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Growth of the Medaka (IV) – Dynamics of Oocytes in the Ovary During Metamorphosis

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ABSTRACT

The present investigation provides information on dynamic aspects of developing oocytes in the ovary during the growth period of the medaka from larva to juvenile. After hatching, the ovary increases in a longitudinal length, in proportion to body growth, although its width exhibits a slightly steeper rise from 13–14 mm total length (TL). In the ovary of larvae measuring 13 mm TL or greater, the largest oocytes enter stage IV in which the chorion (egg envelope) with attaching and villi (non-attaching filaments) begins to form. When body size reaches 14 mm TL, the composition of developing oocytes (follicles) in an ovary shifts from the previtellogenic to the early vitellogenic patterns. After larvae attain a body size of more than 15 mm TL, the largest size of oocytes continues to increase steeply with great variation among individuals as body growth progresses. It was also found that the total number of oocytes in an ovary continues to increase in proportion to body growth during metamorphosis. Moreover, the metrical data reveal that small oocytes of 45 μm in diameter represent a dominant population in the oocyte composition in each ovary, irrespective of body size, and that the ovary always retains oogonia and small oocytes 9–18 μm in diameter in the range of 9–15 per cent. It is conceivable that recruitment of new oocytes may occur directly from oogonia in the germinal nests (germinal cradles) that are located in the epithelial regions surrounding large oocytes and at the edge of the luminal wall of the ovary.

Keywords: oocyte number, oocyte composition, ovary, metamorphosis, larva, medaka

INTRODUCTION

The great variety of reproductive strategies found in teleosts has incited many species-specific studies (Bobe *et al.*, 2008). A tiny freshwater teleost, the medaka *Oryzias latipes* is a cyclical breeder in which the circadian growth and maturation of oocytes are photoperiodically regulated by the combined action of the steroid hormones, follicle stimulating hormone (FSH) and lutenizing hormone (LH), on the follicle cells which completely envelop the surface of the oocyte. In the ovary showing a circadian rhythm, oocytes develop in order from one primitive stage to the following older stages unceasingly throughout the spawning season. Accordingly, the stage composition of developing oocytes in the ovary of spawning females is always maintained in the same pattern. Many investigators have studied morphological and physiological process of oogenesis in the ovary of mature fish of this species (Egami, 1954; Yamakawa, 1959; Yamamoto, 1962; Yamamoto, 1964; Yamamoto and Yoshioka, 1964; Iwamatsu, 1973; Iwamatsu *et al.*, 1988). However, gonadal development and oogenesis in immature fish corresponding to body size and metamorphosis from the larva to the juvenile have hardly been investigated. In particular, little is known with regard to the dynamics of oocyte growth, the oocyte number and oocyte composition in an ovary during the growth period of the larva. The present author was conscious of the need to examine these events.

In the early growth stage shortly after hatching, the medaka (*Oryzias latipes*) female already has oocytes in a primordial gonad, but the size and number of oocytes are still small. A rapid proliferation of primordial germ cells (PGCs) is already perceptible in the late stages of embryonic development (Satoh and Egami, 1972). In the medaka, the differentiation of oocytes takes place between the 6 and 11 mm stages during the period of metamorphosis (Kawamoto, 1973; Yamamoto,1975), and oocyte development is closely correlated with body growth which is significantly affected by stocking density, dietary restriction and photoperiod (Davis *et al*., 2001). In addition, individuals exhibit different growth rates, so that the gonads vary in the degree of development, even if the age is the same (Kanamori *et al*., 1985). It is therefore difficult to determine growth stages by times

and days after hatching even if the environmental factors are controlled during the growth period (Iwamatsu *et al*., 2003). In the present meristic study examining the developmental processes of oocytes in the ovary, the stages of body growth are represented by total length (TL: the distance from the anterior tip of the lower oral jaw to the posterior edge of the caudal fin) rather than age.

Although the morphological changes in the ovary in the medaka during the growth period from larva to adult have been reported by Yoshioka and Shimaya (1976), little meticulous basic study has been conducted on the changes in the meristic relationship between the number and composition of oocytes in an ovary. Therefore, the present study addresses the issue of the changes in the number and composition of developing oocytes (follicles) within an ovary during the period of metamorphosis. Resolving this issue should contribute to clarifying gonadal sex differentiation.

MATERIALS and METHODS

The medaka fish, *Oryzias latipes* (d-*rR* strain), used in the present study were reared in a rectangular glass aquarium (60×35×30 cm, 3 females and 2 males per about 60 liters of water) with an appropriate feeding regimen and reproductive conditions (L14: D10, 26–28°C). During the rearing period, fish were fed four times a day a balanced diet containing one part each of shrimp powder and parched barley flour, supplemented with an equal volume of powdered diet for the goldfish (Ryukin). Mature sex-reversed males with the genotype X^rX^r were obtained by treating early embryos with 2.5 ng/ml 17α-methyldihydrotestosterone (17α-methylandrostan-17β-ol-3-one, Sigma) for 24 hr (Iwamatsu *et al*., 2006; Kobayashi *et al*., 2011). White females of the genotype X^rX^r mated with the fertile sex-reversed white males (X^rX^r) and spawned every morning producing all female progeny. After hatching larvae were reared in a rat PC (polycarbonate) cage under the same breeding conditions as those for adult fish. They were fed the balanced diet in rainwater containing green algae. This experimental system allowing us to produce all female-progeny is very convenient for examining the dynamics of gonadal development in genetic females before and after gonadal sex-differentiation.

