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Biology and culture of the clown loach *Chromobotia macracanthus* (Cypriniformes, Cobitidae): 2- Importance of water movement and temperature during egg incubation

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Abstract – In comparison to older life stages, the embryonic stages of fishes generally have narrow tolerance ranges for environmental conditions, as regards water quality, temperature and mechanical shocks. The knowledge of these factors is indispensable to appraise the threats brought about by climate or anthropogenic changes upon their resilience, and to define adequate ways of incubating their eggs for an efficient propagation of the species under controlled conditions. Clown loach eggs have a narrow thermal tolerance range in comparison to other tropical and temperate fishes. Hatching occurs at 22−30 °C, and non-deformed larvae can only be obtained at 23.8−30.2 °C. Furthermore, the thermal tolerance of any particular progeny was found dependent on the maintenance temperature of the female parent, thereby making the actual tolerance no broader than 4.5 °C. The (log-log) relationship between the duration of the incubation period and temperature was characterized by a shallow slope, which is more typical of coldwater fishes, as is a narrow thermal tolerance range. On the other hand, clown loach hatched more rapidly (20 h at 26 °C) than predicted by existing models on the basis of water temperature and egg diameter, a feature that is shared by other warmwater fishes producing eggs that undergo a strong swelling process (about three times the ova diameter in clown loach). Clown loach embryos are strongly sensitive to mechanical shocks, but their development is not viable either in protracted steady state conditions, in absence of water movement, as they develop various deformities (e.g. pericardial oedema). This is thought to originate from a hypoxic microenvironment around the embryo, as a consequence of an oxygen gradient developing inside and outside the egg, since the boundary diffusion layer is not refreshed by water movement. This issue is worsened by strong egg swelling and incubation at warm temperature.

Keywords: Tropical freshwater fish / Clown loach / Egg incubation / Ontogeny / Temperature / Aquaculture / Ornamental fish / Indonesia

1 Introduction

Chromobotia macracanthus (Bleeker 1852), a riverine cobitid that is endemic to the islands of Borneo and Sumatra (Indonesia), is a key species of the international market of ornamental freshwater fish, with several tens of millions of juveniles captured in the wild and exported worldwide (Ng and Tan 1997). This exploitation is viewed as presumably overintensive (Olivier 2001) and likely to jeopardize wild stocks, and thus the fishery itself, which is essential to the livelihoods of many families or communities in these regions. This context has fostered the development of an international research effort aiming at the development of reliable technologies for the artificial propagation of clown loach in captivity. The effect of maintenance conditions on the sexual maturation of broodfish, the criteria for identification of ripe fish and the efficiency of hormone-induced breeding treatments have been evaluated in the first article of this series (Legendre et al. 2012). The present article focuses on egg incubation, which was the second bottleneck in the implementation of its artificial propagation, as preliminary trials produced low and variable hatching rates and very high proportions of deformed hatchlings (authors' unpubl. data).

Many critical periods of the ontogeny take place (determination of body axes) or start intra ovo (organogenesis). They depend on intricate processes, which can be compromised by a brief exposure to unfavorable conditions. Environmental constraints refer essentially to illumination (especially ultraviolet irradiation, Vetter et al. 1999; Dethlefsen et al. 2001), mechanical shocks (Jensen and Alderdice 1983), hypoxia (Shang and Wu 2004; Miller et al. 2008), carbon dioxide (Ishimatsu et al. 2004) and nitrogenous products (Holt and Arnold 1983). The

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sensitivity to any particular environmental stressor can vary substantially between developmental stages, e.g. the effects of mechanical shocks are highest during gastrulation (Jensen and Alderdice 1983), whereas those of hypoxia are maximal during the hours preceding hatching (Rombough 1988). Temperature is presumably the most pervasive environmental factor, as it governs the metabolic rate of embryos and their sensitivity to most aforementioned stressors. In general, the thermal tolerance range is much narrower in fish embryos than in older life stages (Rombough 1997), but there are substantial variations between taxa and climates (e.g. <8 °C in the gadid *Gadus macrocephalus*, Forrester 1964; 17 °C in the cyprinodontid *Cyprinodon macularius*, Kinne and Kinne 1962). Temperature is also the main factor controlling the duration of the incubation period, which is of importance for hatchery management. Models have been proposed that predict the duration of the incubation period of fish eggs on the basis of water temperature and egg diameter (marine fishes, Pauly and Pullin 1988; temperate freshwater fishes, Teletchea et al. 2009). However, an overview of the literature on the development of tropical teleosts indicates that the duration of the incubation period is much more variable than in temperate freshwater or marine species, e.g., at 25 °C, 17 h in the characid *Astyanax bimaculatus* (Sato et al. 2006) versus 110 h in the gobiid *Pomatoschistus microps* (Fonds and Van Buurt 1974), although both species produce eggs of similar diameter (about 1 mm). Clown loach could be a particularly interesting model in the field of thermal biology of eggs, in view of the unusual latency-to-temperature relationship found in this species after hormonal induced ovulation (Legendre et al. 2012).

The present study examined the effects of water temperature on the hatching dynamics and viability of clown loach embryos under controlled conditions. It also analysed the effects of water movement, which rapidly turned out to influence critically the survival and development of embryos in this species.

2 Materials and methods

2.1 Fish origin and maintenance

Experiments were carried out at the Research and Development Institute for Ornamental Fish Culture (BP2BIH) at Depok (West Java, Indonesia). All broodfish were wild individuals purchased from fishermen around the village of Beringin Teluk on the River Musi (upstream of Sekayu City, Province of South Sumatra, 2° 40′ S–103° 10′ E) during the reproductive season (rainy season, from September to November). Following capture, sexually mature individuals were maintained in groups in wooden cages (0.5 m^3) immersed into the river, then conditioned in plastic bags with a small volume of water and oxygen atmosphere for transportation (car and plane, 10–12 h of travel) to the Depok research station. They were transferred into indoor 200-L quarantine aquaria (ambient temperature, ranging from 26 ± 1 °C to 29 ± 1 °C, depending on time of the year; feeding with earthworms) then into an indoor thermoregulated water recirculation system (10-m^3 tanks, mechanical filter, biological filter, UV-sterilizing system; water flow of 8.2 $m^3\ h^{-1}$, constant water temperature = 26 ± 0.5 °C, 12L:12D, artificial red light of 5–10 lx intensity during the day). They were fed six days a week, twice a day, with commercial pellets (35–40% crude proteins; feeding level of 0.5–1.0% of fish biomass) during the morning, and with earthworms in the evening (feeding level >10% of fish biomass). Previous studies have shown that broodfish of *C. macracanthus* maturing in the wild in the Musi River or in such controlled conditions in the recirculation system displayed similar reproductive traits in terms of quantity and quality of gametes (for more details see Legendre et al. 2012).

