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Original Contribution

Chelonid Alphaherpesvirus 5 DNA in Fibropapillomatosis-Affected *Chelonia mydas*

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Abstract: Fibropapillomatosis is a panzootic and chronic disease among *Chelonia mydas*—usually associated with anthropogenic impacts. This study contributes towards understanding fibropapillomatosis implications for *C. mydas* populations as a reflector of environmental quality, via prevalence and histological, molecular and blood analyses at a World Heritage site in southern Brazil. Sixty-three juvenile *C. mydas* (31.3–54.5 cm curved carapace length–CCL) were sampled during two years. Eighteen specimens ($\sim$ 29%) had tumours (which were biopsied), while 45 had none. Degenerative changes in the epidermis and Chelonid alphaherpesvirus 5 DNA detection with three variants support a herpesvirus infection. Phylogenetic analysis indicated that variants A and B were similar to a herpesvirus lineage from the Atlantic group, but variant C was similar to a herpesvirus from the eastern Pacific lineage and represents the first published case for marine turtles off Brazil. Significantly lower levels of seven blood parameters, but greater numbers of eosinophils, were observed in tumour-afflicted animals. These observations were attributed to metabolism efficiencies and/or differences in diet associated with temporal-recruitment bias and disease development, and greater non-specific immune stimulation. While most animals had adequate body condition independent of disease, longer-term studies are required to elucidate any protracted population effects.

Keywords: Green turtles, Blood parameters, Molecular analysis, Histology, Diseases, Environmental sentinel

Introduction

Seven species of marine turtles exist; all with varying global distributions, but common ecological importance in maintaining oceanic ecosystems (Spotila 2004). Their

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importance extends to indicating hazards to human health as environmental sentinels (Domiciano et al. 2017). Owing to pollution, and/or habitat degradation, climate change and fishing mortality, most species have population statuses collectively listed as either 'Vulnerable', 'Endangered' or 'Critically Endangered' (IUCN 2018). Of particular concern is the cosmopolitan green turtle, *Chelonia mydas* (Linnaeus 1758), which mostly frequents neritic zones following oceanic recruitment and transition from an omnivorous to predominantly herbivorous diet (Gama et al. 2016). Their near-shore proximity renders all *C. mydas* susceptible to anthropogenic threats—which are exacerbated off industrialised and urbanised coasts. One such area of concern in Brazil is the Paranaguá estuarine complex—PEC (25°20′ to 25°35′S, 48°17′ to 48°42′W), which is an important foraging ground for juvenile *C. mydas* (Gama et al. 2016). Although a World Heritage site (UNESCO), the PEC is heavily impacted by artisanal fisheries, harbours, urbanisation and pollution derived from industrial and agricultural sewages. These factors have been demonstrated to affect the morbidity and mortality of various marine sentinels, as stated previously for small cetaceans in the same area (Domiciano et al. 2016). Potential consequences for *C. mydas* range from mortality to less obvious acute and chronic sublethal impacts encompassing various diseases, including fibropapillomatosis–FP; the implications of which have not yet been assessed for most local populations (Domiciano et al. 2017).

Fibropapillomatosis is a panzootic chronic disease closely associated with a virus (Chelonid alphaherpesvirus 5; 'ChAHV5') and is characterised by external and internal tumours that potentially could impair swimming, feeding, reproduction and internal-organ function (Work et al. 2004). The disease prevalence and severity are usually spatially distinct (Jones et al. 2016). Evaluating ChAHV5 DNA, tumour histology and blood parameters are important precursors towards understanding FP pathogenesis (Jones et al. 2016; Santos et al. 2015). The associated strains, evolution of the disease and the health effects detected via blood profiles of *C. mydas* can interconnect the species to environmental quality (Aguirre and Lutz 2004; Herbst et al. 1999; Morrison et al. 2018; Whiting et al. 2007).

Like for all diseases, understanding the extent of possible lethal and sublethal indications associated with FP requires adequate comprehension of the range of health parameters in *C. mydas* populations. Such assessments have been made (via blood profiles) for healthy free-ranging *C. mydas* (Bolten and Bjorndal 1992; Flint et al. 2009; Hamann et al. 2006; Hasbún et al. 1998; Labrada-Martagón et al. 2010; Lewbart et al. 2014; Page-Karjian et al. 2015; Swimmer 2000; Whiting et al. 2007) and unhealthy/rehabilitated animals (including captive) (Flint et al. 2009; March et al. 2018; Swimmer 2000; Whiting et al. 2007). More specifically, assessments of rehabilitated, captive and free-ranging *C. mydas* implied variable immunosuppression, anaemia, uraemia, hypoglycaemia, low cholesterol, lymphocytopenia and hypoproteinaemia associated with FP (Aguirre et al. 1995; Aguirre and Balazs 2000; Kage-Karjian et al. 2014; Santos et al. 2015; Swimmer 2000; Work and Balazs 1999; Zwarg et al. 2014).

Although important, caution must be taken when comparing blood parameters between free-ranging and captive/rehabilitated *C. mydas*, because sample variability of the latter may reflect short-term dietary changes, stressful conditions and/or different health problems (Bolten and Bjorndal 1992; Herbst and Jacobson 2003; Swimmer 2000). Further, even among free-ranging animals, there often exists substantial intra-specific variation among blood parameters; possibly reflecting extrinsic variables, including spatio-temporal differences in diet and environmental conditions and/or intrinsic features like gender, genetics, nutritional status and life stages, and concomitant disease (Aguirre and Balazs 2000; Herbst and Jacobson 2003). Uncertainty concerning the key extrinsic or intrinsic mechanisms supports the wide-scale monitoring of divergent populations of *C. mydas* (Aguirre and Balazs 2000). Particularly, broad assessments of afflicted 'in situ' populations might facilitate a clearer understanding of the implications of FP among *C. mydas* and progress hypotheses concerning likely causal factors, and therefore mitigation.

