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Reproduction and development of the dark-sided frog *Hylarana nigrovittata* sensu lato at the Cologne Zoo

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Abstract. We report on the keeping and breeding of the dark-sided frog at the amphibian breeding unit of the Cologne Zoo Aquarium. *Hylarana nigrovittata* sensu lato represents a species complex, with the current research referring to a population from northern Thailand. Oviposition in captivity commenced in accordance with the natural habitat's dry season, but continued year-round at Cologne Zoo. The crucial factor, which was introduced in order to increase reproduction in captivity, was an increase in misting, in combination with raised water levels. Oviposition also occurred under drier conditions, but to a lesser extent. Development depends on water temperature and generally takes at least 2.0–2.5 months to metamorphosis, at water temperatures of 22–26°C. We provide here for the first time an overview of characteristic developmental stages for the dark-sided frog complex.

Key words. Amphibia, Anura, captive breeding, larval development, *Hylarana nigrovittata* complex, northern Thailand

Introduction

The dark-sided frog was first described as *Limnodytes nigrovittatus* by Blyth in 1856, based on a specimen found in Myanmar. It is today recognised as a representative of the anuran genus *Hylarana* that currently comprises 86 species (Frost 2009). According to the latter author, *Hylarana nigrovittata* is widely distributed, from Nepal and West Bengal (India) to Yunnan (China), Vietnam and south to Malaya.

The dark-sided frog is a medium-sized true frog, of the family Ranidae, growing to a maximum size of 75 mm. *Hylarana nigrovittata* is brown above, with a characteristic dark face and sides. The flanks may be marbled, with large scattered tubercles. The tympanum is distinct and a dorsolateral fold is present. Rictal and humeral glands are present as well. The species lives in lowland forests along streams and rivers, and can be found up to 700 m above sea level. Although primarily nocturnal, the dark-sided frog may also be active during daytime. The dark-sided frog is a dry season breeder and can be found near shallow water in slow-moving sections of streams, on sand, gravel, stones or roots (Taylor 1962, Heyer 1973, Manthey & Grossmann 1997, Ziegler 2002, Neang & Holden 2008). Recent taxonomic research has revealed that the dark-sided frog represents a complex of cryptic species of only regional occurrence (Stuart 1999, Ohler et al. 2002, Gawor et al. 2009). At a time of global amphibian decline, it is essential that we increase our knowledge of natural history and reproduction, especially the factors that induce breeding. In particular, there is an increased need to study threatened or barely known species, such as previously overlooked, cryptic taxa that often have geographically limited distribution ranges. Such research is crucial in order to be better prepared for the conservation of both adults and larvae, both in nature and in captivity. Although a comprehensive revision of *H. nigrovittata* sensu lato, i.e. the dark-sided frog complex, is still lacking, Gawor et al. (2009) were able to show that molecular divergences and morphological differences in larval and adult stages render the individuals from northern Thailand at least specifically distinct from central Vietnamese frogs. We herein describe the successful keeping, breeding and development of Thai *H. nigrovittata* at the amphibian breeding department of the Cologne Zoo Aquarium.

Materials and methods

Collection and measurements

The basis for the captive breeding of *Hylarana nigrovittata* was a couple (Fig. 1) of about 36–40 mm snout–vent length, purchased in Germany, with northern Thailand having been given as their place of origin (see Gawor et al. 2009). Measurements of larval stages were taken with callipers. Developmental stages are given according to Gosner (1960) as reproduced in McDiarmid & Altig (1999). Morphological terminology follows McDiarmid & Altig (1999), Dubois (1995) and Grosjean (2005); abbreviations are: TL = total length; BL = body length; TaL = tail length.

