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Elemental analysis of cetacean skull lesions associated with nematode infections

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ABSTRACT: The elemental composition of both healthy and eroded cetacean skulls associated with nematode infections was evaluated. A total of 27 samples of eroded and non-eroded prepared museum cetacean skulls were characterised by elemental (CHN), X-ray fluorescence, and X-ray diffraction methods. The inorganic composition and crystal line structure (hydroxylapatite-like minerals) were similar for both types of skull samples, but the CHN values clearly differed. The results suggest that the carbon-rich fraction is lost in eroded areas, probably as a result of glycosaminoglycan-degrading *Crassicauda* enzymes.

KEY WORDS: Cetacean · Skull lesions · *Crassicauda*

INTRODUCTION

Nematodes infesting tissues and organs of cetaceans are most commonly members of the family Crassicaudidae (Dailey 1985). In numerous cases *Crassicauda* infections are believed to produce lesions in the skull of cetaceans, primarily in members of the Delphinidae (Dailey & Perrin 1973, Robineau 1975, Dailey & Stroud 1978, Dailey & Walker 1978, Perrin & Power 1980, Raga et al. 1982). Bone lesions have been reported in both stranded and captured dolphins in different oceans and seas of the world (Dailey & Perrin 1973, Raga et al. 1982). The absence of residual bone lesions in older animals has been interpreted as strong evidence indicating that parasitism may constitute a major factor in the natural mortality of small cetaceans (Perrin & Powers 1980, Walker & Cowan 1981, Geraci & St. Aubin 1987). Whatever the proximate cause of mortality, understanding the causal relationships concerning parasitic-caused skull lesions will require a better understanding of both the host lesion and the associated parasites. Recently, a long-term macroscopic examination of cetacean cranial gross lesions in specimens from the coast of Galicia (NW Spain), an important stranding area (López et al. 1998), allowed us to carry out an elemental analysis of skull lesions which appeared as eroded areas in prepared museum skulls.

MATERIAL AND METHODS

A total of 27 prepared museum skulls belonging to immature bottle-nosed dolphins *Tursiops truncatus* were macroscopically examined for skull lesions (Table 1). Trabecula-like bone lesions consisting of sunken surfaces with duct systems, 10×5 to 50×40 mm in size in the orbitary (frontal-orbitosphenoid) skull region, were classified as eroded areas (Fig. 1). Non-eroded bone areas were also sampled as controls. For each specimen, a comparison of eroded to non-eroded skull areas was made.

Eroded and non-eroded skull samples obtained following the procedure of Benneth & Oliver (1992) were examined by X-ray fluorescence and X-ray powder diffraction to determine the composition of the inorganic fraction and its single crystal line structure. Measurements were carried out on a Siemens D-5000 powder diffractometer at room temperature using graphite-monochromatized $CuK_{\alpha 1}$, 2 radiation (λ = 1.54056 Å). The skull organic composition was esti-

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Table 1. *Tursiops truncatus.* Data of samples used for elemental analysis of skull lesions. TBL: total body length in cm; M: male; F: female; SL: skull length in mm; SW: skull width in mm; Sample location; CEMMA: Coordinadora para o Estudio dos Mamíferos Mariños; SGHN: Sociedade Galega de Historia Natural

