Fibropapillomas in a Loggerhead Sea Turtle (*Caretta caretta*) Caught in Almofala, Ceará, Brazil: Histopathological and Molecular Characterizations

April S. Rossi[1], Marco A. Gattamorta[1,2,3], Fabiola E.S. Prioste[1,4], Eduardo H.S.M. Lima[5], Maria T.D. Melo[5], Paulo E. Brandão[6], Sheila O. de Souza Silva[6], Fernanda M. da Silveira[5] & Eliana R. Matushima[1,4]

[1]Research Group on Fibropapillomatosis in Sea Turtles, Universidade de São Paulo, Brazil (E-mail: smara.rossi@gmail.com); [2]Escola Superior de Agricultura Luiz de Queiroz and Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Brazil; [3]Faculdades Metropolitanas Unidas, Brazil (E-mail: mgattamorta@uol.com.br); [4]Laboratório de Patologia Comparada de Animais Selvagens, Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil (E-mail: fabiolaprioste@gmail.com; elianamatushima@gmail.com); [5]Fundação Centro Brasileiro de Proteção e Pesquisa das Tartarugas Marinhas, Brazil (E-mail: eduardo.lima@tamar.org.br, thereza.damasceno@tamar.org.br; fernanda.mendes@tamar.org.br); [6]Applied Molecular Biology and Serology Laboratory, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil (E-mail: paulo7926@usp.br; sheilacateq@usp.br)

There are many threats to marine turtles, including habitat destruction, pollution, coastal development and fishing activities. Additionally, diseases such as fibropapillomatosis (FP), which is characterized by the development of skin tumors (Adnyana *et al.* 1997; Bugoni *et al.* 2001; Marcovaldi & Thomé 2000; Mast *et al.* 2005; Oravetz 2000) may directly or indirectly impact sea turtles. The prevalence tends to be higher in marine environments under the impact of human activities. Environmental pollutants such as organochlorine and organophosphate compounds, carbamates, selenium and heavy metals seem to be possible factors in the pathogenesis of FP (Aguirre *et al*. 1994; Aguirre & Lutz 2004; Chaloupka *et al.* 2009; Ene *et al.* 2005; Herbst 1994; Herbst & Klein 1995; Keller *et al*. 2014; Miao *et al*. 2001). Furthermore, tumors are most often observed when marine turtles are under stressful environmental conditions as well as when they are in regions with low water quality and in the presence of contaminants and toxins (Formia *et al*. 2007). It has been shown that green sea turtles (*Chelonia mydas*) use coastal areas more than other marine turtle species (Hirth 1997), which may explain why this species seems more susceptible than other sea turtles. Studies on the east coast of Florida in 1998 and 1999 compared and examined initial and recapture photographs; results indicated that 88% of recaptured green sea turtles showed regression of FP tumors (22 of 25 recaptured turtles). In Brazil, studies in the coastal region of Niterói, RJ, between July 2008 and August 2013, also documented fibropapilloma regression: 233 green sea turtles were captured and seven of them showed clear signs of regression of at least one tumor (Hirama & Ehrhart 2007; Tagliolatto 2013). Field workers have reported lesions like fibropapillomas in loggerhead sea turtles (*Caretta caretta*) from the Indian River Lagoon, Florida Bay and the Florida Keys (Florida, U.S.) and in Australia; olive ridley sea turtles (*Lepidochelys olivacea*) from the Pacific coast of Costa Rica and flatback sea turtles (*Natator depressus*) from Australia. Histopathological tests were confirmed in loggerhead and hawksbill sea turtles (*E. imbricata*; Ene *et al*. 2005; Herbst 1994).

Studies reveal that FP has multifactorial etiology in which several biological, genetic and environmental cofactors could play a significant role in the pathogenesis. Additionally, a viral etiology could be at play, considering the alphaherpesvirus chelonid fibropapilloma-associated herpesvirus (CFPHV) (Ene *et al.* 2005; Herbst *et al.* 1998; Lackovich *et al.* 1999; van Houtan *et al.* 2010; Work *et al.* 2009).

