

Gonadal morphology in the self-fertilizing mangrove killifish (*Kryptolebias marmoratus*)

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Abstract We conducted anatomical and histological observations of the gonads in the self-fertilizing mangrove killifish (*Kryptolebias marmoratus*) to investigate self-fertilizing mechanism of this species. The gonad has a bilobed structure. The elongated-gonadal lumen (GL) along the dorsal surface of the gonad connects to the common genital sinus. The elongate testicular region is closely attached to the GL. Among the ovulated eggs in the GL, those in the anterior part of GL have micropyles but no perivitelline space (not yet fertilized) while those in the posterior part of GL are fertilized. In our histological analysis, we found free sperms in the posterior area of the GL. We conclude that ovulated eggs may be self-fertilized in the posterior GL.

Key words Self-fertilization · Histology · Hermaphrodite · *Rivulus marmoratus* · Gonad

自家受精魚マングローブキリフィッシュ (*Kryptolebias marmoratus*) の生殖腺の形態

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マングローブキリフィッシュ (*Kryptolebias marmoratus*) の生殖腺の解剖学および組織学的な観察を行い、本種の自家受精機構を考察した。生殖腺は二葉に分かれ、生殖管は生殖腺背面を通り泌尿生殖口へ達した。精巣組織は生殖管に隣接していた。生殖管内に排卵された卵のうち、生殖管前方の卵には囲卵腔がなく卵門を有しており未受精であったが、生殖管後方の卵は受精していた。組織学的観察から、生殖管後方で排精の起こっていることが明らかとなった。排卵後に卵が生殖管を通る段階で自家受精が起こると考えられた。

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The mangrove killifish, *Kryptolebias marmoratus* (family Rivulidae), is the only known self-fertilizing hermaphroditic vertebrate (Harrington, 1961; Warner, 1978), and it is of interest for its unique reproductive biology (Harrington, 1968, 1975). Costa (2004) reexamined the taxonomy of the Rivulidae and recognized *Rivulus marmoratus* as a synonym of *K. marmoratus*. This species inhabits brackish mangrove habitats from Brazil to Florida (Harrington and Rivas, 1958). Individuals are reported to be either hermaphrodites which produce both sperm and ova simultaneously, or secondary males which develop from hermaphrodites by loss of ovarian tissue, or primary males which develop directly to produce sperm throughout the rest of their lives (Harrington 1971; Soto et al., 1992). Both primary and secondary males are quite rare in the field (at most 24% at one site in Belize, see Turner et al., 1992) and the laboratory (less than 5 %, Sakakura and Noakes, 2000).

The self-fertilization of this species had confirmed only by the observation of oviposited fertilized eggs from fish kept individually (Harrington, 1961). Genetic studies have also confirmed that all reproduction of hermaphrodites in this species is by internal self-fertilization (Harrington and Kallman, 1968). A previous histological study (Soto et al., 1992) revealed that the gonad of *K. marmoratus* is a bilobed structure with the posterior tips of the lobes fused and a genital duct. Soto et al. (1992) detailed the maturation process of both ovarian and testicular region. Kweon et al. (1998) observed the ultrastructure of spermatozoa of hermaphrodites in this species and found that the general features of spermatozoa are similar to those of externally fertilizing species. However, the mechanism of self-fertilization in the mangrove killifish is still unclear. Therefore, we conducted detailed histological and anatomical observations on ovarian structure to describe the structure of the ovotestis in relation to the self-fertilizing mechanism of this species.

Materials and Methods

Experimental fish.—We used a total of 29 matured fish, from which we had recovered oviposited fertilized eggs during daily observations, in this study. They were 1 or 2 years old and within the range of standard length (SL) of 18.8 to 23.6 mm. The fish were from the fifth or later generation from a single lineage (PAN-RS), obtained from Dr. W. P. Davis of the U.S. Environmental Protection Agency, Gulf Breeze, Florida, U.S.A. PAN-RS individuals were the descendants of a single hermaphrodite collected near Bocas del Toro, Republic of Panama, in 1994 (previously described to have

originated in Florida, U.S.A., by Sakakura & Noakes 2000, Grageda et al. 2004). Fish were held individually in translucent plastic containers (FisherBrand Collection Containers, Fisher Scientific) containing about 60 ml of brackish water. We constituted the brackish water from distilled water and marine salt (Instant Ocean, Aquarium Systems). We changed water in all containers weekly. We held all fish in a climate – controlled room (12 : 12 hours light : dark photoperiod, 25°C, pH 8.0, 17 ppt salinity) in the Hagen Aqualab at the University of Guelph, Canada and in a temperature - controlled incubator at Nagasaki University, Japan. We fed all fish newly hatched brine shrimp (*Artemia franciscana*) nauplii and checked containers for egg oviposition once daily.

