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Effects of Oral Vitamin D_3 Supplementation on Growth, But Not on Development of Shell Deformities in Juvenile Eastern Spiny Softshell Turtles (*Apalone spinifera spinifera*)

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Abstract

The eastern spiny softshell turtle (SST: *Apalone spinifera spinifera*) is listed as endangered within its range in Canada and, since 2014, has been the focus of a head-start program aimed at increasing the juvenile survival rate over their first winter. From 2017 to 2020, shell deformities have been observed among the SSTs in this program, raising concerns about the potential underlying risk factors for musculoskeletal disease, including vitamin deficiencies. This study aimed to assess the impact of dietary vitamin D_3 (cholecalciferol) levels on shell deformities in captive-raised juvenile SSTs. Twenty-two juvenile SSTs from four distinct nests were selected and divided into two groups. Both groups experienced identical environmental conditions, and their diets differed only in vitamin D_3 content. The control group received standard dietary levels (low: LVD) at 1,980 IU/kg/ration, and the other received an added dietary supplementation (high: HVD) at 6,000 IU/kg/ration. Morphometric measurements and shell deformity severity assessments were conducted at 2-wk and 12-wk intervals, respectively, over the course of 1 yr. The HVD group exhibited significantly greater increases in body weight ($P < 0.001$) and carapace width ($P = 0.004$) than the LVD group by the conclusion of the study. Specifically, HVD females gained the highest body weight ($P = 0.001$), with LVD males displaying the lowest weight gains. Shell deformities were more prominent in females ($P = 0.043$) but were not different between the treatment groups. Although these findings suggest a role for oral vitamin D_3 in the growth and development of juvenile SSTs, it appears that the occurrence of shell deformities in these turtles is more closely associated with sex and growth rate, rather than oral vitamin D_3 supplementation. Further investigations are warranted to understand the nutritional needs and potential risk factors influencing shell deformity development in this endangered species comprehensively.

Keywords: *Apalone spinifera*, cholecalciferol, shell disease, spiny softshell turtle, vitamin D_3

Introduction

With a wide range across eastern North America, the spiny softshell turtle (SST; *Apalone spinifera*) is listed as endangered within its range in Canada by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2016) and has been a Schedule 1 species on Canada's Species at Risk Act since 2005 (Species at Risk Act, 2023). Habitat loss has caused the fragmentation and isolation of subpopulations, leading to the general decline of this species' population and distribution (Fletcher, 2002). The reproductive success of the only eastern spiny softshell turtle (SST; *A. s. spinifera*) population in Québec has been greatly impacted by the detrimental effects of flooding and by predation, specifically targeting eggs during the incubation and hatching periods, rather than predation of hatchlings postemergence (Galois *et al.*, 2002; Équipe de rétablissement des tortues du Québec, 2020). To improve hatching success, the incubation of nest-collected eggs was implemented in 2010 at the Zoo de Granby. In 2014, a head-start program was initiated to increase the survival rate of juveniles, as has been done in other species of freshwater turtles (Haskell

et al., 1996; Vander Haegan *et al*., 2009; Eiby and Booth, 2011; Burke, 2015; Mullin *et al*., 2023). This initiative entails the overwintering of a group of hatchlings from the incubated broods, which allows for improved monitoring of postrelease survival and movement with telemetry (PicoPip, Lotek Wireless, Newmarket, ON, Canada) in the following spring.

Significant mortalities due to gastro-duodenal perforations began to occur in the first cohorts of the head-start program, accounting for 41% of the observed mortalities in the 2014 and 2015 cohorts combined. Excessive amounts of food ingested and provision of abrasive food items such as sun-dried red shrimps (*Solenocera melantho*) were then identified as potential risk factors (Couture *et al*., 2017). Reduced gastro-duodenal perforation-related mortality was associated with subsequent changes to the feeding regime, including the substitution of sun-dried red shrimps with freeze-dried *Gammarus* sp., moistening food, limiting feeding time, and adding fast days. A follow-up cohort study (Ferrell *et al*., 2019) further identified dried turtle pellets as a risk factor for gastroduodenal perforations, possibly due to their effects on gastric pH and pepsin/bile acid concentrations in the lumen of the stomach, as seen in other animals fed finely ground pelleted diets (Mößeler *et al*., 2014). The compared gel mixture was associated with a higher growth rate, presumably due in part to a lower occurrence of chronic GI lesions, and was therefore the recommended base for the diet. According to institutional medical records in Species 360 ZIMS (2023), no further gastro-intestinal perforations have been observed since all of the program's turtles were switched entirely to the gel-mixture diet in 2018. However, carapace deformities were noticed starting with the implementation of the gel-mixture diet, and have been consistently observed since, prompting questions on the long-term growth impacts and potential consequences of the modified, and sometimes reduced, intracoelomic space in the most severely affected animals.

