ON THE EFFECTS OF ENVIRONMENTAL FACTORS UPON THE REPRODUCTION OF FISHES

I. The Effects of Day-length on the Reproduction of the Japanese Killifish, *ORYZIAS LATIPES***

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Introduction

It has been well known for a long time that environmental factors control reproduction among mammals, birds and other vertebrates. Bissonnette (1935) is a pioneer in this field of study; he demonstrated that light could initiate the sexual cycle of birds. Since that very many studies on the same line have been performed by a number of investigators, but only a few of them have treated of the problem using fishes as material.

Hoover and Hubbard (1937), Hazard and Eddy (1951) and Corson (1955) demonstrated the premature spawning of *Salvelinus fontinalis* by artificially alternating photoperiod.

Burger (1939) in *Fundulus heteroclitus* and Bullough (1939) in *Phoxinus laevis* have given information concerning the effects of light on the sexual cycle of fish. They support the theory that light plays the main role in controlling the sexual cycles of fish.

Harrington (1950, 1956, 1957) reported the results of his studies concerned with the effects of day-length on the sexual cycles of cyprinid fish. But his works are confined to the relation between the time of exposure to light and the onset of the spawning cycle in out-of-spawning season. He named the period from mid-July to early-November the "refractory period" in which the fish does not react to stimulus of light manipulations.

Up to the present, the experiments on the effects of light upon fish reproduction have been confined to only a few species. This number may be too small for formulation of a general pattern. Moreover, almost all experiments of which the results have been published presented different evidence from different species, so that a generalization of experimental results is impossible.

The present author planned the following described experiment in order to ascertain the effect of day-length on egg production of *Oryzias latipes* during the

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natural spawning season and on gametogenesis in out-of-normal spawning season.

Before going further the author's hearty thanks are gladly offered to Professor Kiichiro Yamamoto of Hokkaido University, for his constant encouragement and valuable advices during the course of this study, and also for his kind correction of the present manuscript. Thanks are likewise offered to Assistant Professor Tatsuro Kubo and Mr. K. Takano for much help in the course of the present study.

Materials and Methods.

The present experiments on the effects of day-length upon egg production were performed at the Faculty of Fisheries, Hokkaido University, Hakodate (latitude 41° north).

Submaximum sized adult Japanese killifish, *Oryzias latipes* were collected from an irrigation pond of Yunokawa hot spring, Hakodate, Hokkaido. Only two-year fish ranging from 28.0 mm to 30.0 mm in body length were used for this study, because the preliminary experiment showed that the number of eggs laid differs with age of fish. Until the beginning of the experiment, the fish were reared in large aquaria placed in the green house. The spawning period in glass aquaria extended from late May to early September. So, the experiments on egg production have been performed during that period. Wild fish of *Oryzias* in Hakodate differ from the same organisms hitherto reported in respect to small number of eggs laid in one spawning season and short duration of spawning season. The fish which recorded the maximum number of eggs laid about 400 eggs in one spawning season. Even at the peak of the season, she laid eggs at the interval of 2 or 3 days and did not show the daily spawning cycle.

According to Terao & Tanaka (1929), in *Oryzias* the number of eggs laid per one female increases in inverse proportion to population density and the maximum number of eggs is obtained from a pair of fish. So, in the present experiments one male and one female were kept together in a glass aquarium containing about 3 litres of water with some water plants. All fish were nurtured on earth worms throughout the experiments. Every morning newly laid eggs attached to the body were removed and their number, weight and diameter were measured. After the experiments were finished, gonads of all fish were fixed in Allen-Bouin's solution. They were imbedded in paraffin, sectioned and stained with Heidenhain's iron haematoxylin, and Delafield's haematoxylin and eosin.

Results

1. The effects of different day-length on egg production during normal spawning season.

Eighteen hours of light per day and artificial autumn day-length which was reduced from 13.0 hours of light to $9\frac{5}{6}$ hours per day were chosen for the experiment. Fish collected on May 20, 1960 were segregated into three groups and subjected to these photoperiods, as well as to natural summer daylight as the control. Pairs consisted of one male and one female were taken out at random from the stock colony and each pair was kept in a separate glass aquarium, which was placed by north-facing windows. The long photoperiod was provided by daylight supplemented by artificial illumination supplied by a 20-watt firefly lamp. Artificial autumn daylight was obtained by means of daylight artificially reduced by means of a light-covered box.

Table 1. Average number of eggs per one female, average number of eggs per spawning and volume of eggs under different day-lengths

Month	Average No. of eggs per one female.	Average spawning interval (day).	Average No. of eggs per spawning	Average volume of an egg (mm^3).	Average total volume of eggs.
Control					
Jun.	59.6	3.59	7.18	2.05	122.18
Jul.	71.4	1.97	4.69	1.92	137.08
Aug.	62.8	2.55	5.36	1.69	106.13
Sep.	6.0	2.62	3.75	1.60	9.60
Total	199.8				374.60
Long photoperiod (18 hours per day)					
Jun.	39.0	3.65	4.75	1.93	75.27
Jul.	101.2	1.73	5.84	1.79	181.14
Aug.	75.6	1.83	4.63	1.57	108.69
Sep.	6.0	3.00	4.00	1.55	9.30
Total	221.8				374.27
Artificial autumn daylight					
Jun.	55.7	1.64	10.90	1.99	110.84
Jul.					
Aug.					
Sep.					
Total	55.7				110.84

