

Full Length Research Paper

Ultraviolet B radiation (UV-B) and the growth and skeletal development of the Amazonian milk frog (*Trachycephalus resinifictrix*) from metamorphosis

Elfi Verschooren¹, Robert K. Brown^{2*}, Francis Vercammen² and Jeffrey Pereboom^{1,2}

¹Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium.

²Centre for Research and Conservation, Royal Zoological Society of Antwerp, Koningin Astridplein 26, 2018 Antwerp, Belgium.

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In captivity some anurans can show poor growth and skeletal development due to inadequate levels of UV-B radiation. From metamorphosis for 84 days the growth and skeletal development of Amazonian milk frogs (*Trachycephalus resinifictrix*) were assessed between treatments receiving daily UV-B (UV/daily), intense UV-B for 30 min every two weeks (UV/boost), or no UV-B (control). At 14 day intervals snout-urostyle length, weight, and X-ray images were taken. At day 84 five morphometric skeletal variables were measured and compared for absolute values and isometry. At day 42 and 84, X-ray images were compared for maximum opacity. At day 84 the UV/daily treatment showed greater snout-urostyle length, weight, condition index, vertebrae length, head width, and femur length than the other two treatments. The head width:snout-urostyle length ratio was greater in the control than in the UV/daily treatment. Calcification did not vary between treatments at day 84. Skeletal development in the control showed symorphic growth of increased head width: head length ratio. The UV-B treatment showed preferential allometric skeletal development of components that increased mobility and feeding ability including head width, and vertebrae and femur length. Overall, these results show that in *T. resinifictrix*; (1) the provision of daily UV-B improved growth and skeletal development, (2) allometric skeletal development occurs during healthy juvenile growth, (3) symorphic skeletal development occurs under conditions of poor growth, and (4) the provision of UV-B may be necessary in both research of the development of some amphibians and in conservation breeding programs or other husbandry.

Key words: Skeletal development, UV-B, anuran, growth, conservation.

INTRODUCTION

An analysis of the 2008 IUCN red list of threatened species shows that nearly 30% of amphibian species are threatened with extinction (Hilton-Taylor et al., 2009). The Amphibian Conservation Action Plan (2005) set priorities for amphibian conservation including the implementation

of conservation breeding programs (CBPs; Gascon et al., 2005). However, nutritional problems and associated pathologies appear to be a major impediment to the implementation of CBPs (Browne et al., 2009a). In particular proper skeletal development seems to be compromised in amphibians as a result of deficient calcium metabolism (King et al., 2010). In diurnal reptiles it is conventional to provide UV-B lighting, in addition to nutritional supplementation with calcium and vitamin/mineral powders, to improve skeleton calcification and health (Ferguson et al., 1996). Vitamin/mineral powders and increasingly nutritional enrichment (gut loading) of feeder invertebrates are also used to improve anuran

*Corresponding author. E-mail: robert.browne@gmail.com Tel: + 32 3 265 33 36. Fax: + 32 3 265 34 74.

Abbreviations: UV-B, Ultraviolet B radiation; CBP, conservation breeding programs.

nutrition (Li et al., 2008). However, the value of UV-B to improve calcium metabolism is largely untested for anurans and other amphibians (Antwis and Browne, 2009).

Poor calcium metabolism may be due to inadequate levels of UV-B radiation or deficiencies in dietary calcium or vitamin D₃ (Antwis and Browne, 2009). In the skin, UV-B radiation (280 to 320 nm) synthesizes provitamin D, which is then converted into previtamin D₃, and then thermally isomerized into vitamin D₃. The vitamin D₃ then enters the blood plasma where it is transported to the liver and hydroxylated into calcidiol (Holick, 2007). Calcidiol is then transported to the kidney and converted into calcitriol, the hormone active in the calcium metabolism (Ferguson et al., 2002).

Calcitriol is necessary for calcium-uptake from the intestine and also regulates the mobilization of calcium ions to the bone matrix. Parathyroid hormone regulates the resorption of calcium from the bone and increases the synthesis of vitamin D₃ into calcitriol. If this cycle dysfunctions the corresponding failure of calcium metabolism results in skeletal abnormalities, low bone calcification, poor growth, and possibly poor reproductive success (Antwis and Browne, 2009; Ferguson et al., 1996, 2002). Consequently, the UV-B/vitamin D₃/calcium cycle is complex and bone calcification involves many glands, hormones, and calcium stores.

