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Sex change of hatchery produced *Amphiprion ocellaris*: Influence of mating system removal on gonad maturation and nesting success

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Abstract

The influence of gonadal maturation and nesting success of the anemone fish *Amphiprion ocellaris* was analyzed through mating system removal. Four experiments viz., (i) Sex change of active male to female in the absence of active female, (ii) Juveniles in the presence of a functional male that was changing sex from male to female, (iii) Juvenile in the presence of a functional female, and (iv) Same length group juvenile fishes (total length: 50 mm) without the presence of adult fishes, were carried out. The gonad of male in experiment (i) showed first perceptible sign of sex inversion after 1 to 2 weeks. After 25 weeks the testicular zone almost disappeared and the gonad looked like maturing ovary with perivitellogenic oocytes and oocytes at various stages of development. The gonad of juveniles in the second experiment showed increased testicular development after 23 to 24 weeks, and testicular area became clearly discernible after 30 weeks. In the third experiment, the gonad of juvenile completed its spermatogenesis after 15 to 16 weeks and showed well-developed testicular zones. In the fourth experiment, the gonad of largest fish showed development of ovarian part after 24 weeks with oogonia and oocytes at various stages, and degeneration of testicular tissues. The observation of nesting success in the above experimental groups showed that spawning was obtained in the third group after 4 months of association; and after 12 to 18 months in the first and second groups, and after 20 to 24 months in the fourth group. The present study shows that in the absence or disappearance of adult female, the active male changes sex to female within a period of 25 weeks. The study also confirms that in the clownfish *A. ocellaris*, the largest and socially dominant fish in a host sea anemone (*Heteractis magnifica*) is generally female, whose gonads are functional ovaries with remnants of degenerated testicular tissues. The second largest fish in the same group is an active male and has gonad that are functioning testis but also possess non-functioning or latent ovarian cells (ovotestis). If the dominant female dies or is experimentally removed from the "queue", the male not only changes sex but also grows at an accelerated rate, and the juveniles also grow faster to become male and fill the size gap of the social group. This adaptation allows continuous reproduction. It is found that social structure plays an important role on the sex changing mechanism.

Keywords: *Amphiprion,* gonad maturation, sex change

Introduction

The mating and social structure of clownfishes are strongly influenced by the distribution pattern of sea anemones as the clownfish depends upon the host anemones in the wild (Moyer and Sawyers, 1973; Allen, 1975; Thresher, 1984; Ochi and Yanagisawa, 1987; Ochi, 1989a, b). The sex reversal is also another peculiar behaviour in anemone fish community, and protandric sex inversion was reported for the first time in *A. bicinctus* and *A. akallopisos* (Fricke and Fricke, 1977). Later, the sex changing mechanisms in *A. melanopus* (Ross, 1978), *A. frenatus, A. clarkii, A. polymnus, A. perideraion* and *A. sandracinos* (Moyer and Nakazono, 1978), *A. clarkii* (Ochi, 1989a; Hattori and Yanagisawa, 1991a), *A. melanopus* (Godwin, 1991), *A. frenatus* (Brusle-Sicard and Reinboth, 1990; Brusle-Sicard *et al.,*1991, 1994; Nakamura *et al*., 1994) and *A.*

percula (Madhu and Madhu, 2005) were reported. Though many fish undergo sex change (Yogo, 1987), there has been little previous work on histological observations on the influence of social structure on protandric sex inversion in clownfishes. The histological details from immature stage to ripe male and female phase of *A. clarkii* (Hattori and Yanagisawa, 1991b), and the existence of social hierarchy in clownfishes (Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Ross, 1978; Ochi and Yanagisawa, 1987; Ochi, 1989a; Hattori and Yanagisawa, 1991a) have been reported. The aim of the present study is to understand the influence of induced social conditions on sex inversion and the gonadal development of juvenile and adults of *Amphiprion ocellaris* under laboratory condition.