Several etiologies, including physical trauma, dietary deficiency, insufficient UV-B exposure, and infections (Boyer, 1998; Hernandez-Divers *et al*., 2009; Boyer and Scott, 2019; Meyer and Selleri, 2019) may result in musculoskeletal deformities in captive turtles. These are, however, often multifactorial, as both diet and environmental conditions play an important role in turtle growth and shell development (Wiesner and Iben, 2003; Chou and Huang, 2013; Hetényi *et al*., 2014). Knowledge about the nutritional requirements of different species of wild and captive softshell turtles is rather limited (Cochran and McConville, 1983; Pierce, 1992; Mahoney and Lindeman, 2017). However, there has been some investigation into the effects of various levels of certain dietary components on the growth of soft-shell turtles intended for human consumption, where high growth rates are desirable. Components investigated include protein (Nuangseng and Boonyaratapalin, 2002; Zhou *et al*., 2013; Wang *et al*., 2014; Kou *et al*., 2022), calcium and phosphorous (Huang *et al*., 2003), and vitamin D_3 (cholecalciferol) (Hetényi *et al*., 2014; Chou and Huang, 2017). Vitamin D_3 can be obtained through environmental UV-B exposure as well as from dietary sources in carnivorous reptile species, for which deficiency is often listed as a risk factor for the development of musculoskeletal and growth abnormalities (McArthur and Barrows, 2004; Boyer and Scott, 2019). Nutritional intake appears to be an important source of vitamin D_3 in carnivorous softshell turtles, and exact requirements have not yet been established, whether within a production context or for conservation projects (McArthur and Barrows, 2004; Chou and Huang, 2017).

This study aimed to evaluate the impact of dietary vitamin D_3 on the development of shell deformities in captive-raised juvenile SSTs during their first year. Considering the accelerated growth rates linked to the head-start program, we suspected that the commonly recommended level of dietary vitamin D_3 in chelonian and reptilian diets (140–2,500 IU/kg; Donoghue, 2006; Chou and Huang, 2017; Boyer and Scott, 2019) might be insufficient to meet the elevated demands during this critical growth phase. We hypothesized that a vitamin D_3–enriched diet would better support growth, reducing the incidence of shell deformities. Additionally, we predicted that increased dietary vitamin D_3 would result in larger and heavier turtles. We also expected that there would be no sex-related differences in the effects of higher dietary vitamin D_3 on weight gain and shell growth in these turtles. This research was part of a broader initiative to optimize head-start husbandry protocols for SSTs.

Materials and Methods

This study was approved by the Université de Montréal's Animal Use Ethics Committee, proposal 21-Rech-2154.

Animals: Between 7 and 21 June 2021, SST eggs were gathered from along the riverbank of Pike River, Quebec, Canada. These eggs underwent an incubation period of 58–62 days at a temperature of 28°C (82°F) and a relative humidity ranging approximately between 70% and 80% (Nature Spirit LLC, Vicksburg, MI, USA). Twenty-two individual turtles originating from four distinct clutches participated in the head-start program for the year 2021. Eleven males and 11 females were identified and divided into each group, with six males and five females ($n = 11$) assigned to the low vitamin D_3 (LVD) group and five males and six females ($n = 11$) assigned to the high vitamin D_3 (HVD) group. Sex was determined at hatching based on shell pattern characteristics, following the methodology outlined by Graham and Cobb (1998). Juvenile SSTs were released after 10 months of hand-rearing, at 314 days of age.

