RESEARCH ARTICLE

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The light-dependent daily cycle of ovulation in the oviparous medaka fish, *Oryzias latipes* (Atherinomorpha: Beloniformes: Adrianichthyidae) artificially pregnant with developing embryos

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Abstract

In the oviparous medaka fish, *Oryzias latipes*, mature spermatozoa that were artificially introduced into the ovarian cavity retaining ovulated eggs could internally fertilize these eggs. This enabled us to examine the effect of ovarian gestation on the ovulation cycle. Most freshly ovulated eggs could be internally fertilized in the ovarian cavity. Yet eggs ovulated 24 h after single insemination remained unfertilized in the ovarian cavity. Artificially pregnant females persisted in a daily cycle of ovulation, which occurred shortly before the onset of light under the present reproductive conditions. Females continuously ovulated a certain number of eggs despite ovarian gestation, that is, the presence of embryos within the ovarian cavity. Repeated cycles of ovulation led to crowding in the ovarian cavity because the group of fertilized eggs, with their hardened egg envelope (chorion or zona radiata), plugged the genital orifice. The development of fertilized eggs was retarded and ceased around the initiation stage of blood circulation, but when they were transferred from the ovarian cavity into regular saline, they regained their ability to develop normally up to hatching. These results show that in oviparous female medaka, ovarian gestation exerted little effect on the time of ovulation and the number of ovulated eggs.

KEYWORDS

gestation, internal fertilization, *Oryzias latipes*, oviparity, ovulation cycle, photodependent reproduction

1 | INTRODUCTION

Atherinomorph fishes exhibit a broad range of reproductive modes including oviparity, ovoviviparity, viviparity, and hermaphroditism (Breder & Rosen, 1966). Many atherinomorph taxa, such as the guppy, *Poecilia reticulata*, the mosquitofish, *Gambusia affinis*, the

mummichog, *Fundulus heteroclitus*, and the medaka, *Oryzias latipes*, have been the focus of reproductive biological investigations for over a century. These studies provide the basis for our understanding of the relationship between ovulation and fertilization in the evolution of reproductive modes (see Parenti, 2005; Parenti et al., 2010). For example, in two live-bearing species of the family

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Poeciliidae—

P. reticulata and *G. affinis*—oocytes mature and are internally fertilized in the ovary but are not ovulated. In *G. affinis*, the next batch of oocytes to mature in the ovary do not start vitellogenesis during this pregnancy (Koya, 2008; Koya & Kamiya, 2000; Mori et al., 2003; Takemura et al., 1987), which strongly suggests that the maturation of oocytes in this viviparous species may be affected by the presence of embryos developing within the ovary. This agrees with the general observation that the ovulation cycle of ovoviviparous and viviparous fishes appears to be affected by a batch of embryos developing normally within the ovary, ovarian cavity, or uterus (Wourms, 1981)

Three oviparous species of the atherinomorph ricefishes, family Adrianichthyidae, are "pelvic brooders" (Kottelat, 1990): *Adrianichthys oophorus*, *Oryzias* (*Xenopoecilus*) *sarasinorum* (see Parenti, 2008), and *O. eversi*. Following fertilization, the developing embryos hang in an abdominal concavity between the elongated pelvic fins of the female and the body. In *O. sarasinorum*, oviposition never occurs when embryos hang in the abdominal concavity of the female (Iwamatsu et al., 2008). In contrast to the ovulation cycles of most species of *Oryzias*, which depend primarily on the diurnal photoperiodicity, the mode of aperiodic reproduction appears to reflect a latent effect of embryos on the activity of the hypothalamo–pituitary–ovarian axis. These females too may have a reproductive system that responds to the presence of embryos.

Internal fertilization—a prerequisite for viviparity—has been recorded rarely in a few oviparous fish taxa, for example, *Myoxocephalus quadricornis* and *O. latipes* (see Wourms, 1981). Although the eggs of *O. latipes* are normally fertilized externally during mating, internal fertilization followed by the laying of fertilized eggs may occur facultatively (Yamamoto, 1975), as first reported by Amemiya and Maruyama (1931). Furthermore, the phenomenon of facultative pregnancy may be encountered occasionally among *Oryzias* species that are normally oviparous. It is biologically interesting as to whether the oviparous female, due to fortuitous internal fertilization, exhibits some modification in the reproductive cycles by gestation, similar to the pregnant manner of the viviparous females.

