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Sex Differentiation and Pubertal Development of Gonads in the Viviparous Mosquitofish, *Gambusia affinis*

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ABSTRACT—The ontogenetic development of gonads from embryo to adult was observed histologically in the viviparous teleost, *Gambusia affinis*. Primordial germ cells (PGCs) appeared in the subendodermal space of the embryo 14 days before birth, and then transferred to the dorsal mesentery to form paired genital ridges 12 days before birth. The PGCs proliferated in the genital ridge, forming gonadal primordia 10 days before birth. All gonadal primordia differentiated to the ovary containing oocytes 2 days before birth, but then redifferentiated to the ovary and testis just after birth. This indicates that the mosquitofish is a juvenile hermaphroditic species. The characteristics of gonadal sex differentiation just after birth were enlargement of the oocytes in females, and invasion of somatic cells from the hilar region to an inner portion of the gonad in males. The paired ovary fused at the basal area 5 days after birth, then on the ventral and dorsal portions, developing into a single ovary 10 days after birth. During this time a single ovarian cavity was formed on the dorsal portion of the ovary. The paired testes fused only at the basal area and became a single testis having two main lobes 10 days after birth. The oocytes gradually developed and began vitellogenesis 100 days after birth, but did not reach maturation until 110 days after birth. Spermatogenic cells formed cysts at 20 days, began meiosis at 70 days, and matured to form sperm balls 90 days after birth. The male fish sexually matured earlier than the female.

Key words: viviparous teleost, primordial germ cell, sex differentiation, gonadal development, juvenile hermaphroditism

INTRODUCTION

The mosquitofish *Gambusia affinis* is a viviparous teleost belonging to the Poeciliidae and is native to North America, ranging from New Jersey to central Mexico. This fish was introduced into many countries including Japan (Sawara, 1974) in the 20th century to control the anopheline mosquito, since it thrives on mosquito larvae (see Kurmholz, 1948). In viviparous poeciliids including the mosquitofish, fully grown oocytes mature but are not ovulated. Instead they are fertilized in the follicles by spermatozoa which were introduced previously by copulation and stored in the ovary. The embryos then develop in the follicles, and fully grown embryos are ovulated and delivered. However, the physiological and endocrinological mechanisms underlying those events (oocyte maturation, fertilization, gestation, and parturition) have not been clarified.

Mosquitofish are suitable as experimental animals because they are hardy and widely distributed. Furthermore, they produce multiple broods in about 22 days (Koya *et al*., 1998) and are easy to rear in small aquaria. This fish has been utilized in various experimental studies, e.g., studies in behavioral ecology, toxicology, and reproductive biology. Recently, they were used in studies on endocrine-disrupting chemicals, to test for masculinization (Drysdale and Bortone, 1989; Bortone and Davis, 1994) and estrogen-like activity (Tolar *et al*., 2001), to investigate the effects on developing embryos in the mother (Dr. Soyano, personal communication), sex differentiation and puberty (Dréze *et al*., 2000). To enhance their value as experimental animals, it is essential to accumulate basic information on their reproductive biology and life history.

To clarify the reproductive life history of mosquitofish, we had already investigated the annual reproductive cycle of a native population and its environmental regulation (Koya *et al*., 1998; Koya and Kamiya, 2000) as well as the ovarian cycle of adult females under rearing conditions (Koya *et al*., 2000). In this paper, we report the ontogenetic development of gonads from embryo to adult, including the appearance of primordial germ cells, gonadal sex differentiation, gonad formation, and pubertal development.

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MATERIALS AND METHODS

Adult mosquitofish (*Gambusia affinis*) which is progeny of the fish collected from natural population at Nagashima town, Mie Prefecture, were kept in 50 l glass aquaria under a 16L:8D photoperiod at 25°C, with each pregnant female isolated in a plastic box (16×9×9 cm^3; Sanran-bako, Nisso Inc., Tokyo, Japan). Under these rearing conditions, the female mosquitofish produced successive broods at an average interval of 22.1±0.46 days (Koya *et al.*, 1998). Developing embryos were obtained from pregnant females, which were killed on a range of days (day 8, 10, 12, 14, 16, 18, 20, and 21) following parturition. Embryo ages were expressed as minus days after birth, as the birthday is 22 days after previous parturition of the mother fish. For the investigation of gonadal differentiation and pubertal development, the newly born fish were reared in 50 L glass aquaria until 110 days after birth. The young of fish were killed on specified days (day 1, 2, 5, 10, 30, 50, 70, 90, 100, and 110) after birth. They were fed once or twice a day during the experimental period.

After anesthetization with ethyl 4-aminobenzoate, ovaries were removed from the body cavity of pregnant mother fish, gently teased apart with tweezers while immersed in physiological saline (L-15 with 10% tryptose phosphate broth; Sigma Chemical Co., St. Louis, MO). Developing embryos within the follicle were carefully separated from connective tissue and oocyte follicles, measured wet weight, and fixed with Bouin's solution. The young of fish were anesthetized with the above-stated reagent, measured for wet weight, and fixed with Bouin's solution. The fixed specimens were embedded in paraffin, consecutively sectioned at 6 μm, and stained with Delafield's hematoxylin and eosin. The developmental stages of oocytes were classified following Koya *et al.* (1998). The diameter of each stage of oocytes was measured, and the total number of oocytes in each ovary was counted. In the testes of young fish, sagittal sections were made, and number of cysts in the largest section of each testis was counted.

All data were presented as mean±SEM. A Student's *t*-test was performed to identify differences in body weight between male and female fish on each sampling day. ANOVA was performed to identify any differences in numerical data among sampling days. Data were then subjected to Fisher's protected least significant difference Test. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Gonadal differentiation (from embryo stage to 30 days after birth)

Embryonic growth

Changes in the wet weight of developing embryos including the surrounding follicle and in newly born fish until 5 days after birth were shown in Fig. 1A. The average embryo weight increased from 3.7 mg on day –12 (12 days before expected birth) to 4.3 mg on day –10, maintained the same level until day –4, and rapidly increased to 7.4 mg on day 0 when the embryos were born. The wet weight of young of fish gradually decreased to 6.6 mg until day 5.