Housing: Each group was housed in a pool containing approximately 100 L of fresh water with 1–2 g/L of salt added, to prevent fungal diseases, at a temperature of 22–26°C (71.6–78.8°F) in a semiaquatic and open vivarium. Turtles were provided artificial UV-B radiation (Exo Terra Swamp Basking Spot 100W, Rolf C. Hagen Inc., Montréal, Quebec,

Canada) delivering a UV index from 1.0 to 2.6, corresponding to Ferguson zones 2–3 (Chattell *et al.*, 2016). UV-B exposure was available in two areas of the habitat: over a basking rock that also served as a thermal hotspot (26–30°C; 78.8–86°F), and above a submerged sandy beach, where turtles' heads could be exposed while their bodies remained buried in the sand. The possible UV-B exposure was adjusted every 3 wk according to the natural photoperiod during the winter (variation of 8.6–15.6 h). Occasionally, some individuals required isolation for brief periods, typically lasting no more than several days, to receive treatment for wounds inflicted by conspecifics. Throughout these isolated periods, consistent with our study protocol, the same treatment diet (HVD or LVD) was administered to these turtles, ensuring their inclusion in the data set for the entire study.

Diet: Each group of 11 turtles was offered a gel-mixture diet for the duration of the program, with an identical standard diet offered from hatching to 80 days of age. For the rest of the program (days 81 [T0] to 314), diet offered to each group only differed in vitamin D_3 supplementation. The LVD (control) group continued to receive the standard diet with a cholecalciferol vitamin D_3 content of 1,980 IU/kg of ration (6,200 IU/kg dry matter), and the HVD (treatment) group received additional supplementation resulting in 6,000 IU of vitamin D_3/kg of ration (18,780 IU/kg dry matter). The diet was formulated using Zootrition™ (Zootrition Software version 2.6, St. Louis, MO, USA) and analyzed at a laboratory (Trouw Nutrition Laboratory, Saint-Hyacinthe, QC, Canada; Table 1) and consisted of gelatin, whole fish, various vegetables (chicory, endive, and zucchini), multivitamins (ReptiVite™ without D_3, Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA), a calcium supplement containing vitamin D_3 (Repti Calcium® with D_3, Zoo Med Laboratories, Inc.), Mazuri®Tortoise Diet No. 5M21 (Mazuri Exotic Animal Nutrition, St. Louis, MO, USA), water, and vitamin D_3 (Vitamin D_3 1,000 IU, Jamieson™, Jamieson Laboratories Ltd, Windsor, ON, Canada) for the HVD group only. The diet was offered to all turtles at ~4% of their body weight (BW) 3–5 days per week, depending on their stage of development and their body condition.

Procedures: The body weight (BW) of the turtles was recorded weekly. Straight carapace length (CL) and carapace width (CW) were measured, and lateral photographs were taken at three different time points: T0, T1, and T2, corresponding to 81, 207, and 305 days of age, respectively. Using the photographs, individual shells were divided into thirds (Fig. 1), with the severity of the deviation being assigned a score of 0–3 (Table 2). A total shell deformity score (SDS) was obtained by adding together the deviation scores for each third of the shell. Each individual was then categorized according to the severity of the shell deformity: Normal (SDS ≤ 1), mild–moderate (SDS 2–4), and marked (SDS ≥ 4, or any individual shell third presenting with an individual score of 3). The scoring process was applied to each individual at each of the three time points by three blinded observers. If interobserver agreement was determined to be good, the mode of the SDS attributed by the three observers was used to attribute a final SDS for each individual at each time point.

Table 1. Dietary analysis results for juvenile eastern spiny softshell turtles (*Apalone spinifera spinifera*) raised in captivity.

Dietary component	Dry matter
Protein, %	50.1
Crude fat, %	22.4
Crude fiber, %	1.81
Sodium, %	0.203
Calcium, %	3.13
Phosphorous, %	1.11
Magnesium, %	0.110
Iron, ppm	205
Zinc, ppm	69.8
Beta-carotene, µg/100 g	73.5
Retinol, µg/100 g	312
Vitamin A (RE), RE/100 g	325
Vitamin A (IU), IU/100 g	1,080
Vitamin D_3, IU/kg	6,200 (LVD)
	18,750 (HVD)

RE, Retinol equivalents; LVD, low vitamin D_3; HVD, high vitamin D_3.

