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Larval and juvenile development of dusky grouper *Epinephelus marginatus* reared in mesocosms

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The larval development of the dusky grouper *Epinephelus marginatus* up to the benthic juvenile stage is described in detail to establish a reference for their larval identification. Development is described in terms of ontogenetic changes in morphology, growth, pigmentation, fin structure and skeletal structure. Larvae were reared in mesocosms at a mean temperature of 24·3° C, salinity of 36·5, dissolved oxygen of 6·4 mg l^{-1} and pH of 8·2. Newly hatched larvae had an estimated total length (L_T) of 2·3 mm. On the second day post hatching the yolk was almost fully absorbed with traces of the oil globule still present, the eyes were already pigmented and mouth and gut functional. At this stage the cranial skeletal elements for feeding and breathing (mouth and gills) and the pectoral-fin support were already present. About 50% of the observed larvae had food in their guts. Pigmentation was very characteristic, consisting of two large chromatophores visible on the edge of the primordial fin, close to the midpoint of the post-anal region of the body and over the midgut and hindgut and post-anal portion of the body. At 2·9 mm L_T the emergence of the second dorsal-fin spine, characteristic of the *Epinephilinae*, was clearly visible. The pre-flexion stage started in larva of 3·2 mm L_T. At 5·5 mm L_T the larvae possessed posterior preopercular angle spines, and the dorsal and pelvic spines presented serrated edges and were pigmented. The water surface-tension-related death of the yolk sac and pre-flexion larvae described in the rearing of several other grouper species did not occur during *E. marginatus* culture. Notochord flexion, with initial ossification of the caudal-fin supporting elements, started at 6·6 mm L_T. At this stage the major melanophores, preopercular, dorsal and pelvic spines and mandibular teeth were already present. Transformation of larvae into juveniles occurred when larvae averaged 13·8 mm L_T. Juveniles with a mean L_T of 20·1 mm started to settle and most of them were benthic with a mean L_T of 26·8 mm.

Key words: morphological development; ontogeny; pigmentation; production system; squamation.

INTRODUCTION

The dusky grouper *Epinephelus marginatus* (Lowe 1834) has a wide distribution, ranging from the eastern and south-western Atlantic Ocean to the western Indian Ocean, and the Mediterranean Sea. It constitutes a very important target for commercial fisheries, game fishing and scuba diving. Solitary and territorial

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individuals can be found from shallow waters to 50 m (Heemstra & Randall, 1993). *Epinephelus marginatus* are sequential hermaphrodites, with protogynous (female–male) and monandric (males being the terminal sex) expressions. Female sexual maturity in the wild is attained between 2·5 kg [*c*. 38 cm in total length (L_T) and 5 years old] and 11 kg (*c*. 57 cm L_T and 7 years old) and only large individuals are males with sexual inversion taking place between 10 and 16 years of age at 80–110 cm L_T (Pierre *et al.*, 2008). This species is known to show low resilience and its vulnerability to human exploitation is high owing to their naturally curious nature, interacting with divers making them especially vulnerable to spear fishing. Because of intensive fishing, *E. marginatus* is listed as an endangered A2d ver 3.1 species by IUCN (2012) and artificial propagation will be an important approach to enhance stocks.

Several attempts at artificial reproduction and larval rearing of *E. marginatus* were undertaken by research groups in Italy and Croatia (Glamuzina *et al.*, 1998; Spedicato & Boglione, 2000; Marino *et al.*, 2001; Marino *et al.*, 2003; Russo *et al.*, 2009) but larval rearing is still a bottleneck (Pierre *et al.*, 2008). Available information on early life stages is scarce with Bertolini (1933) reporting planktonic eggs tentatively attributed to the dusky grouper *Epinephelus guaza* (Jordan & Evermann 1896) as well as two juveniles of 53 and 95 mm L_T (*E. guaza* is a synonym of *E. marginatus*). Glamuzina *et al.* (1998) described and illustrated eggs and early larval development of laboratory-reared *E. marginatus* but only until the end of the yolk-sac phase in larvae of 2·63 mm L_T. The early life history is still insufficiently known and this study attempts to fill this gap by describing the growth and the morphological development from larval to demersal juvenile. Based on these descriptions the key changes that define ontogenetic intervals during development are examined and discussed. The aim is to improve larval rearing techniques and to assist the identification of Atlantic and Mediterranean grouper larvae and juveniles.

MATERIALS AND METHODS

Fertilized eggs of *E. marginatus* were obtained from a captive broodstock maintained for 5 years in two indoor tanks of 10·6 m^3 each and at a mean density of 4·0 kg m^{-3} at the facilities of the Aquaculture Research Station (EPPO), Portuguese Institute for the Sea and Atmosphere (IPMA), Olhão, Portugal. A diet of fresh and frozen Patagonian squid *Loligo gahi* and European pilchard *Sardina pilchardus* (Walbaum 1792) were hand-fed *ad libitum* once a day. Water temperature ranged between 12° C in winter and 25° C in summer. During the spawning period the temperature range was 22·5° C + 0·5° C. Females were hormonally induced to spawn and males were obtained from hormonally sex inverted juveniles. Eggs were collected in sterile plastic containers and transferred to 200 l cylinder-conical fibreglass incubation tanks with a flow-through of sea water filtered through sand and 100 µm polypropylene filter bags (Pentek BPHE-420-1.5 Bag Filter System; www.pentekfiltration.com), UV sterilized ($T = 21°$ C, pH = 8·0 and salinity = 37·5) and gently aerated (Cunha *et al.*, 2009).

