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Recent advances in induced breeding of the dusky grouper *Epinephelus marginatus* (Lowe, 1834)

G. Marino*, E. Azzurro*, M.G. Finoia*, M.T. Messina*, A. Massari** and A. Mandich**

*ICRAM, Via Casalotti 300, Rome, Italy

**Dept. of Biology, University of Genoa, Viale Benedetto XV, 5, Genoa, Italy

SUMMARY – The dusky grouper, *Epinephelus marginatus*, is a popular recreational and food fish in the Mediterranean Sea which has been seriously depleted in many areas. We have studied the feasibility of its reproduction in captivity for aquaculture diversification and for stock enhancement since 1995. This paper briefly analyses the key areas of broodstock husbandry, ovarian development, hormone induced spawning, sex inversion and first larval rearing.

Key words: *Epinephelus marginatus*, dusky grouper, reproduction, spawning induction, sex inversion, egg, larvae.

RESUME – "Progrès récents dans le reproduction induite du mérout *Epinephelus marginatus* (Lowe, 1834)". Le mérout, *Epinephelus marginatus*, est un poisson bien connu à niveau récréatif et alimentaire de la Mer Méditerranée qui a subi une forte déplétion au cours des années. A partir de 1995, on a étudié la faisabilité de sa reproduction en captivité pour sa diversification en aquaculture et pour améliorer le niveau de sa production en stock. Ce document a pour but d'analyser les secteurs clés de l'élevage des géniteurs, du développement ovarien, de la ponte des œufs induite hormonalement, de l'inversion sexuelle et de l'élevage larvaire initial.

Mots-clés : *Epinephelus marginatus*, mérout, reproduction, ponte des œufs induit hormonalement, inversion sexuelle, œuf, larve.

Introduction

Owing to an increasing demand for food fish, high market value, fast growth rate, and disease resistance, there is strong interest in grouper aquaculture throughout the world. At the same time, in many tropical and temperate areas, overfishing and environmental degradation are depleting wild grouper populations (Sadovy, 1993) and studies on grouper biology are thus in progress in order to provide a basis for fisheries management (Huntsman and Shaaf, 1994). This is true also for the dusky grouper, *Epinephelus marginatus*, one of the largest fish in the Mediterranean, which has undergone a dramatic decline in the wild population over the past few decades (Garcia and Castello-Orvay, 1995; Zabala *et al.*, 1997b). This species was recently included among the endangered teleost listed in Annex 3 to the Berne Convention (1996), for which management measures must be planned and culture techniques developed in order to enhance endangered stocks (FAO, 1997).

The present paper analyzes the main constraints and recent advances encountered in broodstock husbandry, induced spawning, artificial sex inversion and first larval rearing trials.

The reproductive biology of *Epinephelus marginatus* in the natural environment

Epinephelus marginatus (Serranidae: subfamily Epinephelinae) is a common, wide-ranging species occurring in the Mediterranean and eastern Atlantic from Britain to South Africa, as well as along the south coast of Brazil (Randall and Heemstra, 1993). Prior to Heemstra's (1991) review, *E. marginatus* was known as *E. guaza* or *E. gigas*.

The reproductive biology of this species has been investigated mainly on the basis of macroscopic observations of gonads, demographic evidences and underwater observations in marine reserve

areas. *E. marginatus* displays protogynous hermaphroditism (Bruslè, 1985), i.e. individuals mature as females and transform into males as they grow larger and older. The species is monandric, since all male gonads pass through a functional female stage (Marino *et al.*, 1998c). According to Chauvet (1988), sex inversion takes place between the 9th and 16th year, peaking at year 12. Bruslè and Bruslè (1976) reported the occurrence of males starting from a body weight of 9 kg, but also exceptional cases of precocious sex change in 3-5 kg individuals. Conversely, Marino *et al.* (1998c) found no males smaller than the smallest functional female and observed a very narrow size range in sex change.

