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The effects of two calcium supplementation regimens on growth and health traits of juvenile mountain chicken frogs (*Leptodactylus fallax*)

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The mountain chicken frog (*Leptodactylus fallax*) is among the 42 % of amphibians threatened with extinction and is dependent upon ex situ populations to recover in the wild. Amphibian captive husbandry is not fully understood and empirical data are required to optimise protocols for each species in captivity. Calcium metabolism and homeostasis are areas of importance in captive husbandry research and have been identified as a challenge in maintaining ex situ populations of *L. fallax*. We trialled two frequencies (twice and seven times weekly) of calcium supplementation via dusting of feeder insects in two groups of *L. fallax* juveniles and measured growth and health effects through morphometrics, radiography, ultrasonography and blood and faecal analysis over 167 days, followed by a further 230 days of monitoring on an intermediate diet informed by the initial dataset. We showed that supplementation treatment did not affect growth or health status as measured through blood analysis, radiography and ultrasonography. More frequent supplementation resulted in significantly more radiopaque endolymphatic sacs and broader skulls. Frogs fed more calcium excreted twice as much calcium in their faeces. The intermediate diet resulted in previously lower supplementation frogs approximating the higher supplementation frogs in morphometrics and calcium stores. Comparison with radiographic data from wild frogs showed that both treatments may still have had narrower skulls than wild animals, but mismatching age class may limit this comparison. Our data may be used to inform dietary supplementation of captive *L. fallax* as well as other amphibians.

Keywords: Amphibia, amphibian, anuran, diet, nutrition, zoo, calcium

INTRODUCTION

The international conservation response (Wren et al., 2015) to the global amphibian extinction crisis (Stuart et al., 2004; IUCN, 2020) involves the establishment of ex-situ populations for conservation, research and education. Unfortunately, data on optimal husbandry practices and natural history are often lacking (Michaels et al., 2014a; Tapley et al., 2015a). In a few species of amphibian, multiple investigation streams are converging to highlight these taxa as emerging conservation model species. The large, terrestrial and Critically Endangered mountain chicken frog (*Leptodactylus fallax*) is endemic to Montserrat and Dominica in the Eastern Caribbean (IUCN SSC, 2017) and is one such species. It has been the focus of well-rounded research and conservation activity in- and ex-situ (Adams et al., 2014; Tapley et al., 2014). Outputs include description and quantification of population trends (Hudson et al., 2016a), disease dynamics and mitigation (Hudson et al., 2016b; Hudson et al., 2019), translocation attempts in the field (Adams et al., 2014), local cultural associations (Nicholson et al., 2020), life history and reproductive data (Gibson et al., 2004), the development of field methods, captive husbandry protocols (Nicholson et al., 2017; Jameson et al., 2019) and the empirical quantification of some of the requirements of this species in captivity from captive populations (Tapley et al., 2015b; Jayson et al., 2018a; b). Importantly, these data streams have been derived from co-ordinated approaches between captive and field components, in line with the One Plan Approach of the IUCN Conservation Planning Specialist Group; however, there is much work to be done to optimise captive husbandry protocols.

The provision of adequate nutrition is a current challenge in amphibian husbandry (Dugas et al., 2013; Gagliardo et al., 2008; Antwis & Browne, 2009; King et al., 2011; Verschooren et al., 2011; Ogilvy et al., 2012; Michaels et al., 2014b; 2015). The physiological requirements of most amphibians for a suite of different nutrients are unknown (Jayson et al., 2018a) and only a narrow range of prey items are available to feed captive

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Table 1. Design, and thermal and photo parameters of enclosures used to house *L. fallax* in this study. Detail is given to heating and lighting arrays due to their impact on calcium metabolism.