Date	Locality	TBL	Sex	SL	SW	No. of lesions	Location
–/–/1983	Esmelle	230	–	480	256	1	SGHN
10 Nov 1990	Xuño	265	–	500	258	0	CEMMA
30 Dec 1990	Santa Mariña	–	–	370	280	6	CEMMA
31 Dec 1990	Louro	290	M	515	272	2	CEMMA
06 Jan 1991	Queiruga	287	M	510	283	3	CEMMA
03 Feb 1991	P. Langost.	265	M	510	283	0	CEMMA
02 Jun 1991	M. do Rostro	–	–	256	270	0	CEMMA
13 Nov 1991	S. Xurxo	250	M	500	274	1	SGHN
12 Jan 1992	M. do Rostro	275	F	470	265	2	CEMMA
24 May 1992	Seaia	–	–	430	225	0	CEMMA
18 Jul 1992	Ponzos	275	F	530	285	6	SGHN
11 Sep 1992	Sanxenxo	132	–	310	160	0	CEMMA
27 Nov 1992	Bouzas	278	M	459	260	2	CEMMA
12 Apr 1993	Sobreira	268	M	475	270	5	CEMMA
30 Jul 1993	Patos	205	M	425	220	0	CEMMA
05 Jul 1994	As Sinas	268	F	500	275	0	CEMMA
23 Jun 1994	Cee	206	M	420	250	0	CEMMA
21 Jan 1995	Lago	268	F	430	270	0	CEMMA
15 Jun 1995	Soesto	244	M	495	270	2	CEMMA
03 Jun 1995	Baldaio	287	F	555	295	4	CEMMA
23 Apr 1996	Ferrol	220	M	450	282	0	SGHN
20 Oct 1996	Louro	249	M	485	260	1	CEMMA
24 Oct 1996	Lapaman	280	M	525	270	1	CEMMA
06 Jan 1997	Corrubedo	154	–	440	278	3	CEMMA
17 Jul 1997	Niñons	–	–	490	290	0	CEMMA
02 Nov 1997	Oia	–	–	510	270	1	CEMMA
24 Nov 1997	Oia	276	–	506	268	1	CEMMA

mated by CHN analyses obtained with an elemental microanalyzer, Carlo Erba Eager 200. Statistical differences in the CHN composition between both healthy and eroded skull samples were assessed using an SPSS WIN 7.5 paired Student's *t*-test.

RESULTS

Lesions were observed in 59.25% of the skulls sampled (range: 0 to 6 lesions in each specimen). Most of the lesions occurred as eroded areas in the frontal-orbitosphenoid and pterygoid regions (Fig. 2), with nasal and orbitary perforations extending into the brain cavity.

Calcium and phosphate minerals were predominant in the inorganic phase of both skull homogenates (Table 2). The powder X-ray diffraction patterns revealed that these calcium phosphate hydroxyde compounds had crystal line structures similar to that of hydroxylapatite, HPA [$Ca_{10}(PO_4)_6$ $(OH)_2$] (Fig. 3), which constitutes the main inorganic phases of the hard tissues of vertebrates. X-ray diffraction patterns of all skull samples were characteristic of moderately crystalline minerals as obtained in a unique crystalline phase.

Table 3 shows the results of the CHN analyses. The carbon percentage in eroded areas was 24% lower than in non-eroded areas ($t = -8.71$; $p < 0.05$). The CHN (i.e., organic phase) mean composition was 33.18% of the total skull sample in non-eroded areas, whereas

Table 2. *Tursiops truncatus.* X-ray fluorescence analysis of inorganic elements (mean ± SD) in non-eroded (n = 11) and eroded (n = 16) skull samples

Element (%)	Non-eroded	Eroded
Na	0.0964 ± 0.01	0.0868 ± 0.01
Mg	0.4240 ± 0.02	0.3920 ± 0.01
Al	0.0690 ± 0.00	0.0866 ± 0.00
Si	0.1560 ± 0.02	0.2190 ± 0.03
P	22.600 ± 0.01	22.600 ± 0.01
S	0.0381 ± 0.00	0.0456 ± 0.00
Cl	0.2310 ± 0.00	0.2430 ± 0.00
K	0.0554 ± 0.00	0.0562 ± 0.00
Ca	33.100 ± 0.39	33.000 ± 0.47
Zn	0.0805 ± 0.00	0.0763 ± 0.00
Sr	0.0361 ± 0.01	0.0363 ± 0.02
Fe	–	0.0265 ± 0.00

Fig. 1. *Tursiops truncatus.* Trabecula-like bone lesions classified as eroded samples for the elemental analysis of skull lesions

this percentage decreased to 27.16% in eroded ones ($t = -7.56$; $p < 0.05$). Values for the C:N ratio were also significantly different from different eroded and non-eroded skull samples ($t = -19.29$; $p < 0.01$).

