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Etienne Baras, R. Ginanjar, Mumtaz Ahmad, A. Permana, A. Priyadi, et al.. Biology and culture of the clown loach *Chromobotia macracanthus* (Cypriniformes, Cobitidae): 4- Thermal biology of embryos and larvae. *Aquatic Living Resources*, 2012, 25 (2), pp.131-142. 10.1051/alr/2012012 . ird-01224108

HAL Id: ird-01224108

<https://ird.hal.science/ird-01224108>

Submitted on 4 Nov 2015

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Biology and culture of the clown loach *Chromobotia macracanthus* (Cypriniformes, Cobitidae): 4- Thermal biology of embryos and larvae

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Received 1st February 2012; Accepted 14 May 2012

Abstract – The knowledge of how fish survive and grow at different temperatures, and how these traits vary between life stages, is essential to evaluate the effects of climate change on wild fish and implement effective strategies in aquaculture. These issues are addressed in this study through a series of experiments that evaluate the effect of temperature (23–34 °C) on the embryos and larvae of clown loach, *Chromobotia macracanthus*. This species is endemic to the rivers of Sumatra and Borneo, highly praised on the ornamental fish market, and has been reproduced in captivity recently. No embryo survived a 24-h exposure to 34 °C until the age of 3 days after hatching (dah); mortality was high at 32 °C at 2 and 3 dah, whereas it was low and similar from 1 to 4 dah at 23–29 °C (<10%). Yolk absorption was proportional to water temperature (Q_{10} °C of 1.69 in the 23–32 °C range), but fish reared at cold temperatures were larger than others at the start of exogenous feeding (5.7 vs. 5.5 mm TL, at 23 and 32 °C, respectively). The survival of larvae fed *Artemia* nauplii *ad libitum* was high at 23–32 °C (80–100%), but almost null at 34 °C. Growth models at different temperatures were produced from weekly measurements in two experiments, and tested by comparing their predictions with the results of a third experiment. Throughout the larval stage, the optimal temperature for growth (T_{opt}°) was close to 29 °C, and departures from T_{opt}° resulted in substantial growth penalties (–30% SGR for –5.1 °C and +3.1 °C). High survival, fast growth (0.7 mm day⁻¹) and limited size dispersal at T_{opt}° are encouraging perspectives for the aquaculture of clown loach. From an ecological perspective, the species has an atypical thermal biology, as it is less thermophilic than other tropical fishes, but more stenothermal than temperate fishes exhibiting similar values of T_{opt}° , both traits being of particular concern in the context of global warming.

Keywords: Tropical freshwater fish / Ornamental fish / Clown loach / Temperature / Survival / Growth / Yolk / Embryo / Larva

1 Introduction

Temperature is undoubtedly the environmental factor that has the greatest impact on fish growth (directing factor, *sensu* Fry 1971), as it affects directly basal metabolism, which influences gut evacuation and food intake. As a result of the effect of temperature on basal and active metabolism, the relationship between temperature and growth in fish feeding exogenously (larvae, juveniles, adults) has a parabolic shape (synthesis in Jobling 1994). The temperature that produces the fastest growth (thermal optimum for growth, hereafter T_{opt}°) varies between species, populations (e.g. Koskela et al. 1997) and life stages. In general, the value of T_{opt}° decreases as fish size increases, by virtue of the allometric growth of exchange surfaces (gills, intestine) and body volume. However, the rate of variation of T_{opt}° with increasing body size varies substantially

between fish species (from –0.2 to –4.7 °C for a 10-fold increase of body mass; synthesis in Baras et al. 2011). The value of T_{opt}° can be further dependent on the feeding level (Kitchell et al. 1977; Hogendoorn et al. 1983) and on the energy or protein content of food (e.g. Elliott and Hurley 1999, 2000; Katersky and Carter 2007). Temperature impacts on growth, but also on size dispersal (Baras et al. 2002, 2011; Baras and Florès 2006), which facilitates cannibalism (Hecht and Pienaar 1993; Baras and Jobling 2002). The mechanisms underlying these patterns are well understood, but the substantial variability between species makes it almost impossible to predict the adequacy of a particular temperature for a particular species or life stage in a particular environment, while such knowledge is indispensable to adjust rearing strategies in an aquacultural context.

In theory, the thermal responses of embryos (either *intra ovo* or after hatching) should be more straightforward than those of fish feeding exogenously. Embryos possess a finite

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food reserve (the yolk), which is readily available, requires no additional expense for foraging or handling, and cannot be disputed by competitors. The rate of yolk absorption increases with increasing temperature, so both the start of exogenous feeding and the death of fish deprived from food should take place at ages that are inversely proportional to temperature. The latter statements have been largely supported by experimental evidences in a broad series of fish species with contrasting origins and egg sizes (yolk absorption: syntheses in Kamler 1992, 2002; death from starvation: Atlantic herring *Clupea harengus*, Yin and Blaxter 1987; Nile tilapia *Oreochromis niloticus*, Rana 1990; Nassau grouper *Epinephelus striatus*, Watanabe et al. 1996; Japanese flounder *Paralichthys olivaceus*, Dou et al. 2005). As a result of the relationship between basal metabolism and temperature, maintenance needs are higher at warm than cold temperatures. As the energy from the yolk is finite, there is less surplus energy for fuelling body growth, so the yolk utilization efficiency (YUE) and the body size of fish at time of full yolk absorption should be inversely proportional to water temperature as well. These trends have been illustrated in many fish species (e.g. American plaice *Hippoglossoides platessoides*, Howell and Caldwell 1984; leopard grouper *Myctoperca rosacea*, Gracia-López et al. 2004; Malabar grouper *Epinephelus malabaricus*, Yoseda et al. 2006). However, there have been as many examples for which the relationship between YUE and temperature was dome-shaped within the thermal range producing no developmental deformities (e.g. ronco croaker *Bairdiella icistia*, May 1974; yellowtail scad *Atule mate*, Santerre 1976; *O. niloticus*, Rana 1990; gilthead seabream *Sparus aurata*, Polo et al. 1991). In other species, temperature was found to have no significant effect on YUE over broad thermal ranges (9–15 °C for sole *Solea solea*, Baynes and Howell 1996; 3.5–17 °C for *C. harengus*, Overnell 1997; 10–19 °C for nase *Chondrostoma nasus*, Kamler et al. 1998; 13–21 °C for the sturgeons *Acipenser brevirostrum* and *A. oxyrinchus*, Hardy and Litvak 2004; 2–10 °C for haddock *Melanogrammus aeglefinus*, Martell et al. 2005). Finally, in the striped snakehead *Channa striata* (Arul 1991) and the lake minnow *Rhyncocypris percnurus* (Kaminski et al. 2006), the body size at yolk exhaustion was slightly longer at cold than at warm temperatures whereas an opposite trend was observed for the fish dry body mass. This brief review highlights the difficulty of predicting whether temperature have a positive, neutral or negative effect on YUE and fish size, although these aspects can be crucial, both in an ecological and in an aquacultural context. A higher dry mass can be essential for resisting periods of food deprivation, whereas a longer body length can be decisive in terms of swimming capacities and access to a larger scope of prey (Planas and Cunha 1999; Yúfera and Darias 2007; Teletchea and Fontaine 2010).

