Main constraints in the artificial propagation of the dusky grouper Epinephelus marginatus (Lowe, 1834): Three years experimental trials on induced spawning and larval rearing

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Main constraints in the artificial propagation of the dusky grouper *Epinephelus marginatus* (Lowe, 1834): Three years experimental trials on induced spawning and larval rearing

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SUMMARY – Although the dusky grouper *Epinephelus marginatus* can be considered a suitable candidate for aquaculture and restocking, its reproductive pattern raises several problems for the broodstock management. Furthermore, the lack of knowledge on larval biology, ecology and physiology determines difficulties in the rearing of this species. This paper reports the results of three years of experiments carried out in the summer of 1996, 1997 and 1998. Females were induced by hormone treatments (HCG and a-LHRH) and individuals in pre-maturation stages were artificially sex-inverted by oral administration of methyltestosterone. The eggs were artificially fecundated and incubated (19-22°C). From the hatching day cultured microalgae were supplied. Different preys were tested: enriched rotifers (small strains) and trochophores. Samples were taken at regular time intervals for fresh examination and SEM observations. The larval rearing lasted 42, 13, and 51 days in 1996, 1997 and 1998, respectively. The 1996 newly hatched larvae (total length: 1.66-2.03 mm) were characterised by two free neuromasts in the rostro-dorsal region. In all the individuals the mouth gape (about 95 µm) and the complete reabsorption of the yolk sac were observed around the 5th day, when the olfactory placode assumed a circular shape. A peak of mortality occurred in each experiment between the 8th and the 12th day. On the 42nd day (1996) the larva was still in a pre-flexion stage, and at the 51st day (1998), larval structure such as dorsal and pelvic spines still persisted. No taste buds have been observed. The very small size of larva as well as mouth gape seems to be a very important, but not the only constraint in *E. marginatus* larviculture.

Key words: *Epinephelus marginatus*, induced reproduction, larval development, sense organs.

RESUME – *"Principales contraintes pour la propagation artificielle du mérou* Epinephelus marginatus *(Lowe, 1834) : Trois années d'essais expérimentaux sur la reproduction induite et l'élevage larvaire". Malgré que le mérou noir* Epinephelus marginatus *peut être considéré une bonne espèce pour l'aquaculture et le repeuplement côtier, ses caractéristiques reproductives posent plusieurs problèmes pour la gestion des géniteurs. D'autre part, la connaissance incomplète sur la biologie, l'écologie et la physiologie larvaires détermine des difficultés pour l'élevage de cette espèce. Le présent travail montre les résultats de trois ans d'expériences conduites en été 1996, 1997 et 1998. Les femelles ont été induites par hormones (HCG et a-LHRH) et les spécimens en phase de pré-maturation ont été inversés artificiellement par l'administration orale de méthyltestostérone. Les œufs ont été artificiellement fécondés et incubés (19-22°C). A partir du jour de l'éclosion des microalgues ont été administrées. Des proies différentes ont été évaluées : rotifères (small strains) enrichis et trocophores. Des échantillons ont été prélevés à intervalles réguliers pour l'examen en frais et pour les observations au SEM. L'élevage larvaire a duré 42, 13, et 51 jours en 1996, 1997 et 1998 respectivement. Les larves à l'éclosion (longueur totale : 1,66-2,03 mm) étaient caractérisées par deux neuromastes dans la région rostro-dorsale. L'ouverture buccale (95 µm environ) et la résorption du sac vitellin ont été observés vers le cinquième jour, lorsque le placode olfactif revêtit une forme circulaire. Un pic de mortalité s'est vérifié dans chaque expérience entre le huitième jour et le douzième jour. Au quarante-deuxième jour (1996) la larve était encore en pré-flexion, et au cinquante et unième jour (1998), les structures larvaires comme l'épine dorsale et pelvienne étaient encore présentes. Aucun bouton gustatif n'a été observé. La dimension très petite de la larve ainsi que l'ouverture buccale semblent représenter une limite très importante, mais pas unique, pour l'élevage larvaire d'*E. marginatus*.