DISCUSSION

Impure forms of HPA, the main mineral of bone and teeth of vertebrates (Bigi et al. 1995), constituted the main inorganic phases of both eroded and non-eroded skull samples. Since HPA skull crystal structures were similar in healthy and eroded skull areas, macroscopic lesions are probably due to altered organic phases in cranial soft tissues. In fact, the C:N ratio (an index believed to reflect the quantity of carbon-rich molecules relative to the quantity of protein; Ferron & Leggett 1994) revealed a decreased composition of the carbon fraction in eroded skull samples. In the extracellular matrix (ECM), major constituents are collagens, non-collagenous glycoproteins and proteoglycans (Kreis & Vale 1993). Within the ubiquitous proteoglycans found on cell surfaces, the glycosaminoglycan (GAG) polysaccharides side chains represents more than 5% of the organic composition of a given bone in an adult mammal (Kreis & Vale 1993). While proteins (N-rich molecules) have a C:N ratio near 3.0 (Harris et al. 1986), GAGs are carbohydrate structures; thus, when these extended sugar chains are present, it is expected that the ratio will increase to a value above 3.0. In

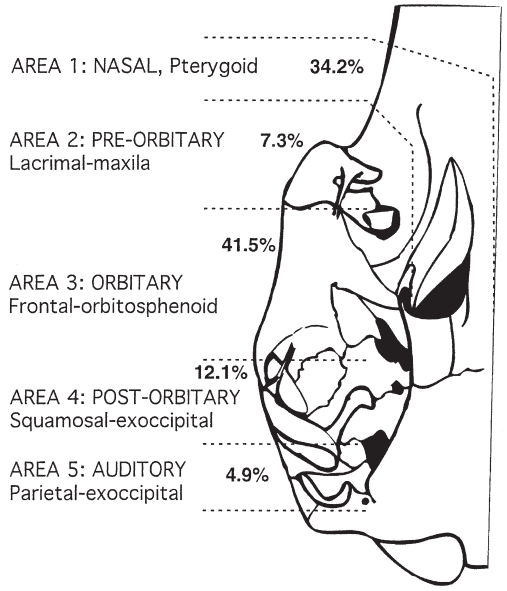

Fig. 2. *Tursiops truncatus.* Frequency of lesions within each area on the ventral skull surface

eroded-skull samples, a C:N ratio was found close to 3.0, which was almost $^1/_2$ of that of non-eroded samples, probably as a consequence of parasite-caused GAG hydrolisation by chondroitinase-like enzymes. Although at present this hypothesis is still rather speculative, other factors support it: (1) Susceptibility of GAGs to digestion by certain bacterial enzymes has traditionally formed an important biochemical criterion by which GAGs are classified (Kreis & Vale 1993); (2) *Crassicauda* spp. infections have frequently been recorded associated with skull lesions during routine necropsies on cetacean carcasses (López et al. 1998); (3) GAG-degrading parasitic enzymes which degrade hyaluronic acids and chondroitin sulphates have been recorded in numerous Nematoda (Sakanari & Mckerrow 1990, Hotez et al. 1994); (4) in migrating Nematoda excretory-secretory (ES) parasitic enzymes are recognised to play an important role in invading host tissues (Fukuda et al. 1990); and (5) adult nematodes use polysaccharides as an energetic substrate (Köhler & Voight 1988). Therefore, it could be that when GAGs are hydrolysed, eroded areas become evident macroscopically due to physical migration of parasitic nematodes through the ECM.

