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The utility of dried blood spots for the assessment of avian vitamin D3 status compared with plasma analysis

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Abstract

There is a high incidence of metabolic bone disease (MBD) in many species kept in captivity. Methods to prevent this disease include diet review and reformulation to balance the calcium, phosphorus and vitamin D_3, as well as ultraviolet-B (UVB) provision to stimulate endogenous production of vitamin D_3. To assess the success of these interventions in raising vitamin D_3 status in the blood, the 'gold standard' is serum analysis. Dried blood spots (DBS), used widely in human samples to assess vitamin D_3, need only a small blood volume and have the potential for syringe free sampling. Using DBS for vitamin D_3 analysis has not been validated in non-human species, though a small but growing literature relies on this unvalidated method. This paper assesses the vitamin D_3 status of 15 captive adult corncrakes (*Crex crex*) using both DBS and 'gold standard' serum/plasma analysis. The serum/plasma analysis provides a reference range (mean ± standard deviation (range): 19.7 ± 6.5 (7.9-30.9) nmol/L) for plasma vitamin D_3 in corncrakes, which ranges are compared with those known for other avian taxa. Although the two datasets (DBS vs. serum/plasma analysis) were highly correlated, analysis of the residuals showed significant systematic and random bias. These data suggest that with the current analytical methods, the DBS method is not good enough at predicting plasma vitamin D_3 and thus not yet a useful diagnostic tool for corncrakes. Therefore, DBS results for vitamin D_3 from non-human blood samples should be treated with caution until validation in a range of taxa can be undertaken.

Background

Many captive vertebrates housed in human controlled environments suffer from metabolic bone disease (MBD; e.g. Jepson 2016), which is often referred to as 'rickets'. This disease occurs when individuals are chronically hypocalcaemic, causing secondary hyperparathyroidism, decalcification of skeletal structures in order to maintain blood calcium levels, and subsequent weakening and deforming of the skeleton (Dittmer and Thompson 2010). Low blood calcium is typically caused by low availability of dietary calcium and/or reduced uptake of calcium from the diet due to low circulating vitamin D_3 levels, due to dietary deficiencies and/or lack of ultraviolet-B (UVB) exposure (Dittmer and Thompson 2010). As such, an important diagnostic tool in the treatment of MBD is the analysis of serum to determine vitamin D_3 status, to assess if changes in diet and husbandry are needed. An assay using a liquid-liquid extraction method and tandem mass spectrometry to analyse a liquid blood sample is widely available (henceforth 'serum analysis'). However, this approach is not useful for very small animals from which sufficient volumes of blood (c. 1ml) cannot be collected, or when working in challenging field environments where liquid blood samples cannot easily be extracted, stored and transported. Dry Blood Spot (DBS) analysis utilises single drops of blood dried onto a paper strip to assess a range of blood parameters. DBS technology was developed, and has been in routine use since the 1960s, for assessing health in human infants (Guthrie and Susi 1963). It has been used in veterinary research since the 1970s to detect antibodies in a presence/absence context (Burridge, Kimber and McHardy

1973; Hopkins et al. 1998), and to quantify toxic heavy metals and organochlorine pesticides, including in wild birds (Lehner et al. 2013; Shlosberg 2012). Quantitative vitamin D_3 analysis from DBS has been extensively validated in humans (Eyles et al. 2009; Eyles et al. 2010; Keevil 2011; McGrath et al. 2010; Wyss et al. 2014), but is not yet validated in non-human taxa. Validation cannot be assumed as factors such as haematocrit, protein content of the blood and viscosity may affect the correlation between serum and DBS results (Eyles et al. 2009). Although laboratories clearly indicate that DBS results for non-human animals are not validated, DBS sampling for vitamin D_3 is becoming increasingly common in an unpublished clinical context with exotic animals (C. Michaels, personal observation). DBS data are also presented in published work (e.g. Michaels et al. 2015; Drake et al. 2017), sometimes without any direct caveat regarding lack of validation for this method. It is therefore of increasing importance to critique the DBS method in non-human animals to test the assumption of transferable technology and to validate DBS vitamin D_3 results in a range of taxa.

