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Gonadal Development and Sex Inversion in Saddleback Anemonefish *Amphiprion polymnus* Linnaeus (1758)

Sukjai Rattanayuvakorn[1], Pisut Mungkornkarn[2], Amara Thongpan[3] and Kannika Chatchavalvanich[4*]

ABSTRACT

Gonadal development and sex inversion of saddleback anemonefish, *Amphiprion polymnus* were described. One-month juveniles had sexually undifferentiated gonads with primordial germ cells aggregated in groups, while two- to three-month juveniles displayed immature hermaphroditic gonads containing early developmental stages of both male and female germ cells, namely spermatogonia, primary spermatocytes, oogonia, and primary oocytes in chromatin-nucleolus stage. Spermatogenesis began at 4 months having testicular tissue comprising of spermatogenic cells in all developmental stages but ovarian cavity was first seen later at 5 months. The male region of ovotestis was peripheral, whereas the female region was more centrally located. There was no connective tissue between ovarian and testicular areas. Six-to 11-month fish had slightly larger ovotestes than those at previous age. Protandric sex inversion first occurred at 12 months. Sex change was characterized by degeneration of male germ cells, deposition of yellow-brown pigment and the formation of vitellogenic oocytes. Before spawning activity began, their gonads contained female germ cells in all stages with numerous vitellogenic oocytes, whereas functional males had both ovarian and testicular tissues. Most females of breeding pairs had mature oocytes in their gonads and began to spawn when their ages reached 14 months.

Key words: gonadal development, sex inversion, saddleback anemonefish

INTRODUCTION

Growth and development of juvenile fish to reach their mature stage are generally determined by the gonadal development. Male and female are distinctly different in their time required for sex differentiation and morphological changes. Most marine fishes are hermaphrodite, i.e., ambiguous in sexual organ identification especially during the juvenile stage (Warner, 1984; Garratt, 1986). Anemonefishes are known for socially controlled protandry with a monogamous mating system and live in colonies within clusters of sea anemones (Fricke and Fricke, 1979; Moyer and Nakazono, 1978; Ross, 1978a, b; Fricke, 1979, 1983). The gonad of juveniles, subadults and functional males are ovotestes but those of females are ovaries (Moyer and Nakazono, 1978; Fricke, 1979). Only the largest two individuals in each colony are reproductively active. The largest fish

1 Institute of Marine Science, Burapha University, Bangsaen, Chonburi 20131, Thailand.
2 Institute of Science, Rangsit University, Patumthani 12121, Thailand.
3 Department of General Science, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
4 Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fsciknc@ku.ac.th

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is a functional female and the second largest one is a functional male (Fricke and Fricke, 1977; Fricke, 1979). These functional female and male live together as a pair in which the female dominates the male. If the functional female disappears or is removed from a colony, the functional male changes sex protandrically and becomes a reproductive female (Shapiro, 1992). Hattori (1991) reported on this type of socially controlled growth and sex change in anemonefish *Amphiprion frenatus* in Okinawa, Japan.

Saddleback anemonefish *Amphiprion polymnus* Linnaeus (1758), is predominantly found in the Gulf of Thailand. It is one of the main species living on the coral reefs in this area. Unfortunately, their number is drastically decreased due to deterioration of natural habitat and the high commercial demand of them as ornamental fish. To replenish its population, attempts are made to raise the anemonefish in artificial conditions and mass production. The first approach to attain this goal was to understand its reproductive system, the stage of gonadal development and timing of their sex inversion. So far, there has been no record on *Amphiprion polymnus* germ cell morphology, gonadal development, sex inversion especially at histological level. This would render a better plan in controlling their population or inducing sex inversion at certain period and condition as well.

