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SHORT COMMUNICATION

Terrestrial larval development and nitrogen excretion in the afro-tropical pig-nosed frog, *Hemisus marmoratus*

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In general, amphibian larvae dispose of nitrogenous wastes as ammonia and change to urea excretion during metamorphosis (Balinsky 1970). Ammonia excretion, however, poses a problem for tadpoles and embryos that live in a limited volume of fluid because it is toxic in higher concentrations. One solution is urea excretion or ureotelism. In amphibians it occurs in species with terrestrial modes of reproduction when larvae develop within the uterus as in the ovoviviparous *Salamandra salamandra* (Schindelmeiser & Greven 1981), when embryos develop in foam nests as in *Leptodactylus bufonius* (Shoemaker & McClanahan 1973), or when offspring develop in brood pouches as in *Gastrotheca riobambae* (Alcocer *et al*. 1992).

Terrestrial reproduction occurs in the afro-tropical pig-nosed frog *Hemisus marmoratus* (Hemisotidae), a fossorial frog that inhabits seasonally dry savanna habitats (Kaminsky *et al*. 1999, Rödel *et al*. 1995, Van Dijk 1997). Females construct underground nests, typically at the beginning of the rainy season before temporary ponds fill, in which they oviposit and attend eggs and tadpoles. Tadpoles remain confined in the nest until they are released by the female during the following rains (Kaminsky *et al*. 1999). Females guide tadpoles to open water by constructing slides if nests remain above the water level (Kaminsky *et al*. 1999). Extended periods of development within terrestrial nests, in which moisture can be provided only by attending females, suggest that pig-nosed frog tadpoles are ureotelic.

In this study we examine the mode of nitrogen excretion in pig-nosed frog tadpoles. We hypothesized that urea excretion should be the dominant form of nitrogen output. We also monitored larval growth and development in the nest to assess whether larval development is arrested during prolonged tadpole tenure in the nest, as reported for *Leptodactylus fuscus* (Downie 1994), and whether females influence arrest.

Research was carried out in the southern Guinea savanna region of the Comoé National Park in the Ivory Coast (8°45′N, 3°49′W) in 1997 and 1998. Amplectant pairs of frogs were collected at a large temporary pond used for breeding (Grafe *et al*. 2004, Kaminsky *et al*. 2004).

Larval development and nitrogen excretion were examined in tadpoles collected from nests constructed by females in large open plastic tubs ($50 \times 39 \times 27$ cm) that were filled with damp soil collected from the same temporary pond from which amplectant pairs were collected (Kaminsky *et al*. 1999, 2004). Tubs were kept in the shade of the gallery forest under ambient conditions (22–38 °C), the soil moistened periodically, and nests left undisturbed for 1 wk until tadpoles had hatched. The nest ceiling was then carefully removed and replaced by a Petri dish to facilitate taking samples repeatedly and the nest covered with several cm of soil to re-seal it.

Larval growth and development was investigated in eight nests. Each week, up to 9 wk after hatching, we removed 8–10 tadpoles from each nest with forceps or plastic pipettes. Fresh mass of each tadpole was determined to the nearest 2 mg using a portable balance (CT-10, Ohaus). Tadpoles were examined under a dissecting microscope and the developmental stage was classified according to Gosner (1960). We also determined the development of the oral discs. Body length of tadpoles was measured with the help of an ocular micrometer. Nest construction took place on different dates within the study season. Since our study season lasted 9 wk we were not able to monitor all nests over the same number of weeks. To determine dry body mass and lipid reserves, tadpoles

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were anaesthetized in a 0.001% solution of MS-222 Sandoz (Sigma), placed in 70% ethanol and transported to the University of Würzburg for lipid extraction. The alcohol samples containing the tadpoles were transferred to small aluminium pans and dried to constant weight at 70 °C. Dry mass was then determined to the nearest 0.1 mg (AE 160, Mettler). Next we extracted lipids from the samples using petroleum ether as a solvent. Samples were then dried again to constant weight at 70 °C to determine the amount of lipid removed by the solvent.

