Early development of the freshwater goby *Orsinogobius croaticus* endemic to Croatia and Bosnia-Herzegovina

by

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Abstract. – Early development of the freshwater goby *Orsinogobius croaticus* (Mrakovčić, Kerovec, Mišetić & Schneider, 1996) endemic to Croatia and Bosnia-Herzegovina, reared under laboratory conditions, is described for the first time. Eggs were pear shaped, 1.4 × 1 mm in size. Hatching occurred 9 to 10 days after spawning, and larvae had a mean T_L of 4.27 mm, with the caudal fin and all three pairs of otoliths developed and clearly visible at hatching. Hatched larvae showed positive phototaxis. The mouth opened on days 2-3 post-hatching (PH), with larvae feeding independently on the third day. The yolk sac was absorbed and notochord flexion began on days 6-8 PH. On day 31 PH, the larvae had a mean T_L of 10.69 mm, and began to show negative phototaxic behaviour, moving towards the bottom of the tank to begin demersal life.

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Résumé. – Description du développement larvaire du gobie d'eau douce *Orsinogobius croaticus* endémique de Croatie et Bosnie-Herzegovine.

Le présent article expose des données inédites sur le développement post-embryonnaire du gobie d'eau douce *Orsinogobius croaticus* (Mrakovcic, Kerovec, Misetic & Schneider, 1996), endémique de Croatie et Bosnie-Herzegovine, élevés en laboratoire. Les œufs sont de forme ovale et mesurent 1,4 × 1 mm. L'éclosion survient entre 9 et 10 jours après la ponte. Les larves mesurent en moyenne 4,27 mm et présentent une nageoire caudale et trois paires d'otolithes bien développés et clairement visibles. Les larves sont attirées par la lumière (phototaxie positive). L'ouverture de la bouche survient entre 2 et 3 jours après l'éclosion, et les larves commencent à se nourrir de manière autonome dès le troisième jour. La vésicule vitelline est résorbée entre le 6e et le 8e jour. La vie larvaire s'achève par une métamorphose au bout de 31 jours et les juvéniles, d'une longueur totale de 10,69 mm, se déplacent vers le fond et effectuent leur transition à la vie démersale.

Key words
Gobiidae
Orsinogobius croaticus
Croatia
Developmental stages
Spawning behaviour

The Gobiidae family has one of the highest numbers of species of all fish families and vertebrates worldwide (Nelson, 2006). They inhabit mostly marine and brackish habitats and only a few species are purely freshwater (Kottelat and Freyhof, 2007). There are few freshwater and euryhaline gobies in western and northern Europe, though they are common in the Mediterranean, Black and Caspian Sea basins, where about 50 goby species are restricted to freshwater or brackish habitats (Freyhof, 2011). The Balkan region is a well-known European biodiversity hotspot; in particular, the Dinaric karst region along the eastern Adriatic coast is an endemism hotspot (Bănărescu, 2004; Smith and Darwall, 2006; Reyjol *et al*., 2007; Oikonomou *et al*., 2014). The freshwater gobies are particularly diverse in the Balkan Peninsula, with several endemic species (Vanhove *et al*., 2012), and the goby fauna here is dominated by the 'sand gobies', consisting of the following genera: *Pomatoschistus* Gill 1863; *Knipowitschia* Iljin, 1927; *Economidichthys*, Biano, Bullock, Miller & Roubal, 1987, *Gobiusculus* Duncker, 1928 and *Orsinigobius* Gandolfi, Marconato & Torricelli, 1986. *Hyrcanogobius* Iljin, 1928 may be synonymous to *Knipowitschia* (Miller, 2004).

The genus *Orsinogobius* has recently been rehabilitated (Geiger *et al*., 2014) due to molecular studies that have indicated the separation of the species *Knipowitschia punctatissima* (Canestrini, 1864) (Miller, 1990), which was formerly called *Orsinogobius punctatissimus* (Gandolfi *et al*., 1985), and the species *Orsinogobius croaticus* Mrakovčić, Kerovec, Mišetić & Schneider, 1996, which was formerly named *Knipowitschia croatica* Mrakovčić, Kerovec, Mišetić & Schneider, 1996, and originally considered to be a subspecies of *O. punctatissimus*. Both species are purely freshwater species inhabiting the rivers of the Adriatic basin; *O. punctatissimus* in the western part of the basin in Italy, while *O. croaticus* inhabits the eastern part of the basin in Croatia and Bosnia-Herzegovina.

