Acta Scientiarum. Biological Sciences
ISSN: 1679-9283
eduem@uem.br
Universidade Estadual de Maringá
Brasil

Ferreira, Caio Henrique; Gonçalves Vieira, Lucélia; Chavauty Valdes, Sady Alexis; dos Santos Mendonça, Juliana; de Abreu Fernandes, Evandro; Quagliatto Santos, André Luiz
Chemical characteristics and thickness of Podocnemis expansa post-hatching eggshells (Testudines, Podocnemididae)
Acta Scientiarum. Biological Sciences, vol. 38, núm. 2, abril-junio, 2016, pp. 195-200
Universidade Estadual de Maringá
Maringá, Brasil

Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v38i2.28019

Chemical characteristics and thickness of *Podocnemis expansa* post-hatching eggshells (Testudines, Podocnemididae)

Caio Henrique Ferreira, Lucélia Gonçalves Vieira*, Sady Alexis Chavauty Valdes, Juliana dos Santos Mendonça, Evandro de Abreu Fernandes and André Luiz Quagliatto Santos

*Escola de Medicina Veterinária, Universidade Federal de Uberlândia, Laboratório de Ensino e Pesquisa em Animais Silvestres, 70910-900, Uberlândia, Minas Gerais, Brazil. *Author for correspondence. E-mail: luceliabio@yahoo.com.br*

ABSTRACT. Knowledge on chemical components of the post-hatching eggshell of reptiles may provide indicators of the quality of the diet offered to females kept in captivity. Therefore, the objective of this study was to investigate the chemical characteristics of the calcareous layer, as well as the thickness of *Podocnemis expansa* post-hatching eggshells. Eggshell thickness was 183±1.405 μm. This value is similar to that of the eggs of other Testudines with flexible eggshells. As for the chemical composition, the following percentages were observed: nitrogen 7.983 ± 0.054; crude protein 49.91 ± 0.324; crude fat 0.068 ± 0.002; mineral matter 20.302 ± 0.807; calcium 13.374 ± 0.647; and phosphorus 0.176 ± 0.003. Knowledge on chemical composition of the eggshell may aid the nutrition of *P. expansa* raised in commercial facilities, once this species is an alternative and promising source of exotic meat.

Keywords: chemical composition, eggs, limestone layer, Amazon turtle.

Aspectos químicos e espessura da casca de ovos de *Podocnemis expansa* pós-eclosão (Testudines, Podocnemididae)

RESUMO. O conhecimento sobre os constituintes químicos da casca de ovos pós-eclosão de répteis pode ser um referencial de qualidade da dieta fornecida para as fêmeas que vivem em cativeiro. Assim, este trabalho visa investigar os aspectos químicos que compõem a camada calcária, bem como a espessura da casca do ovo de *Podocnemis expansa* no período pós-eclosão. A espessura média da casca dos ovos foi de 183 ± 1,405 μm. Esta espessura é condizente com ovos de outros Testudines com casca flexível. Para os constituintes químicos, foram encontrados os seguintes valores médios percentuais: nitrogênio 7,983 ± 0,054, proteína bruta 49,91 ± 0,324, gordura bruta 0,068 ± 0,002, matéria mineral 20,302 ± 0,807, cálcio 13,374 ± 0,647 e fósforo 0,176 ± 0,003. Este conhecimento sobre a composição química da casca poderá auxiliar na criação em escala comercial dessa espécie, uma vez que a *P. expansa* tem se apresentado como fonte alternativa e promissora de carne exótica.

Palavras-chave: composição química, ovos, camada calcária, Tartaruga-da-Amazônia.

Introduction

The eggshell is a fundamental structure in the incubation period. It protects the embryo against microorganisms, controls the flow of gases and water through its pores, and is a source of calcium for the embryo during their development. Successful performance of these roles depends on adequate structural formation and composition (Sahoo, Sahoo, & Mohanty-Hejmadi, 1998; Zhou et al., 2011).