Gonadal sex differentiation

On day –14, the embryo had a notochord, and the neural tube was just before shutdown (Fig. 2A). The intestine, which was composed of a single-layered epithelium, optic cup, and lens had differentiated. Several primordial germ cells (PGCs) were observed on both, the right and left side

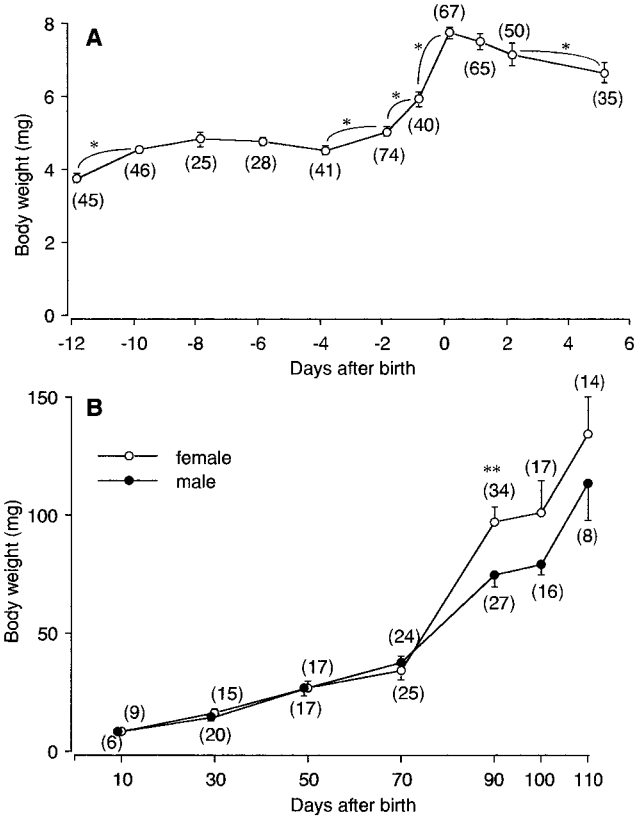


Fig. 1. Changes in body weight of embryos before and after birth (A), and young fish from 10 days after birth (B) in mosquitofish. Figures in parentheses indicate the number of fish counted. *Significantly different from each other ($P<0.05$). **Significantly different between male and female ($P<0.05$).

between the lateral mesoderm and yolk syncytial layer on day –14 (Fig. 2A). The PGCs at 12–15 μm in diameter were larger than the other somatic cells. The nucleus was 5 μm in diameter and was barely stainable. The cytoplasm was stained faintly with eosin. On day –12, the embryos had vertebra and intestines which dangled into the coelom by the mesentery (Fig. 2B). Melanin was deposited on the eyeball. The PGCs were concentrated on both the right and left sides of the basal part of the mesentery, forming small clusters (Fig. 2B). On day –10, several PGCs and somatic cells formed a pair of gonadal primordia hanging from the dorsal wall of the coelom (Fig. 2C). The division of gonial germ cells was often observed (Fig. 2D), and the number of germ cells increased. From days –8 to –6, the division of germ cells was frequently observed (Fig. 2E). The clusters of gonial germ cells were sparsely surrounded by somatic cells. On day –4, a pair of gonads were suspended from the dorsal coelom at the base of the mesentery by a mesentery-like membrane (Fig. 2F). In 3 of every 5 embryos, a few germ cells began meiosis and developed into oocytes. The oocytes were 13 μm in average diameter with a round nucleus 5 μm in diameter including a large nucleolus. On day –2, all 32 embryos observed included oocytes in their gonads, indicating a differentiating ovary (Fig. 2G). The

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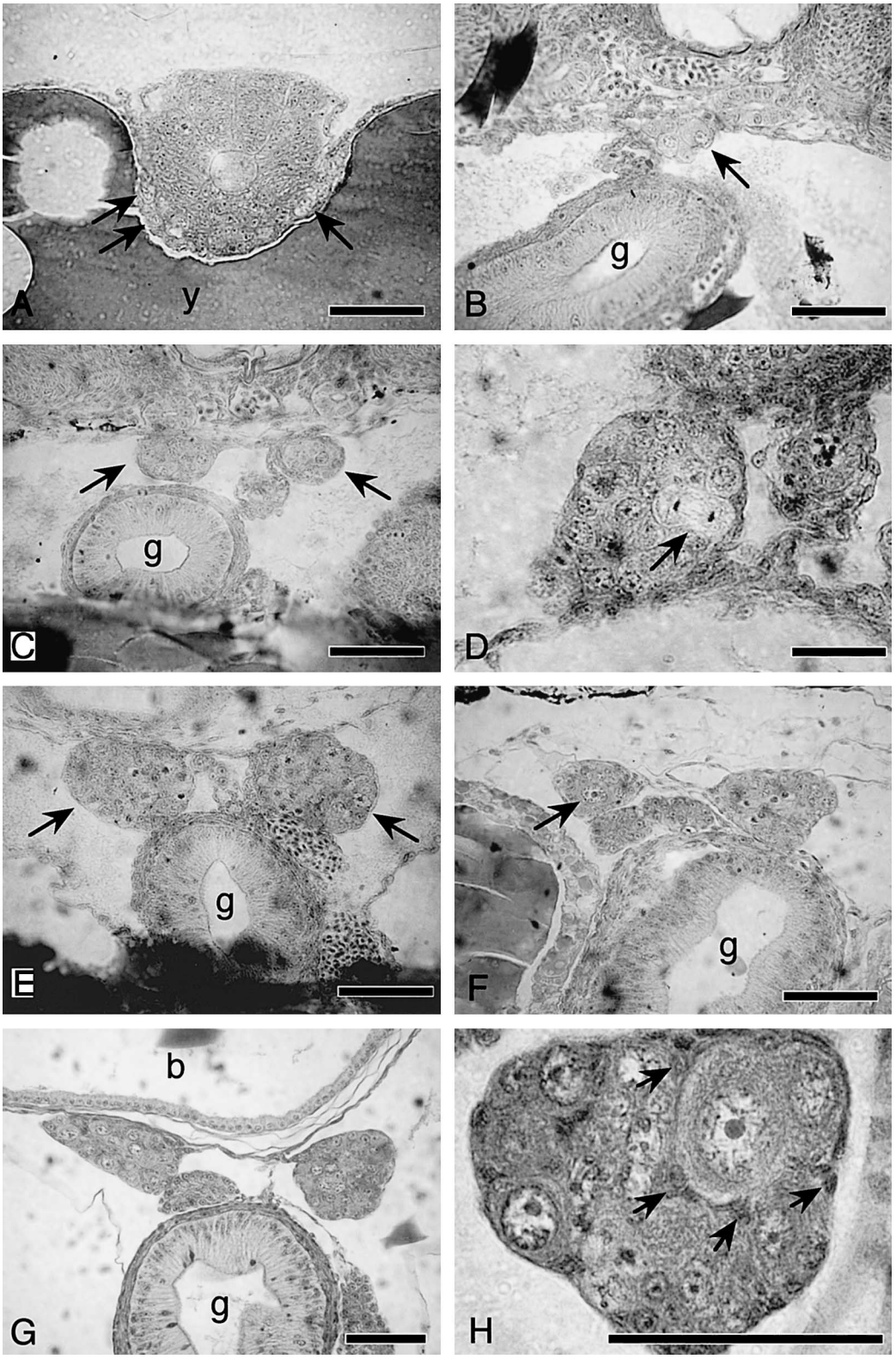


Fig. 2. Photomicrographs of cross sections of embryos in mosquitofish. (A) Primordial germ cells (arrows) in a neurula embryo on day –14. (B) Genital ridge (arrow) on the dorsal coelomic wall on day –12. (C) Paired gonadal primordia (arrows) on day –10. (D) Dividing germ cell (arrow) in the gonadal primordia on day –10. (E) Paired gonadal primordia (arrows) on day –6. (F) Paired gonadal primordia on day –4. Arrow indicates oocyte beginning meiosis. (G) Paired ovaries on day –2. (H) High magnification photo of the ovary on day -2. Arrows indicate interstitial cells around oocytes. b, swim bladder; g, gut; y, yolk plate. Scale bars: A, B, C, E, F, G, and H, 50 μm; D, 25 μm.