MATERIALS AND METHODS

Ten couples of sexually mature male and female saddleback anemonefishes, *Amphiprion polymnus* Linnaeus (1758) with the average 86 cm SL (standard length), 16.18 mg (SL range 55-113 cm, body weight range 8.5-28.2 mg) were collected from the Gulf of Thailand. They were raised in the laboratory at Burapha University, Chonburi Province. The rearing conditions were set to mimic the saddleback natural habit. They were kept in a tank of 30 × 60 × 40 cm in size. The aerated sea water in the tank was controlled at 25-28 °C, having 30-32 ppt salinity, 0.02 ppm ammonia, 0.01 ppm nitrite, 10 ppm nitrate. The pH varied between 6.5 and 7.5. Egg-laying materials comprised of bivalve shell, bark rock, coral and seaweeds. Broken ceramic plates were also put in the tank as lining for home surrounding, while sea anemones *Heteractis crispa* and *Stichodactyla haddon* were added to mimic the natural environment of this fish. They were fed with brine shrimp, finely chopped fish, shrimp, clams and dry algae flakes *ad libitum*. F1 offspring were reared in a tank of 75 × 150 × 80 cm. When their ages reached 5 months, groups of three fish each were collected and reared in separate container of 30 × 60 × 40 cm until F1 offspring at the age of 12-14 months laid eggs.

Sample preparation

Five to ten juveniles starting from the age of 1-month were monthly sampled_until they reached 14-month of age. They were anaesthetized with quinadine. Their gonads were excised and fixed. The gonads of specimen larger than 40 mm SL were removed and fixed in Bouin's solution for 24 h. The whole body without head and tail of smaller than 40 mm SL were fixed in Bouin's fluid**,** and decalcified for 3 days. They were processed using a standard paraffin method and serially sectioned at 6 ?m thick. Sections were stained with hematoxylin - eosin and examined under a light microscope.

RESULTS

Gonadal development of *Amphiprion polymnus*

Since saddleback anemonefish *Amphiprion polymnus* was a protandrous hermaphrodite, its gonad was found to contain both male and female germ cells at the early stages of development. Primordial germ cells were found when they were 1 month old. These cells were aggregated in a group of 8-15 cells enclosed with

thin membrane (Figure 1A, B). The primordial germ cells were spherical, about 6.1 μm in diameter, with large nucleus and scanty cytoplasm (Figure 1 B). Each aggregation was enclosed by thin connective tissue and attached to the abdominal cavity by the dorsal mesentery. Gonads at this stage of unidentified male or female were defined as indifferent gonads.

When the juveniles reached 2-3 months old, gonadal lamellae were found parallel along the abdominal cavity beside the small intestine (Figure 1C). The gonads contained both male and female germ cells, namely spermatogonia, oogonia, some primary spermatocytes and primary oocytes in chromatin-nucleolus stage (Figure 1D, E, F). These spermatogonia were spherical, about 6.4 μm in diameter, with round nuclei and clear cytoplasm. The spermatogonia were enclosed by