The herpesvirus detected in fibropapilloma belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Scutavirus,* and is named chelonid herpesvirus 5 (ChHV-5). According to Herbst *et al.* (2004), phylogenetic analysis of ChHV-5 identified two major clades each with Atlantic and Pacific representatives. This herpesvirus has been identified in 100% of tumors induced by inoculation of tumor cell infiltrates (Ene *et al.* 2005).

In Brazil, many studies have been conducted to understand the physiopathology of FP. The aims of the present study were to characterize FP and detect CFPHV by histopathological and molecular analyses in tumors obtained from one loggerhead sea turtle caught at Boca da Barra, Ceará, Brazil.

A loggerhead sea turtle was captured incidentally by fishermen in fishing weir number 16 (Longitude: -39.82199°, Latitude: -2.89348°) at Boca da Barra, district of Almofala, Western Coast of Ceará, Brazil (Fig. 1) on 31 March 2010 (straight carapace length = 79 cm; Inconel flipper tag numbers BR66579 and BR66580; sex not

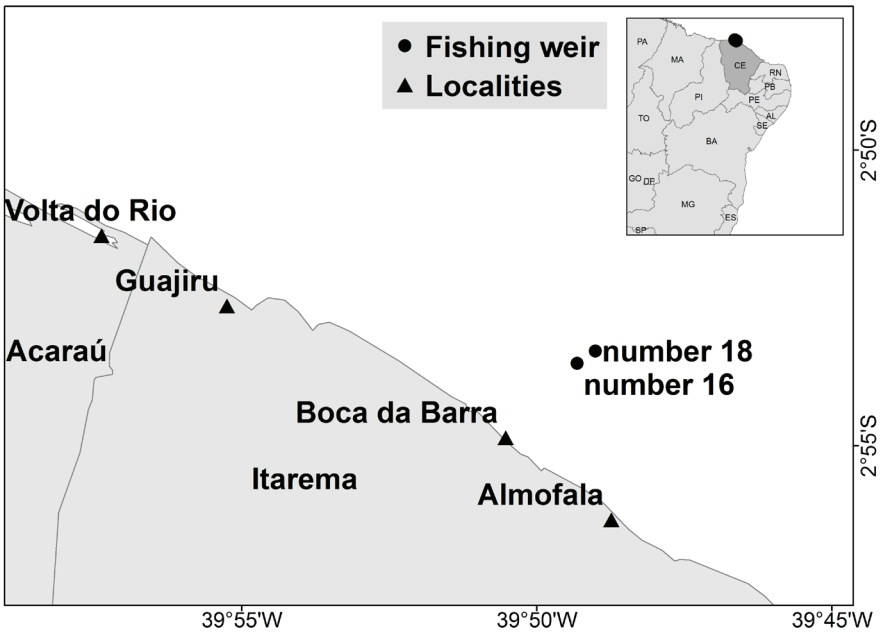

Figure 1. Fishing weirs number 16 and 18 in Boca da Barra, Ceará, Brazil, where the loggerhead sea turtle was captured.

Figure 2. Loggerhead sea turtle with tumors, captured at Boca da Barra, Almofala, Ceará, Brazil. Photo credit: Image bank of Projeto TAMAR/Ceará.

determined). After capture, the turtle was delivered to the Brazilian Sea Turtle Conservation Program (Projeto TAMAR-ICMbio), and released soon after. On 26 October 2012, the same loggerhead (84.6 cm SCL and 64 kg; classified as female) was recaptured at Boca da Barra in fishing weir number 18 (Fig. 1) (Longitude: -39.81682°, Latitude: -2.89007°) and cutaneous growths, similar to fibropapillomas found in green turtles, were observed. In this region, the fishing weirs are located at depths between 5 and 6 m (1-2 miles offshore) and set in an area where there are algae associated with marine gravel. The Ceará coast of northeastern Brazil extends 573 km along the Equatorial South Atlantic Ocean. This area is characterized by low industrial development and is considered an important feeding area for green sea turtles. Observational reports of loggerheads, olive ridleys, hawksbills and leatherbacks (*Dermochelys coriacea*) exist for this area (Lima *et al.* 2007; Lima *et al.* 2013; Marcovaldi 1993). The major threat to marine turtles in this region is the high rate of incidental capture in fishing weirs, gillnets and trawl nets (Lima *et al*. 1999; Lima *et al.* 2013; Marcovaldi & Marcovaldi 1999).

