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Larval development of *Gobius xanthocephalus* with genetic validation of larval identification

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Larval development of *Gobius xanthocephalus* with descriptions of the ontogeny of the main structures, changes in pigmentation patterns and allometric relationships are presented for the first time. No previous descriptions of *G. xanthocephalus* larvae are available and therefore field collected larvae were compared with larvae obtained under controlled conditions. The DNA of adults, juveniles and larvae was compared in order to validate identifications. The overall developmental sequence obtained agrees with those described for other gobies. Pigmentation patterns were, however, distinct from those of other sympatric Gobiidae allowing a clear identification of *G. xanthocephalus* larvae. Otolith microstructure analysis revealed a linear age and body length relationship, with an estimated larval growth rate of 0·28 mm day^{-1}.

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Key words: DNA validation; *Gobius xanthocephalus*; larval description; ontogenetic development.

INTRODUCTION

Gobius xanthocephalus Heymer & Zander is one of the most common gobies found along the Portuguese continental western and southern rocky shores (Henriques *et al.*, 1999). Heymer & Zander (1992, 1994) found this species on the French Mediterranean coast and have suggested the Canary Islands and Ria de Arosa (north-west Spain) as other probable locations.

Gobius xanthocephalus has long been misidentified as *Gobius auratus* Risso and *Gobius luteus* Kolombatovic until Heymer & Zander (1992) clarified this issue. Almeida & Arruda (1998) and Henriques *et al.* (1999) confirmed that fish previously described as *G. auratus* in Portuguese waters were actually *G. xanthocephalus*. The morphology and ecology of the adults has been studied (Heymer & Zander, 1992, 1994; Almeida & Arruda, 1998; Beldade *et al.*, 2006) although the larval stages have not been described.

The extent of the breeding season of *G. xanthocephalus* is not known. Nevertheless, in Portuguese waters, breeding has been observed by divers in May and

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juveniles have been seen recruiting from May to October (unpubl. data). Like other gobiid species, it spawns demersal eggs, which are laid in a single layer under stones and shells and are guarded by the male.

Besides *G. xanthocephalus*, there are five other *Gobius* species that breed at the Arrábida Marine Park (Henriques *et al.*, 1999): the giant goby *Gobius cobitis* Pallas, the black goby *Gobius niger* L., the rock goby *Gobius paganellus* L., the red-mouthed goby *Gobius cruentatus* Gmelin and the Steven's goby *Gobius gasteveni* Miller. Descriptions of larval development are available only for the first three species: *G. cobitis* (Spartà, 1950; Gil *et al.*, 1997), *G. niger* (Lebour, 1919; Petersen, 1919; Ballard, 1969; Iglesias, 1979) and *G. paganellus* (Hefford, 1910; Lebour, 1919; Spartà, 1934; Borges *et al.*, 2003). The larvae of *G. cruentatus* have only been described at hatching (Gil *et al.*, 2002), and there are no available data on larval stages of *G. gasteveni*.

The correct identification of eggs and larvae is a prerequisite for ecological and taxonomic studies of the pelagic stage of fishes (Leis & Rennis, 1983; Powles & Markle, 1984; Leis & McCormick, 2002). Given the difficulty in distinguishing larvae of related species from plankton collections, more detailed descriptions are in great need.

In this study, larval development of *G. xanthocephalus* is described for the first time, based on plankton collections. Newly hatched larvae under controlled conditions and DNA analysis were used to validate identifications. The age and body length relationship and larval growth rate were determined from otolith microstructure analysis.

MATERIALS AND METHODS

LABORATORY REARED LARVAE

Three pairs of adult fish were collected at the Arrábida Marine Park (38°27′ N; 9°01′ W), 30 km south of Lisbon, Portugal on 15 June 2005, and maintained in two 250 l tanks at Instituto Português de Investigação das Pescas e do Mar (IPIMAR). The bottom of the tanks was covered with a layer of sand, and some large stones and plastic half flowerpots were provided as shelters and spawning sites. Tanks were illuminated with a 60 W fluorescent light from 0600 to 2100 hours at a mean ± S.D. temperature of 17·4 ± 0·5° C. Fish were fed daily with shrimp and mussels. Newly hatched larvae were obtained from three batches, spawned on 29 July, 19 September and 10 October 2005. Embryonic development lasted 9 days at a mean ± S.D. temperature of 17·8 ± 0·5° C for the first batch, at 17·4 ± 0·4° C for the second and at 17·3 ± 0·4° C for the last batch. After hatching, larvae were collected by aspiration with a pipette and anaesthetized with MS222. Larvae were observed and photographed with a digital camera under a stereomicroscope equipped with an eyepiece micrometre, to the nearest 0·1 mm. The L_N was measured according to Leis & Carson-Ewart (2000). Larvae were then preserved in 4% saline formalin buffered with sodium borate for several months prior to being measured again. Adult fish were returned to the Marine Park after the experiment.