Captive management and breeding

The initial pair was maintained in a terrarium at the amphibian breeding unit of the Cologne Zoo Aquarium,

which is not accessible to the public. Terrarium measurements were 140 × 60 × 60 cm (length × width × height). The base of the terrarium consisted of two sections: soil and an artificial stream bordering the front glass panels, measuring up to 20 cm width and 4–5 cm in water depth (Fig. 2). Below the stream was an approximately 10 cm high water tank, inaccessible to the frogs due to layers of wire mesh, a filter mat and cement. For filtering, we used an external Eheim filter type 2224 (http://www.eheim.de) with three litres of filter volume. The stream was separated from the land area by gravel and swamp plants in a flat transition, cemented with large stones. The land substrate consisted of local soil (ca. 10–15 cm deep) with a top layer of leaf litter. In addition roots and plants were added in order to provide hiding places for the frogs. Rear and side glass panels of the terrarium were panelled with Juwel structure rear panels (http://www.juwel-aquarium.de). To maintain a high level of humidity, large sections of the ventilation grids were covered. Fluorescent tubes (Namiba compact lights, UV Replux, 36 W; http://www.namibaterra.de) were used for illumination. The photoperiod was set to daylight between 7:00 to 18:00 h each day. During both day- and nighttime, the average air and water temperatures in the terrarium were 24–25°C.

Adult frogs were fed a diet of farmed house crickets and small locusts every two to three days. Froglets were fed flies and house crickets every second day. All food was dusted with minerals and vitamins (Calcamineral) before being fed to the frogs. As the dark-sided frogs were mostly hidden during daytime and only started to become active at dusk, food was only dispensed in the late afternoon.

Results

First reproduction phase (2006)

The first oviposition event (8–9 October) occurred during the night less than two weeks after the introduction of the

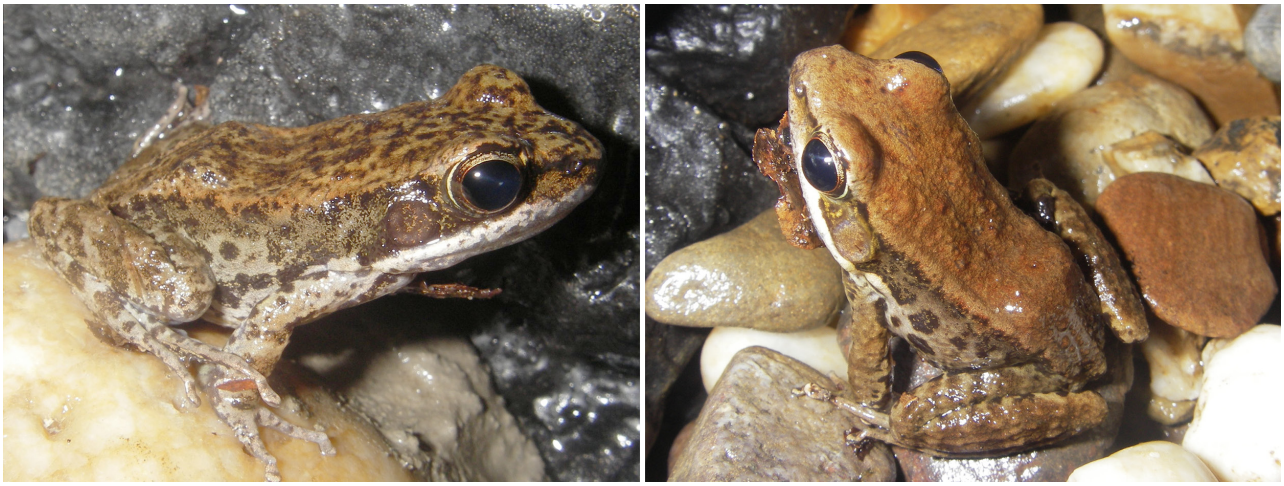

Figure 1. The initial pair of *Hylarana nigrovittata* in the terrarium at the Cologne Zoo. Photo: T. Ziegler, Cologne Zoo.

Figure 2. *Hylarana nigrovittata* terrarium at the Cologne Zoo with the artificial stream in the foreground. Photo: D. Karbe, Cologne Zoo.

