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Evidence-based practice

Failure to improve calcium content of earthworms *Dendrobaena veneta* through three methods of gut-loading.

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Abstract

The diets provided to many captive insectivores are deficient in calcium and high in phosphorus, which can lead to nutritional disease. Husbandry professionals may address this imbalance through supplementation, but the efficacy of different methods varies between invertebrate taxa. Earthworms are frequently used for aquatic and fossorial insectivores and this along with their rapidly shed mucus layer makes dusting with supplements ineffective; gut loading is likely the only available route to improving nutritional quality. Moreover, earthworms are often considered a good source of calcium, though data exist only for some taxa and results are mixed with regards to calcium content. The present study analysed the calcium and phosphorus content of *Dendrobaena veneta* earthworms, a species commonly commercially reared and sold for insectivore food, gut loaded on three diets (fresh vegetables, fortified instant porridge oats and a commercial gut loading diet) and quantified the zinc, copper and magnesium content of fasted worms. *Dendrobaena* worms contained sufficient zinc, copper and magnesium to meet the general requirements of domestic birds, mammals and other vertebrates for these metals. However, calcium and calcium:phosphorus ratios of worms were deficient and did not improve after being offered fortified diets. Insufficient calcium in the diets, unpalatability of food and habituation effects also potentially contributed to this result. Unless better means of improving calcium content of *Dendrobaena* can be developed, husbandry professionals should be circumspect in their use of this species in a diet and ensure that dietary items with sufficient calcium are also provided.

Background

Many vertebrate taxa are at least partially insectivorous and, when maintained in captivity, often require invertebrates on which to feed. Although a number of feeder invertebrate species are available in sufficient numbers to form staple dietary items, the range of feeder insects available is still highly limited taxonomically and nutritionally compared with wild diets (Augustine et al. 2016; Jayson et al. 2018). Although some taxa (especially amphibians) can obtain calcium both from the environment and from food, the diet remains the principal calcium source for most vertebrates (Baldwin and Bentley, 1980). Feeder invertebrates are frequently deficient in calcium and abundant in phosphorus (Allen and Oftedal 1989; Allen et al. 1993; Frye 1997; Barker et al. 1998; Finke 2002; Oonincx and Dierenfeld 2012; Michaels et al. 2014; 2015). Calcium deficiency and inverse calcium:phosphorus ratios in the diet causes acute and chronic health issues as the body is deprived of calcium ions required for structural and neurological function (Allen et al. 1993; Frye 1997; Mader 2006; Antwis and Browne 2009; Hoby et al. 2010), and for growth, especially in young animals (Bilby and Widdowson, 1971). The calcium content of feeder items can be improved by both gut-loading. i.e. feeding with a high calcium diet prior to being offered to insectivores (Allen and Oftedal 1989; Finke, 2003; Michaels et al. 2015), and dusting with calcium-rich powder (e.g. Michaels et al. 2014).

Although the latter is generally more effective in boosting calcium content (Michaels et al. 2014), certain feeder items are unsuitable for dusting either due to their own physical characteristics or due to the medium in which they will be offered to predators. Earthworms have soft, mucous skins and are also frequently used as staples for aquatic and fossorial predators (e.g. Tapley et al., 2019), making them doubly unsuitable for dusting with mineral powders, which are rapidly shed into the environment before and during ingestion (personal observation, CM).