O. croaticus, the Vrgorac goby, is endemic to Neretva drainage in Croatia and Bosnia and Herzegovina. In Croatia, as a native species, it inhabits the Matica River and its springs, and several smaller water bodies associated within

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the Neretva River system. On the IUCN Red List, the global threat status was assessed as vulnerable (VU) (Crivelli, 2006), whereas the regional threat status was assessed as critically endangered (CR) due to its extremely limited range (Mrakovčić *et al*., 2006). Its biology has been the subject of recent study (unpubl. data), and to date, only its reproductive biology (Zanella *et al*., 2011) has been described. This study has revealed the presence of this species in nearby watercourses in neighbouring Bosnia-Herzegovina, and thus, based on the expansion of its range to include additional waters, its listing has been proposed to be downgraded from CR to endangered (EN) (Horvatić *et al*., 2017).

Laboratory rearing of larvae of the Gobiidae species has been widely performed to obtain better insight into the reproductive and development traits of these species and for the purposes of comparative embryology and research in evolutionary development (Archambeault *et al*., 2015). The description of the developmental sequence of larval development of goby species is important to fill in the gaps in the knowledge of their biology.

The early development stages have not been described for either of the freshwater *Orsinogobius* species, though there are some data available on the early development of the related species in the genus *Knipowitschia*; *i.e*. for the euryhaline species *K. caucasica* (Berg, 1916) where egg size, size at hatching, duration of development in days and larval description was provided (Daoulas *et al*., 1993; Economou *et al*., 1994b) and *K. longecaudata* (Kessler, 1877), where only egg size is stated (Miller, 1986).

The objective of this study was to present the first detailed data on the larval development for *O. croaticus*, from spawning to juveniles in laboratory reared fish. The breeding behaviour is also described. Data on the larval development are compared with other sand goby species.

MATERIALS AND METHODS

Fish were captured by electrofishing (Hans Grassl 2.5 kW) in the Matica River in January 2007 (Fig. 1). This river is 15 km in length, with flow seawards during periods of high water, and reverse flows during periods of low water. This is a typical karst river, fed by numerous springs, and draining into sinkholes, with an artificial channel (160 m

