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Ultrastructure and in vitro Steroidogenesis of the Gonads in the Protandrous Anemonefish *Amphiprion frenatus*

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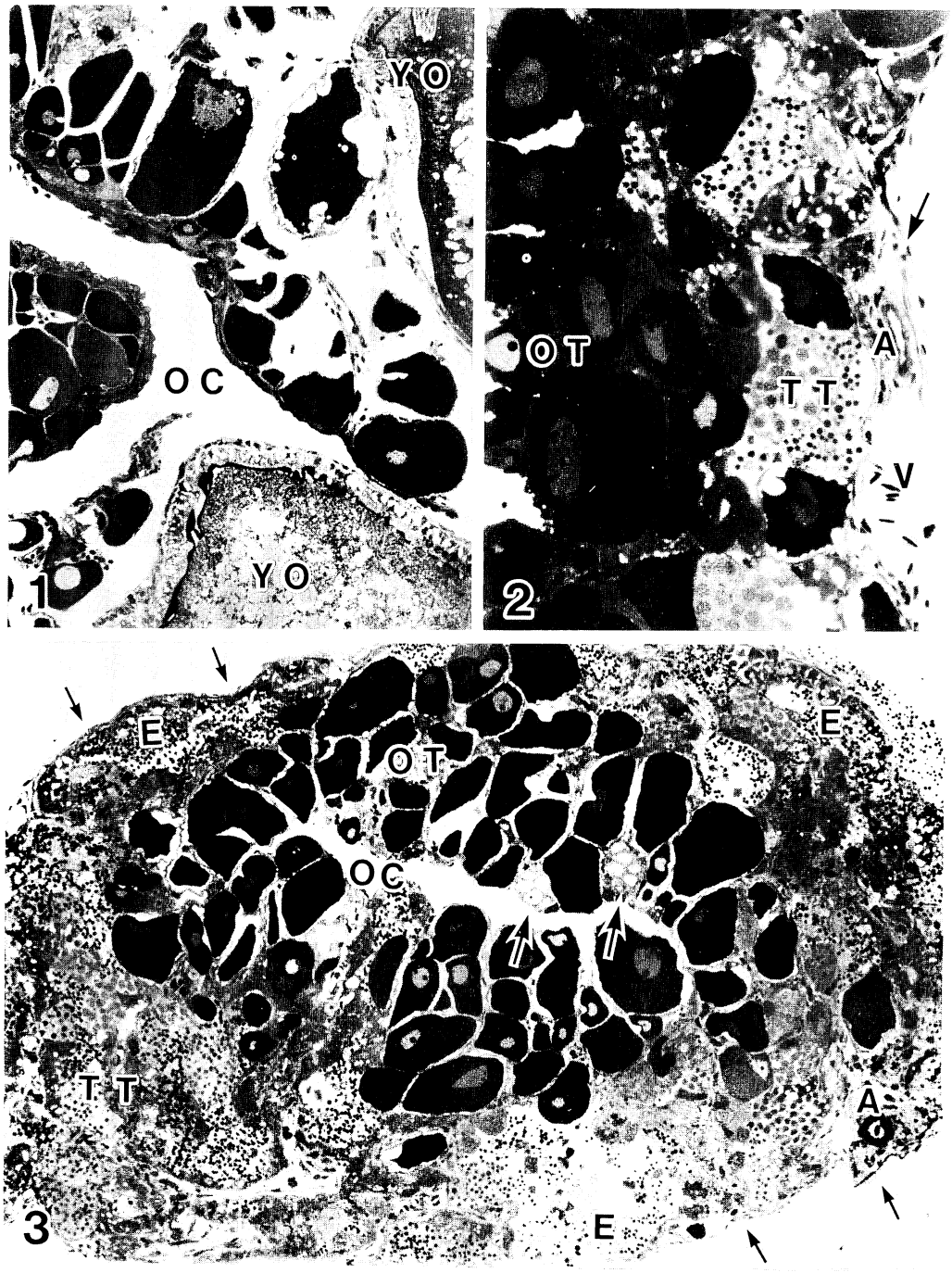
Abstract In order to clarify the basic questions with regard to sex change in the protandrous anemonefish *Amphiprion frenatus*, gonadal structure of females and males, distribution and activity of steroid producing cells and the ability of gonads to produce sex hormones were examined. Three fish out of 29 had ovaries with many previtellogenic and vitellogenic oocytes and an ovarian cavity, but they have not testicular tissue. Therefore they were identified as females and the rest were identified as males. They had hermaphroditic gonads with both testicular and ovarian tissues. Both tissues localized separately, though there was no boundary tissue between them. Ovarian tissue consisted mainly of many oocytes at the peri-nucleolus stage. However, most of these oocytes were degenerating. Individual fish varied greatly as to the level of spermatogenesis in testicular tissue. Some fish had a large amount of active spermatogenic tissue, while others had a small amount of inactive spermatogenic tissue. There is highly positive correlation in ultrastructural level between the activities of the steroid producing cells in the testicular tissue and in the ovarian tissue. There is also highly positive correlation between the activity of the steroid producing cells and spermatogenesis. In vitro incubation of gonads with salmon gonadotropin revealed that male gonads had higher levels of 11-ketotestosterone production than did female gonads, while female gonads had higher levels of testosterone and estradiol-17β than did male gonads.

