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**Influence of feeding and UVB exposition
on the absorption mechanisms of Calcium in the gastrointestinal
tract of veiled chameleons (*Chamaeleo calyptratus*)**

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Summary

The aim of this study was to investigate the influence of feeding and UVB exposition on the occurrence and distribution patterns of vitamin D receptors (VDR) and calbindin D28k (Cb-D28k) in the gastrointestinal tract of veiled chameleons.

Thus, 56 hatchlings were divided into 6 treatment groups: UV (with UVB exposure); No (no supplements); CaAUV (with calcium (Ca), vitamin A (vitA), UVB exposure); CaA (Ca, vitA); CaADUV (Ca, vitA, vitD, UVB exposure); CaAD (Ca, vitA, vitD) and reared for 6 months on locust-based diets. Finally, tissue sections of stomach, duodenum, ileum and colon were stained immunohistochemically (IHC) against Cb-D28k or VDR and assessed semi-quantitatively.

VDR stained stronger in the luminal epithelium of duodenum than ileum, with more intense signals localized at the base of the villi when compared with the tip. Cb-D28k was mostly targeted to the luminal epithelium of the duodenum. In CaADUV and CaAD (groups with all supplements) Cb-D28k staining was stronger than in UV group.

The important role of duodenum in the active transcellular absorption of Ca in veiled chameleons is suggested. Whereas the expression of Cb-D28k seems to be regulated by dietary supplementation with vit. D and A, the expression of VDR appears higher in animals not supplemented with Ca or vit D and A. The latter may be due to the decreased Ca concentrations, causing mobilization of vitamin D in the skin of animals exposed to UVB only.

Keywords: veiled chameleon, calcium regulation, gastrointestinal tract, nutrition

Zusammenfassung

Der Einfluss der Fütterung sowie einer UV-B-Exposition (UVB) auf das Vorkommen und das Verteilungsmuster von Vitamin-D-Rezeptoren (VDR) und Calbindin D28k (CB) im Magen-Darm-Trakt des Jemenchamäleons wurden untersucht. 56 Jungtiere wurden in 6 Gruppen eingeteilt: UV (nur UVB); No (ohne Zusätze); CaAUV (Calcium (Ca), Vitamin A (VA), UVB); CaA (Ca, VA); CaADUV (Ca, VA, Vitamin D (VD), UVB); CaAD (Ca, VA, VD). Die Tiere wurden 6 Monate mit einer Diät auf Heuschreckenbasis aufgezogen. Magen, Duodenum, Ileum und Kolon wurden entnommen und Gewebeschnitte semiquantitativ immunhistochemisch auf CB und VDR untersucht. Im Oberflächenepithel war die VDR-Immunreaktion im Duodenum stärker als im Ileum und an der Basis der Zotten höher als an der Spitze. CB wurde hauptsächlich im Oberflächenepithel des Duodenums beobachtet. Die beiden mit sämtlichen Zusätzen (CaADUV, CaAD) behandelten Gruppen zeigten eine höhere CB-Immunreaktion als die Gruppe lediglich mit UVB-Exposition. Das Duodenum scheint bei der aktiven transzellulären Absorption von Ca beim Chamäleon eine wichtige Rolle zu spielen. Während die Expression von CB durch den Zusatz der Vitamine D und A reguliert zu werden scheint, wird der VDR dann hochreguliert, wenn die Tiere nicht mit Ca sowie den Vitaminen A und D versorgt wurden. Letzteres könnte an den verminderten Ca-Konzentrationen liegen, die eine Aktivierung von Vitamin D in der Haut bei Fehlen jeglichen Futterzusatzes aber unter UVB hervorzurufen vermögen.

Schlüsselwörter: Jemenchamäleon, Calcium-Stoffwechsel, Magen-Darm-Trakt, Fütterung

Influence of feeding and UVB exposition on the absorption mechanisms of Calcium in the gastrointestinal tract of veiled chameleons (*Chamaeleo calyptratus*)

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Running head: Calcium absorption in chameleons

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Introduction

The global trade of chameleons is still predominantly composed of animals caught in the wild. In recent years, successful captive breeding of several chameleon species has been possible due to the increased knowledge of chameleon husbandry (Carpenter et al., 2004). It is widely accepted that inadequate diet composition in regard to vitamins and/or minerals can lead to different metabolic diseases (e.g. nutritional metabolic bone disease), that are characterized by functional and morphological changes in bone that result from dietary calcium (Ca) and phosphorus (P) imbalance, vitamin A and vitamin D deficiency in the diet, or a lack of UVB light exposure followed by the effects of secondary hyperparathyroidism (Coke, 1998; Abate et al., 2003, Hoby et al., 2010).

Ca is an important mineral with multiple functions. In chameleons, 1% of body Ca found outside the bone is sufficient for maintaining good health, where it regulates enzyme activation, muscle contraction, nerve transmission, heartbeat, blood clotting, etc. (Donoghue, 2002). It is well recognized that the intestinal absorption of calcium occurs by two distinct mechanisms, a saturable active transport process and a non-saturable passive diffusion process. Bronner et al. (2003) suggest that the major mode of calcium absorption is by vitamin D-independent passive diffusion and not by active transport in mammals. Moreover, Bronner et al. (1988) state that in the ileum of rats, calcium is absorbed only by the passive pathway, which they consider to be a vitamin D independent process.