Mots-clés : Epinephelus marginatus*, reproduction induite, développement larvaire, organes sensoriels.*

Introduction

The species of the *Epinephelus* genus are among the most heavily exploited fish in the Mediterranean and the dusky grouper (*Epinephelus marginatus*, Lowe, 1834) is the most well known

member. Though quantitative estimates of the species' status are not available for several Mediterranean areas, various authors reported a general decline and rarefaction of the species (e.g. Bruslè, 1985; Coll *et al*., 1995). There is thus concern about sustainability of the fishery and ultimately, the conservation of the species, which has been included in the list of marine organisms that need management measures (Annex 3 of Bern Convention).

Like other groupers, *E. marginatus* is a proterogynous hermaphrodite, first maturing as female at about 4-5 kg (Bruslè, 1985), when the individuals are about 10-12 years old (Kara and Derbal, 1995). Because of this characteristically slow growth, natural sex-inversion occurs much later, usually at a size of about 9-10 kg, and can be affected by social and environmental factors (Shapiro, 1987).

Although the dusky grouper can be considered a suitable candidate for aquaculture (Barnabé, 1974; Fernández Vilar *et al*., 1993) and restocking programs, its reproductive pattern raises several problems for the broodstock management. Females need to be hormone induced even if farmed for long time (Spedicato *et al*., 1995, 1998a). Moreover, as in many grouper species, the availability of wild males represents a constraint for reproduction in captive conditions, though this problem can be effectively circumvented by the artificial sex inversion (Glamuzina *et al*., 1998a; Marino *et al*., 1998; Spedicato *et al*., 1998b,c), limiting the capture of old specimens. The research works performed so far on the experimentally induced spawning allowed some preliminary description of the embryonic (Glamuzina *et al*., 1998b) and larval development (Spedicato *et al*., 1998c,d) of dusky grouper, as well as of the sensory ontogenesis (Boglione *et al*., 1999b). But the failures of both larval rearing trials compel us to not consider such ontogenetic descriptions as the normal ones.

In such framework, the lack of detailed knowledge on larval biology, ecology and physiology determines difficulties in the rearing and management of the species.

This paper aims at giving a contribution to highlight the main constraints occurring in the artificial propagation of this fish, on the basis of three years experiments, from 1996 to 1998, carried out on the dusky grouper broodstock established at the COISPA laboratory (Spedicato and Lembo, 1996).

Material and methods

The breeders were maintained in 16 m^3 tanks (water renewal: 5-6 volumes/day), under natural day-length and temperature, at a density of 4-5 kg/m^3. They were fed squids *ad libitum*. Spontaneous spawning did not occur after the temperature increase from 16 to 23°C during four months (from April to July). Consequently the females were induced by hormone injection after oocyte sampling by catheter. Different treatment protocols were applied, administering Human Chorionic Gonadotropin (HCG) and a-LHRH (buserelin), at specific doses ranging from 800 to 2000 IU/kg and from 15 to 31 µg/kg, respectively. Individuals in pre-maturation stages (weighing from 1.6 to 2.55 kg) were artificially sex-inverted by oral administration of methyltestosterone (specific dose: 1 mg/kg, for 14 or 15 weeks), resulting in fluent males whose sperm showed fecundating capability (Spedicato *et al*., 1998a).

The eggs obtained from the treated females (200-250 g, according to the trial) were artificially fecundated by the "dry method" in the three different experiments (August-July of 1996, 1997 and 1998), using both natural and artificially sex-inverted males (1.5-2 ml of sperm according to the egg amount). The floating eggs were incubated at 19-22°C, according to the experiment, under a moderate aeration and a drip flow of water in 1.5 m^3 tanks. Larval rearing temperature ranged between 19.5 and 23°C, according to the trial. From the hatching day cultured microalgae *Isochrysis galbana* and *Tetraselmis suecica* were supplied. Different live preys were used in the three different trials and namely: enriched (commercial emulsion DHA Selco) *Brachionus plicatilis* (small strains) and *Cassostrea gigas* trochophores as first feeding, followed by enriched Artemia nauplii (Fig. 1). The rotifer concentration in the rearing units was ranging between 10 and 20 individuals/ml, and that of trochophores between 20 and 25 individuals/ml.

