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Larval development, stages and an international comparison of husbandry parameters of the Vietnamese Mossy Frog *Theloderma corticale* (Boulenger, 1903) (Anura: Rhacophoridae)

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ABSTRACT

We describe the larval development and stages of the locally threatened Vietnamese Mossy Frog *Theloderma corticale*, which is endemic to northern Vietnam. Diagnostic morphological characters are provided for Gosner (1960) larval stages 1-46. This is to our knowledge the first larval staging for the rhacophorid anuran genus *Theloderma* in general. As guideline for further breeding engagement with *Theloderma* representatives in an international scale, based on the species *T. corticale* as husbandry analogue, we further oppose larval development, captive reproduction and husbandry management both achieved under tropical conditions at the Amphibian breeding station of the Institute of Ecology and Biological Resources in Hanoi (Vietnam), and in Europe, at the amphibian breeding unit at Cologne Zoo (Germany). Observed ovipositions at Cologne Zoo took place from March to September and were initiated after increase of temperatures and humidity (increased spraying) subsequent to a hibernation phase in combination with raised water levels. The developmental time observed for *T. corticale* at 20°C was about 4.5 months. For providing a recent captive management overview, we furthermore compare our husbandry experiences and data on the reproductive biology of *T. corticale* with data from the literature.

Key words: Amphibia, mossy frogs, captive breeding, tadpole staging, developmental biology

INTRODUCTION

The rhacophorid anuran genus *Theloderma* is characterized amongst others by the presence of Y-shaped terminal phalanges together with calcified warts on the dorsum. These warts, combined with a cryptic color pattern, dissolve the anuran body shape on tree bark and rocks, overcasted by lichen or moss. *Theloderma* representatives are nocturnal forest dwellers, which can be found on vegetation and fallen logs as well as in tree holes, karst caves, and the environment of streams and cascades (Liem, 1970; Manthey & Grossmann, 1997; Orlow, 1997). Currently, 23 species of *Theloderma* are recognized with a wide distribution range from northeastern India and Sri Lanka through Myanmar, Thailand, Laos, and Cambodia to southern China and Indochina to Malaya and Sumatra. In Vietnam, 16 *Theloderma* species are recorded at time (Nguyen V. S. *et al*., 2009; Frost, 2011; Rowley *et al*., 2011; Orlov *et al*., 2012), of which one species is listed as endangered, two as near threatened, three as least concern, and the remaining ones as data deficient (IUCN, 2012). In general, morphological and ecological data of *Theloderma* species are incomplete, in particular with respect to their larval stages. So far, larval descriptions are only available for 39 percent of the known *Theloderma* species, with the tadpoles of the endangered species *T. bicolor* and the data deficient species *T. corticale* having been described only recently (Gawor *et al*., 2012a).

However, knowledge about tadpole morphology is crucial for the proper determination of anuran larval stages in the field both for batrachofaunal inventories and habitat assessments, which are important data sets for potential conservation actions. In general, data on amphibian reproduction and development are important to know, in particular in times of the global amphibian crisis, with conservation breeding programs being one measure to preserve threatened amphibian species (Griffiths & Pavajeau, 2008; McGregor Reid & Zippel, 2008; Browne *et al*., 2011; Ziegler *et al*., 2011; Zippel *et al*., 2011). But prior to conservation breeding efforts, the triggers for mating and egg deposition in captivity and the optimum environmental conditions for successful development have to be identified, which can be long-lasting undertakings at least for certain, difficult to breed amphibian taxa (e.g., Gawor *et al*., 2012b). It thus was the major aim of the Amphibian Breeding Station of the Institute of Ecology and Biological Resources (IEBR) in Hanoi (Vietnam) to keep and breed poorly known or threatened amphibian taxa from Vietnam to learn more about their husbandry management, reproductive biology and development (Ziegler & Nguyen, 2008; Nguyen Q. T. *et al*., 2009; Ziegler *et al*., 2011).

Data on the reproduction and in particular regarding the development and larval stages of *Theloderma* are relatively scarce or even not available. General information on the reproductive biology in the genus *Theloderma* were provided by Orlow (1997) and Orlov *et al*. (2010). General keeping and breeding reports were published by Kunz (2009), Kunz *et al*. (2010), and Bagaturov (2011); husbandry papers dealing with particular species were provided to our

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knowledge by Orlov & Ryboltovskv (1999), Ryboltovsky (1999a, b), and Dunce (2004) for *T. stellatum*. Information on larval morphology were published so far by Bourret (1942), Leong & Lim (2003), Inthara *et al.* (2005), Rowley *et al.* (2011), Gawor *et al.* (2012a), and Orlov *et al.* (2012).

We herein document for the first time larval staging in the genus *Theloderma*. Based on the locally threatened species *T. corticale*, which is endemic to northern Vietnam, we describe its development and larval stages. In nature, this species inhabits steep rocky cliffs in primary forests at higher elevations; reproduction takes place in rock cavities and tree holes containing water (Orlow, 1997; Nguyen V. S. *et al.*, 2009; IUCN, 2012). As guideline for further breeding engagement with *Theloderma* representatives in an international scale we further oppose husbandry management, captive reproduction, and larval development of *T. corticale* as husbandry analogue for congeners both under tropical conditions in the Amphibian breeding station in Hanoi, and in Europe, at the amphibian breeding unit at Cologne Zoo in Germany. For providing a recent captive management overview for *T. corticale*, we furthermore compare our husbandry and breeding results with data from the literature.

MATERIAL AND METHODS

Captive management at the Amphibian Breeding Station, Hanoi (Vietnam)

During data acquisition at the Amphibian Breeding Station in Hanoi (June to July 2010) *Theloderma corticale* individuals (origin: Tam Dao, northern Vietnam) were kept in two air-conditioned rooms: 11 wild caught adults (8 males, 2 females, 1 unknown sex), six male subadults (offspring: older than 1 year), 57 juveniles (offspring: 1-3 months old), and tadpoles in different developmental stages. Sexes were kept together year-round.

Adults, froglets and larvae of *T. corticale* were kept in basins with the measurements L97.5 x W39.0 x H47.5 cm (Length x Width x Height). The basins were made of concrete and bricks (Figure 1). The water level in each basin was on average 6.0 cm. The terrestrial part in the middle of each basin was formed by different sized lime stones, halved bamboo canes (30.0-50.0 cm length), one or two clay pots as well as one or two Xaxim plates (L20.0 x W20.0 x H3.0 cm). Moreover, some single shells of Mangosteen fruit were placed in the water.

The tops of the basins were half covered with a glass pane and half with bamboo. No artificial illumination was applied, so enclosures were rather dark. The air temperature in both rooms fluctuated between 26-29 °C at daytime (air condition was switched off during night), relative humidity varied between 55-90% and water temperature ranged between 24-28 °C. On average, the water parameters (water extracted from a fountain) were: pH 8, KH 8, GH 10. The NO_3 (nitrate) value ranged between 0-25 mg/l and the NO_2 (nitrite) value between 0-5 mg/l.