Considering the above, our aims here were threefold. First, we sought to confirm the prevalence of FP among *C. mydas* at the previously unassessed southern range of their Brazilian distribution in the PEC by assessing tumour histology and the presence of Chelonid alphaherpesvirus 5 DNA. Second, we tested the hypothesis of no differences in the blood profiles of free-ranging *C. mydas*, with and without FP tumours. And third, using the various information, we propose strategies for future research and monitoring the broader effects of FP among *C. mydas* populations as a precursor to examining direct or indirect causal relationships with environmental factors.

Methods

Field Sampling

Chelonia mydas were targeted during seven days in April 2014 and 13 days in May and June 2016 using a surface-set gillnet (300-mm mesh) diurnally deployed in the PEC (Fig. 1). Each *C. mydas* was disentangled and manually restrained onboard, while blood (2–3 ml) was withdrawn from the occipital sinus, using a heparinised 20-gauge needle and 3.0-ml disposable syringe within < 60 min of capture. Glucose (mg dL^{-1}) was immediately measured in the whole blood sample using the One Touch® Ultra® 2 glucometer, before the remainder was placed into two 5-ml tubes, containing either lithium heparin anti-coagulant or gel separator with clot activators and stored on ice prior to laboratory processing.

All specimens were measured for curved carapace length (CCL to the nearest 0.1 cm) and weighed (body mass; BM to the nearest 0.1 kg). Body condition was estimated and classified as 'good', 'fair' or 'poor' (based on the plastron, eyes, muscular mass and fat deposits) (Torezani et al. 2010). Additionally, the body condition index (BCI) was derived (Labrada-Martagón et al. 2010).

Small sections of tumours were excised. Prior to sample removal, skin and subcutaneous tissue were infiltrated with lidocaine hydrochloride 2% (2–5 mg/kg), which was sufficient to preclude any subsequent reflex response. Excised samples were fixed in 10% buffered formalin solution for histology or stored at − 80°C for subsequent molecular analyses. After sampling, individuals were tagged on the trailing edge of their front flippers with Inconel metal tags (National Band and Tag Co., USA; style 681) and released.

A field permit was granted by the Ministry of Environment—MMA (SISBIO 43443-3). The field permit (SISBIO 43443-3) encompassed the legislated ethics approval for catching and sampling live sea turtles.

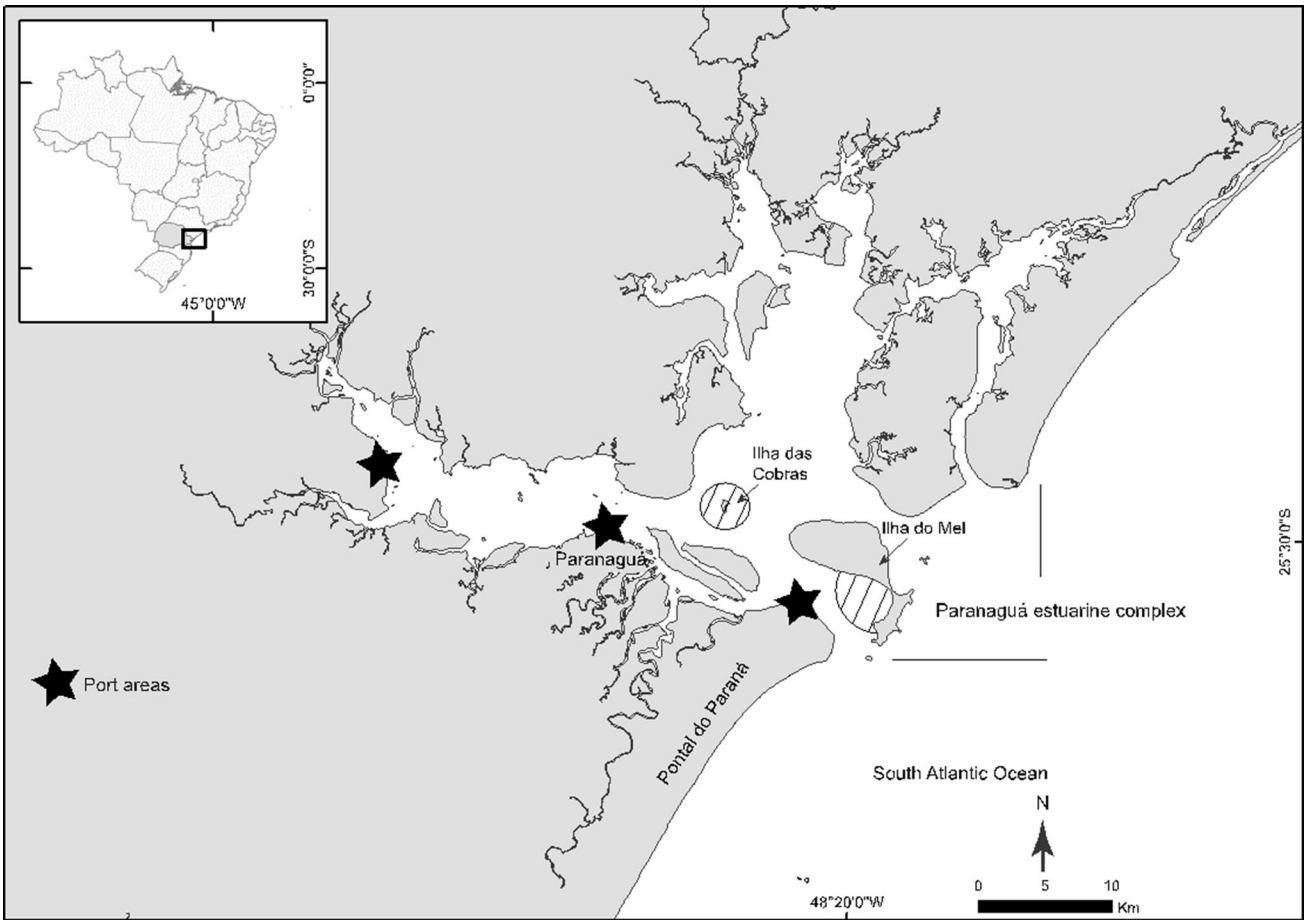


Fig. 1. Map of the Paranaguá estuarine complex, Brazil, and hatched areas indicating where *Chelonia mydas* were intentionally caught.