First sexual maturity is reached late, when females are 5 years old and 38 cm long (Chauvet, 1988). Mat_{50} was found to be 43.8 cm SL in females and 81.3 cm SL in males (Massari *et al.*, 1998). Some rare cases of precocious ovarian activity were reported by Spartà (1935) and Bruslè and Bruslè (1976), who found sub-mature females weighing 2 and 3 kg, respectively. Large sized females (>15 kg) were also found. Few data are available on ovarian morphology and development patterns. Bruslè (1985) described a synchronous development of the ovary, whereas maturation in shifts, by the recruitment of "waves" of previtellogenic oocytes, was described by Bouain and Siau (1983). A group synchronous ovarian type was determined by Massari *et al.* (1998) on the basis of the frequency distribution of vitellogenic oocytes. Multiple spawning was also identified by the authors in relation to the presence of postovulatory follicles in maturing ovaries.

The eggs of *E. marginatus* have been described (Spartà, 1935; Barnabè, 1974; Skaramuka *et al.*, 1989; Zabala *et al.*, 1997b) in the range of 750-810 μm in diameter with a single oil globule of 200 μm .

The spawning season in the Mediterranean extends from June through September, and is probably dependent on water temperature. Females in vitellogenesis have been found off the Tunisian coast in April (Bouain and Siau, 1983; Massari *et al.*, 1998) and mature females occur from the end of May until September with a peak in July-August (Barnabè, 1974; Bouain, 1980; Bruslè, 1985; Chauvet, 1988; Massari *et al.*, 1998). In Spanish waters, reproductive activities start in July and spawning takes place by mid August, declining rapidly afterwards (Zabala *et al.*, 1997a). In the north Adriatic Sea, spawning takes place in the second half of August and in early September (Skaramuka *et al.*, 1989).

Like other groupers, this species exhibits complex social and behavioral patterns (Louisy, 1996). Dusky grouper juveniles colonize grottos and rocky breaks of shallow water with a size-dependent bathymetric distribution. Adults inhabit deeper water down to depths of 250 meters and are considered as sedentary fish, living around a permanent hole (Chauvet and Francour, 1990). However, some evidence indicates that dusky grouper cluster in specific sites during the reproductive period (Bruslè, 1974; Zabala *et al.*, 1997b). These gatherings differ from the large spawning aggregations of tropical groupers. Males are strictly territorial, polygynous and display a silver streaked color pattern during reproductive activity (Louisy, 1996). Dominant males are aggressive towards neighboring males and females (Zabala *et al.*, 1997a). Sex ratio (3:1 females/males) during reproductive aggregation is biased toward females (Marino, 1997; Zabala *et al.*, 1997a).

Spawning in the natural environment was described for the first time by Zabala *et al.* (1997a): it occurs as single female/male pairs and gamete release following an ascending "rush" (6-8 m). Spawning was observed one hour after sunset, when the moon was in a new lunar phase and water surface temperature was 25°C.

Inducing breeding and larval rearing of dusky grouper in captivity: Main constraints

Methods for the controlled breeding and larviculture of grouper species have been developed since 1970, especially in Southeast Asia and in tropical and subtropical western Atlantic regions. However, grouper aquaculture is still far from full commercialization owing to the shortage of fingerlings from the wild and the lack of reliable technology for hatchery production (Lim, 1993; Kuo, 1995; Watanabe *et al.*, 1995).

The aquaculture of the Mediterranean dusky grouper is still in the early stages of development (six ASFA reports since 1995), but available data indicate that biological and technical constraints,

identified for other epinephelids, also apply to *E. marginatus*. Some of these constraints have already been overcome, while other aspects of controlled breeding and larviculture have still to be investigated fully. Among others, the following aspects were found to represent key factors:

(i) Dusky grouper is a protogynous monandric hermaphrodite, reaching first sexual maturity late. Since it is not a fast growing species, in order to form broodstocks, sexually mature fish must be collected from the wild and adapted to captivity (Spedicato *et al.*, 1995; Spedicato and Lembo, 1996). Otherwise, the fishing of healthy fish is extremely difficult since they inhabit deep waters and suffer serious trauma and damages during capture, increasing in severity with fish size (Di Marco and Marino, 1998; Di Marco *et al.*, 1998).

(ii) To overcome the shortage of large males (and small quantity of milt expressed by natural males), it is necessary to develop protocols for precocious sex inversion. Successful attempts at induced sex change by administering 17 α -methyltestosterone have been recently reported by Glamuzina *et al.* (1998a), Marino *et al.* (1998b) and Spedicato *et al.* (1998a).