Enclosure	Animals held	Study days occupied	Dimensions (LxWxH)/m	Furnishings	Heating and Lighting array	Thermo- and photo-gradients
1	Parents ---------- Study animals	N/A ---------- 200-398	2 x 2 x 3	Artificial planting, over-turned plastic dog-beds, clay-lined nest box. Water dishes provided. Bark chip substrate.	300 W Ultravitalux lamp (Osram, Germany) LightWave T5 lighting unit (Growth Technology, UK) with 2x 54W D3+ 12 % T5 HO lamps (Arcadia, UK) and 6 x 54W Prolite T5 HO lamps (Phillips, Germany).	Basking zone temperature: 29–31 ˚C Ambient temperature: 26–27 ˚C Cool zone temperature: 23–25 ˚C Nocturnal ambient temperature: 21–23 ˚C UVI: 0–3 correlated with heat Photoperiod: 12:12
2	Study animals	0-200	0.875 x 0.655 x 0.705	Leaflitter and cork flats. Water dish provided Peat-free compost substrate.	160 W mercury vapour lamp (Arcadia, UK) LightWave T5 lighting unit with 1 x 24 W 12% T5 HO D3+ 12 % T5 HO lamp (Arcadia, UK) and 3 x 24WProlite T5 lamps (Phillips, Germany)	Basking zone temperature: 29–34 ˚C Ambient temperature: 25–26 ˚C Cool zone temperature: 23 ˚C Nocturnal ambient temperature: 23 ˚C UVI: 0–3 correlated with heat Photoperiod 12:12

UVI = UV Index

amphibians (Michaels et al., 2014b). Calcium metabolism is a case in point as it relies on the appropriate replication of sunlight (specifically UVB radiation and heat) and the provision of dietary calcium, phosphorus and vitamin D3 sources in appropriate amounts and combinations (Antwis & Browne, 2009; Baines et al., 2016). This is further complicated by the tendency for commercially raised invertebrate species to be calcium-deficient in both absolute terms and relative to phosphorus content (Barker et al., 1998; Finke et al., 2002; Jayson et al., 2018a). In *L. fallax*, comparison of wild and captive diets (Jayson et al., 2018a) and empirical refinement of UVB provision (Tapley et al., 2015b) have demonstrated that supplementation of diets with calcium along with appropriate provision of UVB lighting is important to meet the needs of the species in captivity. However, empirical data regarding the optimal amount of supplementation are lacking, and the complexity of factors influencing calcium metabolism precludes simple replication of wild diets (see Michaels et al., 2014b; 2015). Moreover, husbandry professionals tend to be particularly focussed on avoiding calcium deficiency for captive amphibians due to the high frequency of nutritional secondary hyperparathyroidism (NSHP) commonly known as nutritional metabolic bone disease (NMBD) in anurans (Wright & Whitaker, 2001), and in *L. fallax* specifically (King et al., 2011; Tapley et al., 2015b). The potential for over-supplementation of calcium, however, has been less explored and is currently unknown. There have been increasing reports of choleliths (or, gall-stones) and cholecystitis in captive *L. fallax* in recent years; in some cases, cholecystitis was severe and likely associated with the cause of death in affected individuals (Jameson et al., 2019). The choleliths analysed to date have been composed of almost pure calcium carbonate ($CaCO_3$) and one hypothesis is that choleliths may form as a result of an excess of calcium in the standard captive diet used for this species (Jameson et al., 2019). It is therefore important to be cautious in attempts to supplement calcium in the diets of these frogs and to conduct empirical work to underpin species-specific captive husbandry (Michaels et al., 2014a). To this end, we present the results of trialling two different calcium supplementation regimens for captive *L. fallax*.

METHODS

Ethics statement

All aspects of this study were conducted within the routine husbandry and health monitoring practices for captive amphibians at ZSL London Zoo; the study was conducted between November 2016 and December 2017. All diets used met required calcium needs for vertebrates (see below). Animals were monitored continuously for clinical signs indicative of negative effects, but none were observed. The methods were reviewed by the Zoological Society of London and it was determined that they required neither internal ethical review nor a Home Office Licence under the Animals Scientific Procedures Act 1981; the project was approved and assigned the reference code ZDZ72.

