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Is there a chance for conservation breeding? *Ex situ* management, reproduction, and early life stages of the Harlequin toad *Atelopus flavescens* Duméril & Bibron, 1841 (Amphibia: Anura: Bufonidae)

[1,3]Anna Gawor, [1]Anna Rauhaus, [1]Detlef Karbe, [1]Karin Van Der Straeten, [2,4]Stefan Lötters, and [1,5]Thomas Ziegler

[1]*Cologne Zoo, Cologne, GERMANY* [2]*Trier University, Biogeography Department, Trier, GERMANY*

***Abstract.*—We report on our experiences with the captive management and *ex situ* reproduction of the Harlequin toad from Suriname (*Atelopus flavescens*) at the amphibian breeding unit of the Cologne Zoo. Egg deposition was stimulated by maintaining *A. flavescens* in a drier environment followed by a period of intensive irrigation. Here we provide for the first time an overview of the larval development from oviposition to metamorphosis, including diagnostic morphological characters according to Gosner. Eggs were arranged in strings and attached to the substrate below the water surface. Larvae hatched about five days after egg deposition and the characteristic abdominal suctorial disc developed about two days later (stages 20-21). Tadpoles are gastromyzophorous and were observed rasping algae. The average time for larval development to stage 41 was 100-130 days. Larval development appears to be dependent on water temperature with faster development at higher temperatures. Concerning color pattern in adults, we observed a slight sexual dimorphism and we were able to recognize individuals due to a constant color pattern. However, color was observed to slightly change over time.**

Key words. Anura; Bufonidae; *Atelopus flavescens*; husbandry; breeding; development; larval stages; adult color pattern; individual recognition

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Introduction

Harlequin toads of the bufonid genus *Atelopus* have a Neotropical distribution. They can be found in humid environments from Costa Rica south along the Andes stretch south to Bolivia and eastwards into the Amazon basin to eastern Guyana. This species-rich taxon is comprised of 113 taxa some of which are undescribed (La Marca et al. 2005). We are aware of additional new species, and taxonomic reviews of several *Atelopus* species complexes are still pending (e.g., Rueda-Almonacid et al. 2005; De la Riva 2011; Frost 2011). Many of these species have a highly restricted geographical distribution. This may be one reason why many *Atelopus* species are among the most hard-hit lineages in ongoing worldwide amphibian population declines and extinctions. *Atelopus* is one of the most threatened vertebrate groups in the world, with the majority of species having undergone dramatic declines within the last three decades. Many of these are so called "rapid enigmatic declines" and several populations and species are now thought to be extinct (La Marca et al. 2005; Stuart et al. 2008). Multidisciplinary conservation strategies are urgently needed (Lötters 2007). *Atelopus* species reproduce in streams and have rheophilic larvae. But apart from this, natural history information is sparse to lacking for most *Atelopus* species (Lötters 1996; Rueda-Almonacid et al. 2005; Karraker et al. 2006; Luger et al. 2009).

Many of the *Atelopus* declines and extinctions are presumably related to the occurrence of the amphibian fungal disease chytridiomycosis (Bonaccorso et al. 2003; Pounds et al. 2006; Lötters et al. 2010), which can occur even in undisturbed environments. As pointed out by Lötters (2007), *ex situ* conservation action, namely conservation breeding, should be considered among the potential measures to rescue these amphibians. This is in agreement with recommendations in the *IUCN Amphibian Conservation Action Plan*, which cites conservation breeding as an option for protection of many amphibians (see also Griffith and Pavajeau 2008; Browne et al. 2011; Lötters et al. 2011a; Zippel et al. 2011). Nevertheless, so far there are only few reports about successful captive breeding and rearing of *Atelopus* species (e.g., Mebs

Correspondence. *Email:* [3]*anna_gawor@gmx.de* [4]*loetters@uni-trier.de* [5]*ziegler@koelnerzoo.de* (corresponding author).

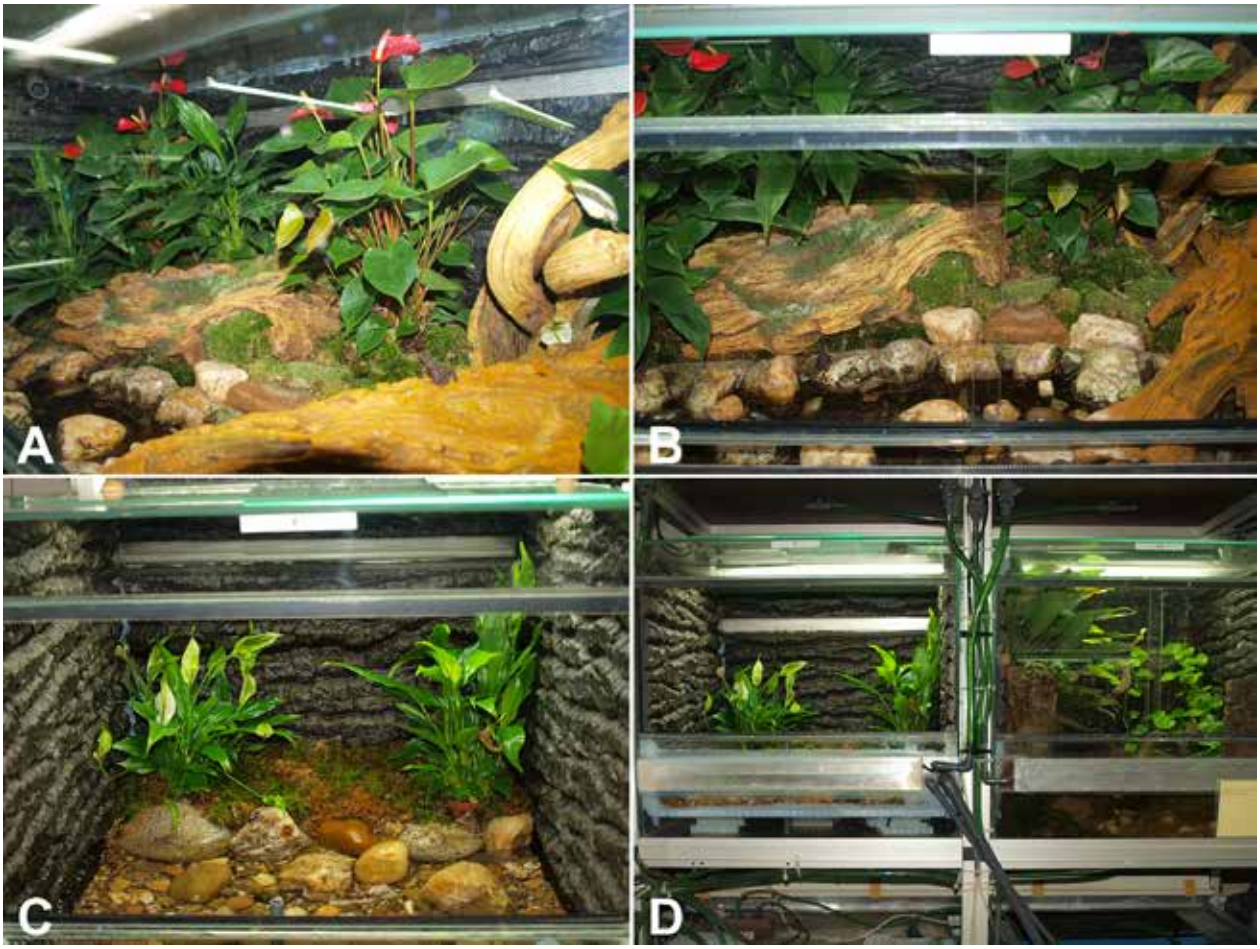

Figure 1. *Atelopus flavescens* terraria in the amphibian breeding unit at the Cologne Zoo from different perspectives (A) - (D); both terraria have artificial streams in the foreground. *Photographs by D. Karbe.*

1980; Heselhaus 1994; Haas 1995; Poole 2006; Siavichay et al. 2011). Likewise, little is known about *Atelopus* reproductive ecology in the wild (Karraker et al. 2006). Thus, it is not only important to widen the number of successfully bred *Atelopus* species, but also to report about any progress in breeding, and to better understand *Atelopus* reproductive biology and *ex situ* management for conservation breeding programs.

