to 9. This value is comparable with those of normal fish. All the fish except one, had some eggs in the ovarian lumen. One of them had 63 eggs and the others had 2 to 5 eggs. But their spawning behavior was normal.

From these results it must be reasonably concluded that the spawning acts of goldfish are not directly concerned with the pituitary gland.

3. Discussion

Spawning in each female goldfish lasts from ten minutes to several hours. During this time the spawning acts occur cyclically.

The goldfish do not spawn unless environmental conditions are suitable for

oviposition even if ovulation occurs under the same conditions. This indicates that the mechanisms involved in the oviposition are entirely different from those involved in ovulation as Egami (1959) and Egami and Nambu (1961) suggested in *Oryzias latipes.*

Among the environmental factors concerned with their oviposition, light is a very important factor, and temperature also effects the oviposition. There seems to be a critical temperature below which the fish will not oviposit. The vegetation or other substitutes facilitates the spawning of female goldfish as shown in the present study. If aquatic plants are absent in the spawning ground, the oviposition is often inhibited. Other environmental factors which affect oviposition or spawning behaviors in fish have been reviewed by Aronson (1957) and by Pickford and Atz (1957). They indicate that the outerstimuli which are transported to the nerve center play important roles in the initiation or duration of oviposition in fish. Nambu and Hosokawa (1964) also suggested that nervous factors take very important roles in the oviposition of *Oryzias latipes.*

Non-ovulated goldfish show no spawning behavior even if they have fully grown eggs. Spawning behavior is only seen in the ovulated fish. The frequency of the oviposition is closely connected with the volume of ripe eggs present in the ovarian lumen. As the eggs in the lumen decrease in number by spawning, the frequency of the oviposition also becomes small. If the ovulated eggs are forced out from fish by mechanical pressure, the fish do not show any spawning behavior even if other conditions are suitable. Therefore it is reasonably assumed that these ripe eggs in the ovarian lumen stimulate the spawning behavior of females via some pathway.

The behaviors concerned with the reproduction seem also to be influenced by the endocrine secretions from the pituitary gland and gonads. Numerous investigators have induced ovulation and sexual behavior in many species of fish prior to the normal breeding period using homoplastic or heteroplastic pituitaries as well as a variety of anterior pituitary extracts and other pituitary-like hormones from other sources. However, in these cases it seems questionable whether the behavior is induced by the direct action of these treated hormones, because the secretions from the fish's own pituitary and gonads could not be ruled out. Noble and Kampf (1936) implanted frog pituitary glands into spayed jewel fish and observed the first stage of courtship. Burger (1941) obtained a frenzy of pre-spawning display in the hypophysectomized male *Fundulus* which received implantations of fresh pituitaries.

Pickford (1952) injected purified fractions of pollack pituitary glands into hypophysectomized male *Fundulus* and observed a characteristic S-shaped spawning spasm within 30 minutes. She concluded that the pituitary hormone must stimulate

the nervous system directly in producing these movements. Wilhelmi *et al.* (1955) and Egami (1959) also demonstrated that neurohypophysial substance induces the rapid occurrence of the spawning reflex, when it is injected into both sexes of adult fish and immature fish. Nevertheless, gonadotrophin and other anterior lobe preparations have little or no such activity (Wilhelmi *et al.* 1955). These spawning reflexs are said to be the same as that seen in natural spawning. Egami (1959) could induce oviposition in ovulated fish by means of the administration of this hormone.

On the other hand, the neurohypophysial hormones are known to be present and identified in several teleost fish (Heller, Pickering, Maetz & Morel 1960, Sawyer & Pickford 1963). Sawyer and Pickford (1963) have stated that the content of oxytocine-like principle in the hypophysis of *Fundulus* changes significantly with the reproductive cycle and in the spawning season it become more abundant. Therefore it is assumed that oviposition in fish is controlled by the neurohypophysial hormones.

However, in the present experiments it was clearly shown that the hypophysectomized goldfish could spawn normally even 5 days after the operation if the fish had ovulated eggs in the ovarian lumen. The eggs spawned were normally fertilized. The spawning frequency was nearly the same as those of intact or mock operated controls. A pituitary gland of teleost is composed of two distinct components *i.e.*, neurohypophysis and adenohypophysis; the former penetrates the later with complicated branches (Scruggs 1951, Hoar 1957, Pickford & Atz 1957, Honma 1960). Therefore it is difficult to separate the two parts with surgical procedure. But from the results of the present study, it may be surmised that the removal of the neurohypophysis may probably have no effect on the oviposition in goldfish.

This result is contradictory to the previous reports that the neurohypophysial hormone is concerned with oviposition and to the report of Barr (1963) that the ovulated plaice did not oviposit after the removal of the pituitary gland.

However, Shirley and Nalbandov (1956) have also found that the oviposition in the hen is not seriously affected by the removal of neurohypophysis. And recently, Tanaka and Nakajo (1962) concluded from the results of their skillful experiments that a prompt discharge of the hormone from the posterior lobe of the pituitary occurs at the time of oviposition and they suggested that the oviposition in the neurohypophysectomized hen is caused by the hormone directly released from the hypothalamus.

In fish, as in other vertebrates, it has been shown that the neurohypophysial hormones are not produced in the neurohypophysis, but mainly in the neurosecretory cells in the nucleus preopticus of the hypothalamus (Sano 1963). Up to date

there seems to be no report concerning the effect of the operation such as surgical or electrical lesions of the hypothalamus or diencephalon which may damage the production of the neurohypophysial hormone in female fish. But in the male all reproductive and parental activities are markedly reduced by extensive hypothalamic injury (Aronson 1957). On the other hand, the production of neurohypophysial hormones in the neurosecretory center of the hypothalamus does not decrease even after hypophysectomy (Enami 1957).

Therefore it may be probable that the oviposition in hypophysectomized goldfish was caused by the hormones directly released from the hypothalamus. What factors are concerned with the secretion of the neurohypophysial hormones from the hypothalamus is still uncertain. Therefore, detailed studies concerning the mechanisms of secretion of neurohypophysial hormones in the hypothalamus are needed to arrive at a general conclusion about the mechanisms of oviposition.

SUMMARY

1. The reproductive cycle of goldfish is repeated with one year intervals. This cycle is composed of six events: 1) oogonial multiplication, 2) first growth phase of oocytes, 3) second growth phase of oocytes, 4) ovulation, 5) oviposition, and 6) degeneration of oocytes in the process of ripening.

2. The oogonia of 12 to 14 micra in diameter were found forming cysts in the wall of the ovigerous folds. The division of oogonia was seen in the ovaries obtained in May, June, July, August, October, and February. The most frequent divisions were seen in spent ovaries.

3. Hypophysectomy inhibited oogonial division, while the treatment with pituitary glands of both goldfish and dog salmon induced oogonial division in hypophysectomized fish.