Most reptile eggshells are organized in layers. The inner layer is organic, made up of fibrous connective tissue. The outer layer is calcareous, made up of different types of minerals (Kitimasak, Thirakhupt, & Moll, 2003) and other components, mainly calcium, magnesium, phosphorus, oxygen, crude fat, protein, and nitrogen (Kitimasak et al., 2003; Cusack, Fraser, & Stachel, 2003). These eggshell elements are organized in a characteristic way in each species. Modifications in the composition and structure may be caused by lixiviation, external moisture, abrasion by soil particles, and extrinsic microbial degradation. Intrinsic characteristics of the egg may also lead to differences in the chemical composition of Testudines eggshells (Mine, 1995).

According with their chemical composition and layout of the components, Testudines eggshells may range from rigid and rough, with little potential for water and gas exchanges, to flexible, which depend much more on the external aqueous medium. This structural characteristic makes it possible for different species to incubate their eggs in different environmental conditions, as well as in different substrates (Packard, Phillips, & Packard, 1992).

Eggshells have long attracted the attention due to their specific properties of gas transport, and to the fact that they are sources of minerals to the embryos (Cusack et al., 2003; Fraser & Cusack, 2002). Therefore, chemical composition, ultrastructure, and porousness of reptile eggshells have been studied by many researchers. Packard, Packard, and Boardman (1982) analyzed eggshell structure and water relationships in reptile eggs; Feder, Satel, & Gibbs (1982) analyzed the resistance of the eggshell membrane and the mineral layer in oxygen and water diffusion in flexible eggshells of *Chelydra serpentine;* Kitimasak et al. (2003) studied the thickness, ultrastructure, and chemical composition of *Chitra chitra* eggshells; Osborne and Thompson (2005) described the chemical composition and structure of the eggshell in three lizard species; Areekijseree, Nuamsukon, Chuen-im, and Narkkong (2010) analyzed the ultrastructure and composition of *Chelonia mydas* eggshell elements; and Kusuda et al. (2013) evaluated the structure of the calcareous layer, and classified Testudines eggshells.

Chemical characteristics of *Podocnemis expansa* post-hatching eggshells have not been elucidated yet. This species is the most well-known in the genus, and it is found in the Orinoco, Essequibo, and Amazon river systems, reaching 13ºS (Roze, 1964; Pritchard & Trebbau, 1984; Iverson, 1992). It is the largest freshwater Testudines species found in South America.

The species *Podocnemis expansa* is one of the main representatives of this order, which is considered the larger freshwater Testudines species in South America, reaching up to 107 cm in shell length and weighting 90 kg. These animals suffer environmental influences (temperature, water, and gas exchanges) that interfere both with their embryonic development and with sex determination (Pough, Heiser, & Janis, 2008). Eggs are laid from September to October, in an average of 100 eggs per nest (Alves-Júnior et al., 2012), which hatch after 54 to 68 days (Ferreira Júnior & Castro 2003).

The commercial raising of *P. expansa* is one aquiculture activity in Brazil that tends to become an interesting alternative for riverine populations, fisherman communities, cooperatives, associations, and rural settlements, reducing overfishing (Luz et al., 2003). Knowledge on the proportion of chemical elements in the post-hatching eggshell is extremely important, as it may be an indicator of the quality of the diet offered to the females. Therefore, the objective of this study was to investigate the chemical characteristics of the calcareous layer, as well as the thickness of *Podocnemis expansa* post-hatching eggshells.

Material and methods

Egg collection site

In October, 2012, a total of 60 eggs of *P. expansa* were collected from two nests in the same beach at the Environmental Protection Area *Meandros do Rio Araguaia*, Brazil (Figure 1). The collection was authorized by license no. 36957-1/2012 SISBIO/ICMBio. Thirty eggs were removed from each nest and placed, in the same position they were found, in containers with vermiculite and water 2:1 v v^{-1}. The procedures carried out in this study were approved by the Ethics Commission for Animal Use of *Universidade Federal de Uberlândia* (CEUA/UFU 055/12). Then, the eggs were transferred to the *Laboratório de Ensino e Pesquisa em Animais Silvestres* of *Universidade Federal de Uberlândia* (LAPAS/UFU), where they were artificially incubated.

Artificial Incubation

The eggs were artificially incubated in plastic trays placed in incubators according to the method described by Verdade, Lavorenti & Packer (1993). The temperature was kept between 28 and 31ºC, and relative humidity between 80 and 100% throughout incubation. Vermiculite was used as the substrate for incubation.