In 2002, a Corncrake Reintroduction programme was established, coordinated by Natural England, Pensthorpe Conservation Trust, Royal Society for the Protection of Birds, and the Zoological Society of London (ZSL) in response to significant population declines in the UK. This programme includes a captive breeding component. Captive bred birds are subject to routine health examinations, including vitamin D_3 status assessment and veterinary checks for MBD. This paper presents quantitative vitamin D_3 data obtained through paired serum and DBS analysis from these captive corncrakes, which are used to validate DBS analysis in corncrakes.

Action

Ethical statement

All husbandry of birds used in this study was routine and in accordance with best practice. All blood samples were taken as part of routine health monitoring practice and no additional blood volume was required for this study. This work therefore did not require further ethical review or Home Office licensing under Animals: Scientific Procedures Act 1986.

Bird husbandry

The corncrakes used in this study were held at ZSL Whipsnade Zoo (Bedfordshire, UK). The birds are managed between summer and winter enclosures, and enclosure rotations happen in March/April and in September. The birds undergo an annual health check in March/April before being transferred to the summer pens. The birds sampled in this study had been held in winter pens since September 2016 and were sampled in April 2017 during their annual health check.

The winter enclosure is a large shed with 24-hour access to an outdoor grassy paddock. In addition to two non-UVB emitting T8 fluorescent lamps mounted on the wall to illuminate the entire shed with ambient lighting, two UVB emitting lamp arrays were placed above the food dishes situated indoors. Each unit consisted of two 54W T5 fluorescent Arcadia bird lamps with 2.4% UVB output with reflectors. Units were positioned to provide a range of 0.9 to 1.4 UV Index (UVi; measured using a Solarmeter 6.5; see Baines et al. 2016) at corncrake head height over an area of approximately 120x45 cm per unit. Lamps were automatically set to a 12:12 photoperiod cycle. The use of artifical UVB lighting and of outside areas by birds was not quantified.

The winter diet consists of Lundi 20 Micro pellet (Bremehr GmbH & Co. KG, Schmiedestrang 33, 33415 Verl, Germany) provided on an ad libitum basis. This was analysed at Sciantec Analytical Services (Stockbridge Technology Centre, Cawood, UK). Vitamin D_3 (cholecalciferol) was 1.26 IU/g on an as fed basis (moisture 6.3%) or 1.34 IU/g, calcium 1.39% and phosphorus 0.79% on a dry matter (DM) basis, with a Ca:P ratio of 1.75. A calcium carbonate supplement (Farm & Stable, Horndean, UK) was added at 4% and the birds received approximately 2 g of live food (mealworms or crickets) per bird per day. The offered diet contained 2.86% calcium, 0.75% phosphorus and 1.23 IU/g vitamin D_3 per gram DM. Intake of ingredients was not measured.

Blood sampling and processing

During routine annual health examinations over two subsequent days in April 2017, blood samples (0.5 ml) were taken by a veterinary surgeon from the medial metatarsal vein or the jugular vein from 15 adult corncrakes in a heparinised syringe. Of the corncrakes sampled, nine were female and six male. DBS were made with four drops (not aliquoted) of the whole blood on Whatman 903 filter paper (GE Healthcare, Whatman Plc, Maidstone, UK) according to guidelines provided by Sandwell & West Birmingham Hospitals NHS Trust Vitamin Laboratory (Birmingham, UK). The remaining blood was placed in heparinised tubes and spun down by centrifuge within 2 hours of the sampling to produce plasma samples. The plasma samples and DBS were sent by first-class post to Sandwell & West Birmingham Hospitals NHS Trust Vitamin Laboratory at ambient temperature within 24 hours of sampling. Prior to dispatch, plasma samples were stored refrigerated while DBS were dried (for 2-4 hours) and stored at ambient temperature.

Vitamin D_3 analysis:

Serum analysis (vitamin D_3 LC-MS/MS Assay) used a liquid-liquid extraction method and tandem mass spectrometry (Waters TQD and Acquity UPLC) to measure 25-hydroxyvitamin D_3. Deuterated internal standards were also measured for 25-hydroxy vitamin D_3. The limit of quantification for the assay (CV less than 15%), was 7.5 nmol/L for vitamin D_3. A minimum of 150 uL of serum/plasma was required for this assay; to produce the sample, heparinised whole blood was spun down by centrifuge.