Statistical analysis: For statistical analyses, the sex group was obtained by grouping animals by sex in each group to obtain four independent groups (HVD females, HVD males, LVD females, LVD males). All statistical analyses were performed using R (R version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria) implemented in RStudio v.1.3 (RStudio®, Boston, MA, USA). Linear mixed models (LMM) were used to measure the interaction between time, group (HVD and LVD), and the interaction between the two and BW. The identity of each individual was added as a random factor within the models, in order to avoid potential pseudo-replication bias due to the fact that the data related to BW were not independent. A model was built for each of the nine dependent variables (BW, CL, CW, SDS) and the variance (ANOVA) of the models explained by the independent variables was analyzed. Type 3 ANOVAs were used when there was an interaction between time and group, and type 2 ANOVAs if there was no significant effect of this interaction. Likelihood-ratio tests (LR) on the ANOVAs were performed on the different LMMs. When independent variables containing multiple modalities had a significant effect on the dependent variable, a *post hoc* test was performed to compare the groups to each other. *P* values were corrected using the Benjamini-Hochberg method. The explanatory variables of sex, group, and sex-group were tested independently for each time point and compared. Spearman's correlation coefficient (φ) was used to evaluate relationships between morphologic measurements (BW, CW, CL) and SDS. For interobserver agreement, an intraclass correlation

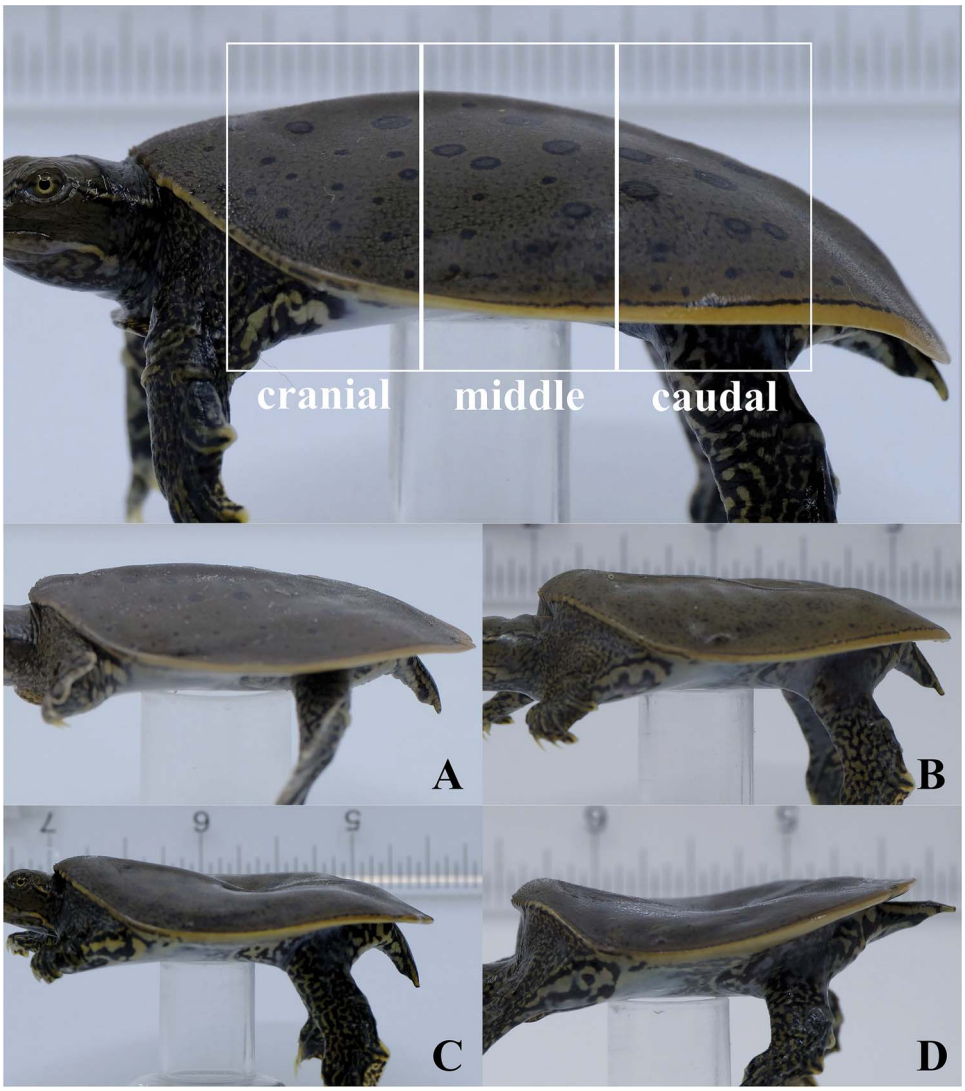

Figure 1. Lateral views of shells of juvenile eastern spiny softshell turtles (*Apalone spinifera spinifera*) divided into cranial, middle, and caudal sections for deformity score classification. Each section is classified as no deformity (0), mild–moderate deformity (1–2), and marked deformity (3), later summed to obtain a cumulative score attributed to every individual at three time intervals (T0, T1, and T2), 12 wk apart. Cumulative shell deformity scores (SDS) were classified as no deformity (SDS < 2) (A), mild–moderate (SDS 2–3) (B), and marked (>4) (C and D).

coefficient (ICC) was estimated for the SDS attributed at each time point. The ICC between observers was estimated using the psych package (Revelle, 2021) by choosing ICC according to the methods by Shrout and Fleiss (1979).

