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The vitamin D system in iguanian lizards

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Abstract

The apparent plasma concentration of vitamin D binding protein (DBP) in an iguanian lizard, *Pogona barbata*, and the affinity of this protein for 25-hydroxyvitamin D_3 ($25(OH)D_3$), 25-hydroxyvitamin D_2 ($25(OH)D_2$), and 1,25-dihydroxyvitamin D_3 ($1,25(OH)D_3$) was found to resemble more closely that of the domestic hen than that of the human. The characteristics of *Pogona* DBP, the pattern of vitamin D metabolites derived from injected radioactive vitamin D_3 and the plasma concentrations of endogenous 25-hydroxyvitamin D (25(OH)D) in a range of iguanian lizards have been examined. The findings suggest that 25-hydroxyvitamin D (25(OH)D) is the major metabolite of vitamin D, and that it may represent the storage form of vitamin D in these species in the same way as in mammals. High concentrations of vitamin D within iguanian embryos and egg yolks suggest a role for this compound in embryogenesis in these species, and perhaps indicates that there is a mechanism for vitamin D delivery to eggs comparable to that found in the domestic chicken.

Keywords: Reptile; Squamate; Lizard; *Pogona*; Egg; Cholecalciferol; Vitamin D; Vitamin D binding protein

1. Introduction

Vitamin D is a secosterol, and is the parent compound of an endocrine system that also includes its metabolites 25-hydroxyvitamin D_3 ($25(OH)D_3$), as the major storage molecule, and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) as the biologically active hormone. Vitamin D under natural conditions is thought to be derived mainly from the photosynthetic conversion of 7-dehydrocholesterol in the skin [19]. The vitamin D so formed is transported to the liver where its first hydroxylation takes place to form $25(OH)D_3$ [29]. $25(OH)D_3$ is further hydroxylated in the kidney to $1,25(OH)_2D_3$ [12,13]. Vitamin D and its metabolites are transported in blood in association with a vitamin D-specific binding globulin (DBP) [7,20]. By retaining vitamin D and its metabolites in the extracellular fluid, DBP limits their catabolism, and allows blood plasma to act as the major storage site for $25(OH)D_3$ [4,14].

The metabolism and functions of vitamin D have been extensively studied in mammals and birds [10], but relatively little is known about the physiology of vitamin D in reptiles. Squamate reptiles (lizards and snakes) have been shown to possess $25(OH)D_3$-1-hydroxylase activity [18], as well as vitamin D-dependent calcium binding proteins in kidney tissue [31]. Intraperitoneal administration of vitamin D_3 is reported to have caused hypercalcaemia in a lizard, *Varanus flavescens* [34], and a snake *Natrix piscator* [32,33]. Vitamin D metabolites have been shown to bind to protein fractions in the plasma of several species of reptiles [15,25].

If captive squamate reptiles are not exposed to UV light they may develop signs of vitamin D deficiency which are similar to those in mammals and birds [2,3]. Calcium deficiency impairs bone formation of the squamate embryo, and the hatchability of squamate eggs [24], and because deficiency of vitamin D may interfere with avian embryonic development [8,12,28] it is likely that vitamin D is also required for squamate embryogenesis. Although it is presumed that vitamin D has a function in squamate reptiles, no detailed studies of the physiology of vitamin D in these animals have been reported.

Abbreviations: DBP, vitamin D binding protein; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)D, 1,25-dihydroxyvitamin D.

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The properties of the plasma vitamin D-binding protein has been investigated in an Australian iguanian, and its affinities for various vitamin D_3 and vitamin D_2 metabolites compared to those of the DBP in human and chicken plasma. These studies suggest that the formation of vitamin D and its metabolism in agamid and iguanid squamates may be similar to that observed in mammals and birds, although with quantitative differences. It is also proposed that vitamin D has a function in iguanian eggs similar to that demonstrated in avian eggs.