Semi-extensive mesocosm experiments were performed outdoors in shaded 3 m^3 circular tanks during July and August 2008 as described in Cunha *et al.* (2009). Sea water was filtered through a 500 µm plankton mesh to seed the tanks with natural plankton and eliminate potential predators. Food abundance was adjusted by daily addition of enriched rotifers (*Brachionus* sp.) initially, and later newly hatched and enriched *Artemia* sp. and dry feed, as detailed by Cunha *et al.* (2009) (Fig. 1). Water temperature ranged from 21·6 to 24·9° C, with

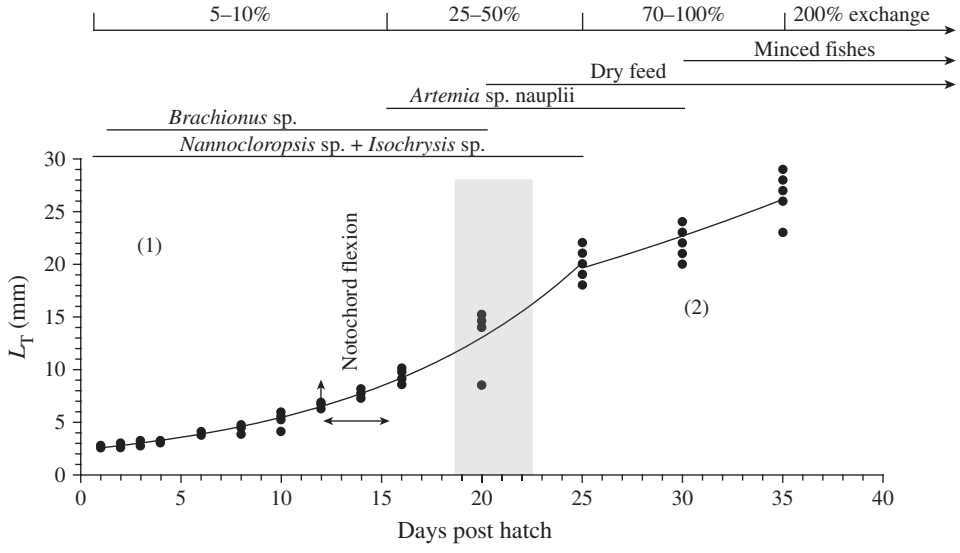

FIG. 1. Semi-extensive rearing of *Epinephelus marginatus* (water exchange and feeding schedule) and larval growth (in total length, L_T) during the rearing experiment. Exponential lines and regression coefficients are given (1; $y = 2{\cdot}296e^{0{\cdot}087x}$; $r^2 = 0{\cdot}987$, $n = 114$) before metamorphosis and (2; $y = 9{\cdot}618e^{0{\cdot}029x}$; $r^2 = 0{\cdot}752$, $n = 31$) during transformation. Shaded area corresponds to the transition period of metamorphosis.

an average of 24·3° C, salinity from 36·7 to 37·7 and dissolved oxygen from 4·3 to 8·2 mg l^{-1} (mean = 5·9 mg l^{-1}).

In order to describe the different developmental stages, samples of 10 individuals were collected daily during the first 4 days, every second day until day 16 and every 5 days until day 35. Larval age (A) is given in days post hatching (dph) and in growing degree day (G_{DD}, °C-day). This last age was calculated according to the study of Neuheimer & Taggart (2007) as the time integral of the mean daily water temperature measured above the 0° C using the formula: $G_{DD} = T_{AVG}\ A$, where T_{AVG} was the mean rearing temperature (24·3° C).

Meristics and morphometric data on larvae were based on 137 specimens. Photographic records of live specimens and measurements were made to the nearest 0·01 mm under a stereoscopic microscope (Carls Zeiss Stemi 2000C; http:/microscopy.zeiss.com). L_T and standard length (L_S) were measured from the tip of the upper jaw to the posterior margin of the caudal-fin and from the tip of the snout to the end of the notochord. For ossification and squamation analysis, 4% buffered formaldehyde preserved specimens were stained with alizarin red S and cleared with 2% potassium hydroxide as described by Gavaia *et al.* (2000), prior to observation and photography. The diagrams were drawn over pictures of stained fish using Paint Shop Pro 9.0 (Jasc Software; www.corel.com) and represent an average stage for the age class.

Stained fish were also used to calculate the average mouth opening (M_O) for each age class. M_O values are equivalent to the distance between the foremost points of the upper and lower jaws at an opening angle of 50° according to Shirota (1970) and Dabrowski & Bardega (1984) and were calculated using the equation: $M_O = \tan(50^\circ)L_M$, where L_M was the length of the lower mandible measured on the stained specimens.

The terminology and stages used for the description of the larval development of *E. marginatus* follow the study of Kendall *et al.* (1984). In this article, length refers to L_T, unless otherwise stated. Simple linear regression using least squares was used to draw the best fit line between larval L_T and age (dph) and correlation analysis used to assess the strength of association.

RESULTS

GROWTH AND DEVELOPMENT OF LARVA AND JUVENILES

Epinephelus marginatus show an indirect ontogeny with rapid larval development characterized by changes in their size and morphology. Growth from hatching until 35 dph occurs in two different phases demarcated by metamorphosis (Fig. 1). Each could be adequately described from the exponential regression of L_T at age (L_{TD}, mm) and A (dph): $L_{Tt} = L_{T0}e^{Gt}$, where L_{Tt} is the total length at age t, G is the instantaneous daily growth coefficient and L_{T0} is the total length at hatching. The two best fitted regression equations (d.f. = 113 and 31, both $P < 0{\cdot}001$) corresponded to two different growing periods: the first (1) with a rate of growth of *c.* 9% day^{-1} and an intercept of 2·3 mm corresponded to the larval stage, and the second (2) with a slower growth rate of *c.* 3% day^{-1} and an intercept of 9·6 mm was the juvenile stage. The intercept of the first equation is the L_{T0} (2·3 mm).

The main ontogenetic and morphological characters of larvae and juveniles are described in Table I and Fig. 2 illustrates several of the stages. The first phase of larval stages is the yolk-sac larva, defined as development until the complete absorption of the yolk sac and oil globule [Fig. 2(a), (b)]. The yolk sac was the first to be absorbed and its exhaustion coincided with the opening of the mouth and anus. After complete absorption of the oil globule, the larvae started on an entirely exogenous diet. This is the beginning of the pre-flexion stage [Fig. 2(c)–(f)]. The flexion stage [Fig. 2(g), (h)] started with the formation of the hypuralia and the upward bending of the posterior portion of the notochord. The proportion between L_S and L_T changed progressively from 95% in larvae before flexion to 82% after larval transformation. The post-flexion stage [Fig. 2(i)] starts when the posterior portion of the notochord flexion is completed (at an angle close to 45°), the end of caudal-fin is truncated and the caudal complex starts to be formed. This stage is defined as the period until the completion of the adult fin-ray count. During this period the continuous finfold disappeared and anal spines and the pectoral-fin rays appeared. During transformation the number of fin rays is complete though there were individual variations on the ray counts [Fig. 2(j)]. After completion of fin structures, the juvenile stage begins with an increase of body length, pigmentation and changes in life style [Fig. 2(k), (l)]. The process of settling occurs in juveniles when the external melanistic pigmentation is already evident [Fig. 2(k)]. Demersal juveniles became bottom dwellers and have adult-like appearance [Fig. 2(l)]. During this phase both pelagic and demersal juveniles coexist.

