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Effect of photoperiod and temperature on gonadal activity and plasma steroid levels in a reared strain of the mummichog (*Fundulus heteroclitus*) during different phases of its annual reproductive cycle

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Abstract

Mummichog, a spring and summer spawning teleost, were exposed to various photoperiod and temperature conditions to investigate the environmental regulation of the annual reproductive cycle. In early spring, latter phases of gonadal development (vitellogenesis in females and active spermatogenesis in males) were effectively accelerated by warm temperature (16 °C) regardless of the photoperiod (11L or 16L), although internal factor(s) may be concerned with triggering the initiation of the development. In late summer, intense gonadal regression which leads to the termination of the spawning period was accelerated by a short day length ($\leqslant$13L) in both yearlings and underyearlings. In underyearlings, however high water temperature and internal factor(s) may also be concerned. In early autumn, early phases of gonadal development (growth of cortical alveolus stage oocytes in females and basal spermatogenesis in males) were induced by moderate or lower temperatures ($\leqslant$22 °C) even under a short day length (11L). From the middle of autumn to early winter, this fish is probably in the "refractory period," and did not progress to the latter phases even under adequate temperature and long day length conditions (22 °C-16L). Mummichog showed a probable circa-annual rhythm of gonadal activity under constant temperature and photoperiod conditions (22 °C-16L): this rhythm may be the basis of the trigger of the gonadal development prior to the spawning period, the termination of the spawning period in underyearlings, and the occurrence of the "refractory period" during autumn. Plasma concentrations of estradiol-17β in females and testosterone in males correlated well with the gonadal development during early spring and the regression during late summer. However, there was no correlation between plasma steroid levels and the degree of progress during the early phases of gonadal development in autumn, suggesting other factor such as direct action of GtH(s) or mediating substance other than sex steroids for these phases.

Keywords: Mummichog; *Fundulus*; Reproduction; Annual cycle; Gonad; Photoperiod; Day length; Temperature; Estrogen; Androgen

1. Introduction

The mummichog *Fundulus heteroclitus* is broadly distributed along the eastern coast of North America, and between locality shows some variations in the reproductive characteristics such as in the spawning period, spawning frequency, and appearance of lunar/semilunar rhythms. Among such variable types, a reared strain of the mummichog originating from Chesapeake Bay (Arasaki strain) has a constant annual reproductive cycle and a clear daily reproductive cycle normally without showing any lunar/semilunar rhythms, and can be used as excellent material for studying the environmental and endocrine control of reproductive cycles of teleosts (Shimizu, 1997).

Gonads of yearling fish of this strain are immature in September. During late autumn and winter, a gradual increase in the GSI of both sexes is observed, and the development of cortical alveolus phase oocytes in females and basal spermatogenesis in males progresses. In late February, a rapid increase in the GSI of both sexes,

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vitellogenesis in females, and active spermatogenesis in males, occur. The spawning period of the yearling fish extends from late March to August. The spawning period of the underyearling fish starts in the same month as the yearlings, but terminate one month earlier. In the midst of the spawning period, each female spawns almost everyday and shows clear daily reproductive rhythm (Shimizu, 1997).

This study is intended to clarify environmental involvement in the controlling mechanism of the annual reproductive cycle of the mummichog, i.e., what environmental (or internal) factors regulate the various reproductive events such as the initiation of the spawning period, termination of the spawning period, and initiation of the early phase of gonadal development during autumn and winter. It is also directed to clarify the endocrine changes which mediate the environmental effects to the reproductive system, and then, to contribute to basic knowledge of the environmental control and endocrine mediation of annual reproductive cycle of spring or spring-to-summer spawning teleosts.

2. Materials and methods

2.1. Fish

Mummichog of the Arasaki strain were reared under natural photoperiod and temperature conditions in outdoor tanks supplied with natural seawater at Arasaki (35°N, 140°E). The maintenance conditions of the stock mummichog are reported previously (Shimizu, 1997). Yearling (1+ year) fish (TL = 70–101 mm) or underyearling (0+ year) fish (TL = 66–78 mm) were used in the experiments.

2.2. Experimental procedure

At different phases of their annual reproductive cycle, 10–20 fish were introduced into each 60 L glass aquarium (one aquarium per each group except for the former half of the Experiment 6) filled with sea water, and were subjected to various photoperiod and temperature regimes as described below. Photoperiod was controlled with a 20 W fluorescent lamp connected to an electric timer, and water temperature with a commercial aquarium thermostat and heater. Aquaria were situated in each shaded room for each specific photoperiod regime. For the lower temperature regimes, aquaria were situated in shaded cold rooms. Water temperature was maintained ±1 °C except during the acclimation period to each temperature regime. The acclimation period, in which the rate of the temperature change was limited to 3 °C/day, occupied the first 1–3 days of the experimental period. Fish were fed with commercial pellets for trout every day. The aquarium water was circulated through a commercial aquarium filter equipped with coral gravel beds, and half of the water was exchanged twice a month.

2.3. Photoperiod and temperature regimes

Since the experiments were designed to clarify the natural conditions controlling the annual reproductive cycle, photoperiod and temperature regimes were selected considering normal conditions of the annual cycle. The regimes used were as follows: 16L8D (hereafter abbreviated as 16L): approximately the longest day length (around summer solstice) in natural habitat of this species; 13L: moderate-short day length during early autumn; 11L: short day length during late autumn, late winter, and early spring; 9L: approximately the shortest day length (around winter solstice) in natural habitat of this species; 7 and 11 °C: low temperatures during winter and early spring; 16 and 22 °C: moderate temperatures during spring, early summer, and autumn; 26 °C: high temperature during summer and early autumn; 30 °C: approximately the higest temperature in natural conditions.

2.4. Sampling

At sampling, fish were anesthetized with tricaine methanesulfonate, blood was taken by cutting the caudal peduncle, after which the fish were killed by cutting the medulla oblongata. The abdominal cavity was opened, and the whole body was fixed in Bouin–Hollande solution. After rinsing with water, the body weight and gonad weight were measured, and the gonadosomatic index (GSI, gonadal weight × 100/body weight) was calculated. Gonads were embedded in paraplast (TM) and prepared for histological observation. Developmental stages of gonads were classified by criteria shown in Table 1. Plasma was separated by centrifuging the blood at 9000*g* for 3 min and stored at −30 °C until the steroid measurements were made.

2.5. Experiment 1 (factor that stimulates the initiation of the spawning period)

The experiment was started on February 2, when the natural water temperature of the outdoor tank was 13 °C. Yearling fish were kept under conditions of 7 °C-11L, 7 °C-16L, 11 °C-11L, 11 °C-16L, 16 °C-11L, and 16 °C-16L for 48 days.

2.6. Experiments 2 and 3 (factor that stimulates termination of the spawning period)

Experiment 2 was started on July 19, when the natural water temperature of the outdoor tank was 24 °C.

