

Research article

Effects of different UV and calcium provisioning on health and fitness traits of red-eyed tree frogs (*Agalychnis callidryas*)

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Abstract

In response to global amphibian declines and extinctions, the IUCN has recommended the establishment of *ex situ* conservation breeding programmes. However, there are a limited number of studies that scientifically assess amphibian husbandry practices, even at a basic level of nutrition and lighting. One component of captive husbandry that is increasingly discussed is the provision of ultraviolet radiation (UVR), which is required for the synthesis of vitamin D₃ and subsequent assimilation of calcium and phosphorus from the diet. Here we used two methods of UV provision (“background UV” and “background UV with UV boost”) and two calcium gut-loading diets (5% and 10%) to assess the effects on a range of fitness measures in the red-eyed tree frog (*Agalychnis callidryas*). We found no effects of either UV treatment or calcium diet on growth, body condition or cutaneous bacterial communities of frogs, although subsequent to the UV boost, frogs had a significantly greater fungal load in comparison to frogs that were not UV-boosted. There were negligible differences in the breeding success of females according to UV exposure. Provision of the UV boost was not demonstrated to provide any real advantages for *A. callidryas* in terms of growth or breeding success. In addition, there were no benefits of a 10% calcium diet over a 5% calcium diet (in conjunction with regular dusting). Further studies that investigate the UV requirements of other amphibian species and ecotypes are required, particularly in conjunction with naturalistic cricket gut-loading diets.

Introduction

Amphibians are experiencing unprecedented global population declines, with 30% (1950 species) of data-sufficient amphibian species threatened with extinction (IUCN 2013). *Ex situ* populations are being established to ensure the persistence of more vulnerable species, with the ultimate aim of reintroduction to the wild once conditions allow (Gascon et al. 2007). Despite a wealth of husbandry knowledge in the zoo and hobbyist communities, there are a limited number of studies that scientifically assess amphibian husbandry practices, even at a basic level of nutrition and lighting. Given the large numbers of individuals required for successful conservation breeding programmes and reintroductions (Griffiths and Pavajeau 2008; Germano and Bishop 2009), it is important to determine which husbandry practices maximise the health and fecundity of captive amphibian populations on reintroduction to the wild.

One component of captive husbandry that is increasingly discussed among amphibian keepers is the provision of ultraviolet radiation (UVR). UVR is emitted at wavelengths of

100 to 400 nanometres (nm), and can be subdivided into UVA (320–400 nm), UVB (280–320 nm) and UVC (100–280 nm). UVB (and some UVA) is required for the photobiosynthesis of vitamin D₃ in most terrestrial vertebrates; D₃ is then converted to calcitriol, one of the hormones involved in calcium and phosphorus assimilation from the diet (reviewed in Antwis and Browne 2009). Vitamin D₃ can also be obtained through dietary intake, although synthesis by UV radiation may be preferable as the process is self-regulating by wavelengths up to 325 nm, thereby avoiding the risk of hypervitaminosis (Holick et al. 1981; MacLaughlin et al. 1982; Webb et al. 1989; reviewed in Antwis and Browne 2009).

A prolonged lack of vitamin D₃, calcium and/or phosphorus causes metabolic bone disease (MBD) in *ex situ* amphibian populations (Densmore and Green 2007). Although not always easily diagnosed in the early stages, there are usually clinical signs of illness in the later stages of MBD, including weakness, tetany, loss of leg function, disfigured mandibles, abnormal posture, inability to raise head, bloating, subcutaneous oedema, cloacal prolapse, multiple fractures and/or reduced

bone density particularly in the vertebrae and limb bones (Zavanella and Losa 1981; Wright and Whitaker 2001; Hibma 2004; Densmore and Green 2007; Klaphake 2010). Conversely, it is well known that excessive exposure to UVR also causes sunburn, cataracts and melanomas in humans, and there is some evidence for other detrimental effects of UVR exposure for eggs and larvae of amphibians (Licht 2003). Therefore it is necessary to find a balance between providing sufficient UVR exposure to captive amphibians for vitamin D₃ synthesis, and avoiding excessive exposure that may have lethal or sub-lethal effects.

Partly due to a lack of quantitative studies, UVR provision for captive amphibians varies widely between institutions according to accepted practice and availability of products. Many UK institutions are moving towards providing amphibians with access to strip lights that provide some level of UV exposure (F. Baines, B. Tapley, G. Garcia, pers. comm.). There is also anecdotal evidence to suggest that frogs exposed to a 20 minute “UV boost” each month from a mercury vapour lamp have greater reproductive success and a lower chance of developing bone abnormalities (D. Sherriff, R. Gibson, pers. comm.), although this method is yet to be scientifically tested.