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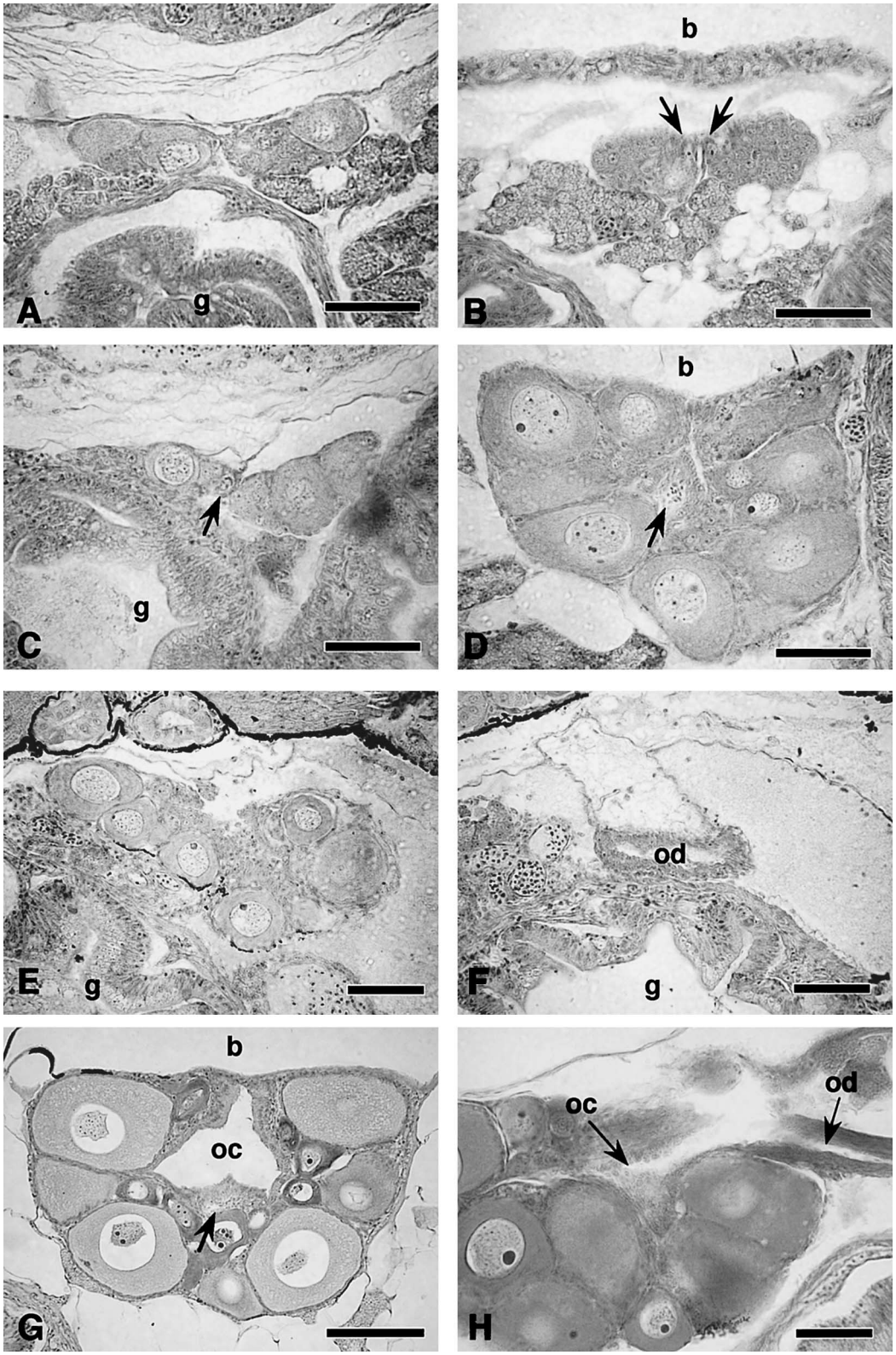


Fig. 3. Photomicrographs of cross (A–G) and vertical section (H) of ovary after birth in mosquitofish. (A) Ovary of newly born fish. (B) Testis of newly born fish. Arrows indicate cluster of somatic cells including blood vessels in hilar region. (C) Fusing ovaries on day 5. Arrow indicates main blood vessel between each ovary. (D) Single ovary on day 10. Arrow indicates main blood vessel beneath single narrow ovarian cavity. (E) Posterior region of the ovary on day 20. (F) Single oviduct behind the ovary on day 20. (G) Single ovary on day 30. Arrow indicates main blood vessel beneath enlarged ovarian cavity (oc). (H) The connecting region of ovarian cavity and oviduct on day 30. The front of fish corresponds to the left of photo. b, swim bladder; g, gut; oc, ovarian cavity; od, oviduct. Scale bars: 50 μm.

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oocytes were surrounded by thin follicle cells. Some interstitial cells were distributed sparsely within the ovary (Fig. 2H). The mesovarium was stretched compared with that on day –4.

In the fry just after birth (day 0), two types of gonads

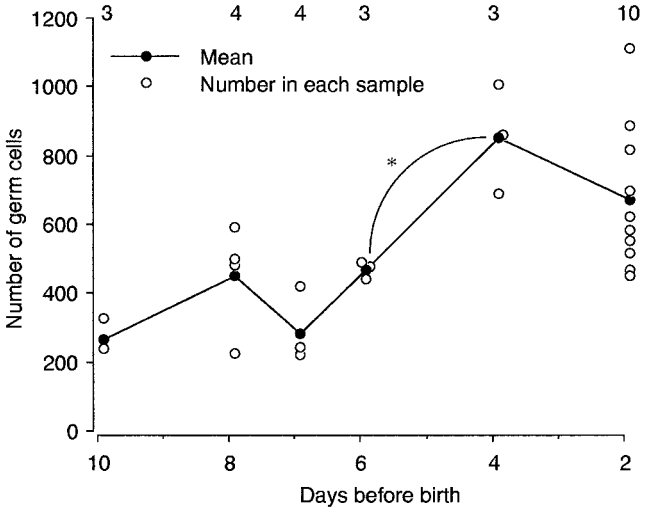


Fig. 4. Changes in germ cell numbers before birth in mosquitofish embryo. Figures above graph indicate the number of fish measured. *Significantly different from each other (P<0.05).

Table 1. Number and mean diameter of oocytes and germ cells in the embryos at four and two days before birth.

Days before birth	Mean diameter of oocytes (μm)	Number of oocytes	Total number of germ cells
4	–	0	–
	–	0	990
	13	ND*	–
	13	5	844
	13	8	672
2	14	5	433
	14	50	871
	14	26	606
	17	100	563
	17	114	532
	18	2	800
	18	3	1092
	18	278	681
	18	116	498
	20	205	450

*ND indicates that the sample could not be obtained accurate value.