It is important to learn more about the reproductive biology and *ex situ* management of *Atelopus* as a basis for the further development of conservation breeding programs. For this purpose, we selected the Harlequin toad (*Atelopus flavescens*; Alonso and Mol 2007) from the Nassau Plateau and its vicinities in Suriname. It was discovered in 2007 and was identified as a color morph of the widely distributed polymorphic *A. flavescens* Duméril and Bibron, 1841 from the eastern Guiana Shield (Lötters et al. 2011b; S. Lötters and colleagues, data not shown). This species is one of the few apparently yet "intact" Harlequin toad taxa with stable populations (Rueda-Almonacid et al. 2005) and is occasionally available via the pet trade. We selected *A. flavescens* as a husbandry analogue species for the threatened genus *Atelopus*; to start with a relatively easy-to-obtain-taxon, which has relatively stable status in nature, and that is suitable for learning more about the husbandry and breeding of *Atelopus* species in general. About six years ago, Cologne Zoo (Germany), together with other European zoos (e.g., Zurich Zoo, Switzerland) and Atlanta Botanical Garden, established a cooperative conservation breeding program. To optimize *ex situ* conditions and to maximize captive reproduction success, field research has also been conducted (Lötters et al. 2011a). Data obtained from field studies finally led to successful *ex situ* deposition of eggs and subsequent larval development of *A. flavescens*. Herein we present our first experiences with the captive management and *ex situ* reproduction of *A. flavescens* at the amphibian breeding unit of the Cologne Zoo with emphasis on a description of mating, egg laying, and larval development.

Material and methods

In December 2006, Cologne Zoo received 15 *A. flavescens*, which originated from the vicinity of the Nassau Mountains, Suriname, from the Atlanta Botanical Garden for developing the international conservation breeding

program. As all individuals turned out to be male, an additional group of 25 males and five females was obtained from the pet trade in December 2008. These animals were probably also derived from Suriname.

To provide vouchers, and to enable further study, some deceased adults were fixed in 40-60% ethanol and subsequently preserved in 70% ethanol and deposited in the herpetological collections of the Department of Herpetology and Ichthyology, Muséum d'histoire naturelle (MHNG), Geneva, Switzerland, and of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn, Germany: MHNG 2727.25-2727.26 (n = 2), ZFMK 92947-92949 (n = 3). In addition, four freshly dead larvae in different developmental stages were fixed in 4% formalin and subsequently preserved in 70% ethanol. The larvae were deposited in the herpetological collection of the ZFMK (ZFMK 92351, deceased 22 December 2010, from first clutch 17 days after egg deposition, stages 24-25; ZFMK 92352, deceased 26 December 2010, from first clutch 21 days after egg deposition, stage 25; ZFMK 92353, deceased 29 December 2010, 24 days after egg deposition, stage 25; ZFMK 92354, deceased 26 December 2011, from second clutch 10 days after egg deposition, stages 22-23).

In addition, one deceased froglet (ZFMK 92350, from the first clutch; deceased 26 April 2011 at day 142, stage 46) and three malformed larvae (ZFMK 92955, deceased 22 December 2010, 17 days after egg deposition) were likewise fixed and preserved. Each preserved tadpole was used for closer character state examination and larval stage determination under a Leica binocular microscope.

After arrival, all adults were immediately photographed in dorsal and ventral views to examine whether individuals could be recognized using their distinctive color patterns. Egg clutches and larvae were photographed daily for documentation of their development. For assignment of developmental stages following Gosner (1960), as reproduced in Altig and McDiarmid (1999), several larvae were temporarily placed in glass vessels and photographed in dorsal, lateral, and ventral views. All photographs were taken with a digital camera (OLYMPUS E-600, DG MACRO 105 mm 1:2:8 object lens, SIGMA).

Abbreviations used are as follows: GH - total hardness, KH - carbonate hardness of water; pH - pH value of water; SVL - snout-vent length; TL - total length of tadpole. Terminology of larval morphology followed Altig and McDiarmid (1999).

Captive management of adults

After six weeks of quarantine, during which specimens were tested and found to be negative for the amphibian chytrid fungus (among other treatments), adult males were permanently maintained at Cologne Zoo in three groups consisting of 12 to 15 individuals in terraria measuring L100 × W60 × H60 cm. The five females were kept together in a terrarium measuring L60 × W45 × H40 cm, as in their natural environment, males and females occupy separate habitats throughout most of the year.

In the native environment, males stay in the vicinity of streams for longer periods or permanently (by implication, Kok 2000; Lötters et al. 2011a), while females have only been found inside the forest at least 25 m away from the closest stream. Females might appear at streams only shortly before mating. Back and side panels of the terrarium were pasted up with structure rear panels (Juwel) for providing a naturally looking environment. In male terraria, floor drains were installed and an artificial stream was constructed, which measured between 15 and 20 cm in width.

The stream was separated from the terrestrial part of the terrarium using 12 cm high glass strips pasted in with silicone. Different elevation levels were created using plastic light grid pieces, which were covered with one cm foam plastic and afterwards set in concrete. In order to provide easy access between land and water parts, as well as to form elevated "calling spots," several stones were placed in the stream before the concrete dried. Subsequently, smaller pebbles were brought in for a more naturalistic arrangement. To be able to reach the tubes of the filtration system and for cleaning, parts of the light grids were not set in concrete but only covered with pebbles. The total water depth in the terrarium was about 10 cm but the maximum depth accessible for the toads (measured from the concrete coat) was about three cm (Fig. 1 A-D).

An Eheim external filtration system (type: 2224, 50 Watt) with a capacity of 700 l/h was attached to the artificial stream. The water parameters were: pH = 7.12, GH = 6, KH = 3, conductivity = 280 µS, temperature = 22-24 °C. These parameters differ in some respects from those measured in the wild in *A. flavescens* stream habitats in French Guiana (Kaw, 7 July 1979: pH = 5, temperature = 25.5-26.0 °C (Lescure 1981); Noragues, 6 February 2010: pH = 6.5, GH < 1, KH < 1, temperature = 25 °C [P. Werner, data not shown]).

The terrestrial substrate in the terraria consisted of leaf litter, covered with forest moss in order to avoid pollution of the streams by ground substrate. A variety of plants (swamp grasses, small sized *Anthurium* sp. and *Spathiphyllum* sp.) completed the terrarium structuring.

Illumination was provided via T5 fluorescent tubes (males: Osram FQ, 865 Lumilux daylight: single-flame 36 Watt, females: dual-flame 24 Watt). The photoperiod lasted between nine and 12 hours; in addition, three room windows allowed for natural light and fluctuation of day lengths.

Daily average temperatures in the terraria measured between 24 and 27 °C throughout the year; the relative humidity ranged between 60 and 100%. In the beginning, terraria with males were fogged several times a day with a humidifier (Lucky Reptile SuperFog). After one year, all terraria were only sprayed once a day with a manual

pump sprayer. In October 2010, a rain system (Namiba's Tropical Rainsystem) with a coarse nozzle insert (Gloria) was installed to amplify the former manual irrigation. The rain system was run five or six times a day for 10 to 20 minutes; about 10 liters of water per 15 minutes were sprayed. At night, no irrigation took place.

Terrarium for egg deposition

Females were transferred to one of the male terraria, which soon led to the first egg deposition within the stream bed (see Results). For better observation, another, completely water-flooded terrarium with rocks breaking through the surface (L60 × W60 × H55 cm) was prepared, intended for subsequent concerted separation of couples for mating. Here, a second egg deposition took place (see Results). A connected adjacent aquarium, equipped with three foam mats and with a capacity of 90 liters (L60 × W60 × H25 cm) served as an external filter. In addition, a constant drop-wise fresh water supply was attached. The water temperature was maintained at about 24 °C by the use of a filter heater.

Plastic light grids were laid out over top of the filtration tubes in order to achieve a maximal water depth of six cm at a water volume of about 36 liters and to hide the filtration system tubes. The light grids were covered with filter fleece, a thin layer of river sand (particle size: 0.2 mm) and several pebbles; the edges of the fleece were sealed with aquarium silicone to prevent the tadpoles from escaping below the ground cover. In the back part of the terrarium, a small artificial shore zone was constructed by layering pebbles and moss. The same type of rain system as used for the male terraria was installed for irrigation. The rain system was run four times a day for 15-30 minutes; during night time, no irrigation took place.