Study animals and husbandry

The study used a split clutch design using 12 juvenile *L. fallax* captive bred at ZSL London Zoo as part of the Mountain Chicken Recovery Programme. These animals were the offspring of a three-year-old captive bred female and a four-year-old captive bred male. These parental animals were housed in Enclosure Type 1 (Table 1). Enclosure temperature and Ultraviolet Index (UVI) gradients (Table 1) were measured using a Ketotek laser thermometer gun (model KY600Y) and a Solartech 6.5 UVI meter.

The study animals hatched from a single clutch laid on 12th May 2016 and metamorphosed between 15th–22nd July 2016. The clutch was randomly split into two groups of six animals each on the 4th November 2016 (day 0). Visible Implant Elastomer tagging was used to identify each individual (Nauwelaerts et al., 2000), along with photographic IDs. From day 0 until day 200, animals were housed in two separate identical enclosures (Enclosure

Type 2, Table 1). On day 200, at approximately 1 year old, the animals were combined in a single enclosure with the same conditions as the parents (Enclosure Type 1, Table 1) although not co-housed with them.

Animals were fed a varied diet following Jayson et al. (2018a). All feeds for adults and pre-experimental juveniles were supplemented with a 1:1 by weight mix of Vetark Nutrobal® (VETARK Professional, Winchester, UK) and powdered calcium carbonate (product code P0302, Cambridge Commodities, Cambridge, UK), which was dusted onto prey items (Michaels et al., 2014b). Nutrobal® is a vitamin and mineral feeding supplement containing calcium carbonate and vitamin D_3, as well as other vitamins, in sufficient levels to mobilise the calcium provided. The 1:1 mixture contains 31.02 % Dry Matter (DM) calcium based on calculations from calcium content analysis of Nutrobal® and calcium carbonate powder (Supplementary Materials). Approximately 2–3 prey items per animal were added to the enclosures at each feed.

Experimental treatments

Two experimental treatments were implemented based on the variations in routine supplementation regimens reported anecdotally by amphibian keepers. The animals were randomly assigned to either a control group (HighCal) or an experimental (LowCal) treatment group. The LowCal group was regarded as the experimental group as it was the treatment that deviated from standard practice. Prior to the start of the study, all frogs were given the control diet. From day 0, HighCal frogs were offered supplemented food every day, whereas LowCal frogs received supplemented food twice a week while still receiving un-supplemented food the rest of the week. Sex was determined towards the end of the study when sexual dimorphism became apparent; the HighCal group comprised four males and two females, the LowCal group comprised two males and four females. The supplement was a powder comprising a 1:1 mix of calcium carbonate and Vetark Nutrobal®. Food items were bagged and coated in the supplement just prior to feeding to maximise supplement powder retention (Michaels et al., 2014b). The animals were fed daily between 0830-0900. The calcium content of the supplemented insect diet offered to HighCal frogs seven times per week and LowCal frogs twice weekly was approximately 3.94 % dry matter (DM) with a calcium:phosphorus ratio of approximately 6.86:1 at the point of feeding (Jayson et al., 2018a). The unsupplemented diet offered to LowCal frogs five times weekly constituted approximately 0.11 % DM calcium and had a calcium:phosphorus ratio of approximately 0.18:1 (Jayson et al., 2018a). Therefore, averaging across supplemented and non-supplemented days, LowCal frogs received a diet containing approximately 1.69 % DM calcium and a Ca:P ratio of approximately 2.03:1.

Data collection

Data collection began on day 0 when LowCal frogs began receiving the experimental treatment. As part of routine health checks carried out on all juvenile individuals of this species, frogs were captured and weighed using digital balances (Salter Housewares, UK) approximately every 14 days, starting on this date. Snout-vent lengths (SVLs) were measured using digital callipers (Transcat, USA) to the nearest millimetre every three months. After data collection on day 167, data were reviewed to inform management moving forwards.

Whole body dorsoventral radiographs (X-ray generator: Ultralight 300 (veterinary X-rays); Processor: Fujifilm FCR Prima II) and coelomic ultrasound images (Ultrasound Machine: Logiq E BT12 Console; Ultrasound probe: L8-18i-RS Wide-band high-frequency linear array with 6.7–18.0 MHz Imaging frequency) were obtained by veterinarians from all frogs on days 0, 87 and 167 as part of routine health checks for this species. Blood samples were collected on the two latter dates (on the initial date the animals were too small for samples to be collected). These procedures, which were conducted under isoflurane induced anaesthesia by veterinary staff, are used to detect the onset of choleliths in the captive population of this species. Blood samples were tested for plasma biochemistry and complete blood count, including plasma glutamate dehydrogenase (GLDH) (an inflammation marker), and plasma calcium and phosphorus levels.