(iii) The specific environmental and social conditions that are favorable to the induction of gametogenesis and to the production of quality eggs have still to be determined.

(iv) Spontaneous spawning does not occur in captivity and hormonal treatments are required to induce final maturation and ovulation in vitellogenic females. Eggs are usually obtained by artificial stripping (Spedicato *et al.*, 1995, 1998b,c; Glamuzina *et al.*, 1998b; Marino *et al.*, 1998a,d). The identification of appropriate hormone treatment and a precise time for collecting eggs are consequently key factors in obtaining quality eggs.

(v) Egg production is rather low and limited to a short period during the year.

(vi) Early larval stages had small body and mouth size and limited yolk reserve. Larval development is very slow and the length of larval period is consequently very long (Bogliione *et al.*, 1998a,b).

(vii) Reliable larviculture techniques and appropriate feeding sequences have not yet been established. In the early larval stages, adult rotifers (S- and L-types) are usually too large as first prey (Marino *et al.*, 1998a; Spedicato *et al.*, 1998c).

(viii) Despite the importance of food quality, with special reference to (n-3) highly unsaturated fatty acids (HUFA), on the larval performance of most grouper larvae (Dehrt *et al.*, 1991), the study of HUFA requirements and enriched larval diets is still only in the early stages (Messina *et al.*, 1999).

(ix) Owing to the above-mentioned difficulties, coupled with lack of knowledge on dusky grouper breeding and larviculture, larval survival at early stages is still low (Glamuzina *et al.*, 1998c; Marino *et al.*, 1998d; Spedicato *et al.*, 1998b).

Inducing breeding and larval rearing of the dusky grouper in captivity: Recent advances

Broodstock husbandry

Wild groupers were caught by long-line around Lampedusa Island in 1994-1996. Forty fish (1.6-17.5 kg body weight, BW) were transported to a commercial hatchery (Ittica Mediterranea, Trapani), individually tagged by passive transponders (Fish Eagle, UK) and kept in two 40 m³ concrete outdoor tanks. Tanks were covered to reduce disturbance and light intensity and pipes were laid to provide hides. Stocking density ranged from 1.1 to 2.1 kg/m³. Fish were fed *ad libitum* four times a week on frozen fish or squid (0.3-1% BW). Vitamin mix was added to trash fish intermittently from April to September.

During the 1995-1997 reproductive seasons, both groups were maintained under natural photoperiod and temperature conditions. From April to October 1998, two controlled thermoperiods were applied in order to mimic the ambient water temperature measured in spawning aggregation areas (data not shown). Variations of water temperature in 1997 and 1998 are illustrated in Fig. 1.

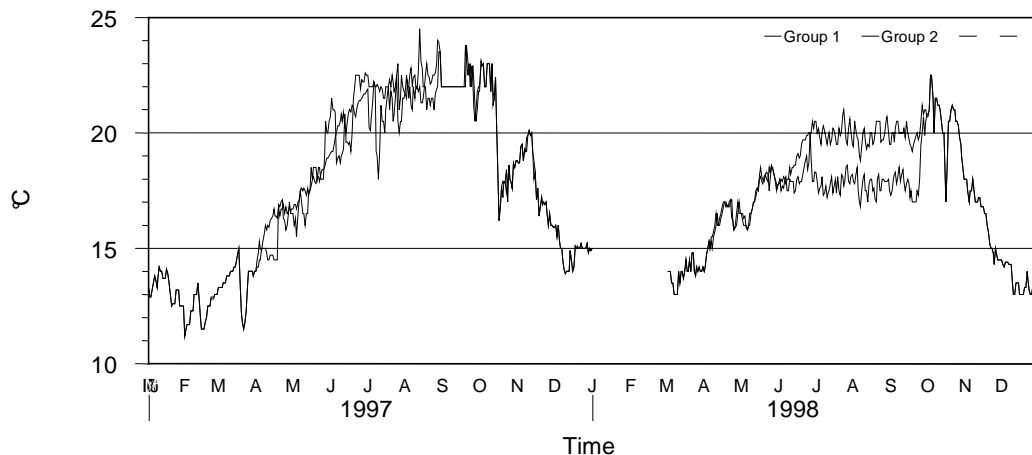


Fig. 1. Water temperature conditions during dusky grouper broodstock maintenance in captivity. 1997: ambient water temperature; 1998: controlled water temperature.