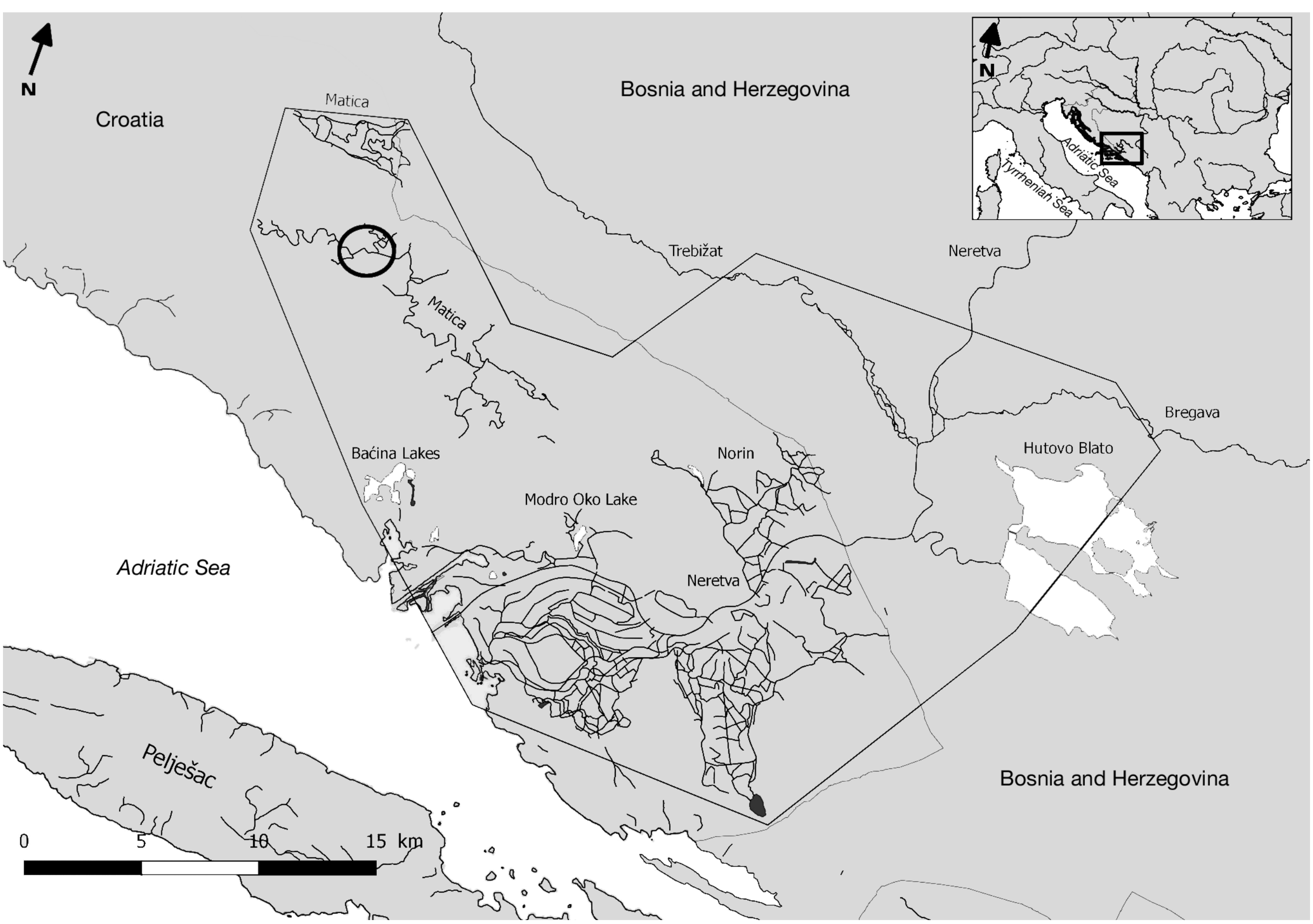

Figure 1. – Geographic distribution of the *Orsinogobius croaticus*. The circle indicates the sampling site.

length) constructed at the downstream section of the river to connect the river to the nearby Baćina Lakes, to prevent winter flooding of the valley. Flow is slow and sediment is silty to sandy (Zanella *et al*., 2011).

The experiment was conducted with wild caught adults. In this river, this species is highly abundant and is the most common fish species present. Regardless, due to its endangered status, a small number of individuals were captured (n = 24) to avoid any threats to the population. Captured fish were placed in aerated containers until their return to the laboratory (approx. 8 h). Adults were captured in January, prior to the start of the natural breeding season, in order to allow fish to acclimate to their new surroundings prior to the start of breeding. *O. croaticus* is a multiple spawner, with an extended breeding season from March to November, and the peak from April to September (Zanella *et al*., 2011).

Rearing conditions

In the laboratory, fish were distributed into three 100 L tanks. Tanks were maintained at ambient temperature (17º-20º) with recirculating water (Resun 1200 water pump, Fluval 2 filter).

Sex was determined by colouration, as sexual dimorphism is present among breeding individuals. Three males and three females were added to each tank (total n = 18; the remaining six fish were males and were instead used in other experiments requiring live specimens). The bottom of the tank was covered with a sand layer, and several large, flat stones positioned as shelter and breeding sites. Artificial nests were created with a dark glass plate as the ceiling for easier manipulation. Water temperature was held constant at 17° ± 1°C. Oxygen content was kept between 8.2 to 8.75 mg O_2/L, pH from 8.11 to 8.41. Photoperiod was kept at 10L:14D. Adults were fed daily with live *Daphnia magna* to the tanks. The study period continued from 1 February to 29 May 2007. Spawning behaviour was observed directly in the tank for the spawning event on 8 March.

