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Induced sex reversal and breeding of greasy grouper *Epinephelus tauvina* (Forskal)

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ABSTRACT

Hormone doses required for induced breeding and induced sex reversal of females to males in the greasy grouper *Epinephelus tauvina* were standardized. Spawning was induced by multiple injection of HCG at doses ranging from 700 and 2400 IU/kg body weight and LHRHa @ 30-40µg/kg body weight. Females with initial mean ova diameter of 450 µm and above responded well to hormone treatment. The ovulation time ranged from 72 to 144 hrs. The fertilization rate was about 19% and hatching rate was 5-10%. Mortality of larvae was noticed on 4th and 7th days after hatching. Females were converted to oozing males in 6 – 9 months by oral administration of 17α methyl testosterone hormone on alternate days @ 2.0 mg/kg body weight.

Introduction

Grouper is the most popular fish species in Indo-Pacific region and cultured extensively in earthern ponds and cages (Taburan *et al.*, 2001). Since, live grouper command high price in the south east Asian countries, there is a growing demand for seed from farmers (Sugama *et al.*, 2002). The short and unreliable supply of seeds from wild due to over exploitation of grouper has restricted the expansion of grouper farming. Hence, development of viable grouper breeding technology has gained lot of importance in the recent years. Although, there are several reports on the breeding and larviculture of groupers, details on controlled breeding and hatchery production of grouper fingerlings for commercial farming is rare due to several technical constraints and poor larval survival (Watanabe *et al.*, 1995). Since breeding of grouper involves various activities such as broodstock development, induced breeding and larval rearing, it is important to standardize all these techniques. Moreover, the viral disease outbreak in shrimp farming has resulted severe set backs in brackishwater aquaculture industry and has urged the researchers to find out an alternate candidate species for farming purposes.

The greasy grouper *Epinephelus tauvina* is one of the most commercially important fish species and it has high demand in both domestic and interna-

tional markets. *E. tauvina* is a protogynous hermaphrodite fish. It first matures as females and change sex to male's later (Tan *et al.,* 1974). In the case of protogynous hermaphrodites, induced sex reversal is considered necessary to ensure the availability of males during spawning season. Converting females to males by hormonal treatment is commonly practiced in grouper breeding. Even though grouper can spawn spontaneously without hormonal intervention, normally, the fish may not spawn naturally due to environmental and other stress conditions. In the absence of natural spawning, it is necessary to use the induced spawning method followed by artificial fertilization. The present study was to determine the minimum effective dose of hormones such as Human Chorionic Gonadotraphin (HCG) and Leutinizing Hormone and Releasing Hormone (LHRHa) required for induced breeding of females and requirement of 17α Methyl Testosterone for effectively converting the females to viable males for breeding purpose.

Materials and methods

Standardization of hormone dose for ovulation of females

A total of 42 fishes of *E.tauvina* with the size range from 2.5 to 6.0 kg were procured from Muttukkadu coastal waters and maintained in 100 tonne capacity RCC tanks during the period between April 1999 and March 2003. Fishes were fed with frozen trash fish (tilapia) @ 5% body weight daily. Broodstock fish holding tanks were cleaned and 80% water exchange was done on alternate days. Water quality parameters such as temperature, salinity, dissolved oxygen, pH and ammonia were recorded and found in the range of 27.5–34°C, 23.0-34.0 ppt, 4.0-5.5 ppm, 7.75-8.10 and 0.07-0.1 ppm respectively. Females were examined through ovarian biopsy for the assessment of maturation from September 1999. A sterile polyethylene cannula with the inner diameter of 0.6-1.0 mm was inserted through the ovipore of the anaesthetized female and the oocytes were taken from the anterior and middle part of the ovary by gentle aspiration. The oocytes were examined and measured under calibrated microscope. Fishes that had mean oocytes diameter of above 450µm were selected for induction of spawning. Four trials were attempted with different doses of HCG hormone (Table 1). These females were in the size range from 500mm - 510mm length and 3.0 - 4.0 kg weight. They were administered with HCG @ 700 IU - 1300 IU /kg body weight and the fishes were observed carefully for sign of hydration and ovulation. The ovulated females were stripped to collect the eggs. These eggs were washed with filtered seawater. Total number of eggs collected from each trial was estimated and recorded.