Calcium metabolism is particularly significant in anuran tadpoles as they go through a stage of “metamorphic climax”. Metamorphic climax involves extensive morphological and physiological changes including adaptation from an aquatic swimming form to a terrestrial tetrapod morphology and the development of the backbone, skull and limbs (Pilkington and Simkiss, 1966). Plasma levels of metabolic calcium increase during metamorphosis and are necessary for skeletal calcification (McWilliams, 2008). After metamorphosis the continued development and calcification of the skeleton depends on balanced calcium metabolism (Antwis and Browne, 2009).

A common nutritional disorder of amphibians and reptiles in captivity is nutritional metabolic bone disease (NMBD) resulting from deficiencies in calcium metabolism (Donoghue, 1998). Nutritional secondary hyperparathyroidism (NSHP) produces NMBD through an excessive production of parathyroid hormone (PTH) in response to hypocalcaemia. Calcium is then reabsorbed from the bones resulting in an increase in serum calcium and decalcification of the skeleton (Fudge, 2000). Low levels of calcium can negatively affect the reproduction of reptiles, both through difficulties in laying and poor egg hatch (Ferguson et al., 1996, 2002), and may have similar effects in amphibians.

Poor nutrition or metabolism may result in symmorphosis with increased growth of the morpho-functional parts of the skeleton that are important to survival including those important for escape from predators or for food ingestion (Peña and Dumas, 2009; Osse et al., 1997). Most

anurans only consume moving prey and head and gape width may display preferred growth as limits to their size also limit the size of ingestible prey (Smith and Petranka, 1987). Similarly, the vertebrae length and femur length are morpho-functional systems that correspond to motility and also display preferred growth (Peña and Dumas, 2009; Osse et al., 1997).

In the current study we assessed the growth and skeletal development of the Amazonian milk frog (*Trachycephalus resinifictrix*) under different UV-B treatments. *T. resinifictrix* is an arboreal tropical species of Hylidae that breeds exclusively in water-filled tree holes. Tadpoles feed on conspecific eggs and vegetable detritus and in nature have a high level of calcium and vitamin D₃ uptake (Schiesari et al., 2003). Adults range in size from 6 to 10 cm with males being smaller than females. Both juvenile and adult *T. resinifictrix* are exposed on leaves or bark during daylight, where living under the forest canopy they are subject to low levels of UV-B radiation. However, due to their sheltered habitat the exposure of *T. resinifictrix* tadpoles to UV-B is probably minimal (Schiesari et al., 2003; Amphibiaweb, 2010).

Methods to provide vitamin D₃ to post-metamorphic anurans in captivity include diet, UV-B lighting, or medicinal oral or topical administration. However, few zoos and breeding facilities currently provide amphibians with UV-B. There are two methods used to provide UV-B to amphibians; daily exposure to moderate levels of UV-B lighting (1 to 30 $\mu\text{W}/\text{cm}^2$) and periodic exposure to high levels of UV-B lighting (UV/boost). The UV/boost method was developed at Chester Zoo, UK, by Douglas Sherriff and Edwin Blake for Dendrobatid frogs. UV/boost is accomplished by placing frogs in a small enclosure with a screen mesh lid. This enclosure is placed underneath a UV-B emitting lamp to provide a UV-B level of 300 to 400 $\mu\text{W}/\text{cm}^2$ (Gibson pers. com.). We compared the provision of UV/daily or UV/boost, with a control without UV-B during the first 84 days from metamorphosis on the growth and condition, skeletal calcification, and five morphometric skeletal variables of *T. resinifictrix*. Morphometric values were compared between treatments to identify allometry and symmorphosis.