Results

One female turtle from the LVD group died from desiccation following an escape from the pool during the study period (between T0 and T1). Consequently, data pertaining to this turtle's morphology and shell characteristics were retained only for T0.

Morphological measurements: Hatchlings exhibited similar mean weights in the LVD group and HVD groups of 7.4 ± 0.4 g (mean ± standard deviation, range: 5.9–8.8 g) and 7.9 ± 0.6 g (mean ± standard deviation, range: 5.9–9.8 g), respectively. The variable "time" was statistically associated with BW, CL, and CW. Body weight (LR $\chi^2 = 6.69$; $P = 0.035$) interacted differently through time between the two groups. The HVD group had a significantly greater CW than the LVD group (LR $\chi^2 = 8.18$; $P = 0.004$). BW, CL, and CW all increased significantly from T0 to T1, T0 to T2, and T1 to T2 ($P < 0.001$) in each group. At time T2, the average BW of the HVD group was significantly greater than that of the LVD group ($P < 0.001$). Body weight was significantly higher in the HVD group and in females (when combined), at T2 ($P = 0.011$), and at T0 ($P = 0.018$), respectively (Fig. 2).

The BW of HVD group females was significantly greater than that of LVD group females ($P = 0.001$) and LVD group males ($P < 0.001$). The BW of HVD group males was significantly higher than those of LVD group females ($P = 0.005$)

Table 2. Shell deformity scoring system used to evaluate the occurrence of shell deformities in juvenile eastern spiny softshell turtles (*Apalone spinifera spinifera*) raised in captivity.

Score	Severity	Definition
0	Normal	The shape of the shell at this third presents a slight convexity, the arc of which is generally the same on all segments and is considered normal for the species.
1–2	Mild–moderate	A subtle or moderately concave, linear, or convex deviation from the expected incurvation of the shell is noted.
3	Marked	An obvious and pronounced convexity or concavity is present.

and LVD group males ($P = 0.001$). There were, however, no significant differences in BW between males and females within each group (HVD, $P = 0.574$; LVD, $P = 0.758$).

Shell deformities: For the observers, an ICC of 0.76 ($P < 0.001$) was obtained, indicating a good reliability of interobserver classification. Therefore, the classification obtained from ≥2 observers was assigned to each individual at each of the three time points. No shell deformities were observed in any of the turtles at T0, and mild–moderate and marked deformities were observed in both groups at T1 and T2, the majority of the latter being observed at T2 (Fig. 3). Overall, SDSs were greater at T1 and T2 as compared to T0 (T1 > T0, $P < 0.001$; T2 > T0, $P = 0.001$) but were not significantly different between T1 and T2 ($P = 0.137$). Shell deformity scores were higher in females than in males ($P = 0.043$) and had moderate, positive correlations with BW ($\rho = 0.418$, $P < 0.001$), CL ($\rho = 0.382$, $P = 0.002$), and CW ($\rho = 0.361$, $P = 0.003$) for both sexes and groups combined. There were no significant differences between the groups or group–sex and SDS.

Discussion

All three morphologic variables (BW, CL, CW) exhibited significant interactions with time, as expected, because the juvenile SSTs used in this study were growing. Initially, BW and CW did not differ between groups at T0 and T1; however, differences emerged over the study duration, with BW showing a significant interaction with time exclusively in the HVD group, and CW being notably higher in the HVD group at T2. These findings substantiate the hypothesis that oral vitamin D_3 supplementation influences the growth and development of juvenile SSTs, consistent with observations in analogous species (Hetényi *et al.*, 2014; Chou and Huang, 2017). Moreover, females outweighed males at T0, but at T2, the difference in BWs was solely attributable to group variation, indicating that although both oral vitamin D_3 supplementation and sex impact the growth and development of juvenile SSTs, the former exerts a more pronounced effect on BW earlier in development. This assertion is reinforced by the absence of intragroup differences, but noticeable intergroup disparities in BW, whereby both HVD males and females were heavier than LVD males and females. The loss of an LVD female at the beginning of the study may have been an attrition bias, which may have reduced the statistical power of the results obtained, potentially affecting the intrasex and intergroup differences observed.