After hatching, eggshells were washed manually to remove residues, such as remaining chorioallantoic membrane and vermiculite, and were used in the analysis of thickness and chemical composition.

Thickness

Eggshell thickness was measured in three random areas of the shell using a pachymeter (Starrett 125 MEB), and the average of the three measurement was used in the analysis.

Analysis of chemical composition

The eggshells were divided into ten groups of six eggshells each, in order to yield enough mass for the analysis. Then, the samples were dried in an incubator oven at 80ºC, ground and prepared for the following analyses: mineral matter and total fat (Association of Official Analytical Chemists, AOAC, 1995), nitrogen and crude protein (American Association of Cereal Chemists, AACC, 1999; AOAC, 2005a and b), and calcium and phosphorus (AOAC, 1984). These analyses were carried out in the *Laboratório de Nutrição Animal of Universidade Federal de Uberlândia*.

The data obtained were analyzed by calculating the mean between the groups and standard error of the mean. Later on, means of the groups were compared using Student's t test. Significance was set at $p < 0.05$.

Results and discussion

The analyses carried out in the eggshells of *P. expansa* showed the main chemical components, as well as their percentage after hatching.

These results are presented in Table 1. Interspecific differences in the chemical composition of recently hatched eggshells and in the percentage of these elements may be explained by the diet of the female, by the different nutritional requirements of the embryo, and by differences in exposure to external agents, once each species has different nesting habits.