DEVELOPMENT OF THE FIN RAYS

The pectoral-fin spines and rays are the first appendicular structures to develop, followed by the spiny dorsal and spiny pelvic fins, almost simultaneously, and later by the caudal-fin, soft dorsal and soft anal simultaneously, and finally the spiny anal. Development of pectoral fins started very early and they are already visible in yolk-sac larvae of 2·7 mm, whereas the transforming larvae already showed the full adult complement of fin rays. The typical Epinephelini kite-shaped larvae resulted from the presence of large second dorsal and pelvic spines that start to develop early in pre-flexion larvae. At the end of flexion stage, the length of these spines attained proportions to the larva L_T that are >40% [Fig. 3(a)]. After this stage the

TABLE I. Meristics and main ontogenetic characters of larval and juvenile *Epinephelus marginatus* reared at a mean temperature of 24° C and based on live specimens. Age is given in days post hatching (dph) and growth degree days (°C-day)

Stage	Age		L_T, mean ± S.D. (mm)	Main morphological and ontogenetic characters
	dph	°C-day		
Yolk sac	0	12	2·5 ± 0·3	Large yolk sac with a single oil globule; large granular cells distributed over the body; eyes transparent; narrow straight gut
	1	25	2·7 ± 0·1	Similar to above but reduced yolk sac; visible digestive track; anus closed [Fig. 2(a)]
	2	48	2·7 ± 0·2	Yolk almost fully absorbed; eyes pigmented; mouth open; visible pectoral-fin; evident digestive glands and urinary bladder; distinct intestine and rectum owing to the presence of intestinal-rectal valve; cap of melanophores over the dorsum of the gut; band of melanophores encircling the myomeres close to the midpoint of the post-anal region [Fig. 2(b)]
	3	71	2·9 ± 0·2	Oil globule still present; cleithra clearly visible; distinct nostrils; beginning of digestive tract rotation and presence of food in the gut; emergence of dorsal-fin spine with the anlage protruding from the myomeres into the fin fold
Pre-flexion	4	95	3·2 ± 0·1	Lipid droplet completely resorbed; larvae start a completely exogenous diet; digestive track almost fully rotated; emergence of pelvic spine complex bud [Fig. 2(c)]
	6	141	3·9 ± 0·1	Dorsal and pelvic-fin buds clearly visible with tips of incipient second dorsal and pelvic-fin spines pigmented [Fig. 2(d)]
	8	188	4·5 ± 0·3	Kite-like larva with elongate second dorsal and pelvic-fin spines [Fig. 2(e)]
	10	237	5·5 ± 0·5	Emergence of posterior preopercular angle spines characteristic of Epinephelinae; serrated and pigmented dorsal and pelvic spines; visible first dorsal spine and emergence of the third dorsal spine; presence of haemoglobin on the heart and xanthophores over the cap of melanophores on the gut [Fig. 2(f)]

TABLE I. continued

Stage	Age dph	Age °C-day	L_T, mean ± S.D. (mm)	Main morphological and ontogenetic characters
Flexion	12	284	6·6 ± 0·2	Beginning of notochord flexion with initial ossification of the caudal-fin supporting elements; conical teeth present; visible third dorsal spine; appearance of the second preopercular spines; emergence of dorsal and anal-fin rays; emergence of chromatophores on the midbrain and cleithral symphysis [Fig. 2(g)]
	14	333	7·6 ± 0·3	Evident flexion of the notochord; emergence of fourth dorsal spine [Fig. 2(h)]
Post-flexion	16	382	9·2 ± 0·6	Urostyle at maximum angle (45°) with the axis of the notochord; posterior part of caudal-fin truncated; disappearance of the continuous fin fold; differentiation of each unpaired fins; formation of pectoral-fin rays; emergence of fifth dorsal spine; emergence of first and second anal-fin spines [Fig. 2(i)]
Transforming	20	481	13·8 ± 2·4	Complete fin structures with X + I dorsal spines and 16 rays in the dorsal-fin, II + I anal spines and nine rays in the anal-fin and 18 pectoral-fin rays [Fig. 2(j)]
Pelagic juvenile	25	604	20·1 ± 1·2	Complete fin structures with XI dorsal spines and 15 rays in the dorsal-fin, III anal spines and nine rays in the anal-fin and 18 pectoral-fin rays; melanistic external pigmentation on the upper surface of the head and a line along the base of dorsal-fin
Settling juvenile	30	727	21·7 ± 1·3	Overall melanistic external pigmentation, squamation forming anteriorly [Fig. 2(k)]
Demersal juvenile	35	849	26·8 ± 1·8	Settled juveniles with barred pattern pigmentation and full squamation coverage with complete lateral line [Fig. 2(l)]

L_T, total length.