The present study aimed at investigating the thermal biology of the clown loach, *Chromobotia macracanthus*, a species that is endemic to the islands of Sumatra and Borneo. It is highly praised on the ornamental market and undergoes substantial fishing pressure in the wild (Ng and Tan 1997), which has fostered the development of research efforts for its artificial propagation (Legendre et al. 2012). Preliminary rearing experiments have revealed a slow and heterogeneous growth

of its larvae, which might originate from inadequate rearing temperatures (Baras et al. 2012). Recent results on the incubation of clown loach eggs have indicated that the thermal range in which viable and normal hatchlings could be obtained was particularly narrow and took place at relatively cold temperatures (24–28 °C), by reference to the thermal regimes of lowland Indonesian rivers (Slembrouck et al. 2012). The present study investigates, through a series of experiments, how temperature affects the survival, growth and size heterogeneity of clown loach embryos and larvae.

2 Methods

2.1 Fish and rearing procedure

All fish used in this study were obtained from the hormonally induced breeding of captive broodfish, following the methods evaluated by Legendre et al. and Slembrouck et al. (2012). Broodfish were wild specimens originating from the River Musi (Sumatra, Indonesia) and acclimated for at least two years in the experimental facilities of BP2BIH (Depok, Java, Indonesia), where all experiments were conducted. Fertilized eggs were incubated in zipper jars at 27 ± 1 °C in an indoor recirculating water system under 12L:12D. During the minutes following hatching, embryos spontaneously ascended the water column, exited the jars and were collected in hapas, where they were maintained at 27.5–28.0 °C until the start of the experiments (different ages, see below). When raised at this temperature, clown loach generally start feeding exogenously at 90–96 h after hatching (hereafter, hah; mean total body length of 5.6 mm, remaining yolk of about 0.04 mm³; Baras et al. 2012). From this age onwards, they were offered freshly hatched *Artemia* nauplii.

In total, six experiments (I–VI; Table 1), of which three on embryos (I–III) and three on larvae (IV–VI), were conducted on fish from three progenies (A, B and C; Table 1). All experiments took place in an indoor recirculating water system, comprising five 225-L tanks in which water temperature was regulated to the desired value (23, 26, 29, 32 or 34 °C) by two 300-W submersed heaters connected to a thermostat (Biotherm 2000) and supply of cold water from a chiller (Resun C1000, 2.700 W). During experiments I and II, which involved the frequent measurement of mortality, fish were housed in 0.3-L containers made of translucent plastic, which were filled with water from the hatchery and placed floating into the aforementioned tanks for controlling their temperature. About 90% of the water was renewed once a day, at the time of measurement. In the other experiments (III–VI), fish were housed in 15-L (28 × 28 × 19 cm) square glass aquaria, also partly immersed into the tanks, but with constant water renewal (about 0.5 volume per hour). Each tank could host a maximum of five 15-L aquaria. Water temperature was measured twice a day (morning, evening). No temperature higher than 34 °C was evaluated, after preliminary observations indicated they were lethal within a few hours, both for embryos and larvae. No temperature lower than 23 °C was tested, as their stability could not be guaranteed on very warm days. This was no major shortcoming however, as these temperatures are lethal for clown loach eggs (Slembrouck et al. 2012) and seemingly never occur in

Table 1. Environmental conditions during the series of experiments (I–VI) on the thermal biology of clown loach. Age is given in hours after hatching (hah) or days after hatching (dah). *TL* and *WM* stand for total body length and wet body mass, respectively.

Exp.	Progeny	Fish age	T° ($^{\circ}\text{C}$)	Volume (L)	n fish	Density (fish L^{-1})	Replicat per thermal treatment	Examination	Sample or all fish	Variables
Embryos										
I	A	2–24, 25–48 49–72, 73–96 hah	23, 26, 29, 32, 34	0.3	30	10	2 (every 24 h)	Final	All	Survival
II	A	12 hah until death	23, 26, 29, 32, 34	0.3	30	10	2	Every 12 h	All	Survival
III	A	12–120 hah	23,26,29,32	15	150	10	3	Every 12 h	Sample	<i>TL</i> , <i>Yolk</i>
Larvae										
IV	B	7–28 dah	23,26,29,32	15	45	3	5	Every 7 days	All	Survival, <i>WM</i>
V	A	5–19 dah	23, 26, 29, 32, 34	15	45	3	3	Every 7 days	All	Survival, <i>WM</i>
VI	C	5–31 dah	23, 26, 29, 32	15	45	3	4	Final	All	Survival, <i>WM</i>

the stretches of the Musi River where clown loach eggs, embryos and larvae can be found (authors' unpublished data). The thermal acclimation of fish was identical in all six experiments. At the time of transfer into the experimental tanks, fish were housed in 0.3-L containers filled with water of the hatchery (27.5 $^{\circ}\text{C}$). The containers were partly immersed in the tanks, the water of which was already at the desired temperature. Depending on the thermal treatment, the water temperature inside the containers attained the desired value within 1–2 h. Thereafter, in experiments III–VI, the fish were transferred into the aquaria, whereas in experiments I and II, they remained in their respective containers.

At the start of each experiment, fish were selected at random and counted. Nevertheless, fish with obvious anatomical deformities or irregular swimming movements were not retained. In experiments IV–VI, only fish having achieved the transition to exogenous feeding, as verified by the presence of nauplii in their guts, were selected.

2.2 Experiments on embryos

In experiment I, it was tested whether the thermal tolerance of clown loach embryos varied between developmental stages. Four consecutive tests (24 h each) were carried out, with two replications per thermal treatment (30 fish per container; temperatures of 23, 26, 29, 32 and 34 $^{\circ}\text{C}$). All fish used in this experiment had been housed in the hatchery at 27.5 $^{\circ}\text{C}$ until the start of the tests. The study did not extend beyond the age of 96 hah, as clown loach reared at 27.5 $^{\circ}\text{C}$ start feeding exogenously at this age (Baras et al. 2012). At the end of the 24-h period, survivors were counted. Fish used on a particular day were never re-used later.

Experiment II aimed examined the resistance of clown loach to food deprivation at 23, 26, 29, 32 and 34 $^{\circ}\text{C}$ (two groups of 30 fish each per thermal treatment). It started at 12 hah and ended when all fish had died. Every 12 h, the containers were placed on a glass table with a neon light underneath, and dead fish were removed with a pipette. Water level was decreased, survivors were counted to ascertain that no fish had decayed and passed unnoticed, and about 85–90%

of the water of the container was renewed, using well oxygenated water from the recirculating system, at the desired temperature.

In experiment III, the dynamics of yolk absorption and body growth of clown loach embryos at different temperatures were examined in order to test whether the efficiency of yolk utilization was temperature-dependent. Large numbers of fish were used here (three replicate groups of 150 fish each; 15-L aquaria, 10 fish L^{-1}) to ascertain that there would be enough fish in all thermal treatments, even in case of high mortality. Every 12 hours, samples of about 20 fish were gently removed with a siphon from each group in each thermal regime, anaesthetised (2-phenoxy-ethanol, 0.35 ml L^{-1}), placed in a small Petri dish under the stereomicroscope (magnification $\times 12$ –25) and photographed in profile view with a digital camera by reference to a finely graduated scale for the measurement of fish body length and yolk sac (see below). Fish with obvious morphological deformities were not retained in the analysis. No sampled fish was re-used for any subsequent observation. The operation was repeated until the age of 120 hah. Fish mortality was not measured in this experiment.