The loggerhead turtle had thirteen tumors (Fig. 2), which were classified according to categories of size: A (<1 cm), B (1-4 cm), C (>4-10 cm) and D (>10 cm) (Work & Balazs 1999) and anatomic region. Three tumors were obtained for histopathological and molecular analyses: (1) collected from the right front flipper (category A), (2) obtained from the neck (Category D) and (3) collected from the left front flipper (non-classified). Before sample collection, the affected regions were cleaned with alcohol. Tumor biopsies were collected using a scalpel blade between the skin and tumor, with a margin of safety avoiding neoplasia recurrence. Povidine® solution was used for asepsis and the bleeding was controlled with gauze compress for several minutes. Tumor biopsies were fixed in 10% neutral buffered formalin for histopathology analysis and subsequently stored in 70% alcohol and frozen at -20 °C until molecular processing. Slides of the tumor samples were stained with hematoxylin-eosin (HE) and prepared at Laboratório de Histologia, Departamento de Patologia, Faculdade de Medicina

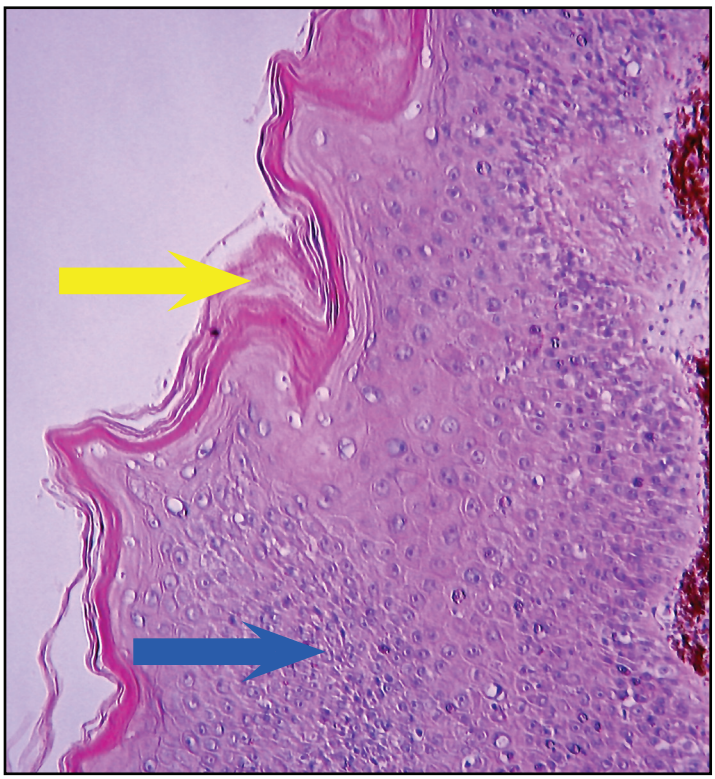

Figure 3. Histopathological section of fibropapilloma from the loggerhead sea turtle stained using HE (obj. 4x). There is a great proliferation of epithelial cells characterized by ballooning degeneration (blue arrow), thickening of the stratum corneum (yellow arrow). Photo credit: Image bank of Research Group on Fibropapillomatosis in Sea Turtles.