The DBS technique measures 25-hydroxy vitamin D_3 by tandem mass spectrometry after derivatisation and liquid-liquid extraction, using in house calibrators and quality control material. The 25-hydroxyvitamin D^3 is extracted from the filter paper by sonicating in water containing deuterated 25-hydroxyvitamin D^3 internal standard. The DBS method is aligned with the laboratory's serum 25-hydroxyvitamin D_3 method which results in blood spot vitamin D levels that are the equivalent of serum levels in human samples. The methods are aligned by assigning values to the in-house calibrators based on the serum 25-hydroxyvitamin D_3 concentrations obtained from the whole blood used to make the calibrators. Serum samples are analysed by tandem mass spectrometry after liquid-liquid extraction using Waters suggested sample preparation and mass spectrometer parameters. The serum method uses commercially available calibrators and internal quality control material. An external quality assurance scheme (DEQAS) is participated in for the serum assay and paired blood spot and serum samples are also used as part of the quality control process for the blood spot assay (Shea and Berg 2016). Centre punches (3 mm diameter) are taken from two blood spots and results generated in nmol/L. It is then checked that the replicates agree to within 15% either side of the mean concentration and the mean concentration is the reported result. For DBS analysis, four blood spots were produced per animal to maximise the chance of producing the minimum of two good quality blood spots that the laboratory required. Each blood spot was approximately 50 uL (0.05 ml), so a total of 200 uL of whole blood (0.2 ml) was needed per bird to produce four blood spots.

Statistics

We compared the vitamin D_3 results from serum and DBS tests, as this form of vitamin D is the end product of photobiosynthesis in response to UVB irradiation, and also the compound present in dietary supplements. To compare the results from the DBS to the 'gold standard' results from the serum analysis several statistical analyses were performed. A Shapiro-Wilks test was performed on each data set to test for normality and Fmax tests to test for homogeneity of variance.

A two-tailed paired t-test compared the DBS and serum results. Pearson's R test was used to calculate the correlation between the two analyses. R^2 value was calculated from the best-fit regression model, with DBS results as the predictor variable. Residuals of DBS versus serum vitamin D_3 results were calculated (i.e. DB minus Serum values) and compared with the serum ('true' values) values in order to check for systematic and random biases (Van Stralen et al. 2008). The mean residual and the standard deviation of residuals were also calculated.

Consequences

Vitamin D_3 results

For vitamin D_3, serum results in nmol/L were mean ± standard deviation (range): 19.7 ± 6.5 (7.9-30.9). DBS results were 30.0 ± 9.9 (14.3-50.5) nmol/L.

For DBS, the laboratory standard practice is to accept replicate measurements of results if they fall within 15% either side of the mean value. All the corncrake results met this criterion. These results indicate that intra-card variability is acceptable according to the standard criteria used for human diagnostic tests.

DBS blood spot quality

Blood spots were of good quality, comparable to good quality human blood spots (Nicola Barlow, personal communication, 7 June 2017), and lacking the straw-coloured serum ring seen by the laboratory in some amphibian species (C. Michaels, personal observation). A good quality blood spot is one where there is a clear, well-defined single spot, where the size of the spot is similar to the size guide on the cards and has fully soaked through to the back of the filter paper, where the blood spots are not merged with one another and where the spots look homogenous. Quality of blood spots was not quantified.

Statistics

DBS and serum results are presented in Fig. 1; residuals are presented in Fig. 2. Variance was not significantly heterogeneous (Fmax=2.44, P (1-tailed)=0.053) and data were normally distributed (W>0.965 for both data sets). DBS gave significantly higher results than serum; t_{14}=5.893, p (two-tailed) <0.001. Results from DBS and serum were significantly positively linearly correlated; R=0.7894, P (two-tailed) <0.001. The regression equation was y=0.5057x + 4.6313, where y is the serum sample result and x is the DBS result; R_2=0.6232. The SE of the regression slope was 0.10908. The slope was significantly different from 1 (t_{13}=-4.53; P<0.001). The SE of the intercept was 0.087.

