

# Use of Micro-Computed Tomography for Dental Studies in Modern and Fossil Odontocetes: Potential Applications and Limitations

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## ABSTRACT

Teeth are important elements in studies of modern and fossil Cetacea (whales, dolphins), providing information on feeding habits, estimations of age and phylogenetic relationships. The growth layer groups (GLGs) recorded in dentine have demonstrated application for aging studies, but also have the potential to elucidate life history phenomena such as metabolic or physiologic events. Micro-Computed Tomography (Micro-CT) is a non-invasive and non-destructive technique that allows 3-dimensional study of mineralized tissues, such as human teeth, and their physical properties. Teeth from extant dolphins (Cetacea: Odontoceti) and some fossil odontocetes were scanned in a Skyscan 1172 Micro-CT desktop system. X-rays were generated at 100 kV and 100  $\mu$ A for extant samples, and at 80kV and 124  $\mu$ A for fossils. 0.5 mm thick aluminum and copper filters were used in the beam. Reconstructed images were informative for most extant species, showing a good resolution of the enamel layer, dentine and pulp cavity. Greyscale changes in the dentinal layers were not resolved enough to show GLGs. Visualization of the internal structure in fossil cetacean teeth depended on the degree of diagenetic alteration in the specimen; undifferentiated enamel and dentine regions probably reflect secondary mineralization. However, internal details were finely resolved for one fossil specimen, showing the enamel, internal layers of dentine and the pulp cavity. Micro-CT has been proven to be a useful tool for resolving the internal morphology of fossil and extant teeth of cetaceans before they are sectioned for other morphological analysis; however some methodological refinements are still necessary to allow better resolution of dentine for potential application in non-destructive age determination studies.

## INTRODUCTION

Teeth are a valuable tool in studies of fossil and extant animals, supplying data on the feeding habits, environmental influences, agonistic and display behaviours, phylogenetic relationships among species, and estimations of age (Ungar 2010). Teeth also form a prominent part of mammal remains in paleontological and archaeological sites because of their tough and resistant composition, thus becoming key elements in the study of biology, functional morphology, systematics and evolution of fossil and recent species (Bergqvist 2003, Hillson 2005).

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Cetaceans have a peculiar dentition when compared to most other mammals. In contrast with the eutherian dental standard, dolphins produce a single set of teeth that remain in place throughout their life (monophyodonty), the teeth are undifferentiated and simplified in shape to cones or pegs (homodonty) and they have a much-increased number of teeth (polydonty) compared to most terrestrial mammals (Flower 1885, Myrick 1991, Ungar 2010). These simplified teeth are covered by a cap of enamel, which is deposited before birth. The bulk of the tooth is composed of dentine, which has a layered deposition cycle. The first layer, the prenatal dentine, is deposited antenatally and represents a record of the foetal life of the animal. Subsequent layers, called postnatal dentine, are accumulated throughout life until the death of the animal or until the pulp cavity is closed. The deposition of cementum, which covers the tooth root, also starts shortly after birth and continues until death. By studying captive and known-age animals, it was demonstrated that dentinal layers, or growth layer groups, correspond to annual increments, thus having the potential to provide the age of the animal (Myrick 1991).

The growth layer groups (GLGs) recorded in dentine have been routinely applied in aging studies, but it is known that they also have the potential to elucidate life history phenomena such as metabolic and physiologic events. It has been shown that layers in dentine and cementum can provide information regarding general health, life history events such as parturition, weaning and achievement of sexual maturation, as well as environmental conditions and other stressors. These conditions are often manifested as mineralization anomalies within the layers (Luque *et al.* 2009). Dentinal growth layer groups consist of alternating poorly- and more highly-mineralized layers throughout postnatal dentine (Hohn 1980). The thickness of growth layers is variable among species, but commonly the first two or three layers are thicker than the following, which become increasingly thinner. Previous studies have reported thicknesses for the first two growth layers of about 700  $\mu\text{m}$  for the bottlenose dolphin (*Tursiops truncatus*), 240  $\mu\text{m}$  for the spinner dolphin (*Stenella longirostris*) and 400  $\mu\text{m}$  for the franciscana (*Pontoporia blainvillei*), while the subsequent layers measured 500-100  $\mu\text{m}$ , 180-80  $\mu\text{m}$  and 200-100  $\mu\text{m}$  on average, respectively (Myrick *et al.* 1984, Hohn *et al.* 1989, Pinedo and Hohn 2000).