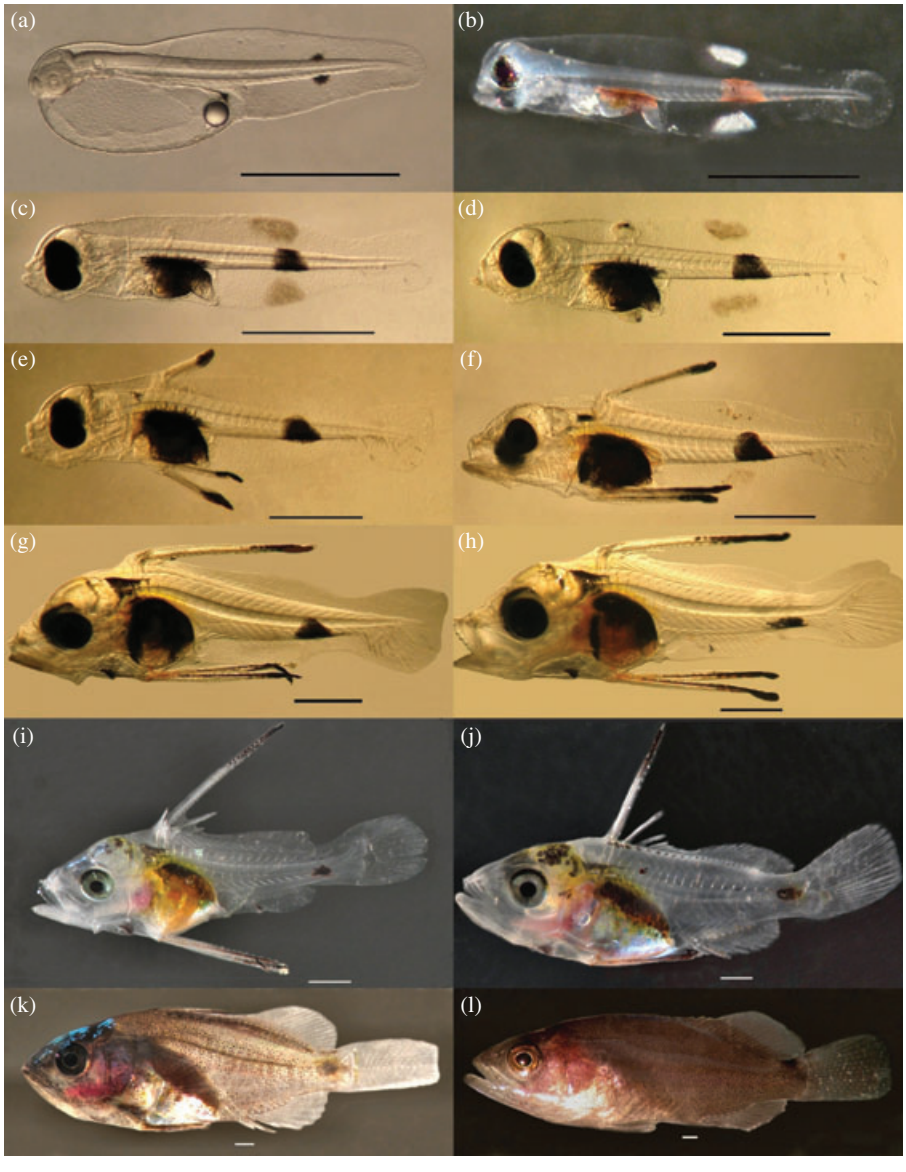

FIG. 2. Different larval stages of *Epinephelus marginatus*. Yolk sac: (a) newly hatched 1 and (b) 2 days post hatching (dph). Pre-flexion: (c) 4, (d) 6, (e) 8 and (f) 10 dph. Flexion: (g) 12 and (h) 14 dph. Post-flexion: (i) 16 dph. Transforming: (j) 20 dph. Juveniles: (k) settling 30 dph and (l) demersal 35 dph. Scale bars 1 mm.

proportion between the length of the second dorsal spine and the length of the pelvic spine remains constant with the second dorsal being slightly longer, up to 20% [Fig. 3(b)]. The first and third dorsal spines are already visible in late pre-flexion larvae. The fourth dorsal spine is visible in late flexion larvae. Transforming larvae possess the adult complement of 10 dorsal-fin spines. Soft caudal-fin rays appear in late

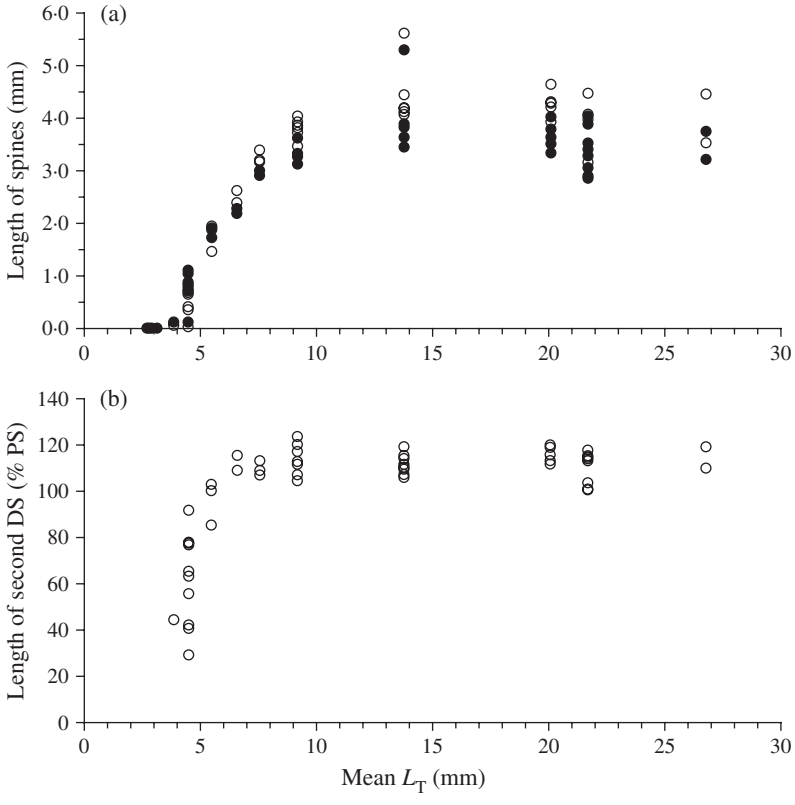

FIG. 3. Changes in length of the second dorsal spine (DS) and pelvic spines (PS) of *Epinephelus marginatus* larvae with growth: (a) relative length to larva total length (L_T) of the second dorsal spine (○) and the pelvic spine (●) and (b) proportion between the lengths of the two spines.

pre-flexion larvae followed by soft dorsal and soft anal-fin rays that are visible in flexion larvae of 6·6 mm. Post-flexion larvae of 9·2 mm already possess the adult complement of eight superior and seven inferior principal rays in the soft caudal-fin. The adult complement of soft dorsal-fin rays (15–16) and soft anal-fin rays (9) are present only in transforming larvae of 13·8 mm and larger. The spiny anal-fin is the last to develop being clearly visible in larvae of 9·2 mm. Pelagic juveniles have complete fin structures with XI dorsal spines and 15 rays in the dorsal-fin, III anal spines and nine rays in the anal-fin and 18 pectoral-fin rays.