Gestation in oviparous fishes provides a model to gain insights into how the mode of reproduction has been modified during evolution. Preliminary studies (Iwamatsu, 2002; Iwamatsu et al., 2005) first demonstrated that, in *O. latipes*, internal fertilization may occur if spermatozoa are artificially introduced into the ovarian cavity, which contains unfertilized eggs shortly after ovulation. In this species, the comparatively thick egg envelope (chorion or zona radiata) begins to harden within 10 min after exocytosis of the cortical alveoli upon fertilization (Iwamatsu, 1969; Iwamatsu et al., 1995; Shibata et al., 2000, 2012). Eggs fertilized in vivo within the ovarian cavity cannot pass through the narrow, short oviduct (or gonoduct) and through the genital orifice. Consequently, fertilized eggs with the hardened egg envelope (chorion or zona radiata) can no longer pass through the genital orifice, they start to develop in the ovarian cavity. Thus, pregnancy can be artificially induced in females of this oviparous fish. Using this species, we performed experiments to

Highlights

- We induced ovarian gestation in the oviparous medaka fish with a unique and novel technique.
- The results indicated that artificially pregnant females of the oviparous medaka fish (*Oryzias latipes*) continuously ovulated a certain number of eggs daily around the onset of light during gestation.

clarify the effects of ovarian gestation on (1) oogenesis, the daily cycle of maturation and ovulation of the oocyte, and (2) the number of ovulated eggs.

2 | MATERIALS AND METHODS

2.1 | Fish culture

Ninety-six mature medaka fish, *O. latipes* (27–39 mm total length [TL]), in which females were white (r, genotype X^rX^r) and males orange–red (R, genotype X^rY^R) of the S-rR strain (Hagino et al., 2001) were used. Females were reared with males in a glass aquarium (60 cm length × 30 cm width × 35 cm height) and fed a diet of a mixture of one part shrimp powder and one part roasted wheat grain powder. Under the growing conditions—water temperature, 26–30°C; illumination of approximately 150 lux at the water's surface; and a 15 h light regime, from 4:00 a.m. to 7:00 p.m.—females containing variously sized oocytes in the ovary continued to spawn eggs following copulatory stimulation by the male every day, year-round.

2.2 | Determination of the time of ovulation

The timing of ovulation and oviposition in *O. latipes* is diurnally photoperiodic, and oviposition is highly responsive to abrupt changes in the onset of light (Egami, 1954; Robinson & Rugh, 1943). The time of oviposition shortly after ovulation was recorded to ascertain the presumable time of ovulation by either frequently observing females with eggs hanging on the belly or noting the developing stage of fertilized eggs. In the medaka, the time of oviposition was shifted toward a new, artificially modified light regime (10:00 p.m. onset of lighting). Preliminary to preparing females that exhibit a different ovulation cycle, we conducted an experiment to ascertain the effect of changing the onset of light on the time of oviposition. The process of shifting the times of oviposition is shown in Figure 1. When the onset of light was shifted from 4:00 a.m. (light period 4:00 a.m. to 7:00 p.m.) to 10:00 p.m. (light period 10:00–7:00 p.m.), the time of oviposition was shifted to around 11:00 p.m. within 4 days after the change of the light cycle. As pointed out by Egami (1954), *O. latipes* females do not always lay eggs immediately after ovulation since, in most cases, oviposition is induced

by copulatory stimulation by males. Moreover, different females laid eggs at different times. Therefore, the time of oviposition is not useful to determine the time of ovulation. Thus, the time of ovulation was established by directly observing eggs outside of the ovary.