2.3 Experiments on larvae

Experiments IV–VI aimed at analysing the effect of water temperature (23–34 $^{\circ}\text{C}$) on the survival, growth and size heterogeneity of larvae (15-L aquaria, 3 fish L^{-1} , 3–5 replications per temperature). Experiments IV and V provided data on growth rates at weekly intervals, which served to modelling growth at different temperatures (see below). The results of experiment VI, on an independent data set, were compared with the values predicted by these models. At the start of each experiment, the wet body mass (*WM*) of fish was measured (nearest 0.1 mg) under anaesthesia (2-phenoxy-ethanol, 0.35 ml L^{-1}) in two independent samples (45 fish each, as in the experimental groups), to avoid any possible interference of measurement on the subsequent performance of the fish under study. Fish were fed *Artemia* nauplii (six daily meals, from 07:00 to 19:00 h). The interval between successive meals (2.4 h) was fixed on the basis of the gut evacuation rates of

clown loach larvae at 32 °C (authors unpublished data). Feeding in excess is a prerequisite for the determination of T_{opt}° (Jobling 1994), so meal size was in slight excess of the maximal ingestion capacities of clown loach (i.e. 15% WM in fish <1 mg, 20% WM in 1–5 mg fish, 25% WM in larger fish; Baras et al. 2012), and was incremented every day, on the basis of previous rearing trials. Dead fish, faeces and excess food were removed with a siphon once a day, about 1 h before the last meal.

At the start of experiment IV, the tolerance of clown loach larvae to repeated handling was unknown, and was tested as follows. At each temperature (23, 26, 29 and 32 °C), five replicate groups of fish aged 7 dah were raised over three weeks. One of the five groups was measured at 14, 21 and 28 dah, another one at 21 and 28 dah, and the three remaining groups at 28 dah only. On days of measurement, feeding was suspended during the entire morning in all aquaria (including those hosting fish that were not measured on this day), so each period of 7 rearing days comprised 6.5 feeding days. In all cases, all survivors were measured to avoid sampling effects. The comparison between the survival and growth rates of fish controlled 0, 1 and 2 times before the final measurement gave an indirect evaluation of whether repeated measurements interfered with the performance of clown loach, and whether this trend varied over the thermal range under study. In view of the outcome of experiment IV (see results), it was decided that measurements in experiment V could be done on a weekly basis in all tanks (three replications per thermal treatment). In experiment IV, the groups raised at 23 °C (slow growth) were raised during an additional week to document the growth of fish of similar size as at the other temperatures. In experiment VI, fish were raised over 26 days without any measurement and thus without any interruption of feeding.

2.4 Measurements and calculations

The total body length (TL) of embryos was measured to the nearest pixel from digital photographs, using the freeware Image J (Abramoff and Magalhaes 2004). The volume (V) of the yolk sac was back-calculated from its length (L), depth (D), after a preliminary study showed that the depth and width of the yolk of clown loach at a particular age were almost identical, and so was their decrease during the ontogeny (Baras et al. 2012). The yolk of clown loach is pear-shaped or strongly conical in its caudal region, so its volume is systematically overestimated with an ellipsoidal model using its maximal extensions (L and D) as diameters (i.e. $V = 1.333\pi LD^2/8$). To correct for this, the perimeter of the yolk was contoured with a hand-drawn closed polygon, and its surface area (S) was calculated by the software. Thereafter, a planar ellipse was produced, with a surface area equal to S , and a ratio between its diameters (D_1 and D_2) equal to the ratio between the values of L and D that were measured on the photograph. The values of D_1 and D_2 substituted those of L and D in the ellipsoidal model for calculating the yolk volume, i.e. $V = 1.333\pi D_1 D_2^2/8$.

Survival was calculated as a proportion (%) of the fish stocked at the start of the experiments. The heterogeneity of a particular dimension (yolk volume, total body length, wet

body mass) was expressed as the coefficient of variation CV (%), i.e. $CV = 100 \text{SD mean}^{-1}$, where SD is the standard deviation of the mean. The specific growth rate (SGR , % $WM \text{ day}^{-1}$) was used to characterise fish growth during experiments IV–VI, i.e. $SGR = (\text{Ln}WM_2 - \text{Ln}WM_1) (T_2 - T_1)^{-1}$, where WM_2 and WM_1 are the wet body masses of fish at times T_2 and T_1 , respectively. Here, $T_2 - T_1$ refers to the number of feeding days (6.5 days per week in experiments IV and V).

For each of the five temperatures under evaluation, a model was constructed between SGR and the mean geometric WM of fish (WM_g) during each rearing week, using the data collected on a weekly basis during experiments IV and V. The value of WM_g was calculated as $WM_g = WM_1 e^{(0.005 SGR[T_2 - T_1])}$. Thereafter, the value of T_{opt}° at a particular WM was obtained by equating modelled values of SGR with temperature, using a third-order polynomial. The calculation was repeated for a series of values of WM over the size interval examined during the present study, so as to describe the ontogenetic variation of T_{opt}° in clown loach.

2.5 Statistics

The survival rates of embryos of different ages at different temperatures in experiment I were compared with two-way analysis of variance (2-way ANOVA). Contingency table analyses were used for comparisons of survival between replicates and between categories (i.e. temperature \times age). Log-logistic regression models were used for estimating the P_{50} of the survival curve to food deprivation at different temperatures in experiment II. Yolk absorption and growth in body length at different temperatures in experiment III were compared with 2-way ANOVA (and Scheffe post-hoc tests for comparisons of means), and modelled with simple logarithmic (yolk) or power regression analyses (growth). In experiments IV–VI, the WM of fish of identical ages but raised at different temperatures were compared with nested analyses of variance and Scheffe F -tests for post-hoc comparisons of means. Survival rates were compared with contingency table analyses. In experiment IV, 2-way ANOVA was used to test whether the survival rate and WM at the end of the experiment were influenced by the frequency of previous measurements (three modalities), and whether this effect was dependent on temperature (four modalities). Null hypotheses were rejected at $p < 0.05$.

3 Results

3.1 Experiments on embryos

3.1.1 Effect of temperature on survival

In experiment I, the survival of clown loach embryos exposed during 24 h to temperatures ranging from 23 to 34 °C was significantly ($p < 0.0001$) dependent on temperature, fish age and their interaction (2-way ANOVA; Fig. 1). At 34 °C, survival was null in fish younger than 72 hah, and very low (16.7%) in older fish. At 32 °C, mortality on days 2 and 3 (53.3% and 26.7%) was significantly higher than at colder temperatures. Within the 23–29 °C thermal range, survival

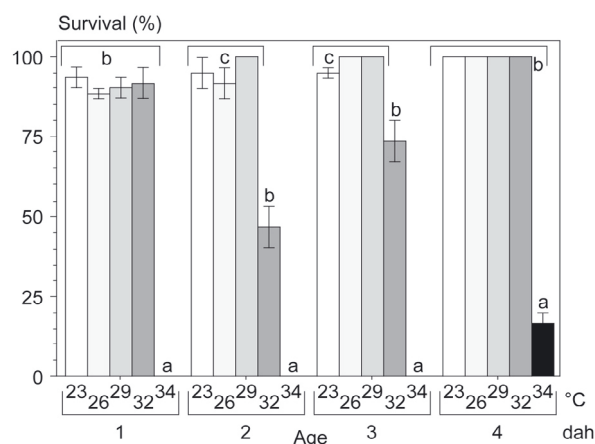


Fig. 1. Survival of clown loach embryos of different ages (1st, 2nd, 3rd or 4th day after hatching, dah) exposed during 24 hours to different temperatures (data from Exp. I). Bars and whiskers are the means and standard deviations of two groups of 30 fish each (experimental conditions as in Table 1). No difference between replicate groups at any age or temperature (contingency table analyses). Bars with different superscripts (a, b or c) differ at $p < 0.05$ (contingency table analyses).

was independent from temperature, but increased with increasing fish age, from about 90% on day 1 to 100% on day 4 (2-way ANOVA, $p = 0.3966$ for T° , $p = 0.0036$ for age and $p = 0.3159$ for the interaction between the two factors).