Eggs

Five fertilization events were recorded during the study period: 1 February, 6 March, 8 March, 28 May and 29 May. When eggs were observed on the underside of the "ceiling" of the nest in the tank, the ceiling was turned over, and the batch was measured and photographed using a digital camera (Canon S80) (see Zanella *et al.*, 2011). Several eggs were removed from the batch by pipetting and transferred to a petri dish with water from the tank, and the ceiling immediately returned to its position. Eggs were measured using the ocular micrometer to a precision of 0.01 mm and photographed using an Olympus E-330 digital camera attached to the Zeiss Stemi 2000-C dissecting scope. Several eggs were pipetted from the batch each day to monitor embryonic development.

Embryonic development

The pipetted eggs from each batch were examined under the dissecting scope to determine the stage of development: 1, Eye development; 2, Heartbeat; 3, Body pigmentation and eye pigment development (Miller, 2004).

Early development

Just before hatching, all adult fish were removed from the tank. From the day of the hatching, several larvae were extracted by pipetting daily, and transferred to a small dish containing the same water. Larvae were anaesthetized using tricaine methanesulfonate (MS222). Larvae were examined, measured for total length (L_T; in mm) at a magnification of 100-220X using the ocular micrometer of the Zeiss Stemi 2000-C dissecting scope (precision to 0.01 mm) and were photographed with the attached Olympus E-330 digital camera. Once examined, larvae were stored in plastic vials, and preserved in a solution of 4% formalin.

On day 3 post-hatching (PH), feeding of the larvae began with the addition of powdered cooked egg yolk mixed with water to the tank. Starting on day 10 PH, larvae were fed with both the powdered egg mixture and with live *Artemia salina* twice daily. From day 15 PH, feeding was only with *A. salina* twice daily.

RESULTS

Spawning behaviour

Typical goby reproductive behaviour includes the following stages: (1) establishing a territory, (2) nest preparation (usually the under-surface of a shell or a stone, or a burrow), (3) pre-spawning or courtship behaviour, (4) spawning on the nest ceiling or walls, with eggs deposited in a single layered patch and (5) parental care of eggs, chiefly by fanning and aggressive behaviour towards intruders (Miller, 1984). In this study, all five behaviours were observed.

Each male in each tank immediately took up position at the entrance to a nest structure. Only one actual spawning event was directly observed. The female, when ready to spawn, approached the nest and displayed her yellow abdomen. The male then exited the nest and began the typical spawning ritual for goby males (as described in Gandolfi *et al.*, 1991). The female entered the nest alone, turned upside down to deposit the eggs on the underside of the nest ceiling. The laying of the eggs lasted between 30 minutes and 1 hour, after which the female left the nest. The male then immediately returned into the nest, and assumed a defensive position, with the start of protective behaviour. It was not determined whether then male released sperm prior to or immediately after the laying of the eggs in the nest. The removable "ceiling" (either glass panel or flat rock) of the nest allowed easy examination of batches.

Until hatching, the male remained in the nest, guarding, aerating and cleaning the fertilized egg clutches. Aeration was achieved by the circular waving of the pectoral fins. Nest fanning in gobiids improves the oxygen supply for the developing eggs (Miller, 1984), and fanning has been reported as positively correlated with egg age, and increases through the breeding cycle (Jones and Reynolds, 1999). For the entire guarding period, males did not leave their nests, and showed highly aggressive behaviour towards other males and females in the tank, biting them on the head or on the dorsal fin if they approached. The only feeding by the male was when he was able to capture prey present directly in front of the nest. Cannibalism was also assumed to have occurred due to decreased numbers of eggs present on the nest roof over time, though it was not determined only unfertilized eggs or also fertilized eggs were consumed, or whether the guarding male consumed the eggs, or whether the eggs were consumed by intruding males or females. After spawning, the female returned to normal life and dedicated no care to the fertilized eggs.

