Asian Herpetological Research 2012, 3(3): 192–197
DOI: 10.3724/SP.J.1245.2012.00192

Reproduction, Development, and Growth Response to Captive Diets in the Shangcheng Stout Salamander, *Pachyhynobius shangchengensis* (Amphibia, Urodela, Hynobiidae)

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Abstract The Shangcheng stout salamander (*Pachyhynobius shangchengensis*) is a poorly known stream dwelling hynobiid salamander from China. We studied the reproduction, development and growth response of the salamander to different diets in captivity. Two females each produced two unique, striated egg sacs, with each containing 18 and 20, and 22 and 32 white eggs with an average diameter of 3.3 mm. Hatchlings (n = 27) had front- and hind-limb buds with interdigit membranes and a large yolk sac, and developed cornified finger and toe tips during further development. Metamorphosis occurred between d 441 and d 454 after hatching at an average total length of 94.8 mm (n = 21). These reproductive and developmental traits fit in well with the ancestral state reconstruction in hynobiids. If corrected for dry mass, feed conversion ratios obtained by feeding bloodworms (Chironomidae) to *P. shangchengensis* larvae and mealworms (*Tenebrio molitor*) to postmetamorphs, were very low (0.33 and 0.34 respectively), equaling a very high mass conversion efficiency.

Keywords *Pachyhynobius shangchengensis*, husbandry, reproduction, nutrition, development

1. Introduction

The Shangcheng stout salamander (*Pachyhynobius shangchengensis*, Fei, Qu and Wu, 1983) is endemic to mountain streams in the Mt. Dabie area in central China (Fei *et al*., 2006). *P. shangchengensis* is classified as vulnerable by IUCN. Although the species has occasionally turned up in the international pet trade, no report concerning its reproduction has been published until now, resulting in significant knowledge gaps (Zhang *et al*., 2006). We present the first data on reproduction of this species, including a description of the hitherto unknown egg sacs, larval, and juvenile development. Additionally, we present the first data on the growth response of *P. shangchengensis* larvae and juveniles to captive diets.

2. Materials and Methods

2.1 Animals, captive husbandry and morphometric characteristics One male and female pair of wildly caught, adult *P. shangchengensis* was acquired through the pet trade (unknown locality) in 2001 and was referred to as Group 1. Both animals showed a uniform dark brown in dorsal color. A second group (Group 2) of pet-

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Received: 16 May 2012 Accepted: 15 August 2012

trade-derived wildly caught, adult animals (four females and one male) was acquired in 2008. These animals possess blackish dorsal ground color with fine blue spots all over the body. Two formalin-fixed, pet-trade-derived specimens, a wildly caught male and female, were included for morphological analyses. The total length (TL) of the six adult females measured 185 ± 11 mm on mean ± SD, the three males averaged 185 ± 14 mm. The largest female and male measured 201 and 196 mm, respectively. The adult males could be easily identified based on their wider heads with much more developed masseter muscles than those of females, a characteristic also visible outside the breeding season.

Group 1 was housed in a glass tank measuring 90 × 40 × 30 cm (length × width × height), with a water depth of 10 cm, coarse gravel as substrate and stone piles as hiding places. Group 2 was housed in a PVC tank of 120 × 90 x 60 cm, with a water depthl of 10 cm, sand as substrate and stone piles as hiding places. The water was unaerated, unfiltered tap water. The tanks were housed in a shaded place and only received dim daylight. Until 2004, the aquarium was exposed to room temperatures fluctuating between 10 and 27°C, following the fluctuations of the temperate Belgian climate. From 2005 onwards, water temperatures fluctuated from 2°C in winter to 18.5°C in summer. Nitrite and nitrate levels were assessed monthly. Water was replaced by regular tap water (pH 7, hardness 14° dH) when nitrite levels were > 0.1 mg/l or when nitrate levels were ≥ 100 mg/l. Feed consisted of earthworms and, occasionally, turtle pellets (Natural Aquatic Turtle Food, ZooMed Labs), and was offered weekly ad libitum.