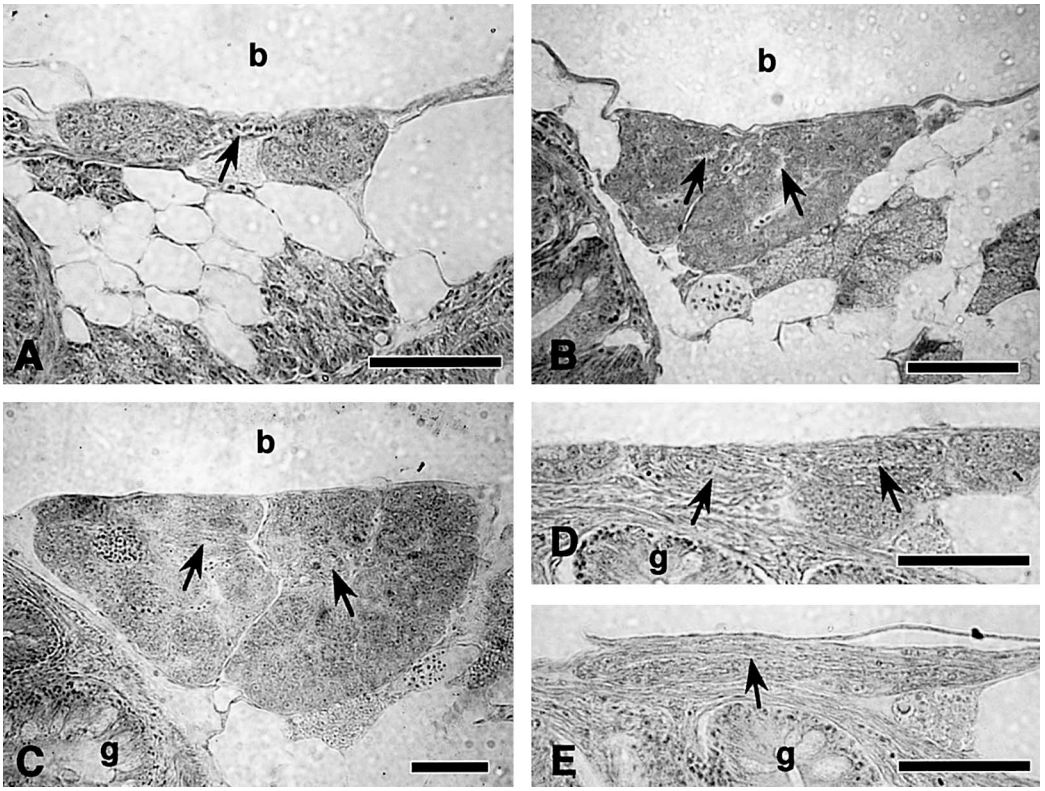


Fig. 5. Photomicrographs of cross section of testis after birth in mosquitofish. (A) Fusing testes on day 2. Arrow indicates main blood vessel between each testis. (B) Testis on day 10. Arrow indicates main blood vessel located between each testicular lobe. (C) Testis on day 20. Arrows indicate main sperm ducts. (D) Posterior region of testis with right and left sperm ducts (arrows) on day 20. (E) Single common sperm duct (arrow) at the posterior end of testis on day 20. b, swim bladder; g, gut. Scale bars: 50 μm.

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were distinguished. One (13 of 21 fry) included many oocytes, often large peri-nucleolus stage oocytes, considered to be ovaries (Fig. 3A). The other (5 of 21 fry) did not include oocytes, but only gonial germ cells within the gonad, and a cluster of somatic cells that had invaded from the hilar region of the gonad and were thought to be testes (Fig. 3B). A blood vessel traversed the basal part of the gonad in both ovary and testis.

Number and size of germ cells

Numbers of all germ cells in the gonadal primordia or gonads were counted, and the changes were shown in Fig 4. Germ cells numbered around 400 and did not change from day –10 to –6, but significantly increased to 800 on day –4, after which their number remained constant until day –2.

Numbers and mean diameters of oocytes in the gonads of days –4 and –2 embryos were counted and were shown in Table 1. On day –4, the average diameter of oocytes was 13 μm, but they were comparatively few in number. On day –2, various size of oocytes from 14 to 20 μm in average diameter were observed. Their numbers also varied, but the embryos with large oocytes (18 μm<) could be divided into two types; those with only a few oocytes (2 and 3, respectively) and those with many (278, 116, and 205, respectively). In the former type of gonad, the total germ cell numbers were comparatively high, i.e., 800 and 1092, respectively. On the other hand, in the latter type, the total germ cell numbers were relatively low at 681, 498, and 450, respectively, and accounted for about a quarter to a half of the total germ cells.

Ovarian formation

On day 5, the mesovarium disappeared, and the basal part of the ovary attached directly to the mesentery. Clusters of somatic cells surrounding blood vessels at the basal part of a paired ovary fused with each other, and a single thick blood vessel was formed in the anterior region (Fig. 3C), but not in the posterior region of the ovary. On day 10, the right and left ovary fused broadly from dorsal to ventral sides, and attached to a swim bladder (Fig. 3D). A single blood vessel in the center of the ovary and a narrow inverse Y-like ovarian cavity were formed above the central blood vessel. Such a fusion was not observed in the posterior region of the ovary at that time.

On day 20, the right and left ovaries fused on the ventral side but not on the dorsal, and the ovarian cavity did not close in the posterior region of the ovary (Fig. 3E). However, farther toward the posterior region a closed oviduct consisting of epithelial cells was formed (Fig. 3F). The oocytes grew to the late peri-nucleolus stage. On day 30, a triangular ovarian cavity on the dorsal side enlarged, and a columnar single-layered epithelium lined the ovarian cavity (Fig. 3G). The oviduct joined with the ovarian cavity at the posterior end of the ovary (Fig. 3H). Thick blood vessels traversed the lower edge of the ovarian cavity.

Testicular formation

On day 2, a pair of testes grew closer to each other, and a single blood vessel was often observed (Fig. 5A). A mesorchium developed from the mesentery. On day 10, the right and left testes fused only in the basal region, and ventral lobes developed, forming a deep groove from the ventral to the dorsal side. The clusters of somatic cells at the basal region of each lobe of the testis showed a tubular arrangement, forming a pair of ducts in the posterior region (Fig. 5B), but remaining indistinct in the anterior region of the testis. By day 20, a pair of sperm ducts were still indistinct in the anterior region of the testis (Fig. 5C). The right and left sperm ducts (Fig. 5D) fused and formed a single common sperm duct at the posterior end of the testis (Fig. 5E), but were indistinct farther toward the posterior region. The formation of cysts by spermatogonia was observed.

