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Biology and culture of the clown loach *Chromobotia macracanthus* (Cypriniformes, Cobitidae): 3- Ontogeny, ecological and aquacultural implications

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Abstract – Hatchlings of clown loach (3.8 mm in total length, *TL*) are pelagic. When reared at 26–28 °C, they become benthic 3 days after hatching (dah), when their swim bladder is inflated. The fin development sequence (pectorals < caudal < dorsal = anal < pelvic) is typical of the clade Ostariophysi. All fins and finrays are fully elongated at 20 mm *TL*, but the finfold persists until 25–26 mm *TL* (start of juvenile stage). Melanophores appear at 3 dah, they form a 5-bar pattern at 5 dah, then two bars (III and V) vanish progressively, producing at 26 mm *TL* the 3-bar pattern that is typical of adults. Throughout the ontogeny, the pigment pattern exhibits a structural regularity (bars spaced at regular intervals), which is interpreted in a functional perspective by reference to the maintaining of crypsis and signalling throughout. Exogenous feeding commences at 4 dah (5.5 mm *TL*). Food intake (*FI*) increases rapidly, from 6% wet body mass (*WM*) at 5.5 mm *TL* to >20% *WM* in fish >7 mm *TL*. Gut evacuation rate (R_g) increases with increasing meal size and fish size, as a result of gut coiling (from 8 to 15 mm *TL*), and is highest at 11 mm *TL* (about 10% *WM* h^{-1} in fish feeding maximally). The allometric increase of *FI* and R_g during the early larval stages is accompanied by increasing capacities for growth, so early sizes differences amplify rapidly during the ontogeny. Nevertheless, growth remains slow (mean of 0.4 mm *TL* day^{-1} from 4 to 29 dah; 0.9 mm *TL* day^{-1} for top growers). By contrast, unfed fish display long resistance to starvation (until 14–15 dah). The combination of slow growth and long resistance to starvation is discussed in respect to the reproductive phenology of the species, as the capacity of making metabolic economies prevails over fast growth for seasonal strategists spawning mainly at the start of the rainy season.

Keywords: Tropical freshwater fish / Ornamental fish / Clown loach / Aquaculture / Ontogeny / Pigmentation / Growth / Food intake

1 Introduction

The understanding of how a species can adapt to environmental conditions requires the capacity of identifying them at any life stage. This can be straightforward in adults, but more difficult at younger life stages, whereas these stages are generally more sensitive than others because of their small size, limited movement capacities, low energy reserves and restricted food spectrum (Balon 1975; Kamler 1992). Genetic tools such as DNA barcoding nowadays bridge this gap (Ward et al. 2009), but nevertheless remain of limited use in many instances, because of their cost for large sample size or destructive nature for very small organisms. Morphological descriptors (including pigmentation) thus remain tremendously useful and central to all identification keys of fish larvae (e.g. Koblitskaya 1981; Pinder 2001). Likewise, the study of fish behaviour during the early stages can provide essential insights into their ecology, for example as regards their propensity to drift, which can be inferred from their use of the water column (e.g. Araujo-Lima and Oliveira 1998; Lucas and Baras 2001). Similarly, the study of their physiology, in particular the dynamics of yolk absorption, resistance to starvation, food intake and growth, can be extremely useful, as these traits intimately condition their survival in the wild (Kamler 2002; Yúfera and Darias 2007). It is difficult to obtain accurate data on these traits in natural environments, whereas this can be achieved among fish born in captivity and raised for aquacultural purposes. All aforementioned variables, of physiological, behavioural or morphological nature, are also central to the concerns of aquaculturists to maximise survival, growth, size homogeneity and food conversion efficiency.

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The present study investigates the ontogeny of the clown loach *Chromobotia macracanthus* (Bleeker 1852), with the objectives of providing information for its propagation in captivity, its univocal identification and better understanding of its ecology in natural environments. Clown loach is a key species for ornamental aquaculture (Ng and Tan 1997), but for which all marketed specimens exclusively come from capture-based fisheries. The current degree of endangerment of this species is largely unknown, but is presumably worth considering in view of its endemic distribution (rivers of Sumatra and Borneo Islands) and intensity of fisheries, which collect 20–50 million juveniles annually. Recent research efforts have enabled the maturation of clown loach broodfish in captivity, the production of functional gametes following hormonally induced reproduction, and the birth of viable embryos and larvae (Legendre et al. 2012; Slembrouck et al. 2012). Here, the ontogeny of clown loach is analysed, focusing on embryos, larvae and young juveniles, which are the main critical life stages in the wild and those of interest for aquaculturists, as the market size for clown loach does not exceed 4–5 cm. In addition to standard morphological descriptors, special attention is dedicated to the pigment pattern of clown loach, which consists in a typical alternation of yellow-orange and black bars in adults, but is also found at much younger life stages. The emphasis is also laid on other traits that could contribute to making the culture of clown loach sustainable, in particular the ontogenetic variations of meal size and gut evacuation rate, which are essential to the optimisation of feeding schedules and costs (Jobling 1994; Houlihan et al. 2001).

2 Materials and methods

2.1 Fish and rearing conditions

Experimental fish were obtained from the hormonally induced ovulation and spermiation of broodfish originating from the River Musi (Sumatra) and held captive in the experimental facilities of the BP2BIH research station (Depok, West Java, Indonesia). All observations were done in indoor recirculating systems, under a day length of 12L:12D, with light intensities of 50–100 and <0.01 lux during the hours of light and darkness, respectively. Oxygen was maintained near saturation (>90%) throughout in all rearing structures. Throughout the experiments in the present study, water quality stood as follows (ranges): pH: 7.75–7.86; hardness: 12.2–18.2 mg L^{-1}; conductivity: 123–254 μS cm^{-1}; total ammonia nitrogen, TAN: 0.002–006 mg L^{-1}; nitrite, $N\text{-}NO_2^-$: 0.001–0.002 mg L^{-1}.

Hormonal-induced breeding and ova fertilization were performed after Legendre et al. (2012). Incubation was carried out at 26–28 °C, which is near optimal for this species, in zugger jars (30 cm in diameter, 35 cm high; flow of 1–2 L min^{-1}), as developmental deformities occur in absence of egg agitation (Slembrouck et al. 2012). Following hatching, embryos ascended the water column in the zugger jars and spontaneously entered hapas (i.e. fine mesh cages) placed in the main tank of the hatchery water recirculation system.

At the age of 36 h after hatching (hereafter hah), 30 fish were randomly collected for the study of resistance to food deprivation. The fish were housed individually in small plastic containers (0.125 L; 6.5 h × 5.0 cm in diameter) that were filled with aerated well water and partly immersed in the main tank of the hatchery (next to the hapas) for maintenance of temperature (range of 25.8–27.5 °C). Housing in isolation was desirable to avoid possible biases originating from necrophagy or cannibalism. Water was changed every two days. Survival was verified every morning and evening by moving gently the fish with a water flow produced by a pipette.

The other fish were transferred into 90-L aquaria (50 × 50 × 36 (h) cm) in an indoor water recirculation system at ambient temperature (mean of 27.5 °C, range of 25.7–29.3 °C). The stocking density was 1.33 fish L^{-1} (120 fish per tank). From the age of 72 hah onwards, freshly hatched *Artemia* nauplii were distributed six times a day (02:30, 06:30, 10:30, 14:30, 18:30 and 22:30 h). At each meal, feeding was supposedly in slight excess, as verified by the presence of live nauplii in the water column 30 min after the distribution of food. Excess food, faeces and dead fish were removed once a day with a siphon.