4. The multiplication of pre-existing oogonia is followed by the first growth phase of oocytes. The oocytes in the first growth phase ranged from 14 to 150 micra in diameter. Pituitary gonadotrophins are not concerned directly with the growth of oocytes in this phase. Thus, the oocytes in this phase continue to grow in hypophysectomized fish.

5. The second growth phase of oocytes represents the period during which yolk formation takes place in the oocyte. The oocytes of this phase vary from 150 micra to 900 micra in diameter. Follicle cells greatly increase in number by mitotic division during this phase.

6. Soon after hypophysectomy, both the decrease in thickness of the follicle cells and the cessation in the division of follicular epithelial cells take place. Then the oocytes with yolk begin to degenerate and become corpora atresia. The process of degeneration of the oocytes after hypophysectomy resembles that of

spontaneous atresia after spawning.

7. The velocity of oocyte degeneration after hypophysectomy depends on the water temperature of the aquaria in which the operated fish are maintained. At 25°C all oocytes with yolk degenerated for about two weeks, at 20°C they degenerated for about three weeks, and below 15°C, 50 to 80 per cent of the eggs with yolks remained in the ovaries even 36 days after.

8. With the passage of time after hypophysectomy, the atretic oocytes are gradually absorbed and the ovaries become filled with only yolkless oocytes.

9. The treatment of hypophysectomized fish with pituitary glands of goldfish or dog salmon causes the division of follicle cells and induces the formation of yolks in the yolkless oocytes.

10. The pituitary gland of fish is assumed to have follicle stimulating hormone (FSH) which stimulates the follicle cells and induces yolk formation.

11. Mammalian gonadotrophins were ineffective for the yolk formation of goldfish. Therefore, FSH of goldfish is considered to be very different qualitatively from FSH of mammals.

12. At ovulation the oocytes are extruded from the follicular envelope to the ovarian lumen with accompanying nuclear changes.

13. Mammalian LH or HCG is very effective for the acceleration of ovulation of goldfish.

14. Hypophysectomy completely inhibits ovulation, but the HCG injection brings about ovulation in hypophysectomized goldfish. This may indicate that the pituitary gland contains an LH-like ovulation hormone.

15. Normal ovulation occurs if hypophysectomy is carried out in fish a few hours before ovulation.

16. Oviposition occurs cyclically after a latent period of 10 to 30 minutes. The frequency of oviposition decreases as the ripe eggs present in the ovarian lumen are reduced in number, and the spawning acts in the female do not take place if no ripe egg is present in the ovarian lumen.

17. The spawning acts are prevented by the lack of males or of aquatic plants even though many ripe eggs are present in the ovarian lumen of the fish.

18. Oviposition takes place normally in the fish without pituitary gland.

19. The significance of neurohypophysial hormone for oviposition is discussed.

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Explanation of Plates

All figures are photomicrographs obtained from sections of the ovaries of goldfish. Fixed in Bouin's solution and stained with Delafield's haematoxylin. *ao*: atretic oocyte; *df*: dividing follicle cell; *dg*: degenerating follicle cell; *do*: dividing oogonium; *ef*: empty follicle; *fc*: follicle cell; *gc*: oogonial cyst; *mc*: micropyle; *mp*: micropylar cell; *tc*: theca cell; *yg*: yolk globule; *yn*: yolk nucleus; *yv*: yolk vesicle; *zr*: zona radiata.

PLATE I

Fig. 1. Portion of the ovary from a fish obtained on May 16. Many atretic oocytes together with empty follicles are found. ×20

Fig. 2. Oogonial cyst found in the same ovary as above. ×790

Fig. 3. Portion of the ovary from a fish obtained on August 15. Atretic oocytes and an oogonial cyst embedded in the wall of the ovigerous fold are found. ×57

Fig. 4. Oogonial cyst found in the same ovary as above. ×810

Fig. 5. Portion of the ovary from a hypophysectomized fish implanted with 34 fresh pituitaries of adult goldfish. ×150