Sex reversal through hormonal manipulation

A total of 11 fishes of *E.tauvina* in the size range from 4.0 to 9.0 kg were maintained in 100 tonne capacity RCC tank from June 2002 to March 2003. Hormone pellets were prepared using 17á Methyl Testosterone (MT) with cholesterol as matrix and cellulose as binder (Sherwood *et al.,* 1988). MT pellets were orally administered to the fishes through feed @ 2 mg/kg body weight on every alternate day from June 2002. On an average (excluding holidays) each fish received 12 doses per month. Fishes were examined at monthly intervals to assess the sex reversal processes taking place in the fish. Milting fishes were counted and the percentage of conversion was calculated on monthly basis. Oozing males were classified as freely oozing and

TABLE 1. *Experiments on standardization of hormone for ovulation of grouper* E. tauvina

Sl No	Date	Females Length (mm)	Weight (Kg)	Initial oocyte diameter (µm)	Males Length (mm)	Weight (Kg)	Hormone dose HCG	LHRHa	Response
1	22.09.0999	510	3.0	468.5 (451-499)	-	-	HCG 700IU /Kg BW	40µ g/Kg BW	Enlarged belly was noticed after 72 hrs of hormone treatments. Stripping was successfully attempted. A total of 4,74,360 eggs were obtai ned with the mean diameter of 780 µm and the range of 676 - 843µm. Eggs could not be fertilized due to non availability of oozing males
2	22.09.1999	500	3.0	463.7 (435-499)	-	-	HCG 1300 IU /Kg BW	40 µg/Kg BW	A total of 4,12,000 eggs were strip ped and the eggs had mean oocyte diameter of 783.6 µm with the size range of 708 – 842 µm. Eggs could not be fertilized due to non availability of oozing males
3	25.10.1999	510	3.0	509 (451-564)	540	3.5	HCG 700 IU /Kg BW for female only	40 µg/Kg BW for female only	Fish spawned after 12 hrs of LHRHa injection. A total of 7,25,000 eggs estimated with the mean dia meter of 805 µm and the range of 710 – 864 µm. However all the eggs were unfertilized.
4	15 09.2001	560	4.0	452 (440-480)	-	-	HCG 1250 IU /Kg BW	40 µg/Kg BW	Stripped a total of 2, 21,000 eggs. Stripped egg had mean diameter of 790.6µm with a range from 724-830 µm Eggs did not fertilize in spite of using the cryopreserved milt.

sparsely oozing. Fishes that had converted in to males were used for obtaining milt to fertilize the ovulated eggs during breeding experiments.

Induced breeding

Three trials were performed for induced breeding of *E.tauvina* (Table 2). Females in the size range of 3.3-7.0 kg having mean oocyte diameter of 462.5-473.5µm were selected. They were administered HCG intramuscularly at a dose that varied between 1150 and 2400 IU/kg body weight. In two trials LHRHa @ 30 – 40 40µg/kg body weight was also administered after HCG dose. In all the trials, fishes were maintained in 20 tonne capacity RCC tanks for ovulation and spawning. After observing the swollen abdomen due to ovulation, the eggs were stripped from females and the free flowing eggs were collected in a sterile plastic bowl. Eggs were fertilized by dry method with the milt obtained from sex reversed males. Males were also administered with LHRHa @ 30µg/kg body weight, 24 hr before milt collection. Stripped eggs were mixed with milt using clean feather and after washing, the eggs were transferred into incubation tank (2.0 tonne capacity FRP tank). After 3-4 hrs of incubation the aeration was stopped and the unfertilized eggs that have settled at the bottom were removed. The incubation tank was provided with filtered seawater flow through facility and aeration. The number of eggs stripped, fertilization rate and hatching rate were determined in each trial. Larval rearing was conducted by providing *Brachionus plicatilis* as initial feed along with *Chlorella* sp. in the larval rearing tank from 48 hrs of post hatch. Larval measurements and survival were recorded.