Sixty fish were randomly sampled in the aforementioned tanks for a more accurate study of fish growth and size dispersal involving the measurement of all fish. Except for stocking density (two 90-L aquaria, with 30 fish each), the environmental conditions were identical as above. The study was not commenced before fish were aged 8 dah (days after hatching), as it was uncertain whether younger and smaller fish could tolerate handling. To minimize operational stress to the larvae, measurements were done on a weekly basis (8, 15, 22 and 29 dah). On days of measurement, all fish were captured, anaesthetised (2-phenoxy-ethanol, 0.35 ml L^{-1}) and their body lengths were measured with a graduated eyepiece under the stereomicroscope (magnification: ×6–25, depending on fish size).

2.2 Morphological observations

Samples for morphological analyses were collected twice a day until the age of 5 dah, at daily intervals until 12 dah and less frequently thereafter, until the transition between the larval and juvenile stages (i.e. when all fins and finrays were developed, and the finfold was fully absorbed). Fish were anaesthetised (2-phenoxy-ethanol, 0.35 ml L^{-1}) and photographed in profile, dorsal and ventral views under the stereomicroscope (magnification: ×6–50). Additional photographs were taken under the light microscope for a more detailed examination of mouth dimensions (magnification: ×50–200). Dimensions were measured to the nearest pixel from digital photographs on the computer, by reference to a finely graduated scale that had been photographed at the same magnification(s). The morphomeristic variables under scrutiny were those that are essential to feeding and locomotion: i.e. fish size (total, fork and standard lengths), yolk dimensions (length, depth and width), body depth, fin length and finray extension, head width and length, mouth width and jaw length. Gape height was estimated from jaw length, on the assumption of a 90° opening capacity (Shirota 1970). The ontogeny and regularity of the pigment pattern was analysed as follows. The centre of each vertical bar was marked on photographs at the level of the lateral line, then the distance between this mark and the fish snout was measured and expressed as a proportion of the fish total body length (hereafter *TL*).

Fish wet body mass (hereafter *WM*) was measured (nearest 0.1 mg) under anaesthesia in fish with empty guts (morning samples), either individually for fish >10 mg or in groups of 5–10 individuals of homogeneous size (nearest 0.2 mm) for smaller larvae, so as to document the relationship between *WM* and *TL*. Following the measure of *WM*, anaesthetised fish were euthanized with an excess dose of anaesthetics (2-phenoxyethanol, 2.0 ml L^{-1}), placed at 105 °C for 12 h then weighed again for the measurement of dry body mass (hereafter *DM*, AOAC 1995). Measurements were done on groups of fish producing at least 30 mg of *DM* (tens of fish for small larvae, at least three individuals for larger fish).

2.3 Food intake and gut evacuation rate

All experiments took place in a thermostated room (26.5–27.0 °C) and used the protocols developed by Baras et al. (2012, in press). The experimental fish had been removed from the rearing tanks during the preceding evening and offered no food overnight in order to ascertain that they would have empty guts on the next morning. Fish were allowed to feed on *Artemia* nauplii during 20 min and then they were transferred in water devoid of food.

The measure of food intake took place about 40 min after the start of feeding, well below the start of defecation in larvae of this species (see results). The fish were anaesthetised (2-phenoxy-ethanol, 0.35 ml L^{-1}), placed in a small (50 mm in diameter) Petri dish filled with the anaesthetic solution under the stereomicroscope and measured with a graduated eyepiece. Their abdominal region was photographed in profile view under magnification ×12–50 (depending on fish size) by reference to a finely graduated scale (0.1 mm). Prior to this study, it had been tested whether accurate estimates of gut content could be obtained from profile views only, by comparing the width and depth of the gut content from photographs of clown loach in ventral and profile views. The ratio between gut content depth (measured from profile views) and width (measured from ventral views) was close to 1 (mean ± SD of 0.98 ± 0.02) and did not vary between fish of different sizes.

The protocol for measuring gut evacuation rates (R_g) was slightly different as it involved sequential observations of the same individual fish, and no anaesthetics was used as it could have interfered with the fish metabolism (and thus with R_g). About 20 minutes after feeding, five or six fish were sampled and housed in isolation in 300-ml plastic containers (12.5 × 8.0 × 3.0 (h) cm) filled with water devoid of food. At the time of observation, an individual fish was gently captured with a pipette, placed in a Petri dish filled with water under the dissection microscope, then about 80% of the water in the Petri dish was pumped with a pipette until gravity gently forced the fish in a lateral recumbent position, The gut region was photographed rapidly, water was poured again and the fish was returned to its enclosure. The operation, which took no longer than 30 s, was applied to the other fish, so all individuals were photographed within less than 3 min. The same sequence was repeated at regular intervals (about 40 min) until gut evacuation had (almost) ended. Thereafter, all fish were anaesthetised and photographed in full for measuring their body length (same protocol as above).

The measure of R_g in the same individual fish was preferred to the measures of gut volumes of different fish at different times after feeding, because the value of R_g can depend strongly on meal size and degree of gut fullness (Jobling, 1994). Nevertheless, it was uncertain whether repeated observations of the same fish interfered with R_g. To test for this possible bias, a post hoc comparison was made between the R_g values obtained with the sequential observations of the same individuals in absence of anaesthesia, and those from different anaesthetised individuals at different times after feeding (same protocol as in Baras et al. 2012, in press). The scope of this particular comparison was restricted to fish with very high food intake. The R_g curves that were produced with the two methods were highly consistent (data not shown), thereby suggesting that the individual protocol was not excessively stressful.

2.4 Calculations and statistics

Morphological variables were expressed as a proportion of the fish total body length (*TL*), to facilitate comparisons between fish of different sizes. *TL* was preferred to the standard body length *SL*, which could not be measured before the notochord was bent. Fish growth was expressed in terms of linear growth (*GL*, mm day^{-1}), which was calculated as $GL = (TL_2 - TL_1)\ (t_2 - t_1)^{-1}$, where TL_2 and TL_1 are the total body lengths (mm) of fish at times 2 and 1, respectively. Size heterogeneity was expressed by the coefficient of variation (*CV*, %), which was calculated as $CV = 100\ SD \times TL_m^{-1}$, where *SD* is the standard deviation of the mean body length (TL_m). The water content of fish (*WC*, % *WM*) was calculated as $WC = 100\ (WM - DM)\ WM^{-1}$, where *WM* and *DM* are the wet and dry body mass of fish (mg).

The volume (*V*) of yolk sac was calculated from its length (*L*), depth (*D*) and width (*W*), which were measured on profile and ventral views of the fish. The yolk of clown loach was pear-shaped or strongly conical in its caudal region, so its volume was systematically overestimated with an ellipsoidal model using *L*, *W* and *D* as diameters (i.e. $V = 0.167\,\pi\,L\,W\,D$). To correct for this, the perimeter of the yolk was contoured with a hand drawn closed polygon, and the surface area (*S*) was calculated with the freeware Image J (Abramoff and Magalhaes 2004). Thereafter, the diameters (D_1 and D_2) of a planar ellipse with a surface area equal to *S* were calculated on the assumption that the ratio between the two diameters had to be identical to the ratio between the actual dimensions of the yolk ($D_1/D_2 = L/D$ for profile views and $D_1/D_3 = L/W$ for ventral views). The values of D_1, D_2 and D_3 substituted those of *L*, *H* and *W* in the ellipsoidal model for calculating the yolk volume.