Fig. 6. Oogonial cyst found in the same ovary as above. ×1130

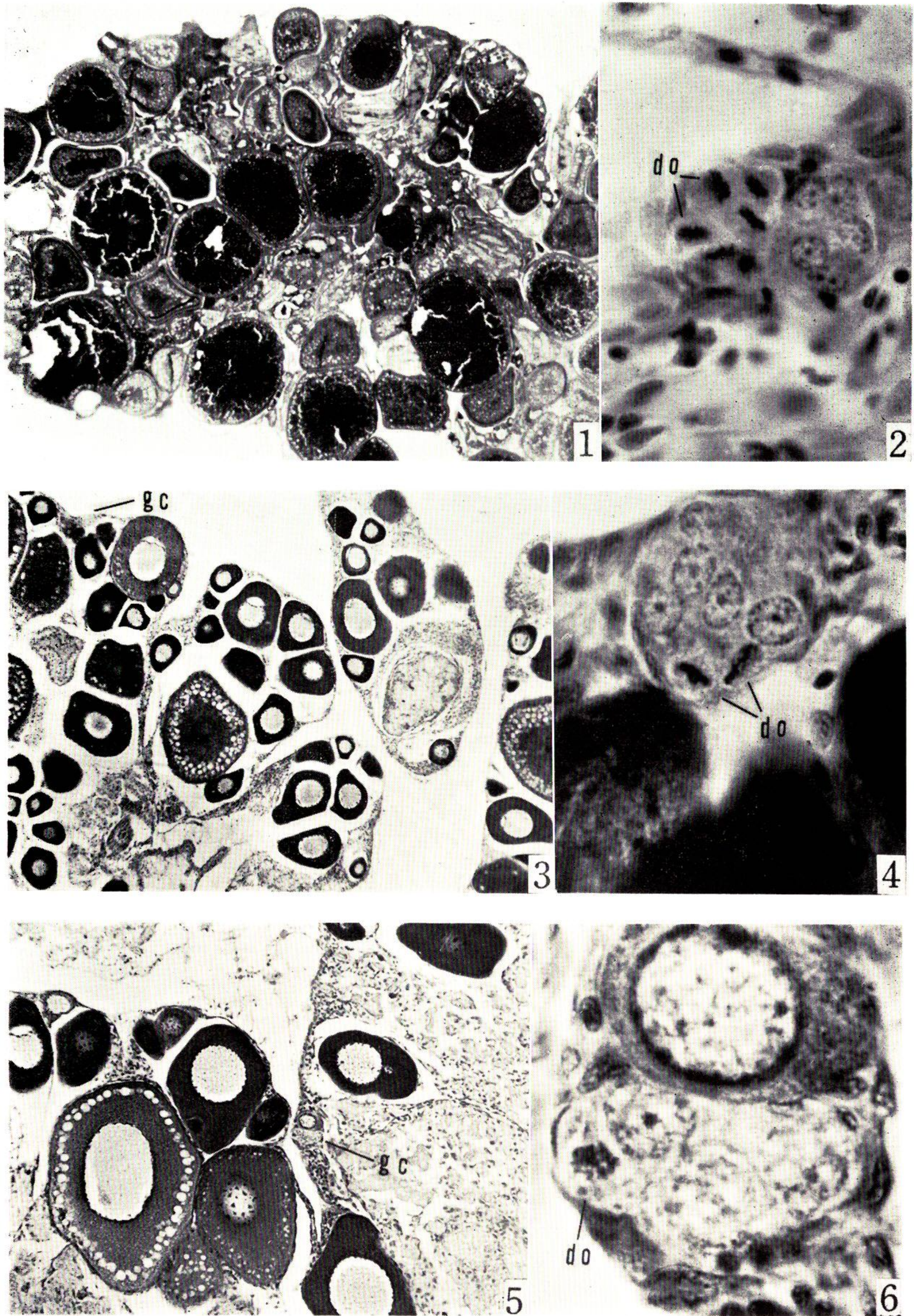

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PLATE II

Fig. 7. Portion of the ovary from the same fish as in Fig. 5. ×150

Fig. 8. Oogonial cyst found in Fig. 7. ×1130

Fig. 9. Portion of the ovary from a hypophysectomized fish injected with suspension of 14 pituitaries of dog salmon. ×82

Fig. 10. Oogonial cyst found in the same ovary as above. ×350

Fig. 11. Oocytes in the chromatin nucleolus stage. ×1900

Fig. 12. Oogonial cyst which contains the oocytes of the chromatin nucleolus stage. ×790

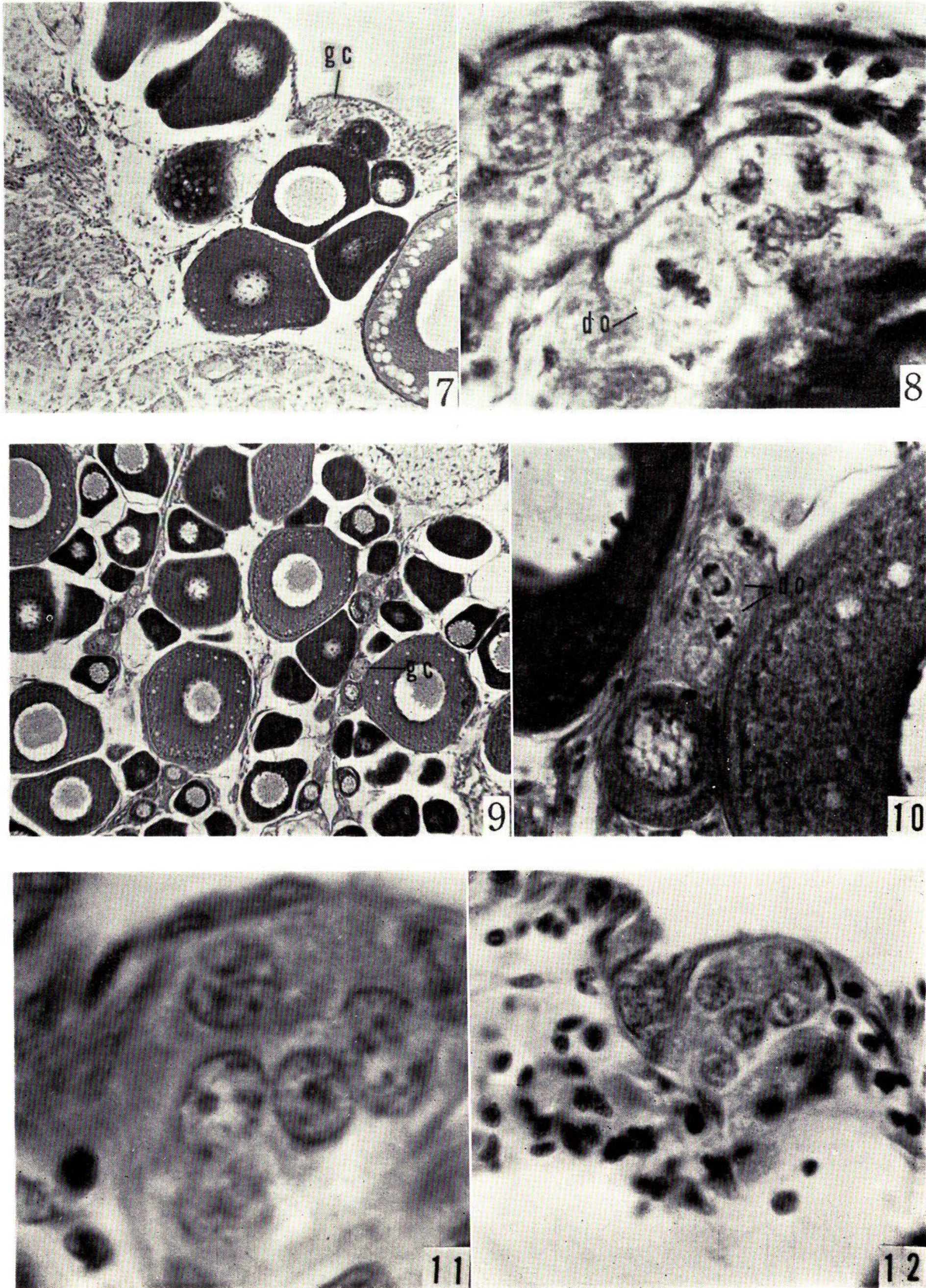

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PLATE III

Fig. 13. Oocytes in the chromatin nucleolus stage. ×1900
Fig. 14. Cyst composed of the oocytes in the chromatin nucleolus stage. ×900
Fig. 15. Oocytes in the chromatin nucleolus stage. ×1900
Fig. 16. Group of chromatin nucleolus stage oocytes. ×810
Fig. 17. Oocytes in the same stage as above. ×1900
Fig. 18. Oocytes in the same stage as above. Crescent-shaped chromatin nucleolus is found. ×810
Fig. 19. Oocyte in the chromatin nucleolus stage. ×810