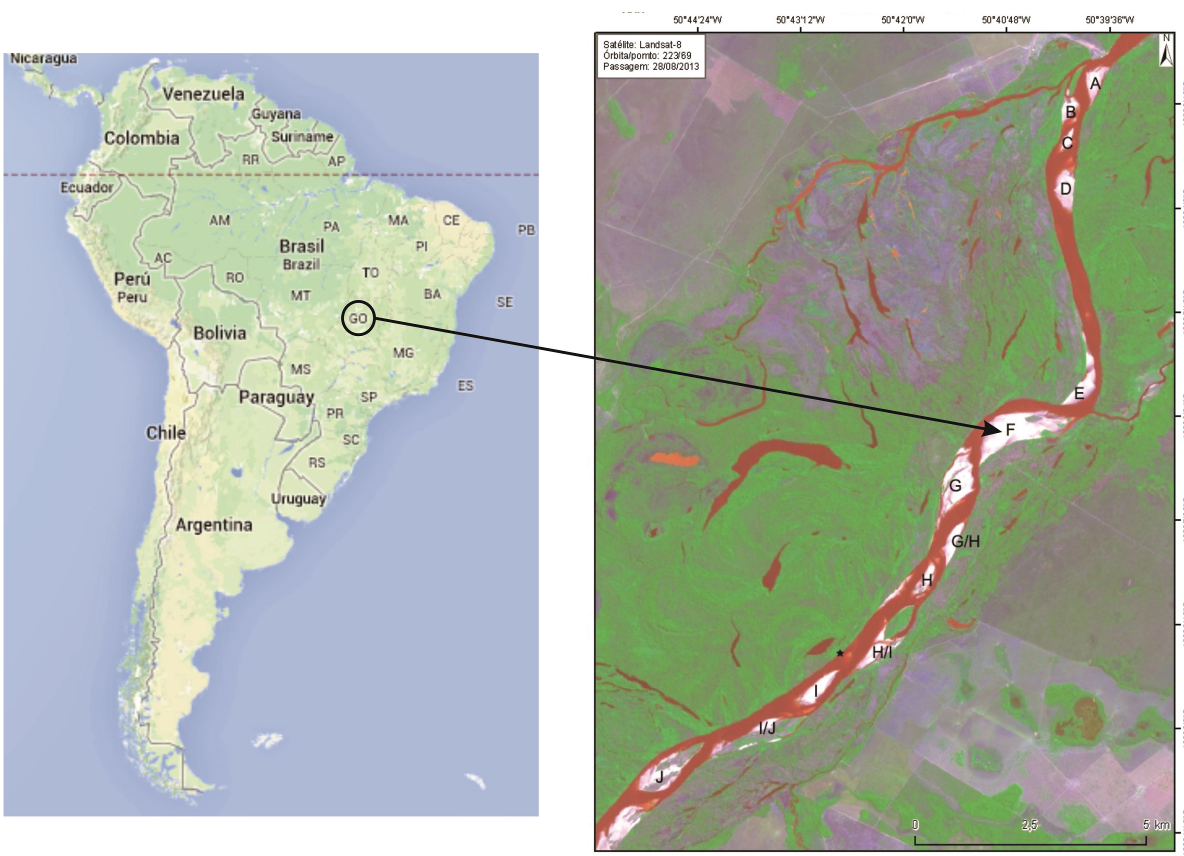

Figure 1. Reproduction area of *Podocnemis expansa* in Conservation Unit in Araguaia River, State of Goiás, Brazil. The arrow indicates the site of egg collection in 2012 (13° 20' 38" S and 50° 38' 05" W).

Table 1. Percent chemical composition of post-hatching *Podocnemis expansa* eggshells.

Group	Nitrogen	Crude protein	Crude fat	Mineral matter	Calcium	Phosphorus
1	8.13	50.7	0.07	18.2	11.93	0.18
2	8.14	50.9	0.06	17.9	11.48	0.18
3	8.24	51.43	0.06	17.98	11.52	0.17
4	8.11	50.15	0.08	17.63	10.99	0.16
5	8.03	51	0.07	17.8	11.48	0.17
6	7.82	48.91	0.08	22.71	15.62	0.18
7	7.83	48.98	0.07	23.01	15.95	0.19
8	7.92	49.1	0.06	22.97	15.26	0.19
9	7.71	48.93	0.06	22.92	15.13	0.17
10	7.9	49	0.07	21.9	14.38	0.17
Mean	7.983	49.91	0.068	20.302	13.374	0.176
Standard Error	0.054	0.324	0.002	0.807	0.647	0.003

Thus, Simkiss (1962) and Cox, Koob, Mecham, and Sexton (1984) stated that eggshell composition is influenced by the external and internal environment. Knowledge about of these aspects, mainly in the case of *P. expansa*, is important for successful reproduction in captivity.

The components of the calcareous layer of the eggshell contribute to its thickness. However, according to Osborne and Thompson (2005), the fibrous layer of the eggshell has a more significant contribution. *P. expansa* has a flexible eggshell, and thickness recorded in the present study was 183 ± 1.405 μm. This parameter was also analyzed by Kusuda et al. (2013), in several Testudines species that had flexible (146 to 268μm) and rigid eggshells (220 to 758μm). The results of the present study show that the thickness observed in *P. expansa* eggshells is in the same range of the values observed for other Testudines with flexible eggshells.

According to Washburn (1982), nutritional factors are the main determinants of egg quality. In relation to the eggshell, nutrition affects its weight, thickness (Nakage, Cardozo, Pereira, Queiroz, & Boleli, 2002), porousness and conductance (Ar, Paganelli, Reeves, Greene, & Rahn, 1974; Nakage et al., 2002). Considering that the eggshell supplies calcium for embryonic and fetal development (Grizzle, Iheanacho, Saxton, & Broaden, 1992), it is necessary that the standard thickness of a given species remains unchanged so as not to alter the availability of this mineral for *in ovo* development. Besides, thickness and, consequently, pore length, influence gas and water exchange through the eggshell (Ancel & Girard, 1992).

Calcium is the main inorganic component of the eggshell. Other minerals are found in much smaller amounts (Sahoo et al., 1998). This is also true for *P. expansa*, although the percentage of this mineral may vary in the different species of Testudines. Solomon and Baird (1976) observed that *Chelonia mydas* eggshells are made up of 20% calcium. Sahoo et al. (1998) reported 9.4% calcium in *Lepidochelys olivacea* hatched eggshells. Kitimasak et al. (2003) reported lower levels in *Chitra chitra* (5.37%). Calcium concentration found in *P. expansa* eggshells (13.37%) is halfway between the values reported for these other two species.