Anatomical and histological observation.—We euthanized three fish with 400 ppm of MS222 (Tricaine, SIGMA) and removed gonads using fine forceps and scissors under a dissecting microscope. After observing the external morphology of gonads, we dissected the gonadal lumen to observe the internal structure and to remove ovulated eggs. We identified developmental stages of ovulated eggs according to the descriptions of Koenig and Chasar (1984).

We examined 26 fish in our histological analysis. They were euthanized with 400 ppm of MS222 and then fixed in Bouin's fixative. We processed all preserved specimens for histological study by dehydrating and embedding in paraffin following the procedure of Cole and Noakes (1997). We made transverse sections serially at 5-7 µm through the entire body cavity (about 2000 sections per fish), mounted sections on glass slides, and then stained them with hematoxylin and eosin. Modifying the former study (Soto et al., 1992), we classified 4 categories for oocyte maturation as: (1) peri-nucleus stage (PN), with nucleoli and cytoplasm stained markedly by hematoxylin; (2) yolk vesicle stage (YV), with pink-stained yolk vesicles; (3) yolk stage (Y1), with eosinophilic cytoplasm; (4) final maturation (Y2), with coalesced yolk and absence of nucleoli. We viewed all gonadal sections for gonadal structure, testicular region and oocyte maturation under a microscope equipped with a camera lucida (BH-2, Olympus, Tokyo, Japan). Gonads of 10 fish, which had no loss of sections during histological process, were traced by camera lucida and the numbers of oocytes in the 4 categories were counted. Then we calculated the mean numbers of oocytes for the 5 regions of gonads divided by the relative distance from the anterior part of the gonad (0-20, 21-40, 41-60, 61-80, and 81-100%, respectively).

Results and Discussion

The mangrove killifish gonad from 3 specimens is a bilobed structure, with the posterior tips of the lobes fused and a genital duct (Fig. 1a). The gonadal lumen (GL) extends along the dorsal area of the gonad and attaches to the common genital sinus. The anterior part of the GL invaginates into the gonad and the tip forms the ovarian cavity where well-developed eggs are attached in one layer. We found ovulated eggs in the GL. Eggs in the anterior part of GL had micropyles but no perivitelline space, and were defined as not fertilized (Fig. 1b). On the other hand, eggs found in the posterior part of GL had blastodiscs and distinct perivitelline space, and so were determined to be fertilized (Fig. 1c).

The histological sections of the gonad from one hermaphrodite (SL 21.0 mm) is shown in Fig. 2. All 26 specimens had the same gonadal structure. In the anterior regions of the gonad, the GL was formed with thin epithelium and was surrounded by both ovarian and testicular tissue (Fig. 2a,e) confirming our anatomical observation that the ovarian cavity is formed in this area, which is common in teleost ovaries (Nagahama, 1983). In the middle regions of the gonad, the GL appears in the dorsal area of gonads, and ovarian and testicular regions are attached separately to the GL (Fig. 2b,f). In the posterior region of the gonad, the testicular region dominates and is attached to the GL (Fig. 2c,g). The common genital sinus of the mangrove killifish has a thick epithelium and a smooth muscle layer (Fig. 2d). Five of our 26 specimens had spermatozoa in the posterior part of the GL which had been discharged from spermatogenic tubules (Fig. 2g,h), as reported in the previous study by Kweon et al. (1998) which found the lumen of efferent ducts filled with free spermatozoa. In all cross sections examined, we could not observe the sperm storage-like structure reported by Kweon et al. (1998), which is often found in viviparous teleosts (Nagahama, 1983). Thus, spermatozoa may be discharged directly into the GL. Oocytes of PN were observed in most ovarian regions but tended to decrease in the anterior part of the gonad. We found oocytes at various stages of maturation throughout the gonad, as has been reported in previous studies (Soto et al., 1992; Cole and Noakes, 1997; Sakakura and Noakes, 2000). From 10 specimens for which we had all cross sections, yolk vesicle stage oocytes (2.4 oocytes in average) were observed in 61-80 % of ovarian regions, whereas Y1 (2.7) and Y2 (2.4) oocytes were found in 41-60 % of ovarian regions. This supports the conclusion that mature oocytes are ovulated into the ovarian cavity in the anterior part of the gonad.