For the measures of gut content and gut evacuation rate (i.e. decline of gut content over time), the stomach and intestine were analysed separately, because of their contrasting shapes (details in Baras et al. 2012, in press). The stomach region has an ellipsoidal shape, so its volume (V_s) was calculated in the same way as for the yolk sac. For the intestinal content, which has a cylindrical shape, the surface of the gut content (S_i) was contoured and its length (L_i) was measured by tracing an open polygon passing in its centre. The S_i/L_i ratio gave

the mean diameter (D_i) of the intestinal content, the volume of which (V_i) was calculated as $V_i = 0.25\,\pi\,L_i\,D_i^2$. The total gut content volume V_g was calculated as $V_g = V_s + V_i$, and expressed as a proportion of the fish *WM*, assuming that a gut content volume of 1 mm^3 weighed 1 mg.

Simple (linear, logarithmic or power) and polynomial regression analyses were used to describe the relationships between body dimensions, *WM* and *TL*, *DM* and *WM*, and between *TL* and fish age. A log-logistic regression model was used for modelling the resistance (P_{50}) to starvation. A stepwise multiple-regression analysis was used to identify the variables (fish size, food intake and their interaction) that influenced significantly the gut evacuation rate. Null hypotheses were rejected at $p < 0.05$.

3 Results

3.1 Morphology

3.1.1 General

The ova used in this experiment averaged 1.18 mm in diameter and 0.88 mg *WM*, with a water content of 66.6%. Hatching took place 19–20 h after fertilization (27 ± 1 °C). Hatchlings averaged 3.8 mm *TL* and their yolk sac (0.49 mm^3) exhibited a marked posterior axial protrusion (yolk extension, Fig. 1a). Until 78–84 hah, before the inflation of the swim bladder, embryos were strongly pelagic, swimming up and down in the water column. Exogenous feeding started at 96 hah. Larvae were strongly thigmotactic and essentially benthic by then. First-feeding larvae averaged 5.5 mm *TL* and 1.2 mg *WM*, with a water content of 87%.

The ontogeny of clown loach is illustrated in Figure 1. The following sections lay the emphasis on the development of the fins, cephalic region, swim bladder, gut, and pigmentation. Morphometric relationships are given in Table 1. The model between *WM* and *TL* in young clown loach larvae has a polynomial nature, as it depicts the steps of allometric growth of body depth and width that take place during the early larval stage of this species (see below).

3.1.2 Fins

At hatching, only a continuous, non-structured finfold was present. Two days later, it was structured in the caudal, dorsal and abdominal regions. The fin development sequence, apparition and completion of finrays are illustrated in Figure 2. Pectoral fins grew first (20 hah, 4.6 mm *TL*), but the first finrays to become differentiated were those of the caudal fin, slightly before the flexure of the notochord, which was conspicuous at about 6.7 mm *TL* and attained its maximal bend (45–50°) at 8.8 mm *TL*. The anal and dorsal fins followed similar developmental patterns, and pelvic fin grew last. All fins had complete finrays and were fully elongated at 20 mm *TL*, but the finfold in the caudal and abdominal regions did not vanish before 26 mm *TL*, which can be regarded as the cut-off size between larvae and juveniles in this species.

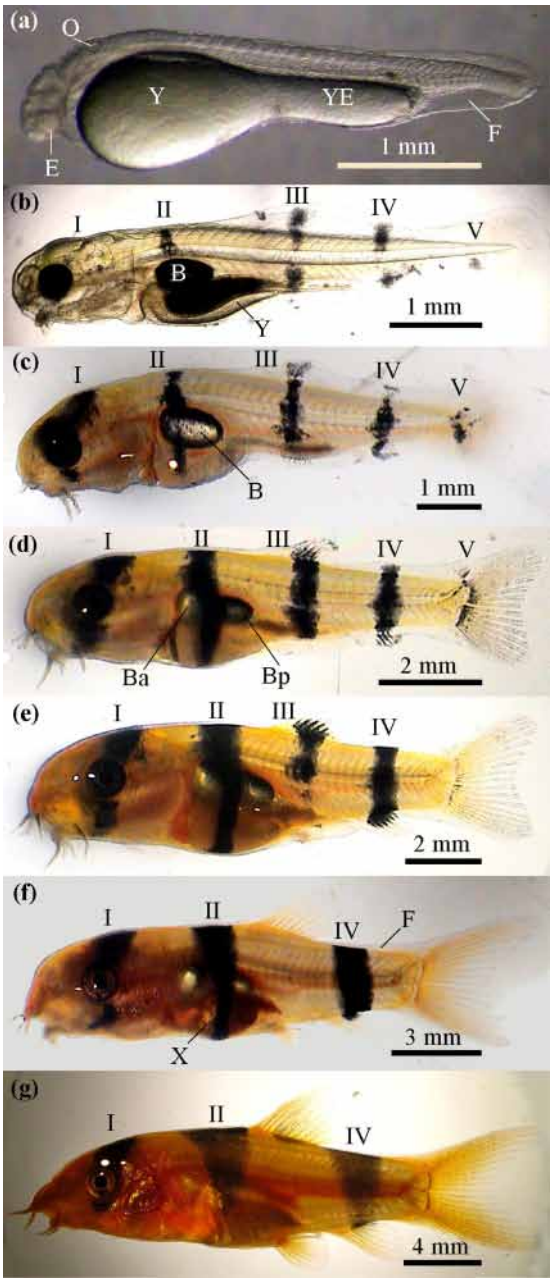

Fig. 1. Ontogeny of clown loach with emphasis on the pigment pattern. I–V: dark pigment bars; B: swim bladder; Ba and Bp: anterior and posterior chambers of the swim bladder after specialization; E: eye; F: finfold; O: otic capsule; X: xanthophores; Y: yolk; YE: yolk extension. (a) 3.7 mm *TL*, 4 h after hatching, no pigment; (b) 5.8 mm *TL*, 5 days after hatching (dah); (c) 7.8 mm *TL*, 10 dah; (d) 10.0 mm *TL*, 14 dah; (e) 13.5 mm *TL*, 21 dah; (f) 17.0 mm *TL*, 28 dah; (g) 26.0 mm *TL*, 37 dah.

3.1.3 Cephalic region

Head length and width attained their maximal dimensions relative to body length (20 and 17% *TL*, respectively) at the start of exogenous feeding (96 hah). The eyes became pigmented at about 12 hah and were fully pigmented at 60 hah (4.4 and 5.2 mm *TL*, respectively). The erectile subocular

Table 1. Morphometric relationships in clown loach larvae and small juveniles. *V* is the volume of the yolk sac (mm^3), A is the age (days after hatching), *FL*, *SL* and *TL* are the fork, standard and total body lengths (mm), *WM* is the wet body mass (mg), *WC* is the water content (%), and TL_M is the total body length of the fastest growing fish in this study (data in Fig. 8), respectively; $p < 0.0001$ for every coefficient in every model.