2. Materials and methods

2.1. Animals and subjects

Lizards for this study were chosen from two closely related families within the suborder Iguania, the Agamidae: eastern water dragon (*Physignathus lesueurii lesueurii*), coastal bearded dragon (*Pogona barbata*), and frilled lizard (*Chlamydosaurus kingii*) and the Iguanidae: green iguana (*Iguana iguana*), and rhinoceros iguana (*Cyclura cornuta cornuta*). Individuals were obtained from a variety of sources, both wild specimens, and captive animals held in various institutions in Australia. All captive animals were housed out of doors, and were able to regulate their own exposure to natural sunlight. Blood from the subcaudal venous sinus was collected into heparinised tubes. Plasma obtained by centrifugation was stored at −20°C until analysis. Human blood was collected into heparinised tubes from the radial vein of healthy male and female laboratory workers and the plasma was stored at −20°C until analysis. Plasma from domestic chickens was obtained from 5-week-old male broiler-type birds.

2.2. Binding protein studies

The concentration of DBP in plasma and its affinity for $25(OH)D_3$, $25(OH)D_2$, and $1,25(OH)_2D_3$ were calculated from competitive protein binding assay, a modification of a method described elsewhere [39]. Briefly, plasma from each species was diluted 1:7500 in barbital acetate buffer (pH 8.6). A standard amount (0.35 pmol; approximately 10 000 dpm) of [^{3}H]$25(OH)D_3$ (Amersham) in ethanol, and increasing concentrations of $25(OH)D_3$ (0.18–11.9 nmol/l), $25(OH)D_2$ (0.18–11.9 nmol/l), or $1,25(OH)_2D_3$ (5–1250 nmol/l) (Hoffman La-Roche, Switzerland) in ethanol were added to successive tubes containing the diluted plasma, so that the final volume of ethanol in each tube was less than 10% of the total volume. The association of ligand and protein was allowed to reach equilibrium at 4°C for 2 h, after which a dextran/charcoal solution was added, and the tubes were centrifuged to remove the unbound ligand. Radioactivity (representing the bound fraction of [^{3}H]$25(OH)D_3$) was estimated in the supernatant.

The maximum binding (and hence the DBP concentration assuming a single class of specific binding sites and a stoichiometric ratio of protein to ligand of 1:1) and the dissociation constant (K_D) of specific binding sites for $25(OH)D_3$ were determined by plotting total bound [^{3}H]$25(OH)D_3$ on the *Y*-axis, and total competing $25(OH)D_3$ on the *X*-axis. It has previously been demonstrated that most species possess a single specific binding protein [15,25], and no species have yet been identified to possess a DBP with multiple vitamin D ligand binding sites. Curves describing total binding were created by fitting a nonlinear regression equation to the data using the Marquardt–Levenberg algorithm in a commercial graphics software package (Sigma Plot v3.0; Jandel Scientific) by the method of Swillens [35]. The regression equation describes the non-specific binding as a linear function and the specific binding using a hyperbolic curve, and was chosen because a relatively high fraction of ligand is bound in the assay procedure (thus violating the rules of standard binding regression analysis). The dissociation constants of the plasma for $25(OH)D_2$ and $1,25(OH)_2D_3$ were determined by a modification of the Cheng and Prusoff technique [26], where the amount of non-labelled competitor required to displace 50% of a radioligand with known K_D (in this case [^{3}H]$25(OH)D_3$) is used to determine the K_D of the competitor.

2.3. Characterisation of circulating vitamin D metabolites

Two adult male Coastal Bearded Dragons (*P. barbata*) received an intraperitoneal injection of approximately 10 000 dpm [4-^{14}C]vitamin D_3 dispersed in homologous plasma (500 μl). Blood (1.5 ml) was collected 96 h later. Vitamin D and its metabolites were extracted from plasma using chloroform/methanol (2:1 v/v) and the lipid extract was fractionated on a 60 × 1 cm Sephadex LH20 (Pharmacia) column using chloroform/hexane/methanol (75:23:2 v/v/v). Radioactivity in 2 ml fractions was measured by liquid scintillation spectrometry.

2.4. Plasma 25(OH)D concentration

Plasma 25(OH)D concentration was determined in partially purified lipid extracts using a competitive protein binding assay modified from a procedure described by Mason and Posen [27]. Dilute human plasma in barbital acetate buffer (pH 8.6) was used as a source of binding protein.