Calcium (and phosphorus) requirements of amphibians are also under-researched, particularly in conjunction with UVR provision. Feeder insects provided to captive herptiles are of notoriously poor quality with respect to mineral content and inverse Ca:P ratios, which can induce the release of parathyroid hormone and subsequent bone degradation (Sax 2001; Finke 2002; Finke 2003; Campbell 2008). Correct dietary Ca:P ratios vary according to species and requirements, but for most animals it lies between 1:1 and 2:1 (Wise et al. 1963; Fledelius et al. 2005). “Gut loading” feeder insects on a fortified diet prior to feeding to insectivores can improve the nutritional quality and, if the diet contains calcium, correct inverse Ca:P ratios (Finke 2002; Finke 2003). Allen et al. (1993) found no significant differences in whole-carcass calcium content of Cuban tree frogs (*Osteopilus septentrionalis*) fed on “low” calcium (1.2%) and “high” calcium (8.2%) gut-loading diets, although even those fed the high calcium diet contained approximately 25% less calcium in comparison to wild frogs, indicating neither diet was sufficiently high. However, frogs in this study were not provided with UVR (although the diets did contain a small amount of vitamin D₃) and so calcium absorption may have been compromised. Similarly, King et al. (2010) found a 4.2% calcium diet was insufficient to prevent MBD developing in captive mountain chicken frogs (*Leptodactylus fallax*), although again no UVR was provided. More comprehensive studies that investigate the interaction between UV and calcium provision on the fitness of captive amphibians are required.

UVR exposure and calcium supplementation for captive amphibians may have other sub-clinical effects aside from influencing bone density, which can be assessed using a range of measures. Along with commonly used morphometrics, the fecundity of frogs can also provide valuable insight into indirect effects of different husbandry conditions. Moreover, it has previously been shown that diet (Antwis et al. 2014a) and the environment (Loudon et al. 2013; Michaels et al. 2014a) can influence the bacterial community associated with the skin of captive amphibians. UV radiation is particularly likely to influence the bacterial community associated with the skin as it is known to kill bacteria and is commonly used in the laboratory setting to sterilise equipment. This may be important as symbiotic bacterial communities are associated with the protection of hosts from infectious diseases, including amphibian chytridiomycosis and bacterial red-leg syndrome (Pasteris et al. 2011; reviewed in Bletz et al. 2013). In addition, the potential use of symbiotic bacteria in probiotic treatments that protect amphibian populations from infectious diseases is becoming increasingly researched (e.g.

Pasteris et al. 2011; reviewed in Bletz et al. 2013). If different husbandry protocols influence the bacteria that exist on the skin, the success of such treatments may be affected.

In this study we used a fully factorial study design with two methods of UV provision (“background UV” and “background UV with UV boost”) and two calcium gut-loading diets (5% and 10%) to assess the effects on a range of fitness measures (growth and morphometrics, body condition, faecal mineral content, cutaneous microbial communities and fecundity) in the red-eyed tree frog (*Agalychnis callidryas*).

Methods

Ethics statement

Prior to commencing, this study was approved by The University of Manchester Ethics Committee and the Chester Zoo Ethical Committee. All methods were non-invasive and did not require a Home Office licence.

Experimental design

Forty *A. callidryas* frogs were used in this study, maintained in groups of four per tank as described below. At the start of the study (immediately post-metamorphosis) frogs were randomly assigned to tanks (it was not possible to sex individuals at this point to ensure equal numbers of each gender in each treatment group). This study comprised two UV treatment groups: “background UV” and “background UV with UV boost”; and two dietary treatments: 5% calcium and 10% calcium gut-loading diets, with a fully factorial study design and ten frogs per treatment group. The treatment groups were as follows:

- Background UV, 5% calcium diet;
- Background UV, 10% calcium diet;
- Background UV with UV boost, 5% calcium diet;
- Background UV with UV boost, 10% calcium diet.

General frog husbandry

Frogs were bred at the University of Manchester from an existing captive population. Frogs were maintained in a walk-in chamber with a day temperature of 25° C and a night temperature of 22° C. Frog tanks were sprayed once or twice daily using an automated spray system. The study outlined here started from metamorphosis, with froglets maintained in groups of four in 37 x 22 x 25cm ExoTerra™ plastic tanks lined with damp paper towels and containing a water dish and a cutting of devil’s ivy (*Scindapsus* sp.). After three months, froglet groups were moved into 30 x 30 x 45cm ExoTerra™ glass tanks, also lined with damp paper towels and containing a water dish and a cutting of *Scindapsus*. Paper towels and water dishes were changed twice weekly. The following month tanks were converted to a more “naturalistic” set-up consisting of clay balls with a depth of approximately 5 cm for drainage, and a coir/soil mixture with a depth of approximately 3 cm, covered with moss and dried oak leaves. *Scindapsus* cuttings were planted into the soil and supported using a length of bamboo cane. Water dishes continued to be changed twice weekly. Throughout the study all tanks were maintained with a ZooMed Reptisun 10.0 UV strip light with reflectors to provide “background UV”, along with a Philips daylight bulb with reflectors for plant growth, both of which were on a 10:14 light:dark cycle. UV lights were “burnt in” for 100 hours prior to use to achieve stable UV outputs (F. Baines, pers. comm.) and were tested monthly using a Solarmeter 6.2 (Solartech Inc, USA) to ensure UV outputs were not different between treatment groups or diminished over the study period, which they were not. The average UV index over the study period for the background UV treatment group was 1.01 (±0.05), and 0.99 (±0.07) for the background UV with UV boost group. At three

Table 1. Proportion of each ingredient in the 5% calcium (1.8% phosphorous) and 10% calcium (3.3% phosphorous) gut-loading diets fed to black crickets (*Gryllus bimaculatus*), which were then fed to *Agalychnis callidryas* frogs.