Samples were taken at regular time intervals for fresh examination, collection of biometrics data and evaluation of stomach contents. Larvae samples were also preserved (fixing agent: 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, followed by osmium tetroxyde postfixation) for scanning electron microscopy (SEM) observations, carried out on specimens previously critical point dried, and then examined in a ZEISS DSM 950 after gold coating.

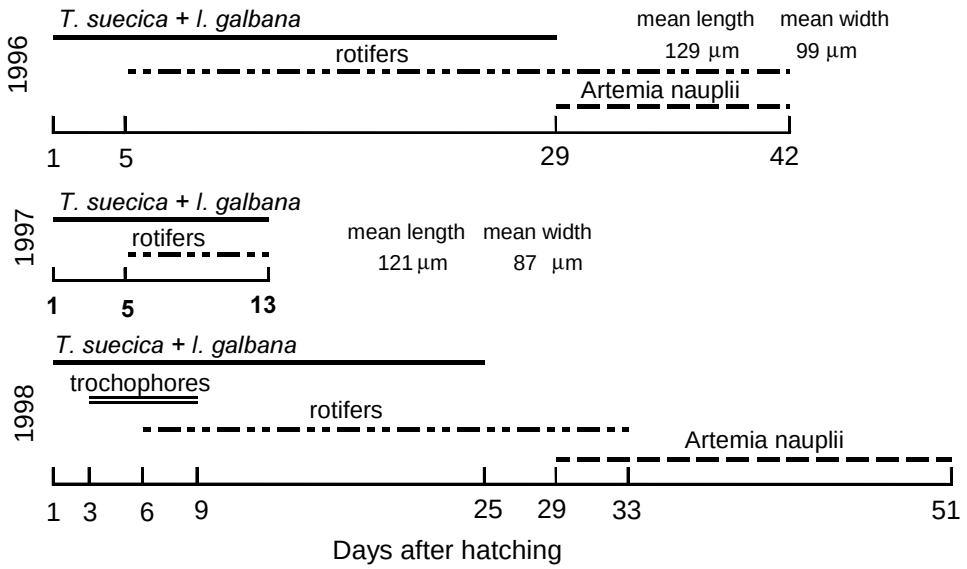

Fig. 1. Feeding schedule of the three experiments from 1996 to 1998. The lorica size of the rotifers used in the different trials is reported. In 1998 larvae were fed rotifers of the same strain as in 1997.

The experimental larval rearing lasted 42, 13 and 51 days in 1996, 1997 and 1998, respectively.

Results and discussion

Natural spawning never occurred in our experimental conditions, even if females were farmed for a long time (more than 4 years). Oocytes of captive dusky groupers, however, attained the different stage of vitellogenesis (initial oocyte diameter: 300-575 µm, Table 1), though final maturation had to be induced by hormone treatments. Both HCG and a-LHRH were effective to this end. Nevertheless, aLHRH-administered females appeared to give better performances in terms of egg quantity that, on the other hand, was negatively affected by the stripping technique applied. This procedure was however necessary, as deposition following hormone treatments was sometimes observed, but generally only of overmature eggs. In Table 1 a summary of some successful results obtained by a-LHRH injections are reported. Indeed, except in a few cases, the amount of viable eggs obtained was generally low and unpredictable.

Besides the problems related to the management of females, the availability of wild males could be a constraint in the propagation of dusky grouper, due to the well-known decline of large specimens of this nearshore species.