Table 1
Criteria used for the histological evaluation of gonadal activity in mummichog

Stage	Ovarian histological characteristics
I (Perinucleolus)	The ovary mainly consists of perinucleolus stage oocytes. Small number of early cortical alveolus stage oocytes may be present
II (Early cortical alveolus)	The ovary mainly consists of early cortical alveolus stage oocytes (cortical alveoli are situated peripherally in almost a single layer). Small number of late cortical alveolus stage oocytes may be present
III (Late cortical alveolus)	The ovary mainly consists of late cortical alveolus stage oocytes which are filled with cortical alveoli
IV (Early yolk globule)	The ovary contains early yolk globule stage oocytes which have particular yolk globules only in the central region
V (Mid yolk globule)	The ovary contains mid yolk globule stage oocytes which have fragmental, partially fused yolk
VI (Late yolk globule)	The ovary contains late yolk globule stage oocytes which are occupied with completely fused yolk mass
VII (Fully mature)	The ovary contains matured oocytes without germinal vesicle. Ovulated eggs may be present
R (Regressing)	The ovary contains many degenerated oocytes
Stage	Testicular histological characteristics
I (Spermatogonia)	Small cysts which mainly consist of spermatogonia occur only at the surface of the testis. Small numbers of spermatocytes may be present
II (Spermatocytes)	Small cysts which mainly consist of spermatocytes occur. Small numbers of spermatids and sperm may also be present
III (Basal spermatogenesis-1)	The testis contains all types of germ cells (spermatogonia to spermatozoa), however, spermatogenic tubules are still undeveloped (thickness of the spermatogenic tubule region is generally thinner than that of the efferent duct region in the middle cross-section)
IV (Basal spermatogenesis-2)	Spermatogenic tubules are considerably developed (thickness of the spermatogenic tubule region is generally thicker than that of the efferent duct region in the middle cross-section). However, efferent ducts contain few matured sperm
V (Fully mature)	Spermatogenic tubules are well developed containing large amounts of all types of germ cells, and the efferent ducts are full of matured sperm
R1 (Regressing-1)	Spermatogenic tubules are considerably shortened although the efferent ducts are full of matured sperm
R2 (Regressing-2)	Spermatogenic lobules containing mainly spermatogonia are situated at the testis surface. Considerable amount of sperm are still present in the efferent ducts

Underyearling fish were kept under conditions of 22 °C-9L, 22 °C-13L, 22 °C-16L, 30 °C-9L, 30 °C-13L, and 30 °C-16L for 28 days.

Experiment 3 was started on July 18, when the natural water temperature of the outdoor tank was about 24 °C. Yearling fish were kept under conditions of 22 °C-13L, 22 °C-16L, and 30 °C-16L for 28 days.

2.7. Experiment 4 (factor that induces early gonadal development during autumn and winter)

The experiment was started on September 18, when the natural water temperature of the outdoor tank was 25 °C. Yearling fish were kept under conditions of 16 °C-11L, 22 °C-11L, 26 °C-11L, and 30 °C-11L for 60 days.

2.8. Experiment 5 (effects of photoperiod on gonadal activity under adequate temperatures during autumn and winter)

The experiment was started on October 18, when the natural water temperature of the outdoor tank was 20 °C. Yearling fish were kept under conditions of 16 °C-11L, 16 °C-16L, 22 °C-11L, and 22 °C-16L for 70 days.

2.9. Experiment 6 (changes in gonadal activity of the fish kept under constant photoperiod and temperature conditions)

The experiment was started on October 18, when the natural water temperature of the outdoor tank was 20 °C. Yearling fish were kept under constant conditions of 22 °C-16L for 558 days. Fish were sampled on February 15 (the following year), March 27, July 12, October 23, January 9 (the subsequent year), and April 29.

2.10. Steroid RIA

Plasma steroids were measured by [^{3}H]RIA as reported previously (Shimizu et al., 1985). Concentrations of estradiol-17β (E_2) were measured in females. In males, testosterone (T) levels were measured; since RIA of 11β-hydroxytestosterone (11β-OH-T, the dominant androgen in the mummichog; Cochran, 1987) has a problem in the availability of the labeled hormone and the specific antiserum, and plasma T levels generally showed similar pattern of changes to that of 11β-OH-T levels in annual sampling of the wild fish (Cochran, 1987).

2.11. Statictics

Data of plasma steroid levels were normalized by logarithmic transformation before the tests. Dunnet's multiple comparison test following one-way ANOVA was used to compare each treated group with the initial control group. For the experiments in which multiple regimes were used for both photoperiod and temperature (Experiments 1, 2, and 5), two-way ANOVA (factorial) was also used among the treated groups to test the effects or interaction of photoperiod and temperature.

3. Results

3.1. Factor that stimulates the initiation of the spawning period (Experiment 1)

Fig. 1 and Table 2 show the changes in GSI and gonadal histology, respectively, according to the treatments in Experiment 1. GSI of both females and males of the initial controls were low: the ovaries contained large cortical alveolus phase oocytes without vitellogenic or older ones. The testes contained lobules containing all types of germ cells although the efferent ducts had few matured sperm. After 48 days of the treatments, there were very little changes in the GSI and gonadal histology in the 7 °C groups. In contrast, the GSI of the 16 °C groups increased prominently regardless of the photoperiod regime, and most fish of both sexes were fully matured. Some females in the 11 °C groups showed an increase in the GSI and accumulation of yolk globules. Males in the 11 °C groups showed an evident increase in the GSI, and some fish attained full maturity.

Fig. 2 shows the changes in plasma steroid levels with the treatments. Both E_2 in females and T in males of the initial controls were low levels. After the treatments, all the 16 °C groups showed a prominent increase in plasma E_2 and T. Both females and males in the 11 °C groups also showed significant increases in plasma steroid levels.

3.2. Factor that stimulates termination of the spawning period (Experiments 2 and 3)

Fig. 3 and Table 3 show the changes in GSI and gonadal histology, respectively, according to the treatments in Experiment 2. Both female and male GSI of the initial controls were very high, and most fish of both sexes were fully matured. After 28 days of the treatments, both female and male GSI decreased prominently in the 13L and the 9L groups regardless of temperature. The ovaries mainly contained early cortical alveoli and perinucleolus stage oocytes (occasionally with degenerated oocytes), and the testes contained small lobules which consisted of spermatogonia and some spermatocytes. In the 30 °C-16L group, major part of the fish showed gonadal regression combined with a decrease in GSI. Major part of the males and a few females in the 22 °C-16L group also showed gonadal regression with decrease in GSI.