Material and Methods

Social groups containing pairs of active fishes (male and female) and two to three juveniles of *A. ocellaris* were collected from the wild without breaking their natural pair bonds. Each social group was stocked and acclimatized separately in 500 l perspex tank along with host sea anemone (*H. magnifica*) prior to the experiment. Water was recirculated with the help of a biological filter. To study the influence of social structure on sex change, four types of experiments (nine replicates) were carried out. In all the rearing tanks (500 l perspex tank), the bottom was covered with coral sand and provided earthen pots for deposition of eggs. The fish were fed with minced shrimp and fish egg mass at the rate of 15% of body weight. Every two weeks, the length and weight of fishes were noted and the presence of mature eggs or sperm was determined by gently pressing the abdomen. During the experimental period, the water temperature ranged from 27 to 29° C. In experiment (i) the female of the pair was removed and the adult male was allowed to remain with a juvenile. The gonads of male were histologically examined during different periods of association. In experiment (ii) juvenile was reared in the presence of a functional male that was changing sex in the absence of adult female and the gonadal conditions of juveniles were histologically examined. In experiment (iii) juvenile fish were allowed to remain with a functional female in the absence of adult male, and the gonadal development of this juvenile fish during different time of association was analysed. In experiment (iv) the hatchery produced juveniles of similar size [total length (TL) = 50 mm] were kept together in an aquarium for 25 weeks without the presence of adults. The testis and ovary of fishes in each experimental group at different duration of association were dissected out from freshly sacrificed specimen and fixed in 10% neutral buffered formalin for histological analysis. After 24 hours of fixation, they were washed overnight under running tap water and stored in 70% ethyl alcohol until further processing. The stored tissues were later dehydrated following the standard procedure in different grades of alcohol series. The tissue were then cleared in chloroform and isopropyl alcohol and impregnated in boiling paraffin and embedded in paraffin wax (melting point 58-60° C). The paraffin wax blocks were catalogued and stored in labelled polyethylene bags. Longitudinal and transverse sections were cut at 3-5 mm thickness using Fuji Optec rotory microtome. Mayors egg albumin (Gray, 1973) was used as the adhesive for fixing the paraffin ribbon with section on the clean dry slides. The sections were deparaffinised, dehydrated and stained with Harris hematoxylin stain and counter stained with eosin (Preece, 1972; Clark, 1981; Venkataramanujam and Ramanathan, 1994). The stained slides were observed under a trinocular microscope (Carl Zeiss) and photographed to record the histological changes in the ovary and testis.

Results

(i) Protandric sex inversion of active male to female in the absence of active female: The histological observation of the gonads of adult male showed spermatids, spermatocyte cysts, etc. (Fig. 1a). The gonad of male on removal of adult female after 1 to 2 weeks revealed the first perceptible sign of sex inversion with only a few numbers of spermatocytes which indicated the decrease of spermatogenesis. After 8 weeks, the number of spermatocytes in the gonad further reduced than in the previous weeks. Degeneration of male germ cells and presence of more number of darkly stained brown bodies, oogonia and oocytes in perinucleolar stage after 12 weeks indicates that male activity was

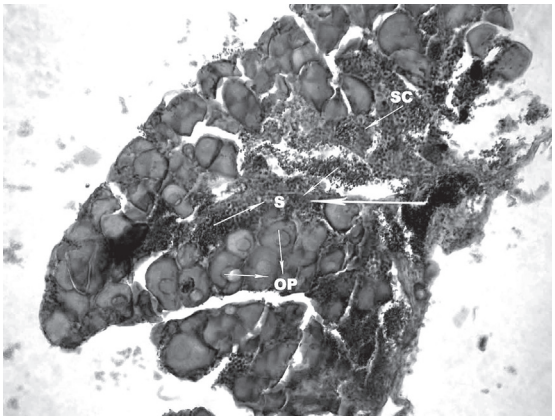

Fig. 1a. Initial stage of active male (Ovotestis). Spermatids (S), Oocytes in perinucleolar stage (OP), Spermatocyte cyst (SC)

