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Gonadal restructuring during sex transformation in the protogynous greasy grouper *Epinephelus tauvina* (Forsskal) (Perciformes: Serranidae)

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ABSTRACT

This paper elucidates the developmental changes in the structure of the gonad during sex reversal in the greasy grouper *Epinephelus tauvina* (Forsskal). Sex transformation was induced artificially by administering the male hormone 17 α-methyl testosterone to mature female fishes by adding the hormone to the feeding regime. The hormone treated fishes became mature males after the administration of a mean accumulated dose of 50-55 mg kg^{-1} body weight of fish, after 9-12 weeks. Sex inversion was induced in both smaller and the larger fish, with no clear relationship with fish size and age. The study was carried out using histological techniques.

Keywords: Gonad restructuring, Greasy grouper, Hormone administration, 17 α-methyl testosterone, Protogynous, Sex transformation

Introduction

Reproductive patterns among members of the family Serranidae are highly varied and include several different sexual patterns like gonochorism, simultaneous and sequential hermaphroditism. Among groupers (sub family Epinephelinae), protogyny is reported to be the most common mode. According to Sadovy and Collin (1995), a better understanding of grouper reproductive biology would greatly facilitate in evaluating the sustainability of the population as well as stock management. Information on annual fecundity, sexual maturity, number of spawns as well as age and size at maturity are useful in management (Collins *et al*., 1996). *Epinephelus tauvina,* the most common inshore species of grouper in the Indian Ocean especially the western Indian Ocean including the Red Sea and the Gulf region and the coast of India, is abundant in the vast stretches of coral reefs and rocky areas, estuaries, mangrove swamps, sandy and muddy bottoms upto depths of 150 m (Heemstra and Randall, 1986).

E. tauvina a protogynous hermaphrodite which does not exhibit any externally distinguishable sexual characters. In protogyny, males may develop directly from the larval/ juvenile stage or may develop from adult females by sex reversal. Being protogynous hermaphrodite, grouper gonad development undergoes sex transition from ovary to intersexual and then to testis; and primordial germ cells and different stages of gametic cells during oogenesis and spermatogenesis are synchronously observed in the transitional gonad (nonfunctional ovotestis).

Large scale development of the grouper culture industry has been hindered by the lack of seed for stocking, which is due to the lack of a standardised method of controlled sex change and also due to the unavailability of mature male broodstock (Yeh *et al*., 2003). Long term husbandry and maintenance of broodstock are time consuming and tedious, and consequently, the male broodstock for propagation is generally obtained by means of induced sex change at a relatively early age. Therefore, induction of sex change in these species has been of great interest to aquaculturists. Androgens including testosterone (T), 11- ketotestosterone (11-KT) and the synthetic 17 α- methyl testosterone (MT), have been used to induce sex reversal. MT has been demonstrated to be the most effective and is widely used for reversal of sex in many teleosts (Chao and Chow, 1990; Lee *et al*., 1996)

Smith (1975) states that gonads of all hermaphroditic serranids are similar in gross appearance. The gonads of groupers are composed of two unequal lobes, in which ovaries, testes and transitional gonads have a central lumen and lamellae of connective tissue projecting in to it. Most ovaries contain islets of testicular tissue. At sexual transition, the oocytes degenerate, the spermatogonia proliferate and the ovary is transformed into a functional testis. Often oocytic remnants persist in the testes, which may be used in the diagnosis of female to male sex change. Thus the transforming gonads contain degenerating tissues of ovary and proliferating tissues of testis.

The method followed by Chen *et al*. (1977) and Chao and Chow (1990) was adopted in the present study on sex

inversion of the greasy grouper *E. tauvina,* using histological and cytological criteria.

Materials and methods

Groupers used in this study were selected from the broodstock maintained in the onshore tanks at the Fisheries Harbour Laboratory of the CMFRI. Stages of maturity of the gonad were taken from fish samples drawn from the experimental tanks where the treatment using the male hormone 17∝–methyl testosterone was carried out.