For preparation of fertilized eggs, spawned females were netted, and fingers were used to pluck off clusters of chorion-hardened eggs that hung from their urogenital pores. Under a stereoscopic microscope (Olympus SZX12) with substage illumination, long attaching filaments on the chorion of each egg were carefully grasped with watchmaker forceps and cut off with the blunt tip of a small glass rod while the egg was pulled away.

For examination of ovaries and oocytes (follicles), fish were anesthetized in a saline solution containing a mixture of 7 parts of phenylurethane and 3 parts of ethanol. Deeply anesthetized fish were laparotomized after body size (TL) was measured, and the ovary was removed in a saline solution that was designed for medaka oocytes (Iwamatsu, 2006). The ovary was fixed in 0.05% glutaraldehyde-saline for more than 24 hr (4°C) after partial incisions were made in the ventral portion. Fixed ovaries were stained with Gimsa's solution for about one minute at room temperature, immediately rinsed once in 0.1 M phosphate buffer solution (pH 7.2) and then put into 0.1 M phosphate-buffered saline containing 0.05% glutaraldehyde and diluted (1/200) Gimsa's solution. The epithelial layer (luminal wall) of the ovarian sac was stained purplish blue, and oocytes were stained light blue. The ovary was carefully dissected, and oocytes were separated as individually as possible using a sharp anatomical needle and a small scalpel under a stereoscopic microscope (×50–63). The sizes (diameter) of all oocytes in an ovary were precisely measured to the nearest micrometer line of a calibrated ocular micrometer ruled to 9 μm diameter. To avoid measuring any oocyte more than once, as soon as its size was measured, each oocyte was carefully sucked out and removed by a small glass pipette. Photomicrographs were made with an Olympus SZX12 microscope equipped with substage illumination and an Olympus automatic camera. Growth stages of fish were assigned following the author's developmental criteria (Iwamatsu, 2004, Iwamatsu *et al*., 2003). The numbers of oocytes in the size intervals were presented as percentages of the total numbers of oocytes in an ovary.

OBSERVATIONAL RESULTS and DISCUSSION

When embryos hatch at 8–10 days post-fertilization, the number of undifferentiated germ cells tends to be much larger in females than in males, which is a well-known-manifestation of the sexual dimorphism in medaka (Hamaguchi, 1982). In larvae about 6 mm TL (about 10 days after hatching) oogoina measuring about 10 μm in diameter are arranged in the peripheral region of the ovary (Satoh, 1974) which contains small oocytes at different stages of the meiotic prophase (Kanamori *et al*., 1985).

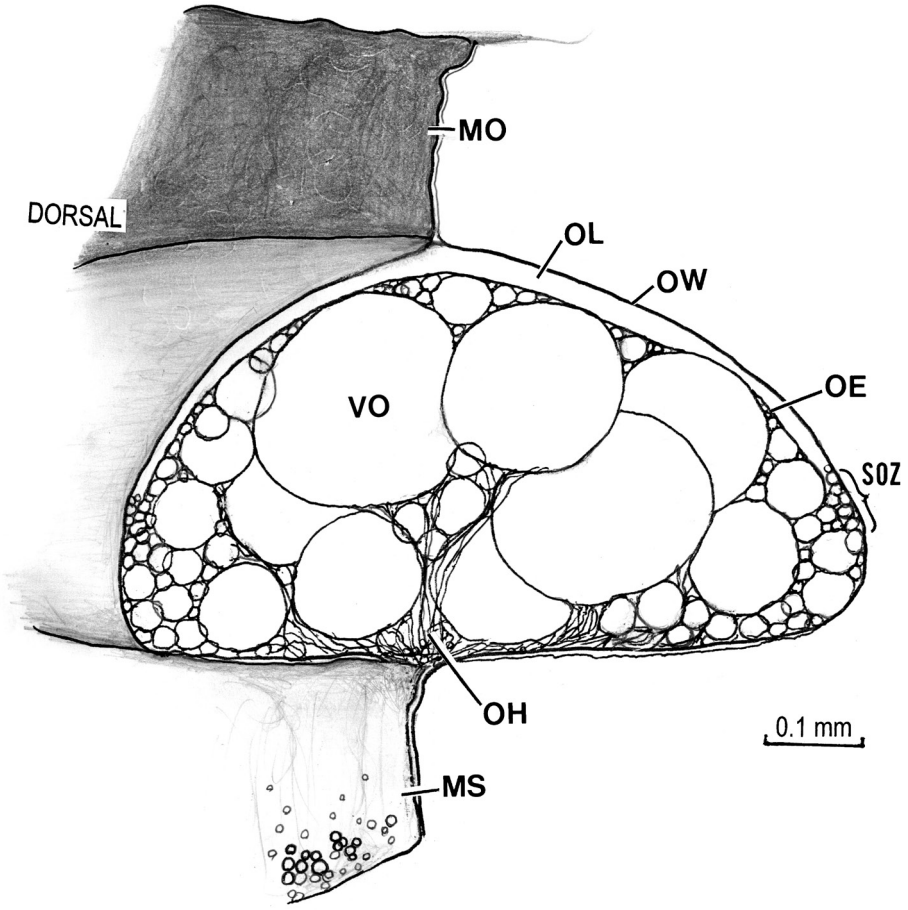

Fig. 1 Schematic diagram of a transverse section of an ovary of a medaka *Oryzias latipes* at the end of metamorphosis.
The ovary contains various oocytes, and small oocytes are located at the ovarian epithelial region (OE) surrounding large oocytes (VO) and at the germinal zone at the marginal edge in the luminal epithetlium (OW). The ovarian lumen (OL) is located on the dorsal side and the loose connective tissue (OH) (fiberous network) is located on the ventral side. Each oocyte is anchored to the basal connective tissue by a few fiberous stalks (cords). MO, mesovarium; MS, mesentery. SOZ, germinal zone.