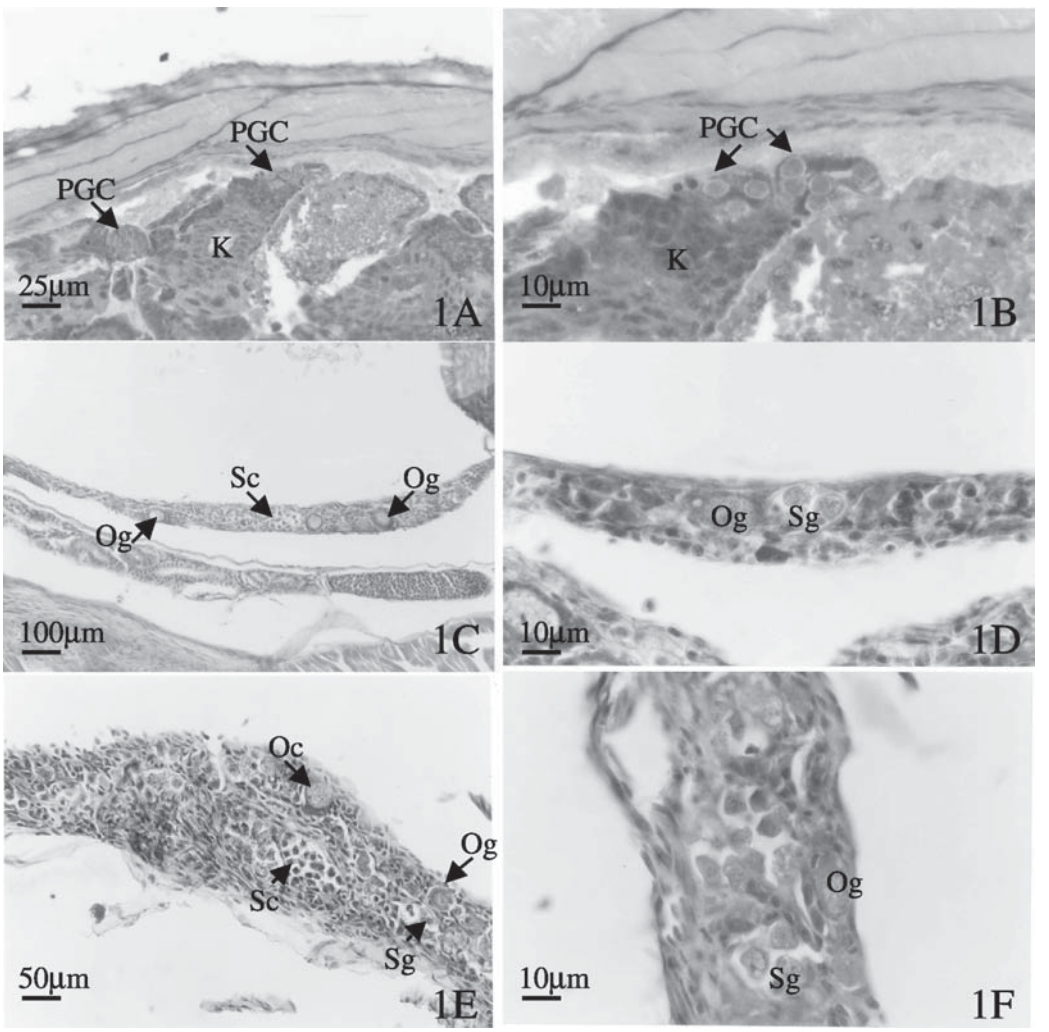

Figure 1 The gonadal development of *Amphiprion polymnus* showing (A) the location of primordial germ cells, (B) group of primordial germ cells developed in gonads of 1-month juvenile, (C) and (D) ribbon-like gonads of 2-month juvenile, (E) and (F) gonads of 3-month juvenile. K, kidney; Oc, oocyte; Og, oogonia; PGC, primordial germ cells; Sc, spermatocytes; Sg, spermatogonia.

their supporting cells. The primary spermatocytes were about 5.6 μm in diameter; their nuclei were at early stage of prophase I. These germ cells gradually multiplied in number by mitosis and formed spermatocyst among stromal tissue. The oogonia, however, were about 10.7 μm in diameter with round or oval nuclei having distinct nucleoli. Primary oocytes were distinguished from oogonia by their larger size and more darkly stained cytoplasm. The germ cells of both sexes were found intermingled in the same area.

Spermatogenesis began at 4 months (Figure 2A, B). Male area of a gonad contained spermatogenic cells in all stages, i.e. spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. The development of these germ cells in spermatocysts was synchronous. As for female area at 4 months, the gonad consisted of oogonia, oocytes in chromatin-nucleolus stage and perinucleolus stage but lack discernable ovarian cavity (Figure 2B). Follicle cells which enclosed perinucleolar oocyte