PIGMENTATION

Head

Most newly hatch larvae display pairs of small punctuate melanophores over the snout, nostril and frontal lobes. Some show a few branched melanophores over the auditory sac. These melanophores disappear with age and there is almost no pigmentation in the head during most of the pre-flexion stage. Eyes appear silvery since their formation in yolk-sac larvae of 2·7 mm. At 4·5 mm, pre-flexion larvae show a small

blotch of internal melanophores over the posterior part of the hindbrain that increases with age. Stellate melanophores appear over the midbrain at the beginning of flexion in larvae of 6·6 mm and increase in number with age forming a cap in post-flexion larvae, whereas the internal melanophores extend ventrally in the nape region in transforming larvae. Melanophores appear for the first time over the operculum in transforming larvae and over the snout and preorbital in early juveniles of 20·0 mm.

Colour appears for the first time on the head region as a pale red (haemoglobin) below the operculum and on the branchial arcs at the end of pre-flexion in larvae of 5·5 mm. Yellow pigmentation (xanthophores) follows and is present in the dorso-posterior surface of the head at the beginning of flexion and with larval development extends ventrolaterally to the eye and operculum, which show a faint silvery shine for the first time in transforming larvae of 13·8 mm. During the juvenile stage the melanophores increase in number and spread onto the head until it is completely covered with melanophores, obscuring the colour pigmentation, with the exception of a red colouration under the operculum.

Pre-anal region

Newly hatched larvae show a small internal cluster of melanophores on the posterior surface of the yolk sac and under the primordial digestive track that increases in size during development. At 2·7 mm they form an internal dorsal shield over the midgut and hindgut gradually expanding upwards along the myosepta and ventrolaterally until completely enveloping the digestive organs in larvae of 5·5 mm. In older larvae the area enveloped by melanophores reduces gradually forming a posteriodorsal melanistic cap over the peritoneum in larvae of 9·2 mm. This shield is clearly visible until external melanistic pigments obscure it during the juvenile stage. Larvae start to acquire internal melanophores on the dorsolateral myomeres posteriorly to the cleithrum and dorsally to the notochord. With development, melanophores extend anteriorly to the perineural sheath and hindbrain, and ventroposteriorly along the perineural sheath during later stages.

First formation of yellow and reddish pigmentation in the pre-anal region occurs in kite-shaped larvae. The yellow pigmentation appears as a film of colour covering the gut. Xanthophoric pigmentation (yellow and orange) spreads lateroventrally covering the entire peritoneal shield in flexion larvae, whereas anteriorly to the gut, the erythrophoric pigmentation (red) becomes more vivid. In post-flexion larvae the yellow pigmentation over the gut spreads towards the head and red pigmentation defines the dorsal vein and aorta on the haemal side of the vertebral column. Silvery colour starts to appear in the ventrolateral region of the peritoneal shield in post-flexion and transforming larva and in early juveniles the outer surface of the peritoneum is totally silver. The colour pigmentation is concealed by external melanistic pigmentation in juveniles $> 21{\cdot}7$ mm.

Post-anal region

At mouth opening the melanophores form a half-circle with the widest margin close to the edge of the primordial fin and some scattered melanophores in the ventral side of the caudal peduncle. As the larvae grow there is a progressive reduction of the dorsal extension of melanophores from the caudal patch that attains its most ventral position at the end of flexion stage. During transformation there is a ventrolateral

reduction of the extension of the caudal patch and upward migration of melanophores so that in transforming larvae the caudal patch is over the midline of the body on the caudal peduncle. Rows of melanophores along the base of dorsal-fin spines and rays appear in transforming larva. These larvae also show an internal row of melanophores along the dorsal side of the spinal chord. Early juveniles start showing external pigmentation with larger concentration of stellate chromatophores close to the dorsal and anal fins and to the caudal peduncle.

Orange chromatophores appear for the first time along the haemal side of the vertebra at the end of flexion stage. In post-flexion larvae they show as a row along the ventral side, close to the insertion of the soft anal-fin. These pigments become more abundant in larger larvae. Yellow pigmentation appears in transforming larvae surrounding the melanistic pigment of caudal patch and, in early juveniles, over the base of the caudal-fin rays and internally along the dorsal surface of the vertebral column. Internal and colour pigmentation as well as the caudal peduncle patch are obscured by musculature and external pigmentation in juveniles of 21·7 mm, with some specimens already showing the barred pattern found on larger juveniles and adults.

Fins

At hatching, *E. marginatu*s larvae possess two small patches of melanophores on the primordial fin. These patches are located on the posterior part of the body below and above the notochord. At the same body location and on the outer portion of the primordial fin, two other patches produce a white hue under white light. The colouration suggests the presence of leucophores on larvae as early as 1 dph. This pigmentation disappeared gradually during the pre-flexion stage. Larvae at the beginning of flexion do not possess such patches of chromatophores on the primordial fin. Yolked larvae at mouth opening show scattered melanophores on the trabeculae of the ventral part of the primordial caudal-fin. The number of pigmented trabeculae on the ventral side of the caudal-fin increases with age, highlighting some principal caudal rays at the beginning of flexion. As the caudal-fin develops, these melanophores are reduced in number and appear on the caudal-fin membrane in transforming larvae and early juveniles. A faint yellow pigmentation starts to appear over the base of the principal caudal rays in larvae undergoing transformation, whereas early juveniles show a continuous band that extends posteriorly into the intraradial membrane that displays some orange chromatophores. Melanistic pigmentation obscures this pigmentation in settling juveniles.

Melanistic pigmentation appears on the fleshy tips of incipient second dorsal and pelvic spines in 3·9 mm larvae and tints the flag-like fin membrane at the distal end of the spines in later larvae. With development there is a proximal extension of pigmentation, more intense in the pelvic spines. Orange chromatophores appear for the first time at the base of pelvic spines in larvae with a pelvic spine (L_{PS}) L_T proportion ($L_{PS}\ L_T{}^{-1}$) > 23%, *i.e.* in larvae > 4·5 mm. This orange pigmentation extends distally with development forming orange patches among the melanistic pigmentation in older larvae. The second dorsal spine shows orange chromatophores close to the flag-like fin membrane and immediately along the spinous process just before flexion. Yellow appears at the base of the three first dorsal spines in post-flexion larvae and transforming larvae have a row of melanistic pigmentation at the insertion of dorsal spines and rays. The incipient second anal spine is marked by a pigmented patch during flexion in larvae of 7·6 mm. These larvae also show a

row of orange chromatophores at the insertion of the anal rays. With development both, the second and first anal spines, become pigmented with intercalated patches of xanthophores and melanophores. Melanistic pigmentation obscures this pigmentation in settling juveniles.