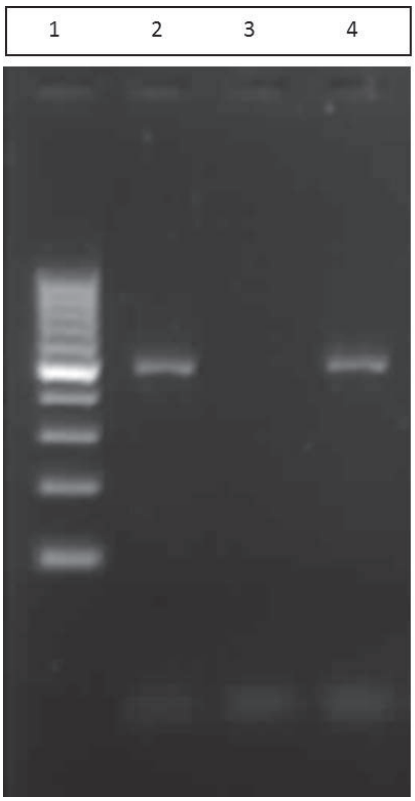

Figure 4. Electrophoreses in 1.5% agarose gel for 480-bp fragments using GTHV 2/GTHV 3 primers. (1) Ladder 100-bp; (2) Positive control; (3) Negative control; (4) Sample of fibropapilloma from *C. caretta*, positive for DNA polymerase of turtle herpesvirus.

Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP).

DNA extraction was carried out according to Chomksinsky (1993). The herpesvirus variant reactions were conducted using methods described by Ene *et al.* (2005). A 2.5-µL DNA aliquot was submitted to PCR in a final 25-µL reaction using the specific primers for DNA polymerase of turtle herpesvirus, GTHV2 (5'- GACACGCAGGCCAAAAAGCGA-3') and GTHV3 (5'-AGCATCATCCAGGCCCACAA-3'), described by Quackenbush *et al.* (2001). The conventional PCR reaction was conducted in 12.625 µL ultrapure water, 2.5 µL of buffer solution for PCR (20 mM of Tris–HCl pH 8.4 and 50 mM of KCl), 4.0 µL dNTP (200 µM each dNTP), 1.25 µL of each primer (0.4 µM of each primer), 0.75 µL of 1.5 mM $MgCl_2$ and 0.125 µL of the enzyme Platinum Taq polymerase (Invitrogen Life Technologies). The sample was denatured at 94 °C for 5 min and then was amplified with 35 cycles (94 °C for 30s, 62 °C for 30s, 72 °C for 30s) and then a 10-min cycle at 72 °C in a thermal cycler. The amplified product in all PCRs was resolved by electrophoresis in 1.5% agarose gel in Tris-borate-EDTA buffer (0.045 M Tris-Borato, 0.5 M EDTA) and a voltage of 1-10 V/cm of gel. The PCR product was a fragment ~480 bp and was purified using a GFX Purification kit (GE Healthcare, UK). The purified product was submitted to an automated sequencing reaction performed using a commercial kit: ABI Prism Big Dye TM terminator - Cycle Sequencing Ready Reaction (Applied Biosystems, CA). The nucleotide sequence was processed using the BioEdit program and aligned with Clustal W (Thompson *et al*. 1994) and the sequence will be submitted to GenBank (www.ncbi.nlm.nih.gov). The molecular analysis was carried out at Applied Molecular Biology and Serology Laboratory, Departamento de Medicina Veterinária Preventiva e Saúde Animal, FMVZ-USP.

The fibropapilloma tumors varied in their appearance, such as color (white, pink and gray) and texture (smooth to verruciform). On microscopic analysis, proliferative lesions in epithelial cells characterized by ballooning degeneration varying from minimal to extensive were observed. Orthokeratotic hyperkeratosis with thickened *stratum corneum* was also verified in formations (Fig. 3). In the basal layer, it was found that vacuolated cells were necrotic and the *stratum spinosum* showed vacuolated cells often related to the underlying basal layer process. Furthermore, a highly vascularized hyperplastic stroma consisting of connective tissue resulting in an increase of dermal thickness was also seen. Wide proliferation of fibroblasts was found in all sections, mainly in the papillary area. Also, we verified a diffuse infiltration of mononuclear cells in the dermis. Many of these characteristics were also described in previous studies, which reported fibropapillomas in *C. mydas* and *E. imbricata* (both species captured on the Brazilian coast), in oral and skin tumors from *C. mydas* caught in the Hawaiian Islands and in *C. mydas* from the Indian River Lagoon, Florida, U.S.A. (D'Amato & Moraes-Neto 2000; Jacobson *et al.* 1989; Work *et al.* 2004; Zwarg *et al.* 2014).