Similar mortality patterns were observed during the first days of experiment II, as all fish exposed to 34 °C had died at 24 hah, and 68.3% of the fish exposed to 32 °C died in between 24 and 48 hah (Fig. 2). Fish at 32 °C here died at slightly younger ages than in experiment I, but here they had been exposed to 32 °C throughout. Some mortality was also observed during the first 2 dah at lower temperatures, essentially, but survival at 3 dah was much higher than at 32–34 °C (75.0, 80.0 and 93.3% at 23, 26 and 29 °C, respectively). These early episodes of mortality took place before the yolk was fully absorbed (see below), and were thus independent from food deprivation. Henceforth, the log-logistic models for modelling the resistance of clown loach larvae to food deprivation were constructed as if the survival rates at 3 dah had been 100% (Fig. 2). Based on these models, the P_{50} of the resistance to food deprivation lied at 9.4, 11.5, 14.6 and 19.3 dah, at 32, 29, 26 and 23 °C, respectively.

3.1.2 Effect of temperature on yolk utilization

In experiment III, yolk volume was significantly dependent on fish age and temperature (2-way ANOVA, $p < 0.0001$), whereas the interaction between the two factors was not significant ($p = 0.1127$). At all temperatures, yolk depletion was curvilinear and best described by a logarithmic equation (Fig. 3a; Table 2), at least until the remaining yolk was about 0.04 mm³ (about the mean yolk remaining at the start of exogenous feeding in clown loach). Thereafter, yolk depletion slowed down, and remnants of yolk were observed at all temperatures until the age of 120 hah. The 0.04-mm³ mark was

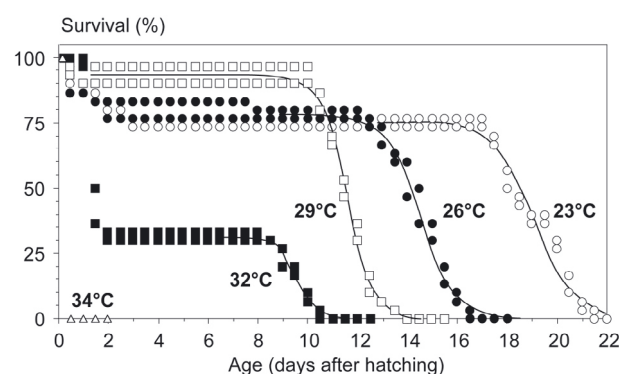


Fig. 2. Temperature-dependent resistance of clown loach to food deprivation. Each symbol stands for the survival (%) in a group of 30 fish (two replications per temperature; experimental conditions as in Table 1; data from Exp. II). Curves were constructed with log-logistic models. Early mortality was not taken into account in the modelling approach, as yolk was not exhausted yet and mortality was independent from starvation.

attained at 116, 96, 80 and 72 hah, at 23, 26, 29 and 32 °C, respectively. Yolk absorption was faster at 32 °C throughout except from 24 to 36 hah, which corresponded precisely to the period of highest mortality at 32 °C in experiment II.

As was the case for yolk volume, fish total body length (TL) in experiment III was significantly influenced by age and temperature (2-way ANOVA, $p < 0.0001$), but not by their interaction ($p = 0.103$). At all temperatures, fish size increased in a curvilinear way that was best described by a power function (Fig. 3b; Table 2). From 12 to 24 hah, fish growth was proportional to temperature. The same trend was observed from 24 to 36 hah, except at 32 °C, where fish showed almost no growth in length during this interval, which corresponded to a period of high mortality (experiment II) and slow yolk absorption (Fig. 3a). Thereafter, fish growth became faster at cold than at warm temperatures, and at 120 hah the sizes of fish were inversely related to temperature (Fig. 3b).

3.2 Experiments on larvae

Two particular issues were encountered during the experiments on larvae. During the first week of experiment IV, *Artemia* nauplii were held at ambient temperature in the experimental hall (35 °C at noon) and did not survive more than a few hours. This resulted in much slower fish growth than in the other two experiments, so data from the first week were not retained for analysing the relationships between WM and SGR . In the rest of experiment IV (and experiments V and VI), nauplii were stored at 8 °C before use. The second issue referred to an unnoticed interruption of water supply overnight in one of the three replicate groups at 23 °C during experiment V, which resulted in higher mortality and slower growth. This group was not taken into account either in any analysis.

During experiment IV, neither survival nor growth was influenced by the frequency of measurements (F_M : 0, 1 or 2 times) or its interaction with temperature ($F_M \times T^\circ$; 2-way ANOVA, survival: $p = 0.756$ and 0.978 for F_M and $F_M \times T^\circ$, respectively; growth: $p = 0.2874$ and 0.4958 , respectively;

Table 2. Models of yolk absorption (upper part) and growth (lower part) in clown loach embryos raised at different temperatures (Experiment III). Models are of logarithmic nature for yolk absorption (i.e. yolk volume (mm³) against log age (hours after hatching, hah)) and of power nature for growth (log size against log age). The models are restricted to a part of the age range, as beyond a certain developmental stage (96 hah), which corresponds at the start of exogenous feeding, yolk absorption slows down substantially and growth is fuelled by exogenous resources (see methods). Values between brackets are the standard errors of coefficients. No model for 34 °C as survival was null.

<i>T</i> ° (°C)	Intercept	Slope	<i>r</i> ²	<i>F</i>	<i>df</i>	<i>p</i> intercept	<i>p</i> slope
Yolk Absorption							
23	1.009 [0.028]	−0.477 [0.016]	0.995	918.9	6	<0.0001	<0.0001
26	1.003 [0.011]	−0.486 [0.006]	0.999	5 676.1	6	<0.0001	<0.0001
29	0.997 [0.011]	−0.501 [0.018]	0.998	2 212.6	5	<0.0001	<0.0001
32	1.029 [0.053]	−0.533 [0.032]	0.989	275.1	4	0.0003	0.0005
Growth							
23	0.621 [0.010]	0.071 [0.005]	0.971	166.9	6	<0.0001	<0.0001
26	0.635 [0.009]	0.062 [0.005]	0.965	137.4	6	<0.0001	<0.0001
29	0.653 [0.009]	0.052 [0.006]	0.957	88.4	5	<0.0001	0.0007
32	0.659 [0.006]	0.044 [0.003]	0.975	197.4	6	<0.0001	<0.0001