Male brooder development

Sex inversion was induced in wild dusky grouper juveniles (1.3-2.0 kg BW) by dietary treatment and injection of 17α -methyltestosterone (MT, Sigma).

MT was included in feed at the rate of 1 mg/kg BW and given to fish by the farm hands or released in the tanks. MT was also intramuscularly injected at the rate of 2 mg/kg BW every 7-10 days (Marino *et al.*, 1998b).

Mature males, through sex inversion, were obtained by both methods of 17α -methyltestosterone administration. Diet treatment was completely successful when fish were individually fed and partially successful (25%) when hormone supplemented feed were released in tanks.

In individually fed fish, natural spermiation was first observed after 14 weeks and 77.5 mg/kg MT intake. Mature males were obtained after the administration of a mean accumulated dose of 85 mg/kg after 14-15 weeks. Sex inversion was induced both in the smaller and the larger fish, with no clear relationship with fish age and size.

When feed blocks were given not to each single fish but released directly into the tank, only 1 out 4 fish sex inverted. The specific MT dose ingested by each fish was probably different owing to differential feed uptake. Social hierarchies formed among the fish could cause lower food uptake and hence lower MT intake in subordinate specimens (Pandian and Sheela, 1995).

Also intramuscular injection of MT induced sex inversion. Spermiation was observed in 3 out 8 treated fish starting from the 14th week of treatment. A total accumulated MT dose of 29.4-32.1 mg/kg BW was effective. The remaining fish had not completed spermatogenesis after 18 weeks. Stress caused by repetitive injections are probably be involved in the lower response to hormone.

Inverted males produced small quantities of sperm (200-750 μ l), with vigorous motile spermatozoa (up to 75%). Sperm was activated 5-10 sec after dilution in seawater and remained motile much longer than 30 min. Successful egg fertilization was obtained using milt from MT inverted males, but fertility rate was lower than in natural males.

Six months after the end of MT administration, sex inverted fish changed back to females (Marino *et al.*, 1998b), thus indicating that gonad changes are not stable and prolonged MT treatment is required.

Reproductive condition of captive dusky grouper females

Sex and reproductive condition of 26 brood fish belonging to two groups were monitored by ovarian biopsies and sex steroid analyses (Mandich *et al.*, 1998a,b) during two reproductive cycles. Three ovarian stages were identified by histological analysis: F1 immature, F2 developing and F3 maturing. Females at stage F1 displayed oogonia and previtellogenic oocytes, both in chromatin and in at the perinucleolus stage (Fig. 2). Female at stage F2 had ovaries with oocytes at the lipid vesicle stage and numerous large sized perinucleolus stage (Fig. 3). Females at stage F3 had vitellogenic oocytes at different stages (Fig. 4). The final stages of vitellogenesis were observed in oocytes larger than 400 μm . Histological data indicated that the ovaries of dusky grouper are of the group synchronous type, with 3-4 batches of oocytes at different stages coexisting in the ovary. The same pattern of ovarian development was described in wild females by Bouain and Siau (1983) and by Massari *et al.* (1998).

