Morphophysiological Analysis of Keratobranchial Bone II located in the Hyoid of the Green Turtle (*Chelonias mydas*) found in Peruíbe, South Coast of Brazil, Mosaic of Conservation Units-Juréia-Itatins and APACIP - Environmental Protection Area-Cananéia-Iguápe-Peruíbe-SP

Análise Morfofisiológica do Osso Keratobranquial II localizado no Hyoid of the Green Turtle (Chelonias mydas) encontrado no Peruíbe, Litoral Sul do Brasil, Mosaico de Unidades de Conservação-Juréia-Itatins e APACIP - Área de Protecção Ambiental-Cananánéia-Iguápe-Peruíbe-SP

DOI: 10.53499/sfjeasv1n4-002

Received in: August 1st, 2021
Accepted in: September 30th, 2021

Edris Queiroz Lopes
PhD in Sciences by the University of São Paulo
Institution: Institute of Marine Biology and Environment – IBIMM
Anatomy of Domestic and Wild Animals - FMVZ / University of São Paulo
Address: Palmares Farm – Santa Cruz das Palmeiras – SP Brasil
Zip Code: 13650-000
E-mail: edris@ibimm.org.br

Tatiane Gonçalves de Lima
Graduating in Biological Sciences from Cruzeiro do Sul University, São Paulo
Institution: Institute of Marine Biology and Environment
Address: Palmares Farm – Santa Cruz das Palmeiras – SP
Zip Code: 13650-000
E-mail: tatiane@ibimm.org.br

Luana Félix de Melo
PhD student in Sciences at FMVZ - University of São Paulo
Institution: Institute of Marine Biology and Environment - IBIMM
Anatomy of Domestic and Wild Animals - FMVZ / University of São Paulo
Address: Av. Prof. Orlando Marques de Paiva, 87 – Butantã – São Paulo – SP
Zip Code: 05508-010
E-mail: luuh-felix@hotmail.com

Rose Eli Grassi Rici
PhD student in Sciences at FMVZ - University of São Paulo
Institution: Institute of Marine Biology and Environment - IBIMM
Anatomy of Domestic and Wild Animals - FMVZ / University of São Paulo
Address: Av. Prof. Orlando Marques de Paiva, 87 – Butantã – São Paulo – SP
ZipCode: 05508-010
E-mail: roseeli@usp.br

ABSTRACT
The green turtle (*Chelonia mydas*) present in tropical seas, uses as a feeding area the coastal region of Peruíbe, has the skull as a relatively large and solid structure, and a strong jaw formed by the junction of small bones as it has very abrasive feeding. By applying scanning electron microscopy techniques, it was possible to identify the presence of a bone structure located in the hyoid in the ventral region of the skull along with the mandible of juvenile individuals of green turtles, and as there is no related research, it was necessary to perform a CT scan, decalcification and histology of the quelonian hyoid, to discover the morphological composition of this new structure, described only in the species *Chelonia mydas*. Thus, the morphology of the structures and its confirmation as a real bone, with characteristic of spongy bone, described as certobranchial II, was confirmed, thus helping researchers to seek other ways to understand the feeding processes of these animals that are going through a series of serious environmental problems and therefore perhaps having to change their eating habits to overcome the high level of pollution that we are finding in the oceans.

Keywords: anatomy, histology, morphology, tomography.

RESUMO
A tartaruga verde (Chelonia mydas) presente nos mares tropicais, utiliza como zona de alimentação a região costeira do Peruíbe, tem o crânio como uma estrutura relativamente grande e sólida, e uma mandíbula forte formada pela junção de pequenos ossos, uma vez que tem uma alimentação muito abrasiva. Aplicando técnicas de microscopia electrónica de varrimento, foi possível identificar a presença de uma estrutura óssea localizada no hióide na região ventral do crânio, juntamente com a mandíbula de indivíduos juvenis de tartarugas verdes, e como não existe investigação relacionada, foi necessário realizar um TAC, descalcificação e histologia do hióide quelónico, para descobrir a composição morfológica desta nova estrutura, descrita apenas na espécie Chelonia mydas. Assim, a morfologia das estruturas e a sua confirmação como um osso real, com característica de osso esponjoso, descrito como certobranchial II, foi confirmada, ajudando assim os investigadores a procurar outras formas de compreender os processos de alimentação destes animais que estão a passar por uma série de graves problemas ambientais e, por conseguinte, talvez tenham de alterar os seus hábitos alimentares para superar o elevado nível de poluição que estamos a encontrar nos oceanos.