Results

Standardization of hormone dose for ovulation of females

Four experiments were conducted in order to standardize the hormone dose. Table.1 illustrates the results of experiments conducted for ovulation and stripping of eggs from females with different dose of HCG. In first trial, one female fish (510 mm/3.0 kg) was injected with 2000 IU HCG @ 700 IU/kg BW and after 48 hrs with LHRHa (40 µg/kg BW) and the fish achieved complete ovulation after 72 hr of first injection. A total of 4.74 lakh eggs were obtained by stripping. The stripped egg had mean diameter of 780µm. In the second trial, the female fish (500 mm/3.0 kg) received HCG @1300 IU/kg BW (4000 IU HCG in 4 days) and LHRHa @ 40 µg/kg BW administered on the 5th day and ovulation was observed only after 144 hrs of first injection and the stripped egg had mean diameter of 783.6µm and a total of 4.12 lakh eggs were estimated. Even though, both trials showed similarity in the size and the number of eggs stripped, in the trial two the fish had taken longer duration of 144 hrs for ovulation despite the higher dose of HCG administered. In trial three, the female fish (510 mm / 3.0 kg) received HCG @ 750 IU/Kg BW (2000 IU administered as single dose) and LHRHa @ 40 µg/kg BW. This fish spawned spontaneously after 48 hrs of HCG injection and 24 hrs after LHRHa administration. A total of 7.25 lakh eggs were accounted. In trial four, the fish received HCG @ 1250 IU/Kg BW (4000 IU HCG administered in three days period) and after 72 hrs with LHRHa @ 40 µg/kg BW and complete ovulation was observed after 96 hrs of first injection. A total of 2.21 lakh eggs were accounted with mean diameter of 790.6µm.

TABLE 2. *Induced breeding experiments of grouper* Epinephelus tauvina

Sl No	Date	Females Length (mm)	Weight (Kg)	Initial oocyte diameter (µm)	Males Length (mm)	Weight (Kg)	Hormone dose HCG	LHRHa	Response
1	23.12.2003	710	7.0	473.5 (448-504)	510 520 540	3.0 3.0 3.5	HCG 1150 IU / Kg BW for female	Nil	Fish spawned spontaneously after 72 hrs HCG injection. A total of 1,12,000 eggs were estimated with mean diameter of 890µm. Even though most of eggs were floating on the water surface initially, after nine hrs all the eggs settled at the bottom of the tank.
2	26.02.2003	530	3.3	462.5 (442-486)	500 540	3.0 3.5	HCG @ 2400 IU/Kg BW	40 µg/Kg BW	Stripping was attempted after 24 hrs for female LHRHa adminis tration. A total of 20,000 eggs were collected Milt was obtained from two males and fertilized the eggs. Fertilization rate was estimated 19.6%. Fertilized egg had mean diameter of 784.4 µm. Larvae hatched between 22-24 hrs. Newly hatched larvae had mean total len gth of 1.78mm. Hatching rate was 5%. Mouth opened at 56 hrs after hatching. However, larvae survived only up to 3rd day.
3	21.03.2003	480	5.0	466.4 (448-498)	510 500	3.0 3.0	HCG @ 1600 IU/Kg BW for female	40 µg/Kg BW for female	A total of 4,78,000 eggs were obtained through stripping. These eggs were fertilized with milt obtained from males. Fertilization rate was 19.8%. Eggs hatched between 22-24 hrs with the hatching rate of 46.2%. Fertilized egg had mean diameter of 790 µm. All larvae died on 7th day

Sex reversal

Table 3 shows the results on sex reversal of females to males achieved through oral administration of 17 á methyl testosterone treatment. Milting fishes were observed only after six months of MT treatment. It is observed that the smaller fishes in the size range of 3.0 to 4.0 kg were sexually converted into oozing males (27%) by oral treatment of MT for six months from June 2002 to November 2002. However, large size fishes of 6.0 kg and above could be converted to males only after nine months (81.0%) of MT treatment (June 2002 to February 2003). The MT dose required for sex conversion of *E.tauvina* has varied from 648 mg (for 3.0 kg fish in six months period) to 1296 mg (for 6.0 kg fish in nine months period) to convert them to oozing males. This works out to 24-36 mg MT per kg per month. However, it is difficult to estimate the actual dose of MT consumed by an individual fish, since all the fishes were maintained as single group in the same tank.