2.3 | Artificial insemination

To prepare a sperm suspension to be used for internal insemination, a male was deeply anesthetized with a mixture of phenylurethane (seven parts) and ethanol (three parts) and transferred to cold (about 8°C) regular saline (NaCl 6.5 mg/ml, KCl 0.4 mg/ml, CaCl$_2$·2H$_2$O 0.15 mg/ml, adjusted to pH 7.4 with N/10 NaHCO$_3$) in a Petri dish (Iwamatsu, 1974). Mature spermatozoa were extruded from the urogenital orifice into the surrounding saline by gently pressing the posterior region of the abdomen with the blunt tip of a glass rod

under a binocular dissecting microscope (Olympus SZX12, ×20). The mass of mature spermatozoa released into the saline (along with a small amount of saline) was immediately collected with a small-tipped glass pipette and transferred to a small (0.5 ml) plastic sample tube. Mature females do not spawn eggs for at least several hours after ovulation when they are isolated from the male before ovulation (Yamamoto, 1975). Therefore, single insemination was conducted by introducing mature spermatozoa into the ovarian cavity containing mature eggs after the estimated time of ovulation. After inserting a fine glass pipette (diameter = 50 μm) through the genital orifice and oviduct of deeply anesthetized females under a dissecting microscope (Figure 2), we injected about 25 μl of sperm suspension (about $7 × 10^4$ spermatozoa/ml) into the ovarian cavity. Inseminated females were anesthetized and killed at given intervals after ovulation, and the number of oocytes and eggs in the ovary was ascertained.
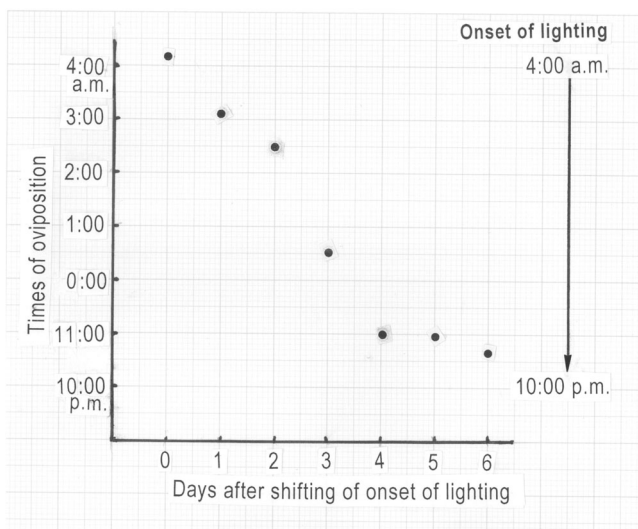
2.4 | Egg collection and examination

Females known to have spawned every morning were isolated from males the day before the experiment was performed. On the first day of the experiment, females that contained ripe eggs in the ovarian cavity were artificially inseminated about 5.5 h (8:00 a.m.) after ovulation. In the gravid females, oocyte maturation and ovulation continued, entrained to the daily cycle of light. At 8:00 a.m. after 1 day, 2 days, and 3 days postinsemination (PI), the gravid females were treated with a lethal dose of anesthetic and then laparotomized; the number of eggs in each ovulation was determined by the respective morphological features of the eggs.

Internal fertilization occurred following single insemination. Then, a group of the first fertilized eggs, with hardened egg envelope, acted as a plug of the short, narrow oviduct, causing subsequently ovulated eggs to accumulate in the ovarian cavity. Females that had been inseminated in vivo were isolated from males in a separate aquarium (about 27°C) PI. The abdomen of these gravid females became greatly distended. About 4 h after the onset of light, that is, at 8:00 a.m. after 1 day, 2 days, and 3 days PI, the females were treated with a lethal dose of anesthetic and then laparotomized and
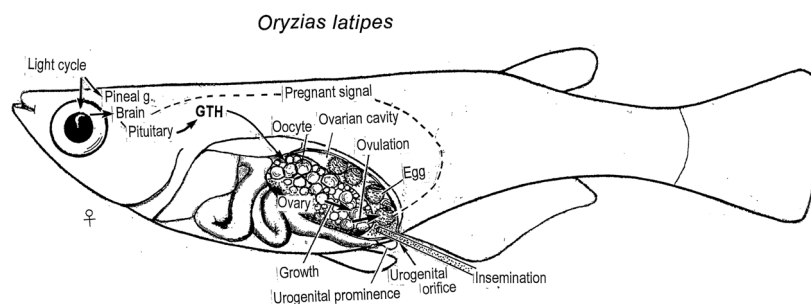