Captive management of larvae

The larvae of the first clutch were left in the artificial stream within the terrarium of the adult males. For maintaining constant water parameters, fresh water was supplied (ca. one drop per second), the first five days for three hours a day and later, constantly. The last surviving tadpole was later transferred into a small gauze aquarium (L16 × W10 × H10 cm), which was suspended into a larger aquarium (L119 × W43 × H30 cm) with the following water parameters: pH = 7.12, GH = 1, KH = 1 or 2, conductivity = 206 µS, temperature = 22.8 °C. An external filter with a capacity of 500 l/h and a universal water pump (Eheim, 600 l/h) was attached. Illumination was provided by a T5 fluorescent tube (Osram FQ, 865 Lumilux daylight: single-flame 54 Watt), which was mounted 70 cm above the water surface. To allow the metamorphosing froglet to leave the water, a ramp of pebbles was placed in one corner of the gauze aquarium.

The larvae of the second clutch remained in the tank that was erected for egg deposition, but in contrast to the first clutch, adult individuals were not housed in the same tank.

Nutrition

Adults were fed two or three times a week during their activity time (daytime); the food consisted of small invertebrates, including fruit flies (*Drosophila* sp.), small house crickets (*Acheta domestica*), and springtails (Collembola). All insects were nourished with high quality food and dusted with mineral and vitamin supplements (Korvimin ZVT + Reptil/Calcamineral) before being fed.

Tadpoles were fed with algae growing on the stones in the artificial streams. In addition, different sorts of fish food (*Spirulina*-tabs, *Spirulina*-powder, Sera-flora, algae-chips) were offered. The fish food was pulverized, mixed with water, applied to flat stones, and inserted into the stream bed after drying.

Results

Pre-mating observations and mating

Throughout the year, males showed calling activity after the daily spraying of the terrarium (Fig. 2 A, B). From the end of September until the end of March or beginning of April the calls occurred more frequently than during the rest of the year, and also occurred beyond the irrigation periods, mostly in the morning. Usually the male that was thought to be the dominant individual in the group, started the calling activities. Individuals could be identified by their characteristic back patterns.

In March 2010, two females each were introduced into two male groups. Three of the females were observed in amplexus after being introduced. The fourth female averted all mating attempts of the males and was removed from the terrarium after four weeks. The axillary amplexus lasted for about five weeks (Fig. 2 C) and the involved males did not appear to feed during this time. After the couples had split up without egg deposition, the three females were removed from the male terraria. Two further trials in May and June 2010 also led to amplexus but without oviposition.

Afterwards, a dry season with reduced water level and minimal spraying was induced. The males discontinued calling and reduced their food intake and their daytime activity by remaining stationary on elevated leaves or under the moss pads. Their legs were often held tightly against their bodies. After three months of dry season (July until September), at the beginning of October 2010 (simulating the small rainy season in the species' natural habitat), a wet season with intensified manual spraying and a higher water level was initiated.

In mid October, one female each was introduced to a male group (with all individuals coming from the second group, received in December 2008). After about three weeks, amplexus took place with both females. From 29 November 2010, the previously introduced rain system was used to amplify the irrigation. During and after the irrigation, all the males were highly active, showing calling activity and preferring to be exposed to the rain, while the couples in amplexus searched for hiding places. At 5-10 minutes after the irrigation, the couples came out and often stayed within the stream. The solitary males frequently importuned the couples in amplexus; one time a male was observed pushing a couple under water for about five minutes.

On 2 December 2010, no irrigation was effected; the next day, the rain system was only run two times, once for five and once for 10 minutes. On 4 and 5 December, again no irrigation was induced. The first oviposition took place during the night from 5 to 6 December, shortly after the reduction of the intensive irrigation. About six weeks later, in the night from 16 to 17 January 2011, a second oviposition occurred, but this time not in the males' terrarium but at the terrarium especially prepared for egg deposition. A few days before, on the 6 January,

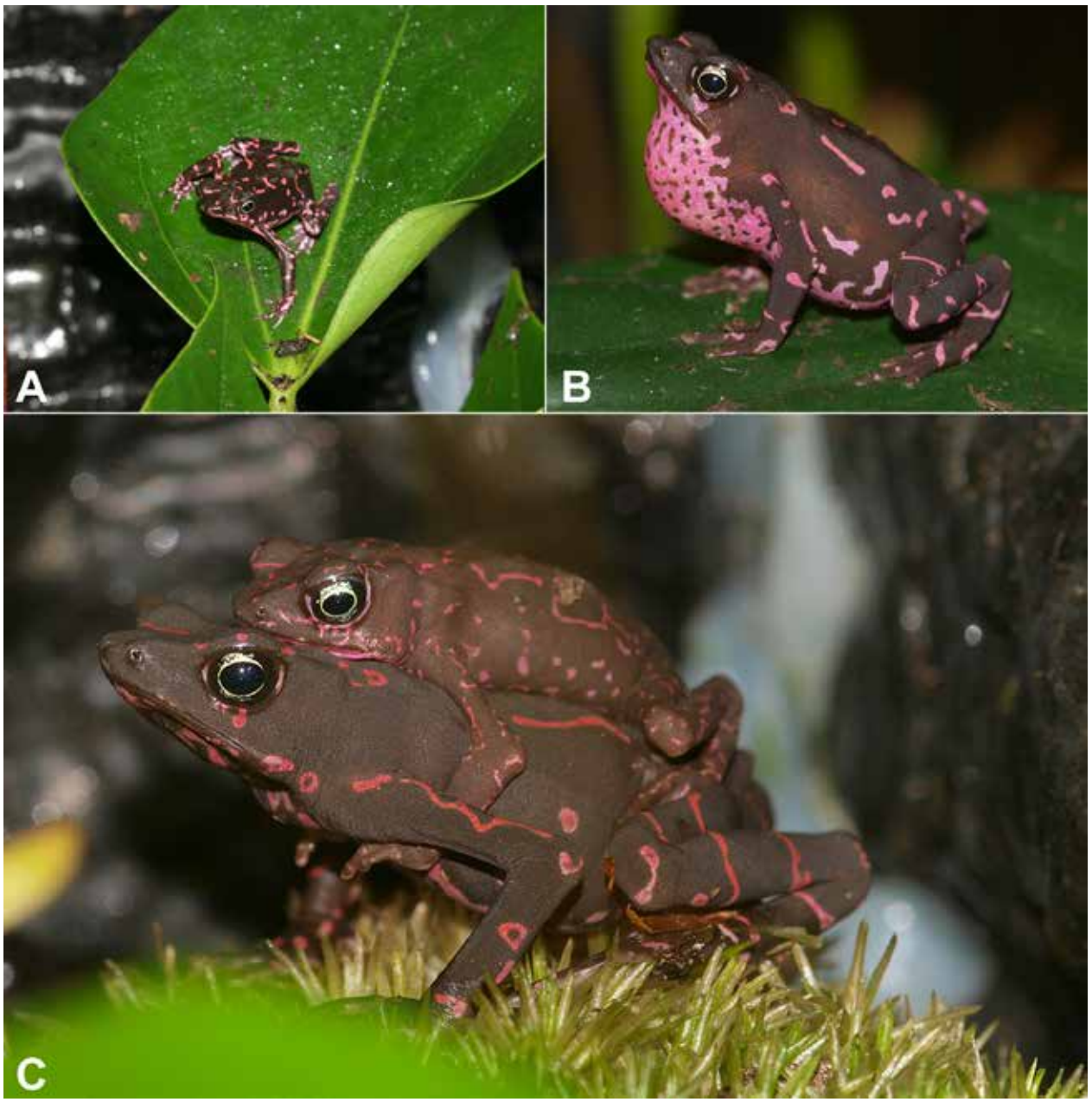

Figure 2. *Atelopus flavescens* at the amphibian breeding unit at the Cologne Zoo: (A) adult male, (B) calling male, and (C) couple in amplexus. *Photograph* (A) (B) *by T. Ziegler and* (C) *by D. Karbe.*

four males had been placed in this terrarium, joined by a female from 10 January onwards. At that time the irrigation system was turned on constantly for 30 minutes daily. Amplexus took place one hour after the female was introduced. The irrigation frequency (see Material and methods) remained unchanged until the oviposition event.

Clutch deposition and description

The first deposited egg clutch (December 2010) consisted of more than 500 eggs, which were arranged in single strings, partly branched (i.e., peripheral rami), and affixed about two cm under the water surface to stones or filamentous algae. The cream-colored eggs (ca. one mm in diameter) were surrounded by a thin membrane and a gelatinous capsule (total diameter ca. three mm) (see Table 1, Fig. 3 A, B). On the third and fourth day after egg deposition, a consistent clockwise rotation of the eggs could be observed; on the fifth day the rotation of the eggs changed direction and started moving counterclockwise. The smallest egg-string (containing 27 eggs) was found to be unfertilized on the fourth day after egg deposition while the other eggs showed discernible development (Fig. 3 C, D).