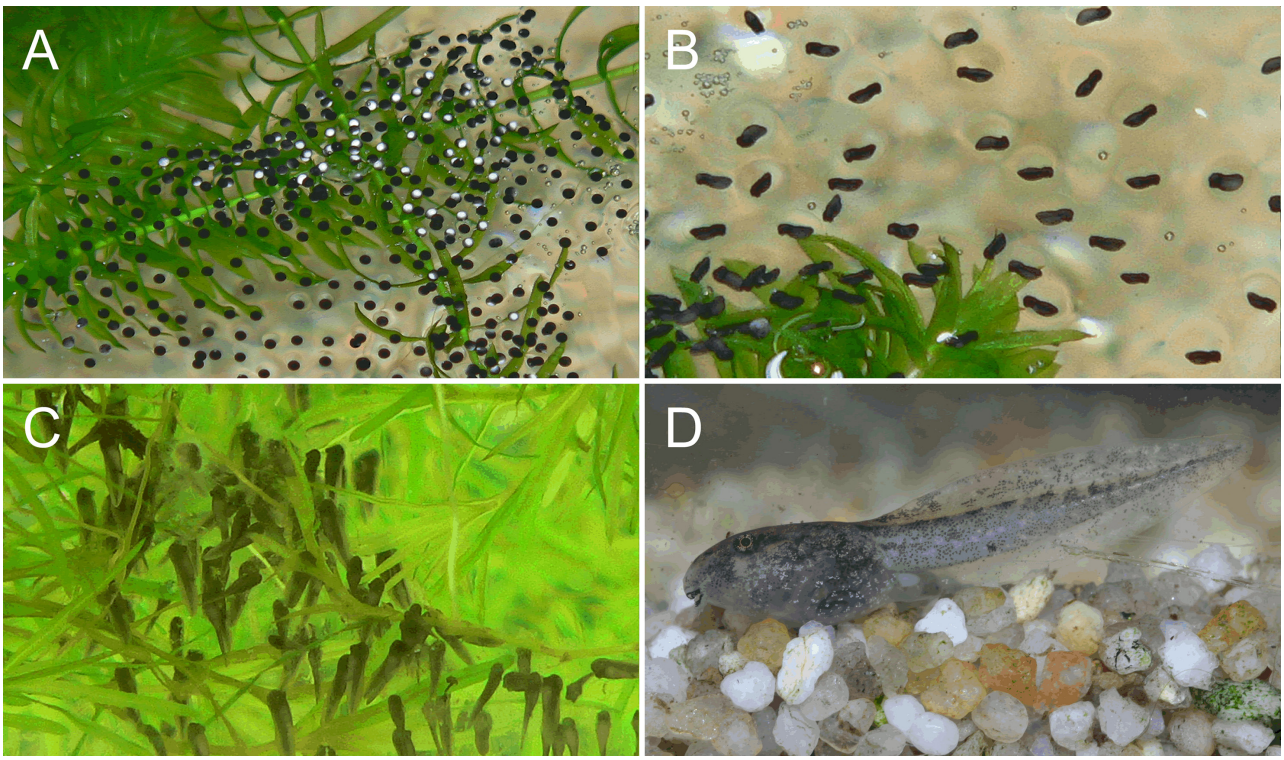

Figure 3. *Hylarana nigrovittata*: (A) freshly deposited spawn on the water surface, 9 October 2006; (B) developing eggs at Gosner stage 18/19, 10 October 2006; (C) freshly hatched larvae at Gosner stage 21/22, 11 October 2006, Photos: D. Karbe, Cologne Zoo; (D) tadpole (18.5 mm) with developing hind legs at Gosner stage 27/28, 28 October 2006, Photo: T. Ziegler, Cologne Zoo.