A variety of earthworm taxa are available as feeder items. The true earthworm genus *Lumbricus* has yielded varying results in terms of calcium content, potentially linked to gut fill (Bilby and Widdowson, 1971; Bernard et al. 1997; Barker et al. 1998; Finke 2002), but this form of worm is not cultured and is only available from wild-harvested sources, presenting sustainability and biosecurity challenges. The widely used compost worm *Dendrobaena veneta* (sometimes referred to as *Eisenia veneta*, as there lacks taxonomic consistency in the literature) is frequently cultured in large numbers for live food and is particularly useful given its rapid reproduction and growth rate, and huge variation in size between hatchlings (c. 10mm) and adults (more than 100mm). Similar species (mainly *Eisenia foetida*) are successful components of diets for fish in aquaculture, but are typically also low in calcium (variable but typically <1.5:1 Ca:P; Sun and Jiang 2007; Muysoka et al. 2018). To be practically useful for feeding captive animals, especially in situations where large numbers of animals need to be fed, feeder preparation through gut-loading must be practical and time-efficient. Crickets and similar insects have been shown to effectively gut-load for calcium in less than 48 hours (Allen and Oftedal 1989; Anderson 2000; Finke et al. 2005), which allows husbandry professionals to prepare these invertebrates for feeding out rapidly. In order to investigate this possibility, the calcium and phosphorus content of *D. veneta* worms was measured and the calcium gut-loading efficacy of standard vegetable, and two high-calcium diets, was assessed over a 48-hour period, which is longer than gut transit time for this species (Piearce 1972). Other elements (zinc, copper and magnesium) were also measured, which are all important trace elements for vertebrate physiology (Robbins 1993; NRC 1994; 2006).

Action

Although earthworms are not protected by the Animals (Scientific Procedures) Act 1986, the methods for this study were reviewed and approved internally by the Zoological Society of London (ZSL) Ethics Committee. Earthworms were euthanised in accordance with best practice (Cooper 2011).

Commercially farmed earthworms *D. veneta* were procured from a commercial dealer (Worms Direct, UK) and maintained in moist coconut fibre, which provides suitable texture, humidity and moisture but is not in itself a food source for the worms. This medium is frequently used to culture this species of worm. Species identification was provided by the dealer and confirmed by external morphological examination (Jones and Lowe 2014). Worms were maintained at 22°C in a dark, climate-controlled incubator for the duration of the study. Worms were fasted for 48 hours (long enough to void the majority of the alimentary canal of most contents in other closely related species; Piearce 1972) and then provided with one of four treatment substrates; Ready Brek instant porridge oats (Weetabix, UK)—dry flakes moistened with reverse osmosis (RO) water into a paste, Nutrogrub (Vetark, UK)—a dry powder moistened with RO water in to a paste, a mixture of fruits and vegetables (sweet potato, carrot and potato sliced into thin pieces in equal proportions by weight), or a control where the worms were not fed. These diets were chosen as they represent a commercially available gut-loading product (Nutrogrub), a readily available product fortified with calcium (Ready Brek) and a diet that is typically provided to cultured *Dendrobaena* earthworms reflecting their natural diet of decomposing plant matter (fruit and vegetables). Food was provided ad libitum on the surface only of the coconut fibre substrate and it was verified that worms were actively feeding on the substrate from beneath. *Dendrobaena* worms feed readily on organic material on the surface of the soil (Piearce 1972). Worms were sampled at 0, 6, 24 and 48 hrs; worms (0.3–0.8 g dry mass) were removed from culture, rinsed thoroughly in reverse osmosis water, drained and then euthanised with carbon dioxide gas (Cooper 2011). Samples were then immediately frozen at -20°C before being sent for mineral analyses (see below).

Five replicates per treatment per time point were used. A sample of each diet and of the coir substrate was also subjected to mineral analysis.