Induced breeding

Three induced breeding experiments were conducted and the results are presented in Table 2 and the embryonic developmental stages are presented in Fig. A – H. In trial 1, a female weighing 7.0 kg and with mean oocyte diameter of 473µm was administered with HCG @ 1150 IU/Kg BW in four daily doses totaling 8000 IU (on the first day 5000 IU and after 24 hr, 1000 IU each day for three days) and maintained along with three oozing males. After 72 hrs of first injection the fish spawned spontaneously releasing 1.12 lakh eggs. About 50% of the eggs were floating. The mean diameter of floating egg was 890 µm with one oil globule measuring 185µm. Cell division was noticed up to nine hours and there after, the eggs settled without further development.

In trial 2, the female was injected with HCG @ 2400/kg BW (on the first day 5000 IU and after 24 hr, 1000 IU each day for three days) and after 96 hrs with LHRHa (40µg/kg BW) and maintained along with two oozing males. Male also received LHRHa @ 40 µg/kg BW, along with female. After 96 hrs of first injection, the fish was stripped and 20,000 eggs could be obtained. Milt obtained from males was used to fertilize the eggs. Fertilization rate was 19.6% with mean egg size of 784.4µm. Larvae hatched out between 22-24 hrs with 5% hatching rate. The newly hatched larvae had mean total length of 1.78mm. Rotifer Brachionus plicatilis in the size range 120 – 180µm were supplied to the larvae as initial feed in the larval rearing tank 48 hr after

TABLE 3. *Experiments on sex reversal of* E. tauvina *through 17á methyl testosterone*

Month	No. of fishes stocked	Percentage of Milt (only traces) oozing fishes	Percentage of Milt (freely) oozing fishes	Total percentage of oozing fishes
July 2002 – November 2002	11	Nil	Nil	Nil
December	11	9.1	18.2	27.3
January 2003	11	9.1	27.3	36.4
February	11	9.1	45.5	54.6
March	11	27.3	54.5	81.8

hatching. However, the larvae survived only for three days.

In trial 3, a female of 5.0 kg weight with mean oocyte diameter of 466.4 µm was administered with HCG @ 1600 IU/ kg BW (5000 IU on the first day followed by 1000 IU each day for three days) with cumulative dose of 8000 IU and after 96 hrs with LHRHa @ 40µg/kg BW. Complete ovulation was noticed after 96 hrs and the fish was subjected to dry stripping. Eggs were fertilized with the milt obtained from sex reversed males which were also administered with LHRHa @ 40 µg/kg BW, 24 hr before obtaining the milt. Fertilized eggs measured 790 µm (mean) in diameter and a total of 4.78 lakh eggs were obtained and the fertilization rate estimated was 19.8%. Larvae hatched out between 22-23 hrs with 10% hatching rate. The size of the newly hatched larvae was 1.74 mm in total length. In spite of supplying the rotifer *B.plicatilis* to the larvae, larval mortality was noticed on the 7th day.

Discussion

In the present study, the mean initial oocytes diameter of grouper selected for induced breeding varied from 452 µm to 509 µm. Several authors have reported in many species of grouper that the minimum diameter of oocyte is essentially required for induction of spawning through hormonal manipulation. Kuo *et al.*,(1988) have reported the oocyte diameter of 500 µm for *E. fario*, 400 µm for *E. malabaricus* (Kungvankij *et al.*, 1986) and 482 – 561 µm for *E. striatus* (Watanabe *et al.*, 1995) for successful induced breeding. Watanabe *et al.*, (1995) have reported that successful spawning were obtained using HCG in combination with LHRHa as priming and resolving doses, suggesting that these hormones can be used for induced breeding of *E.*