initial couple into the terrarium at Cologne Zoo. Neither amplexus nor mating calls had been witnessed or heard prior to this event. The eggs were deposited on the water surface in the bank region of the artificial stream. They were black, measured about 1 mm in diameter and were enveloped in a transparent jelly sheath. Immediately after discovery, the strongly glutinous eggs were transferred to an aquarium (120 × 44 × 26 cm) with a substrate of sand for further observation (Fig. 3A). The aquarium had an external filter attached. Water temperature was 26°C, pH-value 8.3, total hardness 6–8°dH, carbonate hardness 2–4, and water conductivity was 320 microsiemens. Two days after oviposition (10 October), early-stage larval development was clearly discernible, with the embryos taking on the typical larva shape (Fig. 3B). On 11 October tadpoles hatched, measuring approximately 2–3 mm. Although predominantly motionless, some first short attempts at swimming were observed (Fig. 3C). Feeding commenced at an age of ten days (18 October). In the beginning, the tadpoles were fed dry staple fish food three to four times daily. To maintain water parameters and hygienic conditions, half of the water volume was exchanged daily. Additionally, faecal matter and food remains were siphoned off every day. After 24 days (1 November), the larvae were also fed particles of bovine heart, dead house crickets and fish, and even consumed perished sibling larvae. At this developmental stage, some larvae had already developed protruding hind limbs (Fig. 3D). On day 27 (4 November), ten larvae were randomly chosen to be measured. Their sizes ranged from 2.2 to 2.8 mm. Thirty-three days after oviposition (10 November), the larvae that had distinctly developed hind limbs were taken out and transferred to two aquaria (120 × 44 × 26 cm); after 18 days, having reached an age of 51 days (28 November), forelimbs began to develop. From day 54 on (1 December), we began to transfer froglets to a terrarium (145 × 60 × 56 cm) that sported land and water sections, interconnected by foam mats to ease leaving the water for land. At the age of 56 days (3 December), approximately 80% of the froglets had entered the land section of the terrarium. The last froglet left the water 71 days after oviposition (18 December). In 2006, further oviposition events were observed on 6 and 18 November.

Second reproduction phase (2007–2008)

In 2007, we observed further instances of oviposition, predominately after phases of increased misting and the subsequent rise of the stream water level. In winter of 2007, reproduction was again investigated within the framework of the senior author's bachelor thesis at Cologne University. Characteristic traits of different developmental stages were documented and on regular occasions, five randomly chosen larvae were measured to document their growth (see Figs. 4–5, Tables 1–2). Oviposition took place on 12 November. Eggs were again transferred to a separate aquarium. Conditions were the same as reported for the year 2006, with the exception that water temperatures were notably lower (22°C ± 1°C). In addition, aquatic plants were now used as interior outfitting. Three days after oviposition, on 15 November 2007, the jelly sheath started dissolving and larval development was distinctly discernible (Fig.