In contrast to the first egg deposition, the second egg deposition, which took place during the night from 16 to 17 January 2011, occurred under the hollow of a large stone. Before egg-laying, the couple had shoved aside smaller pebbles from the deposition place. The clutch consisted of more than 400 eggs of about the same size as in the first clutch, and of which ca. 10% were unfertilized.

Two deceased adult females contained large yellowish-orange eggs: ZFMK 92947 (SVL 33.6 mm) had eggs with 1.2 mm maximum diameter; ZFMK 92948 (SVL 30.2 mm) had eggs with 1.3 mm maximum diameter.

Larval development and stages

Hatching of tadpoles from the first clutch started seven days after egg deposition (12 December 2010). All larvae hatched during the night and were found next to the eggs the next morning where they remained for the following days; first movements of the tails could be noticed on the day after the hatch (stage 20). The larvae had a total

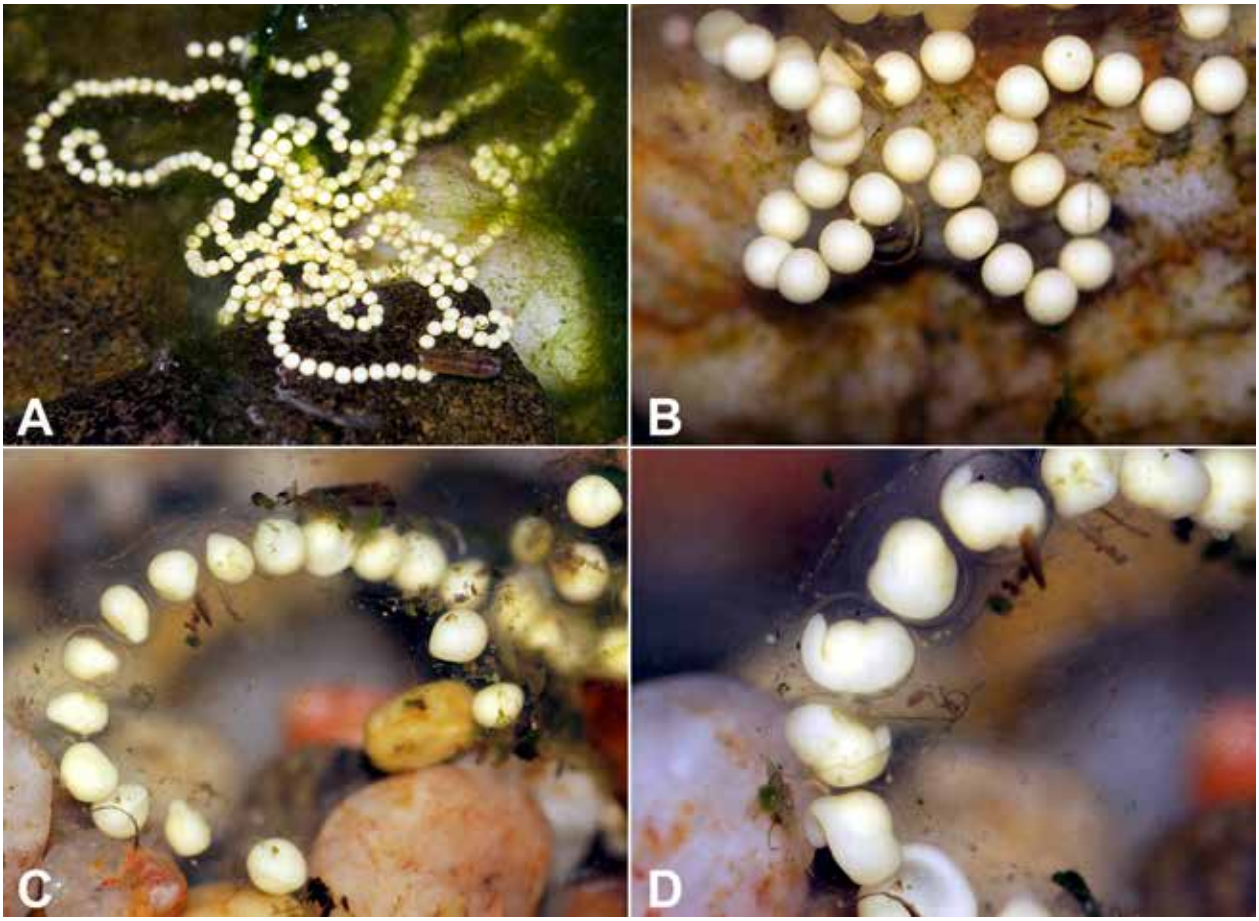

Figure 3. First clutch of *Atelopus flavescens* at the amphibian breeding unit at the Cologne Zoo: (A) freshly deposited spawn under water surface on stones or filamentous algae (5 to 6 December 2010), (B) cream-colored eggs one day after deposition (6 December 2010), (C) developing embryos at Gosner stage < 18 (9 December 2010), (D) embryos at stage 19 (10 December 2010). *Photographs by D. Karbe.*

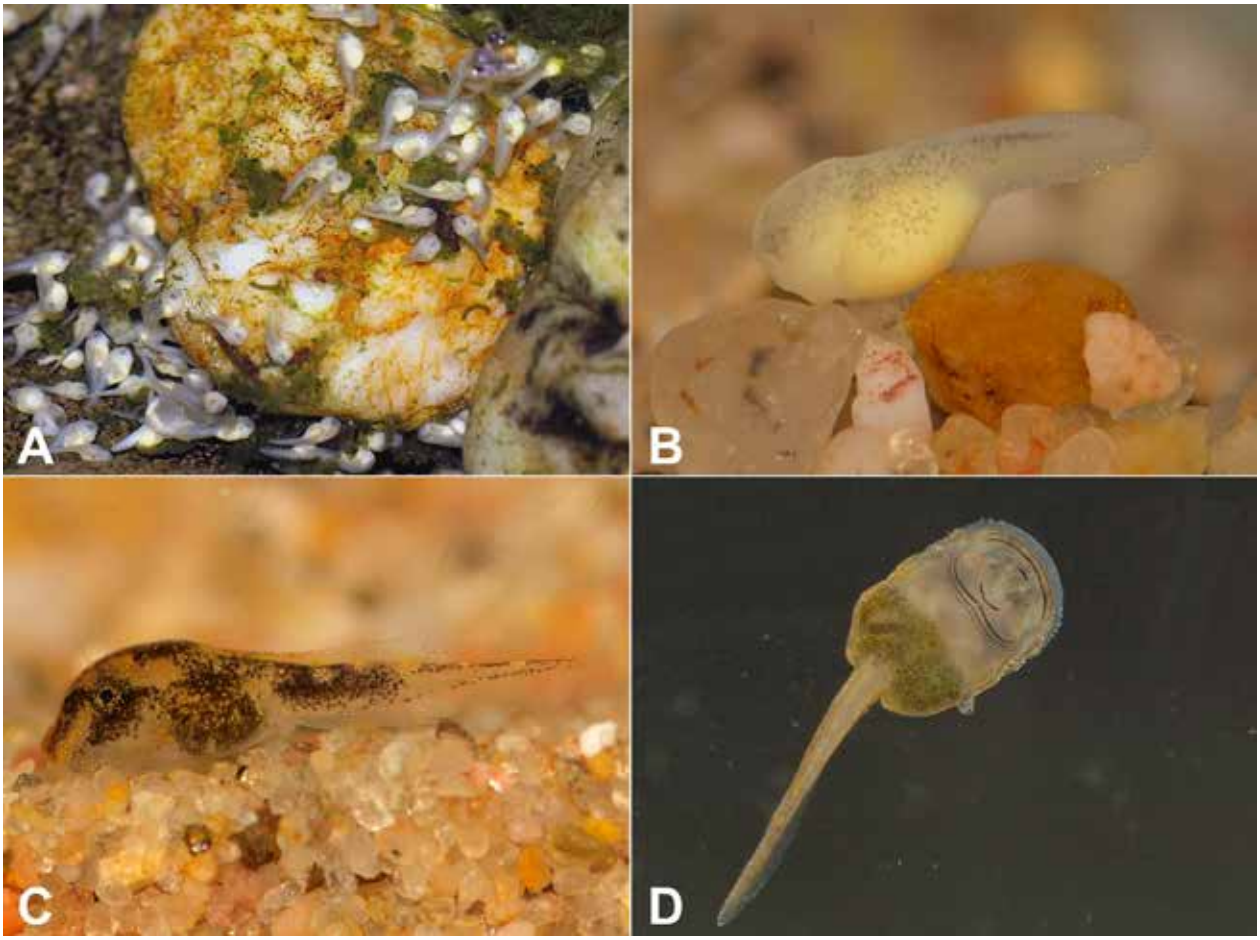

Figure 4. Hatched larvae of *Atelopus flavescens* (from first egg deposition): (A) - (B) hatchlings at Gosner stage 20 (13 December 2010), (C) lateral view of tadpole at stages 24-25 (27 December 2010, 22 days after egg deposition), (D) ventral view of tadpole at stage 25 (3 January 2011, 29 days after egg deposition). *Photographs by D. Karbe.*