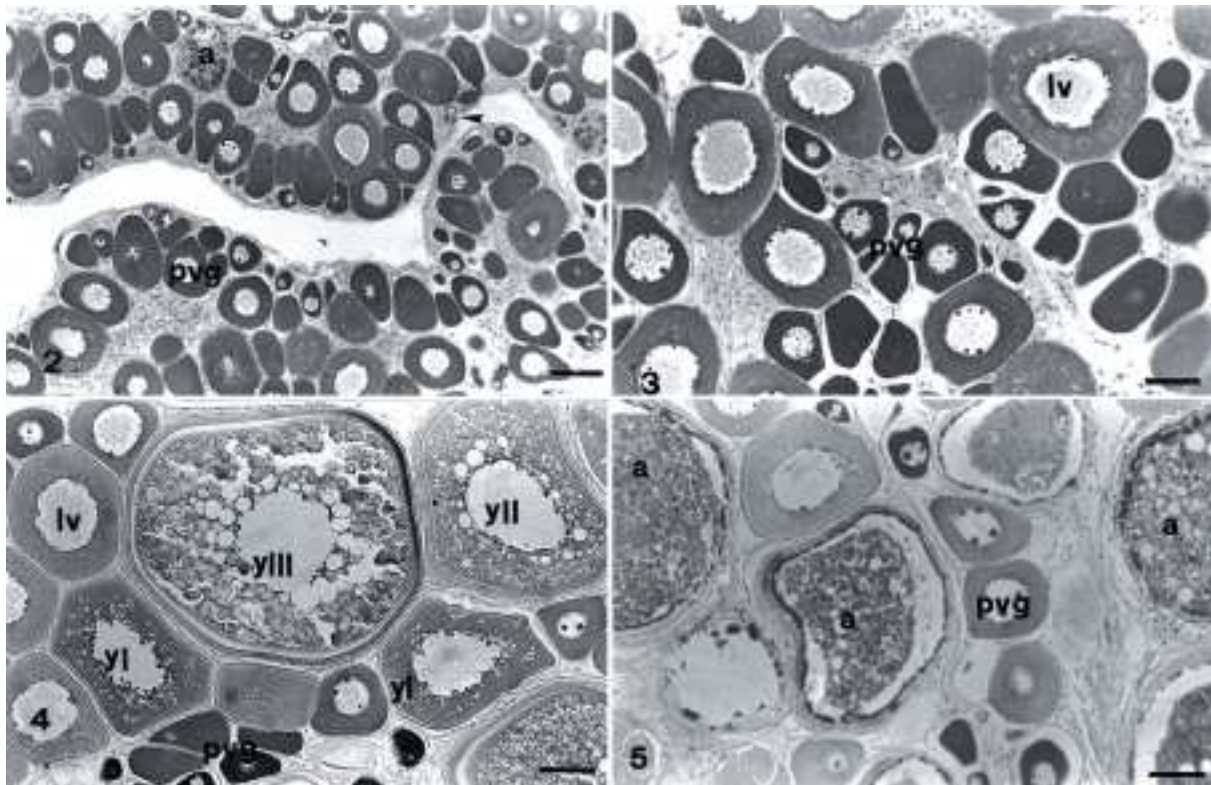


Fig. 2. Biopsy of a female in stage F1. Oogonia (arrowhead) and previtellogenic oocytes (pvg) of different size are scattered along the edge of the ovigerous folds. Atresia (a) of a previtellogenic oocyte is present. Toluidine blue, bar = 100 μm .

Fig. 3. F2 stage. Many oocytes in lipid vesicle stage (lv) are distributed amongst small and large previtellogenic oocytes (pvg). Toluidine blue, bar = 50 μm .

Fig. 4. Biopsy of F3 female. Previtellogenic oocytes (pvg), lipid vesicle (lv) and yolk granules I, II and III (yl, yll, ylll) oocytes are present. Mann Dominici, bar = 50 μm .

Fig. 5. F6 female. Atresia (a) of vitellogenic follicles near previtellogenic oocytes (pvg) at the end of the reproductive season. Mann Dominici, bar = 50 μm .

Sexual maturity was observed in one female 2.5 kg BW (1.6 kg at capture), indicating that first sexual maturity can be reached under captivity conditions. Functional females with vitellogenic

oocytes were found in the range 2.5-5.4 kg BW and represented about 30% of all females in both reproductive cycle. The remainder showed previtellogenic oocytes or vitellogenic oocytes at early vitellogenesis that did not proceed to the final stage of vitellogenesis during the season.

No clear-cut differences in ovarian development were observed in 1997 and 1998 in relation to water temperature, as the percentage of mature females did not vary substantially. In both seasons early signs of vitellogenesis were first observed in late April when the temperature was about 17°C. The final stages of vitellogenesis were observed in the range of 19-23°C water temperature. Vitellogenic oocytes underwent atresia in August-September, at the end of the reproductive season (Fig. 5).

Induced spawning and artificial fertilization trials

Twenty-seven induced spawning trials were carried out in vitellogenic females using different hormone preparations (Marino *et al.*, 1998a).

hCG (Profasi, Serono) was administered via multiple injections, 24 h apart, at a dosage of 2000 UI/kg.

Sea bass pituitary was acetone dried and used at 9 mg/kg BW in combination with hCG (2000 UI/kg).