2.5. Measurement of vitamin D_3 in eggs by high performance liquid chromatography (HPLC)

Two complete yolks collected from the ovary of a green iguana (*I. iguana*) post mortem, and an entire eastern water dragon (*P. leseurii*) embryo at hatching, were analysed for vitamin D content by high performance liquid chromatography. Vitamin D in squamate eggs was partially purified, and then quantified by a method adapted from Kobayashi et al. [21]. Samples were saponified, and the unsaponifiable material extracted with light petroleum. Preliminary purification was performed on silica Sep Pak cartridges (Waters). Preparative HPLC was performed on a reversed phase C18 column (Ultrasphere ODS 5 mm, 10 mm × 25 cm; Beckman) using 100% methanol as the mobile phase. Analytical HPLC was performed on a straight phase silica column (Ultrasphere Si 5 mm, 4.6 mm × 25 cm; Beckman), with a mobile phase of 0.4% isopropanol in hexanes. An estimate of the efficiency of the extraction procedure was made from the recovery of approximately 2000 DPM of [1,2-^{3}H]vitamin D_3 added to each sample prior to saponification.

2.6. Measurement of calcium in plasma and solid tissues

An eastern water dragon embryo at hatching, and six developed yolks from the ovary of a female green iguana, were analysed for calcium content. Lipids were extracted from solid tissues by refluxing with ethanol, before combustion of the inorganic material at 600°C in a muffle furnace. The resultant ash was dissolved in 50% HNO_3. Plasma samples from squamates were diluted in 5 g/l KCl. The calcium content of the dissolved ash, and diluted serum samples was measured by flame atomic absorption spectroscopy. Reference sera, obtained from Boehringer Mannheim, were included as internal quality controls.

2.7. Data analysis

All data are presented as mean ± S.E.M. Statistical analysis of data was performed using standard Pearson's correlation, one-way analysis of variance (ANOVA) and Student's *t*-tests.

3. Results

3.1. Binding studies

Examples of homologous binding curves determined from the equilibrium binding of [^{3}H]25(OH)D_3 and 25(OH)D_3 to dilute plasma from human, chicken, and an iguanian (*P. barbata*) are shown in Fig. 1. Fitted curves all resulted in a coefficient of multiple determination (R^2) greater than 99%. Values for plasma DBP concentration in each of the species, and K_D values for 25(OH)D_3, 25(OH)D_2 and 1,25(OH)$_2$$D_3$ are presented in Table 1.

The concentration of DBP in human plasma was significantly higher than that in chicken plasma ($P < 0.001$) and also significantly higher than that in squamate plasma ($P < 0.005$) There were no statistically significant differences in the plasma DBP concentrations between *Pogona* and chicken plasma, nor between the K_D values for [^{3}H]25(OH)D_3 in all three species. However, K_D was significantly higher for 25(OH)D_2 than for 25(OH)D_3 in the chicken ($P < 0.05$) and in