METHODS

General

The experiment was conducted at the Centre for Research and Conservation, Royal Zoological Society of Antwerp, Belgium. Amazonian milk frog (*T. resinifictrix*) tadpoles from captive adults were fed spirulina powder and fish food (SERA, Heinsburg, Germany, D 52515). At tail bud absorption (Gosner stage 45; Gosner, 1960) the metamorphs were placed into experimental treatments with the period of study extending for 84 days. Due to ethical standards of the European Association of Zoos and Aquariums the *T. resinifictrix* could not be euthanised at the study's conclusion and molecular assays of vitamin D₃ were not taken

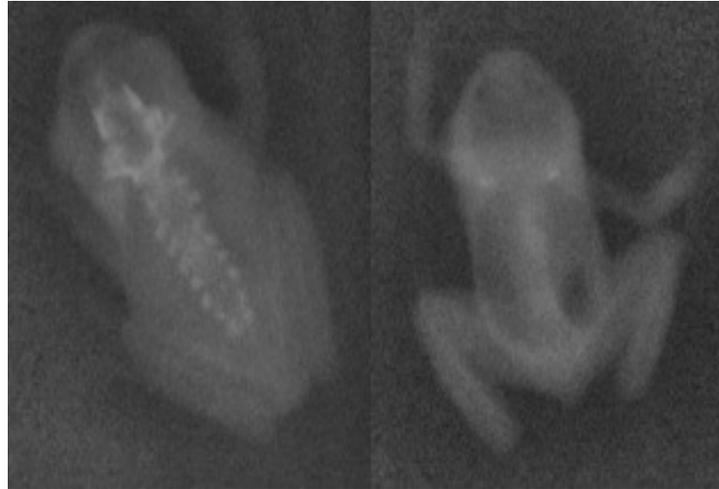


Figure 1. X-rays (left) of the Amazonian milk frog (*T. resinifictrix*) and (right) Golfo Dulce poison-dart frog (*P. vittatus*) at metamorphosis.

(Figure 1).

Experimental array

A large plastic box (41W × 34L × 17H cm) was used for each treatment to contain the individual plastic food containers (11W × 11L × 4H cm) housing each animal replicate. Each individual plastic food container held a sponge (11 cm × 5 cm × 2 cm), 2 cm of water change three times a week, and was covered by fine curtain material (1 mm × 1 mm mesh) held with a rubber band. Three times a week *T. resinifictrix* were fed live crickets (*Acheta domestica*; stage 1 to 4) dusted with fine calcium and vitamin/mineral powder (Exo Terra, HAGEN Deutschland GmbH & Co. Germany).

The study included two UV-B treatments (UV /daily and UV /boost) each of six individuals and a control treatment of eleven individuals. Treatment 1, the UV/daily treatment exposed *T. resinifictrix* to 12 h UV-B per day from a Zoo Med Reptisun 5.0 UV-B light - 14W, 375 mm/15" (<10 $\mu\text{w cm}^{-2}$). Treatment 2, the UV/boost treatment exposed *T. resinifictrix* to high levels of UV-B (>50 $\mu\text{w cm}^{-2}$) for a 30 min period every two weeks from a Zoo Med Reptisun 10.0 UVB light – 15 W, 475 mm/18". The control group was exposed to lighting from a Philips TL-D lamp (15W/33-640) that did not provide UV-B. A UV-B opaque plastic board was placed between the UV/daily and the other treatments to block incidental radiation. Each treatment box was fitted with an aluminum foil hood to increase lighting levels. UV-B levels were measured with a Zoo Med ST-6 Digital Ultraviolet Radiometer (range 280 to 325 nm, peak 290 nm, and accuracy $\pm 10\%$).

Responses

Every two weeks individual replicates were measured for weight to 0.1 g (CHAUS Scout Pro scale) and snout-urostyle length to (0.1 mm) with dial Vernier calliper. To accurately weigh small *T. resinifictrix* absorbent paper towel was placed on the plate of the scale. The scale was then zeroed and the *T. resinifictrix* was weighed. After weighing, any increase in the paper with water was deducted from the total weight (Browne and Antwis, 2009). Repeated measurements were taken twice. Condition index (CI)

was calculated as, $CI = (\text{weight}/\text{length}^3) \times 100$ (Browne and Edwards, 2003). Two X-rays of each *T. resinifictrix* were taken at day 42 and 84 using a portable X-ray machine (RX Model AJEX 9020H) at the University of Antwerp. X-rays were taken from a Golfo Dulce poison-dart frog (*Phyllobates vittatus*) at Gosner stage 45 and X-rays were compared between *T. resinifictrix* and *P. vittatus*.

