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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Cetacean Morbillivirus Infection in a Killer Whale (*Orcinus orca*) from Brazil

Kátia R Groch[*], Hassan Jerdy[†], Milton CC Marcondes[‡], Lupércio A Barbosa[§], Hernani GC Ramos[‡], Larissa Pavanelli[‖], Luz Alba MG Fornells[¶], Marina B Silva[†], Giliane S Souza[†], Milton M Kanashiro[†], Pollyana Bussad[†], Leonardo S Silveira[†], Samira Costa-Silva[*], Dominique J Wiener[#], Carlos EPF Travassos[†], José L Catão-Dias[*] and Josué Díaz-Delgado[*]

[*] *Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo,* [†] *Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro,* [‡] *Instituto Baleia Jubarte, Caravelas, Bahia,* [§] *Instituto ORCA, Vila Velha, Espírito Santo,* [‖] *Instituto Mamíferos Aquáticos, Salvador, Bahia,* [¶] *Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil and* [#] *Department of Veterinary Pathobiology, College of Veterinary Medicine & Biomedical Science, Texas A&M University, College Station, Texas, USA*

Summary

We provide pathological, immunohistochemical and molecular evidence of cetacean morbillivirus (CeMV) infection in a live-stranded adult female killer whale (*Orcinus orca*), which stranded alive in Espírito Santo State, Brazil, in 2014. Although attempts were made to release the animal, it stranded again and died. The main pathological findings were severe pulmonary oedema, pleural petechiation, multifocal, lymphoplasmacytic meningoencephalitis and leptomeningomyelitis with perivascular cuffing and gliosis, chronic lymphocytic bronchointerstitial pneumonia and multicentric lymph node and splenic lymphoid depletion. Other pathological findings were associated with the 'live-stranding stress response'. Immunohistochemical analysis revealed multifocal morbilliviral antigen in neurons and astrocytes, and in pneumocytes, histiocytes and leukocytes in the lung. CeMV was detected by a novel reverse transcriptase polymerase chain reaction method in the brain and kidney. Phylogenetic analysis of part of the morbillivirus phosphoprotein gene indicates that the virus is similar to the Guiana dolphin (*Sotalia guianensis*) morbillivirus strain, known to affect cetaceans along the coast of Brazil. To the authors' knowledge, this is the first report of morbillivirus disease in killer whales.

Keywords: cetacean morbillivirus; cetacean pathology; killer whale; marine mammal disease

Cetacean morbillivirus (CeMV; Genus *Morbillivirus*, Family *Paramyxoviridae*; Order *Mononegavirales*) is a significant threat to cetaceans worldwide. CeMV is subclassified into three well-characterized strains, porpoise morbillivirus (PMV), dolphin morbillivirus (DMV) and pilot whale morbillivirus (PWMV), and two presumptive more recent strains, including the novel Guiana dolphin (*Sotalia guianensis*) morbillivirus strain (GDMV), which affects cetaceans off the coast of Brazil (Groch *et al*, 2014; Van Bressem *et al*, 2014). Morbilliviral infection has been documented by immunohistochemistry (IHC) or PCR in at least 22 different cetacean species, including mysticetes

Correspondence to: Kátia R Groch (e-mail: katia.groch@gmail.com).

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and odontocetes (Van Bressem *et al*, 2014; Jacob *et al*, 2016; Groch *et al*, 2019).