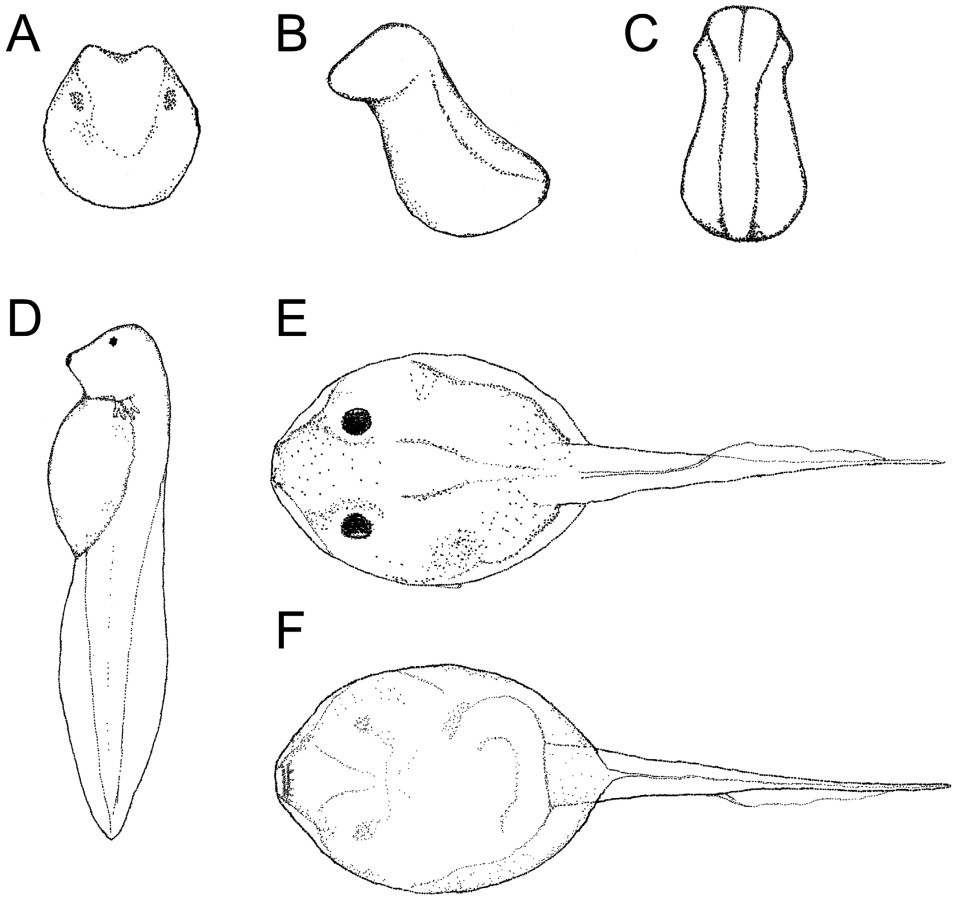

Figure 4. *Hylarana nigrovittata*: (A) egg at Gosner stage 13/14; (B) and (C) the embryo begins to develop larval traits, Gosner stage 18/19; (D) freshly hatched larvae at Gosner stage 21/22; (E) and (F) larvae at Gosner stage 25. Drawings: A. Gawor, Cologne Zoo.

4B, C). The head and tail regions of the dark grey squirming larvae (3.9–5.2 mm in total length) were clearly recognisable. Larvae hatched between four and five days after oviposition (Fig. 4D).

The larvae remained predominantly motionless in a vertical position at this developmental stage. At the age of eight days, tadpoles attached themselves to the glass panes of their aquarium or moved to the bottom of the aquarium. The oral disc was beginning to develop, but the keratodont row was not yet distinguishable. Between nine and 14 days after oviposition (21–26 November), a distinct increase in both tadpole size and activity was observed. In addition, the previously uniformly coloured body took on a greyish-olive marbled appearance. Distinct movements of the oral disc were observed in larvae attached to the aquarium panes during algae grazing. At this stage, the spiracle was clearly discernible on the left side of the body (Figs. 4E, F).

Seventeen days after oviposition, the tadpoles were first fed fish food (TetraMin), as described for the first reproduction phase. Accelerated larval growth was observed, with the oral disc becoming more prominent and the body shape taking on an oval form. Between the age of 24 and 40 days, the tail grew distinctly longer and stronger, and the larvae became darker. After 48 days (29 December) we transferred the larvae to another aquarium, where the land and water sections were interconnected by foam mats, plants and roots. The first metamorphosing tadpoles were discovered at an age of 56 days (6 January 2008). Hind legs were fully developed, including toes. Although forelegs had not yet protruded, they were easily discernible through the integument (Figs. 5A–C). The body was now elongated and less transparent, with a dark dorsolateral band visible on both sides. It is interesting to note that some other larvae were still in Gosner developmental stages 35–36 at that time. The first froglets were discovered on 16 January on the land section of the aquarium, at the age of 66 days. In contrast to the adults, the body was uniform brown dorsolaterally. When approximately 50% of the froglets had moved onto land, the remaining larvae were still in or below Gosner stage 42. The last instance of oviposition in our dark-sided frog couple was observed on 25 May after an extended phase of increased misting several times daily.