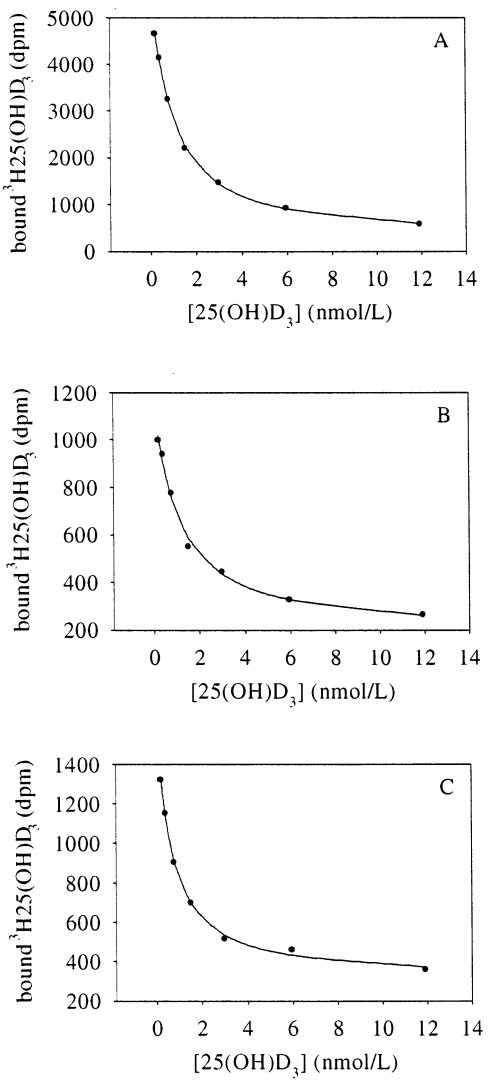

Fig. 1. Examples of homologous displacement curves for [^{3}H]25-hydroxyvitamin D_3 (25(OH)D_3) and 25(OH)D_3, from which plasma vitamin D binding protein (DBP) concentration and K_D for 25(OH)D_3 were calculated. Points represent duplicate measurements. R^2 for all binding curves in the study was greater than 99%. (A) Human plasma; (B) chicken plasma; (C) bearded dragon (*Pogona barbata*) plasma.

Table 1
Vitamin D binding characteristics of human, chicken, and squamate plasma[a]

	Plasma DBP (μmol/l)	K_D for 25(OH)D_3 (nM)	K_D for 25(OH)D_2 (nM)	K_D for 1,25(OH)$_2D_3$ (nM)
Human ($n = 3$)	$2.4^{a} \pm 0.16$	$0.16^{c,1} \pm 0.039$	$0.15^{d,1} \pm 0.020$	$109^{f,2} \pm 22.5$
Domestic chicken ($n = 3$)	$0.48^{b} \pm 0.12$	$0.33^{c,1} \pm 0.19$	$4.02^{e,2} \pm 0.89$	$100^{f,3} \pm 22.4$
Bearded dragon (*P. barbata*) ($n = 3$)	$0.70^{b} \pm 0.13$	$0.25^{c,1} \pm 0.073$	$4.04^{e,2} \pm 0.73$	$310^{g,3} \pm 10.6$

[a] Different letters within columns indicate significant differences (see text); different numbers across rows indicate significant differences (see text).

Pogona ($P < 0.01$), but not in the human plasma. In all three species K_D for 1,25(OH)$_2D_3$ was significantly higher than for either of the other two metabolites ($P < 0.05$), although *Pogona* plasma had a significantly higher K_D for 1,25(OH)$_2D_3$ than either of the other two species ($P < 0.005$).

3.2. Plasma metabolites of vitamin D

The Sephadex LH20 chromatographic pattern of radioactivity from plasma lipid of *P. barbata* injected intra-peritoneally with [^{14}C]vitamin D_3 is shown in Fig. 2. The first peak eluted with standard [1,2-^{3}H]vitamin D_3. The second peak co-chromatographed with standard [^{3}H]25(OH)D_3, and represents the major peak found in *Pogona* plasma.

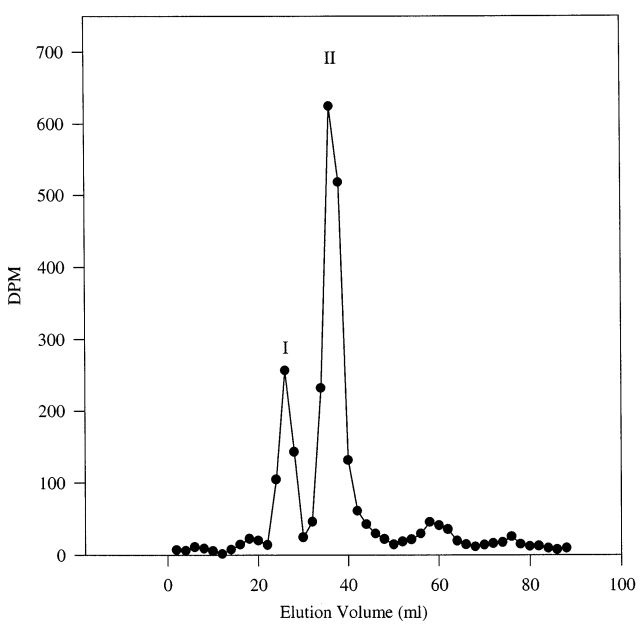

Fig. 2. Pattern of elution of radioactivity from a Sephadex LH20 column after application of plasma from a bearded dragon (*Pogona barbata*) previously dosed with [^{14}C]cholecalciferol by intraperitoneal injection. Peak I eluted with standard [^{14}C]cholecalciferol. Peak II eluted with standard [^{3}H]25-hydroxycholeaclciferol.