The killer whale (*Orcinus orca*) is the most widely distributed marine mammal species with records from all oceans and most seas, primarily coastal and temperate waters with high productivity (Forney and Wade, 2006). Although they are known to be present along the southeastern coast of Brazil, sightings and strandings are only occasional (Dalla Rosa and Secchi, 2007; Vianna *et al*, 2016; Ott *et al*, 2017). Current knowledge on the health and disease status of free-ranging killer whales in Brazilian waters is very limited, although anthropogenic and non-anthropogenic ('natural') causes have been recorded (Dalla Rosa and Secchi, 2007; Laeta *et al*, 2019). The only evidence of CeMV infection in killer whales has been antibody titres in an animal that stranded along the coast of Washington State in the USA in 2002 (Rowles *et al*, 2011). The spread of CeMV has been simulated over a social network of the endangered free-ranging killer whale population in the northeastern Pacific (Weiss *et al*, 2020) using a stochastic individual animal-based 'susceptible-infected-removed' model. This modelling suggested that populations with strong social preferences might be vulnerable to disease outbreaks, resulting in large scale mortality (Weiss *et al*, 2020). The aim of the present study is to report the pathological, immunohistochemical and molecular analyses results of morbilliviral infection in a live-stranded killer whale from Brazil.

A 5.2 m, adult female killer whale stranded alive on Praia dos Castelhanos, Anchieta, Espírito Santo State, Brazil (S 20°50′, W 40°37′), in August 2014. Physical examination revealed moderate body condition and multiple skin lesions including intraspecific 'tooth rakes', 'cookie cutter' shark (*Isistius* spp) bite wounds, chronic healed wounds and stranding-associated linear erosions and abrasions. A few whale lice (morphology compatible with *Isocyamus* spp) were also noted. Blowhole swab samples were collected, preserved in Stuart transport medium and refrigerated for bacteriology. Several attempts were made to refloat the animal to deeper waters. However, it was unable to maintain buoyancy and stranded again, dying on the following morning. A complete necropsy was performed shortly afterwards.

Samples of cerebrum, cerebellum, kidney, pleural effusion and tracheal fluid were collected in sterile vials and frozen at −20°C for virology. Representative samples of the brain (cerebrum, brainstem and cerebellum), cervical spinal cord, lung, heart, liver, spleen, stomach, pancreas, uterus, vulva, skin and longissimus dorsi muscle were collected and fixed in 10% neutral buffered formalin. The tissues were processed routinely and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE) for histopathological examination.

Sections of brain, spinal cord, lung, liver, spleen and stomach were subjected to immunohistochemistry (IHC), using a monoclonal IgG2B (kappa light chain) antibody against the nucleoprotein of canine distemper (CDV) morbillivirus (1:200 dilution; CDV-NP MAb, VMRD, Pullman, Washington, USA) as described (Groch *et al*, 2020b) The avidin—biotin—peroxidase complex (Elite ABC Kit, Vector Laboratories, Burlingame, California, USA) method was used followed by visualization with 3-amino-9-ethyl-carbazole (AEC, MilliporeSigma Corporate, St. Louis, Missouri, USA). Sections were counterstained with Mayer's haematoxylin. Positive controls were lung and lymph node sections from GDMV-positive Guiana dolphins or lung from a CDV-positive dog. As negative controls, sequential sections of the positive control tissues were incubated with non-immune mouse IgG antibody instead of primary antibody.

Blowhole swab and fresh lung tissue samples, preserved at 4°C, were submitted to an independent laboratory for routine bacteriological analyses.

For molecular analysis, a novel, sensitive quantitative reverse transcription-polymerase chain reaction (RT-qPCR) method was employed (Groch *et al*, 2020a). Briefly, viral RNA was extracted from frozen brain, cerebellum, kidney, pleural effusion and tracheal fluid by using TRIzol Reagent (Invitrogen, Life Technologies Corporation, Carlsbad, California, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA) and PCR was performed using Power up SYBR Green Master Mix Kit (Life Technologies) and forward (PAN-F: 5′- CCTCTAACA GGGGATCT(A/G)CTC -'3) and reverse primers (PAN-R: 5′- CCTGTGCCCTTTTTAATGGA -'3), which target the CeMV phosphoprotein gene as described (Groch *et al*, 2020a). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Chatsworth, California, USA) and directly sequenced. Frozen lung from GDMV-positive (Groch *et al*, 2014) and GDMV-negative Guiana dolphins were used as positive and negative controls, respectively. MEGA7 (Kumar *et al*, 2016) was used to construct a neighbour-joining phylogenic tree based on the sequenced amplicon from this study (GenBank accession no. MT647723) and 11 other GenBank sequences representing six previously reported morbillivirus species.