length of 3.9 mm and a tail length of 1.9 mm. We noticed dark pigmentation in the form of irregular blotches, reaching from the lateral and dorsal sides to the tail region. The ventral side lacked pigmentation. The prospective eye region was already visible at this stage (Fig. 4 A, B). Ten days after egg deposition (stage 21), the abdominal suctorial disc was discernible. The nostrils were indicated by two white spots, the developing eyes by two black spots. The lateral and dorsal blotches darkened and expanded to the ventral side. On day 14 (stages 21-23), the first larvae were seen swimming. One day later, most of the larvae were well distributed over the available space; they covered short distances swimming and adhered themselves to the substrate with their abdominal suctorial disc, which now covered three fourths of the ventral side. The yolk reservoir was completely absorbed and the oral disc was not completely developed. Sixteen days after egg deposition first feeding was observed (stage 24-25, see Fig. 4 C, D). Tadpoles were able to stay adhered while moving and feeding, classifying them as belonging to the gastromyzophorous type of rheophilic anuran larvae (Altig and McDiarmid 1999).

Tadpoles fed on algae growing on the stones in the artificial streams. However, we could not confirm uptake of the pulverized and mixed fish food that was provided. The dark pigmentation increased forming connected blotches. In addition, several golden spots showed up at the dorsal body side. In some of the larvae, the eyes were well discernible and the vent tube could be distinguished for the first time. The vent tube, which measured about 0.1 mm at this time, grew longer during development and showed a golden spotted coloration from day 23. Depending on the lighting, the heart was visible under the skin surface.

Twenty-two days after egg deposition, first excretion of feces could be detected. Twenty-five days after egg deposition, a tadpole at stage 25 was carefully inspected under a binocular microscope. Here, papillae at the edges of the oral disc, which covered more than two thirds of the ventral side at this time, were discernible, as well as the tooth rows in the oral disc (labial tooth row formula was 2/3). The body surface was covered with a large number of golden spots; the dark ventral pigmentation had reduced to smaller, isolated blotches. Twenty-six days after egg deposition, intestines were visible. On day

Table 1. Developmental stages of *Atelopus flavescens* bred at the Cologne Zoo from Gosner stage 1 to the completion of metamorphosis including diagnostic features according to Gosner (1960); TL = Total length (mm), labial tooth formula = number of tooth rows per upper/lower labium, SVL = snout-vent length (mm); water temperature = 22-24 °C; (1) = larger tadpole, (2) = smaller tadpole, as explained in text.

Age (days)	Gosner stage	Diagnostic features	
1	**1-12**	egg clutch arranged in branched strings; eggs cream-colored; diameter of single egg without transparent jelly capsule about 1 mm	**Embryonic stages**
2-5	**13-19**	embryos assume larval shape with head region set off from tail; yolk reservoir present; larvae uniform yellowish	
7-8	**20**	larvae hatched; elongation of body and tail; development of recognizable head; formation of greyish pigmentation pattern begins on upper region of head, body and tail; tail fins become transparent	**Hatchlings**
10-15	**21-23**	free-swimming larvae: tail longer than body; body ovoid in dorsal view, laterally depressed; increase of pigmentation on body and tail; eye region begins to develop; nares present; spiracle sinistral, laterally situated; oral disc differentiation begins; abdominal suctorial disc extending from posterior labium until half of body; vent tube present; yolk reservoir absorbed on day 15	
16-43	**24-25**	feeding tadpoles: TL > 5.0 mm: golden blotches on body and tail appear; eyes clearly discernable; oral apparatus completely developed on day 22: upper and lower beak slightly keratinized to distal edge, labial teeth present (labial tooth row formula 2/3), upper labium with marginal papillae; abdominal suctorial disc rounded, extending from posterior labium for more than half the body length; elongation of spiracle; intestinal coils visible through integument > day 26, stage 25	
46	**(1) 26**	(1) TL > 7.0 mm; appearance of hind limb buds in larger tadpole	**Larvae – Metamorphosis**
65	**(2) 26**	(2) TL > 7.0 mm; appearance of hind limb buds in smaller tadpole	
75-79	**(1) 28**	(1) TL > 10.0 mm; length of hind limbs ≥ basal width	
83	**(1) 30** **(2) 27**	(1) length of hind limbs = two times basal width; appearance of pigments on hind limbs; (2) length of hind limbs ≥ one half basal width	
86	**(1) 31**	(1) ongoing developing of limb buds: foot paddle shaped	
90-95	**(1) 33-34**	(1) development and differentiation of toe 2-4	
97-101	**(1) 36-37** **(2) 28-29**	(1) development and differentiation of toe 1-2, begin of toe separation; pigmentation of hind limbs darkens; forelimbs visible through integument > day 101; atrophy of vent tube; (2) length of hind limbs ≥ one half basal width	
103-106	**(1) 37-41;**	(1) mouthparts and abdominal suctorial disc atrophy; spiracle still present; changes of metamorphosis begin; disappearing of tadpole on day 112	
109	**(2) 34**	(2) toes development	
119-122	**(2) 36-37**	(2) TL > 13.0 mm; growing and separation of toes (toes completely separated on day 122); forelimbs visible through integument	
129-130	**(2) 40-41**	(2) changes of metamorphosis begin: mouthparts, abdominal suctorial disc and spiracle atrophy; vent tube gone; tail atrophy begins; forelimbs pigmented, increased in length	
131	**(2) 42**	(2) forelimbs emerged; mouth anterior to nostril, tail mostly reduced	
133-134	**(2) 43-44**	(2) mouth between nostril and eye; tail greatly reduced	
139-140	**(2) > 46**	(2) SVL 6.0 mm; tail resorbed; forelimbs malformed	

30, we noticed a large decrease in the number of larvae in the stream, but no dead larvae were found.

On day 43 after egg deposition, only two larvae were detectable in the stream. Both were in different developmental stages and later died at different stages. In the following, we first describe the development of the larger larva from day 43 onwards (see Table 1, Fig. 6), and subsequently the development of the smaller larva.

On day 46 after egg deposition, the larger larva began to develop hind limb buds (stage 26). After 75 days (stage 28), this larva measured 10 mm total length (TL). The hind limb buds were clearly visible at this time (Fig. 5 A). On day 83 (stage 30) dark pigmentation had developed on the hind limb buds. These were followed by golden spots, which appeared at day 89, and a rust brown coloration appearing four days later. Development of toes began at day 90. Five days later (stages 33-34), the coloration of the spots on the body surface partly turned from golden into a rusty brown. On day 97 (stages 36-37), separation of toes started. After 101 days, developing forelimbs were visible under the skin surface. From day 105 (> stage 39), hind limbs were actively used to support locomotion and from day 112 on, the development of this tadpole could not be documented anymore as it disappeared (and apparently died).