After day 167, all animals were switched to a supplementation regimen intermediate to the experimental treatments as preliminary assessment of data suggested that either extreme may be suboptimal (see Discussion). The intermediate supplementation regimen involved supplementing live food four times weekly; the diet offered therefore had an average calcium content of approximately 2.30 % DM and an approximate Ca:P ratio of 4.0:1 (Jayson et al., 2018a). Final SVL and bodyweight measurements were recorded on day 396 and final radiographs on days 396–8.

Radiographs were analysed using ImageJ (Schneider et al., 2012) following the methods used by Michaels et al. (2015). SVL, femur length, femur width, skull length and skull width were measured. ImageJ was used to calculate the standardised mean greyscale value of the cranial and paravertebral endolymphatic sacs, which are used to store calcium in anuran amphibians (Stiffler, 1993). A polygon was drawn around the paravertebral calcium sacs and the mean greyscale value calculated using the inbuilt program function (following Michaels et al., 2015). To standardise for variation in greyscale value between radiographs, this value was divided by the mean greyscale value of a 52 x 52-pixel square sampled from the radiograph background to generate a standardised mean greyscale value for each frog. Intra-observer reliability of this method has already been established with the same observer by Michaels et al. (2015).

Dorsoventral radiographs from seven wild collected adult *L. fallax* were provided by JD King; these radiographs are referred to by King et al. (2011) and are derived from animals collected in Dominica in 2001 and maintained for c. 3 years in captivity in the USA before radiographs were taken. ImageJ was used to calculate skull length:width and femur length:width ratios from these radiographs.

Fresh faeces were collected daily from enclosures from day 180 to day 186 (inclusive) to ensure that

material from supplemented and non-supplemented feeds were represented; this was repeated for three consecutive weeks. Faecal samples were pooled for each week/treatment combination and frozen at –20˚C. They were then transported frozen to Manchester Metropolitan University where they were analysed for calcium and phosphorus content by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (see Michaels et al., 2014b; 2015).

Statistics

All statistics were conducted using SPSS 25.0 (IBM) for Windows 10. Non-parametric tests were used because of the small sample size. Mann-Whitney U tests were performed to compare SVL, bodyweight, skeletal measurements and greyscale between treatments at each timepoint. Ultrasound findings and blood results were compared qualitatively. A Friedman test for repeated measures was used to compare skull proportions within treatment groups between the final two radiographs. A Kruskall-Wallis test was used to compare skeletal proportions between wild, HighCal and LowCal frogs. A Mann-Whitney U test was used to compare mean plasma glutamate dehydrogenase (GLDH) activity for each frog between treatments. Mean results from faecal data were calculated for comparison between treatments, but N was too small for statistical analysis.

RESULTS

Summary morphometric and radiographic data are presented in Table 2 and Figure 2. P values for bodyweight data, where 13 tests were performed, were not corrected as no comparisons were significantly different with an alpha of 0.05. There were no significant differences between treatment groups for SVL, bodyweight, or femur length:width ratio. Frogs were not significantly different between treatments in terms of skull length:width ratio for the first two radiographs, but HighCal animals had a significantly lower ratio (U_5=3, p < 0.05) on the third radiograph, before diets were changed to the intermediate regimen. A significant change in skull proportions was observed in animals in the LowCal group in that they became relatively broader within-group between the penultimate and final radiographs (X^2_5 = 6, p=0.014). There was no significant change over the same timeframe in HighCal animals (X^2_5 = 0, p > 0.999) and after eight months on the intermediate diet at the final radiograph, no significant difference between treatments was apparent (Fig. 1B). Wild collected frogs had significantly proportionately broader heads than either captive experimental groups on day 167 (H_2=14.9684, p = 0.00056). Wild collected frogs had significantly proportionately thicker femora than either captive experimental groups (Table 2).