To maintain optimum water parameters and hygienic conditions, feces and food remains were siphoned off every day. Eggs were daily sprayed with water in order to prevent drying-out until hatching.

Captive management at Cologne Zoo (Germany)

In July 2010, Cologne Zoo received 20 juvenile specimens of *Theloderma corticale* (aged about three months) as offspring from the Amphibian Breeding Station in Hanoi. After six weeks of quarantine, during which the frogs were tested and found to be negative for the amphibian chytrid fungus, three specimens, which later on turned out to be two males and one female, remained at Cologne Zoo; the other individuals subsequently were provided to other zoos for complementing or building up breeding stocks.

After quarantine, the three juvenile *T. corticale* were maintained at Cologne Zoo's amphibian breeding unit in a terrarium with measurements L100 x W50 x H60 cm, divided into an aquatic and a terrestrial section. Temperatures ranged between 23-25 °C. In September 2011, the frogs were transferred to a cooler room within the amphibian section, where temperatures were reduced to 15 °C via an air conditioning system in order to simulate a hibernation period as it occurs in the natural habitat, where temperatures can fall down to 4-5 °C (Ryboltovsky, 1999a). The terrarium in the cooler unit measured L100 x W60 x H50 cm. It was completely water-flooded with a water level of 5.5 cm and structured by climbing, hiding and sitting opportunities such as artificial roots, plants (*Anubias* sp.) and pieces of mangrove wood. The back wall was pasted up with a structure rear panel (Juwel) for providing a naturally looking environment as well as additional climbing structures. Illumination was supplied by a T5-fluorescent tube (54 W), which was attached to a clock-timer; the photoperiod lasted for 10 hours. During the artificial cold spell, the former daily water spraying with a manual pump sprayer was reduced to a minimum; feeding intervals and quantity also were reduced. From February 2012 onwards, the room temperature was gradually raised to 20-25 °C. Furthermore, water spraying was increased and the water level elevated to 7 cm. The photoperiod was extended to 12 hours. From October to December, temperature was lowered step by step down to ca. 18 °C and sexes were separated. Illumination was reduced to 10 hours / day at the end of October.

Egg clutches remained in the adult terrarium; eggs that were fallen into the water were incubated in plastic basins or petri dishes with low to medium water level.

After hatching, larvae were kept in a subdivided plastic tank with measurements L60 x W41 x H22 cm and a water level of 12.5 cm. Water parameters were: GH 20, KH 9, NO_2 0, pH 9.0.

The ground was covered with river sand; PVC-tube-pieces (about 10 cm length) and stones served as hiding opportunities. In order to maintain a constant water quality, an external filtration system (Eheim type 2322) with a capacity of 500 l/h was attached. In addition, partly water changes took place from time to time for the disposal of food remains etc. A heating element in the filter (150W) ensured a constant water temperature of 20 °C. In order to protect the tadpoles from being injured by suction, a piece of nylon fabric was pulled

over the filter pipe (Figure 1). For maintaining stable water parameters, sea almond (*Terminalia catappa*) leaves were added to the water. Shortly before metamorphosis, tadpoles were transferred to a terrarium with measurements L100 x W60 x H50 cm, divided into a land and a water part. For optimum keeping conditions, the water from the plastic tank (in which the tadpoles had previously been reared) was filled into the water part of the terrarium. The water temperature of 20 °C was again maintained by a heating element in the attached filtration system (Eheim type 2322). Further clutches were reared in water with less hardness (GH 7-8, KH 3-4) and at somewhat higher temperatures (ca. 25 °C). The ground substrate of the terrestrial section consisted of a 6 cm thick layer of leaf litter, covered with moss. An artificial root, protruding from the water to the land part, was brought in to serve as a bridge and to facilitate the entering of the land section for freshly developed froglets. Back and side walls were pasted up with structure rear panels; another artificial root on the land part provided for hiding space.

Nutrition

Adults and subadults were supplied with a diet of farmed house crickets (*Acheta domestica*) every second to every third day both in Hanoi and in Cologne. The insects were nourished with high quality food and dusted with mineral and vitamin supplements (Korvimin ZVT + Reptil / Calcamineral) before being fed. Larvae were provided daily with fish food flakes (Tetramin +) and at a later stage also with blood worms. Metamorphed frogs were supplied with small house crickets (from about 2 mm onwards); first feeding was introduced seven days after the froglets had entered the land section.

Data acquisition and staging methodology

Mating, egg deposition and larval development data were recorded from 2nd June to 28th July 2010 at the Amphibian Breeding Station Hanoi, and since 8th March 2012 at Cologne Zoo. Eggs, embryos and larvae were measured with a digital caliper to the nearest 0.1 mm (Hanoi) or photographed on millimeter paper (Cologne). For the documentation of characteristic morphological traits of developmental stages and the determination of larval stages according to Gosner (1960), as reproduced in Duellman & Trueb (1994) and McDiarmid & Altig (1999), five of the larvae were temporarily placed in glass vessels and photographed in dorsal, lateral, and ventral views every second day (Hanoi) or at least weekly (Cologne). All photographs were taken with digital cameras (OLYMPUS E-600, DG MACRO 105 mm 1:2.8 object lens, SIGMA; Pentax K-x, DG MACRO 105 mm 1:2.8 object lens, SIGMA). Terminology of clutch structure and larval morphology followed McDiarmid & Altig (1999), Altig (2007) and Altig & McDiarmid (2007). Water parameters were conducted using SERA water tests.

Abbreviations

GH – total hardness; H – height; L – length; KH – carbonate hardness of water; pH – pH value of water; TL – total length of tadpole; W – width.

RESULTS

Herein we describe the tadpole development and larval stages of *Theloderma corticale* (see also Figures 2-7, Tables 1-2), followed by a comparison of husbandry parameters (own data versus information from literature: Tables 3-4) and a brief summary of our reproductive ecological observations (see also Figure 8) opposed to data from literature (Table 5).

Development and larval stages

The development of the embryonic and larval stages to the completion of metamorphosis was studied in detail on the basis of seven tadpoles from the first deposited egg clutch maintained at temperatures of 20 °C at Cologne Zoo.

Embryonic development was observable three days after oviposition, but the characteristic larva shape with distinct head and tail was only detectable at day five, six, or seven, respectively.