To examine whether the presence of females influenced the developmental rate of tadpoles, one nest was sampled on three occasions, once a week, with a total of 20 (8, 6 and 6) tadpoles removed. Tadpoles were briefly rinsed, placed in a Petri dish with moist filter paper, buried under a 5 cm layer of soil and their development then followed for 2 d in the female's absence.

Total nitrogen excretion and the relative amounts of urea and ammonia produced by pig-nosed frog tadpoles were determined from six nests 4 wk after tadpoles had hatched. Five tadpoles were removed from each nest, weighed individually and placed in 60 ml of water for 6 h. The water was then sampled for urea and ammonia using test kits available from Merck (Darmstadt, Germany; article numbers 114400 and 114843) and tadpoles then held overnight in an aquarium and fed commercially available fish food (TetraTabiMin). This procedure was repeated over the next 3 d resulting in measurements of nitrogen excretion between 0–6, 24–30, 48–54 and 72–78 h after removal from nests. Nitrogen excretion per gram per day was calculated based on the weight of the group of tadpoles tested and by scaling up values from 6 h to 24 h.

For multisample non-parametric data we used the Kruskal–Wallis and Friedman tests. The Wilcoxon–Wilcox test was used as a *post hoc* test for the Friedman test to examine differences between samples. Descriptive statistics are given as mean ± SD unless stated otherwise.

Tadpoles reached developmental stage 25 and had fully differentiated oral disks 1 wk after hatching. Tadpoles remained at this stage throughout their tenure in the nest. In two nests that were monitored the longest, developmental arrest lasted 56 d until they were released into water. Tadpoles that had been separated from their mother were still in stage 25 after 2 d. In contrast, tadpoles taken directly from their nest and placed in water began to feed within a few minutes and resumed development.

The average number of tadpoles in a nest, shortly after hatching, was 201 ± 26 (n = 10). Mortality in the nest, although not quantified, appeared to be low. Tadpoles from one nest that we released after 5 wk still contained 182 tadpoles. A second nest still had 202 live tadpoles after 9 wk.

Dry mass of tadpoles dropped significantly over time (Figure 1; Kruskal–Wallis test, $H_9 = 25.5$, $P < 0.01$), decreasing a median of 0.36 mg (range, −0.22–0.56) each week over the course of 9 wk.

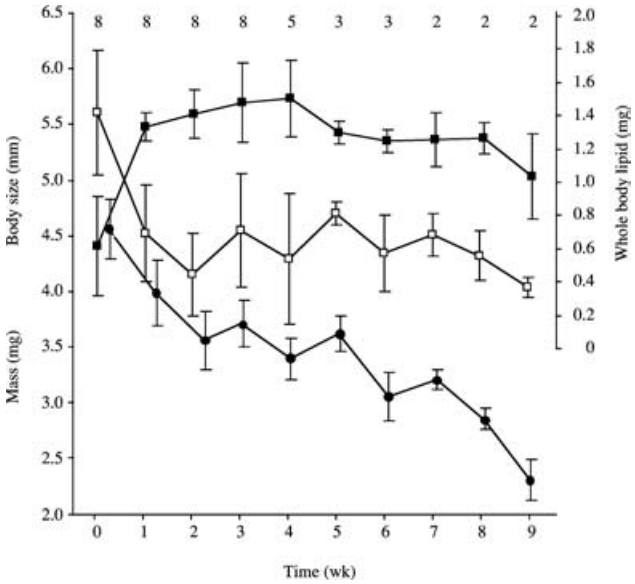

Figure 1. Dry mass (closed circles), body length (closed squares) and whole body lipid content (open squares) of pig-nosed frog tadpoles developing in underground nests. Values show mean ± SD across nests. Numbers above error bars indicate the number of nests sampled.