DEVELOPMENTAL SERIES

Sampling methods

Larvae used for the developmental description were collected at the Arrábida Marine Park. Between 26 June and 27 July 2001, 32 samples were collected over the adults'

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habitats using a plankton net (300 mm mouth diameter and mesh-size 350 μm, diameter:length ratio = 1:3) attached to an Apollo AV-1 underwater scooter (Apollo Sports, U.S.A., Inc., Everett, WA, U.S.A.). Trawls of 5 min were performed at a speed of 2·8 km h^{-1} (1·5 knots). The volume of filtered water per trawl was measured by a Hydrobios flowmeter (Kiel–Holtenau, Germany) attached to the mouth opening of the net (mean ± S.D. volume = 6·96 ± 1·29 m^3). Larvae were immediately preserved in 4% saline formalin buffered with sodium borate for at least 1 month.

A light trap adapted from Sponaugle & Cowen (1996) (entrance funnel opening 20·0 mm diameter, plankton mesh-size 500 μm), was used on 22 July 2003 at the same area and four 1 h samples were collected. Since larger larvae can avoid plankton nets, this method was used to try to complete information for the developmental series.

Larval identification

After sorting from the plankton collections, 375 larvae were identified as *G. xanthocephalus* through the 'series' method (Neira *et al.*, 1998), by comparison with the descriptions available for other *Gobius* species and using meristic counts of the more developed larvae. Identification of the smallest larvae was confirmed by comparison with the laboratory-reared larvae.

Their body length (L_B), defined as L_N in pre-flexion and flexion larvae and as standard length (L_S) in post-flexion larvae was measured to the nearest 0·1 mm, as well as their total length (L_T), both according to Leis & Carson-Ewart (2000). All measurements were performed under an Olympus stereomicroscope (Olympus Corporation, Tokyo, Japan) equipped with an eyepiece micrometre.

For the descriptions, a sub-set of 124 larvae was used. In order to ensure that all size classes were equally sub-sampled, 10 individuals (when possible) were randomly selected within each size class of 0·5 mm interval L_B classes, ranging from 3·0 to 11·0 mm L_B (3·1–13·1 mm L_T).

Samples caught with the light trap contained mainly juveniles (Kendall *et al.*, 1984). The smaller specimen caught by this method was 15·0 mm L_B (18·5 mm L_T), and was used to complete the ontogenetic description.

Ontogenetic development

The 'dynamic approach' method recommended in Neira *et al.* (1998) was applied to describe the main ontogenetic events. These included notochord flexion, fin and gill filament development, vertebral ossification and presence of teeth and pigmentation patterns. Each characteristic was considered to be 'present' from the L_B class at which it appeared in all larvae.

Morphometrics

For the morphometric analysis several measurements (besides L_B) were taken to the nearest 0·1 mm as described above: L_T, pre-anal length (L_{PA}) and head length (L_H), according to Leis & Carson-Ewart (2000); head depth (D_H) as described in Olivar (1986); and body depth at anus (D_{BA}), following Neira *et al.* (1998). Allometric relationships between these measurements and L_B were calculated using the allometric law described in Krickeberg *et al.* (1971). Eye diameter (D_E) was measured using the same method (Leis & Carson-Ewart, 2000) but to the nearest 0·001 mm and allometrically related to L_H. The relationship between L_B and L_T was used to extrapolate the L_T classes of the *G. xanthocephalus* larval developmental sequence described here with the purpose of comparing this information with the results obtained for other gobiid species.

This information was further used to classify body shape, head size and eye size according to Leis & Carson-Ewart (2000) and gut size following Neira *et al.* (1998).

VALIDATION OF IDENTIFICATIONS: DNA ANALYSIS

In order to confirm the identification of the larvae used in the developmental series, DNA from four adults, four juveniles and three larvae (all from field collected samples

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preserved in 70% ethanol) was analysed. Total genomic DNA was extracted from specimens preserved in ethanol by an SDS and proteinase-K based protocol adapted from Sambrook *et al.* (1989). Two mitochondrial genes were sequenced: 12S rDNA and D-loop (mitochondrial control region) in all specimens. The choice of these genes was based on their common use in phylogeny and their different mutation rates (Kocher & Stepien, 1997).