Ingredient	5% calcium diet	10% calcium diet
Organic soya flour ¹	15	15
Organic wheat flour ¹	15	15
Organic spirulina ²	10	10
Organic corn flour ¹	28	13
Organic vegetable oil ³	15	15
Nutrobal ⁴	2	2
Mineral mix ⁵	15	30

¹Infinity Foods Co-operative Ltd., UK.

²EverTrust Ltd., UK.

³Sainsburys, UK.

⁴Nutrobal vitamin powder, VetArk, UK.

⁵Combination of Equimins Limestone Flour (39.8% Ca; Equimins Ltd., UK) and Equimins Egg Shell Improver (24.5% Ca, 18.2% P) in a ratio of 1: 1.333 to give a mineral mix with a Ca: P ratio of 3:1.

months post-metamorphosis, frogs were marked using visible implant elastomer dye (VIE; Northwest Marine Technology, USA) in the skin of the tibiofibular using a combination of colours and legs in order to identify each frog individually (each frog received only one mark in either the left or right leg).

UV treatments

All frogs were maintained with background UV (as described above) from metamorphosis until the end of the study. The UV boosts did not commence until month 3 as prior to this froglets may have been susceptible to desiccation during the boosting process due to their small size. After three months, two frogs per tank (identifiable by VIE markings) were randomly assigned to each of the two UV treatment groups. Monthly UVR boosts were conducted every 28 days (= 1 “month”) starting from month 3. This consisted of placing frogs receiving “background UV with UV boost” in a 37 x 22 x 25cm ExoTerra™ plastic tank covered with 0.2 cm nylon mesh, and leaving them to settle for 30 to 60 minutes. Frogs then received a “UV boost” for 20 minutes from a 300-watt Osram™ Ultra Vitalux mercury vapour sun lamp suspended 40cm above the base of the tank. The Ultra Vitalux bulb was burnt in for 100 hours prior to the start of the study, and prior to each boosting session it was switched on and left to warm up for 30 minutes. Frogs in the background UV group were not provided with a sham UV boost (i.e. a simulated boost with the UV filtered out) as this would not reflect normal husbandry practices.

Gut-loading diets

Calcium gut-loading diets were hand-made according to Michaels et al. (2014b), with calcium and phosphorus contents of the diets adjusted to achieve 5% and 10% calcium contents while maintaining a calcium: phosphorus ratio of 3:1 (see Table 1; calcium diets contained 1.8% and 3.3% phosphorus respectively). This Ca: P ratio was chosen because preliminary studies showed this translated into a 1:1 – 2:1 ratio in gut-loaded crickets, which is the usual recommended ratio for feeder diets (Allen and Oftedal 1989). Diets were stored in air-tight containers at -20°C until use. Each tank of frogs received black crickets (*Gryllus bimaculatus*) gut loaded on one of the two calcium diets for 24 hours prior

to feeding out (5 tanks/diet = 20 frogs/diet). Black crickets fed on the 5% calcium diet contained approximately 0.8% calcium (80,000 mg/kg), and those fed on the 10% calcium diet contained approximately 1.2% calcium (120,000 mg/kg; Michaels et al. 2014b). Frogs were fed three or four times weekly, and crickets were dusted with Nutrobal (VetArk, UK) twice weekly. After six months frogs were fed twice weekly, and crickets dusted once weekly. Frogs were fed to satiation, as indicated by a small excess of crickets in the tank the following morning after feeding.

Faecal mineral analyses

To test for differences in the absorption of calcium between frogs fed the two different diets, faecal samples were spot-collected 3–6 days before the UV boost at month 14 (as frogs receiving both UV treatments were mixed within tanks it was not possible to discriminate faeces according to this). These were analysed for mineral contents using atomic emission spectrometry as described in Michaels et al. (2014b). Differences in faecal calcium content, phosphorus content, and Ca:P ratios were analysed according to calcium gut-loading diet using a t test in JMP 10[®].

Morphometrics

Snout-vent length (SVL) of the frogs was measured at the start of the study (month 0) and at month 3, on which day the UV boosts were started. Frogs were photographed on 2mm squared graph paper and SVL measured in ImageJ (available at <http://rsb.info.nih.gov/ij>, accessed January 2014). After the monthly UV boosts were started, SVL and body mass data were collected every three months. Body mass was measured by weighing frogs to two decimal places. At month 15 (the end of the study), head width and tibiofibular length of frogs were also measured from photographs in ImageJ.