Tumour Histology and Molecular Analysis

In total, 26 tumours from ten animals that had been fixed in 10% buffered formalin solution were routinely processed, embedded in paraffin and stained with haematoxylin and eosin for histopathology analysis. Previous described histological features were evaluated as proposed by Herbst et al. (1999) and used to confirm the FP diagnosis.

For each of 12 frozen tumours from seven animals, DNA was extracted using the commercially available 'Qiagen DNeasy blood and tissue kit' (Qiagen Sample and Assay Technologies, Hilden, Germany) in accordance with the manufacturer's instructions. Five microlitres of the extracted DNA was subjected to the polymerase chain reaction (PCR) with the turtle-specific herpesvirus primers GTHV2 (5′GACACGCAGGCCAAAAAGCGA3′) and GTHV3 (5′AGCATCATCCAGGCCCACAA3′). The PCR mixture (45 µl) comprised 1 µl (20 pmol) from each of the above primers; 200 mM of each dNTP (Invitrogen, Life Technologies, Carlsbad, USA); 2.5 units of Platinum Taq DNA polymerase (Invitrogen, Life Technologies, BR); 1 × PCR buffer (20 mMTris–HCl pH 8.4 and 50 mM KCl); 1.5 mM of $MgCl_2$; 2.5% DMSO; and ultrapure sterile water to a final volume of 50 µl. Amplification was performed in a thermal cycler (Swift MaxPro, Esco Healthcare, USA) with the previous described cycling profile and following Quackenbush et al. (2001). The PCR products were analysed by electrophoresis in 2% agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 mg/ml), and visualised under UV light.

In order to ensure the specificity of the primers, the products of PCR of nine samples from six animals were submitted for sequence analyses. The PCR products were purified using a PCR DNA and Gel Band Purification kit (Invitrogen, Life Technologies, BR) and quantified using a Quant-iT^{TM} dsDNA BR Assay kit (Invitrogen, Molecular Probes, Eugene, OR, USA) in the Qubit ^{TM} Fluorometer (Invitrogen, Molecular Probes, Eugene, OR, USA). The direct sequencing was performed by using a BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, USA) with the forward and reverse corresponding primers in a 3500 Genetic Analyser (Applied Biosystems, Carlsbad, USA), and all according to the manufacturer's instructions.

Blood Profiles of Fibropapillomatosis-Afflicted and Non-Afflicted Specimens

Heparinised blood samples were analysed for total erythrocyte (He-10^6 μL^{-1}), and leucocyte (WBC-$10^3 \mu L^{-1}$) counts within 10 h using Natt-Herrick's diluents (1:200) and a Neubauer hemocytometer. Packed cell volume (PCV-%) was determined by centrifuging heparinised blood in microhematocrit tubes (14,800 × g for 5 min, model H-240 DIG; Centribio). Plasma proteins (g dL^{-1}) were detected using a refractometer (Vet 360 TS meter, Reichert Analytical Instruments). Blood smears were fixed and stained with Diff-Quick (Laborclin©), and the differential white blood-cell count was determined via optical microscope lens. Thrombocytes were counted by 100 leucocytes.

Serum-gel tubes with whole blood were centrifuged (1800 × g for 10 min, model 80-2B; Centribio) and the serum frozen (− 20°C) for three months. Serum parameters were measured with commercial kits (Siemens®) and processed using the Dimension© Clinical Chemistry System for: alanine aminotransferase (ALT) (U L^{-1}), aspartate aminotransferase (AST) (U L^{-1}), creatine kinase (CK) (U L^{-1}), gamma glutamyl transpeptidase (GGT) (U L^{-1}), alkaline phosphatase (ALP) (U L^{-1}), albumin (g dL^{-1}), urea (mg dL^{-1}), creatinine (mg dL^{-1}), uric acid (mg dL^{-1}), lactate (mmol L^{-1}), cholesterol (mg dL^{-1}), triglycerides (mg dL^{-1}), magnesium (mg dL^{-1}), calcium (mg dL^{-1}) and phosphorus (mg dL^{-1}). Electrolytes were measured by the electrode ion selective method, using a Cobas® b 121 gas analyser, including: sodium (mmol L^{-1}), potassium (mmol L^{-1}), chloride (mmol L^{-1}) and ionised calcium (iCa; mg dL^{-1}).

Data Analyses

The frequency of tumour histological features was described and compared to FP tumours from other locations. For the analyses of molecular data, the nucleotide (nt) sequences of polymerase gene of ChAHV5 sequenced here were compared with partial sequences deposited in the GenBank database using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide quality analysis and contig assembly of the DNA polymerase gene of the ChAHV5 sequences were performed using Phred and CAP3 software (http://asparagin.cenargen.embrapa.br/phph), respectively. The phylogenetic tree was based on complete sequence of nt of polymerase gene and was obtained using the neighbour-joining method in MEGA V7 software

(Kumar et al. 2016). The bootstrapping probabilities were calculated using 1000 replicates (Saitou and Nei 1987). The gene sequences described in the present study have been deposited in the GenBank database under accession numbers: MH144348–MH144350.

All physiological variables and CCL, BM and BCI, were separately analysed using linear mixed models (LMM) that included 'years' and 'sampling day within years' as random, and 'FP tumours' (present/absent) as fixed factors (CCL was a covariate in all physiological models). Absolute data were checked for normality (Q–Q plots) and log-transformed as required, while percentage data were arcsine square-root transformed. The significance of blood parameters between turtles with or without tumours was determined using a Wald-*F*. All LMMs were fitted using ASReml in R. Comparisons of blood parameters with other *C. mydas* populations were prioritised according to free-ranging juveniles, and especially from those studies that evaluated FP-afflicted and non-afflicted animals.