2.2 Induced breeding and gametes management

Broodfish selection, hormonal treatments, gamete collection and artificial fertilization were performed as follows (detailed protocols in Legendre et al. 2012). Broodfish were anesthetized (2-phenoxy-ethanol, 0.3 ml L^{-1}) prior to all manipulations. The mature females were chosen after intraovarian biopsy on the basis of a modal diameter of oocytes >1.02 mm. A priming injection of hCG (human chorionic gonadotropin; Organon, France) at 500 IU kg^{-1} was given 24 h before an ovulatory injection of Ovaprim® (Syndel Laboratories, Qualicum Beach, BC, Canada) at 0.6 ml kg^{-1} body weight. Selected males were producing milt at stripping and were treated with a single Ovaprim injection (0.4 ml kg^{-1}) 16 to 24 h before sperm collection. Selected broodfish were held in 200-L tanks (1 or 2 fish per tank for females, and groups of 3-6 individuals for males) at ambient temperature (26 ± 1 to 29 ± 1 °C). A few hours before ova collection, sperm was collected by stripping directly in a syringe containing an immobilizing solution (155 mM NaCl, dilution rate 1:5) and kept in crushed ice (4 °C) until use. Sperm motility was systematically evaluated under a microscope (×100) and only sperm with over 70% motile spermatozoa was used for fertilization. When ovulation was observed following gentle stripping, ova were collected and immediately fertilized, using diluted sperm from two to five males pooled in equivalent volumes (about 10^6 spermatozoa per ovum, activation with commercial spring water). Each batch of fertilized eggs was transferred into a plastic container filled with 300 ml of commercial spring water.

The proportion of fertilized eggs was calculated from counts under the stereomicroscope (×25) in between 2 and 4 h after fertilization (hereafter, haf). At this stage, fertilized eggs had generally attained the morula or early gastrula stage depending on temperature, and could be discriminated without ambiguity from non-fertilized eggs. After hatching, the hatching rate (proportion of fertilized eggs that developed until hatching) and the proportions of non-deformed free embryos (number of non-deformed embryos relative to the number of hatchlings) were determined for each batch of eggs by counts over an illuminated table.

2.3 Experimental design for egg incubation

Several experiments were conducted to evaluate the effects of water temperature and egg movements on the development of embryos and the success of incubation. In total, the progenies of 16 females of 87–368 g body weight (BW) were used in the incubation experiments. Eggs were incubated until

hatching, either in plastic containers or in zugger jars, depending on experiments (see below).

2.3.1 Effects of water temperature

The progenies of nine females were exposed to seven incubation temperature (18, 20, 22, 24, 26, 28 and 30 °C). For each progeny and temperature, four batches of fertilized eggs were used: (1) two replicate batches of about 500 eggs each, which were fertilized in plastic containers, left floating in the incubation systems over 30 min for progressive acclimation to the experimental temperature, then transferred into 5-L funnel-type zugger jars (30 cm in diameter, 35 cm high; flow of 1−2 L min^{-1}); and (2) two other replicate batches of about 150 eggs each, which were maintained in the 300-ml plastic containers and left floating at the surface of the water until hatching, and served essentially at determining the duration of the incubation period at the different temperatures. For each temperature tested, the two latter batches served exclusively to observe the embryonic development and time of hatching, so as to examine the hatching rates in the zugger jars in due time (i.e. any collect from the zugger jars and counting might have been destructive and could have biased the results if done in advance of hatching). Zugger jars were set in an indoor recirculating water system comprising seven 225-L tanks (75 × 75 × 40 (h) cm).The inner diameter of the base of the funnel was set at 2.5 cm, after preliminary trials with finer inlets resulted in excessive egg agitation that caused the rupture of the egg chorionic membrane and death of embryos. In each tank, water temperature was maintained at the desired value (±0.3 °C) by two 300-W submersed heaters connected to a thermostat (Biotherm 2000) and a supply of cold water from a water chiller (Resun C1000, 2700 W). The nine progenies used in this particular experiment were all obtained from female broodfish having matured in the wild and transferred into the quarantine aquaria for less than 10 days. As these fish were shipped at slightly different times of the year, the water temperature in the quarantine aquaria differed slightly and varied between 26 ± 1 and 29 ± 1 °C.

The duration of the incubation period in each batch was set at mid of the P_5 and P_{95} of the hatching dynamics, which were estimated by counts of hatchlings and remaining eggs at 20-min intervals (same principle, though on a looser time scale than for the hatching kinetics, see below). It turned out rapidly that the eggs in the plastic containers systematically produced very high proportions (>90%) of deformed, non viable hatchlings, in contrast to the situation in the zugger jars. As the egg density in the plastic containers was low (about 0.5 egg ml^{-1}) and as the incubation period was short (see results), water quality was presumably not the major factor behind this issue. This was empirically supported by occasional measurement of oxygen, ammonia and nitrites, which did not reveal strong signs of water quality degradation. Henceforth, it was suspected that the absence of egg agitation during one or several critical developmental stages had been critical, and caused us to examine the importance of this factor in another series of dedicated experiments. This also caused us to consider exclusively the results from the zugger jars to examine the effects of incubation temperature on the hatching rate and proportion of non-deformed free embryos in each progeny.