FIGURE 1 Shifting the timing of oviposition by changing the onset of lighting. The time of oviposition shortly after ovulation was recorded as described in Section 2. When the beginning of the light period was changed from 4:00 a.m. to 10:00 p.m., the times of oviposition were shifted from just after 4:00 a.m. to around 11:00 p.m. within 4 days

FIGURE 2 Illustration of artificial insemination for internal fertilization in *Oryzias latipes* female. Spermatozoa were introduced into the ovarian cavity, which was filled with ovulated eggs. A fine glass pipette containing spermatozoa was inserted through the genital orifice into the ovarian cavity of the female, in which eggs were internally fertilized. GTH, gonadotropin-releasing hormone

their ovaries were isolated and transferred to regular saline. Using a dissecting microscope, we examined the meristic and morphological features of ovulated eggs and full-grown oocytes in an ovary: preovulatory oocytes, freshly ovulated, overripe and cytolytic eggs, and developing eggs (embryos). After attachment filaments of the egg, the envelope was removed, and developing eggs were further incubated in Petri dishes in tap water containing $2 \times 10^{-4}\%$ methylene blue to prevent fungal growth. At the beginning of incubation, the developmental stage of each embryo was noted, using Iwamatsu's developmental staging (Iwamatsu, 1994, 2004).

Data were analyzed by Student's t-test and one-way analysis of variance and $p < 0.05$ was considered to be significant.

2.5 | Ethical care

The S-rR strain fish used were maintained in the tanks of the laboratory of the Aichi University of Education. We followed the Guidelines for the Use of Fishes in Research as adopted by the American Fisheries Society, American Institute of Fishery Research Biologists, and American Society of Ichthyologists and Herpetologists (UFR Committee, 2013).

3 | RESULTS

3.1 | Time of ovulation

To determine the time of ovulation, ovaries were isolated from 11 lethally anesthetized females during the expected time of ovulation, which was estimated from the time of oviposition. At 2:00 a.m. (2 h before the onset of light), three females contained freshly ovulated eggs, but another four females exhibited no ovulation and contained preovulatory oocytes that had undergone the breakdown of the germinal vesicle in the ovary. At 3:00 a.m., all of the remaining four females examined had already ovulated eggs. This result was consistent with that of Takano et al. (1974). Judging from these observations, the time of ovulation under the reproductive conditions was estimated to be about 1.5 h (2:30 a.m.) before the onset of light (4:00 a.m.)

3.2 | Oocyte maturation and ovulation in pregnant females

In four females (28–34 mm TL: n = 4), 24 h-PI unfertilized eggs, overripe eggs, and developing embryos were crowded in the ovarian cavity; the numbers of each group of eggs were 31.8 ± 8.0 (range 10–47). The ovary contained full-grown oocytes (diameter = 810–1100 μm). Fertilized eggs (the rate of fertilization was 92.5 ± 2.8%, range 73.3%–100%) developed into embryos of Stages 14–19 (26–28°C), compared with that normal embryo of developmental Stage 17 (26°C). Eggs freshly ovulated 24 h after insemination remained unfertilized, probably due to the loss of fertilizing capacity of the spermatozoa.