The present study also found that in the medaka, oogonia or the youngest oocytes (18–36 μm in diameter) distribute in a germinal zone in the boundary layer between the germinal epithelium and the luminal epithelium (wall) of the ovarian lumen (Figs. 1 and 2) or as clusters at the ovarian surface region surrounding large oocytes. In all ovaries of adults, most oogonia cluster together and form so called "nests" (Yamamoto, 1962), or "germinal cradle" (Nakamura *et al*., 2011). Thus, the sexually undifferentiated germ cells persist throughout gonadal development, inexhaustibly supplying germ cells for oogenesis (cf. Saito and Tanaka, 2009). In syngnathans, oocytes are formed from oogonia within the germinal ridge (Selman and Wallace, 1989).

Small oocytes of the chromatin-nucleolus stage and early peri-nucleolus stage are found in the ovaries of all larvae. The oocytes in follicles 30–90 μm in diameter each have a comparatively large nucleus and a yolk nucleus (Balbiani's body) in the cytoplasm (Kobayashi and Iwamatsu, 2000) and are surrounded by flattened follicle cells (Yamamoto, 1963; Iwamatsu *et al*., 1988). In larvae 12–13 mm TL in which prominent urogenital papillae are developing, previtellogenic oocytes of stage III measuring 91–120 μm in diameter first appear, as well as oogonia and major small oocytes of stage I–II. When the size of oocytes reaches about 100 μm (Tsukahara, 1971) , or 115 μm (Iwamatsu, 1992) in diameter, there are electron dense and amorphous veruciform structures among the granulosa cells on the oocyte surface (Iwamatsu *et al*., 1988). In ovaries of larvae more than 14 mm TL, the stage IV oocytes 120–150 μm in diameter (Iwamatsu *et al*., 1988; Nakashima and Iwamatsu,1989; Iwamatsu, 1992) are enveloped by the chorion in a continuous layer 200–400 nm thick with many villi (non-attaching filaments) on the entire surface and attaching filaments at the vegetal pole region. Although mitotic figures are rarely observed, the follicular cells surrounding the oocyte increase sharply in number as oocytes grow from stage IV to stage V (Iwamatsu and Nakashima, 1996). At the animal pole region of the follicle layer surrounding the stage V oocyte, a micropyle cell is found (Nakashima and Iwamatsu, 1989).

1) Morphological change in the ovary during the growth period

In young fish 17–18 mm TL, the ovary consists of various sized follicles, blood vessels, nerves and connective tissue elements (Fig. 1). On the dorsal side of the ovary, the luminal wall of the ovarian lumen connects at the median surface to the mesovarium that attaches to the dorsal wall of the peritoneum. On the ventral side of the ovary, the median surface of the ventral connective tissue with its loose, complex configuration connects with the mesentery and attaches to the gut. The fiberous cords extending from the surface of each oocyte pass among various sized oocytes towards the ventral connective tissue. Each follicle