2.2 Egg, larval and juvenile development Egg sacs were transferred to a 40 × 20 × 10 cm plastic tank, containing 2 L of water from the parents' tank and observed daily. After hatching, all larvae were housed individually in plastic containers containing 3 L of tap water that was replaced weekly. Larvae were fed ad libitum with *Daphnia*, *Chironomus* larvae (bloodworms), *Tubifex*, pieces of earthworms and/or turtle pellets (ZooMed Labs). After metamorphosis, the juveniles were further kept individually and offered a land area with hiding place. Feed items consisting of bloodworms, chopped earthworms and/or turtle pellets were offered three times weekly to metamorphosed juveniles. On September 21st, 2010, at an average length ± SD of 149 ± 15 mm, a transponder (AL-VET ID ISO MINI) was introduced in the coelomic cavity of all reared juveniles under isoflurane anesthesia for individual identification. From then onwards, the juveniles were kept in groups of 4–9 animals. Larvae and juveniles were measured on a regular basis to the nearest 0.1 mm by taking dorsal pictures in a transparent container on graph paper.

2.3 Growth characteristics of *P. shangchengensis* larvae and juveniles fed on a defined diet A first experiment was conducted in September 2006 using ten 42.5 ± 1.8 mm long larval salamanders (TL). The animals were fed with aquatic larvae of the non-biting midges of the family Chironomidae (bloodworms) ad libitum. The average weight of bloodworms was recorded and their nutrient composition was determined through proximate analysis, including dry matter (DM), ash, crude protein (CP), crude fibre (CF) and ether-extract (EE), which were measured as described by AOAC (1995). Nitrogen-free extract (NFE) resulted from the difference between DM and the sum of CP, CF, EE and ash. Gross energy (GE) was calculated with the formula of Schiemann *et al*. (1971): GE (kJ/kg) = 23.9 CP + 39.7 EE + 20.0 RC + 17.4 NFE, with nutrient concentrations expressed in g/kg.

The number of bloodworms consumed was counted daily for 8 consecutive d. The larvae of this salamander were measured to the nearest 0.1 mm and weighed to the nearest 1 mg before and at the end of the experiment.

In a second experiment, conducted in August 2008, six metamorphosed juveniles (119.9 ± 3.4 mm TL) were fed with *Tenebrio molitor* larvae and six juveniles (113.3 ± 6.4 mm TL) were fed on commercial turtle pellets ad libitum for 14 d. The average worm and pellet weight and composition were determined, and the number of feed items consumed was counted daily for 14 consecutive d. Salamanders were weighed to the nearest 1 mg and measured to the nearest 0.1 mm before and at the end of the experiment.

Feed conversion ratios were calculated by dividing the intake of dry mass of a given diet by the weight gain over the experimental period. Energetic conversion ratio was calculated as the gross energy intake (in J) necessary for a 1 mg increase in weight.

Statistical comparisons using independent samples *T* tests (df = 11, equal variances) were applicable only between the two age-matched groups (mealworm diet versus pellets).

3. Results

3.1 Reproduction Mating behavior was sporadically observed in both males. In the days preceding the deposition of the egg sacs, the males were sometimes seen lining up with the females, undulating their body while keeping it parallel to that of the female. No further interaction was observed. On May 28th, 2006 and on April 11th, 2011, two pairs of egg sacs were produced by the female of the first breeding group at a water temperature of 12°C and the one of the second group at 11°C, respectively. The egg sacs were attached on the underside of flat stones. The egg sac envelopes were dull white with a bluish sheen and were longitudinally striated (Figure 1). The egg sacs deposited in 2006 measured 57 mm in length × 17 mm in width at the widest point and 53 mm x 16 mm, and contained 32 and 22 eggs, respectively. In 2011, the egg sacs measured 38 mm × 16 mm and 44 mm × 14 mm, and contained 18 and 20 white eggs, respectively. The diameter of the eggs measured 3.3 ± 0.2 mm (mean ± SD).

Figure 1 Freshly laid egg sacs of *P. shangchengensis*, deposited in captivity in 2006.