According to Sahoo et al. (1998), egg content (yolk and albumen) in *L. olivacea* supplies 40% of the calcium necessary for embryo development. The rest of the requirement is supplied by the eggshell, which undergoes a reduction in calcium concentration between the moment the egg is laid and hatching. Transfer of calcium from the eggshell to the *L. olivacea* embryo starts around the 40th day of development, therefore, the concentration of this mineral in the eggshell decreases from 21 to 9.4% from the beginning to the end of incubation. According to these authors, other Testudines, Crocodylia and birds also use eggshell calcium as a secondary source for embryonic development. Additional studies are necessary to evaluate calcium dynamics in *P. expansa* eggshells, and to understand its participation in embryonic development. Regardless of that, it is a well-known fact that calcium used in eggshell production comes from the diet. Therefore, adequate nutrition of the females is important for eggshell quality, successful hatching, and high offspring quality (Pedroso, Moraes, Ariki, & Kronka, 1999).

Phosphorus is found in small amounts in *P. expansa* eggshells (0.176%). Analyses of phosphorus concentration in *C. mydas* eggshells found 1% of this element (Solomon & Baird, 1976). In *L. olivacea*, Sahoo et al. (1998) showed absence of phosphorus in the eggshells of this species. In the present study, phosphorus values were halfway between those species cited above. These values corroborate the findings of Packard et al. (1992), who stated that there is a small amount of phosphorus in the eggshell, and that the yolk is the only significant source of this mineral for embryonic development.

According to Dennis et al. (1996) and Cusack et al. (2003), during the development of the egg in the oviduct, phosphorus is incorporated to the eggshell at very low concentrations. However, until the moment of oviposition, there is a gradual increase in the concentration of this element. Phosphorus takes part in the formation of aragonite (Fraser & Cusack, 2002), one of the main forms of calcium carbonate in reptile eggshells (Bessler & Rodrigues, 2008). Low levels of phosphorus increase eggshell resistance and may increase hatching rate (Jackson, Hellwig, & Waldroup, 1987).

The main components of the organic matter of the eggshell membrane are proteins and small amounts of fat (Burley & Vadehra, 1989). Cox et al. (1984) evaluated the chemical composition of Squamata eggshells and detected that protein concentration gradually decreased during incubation. Changes in the levels of protein in the eggshell may also lead to changes in bound calcium (Cox et al., 1984). *P. expansa* eggshells showed 49.91% crude protein. It is important to know protein concentrations in post-hatching *P. expansa* eggshells of other oviposition areas to determine reference values for the species.

Chemical composition of reptile eggshells is responsible for some functions, such as the transport of gas and liquids (Lynn & Brand, 1945; Osborne & Thompson, 2005). Crude fat is one of the elements that contributes with this function. Post-hatched *P. expansa* eggshells showed 0.068% fat. Reptiles that have flexible eggshells, such as *P. expansa*, are more sensitive to environmental variations in humidity. Therefore, their embryonic metabolism is directly influenced by water availability (Packard et al., 1982). Adequate concentrations of crude fat in *P. expansa* eggshells may be an important element to prevent excess loss of water during incubation.

Conclusion

The average thickness of the egg shell was 183 ± 1.405 μm. This value is consistent with eggs of other Testudines with flexible egg shell. A calcium value of 13.37% was found in the shells of *P. expansa* eggs, and this amount is considered intermediate according to the values found in other species of Testudines. Among the minerals, the phosphorus showed the lower percentage, only 0.176%. The amount of other constituents in the egg shell, although variable among species, is as expected for the shell type of *P. expansa* eggs.

References

Alves-Júnior, J. R. F., Lustosa, A. P. G., Bosso, A. C. S., Balestra, R. A. M., Bastos, L. F., Miranda, L. B., & Santos, A. L. Q. (2012). Reproductive indices in natural nests of giant Amazon river turtles *Podocnemis expansa* (Schweigger, 1812) (Testudines, Podocnemididae) in the Environmental Protection Area Meanders of the Araguaia river. *Brazilian Journal of Biology, 72*(1), 199-203.

American Association of Cereal Chemists [AACC]. (1999). *Approved methods of analysis of AACC* (9th ed.). St. Paul, MN: AACC.