Sixty-five days after egg deposition, the smaller larva began to develop hind limb buds (stage 26, a stage which had been reached by the aforementioned larger larva already 19 days before, i.e., 46 days after egg deposition; see Table 1). On day 75, this tadpole measured seven mm TL, and on day 100, slightly pigmented hind limb buds were clearly visible without the use of a hand loupe

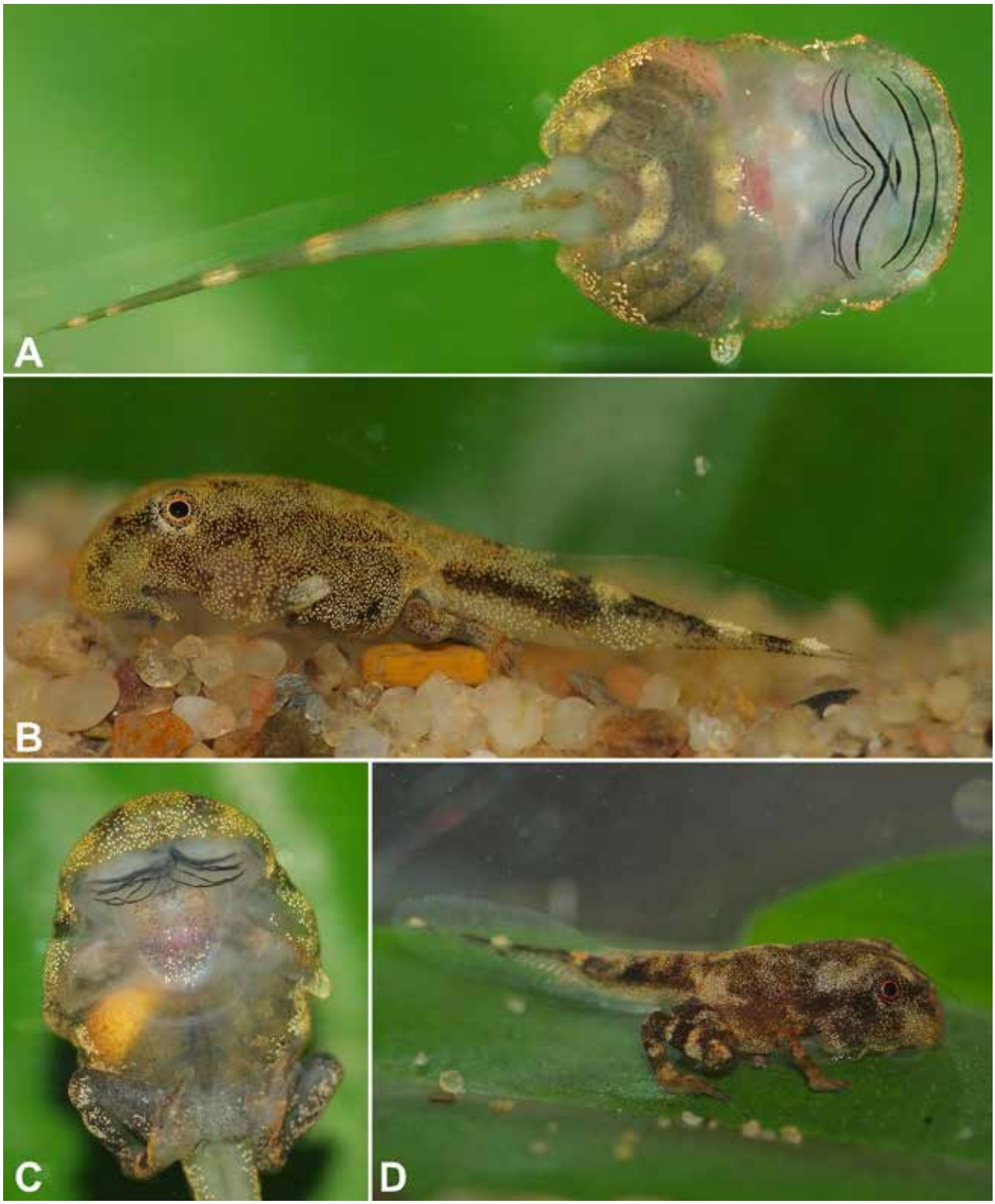

Figure 5. Tadpoles of *Atelopus flavescens*: (A) ventral view of larva at Gosner stage 28 (22 February 2011, 79 days after egg deposition; from first clutch; larger larva), (B) lateral view of tadpole at stages 34-36 (22 April 2011, 96 days after egg deposition; from second clutch), (C) ventral view of tadpole at stage 41 (26 April 2011, 100 days after egg deposition; from second clutch), (D) tadpole at stage 42 (15 April 2011, 131 days after egg deposition; smaller larva). *Photographs by D. Karbe.*

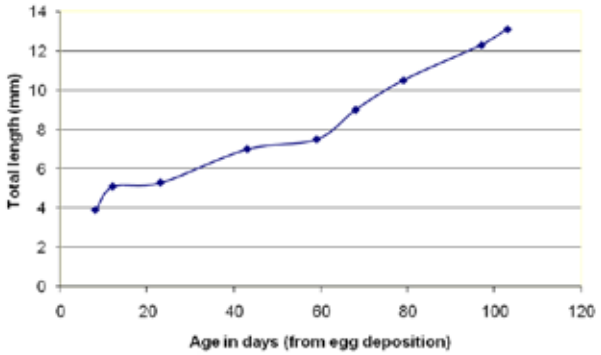

Figure 6. Total length (mm) of larger tadpole of *Atelopus flavescens* from first clutch in relation to age in days; water temperature 22-24 °C.

(stages 28-29). After 119 days (stages 36-37), this larva had reached TL 13 mm. Hind limbs, which were tightly attached to the tail at this stage, measured about 2.5 mm, and were rusty-brown in coloration. On day 122 the legs, with all toes being separated, could be moved and the fore limbs were already discernible.Two days later, the larva was transferred into a separate aquarium (see Material and methods). In order to provide food resources, some stones overgrown with algae were added. After 129 days (stages 40-41), the fore limbs were pigmented and well recognizable under the skin surface; the intestine was less distinct. At that time the tadpole remained near the stream substrate more frequently. The dorsal pigmentation gradually changed: the bigger blotches were still dark, while the coloration of the smaller spots turned