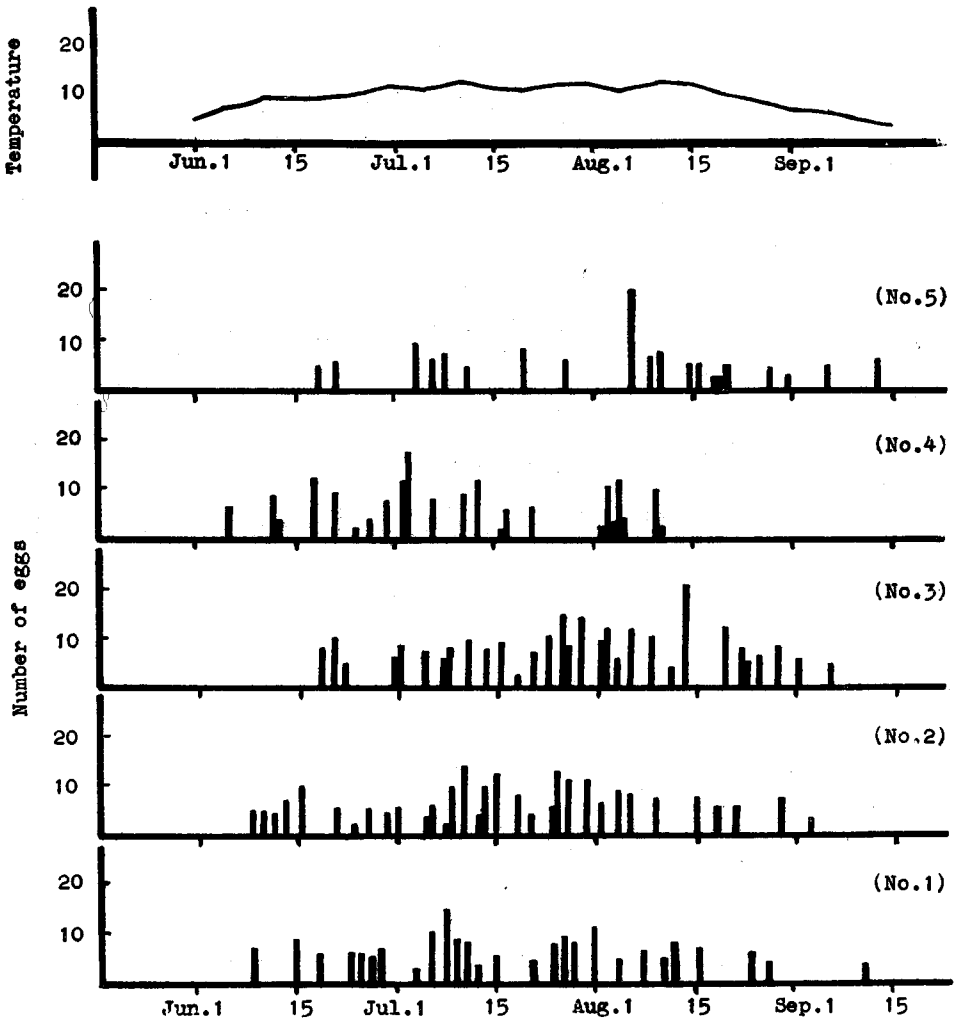
Each group consisted of 15 pairs. They all were kept at natural room temperature. The changes of water temperature and day-length in each group are indicated in Text-figure 4. It is well established that the Japanese killifish is accustomed to lay its eggs within a few hours after dawn (Egami, 1959). By the change of photoperiod, the spawning cycles of this species were easily put out of order owing to the breaking of the relationship between the mechanisms of spawning acts and the stimulus of light (Robinson and Rugh, 1943). In the early phase of the experiments, therefore, the fish of the experimental groups were made to lay their eggs at a different time (at 5–10 a.m.) from the controls.

Eleven fish in the control group began to lay their eggs in early June and ended laying in early September. Three typical reproductive cycles of them are shown schematically in Text-figure 1, 1–3. But remaining 4 fish showed different reproductive cycles from the above ones (Text-figure 1, 4–5); spawning period is short and the total number of eggs laid is small. The figure shows that as water temperature rises, spawning interval shortens, and the number of eggs laid in each month increases as a rule. Average values of those results are summarized in Table 1. From the data, it is clear that active spawning in this species covers the period from July to the middle of August. The average maximum number of eggs per month was observed in July.

On the other hand, in the 9 fish exposed to long photoperiod the total spawning period was slightly prolonged about two weeks until mid-September. Three typical reproductive cycles of them are shown in Text-figure 2, 1–3. They laid a large number of eggs in early July. Conversely in late June when the control fish spawned many eggs, they laid only a small number. The seasonal changes in spawning of 6 other fish, however, were almost the same as in the control group (Text-figure 2, 4–5).