SQUAMATION

The scales are dermal structures composed of a mineralized tissue formed by cells similar to those in bone. These structures are formed late in development, after metamorphosis. Scales were first observed in specimens > 20 mm as a small patch located posterior to the head, and dorsally to the pectoral-fin partially covering the abdomen [Fig. 4(a)]. In juveniles of 20 mm the modified scales that compose the lateral line and were under initial differentiation start to appear on a discontinuous pattern posterior to the dorsal portion of the cleithrum. Juvenile specimens of 22 mm show a squamation coverage extending ventrally and posterior to the middle portion of the body. The lateral line also extends posteriorly, maintaining a discontinuous pattern [Fig. 4(b)]. At 27 mm the squamation extends posteriorly and ventrally under the pectoral fins and a small patch of scales is visible on the head covering the skull [Fig. 4(c)]. The lateral-line scales now form a continuous row, extending almost to the base of the caudal peduncle. A complete squamation pattern was observed in juvenile individuals at 35 mm (50 dph), which display a full body covered with scales and a clearly differentiated lateral line extending from the posterior portion of head to the caudal-fin (Fig. 5).

DISCUSSION

NORTH-EASTERN ATLANTIC OCEAN AND MEDITERRANEAN SEA EPINEPHELINAE EARLY LIFE HISTORIES

From the six species of *Epinephelinae* found in the north-eastern Atlantic Ocean, four belong to the genus *Epinephelus*, the white grouper *Epinephelus aenaeus* (Geoffroy Saint-Hilaire 1817), the dogtooth grouper *Epinephelus caninus* (Valenciennes 1843), the goldblotch grouper *Epinephelus costae* (Steindachner 1878) and *E. marginatus*, and two to the genus *Mycteroperca*, the island grouper *Mycteroperca fusca* (Lowe 1838) and the mottled grouper *Mycteroperca rubra* (Bloch 1793) (Heemstra & Randall, 1993). For these species, descriptions were found only for early development stages of *E. marginatus* (Glamuzina *et al.*, 1998; Dantart *et al.*, 1999; Spedicato & Boglione, 2000; Marinaro *et al.*, 2005) and *E. costae* (Glamuzina *et al.*, 2000) although *E. aenaeus* has been reared artificially in Israel for some time (Koven *et al.*, 2007).

The eggs and early larvae of *E. costae* are comparatively larger than those of *E. marginatus*. There are differences in L_T of *E. marginatus* larva at the absorption of the oil globule between experiments, but the larvae reported in this work were larger. It was briefly referred to by Boglione *et al.* (2009) that scales in *E. marginatus* were present in specimens after 18 mm. No other reference to location or developmental progression of squamation development, however, was made. Spedicato & Boglione (2000) could not find any signs of squamation in larvae of 14·7 mm 51 dph. According

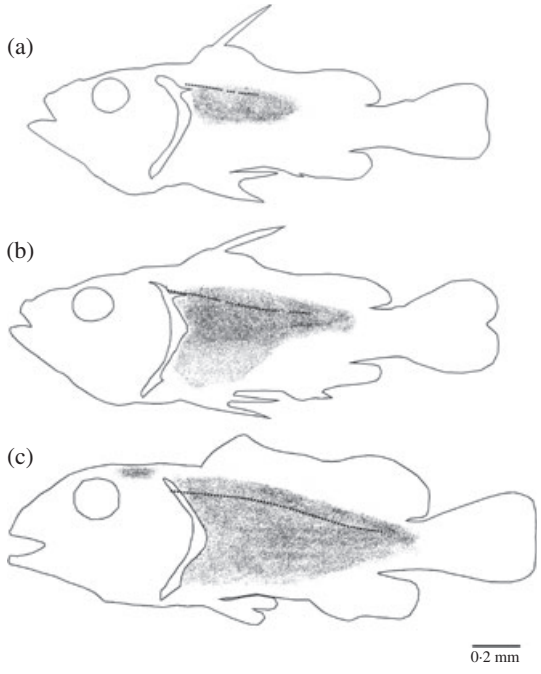

FIG. 4. Diagrammatic representation of squamation in juvenile *Epinephelus marginatus*: (a) 25, (b) 30 and (c) 35 days post hatching (**.....**, lateral line).

to this work, larvae of this size were still transforming and therefore it is not to be expected that they would possess scales. These results are in agreement with that observed for *E. fasciatus* development where the first appearance of scales was observed in specimens between 17 and 20 mm (Kawabe & Kohno, 2009). In the leopard grouper *Mycteroperca rosacea* (Streets 1877) squamation appeared only in individuals of 24·7 mm and at 30 mm the entire body was covered with scales (Martinés-Lagos & Gracia-López, 2009).

ONTOGENETIC INTERVALS AND LARVAL REARING TECHNIQUES

Early development of *E. marginatus* has two periods with different growth rates, the larval and the juvenile stages (Fig. 6). The larval stage, from first feeding to the end of metamorphosis, has a higher daily increase in L_T than the juvenile stage. Larval development is characterized by changes in size and morphology from which stout, elongate, serrate and pigmented dorsal and pelvic spines are the main characteristics (Kendall, 1984; Leis, 1987). After completion of fin structures, the juvenile stage occurs The growth rate reduces while squamation, pigmentation and changes in life style take place.