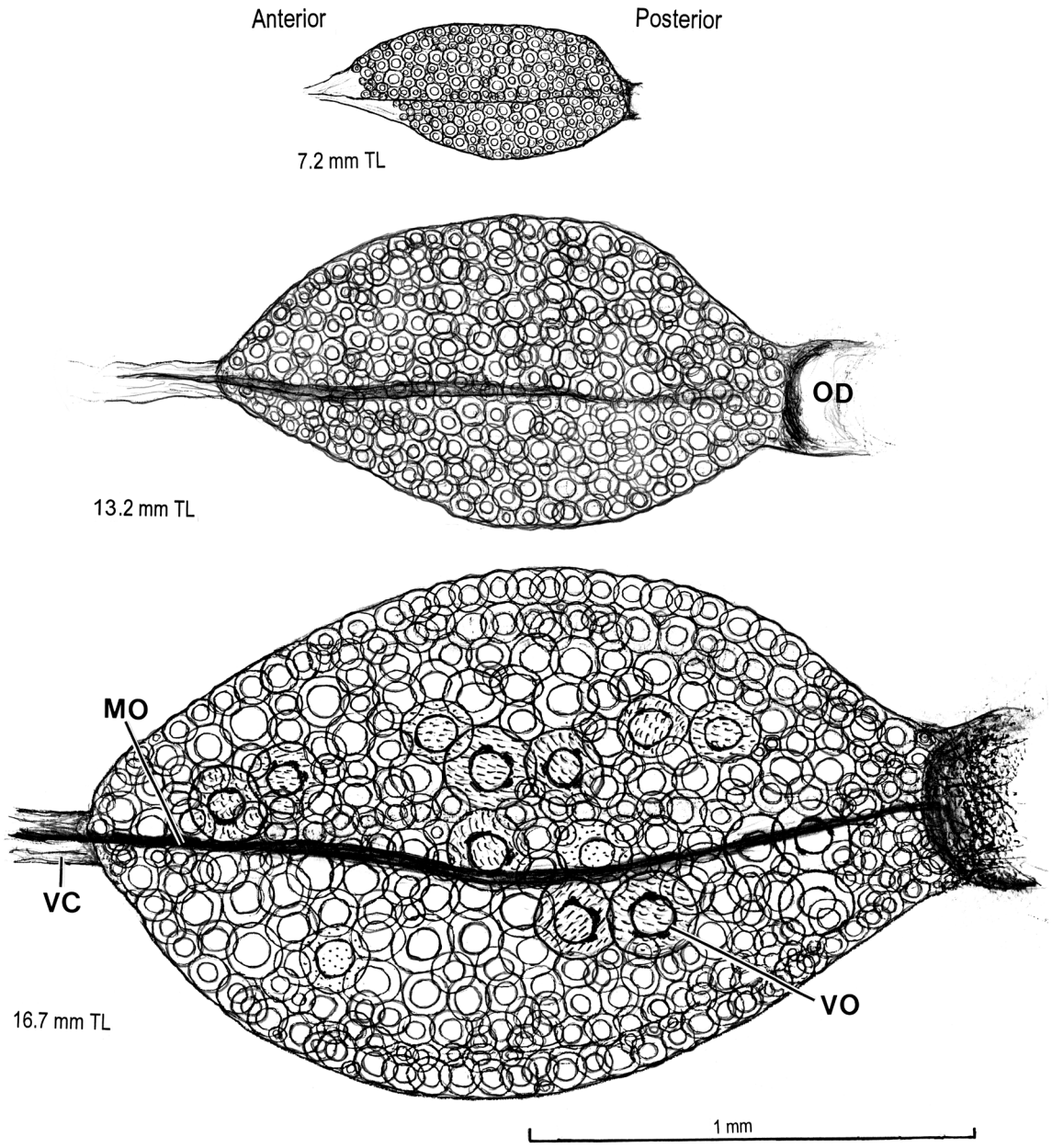

Fig. 2 Changes in the ovary of during the growth period.
OD, oviduct; VC, the cord of blood vessels. Other abbreviated symbols see Fig. 1.

larger than about 30 μm in diameter is anchored in the connective tissue by fiberous stalk-like structures (cords) (Iwamatsu and Nakashima 1996). The connective tissue of the ventral side may correspond to the central cord surrounded by smooth muscle, nerve, abundant collagen, and stromal cells seen in the ovary of *Fundulus heteroclitus* (Brummette *et al*., 1982). After ovulation, the post-ovulatory follicle is stretched towards the connective tissue of the ventral cortex (ovarian hilus) by the fiberous cords. Consequently, the post-ovulatory follicles are temporarily found near the intricate nets or cords in the connective tissue (Iwamatsu *et al*., 1988), which contains fibroblast cells, a network of blood vessels and associated nerves. Along the median ventral region of the ovary, the blood vessels (ligaments) extend from the anterior end of the ovary to the duct Cuvie through the anterior wall of the peritoneum. The ovarian lumen connects posterior with a short tubular oviduct which leads to the genital opening located at posterior region of the urogenital papilla (protuberance). During metamorphosis, a dorsoventral thin-shaped ovary consisting of a monolayer of small oocytes transforms to a thick oval ovary containing oocytes of various sizes.

In larvae 5.0–5.5 mm body length (probably TL) 6–8 days post-hatching, the ovary is located on the gut underneath the mesonephros ducts. The ovary is enveloped by a single layer of peritoneum and located at the left side of the coelon (Yoshioka