The 12S rDNA is a slowly evolving gene (by mitochondrial standards) that usually shows little intraspecific variation, but differs sufficiently between closely related species to discriminate them reliably (Henriques *et al.*, 2002; Almada *et al.*, 2005). D-loop, on the other hand, has a very high mutation rate and is often used to study intraspecific variability (Fauvelot *et al.*, 2003; Astolfi *et al.*, 2005). Thus, if larvae and juveniles share a given haplotype with the adults, which were unambiguously identified as *G. xanthocephalus*, there will be a high level of confidence that the different forms belong to the same species. Part of the mitochondrial 12S gene (390 bp) was amplified, using the primers 12SFor 5′-AAC TGG GAT TAG ATA CCC CAC-3′ and 12SRev 5′-GGG AGA GTG ACG GGC GGT GTG-3′ (Almada *et al.*, 2005). The polymerase chain reaction (PCR) conditions followed those in Almada *et al.* (2005). A fragment of 388 base pairs (bp) of the D-loop gene was amplified, using the primers L-PRO1 5′-ACTCT CACCC CTAGC TCCCA AAG-3′ and H-DL1 5′-CCTGA AGTAG GAACC AGATG CCAG-3′ (Ostellari *et al.*, 1996), corresponding approximately to the positions 15593–15977 of the mitochondrial genome of the gobiid *Gymnogobius petschiliensis* (Rendahl) (GenBank accession number NC_008743). This fragment includes most of the first half of the control region. The PCR conditions followed those in Stefanni (2000). Sequencing reactions were performed by Macrogen Inc. (Gasan-dong, Geumcheon-gu, Seoul, South Korea) in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM BigDyeTM terminator cycle sequencing kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems, Foster city, CA, U.S.A.), following the protocols supplied by the manufacturer and the same primers used for PCR. Sequences were aligned in Clustal X (Thompson *et al.*, 1997) followed by visual inspection. All sequences have been deposited in GenBank (accession number DQ382237–DQ382251 for 12S gene and DQ382252–DQ382266 for D-loop gene).

OTOLITH MICROSTRUCTURE

In order to analyse the relationship between age and size, otoliths from 15 larvae, from 5·0 to 11·0 mm L_B classes (5·5–13·1 mm L_T) were removed and treated following Secor *et al.* (1992). Sagittal otoliths were extracted and then fixed to a glass blade with Crystal Bond (Aremco Products, Inc., Valley Cottage, NY, U.S.A.). Polishing was performed with 3·0 and 0·3 μm lapping film (3M products, St Paul, MN, U.S.A.) along the sagittal axis. For each otolith, daily increments were counted three times under an Olympus microscope with ×1000 magnification, using transmitted light. Although the increment deposition pattern has not been validated for this species, daily deposition is known in other gobiids (Iglesias *et al.*, 1997; Hernaman *et al.*, 2000; Shafer, 2000). No increment discontinuities or double rings were present. The first otolith increment was considered to form at hatching as described for other gobiids (Sponaugle & Cowen, 1994). Age (in days) was therefore considered to correspond to the number of increments in the otolith.

RESULTS

ONTOGENETIC DEVELOPMENT

The newly hatched laboratory-reared larvae (Fig. 1) measured 2·8 ± 0·2 mm L_B (mean ± S.D.; range = 2·7–3·1 mm, n = 3). After fixation and preservation mean ± S.D. L_B was 2·2 ± 0·2 mm (range = 1·8–2·4 mm, n = 20) resulting in

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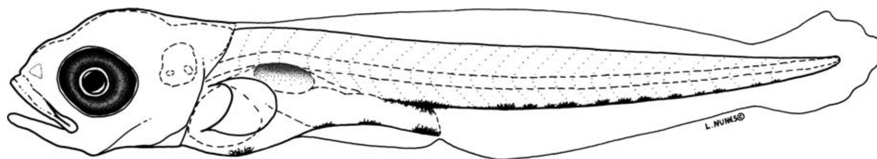

FIG. 1. Newly hatched larva reared under controlled conditions (L = 3·1 mm body length).

a shrinkage of *c.* 20%. The mouth and anus were open, the lips were formed and the jaws were differentiated. The yolk was almost fully absorbed. Eyes were fully pigmented, the liver developed and the opercula were open, with four branchial arches. The gas bladder was filled. Pectoral fins were differentiated and the finfold was complete. Sagittae and lapilli otoliths were visible.

The dorsal membrane of the gas bladder was heavily pigmented. Ventrally, there were some melanophores under the liver, gut and anus, one above the anus and a post-anal row of similar, slightly ramified melanophores which were regularly spaced, from the anus to the caudal peduncle (Fig. 1).

Larvae from the plankton collections in different developmental stages are shown Fig. 2. The smallest larvae caught in the plankton [Fig. 2(a)] was at a developmental stage similar to that of the newly hatched larvae reared in the laboratory (Fig. 1), although the former was somewhat bigger and no yolk was present. Pigmentation patterns were also similar. The same pattern of melanophore distribution was maintained until *c.* 11·0 mm L_B (13·1 mm L_T), with a slight decrease in the intensity and number of ventral pre-anal melanophores.

The sequence of the main ontogenetic events is presented in Fig. 3. Caudal fin rays were the first to begin differentiation, immediately after the start of notochord flexion, between 4·0 and 4·9 mm L_B (4·3–5·4 mm L_T). By this time, pigmentation appeared on the ventral base and rays of the caudal fin and then spread dorsally during larval development.