Palavras-chave: anatomia, histologia, morfologia, tomografia.

1 INTRODUÇÃO

The green turtle (*Chelonia mydas*) is present in tropical seas (ERNEST & BARBOUR, 1989), and uses as a feeding area the littoral region of Peruíbe, located in the southern coast of São Paulo that is within the areas of Conservation Units Cananéia - Iguape - Peruíbe, Mosaic of Conservation Units Juréia - Itatins and Ecological Station Tupiniquins, belonging to APAMLC (Apa Marinha Litoral Centro) where the specimens of this study were collected (LOPES et al, 2018).

The sea turtle has a solid skull and has no temporal openings, it is formed by the junction of several small bones that aim to protect its brain (ROMER, 1956). The skull is formed by the neurocranium, the internal box that houses and protects the brain and the splancnocranium, a bony superstructure that is the external part with the function of covering, protecting, and housing the neurocranium, the sense organs, provides the muscle attachment for the muscles of the jaw, throat, and neck (WYNEKEN, 2011). Thus the skull is a relatively large structure with a strong jaw, formed by the junction of small bones.

The types of bones are characteristic for each species and another interesting factor is that turtles do not have teeth but rather horny beaks (also called ranfotecae) in both the upper and lower jaws. They differ according to diet and can be used to identify species (WYNEKEN, 2001).

Computed tomography (CT) is a non-invasive method of imaging diagnosis that has been the focus of research to develop new methods of study and treatment in order to assist in the conservation of sea turtle species, as can be cited the use in rehabilitation processes. By presenting high sensitivity, it allows the visualization of various organs, providing information about their shape, as well as the characterization of small changes, in addition, it is possible to provide information on bone morphology, consequently enabling the acquisition of biomechanical parameters, as it characterizes the loss of bone mass (ALVES, 2004; OLIVEIRA et al., 2012). It is widely used to detect skeletal and soft tissue disorders in chelonians (GUMPENBERG & HENNINGER, 2001). This occurs because turtles present alterations in bone mineral metabolism regardless of whether they are free-living or captive animals (ADAMS, 2009).

In application of the CT technique in the ventral region of the skull along with the mandible of juvenile green turtle individuals, the presence of a bony structure located in the second ceratobranchial horn of the hyoid bone was observed (LOPES et al., 2019) and since there is no related research it was necessary to perform histology of the chelonian hyoid to find out the morphological composition of this new structure, described only in the species *Chelonia mydas* by Lopes and collaborators (2019) called ceratobranchial bone II.

Thus the present study aimed to describe the microscopic part of the new hyoid bone structure, located in the 2nd branchial horn through the bone decalcification technique, with the histology analyzed by light microscopy.

2 MATERIAL E MÉTODOS

2.1 ANIMAIS E OSSOS

Two specimens of green turtles (*Chelonia mydas*), found stranded dead on beaches within the Environmental Protection Area - Cananéia - Iguápe - Peruíbe and Conservation Units of the Forest Foundation of the State of SP, collected by the SOS Turtle Project, with a license from Tamar ICMBio-50132 and authorization of the Ethics Committee - 003/19 CEUA-IBIMM, were used in this study. The skull dissection was performed using a scalpel, sectioning the skin and muscles to access the tongue, after which the muscles and cartilage of the tongue were separated to approach the hyoid bone. The bones were collected, photographed, with an average length of 0.5 cm, and stored in 70% alcohol.