Body condition indices (BCI) for each frog at months 3, 6, 9, 12 and 15 were calculated from the SVL and body mass measurements using the equation $BCI = M [L_0/L]^R$, where M is the mass, L is the SVL for that individual, L_0 is the arithmetic mean of the SVLs for the whole population (separate for males and females), and R is the scaling component and is equivalent to the regression value (R value) of M on L for the whole population (Peig and Green 2009; MacCracken and Stebbings 2012). This measure of body condition allows for the allometric relationship between body length and mass and has been shown to accurately represent actual body condition and energy stores of frogs (Peig and Green 2009; MacCracken and Stebbings 2012).

The final number of frogs (males and females) in each treatment group is shown in Table 2 (genders were assigned based on obvious size differences at the end of the 15 month study). Six frogs died before the end of the study (one frog remained small during the study and died after four months, one frog rapidly lost body condition and died of unknown causes, and four frogs died from a skin infection at about 8 months old; see Table 2 and

Table 2. Final number of *Agalychnis callidryas* frogs in each treatment group at the end of the study (month 15).

Treatment group	Number of males	Number of females	Total number of frogs
Background UV, 5% calcium diet	7	1	8
Background UV, 10% calcium diet	8	1	9
Background UV with UV boost, 5% calcium diet	3	4	7
Background UV with UV boost, 10% calcium diet	7	3	10

Discussion) and one frog had asymmetrical hips and may not have grown optimally, and so none of these individuals was included in the repeated measures analyses for morphometric data. All analyses were conducted in JMP 10[®]. Differences in SVL of frogs for month 0 and month 3 according to diet were analysed using a t test. From months 3 to 15, differences in SVL length, body mass and BCI according to UV treatment, diet and their interaction were analysed using repeated measures ANOVAs for males only, as some dietary treatment groups only had one female (see Table 2). Differences in SVL length, body mass and BCI were also analysed using repeated measures ANOVAs for UV treatment alone for both genders. Differences in relative growth rate for SVL and BCI (from month 3 to month 15), final SVL, final BCI, final head width, and final tibiofibular length using data from month 15 were also analysed according to UV treatment alone (both genders separately) using t tests, and for UV treatment, diet and their interaction (for males only) using two-way ANOVAs.

Microbial communities

Three weeks after the UV boost in month 10 ("pre-boost"), and again the day after the subsequent UV boost ("post-boost"), microbial communities were collected from frogs according to Antwis et al. (2014a). Briefly, frogs were rinsed twice on each surface using sterile bottled water prior to swabbing, and dorsal and ventral regions of the body were swabbed separately using sterile Eurotubo® collection swabs (Deltalab, Spain). Swabs were placed into 1.5 ml sterile screw-top tubes containing 1 ml of 1M NaCl₂ for immediate plating out. Sterile gloves were worn throughout handling and changed for each frog to minimise cross-contamination. Care was taken to ensure frogs were not harmed during the swabbing process, and individuals were monitored for two weeks post-swabbing for signs of distress or injury in response to the swabbing, of which none were observed. Samples were diluted to 10⁻¹ and plated out on low-nutrient R2A agar media (Lab M Ltd., United Kingdom), sealed with parafilm and incubated at 25°C. New morphologically distinct bacterial colonies ("morphotypes") and fungal colonies were counted until day 8, after which negligible new colony growth was observed.

Bacterial counts were multiplied by the dilution factor of 10, and the dorsal and ventral surfaces summed for each frog to give a total bacterial community associated with each individual. For each sampling point, overall bacterial community composition was analysed for differences according to diet, UV and their interaction using the Adonis function in RStudio®. The effect of sampling point, diet, UV treatment and all possible interactions on species richness (the number of different morphotypes isolated from each individual) and total abundance (total number of bacterial colonies isolated from each individual) were analysed using two-way ANOVAs in JMP 10[®] (data for bacterial abundance were log transformed to achieve a normal distribution).

Fungal colonies counts were also multiplied by the dilution factor of 10 and summed between surfaces for each frog. A normal distribution could not be obtained for the raw data or log-transformed data and so data were analysed in R using a general linear mixed model with Poisson distribution (with frog included as a random effect to account for repeated measures from the same individual) to test for effects of sampling point, UV treatment, diet and all possible interactions.

Breeding trials

Rain chambers were constructed using 18" x 18" x 18" ExoTerra™ vivariums containing cheese plants (*Monstera deliciosa*) and large cuttings of devils ivy (*Scindapsus* sp.), with the bottom of the tank flooded with approximately 4 inches of water. Holes were pierced into a length of ¾-inch flexible plastic tubing (about 1.5 m) for each rain chamber. These were coiled, fixed to the roof of the tank, and attached to a water pump in the base (New-Jet 400).