At necropsy, the main gross findings were meningeal and neuroparenchymal encephalic congestion, diffuse, bilateral pulmonary distension with

abundant frothy fluid in the trachea, bronchi and lung parenchyma, multifocal pleural petechiae, multifocal haemorrhagic plaques in the antimesenteric aspect of the small intestine, right scapulohumeral haemarthros, lack of ingesta and sand in the blowhole and proximal nasal passages.

Microscopically, the main lesions were confined to the central nervous system (CNS) and the respiratory and lymphoid systems. CNS lesions were characterized by mild to moderate, multifocal, chronic lymphoplasmacytic meningoencephalitis and leptomeningomyelitis with perivascular cuffing, acute neuronal degeneration (primarily in the cerebellum and rarely in telencephalic regions), astrocytosis and microgliosis (Figs. 1 and 2). Perivascular oedema, haemorrhage and ceroid-lipofuscinosis were also seen. In the lung, there was mild, multifocal, acute neutrophilic bronchopneumonia with moderate numbers of intra-alveolar 1–2 μm coccobacillary bacteria, fibrin, oedema and haemorrhage, as well as mild to moderate, multifocal, chronic lymphocytic bronchointerstitial pneumonia (Fig. 3) and alveolar histiocytosis. In the lymphoid system, there was lymph node and splenic lymphoid depletion. Other relevant microscopic findings in this case were associated with the 'live-stranding stress response' (LSSR) and included acute centrilobular hepatic congestion with individual hepatocellular and lobular necrosis, and intrahepatocytic eosinophilic globules. There was acute segmental, hyaline cardiac and skeletal myodegeneration and myonecrosis characterized by contraction band necrosis, oedema and rare haemorrhage.

IHC analysis revealed intense multifocal granular intracytoplasmic immunolabelling of morbilliviral antigen in neurons (Fig. 1) and occasional astrocytes. Morbilliviral antigen was also detected in pneumocytes, histiocytes and intravascular leucocytes in lung tissue. Heart, liver, spleen, stomach, pancreas, uterus, vulva and skin were immunonegative.

Microbiological analysis and antibiotic sensitivity testing identified *Enterobacter cloacae*, which was resistant to cephalothin, ampicillin, amoxicillin-clavulanic acid and cefoxitin, in the blowhole swab. No bacterial growth was detected in lung tissue.

Molecular analysis identified CeMV genetic material in the kidney and brain, at the 32nd and 39th RT-qPCR cycles, respectively. The assay amplified a 206 bp fragment of the CeMV P gene with a melting temperature of 81°C. Phylogenetic analysis, based on the sequenced amplicon, showed that the sample shared 99% nucleotide and 97% amino acid identity with GDMV sequences (Fig. 4). No morbilliviral genetic material was amplified from lung tissue. Based on these findings, a diagnosis of morbilliviral infection was determined.