According to histopathological examination, the cutaneous growths correspond to papillomas or fibropapillomas according to their epithelial and/or stromal proliferation. The presence of herpesvirus was confirmed with the agarose gel, and the amino acid sequence of the fibropapilloma sample suggested that it was a fragment of DNA polymerase from ChHV-5, according to GenBank (Fig. 4). However, new DNA amplification and sequencing has been conducted for the molecular characterization of ChHV-5, because some parts of the antisense sequence presented low quality in the electropherogram analysis. The molecular characterization of alpha-herpesvirus in marine turtles is part of a study conducted by the Research Group on Fibropapillomatosis in Sea Turtles - USP.

Fibropapillomas are less common in loggerheads, so more studies about the herpesvirus variants are needed for this species. Rodenbusch et al. (2012) detected the ChHV-5 in a fibropapilloma of a green turtle, caught on the coast of Rio Grande do Sul, RS-Brazil; this first report formalized the characterization of this variant in green sea turtles found in that area. Our study enabled us to characterize the tumors in the sampled loggerhead sea turtle and to detect if the herpesvirus variants were the same ones as those found in green sea turtles. This information adds knowledge about fibropapillomatosis in loggerheads that use the coast of northeastern Brazil.

Acknowledgements: We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2012/14319-6) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. The authors wish to thank Projeto TAMAR-ICMBio, which is historically a collaboration between the Brazilian government and Fundação Pró Tamar, officially sponsored by Petrobrás and Sueli Akemi Taniwaki from the Applied Molecular Biology and Serology Laboratory, FMVZ-USP for help in molecular analysis.

ADNYANA, W., P.W. LADDS & D. BLAIR. 1997. Observations of fibropapillomatosis in green turtles (*Chelonia mydas*) in Indonesia. Australian Veterinary Journal 75: 737-742.

AGUIRRE, A.A., G.H. BALAZS, B. ZIMMERMAN & T.R. SPRAKER. 1994. Evaluation of Hawaiian green turtles (*Chelonia mydas*) for potential pathogens associated with fibropapillomas. Journal of Wildlife Diseases 30: 8–15.

AGUIRRE, A.A. & P.L. LUTZ. 2004. Marine turtles as sentinels of ecosystem health: Is fibropapillomatosis an indicator? EcoHealth 1: 275-283.

BUGONI, L., L. KRAUSE & M.V. PETRY. 2001. Marine debris and human impacts on sea turtles in southern Brazil. Marine Pollution Bulletin 42: 1330-1334.

CHALOUPKA, M., G.H. BALAZS & T.M. WORK. 2009. Rise and fall over 26 years of a marine epizootic in Hawaiian green sea turtles. Journal of Wildlife Diseases 45: 1138-1142.

CHOMKCZYNSKI, P.A. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and protein from the cell and tissues samples. Biotechniques 15: 532-537.

D'AMATO, A.F. & M. MORAES-NETO. 2000. First documentation of fibropapillomas verified by histopathology in *Eretmochelys imbricata*. Marine Turtle Newsletter 89: 12.

ENE, A., M. SU, S. LEMAIRE, C. ROSE, S. SCHAFF, R. MORETTI, J. LENZ & L.H. HERBST. 2005. Distribution of chelonid fibropapillomatosis associated herpesvirus variants in Florida: molecular genetic evidence for infection of turtles following recruitment to neritic developmental habitats. Journal of Wildlife Diseases 41: 489-497.

FORMIA, A., S. DEEM, A. BILLES, S. NGOUESSONO, R. PARNELL, T. COLLINS,, G.P. SOUNGUEST, A. GIBUDI, A. VILLARUBIA, G.H. BALAZS & T.R. SPRAKER. 2007.