During the experimental period, fishes were maintained in 5 numbers of 4 m^3 circular cylindroconical FRP tanks of 5 t capacity each and provided with seawater recirculation facilities. Control groups were also maintained in a similar tank. Size distribution of fishes in both control and experimental groups was similar *viz.,* 380-600 mm in total length. Each fish was weighed to the nearest milligram and the total length and standard length of fishes were measured to the nearest millimeter. Fishes were subjected to ambient photothermal conditions and were fed *ad libitum* with raw/frozen fish, once a day. The male hormone 17 α – methyl testosterone (Ms. Argent Chemicals, USA) was made into pellets using cholesterol matrix and a cellulose binder as per Sherwood *et al.* (1988). The hormone pellets implanted into prey fish were administered orally to the selected experimental females, three times a week at the normal feeding time. Hormone was administered at an average dose of 3 mg kg^{-1} body weight. Diet treatment was by feeding the fishes individually. The fishes were examined periodically for the presence of milt. An initial sampling was carried out at the start of the experiment. Subsequent samplings were carried out on the 10^{th}, 30^{th}, 40^{th} and the 80^{th} day after start of hormone application.

During sampling, gonads were removed and fixed in neutral buffered formalin for preparation and analysis employing standard histological techniques (Bullock *et al.*, 1996)**.** Sections were cut at 5-7 µm and stained using haematoxylin and eosin. These sections were examined under a compound microscope to determine the sex and stage of reproductive maturity of each fish as per Mackie (2000). Photomicrographs of the histological preparations of the ovary were taken using a binocular microscop (Leitz, Germany) Leitz.

Sex and maturation stages were defined based on the most advanced developmental stage of oocytes, proportion of spermatogenic cysts, appearance of sperm sinuses and presence of sperm.

Results and discussion

Three stages *viz.,* (1) the early transitional or the degenerating ovary, (2) the bisexual and (3) late transitional could be distinguished from the extent of degeneration of female germ cells, the proportion of male and female tissues, the presence of male cysts in the lobules and the development of sperm sinuses along the gonad wall.

The hormone treated fishes became mature males after the administration of the male hormone 17 α-methyl testosterone at mean accumulated dose of 50-55mg kg^{-1} body weight per fish, for a period of 9-12 weeks. Sex inversion was induced in both smaller and the larger fish, with no clear relationship with fish size and age. The gross appearance as well as physiological details of the gonads at various stages of hormone treatment are discussed here.

The degenerating ovary after 10 days of hormone treatment

Morphologically, the gonad appeared similar to the ovary, measuring 8.5 to 9.2 cm in length and histological examination revealed that the ovary contained oocytes in previtellogenic stage, primary oocytes and atretic oocytes. The total quantity of MT administered to each fish was around 27.5 mg. The ovarian lamellae at this stage contained vitellogenic and previtellogenic oocytes in alpha and beta stages of atresia; the outer cytoplasm was less basophilic and had split from the central mass of cytoplasm and also the nucleus appeared necrotic. Yellow brown bodies were also common (Fig. 1), but the gonads began to show early transitional characteristics like the emergence of eosinophilic granulocytes in the gonadal connective tissue.

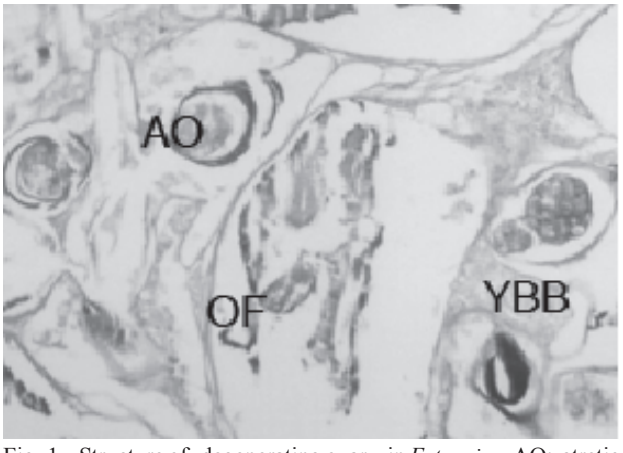

Fig. 1. Structure of degenerating ovary in *E. tauvina.* AO: atretic oocytes, OF: empty ovarian follicle, and YBB: yellow brown bodies; X 100

The transforming or bisexual gonad after 30 days of hormone administration

The ovaries appeared loose walled, more elongated and compressed structure, indicating signs of sex transformation. Atresia and degeneration of the perivitellogenic and chromatin nucleus stage oocytes had taken place, which were intermingled with spermatogonial tissue. As atresia continued, the oocytes became reduced in size and filled with vacuoles and the spermatogonia as

well as spermatocytes increased considerably. The quantity of hormone administered was 62 mg. Eosinophilic granulocytes were seen in plenty in the connective tissue of gonads. At this stage, the gonads were differentiated into lobules eventhough there were still some oocytes in the gonads and spermatogonia and spermatocytes also had formed in the lobules; the oocytes decreased in number and spermatogonia and spermatocytes increased gradually. (Fig. 2 a, b.) Morphologically the MT treated fish became smaller in size.