At 48 h PI, six females (28–35 mm TL) contained 15.8 ± 2.0 full-grown oocytes (o; Gestation Day 0: follicular diameter = 840–1080 μm) in their respective ovaries. In the ovarian cavity, unfertilized (u), overripe (or), and cytolytic eggs (c), as well as developing embryos (e) of Stages 16–23 with 0–12 somites could be identified and grouped into four types on the basis of morphological features and developmental stages (Figures 3 and 4a). The ripe eggs (u; Day 0: n = 16.2 ± 3.6) freshly ovulated 48 h PI remained unfertilized and displayed cortical alveoli except in a restricted region at the animal pole, as well as oil droplets evenly distributed throughout the whole cortical cytoplasmic layer. These eggs displayed no decrease in cortical alveoli due to spontaneous exocytosis in the process of aging. Overripe eggs (or; n = 15.2 ± 3.1) that ovulated in 24 h PI (Day 1) had a decreased number of somewhat enlarged oil droplets haphazardly gathered in the vegetal hemisphere and fewer cortical alveoli, a result of spontaneous exocytosis into the perivitelline space. On the basis of these morphological features of eggs, it is easy to distinguish between freshly ovulated (u) and overripe (or) eggs (Figure 3). When females were inseminated and examined 48 h PI, 89.3 ± 5.7% (range 73.3%–100%) of the eggs were internally fertilized. Residual, unfertilized eggs appeared to be cytolytic. Cytolyzed eggs (c in Figure 3) exhibited a single oil droplet and an aggregation of cytoplasmic components. Fertilized eggs were in a range of developmental Stages 20–23, suggesting that they were about 5 h out of synchrony with each other (Iwamatsu, 1994, 2004), perhaps indicating the lack of synchronization of in vivo fertilization. The number of developing embryos, including the cytolytic eggs, on Day 2 was 17.2 ± 2.0. There was no significant difference (p > 0.05) between the mean numbers of full-grown oocytes (o) and freshly ovulated eggs (u) in an ovary (Figure 4a).
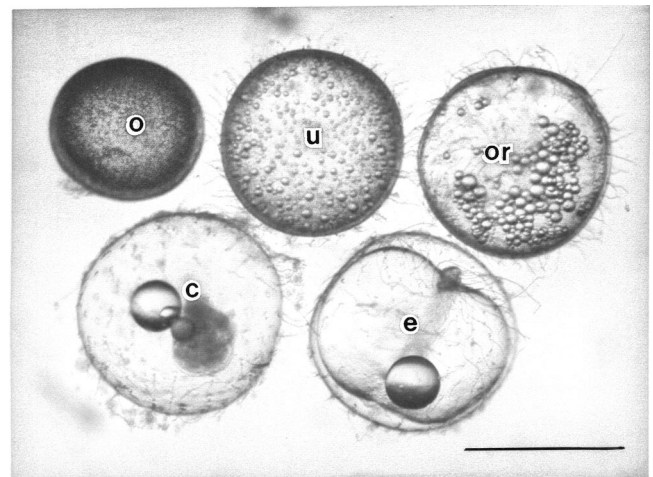

FIGURE 3 Morphological features of a full-grown oocyte and eggs in the ovary of a female 48 h after single insemination. Cytolytic eggs (c, 48 h postovulation, PO) had a slightly distended egg envelope and exhibited a single oil droplet and an aggregation of cytoplasmic components. Scale bar = 1 mm. e, embryo at Stage 16–23 according to Iwamatsu (1994, 2004) (48 h PO); o, full-grown oocyte about 20 h before ovulation; or, overripe egg (24 h PO); u, freshly ovulated unfertilized egg (4 h PO)
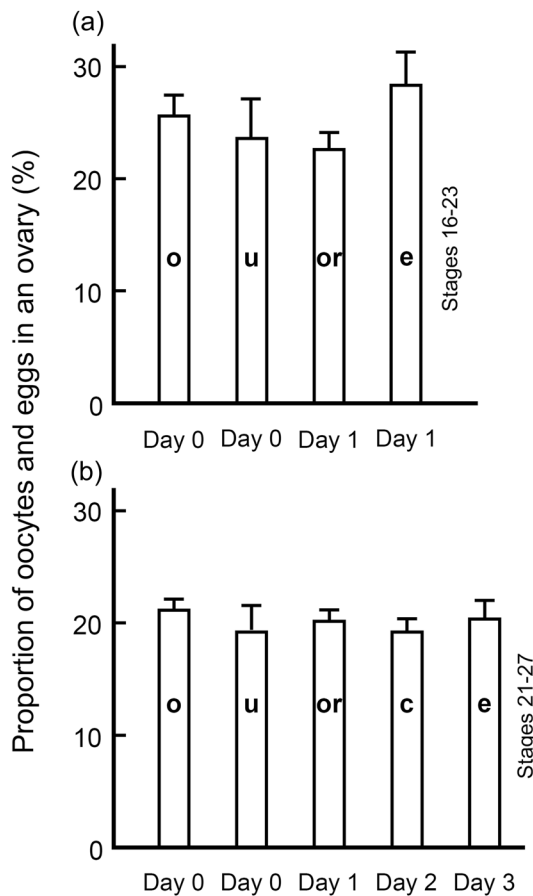
FIGURE 4 Proportions of full-grown oocytes and ovulated eggs (mean ± S.E) in an ovary of *Oryzias latipes* female pregnant with developing embryos for 48 (a) and 72 h (b) postinsemination. Day 0, 1, 2, and 3 represent the day of the ovulation, the next day of the ovulation, and 2 and 3 days after the ovulation, respectively. The mean values are not significantly different ($p > 0.05$) in the analysis of variance. c, cytolytic eggs; e, embryos; o, full-grown oocytes; or, overripe eggs; u, unfertilized eggs