Gill buds and olfactory pits were discernible six days after egg deposition (stage 18/19), when also the first slight pigmentation occurred; gill circulation started after eight days (stage 19/20). On day nine after egg deposition, the tail was distinctly flattened and elongated (stage 20). 11 days after egg deposition, the eye region was slightly discernible and two days later clearly visible (stage 21). In addition, the mouth region started to develop. On day 14 after egg deposition (stage 21/22), tail fins were transparent, fin circulation began and the first three larvae hatched from the eggs that were fallen into the water. At that time, the larvae had a length of about 11.3 mm; the yolk reservoir was still distinct. Two of the larvae died on the next day. From day 16 onwards, the pigmentation increased and became progressively darker and the operculum covered the external gill bases (stage 22/23). Due to continuing growth, the larvae gradually started to coil up inside the vitelline membrane.

19 days after egg deposition (stage 24) the external gills were completely atrophied, the dorsal pigmentation was light greyish with dark spots. Numerous blood vessels were noticeable on the lateral and ventral body sides and on the tail. From day 22 onwards (stage 24/25), the dark pigmentation started to extend laterally. The yolk sac was visibly reduced and the larvae were strongly coiled up due to their size. As most of the larvae failed to hatch by day 25 (stage 25), even though larvae of *T. corticale* have been reported to hatch at an age of 10-17 days (Orlov & Ryboltovsky, 1999, Ryboltovsky, 1999a, b, Kunz *et al.*, 2010), they were assisted to hatch by rupturing the embryonic membranes artificially with needle and forceps on the days 25 and 26, respectively. Larvae from subsequent ovipositions hatched independently after 14-22 days (depending on temperature). Larvae from eggs that were fallen into the water hatched at temperatures of 20-22 °C at days 14-15 with a size of 14 mm. Eggs that were deposited at land hatched 2-3 days later with a size of ca. 19 mm. The larvae that hatched at land immediately started to swim (see below), whereas larvae that were hatched in the water initially remained laying on the side. However,

Figure 1. a) *Theloderma corticale* husbandry basin at the Amphibian Breeding Station in Hanoi (after cleaning, with low water level); b) just installed tadpole rearing container for two larvae groups at Cologne Zoo in front of the adult terraria, with filter system, and c) equipped and with tadpoles.

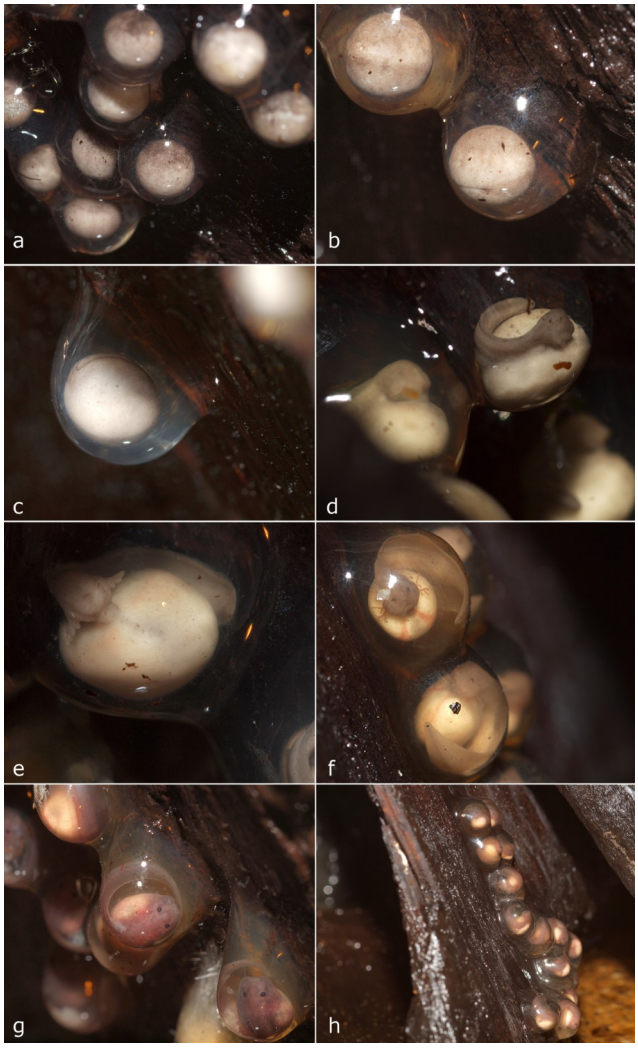

Figure 2. First clutch of *Theloderma corticale* at the amphibian breeding unit at the Cologne Zoo: a) – c) developing embryos in stages 1-16; d) embryos in stages 18 to 19; e) – f) embryos in stages 20 to 22; g) – h) larvae in stages 23 to 25.

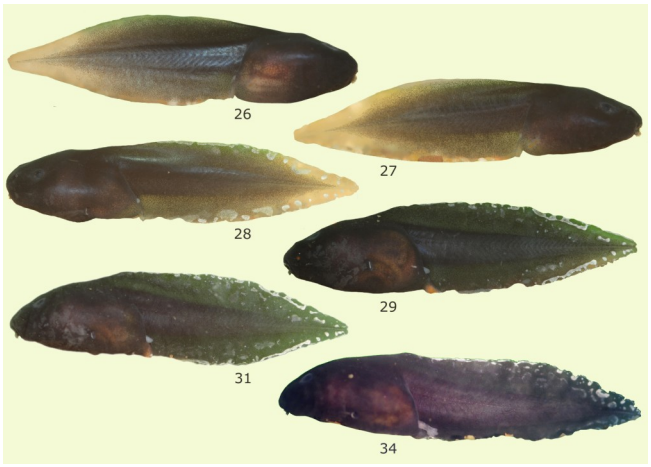

Figure 3. *Theloderma corticale* larvae at the amphibian breeding unit at the Cologne Zoo in stages 26 to 34.

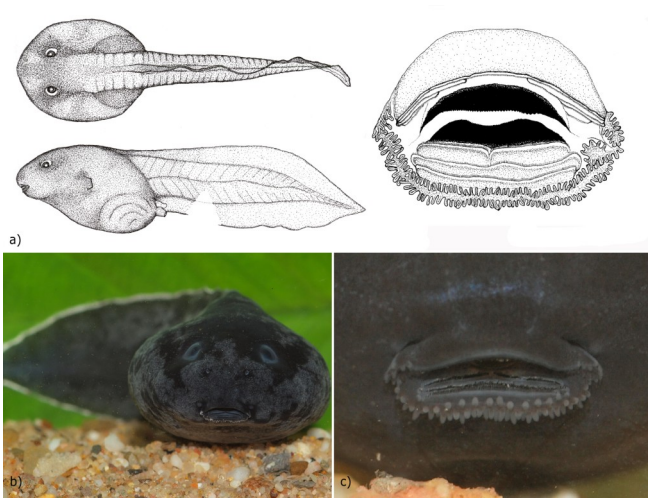

Figure 4. Overall tadpole morphology and oral disc of *Theloderma corticale*: a) drawings based on preserved specimen from the amphibian breeding station Hanoi (stage 32) in dorsal and lateral view (part of lower tail fin including musculature cut for tissue sample) as well as oral disc; from Gawor *et al.* 2012a), and in life at Cologne Zoo: b) stage 36 at an age of 90 days c) stage 28 at an age of 39 days.