2.3.2 Effects of egg agitation

Two series of experiments were carried out to test for the importance of egg movement during the incubation period. In the first series, batches of fertilized eggs placed in plastic containers filled with 300 ml of commercial spring water (about 150 eggs per container) were either left immobile (two replicate batches per female) or moved continuously until hatching (two other replicates). Continuous movement was obtained by placing the containers on an oscillatory plate (Heidolph Duomax 1000), tuned to produce egg horizontal movements with an amplitude of 8 mm and an average speed of 7.5 mm s^{-1} (as measured with a digital video camera). Water was not renewed during the incubation period so as to maintain complete egg immobility in the "immobile" treatment. The hatching rates and proportions of deformed individuals were measured in each batch, soon (a few minutes or tens of minutes) after all embryos had hatched. All trials were conducted at 26.0 ± 0.5 °C, which was found adequate for all progenies evaluated during the study on water temperature (see results). The experiment was repeated on the progenies of nine female broodfish (of which two also served to the second series of experiments, see below) fertilized with pooled sperm from at least three male broodfish.

The second series of experiments aimed to determine when (at which period(s) of the embryonic development) the absence of egg movement impacted significantly on the hatching rate and presence of morphological deformities in hatchlings. It was conducted on the progenies of two female broodfish (20 batches of eggs per progeny, 300-ml plastic containers, 150−200 eggs per batch, no change of water, 26 °C), using the following design. Within the 5 minutes following fertilization, 10 batches of eggs were placed on the oscillatory plate (same tuning as above), whereas the 10 other ones were left immobile. At 2 haf, one immobile batch and one agitated batch were selected at random and moved to the other treatment. Thereafter, these batches were left unchanged in their new situation until hatching. The operation was repeated every 2 h until hatching (about 19 haf at 26 °C), thereby producing two complementary time series (i.e. eggs agitated or left immobile from fertilization until the age of 2, 4, 6, ... 20 haf). At the time containers were moved from a situation to another, several photographs were shot for a rough characterization of the developmental stage and search for obvious morphological deformities.

2.3.3 Follow up of embryos development and hatching kinetics

In order to pinpoint the information on incubation dynamics, the hatching kinetics of two progenies were followed at close intervals (i.e. hatchlings were removed with a pipette and counted) to determine the following indicators: total hatching period (delay between first and last hatching events), median (P_{50}) hatching time (time at which 50% of the embryos had hatched) and how long it takes to hatch for 50% ($P_{75}-P_{25}$) or 90% ($P_{95}-P_5$) of the embryos. The experiment was conducted exclusively on eggs at 26.0−26.5 °C, which was ultimately found as optimal for incubating clown loach eggs (see results).

2.4 Statistical analyses

Values are means ± SEM (standard error of the mean). One-way non parametric analysis of variance for paired data was used for comparisons of mean hatching rates or proportions of non-deformed free embryos in the experiments on the effect of temperature and egg agitation (transfer from immobility to movement and vice versa). Wilcoxon sign test for paired data was used for comparing the hatching rates and proportions of non-deformed free embryos obtained from eggs constantly incubated in immobile or agitated conditions. Simple regression analyses were used to test for the correlation between hatching rates and proportions of non-deformed hatchlings and for the effect of temperature on hatching time. Hatching kinetics and relationships between age at transfer and hatching rate (or proportion of non-deformed hatchlings) were modelled with log-logistic regression analyses. All tests were done using Statistica (6) software. Null hypotheses were rejected at $p < 0.05$.

3 Results

The mean diameter of striped ova ranged from 1.08 to 1.36 mm. Following the transfer in water, the eggs underwent a rapid swelling process, the extent of which depended on water conductivity (e.g. 3.24–4.08 mm and 2.70–3.40 mm final egg diameter after swelling in water of 18 and 175 μS cm^{-1}; i.e. 300% and 250% of the initial ova diameter, with a corresponding volume increase of 27 and 15.5 times, respectively). The bulk of swelling took place within the first 40 min after fertilization, but some further enlargement of the perivitelline space was observed during the next 4 h. Similar swelling was observed in non-fertilized eggs, and in all cases, it caused the eggs to become semi-buoyant. Fertilized eggs were almost spherical and their chorionic membrane did not show any adhesive properties. The yolk of clown loach eggs was greenish-yellowish and contained no oil globule. When eggs were incubated in favourable conditions (see below) at a constant water temperature of about 26 °C, the first embryonic division occurred after about 40 min and the morula stage was attained after 90–120 min. Gastrulation (ring stage) was conspicuous at about 4 haf and was completed (blastopore closure) 3–4 h later. The cephalic area was conspicuous at 10 haf, and the first somites were observed during the next hour. The elongation of the embryo's body during the following hours caused the yolk sac to acquire an ellipsoidal shape (12 haf) then a pear shape from 15–16 haf onwards. This stage coincided with more frequent flexures of the embryo's body, and hatching took place between 18 and 22 haf.

3.1 Effect of water temperature on survival and development

In every progeny under evaluation, the embryonic development of eggs incubated at 18 °C in zugger jars was halted before the morula stage, and free embryonic cells were observed in the perivitelline space. A similar situation was observed at 20 °C, except that the embryonic development continued, at least in some eggs, until the start of gastrulation.