Mineral analyses were conducted via Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) at the Manchester Metropolitan University (MMU). All samples were dried from frozen at 60°C for 48 hours. Dried samples were transferred to polyethylene sample bags for storage before digestion. Samples were homogenised and 0.25 g (dry mass) was transferred to a microwave digestion vessel. As well as the samples, five blanks were made. To each vessel 8 ml nitric acid (>68% PrimerPlus—Trace analysis grade) was added and left for two hours to digest. For half the samples, the tubes were then transferred to the microwave (CEM Mars Xpress 5) and run on the following cycle: 1. 5-min ramp and 5 min hold at 90°C; 2. 10-min ramp and 10 min hold at 170°C. The cycle was repeated again to ensure complete digestion. After this 2 ml of hydrogen peroxide (>30% w/v) was added and left for 30 min. This method resulted in excess frothing of the samples, and samples from four treatments and three time points were lost (four 0-hour samples, from Nutrobal (n=1), Ready Brek (n=1) and Standard (n=2) diets; two 6-hour samples from Control (n=1) and Standard (n=1); two 24-hour samples from Nutrobal (n=1) and Control (n=1)). The second half of the samples were processed such that the microwave cycle occurred after hydrogen peroxide addition, which mitigated frothing. The processing was otherwise identical and there was no effect of the difference in the results derived. After digestion, 5 ml ultrapure water was added to each vessel to dilute. Each sample was then filtered. Samples were filtered into a 100 ml volumetric flask. Before filtering, the vials were acid washed for 24 hrs (2 ml >68% PrimerPlus Nitric acid was added to each vial and then filled to the top with de-ionised water). To filter each sample, a funnel and filter paper was placed into a 100 ml flask, and the contents of a vessel slowly poured in. The digestion vessel was washed repeatedly with de-ionised water to ensure all the sample was filtered. The flask was then made up to 100 ml using de-ionised water and shaken. Subsequently, 13 ml was transferred into each of two falcon tubes, to be used during the ICP analysis using a Thermo scientific iCAP6300 Duo. Data for calcium and phosphorus were collected for all samples, and copper, zinc and magnesium were measured in fasted worms only, as these were not targets for gut loading. Phosphorus was measured at 177.495 nm, calcium was measured at 422.673 nm.

All statistical analyses were performed in SPSS for Windows. Calcium and phosphorus data were normally distributed (Shapiro-Wilkes test, Ca: W_{71}=0.97, P=0.16; P: W_{71}=0.98, P=0.25) with homogenous variance (Levene's test, $W_{17, 47}$=0.001, P=0.99). General Linear Models (GLMs) were used to analyse calcium, phosphorus and Ca:P ratio in samples including treatment, and time from start as variables. Initially, an interaction term was included using Type 1 sums of squares. If non-significant, this was subsequently removed from the model and Type 3 sums of squares were used. Post-hoc Tukey tests were employed on significant variables from the final model used.

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Table 1. Calcium and phosphorus content of substrate (coir) and diets used to maintain earthworms in this study.

Substrate/diet	Mean Ca:P ratio	Mean P (mg/kg DM)	Mean Ca (mg/kg DM)
Coir	17.44	286.40	4,995.23
Standard	0.70	1,594.47	1,121.83
Ready Brek	2.55	4,279.35	10,928.51
Nutrogrub	14.37	3,657.52	52,578.67

Consequences

Earthworm activity and physical changes to food preparations indicated that worms fed successfully on all diets to at least some extent. All values reported are dry matter (DM) values.

Mean elemental contents of earthworm substrate and diets are presented in Table 1 and mean elemental contents of earthworms are presented in Table 2. There were no significant interaction terms in the models for any of the three dependent variables, so interactions were not included in any final model; main effects only and Type 3 sums of squares were used. There was no significant effect of treatment on any of the dependent variables (Ca: $F_{3,71}$=1.436, P=0.240; P: $F_{3,71}$=0.564, P=0.641; Ca:P ratio: $F_{3,71}$=1.759, P=0.164). There was no significant overall effect of time from start for calcium ($F_{3,71}$=1.494, P=0.225), but a significant effect was found for phosphorus ($F_{3,71}$=4.136, P=0.010) and Ca:P ratio ($F_{3,71}$=4.344, P=0.008); these were very small effects, however, and did not reflect a directional trend over the course of the study (Figure 1).