Results

Field Sampling

Forty-three *C. mydas* were caught in 2014: ten (~ 23%, mean CCL and BM ± SE of 43.51 ± 1.40 cm and 10.42 ± 0.94 kg) had mostly small (< 1.5 cm) external tumours; and 33 (41.06 ± 0.81 cm CCL and 8.58 ± 0.52 kg) had no visible tumours. In 2016, 20 *C. mydas* were caught: eight (40%, 42.35 ± 3.03 cm CCL and 12.14 ± 3.75 kg) had tumours and 12 (37.79 ± 0.95 cm CCL and 8.70 ± 1.20 kg) did not. Neither CCL nor BM significantly varied between FP-afflicted or non-afflicted groups (LMM, $p > 0.05$). All except for one individual (which had no tumours and was 'fair') were in good body condition, and BCI remained similar between individuals without (mean ± SE of 1.21 ± 0.02 in 2014 and 1.68 ± 0.21 in 2016) or with tumours (1.24 ± 0.03 in 2014 and 1.35 ± 0.23 in 2016) (LMM, $p > 0.05$; Table 1). Three tumour-afflicted animals were recaptured: one animal in 2014, first sighted without tumours in São Paulo state, south-eastern Brazil in 2012, and two animals in 2016 at the same location two days apart.

Tumour Histology and Molecular Analysis

Microscopical analyses of most small cutaneous tumours were performed despite body localisation in four *C. mydas* (40%; 4/10) captured in 2014 (mean = 3 tumours per *C. mydas*; two to six tumours; $n = 13$) and six *C. mydas* (75%; 6/8) captured in 2016 (mean = 2 tumours per *C. mydas*; one to three tumours; $n = 13$). The lesions were classified as FP with both epidermal and dermal hyperplasia, and orthokeratosis (96.1%), basal cell degeneration (80.8%), lymphocyte infiltrate (65.4%), ulceration (65.4%), dermal–epidermal cleft (61.5%), cornified inclusion cysts (38.5%) and ballooning degeneration (15.4%) were also observed. Spirorchiid eggs were observed in six animals (60%) associated with mild-to-severe granulomatous inflammation. Superficial bacteria were observed in two cases (20%), but eosinophilic intranuclear inclusion bodies (EII) were not observed.

The PCR was performed for five *C. mydas* caught in 2014 (including four animals that were microscopically evaluated) and for two *C. mydas* caught in 2016 (both were microscopically evaluated). The DNA of Chelonid alphaherpesvirus 5 was detected in all samples ($n = 12$ tumours, mean = 1.7 tumours per *C. mydas*), and sequence analyses of nine samples from six *C. mydas* indicated three genetic variants of ChAHV5 DNA (GenBank accession number MH144348-50). One variant was observed in five animals (variant A = 1 *C. mydas*, variant B = 3 *C. mydas* and variant C = 1 *C. mydas*), but two variants (A and B) were observed in one *C. mydas* in different tumours. The phylogenetic analysis indicated clustering with ChAHV5 DNA from other marine turtles from Brazil, that were closely related to the Atlantic group, except for the variant C, which was closely related to eastern Pacific group (Fig. 2).

Blood Profiles of Fibropapillomatosis-Afflicted and Non-Afflicted Specimens

Blood samples were collected from all except one *C. mydas* (tumour free), and the number of samples evaluated varied according to the tested parameters (Table 1). Compared to tumour-free individuals, those with tumours had significantly lower cholesterol (mean ± SE; 106.90 ± 9.34 vs 58.11 ± 7.63 mg dL^{-1}), urea (mean ± SE; 44.93 ± 6.96 vs 29.11 ± 7.65 mg dL^{-1}), creatinine (mean ± SE; 0.26 ± 0.02 vs 0.14 ± 0.04 mg dL^{-1}), albumin (mean ± SE; 0.52 ± 0.07 vs 0.46 ± 0.07 g dL^{-1}), ALP (mean ± SE; 23.02 ± 2.08 vs 15.83 ± 1.99 U L^{-1}), phosphorus (mean ± SE; 6.25 ± 0.39 vs 5.10 ± 0.46 mg dL^{-1}), glucose (mean ± SE; 60.61 ± 3.94 vs 51.18 ± 3.71 mg dL^{-1}), but greater absolute numbers of eosinophils (mean ± SE;

0.71 ± 0.22 vs 1.85 ± 0.73 μL^{-1}) (LMM, $p < 0.05$; Table 1).

Discussion

The results from this study contribute towards understanding the health implications of FP among juvenile *C. mydas* in the south-western Atlantic Ocean, including some of the distinguishing physiological features. Monitoring this cohort is a valuable conservation tool because post-recruits have been reported to be at greater risk of disease than their larger immature conspecifics; possibly owing to stress associated with their habitat and diet shift (from open ocean to near shore) and disparate environmental threats (Flint et al. 2009). The observations can be discussed with previous assessments according to possible intrinsic and extrinsic variability, and ultimately used to direct future work, but first the classification of FP requires consideration.

The histological and molecular assays characterised FP and the presence of the ChAHV5 DNA in the sampled tumours. Greater frequencies of dermal–epidermal cleft and ulceration were observed here than in *C. mydas* tumours sampled off Hawaii and Florida (Herbst et al. 1999). Although degeneration changes that resulted in separation between dermis and epidermis and consequent necrosis and ulcers are usually features of advanced stages in larger tumours (Herbst et al. 1999), these were also observed in small tumours here. Another noteworthy aspect is that EII presence typically is skewed towards smaller tumours (Monezi et al. 2016; Work et al. 2015), but was not observed in the evaluated samples. Therefore, although ChAHV5 DNA was detected in all evaluated samples, this did not appear concurrent with EII. Such a result is consistent with the pathogenesis of herpesvirus infection, whereby virions are not always present during tumour development (Herbst et al. 1999; Work et al. 2015).