3.3. Concentrations of circulating 25(OH)D_3 and calcium in squamate plasma

Table 2 displays the results of biochemical analyses performed on plasma collected from lizards, and compares them with values for humans, and domestic chickens. The data for the lizards combines values determined from individuals from five iguanian species (*P. lesueurii*, *P. barbata*, *C. kingii*, *I. iguana*, and *C. cornuta*). There was no sex- or species-dependent difference between mean values of plasma 25(OH)D concentration among the lizards. However, it was found that the concentration of 25(OH)D in lizard plasma was significantly higher than that found in healthy adult humans and chickens ($P < 0.0001$). Human plasma concentrations of 25(OH)D compared well with previously reported results [9] and were significantly higher than those for chickens ($P < 0.005$). There was no significant difference in mean plasma calcium levels on the basis of sex, or species, among the iguanians. Unusually high plasma calcium concentrations found in a few females were believed to be associated with vitellogenesis and were omitted from statistical analyses. These high plasma calcium concentrations are presumably due to chelation to the yolk protein, phosvitin, during transport to the ovarian follicles.

There was no correlation between plasma calcium concentration, and plasma 25(OH)D concentration (Pearson's correlation coefficient = 0.052).

Table 2
Plasma biochemistry for humans, chickens, and iguanians

	Plasma 25(OH)D concentration (nmol/l)	Plasma calcium concentration (mg/l)
Iguanian	105 ± 14 ($n = 26$)[c]	104 ± 3.7 ($n = 27$)
Human	56 ± 2 ($n = 61$)[a,d]	85–105[e]
Chicken	45 ± 4 ($n = 40$)[b]	ND

[a,c] Indicates statistical significance ($P < 0.0001$) from all other species.

[b] Indicates statistical significance ($P < 0.005$) from human and squamate values.

[d] From previous study in this laboratory (Trube, personal communication).

[e] From The Merck Manual 13th edition. Berkow R, editor. Rahway, N.J.: Merck Sharp and Dohme Research Laboratories, 1977.

3.4. Vitamin D and calcium in squamate eggs

The vitamin D content, as determined by HPLC, of a water dragon (*P. lesueurii*) embryo at hatching was found to be 100 ng/g. The average vitamin D content of two undeveloped green iguana (*I. iguana*) yolks was 74 ng/g. The total calcium content of a water dragon embryo as determined by atomic absorption spectrometry was 25.9 mg, while that of six green iguana yolks was 38.7 ± 1.5 mg (mean $\pm$ S.E.M.).

4. Discussion

Vitamin D has been identified in species as diverse as plants [30], gastropods [23], and fish [1,36], and has long been known to be present in birds and mammals. 25(OH)D-1-hydroxylase activity has been demonstrated in many species of fish, amphibia, reptiles, birds and mammals [15,17,38] suggesting that the vitamin D endocrine system is of early evolutionary origin, and has been conserved over a wide range of vertebrate and invertebrate groups. However, qualitative differences in the vitamin D system have been identified between some of these groups. The active biological metabolites of vitamin D in the land snail have been identified as $25(OH)D_3$ and an as-yet unidentified compound designated metabolite E [23]; a role for $1,25(OH)_2D_3$ has yet to be determined in this species. $25(OH)D_3$-1-hydroxylase activity, found primarily in the kidney of mammals [12], has been shown to be present in the liver of several species of fish [22,37]. It is possible that other variations in the physiology of vitamin D may occur in other vertebrate groups, for although vitamin D has been extensively studied in humans and domestic animals [10], relatively little work has been reported in other species.

Vitamin D metabolites may bind to a variety of non-specific proteins in plasma, but are mostly associated with a specific carrier, vitamin D-binding protein (DBP) [7], shown to have the electrophoretic mobility of an α-globulin in mammals, reptiles, and some birds [15,25]. Its binding properties, and its concentration in plasma have some influence on the rate of metabolism and biological function of vitamin D since these determine the quantity of free ligand in plasma, from which uptake by cells may occur [4]. The ability of DBP to bind tightly to 25-hydroxylated metabolites of vitamin D is the main reason for these metabolites being retained in the circulating blood.