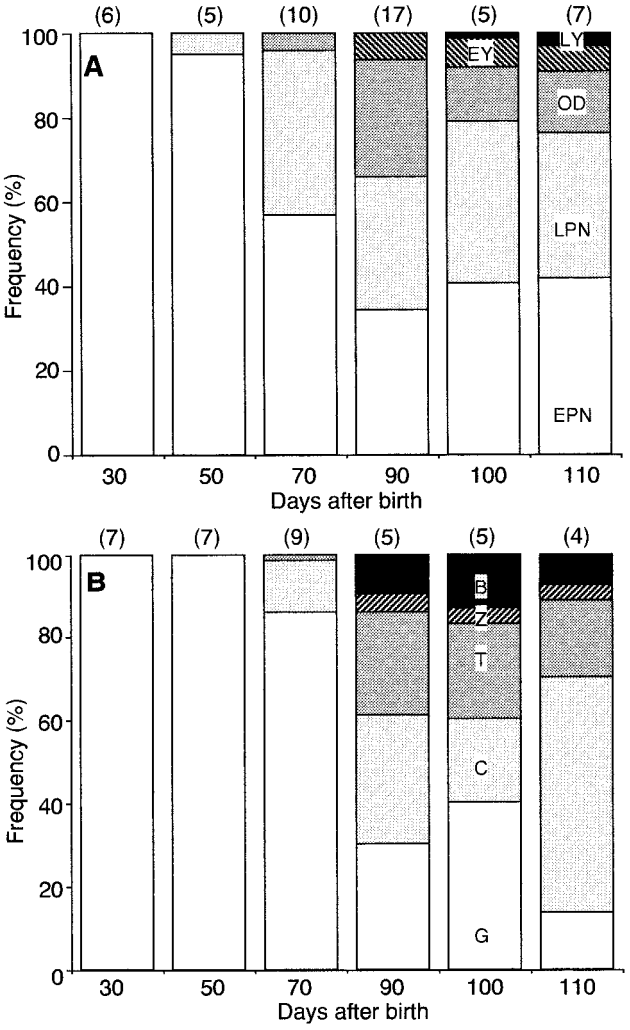


Fig. 6. Changes in stage composition of oocytes in the ovary (A), and spermatogenic cyst in the testis (B) during experimental period in mosquitofish. Figures in parentheses indicate number of fish counted. B, sperm balls; C, spermatocytes; EPN, early peri-nucleolus stage; EY, early yolk globule stage; G, spermatogonia; LPN, late peri-nucleolus stage; LY, late yolk globule stage; OD, oil-droplet stage; T, spermatids; Z, spermatozoa.

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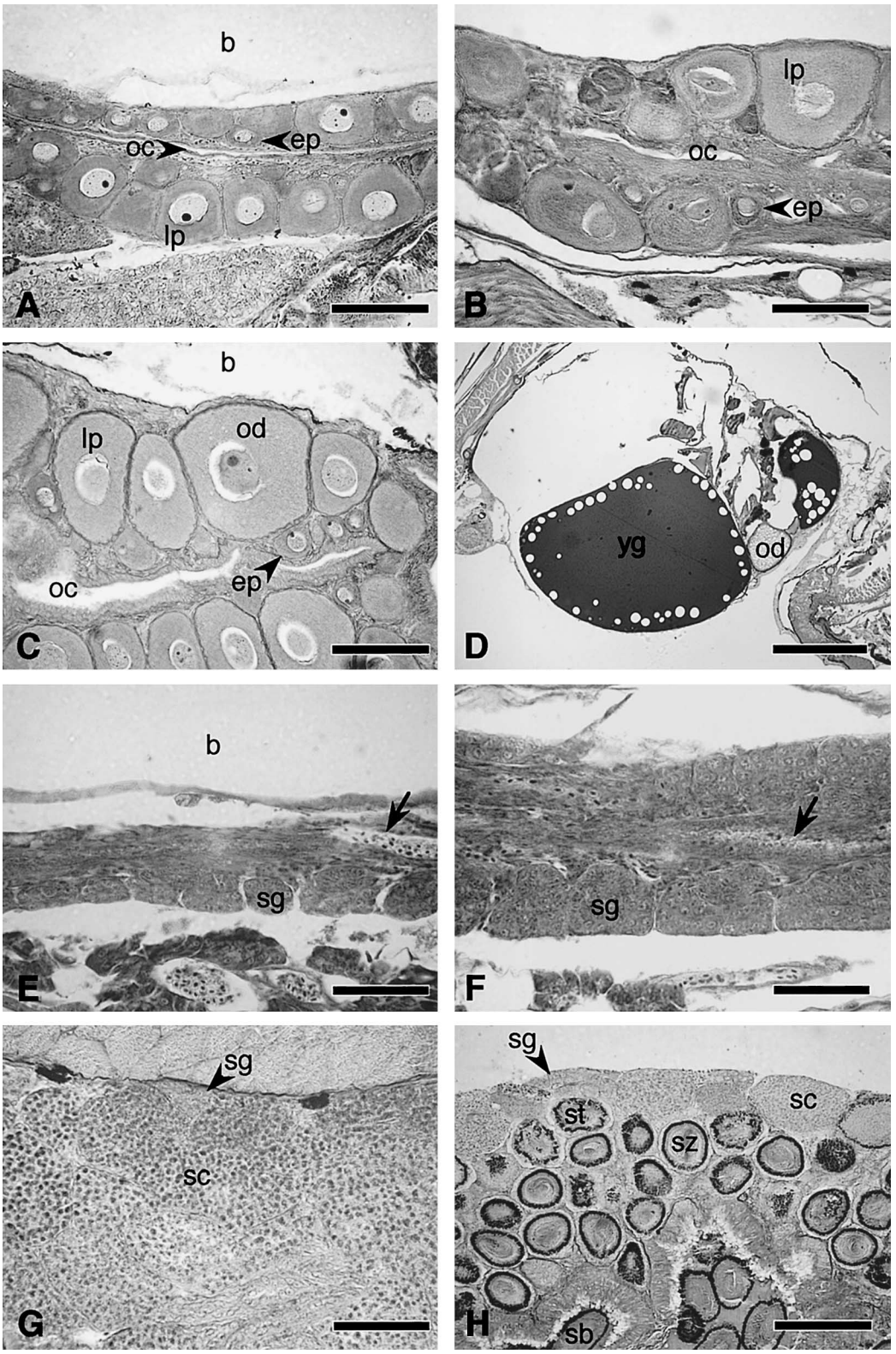


Fig. 7. Photomicrographs of sagittal section of gonads in young mosquitofish. (A) Ovary on day 50. (B) Ovary on day 70. (C) Ovary on day 90. (D) Ovary on day 100. (E) Testis on day 30. Arrow indicates main blood vessel. (F) Testis on day 50. Arrow indicates main blood vessel. (G) Testis on day 70. (H) Testis on day 90. b, swim bladder; ep, early peri-nucleolus stage oocyte; lp, late peri-nucleolus stage oocyte; oc, ovarian cavity; od, oil-droplet stage oocyte; sb, sperm ball; sc, cyst of spermatocytes; sg, cyst of spermatogonia; st, cyst of spermatids; sz, cyst of spermatozoa. Scale bars: A, B, C, D, E, F, and G, 50 μm; H, 500 μm.