Body length of tadpoles changed significantly over time (Figure 1; Kruskal–Wallis test, $H_8 = 25.8$, $P < 0.01$). There was a significant increase in body length by an average 1.1 ± 0.5 mm (range, 0.4–1.8) during the first week (Mann–Whitney U-test, $Z = -3.36$, $P < 0.01$, $n_1 = n_2 = 8$). Body length did not change in the following weeks (Kruskal–Wallis test, $H_7 = 9.1$, $P > 0.05$).

Lipid reserves dropped significantly over time (Figure 1; Kruskal–Wallis test, $H_9 = 23.6$, $P < 0.01$). During the first week lipid content of tadpoles decreased significantly by an average 0.7 ± 0.4 mg (range, 0.3–1.3; Mann–Whitney U-test, $Z = -2.94$, $P < 0.01$, $n_1 = n_2 = 8$) whereas no change took place in the following weeks (Kruskal–Wallis test, $H_8 = 7.9$, $P < 0.44$).

Urea excretion accounted for 84.0% of total nitrogen output after larvae were placed into water for the first time. Urea excretion remained the dominant form of nitrogen output throughout the measurement period of 78 h averaging 75.9 ± 6.0% (Figure 2). The total amount of urea excretion dropped significantly over the course of the experiment (Friedman ANOVA, $\chi^2_3 = 8.8$, $P < 0.05$). The drop was significant only between the first (0–6 h) and second (24–30 h) measurement (Wilcoxon–Wilcox, $P < 0.05$). There was no significant change in urea excretion between 24–78 h (Wilcoxon–Wilcox, $P > 0.05$).

Ammonia excretion accounted for 16.0% of total nitrogen excretion after nest removal (0–6 h) and did

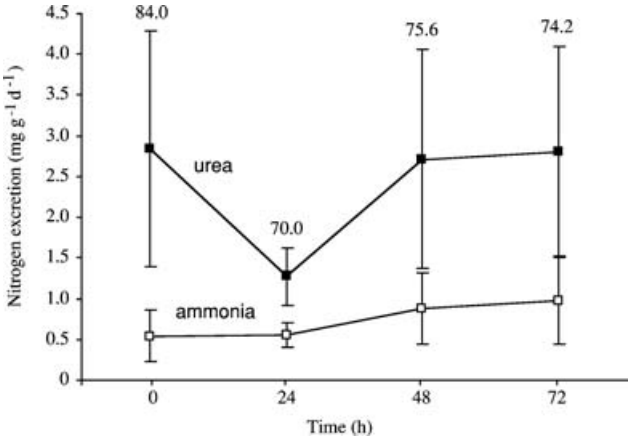

Figure 2. Rates of urea (closed squares) and ammonia excretion (open squares) of pig-nosed frog tadpoles 4 wk after hatching. Values show mean ± SD across nests. Numbers above error bars indicate percentage of nitrogen excreted as urea.

not change significantly throughout the measurement period (Friedman ANOVA, $\chi^2_3 = 6.2, P > 0.05$; Figure 2). Average ammonia excretion over the measurement period was 24.1 ± 6.0% of total nitrogen output.

Four-week-old tadpoles of *Hemisus marmoratus* predominantly excreted urea suggesting that tadpoles are ureotelic while developing in underground nests in which water is not readily available. Urea accounted for an average 76% of total nitrogen excretion over the 78-h measurement period. The initial high value of urea excretion immediately after removal of tadpoles from nests is probably a result of urea voided that had been accumulated during larval development in the nest. Accumulated urea probably serves as an osmolyte to ensure a favourable osmotic gradient between the tadpole and its dry environment.

Ureotelism has also been documented in tadpoles of *L. bufonius*, *S. salamandra* and *G. riobambae* (Alcocer *et al.* 1992, Schindelmeiser & Greven 1981, Shoemaker & McClanahan 1973). The high percentage of urea excreted even after 72 h and after having been fed suggests that pig-nosed frog tadpoles may resemble *G. riobambae* in remaining ureotelic throughout larval development (Alcocer *et al.* 1992). As in *G. riobambae*, the adaptive significance of urea excretion throughout larval life may lie in increased survival during periods of low water levels in drying ephemeral ponds. The switch to ureotelism at early developmental stages well before metamorphosis in *Scaphiopus couchi* is also thought to aid survival under situations of water stress (Jones 1980). In contrast, *L. bufonius* tadpoles show reduced levels of urea excretion after they are transferred to water and fed, with levels rising again only during metamorphosis (Shoemaker & McClanahan 1973).