Discussion

Based on our observation, keeping and breeding dark-sided frogs in captivity is easily accomplished. Oviposition at the Cologne Zoo took place the whole year round. Reproduction was stimulated by increased misting (intensive daily spraying versus spraying every three days) combined with the consequent higher water levels (5 cm versus 2–3 cm) in the artificial stream. The shortest intervals between two oviposition events were only between two and four

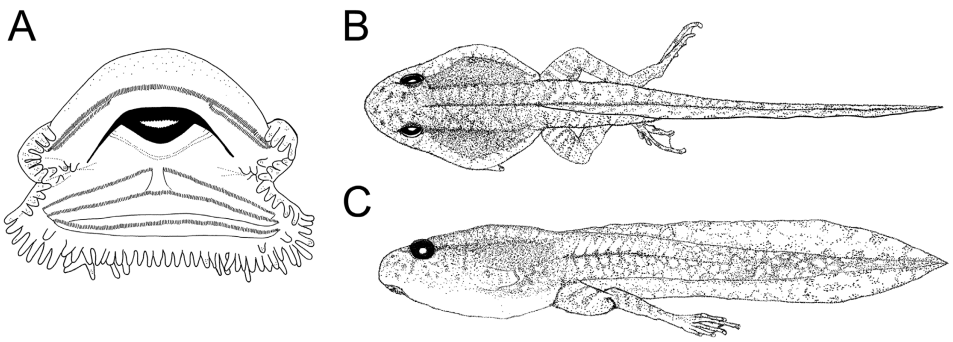

Figure 5. Larvae of *Hylarana nigrovittata* at Gosner stage 41, after Gawor et al. (2009): (A) oral disc, (B) dorsal view, (C) lateral view. Drawings: A. Gawor, Cologne Zoo.

weeks (with a minimum of 12 days between oviposition events), which suggests a high reproduction rate for this species. To slow down reproduction, we reduced the water level in the aquarium to a minimum and sprayed infrequently. However, our couple mated and laid eggs every 2.5–3 months even under these conditions. After finalising the current research, some of the offspring of *Hylarana nigrovittata* were transferred to other facilities and zoos.

Heyer (1973) reported that under natural conditions, *H. nigrovittata* is a dry season breeder. In northern Thailand (Chiang Mai), rain and humidity distinctly decrease in September and October, with the driest period lasting from December through March. Rain and humidity levels increase again in April and May. The first instances of oviposition in captivity reported here correspond to the dry season in the natural habitat in northern Thailand. However, extensive thunderstorms occur even in the natural dry season in northern Thailand, which could explain the increased reproduction rate in response to increased misting. We attribute the cessation of reproduction in our breeding pair from May 2008 to age, as one of them recently died with no evidence of disease.

Table 1. Larval development of *Hylarana nigrovittata* at the Cologne Zoo from oviposition to Gosner stage 25; water temperature = 22±1°C.