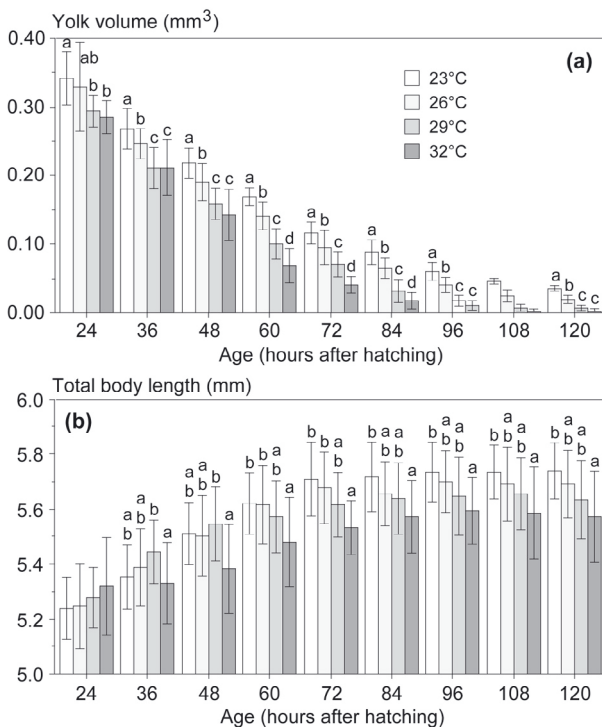


Fig. 3. Temperature-dependent dynamics of yolk absorption (a) and growth (b) in clown loach embryos (data from Exp. III). Bars and whiskers are the mean and standard deviations of yolk volume (a) or total body length (a) in samples of at least 10 fish (see methods). Within each graph, bars that share a superscript in common do not differ significantly, whereas other comparisons differ at *p* < 0.05 (Scheffe post hoc tests for comparisons of means).

Figs. 4 and 5a). This finding supports the view that the results from experiments IV and V could be pooled for modelling the growth of clown loach.

The experimental temperatures during experiments IV and V departed slightly from the desired temperatures but were consistent throughout, and between experiments, i.e. 23.1, 26.0, 28.9, 31.8 and 33.9 °C. The latter values were used to calculate the ontogenetic variations of *T*_{opt}° in clown loach larvae,

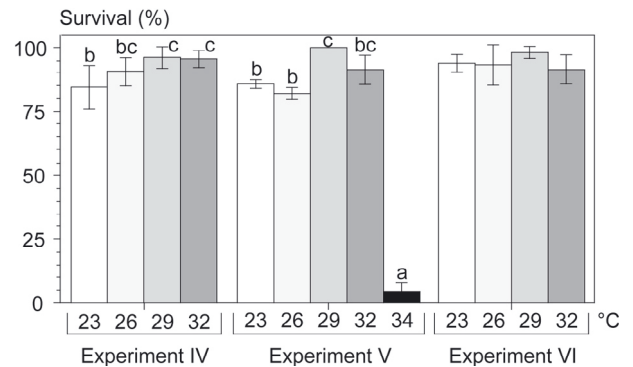


Fig. 4. Survival of clown loach larvae at the end of experiments IV, V and VI, depending on water temperature. Bars and whiskers are the means and standard deviations of five, three and four replicate groups, respectively (experimental conditions as in Table 1). For each experiment, bars that share a superscript in common do not differ significantly, whereas other comparisons differ at *p* < 0.05 (contingency table analyses; the absence of scripts for experiment VI indicates that survival did not differ between any thermal treatment).

but for the sake of clarity, we refer to the target temperatures (23, 26, 29, 32 and 34 °C) in the text.

3.2.1 Effect of temperature on survival

Mortality at 34 °C in experiment V was very high, as only 6 of 135 fish (4.4%) survived until the age of 21 dah (Fig. 4). At temperatures ranging from 23 to 32 °C, survival was high (80–100%) and did not differ between experiments, but varied significantly between temperatures (2-way ANOVA, *p* < 0.0001 for *T*°, *p* = 0.101 for experiment and *p* = 0.318 for the interaction between factors). The overall pattern was dome-shaped, with the highest survival at 29 °C in all experiments (mean ± SD, 98 ± 3% over 12 tanks).

3.2.2 Effect of temperature on growth

There were no significant differences between replicate groups at any temperature in any experiment, except for the

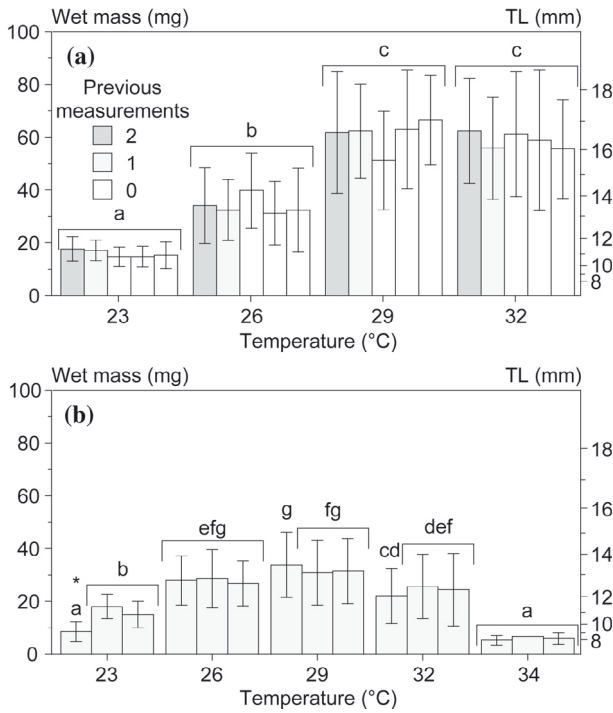


Fig. 5. Effect of water temperature (23–32 °C) on the growth of clown loach larvae in experiments IV (a; three rearing weeks) and V (b; two rearing weeks). Bars and whiskers are the means and standard deviations of fish wet body mass in each aquarium. Correspondence with the total body length of fish (*TL*) is given on the right vertical axis, using the *WM-TL* models given by Baras et al. (2012). In each graph, bars that share at least one superscript in common do not differ at $p < 0.05$ (Scheffe post hoc tests for comparisons of means). The symbol * indicates a temporary interruption of water supply. In Figure 5a, different shades refer to fish that had been measured 0, 1 or 2 times before the final measurement, thereby emphasizing that handling on a weekly basis had no significant effect on the growth of clown loach larvae.

group at 23 °C with an interruption of water supply during experiment V. The final *WM* of fish in experiments IV and V varied significantly dependent on temperature (one-way ANOVA, $p < 0.0001$; Fig. 5). During experiment V, growth was slowest in the groups raised at 34 °C. Within the 23–32 °C range, growth was always slower at 23 °C than at warmer temperatures, and the fastest growth was always observed at 29 °C.

At each temperature, the *SGR*-to-*WM* relationship was best modelled with a power (log-log) relationship (Table 3, Fig. 6). The intercept of the equation at 29 °C was the highest and its slope the lowest, thereby indicating that the growth of clown loach over the size range under study would be faster at 29 °C than at colder or warmer temperatures. The ontogenetic variation of T_{opt}° , that is derived from the five *SGR*-to-*WM* models, is shown in Figure 7, together with the variation of SGR_{max} (i.e. at T_{opt}°). From 2 to 50 mg *WM*, the SGR_{max} of clown loach would decrease from 34.7 to 15.3% *WM day*⁻¹, while their T_{opt}° would pass from 29.5 to 29.1 °C.