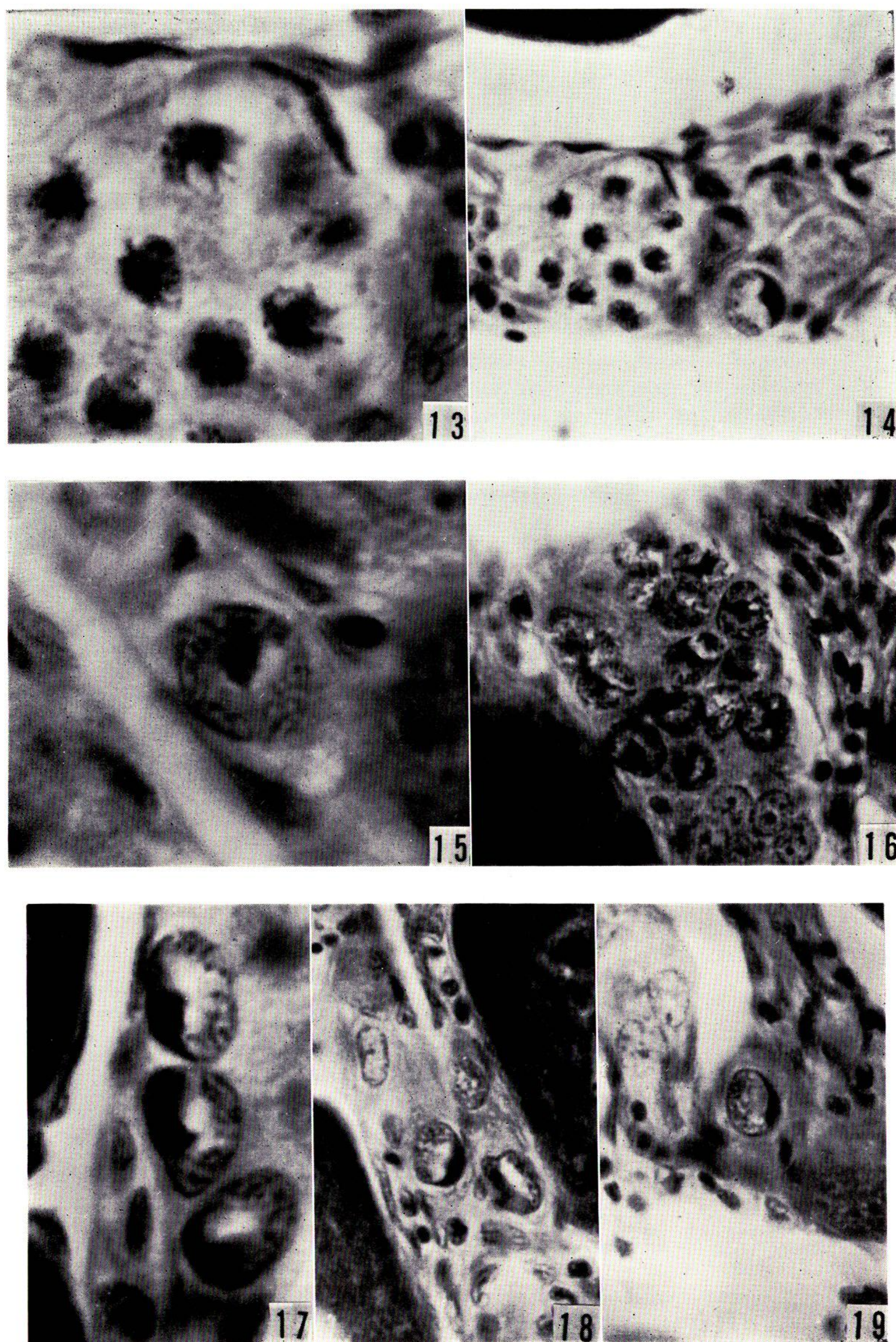

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PLATE IV

Fig. 20. Oocyte in the early peri-nucleolus stage. ×810

Fig. 21. Oocyte in the early peri-nucleolus stage. ×810

Fig. 22. Oocytes in the early peri-nucleolus stage. The cytoplasm becomes stained deeply with haematoxylin. ×259

Fig. 23. Oocyte in the late peri-nucleolus stage. Yolk nucleus is visible. Heidenhain's haematoxylin-light green preparation. ×220

Fig. 24. Oocyte in the early yolk vesicle stage. ×220

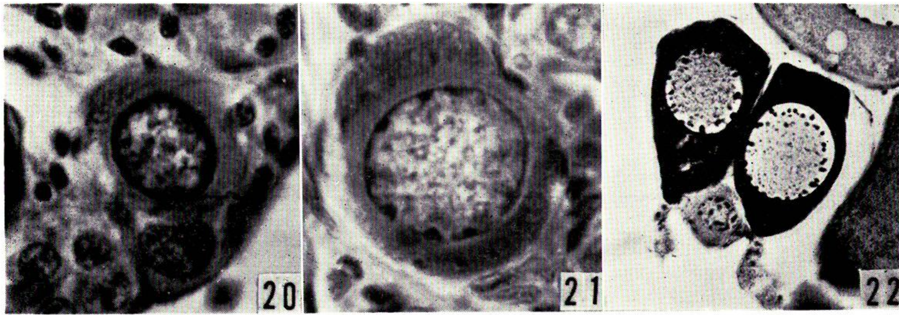
Fig. 25. Oocyte in the late yolk vesicle stage. Zenker's solution and Delafield's haematoxylin-eosin preparation. ×120

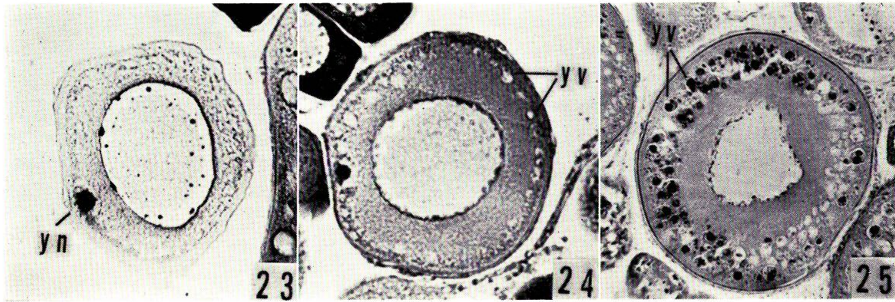
Fig. 26. Oocyte in the primary yolk stage. Heidenhain's haematoxylin-light green. ×105

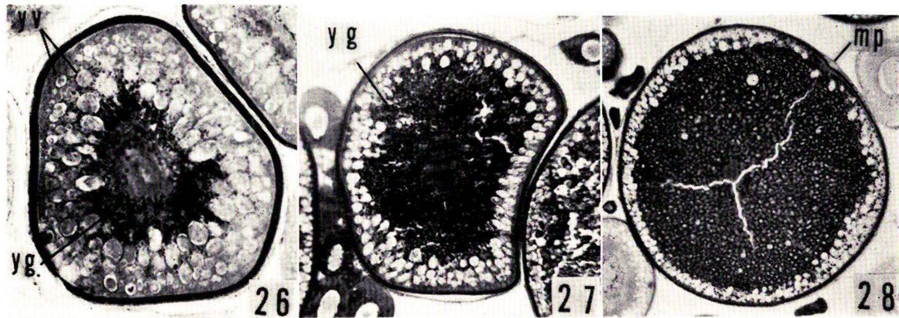
Fig. 27. Oocyte in the secondary yolk stage. Mallory's stain preparation. ×60

Fig. 28. Oocyte in the tertiary yolk stage. Mallory's stain preparation. ×42

Fig. 29. Portion of the ovary from a fish which was hypophysectomized and reared for 25 days at 20°C. ×46