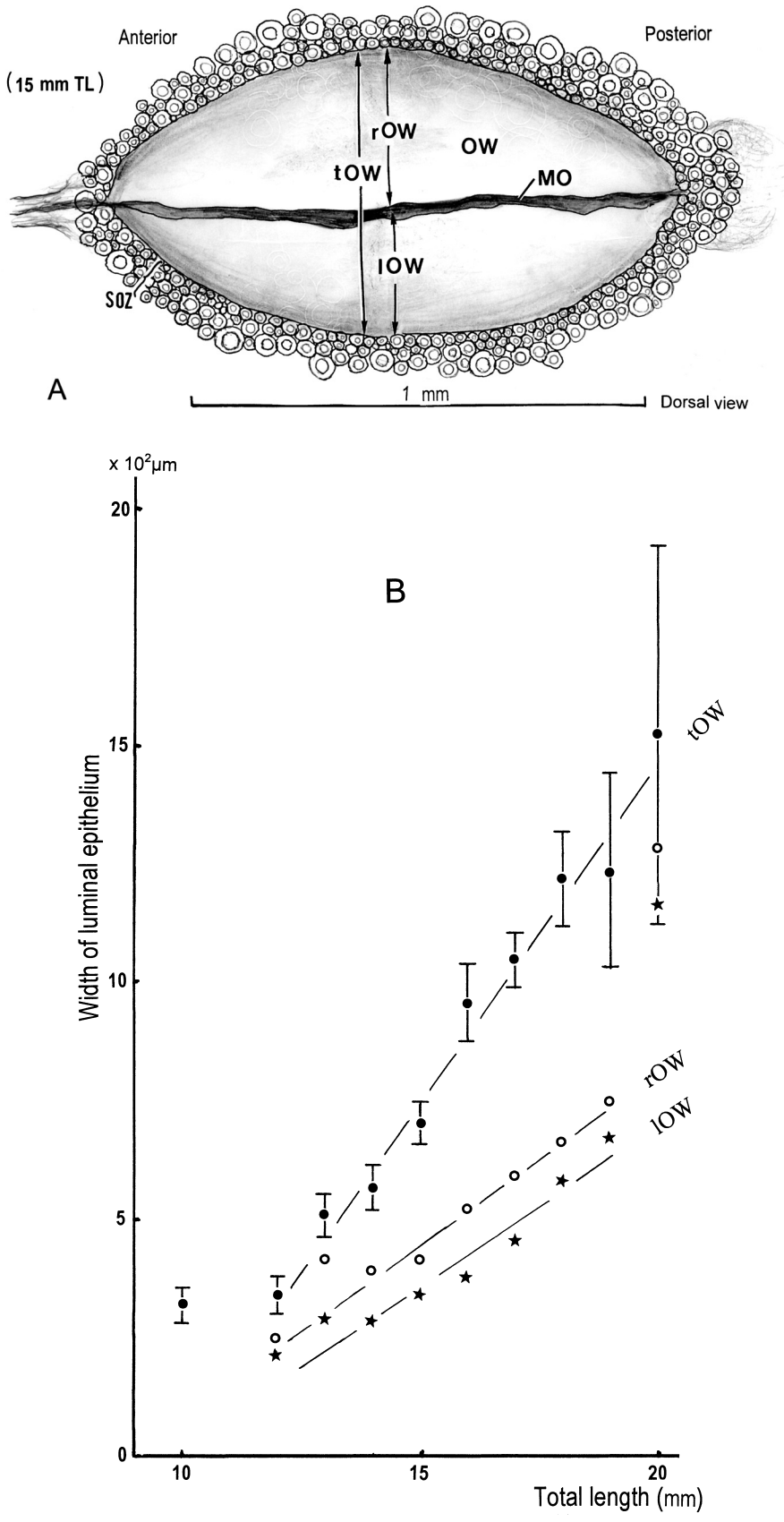

Fig. 3 Changes in the width of the luminal epithelium of the ovary during the growth period.
The upper diagram (A) illustrates the luminal epithelium in the dorsal view of the ovary. The graph (B) shows changes in the total width (tOW), the left (lOW) and the right (rOW) widths of the luminal epithelium.

and Shimaya, 1976).

In larvae 6.4 mm and 7.5 mm TL, ovaries measure 557.8 ± 31.2 μm and 637.1 ± 27.6 μm in length, 106.9 ± 23.0 μm and 276.4 ± 43.0 μm in width and 37.5 ± 5.0 μm and 71.2 ± 11.0 μm in thickness, respectively. These early ovaries attached to the dorsal side of the gut have a bilobed shape with a median groove (midline) (Fig. 2) allowing recognition of the left and right lobes of the ovary. The ovarian lumen and oviduct are not yet formed in larvae 7.5 mm TL. The present data indicate that the length and the width of the left and right lobes of the ovary are asymmetric with the right lobe larger than the left lobe (Fig. 2). This result is consistent with the bilateral asymmetry of the gonad primordium (*O. latipes*: Gamo, 1961) and bilaterally asymmetric distribution of PGCs (*O. celebensis*: Hamaguchi, 1983) in embryos. However, Yoshioka and Shimaya (1976) reported that the length of the ovary in larvae 8 mm body length is greater in the left lobe (214–272 μm) than in the right (111–152 μm). When the larvae are 9 mm TL, the bilobulated feature of the ovary is no longer detectable. When body size reaches 9.5 mm TL, an ovarian lumen begins to form by separation of the ovarian wall from three layers of peripheral epithelial cells (Hosokawa and Nambu, 1971) of the ovarian stroma. Formation of the ovarian lumen begins to form at the anterior region of the ovary in larvae 9.5–10 mm TL, which has also been reported (Onitake, 1972; Yoshioka and Shimaya, 1976; Suzuki and Shibata, 2004). Small oocytes as well as oogonia exist at the edge of the luminal wall at this stage. The width of the luminal wall increases correspondence with body growth (Fig. 3). In larvae 10 mm TL, the average width of the luminal wall connecting with the mesovarium at the median surface is 325.0 ± 47.9 μm, and the width of the right luminal wall is larger than the left. In larvae 11 mm TL, the connection of the ventral surface of the ovary with mesentery is also observable. The length of the ovary increases in proportion to growth of the body, while the width of the ovary begins to increase steeply beginning at 14 mm TL (Fig. 4).

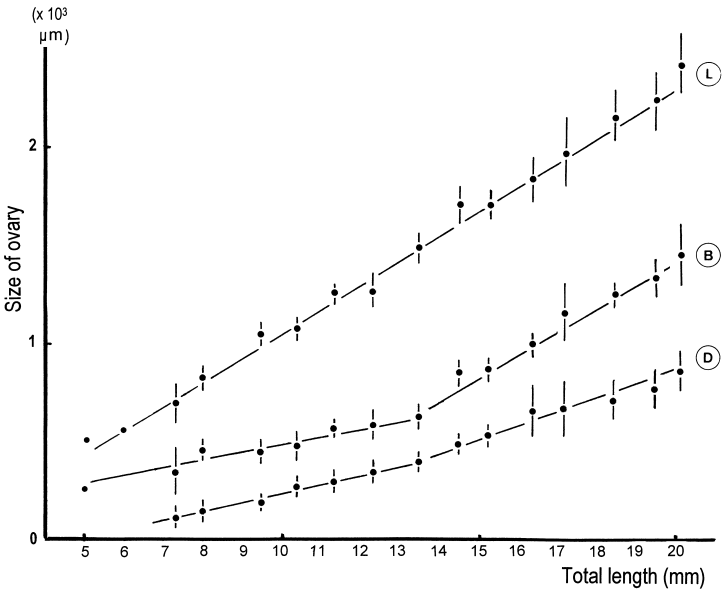

Fig. 4 Morphometric changes in the ovary during metamorphosis.
Figure indicates the increase in length (L), width (B) and thickness (D) of the ovary. Note the edge of the luminal epithelium with a germinal zone (SOZ).