Active Ca absorption in the intestine of chicken is regulated by the active metabolite of vitamin D (1,25(OH)₂ vitamin D₃ or calcitriol), which binds to the vitamin D steroid hormone receptor (VDR) (Pike and Haussler, 1979). Vitamin D on the other hand regulates the synthesis of two cytosolic calcium-binding proteins, calbindin D28k (Cb-D28k) and calbindin D9k (Cb-D9k) which seem to play a role in Ca-ion translocation within the cell (Bronner et al., 1988). The distribution patterns of these two proteins appear to be different between species: Cb-D28k was observed in duodenal absorptive cells of birds (Kallfeltz et al., 1967), while Cb-D9k was found in the intestines of different mammals (Hitchman and Harrison, 1972; Liesegang et al., 2007; 2008). To date, there is no information on the role of Cb-D28k and VDR in the calcium absorption in reptiles.

The question of whether reptiles depend on dietary vitamin D or are capable of UVB-mediated endogenous vitamin D synthesis is controversially discussed (McWilliams, 2005). The results of Hoby et al. (2010) indicate that in the veiled chameleon, the endogenous synthesis of vitamin D is more important compared to the dietary ingestion of vitamin D. The endogenous synthesis of vitamin D₃ occurs as a result of the photosynthetic conversion of 7-dehydrocholesterol to pre-vitamin D₃. Pre-vitamin D₃ is converted to vitamin D₃ via a temperature dependent process. Holick et al. (1995) stated that 50% of pre-vitamin D₃ in lizards is converted to vitamin D₃ in 8 and 72 hours at the temperature of 25°C and 5°C, respectively. At this point, the hormone is transported to the liver, where it is hydroxylated to 25-hydroxycholecalciferol (25(OH) D₃ or calcidiol) by the enzyme 25-hydroxylase and then is further hydroxylated in the kidneys by the enzyme 1 α -hydroxylase to the main biologically active form of vitamin D₃ (1,25(OH)₂ vitamin D₃). In addition to vitamin D₃ and calcium, vitamin A plays an important role for growth, reproduction and bone health (Olson, 1984). Vitamin A supplementation appears to be an important factor in the

health and husbandry of chameleons (Ferguson et al., 1996). Many insects have been found to be poor sources of vitamin A (Barker, 1997; Bowers and McCay, 1940; Dierenfeld et al., 1995; Hoby et al., 2010; Martin et al., 1976; Pennino et al., 1991). Therefore, insects fed to captive animals are often supplemented with vitamin A to improve their nutritional content (Sabatini et al., 1998). While it appears necessary to compensate for a lack of vitamin A in an insect-based diet, the exact amount of this vitamin requires further study. To date, vitamin A requirements have only been established for mammals and birds (Robbins, 1993). High levels of dietary vitamin A interact with vitamin D₃ and may affect the utilization of vitamin D₃. When administered in high dosages, both vitamins can be potentially toxic (Abruto et al., 1998).

The present study examines mechanisms of Ca absorption in veiled chameleons (*Chamaeleo calytratus*) through the detection of VDR and Cb-D28k using semi-quantitative immunohistochemistry (IHC). Furthermore, the influence of different diets on parameters of the intestinal calcium absorption of the growing chameleons is studied.

These investigations are important for animal welfare, both for the individual animal and the species as a whole. Breeding in human care may be improved because of better husbandry, feeding conditions and nutrient composition due to better knowledge of the physiology of these animals.

Materials and Methods

Experimental Design

The study was carried out using 56 hatchlings of chameleons (27 females and 29 males) that were randomly divided into 6 groups as described previously (Hoby et al., 2010):

Group UV: with UVB exposure; n=10

Group No: no supplements; n=10

Group CaAUV: with calcium (Ca), vitamin A and UVB exposure; n=9

Group CaA: with Ca and vitamin A n=9

Group CaADUV: with Ca, vitamin A, vitamin D and UVB exposure; n=9

Group CaAD: with Ca, vitamin A and vitamin D; n=9

The study was performed in accordance with the Swiss animal welfare regulations and approved by the cantonal veterinary office (permit no. 2172). The animals were randomly divided into six groups. The chameleons were kept solitary in cylindrical plastic terraria (diameter 31 cm, height 42 cm) with ventilation slots (4 x 15 cm) at the sides covered by a cloth mesh (covered aperture of 2 x 2 mm single position). Regardless of the light source the temperature was kept between 22 and 30°C (vertical gradient and day-night gradient) and humidity between 40 and 65% (D HygroLog - 1.0, Rotronic AG, Bassersdorf, Switzerland). The general condition and behavior of the chameleons were checked daily. Criteria were defined to interrupt the experiment for the euthanasia of poorly performing chameleons.

All animals were reared for 6 months on locust-based diets. Additional calcium was administered to the locust nymphs (*Locusta migratoria*) by gut-loading for 48 hours (12% Ca per kg wet weight). These nymphs were offered to Ca groups (CaAUV, CaA, CaADUV, CaAD). Vitamin supplementation was provided by dusting the locust nymphs with vitamin A (250,000 IU/kg powder (75 mg/kg)) before feeding them to the groups CaAUV, CaA, CaADUV, CaAD and with vitamin D (25,000 IU/kg of powder (0.625 mg/kg)) to the groups CaADUV, CaAD. An UVB light source (Replux UV-plus 23W, Namiba Terra) was used in groups UV, CaAUV and CaADUV.

At the end of the study, with an age of 6 months, the chameleons were anesthetized using isoflurane (induction chamber with 5 vol% isoflurane in oxygen 0.6 L/min, anesthesia maintenance with mask) and euthanized by intracardially applied sodium pentobarbital (324 mg/kg body weight, Vetanarcol, Veterinaria). Thereafter, the samples of the gastrointestinal tract were taken.