Frogs in the HighCal and LowCal groups did not have significantly different mean greyscale values of the paravertebral endolymphatic sacs at the start of the experiment (day 0), but for the following two radiographs on day 87 and day 167, frogs in the HighCal group had significantly higher greyscale values than frogs in the LowCal group (U_5 = 1, p < 0.05; U_5 = 0, p < 0.05, respectively). On days 396-398 (which were 229-231 days after changing to the intermediate diet), the statistically significant difference between groups seen at earlier timepoints was no longer apparent (Fig. 1A). Mean phosphorus content of faeces (collected days 180-86 inclusive) was similar between the treatment groups (2385 vs. 2241 mg/kg), but calcium content of faeces from frogs in the HighCal group was double that of frogs in the LowCal group (33,381 vs. 13,394 mg/kg).

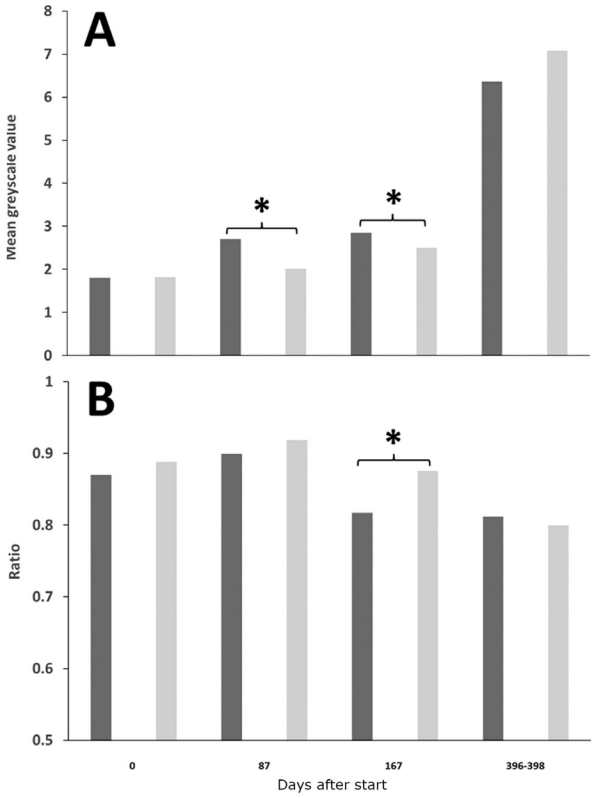

Figure 1. Medians of mean greyscale of the cranial and paravertebral endolymphatic sacs (bar chart A) and skull length:width ratio (bar chart B) of frogs in the HighCal group (dark grey bars) and LowCal group (light grey bars) measured from radiographs taken over the duration of the study. * indicates a statistically significant difference between the HighCal and LowCal groups.

All ultrasound examinations showed grossly normal internal structures with no distinct choleliths, apart from in two animals within the HighCal group, where potential small (< 0.6 mm diameter) stones or echodense aggregations were detected on day 167, but which were not detectable in any of their previous or later ultrasound exams and were not visible on radiographs at any point.

Blood analyses (plasma biochemistry and complete blood counts) were performed from 1–4 times throughout the study, depending on the success of attempted blood draws at each health check event. All findings were unremarkable in comparison with available reference ranges for *L. fallax* (Species360,

Table 2. Median morphometric and radiographic data for *L. fallax* in the HighCal and LowCal treatment groups at the start (day 0) and end (days 396-8) of the study, and morphometrics of wild-collected conspecifics derived from radiographs.