[D-Trp⁶] GnRH (Triptoreline, Decapeptyl, Ipsen Biotech) in microspheres, was suspended in 0.2% Tween 20 (Sigma), dissolved in saline solution and administered at 40 µg/kg.

In four trials hCG in multiple injections was used in females with 400-560 µm oocytes. The females did not respond to hormone treatment and egg overmaturation was also observed in 2 out of 4 injected fish after 74 h. hCG has been widely used to induce ovulation in grouper species (Tucker, 1994), but good results are usually achieved when injection is performed in fully mature females. In dusky grouper, hCG efficiency was verified *in vitro* by assaying 17β-E production by vitellogenic follicles (Mandich *et al.*, 1998b). In this study, the lack of response in hCG injected females could be related to our inability to identify a appropriate time for stripping and/or to repeated handling for multiple treatments, inducing the retention of ovulated eggs.

hCG turned out to be more effective when administered together with sea bass pituitary extracts, according to previous experiments with tropical grouper (Kuo, 1995). Good quality eggs were obtained by stripping 54 h after the first injection in one female, and voluntary spawning was observed in a female after 70 h at 22.1°C.

There are only a few reports on induced spawning by GnRH_a in groupers (Tucker, 1994; Hassin *et al.*, 1997). In dusky grouper, GnRH_a in microspheres seems to be an appropriate treatment to induce final maturation and ovulation of oocytes 350-520 µm. At 40 µg/kg it was effective in 13 out of 20 treated females. Multiple ovulations were recorded in some females, however the first spawning gave the best results as regards number and quality of eggs.

Males continued spermiating from late May until the end of August, although milt was in very short supply. Milt production was sustained with multiple treatment of GnRH_a in microspheres (10 µg/kg). However, injecting males increased short term supply but milt production regressed more quickly, as already reported in *E. striatus* injected with hCG (Tucker *et al.*, 1991).

After hormone treatment, 2-4 females were usually transferred to a 20 m³ tank with one spermiating male. The optimal stripping time after GnRH_a treatment was determined as 68-70 h at 18-19°C. Eggs were artificially fertilized using the dry method. Eggs were transparent and spherical, with a single oil globule and unsegmented yolk. Mean egg diameter was 839 ± 9.9 µm, smaller than that reported by Spedicato *et al.* (1998c) and close to the 846 ± 41 µm found by Glamuzina *et al.* (1998c). Mean dry weight was approximately 66 µg. Fertilization rate ranged from 22 to 98% and hatching rate from 0 to 89.5%. Embryonic development lasted 50-52.5 hours at 18-18.5°C.

Larval rearing trials

Two hatchery experiments were carried out using cryopreserved *Crassostrea gigas* trochophores (Trochofeed, Innovative Aquaculture Products, Canada, size 20-40 μm) and two S-type rotifers (*Brachionus plicatilis*) to evaluate first feeding regimes suitable for growth and survival of dusky grouper larvae. Green water technique and small rearing volume (1500 l) were used. UV-treated filtered seawater was supplied at 19-20.5°C. The microalgae *Nannochloropsis oculata* was introduced to the tank daily (200,000 cells/ml) from d1 to improve visual perception and feeding efficiency (Lim, 1993).

In trial 1 cryopreserved trochophores were enriched for six hours on *N. oculata* and supplied at 30 cells ml density from d4. S-type rotifers (mean lorica length 210 μm , range 120-280) were supplied at 30 ind/ml density from d5.

In trial 2 oyster trochophores were introduced daily (30 cell/ml) immediately after thawing from d3. Baby S-type rotifers (mean lorica length 135 μm), selected as those passing through a 100 μm mesh screen and retained on a 40 μm mesh, were supplied at 30 ind/ml density at the same time.

In both trials larval growth and survival were measured at hatching and on d1, d3, d4, d5, d8, and d12 d15. During the trial 2, samples of thawed trochophores, rotifers, egg and larvae were analysed for total lipids and fatty acids determination by HPLC (Table 1).