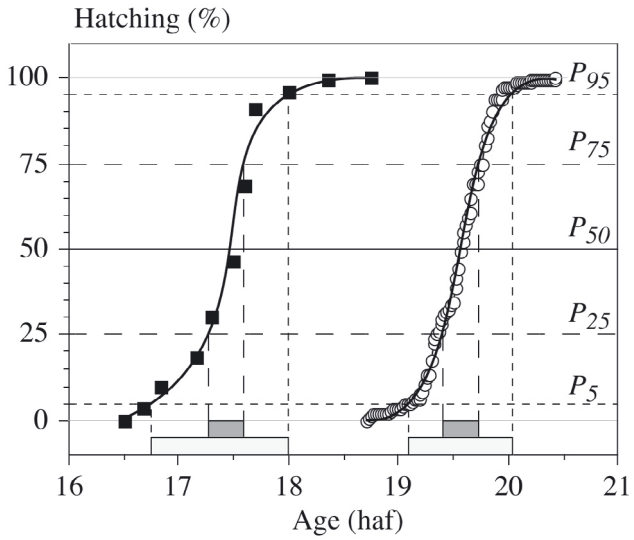

Fig. 1. Hatching dynamics in two progenies of clown loach incubated at 26 °C (open circles; 153 eggs, minute by minute counts) and 26.5 °C (closed squares, 339 eggs, 11 periodic counts). The grey areas at the bottom of the graph illustrate how long it takes to hatch for 50% (dark grey) or 90% (light grey) of the eggs. Curves were produced with log-logistic regression analyses (26 °C: $r^2 = 0.981$, $df = 100$; $p < 0.0001$; 26.5 °C: $r^2 = 0.956$, $df = 8$; $p < 0.0001$). "haf": hours after fertilization.

At all other temperatures (22–30 °C), hatching was observed, after variable durations of incubation: 24–27 haf at 22 °C, 21–24 haf at 24 °C, 18–22 haf at 26 °C, 17–19 haf at 28 °C and 16–17 haf at 30 °C (range of durations for all duplicate batches from all progenies tested). When using the centre of the range of incubation duration at each temperature, this gives an excellent ($r^2 = 0.99$, $df = 4$) relationship between temperature (T, °C) and the duration of incubation (DI, h), i.e. $\log DI = 3.303 - 1\,414 \log T$.

Hatching dynamics were quantified at temperatures close to 26 °C only, as this thermal regime turned out to be the best for incubating clown loach eggs (see below). In the two progenies that were examined, hatching took place at slightly different times (P_{50} of 17.5 haf at 26.5 °C and 19.3 haf at 26 °C). However, the hatching dynamics were most similar as the delays between the first and last hatching events were about 1.7 h in both cases (Fig. 1). Hatching in each progeny was strongly synchronous, as 50% (P_{25}–P_{75}) and 90% (P_5–P_{95}) of the embryos hatched within no more than 26 ± 1 min and 57 ± 7 min, respectively.

Egg quality was highly variable between progenies, as indicated by contrasting fertilization rates (31–83%), hatching rates (H of 26–93%) and proportions of live non-deformed hatchlings (NDH of 7–91%; Fig. 2a). There was a significant positive correlation between H and NDH ($r^2 = 0.90$, $p < 0.0001$, $df = 8$). Hatching occurred over the entire 22–30 °C range in three of nine progenies, and in more restricted thermal ranges (4 or 6 °C) in others (Fig. 2a), but systematically at 26 and 28 °C. At 22 °C, all hatchlings were deformed and at 30 °C non-deformed hatchlings were observed in two progenies only and in low proportions, whereas their occurrence was almost systematic and their proportions much higher at intermediate

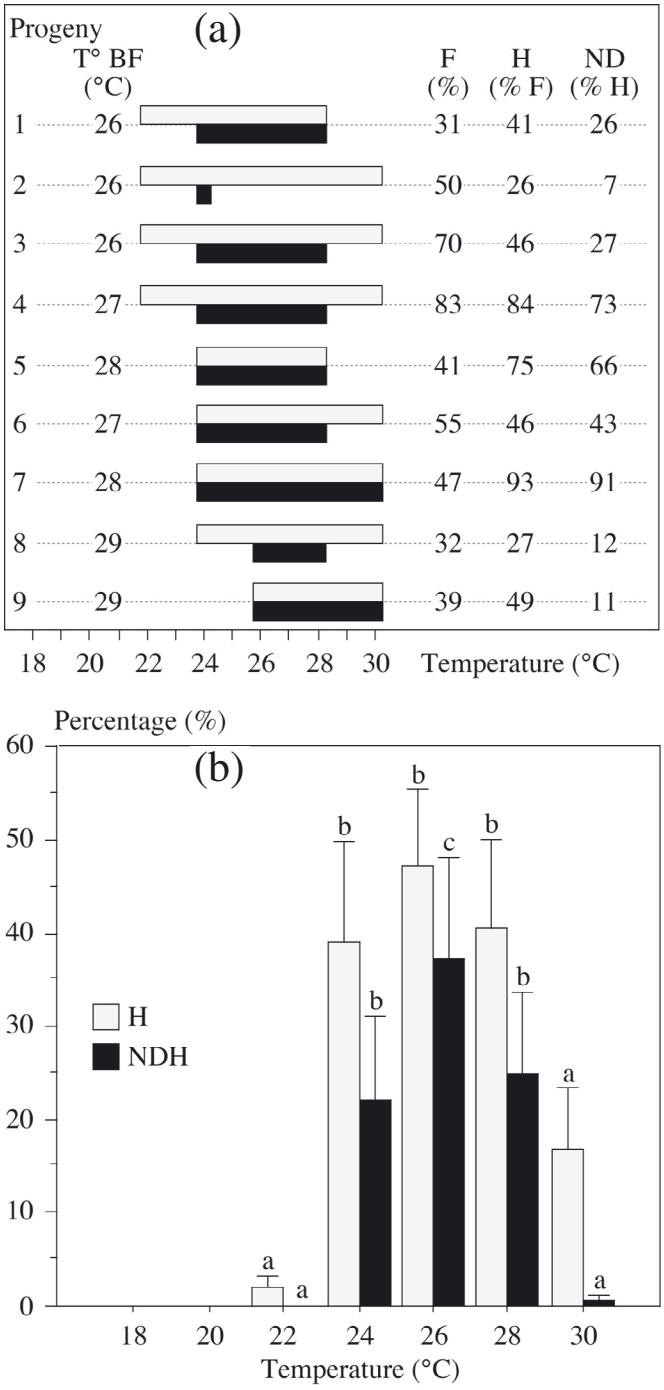

Fig. 2. (a) Thermal range over which hatching occurred (dotted bars) and produced at least some live non-deformed fish (dark bars) in nine progenies of clown loach. $T°$ BF is the maintenance temperature of broodfish during the final stages of maturation, F is the proportion of fertilized eggs, H is the proportion of hatchlings among fertilized eggs and *NDH* is the proportion of live non-deformed fish among hatchlings; the values of F, H and *NDH* on the right side of the graph refer to the highest proportions observed for a particular progeny, irrespective of the water temperature. (b) Mean ± SEM values of H and *NDH* at 18–30 °C in the nine progenies under evaluation. For each variable (H and *NDH*), bars with different superscripts differ at $p < 0.05$ (non parametric repeated measures ANOVA and sign-tests).