3.2 Development Whereas the eggs in egg sacs spawned in 2006 developed normally, those in 2011 did not show any signs of development and were heavily covered by fungal growth after 21 d. Therefore, further data were based on the egg sacs deposited in 2006. The first signs of egg development recognized through the egg sac envelope were observed on d 16 after deposition, and the first larval movements on d 28 after deposition. Fungal growth was noticed on d 19 of development in the tips of both egg sacs. Affected eggs were surgically excised. No further moulding occurred. On d 36 after deposition, the first larva hatched, followed by 7 larvae on d 41 and 19 larvae on d 42 after deposition. The average temperature ± SD during larval development was 14.8 ± 1.6°C. Larvae were characterized by the presence of interdigital membrane, the absence of balancers, the presence of a large yolk sac and the presence of front- and hindlimb buds (Figure 2), and measured 20.9 mm on average (± 1.6 mm SD).

On d 14 after hatching, the yolk sacs were no longer visible and the larvae were fed for the first time with

Figure 2 Larva of *P. shangchengensis* on d one after hatching in captivity.

Daphnia. Afterwards, mainly *Chironomus* larvae, *Tubifex* and pieces of earthworms were fed ad libitum. Turtle pellets were accepted from an age of 420 d after hatching by 6 of the 21 larvae. Six of the 27 larvae died during rearing to (sub) adulthood due to unknown reasons. The growth curve of the larvae is depicted in Figure 3. Larvae had cornified finger and toe tips, and a dorsal fin beginning just cranially of the tail base (Figure 4). All larvae metamorphosed between d 441 and d 454 after hatching at 94.8 ± 4.4 mm.

Juveniles possessed blackish ground color with intensive blue dots all over the body (Figure 5). Although the terrestrial refuge was occasionally used, the juveniles

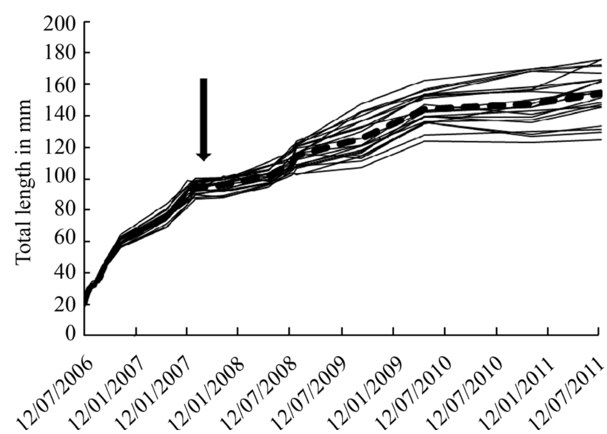

Figure 3 Growth curves of larvae and juveniles of *P. shangchengensis* reared under captive conditions. Measurements are expressed as total length in mm. The dashed line represents the average growth curve. The arrow indicates the time of metamorphosis.

Figure 4 Larva of *P. shangchengensis*, reared in captivity, on d 112 after hatching.

Table 1 Chemical composition (% as-fed basis) of the feed items fed to the salamanders.

Feed item	DM	CA	CP	EE	CF	NFE	GE
Bloodworm	10.64	0.80	7.17	0.30	0.71	1.66	2.30
Mealworm	42.61	1.21	20.70	10.84	2.90	6.96	11.00
Turtle pellet	88.36	6.39	33.10	5.52	5.42	37.93	17.60

DM: Dry matter; CA: Crude ash; CP: Crude protein; EE: Ether extract; CF: Crude fibre; NFE: N-free extract; GE: Gross energy (MJ/kg).

Figure 5 Juvenile of *P. shangchengensis*, reared in captivity, shortly after metamorphosis.

generally lived in the water. Growth curves of the juveniles are shown in Figure 3. A trend to a positive correlation (Pearson correlation coefficient 0.404, $P = 0.07$) existed between TL at hatching and that at approximately five years of age (Figure 6).