Discussion

No previously published vitamin D_3 levels are known from Rallidae, and published results from different avian taxa (Table 1) show high interspecific, intraspecific and inter-context variation even within families, likely as a result of different evolutionary trajectories as well as different environmental contexts. These data for corncrakes may therefore be useful as a reference range for this species, but should be used with caution. At the latitude at which the birds were housed, insufficient UVB radiation penetrates during winter months to enable cutaneous vitamin D_3 synthesis in humans as well as, presumably, other taxa (Holick et al. 2007). This means that the birds are likely to have been dependent entirely on vitamin D_3 provided in the diet and synthesised in response to exposure to artificial UVB light. The diet provided meets the recommended levels of vitamin D_3 and calcium:phosphorus ratio for poultry (National Research Council 1994). It is therefore important to stress that although the values here are from adult birds without clinical signs of MBD, they do not necessarily represent the values found in wild birds or the values that might be detected in birds at different times of the year.

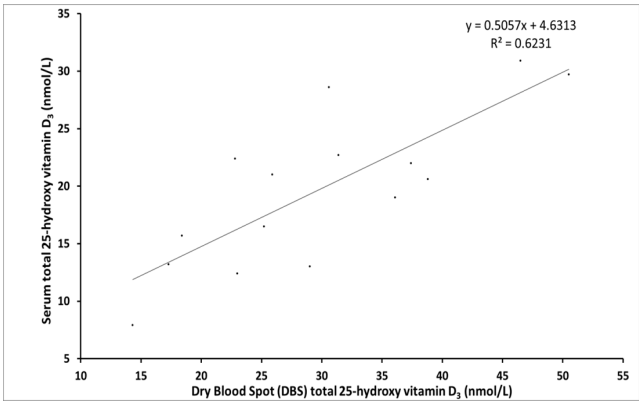

Figure 1. Regression model showing correlation of DBS and serum results for vitamin D_3 in the blood of corncrakes; the equation of the line of best fit and R^2 value are inset.

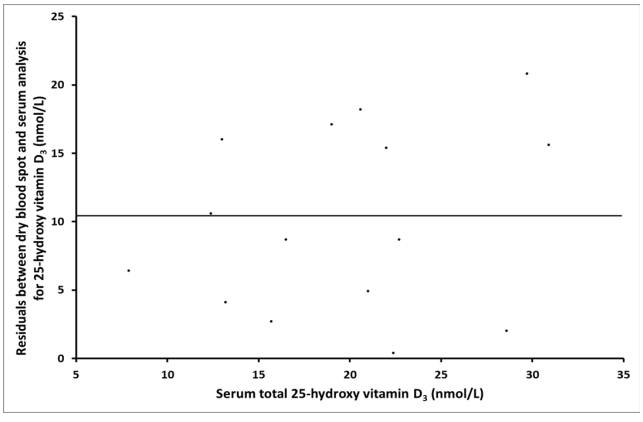

Figure 2. Residual plot of Dry Blood Spot analysis, against serum analysis (or 'true') results for vitamin D_3 results from the blood of corncrakes. The line indicates the mean residual of 10.1 nmol/L.

Although there is a significant correlation between DBS and serum datasets, this does not necessarily indicate good calibration of the DBS method. The correlation coefficient does not allow detection of systematic and random biases in the data (Van Stralen et al. 2008). The term 'calibration' is used here, rather than 'agreement', as the serum method provides true values as opposed to another estimate of unknown accuracy (Van Stralen et al. 2008).

In order to test for biases, we calculated the residuals of the DBS vs. serum analyses and compared them with the serum values; this is similar to a Bland-Altman plot, but rather than using the mean of the two tests as the most likely accurate baseline, instead uses the values obtained from LC-MS/MS Assay serum analysis, which is the currently accepted gold standard for measuring serum 25(OH)D (Binkley and Sempos 2014; Ouweland 2017). The residual plot (Figure 2) shows that there are systematic and random biases in the DBS results. The mean residual of 10.1 nmol/L indicates a systematic bias of this magnitude. In other words, the DBS method overestimates on average 10.1 nmol/L (51% of mean serum value). A simple correction factor is not possible, however, as the standard deviation of residuals, visualised in the spread around the mean line, is 6.6 nmol/L (33% of mean serum value), indicating non-negligible random bias. There is therefore an approximately 95% chance that the true value lies within +/- 13.2 nmol/L (2 x SD) of the predicted value. For example, bird 3992 had a DBS result of 29 nmol/L. Using the regression equation, it can be predicted that the true value is c. 95% likely to fall somewhere within the range 15.8-42.2 nmol/L (in fact the serum value is 16 nmol/L). This range is not dissimilar to the total range of the serum data. These results suggest that the DBS blood test may not be accurate enough in estimating true vitamin D_3 blood values to be clinically useful for corncrakes. This is further supported by the relatively low R^2 value (0.62), indicating substantial spread around the best fit line. For comparison, in a sample of 16 human DBS vs. serum samples analysed at the same lab (Nicola Barlow, personal communication, 7 June 2017), the R^2 value was 0.9771, the standard deviation of residuals was 10% of the mean serum value and the mean residual was <2% of the mean serum value. All of these results indicate a much better predictive power of DBS for human samples in a dataset of a similar magnitude to that for the corncrakes. Moreover, the linear regression slope (=0.5057) for corncrake values was significantly different from 1, indicating that the two values do not scale together.