Eggs

Females laid eggs in five nests (two tanks with two spawning events, one tank with one spawning event) over the course of the study period. Of these, four were successfully fertilized and resulted in the hatching of larvae. The number of eggs per nest ranged from 190 to 254. Eggs were laid in a single layer, in a batch ranging in size from 2.5 × 2.5 cm to 3.7 × 1.8 cm. Eggs were attached to the ceiling via the adhesive filament at the basal end, and turned in such a way that the chorion was upright (Mazzoldi, 1999). Eggs were pear-shaped, with a size of 1.4 ± 0.2 x 1 ± 0.1 mm (Fig. 2).

Embryonic development

The first stage, eye development, was observed between 4 and 5 days following fertilization, heartbeat was observed on day 6, and the final phase was observed on days 7 and 8. Eggs hatched on days 9 and 10 following fertilization, tail first (Fig. 3).

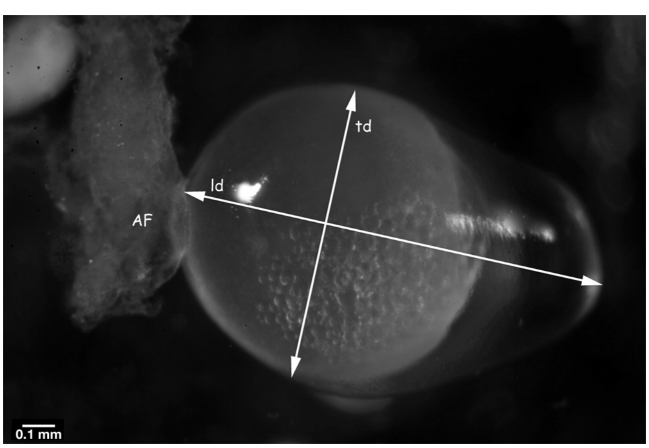

Figure 2. – Fertilized egg of the *Orsinogobius croaticus*. AF: adhesive filament; ld: longitudinal diameter; td: transverse diameter.

Early development

Upon hatching (Fig. 4A), larvae had a mean L_T of 4.27 ± 0.50 mm. The yolk sac was 0.66 mm x 0.25 mm. The caudal fin and all three pairs of otoliths were formed and clearly visible. This marked the start of the transitional phase of the larvae with yolk sac (Kendall *et al.*, 1984). The mouth was partially open but not functional. Larvae were positively phototaxic and remained exclusively in the upper water column, near the surface. Pigmentation was observed along the dorsal side of the head and in the form of two thin lines along the dorsal part of the body, and somewhat less along the ventral side. Star-shaped melanophores were present on the yolk sac.

On days 2-3 PH, mean L_T was 5.15 ± 0.55 mm and the yolk sac was reduced by half (Fig. 4B). The mouth was completely opened. The digestive tract was completely formed and partially filled in some specimens, following the start of feeding on day 3 PH, implying that independent feeding had begun. Pigmentation appeared dorsally side, less ventrally, grouped into two parallel lines of melanophores. Melanophores in the yolk sac area expanded, and pigmentation began to appear ventrally. On days 4-5 PH (Fig. 4C), L_T was 5.38 ± 0.07 mm, and the yolk sac was fully absorbed. The pectoral fins began to appear, and thickening of the tissue was observed where the pelvic disc would develop. Pigmentation increased dorsally, particularly on the head and expanded to the lateral sides of the caudal peduncle. Yellow pigmentation began to appear. The bones of the operculum started to form. On days 6-8 PH (Fig. 4D), L_T was 5.58 ± 0.40 mm. The digestive tract was fully developed and filled. The urostyle began to bend upwards. The tissue at the site of the second dorsal fin thickened. Pigmentation increased, with spots of yellow pigment appearing between melanophores, particularly dorsally on the head. Pigmentation