3.3 Growth characteristics of larval and juvenile *P. shangchengensis* fed with a defined diet The composition of the feed items is summarized in Table 1. Growth characteristics are summarized in Table 2. Although dry matter intake of the pellet feed was significantly higher than that of mealworms ($P = 0.001$), this did not result in significantly increased growth. Feed conversion ratio was significantly higher whereas energy conversion ratio was significantly lower for the pellet diet ($P < 0.001$ and $P = 0.02$, respectively).

4. Discussion

The egg sacs of *P. shangchengensis* are unique within the Hynobiidae by the combination of striation, compact form with blunt ends and presence of relatively few but large eggs (Zhang *et al*., 2006). Moreover, the larval period, admittedly under artificial circumstances, took more than one year with metamorphosis taking place at a relatively large total length of approximately half of the adult length. This observation is confirmed by field observations on two larvae on December 31st, 2007 at a water temperature of 6.4°C (Nishikawa and Jiang, pers. observ.). The compact shape and markedly tough envelope of the egg sacs, relatively few but large eggs, larvae without balancers, cornified finger and toe tips, and the long larval period coincide with the salamanders' stream habitat. With an average diameter of 3.3 mm, eggs were similar in size to those of *Ranodon* (Kuzmin and Thiesmeier, 2001), *Paradactylodon* (Reilly, 1983; Sparreboom, 1979; Ebrahim *et al*., 2004) and *Batrachuperus* (Liu, 1950). As such, these traits seem to fit in well with the ancestral state reconstruction in hynobiids as proposed by Zhang *et al*. (2006).

Interestingly, a positive correlation existed between larval length at hatching and total length at the age of five years. Indeed, the smallest hatchlings were considerably smaller after five years than the larger hatchlings. This finding indicates that body size at hatching might affect individual's lifetime fitness.

The taxonomic status of *Hynobius yunanicus* (Chen, Qu and Niu, 2001), which is sympatric with *P.*

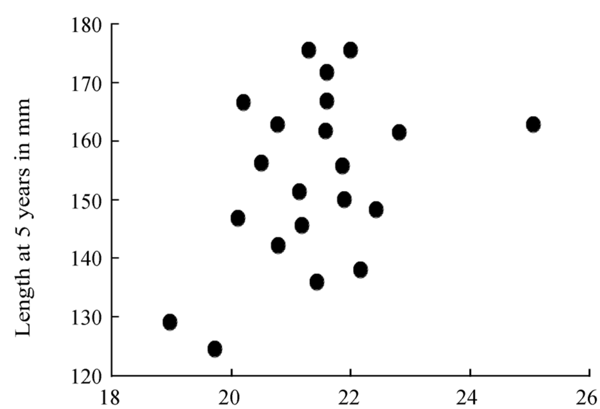

Figure 6 Total length at hatch versus length at approximately five years of age (in mm) in *P. shangchengensis*.

Table 2 Growth parameters of larval or juvenile *P. shangchengensis* after fed with defined diet during 8 or 14 d, respectively.

Feed item	Life stage	Initial weight (mg)	Δ weight (%)	Δ length (%)	DM uptake (mg)	FC_{DM}	E (J) / Δ W (mg)
Bloodworm	Larva	43 ± 2	33.2 ± 3.9	7.8 ± 2.7	49 ± 5	0.33 ± 0.04	7.1 ± 0.9
Mealworm	Juvenile	5979 ± 862	31.5 ± 8.7	4.4 ± 1.1	636^{*} ± 195	0.34^{*} ± 0.04	8.8^{*} ± 1.0
Pellet	Juvenile	8415 ± 475	25.9 ± 8.7	5.9 ± 1.9	1134^{*} ± 216	0.54^{*} ± 0.08	10.7^{*} ± 1.6

DM: Dry mass; FC_{DM}: Feed conversion ratio as determined on DM; E: Energy uptake; W: Weight; J: Joule. The increase (Δ) in weight or length during the trial is presented as % increase compared to the initial weight or length. An asterisk depicts a significant difference between animals fed with mealworm or pellet diet ($P < 0.05$).