At 5·5 mm L_B (6·1 mm L_T), anal and second dorsal fin rays were starting to develop [Figs 2(b) and 3], one or two melanophores appeared immediately anteriorly to the angle of the throat and another one or two posterior to it. A double ventral row of melanophores was visible at the anterior portion of the anal fin insertion. Through development this double line would spread to the whole length of the anal fin insertion. Posterior to this line, a single line could be seen, ending at the caudal peduncle. At *c.* 6·5 mm L_B (7·4 mm L_T), the 13–15 principal caudal rays were all present, ossification of vertebrae was completed (Fig. 3) and all larvae had conspicuous pigmentation in the otolith capsule [Fig. 2 (c), (d)]. At 8·5 mm L_B (9·9 mm L_T), when all anal and second dorsal fin rays (A = 14–15 and D2 = 15–16) were present in all larvae, caudal fin pigmentation was evident as a clear spot at the central portion of the fin rays' base [Fig. 2(c), (d)]. At 11·0 mm L_B (13·1 mm L_T) [Figs 2(d) and 3], all fins and associated major internal structures were present, a melanophore was visible in the angle of the lower jaw, and dorsal fin pigmentation was initiated. The first dorsal fin was the last to achieve full development (Fig. 3), exhibiting six rays at 15·0 mm L_B (18·5 mm L_T) [Fig. 2 (e)]. At this stage, the caudal fin had 17 segmented rays, six of which were branched. Ventral fins reached the anus, with five segmented and branched rays and a marginal non-segmented and unbranched one. The pigmentation pattern changed completely at this stage: the

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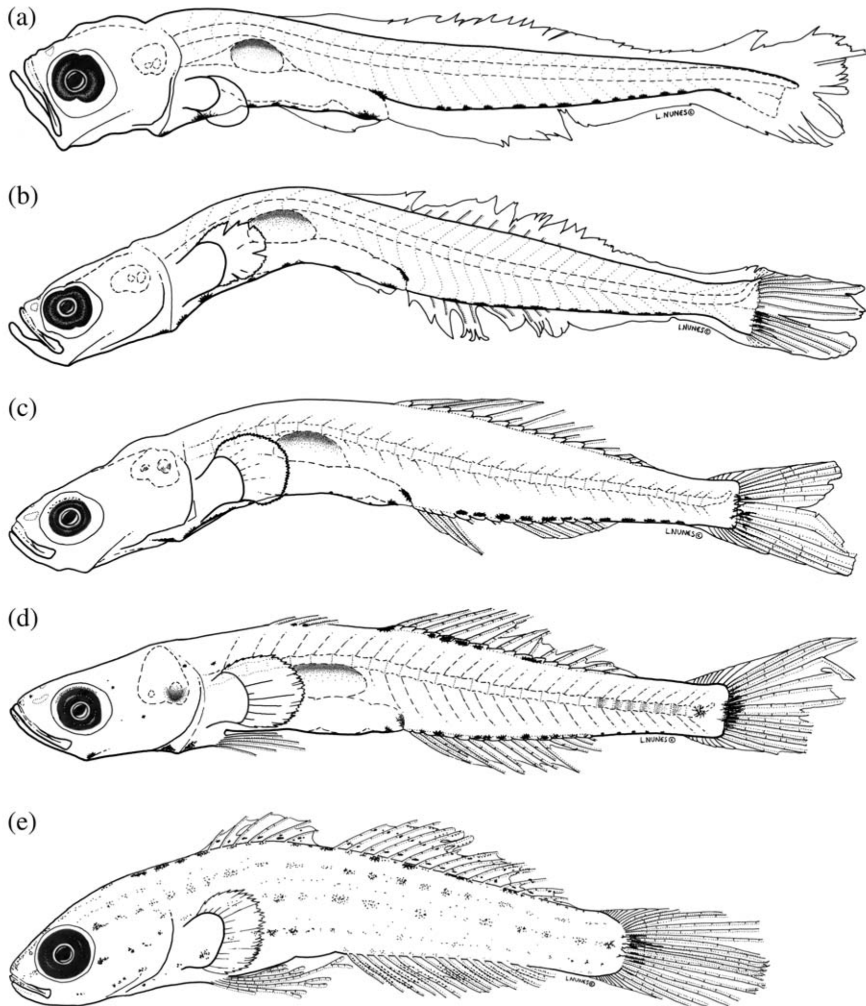

FIG. 2. Early life stages of *Gobius xanthocephalus* collected in the plankton. (a) Pre-flexion larva (3·95 mm body length, L_B) with hypurals developing, (b) post-flexion larva (5·6 mm L_B) with caudal rays differentiated and anal and second dorsal fin rays starting to develop, (c) larva with otolith capsule and caudal fin pigmentation, exhibiting all anal, second dorsal and caudal fin rays (although not all segmented) and ossified vertebrae (8·0 mm L_B), (d) larva with vertebral pigmentation and ventral fin rays formed (11·1 mm L_B) and (e) larva with dermal pigmentation and all fin formula completed (15·0 mm L_B).

body was pigmented with dermal melanophores from the head to the caudal peduncle forming 'stripes' along the body axis and the anal and dorsal fins were pigmented at the base and on the membrane between rays [Fig. 2(e)].