In nine females examined 72 h PI (Gestation Day 3), eggs present in the ovarian cavity could be divided into five groups on the basis of their morphological features and developmental stages (Stages 21–27 with 6–24 somites). Females contained developing embryos (e; Day 3, $n = 14.8 \pm 2.5$), cytolytic eggs (c; Day 2, $n = 13.8 \pm 2.2$), overripe eggs (or; Day 1, $n = 14.3 \pm 2.0$), unfertilized eggs (u) in the ovarian cavity, and full-grown oocytes (o; follicular diameter = 817–1144 μm) in the ovary (Figure 4b). Most eggs ($96.0 \pm 4.0\%$, range 78.0%–100%) were internally fertilized by insemination. Unfertilized eggs showed cytolytic states with a large oil droplet within the expanded egg envelope. There was no appreciable difference between the number of full-grown oocytes (o; Day 0, $n = 14.6 \pm 1.7$) and freshly ovulated eggs (u; Day 0, $n = 14.1 \pm 2.3$) (Figure 4b). These observations suggest that the difference in the proportions of the number of daily ovulated eggs in an ovary was not significant ($p > 0.05$). The total number of these oocytes that were subsequently ovulated was not trending downward, even though the ovarian cavity was overcrowded by the presence of unfertilized, developing, and cytolytic eggs. In each experimental group,

the observation that freshly ovulated eggs remained unfertilized 24 h PI, suggests that spermatozoa retained in the ovarian cavity for 24 h PI were no longer capable of fertilizing eggs.

Embryos obtained 72 h PI regained normal development and hatched after they were transferred to tap water, although the embryos that were retained in the ovarian cavity for 10 days PI were retarded and ceased developing around Stage 29. Thereafter, the larvae were reared outdoors on a regular diet until they reached maturity. Their body colors were evidence that the eggs of the white (r) females (X^rX^r) were in vivo fertilized by spermatozoa bearing X^r- or Y^R-sex chromosome of a heterozygous orange-red male (X^rY^R). The progenies were white (r) females (X^rX^r) and orange-red (R) males (X^rY^R) in equal numbers. This result suggests that eggs (X^r) in the ovarian cavity of white females (X^rX^r) were fertilized by X^r- or Y^R-chromosome-bearing spermatozoa of orange-red (R) males (X^rY^R), and further suggests there is no difference between X^r- and Y^R-spermatozoa in their ability to fertilize in vivo.

3.3 | Time of ovulation in gravid females

The time of ovulation was determined by directly observing eggs ovulated in the ovarian cavity before and after the onset of light (4:00 a.m.). On Day 0, that is, the day before artificial insemination, the females laid eggs around the onset of light. During gestation, the time of ovulation remained mostly unchanged, that is, within 10 min after the onset of light.

To ascertain whether pregnant females also maintain a light-dependent daily cycle of ovulation in different reproductive conditions (light period was 21 h, 10:00–7:00 p.m.), the time of ovulation was examined in the female in which the time of oviposition was previously modified to around 11:00 p.m. for a week under the new reproductive conditions that artificially changed the onset of lighting from 4:00 a.m. to 10:00 p.m. In the new reproductive conditions (light period 21 h, 10:00–7:00 p.m.) that artificially changed the onset of light from 4:00 a.m. to 10:00 p.m., most gravid females (Day 1, Day 2, and Day 3) contained freshly ovulated eggs in the ovarian cavity shortly after the beginning of the light period (Figure 5). The time of ovulation in gravid females was slightly delayed. Yet, modification of the daily cycle of lighting did not disrupt the timing of ovulation relative to the beginning of the light cycle.