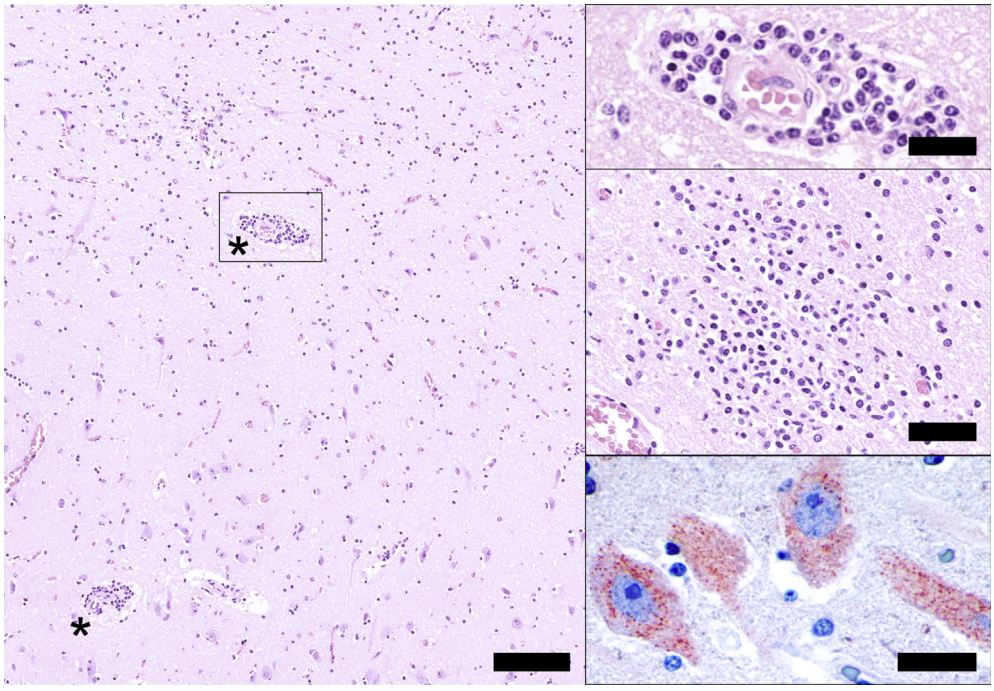

Fig. 1. Killer whale, cerebral cortex, non-suppurative encephalitis, morbillivirus infection. Multifocal lymphocytic perivascular cuffing (asterisks). HE. Bar, 100 μm. Inset (right upper): detail of lymphocytic perivascular cuffing (squared area in main figure). HE. Bar, 25 μm. Inset (right middle): focal gliosis. HE. Bar, 50 μm. Inset (right lower): immunolabelling of morbilliviral antigen in neuronal soma. IHC. Bar, 25 μm.

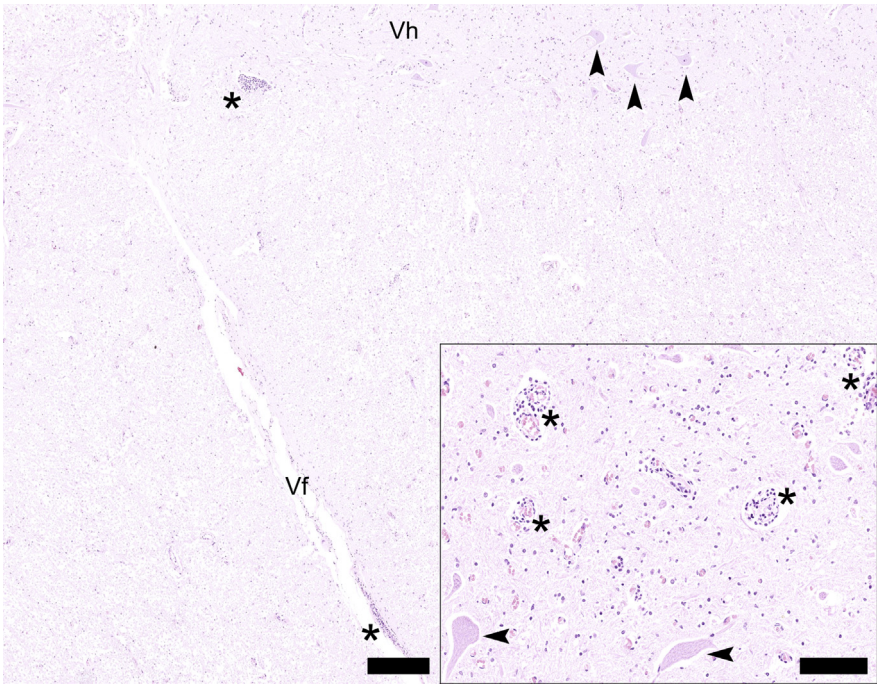

Fig. 2. Killer whale, spinal cord, non-suppurative encephalitis, morbillivirus infection. Discrete lymphocytic perivascular cuffing (asterisks) at white–grey matter interface in ventral horn (Vh). Ventral fissure (Vf). Neurons (arrowheads). HE. Bar, 250 μm. Inset: multifocal discrete lymphocytic perivascular cuffing (asterisks). Neurons (arrowheads). HE. Bar, 100 μm.