The first phase of larval stage is a yolk-sac larva, defined as the development until the complete absorption of the yolk sac and oil globule. It is a very characteristic larva with three small patches of chromatophores, one on the posterior surface of the yolk sac and below the primordial digestive tract, and the other two located on

the primordial fin above and below the notochord and on the posterior quarter of the body. The yolk sac was the first to be absorbed and its exhaustion coincided with the opening of the mouth and anus. The larvae then start to feed exogenously, but the diet is mixed since the oil globule still nourishes the larva. The mouth has a very small opening (mean ± s.d. 216 ± 200 μm). Based on the calculated mouth opening the predicted food size [assuming a length:size of prey *v*. gape of 0·25–0·5 as appropriate (Shirota, 1970; Fernández-Díaz *et al.*, 1994; Busch, 1996; Munk, 1997)] should be between 55 and 110 μm. These values are much lower than the size range of rotifers used in aquaculture (Conceição *et al.*, 2010). It was during the late yolk-sac phase that the eyes became pigmented and the larva acquired part of the pigmentation pattern that characterizes most Epinephelini larva. These are the clusters of melanophores forming a dorsal shield over the gut and the cluster on the ventral surface of the tail (Powell & Tucker, 1992; Sawada *et al.*, 1999; Baldwin *et al.*, 2000). In the case of *E. marginatus*, this last cluster surrounds the caudal peduncle. At this stage *E. marginatus* larvae disappear from the upper layers, suggesting negative phototactic behaviour. This is the opposite from that described for several yolk-sac larvae from the *Epinephelus* genus such as blacktip grouper *Epinephelus fasciatus* (Forskål 1775) (Kawabe & Kohno, 2009), convict grouper *Epinephelus septemfasciatus* (Thunberg 1793) (Tsuchihashi *et al.*, 2003), longtooth grouper *Epinephelus bruneus* Bloch 1793 (Sawada *et al.*, 1999), Hong Kong grouper *Epinephelus akaara* (Temminck & Schlegel 1842) (Yamaoka *et al.*, 2000) and greasy grouper *Epinephelus tauvina* (Forskål 1775) (Lim, 1993) that tend to get trapped at the water surface of the larval tank. The heavy larval mortality reported in several grouper species by authors such as Sawada *et al.* (1999), Yamaoka *et al.* (2000) and Kawabe & Kohno (2009) as occurring at this stage did not occur with *E. marginatus*.

The pre-flexion stage occurs after complete absorption of the oil globule. The larvae start a completely exogenous diet with mouth openings 400 ± 24 μm (mean ± s.d., $n = 5$) and the intestine fully rotated. *Epinephelus marginatus* share a similar gut development with orange-spotted grouper *Epinephelus coioides* (Hamilton 1822) at the onset of feeding (Quinitio *et al.*, 2004), though it seems to

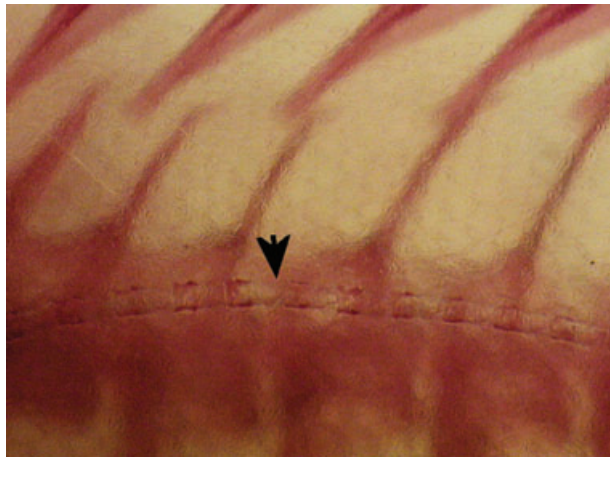

FIG. 5. Detail of the lateral line in a juvenile 50 days post hatching.