However, since the first trial carried out in 1996 (Table 2), we obtained the induced sex-inversion of individuals in pre-maturation stages. The experiments were successfully repeated in the following years, using fish weighing from 1.6 to 2.55 kg and applying the oral administration of methyltestosterone at the specific dose of 1 mg/kg, for 15 weeks, as this exposure time gave the best results. Nevertheless, another problem of the captive dusky grouper breeders concerns the spawning behaviour. In our experimental conditions, the natural males were fluent during the reproductive season, but they never showed the characteristic colour pattern and behaviour as described in the wild by Zabala *et al.* (1997).

The fecundation of *E. marginatus* eggs was achieved in the three trials with rates ranging from 25 (1997) to 30% (1996 and 1998). The mean egg diameter was 871 µm and the oil droplet about 175 µm. The incubation lasted from 40 (1998) to 50 (1996) hours, according to the temperature. The hatching rate was about 60, 50 and 65% in 1996, 1997 and 1998, respectively. In Fig. 2 the two-blastomers, morula and embryo stages are shown.

Table 1. Summary of some successful hormone treatments for induced spawning in dusky grouper females

Fish	Hormone	Body weight (kg)	Total length (cm)	IOD† (µm)	Injection dose (µg)	Specific dose (µg/kg)	Remarks
1	a-LHRH	2.5	51	300-518	25 50	30	Stripping Viable eggs = 20%
2	a-LHRH	3.6	60.5	300-430	30 60	30	Stripping Viable eggs = 70%
3	a-LHRH	11	85	345-575	110 220	30	Stripping Viable eggs = 20%
4	a-LHRH	2.8	52.5	370-518	32 55	31	Stripping Viable eggs = 20%
5	a-LHRH	10.8	82	370-450	110 220	30	Stripping Viable eggs = 20%
6	a-LHRH	4.9	65	370-518	52 100	31	Stripping Viable eggs = 50%
7	a-LHRH	4.3	60.5	414-506	28 56	20	Stripping Viable eggs = 40%
8	a-LHRH	8.5	72.5	345-483	56 112	20	Stripping Viable eggs = 30%
9	a-LHRH	6	68.5	414-506	40 80	20	Stripping Viable eggs = 50%

†IOD = Initial oocytes diameter.

Table 2. Protocol of methyltestosterone administration and results obtained in the trial carried out in 1996 (April 22-July 28; April 15-July 28)

Fish	Exposure time (weeks)	Weight (kg)	Daily specific dose (mg/kg)	Sperm production at the end of treatment	Spermatozoa motility stage††	Spermatozoa concentration (cell/ml)	Sperm production after 30 days
1†	14	2	1.1	Fluent (stripping)	V	6×10^9	Fluent (stripping)
2†	14	2.1	1	Mature	O	–	–
3†	14	2.1	1	Fluent (stripping)	V	8.6×10^9	Fluent (stripping)
4†	14	1.95	1.12	n. d.†††	n. d.	n. d.	–
5†	15	2.2	0.97	Fluent (stripping)	V	6×10^9	Fluent (stripping)
6	15	2.35	0.91	Fluent (stripping)	II	5.9×10^9	Fluent (stripping)
7†	15	2.5	0.88	Fluent (stripping)	V	n. d.	–
8	15	2.1	1	Fluent (stripping)	IV	4.7×10^9	Fluent (stripping)

†Fish treated for 2 consecutive years.
††Determined according to Chamberyon and Zohar (1990).
†††n. d. = Not determined.

The newly hatched larvae (total length ranging from about 1.66 to 2.03 mm) were characterised by two melanophores around the caudal part of the notochord and 1 melanophore caudally to the oil drop. Two free neuromasts were present in the rostro-dorsal region. Two arched olfactory placodes were rostrally present. On the 2nd day all the examined larvae showed pectoral fin buds, and numerous mucous cells were densely packed all around the cephalic region. Around the 4th day some macroscopic features of the larvae were modified, such as the augmented pigmentation of the eyes and of the dendritic expansion of the melanophores. In all the individuals the mouth gape (about 95 µm), the complete reabsorption of the yolk sac, gut peristalsis, pervious anus and opercle formation

were observed around the 5th day. At this stage (Fig. 3), the olfactory placodes had assumed a solid circular shape. Rotifer predation was ascertained only in a low percentage (about 30%) of the 8 days old larvae.