The emergence of shell deformities in these SSTs has historically been perceived as a developmental rather than congenital process, possibly linked to factors associated with growth rate (Boyer and Scott, 2019; Meyer and Selleri, 2019). This hypothesis is supported by the absence of deformities at T0 and the subsequent increase in SDSs over time. Furthermore, the lack of significant differences in SDS between T1 and T2 may suggest that the highest risk period for shell deformity development in juvenile SSTs within this study population is early in their life (between 81 [T0] and 207 days [T1] of life). Disparities in SDSs were attributed to size and sex, rather than to oral vitamin D_3 supplementation, despite the noted influence of oral vitamin D_3 supplementation on BW and CW. This finding does not support our hypothesis that higher dietary vitamin D_3 would reduce the occurrence of shell deformities. Instead, it suggests that sex and growth rate are likely more significant risk factors for shell deformity development in juvenile SSTs than oral vitamin D_3 supplementation alone. Vitamin D_3 levels were not measured in the animals used in this study, and as such, it remains unclear whether the animals had sufficient baseline levels of cholecalciferol or if the oral supplementation provided any measurable effect on circulating cholecalciferol. Furthermore, as UV-B exposure may play a significant role in the synthesis of cholecalciferol in turtles (Chou and Huang, 2013; Chattell *et al.*, 2016), the relative contribution of dietary intake versus UV-B–mediated synthesis in the production of metabolically active vitamin D_3 in turtles requires further investigation. Future studies on cholecalciferol supplementation should not only assess circulating cholecalciferol levels before and after supplementation, but also account for the potential influence of UV-B exposure. Although morphological measurements exhibited a positive correlation with SDSs, the moderate strength of these correlations implies the involvement of additional nutritional or environmental factors not assessed in this study.

Much remains to be elucidated regarding the specific nutritional requirements of captive juvenile spiny softshell turtles, although prior investigations have highlighted the significance of protein, vitamin D_3, calcium, and phosphorus in the growth and development of softshell (Nuangseng and Boonyaratapalin, 2002; Zhou *et al.*, 2013; Wang *et al.*, 2014; Chou and Huang, 2017) and other turtle species (Wiesner and Iben, 2003; Hetényi *et al.*, 2014). The dietary composition provided in this study encompassed various nutritional elements falling within reported optimal ranges for other softshell turtle species, with the exception of a high dry protein content and elevated vitamin D_3 levels.