Breeding trials were conducted immediately after the UV boost in both months 13 and 14. Given that some treatment groups only contained one female (see Table 2), frogs were grouped according to UV treatment and randomly assigned to breeding chambers, with 2–4 females and 4–6 males per tank, and a total of eight frogs per tank (all females were used for both breeding trials). Frogs were left in the breeding chamber for three consecutive nights for each breeding trial. To determine differences in fecundity according to UV treatment, eggs were counted and averaged for the number of females in each tank. For full clutches, differences in average clutch sizes according to UV treatment were compared using a t test in JMP 10[®].

Results

Faecal mineral analyses

Frogs fed a 10% calcium diet had significantly higher calcium content in their faeces ($t_{17} = 2.00$, $p = 0.03$) than frogs fed the 5% calcium diet, with almost double the mean concentration of

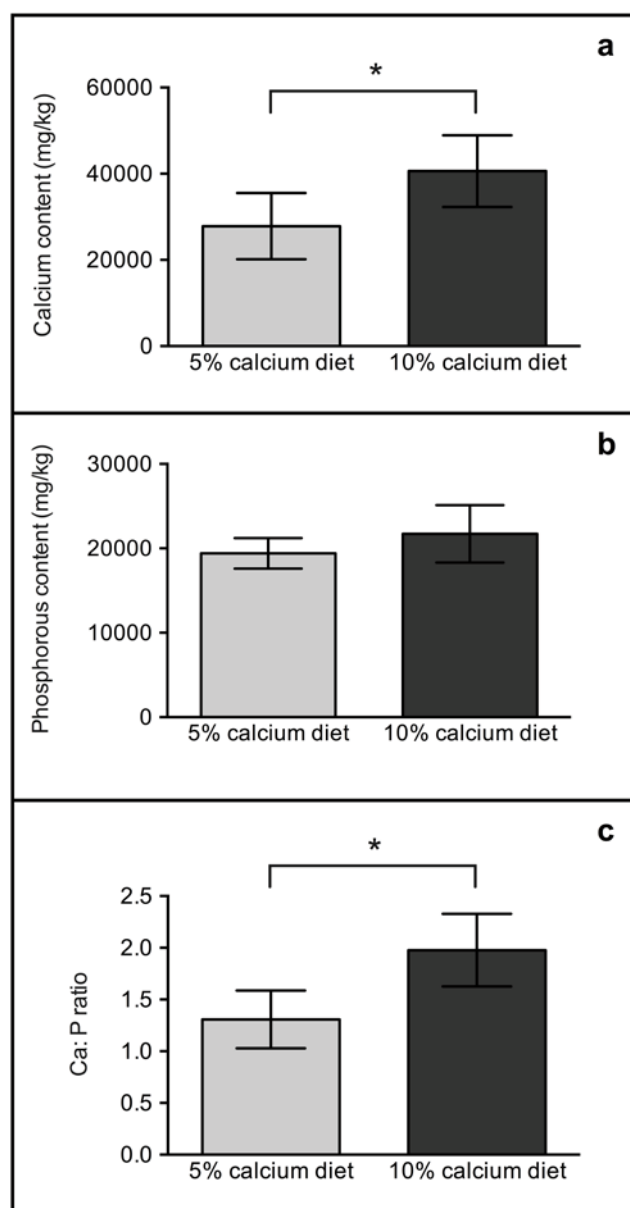


Figure 1. Average calcium (a) and phosphorus (b) content of faeces collected from *Agalychnis callidryas* frogs fed a 5% and 10% calcium diet, along with average Ca:P ratios (c). Error bars show ± 1 S.E.M. Asterisks indicate a statistically significant difference.

Table 3. Results of statistical analyses of morphometrics and body condition of *Agalychnis callidryas* during the 15-month study period.