Size range (*TL*)	Restriction	Equation	Statistics
3.7–5.6 mm	Period of exclusive endogenous feeding	Yolk sac volume (mm^3), $V = 0.474 - 0.103\ A$	$r^2 = 0.907$, $df = 67$
6.7–26.0 mm	After flexure of the notochord	$FL = 0.924 + 0.850\ TL$	$r^2 = 0.999$, $df = 67$
		$SL = 1.183 + 0.710\ TL$	$r^2 = 0.997$, $df = 67$
5.6–12.9 mm	Excluding yolk sac embryos	$\log WM = -11.500 + 32.702 \log TL - 31.115 (\log TL)^2 + 10.973 (\log TL)^3$	$r^2 = 0.981$, $df = 391$
13.0–26.0 mm	None	$\log WM = -1.704 + 2.868 \log TL$	$r^2 = 0.970$, $df = 521$
5.6–26.0 mm	Excluding yolk sac embryos	Water content (%), $WC = 88.44 - 0.212\ TL$	$r^2 = 0.923$, $df = 15$

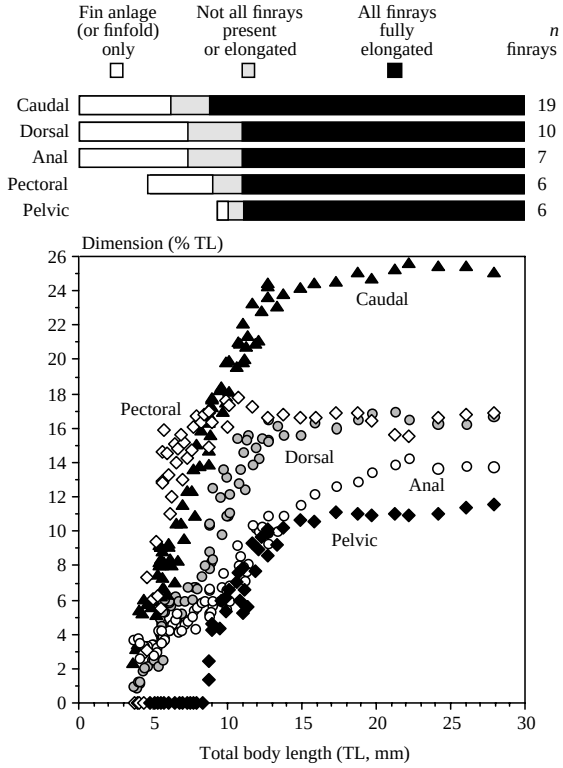

Fig. 2. Fin development in clown loach. The upper part of the figure indicates when finrays start developing and are fully elongated. The lower part shows fins' elongation as a function of the fish total body length. Dimensions are equated to fish size rather than age, owing to the marked growth heterogeneity in clown loach. Symbols refer to individual fish. Closed triangles: caudal fin ($n = 88$); open circles: anal fin ($n = 80$); grey circles: dorsal fin ($n = 80$); open diamonds: pectoral fins ($n = 76$); closed diamonds: pelvic fins ($n = 76$).

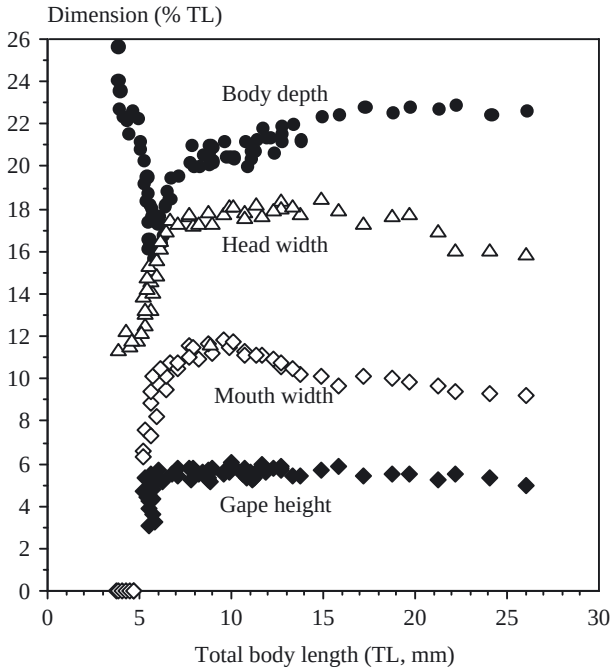

Fig. 3. Ontogenetic variations of gape height (closed diamonds, $n = 76$), mouth width (open diamonds, $n = 54$), body depth (closed circles, $n = 80$) and head width (open triangles, $n = 54$), which also corresponds to maximal body width over the size interval under study. Gape height is calculated from jaw lengths, for a gape opening of 90°.

spines that are found in adults were not observed in fish ⩽26 mm *TL*. The gill arches appeared at 30 hah (4.9 mm *TL*) and the primary gill lamellae about 24 h later. Secondary gill lamellae were not observed before larvae attained 6.7 mm *TL*.

The mouth opened at 30 hah (4.9 mm *TL*) and one day later the upper and lower jaws had already attained their longest dimensions relative to fish size (i.e. 4.0 and 3.2% *TL*, respectively), producing a gape of 5.5–6.0% *TL* for a 90° opening (Fig. 3). By contrast, mouth width continued growing until fish were 7.5–8.0 mm *TL* and attained 11% *TL* by then. At the start of exogenous feeding (96 hah, 5.5 mm *TL*) gape height and mouth width thus do not exceed 0.25 and 0.33 mm, respectively, which is small by reference to the size of *Artemia* nauplii (on average 0.60 mm in length and 0.15 mm

in diameter, excluding appendages) and can complicate feeding (see Sect. 3.4 on food intake).

Clown loach grew four pairs of circumoral barbels. The pair of lateral maxillary barbels started growing in between 60 and 72 hah (5.2–5.4 mm *TL*), whereas the two pairs of central maxillary barbels and the pair of central mandibular barbels were not observed before 6.0 and 6.5 mm *TL*, respectively.

3.1.4 Thoracic and abdominal regions

Fish body depth at hatching was 26–27% *TL*. It decreased rapidly as embryos absorbed their yolk and averaged 15–16% *TL* at the start of exogenous feeding (Fig. 3). Thereafter, it increased in a curvilinear way, up to a plateau at 22–23% *TL* in fish greater than 12.5 mm *TL*. The swim bladder started developing and became pigmented between 48 and 54 hah (5.1–5.2 mm *TL*) and one day later it was inflated (Fig. 1b). Until larvae were 8.0 mm *TL*, the swim bladder exhibited no specialisation. Thereafter, the anterior (hearing-specialised) chamber started differentiating. Its development could not be traced accurately in absence of micro-dissection, because of the development of pigmentation in this part of the thoracic region.

At the start of exogenous feeding, the gut of clown loach was straight (Fig. 4a). Gut coiling started at 7.8–8.0 mm *TL* (Fig. 4b). Thereafter, the stomach progressively acquired the shape of a pouch, while the pyloric bulb was progressively bent upwards and migrated slightly cranial, as far as under the centre of the swim bladder at 15 mm *TL* (Fig. 4d).

3.1.5 Pigmentation

No pigmentation was observed before 72 hah (5.3 mm *TL*). Then, six patches of melanophores appeared almost simultaneously in three body regions on each side of the body (at the level of the swim bladder, slightly cranial and slightly caudal to the anus), with a dorsal and a ventral patch in each region (Fig. 1b). A seventh patch of melanophores appeared ventrally close to the caudal end of the notochord at 100 hah (5.5 mm *TL*), and an eighth patch on the dorsal part of the head, slightly behind the eyes, at 120 hah (5.6–5.7 mm *TL*). In the meanwhile, the six other patches had extended ventrally and dorsally, including over the finfold, and merged to form three vertical bars. At 6.2 mm *TL*, the vertical bars on the left and right sides of the fish had merged in their dorsal region to form saddles. At this size, larvae exhibited five saddles, which lied over the eyes (I), the swim bladder (II), the dorsal (III) and anal fins (IV), and over the hippural complex of the caudal fin (V). Saddle V was always less pigmented than the four others. It vanished progressively and was no longer conspicuous in fish >11 mm *TL*. Saddle III was still complete by then (Fig. 1d). Thereafter, it vanished progressively in its ventral region and only a small patch of melanophores persisted on the basis of the dorsal fin and underlying epaxial muscles in fish >15–16 mm *TL*. This patch had almost completely disappeared at 26 mm *TL* (Fig. 1g). It is worth noticing that although melanophores vanished from two body regions during the ontogeny of clown loach, the saddles were spread at almost regular intervals throughout (Fig. 5).