Fibropapillomatosis confirmed in *Chelonia mydas* in the Gulf of Guinea, West Africa. Marine Turtle Newsletter 116: 20-22.

HERBST, L.H. 1994. Fibropapillomatosis of marine turtles. Annual Review of Fish Diseases 4: 389-425.

HERBST, L.H. & P.A. KLEIN. 1995. Green turtle fibropapillomatosis: challenges to assessing the role of environmental cofactors. Environmental Health Perspectives 103: 27-30.

HERBST, L.H., E.C. GREINER, L.M. EHRHART, D.A. BAGLEY & P.A. KLEIN. 1998. Serological association between spirorchidiasis, herpesvirus infection, and fibropapillomatosis in green turtles from Florida. Journal of Wildlife Diseases 34: 496-507.

HERBST, L., A. ENE, M. SU, R. DESALLE & J. LENS. 2004. Tumor outbreaks in marine turtles are not due to recent herpesvirus mutation. Current Biology 14: 697-699.

HIRAMA, S. & L.M. EHRHART. 2007. Description, prevalence and severity of green turtle fibropapillomatosis in three developmental habitats on the east coast of Florida. Biological Sciences 70: 435-448.

HIRTH, H.F. 1997. Synopsis of the Biological Data on Green Turtle *Chelonia mydas* (Linnaeus1758). US Fish and Wildlife Service Biological Report, Washington DC USA. 126 pp.

JACOBSON E.R., J.L. MANSELL, J.P. SUNDBERG, L. HAJJAR, M.M. REICHMANN, L.M. EHRHART, M. WALSH & F. MURRU. 1989. Cutaneous fibropapillomas of green turtles (*Chelonia mydas*). Journal of Comparative Pathology 101: 39-52.

KELLER, J.M., G.H. BALAZS, F. NILSEN, M. RICE, T.M. WORK & B.A. JENSEN. 2014. Investigating the potential role of persistent organic pollutants in Hawaiian green sea turtle fibropapillomatosis. Environmental Science & Technology 48: 7807-7816. (DOI: 10.1021/es5014054).

LACKOVICH, J.K., D.R. BROWN, B.L. HOMER, R.L. GARBER, D.R. MADER, R.H. MORETTI, A.D. PATTERSON, L.H. HERBST, J. OROS, E.R. JACOBSON, S.S. CURRY & P.A. KLEIN. 1999. Association of herpesvirus with fibropapillomatosis of the green turtle *Chelonia mydas* and the loggerhead turtle *Caretta caretta* in Florida. Diseases of Aquatic Organisms 37: 89-97.

LIMA, E.H.S.M., C.J. LAGUEUX, D.W. CASTRO & M.A. MARCOVALDI. 1999. From one feeding ground to another: green turtle migration between Brazil and Nicaragua. Marine Turtle Newsletter 85: 10–11.

LIMA, E.H.S.M., M.T.D. MELO & M.H. GODFREY. 2007. Incidental capture of a leatherback along the coast of Ceará, Brazil. Marine Turtle Newsletter 117: 11.

LIMA, E.H.S.M., M.T.D. MELO & M.H. GODFREY & P.C.R. Barata. 2013. Sea turtles in the waters of Almofala, Ceará, in northeastern Brazil, 2001-2010. Marine Turtle Newsletter 137: 5-9.

MARCOVALDI, M.A. 1993. A new initiative to protect green turtles at an important foraging ground in Ceará, Brazil. Marine Turtle Newsletter 63: 13-14.

MARCOVALDI, M.A. & G.G. DEI MARCOVALDI. 1999. Marine turtles of Brazil: the history and structure of Projeto TAMAR-IBAMA. Biological Conservation 91: 35–41.

MARCOVALDI, M.A. & C.A. THOMÉ. 2000. Reducción de las amenazas a las tortugas. In: ECKERT, K.L., K.A. BJORDNAL, F.A. ABREU-GROBOIS, M. DONNELLY (Eds.). Técnicas de investigación y manejo para la conservación de las tortugas marinas. Washington: IUCN-SSC Marine Turtle Specialist Group. pp. 187-191.