Parameter	Day	HighCal median	LowCal median	Wild-collected
Bodyweight (g)	0	30	24.5	N/A
	396–8	39.5	33.5	
SVL (mm)	0	47.5	38.5	N/A
	396-8	56.5	46	
Femur length/width	0	22.799	23.815	15.60
	396–8	18.316	18.157	
Skull length/width	0	0.870	0.888	0.65
	87	0.900	0.919	
	167*	0.817	0.875	
	396–8	0.811	0.800	
Adjusted mean greyscale of endolymphatic sacs	0	1.808	1.820	N/A
	87*	2.705	2.010	
	167*	2.845	2.502	
	396-8	6.362	7.080	

SVL = snout-vent length. * denotes where High- and Low- Cal groups were significantly different from one another. Beginning and end data only are given for parameters where no significant differences were found between treatments throughout the study.

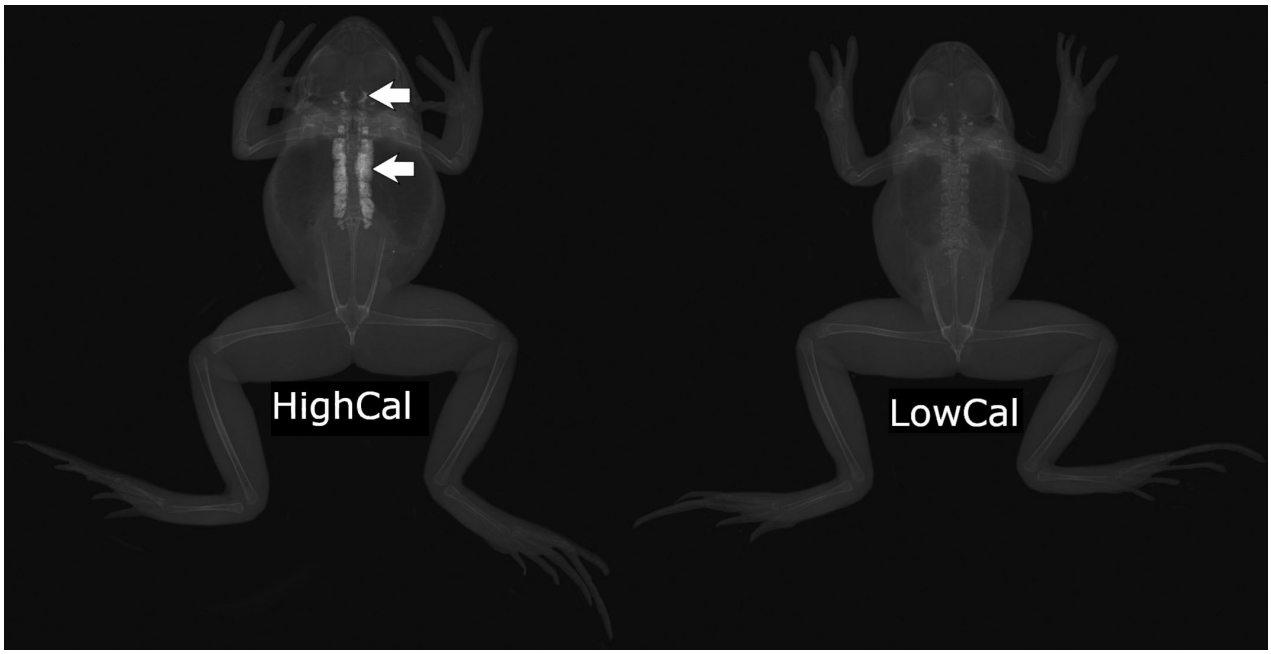

Figure 2. Representative dorsoventral radiographs of frogs from the HighCal and LowCal groups, taken on 20 April 2017. The more radiodense cranial and paravertebral endolymphatic sacs, indicating greater calcium stores, in the frog from the HighCal group are indicated with white arrows. Please note that this is a composite image for illustrative purposes and is not suitable for further analysis.

2020), except for a large variation in the range of GLDH results for which no healthy reference values in amphibians currently exist. Mean (±SD) GLDH across all measurements for all frogs in each treatment was 61.7 (±58.4) U/L for the HighCal group and 74.0 (±78.3) U/L for the LowCal group. A Mann-Whitney U test comparing the mean GLDH value for each frog across treatments showed that there was no significant difference in this parameter between groups, however (U_5=13, $p<0.05$). Compared to expected values in mammals and birds some results appear elevated, but these do not appear to be associated with any appreciable hepatic, gall bladder or other abnormalities in the frogs studied. The number of frogs presenting possible cholethiasis on ultrasound scan was too small for a statistical approach to testing for association between GLDH and signs of cholelithiasis; nevertheless, the frogs with the suspected choleliths did not have more elevated GLDH levels than the rest of the frogs.