MORPHOMETRICS

A pronounced positive allometry was found between D_{BA} and L_B ($D_{BA} = 0{\cdot}06\ L_B^{1{\cdot}27 \pm 0{\cdot}07}$; $r = 0{\cdot}96$; $n = 125$). Growth was thus faster in body depth

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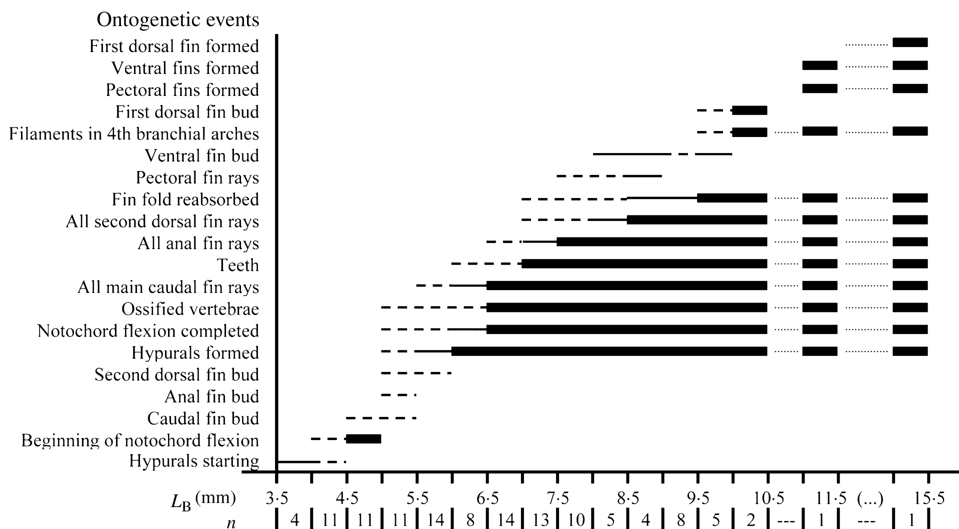

FIG. 3. Main ontogenetic events of *Gobius xanthocephalus* larval development. Event occurring in <75% of larvae of the body length (L_B) class (- - -), between 75 and 99% (——), and in 100% of larvae (▬) (n = number of specimens for each size). ······, the absence of larvae in the respective L_B size classes.

than length. A strong positive allometry was also found between L_B and the following measurements: L_T ($L_T = 0{\cdot}94\ L_B^{1{\cdot}10 \pm 0{\cdot}02}$; $r = 1{\cdot}00$), L_{PA} ($L_{PA} = 0{\cdot}43\ L_B^{1{\cdot}12 \pm 0{\cdot}02}$; $r = 0{\cdot}99$), L_H ($L_H = 0{\cdot}18\ L_B^{1{\cdot}15 \pm 0{\cdot}04}$; $r = 0{\cdot}98$) and D_H ($D_H = 0{\cdot}12\ L_B^{1{\cdot}09 \pm 0{\cdot}05}$; $r = 0{\cdot}97$). One single negative allometry was found, between D_E and L_H ($D_E = 0{\cdot}29\ L_H^{0{\cdot}66 \pm 0{\cdot}04}$; $r = 0{\cdot}93$).

Changes in body shape and in head, gut and eye size through development are shown in Table I. There was a visible change of the overall body shape with growth, with most small larvae having very long bodies [65% of larvae <5·5mm L_B (6·1 mm L_T)], and larger larvae exhibiting long bodies (Leis & Carson-Ewart, 2000). The D_E in relation to L_H also changed with growth since D_E varied little throughout development. Smaller larvae had big eyes while larvae

TABLE I. Changes in body shape and head, gut and eye size between 3·0 and 15·0 mm body length (L_B) larvae: variation range and mean ± S.D. (n = 125 larvae)

	Range	Mean ± S.D.
Body shape	Very long → long	Long
100 $D_{BA}\ L_B^{-1}$	7·2–23·5	10·7 ± 1·9
Head size	Moderate	Moderate
100 $L_H\ L_B^{-1}$	20·0–29·2	23·6 ± 1·8
Gut size	Moderate to long	Long
100 $L_{PA}\ L_B^{-1}$	46·8–59·5	54·2 ± 2·9
Eye size	Big → small	Moderate
100 $D_E\ L_H^{-1}$	49·7–18·3	26·3 ± 5·2

D_{BA}, body depth at anus; D_E, eye diameter; L_H, head length; L_{PA}, pre-anal length.