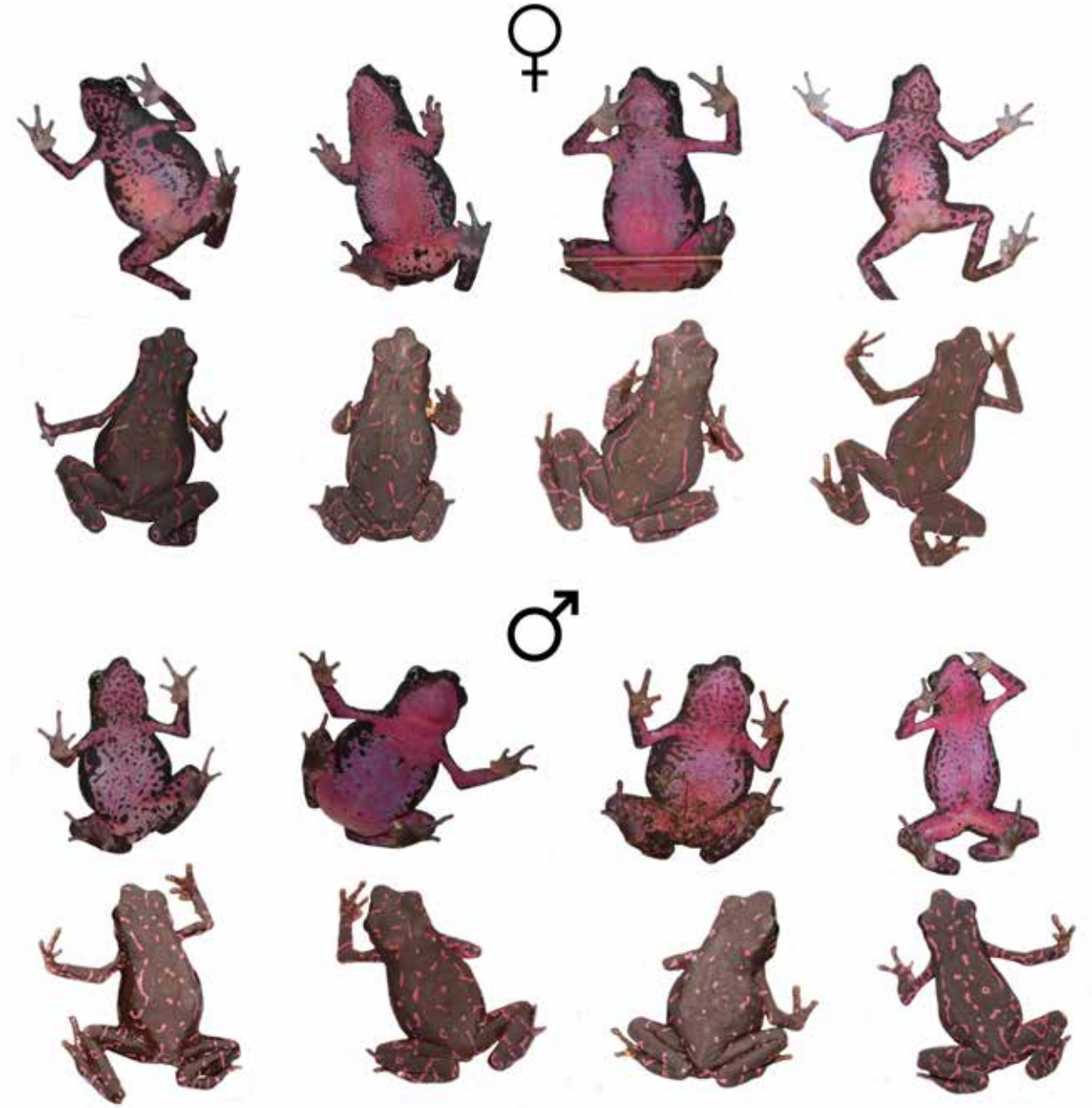

Figure 7. Color patterns of *Atelopus flavescens* at the amphibian breeding unit at the Cologne Zoo: Four females (above) and males (below) in ventral and dorsal views. *Photographs by D. Karbe.*

from golden into a yellowish taupe. The ventral side was partly transparent; the inner surface of the legs was dark pigmented, with several black spots. The soles of the feet were colored rusty brown. 131 days after egg deposition (stage 42, Fig. 5 D), the fore limbs started to protrude, but were malformed (the so-called spindly leg syndrome). One stuck out at a 90 degree angle and the other was angular and could not be stretched. The abdominal suctorial disc as well as the oral disc were reduced and had completely disappeared three days later; the tail also started to resorb.

At day 137 the froglet, which measured 6.0 mm SVL, tried to move out of the water and onto the land for the first time, but it could not stand erect due to the fore limb malformations. Two days later, the tail was completely resorbed (stage 46). Subsequently, no intake of the provided food (spring tails) could be observed. The froglet died at day 142 after egg deposition. Its color had not changed further by that time, i.e., the purple coloration of adults had not appeared by that time.

The development of larvae from the second egg deposition is summarized in Table 2. In this second reproduction phase, larval development could be observed until day 100 (stage 41) before the last tadpoles disappeared.

Individual recognition based on color pattern

By taking photographs of every adult individual and comparing them regularly, we observed that specimens maintained their individual color pattern. The dorsal pattern differed in number, arrangement, size, and shape of the pink-colored spots, stripes, and circles on a dark-brown background. The ventral pattern varied in the arrangement of irregular dark-brown to black blotches on a purple background (Fig.7).

Table 1. Comparison of developmental time (age in days) including stages according to Gosner (1960) between first reproduction phase (5 to 6 December 2010, water temperature 22-24 °C) and second reproduction phase (17 January 2011, water temperature 24 °C) of *Atelopus flavescens* bred at the Cologne Zoo.

Gosner stage	Age in days (first breeding)	Age in days (second breeding)
1-12	1	1
13-19	2-5	2-4
20-21	7-8	5-6
21-23	10-15	7-11
24-25	16-43	16-38
26	46 and 65	39
27-28	–	41
28-29	< 101	51-62
30-32	–	80
32-33	–	83
34-39	–	87-96 (Fig. 5 B)
41	> 106-130	100 (Fig. 5 C)

We also observed that the individual patterns did not change with age. Based on the comparison of photographs taken over two years we were able to determine that the pattern remained the same, but the dorsal coloration changed slightly from dark brown to dark grey or almost black, while the coloration of the spots, stripes, and circles turned from pink to yellowish-white over time (Fig. 8). We also observed a potential slight sexual dimorphism. Compared with the gular region of the females, the throat region of the males appeared to be more intensively purple-colored.

Figure 8. Individual recognition of a male *Atelopus flavescens* based on color pattern, but note the change in color (photographs taken 12 July 2009 and 31 July 2011, respectively). *Photographs by D. Karbe.*

Discussion

During the husbandry and breeding of *A. flavescens* at Cologne Zoo we identified a several months long dry period as a trigger for reproduction. This was done to mimic the dry season in the natural habitat, and was followed by a period of intensive irrigation. In the wild, *A. flavescens* reproduce with the beginning of rains (i.e., October/November to January; April/May to July; Lescure 1981; Boistel et al. 2005; Lötters et al. 2011a). As a reaction to the artificially induced drier period, the toads showed reduced activity, and we often observed them with their limbs closely pressed to their bodies. This posture was probably a reaction to the low humidity because the reduction of the body surface area minimizes water loss from evaporation.

There is little known about the reproductive phases in *Atelopus* species in the wild but the break of the short rainy season is apparently favored for breeding by several species. This may be explained by the fact that Harlequin toads breed in streams and that generally the risk of being washed away by the current is limited when rains are not too heavy (Lynch 1986; Lötters 1996; Karraker et al. 2006). This may be especially important in montane habitats. In lowland populations, like those of *A. flavescens*, it seems that all kinds of rains (with previous drier phases) can trigger species to start reproductive behavior as breeding apparently also takes place during the long rainy season (Boistel et al. 2005). As in the related Guianan *A. hoogmoedi* (Luger et al. 2009), *A. flavescens* males remain at streams in high density for most or all of the year, while females are found at larger distances from streams (Lötters et al. 2011a). Keeping the sexes separate from each other and introducing females to male groups may have triggered the toads to breed.

After increased irrigation, couples in amplexus came out of their hiding places and remained within the stream for some time. Because egg deposition did not take place immediately, and because we also observed the same couples in amplexus in different parts of the stream, we thought that the *A. flavescens* might have been searching for optimum oviposition places. Karraker et al. (2006) reported that in the Panamanian *A. zeteki*, oviposition sites were apparently carefully chosen. Prolonged amplexus, even for weeks, is common in *Atelopus* species and has been reported in wild populations of many species (Lötters 1996).

Whereas the first oviposition was done in the open water, the second oviposition took place below a larger stone. Such hiding places were missing in the stream environment within the first reproduction. Perhaps, shelter within the water body should be offered during captive management. Interestingly, Poole (2006) pointed out that *A. zeteki* eggs may show some light sensitivity. This needs further investigation, especially since Lescure (1981) found an *A. flavescens* clutch below, and Boistel et al. (2005) found one on top, of a rock in the wild. However, other *Atelopus* species apparently perform both oviposition on top of or below submerged rocks (Karraker et al. 2006).

A clutch of *A. flavescens* reported by Boistel et al. (2005) contained fewer eggs (ca. 250) than those obtained in captivity by us, but the clutch geometry was similar with several peripheral rami. These apparently function to stabilize eggs in the stream current and have also been reported in *A. subornatus* from Colombia (Lynch 1986), while in *A. zeteki*, Karraker et al. (2006) described egg strings to be "wrapped back up on themselves creating two or more layers." Clutch size appears to be quite variable within and among *Atelopus* species, as summarized by these aforementioned authors.

Eggs known from other *Atelopus* species are similar in color but most of them are larger than those described here (Karraker et al. 2006) including those of *A. flavescens*. Lescure (1981) referred to an ovum diameter of > 1.5 mm versus ca. one mm only.

Larval stages of several *Atelopus* species have been described (e.g., Lötters 1996). Tadpoles obtained under captive conditions are consistent with those of *A. flavescens* collected in the wild (Lescure 1981; Boistel et al. 2005). In contrast, little information is available on larval development in Harlequin toads. Like in other species (summarized by Karraker et al. 2006), *A. flavescens* embryonic development is short (for comparisons, *A. cruciger* 3-4 days at 20 °C; *A. varius* six days at unknown temperature; *A. zeteki* 7-11 days at 22 °C) and hatchlings measure few millimeters only. Similar to observations by Karraker et al. (2006) on freshly hatched *A. zeteki*, the abdominal suctorial disc developed several days after hatching in *A. flavescens* (i.e., Gosner stage 21) allowing them to adhere to the substrate.

Regarding further larval change until metamorphosis, to the best of our knowledge, there is no information on other Harlequin toads for comparison. Only Lindquist and Hetherington (1998) described metamorphs of *A. zeteki* in Gosner stage 46 and older. They were larger (8.4-17.1 mm SVL) than the single specimen obtained by us. Similar to *A. zeteki,* freshly metamorphosed *A. flavescens* apparently have camouflage coloration rather than any brilliant colors.