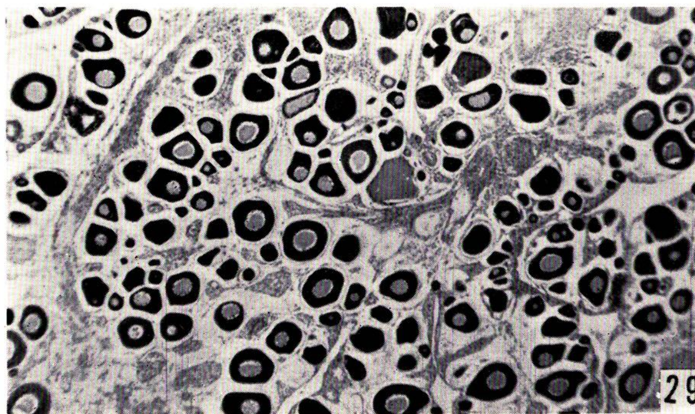


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PLATE V

Fig. 30. Follicular epithelium of an oocyte from an intact fish. ×445

Fig. 31. Follicular epithelium of an oocyte from a hypophysectomized fish. ×490

Fig. 32. Follicular epithelium of an oocyte from a hypophysectomized fish. ×390

Figs. 33, 34. Degenerating follicle cells of oocytes from hypophysectomized fish.
Fig. 33 ×390; Fig. 34 ×1200

Fig. 35. Follicle cells of an oocyte from an intact fish. A dividing follicle cell is visible. ×1130

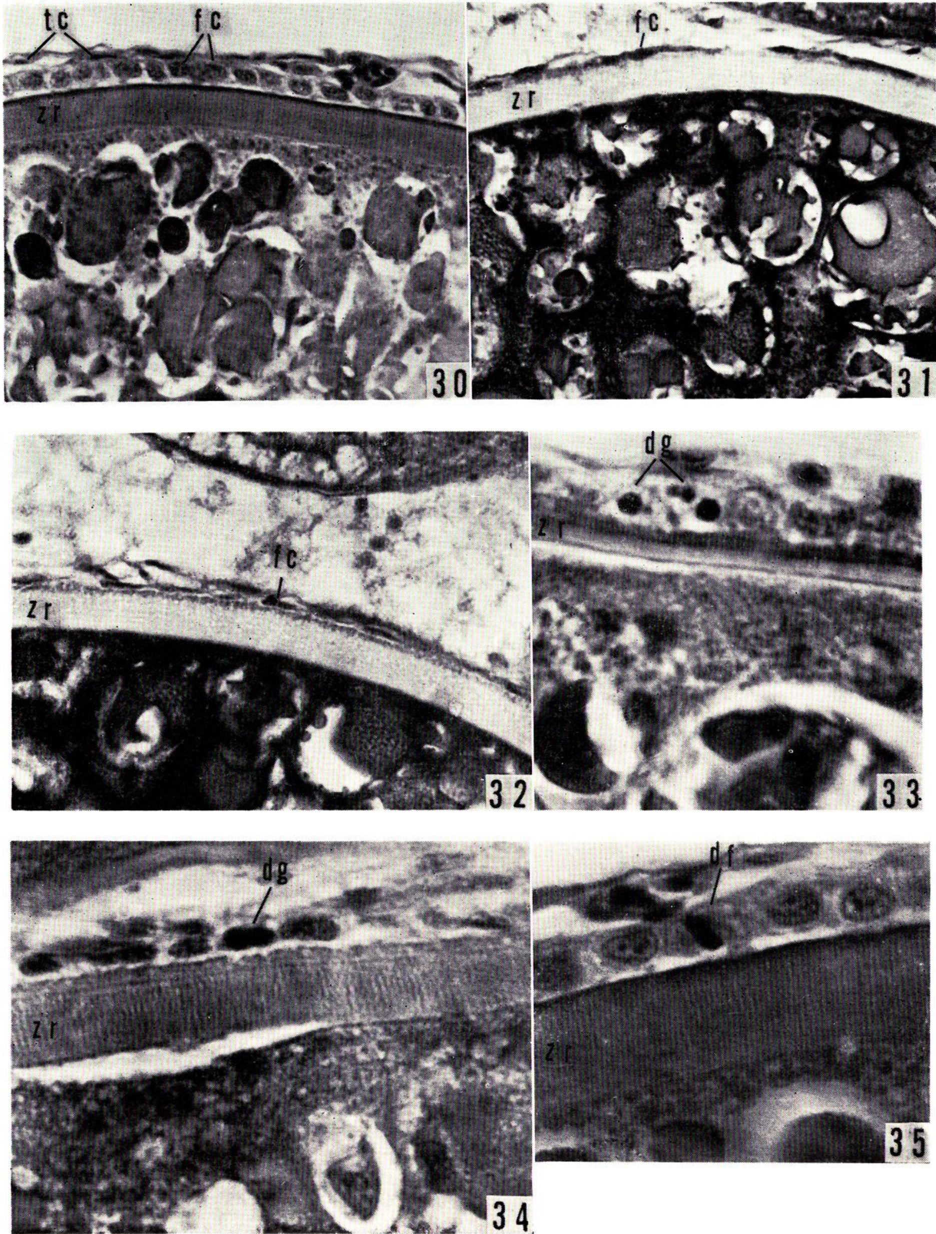
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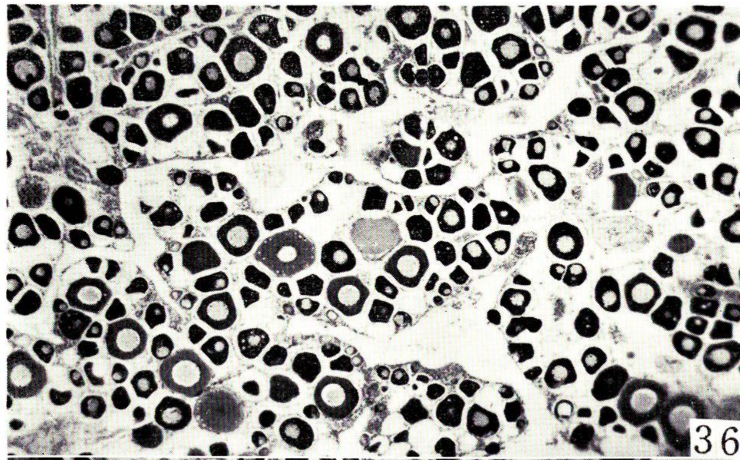
PLATE VI

Fig. 36. Portion of the ovary from a hypophysectomized fish which was kept for nine weeks after operation. ×46

Fig. 37. Portion of the ovary from a hypophysectomized fish which was kept for ten weeks after operation. ×46