The dataset used here, although large enough for analysis, is not vast, but was comparable to the sample sizes used in many studies of non-model avians (e.g. Drake et al. 2017 with 31

Table 1. Serum vitamin D_3 values from a range of avian taxa under a range of wild and captive environmental conditions. All data were obtained using forms of the serum analysis method. [a]LC-MS/MS Assay; [b]radioimmuno assay (RIA) [c]chemiluminescent immunoassay (CLIA) [u]unknown *95% frequency interval given rather than range

Species	Family	Context	Values in nmol/L	Reference
Crex crex[a]	Rallidae	Captive animals	mean ± standard deviation (range), n	This study
Rhynchopsitta pachyrhyncha[b]	Psittacidae	Captive zoo adults; deemed healthy	19.7 ± 6.5 (7.9-30.9), *15*	Howard et al. (2004)
Parus major[a]	Paridae	Free living nestlings	19.04 ± 13.24 (5.2-51)*, *45*	Ruiz et al. (2016)
Leptoptilos crumeniferus[c]	Ciconiidae	Captive nestlings with UVB provision	167.5 (123.75-226.5)*, *15*	Schaftenaar and Leeuwen (2015)
		Captive nestlings without UVB provision	Undetectable, *2*	
Strigops habroptila[a]	Strigopidae	Free living adults, supplemented diet	median 8.00 (95% CI=4.76–8.45), *15*	Chatterton et al. (2017)
		Free living adults, unsupplemented diet	median 0.00 (95% CI=−0.16–0.48), *31*	
		Free living adults (non-breeding)	4.9 (1–14), *10*	von Hurst et al. (2016)
Spheniscus humboldti[b]	Spheniscidae	Captive adults	3.7 ± 2.4 (1-10), *14*	Adkesson and Langan (2007)
Platalea alba[b]	Threskiornidae	Captive (no sunlight)	9.9 ± 2.7, *9*	Woodhouse and Rick (2016)
		Captive (sunlight)	20.8 ± 3.9, *9*	
		Captive (no sunlight, low artificial UVB)	19.0 ± 5.6, *14*	
		Captive (no sunlight, high artificial UVB)	16.5 ± 7.2, *18*	
Falco peregrinus x rusticolus[c]	Falconidae	Captive male adults, whole prey diet	19.77 ± 14.15 (13.2-26.3), *10*	Kubiak and Forbes (2012)
		Captive male adults, 10% red meat diet	16.15 ± 19.06 (8.1-38.4), *10*	

animals from 14 species with 1-4 individuals per species), which represent the most likely users of DBS testing for vitamin D_3. Relatively homogenous data without large variation may limit the conclusions that can be drawn from the Bland-Altman analyses used here. However, given that validation of the DBS method must be performed within one species, and given that by definition vitamin D_3 level variation within one species is likely to be relatively constrained, it may be difficult to address this in future work, although larger sample sizes may help if available. Given that this technique may be applied to small populations of birds of a range of species, acquiring huge datasets as could be available for domestically reared avian taxa may be impractical or impossible. Though not common practice for human DBS, aliquoting exactly 50 uL of whole blood per DBS could minimise variability (Kvaskoff 2012), as well as strictly standardising drying and storage of DBS.