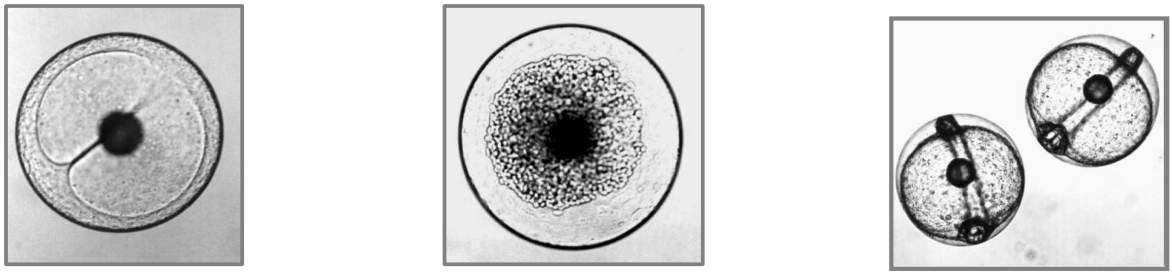

Fig. 2. Two-cells, morula and embryo stages of the artificially fecundated eggs of *E. marginatus*.

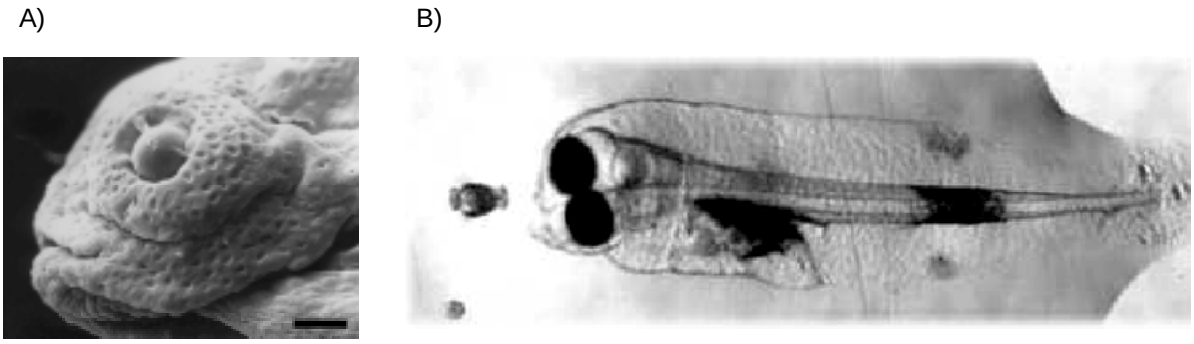

Fig. 3. (A) SEM micrograph of cephalic region and (B) *in vivo* photograph of a 5-days old larva. In (B) the eye-pigmented larva is trying to seize a too big rotifer. The characteristic chromatophores pattern is visible.

In each experiment, peaks of mortality occurred between the 8th and the 12th day. In the 1996 experiment, only 1% of larvae survived after the 12th day, and none after the 42nd day. In the 1997 experiment none larva survived after the 13th day. In the 1998 trial only few larvae survived after the 30th day and the last one died on the 51st day. Larvae older than 24 days (total length about 4.9 mm) showed a pair of elongated dorsal and pelvic fin rays with melanophores on the terminal tract of these rays. On the 42nd the larva was yet in a pre-flexion stage, with the primordial median finfold reabsorbed in the median region. There were 4 neuromasts in the rostral region, whilst 7 free neuromasts formed the trunk lateral line. No chemical receptors (taste buds) have been observed. A larva surviving until the 51st (total length about 14.7 mm) day still displayed elongated fin rays, a transparent body with a small melanophore placed in the middle part of the caudal peduncle and no scales (Fig. 4). At this stage the flexion of the notochord had been attained, as well as dorsal, anal and pelvic fin formation. The anal fin had two thin spines and the elongated pelvic fin rays showed thin protuberances. The head region accounted for almost half the body.