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Pubertal development (from 30 to 110 days after birth)

Growth

Changes in the wet weight of young male and female fish were shown in Fig. 1B. The average weight of both males and females gradually increased from days 10 to 70, and rapidly increased to day 90, by which day the average weight of females was significantly higher than that of males. From day 90 to 110, the weights of both sexes continued to increase, with the average weight of females remaining consistently higher than that of males.

Ovarian development

Changes in the composition of each stage of oocytes in the ovary were shown in Fig. 6A. The ovary was occupied by early peri-nucleolus stage oocytes on day 30, and late peri-nucleolus stage oocytes appeared on day 50 (Fig. 7A). On day 70, oil-droplet stage oocytes appeared, the percentage of late peri-nucleolus stage oocytes increased to 40%, and that of early peri-nucleolus stage oocytes decreased to 55% (Fig. 7B). By day 90, the percentage of oil-droplet stage oocytes increased to 25% (Fig. 7C), and early yolk-globule stage oocytes appeared. On day 100, late yolk-globule stage oocytes appeared (Fig. 7D), and the percentage of each stage of oocyte had stabilized by day 110.

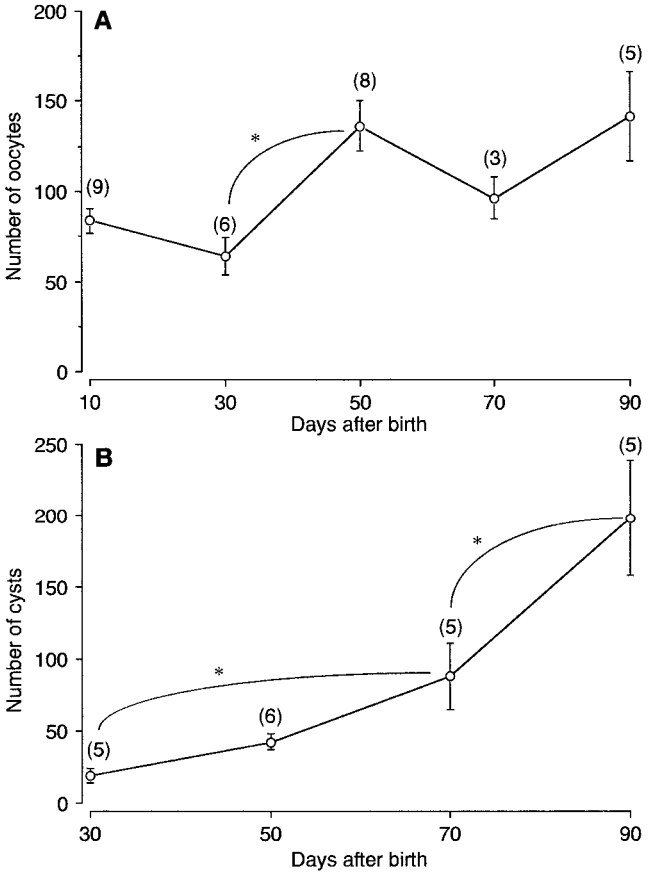


Fig. 8. Changes in total oocyte numbers in each ovary (A) and total cyst numbers in largest sagittal section of each testis (B) during experimental period in mosquitofish. Figures in parentheses indicate number of fish counted. *Significantly different from each other (P<0.05).

Changes in the total number of oocytes after the early peri-nucleolus stage in each ovary were shown in Fig. 8A. The number of oocytes remained at under 100 until day 30, significantly increasing to 136±14.3 by day 50, and holding steady until day 90 (141.6±24.7).

The diameters of oocytes in each ovary were measured, and the relative frequency distribution of diameters on each sampling day were shown in Fig. 9. On days 10 and 30, one group of early peri-nucleolus stage oocytes 20–40 μm in peak diameter were observed. On day 50, in addition to the early peri-nucleolus stage oocytes, late peri-nucleolus

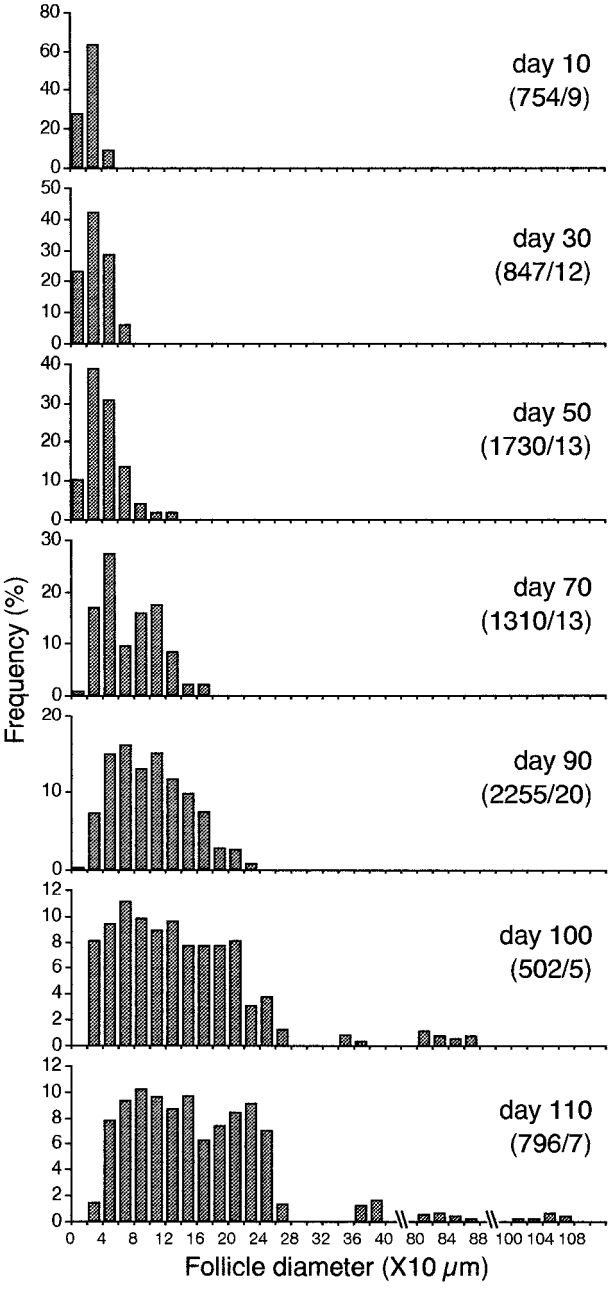


Fig. 9. Changes in frequency distribution of oocyte diameter during pubertal development in mosquitofish. Figures in parentheses indicate number of oocyte/number of fish measured.

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stage oocytes over 80 μm in diameter appeared, and reached a second peak of 100–120 μm in diameter on day 70. By day 90, peaks of frequency became unclear, with oocytes from 40 to 180 μm in diameter randomly observed. By day 100, most of the ovary was occupied by oocytes from 20 to 200 μm in diameter ranging from the early perinucleolus to the oil-droplet stage, with early and late yolk-globule stage oocytes observed only sporadically. The largest oocytes were 880 μm in diameter. By day 110, the diameter of oocytes showed a pattern similar to day 100, with the largest of them reaching 1000 μm in diameter. The yolk-globule stage oocytes were distributed among groups of various sizes, and even in each fish two or three groups of oocytes were observed.