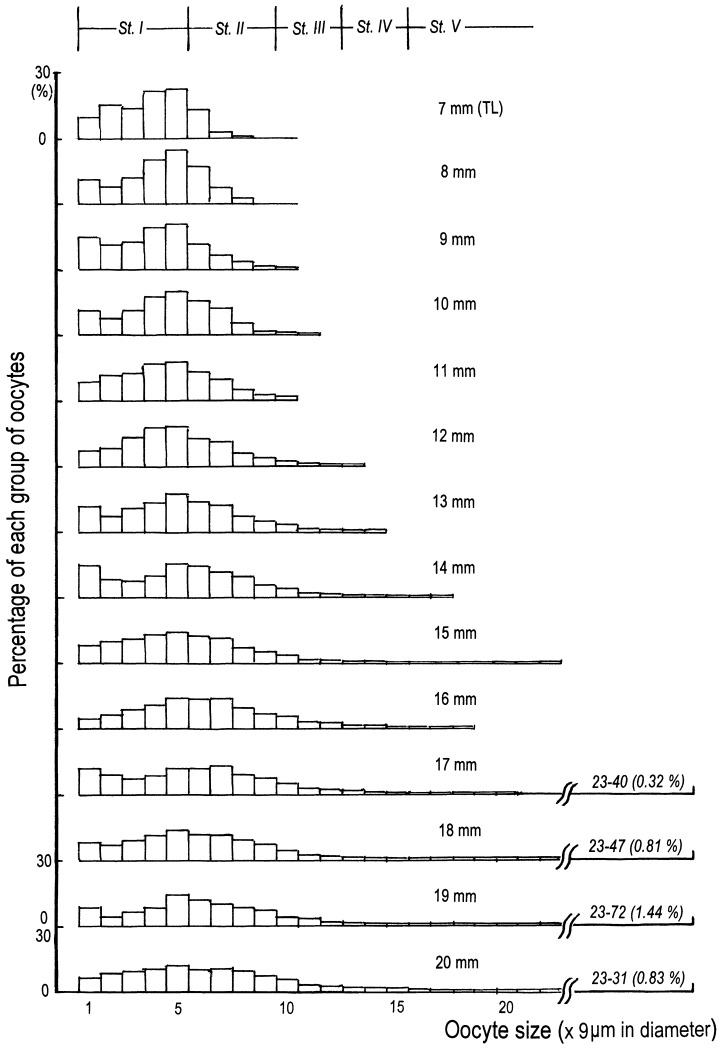

Fig. 5 Oocyte composition in an ovary during the growth period.
The developmental stages of oocytes are listed at the top of the figure.

2) Change in oocyte composition and number in the ovary during the growth period

a. Change in oocyte composition in the ovary

Distinct sex differentiation of the gonad takes place after hatching, and becomes apparent by its meiotic activity. Shortly after hatching, larvae 4.5 mm TL have only oogonia (15–20 μm in diameter: Satoh, 1974; Kobayashi and Hishida, 1992; 12–19 μm in diameter: Hamaguchi, 1982), while in larvae 6.0 mm TL (about 10 days after hatching) some oogonia have differentiated into oocytes (Kawamoto, 1973). Consequently, when larvae have grown to about 6 mm TL, the ovary contains oogonia and many young oocytes most of which are approximately 20 μm in diameter and at the zygotene or pachytene stage of meiosis. According to Kanamori *et al.* (1985), these young oocytes begin to be surrounded by centrally located somatic cells.

The frequency distribution of oocytes was examined in ovaries obtained from larvae of different body sizes. The percentages of the population of the different sized oocytes within an ovary are shown in figure 5. The ovaries of larvae 7–8 mm TL (about 20 days after hatching) mostly possess a population of previtellogenic oocytes 36–45 μm in diameter, surrounded by a single layer of flattened granulosa cells. The ovaries of larvae smaller than 8 mm TL still contain only small oocytes less than 90 μm in diameter.

Until 11 mm TL, larvae have a synchronous population of growing oocytes which may be arrested at stage III. In the ovaries of larvae 12 mm TL, late previtellogenic oocytes of stage IV 91–120 μm in diameter first appear (Fig. 5). The change in oocytes with the largest diameter during the growth period is shown in figure 6. In larvae 7 and 8 mm TL, the maximum size of oocytes within an ovary is 49.5 $\pm$ 8.1 (range 36–90) μm and 56.6 $\pm$ 9.0 (range 54–90) μm in diameter (n = 8), respectively. About 88.6% of total oocytes in each ovary of these larvae are also less than 63 μm in diameter. The diameter of the largest oocytes increases from 81.0 $\pm$ 2.9 (range 72–99) μm in larvae measuring 10 mm TL to 103.9 (range 81–126) μm in larvae 13 mm TL. When the body size of larvae attains about 12 mm TL, the diameter of the largest oocytes is 101.3 $\pm$ 3.3 (range 81–126) μm and the flattened