Fig. 38. Portion of the ovary from a hypophysectomized fish which was injected with suspension of 26 adult pituitaries of goldfish. ×46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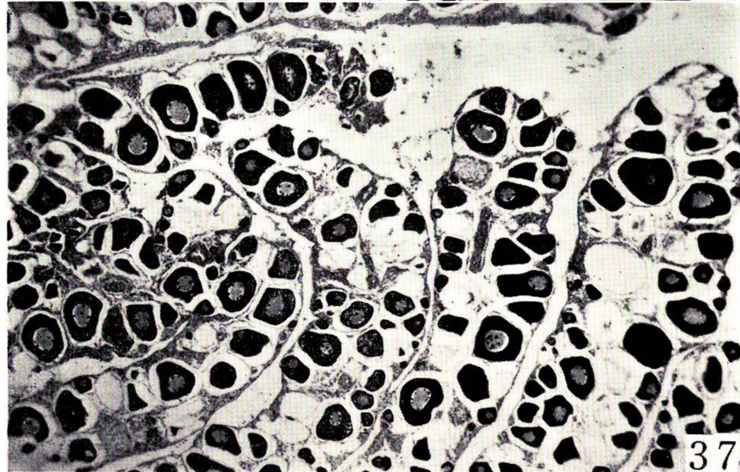
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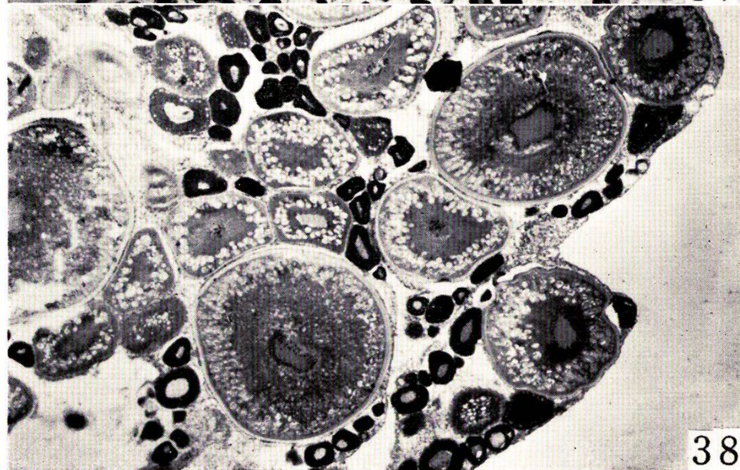
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PLATE VII

Fig. 39. Portion of the ovary from a hypophysectomized fish implanted with 34 fresh pituitary glands of goldfish. ×70

Fig. 40. One of the dividing follicle cells found in the same ovary as in Fig. 39. ×600

Fig. 41. One of the dividing follicle cells found in the ovary of a hypophysectomized fish which was injected with the suspension of pituitary glands of dog salmon. ×320

Fig. 42. Portion of the ovary from a hypophysectomized fish injected with 25 units of HCG once a day for ten successive days. ×30

Fig. 43. Portion of the ovary from a hypophysectomized fish injected ten times with 50 units of HCG every three days. ×40

Fig. 44. Portion of the ovary from a hypophysectomized fish injected with 25 units of HCG once a day for ten days continuously. ×30

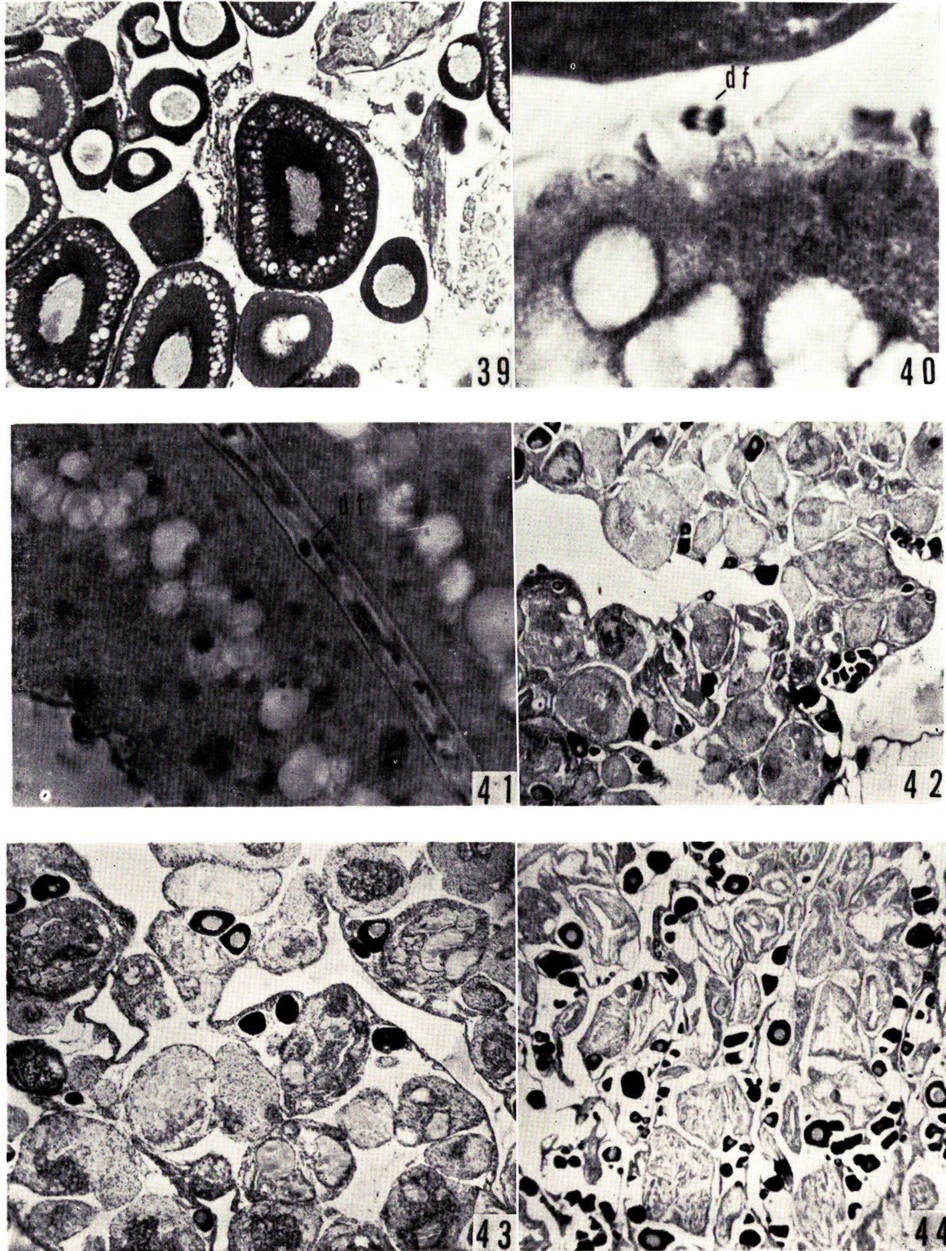

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PLATE VIII

Fig. 45. Portion of the ovary from a hypophysectomized fish injected with HCG (10 u.) and PMS (25 u.) once a day for ten successive days. ×30