To the best of our knowledge, the only reported evidence of morbillivirus infection in killer whales was the detection of neutralizing serum antibody titres against DMV, PMV and CDV in a killer whale that had stranded on the coast of Washington State, USA, in 2002, indicating previous exposure to morbillivirus (Rowles *et al*, 2011). The current case confirms the susceptibility of killer whales to CeMV infection

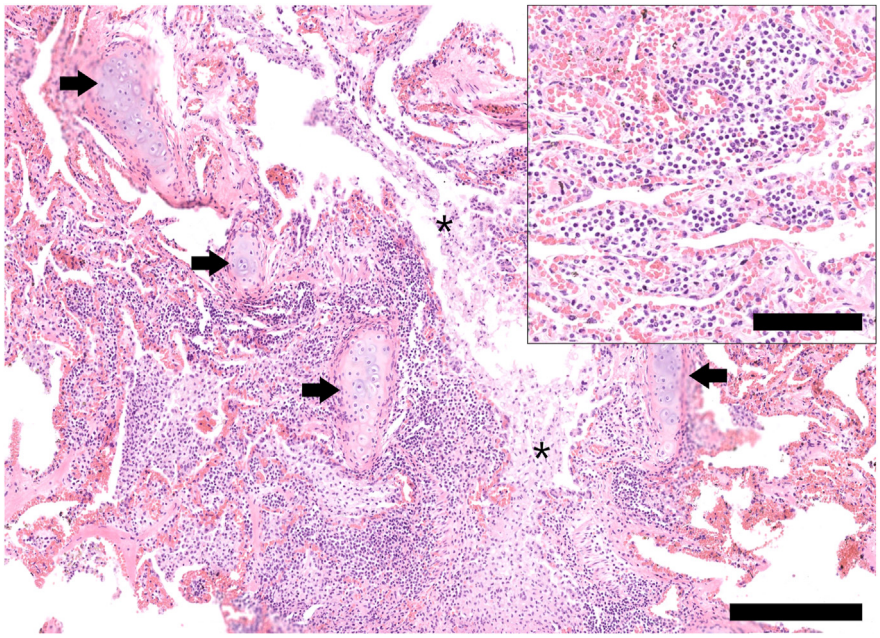

Fig. 3. Killer whale, lung, bronchointerstitial pneumonia, morbillivirus infection. Mononuclear cell infiltration in peribronchial and peribronchiolar tissue and alveolar septa. Intrabronchial and intrabronchiolar cellular exudate, primarily histiocytes (asterisks). Bronchiolar cartilage (arrows). HE. Bar, 250 μm. Inset: detail of alveolar septal mononuclear cell infiltrate. HE. Bar, 100 μm.