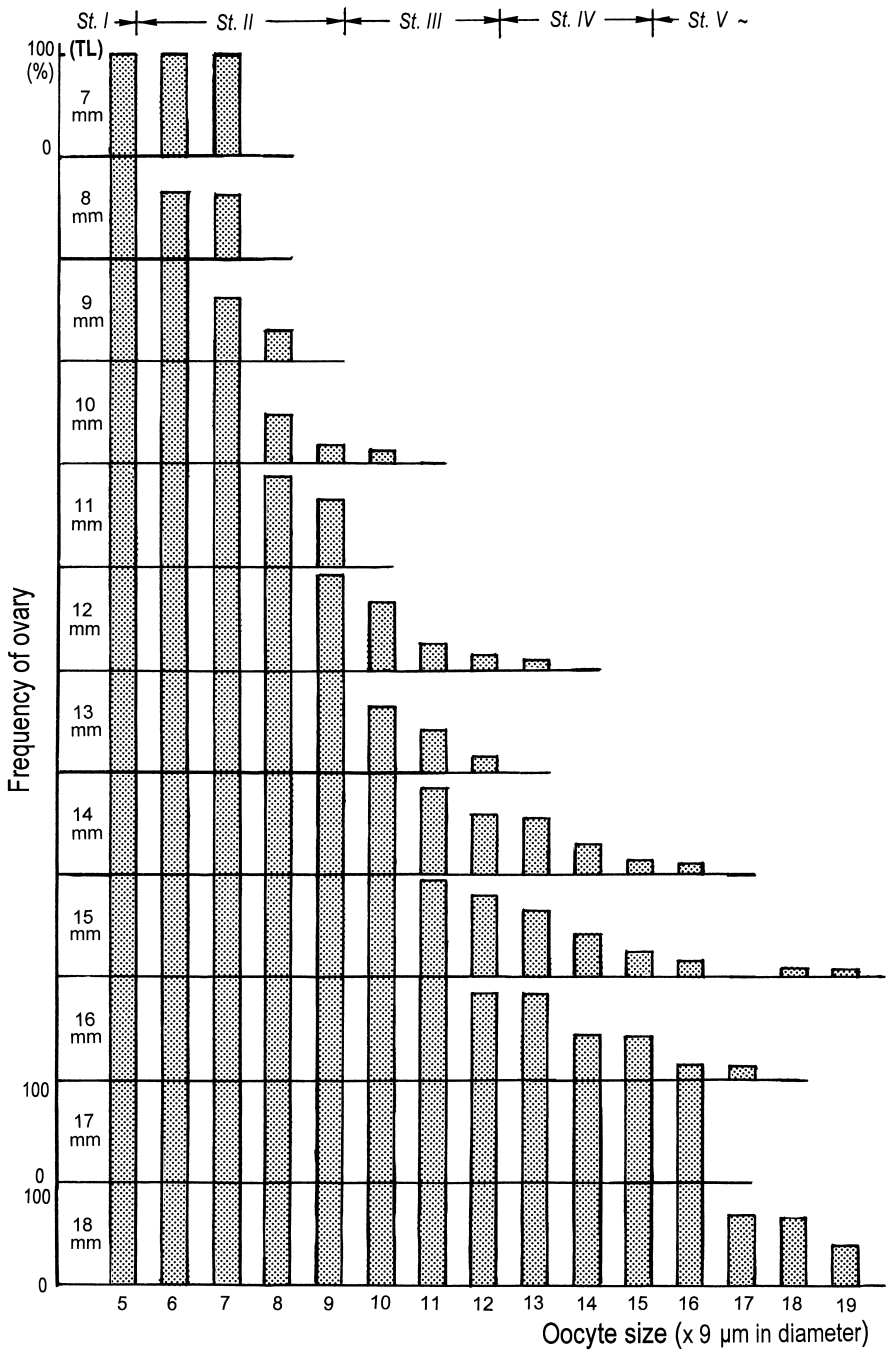

Fig. 6 Frequency distribution of ovaries containing various sized oocytes during the growth period.

follicular cells surrounding the oocyte gather to one side (Iwamatsu and Nakashima, 1989). Before the body size reaches 14 mm TL, the size of most oocytes remains smaller than 110 μm in diameter, but the polarity of the follicle (granulosa) cell distribution is first realized. When larvae attain a body size of more than 14 mm TL, the ovary contains stage V oocytes of the early vitellogenic phase, and oocyte composition in each ovary has the vitellogenic pattern (Figs. 5 and 6). Thus, oocyte composition begins to change from the previtellogenic to the vitellogenic pattern in the period between 13 and 14 mm TL. In the transitional period between larva and juvenile, the larvae gradually attain meristic features of the adult by 15 mm TL. In larvae larger than about 16 mm TL, stage IV oocytes with oil droplets surrounding the nucleus are distributed in the central region of the ovary (Fig. 2). All individuals larger than about 17 mm TL have vitellogenic oocytes more than 153 μm in diameter. Further, in larvae 20 mm TL the size of vitellogenic oocytes extends up to 350 μm in diameter.

The present study also found that small stage I oocytes with diameters of 45 μm are always present as a dominant population in every ovary, regardless of the body size. This seems to be attributable to either a rapid development from 36 μm-diameter oocytes to the next larger oocytes, or slow development from 45 μm-sized oocytes to the next, because the proliferation of developing oocytes is arrested.

During the growth period, a stock of oogonia and small oocytes 9–18 μm in diameter always exists in all ovaries. In *Fundulus heteroclitus*, newly formed oocytes occur in germinal nests (cradles), which appear to be embedded in the wall of the ovigerous lamellae (Selman and Wallace, 1986). In *Oryzias latipes*, oogonia or small oocytes are also found in the germinal zone in the edge of the wall of the ovarian lumen. Oogenesis appears to continue by recruitment from these germ cells throughout the life-history.

At present, it remains to be clarified how the pattern of oocyte composition in an ovary persists stably. In females larvae, the composition of oocytes from stage I to stage III seems to have a stable pattern in the ovary with correspondence to the growth stage of the body. During the body growth period, the transition of oocyte composition from the previtellogenic to the vitellogenic pattern in an ovary reflects physiological changes taking place in developing oocytes.

b. Change in oocyte number in the ovary

During the growth period, the real size of all oocytes within an ovary was examined. In undifferentiated gonads of the newly hatched medaka about 4.5–5 mm TL, the total number of germ cells has so far been examined by use of traditional paraffin sections: the average number of germ cells in an ovary is 117.8 (Tsuzuki *et al*., 1966), 100 (Hamaguchi, 1979) or more than 200 (Satoh and Egami, 1972) within 24 hr post-hatching, about 100 (Hamaguchi, 1979) or beyond 700 (Satoh and Egami, 1972) on the 13th day and 300 (Hamaguchi, 1979) or about 1,000 (Satoh and Egami, 1972) on the 20th day after hatching, although the body size is not described in these reports. When this sectioning method is used, an oogonium or oocyte more than 10 μm in diameter must be cut just in half or nearly half by 5 μm thickness sections and counted by a careful examination of all the serial sections. Therefore large oocytes could frequently be overcounted.