Time point in study	Individuals included in analysis	Parameter	Model	t or F value and degrees of freedom	P value
0 months	All	SVL	Diet	$t_{1,37} = 0.08$	0.93
3 months	All	SVL	Diet + UV treatment + diet*UV treatment	$F_{3,35} = 0.67$	0.58
3 months	All	Body mass	Diet + UV treatment + diet*UV treatment	$F_{3,35} = 0.81$	0.50
3 months	All	Body condition index	Diet + UV treatment + diet*UV treatment	$F_{3,35} = 0.89$	0.46
3–15 months (repeated measures)	Males only	SVL	Diet + UV treatment + diet*UV treatment	$F_{3,20} = 0.21$	0.89
3–15 months (repeated measures)	Males only	Body mass	Diet + UV treatment + diet*UV treatment	$F_{3,20} = 0.47$	0.71
3–15 months (repeated measures)	Males only	Body condition index	Diet + UV treatment + diet*UV treatment	$F_{3,20} = 0.52$	0.67
3–15 months (repeated measures)	All	SVL	UV treatment	Males: $F_{1,22} = 0.63$ Females: $F_{1,7} = 0.12$	Males: 0.44 Females: 0.75
3–15 months (repeated measures)	All	Body mass	UV treatment	Males: $F_{1,22} = 0.01$ Females: $F_{1,7} = 0.02$	Males: 0.92 Females: 0.89
3–15 months (repeated measures)	All	Body condition index	UV treatment	Males: $F_{1,22} = 0.03$ Females: $F_{1,7} = 0.03$	Males: 0.85 Females: 0.86
15 months	Males only	SVL	Diet + UV treatment + diet*UV treatment	$F_{2,23} = 0.12$	0.95
15 months	Males only	Mass	Diet + UV treatment + diet*UV treatment	$F_{2,23} = 0.46$	0.71
15 months	Males only	Body condition index	Diet + UV treatment + diet*UV treatment	$F_{2,23} = 0.47$	0.70
15 months	Males only	Tibiofibular length	Diet + UV treatment + diet*UV treatment	$F_{2,23} = 0.87$	0.47
15 months	Males only	Head width	Diet + UV treatment + diet*UV treatment	$F_{2,23} = 0.44$	0.72
15 months	Males only	Relative growth	Diet + UV treatment + diet*UV treatment	SVL: $F_{2,23} = 0.86$ Body condition index: $F_{2,23} = 0.18$	SVL: 0.48 Body condition index: 0.91
15 months	All	SVL	UV treatment	Males: $t_{23} = 0.28$ Females: $t_8 = 0.44$	Males: 0.78 Females: 0.72
15 months	All	Body mass	UV treatment	Males: $t_{23} = 0.42$ Females: $t_8 = 1.18$	Males: 0.68 Females: 0.37
15 months	All	Body condition index	UV treatment	Males: $t_{23} = 0.57$ Females: $t_8 = 1.57$	Males: 0.57 Females: 0.24
15 months	All	Tibiofibular length	UV treatment	Males: $t_{23} = 0.66$ Females: $t_8 = 1.26$	Males: 0.51 Females: 0.26
15 months	All	Head width	UV treatment	Males: $t_{23} = 1.23$ Females: $t_8 = 0.01$	Males: 0.23 Females: 0.99
15 months	All	Relative growth of SVL	UV treatment	Males: $t_{23} = 0.31$ Females: $t_8 = 1.83$	Males: 0.76 Females: 0.24
15 months	All	Relative increase in body condition index	UV treatment	Males: $t_{23} = 0.33$ Females: $t_8 = 1.84$	Males: 0.75 Females: 0.27

calcium (approximately 40,600 mg/kg (4.06%) and 21,400 mg/kg (2.14%) respectively; Figure 1a). There were no significant differences in the phosphorus content of faeces according to diet ($t_{17} = 0.79$, $p = 0.22$), with similar mean concentrations in both the 5% and 10% diets (18,700 mg/kg (1.87%) and 21,700 mg/kg (2.17%) respectively; Figure 1b). As a result, faeces from frogs fed a 5% calcium diet had on average nearly half Ca:P ratio of frogs fed

the 10% diet, (1.09 and 1.98 respectively), which was significantly lower ($t_{17} = 2.21$, $p = 0.02$; Figure 1c).

Morphometrics and body condition indices

There were no significant differences in morphometrics or body condition of frogs according to UV treatment, diet, or their interaction for either gender at any time point in the study (see

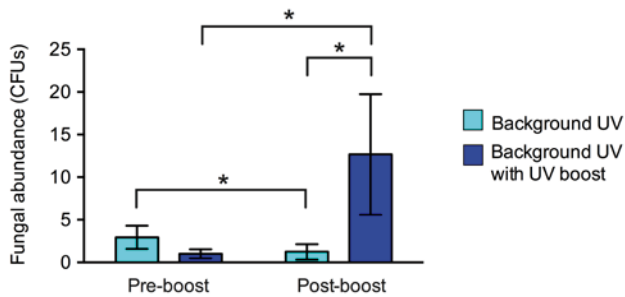


Figure 2. Abundance of fungi associated with the skin of *Agalychnis callidryas* frogs receiving two different UV treatments (background UV, and background UV with UV boost) before and after the UV boost was conducted. Error bars show ± 1 S.E.M. Asterisks indicate a statistically significant difference.

Table 3 for details of statistical outputs). At the start of the study the average SVL for frogs fed the 5% diet was 23.0mm (± 0.58), and 23.0mm (± 0.60) for frogs fed the 10% diet. At the end of the study, the average SVL for males fed the 5% diet in the background UV treatment was 49.7mm (± 2.81), for males fed the 10% diet in the background UV treatment the average SVL was 49.1mm (± 2.19), and for males in the background UV with UV boost the average SVL for the 5% and 10% calcium diets were 49.3mm (± 3.06) and 49.9mm (± 2.04) respectively. Females that received background UV only had an average SVL of 57.5mm (± 4.95) and those that received the background UV with UV boost had an average SVL of 59.1mm (± 3.72).