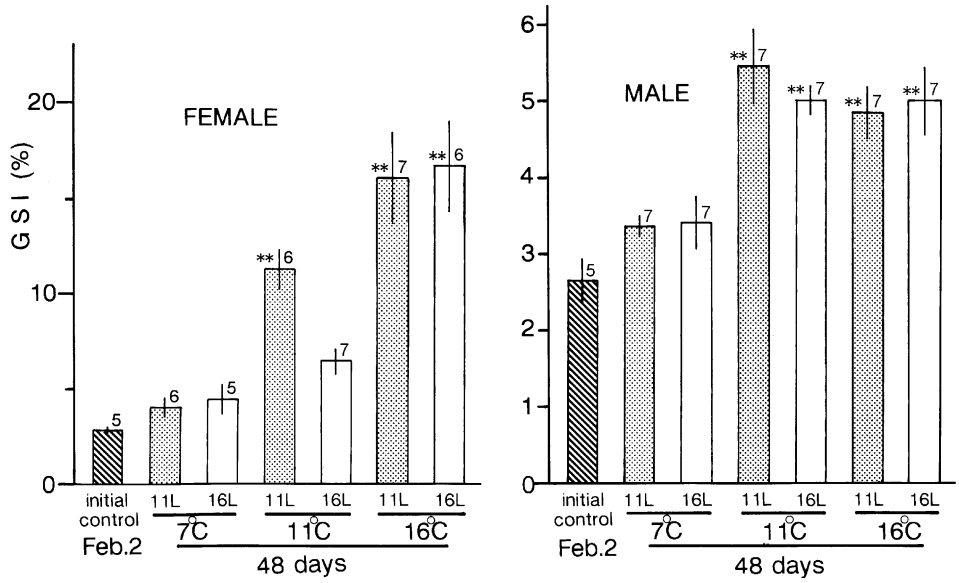

Fig. 1. Effects of various photoperiod and temperature regimes on the GSI of mummichog during early spring (Experiment 1). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. **Significantly larger than the value of the initial controls ($p < 0.01$; by Dunnet's multiple comparison test). Interaction between photoperiod and temperature was not detected ($p > 0.05$) by two-way ANOVA for both female and male GSIs. Significant effects of temperature were detected ($p < 0.05$) by two-way ANOVA for both female and male GSIs.

Table 2
Effects of various photoperiod and temperature regimes on the gonadal histology of the mummichog during early spring (Experiment 1)

Stage	Number of fish at each stage						
	Initial control	7 °C-11L	7 °C-16L	11 °C-11L	11 °C-16L	16 °C-11L	16 °C-16L
Ovary							
I							
II							
III	5	4	4		1		
IV		2	1	2	3		
V				2	3		
VI				2			
VII						7	6
R							
Testis							
I							
II							
III							
IV	5	7	7	3	4		
V				4	3	7	7
R1							
R2							

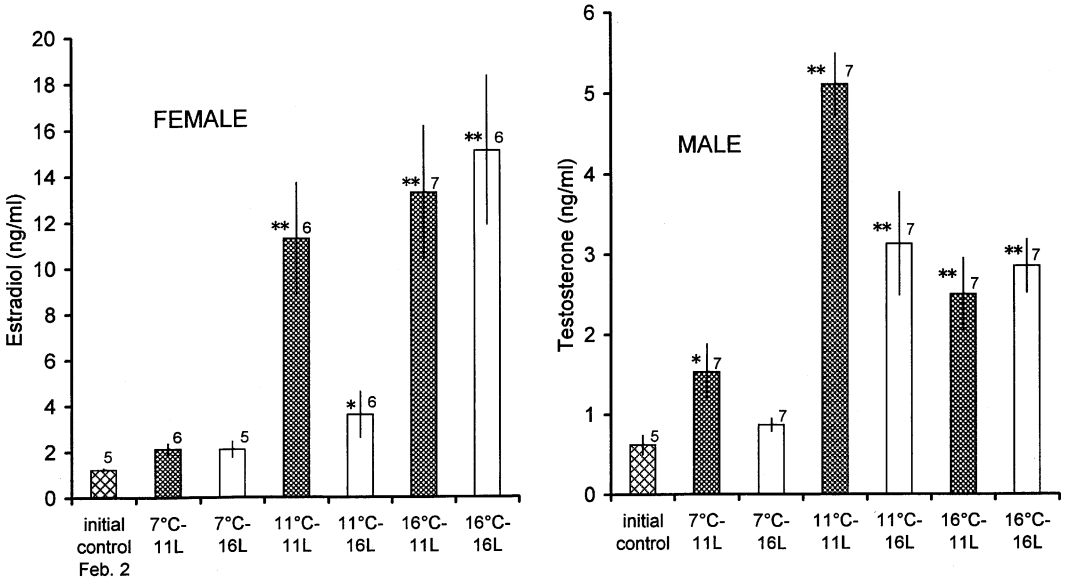

Fig. 2. Effects of various photoperiod and temperature regimes on plasma steroid levels of mummichog during early spring (Experiment 1). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. *,**Significantly larger than the value of the initial controls ($p < 0.05$ and 0.01, respectively; by Dunnet's multiple comparison test after logarithmic transformation). Interaction between photoperiod and temperature was detected ($p < 0.05$) by two-way ANOVA for both estradiol and testosterone levels.

Fig. 4 shows the changes in plasma steroid levels with the treatments. E_2 in females and T in males showed high values in the initial controls. After the treatments, all the 9L and the 13L groups showed an intense decrease in plasma E_2 or T. The 30 °C-16L group also showed a significant decrease in plasma steroid levels in both sexes.

Fig. 5, Table 4, and Fig. 6 show the changes observed in Experiment 3. Yearling fish showed same gonadal regression and decrease in plasma steroid levels in the short day group (22 °C-13L) as was also observed in the underyearlings. However, the yearlings scarcely showed gonadal regression or decrease in plasma steroid levels in the long day length groups (22 °C-16L and 30 °C-16L).

3.3. Factor that induces early gonadal development during autumn and winter (Experiment 4)

Fig. 7 (upper) and Table 5 show the changes in GSI and gonadal histology, respectively, according to the treatments. GSI of both females and males was very low in the initial controls: the ovaries mainly consisted of perinu-