Heterogeneity among ChAHV5 variants has been observed across large geographical scales and may be partially explained by the complex life cycle of *C. mydas* (Patrício et al. 2012). Feeding grounds usually are cohabitated by juveniles from different nesting areas (e.g. those off Paraná comprise individuals from 12 nesting sites, but predominantly Ascension Island, Suriname and Sao Tome in Africa; Coelho et al. 2018), where they can be infected, and this might explain the Atlantic-group variant similarities (Monezi et al. 2016; Rodenbusch et al. 2014) observed here—except for variant C which is closest to the eastern Pacific group (Patrício et al. 2012). These observations represent the first published account of a Pacific variant in regional *C. mydas* and so increase our knowledge to a possible circulating strain off Brazil (Monezi et al. 2016; Rodenbusch et al. 2014). Similar to a variant detected in five *Lepidochelys olivacea* and two *C. mydas* in the Pacific Ocean, and two *Caretta caretta* and three *C. mydas* in the North Atlantic Ocean, variant C might not be uncommon and either represents an independent lineage sharing a common ancestor with all other identified virus variants or, more plausible, is a result of a previous recombination event that involved divergent oceanic strains (Greenblatt et al. 2005; Herbst et al. 2004; Morrison et al. 2018; Patrício et al. 2012; Ramirez 2017). The latter may have occurred during migration around the Cape of Good Hope or, perhaps less likely considering the known gene-flow detection of *C. mydas* populations, at the Isthmus of Panama (Bourjea et al. 2007; Naro-Maciel et al. 2008). As suggested in previous studies (Herbst et al. 2004; Jones et al. 2016), detecting the Pacific variant at a new region (Brazil) may facilitate understanding of the zootiology and pathogenesis of the virus.

The prevalence of confirmed FP here (29%; 2014 and 2016) was lower than that among individuals caught off the more northern Rio de Janeiro (∼ 43%; $n = 246$; Tagliolatto et al. 2016) and Espírito Santo (∼ 34%; $n = 640$ and 58%; $n = 163$; Torezani et al. 2010; Santos et al. 2010), but greater than to the south, off Santa Catarina (∼ 10%; $n = 82$; Proietti et al. 2007). These differences might imply a latitudinal environmental gradient, although previous studies have shown that FP prevalence can vary substantially in time (e.g. between years) and space, even among immediately adjacent regions. The reasons such variability are not fully understood, but have been correlated with residency in areas of reduced water quality (Jones et al. 2016).

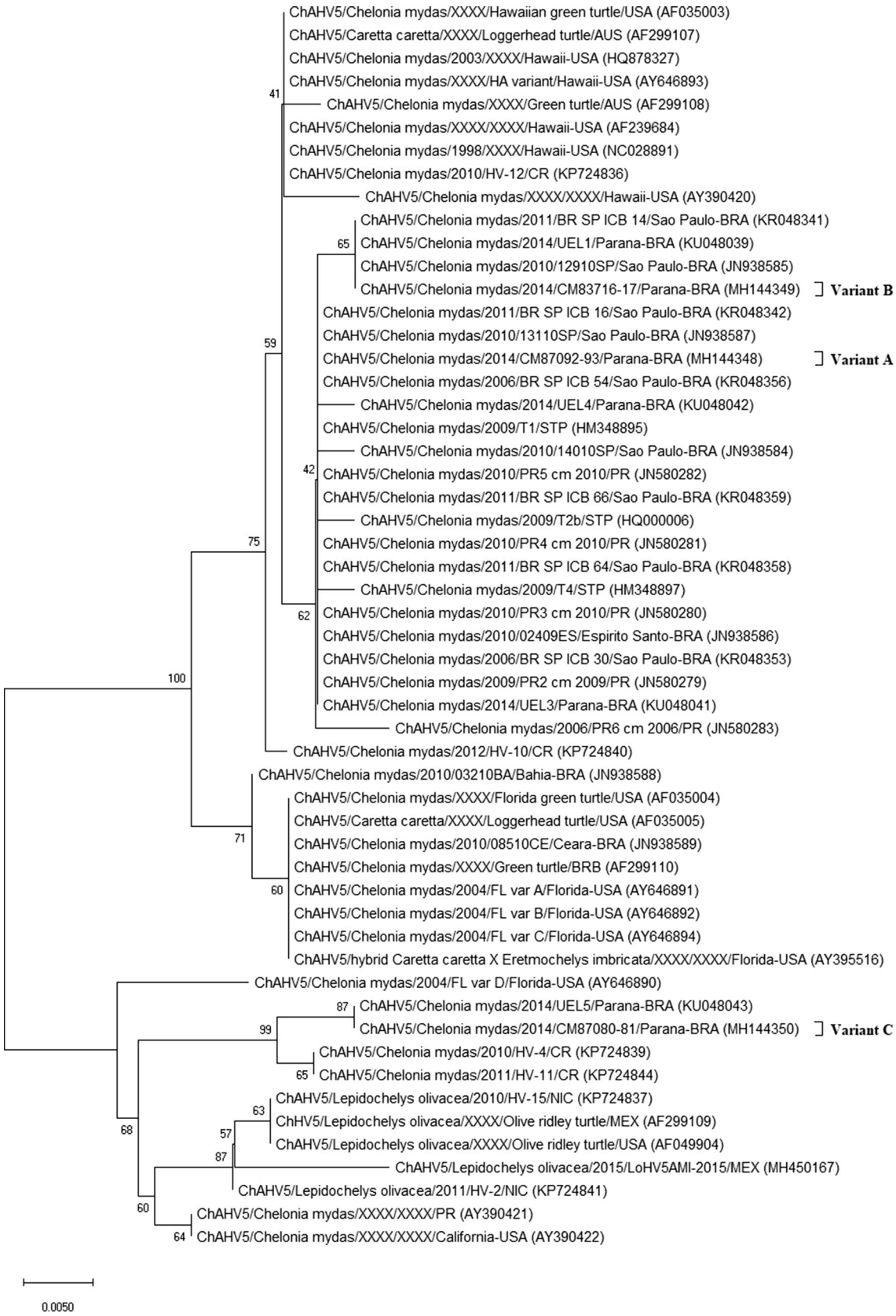
Greater consistency was observed among the general presentation of tumours here and throughout previous regional studies. Supporting this statement, most FP afflicted animals had apparent good health, and like in previous studies (Santos et al. 2010, 2015; Work et al. 2004) we failed to observe a significant difference in BCI among non-afflicted and afflicted *C. mydas*, although the utility of BCI for such assessments is by no means definitive (Work et al. 2004).