The self-fertilization process in the mangrove killifish can be summarized as follows; oocyte maturation occurs around the ovarian cavity, and then they are ovulated into the ovarian cavity. Ovulated eggs are transferred individually in the GL and internal self-fertilization may occur with the spermatozoa discharged directly into the GL. Most reproductive functions, from gametogenesis to behavior, are dependent upon the

endocrine system (Redding and Patiño, 2000). However, Minamimoto et al. (2006) investigated blood-circulating sex steroid levels and the ability for sex steroid production from gonads of *K. marmoratus*. They showed that this species secretes estrogen (17 β -estradiol), androgen (11-ketotestosterone), and progestin (17 α -20 β -dihydroxy-4-pregnen-3-one) synchronously, which is unusual in teleosts. Moreover, the mangrove killifish does not have clear diel rhythms in terms of spawning and ovulation (Grageda et al. 2005). Therefore, the triggering mechanisms of ovulation, self-fertilization, and spawning (oviposition) are still unclear and will be the subject of future research.

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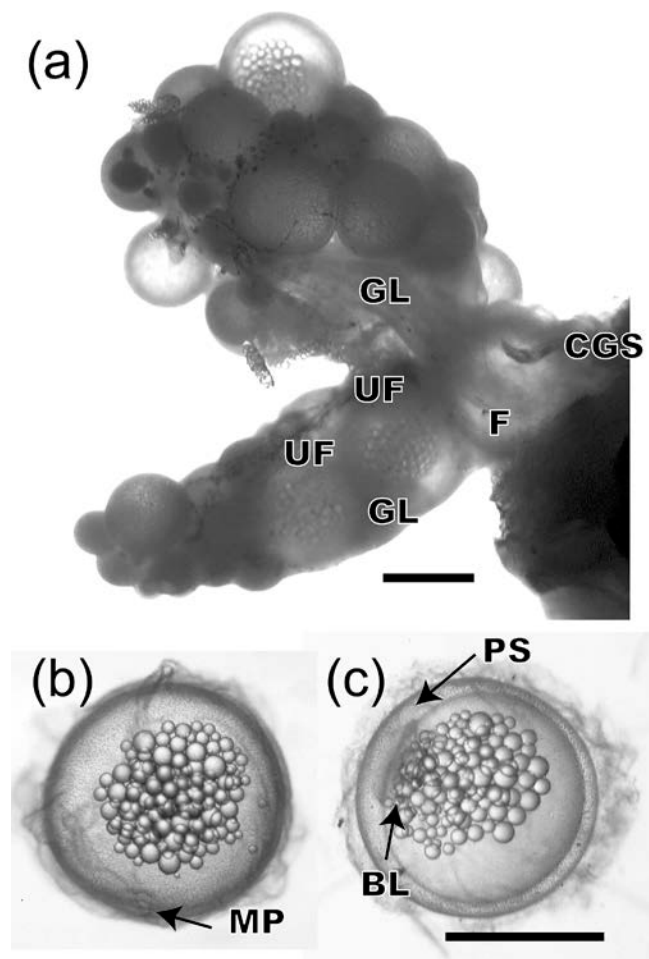


Fig. 1. Dorsal view of the gonads (a), eggs found in GL (b), and fertilized egg (c) of *Kryptolebias marmoratus*. CGS, common genital sinus; F, fertilized egg; GL, gonadal lumen; UF, unfertilized egg. Arrows indicate blastodisc (BL), micropyle (MP), and perivitelline space (PS). Bar 1 mm