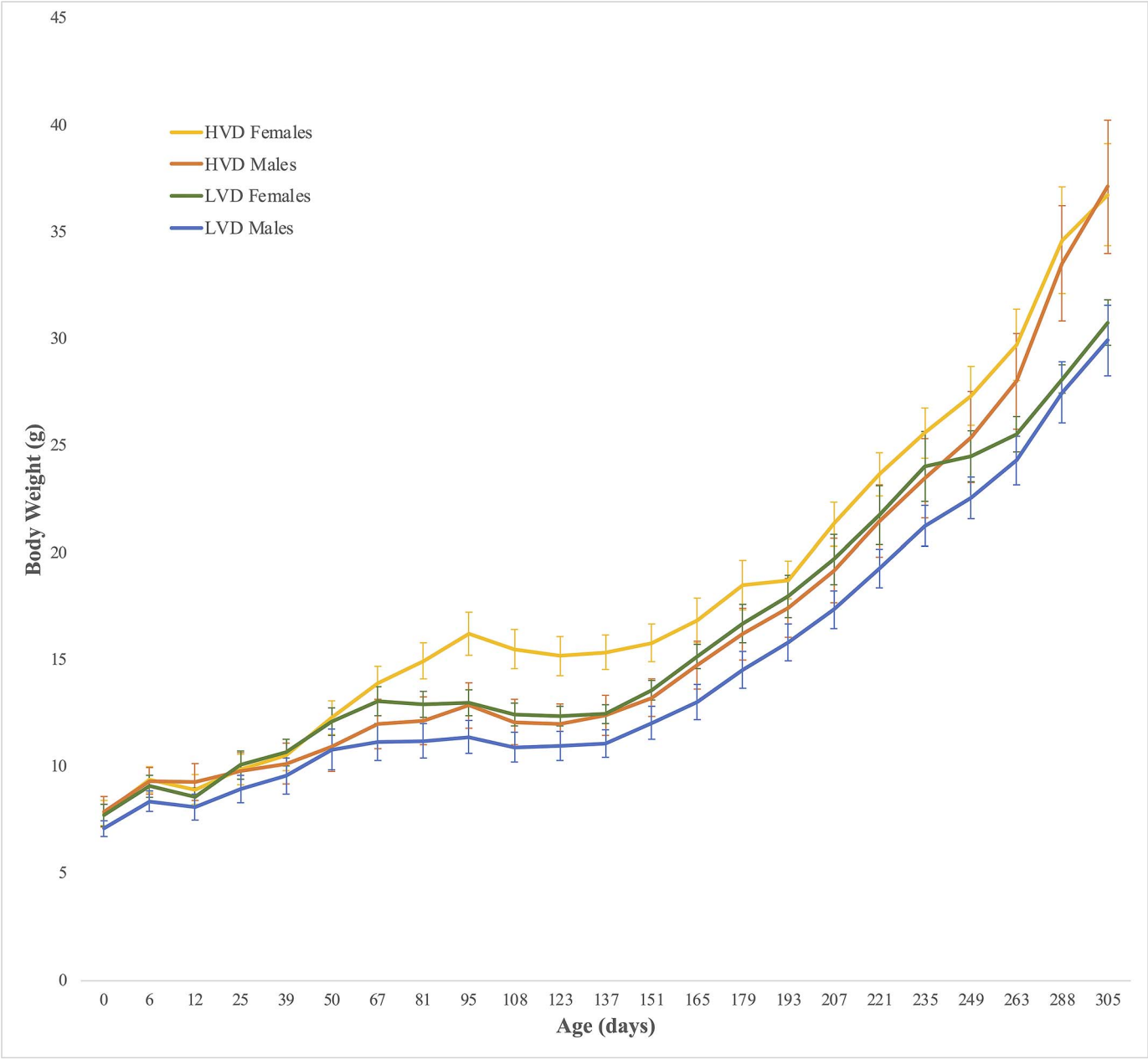

Figure 2. Evolution of the body weight of different sex groups (high vitamin D_3 [HVD] or standard vitamin D_3 [LVD]) of individual juvenile eastern spiny softshell turtles (*Apalone spinifera spinifera*) over the course of 305 days posthatching. Study diets were started at 81 days of age (T0) and various measurements taken at T1 and T2. Error bars represent standard deviation.

Specifically, the diet used in our study contained 50.1% crude protein, compared to a reported range of 27.0–46.5% in previous studies (Nuangseng and Boonyaratapalin, 2002; Zhou *et al*., 2013; Wang *et al*., 2014; Kou *et al*., 2022), and 6,000 IU/kg of vitamin D_3, compared to reported dietary levels ranging from 140–2,500 IU/kg (Donoghue, 2006; Chou and Huang, 2017; Boyer and Scott, 2019). The intentional vitamin D_3 supplementation administered to the HVD group was lower than levels utilized in a prior study on excessive oral vitamin D_3 supplementation of up to 50,000 IU/kg in Hermann's tortoise (*Testudo hermanni*; Hetényi *et al*., 2014), and was considered to be safe, based on the current literature on similar species (Donoghue, 2006; Chou and Huang, 2017; Boyer and Scott, 2019). Although the relatively high protein provision might have impacted the growth rate and development of the study population, it is important to note that this level was consistent across both experimental groups. Moreover, adverse effects on growth rate may be discernible, particularly when dietary protein levels are low (Nuangseng and Boonyaratapalin, 2002; Zhou *et al*., 2013; Wang *et al*., 2014; Kou *et al*., 2022), contrasting with the observed accelerated growth rates linked to relatively high dietary protein content (50%–55%) in softshell turtles (Nuangseng and Boonyaratapalin, 2002). Consequently, we contend that this factor does not undermine the