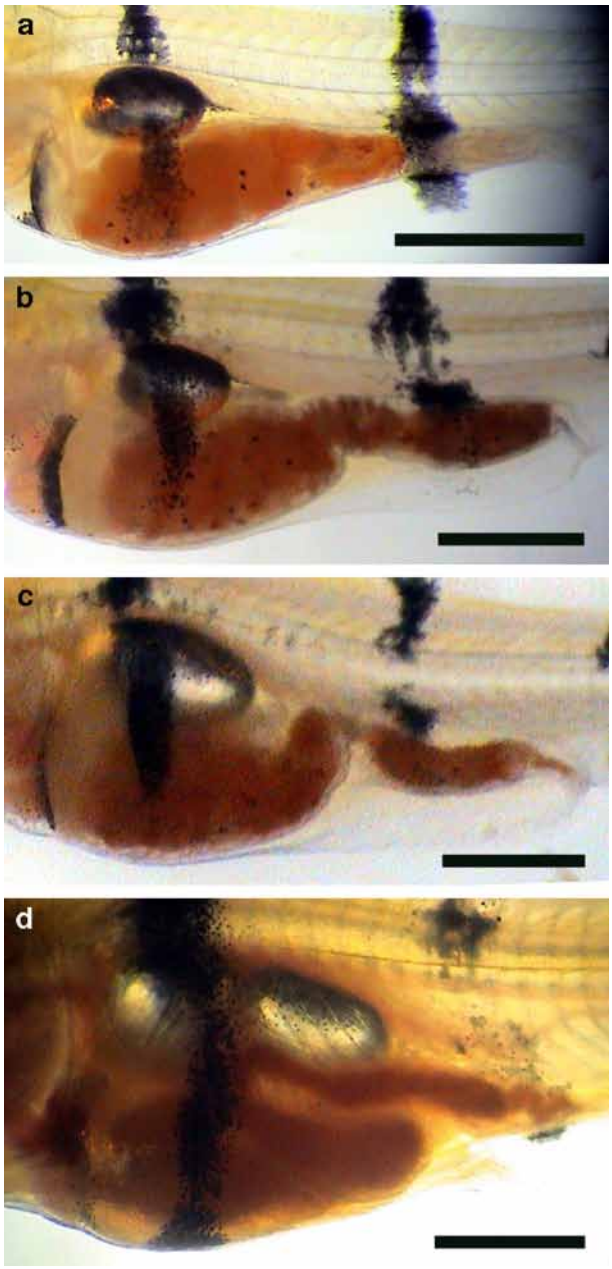

Fig. 4. Gut coiling in clown loach. (a): 6.5 mm *TL*; (b): 8.0 mm *TL*; (c): 9.0 mm *TL*; (d):12.4 mm *TL*. On every photograph, the horizontal bold bar in the lower right corner stands for 1 mm.

3.2 Yolk absorption and resistance to food deprivation

Before the start of exogenous feeding (96 hah), embryos absorbed their yolk at a steady rate (on average 0.103 mm^3 day^{-1}; Fig. 6, Table 1). By contrast, their growth in length was strongly curvilinear during this period. Until 30 hah growth averaged 1.0 mm *TL* day^{-1}, whereas it amounted to 0.25 mm *TL* day^{-1} during the next 24 h, and less than 0.15 mm *TL* day^{-1} until the start of exogenous feeding at 96 hah. Thereafter, the rate of yolk absorption decreased rapidly, and remnants of yolk were occasionally observed until 178 hah.

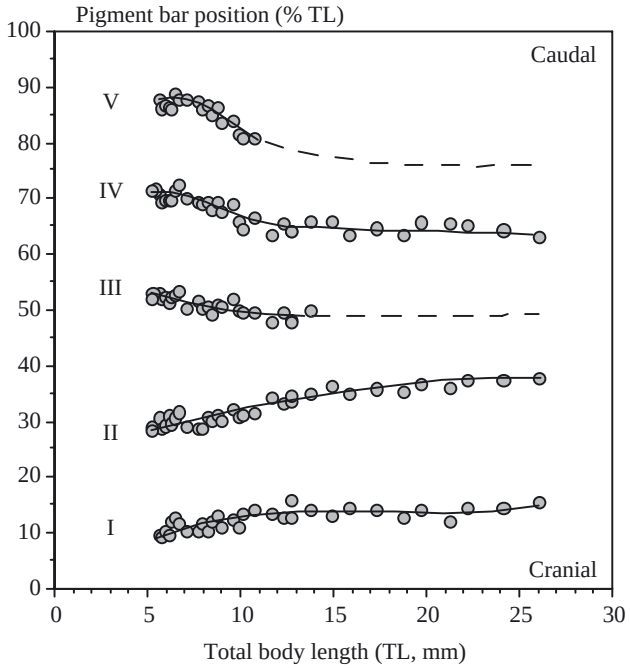

Fig. 5. Ontogenetic variation of the pigment pattern in clown loach. The five dark bars (I–V) are numbered in cranio-caudal sequence. Symbols refer to the position of the centre of dark bars on the lateral line, by reference to the fish total length. Curves were produced by interpolation. The dashed curves illustrate where the centres of bars III and V would have lied if they had not vanished during the ontogeny. Measurements in 30 fish (5.3–26.0 mm *TL*).

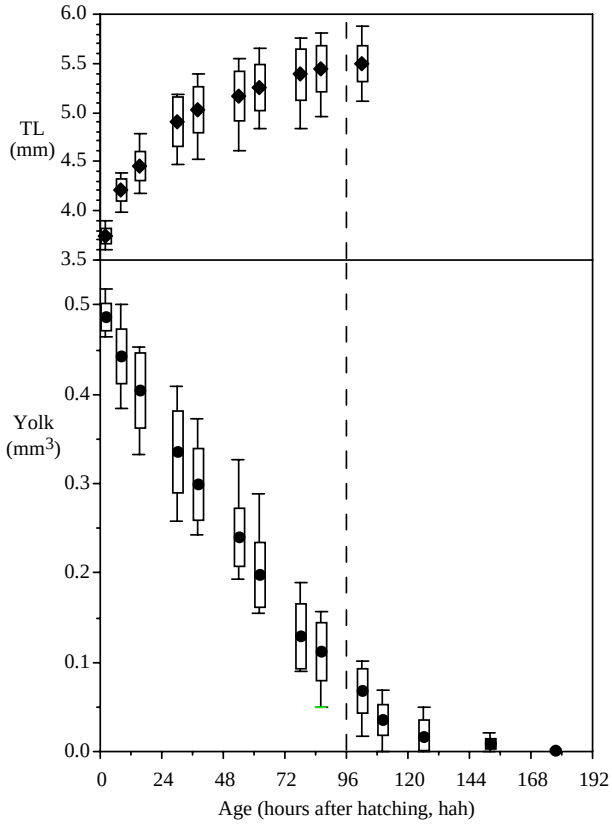

Fig. 6. Dynamics of yolk absorption and growth in clown loach. At each age, the closed circle stands for the mean, the box for the standard deviation, and the whiskers indicate the minimal and maximal values over a sample of 5–10 fish. The dashed vertical line indicates the start of exogenous feeding.