Testicular development

Changes in the composition of each stage of spermatogenic cysts (spermatogonia, spermatocytes, spermatids, spermatozoa, and sperm balls) in the testis from days 30 to 110 were shown in Fig. 6B. On day 30, testes were occupied exclusively by spermatogonia. Cysts were formed inside testicular lobules which appeared on the ventral side of the testes (Fig. 7E). On day 50, the testes increased in size, but meiotic division had not yet occurred (Fig. 7F). On day 70, spermatocytes appeared (Fig. 7G), and spermatids were also observed in some fish. By day 90, all stages of spermatogenic cysts were observed (Fig. 7H). There were many sperm balls in the sperm duct. On day 100, the composition of each stage was similar to that on day 90. On day 110, the percentage of spermatocytes increased and that of spermatogonia decreased.

Changes in the total number of cysts in each testis were shown in Fig. 8B. The number of cysts gradually increased from 19.2±4.7 on day 30 to 88.0±23.3 on day 70, and then rapidly increased to 198.4±40.1 on day 90.

DISCUSSION

Sex differentiation

In zebrafish, PGCs originated in four cells which appeared in the early cleavage period, proliferated during gastrulation, migrated from the marginal to the dorsal part of the embryo, and then accumulated into two groups to the right and left of the subendodermal space (Yoon *et al*., 1997; Nagai *et al*., 2001). Similar accumulations of PGCs in the subendodermal space were observed in the medaka (Gamo, 1961; Hamaguchi, 1982) and rosy barb (Gevers *et al*., 1992). In the present study, the PGCs of mosquitofish were observed between the lateral mesoderm and the yolk syncytial layer of the embryo. In the medaka, after accumulating in subendodermal space, the PGCs were uptaken by the lateral mesoderm and transferred to the dorsal mesentery where they formed gonadal primordia (Hamaguchi, 1982). Among viviparous poeciliid fish, PGCs were also observed in the lateral mesoderm of embryos in the guppy (Dildine, 1936) and platy (Wolf, 1931). The PGCs of mosquitofish formed a pair of genital ridges on both side of the dorsal mesentery in the present study. Thus, the behavior of mosquitofish PGCs before and after the formation of gonadal primordia resembled that of other teleosts as well as that of zebrafish and medaka.

In medaka, the proliferation dynamics of germ cells after the formation of gonads showed a sex-dependent difference before the structural sex differentiation of gonads, i.e., the number of germ cell of expected females was twice that of expected males (Hamaguchi, 1982). In mosquitofish, the PGCs began mitotic division after formation of the gonadal primordia, and the number of germ cell increased around twice until day –4. On day –2, oocytes of the chromatin nucleolus stage were observed in all fish examined, indicating that all embryos in the ovary of their mother feminized just before birth, confirming that the mosquitofish is a hermaphroditic species. Although all embryos had oocytes during this period, two types of gonads were distinguishable by the number of their oocytes and total germ cells, suggesting that the sex differentiation of germ cells may have already occurred as in the medaka. In mosquitofish it is thought that gonads with many oocytes and only a small number of total germ cells developed into an ovary, whereas gonads with a small number of oocytes and many germ cells developed into a testis. Thus, it is a peculiar characteristic of mosquitofish that the proliferation of germ cells is more pronounced in males than in females.

It is well known that many teleost species show juvenile hermaphroditism (Atz, 1964). In the present study, temporary hermaphroditism was observed in mosquitofish for a few days just before birth. However, this phenomenon was not observed in the same species captured in Nagasaki Prefecture, Japan (Dr. Soyano, personal communication). In the guppy, a similar hermaphroditism has been reported in one study (Dildine, 1936), whereas another did not observe it (Goodrich *et al*., 1934). Thus, the hermaphroditism of poeciliid fish does not seem to be a universal phenomenon. The period of hermaphroditism in poeciliid fish is much shorter than that of other juvenile hermaphroditic species; e.g., 10–15 days in the zebrafish (Takahashi, 1977), about 20 days in the Sumatran barb (Takahashi and Shimizu, 1983), and 2 to 4 months in the common carp (Davies and Takashima, 1980). When the embryos feminize in the ovary of the mother mosquitofish, the mother has already begun active vitellogenesis of the next clutch of oocytes (Koya *et al*., 2000). It is generally accepted that in teleosts vitellogenesis is promoted by estrogen, including in viviparous species (Nagahama *et al*., 1991; Korsgaard, 1994; Koya *et al*., 1997). Sex differentiation into females has been induced by exogenous estrogen treatment (Nakamura and Takahashi, 1973; Takahashi, 1975a; Iwamatsu, 1999). Furthermore, it has been suggested that endogenous estrogen induces the formation of ovaries during normal sex differentiation by the presence of steroidogenic enzymes (Nakamura *et al*., 1998). The feminization of embryos just before birth may not be a phenomenon which programmed previously, but may rather be due to the maternal estrogen in poeciliid fish.

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In the present study, clear gonadal sex differentiation was observed just after birth by the rapid growth of oocytes in females and the invasion of somatic cells to the inner portion of the gonad in males. Similar signs of sex differentiation have been reported in mosquitofish (Itahashi and Kawase, 1973) and in other poeciliidae such as the guppy (Goodrich *et al.*, 1934; Dildine, 1936; Takahashi, 1975b) and swordtail (Essenberg, 1923). Morphological signs of gonadal sex differentiation in teleosts include changes in germ cells (e.g., in the medaka, Satoh and Egami, 1972, and three-spined stickelback, Shimizu and Takahashi, 1980) and in somatic cells (e.g., in the tilapia, Nakamura and Takahashi, 1973, and puffer, Matsuura *et al.*, 1994). In mosquitofish, changes in both germ cells and somatic cells can be initial characteristics of sex differentiation of the gonad.

Formation of gonads

Although the gonad of teleosts is usually a paired organ, that of poeciliids is a single organ. The supposed process of formation of a single ovary based on the present observations was illustrated in Fig. 10. The paired ovaries 2 days before birth hung from the basal part of the mesentery by mesovariums (Fig. 10A), but then ovaries grew closer together (Fig. 10B) and finally fused with each other at the hilar region, putting the mesentery between each of the ovaries 5 days after birth (Fig. 10C). At this time, it is thought that each mesovarium also fuses or is absorbed with another mesentery. In the guppy, since the ovaries lacked a distinct mesovarium, paired ovaries joined with each other at the root of the mesentery (Miyamori, 1964). In mosquitofish, ovarian stroma proliferated and each developed dorsally and ventrally after the fusion of each ovary (Fig. 10D). Ventral stroma fused along the mesentery, the lateral side of the dorsal stroma elongated upward along the dorsal coelomic wall and fused at the top of the ovary, forming an ovarian cavity 10 days after birth (Fig. 10E). This process is similar to that of guppies (Miyamori, 1964). It is thought that the inverse Y-like ovarian cavity provides evidence of this anticipated transformation (Fig. 10F). In swordtails, the ovarian cavity seems to originate from the median surface of the fusing ovaries (Essenberg, 1923). Thus, there is species specificity in the formation process of the ovarian cavity