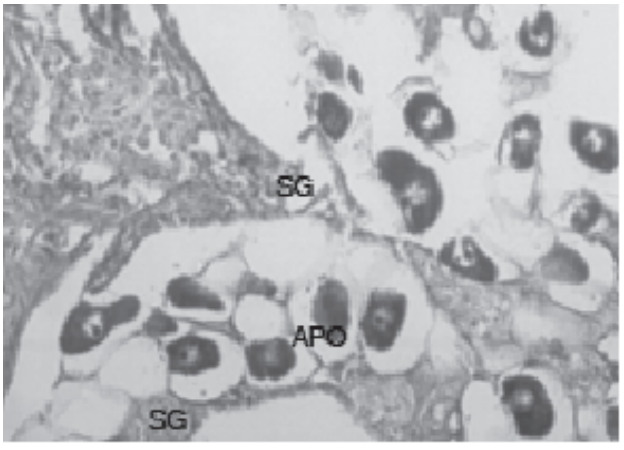

Fig. 2 a. Transforming gonad in *E. tauvina* showing atretic previtellogenic oocytes (APO) and spermatogonial cells (SG) in the transforming gonad; X 200.

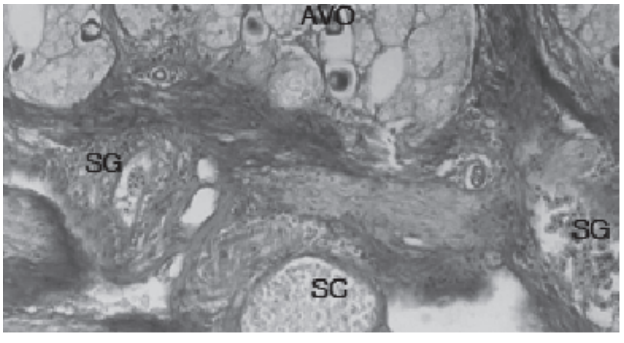

Fig. 2 b. Transforming gonad in *E. tauvina* showing atretic vitellogenic oocytes (AVO), spermatogonial cells (SG) and spermatocytes (SC) in the transforming gonad; X 200.

After 40 days of hormone administration

Morphologically, fishes administered the male hormone testosterone for a period of 40 days became slimmer, compared to the females in the control group. The quantity of hormone administered by this time was 86 mg, which was to the tune of 26.9 mg kg^{-1} body weight per fish. At this stage the gonad appeared very much similar to the testis, with a total length of 6.8 - 7.6 cm in fresh condition . The two lobes of the gonad, flat and leaf like, were still of subequal length as in the ovarian stage. Histologically, it was found that sex change was almost complete. Catheterisation was not possible indicating that the oviduct was closing and sperm duct was being formed. The presence of spermatogonia, spermatogonial crypts and primary spermatocytes along with a few degenerating primary oocytes or atretic bodies were noticeable. The late transitional gonad at this stage consisted of 95% testicular tissue and only 5% residual ovarian tissues. Many spermatogenic cysts containing spermatogonia, spermatocytes and spermatids were seen scattered in the testicular tissue. The transitional gonad at this stage can be designated as a non-functional male (Figs 3 a, b).

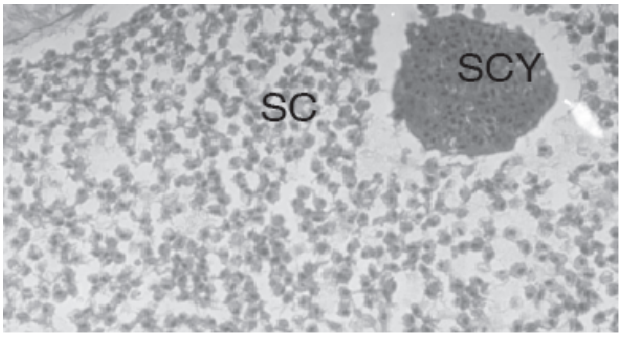

Fig. 3a. Transitional gonad in *E. tauvina* showing spermatocytes (SC) and spermatogenic cysts (SCY) in thelate transitional gonad; X 400.