Ancel, A., & Girard, H. (1992). Eggshell of the domestic guinea fowl. *British Poultry Science, 33*(5), 993-1001.

Ar, A., Paganelli, J. C. V., Reeves, R. B., Greene, D. G., & Rahn, H. (1974). Rahn the avian egg: water vapor conductance, shell thickness, and functional pore area. *The Condor*, 76, 153-158.

Areekijseree, M., Nuamsukon, S., Chuen-im, T., & Narkkong, N. A. (2010). Microanalysis on ultrastructure and elemental composition of green turtle (*Chelonia mydas)* eggshells. *Journal Microscopy Society of Thailand, 24*(2), 78-82.

Association of Official Analytical Chemists [AOAC]. (1984). *Official methods of analysis of the Association of Official Analytical Chemists* (14th ed.). Airlington, TX: AOAC.

Association of Official Analytical Chemists [AOAC]. (1995). *Official methods of analysis of the Association of Official Analytical Chemists*. *Method 900.02.* Airlington, TX: AOAC.

Association of Official Analytical Chemists [AOAC]. (2005a). *Official methods of analysis of the Association of Official Analytical Chemists. Method, 2001.11* (18th ed.). Gaithersburg, MD: AOAC.

Association of Official Analytical Chemists [AOAC] (2005b). *Official methods of analysis of the Association of Official Analytical Chemists*. *Method, 936.15* (18th ed.). Gaithersburg, MD: AOAC.

Bessler, K. E., & Rodrigues, L. C. (2008). Os polimorfos de carbonato de cálcio: uma síntese fácil de aragonita. *Quimica Nova, 31*(1), 178-180.

Burley, R. W., & Vadehra, D. V. (1989). *The avian egg: chemistry and biology*. New York, NY: John Wiley and Sons.

Cox, D. L., Koob, T. J., Mecham, R. P., & Sexton, O, J. (1984). External incubation alters the composition of squamate eggshells. *Comparative Biochemistry and Physiology, 79*(3), 481-487.

Cusack, M., Fraser, A. C., & Stachel, T. (2003). Magnesium and phosphorus distribution in the avian eggshell. *Comparative Biochemistry and Physiology Part B, 134*, 63-69.

Dennis, J. E., Xiao, S. Q., Agarwal, M., Fink, D. J., Heuer, A. H., & Caplan, A. I. (1996). Microstructure of matrix and mineral components of eggshells from white leghorn chickens (*Gallus gallus*). *Journal of Morphology, 228*(3), 287-306.

Feder, M. E., Satel, S. L., & Gibbs, A. G. (1982). Resistance of the shell membrane and mineral layer todiffusion of oxygen and water in flexible-shelled eggs of the snapping turtle (*Chelydra serpentina*). *Respiration Physiology, 49*(3), 179-191.

Ferreira Júnior, P. D., & Castro, P. T. A. (2003). Geological control of *Podocnemis expansa* and *Podocnemis unifilis* nesting areas in Rio Javaés, Bananal Island, Brazil. *Acta Amazonica, 33*(3), 445-468.

Fraser, A. C., & Cusack, M. (2002). Investigation of partridge eggshell cuticle using SEM and EDX. *Microscopy and Analysis (UK), 88*, 15-16.

Grizzle, J., Iheanacho, M., Saxton, A., & Broaden, J. (1992). Nutritional and environmental factors involved in egg shell quality of laying hens. *British Poultry Science, 33*(4), 781-794.

Iverson, J. B. A. (1992). *Revised checklist with distribution maps of the turtles of the world* (2nd ed.). Richmond, IN: Eartham College.

Jackson, M. E., Hellwig, H. M., & Waldroup, P. W. (1987). Shell quality: potential for improvement by dietary means and relationhips with egg size. *Poultry science, 66*(10), 1702-1713.

Kitimasak, W., Thirakhupt, K., & Moll, D. L. (2003). Eggshell structure of the siamese narrow-headed softshell turtle *Chitra chitra* Nutphand, 1986 (Testudines: Trionychidae). *Sciences Asia, 29*, 95-98.