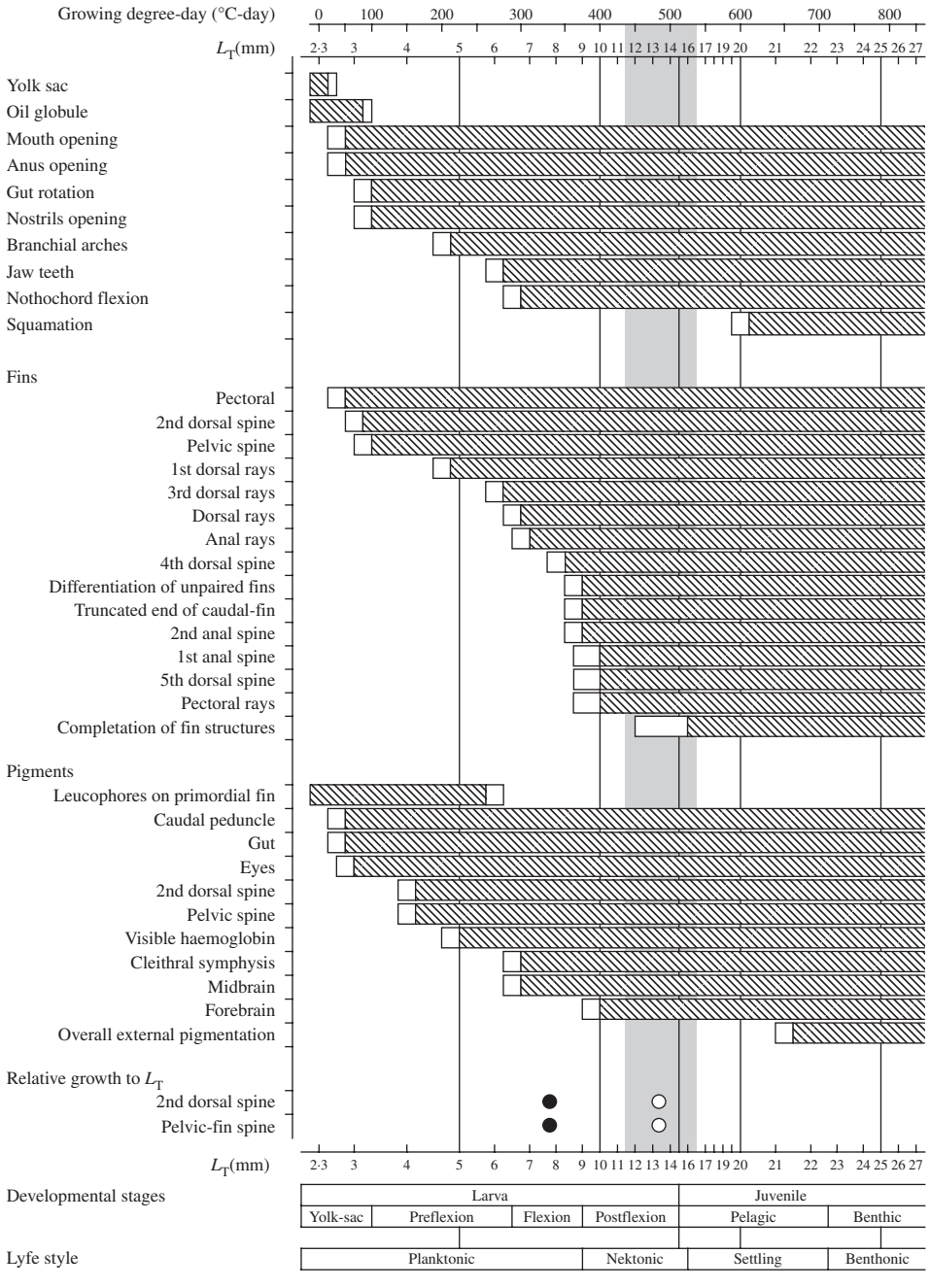

FIG. 6. Schematic representation of the development of selected morphological characters and ontogenetic events during larval rearing in *Epinephelus marginatus*: □, individual variability for specific event or character; ▨, no individual variability for specific event or character; ●, peak value of body proportion in relation to total length (L_T); ○, attainment of constant values in relation to L_T; ▩, period of metamorphosis.

develop much faster owing to the higher growth observed in *E. marginatus*. It is important at this time that the larvae have food particles available ranging from 100 to 200 μm. After spending most part of the pre-flexion stage away from the surface the larvae reappeared at the surface of the tanks as kite-like larvae. During the pre-flexion period, branchial arches and the second dorsal and pelvic spines start to develop along with melanophores on their tips. Haemoglobin is perceptible in the branchial arches. Major spines such as the first and third dorsal spines, the spinelets on the second dorsal and pelvic-fin spines, the posterior preopercular angle spine and the jaw teeth start to develop. The mouth opening is 576 ± 53 μm (mean $\pm$ s.d., $n = 4$) allowing the larvae to feed on particles ranging from 144 to 288 μm. Rapid increase of jaw size and mouth opening allows increasing size of live prey, and it is possible to feed the larvae with rotifers at this time. As larvae are still small they exhibit short swimming time, mainly moving forward and with a tendency to gather in small patches.

The flexion stage starts with the formation of the hypuralia and the upward bending of the posterior portion of the notochord. The second dorsal and pelvic spines grow in length attaining a peak during this stage; dorsal and anal-fin rays start to form and the differentiation of the unpaired fins begins. The larvae change from limited forward movement to active swimming. Example are shown by *E. fasciatus* (Kawabe & Kohno, 2009), honeycomb grouper *Epinephelus merra* Bloch 1793 (Sazaki *et al.*, 1999) and *E. coioides* (Narisawa *et al.*, 1997), when the larvae drift in the water the second dorsal and pelvic spines are spread, but when they swim quickly the fin spines are folded down. According to Kawabe & Kohno (2009) this could indicate that the long fin spines play a role in maintaining position in the water column rather than having a function in swimming. Besides their role in the maintenance of buoyancy, the elongate spines also act as an antipredator mechanism (Moser, 1981). The mouth opening is now 1337 ± 86 μm (mean $\pm$ s.d., $n = 5$) accepting food particles of 334–669 μm. It is necessary at this phase to feed the larva with plenty of rotifers and *Artemia* sp. nauplii owing to their rapid increase in food requirements. The results from Russo *et al.* (2009) indicate that after 7·9 mm the limiting mechanical factor for prey ingestion is the mouth. The authors did not state the larval stage at that L_T but this work indicates that it should be at the end of flexion. Therefore, it seems that after flexion, the larvae are able to eat other supplied food particles, including *Artemia* sp. nauplii.

When the posterior portion of the notochord flexion is complete (at an angle *c*. 45°), the caudal complex starts to be formed, the end of caudal-fin is truncated and the larvae enter the post-flexion stage. This stage ends when the fin-ray count is similar to that of the adult. During this stage the continuous fin fold disappears and anal spines and the pectoral-fin rays appear. Second dorsal and pelvic spines cease growth and attain constant values in relation to body length during this stage. The melanophores on the caudal peduncle, so characteristic of Epinephelini, are now at the midpoint after migrating from a more ventral position. The larvae in the mesocosm swam actively pursuing food, although some remained close to the tank edge. They ate mainly *Artemia* sp. nauplii because most of the other food particles were too small for their needs. It is very important at this stage to increase the amount of enriched *Artemia* sp. nauplii owing to larval growth requirements and to prevent high mortalities observed during the transformation from larvae to juveniles.

During transformation the number of fin rays is complete although there are individual variations in ray counts. Major body proportions in relation to L_T attain peaks and the number of melanophores increases. Most larvae swim actively during the day and at night formed schools near the tank edge. Gradual acclimation to feeding on formulated diet should be started at this phase as *Artemia* sp. nauplii seem to be too small for larval requirements.

During the juvenile stage, lengths of the second dorsal and pelvic spines become shorter relative to the body length, the major head spines disappear and the external melanistic pigmentation develops further. Pelagic juveniles do not have external melanistic pigmentation and the scales start to appear. Scales are involved in protection, serve as a calcium deposit and simultaneously with pigmentation are strongly correlated to changes in behaviour of juveniles (Fukuhara, 1988; Fukuhara & Fushimi, 1988; Pinto *et al.*, 2009). In this work, scales were first observed in specimens >20 mm. Kawabe & Kohno (2009) recommend the use of frequent and sufficient feeding on formulated feed, because fish species show a greater stress endurance than those not fed on formulated diet. When the length of second dorsal and pelvic-fin spines shortens and the external melanistic pigmentation becomes more evident, the juveniles start the process of settling. This is similar to that described for several *Epinephelus* species such as *E. fasciatus* (Kawabe & Kohno, 2009), *E. akaara* (Fukuhara & Fushimi, 1988) and *E. tauvina* (Hussain & Higuchi, 1980). Settlement occurs and demersal juveniles become bottom dwellers. During this phase both pelagic and demersal juveniles coexist. As demersal juveniles already possess great tolerance to handling and to changes in the water temperature (Fukuhara & Fushimi, 1988), it is advisable to reduce stock density by size grading to avoid cannibalism.

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