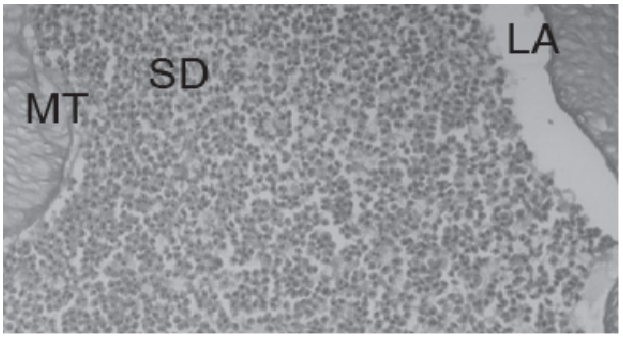

Fig. 3 b. Late transitional gonad in *E. tauvina* showing spermatids (SD), muscular tunica (MT) and lamellae (LA) in the late transitional gonad; X 200.

After 80 days of hormone administration

E. tauvina of 56-62 cm total length, weighing 3.5-5.2 kg became functional male and spermiated between the 79^{th} and 87^{th} day after commencement of feeding the male hormone 17α - methyl testosterone. The total quantity of hormone administered to each fish during this period was at an average rate of 50.35 mg kg^{-1} body weight per fish. The testes further elongated and measured 10.2- 11.4 cm in length, the two lobes were of equal length, flat and leaf like. Morphologically the two lobes were elongated longitudinally, with smooth dorsal surface, producing a furrowed appearance. Histologically in the fully mature, spermiating testes, testicular tissues almost filled the gonads, its seminiferous tubules packed with sperms

either in multitude of crypts and as collection of tailed sperms (Fig. 4). Collecting sinuses were dense with the collected tailed sperms and the gonad moderately distended. Crypts of spermatogonia and spermatocytes were not observed in this stage of fully ripe testis**.** The connective tissue membrane surrounding the crypts broke down as sperms reached maturity. Some empty lumen were also noticed and some lobular walls surrounding the cysts broken down, suggesting some to have spawned from the fish (Fig. 5).

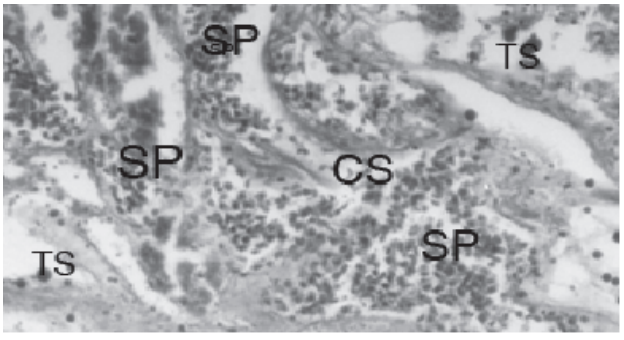

Fig. 4. Testis of a sex transformed male *E. tauvina* showing spermatozoa (SP) and tailed sperms (TS) in collecting sinuses (CS); X 400.

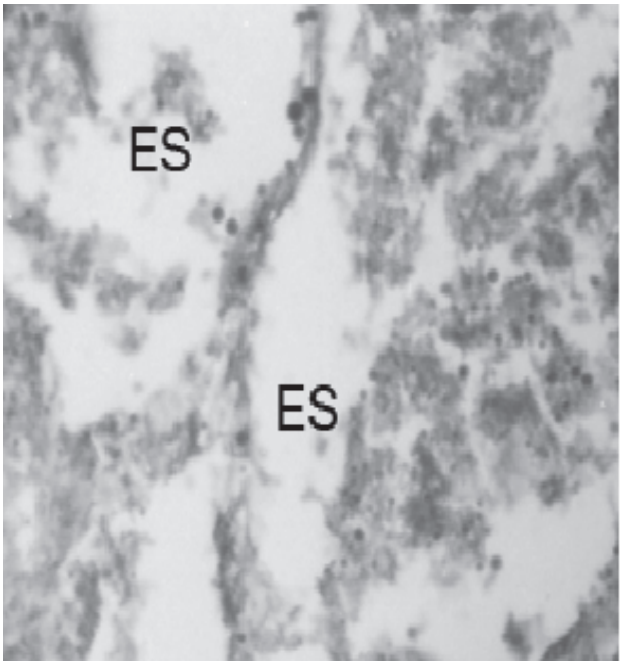

Fig. 5. Testis of a spawned male *E. tauvina* showing empty collecting sinuses (ES); X 400.