Many fish have life histories that include sex change (Atz, 1964; Yogo, 1987). The anemonefish *Amphiprion* changes sex from male to female (Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Fricke, 1979; Ochi, 1989; Hattori, 1991; Hattori and Yanagisawa, 1991). It is highly possible that endogenous steroid hormones are responsible for the sex change (Nakazono, 1991). Data with regard to the role of endocrine system on the protogynous sex change in *Monopterus albus* has been accumulating (Yeung et al., 1993a, 1993b, 1993c). However, little information is available as to the role of endogenous steroid hormones in the protandrous sex change.

In the present study, basic issues regarding sex change, such as gonadal structures of males and females, distribution and activity of steroid producing cells (SPCs) in the gonads and the ability of gonads to produce sex hormones, are examined in the protandrous one-band anemonefish *A. frenatus*.

Materials and Methods

Twenty-nine individuals (53.3–86.7 mm in total length) of the anemonefish, *Amphiprion frenatus*, were purchased from a pet shop which stocks tropical marine fish on 20 and 27 July, 1991. After measurements of total length and body weight, blood samples were collected individually by cutting off the caudal parts. After anesthetized, gonads were fixed with Bouin's solution for observation by light microscope. They were embedded in paraffin, sectioned at 8 micrometers, and stained with hematoxylin and eosin. For electron microscopic observation gonads were fixed with Karnovsky's solution for 2 h at 4°C. After rinsing with 0.1 M cacodylate buffer (pH 7.4), they were postfixed with 1% OsO_4 in the same buffer for 2 h at 4°C. They were then immersed in saturated uranyl acetate for 2 h at 4°C. After dehydration they were embedded in epoxy resin. One-micrometer



sections for light microscopic examination were stained with 1% toluidine blue in 0.1 M phosphate buffer (pH 7.4). Ultrathin sections were stained with lead citrate and were observed using a Hitachi H-7000 electron-microscope.

In vitro production of gonadal steroids in response to gonadotropin was measured in gonadal fragments from fish in both male and female phases. Each

gonadal fragment weighing 1–2 mg (male gonad) and 3–7 mg (female gonad) was minced and then incubated in 1 m*l* of Leibovitz L-15 medium with 10 μg salmon gonadotropin (SGA; Syndel Lab., Canada) for 24 h at 25°C. Three replicates were collected from each individual. Following incubation all media were frozen at −20°C and the tissue weighed. The development of gonads used in the experiment was determined by histological examination. Estradiol-17β (E_2), testosterone (T) and 11-ketotestosterone (11-KT) levels in the incubation media and serum samples were measured by radioimmunoassay, according to the methods of Kagawa et al. (1982) (E_2, T) and Ueda et al. (1985) (11-KT). Cross-reactivities of the antibodies to major steroids had been reported by Hourigan et al. (1991).