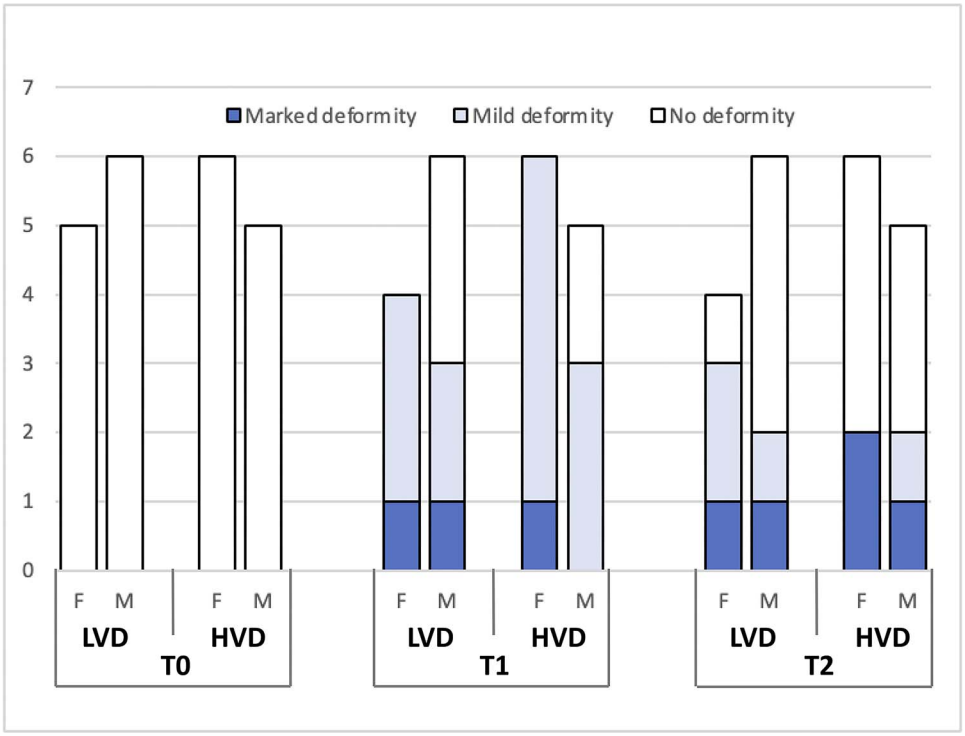

Figure 3. Frequency of occurrence and severity of shell deformities in male (M) and female (F) juvenile eastern spiny softshell turtles (*Apalone spinifera spinifera*) fed a low vitamin D_3 (LVD) and high vitamin D_3 diet (HVD), classified at three different time points (T0, T1, T2) 12 wk apart.

conclusions drawn regarding the effects of supplementation, but that it may have affected the overall growth rate of both groups.

This study faced limitations primarily stemming from a small sample size, constrained by the number of turtles available for selection within our institution's head-start program ($n = 22$). Furthermore, the treatment diets were offered as of 81 days of age (T0), which may have reduced any possible effect of dietary vitamin D_3 on the growth and development of SSTs in this study. Despite efforts to regulate environmental conditions and nutritional components beyond vitamin D_3, inherent individual variations in food consumption and habitat preferences remained uncontrolled and unaccounted for. It is worth noting anecdotally that within each group, evidence of social hierarchies was observed, leading certain individuals to outcompete others for food and favored habitat intermittently, potentially influencing the observed study outcomes. Consequently, these social dynamics and individual discrepancies could have impacted the results. Further investigations are imperative to consider a broader spectrum of environmental, nutritional, and social variables, beyond growth rate and sex, to understand the factors contributing to shell deformity development in juvenile SSTs comprehensively.

Conclusions

The occurrence of shell deformities in juvenile spiny softshell turtles is a multifaceted phenomenon, for which the onset is detected between 81 and 207 days of age. Shell deformities in this population predominantly stem from disparities in growth rate and sex, with female and larger, heavier turtles at the study's conclusion exhibiting a greater propensity for shell deformities. Although oral vitamin D_3 supplementation does influence the growth and development of this species, it does not statistically account for the emergence of shell deformities in our study population. The evaluation of potential associations may have been influenced by our sample size, as statistically significant connections were established between group and growth rate, as well as size, but not for SDSs, despite SDSs having a greater prevalence in larger individuals. Given the persistent observation of this condition within the study population, future investigations are imperative to elucidate additional potential risk factors such as dietary protein and UV-B possibly contributing to the development of shell deformities in this nationally endangered species.

Disclaimer: The authors have no conflicts to disclose.

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