Immunohistochemistry

The Formalin-fixed tissue samples were rinsed in tap water for 24 h, stored in 70% alcohol, finally cut into small pieces and placed in small Histosetts (Histosette 45° M498-4, Simport, Canada). All samples (1 Histosette per chameleon) were subsequently transferred into a tissue infiltration station (Leica TP 1020®) to undergo the following 4-hour program steps: 2x 70% alcohol, 1x 80% alcohol, 2x 96% alcohol, 3x 100% alcohol, 2x xylene, 2x paraffin. Finally, tissue blocks were embedded in paraffin (Histowax®, Leica) in an embedding station (EG 1160®, Leica). A rotary microtome (RM 2165®, Leica) was used to cut 3 µm sections. These were mounted on adhesion slides (Super Frost® Plus, Menzel-Gläser, Braunschweig, Germany), dried overnight at 37°C in an incubator (BE 400/3 TWW, Memmert GmbH, Schwabach, Germany) and used for IHC. All tissues were assessed according to classical morphological and light microscopy analysis to test for proper sampling and preservation.

Immunohistochemical methods

The localization of VDR and Cb-D28k by immunohistochemistry (IHC) was performed on sections of stomach, duodenum, ileum, and colon. For detection of the VDR, the rat monoclonal (9A7) anti-VDR antibody ab8756 (Abcam, Cambridge, UK) was used as primary antibody (1:50 dilution), followed by a secondary antibody anti-rat IgG:HRP complex developed in goat (Sigma-Aldrich Chemie GmbH, Buchs, CH; 1: 100 dilution). As primary antibody for detection of Cb-D28k, monoclonal anti-Cb-D28k (SWANT, Bellinzona, CH; 1: 150 dilution), was used and as a secondary antibody anti-mouse IgG (horse serum) (Vector Labs, USA; 1: 100 dilution) was used. Previously published protocols for IHC were used (Liesegang et al., 2008; Sidler-Lauff et al., 2010; Schroeter-Vogt, 2011). Duodenum of chicken was used as positive control. Negative controls were performed by omitting the primary antibodies and the application of TBS buffer and non-immune serum at the same protein concentration (Sidler-Lauff et al., 2010).

Semiquantitative evaluation of VDR immunoreactivity

All immunohistochemically stained tissue sections were assessed and evaluated under the microscope at 400x magnification (LEICA DM RXE, Leica Microsystems AG, Switzerland). To conduct a semi-quantitative assessment of VDR concentrations in the surface epithelium of all intestinal sections, each of the three sections were tested in 250 to 350 nuclei in different fields. From duodenum (DD), ileum (IL) and colon (CO) – on the surface epithelium from the base and to the tip of the villi three fictive lines were taken by separating the villi in three parts; base/bottom, middle, and the tip. Dependent on the grade of the brown color, enterocyte nuclei were counted in five separate categories: negative, very weak, weak, middle-strong, and strong color. After the visual grading of staining or color intensity (CI) – CI0 = negative; CI0,25 = very weak; CI1 = weak; CI4 = middle-strong positive; CI9 = strong positive – and frequency of the immunoreactive score (IRS) could be calculated by multiplying the relevant number of nuclei with an exponential conversion factor for the color intensity (CI) and adding the individual products. The following formula was used:
$$IRS = 0 \times n(CI0) + 0,25 \times n(CI0,25) + 1 \times n(CI1) + 4 \times n(CI4) + 9 \times n(CI9)$$
 (Liesegang et al., 2007; 2008).

Microscopic quantification of Calbindin-D28k – immunoreactivity

Evaluation of the sections was done with a computerized research microscope (LEICA DM RXE, Leica Microsystems AG, Switzerland). Slides were assessed quantitatively with a computerized digital camera analyzing the mean color intensity (i.e. grey scale values) of cells or tissues (AnalySIS Pro® - Version 5.0, Soft Imaging System GmbH). This procedure was always performed under standardized conditions, thus eliminating primarily errors normally occurring during visual grading of histochemical color intensities. Digitalized pictures were taken, white balance was performed (i.e. measurement in a tissue-free district of the section thus representing 100% transmission), randomly selected regions of interest were defined containing immunopositive structures and color intensity was measured. After assessing seven to nine tissue areas within the tissue, for each immunopositive structure per slide the mean value per pixel per structure and animal was calculated. Grey scale values (GS or GSV) were transformed into extinction (E) which is proportional to dye concentrations at the level of the sections ($E = \text{Lg}1(\text{GS}/256)^{-1}$) (Schroeter-Vogt, 2011) and then this value was used for further statistical analysis.

Statistical analysis

The aim of the statistical analysis was to assess if the response variables VDR and Cb-D28k were significantly associated with the six different treatment groups, the three different intestinal sections (for VDR) and the three different parts of the villi. In order to account for the presence of correlation due to multiple observations taken from the same animal, linear mixed models (with random effects) were chosen for VDR and Cb-D28k in the duodenum and a linear model approach for Cb-D28k in the colon. The response variable Cb-D28k was transformed to a logarithmic scale in order to improve the approximate normality of the observations. The results of the linear (mixed) models are given in the form of p-values (derived from type III tests) that is the significance of each partial effect with all other effects in the model.

All statistical analyses were performed using SPSS statistics software, Version 21.0. (IBM Corporation, Armonk, NY, U.S.A.). The plots were created with the free software R (R Core Team, 2013)

Results

Immunoreactions of Vitamin D receptor

Immunoreactions for VDR were detected in the duodenum (DD), ileum (IL) and colon (CO) in all tested veiled chameleons of all groups. In luminal surface epithelium (villi intestinales (VI)) and tunica muscularis (TM), immunoreactive cells (brown staining) were evident. Also, both nucleus and cytoplasm of enterocytes reacted positive. The goblet cells were entirely negative and not considered in this study. No immunoreactions were present in the stomach of all animals in all groups.