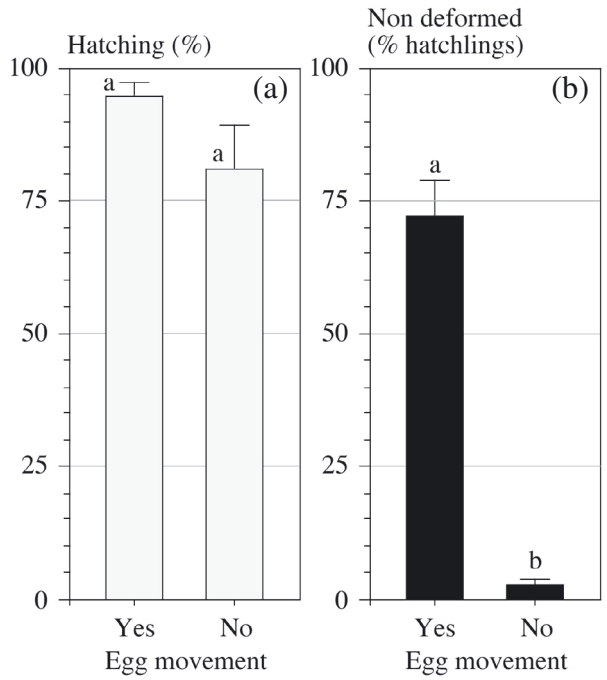

Fig. 3. Effect of egg movement on hatching rate (a) and proportion of live non-deformed fish among hatchlings (b). Eggs from nine progenies incubated at 25–27 °C in 300-ml plastic containers either left immobile or moved continuously from fertilization to hatching. In each graph, bars with different superscripts differ at $p < 0.05$ (sign tests for paired data).

temperatures. Repeated measures ANOVA indicated that the mean hatching rate was significantly higher in the 24–28 °C range than at lower or warmer temperatures ($F_{4.8} = 10.84$, $p < 0.0001$; Fig. 2b). A similar pattern was obtained for *NDH* ($F_{4.8} = 7.84$, $p < 0.0001$), except that a significantly higher score was attained at 26 °C.

3.1.1 Effect of egg movement during incubation on hatching rate and embryo viability

Comparisons between eggs incubated in zugger jars and sibling eggs placed in stagnant water in immobile plastic containers did not reveal any significant difference between hatching rates (Sign test, $z = 1.33$, $p = 0.1820$, $df = 8$). By contrast, the proportions of non-deformed hatchlings in stagnant water were about 12 times lower than in the zugger jars (Sign test, $z = 2.67$, $p = 0.0076$, $df = 8$). Deformities essentially consisted in spinal curvature, no or incomplete yolk extension, short tail, and mild or severe pericardial oedema.

Additional comparisons in standardised conditions (i.e. eggs in small plastic containers either left immobile or placed on an oscillating plate throughout the incubation period) provided further evidence that the absence of egg movement during the incubation did not affect hatchability (H of 81.1 ± 8.1 versus 94.8±2.6%, Sign test, $z = 1.48$, $p = 0.1386$, $df = 8$), but significantly impacted on the embryonic development and viability of hatchlings (*NDH* of 2.9 ± 1.4 versus 72.2 ± 6.7%, Sign test: $z = 2.67$; $p < 0.0077$, $df = 8$; Fig. 3).

Attempts to identify which periods of the embryonic development were critical and required egg movement are shown in Figure 4. It is worth noting that the two progenies used in this particular experiment had the lowest hatching rates of all nine progenies under study for eggs incubated in absence of movement throughout the duration of incubation (23 and 53% versus mean of 81%, Fig. 3). By contrast, the corresponding hatching rates among eggs moved throughout did not depart from the mean score over nine progenies (98 and 99% versus mean of 95%). In both progenies, eggs incubated in absence of movement until the age of 10–12 haf then gently agitated until hatching, exhibited hatching rates close to 100%. This proportion dropped rapidly for any additional 2 hours in absence of movement (Fig. 4a). Conversely, the eggs that were agitated over the first 10–12 h then left immobile until hatching, had low or medium hatching rates, whereas the hatching rate was near maximal when the eggs had been agitated until 14–20 haf. These findings clearly indicated that the absence of egg movement during the final hours of the embryonic development intra ovo was likely to compromise hatching.

The importance of egg movement during the developmental stages prior to hatching was further attested by the corresponding variations in the proportions of live, non-deformed individuals among hatchlings (Fig. 4b). As many as 80–90% of non-deformed hatchlings were obtained when eggs were moved continuously until hatching, whereas this proportion dropped to 10–40% (depending on progeny) if movement was halted 2 or 4 h earlier, and to 0% for ages ⩽12 haf. The mirror situation (transfer from immobility to movement) revealed that egg movement was also indispensable during the first hours following fertilization. The proportion of live non-deformed fish decreased rapidly if the eggs were not moved during the morula and early gastrula stages (2–4 haf) and dropped to less than 10% if gastrulation (4–8 haf) took place in absence of movement.