Microbial communities

At three weeks into the boost cycle, the Adonis analysis showed there were no significant differences in the overall bacterial community according to UV treatment ($F_{1,27} = 0.51$, $p = 0.83$), calcium diet ($F_{1,27} = 1.04$, $p = 0.41$) or their interaction ($F_{1,27} = 0.64$, $p = 0.72$). There were also no significant differences in the overall bacterial communities associated with frogs one day after the UV boost according to UV treatment ($F_{1,27} = 0.59$, $p = 0.72$), calcium diet ($F_{1,27} = 0.82$, $p = 0.53$) or their interaction ($F_{1,27} = 0.53$, $p = 0.76$). The two-way ANOVA model including time point, diet, UV treatment and all possible interactions was not statistically significant for species richness ($F_{7,53} = 1.45$, $p = 0.20$) or bacterial abundance ($F_{7,53} = 0.66$, $p = 0.70$). That is, overall there were no significant differences in bacterial communities associated with frogs as a result of difference in calcium diet or UV exposure either before or after the UV boost.

Fungal abundance was not significantly affected by time point ($X^2 = 1.22$, d.f. = 1, $p = 0.27$), UV treatment ($X^2 = 1.99$, d.f. = 1, $p = 0.16$) or diet ($X^2 = 0.45$, d.f. = 1, $p = 0.50$), although there was a significant interaction between time point and UV treatment ($X^2 = 30.05$, d.f. = 1, $p < 0.001$). The model was re-run to include only time and UV treatment, which was statistically significant for time ($X^2 = 16.10$, d.f. = 1, $p < 0.001$) and the interaction between time and UV treatment ($X^2 = 83.76$, d.f. = 1, $p < 0.001$) but not for UV treatment alone ($X^2 = 0.43$, d.f. = 1, $p = 0.51$). A Tukey's posthoc analysis showed frogs receiving background UV experienced a significant decrease ($p < 0.001$) in fungal abundance over time, whereas frogs receiving background UV with the UV boost experienced a significant increase in fungal abundance from pre-boost to post-boost ($p < 0.001$; Figure 2). Immediately post-boost,

the fungal load associated with frogs that received the UV boost was significantly greater than those that received background UV only ($p = 0.05$; Figure 2).

Breeding trials

During the breeding trials, two clutches were laid from the two females in the background UV group (1 clutch/female; average of 59 eggs/female), and four clutches from the seven females in the background UV with UV boost group (0.57 clutches/female; average of 46 eggs/female). There was no statistically significant effect of UV treatment on clutch size ($t_5 = 0.76$, $p = 0.56$) although the average clutch size was slightly higher for frogs that received background UV with UV boost than for those exposed to background UV alone (74 and 59 respectively). Therefore, females in the background UV treatment had a higher average number of clutches and eggs per female, but females in the background UV with UV boost had a higher average number of eggs per clutch.

Discussion

In this study we aimed to determine whether the UV boost had any effect on the growth, body condition, faecal mineral content, cutaneous microbial communities and fecundity of *A. callidryas* in comparison to the provision of daily background UV, and how this interacted with two different calcium gut-loading diets (5% and 10%).

Faecal analyses

Faecal samples showed frogs fed the 10% calcium diet had twice the calcium content and double the Ca:P ratio of those fed the 5% calcium diet. Frogs fed the 5% calcium diet received crickets containing approximately 0.8% calcium while faecal samples contained only about 0.03% calcium, and frogs fed the 10% calcium diet received crickets that contained approximately 1.2% calcium while faecal samples contained about 0.04% calcium. This indicates the frogs assimilated the vast majority of the calcium from the crickets. In addition, both groups had an average faecal Ca:P ratio greater than 1:1, suggesting both groups had sufficient calcium to avoid MBD resulting from inverse Ca:P ratios. None of the frogs in this study exhibited clinical signs of MBD even after 15 months and most frogs successfully bred, suggesting MBD was not present in the population, although we did not radiograph frogs as there were no other signs of MBD and so did not have cause to. It was not possible to control for differences in faecal mineral content according to UV treatment as tanks contained frogs from both groups. However, within a dietary treatment there were similar numbers of frogs in UV treatment group (see Table 2) and so the results are likely to be reliable. The diets used in this study were relatively manipulated (see Table 1), and it would be worth investigating the calcium content and resulting effects on amphibian fitness for more naturalistic cricket gut-loading diets (e.g. fresh fruit and vegetables), particularly given there are other beneficial nutrients to consider when formulating captive amphibian diets (e.g. carotenoids; Ogilvy et al. 2011; 2012).

Morphometrics

There were no differences in the growth, final morphometrics or body condition of *Agalychnis callidryas* receiving the different UV treatments or calcium diets in this study. Given the UV boost provides a relatively high dose of UV each month, greater differences in morphometrics would be expected if background UV alone was insufficient. Due to the inability to sex this species as juveniles, there were only two females in the background UV group, compared to seven in the background UV with UV boost group. With a larger sample size differences in morphometrics might be expected for females than males, as they may have greater UV

requirements due to their larger size and necessity for egg production. However, an unpublished study (with a larger sample size) by our research group found sub-adult male and female *A. callidryas* that received either background UV with a UV boost or background UV only showed no significant differences in growth during the seven-month study. Therefore we are confident that the UV boost does not provide a benefit in terms of growth for either gender of this species.