In comparing larval development between the first and the second reproductive events, we observed slightly faster development (1-2 days) of larvae from the second egg deposition. This might be due to the more constant and somewhat higher water temperatures during the second reproductive event (24 °C) compared to the water temperatures of the first (22-24 °C).

In both reproductive events a noticeably large number of larvae disappeared. Similar observations were made by Heselhaus (1994) on *A. zeteki* (under the name *A. glyphus*) and Haas (1995) on *A. pulcher*. We cannot explain this. Because in our first reproductive event the adult males remained in the terrarium with the larvae, it cannot be ruled out that adults preyed on the tadpoles (see also

Poole 2006). However, such behavior was not observed during the daytime, and we consider cannibalism can be largely ruled out as *Atelopus* species are known as microphagous anurans feeding on land and preying on ants, mites, and termites (e.g., Lötters 1996).

In the terrarium for egg deposition, where larvae from the second reproductive event were maintained separate from adults, a few dead larvae could be found in the water (already eroded by snails). However, dead larvae never were found in the filtration system, which then would have been an indication that weak larvae might have been absorbed by the filtration system. Here, a possible reason for the abrupt decrease in numbers of larvae, assuming that the missing larvae had died, could be an insufficient oxygen concentration in the water (e.g., due to a shortage of current/air inclusion).

Dissolved oxygen in water is critical to larval development in *Atelopus*, including lowland species. Lescure (1981), Coloma and Lötters (1996) and Lindquist and Hetherington (1998) measured relatively high concentrations in the larval habitats of *A. flavescens*, *A. balios*, and *A. zeteki*, respectively. Lötters (1996) argued that due to their gastromyzophorous diet and occurrence in streams, tadpoles in later stages, when lungs have developed, only receive oxygen from the water through their skin. However, many of the tadpoles in our study disappeared in earlier stages and apparently coped well with oxygen conditions in the terrarium.

Another possibility may involve temperature or water chemistry, as pH, GH, and KH values measured during our efforts to rear *A. flavescens* tadpoles differed somewhat from those taken in a stream where this toad breeds in French Guiana (see above). Temperature was similar to that recorded in the wild, but differed from that measured by Boistel et al. (2005), which was only 20 °C.

Apart from this, changes in water conditions or a lack of food resources could represent possible causes for mortality. An argument for lack of food resources causing mortality could be the observation that the decrease in numbers of larvae always occurred after the development of the intestinal loops. We could observe the grazing of algae, but we never observed larvae feeding on the ground fish food applied to stones, as described by Poole in *A. zeteki* tadpoles (2006). Interestingly, she also mentioned that tadpoles stopped feeding at suboptimal temperatures.

It is also possible that there are particular species of algae occurring in the natural habitat, which would have to be provided to successfully rear the tadpoles. We do not exactly know what Harlequin toad larvae feed on (Lötters 1996). Apart from ingesting visible algae, they may also feed on diatoms or bacteria. The density of these organisms may decrease with higher temperatures. Further research is urgently required to answer these questions.

The cause of the malformed legs in the only froglet can also not be explained at this time. The underdevelopment of the forelimbs (arthrogryposis), which is also known as "matchstick legs" or "spindly leg syndrome" (SLS), is a common malformation in anurans and is manifest in thin and stiff forelegs with underdeveloped musculature. In some cases, one or both forelimbs can be completely missing. Affected froglets do not feed and die of starvation after a short time. Causes of the disease have not yet been determined, though genetic factors as well as environmental factors like water temperature, pH value, or malnourishment of tadpoles or parents have been suggested (Köhler 1996). Regarding the high tadpole loss rate after development of the intestinal tract, we cannot exclude the possibility that our larvae were undernourished, although most studies, which regard the disease as diet-related, suggest that insufficient nutrition of the parents and not of the tadpoles (e.g., Heselhaus 1983; Glaw 1987; Krintler 1988) may play a role. Thus, as a consequence, captive bred amphibians in many cases do not seem to be ecologically and physiologically equivalent to offspring from natural populations in the wild.

Concerning individual recognition based on color pattern, we were able to document that the individual pattern remains constant (even if the color of the pattern may change slightly over time); whether this change in color is due to age or environmental factors such as food deserves further study. Because color patterns remain stable, individual photography can be used as a reliable individual recognition method. The advantage of such a method is that it is non-invasive and applicable in the field to various amphibian species (e.g., Kopp-Hamberger 1998; Beukema 2011). We have successfully used this method in an *A. flavescens* population at Noragues, French Guiana (authors' data not shown). Finally, concerning a potential sexual dimorphism in color pattern, further research is required to confirm our preliminary observations.

Outlook

In summary, the seasonal alternation of dry and wet phases appears to be important for successful reproduction of *A. flavescens*. Another relevant factor for the initiation of reproductive activity may be the initial separation of the sexes. A separate terrarium for egg deposition also seems to be advantageous. However, many unanswered questions regarding the successful rearing of *Atelopus* tadpoles still remain.

We recommend a clearly arranged aquatic part of the terrarium for detecting any decrease in tadpole numbers in time, and the placing of appropriate measures for its prevention such as tadpole relocation. We also recommend removing the tadpoles from the adult terrarium and providing them with adequate water amount, under constant control of water conditions and oxygen-content. To ensure sufficient nutrition, algae cultivation should be started ahead of time.

While there are still aspects related to larval rearing that need to be worked out, Cologne Zoo is the only cooperating institute that has so far succeeded in stimulating oviposition and larval development of *A. flavescens*. This highlights the difficulties faced by conservation breeding programs and the necessity of research to evaluate the optimum conditions for reproduction. It is therefore even more important that as many amphibian keeping institutions as possible engage in such programs and research and then subsequently publish their results, because only those experiences will enable the successful, sustainable, and long-term breeding of amphibians in captivity (see also McGregor Reid and Zippel 2008; Ziegler et al. 2011). Finally, husbandry management must not be regarded separately, but should be ideally combined with field research to achieve optimum basic data for successful *ex situ* conservation breeding (e.g., Luger et al. 2009; Lötters et al. 2011).

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Anna Gawor completed her master thesis at the Faculty of Mathematics and Natural Sciences at the University of Bonn in collaboration with the Cologne Zoo and the Institute of Ecology and Biological Resources in Hanoi, Vietnam in 2011, focusing on the biodiversity of the herpetofauna of the Bai Tu Long National Park. Since 2007, she has been involved in various projects at the Cologne Zoo dealing with tropical batrachology, resulting so far in five scientific publications. Her interests comprise systematics, ecology, and diversity of amphibians, in particular reproduction of anurans, monitoring of larval development as well as larval morphology.

Anna Rauhaus started her career at the Aquarium/Terrarium Department of the Cologne Zoo in May 2011. She finished her apprenticeship as zoo keeper in the year 2010. Her focus of expertise is herpetology and behavioral training.

Detlef Karbe has been employed at the Cologne Zoo since 1974. He has worked for 20 years as a gardener in the Aquarium/Terrarium Department and then continued on as a zoo keeper. His main focus of work is the construction of amphibian facilities and the husbandry and breeding of anurans and salamanders.

Karin van der Straeten has been employed at the Cologne Zoo since 1970. She is a zoo keeper with a focus on amphibians and reptiles. During her career she has successfully bred more than ten species of amphibians. She is head keeper in the Terrarium Department.

Stefan Lötters is an assistant professor at Trier University with focal research on ecology, evolution, and systematics of amphibians from the Amazon and Congo basins. Harlequin toads are among his key groups. Before, he did research postdocs at the universities of Amsterdam and Mainz where he started to engagement in amphibian conservation projects. Stefan has also contributed to the global *IUCN Amphibian Conservation Action Plan*.

Thomas Ziegler has been the curator of the Aquarium/Terrarium Department of the Cologne Zoo since 2003. He completed his herpetological Ph.D. in the year 2000 at the Rhineland Friedrich Wilhelms University Bonn. Thomas so far has conducted herpetological field work in South America (Paraguay) and South East Asia (Vietnam, Laos). Since 1994, he has published 252 papers and books, mainly dealing with herpetodiversity. His main research interests include diversity, systematics, and zoo biology of amphibians, geckos, monitor lizards, snakes, and crocodiles. Since February 2009, he has been an associate professor at the Zoological Institute (Biocentre) of Cologne University.