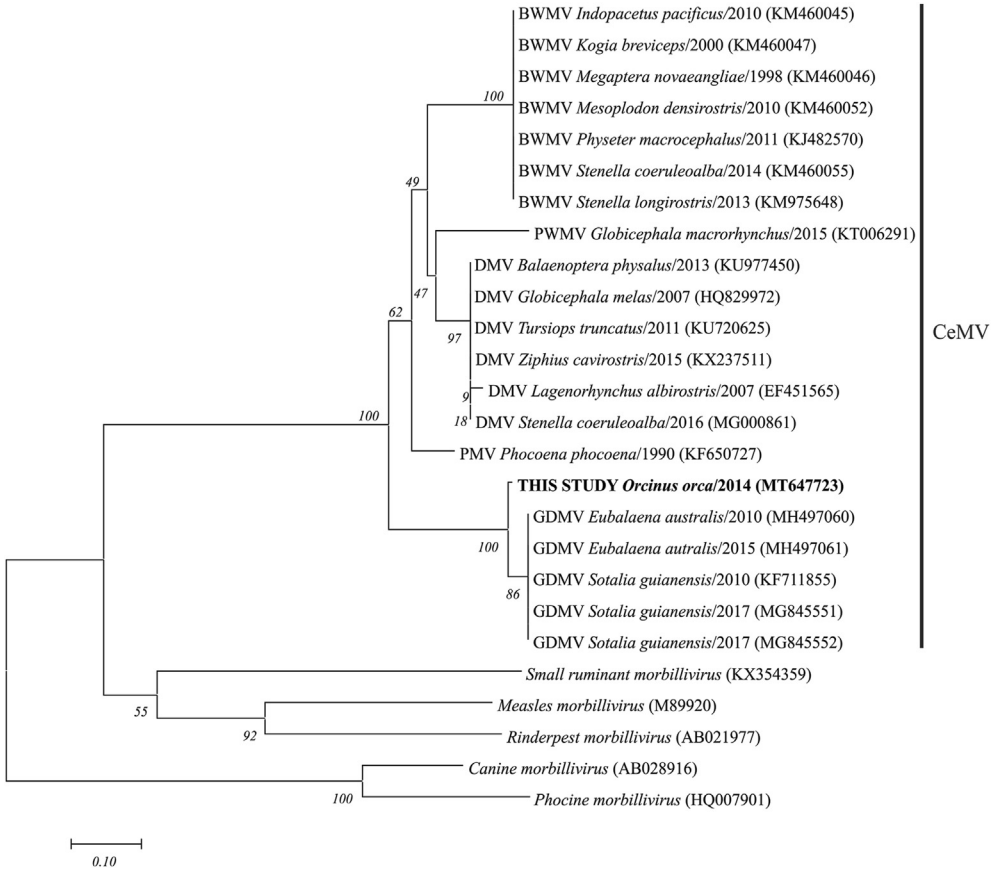

Fig. 4. Neighbour-joining phylogenetic tree with 26 amino acid sequences (60 positions) of morbillivirus phosphoprotein gene from the killer whale (*Orcinus orca*) stranded in Espírito Santo, Brazil (this study), and those of other previously described morbillivirus species. Other recognized cetacean morbillivirus (CeMV) strains are included for comparison. Strain abbreviations are followed by species, year of stranding and GenBank accession number. Bootstrap values (1,000 replicates) are indicated at the internal nodes. Scale bars indicate amino acid substitutions per site. BWMV, beaked whale morbillivirus; DMV, dolphin morbillivirus; GDMV, Guiana dolphin morbillivirus; PMV, porpoise morbillivirus; PWMV, pilot whale morbillivirus.

and widens the known host species spectrum for GDMV. This killer whale appears to have had a subacute systemic presentation of CeMV infection (Van Bressem *et al*, 2014; Díaz-Delgado *et al*, 2019b) characterized by lesions of lymphoplasmacytic meningoencephalitis and leptomeningomyelitis with gliosis, and chronic lymphocytic bronchointerstitial pneumonia and lymph node and splenic lymphoid depletion associated with detection of morbillivirus antigen or genome in CNS, lung, and kidney tissues (Van Bressem *et al*, 2014; Díaz-Delgado *et al*, 2019b). Two plausible explanations for the negative results in most of the tissues tested are prolonged formalin fixation time (>4 weeks) and a heterogeneous distribution of virus in infected tissues. It is possible that the morbillivirus infection interfered with foraging capacity, which could explain the absence of food in the stomach and the moderate body condition. The CNS lesions could have played a role in the stranding of the animal, followed by LSSR (Herraez *et al*, 2013; Camara *et al*, 2019) and death.