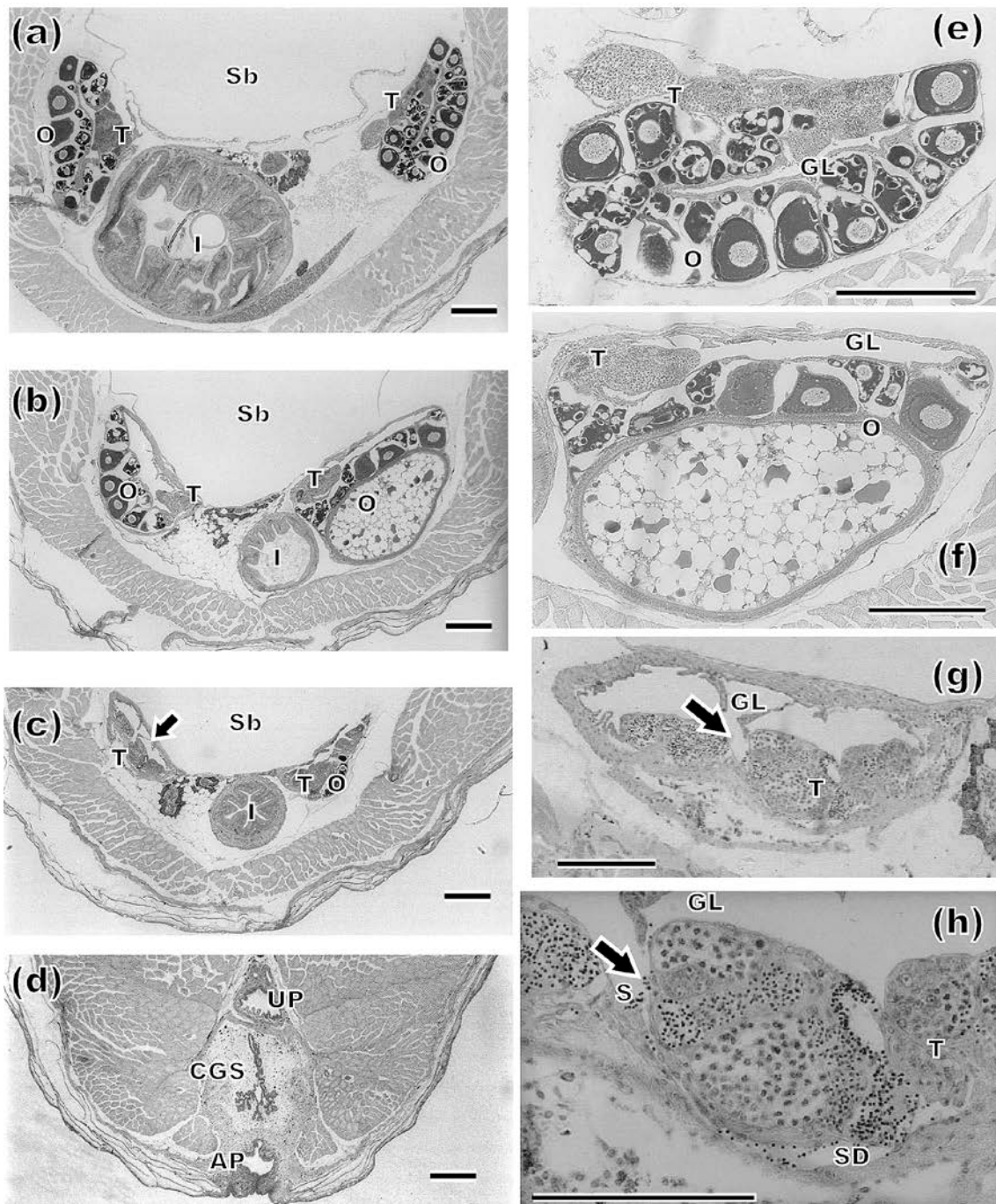


Fig. 2. Cross sections of the gonad of *Kryptolebias marmoratus* (21.0 mm SL). (a,e) anterior part, (b,f) middle part and (c,g,h) posterior part. AP, anal pore; CGS, common genital sinus; GL, gonadal lumen; I, intestine; O, ovarian region; S, spermatozoon; Sb, swim bladder; SD, spermatogenic tube (sperm duct); T, testicular region; UP, urinary pore. Bars 200 μ m for (a-f), 10 μ m for (g,h)