Kusuda, S., Yasukawa, Y., Shibata, H., Saito, T., Doi, O., Ohya, Y., & Yoshizaki, N. (2013). Diversity in the matrix structure of eggshells in the Testudines (Reptilia). *Zoological science, 30*(5), 366-374.

Luz, V. L. F., Stringhini, J. H., Bataus, Y. S. L., Fernandes, E. S., Paula, W. A., Novais, M. N., & Reis, I. J. (2003). Rendimento e composição química de carcaça da Tartaruga-da-Amazônia (*Podocnemis expansa*) em sistema comercial. *Revista Brasileira de Zootecnia, 32*(1), 1-9.

Lynn, G., & Brand, T. V. (1945). Studies on the oxygen consumption and water metabolism of turtle embryos. *The Biological Bulletin, 88*, 112-125.

Mine, Y. (1995). Recents advances in the understanding of egg white protein functionally. *Trends in Food Science & Technology, 6*(7), 225-232.

Nakage, E. S., Cardozo, J. P., Pereira, G. T., Queiroz, S. A., & Boleli, I. C. (2002). Efeito da forma física da ração sobre a porosidade, espessura da casca, perda de água e eclodibilidade em ovos de perdiz (*Rhynchotus rufescens*). *Revista Brasileira de Ciência Avícola, 4*(3), 227-234.

Osborne, L., & Thompson, M. B. (2005). Chemical composition and structure of the eggshell of three oviparous lizard. *Copeia,* 2005(3), 683-692.

Packard, M. J., Packard, G. C., & Boardman, T. J. (1982). Structure of eggshells and water relations of reptilian eggs. *Herpetologica, 38*(1), 136-155.

Packard, M. J., Phillips, J. A., & Packard, G. C. (1992). Sources of mineral for green iguanas (*Iguana iguana*) developing in eggs exposed to different hydric environments. *Copeia*, *1992*(3), 851-858.

Pedroso, A. A., Moraes, V. M. B., Ariki, J., & Kronka, B. (1999). Efeito de níveis dietéticos de cálcio e fósforo disponível sobre o desempenho e qualidade dos ovos de codornas japonesas. *ARS Veterinaria, 15*(2), 135-139.

Pough, F. H., Heiser, J. B., & Janis, C. M. (2008). *A vida dos vertebrados* (4a ed.). São Paulo, SP: Atheneu.

Pritchard, P. C. H., & Trebbau, P. (1984). *The turtles of Venezuela*. Oxford, MI: Society for the Study of Amphibians and Reptiles.

Roze, J. A. (1964). Pilgrim of the river. *Natural History, 73*(7), 35-41.

Sahoo, G., Sahoo, R. K., & Mohanty-Hejmadi, P. (1998). Calcium metabolism in olive ridley turtle eggs during embryonic development. *Comparative Biochemistry and Physiology*, *121*(1), 91-97.

Simkiss, K. (1962). The source of calcium for the ossification of the embryos of the giant leathery turtle. *Comparative Biochemistry and Physiology,* 7(1-2), 71-74.

Solomon, S. E., & Baird, T. (1976). Studies on the egg shell (oviducal and viposited) of *Chelonia mydas*. *Journal of Experimental Marine Biology and Ecology*, 22, 145-160.

Verdade, L. M., A. Lavorenti, & Packer, I. U. (1993). Manejo reprodutivo do jacaré-de-papo-amarelo (*Caiman latirostris*) em cativeiro. pp.143-151. In L. M., Verdade, I.U. Packer, M. B. Rocha, F. B., Molina, P. G. Duarte, & L. A. B. M. Lula, (Eds.), *Anais do 3o. Workshop sobre Conservação e Manejo do Jacaré-de- Papo-Amarelo (Caiman latirostris)* (p. 143-152). Piracicaba, SP: Esalq.

Washburn, K. W. (1982). Incidence, cause, and prevention of egg shell breakage in commercial production. *Poultry Science, 61*(10), 2005-2012.

Zhou, J., Wang, S., Nie, F., Feng, L., Zhu, G., & Jiang, L. (2011). Elaborate Architecture of the Hierarchical Hen's Eggshell. *Nano Research*, *4*(2), 171-179.

Received on June 1, 2015.
Accepted on June 14, 2016.