The poor calibration is possibly due to differences in the composition of avian and human blood, including haematocrit, protein content and clotting factors, which may result in non-even spreading of blood constituents on the blotting paper developed for human use (Eyles et al. 2009). If this is the case, work to find suitable test paper and calibrators validated for birds could make this method viable, subject to further validation for individual species. Shlosberg et al. (2012) used the same filter paper as in this study (Whatman 903) to quantify levels of heavy metals in DBS for vultures (*Gyps fulvus*) and put in place daily validation and quality control using spiked human whole blood as standards. Using avian blood with known concentrations of analytes as a standard may be an option to improve validation when using avian DBS in quantitative studies with small sample sizes.

Our results suggest that researchers should be cautious when applying DBS methodology to sample populations of birds, or other non-validated taxa, without prior validation of the method in the given species and population. In our sample, although the values do correlate, there is not sufficient predictive power to use this method for meaningful clinical or experimental work due to the degree of error. This may be especially important, therefore, for clinicians and researchers working with small species, where large serum samples cannot be acquired, or in environments where transport of viable serum samples is not possible. Under these conditions, results from DBS samples should not be treated as reliable, but rather as a rough guide to vitamin D_3 levels at the very most.

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References

Adkesson M.J., Langan J.N. (2007) Metabolic bone disease in juvenile Humboldt penguins (*Spheniscus humboldti*): investigation of ionized calcium, parathyroid hormone, and vitamin D3 as diagnostic parameters. *Journal of Zoo and Wildlife Medicine* 38: 85–92.

Baines F., Chattell J., Dale J., Garrick D., Gill I., Goetz M., Skelton T., Swatman M. (2016) How much UV-B does my reptile need? The UV-Tool, a guide to the selection of UV lighting for reptiles and amphibians in captivity. *Journal of Zoo and Aquarium Research* 4: 42–63.

Binkley N., Sempos C.T. (2014). Standardizing Vitamin D Assays: The Way Forward. *Journal of Bone and Mineral Research* 29: 1709–1714.

Burridge M.J., Kimber C.D., McHardy, N. (1973) Detection of antibodies to Babesia bigemina in dried blood samples using the indirect fluorescent antibody test. *Annals of Tropical Medicine & Parasitology* 67: 191–195.

Chatterton J., Pas A., Alexander S., Leech M., Jakob-Hoff R., Jensen B.P., Digby A. (2017) Concentrations of calcium and 25-hydroxycholecalciferol (vitamin D3) in plasma of wild kākāpō (*Strigops habroptilus*) living on two islands in New Zealand. *New Zealand Veterinary Journal* 65: 198–203.

Dittmer K.E., Thompson K.G. (2011) Vitamin D metabolism and rickets in domestic animals: a review. *Veterinary Pathology* 48: 389–407.

Drake G.J., Shea R.L., Fidgett A., Lopez J., Christley R.M. (2017) Provision of ultraviolet basking lights to indoor housed tropical birds and their effect on suspected vitamin D3 deficiency. *Journal of Zoo and Aquarium Research* 5: 151–157.

Eyles D., Anderson C., Ko P., Jones A., Thomas A., Burne T., Mortensen P.B., Nørgaard-Pedersen B., Hougaard D.M., McGrath J. (2009) A sensitive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. *Clinica Chimica Acta* 403: 145–151.

Eyles D., Morley R., Anderson C., Ko P. Burne T., Permezel M., Mortensen P.B., Nørgaard-Pedersen B., Hougaard D.M., McGrath J.J. (2010) The utility of neonatal dried blood spots for the assessment of neonatal vitamin D status. *Paediatric and Perinatal Epidemiology* 24: 303–308.

Guthrie R., Susi A. (1963) A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32: 338–43.

Holick M.F., Chen T.C., Lu Z., Sauter E. (2007) Vitamin D and skin physiology: A D-lightful story. *Journal of Bone and Mineral Research* 22: 28–33.

Hopkins J.S, Chitambo H., Machila N., Luckins A.G., Rae P.F., van den Bossche P., Eisler M.C. (1998) Adaptation and validation of antibody-ELISA using dried blood spots on filter paper for epidemiological surveys of tsetse-transmitted trypanosomosis in cattle. *Preventive Veterinary Medicine* 37: 91–99.

Howard L.L., Kass P.H., Lamberski N., Wack R.F. (2004) Serum concentrations of ionized calcium, vitamin D3, and parathyroid hormone in captive thick-billed parrots (*Rhynchopsitta pachyrhyncha*). *Journal of Zoo and Wildlife Medicine* 35: 147–153.