Immunoreactivities were a little higher in DD compared to IL and CO (Fig. 1). Immunoreactive scores for VDR did not differ significantly between groups ($p = 0.814$), but they were significantly different between intestinal segments (Fig. 2). Immunoreactions were significantly higher in DD compared to IL ($p < 0.01$, Fig. 2). There were significant differences between the three regions assessed in intestinal villi (Fig.3). At the base of the villi, the immunoreactive score was significantly higher compared to the tip (basal versus tip: $p = 0.002$ or $p < 0.01$, Fig. 3).

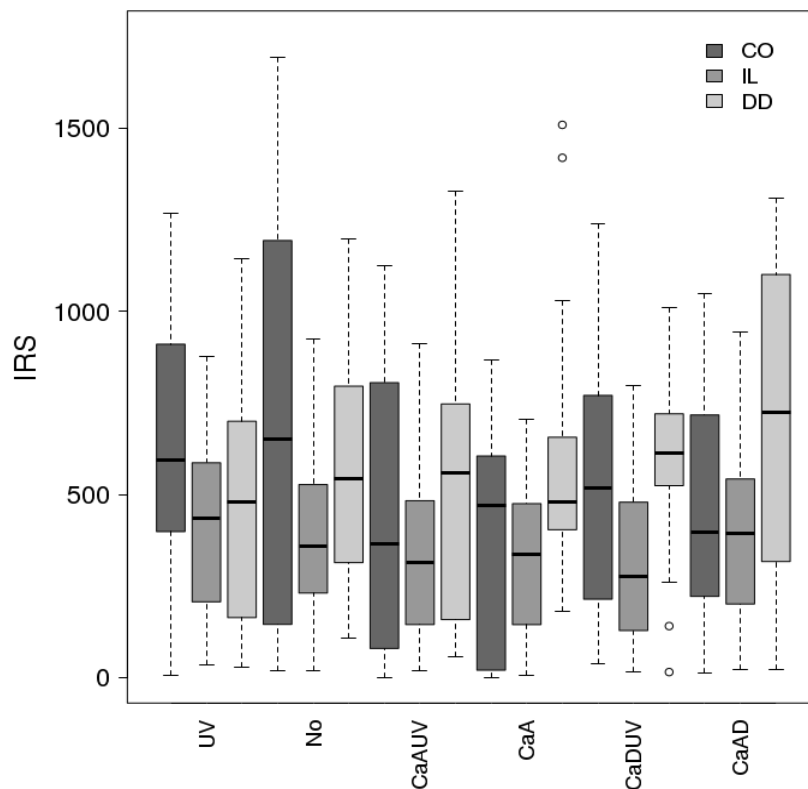


Figure 1: Comparison across treatment groups and organs on VDR expression in veiled chameleons (n=56).

Group UV: with UVB exposure; Group No: no supplements; CaAUV: with calcium (Ca), vitamin A and UVB exposure; CaA: with Ca and vitamin A; CaADUV: with Ca, vitamin A, vitamin D and UVB exposure; CaAD: with Ca, vitamin A and vitamin D. IRS - Immunoreactive Score; VDR - Vitamin D-receptors; n=56 - number of treated veiled chameleons. CO - Colon; IL - Ileum; DD - Duodenum.

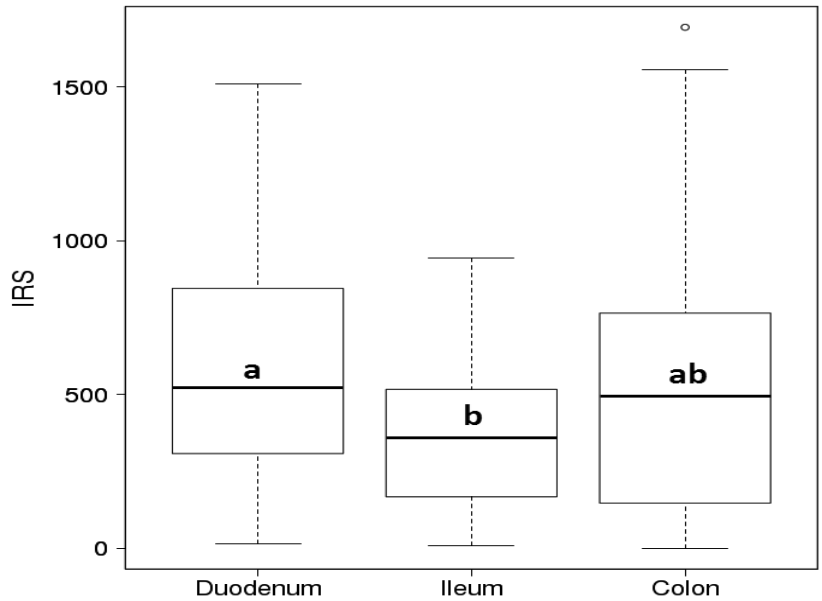


Figure 2: VDR expression of intestinal segments in veiled chameleons (n=56) with different feeding regime and UV exposure.