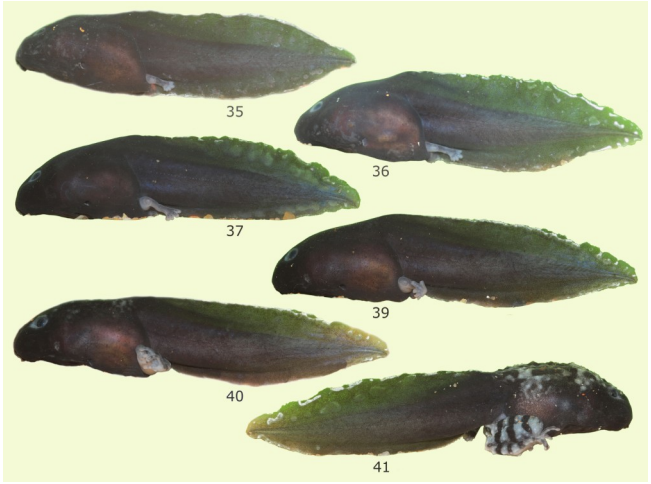

Figure 5. Tadpoles of *Theloderma corticale* at the amphibian breeding unit at the Cologne Zoo in stages 35 to 41.

latter tadpoles still had a considerable yolk reservoir. The eggs / larvae that developed in water showed comparably lower hatching / survival rates.

Shortly after hatching, the tadpoles were able to swim and fled quickly when getting disturbed, taking shelter in the provided PVC-tubes. At this time, the yolk reservoir was completely resorbed and the development of the mouth parts was obvious. On both sides of the oral disc were noticeable bulges, which reduced with the further development of the mouthparts and disappeared ten days later. The whole pigmentation had darkened, except for the transparent tail fins with large blood vessels and the white venter, where the intestinal loops were clearly visible. On the 30th day after egg deposition, marginal papillae and jaw sheaths were already apparent.

On day 35 after egg deposition, the unpigmented hind limb buds were noticeable (stages 26-28). The oral disc was fully developed, the tooth rows (labial tooth row formula 4(2-4)/3(1), see Gawor *et al.*, 2012) were discernible and all tadpoles could be observed feeding. At that time the tadpoles measured between 28.5 and 33.0 mm; most of the body was uniformly dark grey to black except for the intestinal spiral region, which was slightly paler. Mouthparts, eye region, nostrils and medially located vent tube were light grey; the tail pigmentation was greyish and mottled at the edges. Lines of neuromasts forming the lateral line system were visible on head, body and tail. The large sinistral spiracle opening as well as the strongly developed tail musculature was conspicuously developed. On the 42nd day after egg deposition (stages 27-29), we noticed that in some of the tadpoles the edges of the tail fins had become fringed and were irregularly covered with white blotches. One tadpole furthermore showed such white parts also on the body surface, but we could not detect any negative influence on the development. Tadpoles had reached lengths of up to 40.5 mm. On day 62 after egg deposition, the white hind limb buds had distinctly grown in length and the food paddle shape began to develop (stage 31). On day 74, indentation between toes 3-4 (stage 33) and 2-3 (stage 34) had started respectively; and 7 days later, the indentation between toes 1-2 occurred (stage 35).

On the 90th day after egg deposition, all tadpoles were in stage 36 (toes 3-5 separated) and measured between 57.5-60 mm. The hind limbs started to bend and had a grey spotted pigmentation on the upper side. The iris coloration began to change from dark brown or almost black towards a golden brown. Toe separation was completed on day 98 (stages 37-39).

On day 106 after egg deposition (stages 40-41), dark stripes had extended over the dorsal parts of the hind legs and toes, and several light spots and blotches occurred on the back of the tadpoles. In some of the tadpoles, bulges of the developing forelimbs were slightly visible under the operculum. Meanwhile, the iris had become equal to the condition in adults (concerning coloration and pattern) and was spotted in different shades of gold. The eyes were surrounded by a light grey area. Mouth parts started to atrophy progressively. At that time, the largest tadpole measured 62.9 mm.

118 days after egg deposition, forelimbs had emerged in most of the tadpoles (stage 42). A bright color pattern with dark black and reddish blotches and stripes on a light grey to blue/greenish basic coloration covered the dorsal body and extended laterally. Fore and hind limbs were striped black and light grey. The skin started to develop the typical rough structure with large, irregular warts on head and body. On the ventral side and legs many small granules became discernible.

120-123 days after egg deposition, tail resorption had started and the adult color pattern was fully developed (stage 43/44). When the froglets entered the land section from day 125 after egg deposition onwards (stage 45), the warty skin structure was completed, but tail stubs were still present. On day 137 after egg deposition, all froglets had reached stage 46 and completed metamorphosis.

Pre-mating, mating and oviposition

Amphibian Breeding Station, Hanoi (Vietnam)

Advertisement calls were heard mainly during the evening and occasionally during daytime (Figure 8). Calling males were seen sitting on Xaxim plates or bamboo canes. Mating (amplexus axillaris) always took place in the water, and could be observed during daytime and in the early evening (Figure 8). During the two-months observation period *T. corticale* spawned seven times. The clutch size varied between 6-36 eggs. The half white and half grey eggs were on average 4-5 mm in diameter (measured without the transparent jelly layer). All eggs were attached in an array to the bottom side of the halved bamboo canes or to the Xaxim plates (Figure 8). Eggs that fell into the water did not develop. Hatching took place after 9-14 days.

Cologne Zoo (Germany)

Reproductive behavior took place after stimulation by increased temperatures and humidity in combination with an elevated water level after the artificially induced winter period from September to February 2012. The first deposited egg clutch was detected on March 8, 2012. At this time, the air temperature was about 19 °C, humidity ranged between 80 and 90 %. Water parameters were: temperature 17 °C, GH 24, KH 11, NO_2 0, and pH 8.4. The first clutch contained 34 eggs, of which 28 were attached to the vertical sides of the mangrove wood, while six were fallen into the water. After the first egg deposition, the room temperatures were lowered to about 16 °C. Subsequent temperature rises to 21-25 °C led to further oviposition events in July (6th and 18th), with clutches consisting of 45 and 32 eggs, and in August (14th and 26th) and September (21st) with 47, 28, and 31 eggs per clutch, respectively. These clutches also were attached to the vertical sides of the mangrove wood or at the rear panel. Sometimes, few eggs (6-12) fell into the water. Only in one case a clutch was directly deposited in the water due to the absence of adequate egg deposition structures. From July to September, amplexi were always observed one day before spawning. After the first oviposition event, the adults were removed from the terrarium, but remained with the eggs in subsequent reproduction phases. Here, one of the adults could often be observed in direct vicinity of the clutch.