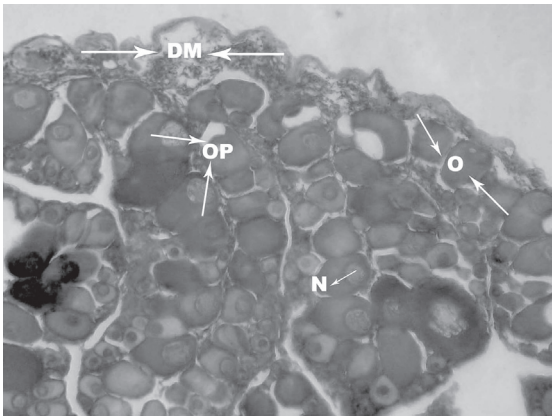

Fig. 1b. Gonadal changes of active male after 12 weeks association with juveniles in the absence of adult female. Degenerating male germ cells (DM), Oogonia (O), Nucleus (N), Oocytes in perinucleolar stage (OP)

about to stop (Fig.1b). After 16 to 17 weeks, the number of small oocytes in the gonad increased. Observation of specimens after 25 weeks showed that the testicular zone had almost disappeared and only a few residual degenerating spermatids and spermatozoa were discernible. At this stage the gonad looked like maturing ovary with perivitellogenic oocytes, a few oogonia, less number of connective tissue and oocytes at various stages of development (Fig. 1c). In this experiment, the nesting success (spawning) occurred 12 to 18 months after association.

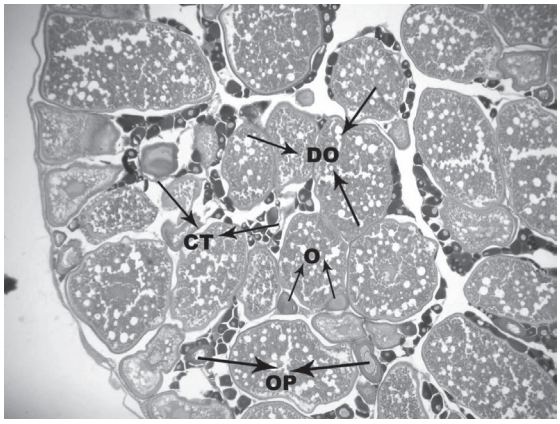

Fig. 1c. Gonadal changes of active male after 25 weeks of association with juveniles in the absence of adult female. Developing oocytes (DO), Oogonia (O), Oocytes in perinucleolar stage (OP), Connective tissue (CT).

***(ii) Juveniles in the presence of a functional male that was changing sex from male to female in the absence of adult female*:** The gonad of juvenile fishes, after removal of adult female, looked like an immature ovotestis with some oogonia, numerous oocytes in perinucleolar stage in female part, and very few spermatocyte cysts and spermatids in the small male area during 19 to 20 weeks in the presence of a functional male (Fig. 2a). After 23 to 24 weeks, the gonads showed increased testicular development. After 30 weeks as sex inversion of the male was

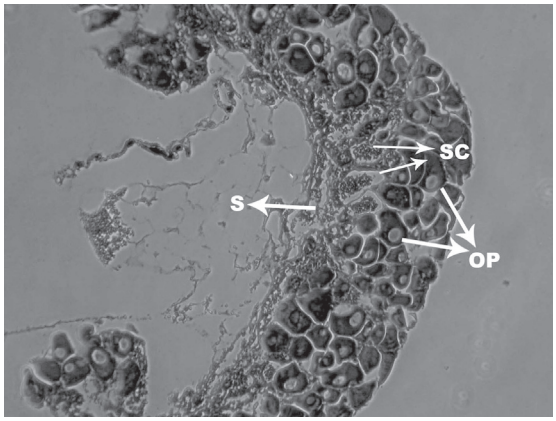

Fig. 2a. Gonadal development of juvenile after 19 to 20weeks in presence of active adult fish that was changing from male to female showing Ovotestis. Oocytes in perinucleolar stage (OP), Spermatocyte cysts (SC), Spermatids (S).

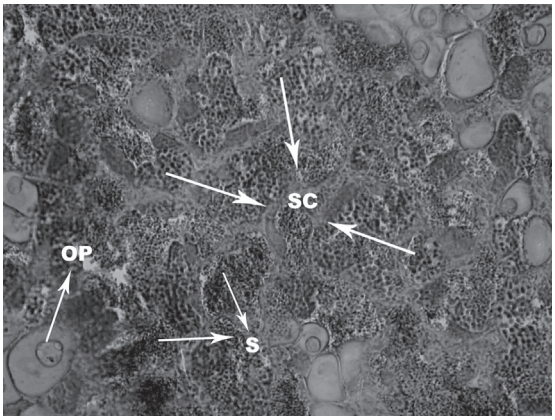

Fig. 2b. Gonadal development of juvenile after 30 weeks in presence of active adult male fish that was changing from male to female; Spermatocyte cysts (SC), Spermatids(S), few oocytes in perinucleolar stage (OP)

nearing completion, the testicular area of juvenile fish became clearly discernible. The presence of numerous spermatocytes gathered in seminiferous cysts and spermatids revealed spermatogenic activity (Fig. 2b). The study revealed that gonadal development of juvenile is dependent on the sex of the associated adult. In this experiment, the juvenile tend to become male showing an active spermatogenesis. In this case, spawning was noticed 12 to 18 months after pairing.