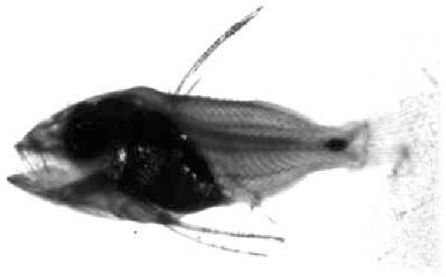

Fig. 4. Dusky grouper larva at the 51st day after hatching.

Conclusions

As in other *Epinephelus* species (e.g. Tamaru *et al.*, 1995) the very small size of grouper larvae, and in turn the so limited mouth gape, seems to be a very important constraint during the early larval rearing. However, regarding *E. marginatus* larviculture, it appeared to be not the only constraint, as suitably sized larval food did not prevent massive mortality during early rearing. The use of trochophores with size compatible with the mouth gape of dusky grouper larvae resulted in survival rate as low as observed in the feeding schedule based only on rotifers. Nor did the high concentration of preys in the rearing tank help in reducing mortality.

The results achieved showed that the dusky grouper larvae had searched preys probably only by sight, and selected them by size, as no gustatory organs and scanty number of mechanoreceptors were available till the 42nd day. Even the olfactory organ seemed to be underdeveloped if compared with other fish larvae (Boglione *et al*., 1988, 1992, 1998, 1999a,b; Cataudella *et al*., 1989). As already observed in some *Epinephelus* species (in *E. tauvina* and *E. fuscoguttatus* by Lavens *et al*., 1991; in *E. coiodes* by Kohno *et al*., 1997) the larval period of dusky grouper was extremely prolonged and longer than reported for other farmed fish (Cataudella *et al*., 1989; Boglione *et al*., 1992, 1998). Indeed, at the 51st day (14.7 mm) from hatching, larval structure such as elongated dorsal and pelvic spines still persisted, as well as the transparency of the body and the absence of scales. The persistence of larval structures, such as the elongated spines, was also reported for the *E. akaara* larvae measuring 19 mm in standard length (Fukuhara and Fushimi, 1988).

In this context, further investigations are necessary to define the exact pattern of larval sensorial ontogenesis to extend the knowledge of the larval feeding behaviour. Therefore, new research efforts are needed in the direction of testing new preys in early feeding. Furthermore, the requirements of the larval rearing environment need to be tested, both in terms of physical parameters (e.g. light), and in terms of volumes, for example, intensive versus large volumes culture system.

Our observations suggest the hypothesis of a dusky grouper larva passively transported by water current (no fin formation till the 42nd day), using the dorsal and pelvic appendage to change direction. It is a passive feeder with some help in individuating food items by sight and selecting by dimensions and not by taste. Although further observations are necessary to validate this hypothesis, it could represent a useful basis for future studies on larval feeding ecology.

Besides the problem related to the biology and ecology of dusky grouper larva, also factors related to the broodstock management need further investigations, in terms of social composition of the reared group and influence of the environmental parameters on maturation and spawning. Indeed, the non-occurrence of natural spawning in captive condition resulted in a low egg quality. Thus, a better understanding of the wild dusky grouper ecology and behaviour, characterised by strong homing and site fidelity (Lembo *et al*., 1999a,b) as well as by delimitation of the territory during reproduction (Zabala *et al*., 1997), might help the knowledge of the mechanisms of adaptation to the captive condition, that can affect the natural spawning process.

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References

Barnabé, G. (1974). La reproduction du mérou *Epinephelus gigas*: Observations préliminaires de terrain. *Aquaculture,* 4: 363-367.

Boglione, C., Bertolini, B., Russiello, M. and Cataudella, S. (1992). Embryonic and larval development of the thick lipped mullet (*Chelon labrosus*) under controlled reproduction conditions. *Aquaculture*, 101: 349-359.