Date	Day	Stages	a) Min.–max. (mean TL ± standard deviation); n = 5 larvae b) Characteristic traits
12. Nov. 07	1		a) egg diameter ca. 1 mm b) eggs black; on water surface, with transparent egg integument, strongly glutinous
15. Nov. 07	4	20	a) 3.98–5.20 (4.69±0.47) mm b) body assumes larval shape, with head region set off from tail region; larvae uniform dark grey; egg integument dissolves
17. Nov. 07	6	21–22	a) 6.80–7.00 (6.96±0.09) mm b) larvae hatched from jelly envelope; body of tadpole dark grey with light-coloured belly, tail grey with transparent upper and lower tail fin; laterally positioned eyes; external gills visible (Fig. 4D)
19. Nov. 07	8	24	a) 7.50–9.00 (8.32±0.66) b) body of tadpole in dorsal view grey; operculum closed on right side, external gills still visible on the left side of the body; body semitransparent in ventral view; oral disc anteroventrally positioned, with developing upper and lower jaw sheath; mouth with few papillae; heart beat and intestinal coils visible through integument
21. Nov. 07	10	25	a) 8.50–10.00 (9.00±0.61) b) body oval shaped in dorsal view, whitish to yellowish, covered with grey to olive-green pigments; spiracle positioned laterally (sinistrally); eyes positioned and orientated dorsolaterally; oral disc with black upper and lower jaw sheath, keratinised tooth rows developing (Fig. 4E, F)
23. Nov. 07	12	25	a) 8.50–10.10 (9.72±0.92) b) larvae still at stage 25, but body pigmented more densely dorsolaterally; tail musculature whitish and slightly pigmented; upper and lower tail fin transparent and without pigmentation; tail generally thin and fine
26. Nov. 07	15		a) 8.50–14.50 (11.59±1.72) b) growing larvae; body heavily pigmented dorsolaterally
28. Nov. 07	17	25	
30. Nov. 07	19		

Table 2. Larval development of *Hylarana nigrovittata* at the Cologne Zoo from Gosner stage 26 to the completion of metamorphosis; water temperature = 22±1°C.

Date	Day	Stages	a) Min.–max. (mean TL ± standard deviation); n = 5 larvae b) Characteristic traits
3. Dec. 07	22	25–26	a) 13.00–16.00 (14.30±1.40) b) appearance of hind limb buds
5. Dec. 07	24	28–29	a) 16.00–17.00 (16.45±0.45) b) growth and development of hind limb buds; body of tadpole still transparent in ventral view, slight pigmentation starting in the region of the throat and chest; oral disc with papillae
7. Dec. 07	26	27–29	a) 15.75–18.00 (17.30±0.96)
10. Dec. 07	29	29–30	a) 16.50–19.00 (18.30±1.10)
27. Dec. 07	46	34–35	a) 24.25–28.00 (25.95±1.89)
29. Dec. 07	48	34–36	a) 22.75–32.00 (26.30±3.48) b) growth of hind limbs, which begin to show slight pigmentation dorsolaterally; tail musculature well-developed at the base
6. Jan. 08	56	39–41	a) 19.50–32.00 (28.50±5.21) b) hind limbs well developed (with single toes discernible); forelimbs visible through skin; a dark lateral band develops on both sides of the body, stretching from snout tip to shortly before hind limbs (Fig. 5B,C)
10. Jan. 08	60	> 41–42	a) 25.00–33.25 (29.45±3.73) b) body elongated, frog shape becomes discernible; reduction of spiracle
12. Jan. 08	62	> 42	a) 18.00–34.50 (27.20±6.15) b) forelimbs emerging
16. Jan. 08			b) first froglets enter land (sit on plants and roots); some of them still with larval tail

Larval development was rapid up to stage 25, followed by increased growth. Stages 25–26 (Fig. 6) were the longest stages. We further observed that development rate was dependent on water temperature. The first cases of metamorphosis occurred after 54 days at a water temperature of 26°C, and the second at a lower water temperature (22°C) nearly two weeks later, after 66 days. In general, the time from oviposition to the completion of metamorphosis was 2–2.5 months. However, even at constant water temperatures, there were considerable differences in individual developmental times. For example, in winter 2006, leaving the water for land took place between 54 and 71 days, with most of the tadpoles having metamorphosed after 56 days. This may have been due to the availability of food and other resources, and influenced by intraspecific competition amongst the tadpoles. However, it might also provide for a continuous supply of freshly metamorphosed froglets to the environment under natural conditions. Although no live cannibalism among larvae was observed, dead larvae were fed upon.