The *SGR*-to-*WM* models in Table 3 were used (successive iterations) to predict the mean *WM* of clown loach at the end of experiment VI (60.6, 117.5, 169.5 and 103.4 mg, at 23, 26,

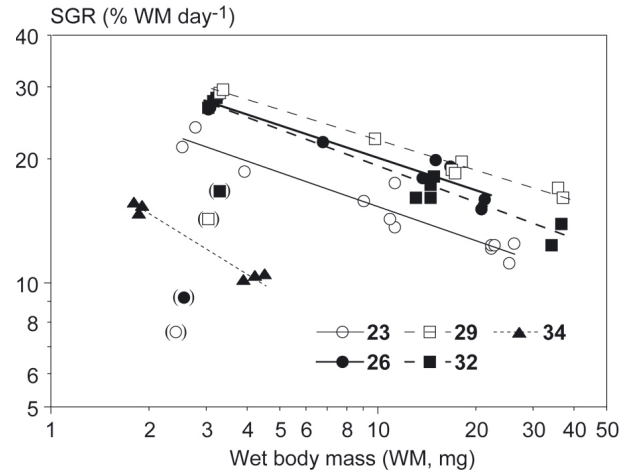


Fig. 6. Size-dependent variation of growth in clown loach raised at different temperatures. *SGR* is the specific growth rate, measured on a weekly basis, and equated to the mean geometric wet body mass (*WM*) of fish during this week (see methods). The equations of the log-log models at the five temperatures are given in Table 3. Symbols between parentheses refer to situations where issues were encountered (detailed explanation in Results section) and were not retained in any model.

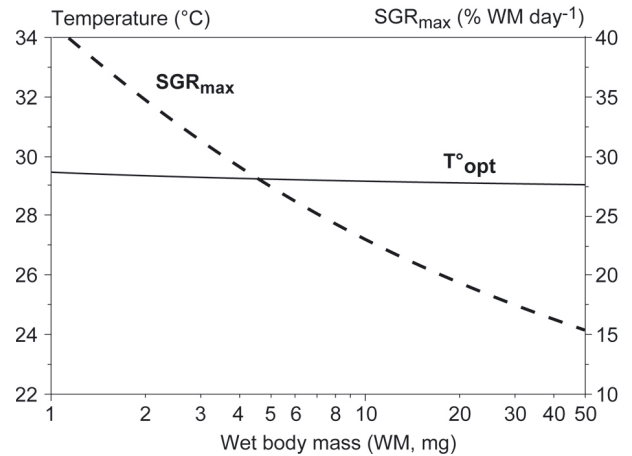


Fig. 7. Ontogenetic variation of the thermal optimum for growth (T_{opt}° ; thin plain line, left vertical axis) and maximal specific growth rate at T_{opt}° (SGR_{max} ; thick dotted line, right vertical axis) in clown loach, as deduced from the models given in Table 3 and illustrated in Figure 6. The models stand as: $T_{opt}^{\circ} = 29.427 - 0.204 \log WM$ and $\log SGR_{max} = 1.618 - 0.254 \log W$. No statistics given, as both models were produced from modelled data ($r^2 > 0.999$ in both cases).

29 and 32 °C, respectively; Fig. 8). There was a good correspondence between the predicted and observed sizes at 26 and 29 °C (mean departure \pm SD, $3.9 \pm 3.3\%$ and $4.4 \pm 2.9\%$, respectively), whereas the predictions were slightly optimistic at 23 ($10.0 \pm 3.6\%$) and 32 °C ($13.6 \pm 8.9\%$). Yet, it is worth noting that the actual temperatures in experiment VI were slightly further from T_{opt}° than in experiments IV and V (22.8 vs. 23.1 °C and 32.1 vs. 31.8 °C). Greater departures to T_{opt}° result in slower growth, especially for temperatures above T_{opt}° , because of the parabolic nature of the relationship between growth and temperature in fish.

Table 3. Variation of specific growth rate (SGR , % WM day^{-1}) as a function of wet body mass (WM , mg) and water temperature (T°) in clown loach (constructed from data from Experiments IV and IV). All models are of power nature, i.e. $\log SGR$ against $\log WM$. SGR is calculated on a weekly basis and equated against the mean geometric WM of fish over the rearing week. Values between brackets are the standard errors of coefficients.

T° ($^\circ C$)	Intercept	Slope	r^2	F	df	p intercept	p slope
23	1.461 [0.0]	-0.274 [0.027]	0.915	107.1	11	<0.0001	<0.0001
26	1.566 [0.0]	-0.262 [0.029]	0.921	81.6	8	<0.0001	<0.0001
29	1.593 [0.0]	-0.246 [0.012]	0.985	455.5	8	<0.0001	<0.0001
32	1.576 [0.0]	-0.294 [0.026]	0.946	123.0	8	<0.0001	<0.0001
34	1.337 [0.0]	-0.559 [0.048]	0.730	108.0	5	<0.0001	0.0303

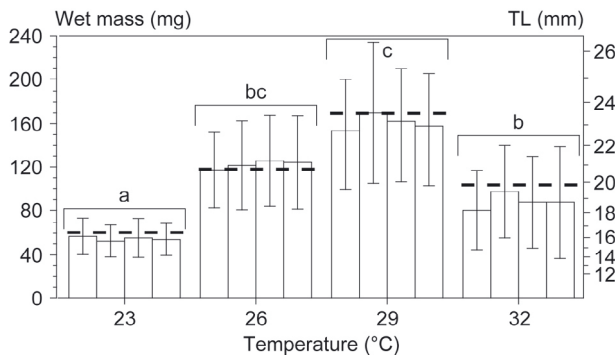


Fig. 8. Comparison between the sizes observed at the end of experiment VI (bars) and those predicted by the temperature-dependent growth models given in Table 3 (horizontal bold dashed lines). As regards observed growth, bars and whiskers are the means and standard deviations of fish wet body mass in each aquarium. Correspondence with the total body length of fish (TL) is given on the right vertical axis. Bars that share at least one superscript in common do not differ at $p < 0.05$ (Scheffe post hoc tests for comparisons of means).

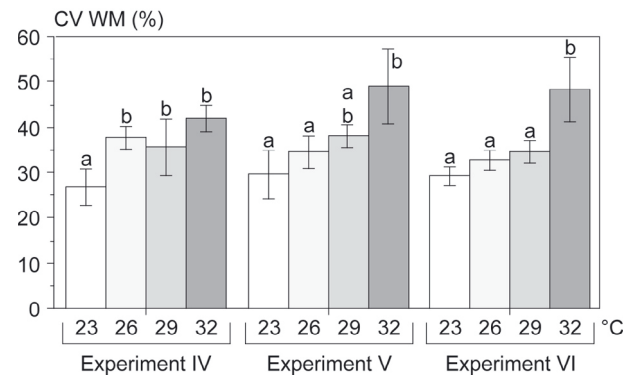


Fig. 9. Effect of rearing temperature on the size heterogeneity of clown loach larvae at the end of experiments IV–VI. $CV WM$ is the coefficient of variation (%) of the wet body mass of fish. Bars and whiskers are the means and standard deviation of $CV WM$ over five, three and four replicates, in experiments IV, V and VI, respectively. For each experiment, bars that share at least one superscript in common do not differ at $p < 0.05$ (Scheffe post hoc tests for comparisons of means).