Boglione, C., Bronzi, P., Cataldi, E., Serra, S., Dini, L. and Cataudella, S. (1999a). Early development of Adriatic Sturgeon *Acipenser naccarii*: Preliminary results, (selected paper). *J. Appl. Ichthyiol.* (in press).

Boglione, C., Cataudella, S., Massa, F., Scorsini, D., Monaco, G. and Bertolini, B. (1988). Development of sea bass (*Dicentrarchus labrax* L.), with particular regard of the chemical and lateral line sense organs. *Journal of Aquatic Products*, 2(2): 163-174.

Boglione, C., Marino, G., Bedin, L., Bertolini, B., Ferreri, F., Fezzardi, D., Finoia, M.G., Fresi, E., Rossi, A., Scardi, M. and Cataudella, S. (1998). Analisi morfo-funzionale dello sviluppo embrionale e della ontogenesi larvale in Teleostei marini da Acquacoltura, in relazione al problema delle malformazioni ed alla ottimizzazione della qualità del novellame. *Biol. Mar. Med*., 5(3): 2289-2298.

Boglione, C., Selmo, C., Contegiacomo, M., Spedicato, M.T. and Cataudella, S. (1999b). Preliminary contribution to knowledge of the sensory ontogenesis of the dusky grouper, *Epinephelus marginatus* (Lowe, 1834). *Marine Life* (submitted).

Bruslé, J. (1985). Exposé synoptique des données biologiques sur les mérous *Epinephelus aeneus* (Geoffery Saint Hilaire, 1809) et *Epinephelus guaza* (Linnaeus, 1758) de l'Océan Atlantique et de la Méditerranée. *FAO Synopsis sur les Pêches*, 129: 1-64.

Cataudella, S., Boglione, C., Crosetti, D., Massa, F., Rampacci, M., Russiello, M. and Bertolini, B. (1989). Breeding technology and larval development of thick lipped mullet (*Chelon labrosus*). *Journal of Aquatic Products*, 3(1-2): 5-16.

Chamberyon, F. and Zohar, Y. (1990). A diluent for sperm cryopreservation of gilthead sea bream, *Sparus aurata*. *Aquaculture*, 90: 345-352.

Coll, J., Renones, O., Moranta, J. and Morales-Nin, B. (1995). Base study for monitoring the reserve effect in the Cabrera National Park, Balearic Islands. An indicator species example: *Epinephelus guaza* (L.). *Rapp. Comm. Int. Mer Médit*., 34: 240.

Fernández Vilar, A., Gracia López, V. and Castello-Orvay, F. (1993). Efecto de la temperatura en el crecimiento del mero (*Epinephelus guaza*, L. 1758) en cautividad. *EAS Special Publication*, 19: 364.

Fukuhara, O. and Fushimi, T. (1988). Fin differentiation and squamation of artificially reared grouper, *Epinephelus akaara*. *Aquaculture*, 69(3-4): 379-386.

Glamuzina, B., Glavic, N., Kozul, V. and Skaramuca, B. (1998a). Induced sex reversal of the dusky grouper *Epinephelus marginatus* (Lowe, 1834). *Aquac. Res*., 29(8): 563-568.

Glamuzina, B., Skaramuca, B., Glavic, N., Kozul, V., Dulcic, J. and Kraljevic, M. (1998b). Egg and early larval development of laboratory reared dusky grouper *Epinephelus marginatus* (Lowe, 1834) (Pisces, Serranidae). *Sci. Mar.*, 62(4): 373-378.

Kara, M.H. and Derbal, F. (1995). Morphométrie, croissance et mortalité du Mérou *Epinephelus marginatus* (Serranidae) des cotes de l'est algérien. *Cah. Biol. Mar.*, 36: 229-237.

Kohno, H., Ordonio-Aguilar, R.S., Ohno, A. and Taki, Y. (1997). Why is grouper larval rearing difficult?: An approach from the development of the feeding apparatus in early stage larvae of the grouper, *Epinephelus coiodes*. *Ichthyol. Res*., 44(3): 267-274.