In terms of evaluated blood parameters, the significantly lower urea, cholesterol, creatinine, albumin, ALP,

Table 1. Summaries of Wald-*F* statistics from linear mixed models assessing the importance of the fixed effect of 'fibropapillomatosis (FP) tumours' in *Chelonia mydas,* for explaining variability among blood physiology parameters, and the observed means (± SE) in the absence or presence of tumours. Some data were [1]log- or [2]arcsine square-root transformed prior to analyses.

		FP not present		FP present	
Variable	Wald *F*	Mean (SE)	*N*	Mean (SE)	*N*
Body condition index (BCI)	0.01	1.32 (0.06)	44	1.00 (0.10)	17
Cholesterol (mg dL^{-1})	17.46***	106.90 (9.34)	44	58.11 (7.63)	18
Triglyceride (mg dL^{-1})	0.40	41.04 (4.98)	43	42.39 (10.38)	18
Urea[1] (mg dL^{-1})	10.57**	44.93 (6.96)	44	29.11 (7.65)	18
Creatinine[1] (mg dL^{-1})	10.92**	0.26 (0.02)	44	0.14 (0.04)	18
Uric acid[1] (mg dL^{-1})	0.00	0.55 (0.08)	44	0.66 (0.11)	18
Total protein (g dL^{-1})	1.46	2.61 (0.14)	44	2.40 (0.22)	18
Albumin (g dL^{-1})	4.52*	0.52 (0.07)	43	0.46 (0.07)	18
Globulin (g dL^{-1})	0.45	2.11 (0.12)	43	1.94 (0.18)	18
Albumin/Globulin	3.19	0.32 (0.07)	43	0.25 (0.04)	18
Alanine aminotransferase (ALT)[1] (U L^{-1})	2.02	4.66 (0.87)	44	3.67 (0.75)	18
Aspartate aminotransferase (AST)[1] (U L^{-1})	2.00	105.45 (15.15)	44	94.44 (15.24)	18
Alkaline phosphatase (ALP)[1] (U l^{-1})	5.81*	23.02 (2.08)	44	15.83 (1.99)	18
Creatinokinase (CK)[1] (U L^{-1})	3.88	692.15 (168.92)	41	274.39 (67.53)	18
Na[1] (mmol L^{-1})	0.48	155.88 (1.72)	44	157.49 (1.83)	18
K (mmol L^{-1})	0.60	3.93 (0.12)	44	4.17 (0.19)	18
Cl[1] (mmol L^{-1})	0.02	113.93 (1.48)	44	113.73 (1.37)	18
Ionised calcium (iCa) (mg dL^{-1})	1.01	1.86 (0.09)	44	1.90 (0.15)	18
Ca (mg dL^{-1})	0.14	6.43 (0.14)	44	6.61 (0.17)	18
Phosphorus (mg dL^{-1})	6.27*	6.25 (0.39)	44	5.10 (0.46)	18
Ca:P	4.01 ($p = 0.05$)	1.26 (0.11)	44	1.50 (0.15)	18
Mg (mg dL^{-1})	1.96	10.95 (0.59)	44	9.86 (0.49)	18
Gamma glutamyl transpeptidase (GGT) (U L^{-1})	1.10	5.41 (0.50)	44	4.00 (0.62)	18
Lactate[1] (mmol L^{-1})	0.12	8.30 (0.84)	44	7.87 (1.22)	18
Glucose (mg dL^{-1})	7.12**	60.61 (3.94)	44	51.18 (3.71)	17
He[1] (10^6 μL^{-1})	0.30	0.18 (0.03)	39	0.19 (0.02)	18
PCV[2] (%)	2.40	25.82 (1.40)	39	22.67 (1.52)	18
Leucocytes (10^3 μL^{-1})	1.08	37.44 (3.40)	39	32.92 (5.66)	18
Heterophils[2] (%)	0.44	74.49 (2.29)	37	73.94 (3.16)	17
Eosinophils[2] (%)	0.65	1.40 (0.38)	37	3.65 (1.20)	17
Lymphcytes[2] (%)	0.01	22.13 (2.15)	37	20.00 (2.41)	17
Monocytes[2] (%)	0.33	1.81 (0.44)	37	2.41 (0.84)	17
Heterophils[1] (10^3 μL^{-1})	0.02	26.78 (2.60)	37	22.70 (3.86)	17
Eosinophils (10^3 μL^{-1})	4.07*	0.71 (0.22)	37	1.85 (0.73)	17
Lymphcytes[1] (10^3 μL^{-1})	1.06	9.53 (1.49)	37	7.30 (1.56)	17
Monocytes (10^3 μL^{-1})	0.17	0.73 (0.19)	37	0.93 (0.42)	17
Thrombocytes[1] (/100)	1.51	157.86 (38.32)	32	146.84 (20.30)	14