Results

Histology

Three fish had ovaries with many previtellogenic and vitellogenic oocytes, and ovarian cavities. No testicular tissue was found in these ovaries (Fig. 1), and thus these individuals were identified as females. All of the remaining individuals were identified as males. They had hermaphroditic gonads with both testicular and ovarian tissues (Figs. 2 and 3). Each tissue type occupied its own locality, but there was no boundary tissue between them. There were some oocytes distributed in the testicular tissue. Five males had ovarian cavities in the central part of gonads (Fig. 3). Although others had not an ovarian cavity, proximal and distal ends of gonads elongated and curved on the side facing the lateral wall. In the gonad without an ovarian cavity, ovarian tissue was distributed on the side facing the lateral wall, and testicular tissue was distributed on the side the mesentery (Fig. 2). In the gonads with an ovarian cavity, ovarian tissue was localized on the periphery of an ovarian cavity, and testicular tissue enclosed outer periphery of ovarian tissue (Fig. 3). Ovarian tissue consisted mainly of many oocytes at the perinucleolus stage and a few cysts of oocytes at the chromatin nucleolus stage. In the gonads with an ovarian cavity, testicular tissue was localized on the periphery of gonad. Individual fish varied greatly in the activity of spermatogenesis in testicular tissue. Some individuals had a small amount of spermatogenic tissue and sperm (Fig. 2). Other fish had a large amount of spermatogenic tissue and sperm (Fig. 3). On the other hand, the amounts of ovarian tissue in each fish were also varied. However, the amount of ovarian tissue did not always have a negative correlation with the amount of testicular tissue and the activity of spermatogenesis. Some blood capillaries were localized in the tissue of the thin gonadal tunica. Some sperm ducts were distributed in testicular tissue (Fig. 3).