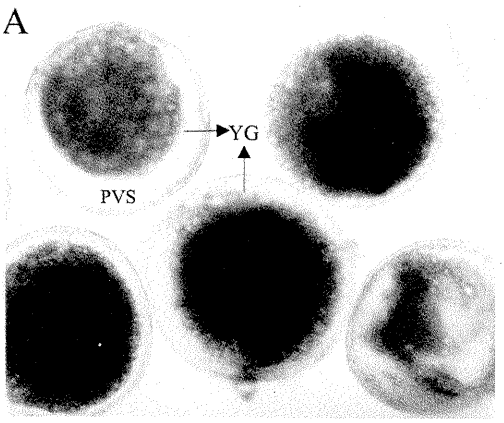

Fig. A. Oocytes (450-474 µm) of *E. tauvina*

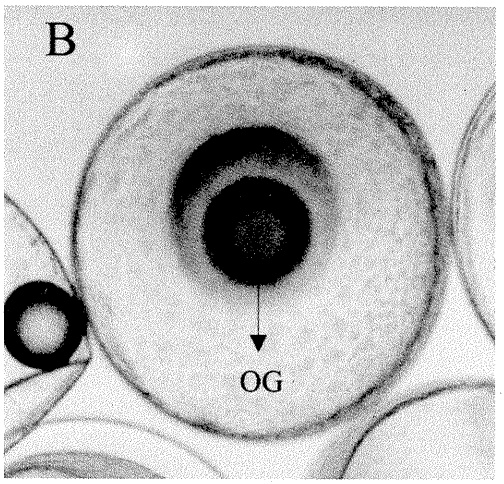

Fig. B. Fertilized egg (784 µm)