2.2 DESCALCIFICAÇÃO

For decalcification of the bone, OSTEOMOLL® Decalcifier - Solution 1 liter (code 101736), diluted to 15%, was used to cover the material with a volume of 10 times the size of the material. To prevent the material from being compromised and to be able to determine when the material was fully corroded, a needle was used for the puncture resistance test, being ready 30 days after the beginning of the process. After this period the material was washed under running water for five days to remove the bluish coloration, so as not to interfere with the staining process.

2.3 MICROSCOPIA DE LUZ (ML)

The samples were fixed in 10% formaldehyde solution for 48 hours, dehydrated in ethanol series in increasing concentrations (70 to 100%), and diaphanized in xylol, each procedure lasting 24 hours, with subsequent inclusion in histological paraffin. The paraffin blocks were hydrated with water and stored in the refrigerator for 16 hours before starting the sections. Sections of 5µm thick were made on a microtome (Leika, German) and stained with hematoxylin and eosin HE. The images were obtained with a Nikon Eclipse E- 80i light microscope from the Centro Avançado em Diagnóstico por Imagem - CADI- FMVZ-USP.

2.4 TOMOGRAFIA COMPUTADORIZADA (TC)

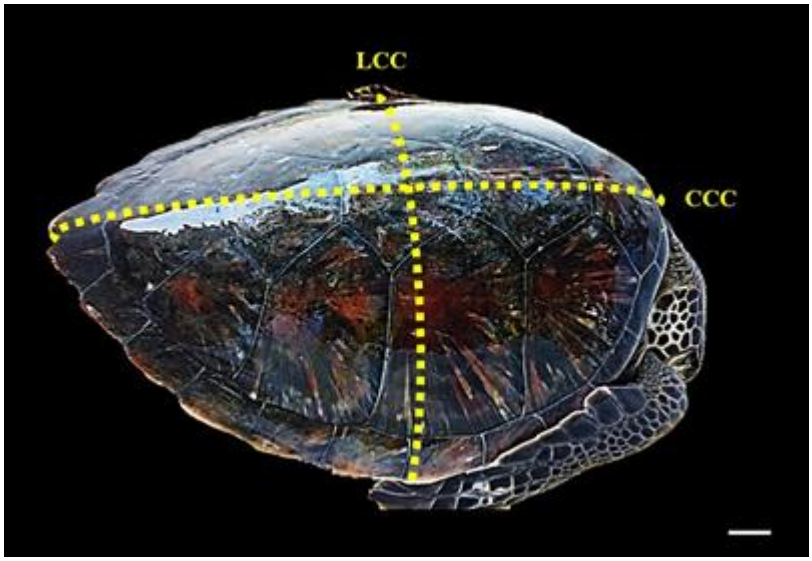
The animals were taken to the radiology and tomography center of the University Hospital of the University of São Paulo, where the images were diagnosed using

Tomography Equipment: Philips Brilliance, 64 rows of detectors. Slice thickness: 1mm. Windows for soft parts and for bone parts were obtained. Soft part windows: Center (WL 60) and Width (WW 400), Bone part windows: Center (WL 300) and Width (WW 1500), Cuts reconstructed in the Axial, Coronal and Sagittal planes. The Volumetric Reconstruction Technique was done with Volume Rendering and the computer programs used for visualization and capture was done using Philips Workstations and Radiant DICOM Viewer. For the hyoid bones only windows for bone parts were used. (HU - University Hospital - Radiology Center - USP).

3 RESULTS

After obtaining the animals, the identification of juvenile green turtle individuals and their biometry was performed by measuring the curvilinear carapace length (CCC), and the curvilinear carapace width (LCC) using a measuring tape (Figure 01).

Figura 01: Fotomacrografia de *Chelonia mydas* com demonstração dos locais medidos para obtenção do CCC e LCC. Barra 1cm.



Together with the measurements and the bodyweight of the animals, using an overhead balance (pesola), the biometric data of the green turtle were obtained (table 01), according to the parameters described by Wyneken (2001).

Tabela 01 – Dados biométricos dos espécimes de *Chelonia mydas*.