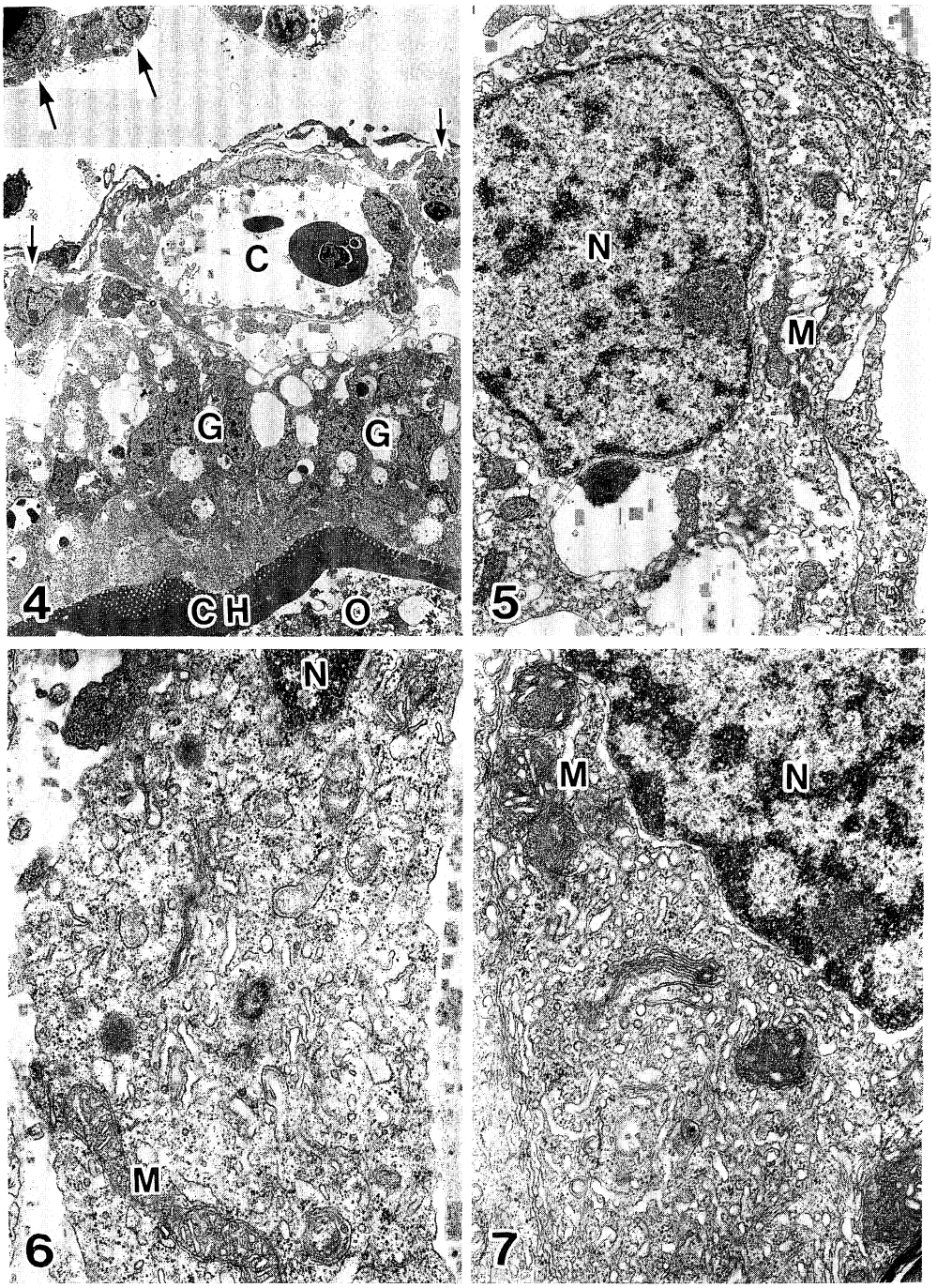
Ultrastructure

Ovary.—Follicle tissues enclosing yolky oocytes in the ovaries consist of inner granulosa cells and outer theca cells including steroid-producing cells (Fig. 4). Granulosa cells had in their cytoplasm a moderate number of round mitochondria with tubular cristae, developed rough endoplasmic reticulum, and some free ribosomes (Fig. 5). Steroid producing theca cells had a moderate number of round and oval mitochondria with tubular cristae, well-developed smooth endoplasmic reticulum, a few Golgi apparatus and free ribosomes (Fig. 6). Clusters of steroid producing cells were found in the interstices among oocytes (Fig. 4). Ultrastructural characteristics of these cells were identical with those of theca cells (Fig. 7).

Male gonad.—Distribution and activity of SPCs in the testicular and ovarian tissues in the male gonads were examined. SPCs were recognized not only in testicular tissue but also in the ovarian tissue. Clusters of SPCs in the ovarian tissue were distributed

Fig. 1. A cross section of an ovary of *Amphiprion frenatus.* Some yolky oocytes (*YO*) are seen together with young oocytes at the peri-nucleolus stage. *OC*—ovarian cavity. ×150.

Figs. 2 and 3. Cross sections of gonads of male *Amphiprion frenatus.* A small amount of inactive spermatogenic testicular tissue (*TT*) is seen on the tunica side of gonad (arrow in Fig. 2). A large amount of active spermatogenic testicular tissue (*TT*) is distributed along the outer periphery of gonad (small arrows in Fig. 3). Ovarian tissue (*OT*) consisting of young oocytes at the peri-nucleolus and chromachin nucleolus stages (large arrows) is present. Ovarian cavity (*OC*) is localized in the central part of gonad. *A*—artery; *E*—efferent duct; *V*—vein. Figure 2. ×370, Figure 3. ×200.



only in the interstices between oocytes (Fig. 8). There were no SPCs in the outer periphery theca layer enclosing the oocytes. Most of the oocytes at the peri-nucleolus stage had irregular cytoplasmic surfaces, allowing spaces to be formed between oocytes and granulosa cells (Fig. 8). These structures indicate degeneration of oocyte. The condition of granulosa cell cytoplasm was poor, and organella