DISCUSSION

We found that the frequency of dietary calcium supplementation of *L. fallax* content affected the radiopacity of endolymphatic sacs, the calcium content of faeces, and the proportions of the skulls of frogs, but not any other measures of growth or skeletal structure. Deficits were eliminated in LowCal animals after change to an intermediate supplementation regimen. Importantly, based on clinical examination, plasma calcium and phosphorus levels, and radiography, none of the frogs in this study was suffering from Nutritional Secondary Hyperparathyroidism (NSHP; often called nutritional metabolic bone disease). In particular, proportions of calcium and phosphorus in the blood, which ultimately become imbalanced in NSHP (Wright & Whitaker, 2001), were normal at around 2:1 in both groups, and no reduction of calcification or pathological deformities or fractures of bones, as reported in NSHP in this species by King et al. (2011) and Tapley et al. (2015b), were apparent. Moreover, both HighCal and LowCal diets met the approximate calcium requirements for vertebrates (Robbins, 1993; NRC, 1994; 2006; Oonincx & Dierenfeld, 2012).

HighCal *L. fallax* had relatively broader skulls (lower median skull length:width ratio) by day 167 in this study, which were not apparent earlier at 87 days. Michaels et al. (2015) found significant effects of high vs. low calcium supplementation on skull length:width ratio after 160 days in *Bombina orientalis* (Anura: Bombinatoridae). Michaels et al. (2015) only performed radiography at a single timepoint so comparing the timeline of skeletal changes to that observed in *L. fallax* is not possible. As in this study, Michaels et al. (2015) found no effect of supplementation on growth rates.

Proportionately broader heads may improve fitness as anuran feeding is gape limited and a wider head may facilitate consumption of a broader range of prey (Emerson et al., 1994). As *L. fallax* is a top vertebrate predator and its diet includes very large food items (Brooks, 1982; Rosa et al., 2012), this effect may be important for animals translocated to the field from captive breeding facilities. Moreover, this result supports the recommendation that skeletal health of frogs is assessed prior to translocation (Tapley et al., 2015b).

The more radiopaque paravertebral endolymphatic sacs in HighCal frogs indicate storage of at least some of the additional calcium provided. These stores of calcium carbonate may be mobilised to provide additional calcium in times of need, such as injury, metamorphosis or to provide a buffer in times of acidosis (Schlumberger & Burk, 1953; Pilkington & Simkiss, 1966; Stiffler, 1993; Warren & Jackson, 2005; Wongdee & Charoenphandhu, 2013) and potentially reproduction or periods of reduced feeding (e.g. breeding males). Increased calcium stores may therefore be interpreted as a benefit of more frequent supplementation. However, as the endolymphatic system in frogs is not fully understood and no data are available on either the status of these sacs in wild *L. fallax*, or their optimum physiological state, it is also possible that excessive radiopacity may also be possible in response to over-supplementation. Mean greyscale values for calcium stores in both groups of animals increased substantially between the penultimate and final radiographs (Fig. 1A). This is following nearly ten months of growth wherein the animals increased in size substantially, which is the likely cause of overall increased radiopacity.