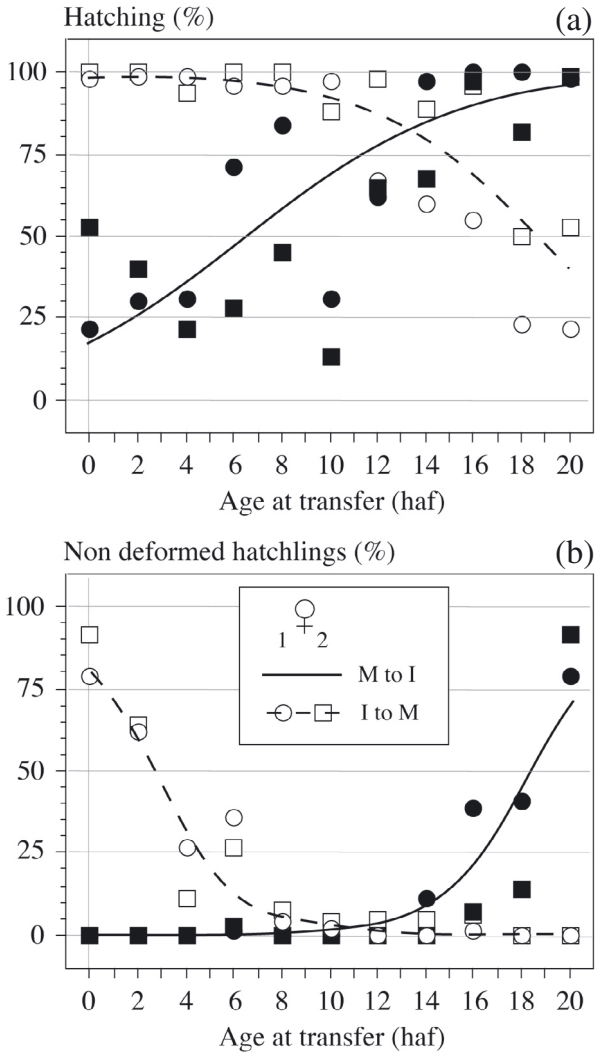

Fig. 4. Effect of the absence of egg movement at different ages (hours after fertilization, haf) on egg hatchability (a) and proportion of non deformed hatchlings in clown loach (b). Circles and squares refer to eggs from two different progenies (1 and 2) that were transferred from situations of continuous movement to immobility (M to I, closed symbols, plain curves) or vice versa (I to M, open symbols, dotted curves) at different ages. Eggs incubated at 26.0 ± 0.5 °C (range) in 300-ml containers, with all hatching events taking place between 18 and 20 haf. Curves were constructed with log-logistic regression models on data from both progenies (statistics, $df = 21$, $p < 0.0001$ in all cases: hatching: $r^2 = 0.593$ and 0.721, for M to I and I to M, respectively; non-deformed hatchlings: $r^2 = 0.855$ and 0.826, for M to I and I to M, respectively).

4 Discussion

4.1 Thermal biology of clown loach eggs

It is well known that the embryonic and larval stages of fishes are always more stenothermal than older stages (Rombough 1997; Rijnsdorp et al. 2009; Pörtner and Peck 2010). Rombough (1997) found that the thermal tolerance range of intra ovo embryonic stages of fishes averaged 11.6 °C. However, an in-depth analysis of the database compiled by Rombough (op. cit., completed with other data from Kamler 1992) indicates that the thermal tolerance range tends to increase with water temperature (Fig. 5). The present study provided evidence that clown loach eggs are more stenothermal than those of other warm-water species, as hatching only occurred from 22 to 30 °C, and non-deformed hatchlings were obtained exclusively from 24 ± 0.2 to 30 ± 0.2 °C, thus a 6.5 °C range. However, it was found here that no single progeny had a thermal tolerance broader than 4.5 °C, and that the range depended on the maintenance temperature of broodfish during the final stages of ovarian maturation (Fig. 2). Such acclimation effects are frequent in fishes (review in Burt et al. 2011). As broodfish in the present study were held at temperatures ranging from 26 to 29 °C only, it cannot be excluded that clown loach eggs could tolerate slightly colder or warmer temperatures if their female parents were held at colder or warmer temperatures than here. It is also possible that the thermal tolerance of progenies issued from different parental combinations varies, as reported in several fish species, e.g. sockeye salmon *Oncorhynchus nerka*, Burt et al. (2012).

In comparison to other tropical fish species (e.g. the catfishes *Clarias gariepinus*, Haylor and Mollah 1995;

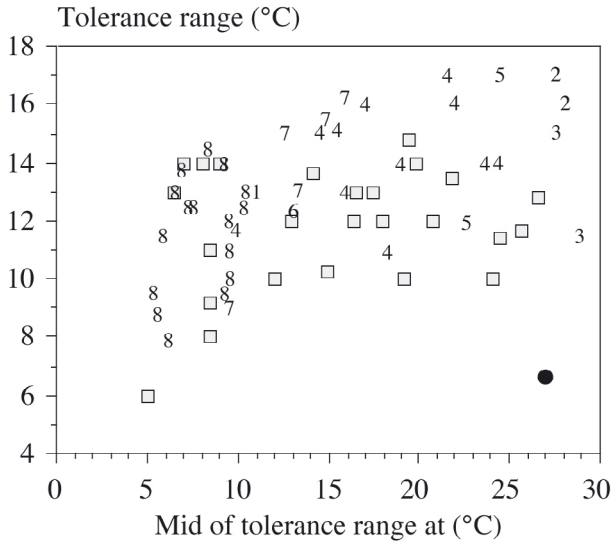

Fig. 5. Variation of the thermal tolerance range during egg incubation in marine (grey squares, 25 species) and freshwater fishes (numbers, 40 species) from different families, in comparison to clown loach (closed circle). For freshwater species: 1: Catostomidae (n = 1); 2: Cichlidae (n = 2); 3: Clariidae (n = 2); 4: Cyprinidae (n = 11); 5: Cyprinodontidae (n = 2); 6. Esocidae (n = 1); 7: Percidae (n = 5); 8: Salmonidae (n = 16). Data from Rombough (1997), essentially, and Kamler (1992).