Other studies have found an effect of UV on growth, but only when one or more treatment groups received considerably reduced or no UV at all. Amazonian milk frogs (*Trachycephalus resinifictrix*) that received exposure for 12 hours a day to a Reptisun 5.0 had significantly greater bone growth and skeletal development than those exposed to a Reptisun 10.0 for 30 minutes a month, or those not exposed to UVR at all (Verschooren et al. 2011). However, *T. resinifictrix* is a tree-hole dwelling species that is unlikely to receive much exposure to UV in the wild, and amphibian species with other ecologies and/or life histories may require greater UV exposure. Oriental fire-bellied toads (*Bombina orientalis*) toads reared using a fully factorial design with UV (10 hours/day from a Reptisun 10.0) or no UV (both groups received vitamin D₃ in the diet) and the same 5% and 10% calcium diets as in the study presented here showed no significant differences in growth, although frogs that received UV had significantly higher vitamin D₃ levels in their blood than those that received no UV exposure (Michaels et al. 2014b). Comparisons of bone density of captive amphibians with wild counterparts could provide more information about the effects of different UV and calcium treatments.

Microbial communities

There was no significant effect of UV treatment or diet on the bacterial community of frogs. This is in contrast to other studies that have found diet (carotenoid-enriched diet) and environment (planted environment) influence the bacterial communities associated with *A. callidryas* (Antwis et al. 2014a; Michaels et al. 2014a). However, in these studies one group of frogs had a complete absence of a particular enrichment (i.e. in the diet study frogs received a carotenoid-free or a carotenoid-enriched diet, and in the environment study frogs were maintained in a plant-free or a planted environment). In contrast, in the study presented here all frogs received UV and calcium in some quantity. Therefore it is possible that a total absence of some enrichment affects the bacterial community, but it is not affected by the degree to which a husbandry aspect is provided. It is also possible that calcium and UV have no effect on the bacterial community, even in the absence of either of these, although this is difficult to test as it may not be ethically sound to provide a UV-free environment to some amphibian species, and it is not ethical to provide a calcium-free diet to any amphibians.

Frogs that received the UV boost experienced a significant increase in fungal growth immediately after the UV boost was administered, as well as a significantly greater fungal abundance in comparison to frogs that received background UV only. The cause of this is unclear as there were no significant disruptions to the bacterial community that may have allowed greater growth of fungi. There is evidence that some fungi can harvest ionising radiation to enhance proliferation (Dadachova and Casadevall 2008). In addition, there may be some unknown physiological mechanism in frogs that allowed an increase in fungi after the UV boost.

Antwis et al. (2014b) found *A. moreletii* exhibited a proliferation in fungi and bacteria on the skin after marking with passive integrated transponder (PIT) tags, although the mechanism was not identified. The skin infection at about 8 months presented as white spots on the dorsal surface, and three out of the four frogs that died were receiving the UV boost. However, the initial onset of the infection was more likely due to an overgrowth of plants in the tanks, as after plants were cut back frogs that exhibited signs of infection cleared up and survived (two frogs receiving the UV boost and one that was not), and deaths occurred indiscriminately throughout the UV boost cycle (i.e. one in the first week post-boost, two in the second week, and one in the final week). It is possible that the fungal proliferation immediately after the UV boost contributed to the infection, or that some other sub-clinical effect of the UV boost caused these frogs to be more susceptible to death from this.

Breeding trials

Frogs exposed to background UV with UV boost had slightly lower breeding success in terms of average number of clutches per females and number of eggs per females, but slightly higher success in terms of average clutch size. However, there were no statistical differences between the breeding success of females according to UV treatment, and within the context of natural variation in egg production of amphibians, the data indicates negligible benefits of providing the UV boost to females of this species. Due to the low number of females, frogs were grouped according to UV treatment for the breeding trials, independent of diet. However, the number of females per diet was roughly similar within each UV treatment group (see Table 2), and so we are confident the results were not confounded by diet.

Conclusions

Overall, provision of the UV boost was not demonstrated to provide any real advantages for *A. callidryas* in terms of growth or breeding success. There is a possibility the UV boost may increase the susceptibility of frogs to skin infections, either via an increase in fungal proliferation or through some other physiological mechanism. The calcium content of the gut-loading diet did not affect the growth of *A. callidryas* frogs, although the calcium content of faeces of frogs fed the 10% calcium diet had twice the calcium content and twice the Ca:P ratios in comparison to those fed the 5% calcium diet. However, the Ca:P ratios of frogs on both calcium diets was above 1:1 and there were no clinical signs of MBD in the population, indicating the 5% calcium diet with regular dusting provided sufficient calcium to frogs. Further studies investigating the UV requirements of other amphibian species and ecotypes are required, particularly in conjunction with more naturalistic cricket gut-loading diets.

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