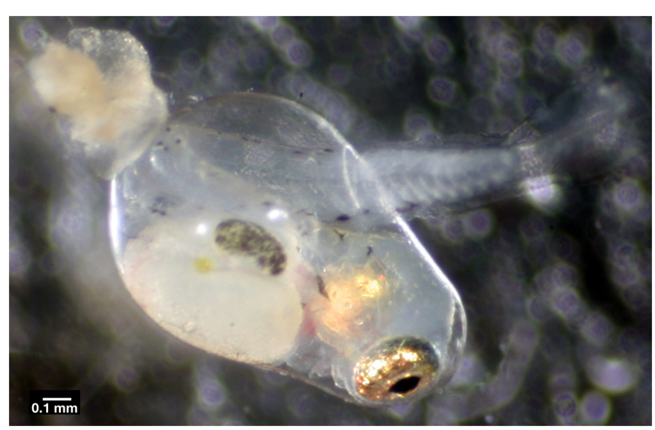

Figure 3. – Hatching of the *Orsinogobius croaticus* larvae, tail first.

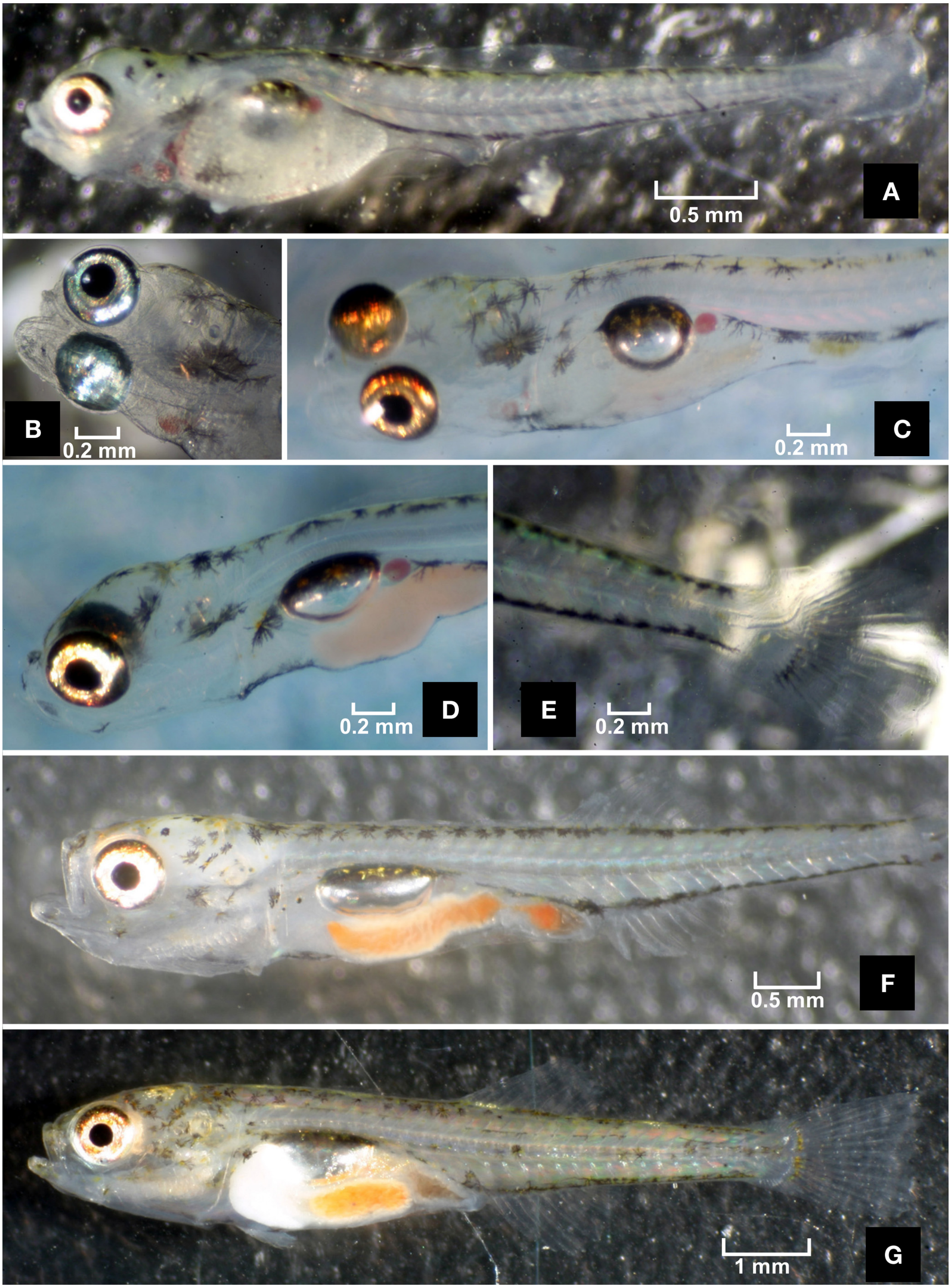

Figure 4. – *Orsinogobius croaticus*. **A**: Upon hatching; **B**: Detail of head and open mouth, day 2 post-hatching (PH); **C**: Developing pigmentation, day 5 PH; **D**: Digestive tract filled, day 7 PH; **E**: Detail of tail fin, with fully bent urostyle, day 12 PH; **F**: Second dorsal and anal fins fully developed, day 20 PH; **G**: Skin less transparent as pigmentation spreads, day 28 PH.

appeared around the mouth. Bones of the head thickened and were less transparent (Fig. 4D).

On days 12-14 PH, L_T was 6.96 ± 0.86 mm and the head became wider than the body (Fig. 4E). The caudal fin was fully developed with 15 rays. The two dorsal fins and pelvic disc began to develop. The urostyle was completely bent. Pigmentation expanded to the area of the tail fin. On day 20 PH, L_T was 7.86 ± 0.50 mm (Fig. 4F). The second dorsal fin and anal fin were fully formed, each with 9 rays. The darkly coloured tissue or "bud" to form the pelvic disc was visible. On day 28 PH, L_T was 9.87 ± 0.77 mm (Fig. 4G). Teeth were visible in the lower jaw. The pelvic disc became clearly visible, but not yet fully developed. The first dorsal fin began to form. Pigmentation became more prominent over the entire body, particularly on the lateral and occipital sections. The skin became much less transparent.

On day 31 PH, L_T was 10.69 ± 0.41 mm. The pelvic disc and first dorsal fin continued to develop. Body colouration was more heavily pigmented. Larvae began to move towards deeper sections of the water column, and parts of the larvae became demersal.

DISCUSSION