X-rays of *T. resinifictrix* at day 42 and at day 84 were measured for five morphometric variables with ImageJ (<http://rsbweb.nih.gov/ij/>). Vertebrae length (M1) was taken as the measurement from the first to the last vertebra, the urostyle and pubis-ischium length (M2) was taken as the measurement from the beginning of the sacral vertebra until the end of the pubis-ischium, the head width (M3) was taken as the measurement from the left side to the right side of the quadratojugal, the head length (M4) was taken as the measurement from the premaxillary until the beginning of the first vertebra, and the length of the femur (M5) was taken as the measurement of the beginning to the end of the femur (Figure 2, Table 1). Ratios of vertebrae length, head width, and femur length, to snout-urostyle length were calculated at day 42 and day 84 (Table 2). The degree of skeletal calcification on day 42 and 84 was taken from the two X-rays of each individual using ImageJ approximated through the mean grayscale of their body outline taken using a polygon (Figure 3).

Statistics

Repeatabilities

The repeatabilities of the length and weight measurements, and the measurements of the variables from the X-rays and the calcium intensity, were calculated using the algorithm, $r = s^2_A/(s^2 + s^2_A)$, with s^2_A being the among-groups variance component and s^2 being the within-group variance component (Lessels and Boag, 1987).

Comparison of treatments

Students t-tests were used to compare treatments for snout-urostyle length, weight, condition index, X-rays intensity,

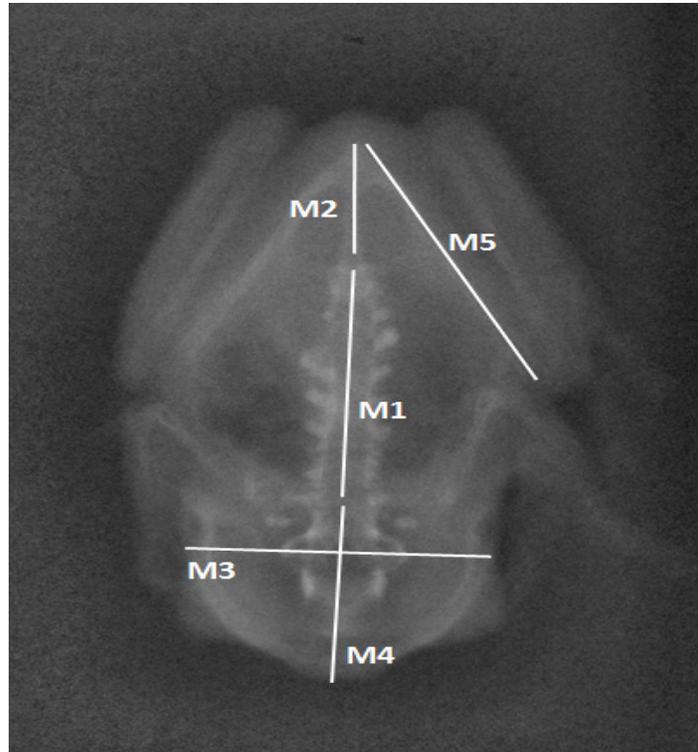


Figure 2. X-ray of an Amazonian milk frog (*T. resinifictrix*) at day 84 showing the morphometric measurements taken at day 42 and day 84. M1 = vertebrae length, M2 = urostyle + pubis-ischium length, M3 = head width, M4 = head length, and M5 = femur length.

and morphometrics and their ratios (Ihaka and Gentleman, 1996).

RESULTS

Repeatabilities

The repeatabilities of the snout-urostyle length and weight-measurements, the measurements of the variables from the X-rays, and the calcium intensity, were high and varied from 0.82 to 0.96 (Table 1).

Snout-urostyle length, weight, and condition index

The snout-urostyle length and weight (Figures 4 and 5), and condition index (data not presented) were significantly higher ($p < 0.05$) in the UV-B/daily treatment than the UV-B/boost or control after 84 days.

Skeletal calcification from X-rays

Figure 1a and b. shows that at Gosner stage 45 the *T. resinifictrix* skeleton showed calcification of the presacral vertebra, frontal-parietal and the prootic region. In contrast, *P. vittatus* had no calcification of the skeleton.