Heterobranchus longifilis, Legendre and Teugels 1991; *Rhamdia quelen*, Rodrigues-Galdino et al. 2009), clown loach is not exceptionally intolerant to cold temperatures but strongly intolerant to temperatures >30 °C. The aforementioned species occur over a rather broad latitudinal range across the tropics, whereas clown loach is restricted to the equatorial region, which is the part of the world where air (and water) temperature exhibits the lowest year-round variations and where climate has been the most stable during the Holocene (Colwell et al. 2008). This context is likely to result in the selection of thermal specialists, as there would be no penalty for being stenothermal. In the very case of clown loach, such stability is presumably further guaranteed by its reproductive ecology, as there are indirect evidences that the species spawns on flood pulses following heavy rainfall (Legendre et al. 2012), when water temperature varies very little between hours of the day and successive days, and as the incubation of its egg is short (20 h at 26 °C). The knowledge on the thermal biology of fish eggs in tropical and equatorial regions is currently too scarce to support or invalid these functional hypotheses.

The duration of egg incubation in fishes is governed by two factors essentially: egg diameter and water temperature (Pauly and Pullin 1988; Kamler 1992; Rombough 1997; Teletchea et al. 2009), i.e. at a given temperature small eggs hatch sooner than large eggs and for a given egg size hatching occurs more rapidly at warm than at cold temperature. Two main (log-log) models have been produced, one for marine species (Pauly and Pullin 1988) and one for temperate freshwater species (Teletchea et al. 2009). The predictions of both models overestimate the duration of incubation in clown loach (by 30 to 40% and 54 to 58%, respectively, depending on temperature), thereby emphasizing that egg incubation is brief in this species. This is not unique, though, as shorter incubation times have been reported in tropical fishes with similar egg diameter (e.g. 11 h at 26 °C in the characid *Brycon cephalus*, Romagosa et al. 2001; 13 h at 27.5 °C in the anostomid *Leporinus frederici*, Sanches et al. 2008), and almost all warmwater or tropical fishes with semi-buoyant eggs and strong swelling hatch much sooner than predicted by any of the two aforementioned models (see the Chinese carps in Teletchea et al. 2009 and a list of tropical species in Rodrigues-Galdino et al. 2009). McDowall (2009) hypothesized that for amphidromous gobies, the shorter the time between fertilization and hatching, the higher the survival for a series of reasons, including shorter exposure to predators, longer transport at the larval stage and greater resistance to starvation as plenty of yolk remains at hatching. This hypothesis also largely applies to freshwater species with drifting eggs and larvae (Lucas and Baras 2001). Species sharing this life style generally trade off the risk of mortality at egg stage against reduced locomotion, as hatching generally takes place at an early developmental stage, when free embryos still have a short caudal region, poorly developed fins or finfold, and a massive yolk, which generally hampers swimming efficiency (Mauguit et al. 2010). However, the latter penalty varies between taxa, as the yolk shape of hatchlings is strongly influenced by phylogeny (Virta and Cooper 2009). In particular, in many Characiformes and almost all Cypriniformes (including clown loach, see Baras et al. 2012), there is a longitudinal yolk extension along most of the abdominal part of the embryo's tail. This trait, which is not shared by other taxa (except for the Anguilliformes, Virta and Cooper 2009), is believed to provide some additional ventral rigidity and result in more efficient swimming, thereby reducing the penalty for hatching at a less developed stage.

The relationship between the duration of the incubation period and water temperature in clown loach was best described by a log-log (power) relationship and characterized by a rather shallow slope, i.e. the incubation period decreased by 12% for any 10% increase in water temperature. This contrasts with most temperate and warmwater species, for which the corresponding variation in the incubation period ranged from 16 to 22% (Cypriniformes; Herzig and Winkler 1986; Kamler et al. 1998; Siluriformes, Haylor and Mollah 1995; Small and Bates 2001; Legendre and Teugels 1991). Slopes as shallow as or shallower than observed for clown loach have been reported among teleosts (5–12%), but essentially in coldwater species (Salmoniformes, Humpesch 1985; Clupeiformes, Pauly and Pullin 1988).

Hatching in clown loach was highly synchronous, as 90% (P_5–P_{95}) of the embryos hatched within less than one hour at 26 °C (Fig. 1). In most fish species documented to date, the length of the hatching period (hereafter LHP) is much longer, but it is well known that this variable is negatively correlated with temperature (Kamler 1992) and most knowledge comes from temperate or coldwater species. In order to enable comparisons between taxa from different climates, the LHP was expressed as a proportion of the mean hatching time (P_{50}), thereby giving a relative value of 5.3% for clown loach at 26 °C. Similar calculations on the data from other authors

indicate much higher values (30–50% in temperate cyprinids; Herzig and Winkler 1986; Kamler et al. 1998; 25% in the cottid *Icelinus quadriseriatus*; 23% in the clariid *Heterobranchus longifilis*, Legendre and Teugels 1991; exception, 5–6% in the sparid *Pagellus erythrinus*, Klimogianni et al. 2004). In echo to the hypothesis by McDowall (2009), it is possible that species having evolved a short hatching time in response to predation threats have also evolved a marked hatching synchrony, for the same reasons.