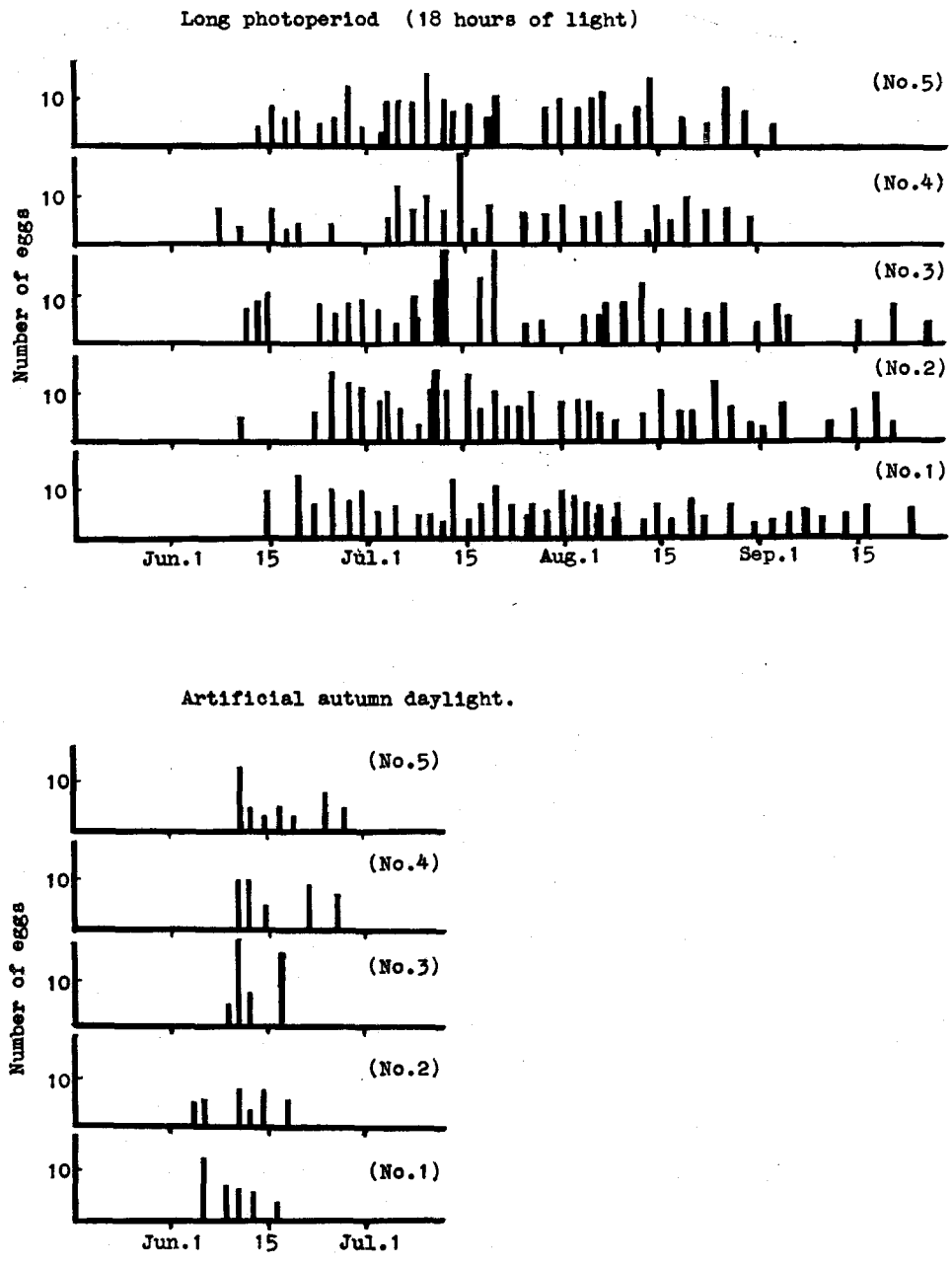
No distinguishable difference between the control and the experimental group was found in the total number of eggs. But the weight and volume of eggs were considerably smaller than those of the controls. Table 1, summarizing the results, indicates that the average volume of an egg in control fish was 2.05 mm^3 in June, 1.92 mm^3 in July, 1.69 mm^3 in August and 1.60 mm^3 in September, while that in experimental ones 1.93 mm^3 in June, 1.79 mm^3 in July, 1.57 mm^3 in August and 1.55 mm^3 in September. Therefore, the average number of eggs laid per fish was slightly larger as compared with the controls, but the average total volume of eggs was nearly the same, showing 374.27 mm^3 in long photoperiod and 374.60 mm^3 in control.

All fish exposed to artificial autumn daylight began to lay their eggs in early June. But the half of them stopped laying in late June and all the remaining



Text-figure 1. Number of eggs and spawning intervals under natural durations of daylight

ones completely ceased laying early in July (Text-figure 2). On July 5 when they had stopped laying, some of the fish were sacrificed and the ovaries were examined histologically. All their ovaries were filled with large yolk-laden oocytes and small yolkless ones. Several yolk-laden oocytes found were in regression (Fig. 1). Regressive oocytes are characteristic of the disappearance of the egg-membrane and hypertrophy of granulose cells. In the early phase of regression, the periphery of the yolk mass began to lose its shape; egg-membrane and yolk in vesicles turn disappeared. In the late phase, there were found aggregated masses of



Text-figure 2. Number of eggs and spawning intervals under different durations of daylight

granulose cells containing several fragments of yolk globules within them. All yolkless oocytes remained intact. From these results, it may be said that the

development of the oocytes more advanced than the yolk stage was influenced by the stimulus of artificial autumn daylight.

2. The effects of day-length on gametogenesis in out-of-normal spawning season.

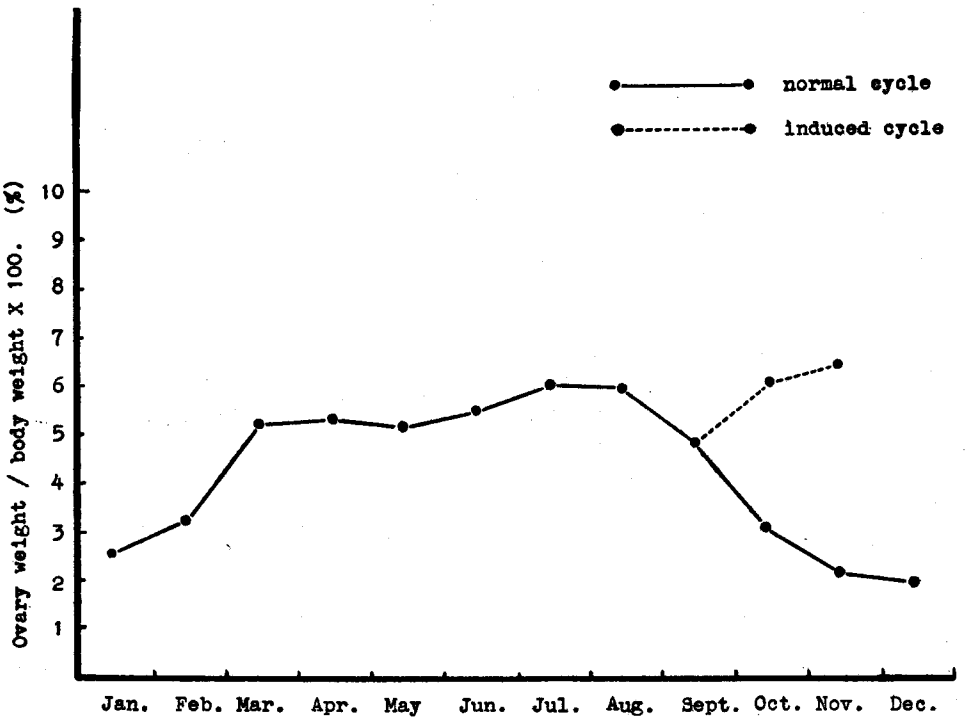
a. Effects on oogenesis.