Fig. 46. Portion of the ovary from a hypophysectomized fish injected with 50 units of PMS once a day for ten successive days. ×20

Fig. 47. Portion of the ovary from a hypophysectomized fish injected with 200 units of PMS once a day for ten successive days. ×40

Fig. 48. Portion of the ovary from a hypophysectomized fish injected with 10 units of Synahorin (HCG+APE) once a day for ten successive days. ×46

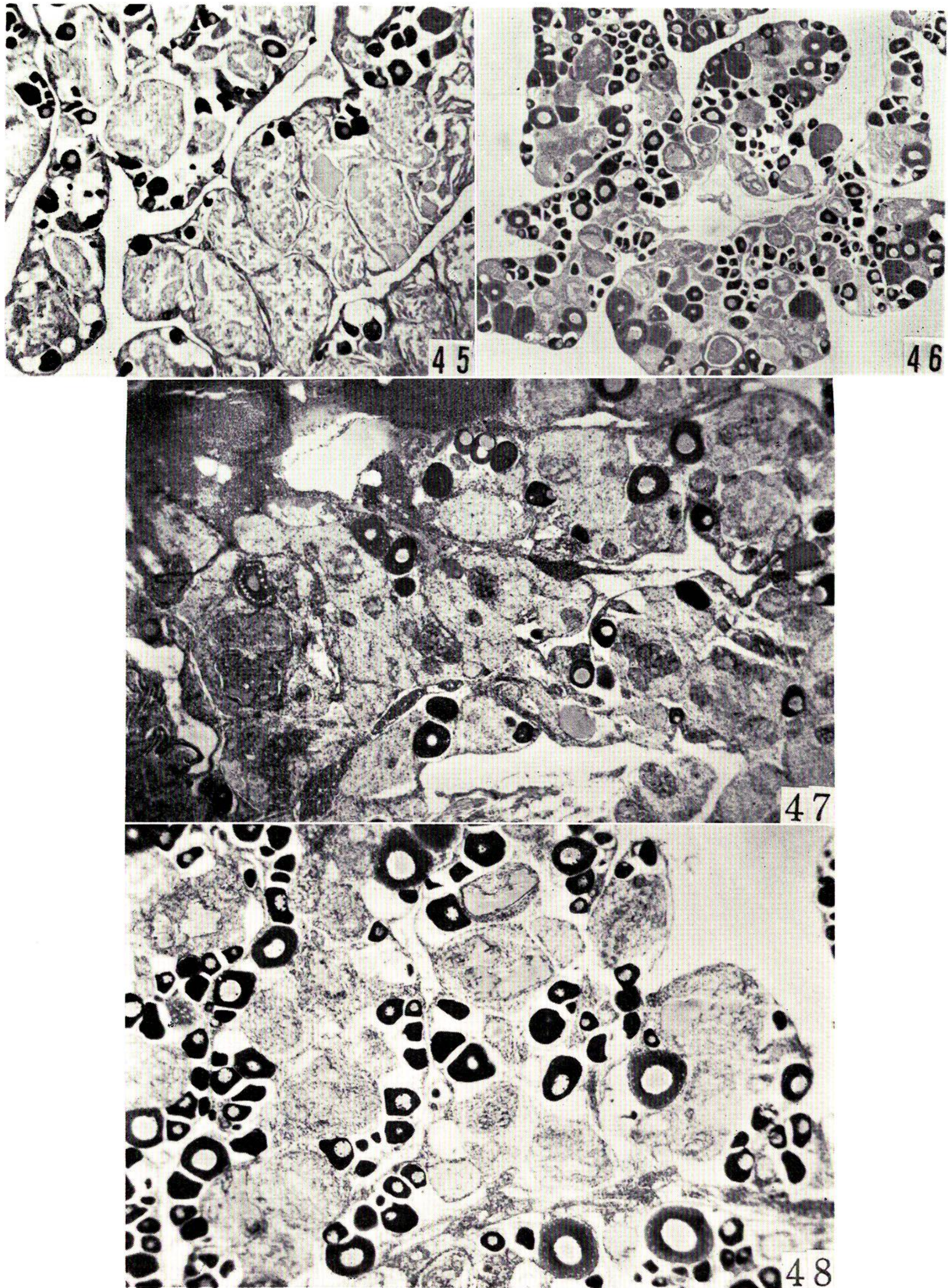

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PLATE IX

Fig. 49. Portion of the ovary from a hypophysectomized fish injected with 10 units of Synahorin once a day for ten successive days. ×57

Fig. 50. Portion of the ovary from a hypophysectomized fish injected with 20 units of Synahorin once a day for ten successive days. ×20

Fig. 51. Portion of the ovary from a hypophysectomized fish injected with 50 units of Praehormone (APE) once a day for seven successive days. ×80

Fig. 52. Portion of the ovary from a hypophysectomized fish injected with 50 units of Praehormone once a day for eight successive days. ×52

Fig. 53. Portion of the ovary from a hypophysectomized fish injected with 5 units of Hypohorin (APE) once a day for ten successive days. ×40

Fig. 54. Portion of the ovary from a hypophysectomized fish injected seven times with 10 units of hypohorin every three days. ×46