(iii) Juvenile in the presence of a functional female and in the absence of adult male: In this group, the functional female was allowed to remain in the aquarium with one juvenile without the presence of a functional male. Gonads of juvenile after 11 to 12 weeks had a testicular area, which was clearly perceptible indicative of the beginning of spermatogenesis. After 15 to 16 weeks, the juvenile had completed spermatogenesis with well-developed testicular zones for future male function (Fig. 3). In this experiment, spawning occurred 16 weeks after pairing. This experiment showed that in the presence of an active female, a juvenile tends to function as an adult male within 4 months in the absence of an active male.

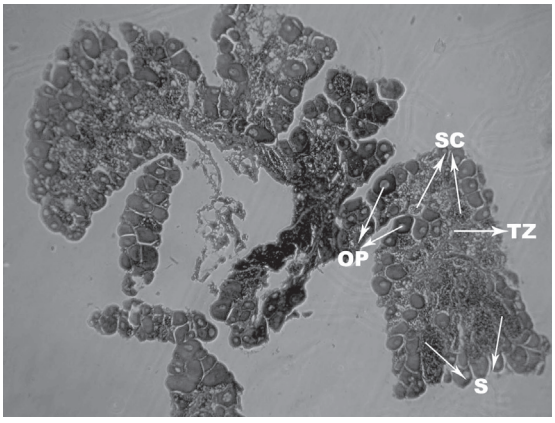

Fig. 3. Gonadal development of juvenile after 12 weeks in presence of active adult female. Testicular zone (TZ), Spermatocyte cysts(SC), Spermatids(S)

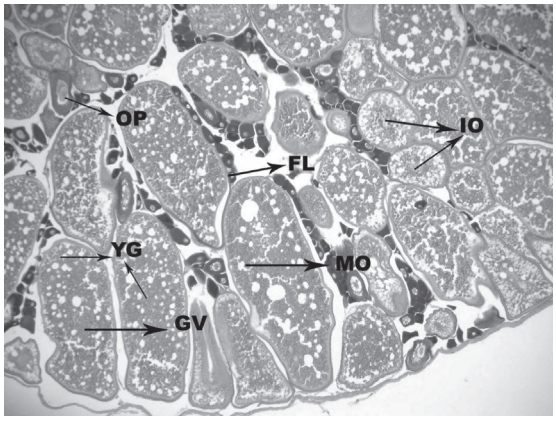

Fig. 4. Gonadal development of largest juvenile after 24 weeks without male phase in absence of adult fishes. Oocytes in perinucleolar stage (OP) Immature oocytes (IO), Germinal vesicle (GV), Yolk granules (YG), Mature oocytes (MO), Follicular cell layer (FL)

(iv) Juvenile fishes without the presence of adult fishes: Hatchery produced juveniles of same age group from different breeding pairs were reared together. After 24 weeks, the testicular tissues degenerated in the gonad of largest fish (TL = 66 mm). Darkly stained brown bodies were noticed in the ovarian zone (Fig. 4). In the ovarian part, oogonia and oocytes at various stages were observed. In the small testicular zone of the second largest fish (TL = 62 mm), the male germ cells were intact and active spermatogenesis was noticed. They were similar to those recognized in the ovotestis of juveniles associated with a female (Fig. 5). The third largest fish (TL = 58 mm) had only a very weak spermatogenic activity and looked like an immature gonad of a juvenile (Fig. 6). In this

experiment, the nesting success was obtained after 20 to 24 months of rearing.