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from 6·5 mm L_B (7·4 mm L_T) exhibited small eyes. Relative head size and gut size varied little through development.

VALIDATION OF IDENTIFICATIONS THROUGH DNA ANALYSIS

In the 12S rDNA only two haplotypes were found, differing by one mutation (one transition). One was shared by nine specimens (three adults, four juveniles and two larvae). The other one was shared by an adult and a larva. Concerning the D-loop gene four haplotypes were present. The most common was found in seven specimens (two adults, four juveniles and one larva). Another haplotype was shared by one adult and one larva (which were the same that presented the second haplotype in the 12s rDNA), while the two remaining ones where found in individual fish. Least frequent haplotypes differed from the most common one by one or two mutations only.

The less common haplotype described for the 12S rDNA and the one shared by one adult and one larva in the control region were also found in sequences of *G. xanthocephalus* deposited in GenBank by other authors (GenBank accession numbers: AF491117 for 12S and AY706982 and 83 for the control region).

The intraspecific genetic distances detected in *G. xanthocephalus* were clearly smaller that those involving comparisons of *G. xanthocephalus* with the closest congeneric sequences available in GenBank. For example, while the largest distance among control region sequences of *G. xanthocephalus* was 0·5% (patristic distance) the smallest distance among *G. xanthocephalus* and the closest relative for which there are sequences in GenBank (*Gobius fallax* Sarato AY706979) was 1·56%.

OTOLITH MICROSTRUCTURE

There was a linear relationship between the number of increments and L_B (Fig. 4). Assuming a daily pattern of increment deposition, the 5·0–11·0 mm

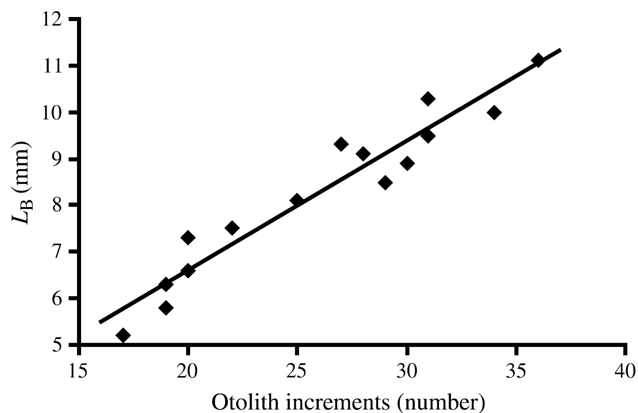

FIG. 4. The relationship between body length (L_B) and the number of increments in the larval otoliths (total number of rings in each otolith). The curves was fitted by: $y = 0·28x + 1·05$ ($r^2 = 0·93$, $n = 15$).

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larval L_B (5·5–13·1 mm L_T) classes studied corresponded to the age range 14–36 days, with a growth rate of *c.* 0·28 mm day^{-1}.

DISCUSSION

The newly hatched larvae showed the typical features characteristic of the Gobiidae (Russell, 1976; Ruple, 1984; Neira & Miskiewicz, 1998; Leis & Rennis, 2000). Although differences between laboratory reared larvae and larvae collected in the plankton may occur (Leis, 2000), in this study the smallest larvae captured in the plankton showed a developmental level and pigmentation pattern similar to that of the laboratory reared ones. They were typified by a slender and elongated body shape with a prominent gas bladder.

The observed advanced developmental level at hatching is typical of marine fishes with male parental care (Thresher, 1984; Sponaugle *et al.*, 2002; Hickford & Schiel, 2003) that spawn demersal eggs. The basic larval development sequence was similar to that known for other species of the same genus: *G. cobitis* (Spartà, 1950; Gil *et al.*, 1997), *G. paganellus* (Hefford, 1910; Lebour, 1919; Spartà, 1934; Borges *et al.*, 2003) and *G. niger* (Lebour, 1919; Petersen, 1919; Padoa, 1956; Russell, 1976). The size at which some ontogenetic events occurred, however, differed among species. While all caudal, anal and 2nd dorsal fin rays were observed in *G. xanthocephalus* from 9·9 mm L_T (8·5 mm L_B), in *G. niger* (Padoa, 1956; Russell, 1976) and *G. paganellus* (Lebour, 1919; Borges *et al.*, 2003) these structures were exhibited from 7·0 to 8·0 mm L_T on and in *G. cobitis* only from 11·0 mm L_T (Gil *et al.*, 1997). Size at settlement also varied from 9·0 mm L_T in *G. niger* (Petersen, 1919; Padoa, 1956; Russell, 1976) to 10·0 mm L_T in *G. paganellus* (Borges *et al.*, 2003) and 13–14 mm L_T in *G. cobitis* (Gil *et al.*, 1997). In *G. xanthocephalus*, the exact size at which larvae start to settle could not be determined in this study. The absence of larvae >13·1 mm L_T (11·0 mm L_B) in the plankton samples may be due to the beginning of the settlement process or to an increased ability of larvae to avoid the net (Cowen, 2002). The smallest *G. xanthocephalus* larva caught in the light trap was 18·5 mm L_T (15·0 mm L_B). It had the typical juvenile pigmentation pattern as well as post-anal scales. Therefore, this specimen may be considered as juvenile (Kendall *et al.*, 1984). These features, together with the fact that light traps usually capture mostly late-stage larvae (Doherty, 1987; Choat *et al.*, 1993), suggest that this specimen could have been already settled. This indicates that *G. xanthocephalus* settlement may occur between 13·1 and 18·5 mm L_T. Beldade *et al.* (2007) estimated an average planktonic larval duration for this species at 30 days with a range of 24–39 days from juveniles between 19 and 25 mm L_T. No data on size at hatching was, however, provided. The range obtained for this species was the greatest of the 10 species studied by these authors, which suggests a high variability on the age at settlement. Given these results, it would be expected that at least some of the specimens analysed in the present study for age estimates were already settled. All individuals, however, were collected from plankton samples and were at a pre-metamorphosis stage of development. Nevertheless, they were collected very close to the substratum which suggests that they were already exploring the nearshore habitats. It is known that early juveniles of some related gobies are hyperbenthic (Kovačić, 2003,