This is the first description of early development of *Orsinogobius croaticus* and the first for the genus *Orsinogobius*. According to Borges *et al*. (2011), embryonic descriptions exist for at least 74 species of gobies, though few data in the literature concern early development of sand gobies: of the Balkan sand gobies, there are data only for the brackish species *Knipowitschia caucasica, Economidichthys pygmaeus,* and *E. trichonis* (Koblitskaya, 1961; Daoulas *et al*., 1993; Economou *et al*., 1994a, b).

The eggs were attached to the ceiling of the nest in a way typical for demersal spawner teleosts (Mazzoldi, 1999). The observed spawning behaviour was typical for goby males (Gandolfi *et al*., 1991). After spawning, nest fanning was also observed, improving the oxygen supply for the developing eggs (Miller, 1984). Fanning has also been reported to be positively correlated to egg age (Jones and Reynolds, 1999).

A nonspherical egg capsule has been noted to be typical for gobies (Skrypzeck *et al*., 2014), and this shape assists in keeping eggs sediment-free, promotes water flow during fanning activities, and condenses the egg clutch for easier male parenting access (Miller, 1984).

A comparative analysis of data for egg size, incubation period and total length at hatching between *O. croaticus* and other sand goby species based on literature reports is shown in table I. *O. croaticus* has a similar egg size to *K. longecaudata*, smaller eggs than *K. caucasica* and *E. pygmaeus* (Holly, 1929), but larger eggs than *E. trichonis* Economidis & Miller, 1990, *Pomatoschistus microps* (Krøyer, 1838) and *P. marmoratus* (Risso, 1810). *O. croaticus* has one of the longest incubation periods. Total length at hatching was similar to that of *K. caucasica*, *E. pygmaeus* and *O. punctatissima*, though total length at hatching was longer than that of *E. trichonis*, *P. marmoratus*, *P. canestrinii* (Ninni, 1883) and *P. microps*. *O. croaticus* retains the yolk sac for a long period (between 5 and 6 days), even though the yolk sac was already reduced by half by the second day. Feeding began on day 3 PH, even though the yolk sac was not yet completely consumed.