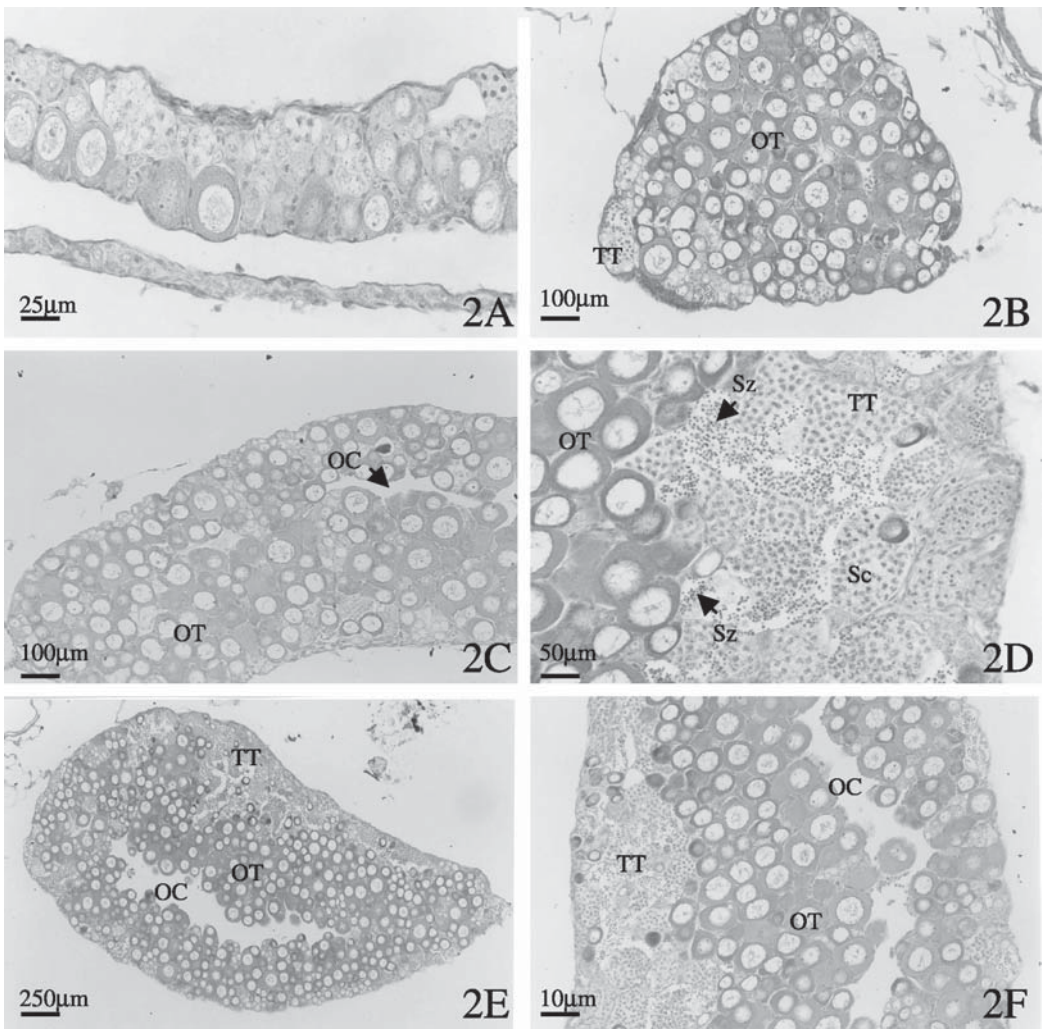

Figure 2 The gonadal development of *Amphiprion polymnus* showing (A) and (B) gonads of 4-month fish, (C) and (D) gonads of 5-month fish, (E) and (F) gonads of 6-month fish. OC, ovarian cavity; OT, ovarian tissue; Sc, spermatocytes, Sz, spermatozoa; TT, testicular tissue.

were clearly seen.

The presence of an ovarian cavity was first seen at 5 months (Figure 2 C). At this same period of time, the testicular tissue of ovotestis was peripherally located, while the ovarian tissue located more centrally (Figure 2D). There was no connective tissue between ovarian and testicular regions. Germ cell types of both sexes were the same as those found at the previous stage (4-months).

Ovotestes of 6-to 11-month fish were similar to those of 5-month but gonadal size was larger than those at previous stages. Testicular tissues and the number of male germ cells had remarkably increased. Ovarian tissues were composed of the same cell types as those found at previous stages but perinucleolar oocytes were increased in number in older fish. Vitellogenic oocytes were still not found.

Sex inversion

Male saddleback anemonefish began to have sex change when their ages were between 12-to14-months. The sex change was characterized by the diminishing of male germ cells whereas the female germ cells were increased in number. The degeneration of male germ cells was also observed (Figure 3A, B, and C). Numerous pyknotic nuclei were observed in deteriorate spermatocyst (Figure 3 D). Late stage of degeneration resulted in the appearance of yellow-brown pigment which usually located at the periphery of gonads (Figure 3D). At the same time, vitellogenic oocytes that contained numerous yolk granules were developed in their gonads (Figure 3E, F). However, advanced stage of vitellogenic oocytes was not found. At this age, some fish still had gonad containing numerous male germ cells in all stages and also female germ cells but oogenic activity did not proceed beyond perinucleolus stage. However, few yellow-brown pigments were found. When the fish reached 14 months, most females of breeding pairs began to have spawning activity. The third and usually the smallest fish of each colony, aged 5-to-14-months remained to be juvenile as long as breeding pairs still persisted.