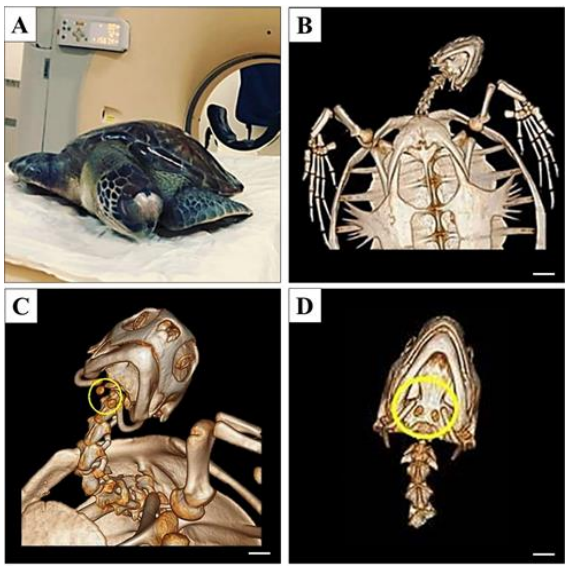
Animal	CCC	LCC	Peso
01	40 cm	35 cm	6,5 kg
02	36 cm	32 cm	4,5 kg

Fonte: Dados obtidos através dos parâmetros utilizados e descritos por Wyneken (2001).

To visualize the bone structures, present in the sea turtle, *Chelonia mydas* specimens were taken for computed tomography (CT) scanning in order to explore, through high-resolution 360-degree images, detailed photos that reconstruct the turtle's entire body three-dimensionally, thus allowing a faithful view of its skeleton that can be analyzed from any angle.

Thus, each turtle specimen was positioned in dorsal decubitus (Figure 02A) in the scanner to obtain and investigate the images. By sectioning the images obtained in the transverse plane and observing the cranial portion in ventral view (Figure 02B), the presence of bony structures located in the body of the hyoid or ceratohyoid can be evidenced, which, being composed of cartilage, does not appear in the images, supported by the two bones of the hyoid process or ceratobranchial bones (figure 02CD).

Figure 02: Photomacrography of *Chelonia mydas.* In A specimen in dorsal decubitus in CT scan. In B computed tomography image of the cranial portion of the turtle. In C and D CT scan image in ventral view of the turtle skull with highlighting in yellow the two bones in the hyoid apparatus, located in the region of the hyoid body. Bar 1 cm.