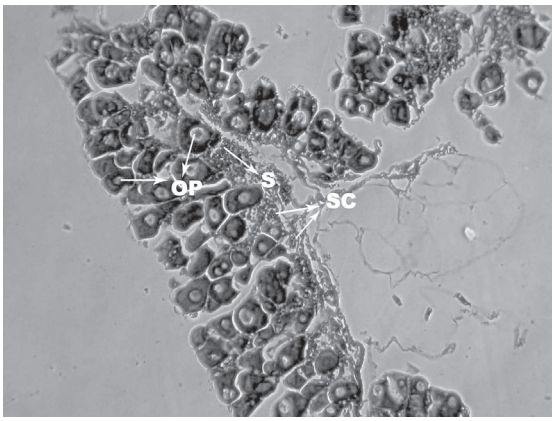

Fig. 5. Gonadal development of second largest juvenile after 16 weeks without presence of adult fishes. Oocytes in perinucleolar stage (OP), Spermatocyte cysts (SC), Spermatids (S)

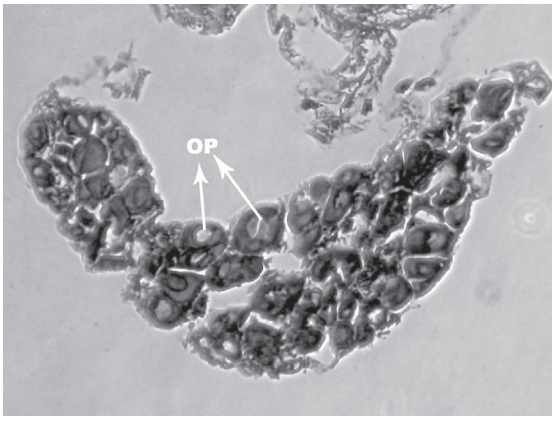

Fig. 6. Gonadal development of third largest juvenile after 24 weeks without presence of adult fishes (immature ovotestis). Oocytes in perinucleolar stage (OP)

Discussion

Histological features of male sex inversion initiated by female removal were characterized by a decrease in spermatogenic activity followed by a degeneration of both male germ cells and their associated cells leading to a disorganization of seminiferous lobules. As male germ cells were degenerating, an oogenic activity was indicated by the presence of numerous oocytes in perinucleolus stage as reported in *A. frenatus* (Brusle-Sicard and Reinboth, 1990). In *Rhabdosargus sarba* (Sparidae), the oogenic activity was found in the enlarging ovarian zone while testis was still functional, followed by a testicular degeneration and the formation of an ovary, in which the heterosexual gonad shows a peculiar pattern that male (ventral) and female (dorsal) areas are distinctly separated by connective tissue (Yeung and Chan, 1987). The gonad of *A. ocellaris* is also an ovotestis, but connective tissue is lacking and the heterosexual germinal elements are in direct contact with each other. In *A. melanopus,* Godwin (1991) noted a differentiation into mature spermatids before their replacement by oocytes, while Hattori and Yanagisawa (1991b) observed that in *A. clarkii,* the spermatocyte cysts are lacking in the transitional gonad. In *A. ocellaris,* the sex change (male to female) was completed within 25 weeks. Residual spermatids and spermatozoa were sparsely observed in the gonad resembling closely the condition of an immature ovary whereas sex inversion was achieved within 30 days in *A. melanopus* (Godwin, 1991).

In the wild population, sex change does not always occur in males of clownfish, which had lost their mating partner as they have an opportunity to get adult female from nearby social groups (Ochi and Yanagisawa, 1987; Ochi, 1989b; Hattori and Yanagisawa, I991a). The gonads of juveniles and males consist of ovarian tissue with only immature oocytes and testicular tissue, whereas functional female had only ovarian tissues in *A. ocellaris,* and in other clownfishes as well (Fricke and Fricke, 1977; Moyer and Nakazono, 1978). In *A. clarkii,* Ochi (1989a) reported that sex change to female is not the best way for an unmated male to increase his future reproductive success because of the loss of time spent on sex change. In the present study, the reproductive success of juveniles of *A. ocellaris* was fast on removal of adult fishes associated with them suggesting that gonadal development of juveniles clearly depends on the sex of the associated adults. On separation of adult female from its male partner, the male starts sex inversion and the juvenile fish remains indifferent until sex inversion of the male is nearly complete. On completion of sex change of male, the associated juvenile showed numerous meiotic spermatocytes in its testicular