Group UV: with UVB exposure; Group No: no supplements; CaAUV: with calcium (Ca), vitamin A and UVB exposure; CaA: with Ca and vitamin A; CaADUV: with Ca, vitamin A, vitamin D and UVB exposure; CaAD: with Ca, vitamin A and vitamin D. IRS - Immunoreactive Score; VDR – Vitamin D-receptors; n=56 – number of treated veiled chameleons; a, b = means with different letters are significantly different ($p < 0.05$)

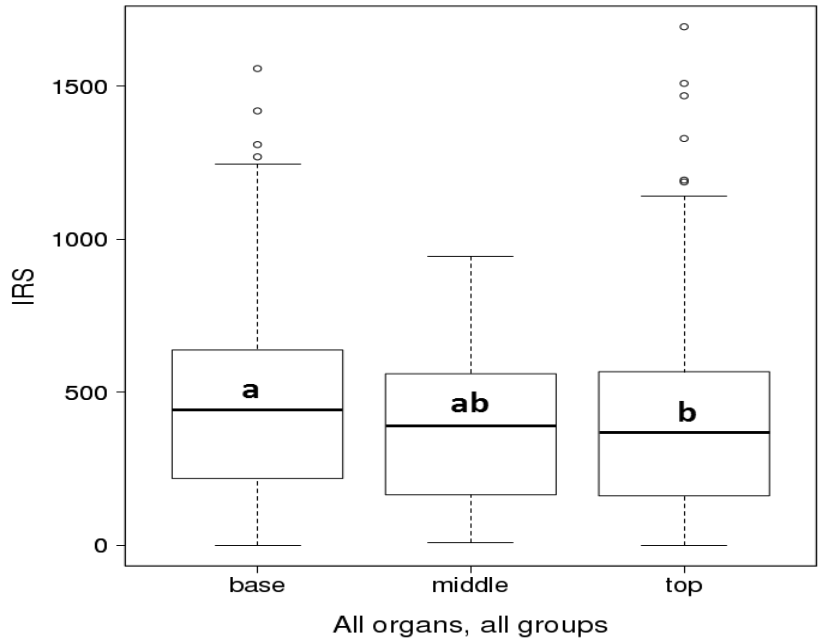


Figure 3: VDR expression between villi regions (base, middle, tip) in veiled chameleons (n=56) with different feeding regime and UV exposure.

IRS - Immunoreactive Score; VDR – Vitamin D-receptors; n=56 – number of treated veiled chameleons; a, b = means with different letters are significantly different ($p < 0.05$)

Immunoreaction of calbindin-D28k

The immunoreaction for Cb-D28k was mainly detectable in DD. Significant differences between treatment groups were found only in DD ($p = 0.007$ – strong evidence of group differences). Higher immunoreactions were detected in the groups receiving all supplements with or without additional UVB exposure (groups CaADUV, CaAD). Statistically significant differences were obtained between group CaADUV versus groups No ($p = 0.027$) and UV ($p = 0.036$) (Fig. 4). There was, however, no significant difference compared to the groups CaAUV and CaA.

The reaction in the CO was weak, and no treatment effect was observed ($p = 0.646$ – using a F-test - no evidence of group differences) (Fig. 5).

In the case of Cb-D28k there were no significant differences in signal intensity between the regions assessed within the villi duodenales. No immunohistochemical reactions were detected in the stomach and IL.

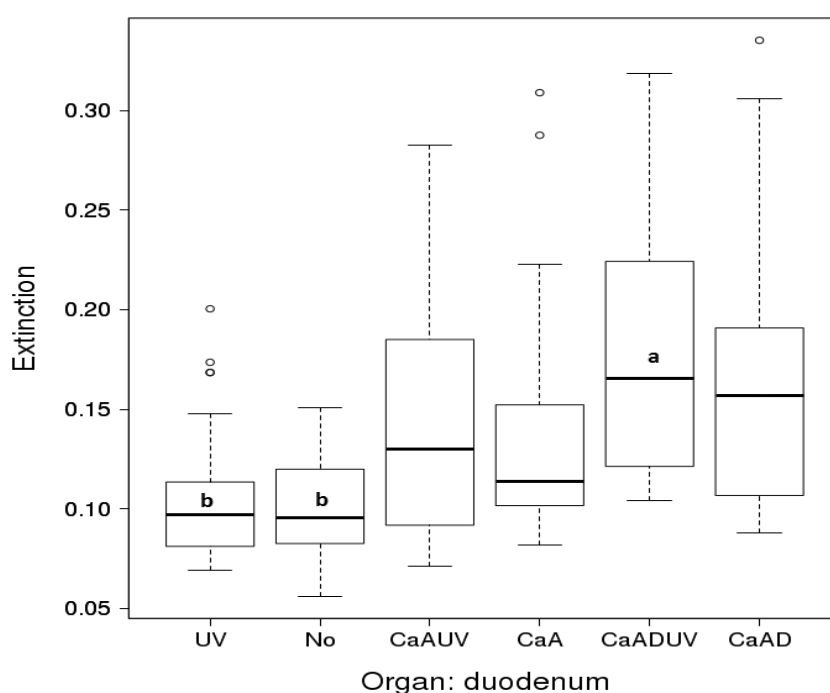


Figure 4: Influence of feeding and UV exposure on Cb-D28k expression in the duodenum.

Group UV: with UVB exposure; Group No: no supplements; CaAUV: with calcium (Ca), vitamin A and UVB exposure; CaA: with Ca and vitamin A; CaADUV: with Ca, vitamin A, vitamin D and UVB exposure; CaAD: with Ca, vitamin A and vitamin D. Cb-D28k – Calbindin-D28k; a, b = means with different letters are significantly different ($p < 0.05$)