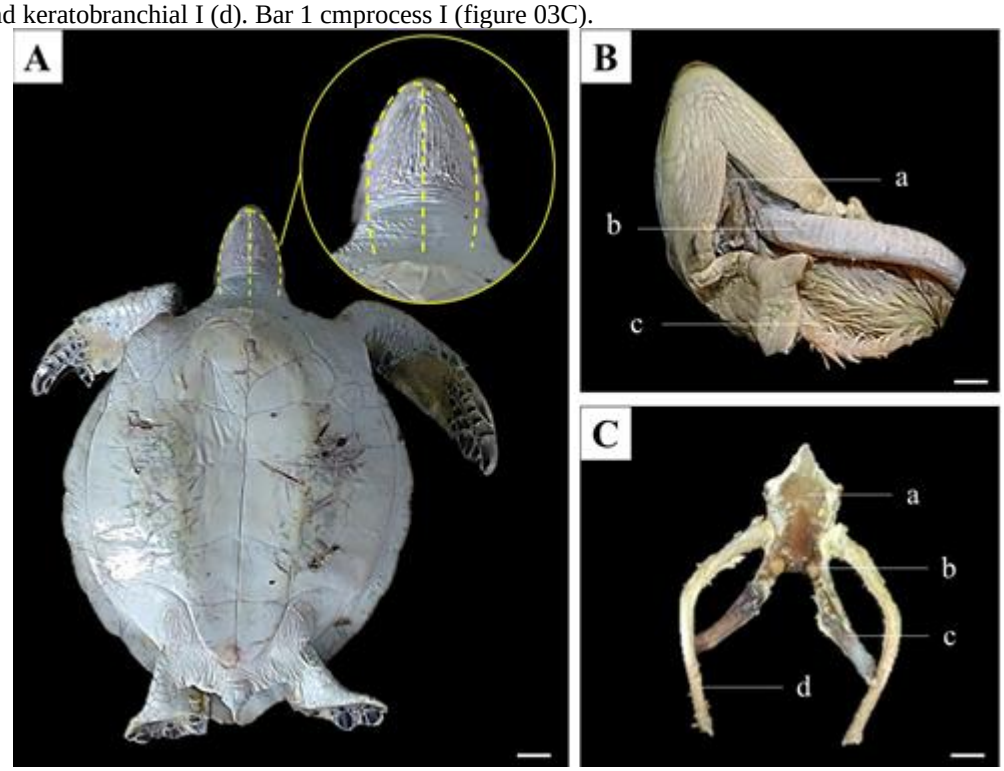


After the location of the keratobranch bones, II present in the hyoid was confirmed using a CT scan, the specimens were sent to necropsy for removal of the structure.

The turtles were laid down in ventral decubitus for access to the ventral region of the skull, with incisions in the medial region, followed by two lateral incisions, shown in yellow (figure 03A), after the first incision it is possible to observe the muscle layer that supports the hyoid, the trachea and the esophagus that in its internal part presents esophageal papillae (figure 03B). After removing the hyoid apparatus, it is possible to

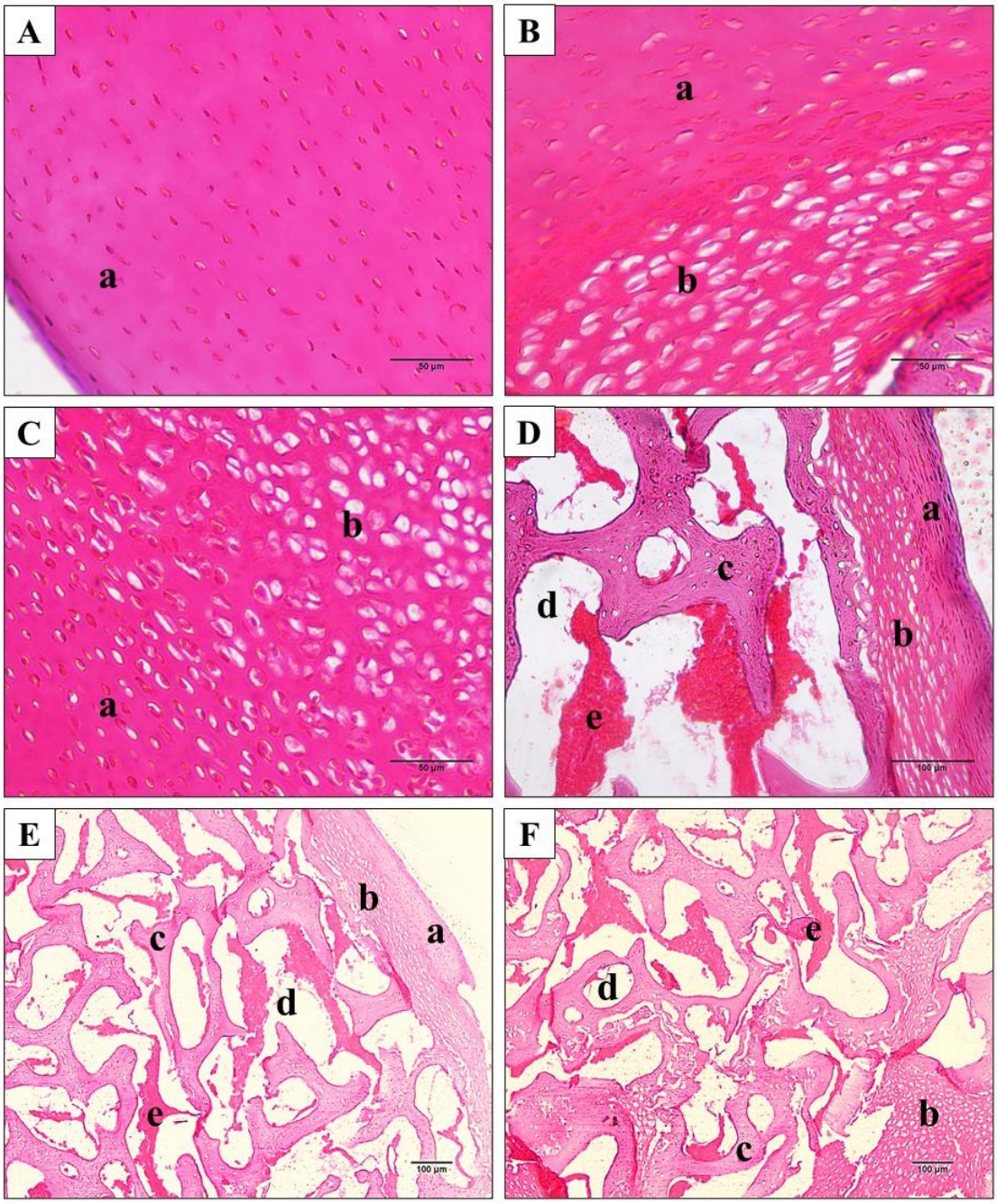
observe all its structures, such as the hyoid body, which in its interior presents the keratobranchial bones II, the hyoid process bone, and the keratobranchial