Table 3. *Tursiops truncatus.* CHN determination (mean ± SD) in non-eroded (n = 11) and eroded (n = 16) skull samples

	Non-eroded	Eroded
Nitrogen (%)	3.55 ± 0.08	5.17 ± 0.06
Carbon (%)	25.38 ± 0.32	18.88 ± 1.01
Hydrogen (%)	4.25 ± 0.03	3.11 ± 0.21
C:N ratio	7.15 ± 0.25	3.65 ± 0.24

Although the data herein reported provide a better understanding of skull lesions, obviously further research is needed. This is especially true concerning *in vivo* cultivation of mature *Crassicauda* adults to biochemically characterise any GAG-degrading parasitic enzyme in the ES nematode products. Furthermore, the collection of fresh fixed cranial tissues (carcasses with Condition 2 as defined by Kuiken & Garcia-Hartmann 1991) is desirable in order to study the tissue and cytochemical structures in both eroded and non-eroded skull areas. Moreover, because of problems associated with the biological interpretation of the C:N

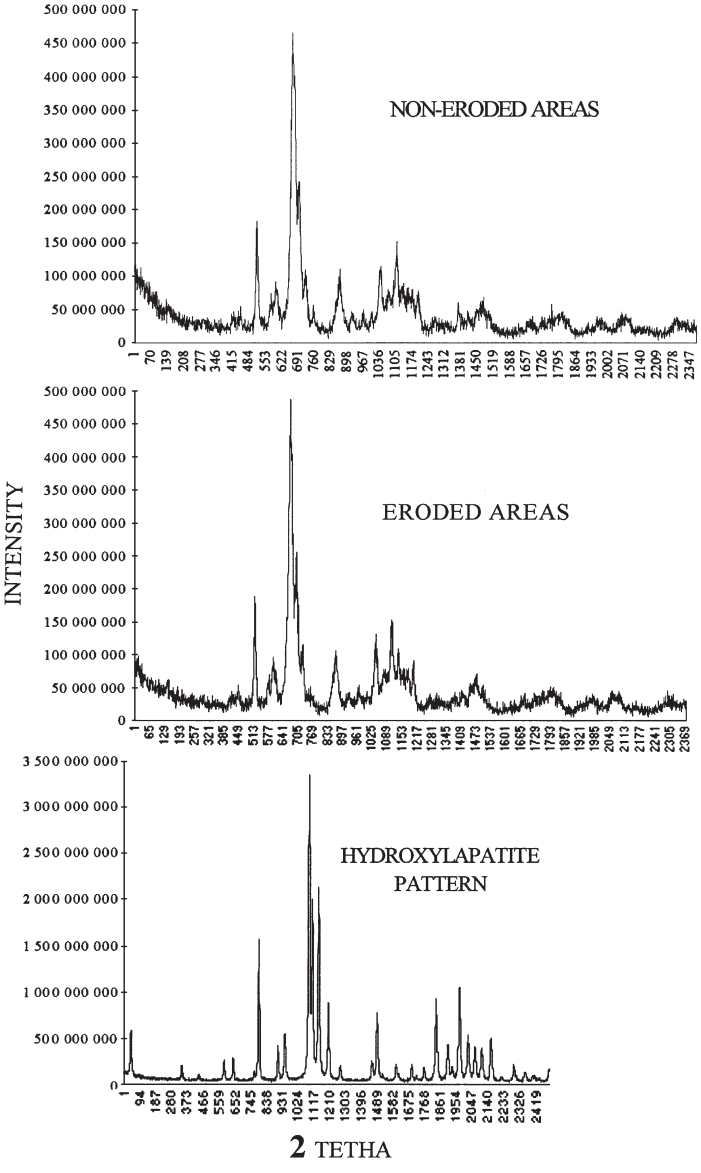

Fig. 3. *Tursiops truncatus.* Powder X-ray diffraction patterns of the skull samples as compared with hydroxylapatite

ratio (Ferron & Leggett 1994) simultaneous measurements of all major molecules are needed in order to assess carbon-nitrogen mobilisation accurately. Finally, while *Crassicauda* nematodes have traditionally been recorded associated with skull lesions, other bacteria and/or viruses may also be present as single or sympatric infections. The existence of multiple etiological agents has also been previously suggested for other parasite-caused diseases in cetaceans (Abollo et al. 1998). A complete etiological examination of eroded skull samples is, therefore, also desirable to delineate the potential role of disease on cetacean mortality and stranding behaviour.

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