In terms of larval pigmentation, *O. croaticus* is most similar to *E. pygmaeus,* with pronounced longitudinal pigmentation in the form of two parallel lines on the dorsal side and less so on the ventral side of the body, with star-shaped melanophores on the occipital part of the head. After hatching, larvae of *O. croaticus* are unpigmented, while larvae of *E. trichonis* have only slight brownish pigment in the abdominal area.

The larval development of *O. croaticus* is somewhat slower than in other sand goby species. Flexion of the urostyle begins on day 8 PH and is completed at day 14 PH at a length of 6.9 mm, while for *K. caucasica* and *E. pygmaeus* that process is complete on day 12 PH at a length of 5.5 mm

Table I. – Analysis of the egg size, incubation period and total length (TL) at hatching for *Orsinogobius croaticus* in comparison to other sand goby species from the literature data.

	Egg size (mm)	Incubation period (days)	TL at hatching	References
O. croaticus	1.4 × 1	9-10	4.3	Present study
O. punctatissima		10-12	3-4	Gandolfi *et al*. (1985)
K. longecaudata	1.7 × 0.8			Miller (1986)
K. caucasica	1.5-2.6 × 0.7-0.9		4.1	Koblitskaya (1961)
Economidichthys pygmaeus	2.3 × 0.9		4	Daoulas *et al*. (1993)
Economidichthys trichonis	0.64 × 0.58		2.2	Daoulas *et al*. (1993)
Pomatoschistus microps	diameter 0.74		2-3.2	Pampoulie (2001); Bouchereau *et al*. (1991)
Pomatoschistus marmoratus	0.8 × 0.6		2.75	Mazzoldi *et al*. (2002)
Pomatoschistus canestrinii			2.2-2.3	Gandolfi *et al*. (1991)

(Koblitskaya, 1961; Daoulas *et al*., 1993). In *E. trichonis,* flexion is complete at a length of 6.5 to 7 mm, though it is not known which day post-hatching (Economou, 1994b). Fin development is also slower. The caudal fin is fully formed with rays around day 10 PH in *E. pygmaeus,* while this is around day 14 PH for *O. croaticus* (Economou, 1994b). The anal fin is formed on day 11 PH (length 5.3 mm) in *K. caucasica*, days 16 to 20 PH (6 to 6.5 mm) in *E. pygmaeus* and only on day 20 PH (7.86 ± 0.50 mm) in *O. croaticus* (Koblitskaya, 1961; Daoulas *et al*., 1993). In *E. pygmaeus,* rays are formed in the dorsal and anal fins on day 10 PH, and both dorsal fins are fully formed on day 20 PH (6-6.5 mm), while on day 20 PH, *O. croaticus* has only the second dorsal and anal fins formed (Economou, 1994b). Only around day 28 PH (approx. 9 mm) does the first dorsal fin and pelvic disc begin to form in *O. croaticus*. Also on day 28, teeth first appear in the lower jaw of *O. croaticus* at a length of 10 mm, as opposed to *K. caucasica,* which develops teeth at a length of 5 mm, and its fins are formed at a length of 10 mm (Koblitskaya, 1961).

After about one month (lengths of 10-11 mm), the larvae of *O. croaticus* begin a demersal life. Similar results were found for *K. caucasica* (9-11 mm; Koblitskaya, 1961) and *K. panizzae* (Verga, 1841) (Gandolfi *et al*., 1991). This suggests that individuals begin to recruit to the adult habitat after about one month of life.

The limitations of this study are primarily due to its execution in laboratory conditions. Though valuable information is provided on the early development of this species, which was previously unknown, it would be interesting to compare these data with larval stages collected in the river. Future behavioural studies of this species would benefit from a larger scale experiment with the video recording of spawning events to enable detection of behaviour of individual fish, particularly females, and to gain insight into the cues that affect female selection of nests.

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