4 | DISCUSSION

Ovulation in teleostean fishes is controlled by gonadotropins secreted by the anterior pituitary gland, which regulate steroid secretion by the ovary or the interrenal gland (Biran & Levavi-Sivan, 2018; Jalabert, 1976). Hypophyseal-gonadotropic control of ovulation via the release of gonadotropin has been demonstrated in many teleosts including the oviparous goldfish, *Carassius auratus* (see Stacey et al., 1979), and carp, *Cyprinus carpio* (see Kime & Dolben, 1985). Such control also occurs in *O. latipes* and is entrained
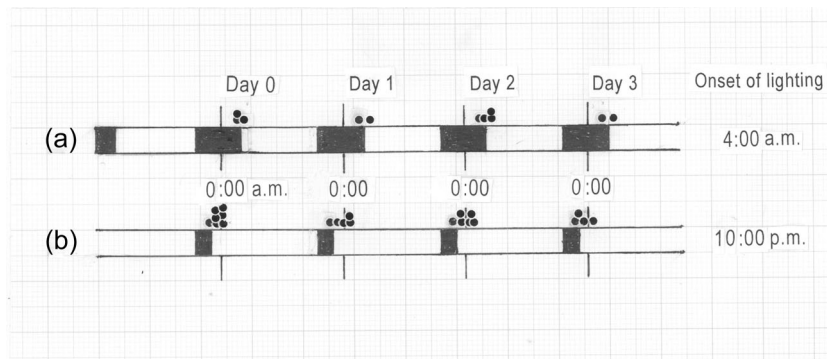
FIGURE 5 Times of ovulation under different light regimes. The time of ovulation was examined in the females in which time of oviposition was previously modified to around 11:00 p.m. for a week after the onset of lighting was shifted from 4:00 a.m. to 10:00 p.m. The beginning of the light period is 4:00 a.m. in (a) and 10:00 p.m. in (b). The solid circles indicate the time of ovulation of each female. The black and white blocks represent the lengths of the dark and light periods, respectively

by a circadian cycle (Iwamatsu, 1978; Takahashi et al., 2019). Rupture of the ovarian follicle to release an oocyte is controlled by proteolytic enzymes in *O. latipes*, although the generality of this ovulation mechanism needs to be investigated among other teleosts (Takahashi et al., 2019).

Hypophyseal-gonadotropic control of ovulation is also likely a general mechanism in fishes that are pelvic brooders and mouth-brooders. In the mouthbrooding cichlid, *Sarotherodon melanotheron*, the presence of eggs in the mouth delays ovulation of the next brood: Specker and Kishida (2000, p. 44) argued "…that the presence of eggs inhibits the pituitary-gonadal axis in both males and females and hypothesize that a chemical signal from the eggs is delaying the initiation of the next brood." Similarly, in females of the pelvic brooding ricefish, *O. (X.) sarasinorum*, oogenesis is retarded and oviposition does not occur in the female when developing embryos are attached to its ventral surface (Iwamatsu et al., 2008; Parenti, 2008). In this species, a chemical signal could emanate from a transient plug-like structure made of a) attachment filaments from the eggs, which extend from the eggs back into the ovarian cavity, b) epithelial cells lining the urogenital pore, and c) capillaries (Iwamatsu et al., 2008).

Here, we report that the presence of embryos in the ovarian cavity of facultatively pregnant *O. latipes* females did not disrupt the daily ovulation cycle on subsequent days, a finding consistent with those reported in two previous studies (Iwamatsu, 2002; Iwamatsu et al., 2008); did not prevent the growth and ovulation of full-grown oocytes; and did not change the timing of ovulation, that is, near the onset of the light phase. Our findings suggest that in these artificially pregnant *O. latipes* females, no signal propagates from the embryos to the brain–pituitary–gonadal axis of the female, because these females continued to ovulate daily.

These results suggest that during evolution, oviparous pelvic brooders developed reproductive systems in response to the presence of embryos, specifically inhibition of ovulation attributable to the presence of embryos, whereas the ovulation cycle of most species of *Oryzias* is governed by diurnal photoperiodicity. Given the

diverse reproductive strategies among species of *Oryzias*, this group of fishes may be ideal in which to study not only the diversity of reproductive strategies among oviparous fishes but also the evolutionary transition from an oviparous to a viviparous mode of reproduction. Studies should also be designed to measure the levels of hormones, including gonadal steroids, that regulate ovulation cycles in pregnant *O. latipes*.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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