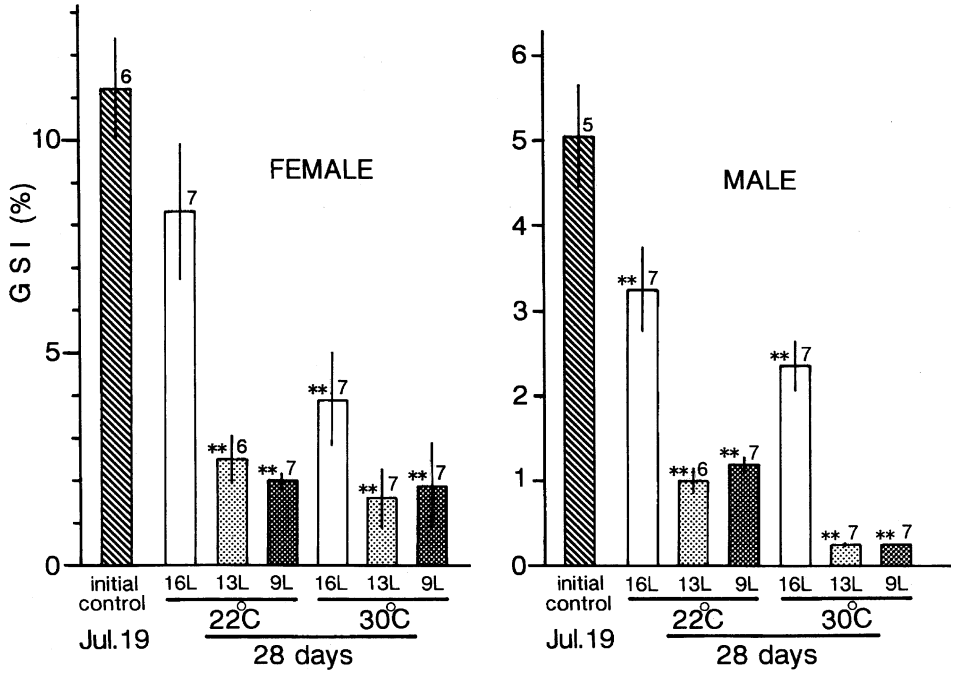

Fig. 3. Effects of various photoperiod and temperature regimes on the GSI of underyearling mummichog during summer (Experiment 2). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. **Significantly smaller than the value of the initial controls ($p < 0.01$; by Dunnet's multiple comparison test). Interaction between photoperiod and temperature was not detected ($p > 0.05$) by two-way ANOVA for both female and male GSIs. Significant effects of photoperiod were detected ($p < 0.05$) by two-way ANOVA for both female and male GSIs. Significant effects of temperature were also detected ($p < 0.05$) by two-way ANOVA for both female and male GSIs.

Table 3
Effects of various photoperiod and temperature regimes on the gonadal histology of the underyearling mummichog during summer (Experiment 2)

Stage	Number of fish at each stage						
	Initial control	22 °C-16L	22 °C-13L	22 °C-9L	30 °C-16L	30 °C-13L	30 °C-9L
Ovary							
I						3	2
II						3	5
III		1	4	5	1	1	
IV					2		
V							
VI	1				2		
VII	5	5			1		
R		*1*	*3*	*2*	*1*		
Testis							
I						5	7
II						1	
III							
IV							
V	5	2			1		
R1		*5*			*5*		
R2			*6*	*7*	*1*	*1*	

cleolus stage oocytes, and the testes of most males contained only spermatogonia and spermatocytes as germ cells. After 60 days of the treatments, both female and male GSI in the 16 °C group increased significantly, while the GSI in the higher temperature groups stayed at low values. The developmental changes of the gonads tended to advance as the experimental temperature decreased.

Fig. 7 (lower) shows the changes in plasma steroid levels of the treatments. E_2 levels in females were very low in the initial controls, and showed almost no change in all groups after the treatments. T in males was undetectable (less than 0.15 ng/mL) or very low values in the initial controls and was also almost undetectable in all the treated groups.

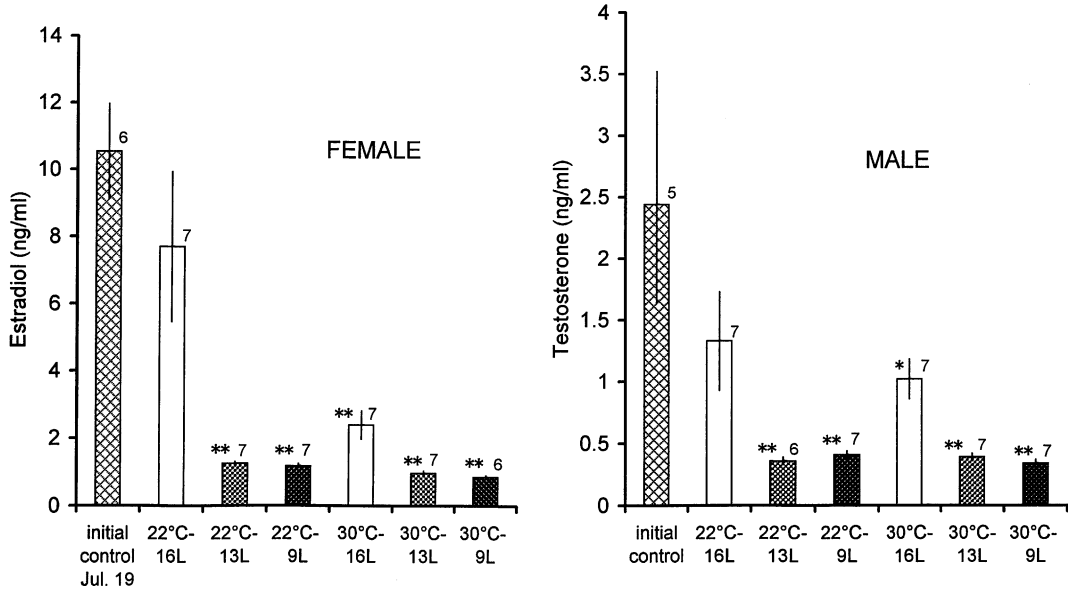

Fig. 4. Effects of various photoperiod and temperature regimes on plasma steroid levels of underyearling mummichog during summer (Experiment 2). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. *,**Significantly smaller than the value of the initial controls ($p < 0.05$ and 0.01, respectively; by Dunnet's multiple comparison test after logarithmic transformation). Interaction between photoperiod and temperature was not detected ($p > 0.05$) by two-way ANOVA for both female and male GSIs. Significant effects of photoperiod were detected ($p < 0.05$) by two-way ANOVA for both estradiol and testosterone levels. Significant effect of temperature was detected ($p < 0.05$) by two-way ANOVA for estradiol levels.

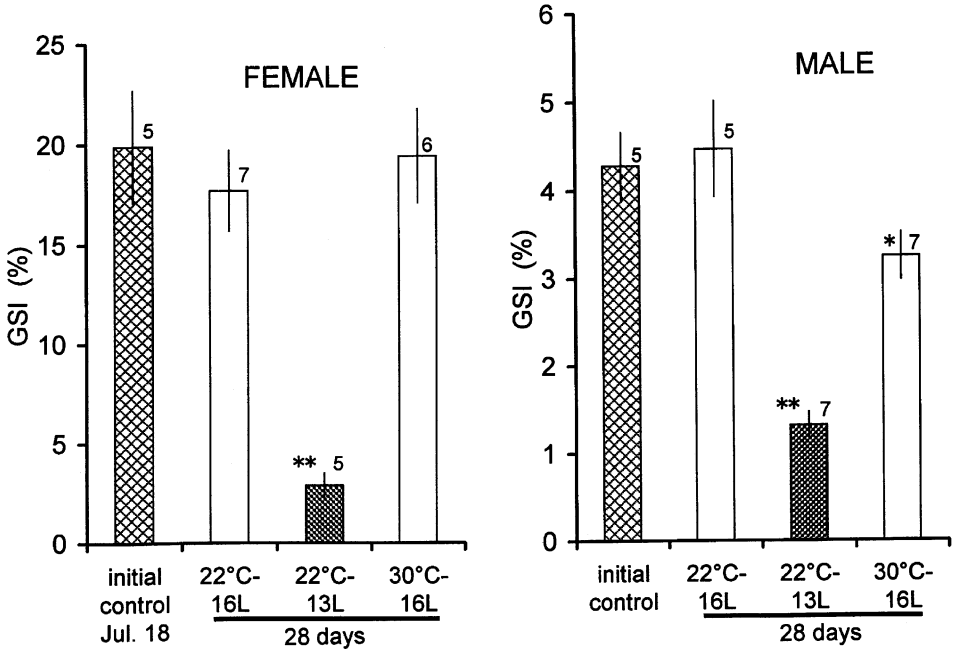

Fig. 5. Effects of various photoperiod and temperature regimes on the GSI of the yearling mummichog during summer (Experiment 3). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. *,**Significantly smaller than the value of the initial controls ($p < 0.05$ and 0.01, respectively; by Dunnet's multiple comparison test).