The calcium content of faeces from HighCal frogs was double that of LowCal frogs. Given that unabsorbed dietary calcium from the gastrointestinal tract was likely the main source of faecal calcium (environmental contributions would be similar between enclosures, and urinary excretion is a near-negligible contribution in anurans; Stiffler, 1993), this suggests that the more frequent dietary supplementation regimen provided more calcium than could be absorbed by *L. fallax*. It is unclear whether this might be because of absolute physiological limitations of *L. fallax* in gastrointestinal calcium absorption, or if other aspects of calcium metabolism are not yet optimised for the species under captive conditions (Tapley et al., 2015b). There have been recent reports of choleliths and cholecystitis in captive *L. fallax* and choleliths analysed to date have been composed of almost pure calcium carbonate ($CaCO_3$) (Jameson et al., 2019). One hypothesis is that choleliths may form due to an excess of calcium in the diet (Jameson et al., 2019). Potential very small choleliths or aggregations of echogenic material in the gall bladder were observed in two individuals from the HighCal group on ultrasound scan on day 167, but these findings were not present on subsequent health checks and were not appreciable on radiographs. Given that supplemented calcium was provided in the form of calcium carbonate in this study, it is possible that excess calcium carbonate in the alimentary canal could have contributed to the presence of choleliths or echogenic material in the gall bladder. However, there is insufficient evidence at this stage to determine whether supplementation regimes influence the development of cholelithiasis in *L. fallax*.

The combined faecal and radiographic data support the management decision to move all frogs to an intermediate supplementation regimen (see Methods), which aimed to provide sufficient calcium to saturate calcium stores but avoid excessive supplementation that could not be absorbed by the gut. Our data suggest that this was successful; after several months on this regimen, differences between treatment groups in skull proportions and mean greyscale value were no longer apparent; the skull proportions of LowCal frogs approximated those of HighCal individuals, while the latter group did not change significantly. This is an important increase in knowledge from Michaels et al. (2015), as it indicates that, at least in this species and in young animals, skeletal and calcium store differences linked to captive diet may be corrected by adjustment of the diet. No further evidence of cholethiasis was seen in any animals after the switch to the intermediate supplementation regimen, but no causative link could be established. Kawamata (1990a; b) reported rapid $CaCO_3$ crystal formation in anuran endolymphatic sacs

after exposure to calcium supplementation, which is consistent with the response seen in this study to changes in supplementation regimen. This suggests that calcium supplementation should be a priority in captive diets and that even a short time with inadequate supplementation may result in depleted stores.

Data concerning the skeletal structure of wild *L. fallax* are lacking. Both HighCal and LowCal frogs had significantly narrower heads and thinner femora than wild-caught animals. Although these effects may be the result of age class (the wild-caught animals were adults), or levels of locomotive or foraging activity influencing bone structure, these data indicate that the wider skulls observed in HighCal frogs and all animals under intermediate supplementation were more similar to the skulls of wild animals than to those of LowCal frogs, further supporting the adoption of the intermediate supplementation regimen. The wild caught frogs had been in captivity for three years, so meaningful comparison of endolymphatic sacs was not possible. Comparison of age-matched captive and wild animals would be preferred to quantify skeletal differences between populations.

Although calcium carbonate is the primary constituent of the supplement used in this study, it also contained vitamin D_3, which facilitates uptake of calcium from the intestines (Antwis & Browne, 2009). Therefore, increased dietary vitamin D_3 provision, as well as higher total dietary calcium, may have contributed to some of the changes observed in the group provided with more frequent diet supplementation in this study. However, Michaels et al. (2015) found that UVB radiation had a more significant effect on serum vitamin D3 levels in anurans than dietary supplementation with a product containing vitamin D_3 and all frogs in this study had access to a similar gradient of UVB radiation. Sufficient blood samples were not recovered from frogs in the present study to measure serum vitamin D_3 levels using validated methods.

The data collected in this study derive from a relatively small sample size with only one enclosure for each treatment group. Statistically, our data may not be extrapolatable to other populations of *L. fallax* if the enclosure is considered the experimental unit. However, as is often the case with non-model and especially conservation dependent species, this limitation is difficult to overcome without creating additional confounding variables. Moreover, the small sample size prevented us from controlling statistically for sex. Sexes could only be determined towards the end of the study rather than when animals were allocated to groups, but both sexes were represented in both populations.

The findings from this study increase knowledge about calcium supplementation for *L. fallax* and highlight that empirical investigation into optimal captive diet preparation is important when developing captive husbandry protocols for amphibians. We recommend that more research into this field is required, particularly further wild data collection so that comparisons between wild and captive frogs can be made in order to better identify and quantify shortcomings and successes in captive diet formulation and husbandry.

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