The present experiment was carried out extending from September 11 to November 10, 1960. On September 11, 1960, fish collected on September 5 were segregated into two groups, experimental and control. Fifteen pairs of fish were placed in an experimental box which was kept at natural room temperature which ranged from 11.5° to 21.0°C.; they were exposed to 18 hours of daylight per day by means of supplementary artificial illumination (long photoperiod). The same number of pairs of fish were held in the control box which was kept at the same temperature as above and exposed to natural autumn daylight which declined during the time from about 13.0 to 10.0 hours of light per day (short photoperiod). Artificial illumination was the same as in the preceding experiment.

On September 11, some fish from the stock colony were sacrificed as initial control. Thereafter, males and females were sampled on October 10 and November 10 from each the experimental and the control group. The largest 30 eggs of each ovary were chosen for measuring of diameter. The frequency distribution of the largest 30 egg diameter from each ovary of 15 females are illustrated in Text-figure 5. On October 3, 1960 spawning was observed in two females under the long photoperiod, 22 days after they were first exposed to the experimental conditions. Thereafter no spawning acts were ever observed in the females of either group.

In the ovaries of initial control, there were found several large yolk-laden oocytes and many yolkless ones (Fig. 2). In addition to them some regressive oocytes were also found. The average diameter of the largest 30 eggs was 390.6 μ. The ovaries from the control group preserved on October 10 contained a lot of normal yolkless oocytes (Fig. 3). Three large oocytes laden with yolk were found in one of these ovaries, but they all were regressive. The ratio of ovary weight to body weight was less than that of the initial controls (Text-figure 3). The average diameter of the largest 30 eggs in these ovaries was about 178.5 μ. The condition of the ovaries obtained on November 10 was very similar to that in October. They were filled mainly with yolkless oocytes (Fig. 4). The average diameter of the largest 30 eggs measured about 252.0 μ. These results showed clearly that *Oryzias* had only oocytes below the stage of yolk vesicles in November.

The conditions of the ovaries of the fish exposed to long photoperiod were very different from those of the fish exposed to short photoperiod. By October 10 large yolk-laden oocytes occupied the main part of the ovaries, and small



Text-figure 3. Seasonal change in the ratio of ovary weight to body weight (%)

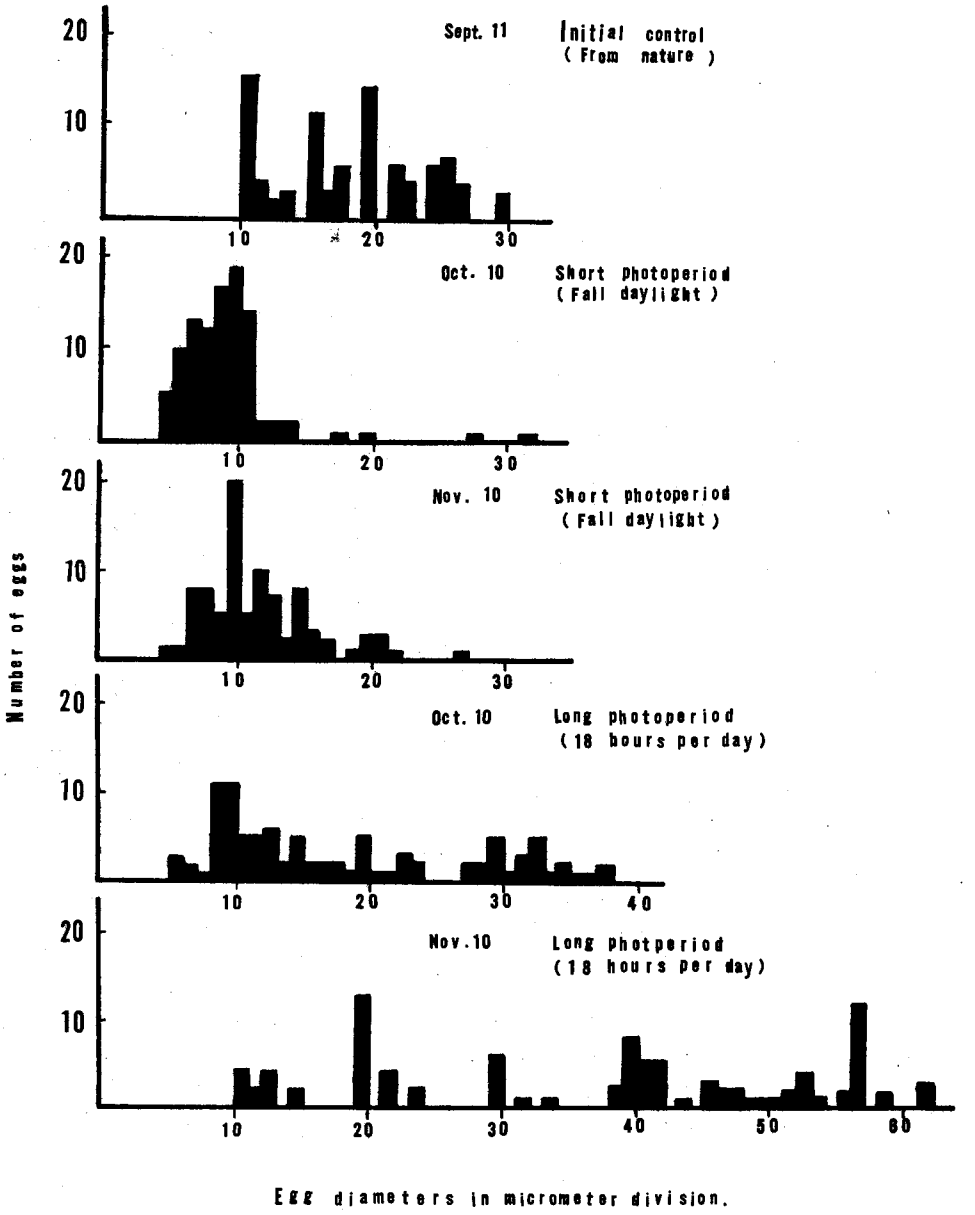
yolkless oocytes filled up the interspaces of them (Fig. 5). The ratio of ovary weight to body weight abruptly increased from 4.9 to 6.4 in a month (Text-figure 3). The average diameter of the largest 30 eggs was 405.3 μ.