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$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ChAHV5/Caretta caretta/XXXX/Loggerhead turtle/AUS (AF299107)
ChAHV5/Chelonia mydas/2003/XXXX/Hawaii-USA (HQ878327)
ChAHV5/Chelonia mydas/XXXX/HA variant/Hawaii-USA (AY646893)
41
ChAHV5/Chelonia mydas/XXXX/Green turtle/AUS (AF299108)
ChAHV5/Chelonia mydas/XXXX/XXXX/Hawaii-USA (AF239684)
ChAHV5/Chelonia mydas/1998/XXXX/Hawaii-USA (NC028891)
ChAHV5/Chelonia mydas/2010/HV-12/CR (KP724836)
ChAHV5/Chelonia mydas/XXXX/XXXX/Hawaii-USA (AY390420)
ChAHV5/Chelonia mydas/2011/BR SP ICB 14/Sao Paulo-BRA (KR048341)
65
ChAHV5/Chelonia mydas/2014/UEL1/Parana-BRA (KU048039)
ChAHV5/Chelonia mydas/2010/12910SP/Sao Paulo-BRA (JN938585)
ChAHV5/Chelonia mydas/2014/CM83716-17/Parana-BRA (MH144349)
Variant B
ChAHV5/Chelonia mydas/2011/BR SP ICB 16/Sao Paulo-BRA (KR048342)
ChAHV5/Chelonia mydas/2010/13110SP/Sao Paulo-BRA (JN938587)
59
ChAHV5/Chelonia mydas/2014/CM87092-93/Parana-BRA (MH144348)
Variant A
ChAHV5/Chelonia mydas/2006/BR SP ICB 54/Sao Paulo-BRA (KR048356)
ChAHV5/Chelonia mydas/2014/UEL4/Parana-BRA (KU048042)
ChAHV5/Chelonia mydas/2009/T1/STP (HM348895)
ChAHV5/Chelonia mydas/2010/14010SP/Sao Paulo-BRA (JN938584)
42
ChAHV5/Chelonia mydas/2010/PR5 cm 2010/PR (JN580282)
ChAHV5/Chelonia mydas/2011/BR SP ICB 66/Sao Paulo-BRA (KR048359)
ChAHV5/Chelonia mydas/2009/T2b/STP (HQ000006)
75
ChAHV5/Chelonia mydas/2010/PR4 cm 2010/PR (JN580281)
ChAHV5/Chelonia mydas/2011/BR SP ICB 64/Sao Paulo-BRA (KR048358)
ChAHV5/Chelonia mydas/2009/T4/STP (HM348897)
62
ChAHV5/Chelonia mydas/2010/PR3 cm 2010/PR (JN580280)
ChAHV5/Chelonia mydas/2010/02409ES/Espirito Santo-BRA (JN938586)
ChAHV5/Chelonia mydas/2006/BR SP ICB 30/Sao Paulo-BRA (KR048353)
100
ChAHV5/Chelonia mydas/2009/PR2 cm 2009/PR (JN580279)
ChAHV5/Chelonia mydas/2014/UEL3/Parana-BRA (KU048041)
ChAHV5/Chelonia mydas/2006/PR6 cm 2006/PR (JN580283)
ChAHV5/Chelonia mydas/2012/HV-10/CR (KP724840)
ChAHV5/Chelonia mydas/2010/03210BA/Bahia-BRA (JN938588)
ChAHV5/Chelonia mydas/XXXX/Florida green turtle/USA (AF035004)
ChAHV5/Caretta caretta/XXXX/Loggerhead turtle/USA (AF035005)
71
ChAHV5/Chelonia mydas/2010/08510CE/Ceara-BRA (JN938589)
ChAHV5/Chelonia mydas/XXXX/Green turtle/BRB (AF299110)
60
ChAHV5/Chelonia mydas/2004/FL var A/Florida-USA (AY646891)
ChAHV5/Chelonia mydas/2004/FL var B/Florida-USA (AY646892)
ChAHV5/Chelonia mydas/2004/FL var C/Florida-USA (AY646894)
ChAHV5/hybrid Caretta caretta X Eretmochelys imbricata/XXXX/XXXX/Florida-USA (AY395516)
ChAHV5/Chelonia mydas/2004/FL var D/Florida-USA (AY646890)
87
ChAHV5/Chelonia mydas/2014/UEL5/Parana-BRA (KU048043)
99
ChAHV5/Chelonia mydas/2014/CM87080-81/Parana-BRA (MH144350)
Variant C
ChAHV5/Chelonia mydas/2010/HV-4/CR (KP724839)
65
ChAHV5/Chelonia mydas/2011/HV-11/CR (KP724844)
ChAHV5/Lepidochelys olivacea/2010/HV-15/NIC (KP724837)
63
ChHV5/Lepidochelys olivacea/XXXX/Olive ridley turtle/MEX (AF299109)
68
57
ChAHV5/Lepidochelys olivacea/XXXX/Olive ridley turtle/USA (AF049904)
87
ChAHV5/Lepidochelys olivacea/2015/LoHV5AMI-2015/MEX (MH450167)
ChAHV5/Lepidochelys olivacea/2011/HV-2/NIC (KP724841)
60
ChAHV5/Chelonia mydas/XXXX/XXXX/PR (AY390421)
64
ChAHV5/Chelonia mydas/XXXX/XXXX/California-USA (AY390422)
0.0050

◀**Fig. 2.** Phylogenetic tree based on 51 partial nucleotide sequences (366pb) of ChAHV5 polymerase gene available in GenBank and three sequences from this study (black square). The tree was constructed using the neighbour-joining method (Saitou and Nei 1987) and the Kimura two-parameter model for nucleotide substitution. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (values < 40% are not shown) (Felsenstein 1985). The scale bars at the bottom of the trees represent the number of nucleotide substitutions per site. The samples were identified according to herpesvirus DNA detected/sea turtle species affected/year of sample/GenBank sample identification/localisation (GenBank accession number). All the analyses were conducted in MEGA7 (Kumar et al. 2016). AUS, Australia; BRA, Brazil; BRB, Barbados; CR, Costa Rica; MEX, Mexico; NIC, Nicaragua; PR, Porto Rico; STP, Sao Tome and Principe; USA, United States of America. XXX: data not available.

phosphorus and glucose, but elevated eosinophils among FP-afflicted individuals, both conflict and support previous assessments. Specifically, elevated blood urea nitrogen (BUN) in severely FP-afflicted *C. mydas* was attributed to greater protein catabolism or dehydration (Santos et al. 2015). Off Hawaii, elevated BUN, but lower cholesterol among diseased *C. mydas* were observed concomitant with poorer body condition and starvation (Aguirre et al. 1995; Aguirre and Balazs 2000).