Figure 03: Photomacrography of *Chelonia mydas*. In A with specimen in ventral decubitus with central and lateral incisions in the ventral region of the turtle's skull highlighted in yellow. In B the ventral region with medial incision of the neck, exposing superficial and deep muscles of the neck (a), trachea (b) and open esophagus (c). In C hyoid apparatus, showing the hyoid body (a), keratobranchial bone II (b), hyoid process (c) and keratobranchial I (d). Bar 1 cmprocess I (figure 03C).



Histology, after decalcification and staining of the material, showed that the structure is bone, with characteristics of cancellous bone, presenting in its outermost layer the mature osteocytes, followed by osteoblasts, and in its internal cavity the elongated trabeculae interposed by the spaces between the growths of the trabeculae, where one can also observe fragments of necrotic bone originating from the bone trabeculae (figure 04ABCDEF).

Figure 04: Photomicrograph of the Ceratobranchial II bone, showing the outermost layer with mature osteocytes (a), and more internally the osteoblasts in maturation (b), the elongated trabeculae (c), characteristic of cancellous bone, spaces of growth of the trabeculae (d), and the fragments of necrotic bone originating from bone trabeculae (e). HE staining.



4 DISCUSSION

The techniques of computed tomography and histology combined with decalcification used in this study contributed to the confirmation of the new structure present in the green turtle hyoid body as a real bone. The decalcification technique, even being time-consuming is an effective method and collaborates for the description of bone structures, thus one can prove what was observed in the scanning electron microscopy of the work

of LOPES and collaborators (2019). In previous works, Kuratani (1988, 1989, and 1997) describes the entire morphology of the development of the hyoid in the skull of the species *Caretta caretta* but does not present at any stage of the development stages the appearance of the structure observed in green turtles. Other authors described the green turtle skull but did not mention the presence of the bony structure found in the hyoid. (WERNEBURG et al., 2011; GARCIA et al., 2012; JONES et al., 2012; WYNEKEN, 2011; ARENCIBIA et al., 2005).

Thus, in all published works on the description of the anatomical structures of the skull of sea turtles, none of the authors in the cited works was able to visualize the structure present in the hyoid apparatus, in the body in the cartilaginous hyoid, called ceratobranchial II in the green turtle.

5 CONCLUSION

The morphology of the structures discovered in the green turtle and its confirmation as a real bone, with characteristic of spongy bone, will help researchers to seek other ways to understand the feeding processes of these animals that are going through a series of serious environmental problems and therefore perhaps having to modify their eating habits to overcome the high rate of pollution that we are finding in the oceans. The methodologies described here will be very useful to professionals as an aid to the tools and methods of diagnostic imaging and histology.

ACKNOWLEDGMENTS

To the Advanced Imaging Diagnostic Center - CADI - FMVZ - USP and to HU - University Hospital - Radiology Center - USP.