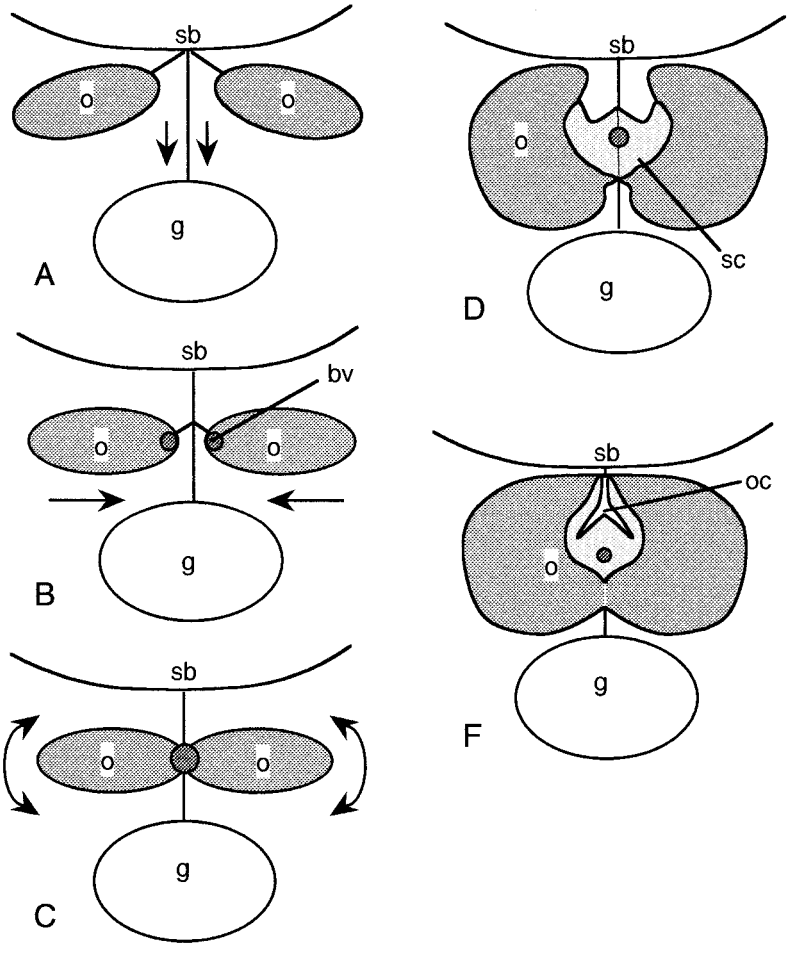


Fig. 10. Summary diagram of formation of single ovary in mosquitofish. (A) Paired ovary hangs from dorsal coelomic wall by mesovarium on day -2. (B) Separate ovaries draw near just after birth. (C) Separate ovaries fuse at hilar region on day 5. (D) Lateral side of dorsal stroma elongate upward along coelomic wall on days 5-10. (E) Elongated lateral sides of dorsal stroma fuse at top of ovary, forming ovarian cavity on day 10. bv, blood vessel; g, gut; o, ovary; oc, ovarian cavity; sb, swim bladder; sc, somatic cell cluster.

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among poeciliids.

There are some reports that the formation of the ovarian cavity begins in the anterior region of the ovary and proceeds in a caudal direction (Nakamura and Takahashi, 1973; Yoshikawa and Oguri, 1978; Shimizu and Takahashi, 1980). In mosquitofish, the formation of the ovarian cavity also began in the anterior region, agreeing well with those reports. The formation of the oviduct began before completion of the fusion of paired ovaries in the mosquitofish. This suggests that the ovarian cavity and the oviduct form separately, but subsequently fuse together.

The fusion of paired testes was observed only at the basal part of each testis in the present study. After fusion, a deep groove remained between each testicular lobe. In mosquitofish, this groove of the testis was visible in adult males (Medlen, 1950). The main sperm duct was formed amid a cluster of somatic cells in the hilar region, agreeing with the reports on other poeciliid fish (Essenberg, 1923; Miyamori, 1964). The formation of the paired main sperm ducts in the mosquitofish seemed to begin in the posterior region of the testis, and proceeded in both the anterior and caudal directions.

Pubertal development

The wet weight of females was higher than that of males on day 90, and that trend continued until the end of the experiment (day 110). In the guppy, females grew larger than males from 9 weeks after birth (Arisaka and Hamai, 1975). By day 90, males had matured but females had not in the present study, suggesting that the delay in male growth after day 90 was due to growth having to share the energy with sexual maturation. It is generally known that females grow larger than males in most viviparous poeciliid fish. Therefore, the different growth patterns after the maturation of male seem to confirm the sexual dimorphism of these fish.

Vitellogenic oocytes appeared on day 90 in the present study. This suggests that the pubertal development of the female had already been triggered 90 days after birth. The rates of each oocyte stage from days 90 to 110 were similar to those of adult mosquitofish (Koya *et al*., 1998). On days 100 and 110, late yolk-globule stage oocytes appeared but did not reach maturation, indicating that female mosquitofish first become pregnant 110 days after birth.

The frequency distribution of oocyte diameters reflected the changes in oocyte stage composition throughout the experimental period. On day 100, vitellogenic oocytes distributed separately from a group of oocytes consisting of those younger than the oil-droplet stage, and they divided into smaller groups, two groups on day 100, and three on day 110. In adult female mosquitofish, vitellogenic oocytes developed sporadically, and it is thought that only prominent oocytes are fertilizable while the remaining ones degenerate (Koya *et al*., 2000). Accordingly, in the first cycle of reproduction also, only the preceding group of vitellogenic oocytes may be fertilizable. Viviparous poeciliid fish are thought to evolve from oviparous ancestors. In oviparous cyprinodontoidei belonging to the poeciliidae, there are many species which repeatedly spawn over a short cycle during the breeding season (Briggs and Egami, 1959; Taylor *et al*., 1979; Emata *et al*., 1991). The sporadic development of vitellogenic oocytes observed in mosquitofish may be an indication of their ancestry.

The formation of cysts in testes was observed 30 days after birth in mosquitofish. The number of cysts increased day by day, and the testis also grew larger, suggesting that their size depends on the number of cysts. Meiosis of spermatocytes was observed after day 70, suggesting that the pubertal development of male mosquitofish has been triggered by 70 days after birth. Mature sperm balls existed in the sperm duct after day 90, indicating that the male mosquitofish are sexually mature by 90 days after birth, which is earlier than the females.

The total number of cysts in each testis rapidly increased from days 70 to 90. In this period, the number of meiotic dividing cysts increased and that of spermatogonial cysts decreased as a result of the rapid progress of spermatogenesis. This indicates that the spermatogonial cysts that decreased with the progression of spermatogenesis continued to be recruited throughout this period.

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