Furthermore, the ovaries preserved on November 10 showed that though the water temperature went on descending from 21.0° to 11.5°C, the developing of the oocytes continued. There were several large yolk-laden oocytes occupying the main part of the ovaries and yolkless oocytes were distributed among them. Degenerated eggs were found in one ovary. But most of the large eggs were intact. The average diameter of the largest 30 eggs was about 798.0 μ. The ratio of ovary weight to body weight remained high increasing as it did also in October.

On the basis of the above facts, it seems probable that day-length above some threshold level is important for egg formation. But the exact value of threshold day-length still remains an open problem.

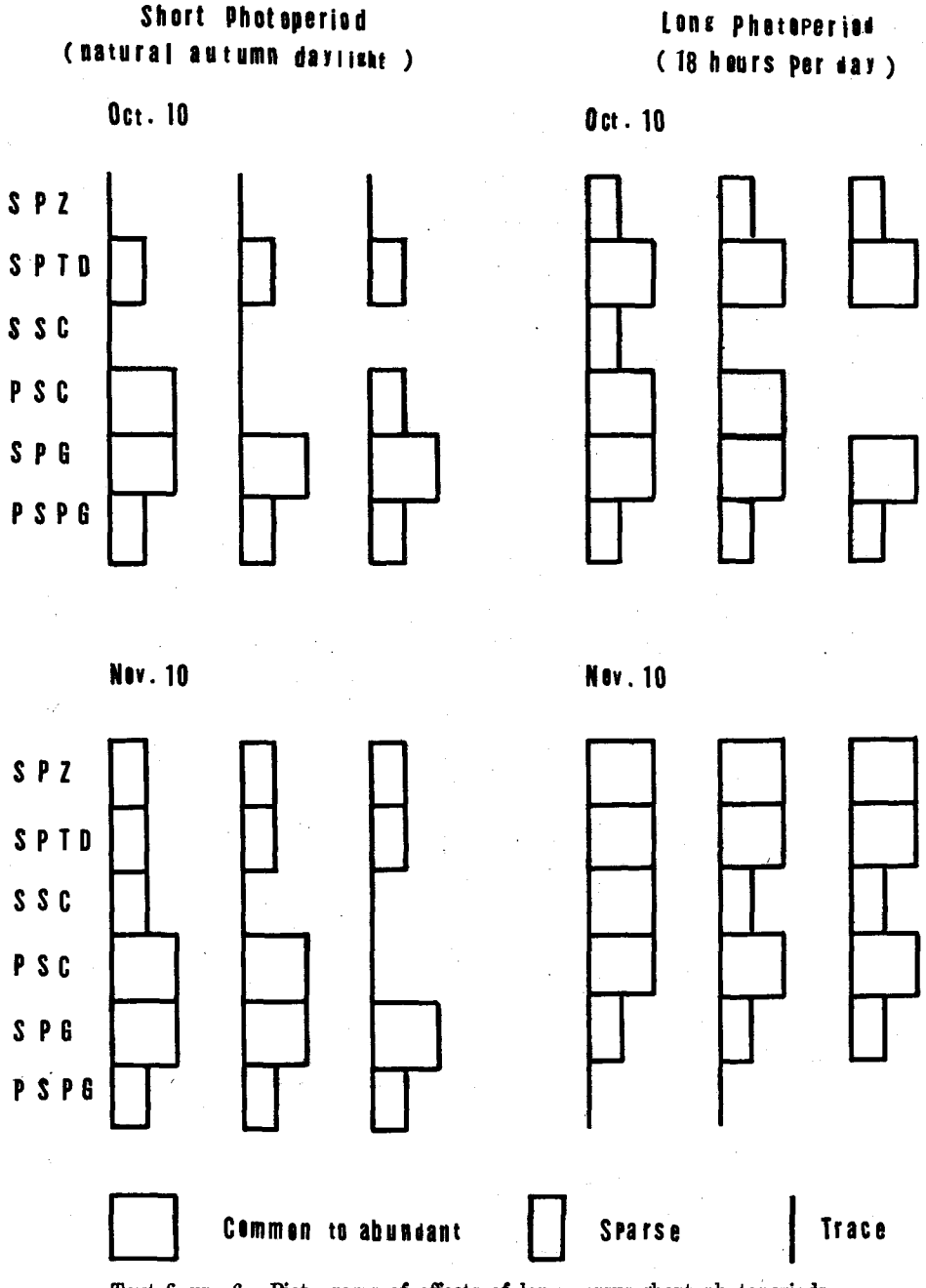
b. Effects on spermatogenesis.