Figure 6. Tadpoles of *Theloderma corticale* at the amphibian breeding unit at the Cologne Zoo: Overview of hind limb development and toe differentiation (stages 28 to 41).

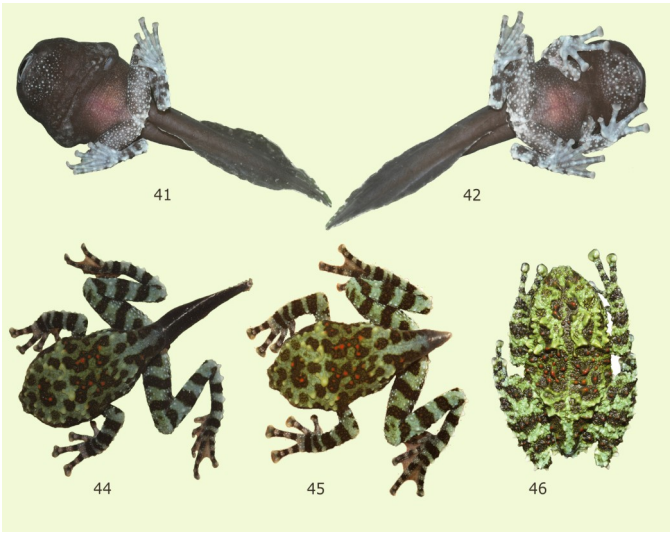

Figure 7. Developmental stages of *Theloderma corticale* bred at the Cologne Zoo from stages 41 to 46 including tail atrophy until the completion of metamorphosis.

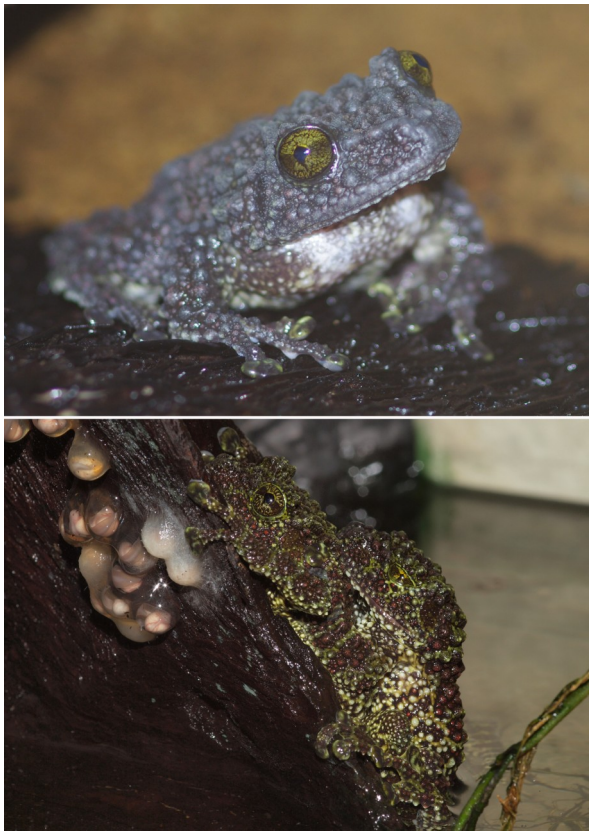

Figure 8. Advertisement call (Hanoi) and amplexus of *Theloderma corticale* beside clutch with developing tadpoles (Cologne).

Table 1. Developmental stages of *Theloderma corticale* bred at the Cologne Zoo from stage 1 to 25 (n = 5-24); stage diagnostic characteristics according to Gosner (1960) are italicised; Age [d] = age in days, TL [mm] = total length in mm, **---** = no data available; [1]could not be verified in this study; [2]could only partially be verified in this study.

	Stage	Age [d]	TL [mm]	Diagnostic features
EMBRYOS	**1-12**	1-2 (n=24)	---	*Fertilisation, cleavage, dorsal lip, yolk plug and late gastrula develop one after the other*[1];
	13-16	3-4 (n=5)	---	*Neural plate, neural folds, elongation rotation, neural tube and gill plates observable in this order*[2];
	17	5 (n=5)	---	*Tail bud and adhesive gland visible*; large yolk sac
	18	5-6 (n=10)	---	*Muscular response, olfactory pits visible*; yolk sac slightly reducing
	19	6-9 (n=17)	---	*Heart beats, gill buds visible*; initiation of pigmentation
HATCHLINGS	**20**	8-11 (n=20)	---	*Gill circulation, tail elongation*; tail distinctly flattened, eye region slightly discernible
	21	13-14 (n=10)	---	*Cornea transparent, mouth opens*;
	22	14-17 (n=20)	---	*Tail fins transparent, fin circulation*; pigmentation increased
	23	18 (n=5)	---	*Labia and teeth differentiate, operculum covers gill base*; larvae gradually start to coil up inside their vitelline membrane
	24	19-22 (n=10)	---	*External gill atrophy, operculum closes on right*; pigmentation gets progressively darker and extends laterally, dorsal pigmentation light greyish with grey spots and numerous blood vessels on lateral and ventral body sides and transparent tail, larvae strongly coiled up due to their size
	25	22-31 (n=21)	19.5 (n=1)	*Mouthparts obvious, spiracle forms on left;* larvae hatched (some were assisted by rupturing the embryonic membranes artificially), oral disc with two lateral bulges, yolk sac completely absorbed, shortly after hatch the tadpoles were able to swim

Table 2. Developmental stages of *Theloderma corticale* bred at the Cologne Zoo from stage 26 to 46 (n = 1-12); stage diagnostic characteristics according to Gosner (1960) are italicised; Age [d] = age in days, TL [mm] = total length in mm, Mean ± SD = mean value and standard deviation, Min – Max = minimum and maximum value, **---** = no data available; [1]could not be verified in this study; [2]could only partially be verified in this study.