Current epidemiological knowledge of CeMV in Brazilian waters includes serological evidence of morbilliviral exposure in Fraser's dolphins (*Lagenodelphis hosei*) (Van Bressem *et al*, 2001) and pathological, immunohistochemical or molecular evidence of infection in Guiana dolphins and Southern right whales (*Eubalaena australis*) (Groch *et al*, 2014, 2018, 2019; Domiciano *et al*, 2016; Díaz-Delgado *et al*, 2019a, b, Groch *et al*, 2020b). Furthermore, the first molecular confirmation of morbilliviral infection in a cetacean from Brazil involved a Guiana dolphin stranded in Espírito Santo State, which is approximately 300 km from the stranding location of the present killer whale (Groch *et al*, 2014). It is reasonable to assume that the virus infects a wider range of cetacean species than initially thought and that cetaceans in this geographical location may have a higher risk of

GDMV infection than other regions. As in other cases, infection may have occurred by respiratory transmission.

The lack of evidence of morbilliviral infection in killer whales was recently discussed in the context of growing concern for increasing pollutant burdens in this species in multiple geographical areas (Desforges *et al*, 2018; Di Guardo and Fernández, 2018). Modelling studies have suggested that high polychlorinated biphenyl (PCB) concentrations in killer whales may be contributing to significant population declines, including in Brazil (Lailson-Brito *et al*, 2012; Jepson *et al*, 2016), and a previous study indicated that organochlorine levels in delphinids from southeastern Brazil are comparable to those reported in cetaceans from highly industrialized regions of the Northern Hemisphere (Lailson-Brito *et al*, 2012). We did not perform toxicological analysis on the current case. However, a combination of high pollutant levels and the finding of morbilliviral disease in killer whales raises concerns for the potential impact on populations. Morbilliviral infection should be included in the differential diagnosis for stranded killer whales that have CNS, respiratory or lymphoid lesions.

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Conflict of Interest Statement

The authors declare no potential conflicts of interest with respect to the research, authorship or publication of this article.

References

Camara N, Sierra E, Fernandez-Maldonado C, Espinosa de Los Monteros A, Arbelo M *et al* (2019) Stress cardiomyopathy in stranded cetaceans: a histological, histochemical and immunohistochemical study. *Veterinary Record*, **185**, 694–704.

Dalla Rosa L, Secchi ER (2007) Killer whale (*Orcinus orca*) interactions with the tuna and swordfish longline fishery off southern and south-eastern Brazil: a comparison with shark interactions. *Journal of the Marine Biological Association of the United Kingdom*, **87**, 135–140.

Desforges JP, Hall A, McConnell B, Rosing-Asvid A, Barber JL *et al* (2018) Predicting global killer whale population collapse from PCB pollution. *Science*, **361**, 1373–1376.

Di Guardo G, Fernández A (2018) Orcas and PCBs. Commentary: Predicting global killer whale population collapse from PCB pollution. *Science* **361**, eLetters, pp. 1373–1376.

Díaz-Delgado J, Groch KR, Ressio R, Riskallah IPJ, Sierra E *et al* (2019a) Comparative immunopathology of cetacean morbillivirus infection in free-ranging dolphins from Western Mediterranean, Northeast-Central, and Southwestern Atlantic. *Frontiers in Immunology*, **10**, 485.

Díaz-Delgado J, Groch KR, Sierra E, Sacchini S, Zucca D *et al* (2019b) Comparative histopathologic and viral immunohistochemical studies on CeMV infection among Western Mediterranean, Northeast-Central, and Southwestern Atlantic cetaceans. *PloS One*, **14**, https://doi.org/10.1371/journal.pone.0213363.

Domiciano IG, Domit C, Broadhurst MK, Koch MS, Bracarense AP (2016) Assessing disease and mortality among small cetaceans stranded at a world heritage site in southern Brazil. *PloS One*, **11**, 0149295.

Forney KA, Wade PR (2006) Whales, whaling, and ocean ecosystems. In: *Worldwide Distribution and Abundance of Killer Whales*, JA Estes, DP Demaster, DF Doak, TM Williams, RL Brownell Jr. Eds, University of California Press, Berkeley, pp. 145–173.

Groch KR, Colosio AC, Marcondes MC, Zucca D, Díaz-Delgado J *et al* (2014) Novel cetacean morbillivirus in Guiana dolphin, Brazil. *Emerging Infectious Diseases*, **20**, 511–513.