The testes of the fish have usually a small number of spermatozoa in any month, but ejaculation of the spermatozoa usually lasts from late May to early



Text-figure 5. Histograms of effects of long versus short photoperiod on the ovaries of female *Oryzias latipes*. The diameters of the largest 30 eggs in one ovary of each fish are plotted. One micrometer divison=21 μ

September. The conditions and procedures of the experiments on the effect of day-length on spermatogenesis were the same as those of the above experiments



Text-figure 6. Pictograms of effects of long versus short photoperiods on the testes of *Oryzias latipes*

SPZ : spermatozoa
SPTD: spermatids
SSC : secondary spermatocytes
PSC : primary spermatocytes
SPG : spermatogonia
PSPG: primary spermatogonia.

with female fish. The effects of the experimental conditions on the testes are summarized in Text-figure 6.

As in the case of the ovaries, the testes exposed to long photoperiod showed marked difference from those of the control. In the testes of the fish exposed to short photoperiod by October 10, there were found a few spermatozoa, secondary spermatocytes, primary spermatocytes and many spermatid, spermatogonia, primary spermatogonia. Primary spermatogonia were arranged on the periphery of cysts. Each cyst was apparently in order and consisted of almost the same cell types.

On November 10, the histology of the testes of the fish exposed to the same daylight as above did not show wide dissimilarity from the testes in October, but many primary spermatocytes were found in every testis (Fig. 8).

On the other hand the testes of the fish exposed to long photoperiod were slightly advanced in ripeness in comparison with those under the condition of short photoperiod. As shown in Figure 9 and Text-figure 6, spermatozoa always increased in amount and secondary spermatocytes began to appear in some testes preserved on October 10.

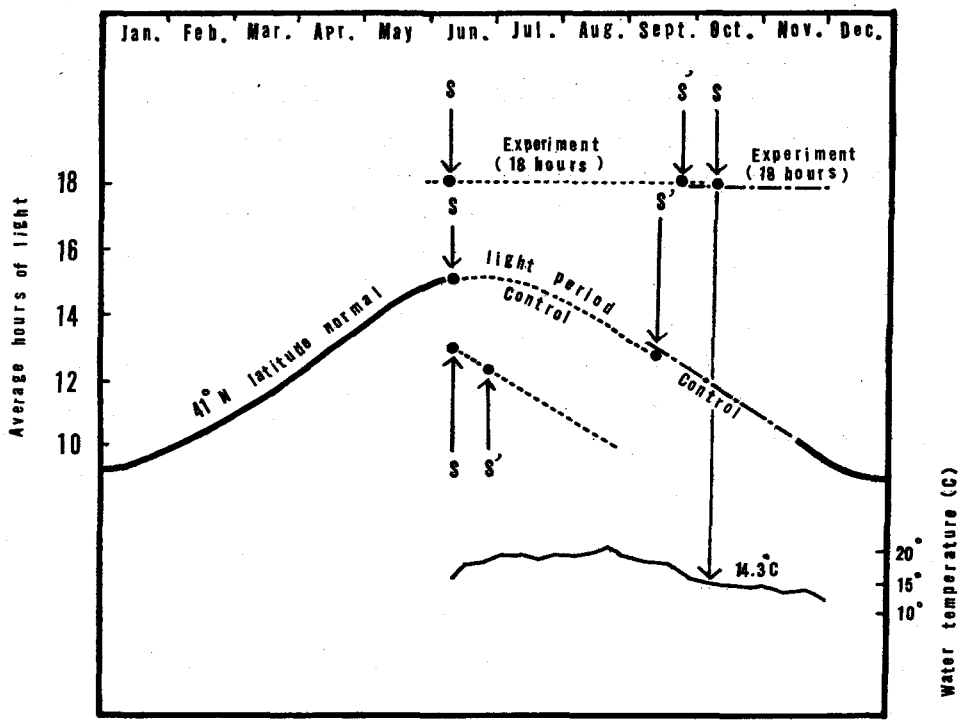
Moreover, marked change of the testes became apparent by November 10 in the testes exposed to long photoperiod. Cells from primary spermatogonia to spermatozoa were found in every testis. In these testes primary spermatocytes and secondary spermatocytes, which were few in the testes of the controls were very common. The round form of cysts was disarranged and spermatozoa formed a large patch in several parts (Fig. 10).

At the same time the testes changed markedly in colour and size; they came to be milky white and remarkably enlarged. This condition of the testes is just the same as the testes immediately before the spawning season in the natural condition.

From the above observations, it is clear that maturation of the testes proceeds in the fish exposed to long photoperiod in harmony with the ovarian maturation.

Discussion

Up to the present time, no experiments have been carried out to disclose the effect of day-length on reproduction of fish in their normal spawning season. The present observations clearly demonstrate that in *Oryzias latipes* exposed to long photoperiods, the end of spawning season was postponed: almost all fish continued to lay eggs until water temperature declined to about 13.0°–14.0°C (by the end of September). From this finding, it is evident that the fish under long photoperiod can lay eggs though water temperature is fairly low. Egami (1959) reported that the growth of oocytes, ovulation and oviposition in *Oryzias* are inhibited almost



Text-figure 4. Experimental conditions in photoperiods and water temperature. First and last spawning observed, S and S'

completely if fish are exposed to a low temperature of 4°–8°C for a period of 2–24 hours. Therefore the threshold water temperature to induce spawning acts of this species may exist within the limits of 8.0°–13.0°C.