Morphometrics from X-rays

Table 1 shows at day 84 the UV-B/daily treatment produced significantly ($p < 0.05$) greater vertebrae length, head width, and femur length than the other treatments. The UV/daily treatment was also biologically significant ($p < 0.07$) higher in snout-urostyle length, and significantly ($p < 0.05$) greater and weight and condition index, than the other treatments. Table 2 shows no significant difference in ratios of head *width to* snout-urostyle length at day 42. However, at day 84 the ratio of head *width/snout-urostyle* length is significantly ($p < 0.05$) greater in the control than the UV/daily treatment.

Skeletal calcification

The UV-B/daily treatment had significantly ($p < 0.001$) higher grayscale level at day 42, with no differences between treatments at day 84 (Table 1, Figure 6).

Skeletal development at Gosner stage 45

There was no skeletal calcification in *P. vittatus*; however, the skeleton of *T. resinifictrix* showed calcification of the vertebra frontal-parietal, and the prootic region (Figure 1).

Table 1. The means \pm SE of five variables measured (M1 to M5) from the X-rays of *T. resinifictrix* at day 84, their repeatabilities, mean grayscale, snout-urostyle length, weight, and condition index, and their comparison through *t*-tests.

	Skeleton morphometrics	Repeatability	UVB/const. (cm)	UVB/boost (cm)	Control (cm)	t-test
(M1)	Vertebrae length	0.94	0.97 \pm 0.01	0.90 \pm 0.02	0.89 \pm 0.01	p < 0.5
(M2)	Urostyle + pubis-ischium length	0.97	0.55 \pm 0.01	0.49 \pm 0.02	0.50 \pm 0.02	NS
(M3)	Head width	0.98	0.94 \pm 0.01	0.87 \pm 0.02	0.91 \pm 0.02	p < 0.05
(M4)	Head length	0.84	0.69 \pm 0.01	0.65 \pm 0.02	0.65 \pm 0.01	NS
(M5)	Femur length	0.82	1.12 \pm 0.017	1.05 \pm 0.03	1.04 \pm 0.01	p < 0.05
Calcium intensity						
	Mean grayscale (ImageJ)	0.98	90.7 \pm 0.7	92.7 \pm 0.7	92.3 \pm 0.8	NS
Snout-urostyle length (mm) and weight (g)						
	Snout-urostyle length	0.96	23.8 \pm 0.4	22.4 \pm 0.5	22.3 \pm 0.4	p = 0.07
	Weight	0.88	1.35 \pm 0.06	1.17 \pm 0.05	1.16 \pm 0.04	p < 0.05
Derived value						
	Condition index		3.07 \pm 0.11	2.75 \pm 0.08	2.73 \pm 0.08	p < 0.05

Table 2. The comparison of the morphometric ratios of vertebrae length, head width, femur length, and their ratio to snout-urostyle length, between the treatments at day 42 and day 84, and their comparison through *t*-tests.

Ratio	UVB/daily	UVB/Boost	Control	p-value
Day 42				
Vertebrae length : Snout-urostyle length	0.042 \pm 0.001	0.042 \pm 0.001	0.041 \pm 0.001	NS
Head width : Snout-urostyle length	0.041 \pm 0.001	0.041 \pm 0.001	0.042 \pm 0.001	NS
Femur length : Snout-urostyle length	0.045 \pm 0.001	0.047 \pm 0.001	0.047 \pm 0.001	NS
Day 84				
Vertebrae length : Snout-urostyle length	0.041 \pm 0.001	0.040 \pm 0.001	0.040 \pm 0.001	NS
Head width : Snout-urostyle length	0.040 \pm 0.001	0.039 \pm 0.001	0.041 \pm 0.001	p < 0.05
Femur length : Snout-urostyle length	0.047 \pm 0.001	0.047 \pm 0.001	0.047 \pm 0.001	NS

Skeletal development in juvenile *T. resinifictrix*

There was no apparent staged difference in the development of skeletal components between treatments. By day 42 the vertebra, frontal-parietal and the prootic region had become more ossified and ossification had extended to the calcaneum and astragalus. The premaxilla, sphenethmoid, and quadrate-jungal had become ossified. Pronounced enlargements at the end of the vertebra had formed, and the maxilla and pterygoid, supra-scapula, humerus, femur and tibiofibula were more calcified (not shown). By day 84 ossification had extended to the pre-sacral vertebra and appearing in the phalanges of back and front feet. Ossification had further increased in most skeletal components except that the enlargements at the end of the vertebra that were reduced (Figure 2).