Groch KR, Díaz-Delgado J, Santos-Neto EB, Ikeda JMP, Carvalho RR *et al* (2020b) The pathology of Cetacean morbillivirus and comorbidities in Guiana dolphin during an unusual mortality event, (Brazil, 2017–2018). *Veterinary Pathology*, in press. https://doi.org/10.1177/0300985820954550 (2017–2018).

Groch KR, Kolesnikovas CKM, de Castilho PV, Moreira LMP, Barros C *et al* (2019) Cetacean morbillivirus in southern right whales, Brazil. *Transboundary and Emerging Diseases*, **66**, 606–610.

Groch KR, Santos-Neto EB, Díaz-Delgado J, Ikeda JMP, Carvalho RR *et al* (2018) Guiana dolphin unusual mortality event and link to Cetacean morbillivirus, Brazil. *Emerging Infectious Diseases*, **24**, 1349–1354.

Groch KR, Taniwaki SA, Favero CM, Brandão PE, Díaz-Delgado J *et al* (2020a) A novel real-time PCR to detect Cetacean morbillivirus in Atlantic cetaceans. *Journal of Virological Methods*, **285**, 113964.

Herraez P, Espinosa de los Monteros A, Fernandez A, Edwards JF, Sacchini S *et al* (2013) Capture myopathy in live-stranded cetaceans. *The Veterinary Journal*, **196**, 181–188.

Jacob JM, West KL, Levine G, Sanchez S, Jensen BA (2016) Initial characterization of novel beaked whale morbillivirus in Hawaiian cetaceans. *Diseases of Aquatic Organisms*, **117**, 215–227.

Jepson PD, Deaville R, Barber JL, Aguilar A, Borrell A *et al* (2016) PCB pollution continues to impact populations of orcas and other dolphins in European waters. *Scientific Reports*, **6**, 18573.

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, **33**, 1870–1874.

Laeta M, Kompanje EJO, Watson A, Souza S, Dittmar K *et al* (2019) Osteochondromatosis (multiple cartilaginous exostoses) in an immature killer whale *Orcinus orca*. *Diseases of Aquatic Organisms*, **134**, 209–213.

Lailson-Brito J, Dorneles PR, Azevedo-Silva CE, Bisi TL, Vidal LG *et al* (2012) Organochlorine compound accumulation in delphinids from Rio de Janeiro State, southeastern Brazilian coast. *The Science of the Total Environment*, **433**, 123–131.

Ott PH, Sucunza F, Wickert J, Danilewicz D, Tavares M (2017) Evidences of attack of a killer whale on a calf southern right whale in southern Brazil. *Mastozoología Neotropical*, **24**, 235–240.

Rowles T, Schwacke L, Wells R, Saliki J, Hansen L *et al* (2011) Evidence of susceptibility to morbillivirus infection in cetaceans from the United States. *Marine Mammal Science*, **27**, 1–19.

Van Bressem MF, Duignan PJ, Banyard A, Barbieri M, Colegrove KM *et al* (2014) Cetacean morbillivirus: current knowledge and future directions. *Viruses*, **6**, 5145–5181.

Van Bressem MF, Van Waerebeek K, Jepson PD, Raga JA, Duignan PJ *et al* (2001) An insight into the epidemiology of dolphin morbillivirus worldwide. *Veterinary Microbiology*, **81**, 287–304.

Vianna TD, Loch C, Castilho PV, Gaidzinski MC, Cremer MJ *et al* (2016) Review of thirty-two years of toothed whale strandings in Santa Catarina, southern Brazil (Cetacea: Odontoceti). *Zoologia (Curitiba)*, **33**, 5.

Weiss MN, Franks DW, Balcomb KC, Ellifrit DK, Silk MJ *et al* (2020) Modelling cetacean morbillivirus outbreaks in an endangered killer whale population. *Biological Conservation*, **242**, 108398.

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