DISCUSSION

Gonadal development of *Amphiprion polymnus* was found closely correlated with age. This differed from that of European eel which related more to body size (Colombo *et al*., 1984; Colombo and Grandi, 1996). The appearance of sexually differentiated germ cells, marking the steps of gonadal differentiation, corresponded to 2-3 months of ages. The first sexually differentiated germ cells of anemonefish were hermaphrodite, having clones of both oogonia and spermatogonia, while those of the European eel were female only (Grandi and Colombo, 1997). Thus, in *Amphiprion polymnus*, gonadal development goes through a hermaphroditic phase before proceeding in two different ways. First, the development of ovotestis proceeds with spermatogenesis of the testicular zone, and the fish function as males. Second, some hermaphrodites change sex at the age of 12-14 months and function as females.

In *A. polymnus*, the male zone of ovotestis was peripheral, while the female part was more centrally located but no connective tissue was found between ovarian and testicular regions. These were the same as those of *A. frenatus* and other anemonefish (Brusle-Sicard and Reinboth, 1990), but were contrast to most protandric teleosts, in which the ovarian and testicular zones are distinctly separated by a well-developed connective tissue (Reinboth, 1962, 1970; Pollock, 1985; Micale and Perdichizzi, 1994). Moreover, a typical sparid gonad was found to consist of a dorsal ovarian zone and a ventral testicular zone (Pollock, 1985; Micale and Perdichizzi, 1994).

Gonads of 1-month juvenile of *A. polymnus* were indif ferent since they consisted of primordial germ cells, having spermatogonia and

oogonia at undifferentiated stages. Gonads of 2-3-month fish were immature hermaphrodite consisting of early stages of both male and female germ cells. This observation revealed that sex differentiation began at 2-3 months. Moreover, the development of male germ cells in a cyst was synchronous as those of *Amphiprion frenatus* (Brusle-Sicard and Reinboth, 1990).

Gonads of 4-month fish had spermatogenesis but had no oogenic activity. This observation indicated that these hermaphroditic gonads stages were actually functional male. Oogenic activity of ovotestis does not proceed beyond the perinucleolus stage, as also seen in the protandric *Amphiprion frenatus* (Brusle-Sicard and Reinboth, 1990). Protandric sex inversion began in 14-month-old fish. However, if the rearing condition was suitable, the hermaphroditic gonad