Although the calcium requirements of most insectivores are poorly known, 4,000–250,000 mg/kg Ca (=0.4 -2.5%) DM and a Ca:P ratio of 1:1-3:1 are estimated minimum dietary requirements for vertebrates (Robbins 1993; NRC 1994; 2006; Oonincx and Dierenfeld 2012). The calcium composition of the diets of wild insectivore species are rarely available, but where available they suggest that the range provided by Robbins (1993) may still be suitable. The wild diet of the mountain chicken frog *Leptodactylus fallax*, for example, has an overall calcium content of 52,900 mg/kg DM and a Ca:P ratio of more than 8:1 (Jayson et al. 2018). Fasted earthworms in this study, by contrast, had a calcium content of approximately 2,000 mg/kg and a Ca:P ratio of approximately 0.3:1. Like many other commercially available live foods (Allen and Oftedal 1989; Allen et al. 1993; Frye 1997; Barker et al. 1998; Finke 2002; Oonincx and Dierenfeld 2012), as well as *Eisenia foetida* (Sun and Jiang 2007; reviewed Muysoka et al. 2018) and commercially harvested *Lumbricus terrestris* (Barker et al. 1998; Finke 2002) earthworms, *D. veneta* are therefore calcium deficient, both in absolute terms and in comparison with phosphorus content. The short-term gut-loading diets offered contained a range of calcium contents, with the highest (Nutrogrub; 52 g/kg Ca) approaching the 65 g/kg shown to be highly effective in improving calcium content in crickets over the same timeframe (Finke et al. 2005). However, these delivered no significant increase of calcium content in *D. veneta* worms, let alone a correction of the calcium levels to within optimal ranges. Although the coir substrate contained calcium (see Table 1), and at higher levels than the standard vegetable diet, this was identical for all treatments. Even if this had contributed to calcium levels, the earthworms were still calcium deficient as a feeder for vertebrates after 48 hours in this substrate.

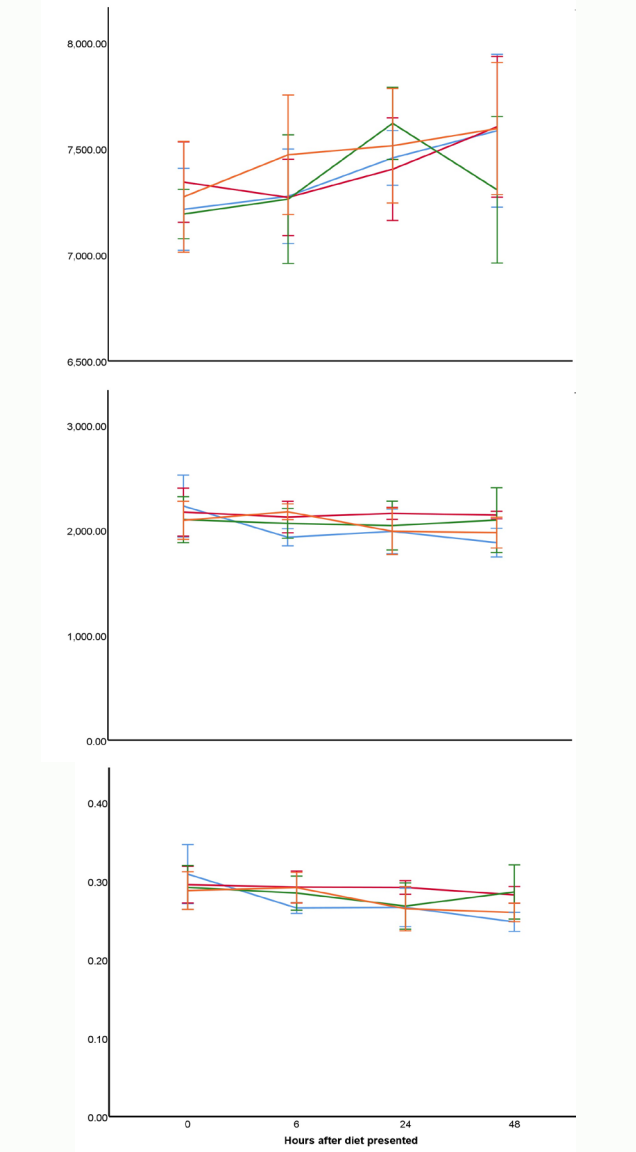

Figure 1. Content of calcium (top) and phosphorus (middle), and Ca:P ratio (bottom), of worms exposed to Nutrobal (red), Ready Brek (green), Standard (orange) and Control (blue) treatments at 0, 6, 24 and 48 hrs post exposure. Error bars represent standard error of the mean.