3.4. Effects of photoperiod on gonadal activity under adequate temperature during autumn and winter (Experiment 5)

Fig. 8 (upper) and Table 6 show the changes in GSI and gonadal histology, respectively, according to the treatments. GSI of both females and males were low: the ovaries mainly contained cortical alveolus phase oocytes, and the testes showed early phases of spermatogenesis. After 70 days of the treatments, female GSIs in the 22 °C groups did not show any significant changes regardless of the photoperiod and temperature. Slight

Table 4
Effects of various photoperiod and temperature regimes on the gonadal histology of the yearling mummichog during summer (Experiment 3)

Stage	Number of fish at each stage			
	Initial control	22 °C-16L	22 °C-13L	30 °C-16L
Ovary				
I				
II				
III			2	
IV				
V				
VI				1
VII	5	7		5
R			*3*	
Testis				
I				
II				
III				
IV				
V	4	4		6
R1	*1*	*1*		*1*
R2			*7*	

increases in the GSI were observed in the 16 °C groups, although the ovarian histology was almost unchanged. Male GSI did not change in the 22 °C groups regardless of the photoperiod. In the 16 °C-16L group, the GSI increased to some extent, and testicular histology showed some advances in spermatogenesis. Slight advance in spermatogenesis was also observed in the 16 °C-11L group.

Fig. 8 (lower) shows the changes in plasma steroid levels with the treatments. Both E_2 in females and T in males were of low value in the initial controls. After the treatments, only males in the 16 °C-16L group showed significant increase in plasma T levels.

3.5. Changes in gonadal activity of the fish kept under constant photoperiod and temperature conditions (Experiment 6)

Fig. 9 (upper) and Table 7 show the changes in GSI and gonadal histology, respectively, according to the treatments. Female GSI of the initial controls showed low values. In the following February, females indicating higher GSI with vitellogenic ovaries appeared although the mean GSI was not significantly different from the value of the initial controls. In late March and the middle of July, all the fish showed very high GSI values. The GSI decreased to a low level again in late October. It remained at a low level still in the subsequent January, then showed a prominent increase in late April. Ovarian histology showed corresponding changes (from cortical alveoli stage to fully matured stage) to the GSI changes. Male GSI showed similar changes to those of females, and the testicular histology also showed corresponding changes to the GSI changes.

Fig. 9 (lower) shows the changes in plasma steroid levels with the treatments. Both E_2 in females and T in males of initial controls were low values. E_2 in females increased during spring and early summer and showed

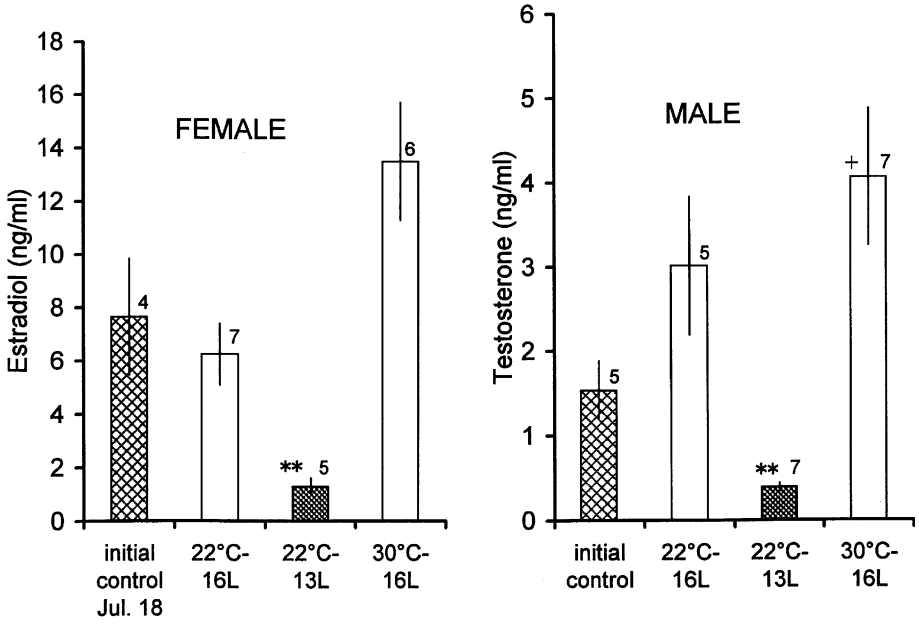

Fig. 6. Effects of various photoperiod and temperature regimes on plasma steroid levels of yearling mummichog during summer (Experiment 3). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. **Significantly smaller than the value of the initial controls ($p < 0.01$; by Dunnet's multiple comparison test after logarithmic transformation). +Significantly larger than the value of the initial controls ($p < 0.05$; by Dunnet's multiple comparison test after logarithmic transformation).

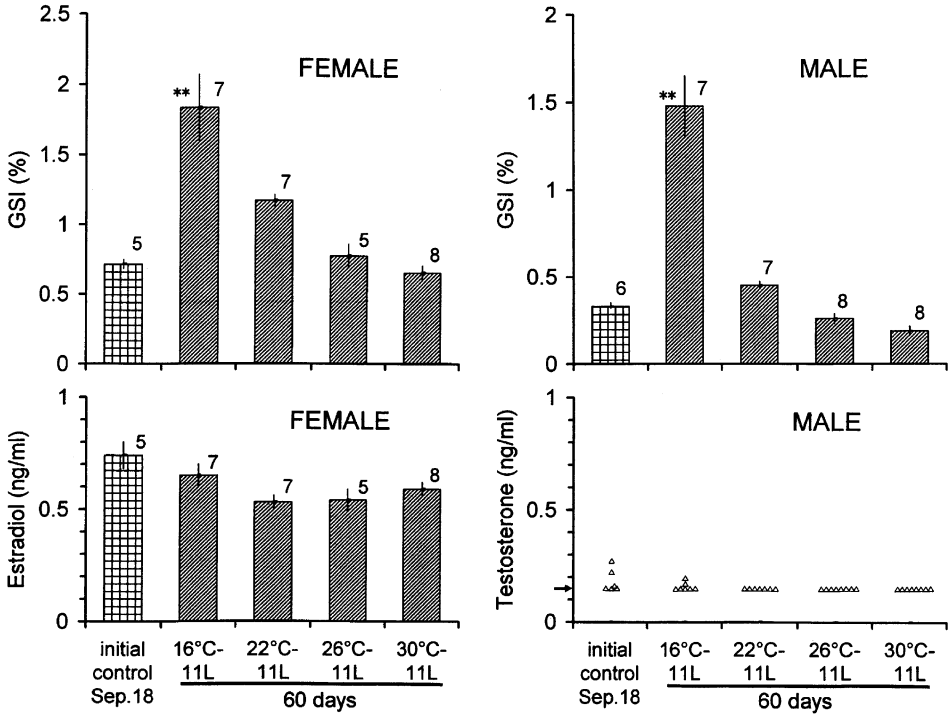

Fig. 7. Effects of various temperature regimes on the GSI (upper) and plasma steroid levels (lower) of mummichog during autumn (Experiment 4). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. Triangles denote the values of individual fish. The arrow indicates the detection limit. *,**Significantly larger than the value of the initial controls ($p < 0.05$ and 0.01, respectively; by Dunnet's multiple comparison test).