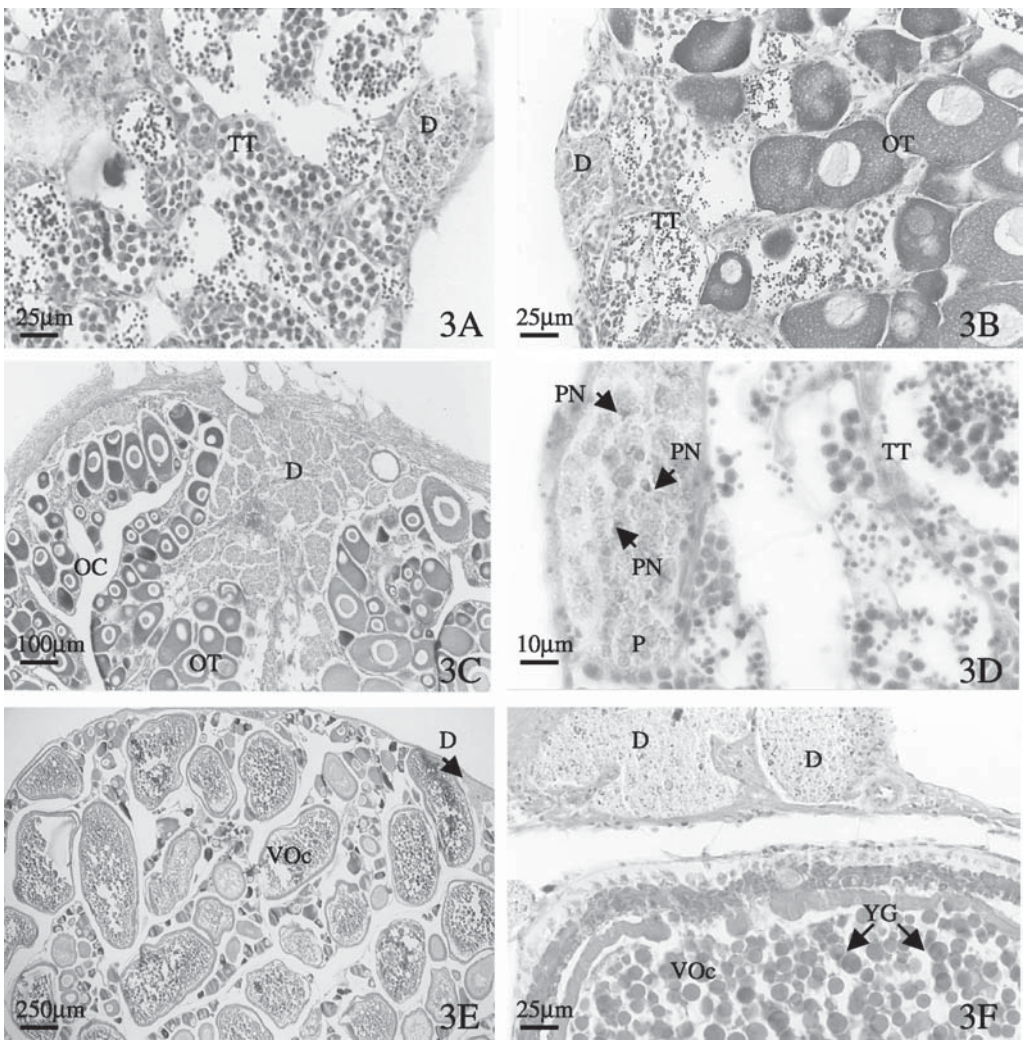

Figure 3 Protandric sex change of *Amphiprion polymnus* showing (A) degeneration of testicular tissue, (B) and (C) increasing of ovarian tissue, (D) pyknotic nuclei and yellow-brown pigment, (E) developing vitellogenic oocytes; (F), vitellogenic oocytes. D, degeneration of testicular tissue; Oc, ovarian cavity; OT, ovarian tissue; P, pigment; PN, pyknotic nuclei; TT, testicular tissue; VOc, vitellogenic oocytes; YG, yolk granules**.**

could change to female at 6-12 months depending on several factors, i.e., the completion of rearing condition or the removal of functional female from a colony (unpublished data) which was similar to other anemonefishes (Fricke, 1979, 1983; Ochi and Yanagisawa, 1987; Ochi, 1989; Hattori and Yanagisawa, 1991; Brusle-Sicard *et al*., 1994). Environmental factors are claimed to play a major role in sex differentiation. High population densities and high temperature are also known to favor male differentiation (D' Ancona, 1957). Temperature and steroid hormone may act on gonadal differentiation in fishes by inducing or inhibiting the production of H-Y antigen early in development, as also seen in chickens and sea turtles (D'Ancona, 1959).

The observation of sex change in *A. polymnus* as characterized by degeneration of male germ cells, deposition of yellowish pigment and the formation of advanced vitellogenic stage oocytes was similar to those occurred in *Diplodus sargus* (Micale and Perdichizzi, 1994). These yellowish pigments were usually found at the periphery of gonads which was the location of the male germ cells, in which the male germ cells had been replaced by masses of yellowish pigment. However, gonads of some fish even at the age over 12 months may still contain numerous male germ cells in all stages. This indicated that these fish might be functional male throughout their lives, but they also could be changed to female if the condition favored the inversion.

CONCLUSION

Histological evidents indicated that the hermaphroditic gonad of *Amphiprion polymnus* under rearing condition had spermatogenic activity for the first time at the age of 4 months and had protandric sex inversion at the age of 12-14 months. Sex inversion in saddleback anemonefish was essential to reproduction. Gonadal development began with hermaphrodite and proceeded to functional male and functional female, respectively. Functional female developed from protandric sex inversion only. Sex inversion, however, depended on external factors and conditions which could be induced at proper stage of development in rearing condition. Without sex inversion, *Amphiprion polymnus* would remain hermaphrodite and functional male all their lives which obstructed their full productive cycle and lower the population in natural environment as well.

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