3.2.3 Effect of temperature on size heterogeneity

No calculation was done for the fish raised at $34^\circ C$, as there were not enough survivors (Fig. 4). Whatever the duration of the experiment and final WM of fish, the values of size heterogeneity ($CV WM$) at the end of experiments IV–VI were temperature-dependent and higher at $32^\circ C$ than at colder temperatures (Fig. 9; 2-way ANOVA; $p < 0.0001$ for T° , $p = 0.468$ for experiment, $p = 0.243$ for the interaction between factors). The lowest values of $CV WM$ were observed at $23^\circ C$, but these were also the fish with the slowest growth.

4 Discussion

4.1 Thermal biology of clown loach: an ecological perspective

This study provided evidence that clown loach, in spite of its equatorial distribution, is highly sensitive to warm temperatures. Embryos did not survive $34^\circ C$ during the first three days after hatching, and the mortality of older fish at $34^\circ C$ was very high, although this temperature was less than $5^\circ C$ warmer than the T_{opt}° of larvae (about $29^\circ C$). It is worth pointing out that the T_{opt}° of clown loach larvae <50 mg WM lies several degrees below those of other freshwater teleosts from tropical regions: i.e.

T_{opt}° of 31 – $33^\circ C$ at 5 – 50 mg in vundu *Heterobranchus longifilis* (Nwosu and Holzlohner 2000), blue tilapia *Oreochromis aureus* (Baras et al. 2002), pirapitinga *Piaractus brachipomus* (Baras and Florès 2006) and striped catfish *Pangasianodon hypophthalmus* (Baras et al. 2011). Similar thermophilic patterns have been reported in small juveniles of freshwater and marine fish species from tropical regions: T_{opt}° of $30.0^\circ C$ at 10 g in *Channa striata* (Qin et al. 1997), $30.0^\circ C$ at 3 g in *O. niloticus* (Azaza et al. 2008), $30.5^\circ C$ at 10 g in rohu *Labeo rohita* (Das et al. 2005), 30.5 – $31.0^\circ C$ at 0.5 – 1.0 g in sharp-tooth catfish *Clarias gariepinus* (Hogendoorn et al. 1983; Britz and Hecht 1987), $31.4^\circ C$ at 0.25 g in gray snapper *Lutjanus griseus* (Wuenschel et al. 2004), $31.5^\circ C$ at 10 g in cobia *Rachycentron canadum* (Sun and Chen 2009) and $32.1^\circ C$ at 5 g in barramundi *Lates calcarifer* (Katersky and Carter 2007). In general, the value of T_{opt}° decreases in fish of increasing size (Jobling 1994), so it is likely that smaller individuals of the aforementioned species (of the same size range as clown loach in the present study) have slightly higher T_{opt}° , and are thus much more thermophilic than clown loach larvae.

Yet, a slightly colder value of T_{opt}° has been reported for the larvae of another tropical species, the atherinid pike silverside, *Chirostoma estor* ($28^\circ C$ at about 30 mg WM), but this species is endemic to a high altitude lake (Lake Patzcuaro, Mexico, 2035 m above sea level; Martínez-Palacios et al. 2002), where

water temperature never exceeds 23 °C, except in inshore areas. In contrast to pike silverside, clown loach is found in lowland rivers, where water temperature can be as warm as 31–32 °C in the main stream (Legendre et al. 2012) and probably warmer in the habitats occupied by larvae and juveniles in the floodplain (inferred from the harvesting practices of local fishermen; Ng and Tan 1997). Proportionally, the value of T_{opt}° in clown loach species resembles more closely those for the larvae and juveniles of temperate or warmwater species: e.g. 26.5 °C at 10–100 mg *WM* in *Chondrostoma nasus* (Keckeis et al. 2001), 28.5 °C at 10 g in the common carp *Cyprinus carpio carpio* (Goolish and Adelman 1984) and 29.5 °C at 40 mg *WM* in goldfish *Carassius auratus* (Kestemont 1995). This brief comparison suggests that the thermal biology of clown loach larvae is not typical of equatorial and tropical species, and resembles more closely that of temperate or warmwater species, at least as regards the value of T_{opt}° .

On the other hand, the degree of eurythermy in clown loach is lower than in fish species from temperate regions. This assertion relies on the comparison between the growth penalties (by reference to the maximal growth at T_{opt}°) incurred by the fish when temperature departs from T_{opt}° . In clown loach of 20–50 mg *WM*, *SGR* is depressed by 30% for temperatures that are either 5.1 °C colder or 3.1 °C warmer than T_{opt}° , thus a 8.2 °C thermal range. The same calculation for temperate fish species (recalculated from the authors' data) gives ranges as broad as 13.3 °C for 10–100 mg *Chondrostoma nasus* (Keckeis et al. 2001), 13.9 °C for 10 g *Cyprinus c. carpio* (Goolish and Adelman 1984) and 15.3 °C for 40 mg *Carassius auratus* (Kestemont 1995). As regards eurythermy, clown loach resembles more closely tropical species, which exhibit narrower tolerance ranges: e.g. 9.4 °C in 250 mg *L. griseus* (Wuenschel et al. 2004), 10.0 °C in 50 mg *P. hypophthalmus* (Baras et al. 2011) and 11.4 °C in 50 mg *O. aureus* (Baras et al. 2002).

Larvae of clown loach are stenothermal by reference to other fishes, but not in comparison to clown loach embryos, which incurred high mortality at 32 °C. It is not infrequent that fish embryos have a narrower thermal tolerance range than larvae or small juveniles (e.g. *O. niloticus*, Rana 1990; salmonids, Elliott and Elliott 2010). To this respect, it is worth remembering that the early developmental stages of clown loach (until hatching) were less thermophilic and more stenothermal, as no egg hatched at temperatures lower than 24 °C or warmer than 29 °C (Slembrouck et al. 2012). In clown loach larvae, T_{opt}° decreased by 0.20 °C for each 10-fold increase of body mass, which is very low in comparison to other tropical and warmwater fishes (review in Baras et al. 2011). Yet, patterns other than a downward shift of T_{opt}° with increasing fish size have been reported in fish larvae, but essentially in species from cold or temperate regions (upward shift in Atlantic cod *Gadus morhua*, Steinarrsson and Björnsson 1999; *C. nasus*, Keckeis et al. 2001; no variation of T_{opt}° in stone loach *Barbatula barbatula*, Elliott et al. 1996).

All in all, the thermal biology of clown loach largely departs from those of other fish species described to date, and which exhibit strong latitudinal or climatic trends. The reasons behind these trends have been discussed extensively, at least in the light of tolerance ranges based on critical or lethal

temperatures (e.g. Rijnsdorp et al. 2009; Pörtner and Peck 2010), and are beyond the scope of the present article. Nevertheless, in the perspective of global warming, the present study suggests that clown loach, as a result of its low T_{opt}° and limited eurythermy, might be at greater risk than many other fish species. Still in an ecological perspective, the thermal biology of clown loach eggs, free embryos and larvae might account for why this species spawns during flood pulses following heavy rainfalls, as the thermal regime during the rainy season is much more favourable to the survival and growth of their embryos and larvae than during the dry season.