Outlook

Stuart et al. (2004, 2008) revealed that about one third of all amphibians were threatened (to different degrees) on a worldwide scale. Presently, more than 6,700 amphibian species are recognised and new species descriptions follow on a regular basis. Unfortunately, for both the latter species and approximately one fourth of the globally known amphibians, i.e., about 1,500 species, natural history data and information about their threat status is deficient. To better understand and thus strengthen our ability to protect barely known and threatened amphibian species, it is essential to invest in amphibian research, both in and ex situ. Further research on the ecology of the adult and the often neglected larval stages is fundamental, both within the natural environment and in holdings in zoological gardens. Only with a strong knowledge base of the ecological processes behind reproduction will we be able to protect amphibian species.

Hylarana nigrovittata is, to our knowledge, not currently listed in any threat category. However, Gawor et al. (2009) recently suggested that this name might actually refer to a species complex, probably consisting of a number of different species (e.g., Matsui et al. 2001, Ohler et al. 2002). Future research must confirm the existence of the so far overlooked cryptic taxa and provide names. It cannot be excluded – due to limited population sizes or geographically isolated occurrences – that particular populations or taxa within this complex are deserving of conservation measures. Further research within the taxonomically poorly understood *H. nigrovittata* complex is therefore crucial.

With this breeding report we hope to further the understanding of the dark-sided frog complex. To date, no other keeping and breeding reports are available for *H. nigrovittata*. We encourage other facilities to start or continue with the breeding of threatened or only poorly known amphibian species, in order to advance our collective knowledge and increase our ability to protect such species. Further natural history studies and breeding reports will substan-

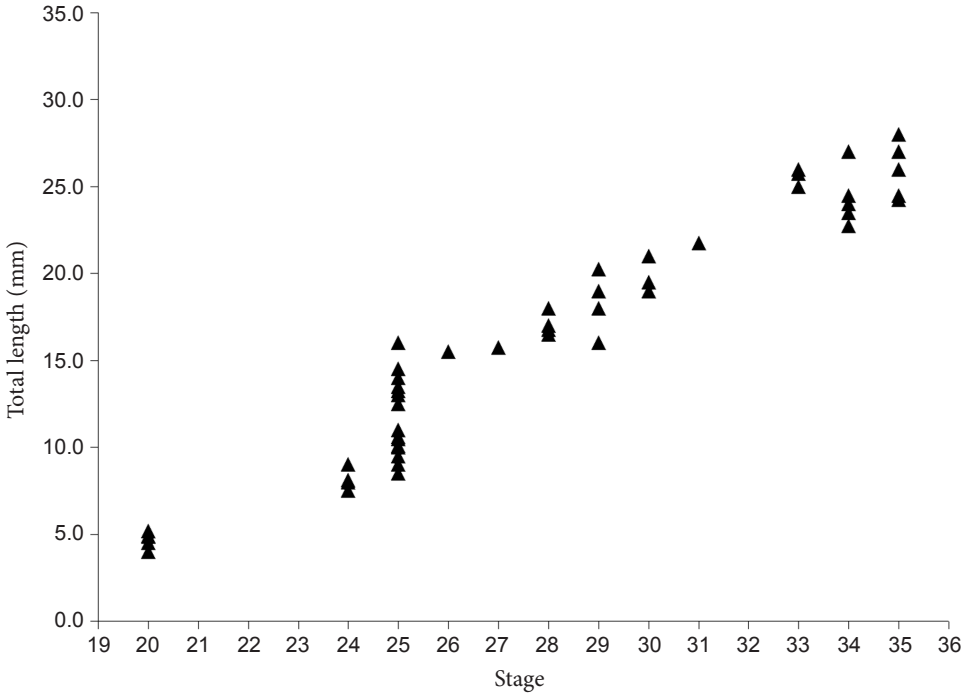

Figure 6. Total larval length of *Hylarana nigrovittata* in relation to different developmental stages after Gosner (1960).

tially add to a better understanding of the most endangered group of vertebrates, the amphibians, and is a vital prerequisite for appropriate in and ex situ conservation measures (i.e., maintaining reserve populations in captivity).

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