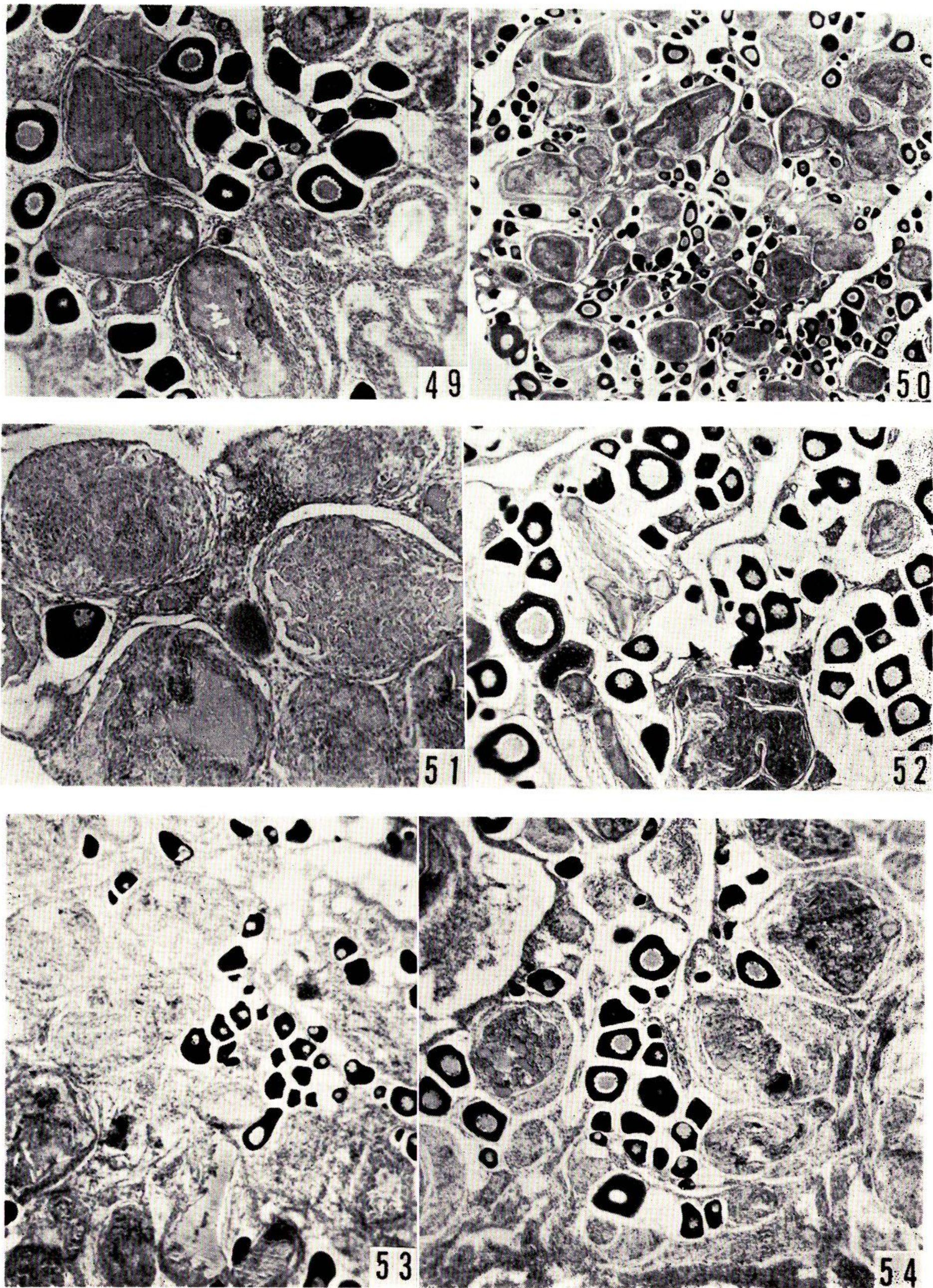
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PLATE X

Fig. 55. Oocyte in the migratory nucleolus stage. Mallory's stain preparation. ×42

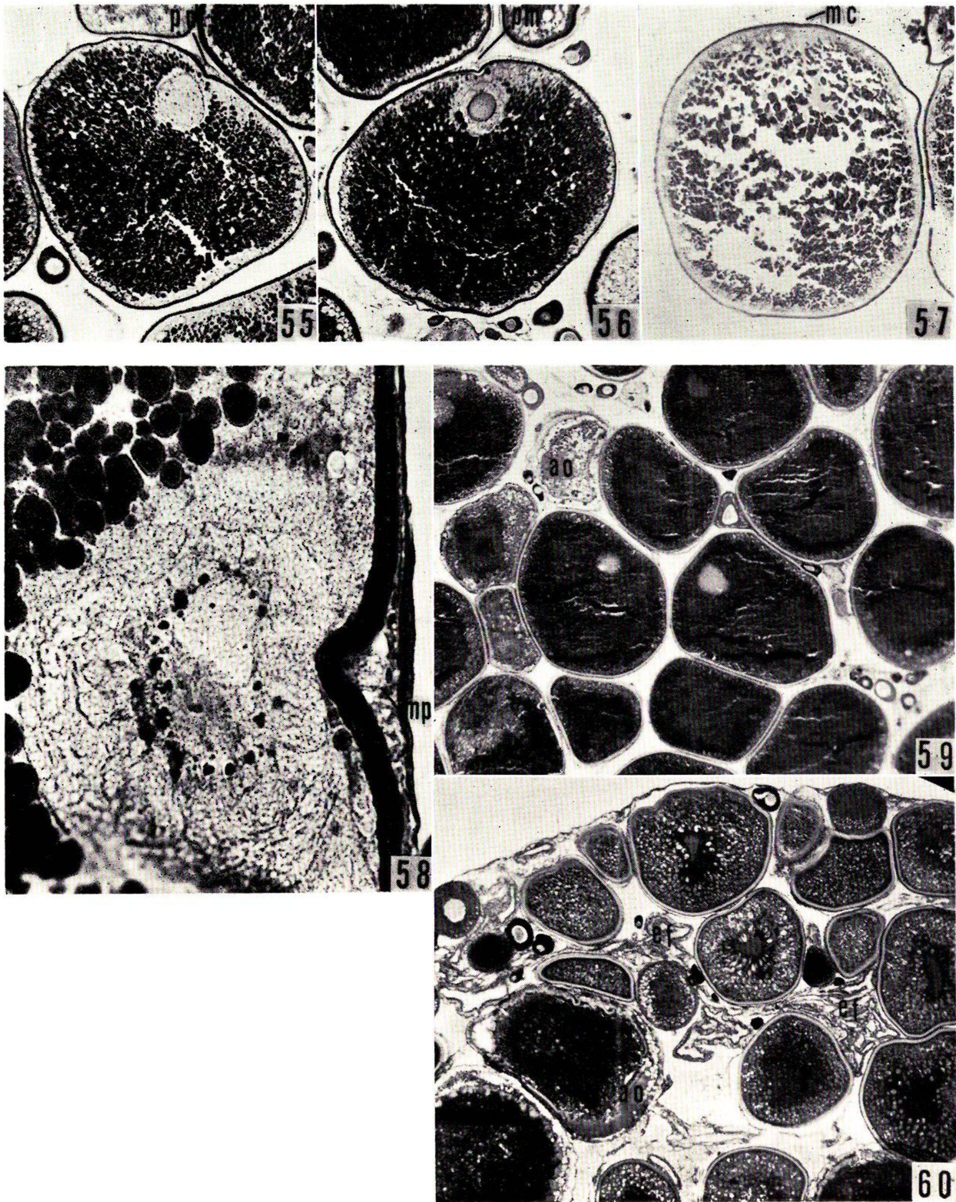
Fig. 56. Oocyte in the pre-maturation stage. Mallory's stain preparation. ×42

Fig. 57. Ripe egg. Mallory's stain preparation. ×40

Fig. 58. Micropyle of an oocyte at pre-maturation stage. ×330

Fig. 59. Portion of the ovary from a fish hypophysectomized on June 19 and killed on June 24. This fish received active courtship of males, but did not ovulate. ×28

Fig. 60. Portion of the ovary from a fish subjected to the mock operation. Many empty follicles are found. ×32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