Groupers of the genus *Epinephelus* primarily display protogynous hermaphroditism. In the greasy grouper *E. tauvina,* inversion of sex is brought about by development of the testicular region and regression of the ovarian part, although externally distinguishing characters are not visible in either stage. As observed by Sadovy and Shapiro (1987) and Brule *et al*. (2003), the main criteria for identifying protogyny in hermaphroditic fishes were clearly observed during the present study, *viz.,* membrane lined central cavities in the testes, sperm sinuses in the gonadal wall and transitional individuals. The gonads contain clusters of spermatocytes, spermatids or spermatozoa with degenerating ovarian tissue; the seminiferous nests could be precocious spermatocytes and sperm cysts present in functional ovaries. Similar to this, scattered sperm cysts were observed in immature and mature ovaries in *Epinephelus fulvus, Epinephelus crenuatus, Epinephelus marginatus* and *Epinephelus flavolimbatus* (Bullock *et al*., 1996).

The results of present study showed that precocious sex inversion in *E. tauvina* can be induced through MT administration. But the mechanism of MT action in the gonadal transformation with complete degeneration of the ovary and its replacement with functional testis is not known. The success and the time duration required for the completion of sex change depend upon the type and dose of hormones, and the manner of hormone administration. Chen *et al*. (1977), Chao and Chow (1990) and Kuo *et al*. (1988) demonstrated in *E. tauvina* and *Epinephelus fario* that for oral administration, a high dosage and long feeding period are necessary. Injection was also given in *Epinephelus suillus* to induce sex change (Tan-Fermin *et al*., 1992). Sex change by the implantation of androgen pellets has been attempted in *E. tauvina* (Chao and Lim, 1991). 17 α - methyltestosterone (MT) has been commonly used in grouper and the dose applied varied in different studies. Oral administration of 145 mg MT kg^{-1} BW for 1 year (Chen *et al.*, 1977) and 120 mg MT kg^{-1} BW for 7 months in *E. tauvina* (Chao and Chow, 1990), 70 mg MT kg^{-1} BW in *E. fario* and 104 mg MT kg^{-1} BW in *Epinephelus salmonoides* (Kuo *et al*., 1988) has successfully induced sex change. Injection of 30 mg MT kg^{-1} BW (0.8–1.5 kg^{-1} BW, six biweekly injections) induced male grouper in *E. suillus* (Tan-Fermin *et al*., 1992). Implantation of MT (0.5 mg kg^{-1} BW) in a silastic capsule for 4 months resulted in functional males in *E. tauvina* (Chao and Lim, 1991)

Primordial germ cells capable of differentiating into oogonia or spermatogonia have been described in the gonads of female, male and sex-inverting *Epinephelus microdon* (Brusle-Sicard *et al.,* 1992). It seems likely that during the MT-induced gonadal transformation in *E. tauvina*, there is extensive proliferation and differentiation of such primordial cells in testicular tissues concomitant with the androgen-induced degeneration of the existing ovarian tissues (Lee *et al.,* 1996). Like other species of the genus *Epinephelus* (Sadovy and Shapiro, 1987; Smith 1975), in *E.tauvina* also the male and female tissues were not separated by connective tissue; but within that region the male and female elements lie side by side, without an intervening connective tissue wall.

Various authors have reported that groupers change sex and transform into males when they grow bigger and older, between ages of 10 and 17 years. (Tan and Tan, 1974, Brusle, 1987). One of the difficulties in defining the time of sex change from the occurrence of transitionals in a population is their low percentage in wild collections (Shapiro 1987; Johnson *et al.,* 1998). Histological studies by Tan and Tan (1974) in *E. tauvina* from South China sea showed that fishes at first maturity in the size range of 450-500 mm were females while male fish with ripe testes were above 740 mm and transitional gonads containing male and female gonadal tissues occurred in fish of 660-720 mm.

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