Yolk volume did not vary much between hatchlings (*CV* of 3.3%), but became strongly heterogeneous afterwards (*CV* of 13–14% from 30 to 66 hah; Fig. 6), thereby suggesting marked individual variations in yolk absorption rates. The sizes of embryos and the remaining yolk volumes at different ages were negatively correlated (r^2 = 0.660), but a better correlation was found between fish age and yolk volume with the same data set (r^2 = 0.907; Table 1). This comparison further supports the view that clown loach embryos absorb their yolk at variable rates, but also that they do not transform yolk equally well. These findings account for why size heterogeneity at the start of exogenous feeding was twice as high as at hatching (*CV TL* of 3.9 versus 2.0%).

The P_{50} survival of larvae housed individually and deprived of food stood at 14.7 dah (Fig. 7). No fish died before 13 dah but none survived beyond 16.5 dah This 3-day range compares with the variation between the ages at which embryos had fully exhausted their yolk (from <120 to 178 hah, Fig. 6).

3.3 Survival, growth and size heterogeneity of larvae and small juveniles

Survival at 29 dah was high in both tanks (26 and 28 out of 30 fish stocked at 8 dah) and no cannibalism occurred. Almost identical growth trajectories were observed in the two replicate groups, so the data were pooled for the description below. During the first days of exogenous feeding, the mean growth rate of clown loach larvae increased rapidly, from 0.15 to 0.5 mm day^{-1} at 5 and 8 dah, respectively (Fig. 8). The average growth rate remained stable around 0.5 mm day^{-1} until 15 dah and slowed down slightly afterwards (0.4 mm day^{-1}). At 29 dah fish averaged no more than 15.4 mm *TL* and 59 mg *WM*. However, in each replicate group, at least one fish was about 10 mm larger than the average fish size, with a steady growth of 0.9 mm day^{-1} thereafter.

Size heterogeneity (*CV TL*) increased with increasing age, from 3% at 4 dah, to 10% at 8 dah (corresponding values of 15 and 36% for *CV WM*), i.e. an average increase of 1.7% day^{-1}. Thereafter *CV TL* continued increasing, but at a much slower pace (0.75% day^{-1}) and attained 26% at 29 dah (corresponding *CV WM* of 84%).

3.4 Food intake and gut evacuation rate

The development of xanthophores in the thoracic and abdominal regions prevented the observation of gut content in fish >15.4 mm *TL* (Fig. 1f), except in an individual with an exceptionally low degree of pigmentation. Food intake (*FI*) varied substantially during the first stages of exogenous feeding (Fig. 9). In fish <5.5 mm *TL* (< 1.3 mg), *FI* never exceeded

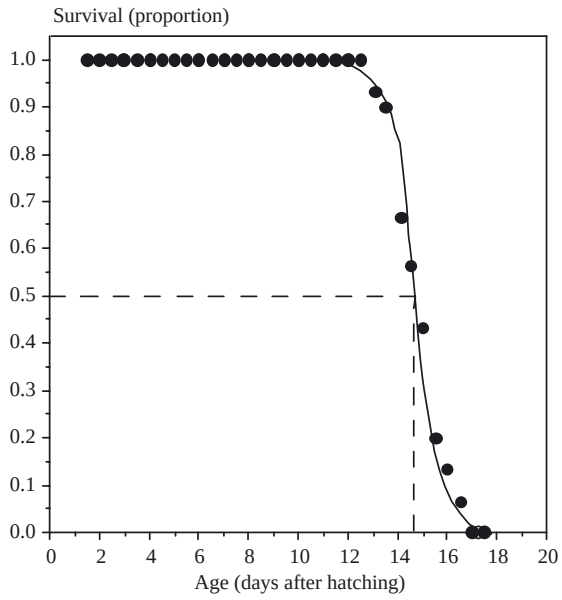

Fig. 7. Resistance to starvation in clown loach embryos and larvae housed in isolation (26.7 ± 0.8 °C, mean ± SD). Curve produced with a log-logistic regression model.

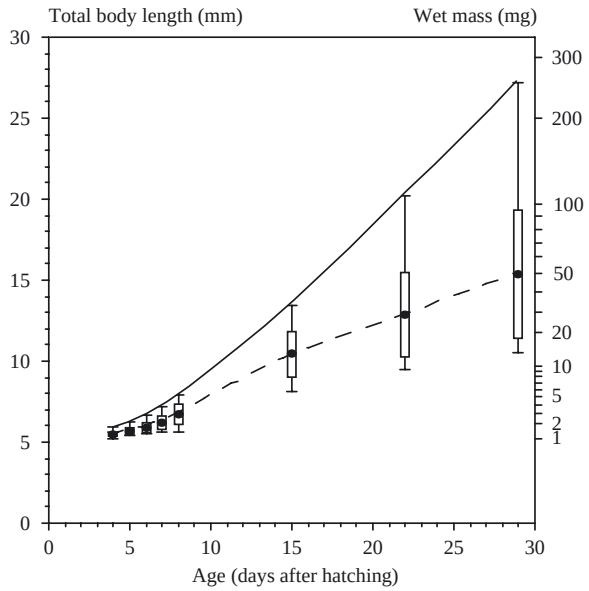

Fig. 8. Growth of clown loach larvae (six daily meals, 28 °C). Symbols and bars are the means and standard deviations, and whiskers show the minimal and maximal total body lengths. Corresponding fish body mass on the vertical right axis. Measures in samples of 20 fish from 4 to 7 dah (stocking density of 1.33 fish L^{-1}) and in all survivors from 8 to 29 dah (2 groups of 30 fish at 0.33 fish L^{-1}) (see methods). Curves produced by interpolation.

6.5% *WM*, whereas it attained 13.5% *WM* at 6.0 mm *TL* (1.8 mg) and 20.0% *WM* at 6.5 mm *TL* (2.3 mg). Thereafter, *FI* continued increasing, but at a much slower pace, and attained 23% at 10 mm *TL* (12 mg). No decrease in *FI* was observed in larger fish, at least until 15 mm *TL*. The examination of gut contents a few minutes after feeding revealed that in fish <5.5–5.6 mm *TL*, the outline and eyes of *Artemia* nauplii in the

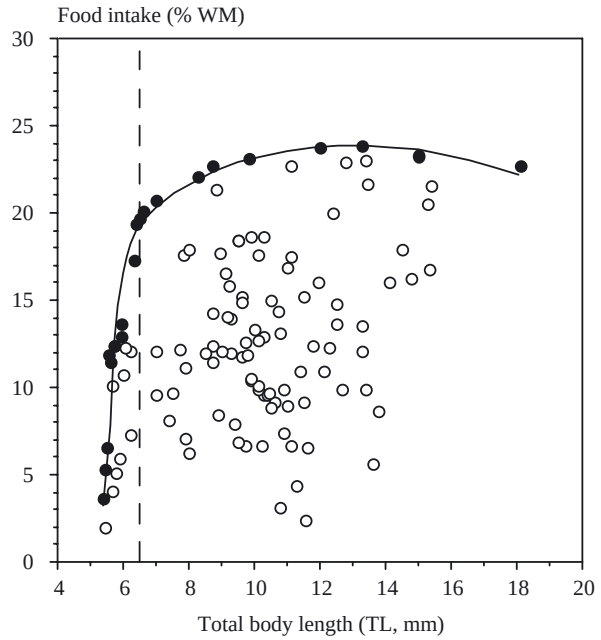

Fig. 9. Size-dependent variations of food intake in clown loach larvae of different sizes. Food intake is expressed as a proportion of the fish wet mass (*WM*). Symbols refer to measurements in individual fish (n = 124). The plain curves were modelled from the highest values of gut content (closed circles). Two separate models were used for fish smaller and larger than 6.5 mm *TL* (vertical dashed line; see Table 2 for equations and statistics).

foregut were never conspicuous, as if they had been chewed or sucked in. By contrast, in fish >5.7–5.8 mm *TL*, the contours of nauplii were sharp and their eyes were visible (Fig. 4a), as if they had been swallowed straight.