Lavens, P., Sorgeloos, P., Jaspers, E. and Ollevier, F. (1991). Larviculture of the greasy grouper (*Epinephelus tauvina* F.) and brown-marbled grouper (*E. fuscoguttatus* F.) in Singapore. Larvi '91. *EAS Special Publication*, 15: 321-322.

Lembo, G., Fleming, I.A., Økland, F., Carbonara, P. and Spedicato, M.T. (1999a). Homing behaviour and site fidelity of *Epinephelus marginatus* (Lowe, 1834) around the island of Ustica: Preliminary results from a telemetry study. *Biol. Mar. Medit*., 6: 90-99.

Lembo, G., Fleming, I.A., Økland, F., Carbonara, P. and Spedicato, M.T. (1999b). Site fidelity of the dusky grouper *Epinephelus marginatus* (Lowe, 1834) studied by hydroacoustic telemetry. *Marine Life* (submitted).

Marino, G., Maricchiuolo, G., Azzurro, E., Massari, A. and Mandich, A. (1998). Induced sex-change of *Epinephelus marginatus*. In: *33rd International Symposium on New Species for Mediterranean Aquaculture*, Alghero (Italy), 22-24 April 1998 (in press).

Shapiro, D.Y. (1987). *Reproduction in groupers*. In: *Tropical Snappers and Groupers: Biology and Fisheries Management*, Polovina, J.J. and Ralston, S. (eds). Westview Press, Boulder, Co, pp. 295-327.

Spedicato, M.T., Contegiacomo, M., Carbonara, P. and Lembo, G. (1998a). Ovarian maturation and spawning in *Epinephelus marginatus* (Lowe, 1834) induced by hormone treatments. In: *33rd International Symposium on New Species for Mediterranean Aquaculture*, Alghero (Italy), 22-24 April 1998 (in press).

Spedicato, M.T., Contegiacomo, M., Carbonara, P. and Lembo, G. (1998b). Induced sex-reversal in the dusky grouper *Epinephelus marginatus* (Lowe, 1834). In: *33rd International Symposium on New Species for Mediterranean Aquaculture*, Alghero (Italy), 22-24 April 1998 (in press).

Spedicato, M.T., Contegiacomo, M., Carbonara, P., Lembo, G. and Boglione, C. (1998c). Artificial reproduction of *Epinephelus marginatus* aimed at the development of restocking techniques. *Biol.*

Mar. Medit., 5(3): 1248-1257.

Spedicato, M.T., Contegiacomo, M., Selmo, C. and Boglione, C. (1998d). First observation on the development and morphology of *Epinephelus marginatus* (Lowe, 1834) larvae obtained by induced spawning. In: *33rd International Symposium on New Species for Mediterranean Aquaculture*, Alghero (Italy), 22-24 April 1998 (in press).

Spedicato, M.T. and Lembo, G. (1996). Formazione di uno stock di riproduttori di *Epinephelus marginatus* (Lowe, 1834): Risultati preliminari. *Biol. Mar. Medit*., 3(1): 430-431.

Spedicato, M.T., Lembo, G., Di Marco, P. and Marino, G. (1995). Preliminary results in the breeding of dusky grouper *Epinephelus marginatus* (Lowe, 1834). *Cah. Options Méditerr.*, 16: 131-148.

Tamaru, C.S., Cholik, F., Kuo, J.C.M. and Fitzgerald, J. Jr. (1995). Status of the culture of milkfish (*Chanos chanos*), striped mullet (*Mugil cephalus*) and grouper (*Epinephelus* sp.). *Review in Fisheries Science*, 3(3): 249-273.

Zabala, M., Gracia-Rubies, A., Louisy, P. and Sala, E. (1997). Spawning behaviour of the Mediterranean dusky grouper *Epinephelus marginatus* (Lowe, 1834) (Pisces, Serranidae) in the Medes Islands Marine Reserve (NW Mediterranean, Spain). *Scientia Marina*, 61: 65-77.