REFERENCES

ADAMS, J.E. 2009. Quantitative computed tomography. European Journal of Radiology, v.71, p.415-424. Disponível em http://www.sciencedirect.com/science/article/pii/S0720048X09004343. Visualizado em 20/04/2019.

ALVES, L. C. 2004. Tomografía Computerizada de la Cavidad Torácica del Perro (*Canis familiares L.*) Mediante Aparato de Sexta Generación y Medios de Contraste Oral y Vascular — Murcia. Doctoral Thesis, Murcia Univ., Murcia, Spain.

ARENCIBIA, A., RIVERO, M. A., DE MIGUEL, I., CONTRERAS, S., CABRERO, A., & ORÓS, J. (2006). Computed tomographic anatomy of the head of the loggerhead sea turtle (*Caretta caretta*). Research in Veterinary Science, 81(2), 165–169.

GUMPENBERG, M.; HENNINGER, W. The use of computed tomography in avian and reptile medicine. Seminars in Avian and Exotic Pet Medicine, v.10, n.4, p.174-180, 2001.

JONES MEH, Werneburg I, Curtis N, Penrose R, O'Higgins P, et al. (2012) The Head and Neck Anatomy of Sea Turtles (Cryptodira: Chelonioidea) and Skull Shape in Testudines. PLoS ONE 7(11): e47852.
KURATANI S (1989) Development of the orbital region in the chondrocranium of Caretta caretta. Reconsideration of the vertebrate neurocranium configuration. Anat Anz 169: 335–349.

KURATANI S, TANAKA S, ISHIKAWA Y, ZUKERAN C (1988) Early development of the hypoglossal nerve in the chick embryo as observed by the whole-mount nerve staining method. Am J Anat 182: 155-168.

KURATANI S, MATSUO I, AIZAWA S (1997a) Developmental patterning and evolution of the mammalian viscerocranium: Genetic insights into comparative morphology. Dev Dyn 209: 139-155.

LOPES, E. Q.; LEITE C. S.; SILVA, C. S. A.; MELO, L. F.; FANNELI, C. Análise do conteúdo alimentar de tartarugas-verdes (*Chelonia mydas*) mortas em encalhes na Costa de Peruíbe, litoral sul de São Paulo. In: Seminário Internacional Oceanos Livres de Plástico, 1, Santos, 7 a 8 jun. 2018. Anais do I Seminário Internacional Oceanos Livres de Plásticos. Santos: Unisanta Bioscience. p. 77-98, 2018.

LOPES, E.Q Et Al, Morphological studies of the green-turtle's hyoid bone composition (Chelonia mydas) found in Peruíbe, Litoral Sul do Brasil, Mosaico de Unidades de Conservação-Jureia-Itatins; International Journal of Advanced Engineering Research and Science (IJAERS) Vol-6, Issue-9, Sept- 2019

OLIVEIRA, JANNINE FORATTINI DE et al. Densitometria da vértebra dorsal, osso pleural e osso neural em tartarugas verdes hígidas por tomografia computadorizada quantitativa.Cienc. Rural, Santa Maria , v. 42, n. 8, p. 1440-1445, Aug. 2012 . Disponível em (http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0103-84782012000800018&lng=en&nrm=iso). Visualizado em 20/04/2019

PÉREZ−GARCÍA, A., DE LA FUENTE, M.S., AND ORTEGA, F. 2012. A new freshwater basal eucryptodiran turtle from the Early Cretaceous of Spain. Acta Palaeontologica Polonica 57 (2): 285–298.

ROMER, A.S. 1956. Osteology of the Reptiles. Krieger Publishing Group. Florida. 772 pp

WYNEKEN, J. 2001. The Anatomy of Sea Turtles. U.S. Department of Commerce NOAA Technical Memorandum NMFS-SEFSC-470, 1-172 pp.

WERNEBURG I. 2012. Temporal bone arrangements in turtles: Na overview. J. Exp. Zool. (Mol. Dev. Evol.) 318:235–249.