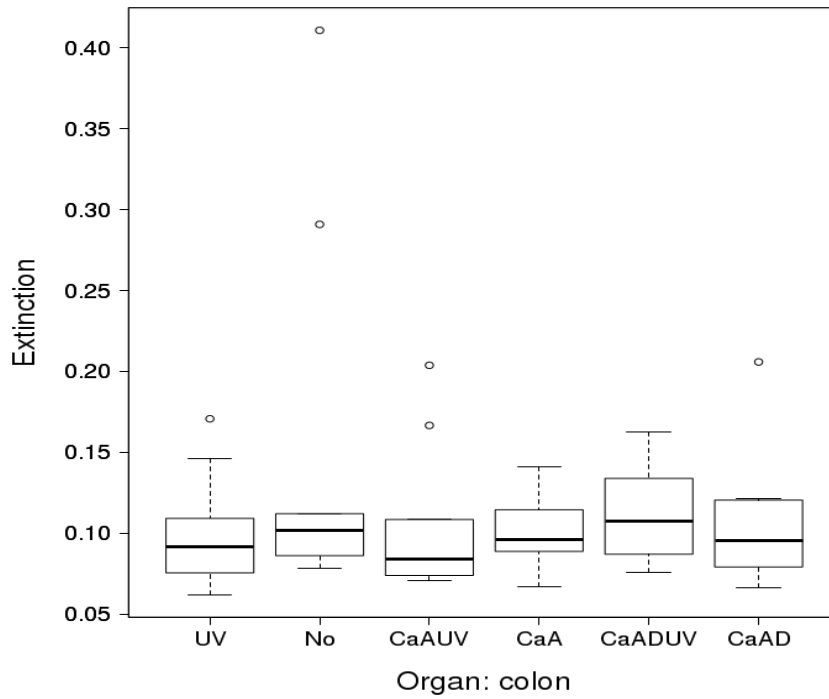
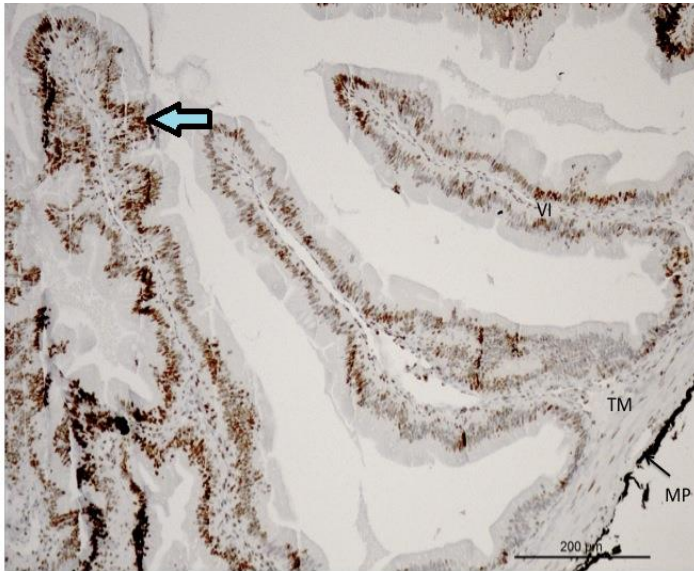
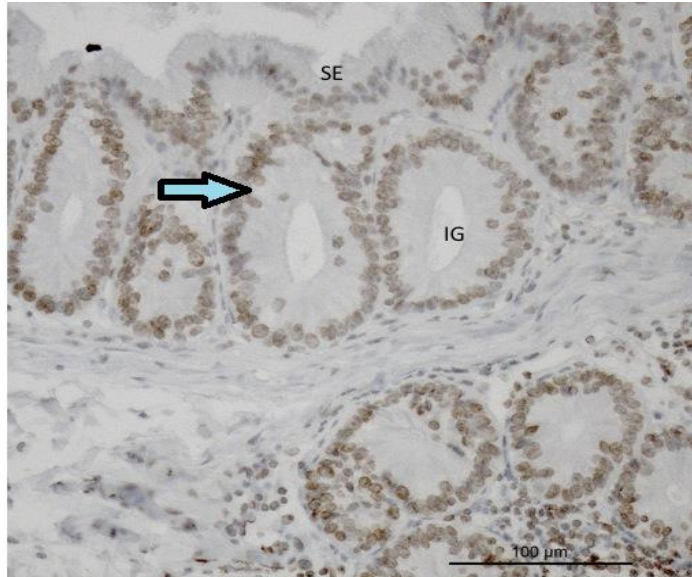


Figure 5: Influence of feeding and UV exposure on Cb-D28k expression in the colon. Group UV: with UVB exposure; Group No: no supplements; CaAUV: with calcium (Ca), vitamin A and UVB exposure; CaA: with Ca and vitamin A; CaADUV: with Ca, vitamin A, vitamin D and UVB exposure; CaAD: with Ca, vitamin A and vitamin D. Cb-D28k – Calbindin-D28k. No statistically significance could be detected ($p > 0.05$).



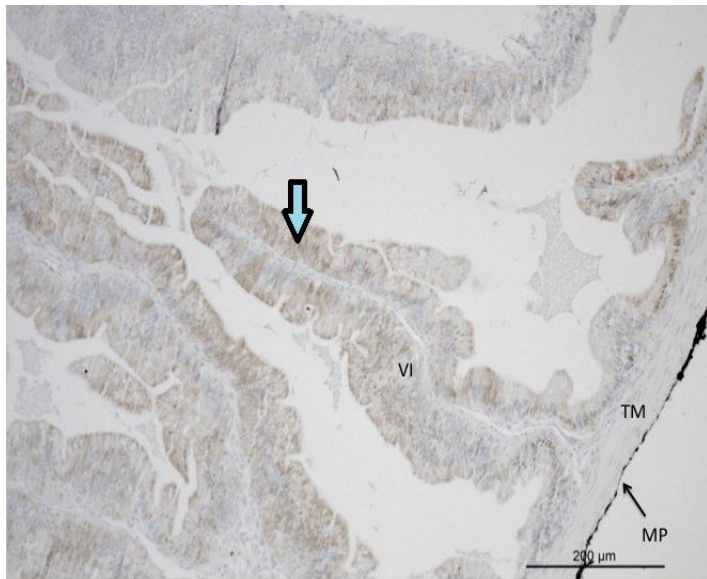
VI: Villi intestinales TM: Tunica muscularis MP: Melanin pigment in mesothelium