Prolonged nest tenure of pig-nosed frog tadpoles was also found by Rödel *et al.* (1995) who observed a nest for 66 d. Such long periods of nest tenure, although observed in semi-natural conditions, may not be unusual because occasional early rains in January or February that stimulate breeding activity and nest construction would require tadpoles to wait until heavy rainfall in March or even April fills the ponds.

Larval development was arrested at stage 25 after 1 wk and was documented to last for 56 d in two nests. Tadpoles of several other anuran species also show developmental arrest, but it is not as prolonged as in pig-nosed frogs. For example, *Heleioporus* tadpoles can delay development up to 20 d (Lee 1967) whereas *L. fuscus* tadpoles that develop in foam nests are known to delay up to 30 d (Downie 1994). Tadpoles developed to stage 27 and stage 28 in *L. mystaceus* and *L. fuscus*, respectively with very slow development thereafter (Caldwell & Lopez 1989, Downie 1994). The mechanism responsible for developmental arrest is not known. However, it has been suggested that urea accumulation may slow metabolism in aestivating frogs (Withers & Guppy 1996). Several Australian frogs do not accumulate counteracting solutes during aestivation (Withers & Guppy 1996) although it is a typical response in many animals to offset the negative effects of urea on enzyme activity (Schmidt-Nielsen 1997). Withers & Guppy (1996) suggest that counteracting solutes are not produced because metabolic depression may be an adaptive side-effect of urea accumulation. Under this scenario, urea accumulation in tadpoles would lead to developmental arrest and the reduction of body urea concentrations after entering water would allow development to resume. Our data on tadpole development in pig-nosed frogs are consistent with this view.

There was no indication of maternal effects on developmental arrest in tadpoles sampled from one nest and observed for 2 d, unless females produce substances with long-term influence on tadpoles. Long-term effects by females have not been documented in any amphibian and it seems unlikely because tadpoles resume development when placed in water and because tadpole survival depends critically on gaining a head start on competitors and predators as soon as they enter the pond (Caldwell & Lopez 1989, Downie & Weir 1997, Linsenmair 1997).

Although mortality of pig-nosed frog tadpoles was not overt within the nest over 56 d, performance and survival may well be compromised in tadpoles with prolonged nest tenure after they enter water. For example, larval arrest in *L. fuscus* tadpoles affects later survival after 20 d in the nest (Downie & Weir 1997).

Freshly hatched tadpoles had a prominent yolk sac, the contents of which were rapidly used up during the first few days of development. The increase in body length and the decrease in whole body lipid content during the first week after hatching suggest that the yolk reserves

were rapidly used for protein synthesis. It appears that larval development rapidly progresses to a stage in which tadpoles can then readily begin feeding should water become available with the next rains. Tadpoles remained in this ready stage for up to 8 wk. Once they are placed in water, tadpoles can double their body length within a few days (Rödel *et al.* 1995). This developmental plasticity allows pig-nosed frog tadpoles to respond adaptively to the unpredictable rains and initial filling of temporary ponds early in the rainy season. This plasticity is an additional key adaptation, next to maternal care and early breeding, that provides pig-nosed frog tadpoles with a head-start against competitors and predators that develop within the same pond (Linsenmair 1997, Rödel *et al.* 1995).

In conclusion, this study has shown that pig-nosed frog tadpoles are able to survive for prolonged periods within underground nests. Their ability to do so is facilitated by excreting nitrogenous waste predominantly as urea and by arresting development until they reach water. The duration of developmental arrest is remarkable for amphibians and provides a flexible response in an unpredictable environment.

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