Stage	Age [d]	TL [mm] as Mean ± SD as Min – Max	Diagnostic features	
26	35 (n=1)	30.0 (n=1)	*Hind limb buds developed (L < ½ D)*; oral disc and tooth rows fully developed, all tadpoles fed, body pigmentation nearly black except for the intestinal spiral region	
27	35-42 (n=5)	30.5 ± 3.4 (n=4) 28.5 – 43.8	*Hind limb buds developed (L ≥ ½ D)*; tail fin transparent with white-dotted edges, lateral bulges on both sides of oral disc reduced	
28	35-53 (n=10)	36.9 ± 1.5 (n=7) 35.0 – 39.5	*Hind limb buds developed (L ≥ D)*; fringed tail fin, lateral bulges on both sides of oral disc gone, lateral organs clearly visible	
29	42-53 (n=6)	44.0 ± 2.8 (n=4) 40.5 – 46.5	*Hind limb buds developed (L ≥ 1½ D)*; black pigmentation with more or less distinct grey patches on body and tail	
30	---	---	*Hind limb buds developed (L > 2 D)*;	
31	62 (n=4)	49.9 ± 1.7 (n=4) 50.0 – 51.0	*Food paddle develops*;	
32	---	---	*Indentation between toes 4-5*;	**LARVAE**
33	74 (n=1)	55.5 (n=1)	*Indentation between toes 3-4*;	
34	74-81 (n=5)	54.7 ± 1.5 (n=5) 52.5 – 56.0	*Indentation between toes 2-3*;	
35	81 (n=2)	57.0 ± 0.0 (n=2)	*Indentation between toes 1-2*;	
36	90-93 (n=9)	59.0 ± 1.3 (n=9) 57.5 – 61.8	*Toes 3-5 separated*; legs begin to bent, slightly darker leg pigmentation	
37	93-98 (n=2)	60.7 ± 1.6 (n=2) 59.5 – 61.8	*All toes separated*; iris colouration begins to change from dark brown/black to golden brown	
38	98 (n=2)	60.0 ± 0.1 (n=2) 59.9 – 60.0	*Metatarsal tubercle*;	
39	98 (n=2)	59.8 ± 1.8 (n=2) 58.5 – 61.0	*Subarticular patches*;	
40	106 (n=5)	60.6 ± 1.6 (n=5) 59.0 – 62.9	*Foot tubercle, vent tube present*; legs and webbed toes with grey and black striped pattern, ventral greyish-white spotting appears, distinct iris pattern appears	
41	118-120 (n=6)	59.0 ± 1.5 (n=6) 56.7 – 61.0	*Forelimbs visible, mouthparts atrophy, vent tube gone*; characteristic body pattern and dermal structure differentiates, larvae display tonic immobility (freezing) when touched lightly	
42	118 (n=2)	56.6 ± 0.1 (n=2) 56.5 – 56.7	*Forelimbs emerge, mouth anterior to nostril*; extension of ventral greyish white spotting	**METAMORPHS**
43	120-125 (n=7)	55.0 ± 2.4 (n=7) 50.6 – 57.2	*Mouth beneath nostril and eye*[1]*, tail atrophies*; adult colouration sets in	
44	123-125 (n=6)	42.9 ± 5.3 (n=6) 33.9 – 48.0	*Mouth beneath eye*[1]*, tail greatly reduced*;	
45	125-131 (n=12)	28.6 ± 1.9 (n=3) 26.4 – 30.0	*Mouth posterior to eye*[1]*, tail stub*;	
46	137 (n=4)	---	*Tail resorbed, metamorphosis complete*; adult colouration fully developed	

DISCUSSION

Generally, the diagnostic characters of the larval stages of *Theloderma corticale* corresponded well with the staging system proposed by Gosner (1960) for *Bufo* (now *Incilius*) *valliceps.* However, considerable differences were noticed regarding the developmental time between the stages (comparable stages occasionally seemed to be longer or shorter in *T. corticale* than in *I. valliceps*).

Larval development was rapid up to stage 25, due to which the stages 1-12 as well as 13-16 were hardly discernible. However, the large yolk sac was noticeable, which was already distinctly developed from stage 17 onwards, and which was completely resorbed in stage 25. By contrast, stages 25-29 were the longest stages and marked by increased growth of the larvae which also resulted in a wide variation in total length. Above this, the hatchlings in stage 25 displayed distinct lateral bulges at both sides of the oral disc.

Despite the fact that the white hind limb buds contrasted strongly with the dark body colour, determination was difficult in early stages, as they were often concealed by a skin membrane at the base of the tail. Hence, the stages 30 (hind limb buds developed, L > 2 D) and 32 (indentation between toes 4 -5) could not be verified during our observation. During the stages 43-45 a wide variation in total length was again observable, when the tail resorption had already set in. Within these stages the tail length was used as the only characteristic for stage determination as the tonic immobility of the metamorphs (reaction to stress) did not allow us to take pictures from lateral view which prevented a detailed observation of the forming of the adult jaws. Metamorphosis was completed after 137 days.

Whether any observed differences in the intraspecific development were caused by, e.g., intraspecific competition or genetic predisposition remains unclear.

Concerning husbandry of *T. corticale* it can be stated that the most important parameters for successful keeping and breeding are sufficient water part for the adults, suitable structures for climbing, hiding and egg deposition, sufficient and high quality feeding (inclusive regular vitamin and mineral nutrients supply), in particular of adults in reproductive phase, and suitable, at best seasonally varying climate, as well as larval rearing tanks of adequate size to prevent possible cannibalism (e.g., Dunce, 2004; Kunz, 2009; Kunz *et al.*, 2010; Bagaturov, 2011; own observations). Although reproduction in captivity seems to take place independent from season (Ryboltovsky, 1999a, b: November to February; own data: March to September), increasing temperature after a cooler period seems to be favourable to stimulate breeding. According to Ryboltovsky (1999a, b), *T. corticale* exclusively hibernate in the water and nearly totally reduce activity including feeding for this period. Further triggers for reproduction are increase of humidity and water level raise. Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b) and Evsyunin (2009) also point to sensitivity of *Theloderma* species regarding disturbance during calling activity, which has to be considered during captive breeding programs. If afore mentioned husbandry parameters are provided, continuous reproduction may be possible, as it takes place in the amphibian station Hanoi since September 2008 (Nguyen Q. T. *et al.*, 2009) and at Cologne Zoo since March 2012.

By comparing keeping parameters in an international scale, breeding certainly is easier to achieve in the country of origin, e.g., due to the predominant climatic conditions, as far as adequate facilities and experienced staff are existent. However, it has to be taken into account that under tropical conditions also natural predators might appear, which may have influence on larval and stock development. We regularly found individuals of the natricine snake *Xenochrophis flavipunctatus* in the amphibian station, which also feeds on amphibians, as well as larger representatives of the spider families Pisauridae and Sparassidae, which possibly prey on tadpoles, although we could not make direct observations of such behaviour.

By comparing the keeping of *T. corticale* in Hanoi and Cologne regarding diseases and mortality, on both sides occasionally bacterial and mycological infections occurred. Continuative studies on this point would certainly be valuable. Both in Hanoi and (predominantly) in Cologne, in some tadpoles of *T. corticale* we observed a white colouration of the tail edges, as it was also mentioned by Kunz *et al.* (2010). While the latter authors described the occurrence and intensity of this colouration as being dependent on nutritional condition, we observed different shapes of the tail edge coloration also under constant husbandry conditions. Furthermore, one of the larvae of the first egg clutch at Cologne Zoo showed such white pigmented areas not only at the tail, but also at the whole body (see Figure 4), which, however, had no negative influence on the development. For a complete clarification of this phenomenon, e.g., whether genetically induced, due to bacterial or mycological infection, further studies will be required.