Figure 6a: Immunohistochemical reaction of VDR in the duodenum of a veiled chameleon with dietary supplementation of calcium, vitamin A, vitamin D and UVB exposure (CaADUV); in luminal epithelium of the villi intestinales (VI) and tunica muscularis (TM), immunopositive cells (brown staining) can be seen. Both nucleus and cytoplasm of enterocytes react positively. Goblet cells react entirely negative. (200x) *No intestinal glands. VDR - Vitamin D-receptors; VI - villi intestinales; TM - tunica muscularis; MP - Melanin pigment in mesothelium; 200x - Scale bar represents 200 μm .



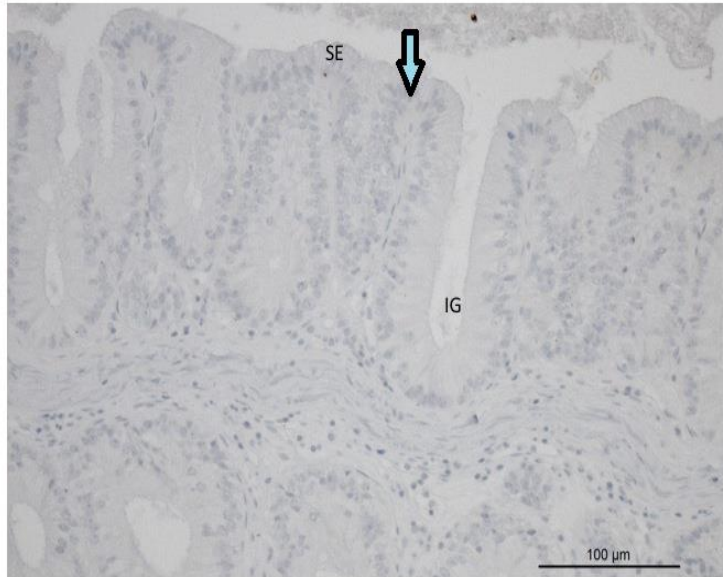
SE: Surface epithelium IG: Intestinal glands

Figure 6b: Immunohistochemical reaction of VDR in the colon of a veiled chameleon supplemented with calcium, vitamin A, vitamin D and UVB exposure (CaADUV). In areas denoted by SE and IG immunopositive cells are seen (brown staining). (100x). VDR - Vitamin D-receptors; SE - surface epithelium; IG - intestinal glands; 100x - Scale bar represents 100 μm .



VI: Villi intestinales TM: Tunica muscularis MP: Melanin pigment in mesothelium

Figure 7a: Immunohistochemical detection of Cb-D28k in the duodenum of a veiled chameleon supplemented with calcium, vitamin A, vitamin D and UVB exposure (CaADUV); luminal epithelial cells of VI are immunopositive (brown staining). (200x). *Note: there are no intestinal glands (IG) in duodenum of veiled chameleon. Ca-D28k – calbindin D28k; VI - villi intestinales; MP – Melanin pigment in mesothelium; 200x - Scale bar represents 200 μm .



SE: Surface epithelium IG: Intestinal glands

Figure 7b: Immunohistochemical reaction of Cb-D28k in the colon of a veiled chameleon supplemented with calcium, vitamin A, vitamin D and UVB exposure (CaADUV). In areas denoted by SE and IG very weakly reacting immunopositive cells are seen (brown staining). (100x)
Ca-D28k – calbindin D28k; SE - surface epithelium; IG - intestinal glands; 100x - Scale bar represents 100 μm .

Discussion

The bulk of information on reptile nutrition is based on observation, not quantification (Carman et al., 2000). Only a few studies have been carried out in reptiles studying calcium homeostasis, although many problems in reptile nutrition are due to an imbalanced diet. In this study, a physiological background on the mechanisms of calcium absorption was studied depending on different feeding regimes.

The main results of the present study were that animals fed with all supplements, i. e. Ca, vitamin A and vitamin D plus exposition to UVB, showed highest duodenal Cb-D28k immunoreaction and hence are indicative for an elevated active Ca transport through the cell. Although it has been shown, that in tissues other than the intestines, like brain, the expression of Cb-D28k is independent or only partially dependent like in the kidneys of chicken (Hall et al., 1990; 1991), 1,25(OH)₂ vitamin D₃ stimulates the expression of Cb-D28k, by increasing the rate of transcription of the corresponding gene in chicken (Ferrari et al., 1990). Since the Cb-D28k gene is a single-copy gene and the vitamin D receptor exists in virtually all tissues, it is possible that the different sensitivity to 1,25(OH)₂ vitamin D₃ depends on the activity of tissue-specific factors. However, the definition of the function, of tissue-specific factors has not been investigated in reptiles and the dependency of the active Ca transport can only be suspected, although no evidence has been described in reptiles until now.