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2004). The same behaviour was observed in late-stage larvae and early juveniles of *G. xanthocephalus* at Arrábida (unpubl. data). Future studies are needed to clarify variation in age, size and developmental stage at settlement and related patterns of settlement marks in the otoliths of *G. xanthocephalus*.

Borges *et al.* (2003) summarized the main morphological characteristics that may help in the identification of larvae of some of the most common coastal gobiids of the eastern Atlantic. Newly hatched larvae of *G. xanthocephalus* are clearly distinguishable from those of other sympatric Gobiidae by their pigmentation pattern and myomere counts (Lebour, 1919; Petersen, 1919; Padoa, 1956; Russell, 1976). No pigmentation occurs at the angle of the lower jaw and there are 28 myomeres. These two features are especially important in the distinction of newly hatched *Gobius* larvae from *Pomatoschistus* spp. and the two-spotted goby *Gobiusculus flavescens* (Fabricius) (Lebour, 1919; Petersen, 1919). Within the genus *Gobius*, the lack of dorsal pigmentation and the presence of a regularly spaced ventral post-anal row of similar melanophores from the anus to the caudal peduncle at hatching distinguish *G. xanthocephalus* from the other described species. They also differ from *G. cobitis* (Spartà, 1950; Gil *et al.*, 1997) and *G. cruentatus* (Gil *et al.*, 2002) by the absence of a conspicuous melanophore, ventral and posteriorly to the otic vesicle, the 'median head cromatophore' described by Petersen (1919).

At intermediate sizes (5·5–11·2 mm L_T and 5·0–9·5 mm L_B), the same distinct pigmentation pattern shown at hatching is exhibited but new melanophores appear. At *c.* 5·5 mm L_T (5·0 mm L_B), a ventral post-anal double row of melanophores appears at the anal fin insertion. *Gobius paganellus* also shows this feature but at 10·0–10·5 mm L_T (Borges *et al.*, 2003). Caudal fin pigmentation also became visible earlier (between 4·3–5·4 mm L_T and 4·0–4·9 mm L_B) than in the other *Gobius* species described (between 9·0 and 11·0 mm L_T in *G. niger*, *G. cobitis* and *G. paganellus*), and a spot is formed in the central portion of the fin base and rays at 9·3 mm L_T (8·0 mm L_B). At 7·4 mm L_T (6·5 mm L_B) the otolith capsule became pigmented in both *G. xanthocephalus*, *G. paganellus* (Borges *et al.*, 2003) and *G. niger* (Petersen, 1919; Padoa, 1956; Russell, 1976). Pigmentation, however, is much more conspicuous in *G. xanthocephalus* than in the other two species.

At larger sizes, species-specific dermal pigmentation patterns develop. By this time, however, the distinction between *Gobius* and other gobiid genera, and between the different *Gobius* species is possible by comparing the meristic counts of fin rays and vertebrae (Miller, 1986; Heymer & Zander, 1992). Nevertheless, there may be some difficulty in separating *G. xanthocephalus* from the Bucchich's goby *Gobius bucchichi* Steindachner and especially from *G. gasteveni*. Larval stages of these two species are unknown but meristic counts are similar to those of *G. xanthocephalus* (Miller, 1986). Even so, *G. bucchichi* never has 16 rays in the second dorsal fin (Miller, 1986). The distinction between *G. xanthocephalus* and *G. gasteveni* was based on a global evaluation of morphological and pigmentation features integrated in the developmental series. Also, *G. xanthocephalus* is much more abundant than *G. gasteveni* at the Arrábida Marine Park and *G. bucchichi* is only present in the south of Portugal.