Figure 7 shows the changes in the total number of oocytes in ovaries during growth. Concomittant with body size, oocytes increase in number and size although the total number of oocytes at each body size shows remarkable individual variation. The total number of oogonia and oocytes increases beyond 100 in some larvae 8 mm TL. When larvae grow from 11 mm TL to 15 mm TL, the total number of oocytes increases from approximately 500 to 1000 in an ovary, respectively. There are statistically significant differences in the average number of oocytes between larvae of 14 mm and 15 mm TL, although the total number of oocytes in an ovary is surprisingly variable among individuals of the same TL. To give another example, some females 13 mm TL contain only a few hundred oocytes in each ovary, whereas others have more than one thousand. In larvae about 20 mm TL (Fig. 7), the oocyte number increases up to about 2,500 and the oocyte composition is similar to that of the adult.

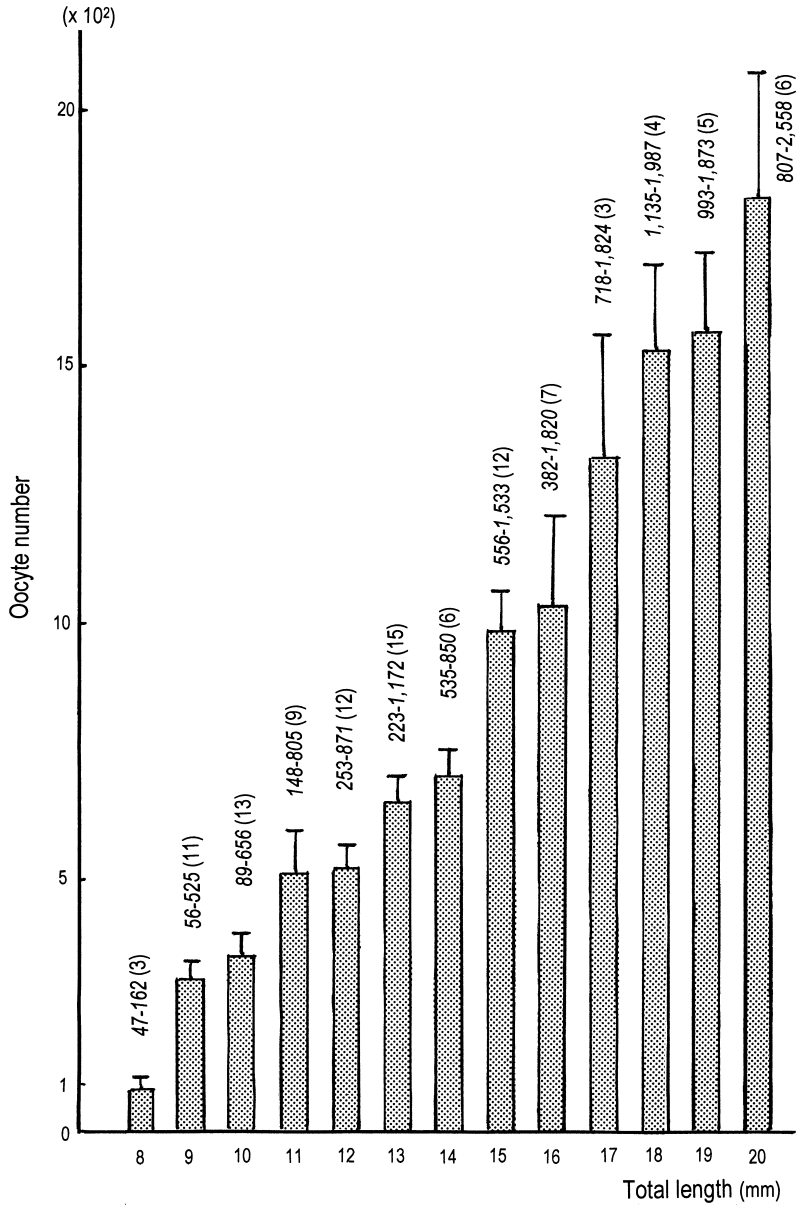

Fig. 7 Changes in the number of germ cells in an ovary during the growth period. Parentheses indicate the number of individuals examined.

Thus, the actual number of oocytes increases in proportion to TL during metamorphosis, but the rate of increase varies among individuals. Why large variations in oocyte number among individuals occur is unknown and remains enigmatic: it is probably a fluctuation due in part to genetic differences among individuals or overpopulation of breeding individuals. The oocyte number is therefore unreliable for determining the age of medaka fish, but oocyte growth has been extensively used to determine the age and rate of body growth in fishes.

To sum up, the present study on morphometric analysis of ovaries during metamorphosis has demonstrated that oocyte composition of larvae shifts concomitantly with body growth to the pattern of the adult, but does not yet give arise to the cyclic changes in reproduction during metamorphosis. The total number of oocytes in an ovary continues to increase during metamorphosis, but the oocyte composition is constantly maintained by recruitment from primary germ cell stocks in the

germinal nests (germinal cradles). In addition, the population of stage I–II oocytes in an ovary is almost constant, regardless of different sizes of the body. Thus, the present data may provide crucial and basic information on the dynamic aspects of the number and composition of germ cells in the ovary during metamorphosis. However, it still remains to be clarified why changes in composition and number of oocytes in an ovary depend on body growth.

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