D. veneta were similar in copper content to commercially harvested *Lumbricus terrestris* (8.66 vs. 8.09 mg/kg) analysed by Barker et al. (1998) but contained substantially less copper than wild collected *L. terrestris* (32.86 mg/kg). *D. veneta* in this study contained substantially less zinc (90 vs. 270 and 231.15 mg/kg) and magnesium (768 vs. 3,100 and 1,900 mg/kg) than wild collected and commercially harvested *L. terrestris* respectively (Barker et al. 1998). Likewise, *D. veneta* contained less copper, zinc and magnesium than ranges for *Eisenia* worms (420–812, 183–1,200 and 2,500 mg/kg, respectively) reviewed by Musyoka et al. (2019). The reason for this is not clear, but may be linked to the elemental content of soils (especially for wild collected *Lumbricus* and for *Eisenia*, the latter of which is often raised in municipal effluent as part of sewage treatment (Musyoka et al. 2019)), and physiological

Table 2. Elemental composition of earthworms in this study. Cu, Zn and Mg were not targets for supplementation and so were only measured in fasted worms. Grand means across all treatments are reported for Ca and P as there was no significant effect of treatment.

Element or ratio	Sample from which mean derived	Mean (SD) mg/kg DM or ratio
Ca	Fasted + experimental at all time points	2,069.86 (206.58)
P	Fasted + experimental at all time points	7,405.44 (291.83)
Ca:P	Fasted + experimental at all time points	0.28 (0.028)
Cu	Fasted only	8.66 (0.66)
Zn	Fasted only	90 (6.51)
Mg	Fasted only	768.71 (85.87)

differences associated with the different habitats and natural food types of *Dendrobaena, Eisenia* and *Lumbricus* in nature (Piearce 1972). In any case, *Dendrobaena* met the minimum requirements for domestic birds and mammals for all three elements (Cu: 1.6–6, Zn: 9.2–30, Mg: 300–1,500 mg/kg) as well as other taxa (e.g. Ferrie et al. 2014) without reaching toxic levels (Robbins 1993). *Dendrobaena* therefore appear to be sufficient dietary items with respect to these elements.

We specifically trialled short-term gut loading in order to test the practicability of rapidly improving the Ca content of feeder worms, which may be a requirement for husbandry professionals dealing with large volumes of both feeders and insectivores. Whereas crickets may be gut loaded with calcium with enough success to convert them from calcium deficient to calcium sufficient prey items within a 48-hour window (Allen and Oftedal 1989; Anderson 2000; Finke et al. 2005), the present did not find similar trends in *Dendrobaena*. The gut transit time for calcium in *D. veneta* is estimated based on data from closely related species to be approximately 0.5–1 day (Piearce 1972), and diet is responsible for almost all calcium uptake in *Dendrobaena* worms (Piearce 1972), so 48 hours of feeding should be sufficient to accumulate the maximum amount of dietary calcium in the alimentary canal. The majority of calcium in earthworms is sequestered in the calciferous glands of the anterior alimentary canal, which rapidly excrete calcium into the oesophageal lumen (Piearce 1972; Ireland 1975). The calcium capacity of these glands varies with earthworm taxon, in association with different degrees of calcium uptake rates in the gut (Piearce 1972). The rate of depletion of the calciferous glands is rapid, so they do not act as calcium stores for much longer than 18–24 hrs (Piearce 1972). *D. veneta* has one of the lowest calcium absorption rates of measured earthworms, and also very low complexity and activity of calciferous glands (Piearce 1972). It is therefore likely that *D. veneta* in this study were unable to absorb or sequester sufficient calcium to significantly increase calcium content of their tissues. Closely related earthworm taxa *Eisenia* sp. have been demonstrated to increase calcium content when living in soils contaminated by large amounts of this element (Ireland 1979), so it is possible that maintenance of *D. veneta* for longer periods, especially over the entire lifespan of a worm, in calcium-rich substrates may yield better results, although in all likelihood will still be limited by calcium absorption and sequestration capacity.