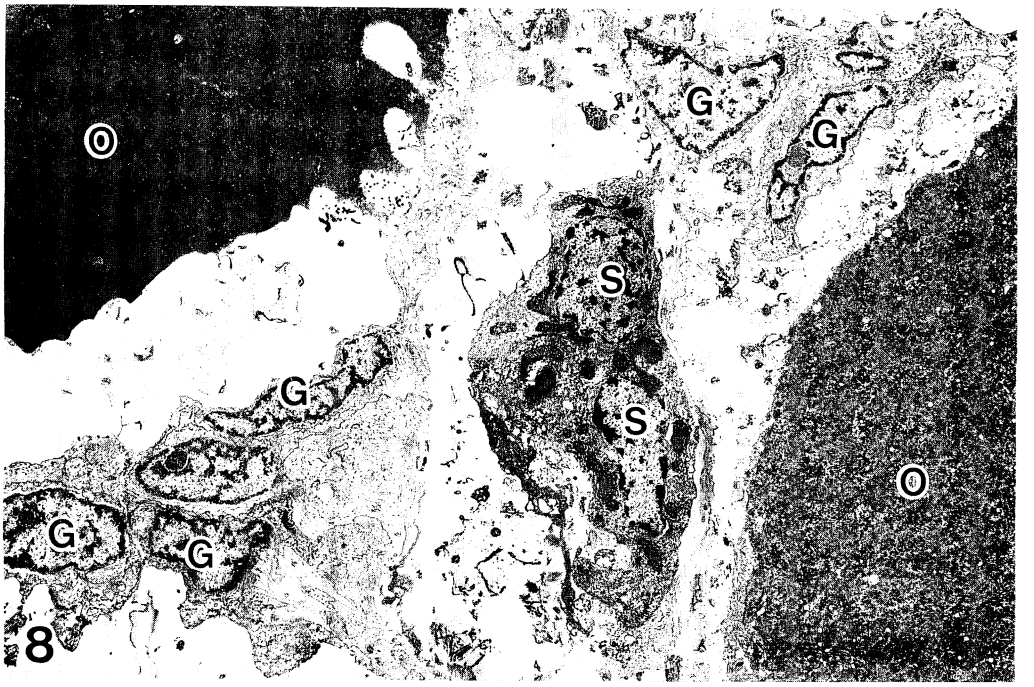


Fig. 8. Electron micrograph of steroid producing cells (*S*) in the ovarian tissue of male gonad. Steroid producing cells are distributed in the interstices among degenerating oocytes (*O*). Narrow space is seen between cytoplasm of oocytes (*O*) and granulosa cells (*G*). ×3,400.

such as mitochondria and endoplasmic reticulum were undeveloped (Fig. 8). SPCs in testicular tissue were localized in the interstices between lobules containing cysts of spermatogenic germ cells (Fig. 9). There was a high positive correlation between the apparent activity of SPCs in the testicular tissue and that of the SPCs in the ovarian tissue. In general, SPCs in the gonads containing active spermatogenic tissue had well-developed mitochondria and well-developed endoplasmic reticulum (Figs. 10 and 11), indicating active steroidogenesis. In contrast, SPCs in the gonads which had only a small amount of inactive spermatogenic tissue had undeveloped mitochondria with poorly developed cristae and undeveloped endoplasmic reticulum (Figs. 12 and 13), indicating inactive steroidogenesis.

In vitro steroidogenesis of gonadal fragments.—In vitro production of T, 11-KT and E_2 by gonadal fragments of fish, both female and male, are shown in Figure 14. Male gonads used in the experiment had large amounts of active spermatogenic tissue together with small amounts of undeveloped ovarian tissue. Ovaries had many developing yolky oocytes and many young oocytes. Ovarian fragments from females produced T (458 pg/mg tissue), 11-KT (51.5 pg/mg tissue) and E_2 (142.3 pg/mg tissue) when they were incubated with SGA. These values are significantly higher than those in the control (T, 120.5; 11-KT, 33.0; E_2, 102.0 pg/mg tissue). On the other hand, male gonadal fragments were also stimulated

Fig. 4. Electron micrograph of the follicle layer enclosing a yolky oocyte (*O*) and interstitial steroid producing cells (large arrows) in an ovary of *Amphiprion frenatus*. Follicle layer consists of inner granulosa cell (*G*) and outer steroid producing theca cells (small arrows). *C*—capillary; *CH*—chorion. ×1,200.

Fig. 5. Electron micrograph of a granulosa cell enclosing a yolky oocyte. The cell has some mitochondria (*M*) with tubular cristae and rough endoplasmic reticulum. *N*—nucleus. ×11,300.

Figs. 6 and 7. Electron micrograph of steroid producing cells in the theca layer (Fig. 6) and in the interstitium (Fig. 7) in an ovary of *Amphiprion frenatus*. Both portions have mitochondria (*M*) with tubular cristae and well-developed smooth endoplasmic reticulum. *N*—nucleus. ×2,070.