Table 5
Effects of various temperature regimes on the gonadal histology of the mummichog during autumn (Experiment 4)

Stage	Number of fish at each stage				
	Initial control	16 °C-11L	22 °C-11L	26 °C-11L	30 °C-11L
Ovary					
I	1			2	8
II	4		2	3	
III		7	4		
IV					
V					
VI					
VII					
R					
Testis					
I	2			3	7
II	3			4	
III	1	2	7	1	1
IV		5			
V					
R1					
R2					

low values in other seasons. T in males showed a similar pattern of change to that of E_2 in females.

4. Discussion

The results of Experiment 1 indicate that the latter phases of gonadal development (vitellogenesis in females and active spermatogenesis in males) which induce the initiation of the spawning period is effectively accelerated by the increase in water temperature during spring. Effect of the photoperiod appears limited in this season because almost no difference occurred in the gonadal activity between the 11L and the 16L groups under the adequate temperature (16 °C). Interaction of photoperiod and temperature was detected by two-way ANOVA

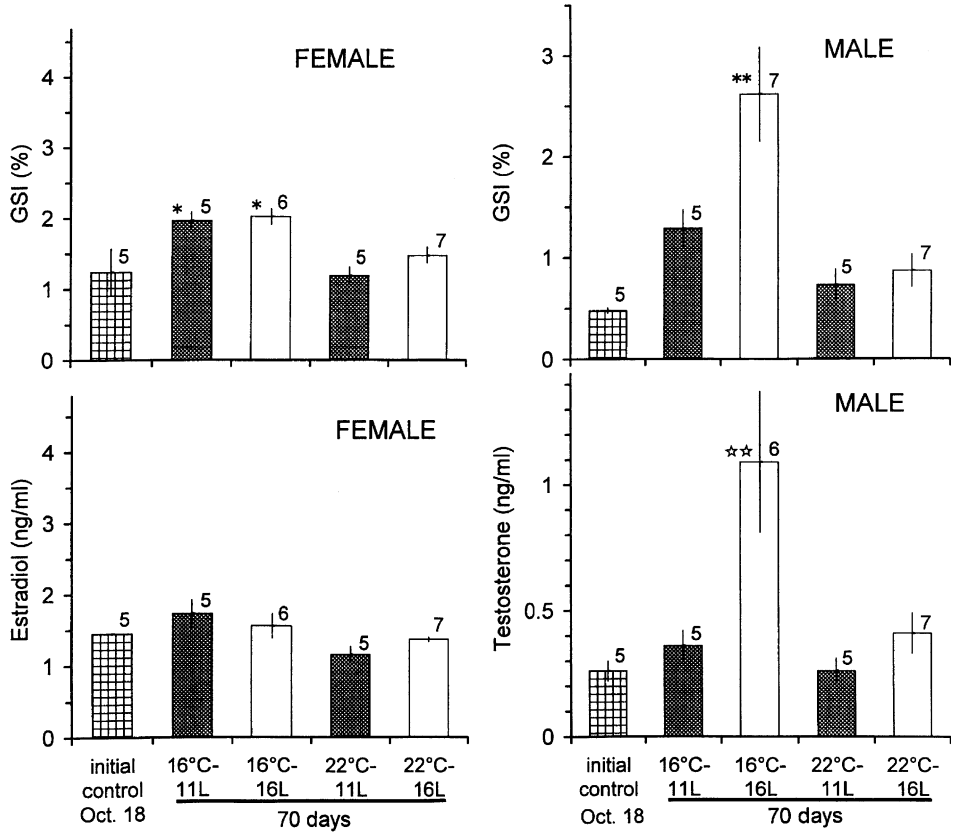

Fig. 8. Effects of various photoperiod and temperature regimes on the GSI (upper) and plasma steroid levels (lower) of mummichog during autumn and early winter (Experiment 5). Columns and bars indicate the means and standard errors, respectively. Figures above columns show numbers of fish examined. *,**Significantly larger than the value of the initial controls ($p < 0.05$ and 0.01, respectively; by Dunnet's multiple comparison test). ☆☆Significantly larger than the value of the initial controls ($p < 0.01$, by Dunnet's multiple comparison test after logarithmic transformation). Interaction between photoperiod and temperature was not detected ($p > 0.05$) by two-way ANOVA for female and male GSIs, estradiol levels, and testosterone levels. Significant effects of temperature were detected ($p < 0.05$) by two-way ANOVA for female and male GSIs, estradiol levels, and testosterone levels. Significant effects of photoperiod were detected ($p < 0.05$) by two-way ANOVA for male GSI and testosterone levels.

for plasma E_2 and T levels in this experiment, mainly because that the levels were considerably higher in the 11L group than in the 16L group under 11 °C. Reason of the paradoxical higher levels in the short day length group is uncertain: they may be caused by a possible slight difference in mean water temperatures in the aquaria, since even a slight difference might cause considerable effects under the conditions near the critical temperature. The increase in water temperature might be the causative factor initiating the spawning period in some cases, especially in habitats where water temperatures in winter decrease below 10 °C. However, water temperature seems not to be the only factor that stimulates the initiation of the spawning period in other cases, including at Arasaki. In Arasaki, mummichog progresses to the latter phases of gonadal development from early spring, and the latter phases do not progress during early and mid winter, although water temperatures seldom fall below 11 °C throughout seasons (Shimizu, 1997). The results of Experiment 1 indicate that the critical temperature for the latter phases of gonadal development is generally situated between 7 and 11 °C, indicating that development could occur even during winter if the temperature is the only causative factor. This case suggests that the initiation of the latter phases is triggered by some internal (endogeneous, possibly neural) factor(s) such as the circa-annual rhythm, and increasing temperature has a strong but only an accelerating effect. The results of Experiment 6 indicate that mummichog actually show annual variations of gonadal activity under constant photoperiod and temperature conditions (22 °C-16L). They fully matured during spring and early summer and had a regressed state during autumn and winter, indicating probable circa-annual reproductive rhythm. Unfortunately, complete proof of a circa-annual rhythm will be very difficult in this species, since it needs detection of a phase shift according to the free-running of the rhythm, and such an experiment will take several years which may be longer than the life span of this fish (about 4 years).