Considering the homogenous body condition among *C. mydas* here, any differences in urea and cholesterol might simply reflect divergent metabolic, digestion and absorption of protein and cholesterol in diseased animals (Stacy and Boylan 2014). An alternative hypothesis involves temporal bias in neritic recruitment and spatial dietary segregation for *C mydas*. Specifically, during recruitment, juveniles transition from an omnivorous to herbivorous diet with concomitantly lower urea and cholesterol (Whiting et al. 2007). Such transition might also account for the lower phosphorus levels in FP-afflicted animals; considering these typically are lower in algae and plants than in animals (Diniz et al. 2012). Possibly, the FP-afflicted individuals here simply were earlier neritic recruits (longer exposed to the aetiological agent and/or environmental factors) than more recently arrived non-afflicted conspecifics (from disease-free oceanic waters).

Although BUN and creatinine are the most common test for monitoring kidney function in mammals, both generally are considered to be of poor diagnostic value in detecting renal disease in reptiles, and the creatinine amount formed in most reptiles is negligible (Campbell 2014). The mean values of urea, creatinine and uric acid observed here were below the mean values observed in other clinically healthy, free-ranging juveniles *C. mydas* populations (Bolten and Bjorndal 1992; Flint et al. 2009; Labrada-Martagón et al. 2010; Santos et al. 2015; Swimmer 2000), which supports the hypothesis that the observed differences between FP-afflicted and non-afflicted animals were more likely associated with the dietary shift than kidney disease.

The mean albumin levels among FP-afflicted and non-afflicted animals here were also lower than those observed among other healthy juvenile *C. mydas* populations (Bolten and Bjorndal 1992; Flint et al., 2009; Whiting et al. 2007). Like in humans and other animals, the albumin concentration is the main factor maintaining the oncotic pressure of blood in reptiles and may reflect adaptations to habitat or variations in nutritional state and health condition (Goldwasser and Feldman 1997; Masat and Dessauer 1968; Whiting et al. 2007). In diseased animals, albumin is considered valuable for prognosis, because during acute and chronic inflammation, the liver produces a large amount of mediators and albumin is not a reactant, so its synthesis may diminish (Doweiko and Nompleggi 1991). Therefore, the lower values observed in animals at the PEC may reflect lower levels of protein in their diet and at least some influence of chronic inflammation in FP-afflicted animals—also observed in unhealthy *C. mydas* elsewhere (Flint et al. 2009; Whiting et al. 2007).

Alkaline phosphatase is widely distributed throughout reptilian bodies, and the plasma activity of this enzyme is not considered to be organ specific (Campbell 2014). Increased activity may reflect increased osteoblastic activity, hyperparathyroidism and bone diseases, and when associated with increases in glucose levels and corticosteroid might imply a stressed condition (Campbell 2014). Although higher levels of ALP were observed in non-diseased than diseased animals, these were similar or lower than among other healthy populations of juvenile *C. mydas* (Bolten and Bjorndal 1992; Labrada-Martagón et al. 2010; Santos et al. 2015). Controversially, ALP deficiency caused by gene mutations in humans has been associated with markedly defective bone mineralisation (Whyte et al. 1996). It is possible that lower ALP levels observed in diseased animals may reflect slow bone development if the condition maintains, but more information is necessary to assess this condition in reptiles.

In general, the blood glucose concentrations of most reptiles are subject to marked physiological variation (Campbell 2014). Compared to other healthy juvenile *C. mydas* populations (Bolten and Bjorndal 1992; Labrada-Martagón et al. 2010; Santos et al. 2015), lower levels of glucose were observed in animals off the PEC, but this was possibly associated with the methodology (i.e. a portable glucometer using whole blood), which usually detects lower levels of glucose than automatic or semi-automatic clinical chemistry analysers assessing plasma/serum blood (Tonyushkina and Nichols 2009). Nevertheless, the glucose levels of FP-afflicted individuals were lower than their non-afflicted conspecifics; which might be associated with foraging state and/or chronic physiological disturbances (Campbell 2014).

Unlike the biochemical parameters, increased eosinophils in tumoured *C. mydas* are more easily attributed to disease. Eosinophils are phagocytic cells particularly involved in destroying parasites and other types of antigen stimulation, so their elevation might imply concomitant diseases in organs other than skin (Stacy et al. 2011). However, high eosinophils concentrations also occur with tissue repair, cancer, and allergic and antitumor activity in mammals (Carretero et al. 2015). No relationships between tumours and immune reaction via eosinophils have been investigated for sea turtles, but these potentially warrant assessment in future research.

Beyond altered levels of blood parameters, key concerns among *C. mydas* with advanced FP are lesions in the eyes, mouth, cloaca and inguinal and axillary areas which could affect vital functions such as foraging, reproduction and migration (Aguirre and Balazs 2000; Work et al. 2004). Other potentially more direct consequences might include greater mortalities owing to fisheries interactions. For example, although few data are available (Chaloupka et al. 2008; Foley et al. 2005), the morphological discontinuities of tumours could increase the possibility of entanglement in gillnets and trawls (both of which are intensely used off South America). Considering commercial fishing is a major source of mortality among sea turtles globally (Domiciano et al. 2017), even marginal increases would have considerable negative impacts on populations. Other less obvious consequences of FP include sublethal effects related to the mobilisation of bioaccumulated contaminants with lipids into the blood stream, which could exacerbate the immunosuppression observed in severely tumoured and emaciated animals (Keller et al. 2014).

The implications associated with the various impacts above support FP being considered a threat for *C. mydas* conservation in the south-western Atlantic Ocean, where the prevalence can reach > 50% of individuals in some populations (Santos et al. 2010; Zwarg et al. 2014). Ongoing studies are required to facilitate a more holistic overview of the various intra- and inter-population effects of the disease among *C. mydas*, along with any correlations between environmental factors and anthropogenic activities. Doing so should help define priorities not only for regional, but also global disease mitigation and improved health parameters (e.g. zoonotic diseases and chemical contamination) for human populations.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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