Apart from the possible biochemical evidence of the action of 1,25(OH)₂ vitamin D₃ on the active transport in the intestine, the findings of an earlier study by Hoby et al. (2010) showing that chameleons fed with all the supplements plus UVB exposure as in the present study were in better health condition and developed no nutritional metabolic bone disease (NMBD), underline the fact that Cb-D28k may play a role in the homeostasis of Ca in growing reptiles. Furthermore, Stanford et al. (2005) drew also the conclusion, that provision of an adequate diet supplemented with vitamin D₃ and Ca plus UVB exposure was essential for the prevention of disorders of Ca metabolism in captive african Grey Parrots (*Psittacus e. erithacus*). In the present study, groups treated only with UV or without any supplements exhibited an impact of these factors on the distribution patterns of VDR and Cb-D28k in the gastrointestinal tract of veiled chameleons. It was shown that in these two groups Ca concentration in blood was low (Hoby et al., 2010), because UVB light might cause activation of vitamin D₃ and also of VDR expression. It is well known, that chameleons can adapt, within limits, to low Ca intakes by increasing absorption and decreasing excretion, as shown in wild-caught chameleons (Donoghue, S. 2002). The VDR and Cb-D28k immunoreactions were differently distributed. While the VDR immunoreactions were present in all six groups without any group effects (UV, No, CaAUV, CaA, CaADUV, CaAD) and significant differences were obvious between intestinal segments (highest immunoreaction in duodenum), the Cb-D28k immunoreactions showed a group effect with highest reactions in the group CaADUV. These findings are in accordance with studies on domestic ruminants (Liesegang et al., 2007; 2008; Sidler-Lauff et al., 2010) indicating that VDRs are highly expressed in duodenum at the site of active, vitamin D-dependent intestinal Ca absorption and that Cb-D28k, which is known for its high affinity for Ca, was possibly enhanced from the presence of all factors in these two groups (CaADUV and CaAD).

In the present study, there was a tendency to increased VDR immunoreactions when animals were not supplemented (groups UV and No). This finding may be due to the low Ca concentrations in the nymph locusts possibly causing endogenous active

vitamin D ($1,25(\text{OH})_2$ vitamin D_3) activation and for this reason in further consequence VDR expression. Nevertheless, this activation was not able to cover the deficiency in these 2 groups where most of the individuals (groups UV and No) developed nutritional metabolic bone disease (Hoby et al., (2010).

The present investigation showed for the first time the expression of Ca-binding protein Cb-D28k in the gastrointestinal tract of veiled chameleons. Detection of Cb-D28k is found mainly on the luminal epithelium of DD, and much less in IL and CO. The same has been reported in a variety of species, including fish, reptiles, amphibians, birds (Cb-D28k), and mammals (Cb-D9k) (Parmentier et al., 1987; Schröder et al., 1996). In rabbits, the most evident Cb-D28k activity is located in the cecum (De Vries de Heekelingen, 2008; Schroeter-Vogt, 2011). Apart from the intestine, Cb-D28k may be detected in many other tissues, including brain (Celio, 1990; Gerfen et al., 1985; Taylor et al., 1968). Cb-D28k was highly expressed in the group provided with all supplements (vitamin D, vitamin A, Ca, (CaAD)), which shows that intestinal Cb-D28k transcription was possibly stimulated by other factors than Ca deficiency. Instead, induction of Cb-D28k may be stimulated by $1,25(\text{OH})_2$ vitamin D_3 (Varghese et al., 1988; Meyer et al., 1995). As for the mechanisms by which $1,25(\text{OH})_2$ vitamin D_3 might regulate the expression and/or function of Cb-D28k, evidence continues to mount (Varghese et al., 1988; 1989; Theofan et al., 1986; Meyer et al., 1995), indicating that the large induction of Cb-D28k messenger observed after treatment with $1,25(\text{OH})_2$ vitamin D_3 may be primarily due to posttranscriptional regulatory mechanisms. To our knowledge, the exact mode of action is not known, however, an indirect effect of $1,25(\text{OH})_2$ vitamin D_3 acting through an intermediate regulatory protein involved in calbindin-D28K messenger accumulation has been implicated (Meyer et al., 1995).

Different studies have shown a dependency of the expression of Ca transport proteins like TRPV5/6 channels (Transient Receptor Potential Vanilloid) on the $1,25(\text{OH})_2$ vitamin D_3 (Hoenderop et al., 2005). As a result, the transcellular Ca transport is a three-step process consisting of passive apical Ca entry through its channels, which are $1,25(\text{OH})_2$ vitamin D_3 -dependent, followed by cytosolic diffusion, which is facilitated by calbindins and, finally, the active extrusion across the basolateral membrane by a high affinity Ca-ATPase and/or a Na^+ -Ca exchanger (Hoenderop et al., 1999). Calbindins regulate the Ca influx across the apical membrane by buffering intracellular Ca and thus controlling feedback inhibition of TRPV5/6 channel activity (Vennekens et al., 2000). This is consistent with the increased $1,25(\text{OH})_2$ vitamin D_3 sensitivity of TRPV5/6 and calbindins observed in vitamin D-deficient animal models and epithelial cell lines. The basolateral extrusion systems NCX1 and PMCA 1b seemed to be less affected (Hoenderop et al., 2005).

The highest immunoreaction for Cb-D28k was recorded in the group receiving all supplements plus UV (CaADUV) whereas the lowest immunoreaction was in the groups that received no supplements and UV (no and UV group) which indicates that calbindin is $1,25(\text{OH})_2$ vitamin D_3 -dependent, accompanied most probably by the expression of the above mentioned channel proteins, which were not ~~not~~ investigated in the present study. This means that expression of Cb-D28k in particular appears to be up-regulated by dietary supplementation of vitamin D and was possibly enhanced by UVB exposure.

From the results of this study, it is concluded that the duodenum plays the most important role in the active transcellular absorption of calcium in veiled chameleons, which is in accordance with many mammals. Furthermore, different feeding and light regimes strongly influence the Cb-D28k expression, but not the VDR expression in the DD.

Further studies will be required to provide in-depth understanding of the physiological processes with regard to Ca homeostasis in the intestines, liver and kidneys of the chameleons followed by RT-PCR and Western-Blot.

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