The results of Experiments 2 and 3 show that extensive gonadal regression is accelerated by short day

Table 6
Effects of various photoperiod and temperature regimes on the gonadal histology of the mummichog during autumn and winter (Experiment 5)

Stage	Number of fish at each stage				
	Initial control	16 °C-11L	16 °C-16L	22 °C-11L	22 °C-16L
Ovary					
I					
II	4	2	3	4	6
III	1	3	3	1	1
IV					
V					
VI					
VII					
R					
Testis					
I					
II					
III	5			3	4
IV		5	6	2	3
V			1		
R1					
R2					

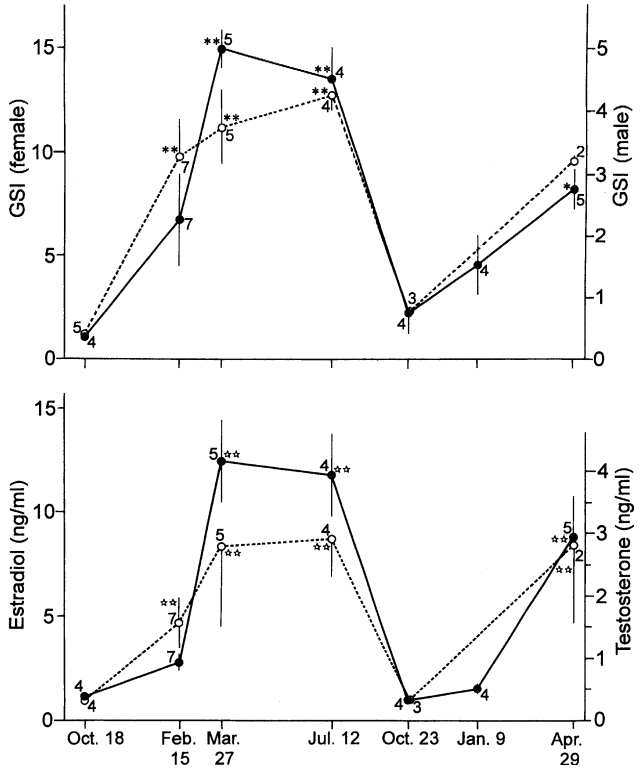

Fig. 9. Effects of a constant photoperiodic and temperature regime (22 °C-16L) on the GSI (upper) and plasma steroid levels (lower) of the mummichog (Experiment 6). Circles and bars indicate the means and standard errors, respectively. The solid lines corresponds to females, and the broken lines to males. Figures by circles show numbers of fish examined. *,**Significantly larger than the value of the initial controls ($p < 0.05$ and 0.01, respectively; by Dunnet's multiple comparison test). ☆☆Significantly larger than the value of the initial controls ($p < 0.01$; by Dunnet's multiple comparison test after logarithmic transformation).

length (9L or 13L) regardless of temperature in both yearlings and underyearlings. In the yearling fish, short day length during late summer is most responsible for the termination of the spawning period (Experiment 3). In the underyearling fish, however, another environmental factor, i.e., high water temperature may also be concerned. Furthermore, some internal factor may be responsible especially in males, since some of the underyearling fish showed regressed gonads under adequate temperature and photoperiod conditions (22 °C-16L). Apparently, these dissimilarities between yearlings and underyearlings cause the difference in the duration of the spawning period.

The results of Experiment 4 indicate that the early phases of gonadal development (growth of cortical alveolus phase oocytes and basal spermatogenesis), which progress during autumn and winter in natural conditions, are induced by lowering temperatures in this season. Although these phases progress even under short photoperiod (11L), the latter phases of gonadal development do not proceed in this season naturally. The results of Experiment 5 indicate that mummichog show very little progress in gonadal development even under adequate temperature-long day length conditions (22 °C-16L). This fish in autumn and early winter is probably in a "refractory period," as reported previously in a wild strain of the mummichog (Taylor, 1986) and in other fish species (Harrington, 1957; Kaya, 1973; Shimizu et al., 1994; Sundararaj and Vasal, 1976; Yoshioka, 1966). The "refractoriness" may be somewhat weakened in males under lower temperatures, because some of the fish of 16 °C-16L groups showed an increase in GSI and gonadal development to some extent. The

Table 7
Effects of a constant photoperiod and temperature regime (22 °C-16L) on the gonadal histology of the mummichog (Experiment 6)

Stage	Number of fish at each stage						
	Initial control	120 days	160 days	267 days	360 days	448 days	559 days
	October 18	February 15	March 27	July 12	October 23	January 9	April 29
Ovary							
I							
II	3	2					
III	1	1			3	2	
IV							
V							
VI		1			1	1	
VII		3	5	4		1	5
R							
Testis							
I							
II	2				1		
III	3	1			2		
IV		5	1				
V		1	4	3			2
R1				*1*			
R2							

appearance of the ''refractory period'' is possibly based on the circa-annual rhythm, as well as the cases of the initiation of the latter phases of gonadal development during early spring and the gonadal regression of underyearling fish under adequate conditions during late summer.

Changes in plasma E_2 and T levels correlated closely with the gonadal changes in Experiments 1–3, indicating that steroid mediation of the latter phases of gonadal development occurs in both sexes. In Experiment 4, however, there was almost no change in the plasma steroid levels according to the progress in the early phases of gonadal development. The early phases may not be mediated by estrogen or androgens in this species. Very low levels of plasma E_2 or T occur during autumn and winter in females or males of the Arasaki strain under natural conditions (Shimizu, 1997), and very low levels of T, 11-ketotestosterone, and 11β-OH-T have been observed during the same period in the wild male mummichog (Cochran, 1987), supporting this suggestion. Direct effect of gonadotropin(s) or mediating substances other than sex steroids may be concerned with these phases, and will be an interesting subject to study.

In this study, the involvement of environmental factors in the normal annual reproductive cycle of mummichog is satisfactorily clarified, although clarifying the involvement of internal factors will need further study. The experiments were designed to clarify the natural conditions controlling the annual reproductive cycle, and regimes which are extremely far from the normal annual cycle (such as high temperature-long day length conditions during winter) were not examined in this study except Experiment 6. Experiments with such regimes will be the next step to study. Occurrence of photoperiodism dependent on temperature (Okuzawa et al., 1989; Razani et al., 1988a,b) and circa-annual changes in photoperiodism occurrence (Baggerman, 1972; Shimizu and Hanyu, 1993) have previously been observed in some teleosts using such regimes, and the former may also exist in mummichog (A. Shimizu, unpublished results).