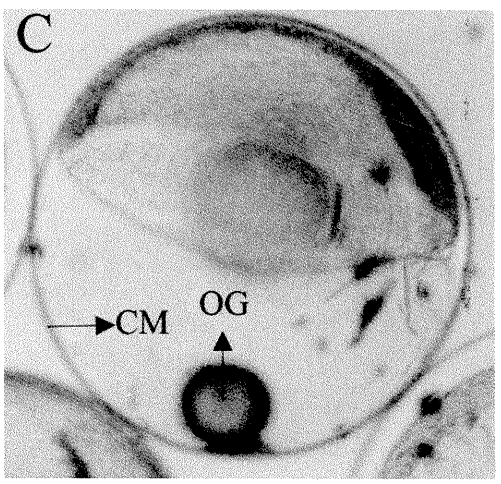

Fig. C. Gastrula stage

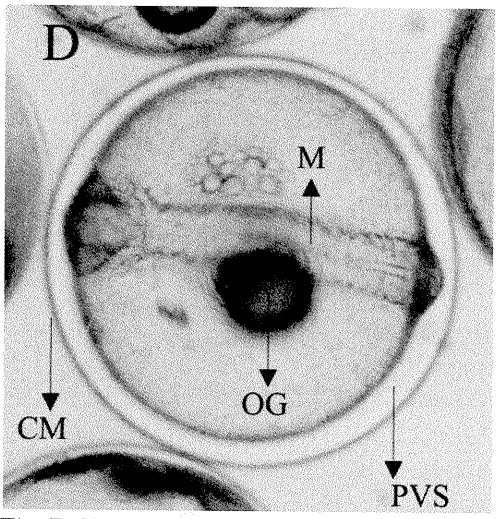

Fig. D. Neurula stage

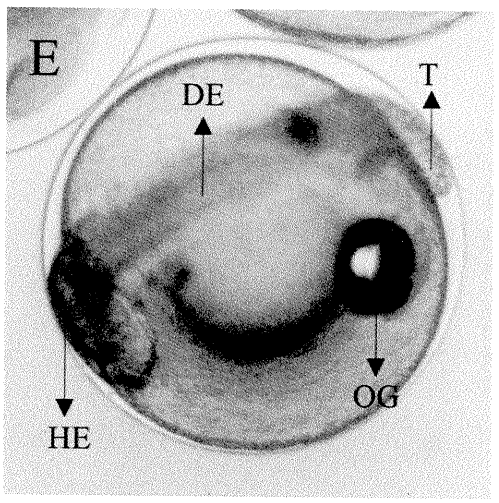

Fig. E. Fully developed embryo stage

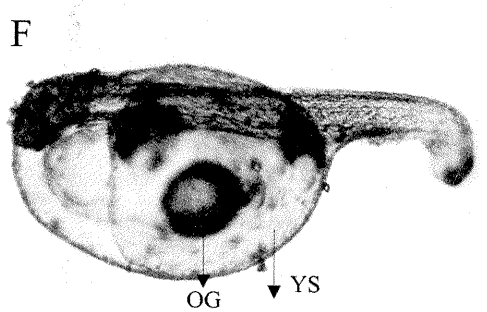

Fig. F. Breaking chorionic membrane and hatching

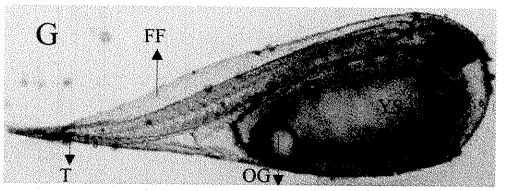

Fig. G. Newly hatched larva

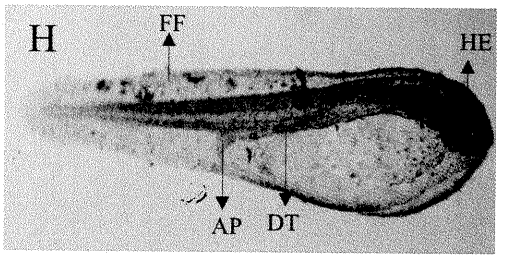

Fig. H. 3 day old larva

tauvina. The dose of HCG and LHRHa hormones used in this study ranged from 700-2400 IU/Kg body weight and 30-40 µg/kg body weight respectively. The HCG and LHRHa are practical agents for inducing ovulation in many species of fresh water and marine fish (Lam *et al.*, 1982). *E. fario* required 2 – 3 injection of HCG @ 900 – 2000 IU/kg body weight for achieving complete ovulation Kuo *et al.*, (1988) and *E. fuscoguttatus* required three injections @ 150-600 IU HCG/kg body weight (Mayunar *et al.*, 1989). In the present study also *E. tauvina* received maximum of 3 injections of HCG (trail 4) for achieving complete ovulation. Time of stripping in relation to ovulation is critical to egg quality and fertilization success (Shelton, 1989). In this study, time of stripping following hormone injections was estimated after observing noticeable swelling of the abdomen, slow swimming of spawner and occurrence of protrusion of the egg mass at the genital opening.

In the present study, ovulation time varied from 72 to 144 hrs after hormone treatment and the egg production varied from 20,000 to 7, 25,000 eggs/spawn-

ing/fish. Egg production also varies greatly with the condition of the brooder and the spawning season. Quinito *et al.,* (1996) have stated that the high variations in the quantity and quality of spawn may be related to fluctuations in environmental conditions and the inconsistent nutritional quality of trash fish fed to the broodstock. In this study, the fertilization rate was varied between 19.6 and 19.8% and the mean egg diameter ranged from 784-790 µm. Tucker *et al.,* (1991) have pointed out that the eggs obtained from induced ovulation sometimes are smaller than the naturally ovulated eggs which may be due to female size or age, location, and season. The hatching rate of *E. tauvina* recorded in this study varied between 5 and 10% and larval survival noticed maximum of up to 6 days. It is reported that mass mortality of grouper larvae during early rearing period may be due to many factors such as size of the rotifer fed to the larvae and its nutritional quality, rearing water quality, tank size, light etc (Kohno *et al.,* 1997). More trials have to be conducted in order to standardize the larval rearing of E.tauvina by providing better rearing conditions and providing with suitable nutritionally enriched and apt size rotifer fed to the larvae as initial feed.

The present result has demonstrated that the milting male grouper can be obtained through oral administration of MT @ 2.0 mg/kg body weight on every alternate day for 6-9 months period for the 3-6 kg size group females. Kuo *et al.,*(1988) have reported that the *E. fario* converted from females to males in 5 months period by feeding the MT @ 0.5 mg/kg body weight on daily basis. Chao and Chow (1990) have stated that the effect of MT treatment can vary according to the feeding response of individual fish. Variations in the percentage of sex reversal and treatment period (6-9 months) recorded in this study may be due to differences in the consumption rate of MT by individual fish since all the fishes were maintained in the same tank. It is evident from the present investigation that the female grouper within the size range from 3-6 kg can be converted in to males through MT treatment and they can be used for the induced breeding purposes. However, the viability tests have to be conducted for milt of converted males in comparison with milt of natural males in order to assess their suitability for fertilizing the eggs.

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