The gut evacuation rate (R_g) was examined in 22 fish ranging from 5.9 to 14.2 mm *TL*. All fish had been selected on the basis of their high *FI* by reference to the curve shown in Figure 9. The decline in gut content over time was curvilinear in fish of all ages and sizes, but was best described by a linear relationship during the first two hours following the start of defecation (Fig. 10). Defecation started earlier and R_g was faster in large than in small fish. Proportionally, size-dependent differences in R_g) were highest around the size of fish at the time of gut coiling (7.8–8.0 mm *TL*; Fig. 4b). A stepwise multiple-regression analysis revealed that R_g was significantly dependent on *TL*, *FI* and their interaction (Table 2). Based on this model, R_g would increase rapidly until about 11 mm *TL* and decrease in larger fish. This steep variation in the capacity of young clown loach to evacuate – and thus consume – food compares well to the curvilinear increase of growth rates observed during this study (Fig. 8).

4 Discussion

4.1 Morphology

The fin developmental sequence in *Chromobotia macracanthus* (Fig. 2) is found in other loaches (Krizanovsky 1949; Kim 1997; Shimizu et al. 1998; Pinder 2001; Kottelat 2004)

Table 2. Models depicting the variations of maximal food intake (FI_M) in clown loach larvae of different sizes (total body length, *TL*), and the effects of fish size and food intake on the gut evacuation rate (R_g). The probabilities in the outer right column refer to the intercept and variables, given in the same sequence as in the equations.

Equation – FI_M (% *WM*), *TL* (mm)	Statistics
Fish <6.5 mm *TL* $FI_M = -1063 + 2606 \log TL - 1568(\log TL)^2$	$R^2 = 0.976$, $df = 10$ $p = 0.0031, 0.0041, 0.0060$
Fish 6.5–15 mm *TL* $FI_M = -34.3 + 107.2 \log TL - 49.8(\log TL)^2$	$R^2 = 0.934$, $df = 12$ $p = 0.0035, 0.0002, 0.0003$
Fish 5.9–15 mm *TL*, R_g (% *WM* h^{-1}) $\log R_g = -4.480 + 0.818 \log FI \log TL + 9.469 \log TL - 5.083 (\log TL)^2$	$R^2 = 0.956$, $df = 21$ $p < 0.0001, <0.0001 <0.0001, <0.0001$

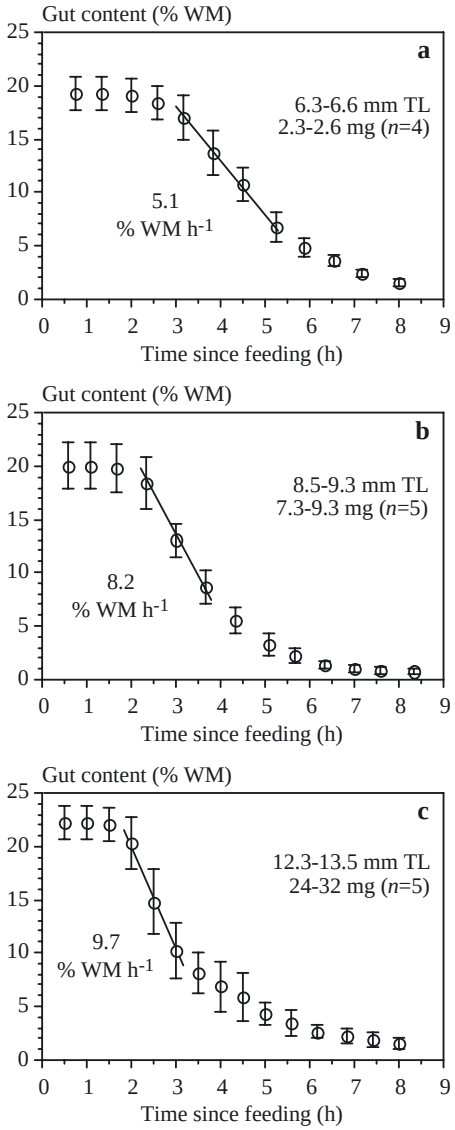

Fig. 10. Decline of gut content volume (% wet body mass, *WM*) in clown loach larvae of different size (total body length, *TL*) fed a single meal. On each graph, symbols and whiskers are the means and standard deviations of the same (four or five) individual fish, having fed near maximally and examined at regular intervals (see Fig. 9 for size-dependent maximal food intake). Only fish with similar sizes are shown here (14 of the 22 fish under examination). The maximal rate of gut evacuation (% *WM* h^{-1}) was calculated on the linear part of the curve, during the first two hours following the start of defecation.

and is shared by all Ostariophysi (E. Baras, unpubl. data). The presence of a yolk extension in clown loach embryos is no diagnostic criterion either, as it is a shared trait among the clade Cypriniformes (Virta and Cooper 2009). By contrast, the early development of the barred pigment pattern in clown loach should make their identification almost univocal from a very young age. It is frequent that skin pigment cells appear during the early ontogeny of fishes (e.g. 2 dah in *Cobitis bilineata*, 3–4 dah in *C. taenia*; Bohlen 1998) and sometimes before hatching (review in Price et al. 2008). However, with the exception of species with very large eggs (e.g. ariid catfishes), it is rare that a pigment pattern be structured at the start of exogenous feeding, and exceptional that it be conserved throughout the ontogeny. The pigment pattern of clown loach passed from five bars in young larvae to three bars in small juveniles (the latter pattern being maintained throughout adulthood). However, at all stages it consisted in an alternation of clear and dark bars spaced at regular intervals (Fig. 5).

Barred or striped pigment patterns serve a broad series of functions, including crypsis, predator evasion or warning, signalling conspecifics or competitors, and it is frequent that a single pattern serves several purposes (Kenward et al. 2004; Ruxton et al. 2004; Price et al. 2008). Whatever the function, pattern repetition and regularity can enhance the effect (Endler 1980; Guilford and Dawkins 1991; Armbruster and Page 1996; McRobert and Bradner 1998), both in mobile and immobile animals (mechanisms in Coren et al. 1999; Kenward et al. 2004). The benefits of structural regularity for a pigment pattern might be one of the reasons why clown loach have evolved developmental mechanisms that enable the (almost) maintenance of a regular distance between bars throughout their ontogeny.