zone (after 24 weeks) whereas, when the adult is a female, the juvenile fish associated with it showed a high spermatogenic activity in the testicular zone after 11 to 12 weeks and its testicular part included all steps of spermatogenesis within 15 to 16 weeks. It may thus be concluded that the association of a juvenile with a single male or female always leads to male differentiation in the gonad of the juvenile, but the time required is quite different. From these investigations on the effect of social relations, it appears that as long as the male has begun changing sex, it prevents testicular development in the juvenile, while the female promotes it. Time spent for the onset of spermatogenic activity is comparatively longer (23 to 24 weeks) in the juveniles which are associated with a functional male that was changing sex from male to female. It is suggested that slow development of testis includes only spermatogonia and their mitotic activity. However the cytological events are faster (15-16 weeks) in the juvenile associated with the functional female and in the absence of adult male. This may also be due to the aggressiveness of the female and monogamous pairing as reported in other clownfishes (Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Ross, 1978; Thresher, 1984; Godwin and Thomas, 1993).

In each aquarium, three juveniles were reared together for 6 months for histological analysis, and at the end, the largest fish exhibited abnormal spermatogenic activity. The presence of several nests of meiotic oocytes and size variation in the ovarian zone suggests the onset of oogenic activity. In the second largest specimen, no alteration of male germ cells was detected and active spermatogenesis was identified. The ovotestis of the third ranking fish was similar to that of all juveniles. The largest anemonefish takes a female orientation skipping the male stage, while the second ranking fish advances towards male differentiation and the third ranking fish remains in a juvenile position, where a monogamous pair is absent. Thus a hierarchy is established within the social group. As shown by Brusle-Sicard *et al.* (1991) and Stahlschmidt-Allner and Reinboth (1991), gonad of juvenile *A. ocellaris* does not necessarily need to pass through a male phase prior to their differentiation as ovary. Therefore, protandry in *Amphiprion* could be facultative. The present study shows that under induced social conditions, the cellular requirements during changes of sexual status, i.e. male sex inversion and juveniles advancing to a male differentiation (following female removal) are mainly attributable to oogonia and spermatogonia. On the other hand, under induced social conditions, i.e. abnormal associations (female; juvenile; juveniles alone without functional adults), the anticipated sex differentiations require not only oogonia but also spermatogonia. Thus, oogonia and spermatogonia appear to be involved in the usual germinal renewal. Pair bonding in most species of clownfishes is very strong and is correlated with the small size of their territories (centered on actinians) which is in turn correlated with the usual social hierarchy that exists in each social group. The present study shows that when a female is experimentally removed from an existing pair, the remaining adult male changes sex and a nonbreeder becomes the male, and due to the social hierarchy, the juveniles (non-breeders) are dominated by both female and male, as also reported in other clownfishes (Allen, 1975; Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Ross, 1978; Ochi and Yanagisawa, 1987*;* Ochi, 1989b; Hattori and Yanagisawa, 1991a). In the absence of adults, the first ranking fish in the non-breeding juveniles of *A. ocellaris* became female without passing through a functional male state. These may be either gonochoristic females or prematurational sex changers as found in *A. clarkii* (Ochi and Yanagisawa, 1987; Ochi, 1989a). However, evidence of genetic control of sex determination in hermaphroditic fishes is poor (Warner, 1978; Shapiro, 1989). The process of sex differentiation in primary males may be conditional in the same way as in *A. ocellaris*. It seems that alternative lifehistory pathways so far found in sequentially hermaphroditic fishes have resulted from flexible sex differentiation, which may be triggered by environmental and social conditions.

The present study concludes that in the absence or disappearance or experimental removal of a partner (adult female) from the queue, the gonad of an active male ceases to function as testis and the egg producing cells become active, and male not only changes sex within a period of 25 weeks, but

also grows at an accelerated rate. Simultaneously, the largest of the non-breeding individual becomes the functional male and this adaptation allows continuous reproduction. The study also confirms that the sex reversal in clownfish *A. ocellaris* is from male to female (protandrous hermaphroditism) in which the largest fish in a social group is female, whose gonads are functional ovaries with remnants of degenerated testicular tissues. The second largest fish in the same social group is an active male and had gonads that not only function as testis but also possess non-functioning or latent ovarian cells. Thus a protandrous (male first) sequential hermaphroditism has been established in *A. ocellaris* and that social structure plays an important role on its sex changing mechanism.

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