Several experiments to examine effects of photoperiod and water temperature on gonadal activity have previously been conducted using wild strains of mummichog. The fish used were taken from the northern area of the geographic range (Burger, 1939) or the middle area (Day and Taylor, 1984) which is near the origin of the Arasaki strain; and the pattern of the annual reproductive cycle is generally resemble each other. They show a similar spawning season during spring and summer, although it begins somewhat later in the northern habitat (Mattheus, 1938) and short-term reproductive fluctuations caused by the lunar/semilunar rhythm were observed in a wild (Delaware Bay) strain (Taylor, 1984; Taylor and DiMichele, 1980; Taylor et al., 1979). The study using the fish captured from Delaware Bay (Day and Taylor, 1984) was carefully designed that the sampling times should be close to predicted peaks in spawning activity (new and full moon), and the results are comparable with those of the present study using the reared strain. Results of the present study are not contradictory to those earlier studies in many cases, suggesting that the basic mechanisms regulating the annual reproductive cycle are common among these strains. However, the conclusions

are not all the same. The earliest studies were conducted on males (Burger, 1939; Mattheus, 1939) and concluded that the photoperiod is not an important factor for reproduction in male mummichog. However, photoperiod is concluded to be the essential factor accelerating termination of the spawning period in both sexes in the present study. Direct comparison of the results is difficult since the early studies did not use constant photoperiod and temperature regimes, and used extreme photoperiod conditions such as DD, LL, 1.5L, or 20.5L which were unnatural. Nonetheless, the deductions of the early studies might not be complete, since the conclusion seems to be obtained without considering the annual variations in the photoperiodic response. More recent and detailed studies (Day and Taylor, 1983, 1984) were conducted on females, and considered such variations. The latter study showed evident photoperiod involvement in the reproduction of female mummichog during winter. They concluded that photoperiod is an important factor accelerating the initiation of the spawning period, and that temperature is not; a conclusion which is not consistent with that of the present study. However, the initiating time of the winter experiments of the study (Day and Taylor, 1984) was December, which is not the actual period of the initiation of the spawning season. Mummichog also showed some photoperiodism in an early spring experiment (Day and Taylor, 1984) which began at a similar time as the actual initiation of the spawning period. Nevertheless, the temperature regime (20 °C) was considerably higher than that of the natural conditions for this period (about 15 °C; Taylor, 1986). It is more likely that mummichog are scarcely responsive to photoperiod during this season under lower moderate, near-natural temperatures (11–16 °C), although they may be responsive under higher temperatures. Preliminary experiments showed that such a tendency is actually present in the Arasaki strain (A. Shimizu, unpublished results). In an experiment conducted near the end of the spawning season (Day and Taylor, 1984), matured females regressed gonads under adequate temperature and long day length conditions, a case which was similar to that of the underyearling fish (a few females and major part of males) in the present study (Experiment 2). Day and Taylor (1984) then suggested the involvement of circa-annual rhythm in the termination of the spawning period in female mummichog. They also suggested such involvement in the initiation of the spawning period. Results of the present study (Experiment 6) strongly support their suggestions, and indicate that the situation is same also in males.

The environmental control of the annual reproductive cycle of this species has common characteristics with those of other spring and spring-to-summer spawning species which have been previously studied, although in many cases species specific variations occur. It seems to be rather a complex of various factors which have also been identified in other species. The rose bitterling *Rhodeus ocellatus ocellatus* spawns during spring and summer, and the tabira bitterling *Acheilognathus tabira* spawns during spring. An increase in water temperature during spring is an important stimulus initiating the spawning period in both bitterling species (Asahina and Hanyu, 1983; Shimizu and Hanyu, 1983), similar to the case of the mummichog. However, these bitterling species do not show an apparent ''refractory period,'' and attain full maturity under moderate temperature-long day length conditions during autumn (Asahina and Hanyu, 1983; Shimizu and Hanyu, 1983, 1991). In contrast, clear ''refractoriness'' during opposite season (spring) to the spawning season is observed in autumn spawning bitterlings, *A. rhombea* (Shimizu et al., 1994) and *Pseudoperilampus typus* (A. Shimizu, unpublished results). Other spring spawning cyprinids, *Carassius auratus* and *Gnathopogon elongatus*, show a common controlling mechanism of the annual reproductive cycle to the spring spawning bitterling (Okuzawa et al., 1989; Razani et al., 1988a,b). However, the appearance of photoperiodism dependent on temperature (strong photoperiodism under higher temperatures and obscure photoperiodism under lower temperatures) is observed in both species and another spring spawning cyprinid *Notemigonus crysoleucas* (De Vlaming, 1975); which case is not clear in the bitterlings. Accelerated gonadal development induced by warm temperatures during spring and evident photoperiodism during autumn and winter are also observed in female sticklebacks *Gasterosteus aculeatus* (Baggerman, 1985; Borg and van Veen, 1982), female medaka *Oryzias latipes* (Awaji and Hanyu, 1988, 1989), mosquitofish *Gambusia affinis* (Koya and Kamiya, 2000), and a small filefish *Rudarius ercodes* (Lee et al., 1984). In male sticklebacks, the situation is complicated because the period of active spermatogenesis (during winter) and that of the sexually active phase (around the spawning season) are completely separated. Nonetheless, the kidney epithelium height which is enlarged by androgens and can serve as an indicator of male sexual activity showed the same responses as the gonadal activity of females (Borg, 1982).

Although variations exist, the importance of warm temperatures for the initiation of the spawning period during early spring and existence of clear photoperiodism (occasionally temperature dependent) or ''refractoriness'' during autumn are probably the common properties in many spring or spring-to-summer spawning teleosts. Presence or absence of summer spawning will be correlated with sensitivity to high temperatures (Shimizu and Hanyu, 1983) or some internal factor(s). Then, there is a question, what is the meaning of the seasonal changes in photoperiodism or the existence of the ''refractory period?'' These may be the mechanism by which spawning only occurs during spring (and

summer) or only during autumn. If the mechanism is absent, the fish may show a double spawning season (spring and autumn) as is previously reported in a dragonet *Repomucenus beniteguri* (Zhu et al., 1989). It seems to be a rather minor case, suggesting that the existence of a double spawning season is not advantageous in many fish species.

Endocrine mediation between environmental factors and the process of gametogenesis is still unclear in multiple spawning teleosts, since there have been few systematic studies conducted on the various phases of the annual reproductive cycle. Knowledge is especially poor for gonadotropins (GtHs). In a study conducted on a spring-spawning cyprinid *Gnathopogon elongatus* during various periods of the year, involvement of gonadotropin was also studied although only one GtH (probably LH) was measured (Okuzawa et al., 1989). Paradoxically high levels of GtH were observed in highly regressed fish under high temperatures in that study. Such high levels were also observed in the goldfish (Gillet et al., 1978; Razani et al., 1988a,b), and the implications of this phenomenon are still not clear. The role of FSH for reproduction is further unclear in multiple spawning teleosts, mainly because of the lack of adequate experimental fish species for this study. Recently, both FSH (GtH I) and LH (GtH II) were purified from the mummichog pituitary gland, and measurement of these hormone levels in this small fish may become possible in the near future (Shimizu and Yamashita, 2002). It will be useful to clarify the endocrine regulating mechanism of annual and short reproductive cycles of multiple spawning teleosts, by using this adequate experimental fish.

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