Table 1. Lipid and fatty acid content in eggs and yolk sac larvae of *E. marginatus*

	Eggs	Larvae				
		D0	D1ph	D3ph	D4ph	D8ph
Total lipid (%)	4	5	5	5	4	3
20:5 n-3 (EPA)	12.18	1.84	2.34	2.19	1.34	5.86
22:6 n-3 (DHA)	32.41	13.30	13.39	4.78	2.54	5.59
EPA/DHA	0.37	0.13	0.17	0.45	0.5	1.04
Saturated	20.95	48.77	48.45	57.1	32.82	31.98
Total (n-3)	45.85	17.93	16.78	14.04	8.55	13
Total (n-6)	16.75	26.15	20.57	15.44	53.52	28.32
HUFA (n-3)	42.22	16.53	16.59	7.48	7.78	12.18
n-3/n-6	2.74	0.68	0.81	0.9	0.15	0.45

The egg hatching rate was 85.5% in trial 1 and 88.9% in trial 2. Early larval rearing had limited success and survival was low. However, differences in larval growth and survival were observed in relation to the feeding regimes utilized. During yolk reabsorption, larvae did not differ in length and survival. At mouth opening (d5), larvae were unable to fed large rotifers supplied in trial 1. Moreover, few larvae displayed trochophores in their gut. Survival fell to 1% on d8 and to 0% on d9.

In trial 2, 30% of examined larvae were ingesting oyster trochophores from d6 onwards. The active feeding of larvae on oyster trochophores was probably due to higher mobility of trochophores when supplied immediately after thawing. Baby screened rotifers were found in 10% of the larvae on d8 and in 20% on d12. However, survival declined on d8 to 22-28% and fell to 0% on d14-d15.

Since trochophores and small size rotifers are actively fed by 20-30% of dusky grouper developing larvae, high larval mortality may be also attributable to factors unrelated to prey availability.

The analyses of the fatty acid composition of grouper eggs revealed the presence of high levels of HUFA n-3 (42.22%). The fatty acid composition was dominated by DHA (22:6 n-3). From hatching to

d5 a decrease of total lipid content was observed in larvae, in agreement with physiological events occurring during first larval development (Rainuzzo *et al.*, 1997).

The fatty acids profile of rotifers and larvae indicated a higher content in EPA than in DHA. Increments of the EPA/DHA ratio in rotifers are known to produce a decrease in larval growth performance (Rodríguez *et al.*, 1998). The increase of EPA/DHA ratio in the larvae from hatching to the last sampling might be related to a nutritional "distress" (Izquierdo, 1996). These preliminary data suggest that *E. marginatus* larvae could require high DHA levels during early development.

Conclusions

(i) Hormone induction of sex inversion seems to be an efficient tool for producing dusky grouper males. However, oral administration of 17 α -methyltestosterone to single fish proved to be very laborious and unsuitable at commercial level. Sex inverted fish change back to females (Marino *et al.*, 1998b), and therefore repeated treatment was required to maintain male sex. To develop practical protocols for the production of dusky grouper males, treatment allowing a slow and prolonged 17 α -methyltestosterone release (Holland *et al.*, 1998) are required.

(ii) In captivity dusky grouper females reach the final stages of vitellogenesis, but the process of final oocyte maturation and spawning do not occur. Moreover, the interruption of vitellogenesis was observed in many females, probably as a result of unfavorable environmental and social conditions (Hassin *et al.*, 1997). No clear-cut differences in ovarian development were observed in relation to water temperature. More exactly, the unbalanced sex ratio in our captive broodstock could result in hormonal failure (Zohar, 1989) and affect ovarian maturation. Further studies are needed to identify environmental and social conditions suitable for ovarian development in captive females.

(iii) Dusky groupers induced to ovulate with [D-Trp⁶] GnRH in microspheres produced eggs with high fertilization and hatching rate. However, since dusky grouper is a fish with a group synchronous ovary, such treatment probably results in the spawning of only a small portion of developing oocytes. The effectiveness of the GnRHa delivery system in inducing ovulation and spermiation have still to be tested.

(iv) Small body and mouth dimension, poor endogenous reserve and low initial feeding rate characterize dusky grouper larvae. High mortality and slow growth are generally observed in the early rearing period. Oyster trochophores and screened S-type rotifers seemed to be adequate sized prey for first feeding, but do not prevent mortality. Appropriately food sequences for early larval stages have to be looked into.

(v) Deficiency in (n-3) HUFA resulted in high mortalities in grouper larvae (Dehrt *et al.*, 1991). Our preliminary data suggest that high DHA levels could be essential in the early development of dusky grouper larvae.

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