shangchengensis, has been the subject of controversy. The both species have recently been placed in synonymy by Xiong *et al*. (2007) and Nishikawa *et al*. (2009). Our finding that the offspring of uniformly dark brown parent *P. shangchengensis* possessed small blue spots all over the body, typical of *H. yunanicus*, confirms the view of Xiong *et al.* and Nishikawa *et al.* that this species has been described based on juveniles of *P. shangchengensis*. Aggressive behavior of males was witnessed on several occasions and was not related to the presence of feed items. Biting wounds inflicted by the males to the females resulted in severe skin lesions then. These wounds typically resulted in exposure of the underlying muscles, but healed well if the bitten animal was isolated for recovery. The severity of the inflicted wounds may well be a consequence of the presence of sexually dimorphic dentition that seems quite capable of slicing due to their chisel- or spear-head crowns (Clemen and Greven, 2009). Besides, the massive head of the adult males is a further indication that the male of this species is well equipped to deliver fierce bites. The reason for this aggressiveness remains to be elucidated in the field. A limited number of suitable reproduction sites and limited nutritional resources may render a reproductive advantage to males that aggressively defend a favorable site. Anecdotal data suggests that males guard and defend the egg sacs (Jamin, pers. comm.).

Pachyhynobius shangchengensis was shown to have a very low feed conversion ratio (FCR) equaling a very high mass conversion efficiency. Although more of the commercial turtle pellets were consumed, the efficiency of this feed item was clearly less compared to that of the invertebrate feed items. Field observations confirm that *P. shangchengensis* mainly consumes aquatic organisms and on one occasion cannibalistic behavior was witnessed (Nishikawa, pers. observ.). If corrected for dry mass, FCRs obtained by feeding invertebrate prey in our study (0.33 for bloodworms in larvae, 0.34 for mealworms in postmetamorphs) were virtually equal. The type of invertebrate feed item and the developmental stage of the salamanders thus appeared not to be of any impact on the nutrient use by the salamanders. Besides, the FCRs in this study were even lower than those obtained in a terrestrial sit and wait predating frog (*Ceratophrys cranwelli*) with FCRs of 0.35 and 0.39 when fed with mice and earthworms, respectively (Grayson *et al.*, 2005), and much lower than the actively swimming, stream inhabiting salmonid fish with an FCR of 0.7–1.4 (Lock *et al*., 2011). This finding supports the hypothesis that aquatic animals have lower maintenance requirements than terrestrial animals, due to lower energy demands for standing and sitting.

Captive breeding programs have been proposed as a last resort to prevent amphibian extinctions during the current amphibian crisis (Stuart *et al*, 2004; Gascon *et al.*, 2007). Stream-inhabiting Asian hynobiid salamanders, belonging to the genera *Batrachuperus, Pachyhynobius, Liua, Ranodon* and *Paradactylodon*, and Asian hynobiid salamanders using mountain streams for reproduction (*Pseudohynobius* and some *Hynobius*) are classified by IUCN either as threatened (11 species as vulnerable, 4 endangered and 3 critically endangered), nearly threatened (2 species) or data deficient (6 species), and are facing similar threats: overcollection, habitat loss and fragmentation as in *Pachyhynobius*. Although these taxa may be interesting candidates for captive maintenance programs, this is hindered by the near absence of reports concerning captive husbandry, nutritional requirements and breeding of these animals. At present, captive breeding has only been reported from the critically endangered *Ranodon sibiricus* (Thorn, 1987, 1994). We provide data concerning captive reproduction of *P. shangchengensis*, which may be useful in captive maintenance programs for this and other Asian stream dwelling hynobiids.

Acknowledgements Jianping JIANG and Kanto NISHIKAWA would like to thank Dr. Xiaohong CHEN for field assistance in Henan Province, China. This work was partly supported by the grants of the Ministry of Education, Science and Culture, Japan (Nos. 20770066, 23770084), the JSPS AA Science Platform Program to Kanto NISHIKAWA, and the National Natural Science Foundation of China (31071906) and the Main Direction Program of Knowledge Innovation of Chinese Academy of Sciences (KSCX2-EW-J-22) to Jianping JIANG.

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