The other noticeable finding obtained in the present study is that the eggs obtained from the fish exposed to long photoperiod were lighter in weight and smaller in volume than those from the controls. Corson (1955) has suggested that the same tendency was obtained in *Salvelinus fontinalis* treated with light manipulation in out-of-spawning season. But further studies have to be made to clear the mechanisms of this phenomenon.

Bullough (1939) demonstrated for *Phoxinus laevis* that in the absence of light, only the early stages of gametogenesis could be induced. The present fish exposed to artificial autumn daylight also stopped laying eggs in early July, though water temperature was certainly suitable for spawning. As no signs of poor feeding were detected in those fish, this can not be attributed to that factor. On several days after the fish stopped laying eggs there were found many yolk-laden oocytes in the way of regression and a few completely degenerated eggs in the ovaries.

Therefore it seems likely that only the early stage of oogenesis is induced in *Oryzias latipes* also under the condition of short photoperiod and that light treatment with long day is necessary for the development of yolk-laden oocytes. But it is uncertain whether declining stimulus of daylight may have that affect or whether only the stimulus of short daylight below a certain levels does so.

Harrington (1957) proposed a theory that the agency of light in the process of maturation of *Notropis bifrenatus* is responsible for the formation of the eggs above the critical diameter values (336 μ). In the present species, oocytes of above 420 μ in diameter appear to respond of the stimulus of exposure to light.

Harrington divided cyprinid reproductive cycle into four periods based on data from sexual maturation and sensitivity to light stimulation. He named a few months after the natural spawning period "a refractory period" in which the fish resist reaction to light manipulation. In *Oryzias latipes* the stimulation of light acted positively on the egg formation in the time corresponding to "refractory period" if water temperature was kept above 11.5°C, resulting in the development of oocytes into maturation stage.

The above facts made it clear that the cyprinodontid fishes showing typical multiplicity of spawning have not such "a refractory period" and the period of sensitivity to day-length is different in different families.

Summarizing the results of experiments which have been carried out to date, it seems evident that, as in the physiology of flower blossum of plants, there are two types of fish to respond to light manipulation, viz., a long day type and a short day type. The former type includes the fishes belonging to the cyprinodontidae and cyprinidae, viz. *Phoxinus laevis* (Bullough, 1939), *Fundulus heteroclitus* (Burger, 1939), *Enneacanthus obesus* (Harrington, 1956), *Notropis bifrenatus* (Harrington, 1957) and the present *Oryzias latipes*. They all begin to breed during the period of increasing long daylight.

The latter type comprises the fishes which begin to breed during the period of declining daylight. They belong to Salmonidae or Plecoglossidae, viz., *Salvelinus fontinalis* (Hoover & Hubbard, 1937), *Oncorhynchus nerka* (Combs et al., 1959) and *Plecoglossus altivelis* (Shiraishi & Takeda, 1961).

Summary

1. The fish maintained at room temperature and exposed to 18 hours of light per day, laid lighter and smaller eggs than the controls.

2. The fish kept at room temperature and exposed to artificial autumn daylight stopped laying eggs by July. There were found in their ovaries many regressive yolk-laden oocytes.

3. In out-of-normal spawning season, the fish kept at room temperature and exposed to long photoperiod succeeded in achieving maturation of eggs.

4. Under the same conditions as above, progressive testicular changes were found in the male exposed to long photoperiod and testes were matured enough to perform spawning acts.

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Explanation of Plates

PLATE I

All figures are photomicrographs from the sections of ovaries.

Fig. 1. Ovary from a fish exposed to artificial autumn daylight, and kept at room temperature. Fixed on June 21, 1960. ×15

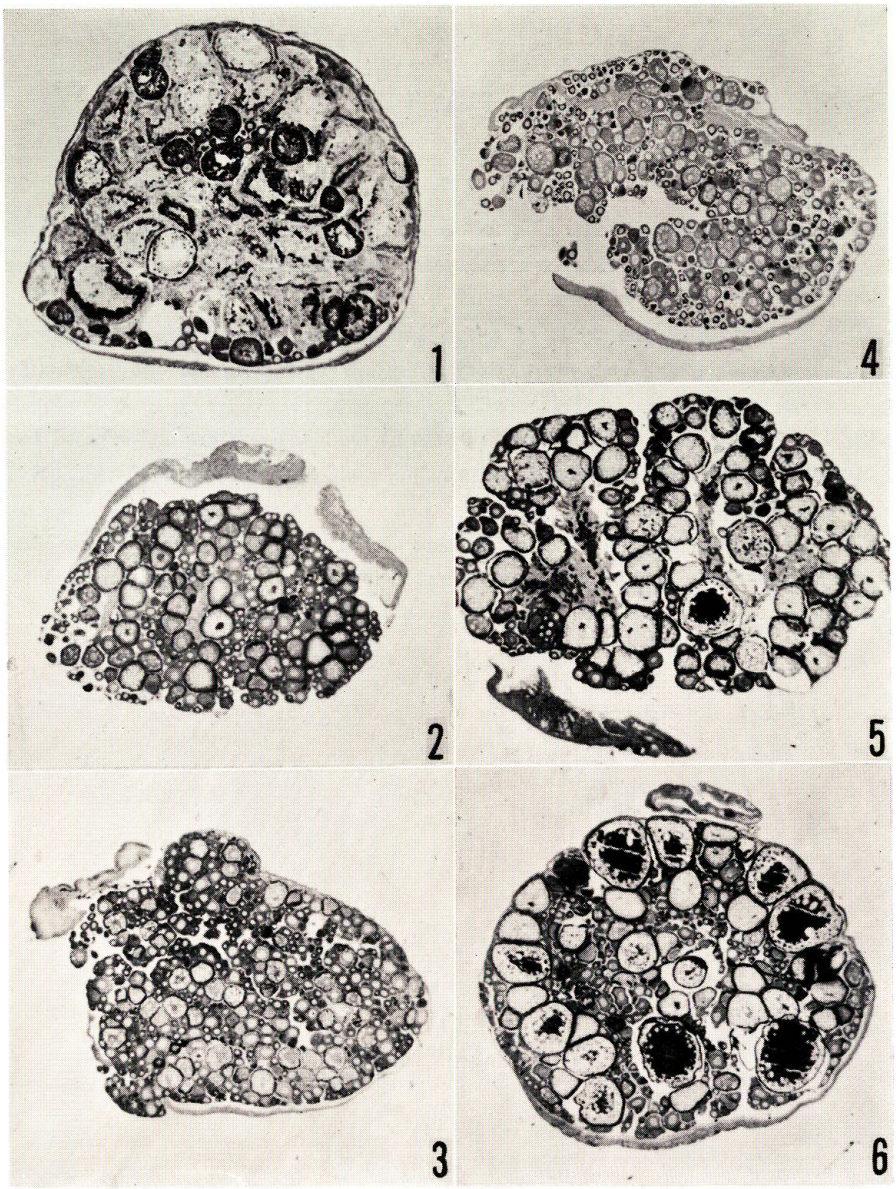
Fig. 2. Ovary from a fish in the initial control. Fixed on September 11, 1960. ×15