In general, our experiences with the husbandry of *T. corticale* largely accorded with the available data from the literature. However, the developmental time until completion of metamorphosis documented in Cologne distinctly exceeded the time recorded by other authors (4.5 months versus 2.5-3 months, e.g., Orlov & Ryboltovsky 1999, Ryboltovsky 1999a, b, Dunce 2004). This deferment might be due to the different water temperature, which was distinctly lower at Cologne (20 °C) compared to data from literature (23-26 °C). Ryboltovsky (1999a, b) reported that tadpoles laid on the ground and did not feed when water temperatures were below 24 °C, but such behaviour could not be observed by us even at temperatures below 20 °C. Also extended development phases are known, which can exceed 6 months, but in which larvae were distinctly larger compared with larvae that metamorphosed earlier (Ryboltovsky, 1999a, b; Dunce, 2004; Orlov *et al.*, 2010; Bagaturov, 2011). However, this seems not to be associated with season or water temperature, because tadpoles of distinctly different sizes may also arise under identical keeping conditions in particular in large groups (Kunz *et al.*, 2010). Concerning temperature and development duration changes, respectively, McDiarmid & Altig (1999) generally report of increasing rates of differentiation and growth with increasing temperatures until an inhibiting temperature is reached .

Table 3. Overview of husbandry parameters for *Theloderma corticale* adults according to Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b), and Kunz *et al.* (2010; including other sources such as Dunce, 2004) combined with own data.

	Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b)	**Kunz *et al.* (2010)**	**own data (Amphibian Station Hanoi)**	**own data (Cologne Zoo)**
Aquaterrarium size (L x W x H)	150 x 40 x 60 cm (for 1 male + 1 female)	minimal 80 x 50 x 40+ cm (for 1 male + 1-3 females)	97.5 x 39 x 47.5 cm (for 11 adults, 6 subadults or 57 juveniles)	100 x 60 x 50, divisible (for 2 males + 1 female)
Land part (L x W)	75 x 40 cm	not necessary	-	-
Water part (L x W)	75 x 40 cm	large water part or completely flooded aquaterrarium	basins completely water-flooded	aquaterrarium completely water flooded
Water depth	15 cm	at least 10 cm	ca. 6 cm	5.5–7 cm
Filtration / water parameters	Aeration, filtration pH 6.7, GH 5-6	-	ca. pH 8, KH 8, GH 10, NO_3 0 -25 mg/l, NO_2 0-5 mg/l	GH 24, KH 11, pH 8.4, NO_2 0
Air temperature	23-26 °C (day) 20-22 °C (night)	24-30°C (day) 20-24 °C (night*)	26-29 °C (day) air condition switched off during night	19**-25 °C (day / night)
Water temperature	20 °C	-	24-28 °C	at time of first egg deposition ca. 17 °C, thereafter ca. 20-22 ° C
Hibernation: time / temperatures	October 18-20 °C (day) 13-15 °C (night)	- 13-20 °C	-	September-February ca. 15 °C
Aquaterrarium equipment (sitting/ hiding/egg deposition):	coarse gravel (land part), ceramic pots, fragments of calcareous stone, pottery crocks, *Anubias lanceolatum*	gravel, calcarous stones etc. (land part), cork pieces, clay flowerpots	different sized lime stones, halved bamboo canes (30-50 cm in length), clay pots, Xaxim plates	mangrove wood, artificial roots, *Anubias* sp.
Nourishment	crickets, cockroaches	insects: juveniles daily, with 1 fasting day per week, subadults every second day, adults every second to third day	crickets (with mineral and vitamin supplements): adults twice a week, froglets every second day	crickets (with mineral and vitamin supplements): adults twice a week, froglets every second day

* - night setback recommended; temperatures should not exceed 26 °C (but husbandry also possible at constant temperatures of 25 °C round the clock)
** - at time of first egg deposition

Table 4. Overview of husbandry parameters for *Theloderma corticale* larvae and juveniles according to Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b), and Kunz *et al.* (2010; including other sources such as Dunce, 2004) combined with own data.

	Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b)	**Kunz *et al.* (2010)**	**own data (Amphibian Station Hanoi)**	**own data (Cologne Zoo)**
Aquarium size (L x W x H)	150 x 40 x 40 cm	young can remain in adult terrarium, but should better be raised separately or in small groups (in aquaria, plastic boxes etc.)	remain in adult basin	60 x 41 x 22 cm
Water depth	20-25 cm	-	ca. 6 cm	12.5 cm
Equipment	-	hiding places (plants such as *Pista stratiotes*, *Taxiphyllum barbieri*, potsherds), leaves of *Terminalia catappa**	-	hiding places (PVC tubes, stones), river sand, *Terminalia catappa* leaves*
Water parameters	24-26 °C	ca. 21 °C	24-28 °C	first group: 20 °C, GH 20, KH 9, NO_2 0,0 mg/l, pH 9.0, filtration second group: 25 °C, pH: 8.3, conductivity: 320 ms, KH 3-4 , GH 7-8, NO_2 0.0 mg/l
Nutrition	meat, fish, liver, boiled eggs, Tetra fish food	fish food flakes, Tubifex worms, blood worms, black mosquito larvae, nettle tea infusion, Spirulina-flakes, cuttlebone/eggshells (calcium supply)	Fish food flakes (Tetramin)	fish food flakes (Tetramin), blood worms
Captive management froglets	aquaterrarium, 28 °C (day) 22 °C (night)	terrarium not too large (for proper feeding), water level max 5 cm	remain in adult basin	terrarium L100 x W60 x H50 cm, water part L50 x W30 x H5 cm, filtration, 20 °C, ground substrate: leaf litter, moss, artificial roots

* - for improvement of water quality

Table 5. Overview of captive reproduction data for *Theloderma corticale* according to Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b), and Kunz *et al.* (2010; including other sources such as Dunce, 2004) combined with own data.

	Orlov & Ryboltovsky (1999), Ryboltovsky (1999a, b)	**Kunz et al (2010)**	**own data (Amphibian Station Hanoi)**	**own data (Cologne Zoo)**
Reproduction trigger	raising temperatures and humidity (15 min. irrigation with warm water in the evening)	raising temperatures*	-	raising temperatures and humidity (spraying), elevated water level (from 5.5 to 7 cm)
Egg deposition months	November-February	throughout year	-	March-September
Egg deposition places	plants/stones/crocks above water	on equipment (branches, cork) above the water surface	bottom side of halved bamboo canes, Xaxim plates	mangrove wood, back wall
Egg number	11-37	10-40 (rarely up to 70), on average 15-20	6-36	28-47
Egg deposition intervalls	every 7-14 days	every 7-30 days, in exeptional cases every second day	2-7 days	at least 12 days
Egg development until hatching	13-17 days at 25-26° C (on land) 7-9 days (in water)	10-15 days**	9-14 days	16-18 days (on land) 14-15 days (in water)
Development time	91 days-6 months	2.5-3 months (sometimes up to 6 months)	-	137 days at 20 °C