Calcium absorption rates and sequestration capacity do not explain why absolutely no increase in calcium was detected in worms, however, as calcium 'loaded' in the gut should also have elevated calcium content irrespective of tissue content. Although evidence of worms feeding on gut-loading diets was observed (reduction of visible food, massing of worms directly beneath food with mouths within or against the diet), it is also possible that insufficient diet was eaten to significantly increase calcium content. Relatively small amounts of food ingested at any one time, or non-selective feeding by the worms such that substantial amounts of coir bedding were also ingested, diluting any effect of gut-loading diet, may explain this result. Food may also have been unpalatable such that worms fed to some degree, but did not intake large quantities, or worms may have required longer to habituate to enclosures (although as feeding was observed, this may have been a relatively minor issue). Separation of uneaten food from the substrate was impossible to allow for weighing it in and out of enclosures, and loss and removal of water and earthworms at different times precluded accurate weighing of the entire culture. Furthermore, although the diets offered as fortified with calcium and, in the case of Nutrogrub, specifically aimed to enhance calcium content of feeder invertebrates, the total calcium content of the artificial diets may have been insufficient (Nutrogrub only barely approaches the optimal concentration range) to elevate calcium content of feeders to levels suitable for insectivore nutrition (Finke 2003; Finke et al. 2005). However, this does not explain why no calcium increase was detected in this study. Trials using different diets, different presentations of food and different proportions of bedding to diet may be useful in exploring this, but these may increase the complexity and logistical considerations of the process, which may reduce applicability in a practical context.

Overall, and irrespective of the precise reason for the observed trends, the results show that as far as calcium content is concerned, commercially farmed *D. veneta* earthworms appear to be a poor choice of feeder invertebrate as a staple diet for vertebrates, when maintained in the short term under normal conditions, even when fed on high calcium gut-load diets under standard culture conditions. The methods used in this study were designed to be easily used in a practical context and as such are subject to logistical limitations. It may be feasible to gut load earthworms using other techniques, but these will necessarily be more involved and require a longer period of assimilation time, neither of which may be practical in a facility working with large numbers of insectivores and with limited resources. From the authors' experience, the only viable means to improve calcium content over a short time period is to dust the worms as they are being ingested by a terrestrial insectivore. Further work to explore the performance of *Dendrobanea* in delivering other key nutrients that might be lacking in captive diets for insectivores reliant on living prey such as vitamin A (Clugston and Blaner 2014) or omega fatty acids (Jayson et al. 2018), and to explore the potential of longer-term exposure to calcium rich diets, and to other forms and presentations of such diets, to improve calcium content, is needed.

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References

Allen M.E., Oftedal O.T. (1989) Dietary manipulation of the calcium content of feed crickets. *Journal of Zoo and Wildlife Medicine* 20: 26–33.

Allen M.E., Oftedal O.T., Ullrey D.E. (1993) Effect of dietary calcium concentration on mineral composition of fox geckos (*Hemidactylus garnoti*) and Cuban tree frogs (*Osteopilus septentrionalis*). *Journal of Zoo and Wildlife Medicine* 24: 118–128.

Anderson S.J. (2000) Increasing calcium levels in cultured insects. *Zoo Biology* 19: 1–9.

Augustine L.K., Terrell A., Petzinger C., Nissen B., Maslanka M. (2016) Nutritional analysis of diet items available to captive and free-ranging hellbenders (*Cryptobranchus alleganiensis*). *Herpetological Review* 47: 63–69.

Baldwin G.F., Bentley P.J. (1980) Calcium metabolism in bullfrog tadpoles (*Rana catesbeiana*). *Journal of Experimental Biology* 88: 357–366.

Barker D., Fitzpatrick M.P., Dierenfeld, E.S. (1998) Nutrient composition of selected whole invertebrates. *Zoo Biology* 17: 123–134.

Bernard J.B., Allen M.E., Ullrey D.E. (1997) Feeding captive insectivorous animals: Nutritional aspects of insects as food. *Nutrition Advisory Group Handbook*, Fact Sheet 3: 1–7.

Bilby L., Widdowson E. (1971) Chemical composition of growth in nestling blackbirds and thrushes. *British Journal of Nutrition* 25: 127–134.