Fig. 3. Ovary from a fish exposed to natural autumn daylight and kept at room temperature. Fixed on October 10, 1960. ×15

Fig. 4. Ovary from a fish exposed to natural autumn daylight and kept at room temperature. Fixed on November 10, 1960. ×15

Fig. 5. Ovary from a fish exposed to long photoperiod, and kept at room temperature. Fixed on October 10, 1960. ×15

Fig. 6. Ovary from a fish exposed to long photoperiod, and kept at room temperature. Fixed on November 10, 1960. ×15



Yoshioka: The Effects of Day-length on Reproduction of Medaka

PLATE II

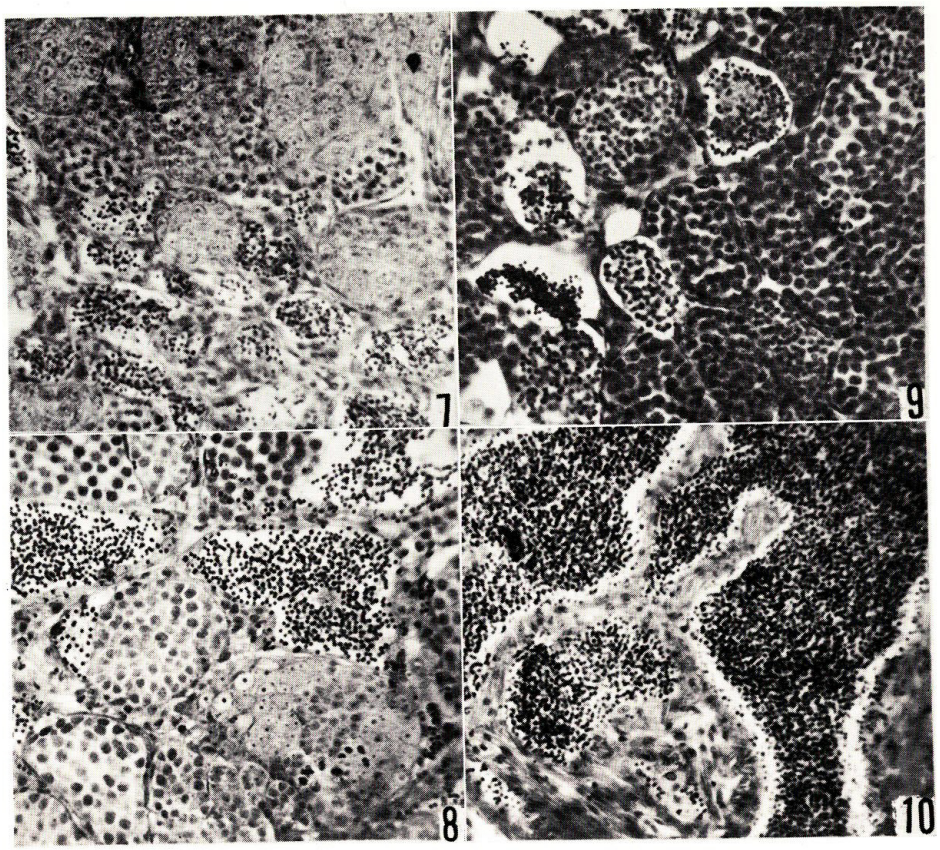
All figures are photomicrographs from the sections of testes.

Fig. 7. Portion of the testis of a fish kept at room temperature and exposed to natural autumn daylight, containing many spermatogonia and a few spermatids. Fixed on October 10, 1960. ×390

Fig. 8. Portion of the testis of a fish kept at room temperature and exposed to natural autumn daylight. Many spermatocytes, a few spermatogonia and spermatids are found. Fixed on November 10, 1960. ×390

Fig. 9. Portion of the testis of a fish kept at room temperature and exposed to long photoperiod. Many spermatocytes, a few spermatids and spermatozoa are found. Fixed on October 10, 1960. ×390

Fig. 10. Portion of the testis of a fish kept at room temperature and exposed to long photoperiod. Many spermatozoa, spermatids and a few spermatogonia are found. Fixed on November 10, 1960. × 390



Yoshioka: The Effects of Day-length on Reproduction of Medaka