4.2 Effect of water movement on clown loach eggs: a matter of hypoxic microenvironment?

This study provided evidence that the incubation of clown loach eggs in stagnant water in absence of movement was highly detrimental to their survival or development. This is not usual, as experiments under similar conditions on several tropical fish species with adhesive (*Clarias gariepinus*, *Heterobranchus longifilis*, *Pangasianodon hypophthalmus*, *Pangasius djambal*) or semi-buoyant eggs (*Pseudoplatystoma punctifer*) did not show any negative effect of steady state condition in stagnant water (Legendre and Teugels 1991; Legendre et al. 2000; Slembrouck et al. 2004; Legendre and Dugué unpubl. data for *P. punctifer*). The realisation of dedicated experiments where eggs were incubated in plastic containers that were either moved or left immobile throughout incubation provided further evidence that these issues did not originate from the absence of water renewal. The effect of movement on fish eggs has been reported in other fishes, but it is generally the other way round: mechanical shocks are highly detrimental to fish eggs, especially during gastrulation, e.g. rainbow trout *Oncorhynchus mykiss* Billard (1976), coho salmon *Oncorhynchus kisutch* Jensen and Alderdice (1983). Clown loach eggs are seemingly sensitive to mechanical shocks as well, as preliminary incubation trials in zugger jars equipped with a fine inlet pipe, which locally produced stronger flows, resulted in early rupture of the chorionic membrane of most eggs. Negative effects of low flows on embryonic development or mortality have been documented in fishes, but generally in species laying egg masses, as eggs at the centre of the mass are subjected to stronger hypoxic environments than those near the periphery, e.g. lingcod *Ophiodon elongatus*, Giorgi and Congleton (1984). Embryos developing in strongly hypoxic environments exhibit almost systematically a pericardial oedema. This deformity has been observed in fishes, e.g. zebrafish *Danio rerio*, Lee et al. (2009) and in other taxa: Grabowski (1970), Webster and Abela (2007); mechanism in Chernoff and Rogers (2010). Here, almost all clown loach embryos incubated in steady state conditions in stagnant water precisely exhibited a mild or severe oedema in the pericardial region. However, the oxygen concentrations in agitated and immobile batches from the same progeny were quite high and similar throughout egg incubation (3.5–5.5 mg O_2 L^{-1}, depending on progeny and fertilization rate). Likewise, the concentrations of nitrogenous products were similar and low (0.006–0.085 mg L^{-1} for N-NH_3 and 0.003–0.004 mg L^{-1} for N-NO_2^-). It cannot be claimed that these environmental conditions were optimal for clown loach eggs, but at least they did not alter substantially the embryonic development in agitated batches.

The ambient medium in non-agitated batches was not hypoxic, but it cannot be excluded that the microenvironment around the embryo became increasingly hypoxic during the ontogeny. Eggs can be viewed as oxygen traps, as the embryo consumes oxygen whereas the chorionic membrane acts as a barrier to oxygen diffusion. An oxygen gradient thus develops gradually inside a "diffusion boundary layer" (DBL), with the lowest oxygen concentration around the embryo (Berezovsky et al. 1979; Pinder and Feder 1990; Ciuhandu et al. 2007; Miller et al. 2008). This can be a major developmental issue, as the aerobic requirements of embryos are met by oxygen diffusion only (Krogh 1941). In steady state situations in stagnant water, the DBL can range several millimeters or centimeters outside the egg capsule (Kranenberg et al. 2000). Oxygen demand increases during ontogeny as a function of embryonic body mass (by one order of magnitude in many cases, e.g. *Gadus morhua*, Davenport and Lönning 1980; *O. mykiss*, Ciuhandu et al. 2007; Miller et al. 2008), thereby making the risk of hypoxia highest during the hours preceding hatching (Rombough 1988). It is assumed that in species laying their eggs in stagnant water, the oxygen layer around the embryo can be refreshed by three mechanisms (1) fanning by the parent(s), (2) natural convection, as a consequence of the excretion of metabolic wastes, which are denser than ambient water and sink, thereby creating a weak current around the egg (about 0.2 mm s^{-1}; O'Brien et al. 1978), and (3) muscular contractions of the embryo, which contribute to rupture the oxygen gradient inside the egg (Kamler 1992). Here, it was observed that clown loach embryos incubated in absence of water movement exhibited very little movement, in contrast to those in agitated water. The exposure of eggs to chronic hypoxia was also found to result in lower metabolism and retarded embryonic development in zebrafish (Shang and Wu 2004) and rainbow trout (Miller et al. 2008). In clown loach, it resulted in pericardial oedema, morphological deformities and fish death in most cases, possibly because the consequences of hypoxia are more severe at warm than cold temperature (oxygen concentration is inversely related to temperature, whereas metabolism increases with temperature). The strong swelling of clown loach eggs (about three times the diameter of ova) is also likely to make the microenvironment near the embryo more hypoxic than in species with smaller perivitelline space (e.g. the catfishes listed at the start of this section).

These considerations could account for why the absence of egg movement and oxygen refreshment of the DBL resulted in morphological deformities during the last hours of egg incubation, when the oxygen demand by embryonic tissues was highest. By contrast, hypoxia can hardly been invoked as the major cause behind the deformities taking place following the absence of egg movement during the first hours of the embryonic development (Fig. 4). The axiation, lateralization and dorso-ventral organization of the embryo take place before gastrulation (Cooper and Virta 2007) and require cortical rotation. Situations where cortical rotation was experimentally prevented systematically resulted in non-viable embryos (Gerhart et al. 1989). However, cortical rotation takes place inside the egg capsule and requires no egg movement, and the

degree of morphological deformation resulting from the absence of cortical rotation is much more severe than observed here (e.g. fully ventralized embryos, lacking all dorsal structures; Gerhart et al. 1989). The reasons why egg movement is indispensable during the early embryonic development of clown loach thus remain to be elucidated.

4.3 Implications for clown loach aquaculture and hatchery management

In view of the detrimental effects of steady state conditions on clown loach eggs, even for restricted periods of time, it is recommended that their eggs be moved gently throughout the duration of incubation, using zugger jars, as here, or any type of egg incubator producing egg movement, provided that it causes no mechanical shocks. The actual tolerance of clown loach eggs to oxygen remains unknown, and by reference with other fish species, it is likely to increase during the course of incubation. It is thus recommended to maintain oxygen as close as possible to saturation, even if concentrations as low as 3.5 mg O_2 L^{-1} were found here to have no negative impact on the embryonic development and hatching rate of agitated eggs.

Clown loach eggs are strongly stenothermal, and their thermal tolerance range is slightly governed by the maintenance temperature of their parents. Henceforth, it is crucial that temperatures in the broodfish maintenance tanks and hatchery be as similar as possible, and ideally as close as possible to 26 °C. In any case, incubation temperatures colder than 24 °C or warmer than 28 °C should be avoided, as departures from this range resulted in dramatically lower hatching rates and higher proportions of deformed hatchlings. In view of the amplitude of daily thermal fluctuations and restricted thermal tolerance of clown loach eggs, it is recommended that incubation be carried out in thermoregulated water systems or thermostatised rooms. When egg incubation is conducted in these conditions, the hatching rate is over 70%, with over 85% of live, non-deformed hatchlings.

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