Clugston R.D., Blaner W.S. (2014) Vitamin A (retinoid) metabolism and actions: What we know and what we need to know about amphibians. *Zoo Biology* 33: 527–535.

Cooper J.E. (2011) Anesthesia, analgesia, and euthanasia of invertebrates. *Ilar Journal* 52: 196–204.

Finke M.D. (2002) Complete nutrient composition of commercially raised invertebrates used as food for insectivores. *Zoo Biology* 21: 269–285.

Finke M.D. (2003) Gut loading to enhance the nutrient content of insects as food for reptiles: A mathematical approach. *Zoo Biology* 22: 147–162.

Finke M.D., Dunham S.U., Cole III J.S. (2005) Evaluation of various calcium-fortified high moisture commercial products for improving the calcium content of crickets, *Acheta domesticus. Journal of Herpetological Medicine and Surgery* 14: 17–20.

Ferrie G.M., Alford V.C., Atkinson J., Baitchman E., Barber D., Blaner W.S., Crawshaw G., Daneault A., Dierenfeld E., Finke M., Fleming G., (2014) Nutrition and health in amphibian husbandry. *Zoo Biology* 33: 485–501.

Frye F.F. (1997) The importance of calcium in relation to phosphorus, especially in folivorous reptiles. *Proceedings of the Nutrition Society* 56: 1105–1118.

Ireland M.P. (1979) Metal accumulation by the earthworms *Lumbricus rubellus, Dendrobaena veneta* and *Eiseniella tetraedra* living in heavy metal polluted sites. *Environmental Pollution* 19: 201–206.

Ireland M.P. (1975) Distribution of lead, zinc and calcium in *Dendrobaena rubida* (Oligochaeta) living in soil contaminated by base metal mining in Wales. *Comparative Biochemistry and Physiology--Part B: Biochemistry and Molecular Biology* 52: 551–555.

Jayson S., Ferguson A., Goetz M., Routh A., Tapley B., Harding L., Michaels C.J., Dawson J. (2018) Comparison of the nutritional content of the captive and wild diets of the Critically Endangered mountain chicken frog (*Leptodactylus fallax*) to improve its captive husbandry. *Zoo Biology* 37: 332–346.

Jones D.T., Lowe C.N. (2014) Key to common British Earthworms. Available from https://www.opalexplorenature.org/sites/default/files/7/file/soil-survey-field-guide-2014.pdf

Michaels C.J., Antwis R.E., Preziosi R.F. (2014) Manipulation of the calcium content of insectivore diets through supplementary dusting. *Journal of Zoo and Aquarium Research* 2: 77–81.

Michaels C.J., Antwis R.E., Preziosi R.F. (2015) Impacts of UVB provision and dietary calcium content on serum vitamin D3, growth rates, skeletal structure and coloration in captive oriental fire-bellied toads (*Bombina orientalis*). *Journal of Animal Physiology and Animal Nutrition* 99: 391–403.

Musyoka S.N., Liti D.M., Erick O., Herwig W. (2018) Utilization of the earthworm, *Eisenia fetida* (Savigny, 1826) as an alternative protein source in fish feeds processing: a review. *Aquaculture Research* 50: 2301–2315.

NRC (1994) *Nutrient Requirements of Poultry*. Washington DC, USA: National Academy Press.

NRC (2006) *Nutrient Requirements of Dogs and Cats.* Washington DC, USA: National Academy Press.

Oonincx D.G.A.B., Dierenfeld E.S. (2012) An investigation into the chemical composition of alternative invertebrate prey. *Zoo Biology* 31: 40–54.

Piearce T.G. (1972) The calcium relations of selected Lumbricidae. *Journal of Animal Ecology* 41: 167–188.

Robbins C.T. (1993) *Wildlife feeding and nutrition, 2nd ed*. London, UK: Academic Press.

Tapley B., Michaels C.J., Gower D.J., Wilkinson M. (2019) The use of visible implant elastomer to permanently identify caecilians (Amphibia: Gymnophiona). *The Herpetological Bulletin* 150: 18–22.