IUCN. (2013) *Guidelines for Reintroductions and Other Conservation Translocations*. Version 1.0. Gland, Switzerland: IUCN Species Survival Commission.

Jepson L. (2016) *Exotic Animal Medicine: a quick reference guide*. Elsevier Health Sciences.

Keevil B. (2011) The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. *Clinical Biochemistry* 44: 110–118.

Kubiak M., Forbes N. (2012) Effects of diet on total calcium, vitamin D and parathyroid hormone in falcons. *Veterinary Record* 171: 504.

Kvaskoff D., Ko P., Simila H.A., Eyles D.W. (2012) Distribution of 25-hydroxyvitamin D3 in dried blood spots and implications for its quantitation by tandem mass spectrometry. *Journal of Chromatography B* 901: 47–52.

Lehner A.F., Rumbeiha W., Shlosberg A., Stuart K., Johnson M., Domenech R., Langner, H. (2013) Diagnostic analysis of veterinary dried blood spots for toxic heavy metals exposure. *Journal of Analytical Toxicology* 37: 406–422.

McGrath J., Burne T., Féron F., Mackay-Sim A., Eyles D.W. (2010) Developmental vitamin D deficiency and risk of schizophrenia: a 10-year update. *Schizophrenia Bulletin* 36: 1073–1078.

Michaels C.J., Antwis R.E., Preziosi R.F. (2015) Impacts of UVB provision and dietary calcium content on serum vitamin D 3, growth rates, skeletal structure and coloration in captive oriental fire-bellied toads (*Bombina orientalis*). *Journal of Animal Physiology and Animal Nutrition* 99: 391–403.

National Research Council. (1994) *Nutrient Requirements of Poultry*: Ninth Revised Edition, 1994. Washington, DC: The National Academies Press.

Ouweland J.M.W., Van Den. (2017). Analysis of vitamin D metabolites by liquid chromatography tandem mass spectrometry. *Trends in Analytical Chemistry* 84: 117–130.

Ruiz S., Espín S., Rainio M., Ruuskanen S., Salminen J.P., Lilley T.M., Eeva T. (2016) Effects of dietary lead exposure on vitamin levels in great tit nestlings - an experimental manipulation. *Environmental Pollution* 213: 688–697.

Schaftenaar W., Leeuwen J.P.T.M. (2015) The influence of ultraviolet-b radiation on the growth of marabou stork (*Leptoptilos crumeniferus*) nestlings in relation to plasma calcium, phosphorus, and vitamin d 3

concentrations. *Journal of Zoo and Wildlife Medicine* 46: 682–690.

Shea R., Berg J. (2016) Self-administration of vitamin D supplements in the general public may be associated with high 25-hydroxyvitamin D concentrations. *Annals of Clinical Biochemistry* 54: 355–361.

Shlosberg A., Wu Q., Rumbeiha W.K., Lehner A., Cuneah O., King R., Hatzofe O., Kannan K., Johnson M. (2012) Examination of Eurasian griffon vultures (*Gyps fulvus fulvus*) in Israel for exposure to environmental toxicants using dried blood spots. *Archives of Environmental Contamination and Toxicology* 62: 502–511.

Van Stralen K., Jager K., Zoccali C., Dekker F. (2008) Agreement between methods. *Kidney International* 74: 1116–1120.

von Hurst P.R., Moorhouse R.J., Raubenheimer D. (2016) Preferred natural food of breeding Kakapo is a high value source of calcium and vitamin D. *Journal of Steroid Biochemistry and Molecular Biology* 164: 177–179.

Woodhouse S.J., Rick M. (2016) The effect of UVB radiation on serum vitamin D and ionized calcium in the African spoonbill (*Platalea alba*). *Journal of Zoo and Wildlife Medicine* 47: 447–456.

Wyss F., Wolf P., Wenker C., Hoby S., Schumacher V., Béchet A., Robert N., Liesegang A. (2014) Comparison of plasma vitamin A and E, copper and zinc levels in free-ranging and captive greater flamingos (*Phoenicopterus roseus*) and their relation to pododermatitis. *Journal of Animal Physiology and Animal Nutrition* 98: 1102–9.