DISCUSSION

We assessed the growth and skeletal development of the Amazonian milk frog (*T. resinifictrix*) under different UV-B treatments. Overall our results show that the UV/daily treatment resulted in improved growth, condition, and skeletal calcification compared with the intermittent or no provision of UV-B. In the UV/daily treatment skeletal growth showed the greatest increase in skeletal components important to feeding (head width), and to mobility (vertebrae length, femur length).

All treatments were fed a standard feeder cricket diet that provided minerals including calcium and vitamins including vitamin D₃. Nevertheless, there was greater skeletal calcification with the UV/daily treatment at day 42 when compared to other treatments. The improved growth of the UV/daily treatment suggest that UV-B

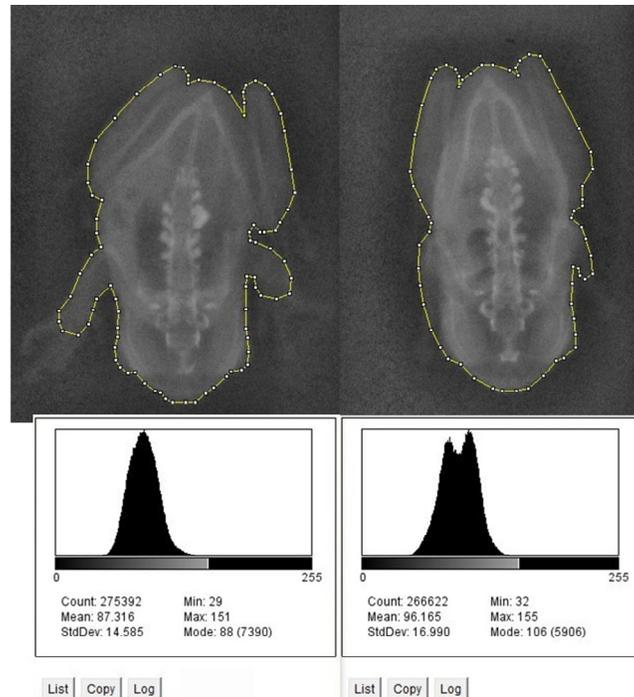


Figure 3. Measuring the calcium intensity of X-rays with ImageJ. By circumfering the shape of the individual and looking at the histogram, the mean grayscale represents the calcium intensity.

provided increased calcium metabolism to that provided by a standard dietary intake of vitamin D₃ or UV/boost. This could be due to UV-B synthesising amounts of vitamin D₃ lacking in the diet or to a basic metabolic requirement for UV-B to provide for calcium metabolism (Antwis and Browne, 2009). Under conditions of poor growth in the control symmorph skeletal development was shown through increased head width ratio. Similar symmorph growth was shown in larval fish with preferential growth of morpho-functional systems that enable improved feeding efficiency. These include an early preference for growth of the vertebral column and parts of the head for feeding (Osse et al., 1997). Peña and Dumas (2009) found similar support for the hypothesis of differential growth patterns for primary functions during early ontogeny.

Our results show that poor calcium metabolism corresponded to reduced overall growth but with the most important skeletal component of head-width the least compromised. In fish during the juvenile stage growth is allometric (Osse et al., 1997). During their juvenile stage up to 42 days *T. resinifictrix* also showed a tendency toward allometric growth in head width ratio. At day 42 the UV-B/daily treatment had significantly higher skeletal calcification and the enlargements at the end of the vertebrae were maximal, suggesting a developmental stage of calcium storage that was most pronounced in the UB/daily treatment. However, at day 84 the skeletal calcification was the same in all treatments.

There was no skeletal calcification in *P. vittatus* at Gosner stage 45. Skeletal calcification of *T. resinifictrix* at Gosner stage 45 included the vertebra, frontal-parietal, and the prootic region. Sheil and Alamillo (2005) recorded extensive skeletal calcification at Gosner stage 45 including the femur and forelimb in *P. vaillanti*. Sheil and Alamillo (2005) considered that arboreal anurans generally have a less calcified skeleton than those of terrestrial species. However, this does not appear to apply to metamorphs, where the arboreal *T. resinifictrix* and *P. vaillanti* showed skeletal calcification, and the metamorphs of the terrestrial *P. vittatus* showed no skeletal calcification. Further studies will show if arboreal species in general have higher calcification at metamorphosis and adults than terrestrial species.