The interspecific comparisons made in this study should be taken with some caution. The existent studies on the development of other *Gobius* species are

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based mainly on measurements of live-reared specimens. The degree of shrinkage that larval fishes suffer with fixation and preservation is species-specific, decreases with larval length (Fey, 1999) and there are no available studies about the effect of formaline solution on the shrinkage of different ontogenetic stages of *Gobius* larvae. As such, unbiased estimates of live measurements were not possible for the more developed larval stages of *G. xanthocephalus*. Yet, the strong shrinkage suffered by the newly hatched larvae indicates an underestimation of *G. xanthocephalus* measurements that must be considered in future interspecific comparisons which should address this issue so that measurements of preserved specimens might be adequately corrected. On the other hand, laboratory-reared larvae, often differ from field-caught larvae concerning their pigmentation or other morphological characteristics, like body proportions and meristic characters (Leis, 2000). Furthermore, the existing descriptions are based on larvae reared under different temperature conditions. Since developmental time decreases with increasing temperatures in many fish species (Blaxter, 1969), the same ontogenetic events may take place in larvae of different sizes at different temperatures. In spite of these words of caution, there are some features of *G. xanthocephalus* larvae that may be used to unequivocally distinguish these larvae from the other *Gobius* species. These are the unique pigmentation pattern in the first developmental stages and, in the more developed larvae, the anal and dorsal fin ray counting.

The ontogenetic index (I_O) proposed by Fuiman (1994) is a fundamental tool to allow interspecific comparisons on the basis of larval size or age at a certain ontogenetic event (Fuiman, 1994; Fuiman *et al.*, 1998). The I_O expresses 'the state of ontogeny of a larva at any point in a developmental sequence', according to the formula $I_O = 100 \log_{10} L_B \log_{10} L_{JUV}^{-1}$, where $L_{juv} = L_B$ at the beginning of the juvenile stage. Assuming that settlement may occur at *c.* 13 mm L_T in *G. xanthocephalus*, the ontogenetic development of some structures can be compared between species using I_O (Table II). Despite the constraints already discussed concerning these interspecific comparisons, most structures seem to develop faster in *G. xanthocephalus* than in *G. paganellus*, *G. niger* and *G. cobitis* (Table II).

TABLE II. Ontogenetic index (I_O) for some ontogenetic events of *Gobius* species, assuming that in *Gobius xanthocephalus* settlement occurs in larvae >13 mm total length (11·0 mm body length)

	Gobius xanthocephalus	*Gobius niger* (Padoa, 1956)	*Gobius paganellus* (Borges *et al.*, 2003)	*Gobius cobitis* (Gil *et al.*, 1997)
Hatching	≤ 42·18	46·86	59·11	65·50
Development of fin rays	≤ 89·38	88·56	90·31	92·13
Initiation of flexion	≤ 61·96		77·82	> 65·50
Complete flexion	≤ 78·03		90·31	77·42
Ossification of vertebrae	≤ 78·03		100·00	> 77·42

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The mutations found in the DNA analysis were consistent with the variability present within species, and the adults analysed were unambiguously identified as *G. xanthocephalus*. Consequently, little doubt remains concerning the correct identification of the described larvae as *G. xanthocephalus*. The two markers used in this study typically display levels of variation among species well above the within species level of variation (Domingues *et al.*, 2005). The results of the present study confirmed this general pattern. Moreover the haplotypes found were shared by larvae, juveniles and adults and even with other conspecifics for which DNA sequences were recently made public. Thus, the probability that the analysed specimens could belong to another species are extremely small.

The use of genetic markers is proving to be a powerful tool for the correct identification of larvae collected in the plankton. For the north-eastern Atlantic Ocean and the Mediterranean Sea there is a considerable number of fish families for which genetic markers are available that can be used to identify individuals, including embryos and larvae, with high levels of certainty [*e.g.* blennids (Almada *et al.*, 2005), tripterygids (Carreras-Carbonell *et al.*, 2005), labrini (Hanel *et al.*, 2002; Henriques *et al.*, 2002) and sparids (Hanel & Sturmbauer, 2000)]. A comprehensive programme using this tool to help in the identification of fish larval forms may resolve many of the identification problems that still persist.

The linear relationship found between age and L_B in *G. xanthocephalus* seems to indicate that the described relationship between the ontogenetic development and body growth actually reflects larval development through time. Future validation of the daily nature of ring deposition in the otoliths, however, is needed to definitely confirm this relationship (Thorrold & Hare, 2002).

The standardization of the methodology used in fish larval descriptions creating objective categories of development, is an essential step in order to allow the full application of Fuiman's I_O (Fuiman, 1994) in comparative studies. Additionally, combining morphological and meristic descriptions with DNA analysis to confirm identity of larvae is a powerful tool to increase the rate of the much needed fish larval descriptions to support ecological, evolutionary and fisheries studies.

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