4.2 Growth depensation

It is generally assumed that siblings possess slightly different capacities for growth, and genuine differences are amplified in the course of dominance hierarchies, resulting in increasing dispersal in fish of increasing size, unless cannibalism emerges, removes the smallest fish and stabilizes size dispersal (Huston and DeAngelis 1987; Hecht and Pienaar 1993; Kubitza and Lovshin 1999; Baras and Jobling 2002; Kestemont et al. 2003). The present study comprised no detailed study of clown loach behaviour or individual growth

trajectories, so it is uncertain whether the marked size dispersal of clown loach under culture conditions was a matter of dominance hierarchies or intrinsic differences in metabolism and capacities for growth. There is some indirect evidence that not all clown loach embryos transformed their yolk with the same efficiency (Fig. 6), which suggests between-individual differences in metabolic rates. However, similar differences can originate from variable yolk composition or resource allocation (e.g. swimming versus growth, Bagatto et al. 2001; for a review, see Kamler 2008).

Nevertheless, the present study provided information that can account for why size dispersal in clown loach is higher than in most cultured fishes, in particular why early size differences can amplify genuinely, even if all siblings possess very similar capacities for growth. Clown loach larvae >5.6 mm *TL* swallow *Artemia* nauplii whole, whereas smaller larvae chew them or suck them in, which is certainly longer and presumably more energy demanding. The transition between the two ingestion modes corresponds to a period of rapid allometric growth of mouth dimensions (Fig. 3). If clown loach larvae are fed *Artemia* nauplii, early size differences are likely to amplify rapidly if the size distribution of larvae at the start of exogenous feeding encompasses the aforementioned cut-off size. This was the case here, and in almost all progenies of clown loach studied since then (authors' unpublished data). Growth depensation further continues because of the allometric increase of food intake (until 7 mm *TL*; Fig. 9) and gut evacuation rate (until 10 mm *TL*; Fig. 10) in parallel with gut coiling (Fig. 4). These genuine differences might account for why size heterogeneity in the growth study increased regularly until all individuals had grown beyond this size (Fig. 8). Clown loach larvae possess no oral teeth, their mouth is small and their body is deep (Fig. 3), so sibling cannibalism is almost impossible, i.e. it could only occur if the cannibal were three times as long as its victim (corresponding *WM* ratio of 25–30), which is much higher than in most cultured species (review in Baras 2012). In absence of cannibalism, size dispersal can continue increasing under culture conditions, especially if rearing protocols are not tailored yet (Kubitza and Lovshin 1999; Kestemont et al. 2003). Further studies are needed to determine the proximal factors (fish density, food density, temperature, etc.) that can limit growth dispersal of clown loach under culture conditions.

4.3 Slow and heterogeneous growth of clown loach: an ecological perspective

Although larvae and juveniles of clown loach were fed abundantly with high-energy feed (*Artemia* nauplii) distributed all day round, their growth averaged 0.4 mm day^{-1} and did not exceed 0.9 mm day^{-1} in top growers. This is a slow growth in comparison to most freshwater tropical fishes in culture conditions, but not in comparison to other cobitids, as this group seemingly comprises no fast growing species. The growth of young clown loach was strongly heterogeneous, for reasons that were debated above. The present study also provided evidence that clown loach larvae could survive protracted periods of food deprivation, as the P_{50} mortality at 26.7 °C did not occur before 14–15 dah (Fig. 7). This is not exceptionally long in comparison to species producing large eggs or living in cold climates (Kamler 2002, 2005), but substantially longer than in most warmwater fishes with similar egg size (Araujo-Lima 1994; Yúfera and Darias 2007).

The combination of these three traits (resistance to starvation, slow and heterogeneous growth) can be interpreted in an ecological perspective. In the River Musi (Sumatra), adult clown loach live in the upper and medium reaches and there are strong indications that they spawn during flood pulses following heavy rainfalls (Legendre et al. 2012). Eggs of clown loach are not adhesive, they undergo substantial swelling and become semi-buoyant (Slembrouck et al. 2012), so they probably enter the drift, as those of many other seasonal strategists with similar characteristics (Lowe McConnell 1987; Araujo-Lima and Oliveira 1998; Lucas and Baras 2001). Free embryos of clown loach in the present study were observed swimming actively in the water column during the first 3 days after hatching. Hence, it is likely that their wild counterparts continue drifting at this age, until they fill their swim bladder, acquire their pigment pattern, shift to benthic behaviour and settle in the recently inundated floodplain. These interpretations are supported by field observations. Because of an increasing shortage of market size specimens in the wild, Indonesian fishermen nowadays harvest clown loach larvae (<10 mm *TL*). Yet, they continue fishing in the same places where they used to collect juveniles in the floodplain, but they focus their fishing effort when water starts receding after a flood pulse, thereby supporting the view that young larvae drift onto the recently inundated floodplain.

Survival in the wild is generally size-dependent and it is frequently assumed that young-of-the-year fishes must have attained a minimal size at the onset of the harsh season (Sibly et al. 1985; Arendt 1997). In the tropics, the larvae of seasonal strategists born at the start of the rainy season can spend months in the floodplain before water recedes and forces them in the main river channel, where predation hazards are high (Winemiller and Jepsen 1998; Lucas and Baras 2001). In view of the length of the growing season, there has probably been little selection pressure on fast growth in these species (Bailey 1988). By contrast, the productivity of the floodplain is generally low at the start of the rainy season, and the first seasonal rainfalls are frequently followed by more or less protracted dry periods, which result in decreasing water levels, habitat fragmentation and temporary food shortage (e.g. Loubens et al. 1992). This context might have favoured the selection of a longer resistance to starvation. In view of the unpredictability of rainfall patterns and associated levels of food availability in the early rainy season, it might be advantageous for early seasonal spawners to produce heterogeneous progenies as regards metabolic rates, as at least a part of the progeny would be adapted to the environment. An analogous way of adapting to unpredictability through intrinsic heterogeneity has been found in annual fishes of genus *Notobranchius* (Cyprinodontidae). Their eggs may enter one or more diapauses of variable durations, postponing hatching at different times of the year, and almost suppressing the risk that an entire progeny dies from hatching in inadequate conditions (Wourms 1972).

4.4 Conclusion, perspectives for the larviculture of clown loach

This study highlighted that the ontogeny of clown loach is particular as regards its pigment pattern, which is well structured before the start of exogenous feeding. It is frequent that the expression of marked pigment patterns varies between social contexts, and this makes clown loach an excellent biological model for the study of social interactions among fish larvae.

In respect to the propagation of clown loach in captivity, survival is expectedly not a major issue, in view of the high fecundity of the species (around 100 000 eggs kg^{-1}; Legendre et al. 2012) and of the low mortality during the embryonic and larval stages in the present study, although the rearing protocols had not been refined yet. Clown loach larvae do not grow rapidly, but this is not a major issue, as they are marketed at a small size (4−5 cm). Nevertheless, improvements in growth rates and size homogeneity are desirable to reduce production costs, especially if broodfish can be reproduced all year round in captivity. Likewise, clown loach in this study consumed large food rations but grew at a slow rate, thereby indicating a low feed efficiency (estimated here as 9−10 mg of *Artemia* nauplii for producing 1 mg of fish). This trait is particularly important because *Artemia* are expensive, so a low feed efficiency could make aquacultural products less competitive than those from capture-based fisheries. Further research in this field is thus needed, essentially by testing the effects of food density, fish density and water temperature, which have been shown to impact on food intake, growth and size dispersal in a broad series of fish species. To this respect, the results on the ontogenetic variations of food intake and gut evacuation rate in the present study provide the bases for tailoring feeding schedules more adequately in respect to the actual feeding capacities of clown loach of different sizes and ages.

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