MAST, R.B., B.J. HUTCHINSON, E. HOWGATE & N.J. PILCHER. 2005. MTSG update: IUCN/ SSC Marine Turtle Specialist Group hosts the second Burning Issues Assessment Workshop. Marine Turtle Newsletter 110: 13-15.

MIAO, X.S., G.H. BALAZS, S.K.K. MURAKAWA & Q.X. LI. 2001. Congener-specific profile and toxicity assessment of PCBs in green turtles (*Chelonia mydas*) from the Hawaiian Islands. Science of the Total Environment 281: 247-253.

ORAVETZ, C.A. 2000. Reducción de la captura incidental en pesquerías. In: ECKERT, K.L.; BJORDNAL, K.A.; ABREU-GROBOIS, F.A.; DONNELLY, M. (Eds.). Técnicas de investigación y manejo para la conservación de las tortugas marinas. Washington: IUCN/SSC Marine Turtle Specialist Group. pp. 217-224.

QUACKENBUSH, S.L., R.N. CASEY, R.J. MURCEK, T.A. PAUL, T.M. WORK, C.J. LIMPUS, A. CHAVES, L. DUTOIT, J.V. PEREZ, A.A. AGUIRRE, T.R. SPRAKER, J.A. HORROCKS, L.A. VERMEER, G.H. BALAZS & J.W. CASEY. 2001. Quantitative analysis of herpesvirus sequences from normal tissue and fibropapillomas of marine turtles with real-time PCR. Virology 287: 105-111.

RODENBUSCH, C.R., L.L. ALMEIDA, F.S. MARKS, M.W. ATAÍDE, M.M. ALIEVI, M. TAVARES, R.A. PEREIRA & C.W. CANAL. 2012. Detection and characterization of fibropapilloma associated herpesvirus of marine turtles in Rio Grande do Sul, Brazil. Pesquisa Veterinária Brasileira 32: 1179-1183.

TAGLIOLATTO, A., S. GUIMARÃES, A. VIDAL, C. MONTEIRO, G. LOBO-HAJDU & H. GITIRANA. 2013. Evidência fotográfica de regressão de fibropapilomas em tartarugas-verdes C*helonia mydas* em Itaipu, Rio de Janeiro, Brasil. In: JORNADA, 6.; reunión de conservación e investigación de tortugas marinas en el atlántico sur occidental (ASO). Resumenes Piriápolis: CICMAR-DINARA, pp. 72-76.

THOMPSON, J.D., D.G. HIGGINGS & T.J. GIBSON. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

VAN HOUTAN, K.S., S.K. HARGROVE & G.H. BALAZS. 2010. Land use, macroalgae, and a tumor-forming disease in marine turtles. PLoS ONE 5: 1-8.

WORK, T.M & G.H. BALAZS. 1999. Relating tumor score to hematology in green turtles with fibropapillomatosis in Hawaii. Journal of Wildlife Diseases 35: 804-807.

WORK, T.M., G.H. BALAZS, R.A. RAMEYER & R.A. MORRIS. 2004. Retrospective pathology survey of green turtles *Chelonia mydas* with fibropapillomatosis in the Hawaiian Islands, 1993-2003. Diseases of Aquatic Organisms 62: 163-176.

WORK, T.M., J. DAGENAIS, G.H. BALAZS, J. SCHUMACHER, T.D. LEWIS, J.C. LEONG, R.N. CASEY & J.W. CASEY. 2009. *In vitro* biology of fibropapilloma-associated turtle herpesvirus and host cells in Hawaiian green turtles (*Chelonia mydas*). Journal of General Virology 90: 1943-1950.

ZWARG, T., S. ROSSI, T.C. SANCHES, M.O. CESAR, M.R. WERNECK & E.R. MATUSHIMA. 2014. Hematological and histopathological evaluation of wildlife green turtles (*Chelonia mydas*) with and without fibropapilloma from the north coast of São Paulo State, Brazil. Pesquisa Veterinária Brasileira 34: 682-688.