4.2 Thermal biology of clown loach: an aquacultural perspective

From an applied perspective, the results of the present study have emphasized that the maintenance of a constant temperature of about 29 °C would be optimal for clown loach, in terms of survival and growth, but also as regards size dispersal. This is further evidence that size heterogeneity can be minimised when rearing fish as close as possible to T_{opt}° , as observed in other fish species (red Florida tilapia, Watanabe et al. 1993; *O. aureus*, Baras et al. 2002; *Piaractus brachipomus*, Baras and Florès 2006; *O. niloticus*, Azaza et al. 2008; *P. hypophthalmus*, Baras et al. 2011). The size dispersal of clown loach larvae in the present study was much lower than during the first rearing trials (Baras et al. 2012) while growth was much faster (*CV WM* 30–35% vs. over 80%, and mean *WM* of about 160 vs. 50 mg, at 31 and 29 dah, rearing temperatures of 29 and 27.5 °C, respectively). In view of the growth observed here at 26 °C (*WM* of about 100 mg at 31 dah), it is unlikely that the slow growth during the first rearing trial was exclusively due to rearing at suboptimal temperatures. The possible role of a family effect cannot be refuted, but its extent is probably limited in view of the consistency of growth rates between progenies in the present study (Fig. 8), and of the similarity between the sizes of the top growers here and in the first rearing trials (330 vs. 270 mg, at 31 and 29 dah, respectively). Instead, it is likely that the feeding schedules during the early rearing trials for clown loach larvae had largely underestimated their ingestion capacities, thereby resulting in an overall slower growth and much greater growth dispersal. Conversely, the relatively fast growth and limited size dispersal in all experiments on larvae (exp. IV–VI) suggest that the feeding schedules in the present study were not inadequate.

Yet, clown loach grew faster here (about 0.7 mm *TL* day⁻¹) than in previous rearing trials, but their overall growth rate remained low in comparison to the young of many fish species. The market size of clown loach is low (about 40 mm *TL*, 750 mg), so slow growth is not a major shortcoming. Based on the *SGR*_{max}-to-*WM* model in Figure 7, this size could be attained at 55–60 dah if fish were raised at T_{opt}° and fed *ad libitum* 6 times a day. Clown loach larvae were fed in slight excess throughout here, so their feed efficiency can just be estimated (about 6.5 *WM:WM* at 29 °C, for fish fed *Artemia* nauplii). In general, the temperature at which feed efficiency is best in fishes lies a few degrees below T_{opt}° (yellow perch *Perca flavescens*, Kitchell et al. 1977; *C. gariepinus*, Hogendoorn et al. 1983). If food conversion is a premium when rearing

clown loach, then temperatures colder than 29 °C should be used. Further experiments, with different feeding levels at different temperatures are needed to pinpoint this. Anyhow, rearing fish in these conditions would result in slower growth, longer production cycles, and a loss of production capacity over an annual cycle.

As regards the embryonic period, yolk absorption in clown loach was faster at warm than at cold temperatures. This general trend has been observed almost systematically in fish, and is interpreted in the light of temperature-dependent metabolism (Kamler 1992; Jobling 1994). The logarithmic nature of the model is in good concordance with the reviews by Pepin (1991) and Kamler (1992), for marine and freshwater species, respectively. Based on yolk absorption rates, the age at the start of exogenous feeding in clown loach raised at 23–32 °C would range from 3 to 4.8 dah, thus a ratio of 1.60 for a 9 °C difference and a $Q_{10}^{\circ\text{C}}$ of 1.69. By contrast, clown loach held at 32 and 23 °C died from starvation on average at 9.5 and 19.3 dah, thus 6.4 and 14.5 days after the respective ages at the start of exogenous feeding, thereby producing a ratio of 2.27 and a $Q_{10}^{\circ\text{C}}$ of 2.49. The $Q_{10}^{\circ\text{C}}$ of clown loach embryos is about 2.1, which is in good concordance with information on embryos of other fishes in the same thermal range ($Q_{10}^{\circ\text{C}}$ from 1.8 to 2.3, mean of 2.06; Kamler 1992). Clown loach embryos resist starvation over longer periods than those of many tropical or warmwater fish species with similar egg size and yolk reserves at hatching. This issue has been interpreted already, in the light of the phenology of reproduction, at the start of the rainy season, unpredictability of rainfall pattern and subsequent variability in food availability (Baras et al. 2012). As an echo to the discussion on ecological aspects, the rapid decrease ($Q_{10}^{\circ\text{C}}$ of 2.49) in the resistance of clown loach to starvation with increasing temperatures can be of particular concern in the case of global warming scenarios. Yet, a 6.5-day interval at 32 °C might look enough to find food. However, the most relevant parameter as regards survival to food deprivation is not the P_{50} of the survival curve, but the point-of-no-return (PNR; i.e. the point beyond which starving fish can no longer recover, even if offered food; Blaxter and Hempel 1963) and which takes place sooner (e.g. *C. harengus*, Yin and Blaxter 1987; Japanese Spanish mackerel *Scomberomorus niphonius*, Shoji et al. 2002; Japanese flounder *Paralichthys olivaceus*, Dou et al. 2005; spotted mandarin fish *Siniperca scherzeri*, Zhang et al. 2009).

When looking at survival only, a temperature of 29 °C is best during the period of yolk absorption (Figs. 1, 2). By contrast, clown loach used their yolk more efficiently at cold than warm temperatures (Fig. 3b), at least on the basis of body length. The dry body mass of fish was not measured here, so it is uncertain whether temperature-dependent differences in body length were accompanied with similar differences in dry mass, or if the water content of the fish was inversely proportional to temperature, as was observed in several fish species (Arul 1991; Kaminski et al. 2006). In clown loach, the size difference after yolk absorption at different temperatures is quite tenuous (5.7 against 5.5 mm *TL* at 23 and 32 °C, respectively), but it takes place during an ontogenetic interval that is pivotal in terms of feeding capacities and growth. In between 5.5 and 6.0 mm *TL*, there is a major allometric increase in jaw

length, which enables clown loach of 6.0 mm *TL* to swallow *Artemia* nauplii whole, whereas siblings of 5.5–5.6 mm *TL* can just suck them in. Concomitantly there is a positive allometric growth of body width and depth, which almost double the relative volume of the stomach (Baras et al. 2012). Hence, the maintenance of clown loach embryos at cold temperatures could produce larvae with a greater readiness to feed on *Artemia* nauplii, which might contribute to alleviate growth heterogeneity during the first days of exogenous feeding. This hypothesis requires experimental validation, especially in view of the results of several studies investigating the long-term effects of water temperature during the embryonic period on the subsequent muscle growth in larvae or juveniles, and reporting that fish incubated at warm temperatures eventually attained larger sizes than others (e.g. Johnston et al. 1998, de Assis et al. 2004; Martell et al. 2005).

Acknowledgements. The authors wish to express their warmest thanks to the technical staff of BP2BIH Depok (Slamet Sugito, Made Agus Widjana, Mertayasa, Moch Hasan, Marjono) for their assistance during the rearing and measurement protocols, as well as to an anonymous referee and the Editor of the Journal for constructive thoughts on a previous version of the article. Mrs. Dominique Caseau-Baras contributed to improve the English style of the manuscript. Etienne Baras is an honorary research associate of the Belgian FNRS.

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