Different species of amphibians are exposed to highly varying amounts of UV-B from extreme levels to no UV-B due to their sun basking, diurnal, crepuscular, nocturnal, or fossorial behaviour. These behaviours may affect their calcium metabolism where species not exposed to UV-B must uptake vitamin D₃ from their diet and species that sun bask may have high relative UV-B thresholds for vitamin D₃ metabolism (Browne et al., 2009b). Within reptiles evidence for high relative UV-B thresholds were shown by Ferguson et al. (2005) where sun-dwelling Anolis lizards had a higher relative UV-B threshold for UV-B induced vitamin D₃ photosynthesis than those that were shade dwelling. In our study the daily exposure of the *T. resinifictrix* per skin area or body

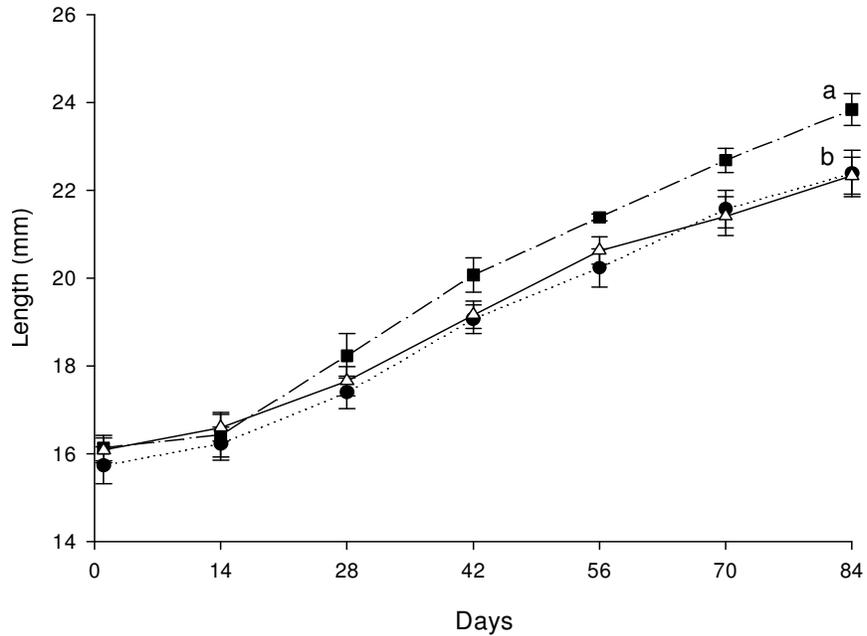


Figure 4. The length (mean \pm SE) of Amazonian milk frog (*Trachycephalus resinifictrix*) in the three treatments from day 0 until day 84. Δ = control, \square = UV constant, \bullet UV= UV boost. $p = 0.07$.