The concentration of the major plasma transport protein for vitamin D compounds in iguanians was calculated to be in the micromolar range, as has been demonstrated in other vertebrate species [5,6]. The value was comparable to that found in chicken plasma, but significantly lower than that found in human plasma. The calculated K_D of iguanian DBP for $25(OH)D_3$ was in the nanomolar range, as has been demonstrated for other species previously [5,6]. DBP has been found to have the highest affinity for vitamin D metabolites which are hydroxylated in the 25 and/or 24 positions while the further addition of a hydroxyl group at the 1-position seems to diminish the affinity of DBP for the vitamin D molecule [14]. Furthermore, Hay and Watson [16] found that plasma from certain fish, reptiles and birds bound $25(OH)D_3$ more efficiently than it did $25(OH)D_2$ (a form of vitamin D derived from ergosterol), which, in animals, can obtain only from dietary sources. In our experiments, human, chicken, and *Pogona* plasma, all demonstrated a lower K_D (and hence a higher affinity) for $25(OH)D_3$ than for $1,25(OH)_2D_3$. In addition, plasma from the chicken and the squamate (but not from the human) demonstrated a higher K_D (and hence a lower affinity) for $25(OH)D_2$ than for $25(OH)D_3$. Human plasma demonstrated equal affinity for both 25-hydroxylated ligands. These data suggest that in iguanian lizards, as in other vertebrate groups, $25(OH)D_3$ is the metabolite of vitamin D most likely to be retained in the blood plasma, and hence acts as a storage metabolite, while $1,25(OH)_2D_3$ is unlikely to remain in the circulation for any appreciable amount of time.

Following intraperitoneal administration of [^{14}C]vitamin D_3 to *Pogona* the major circulating labelled metabolite appeared to have the same chromatographic properties as $25(OH)D_3$. Furthermore, we observed that in iguanian lizards able to regulate their own exposure to natural sunlight, the average 25(OH)D concentration in plasma was 105 ± 14 nmol/l, which is somewhat lower than that previously reported in wild green iguanas in Costa Rica (365 nmol/l) [2]. However, it was significantly higher than that found in other vertebrate species (Table 1), and may reflect the habit of many iguanian species of basking for long periods in sunlight. These high concentrations support the view that 25(OH)D represents the naturally occurring major circulating metabolite in these species. There was no significant correlation between plasma 25(OH)D concentration and plasma total calcium concentration. The apparent lack of a relationship between these two biochemical parameters helps to confirm the view that this vitamin D metabolite in iguanians operates not as a regulated, biologically active metabolite, but as a storage form of vitamin D, as suggested for other vertebrate species.

It is well established that vitamin D is required for embryogenesis and hatching of avian eggs [8,28]. Furthermore, it has been demonstrated that in chickens, vitamin D_3, and not the major circulating metabolite, $25(OH)D_3$, is selectively transported into the egg [11]. The vitamin D concentrations in green iguana (*I. iguana*) yolks (74.1 ng/g) (mean, $n = 2$), and that of an

eastern water dragon (*P. lesueurii*) embryo (99.5 ng/g) as measured by HPLC, were found to be very similar to that of the domestic chicken yolk (50–100 ng/g yolk) [11], although the sample numbers were limited. Such high concentrations of vitamin D in the egg yolks of iguanians suggest a role for this compound in reptilian embryogenesis as demonstrated in the developing avian embryo [8,28]. The fact that iguanians and chickens both have large concentrations of vitamin D in their eggs suggests that they may have similar selective transport mechanisms, demonstrated in chickens to involve the plasma vitamin D binding protein [11], to deliver vitamin D to their eggs.

These studies have shown that iguanians possess a vitamin D endocrine system that is physiologically similar to that of birds and mammals. These lizards have been shown to possess specific plasma binding of vitamin D metabolites that appears to resemble more closely that of birds than of mammals in its capacity and binding characteristics. Iguanians seem to share the same metabolic pattern as other vertebrate groups, and like birds, it seems that vitamin D may play a role in the development of iguanian embryos. Indeed the similarity between these two vertebrate groups with respect to vitamin D content of egg yolks, and between certain characteristics of the plasma DBP seem to suggest a common mechanism of incorporation of vitamin D into the egg. A thorough understanding of the physiology of vitamin D in other vertebrate groups will help to highlight specific adaptations and assist in our understanding of their implications.

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