* - may also reproduce year-round under constant climatic conditions; but several weeks cooling during winter time is recommended, or at least separation of sexes for some months (for female recovery)
** - depending on temperature

Egg clutches are usually deposited on land, but sometimes oviposition can take place in the water part, e.g., due to lack of egg deposition opportunities. According to Ryboltovsky (1999a, b), it is only important to ensure a sufficient aeration in that case, because otherwise clutches develop worse or die off. Ryboltovsky (1999a, b) and Dunce (2004) described an earlier hatch in clutches deposited into the water (7-9 versus 13-15 days); larvae were also smaller (8-11 mm versus 15 mm) and in an earlier developmental stage. In Cologne, eggs that were fallen or deposited into the water were incubated into plastic or petri dishes with low water level as it was painted by Kunz *et al.* (2010), but we noticed a lower survival rate compared to clutches deposited on land (see also Dunce, 2004). Larvae from these eggs also hatched 2-3 days earlier than on the land part (but not until days 14-15; probably due to lower water temperatures), were about 5 mm smaller and did not swim from the beginning but laid on the side for the first time. In the amphibian station in Hanoi, no development could be documented for eggs fallen into the water, probably due to reduced oxygen content in the water and absence of filtration.

Leaving the adults with the egg clutch seems to be a further advantageous aspect for egg development. Tapley (2009) observed adults of *T. stellatum* frequently staying next to the clutches and supposed, at least for this species that secretions of the adults could serve as protection from fungal infections. We also found the adults of *T. corticale* often sitting close to the eggs, but it cannot be certainly stated whether larvae from the first clutch at Cologne did not hatch independently during the first 25-26 days due to absence of the parents or if the delay was caused by the low temperatures compared to the literature. At least, larvae from later egg clutches, where the adults remained in the terrarium, hatched on their own; however, further research on this aspect seems to be necessary.

Concerning the development of eggs that were fallen or deposited into the water there are controversial information available about the water quality and parameters except for the aforementioned importance of well-aerated, i.e. highly oxygenated water. According to Kunz (2009) using fresh tap water as well as performing frequent water exchange has led to successful husbandry; in contrast, loss occurred with too high or low frequent water exchange. Therefore, at the Cologne Zoo water exchange was conducted only occasionally, and concerning larval husbandry an external filtration system was used in order to maintain a constant water quality. Moreover, Kunz (2009) and Kunz *et al.* (2010) recommended to add tropical almond leaves (*Terminalia catappa*) into water (see also Dunce, 2004), which was started with the fourth egg deposition at the Cologne Zoo. According to Kunz *et al.* (2010) tannins and other bioactive substances added to the water have an antibacterial effect, lower the pH-value and promote healing of fungal infection and inflammation. However, Bagaturov (2011) recommended either introducing large branches and pieces of cork bark, or adding boiled leaves or bark of common oak (*Quercus robur*). He discouraged from too frequent water exchange to ensure accumulation of abundant organic elements and humic acids, in particular tannins.

According to Bagaturov (2011), a sufficient concentration of tannins in the water is indispensable for guaranteeing the survival of the tadpoles until metamorphosis. Based on our experiences at the amphibian station in Hanoi and following the suggestions of Ryboltovsky (1999a, b) the breeding unit at Cologne eventually switched to soft water ($GH \leq 8$, $KH \leq 4$) in order to optimise rearing conditions. After Arinin (2009) and Evsyunin (2009) (as cited in Kunz *et al.* 2010) the quality of (tap) water, which may vary widely from one region to another (e.g., regarding total hardness), is crucial for successful tadpole rearing and particularly during settling-in periods. However, further research is required in this regard, because in its natural habitat *T. corticale* occurs in karstic areas usually characterised by high water hardness. In this respect, Orlow (1997) especially mentioned caves behind waterfalls in karstic rock formations as well as crevices in rocky river canyons.

A further not yet universally accepted fact regarding the reproductive biology of *T. corticale* is a possible temperature-dependent sex determination (Kunz *et al.*, 2010). Thus, lopsided gender ratios with males comprising up to 99 % of the overall offspring were recorded not only for *T. corticale* but also other *Theloderma* species. Furthermore, Bagaturov (2011) mentioned that lower temperatures had a beneficial effect on the sex ratios of *T. bicolor* and *T. corticale*, as the proportion of female progeny increased under cooler conditions. At temperatures of 17-19 °C ratios of about 30% males and 70% females were obtained. For *T. asperum* and *T. stellatum* balanced sex ratios were also observed at room temperatures (Bagaturov, 2011). According to Dunce (2004), the phenomenon of male overproduction is not related with water temperature or quality during egg and tadpole development. However, the influence of unusually high or low temperatures on the development and in particular the sex determination of amphibians has not been sufficiently investigated yet. To this day, temperature-induced sex determination has only been reliably established for few amphibian species: *Bufo bufo* LINNAEUS, 1758 (Piquet 1930); *Rana temporaria* LINNAEUS, 1758 (Witschi 1914, 1929a); *Triturus cristatus* (LAURENTI, 1768) (Wallace *et al.*, 1999; Wallace & Wallace, 2000); *Lithobates catesbeianus* (SHAW, 1802) (Hsü *et al.,* 1971); *L. sylvaticus* (LECONTE, 1825) (Witschi, 1929b); *Pleurodeles waltl* MICHAHELLES, 1830 (Dournon & Houllon, 1984, 1985, Dournon *et al.*, 1990); *P. poireti* (GERVAIS, 1835) (Dournon *et al.*, 1984, 1990); *Rana japonica* BOULENGER, 1879 (Yoshikura, 1959, 1963); and *Hynobius retardatus* DUNN, 1923 (Uchida, 1937a, b).

Outlook

Due to their unusual appearance and interesting biology, mossy frogs of the genus *Theloderma* are popular amphibians both in the pet trade and in collections of Zoological Gardens. *T. corticale* is one of the few regional anuran species for which there is a specific demand in the global pet trade (IUCN 2012). As the species is endemic to northern Vietnam, where it is locally threatened, we recommend both *in situ* conservation measures (first and

foremost habitat protection) and in addition *ex situ* breeding efforts for maintaining a stable *ex situ* population, also to fulfill the needs of the pet trade, independent from wild caught specimens. One such measure recently was undertaken at the amphibian station in Hanoi while providing offspring to European zoo facilities for widening or even building up breeding programs, which already were successfully conducted amongst others at Cologne Zoo. However, data on reproductive and developmental biology as well as breeding reports are only available for a limited number of *Theloderma* species. Thus we herein intended to provide an overview of husbandry parameters and for the first time larval staging tables for the genus based on the species *T. corticale*, which thus may serve as a husbandry analogue for the still unexplored or more heavily threatened and not yet bred congeners.

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