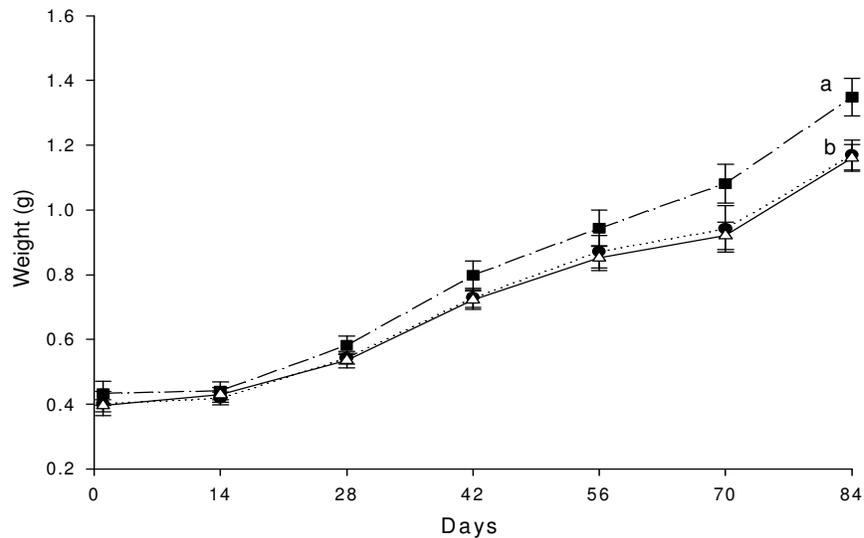


Figure 5. The weight (mean \pm SE) of Amazonian milk frog (*T. resinifictrix*) in the three treatments from day 0 until day 84. Δ = control, \square = UV constant, \bullet UV= UV boost. $p < 0.05$.

mass could not be calculated as they spent some time at the sides of the sponge thus exposing their sides. However, exposure was comparable to that of expected of a diurnal exposed anuran under a forest canopy. Apparently healthy reptiles and birds in captivity have produced dead but fully developed embryos that appear normal except for poorly mineralized skeletons due to

low calcification of the skeleton caused by hypovitaminosis D in the hen and consequently in egg yolks. This syndrome was cured in the panther chameleon (*Furcifer pardalis*) by provision of UV-B to the female prior to oviposition (Ferguson et al., 1996, 2002).

Our study did not show any pathology in any treatments. However, captive anurans have developed

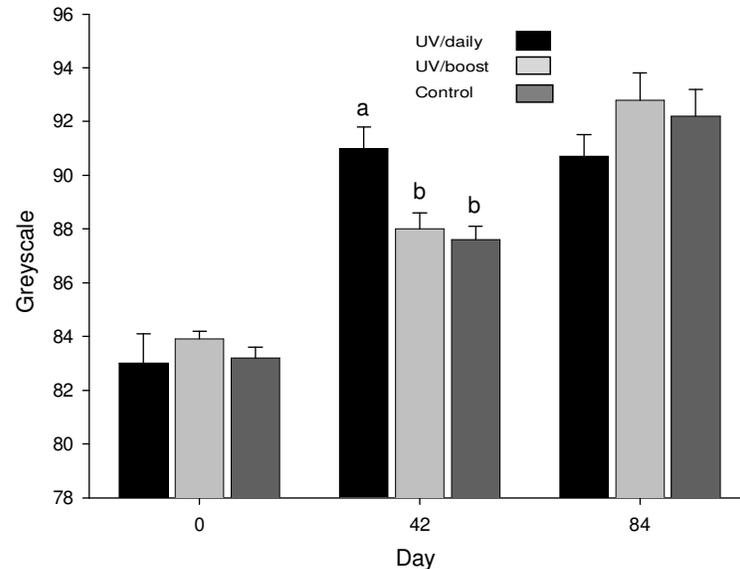


Figure 6. The mean greyscale (mean \pm SE) of Amazonian milk frog (*T. resinifictrix*) on days 0, 42 and 84.

nutritional metabolic bone disease (NMBD) including poor calcification and skeletal malformations (King et al., 2010). King et al. (2010) compared adult wild-caught against captive-bred mountain chicken frogs (*Leptodactylus fallax*) for NMBD and found NMBD with gross skeletal abnormalities in the UV-B deprived, but calcium and vitamin/mineral powder supplemented, captive population of *L. fallax* but not in wild-caught frogs. Similarly, green and golden bell frogs (*Litoria aurea*), a sun basking species, showed gross NMBD and skeletal abnormalities when raised with the provision of calcium and vitamin/mineral powders but without the provision of UV-B (Browne et al., pers com). When *T. resinifictrix* are kept without the provision of UV-B in captivity superficially normal skeletons develop (Antwis and Browne, 2009) and the frogs reproduce successfully for at least two generations (Dale McGinnity, pers. comm.). Nevertheless, our results show that in captivity *T. resinifictrix* can benefit by the provision of UV-B. Because of the potentially beneficial effects of UV-B on amphibian health, UV-B lighting should be considered for captive amphibians that may experience UV-B in their natural environments. In captivity UV-B light should also be provided with visible light to facilitate photo-reactivation during the synthesis of calcidiol (Licht and Grant, 1997). Many amphibians prefer cooler conditions and therefore low power UV-B fluorescent tubes should be provided rather than high temperature UV-B lights.

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