Current techniques for age determination and internal morphological study in cetaceans are laborious and involve destructive sectioning, decalcifying, staining, and mounting tooth sections, followed by extensive microscope analysis (*e.g.* Hohn *et al.* 1989). These techniques have been in use for more than 30 years and have been adapted to suit both species with larger and smaller teeth (Hohn *et al.* 1989, Myrick 1991, Lockyer 1995). Besides the potential financial and laboratory time constraints related to the use of

established sectioning techniques, they also have the disadvantage of being destructive. This may not seem a problem when considering extant species and their numerous teeth, but it becomes crucial when dealing with fossils. Many fossil cetaceans are known only from the holotype, and commonly skulls and teeth are the only skeletal elements available for describing the species (Fordyce 2009). Thus, there is a clear limitation for studies using destructive techniques when most fossil specimens are considered rare and unique.

Micro-Computed Tomography (Micro-CT) is a non-invasive and non-destructive technique that allows 3-dimensional (3-D) study of mineralized tissues and their physical properties. Micro-CT scanners reconstruct digital cross sections (slices) of an object, which can be stacked to create 3-D volumes. The resulting 3-D volumes can be used to generate computerized images of specimens that can be manipulated, sectioned, prepared, dissected and measured to reveal both internal and external morphology. Such methods allow access to internal morphological information of fragile, rare, valuable or small specimens, including both extinct and extant species (Kim *et al.* 2007, Swain and Xue 2009, Abel *et al.* 2012). Besides allowing visualization of hidden structures and details, Micro-CT is also useful to investigate fine morphological variation within specimens and to perform advanced morphometric analyses (Rossi *et al.* 2004).

Much information can be obtained from Micro-CT, as the slices can be recreated in any plane, and the data can be represented as 2-D or 3-D images. The internal and external anatomy of the object can be demonstrated simultaneously or separately, and the images can be assessed both qualitatively and quantitatively (Kim *et al.* 2007). Recent technological improvements have allowed Micro-CT systems to increase the spatial resolution and slice thickness to the micron scale, which provides refined detail (Plotino *et al.* 2006, Swain and Xue 2009).

Micro-CT has mostly been used in odontology for qualitative dental studies in humans, although quantitative approaches have been developed in recent years as research has expanded to consider other mammal groups. Besides morphological study of rare and valuable fossil specimens (*e.g.* McErlain *et al.* 2004, Abel *et al.* 2012, Davis 2012), Micro-CT has been used in diverse studies of, for example, tooth morphometrics (*e.g.* Kim *et al.* 2007), inference of mineral density and concentration (*e.g.* Clementino-Luedemann and Kunzelmann 2006, Park *et al.* 2010), investigations on the development of dental pathology and paleopathology (*e.g.* Rossi *et al.* 2004), root morphology (*e.g.* Plotino *et al.* 2006), and enamel thickness (*e.g.* Swain and Xue 2009).

At present, Micro-CT applications in marine mammal dental studies are virtually nonexistent and unexplored. This paper aims to demonstrate the potential applications of Micro-CT in dental studies of fossil and living cetaceans, outlining the methodological approach, the advantages, and also the limitations of this technique in comparison to other methods.

## MATERIAL AND METHODS

Dental samples of both extant and fossil cetaceans were used in this study (Table 1). For extant species, materials come from deceased-stranded or accidentally entangled animals, normally processed by water maceration and preserved dry or stored in ethanol. Fossils were mostly collected in the Waitaki Valley in Otago and South Canterbury, New Zealand, and represent a variety of Oligocene cetaceans. One of the specimens was from Pliocene sediments of Caldera, Chile. After preparation by physical removal of the associated matrix, fossil specimens were preserved dry.

**Table 1:** Species and specimens analyzed and collection number.

<b>Species</b>	<b>Status</b>	<b>Collection number</b>
<i>Stenella coeruleoalba</i>	Extant	UFSC 1344
<i>Globicephala</i> sp.	Extant	REF 6.5.76.1
<i>Sotalia guianensis</i>	Extant	MCN 060
<i>Inia geoffrensis</i>	Extant	IEPA 1899
<i>Tursiops truncatus</i>	Extant	UFSC 1349
<i>Pontoporia blainvillei</i>	Extant	UFSC 1310
Delphinoidea, Unnamed sp.1	Extinct	SGO-PV-754
Delphinoidea, Unnamed sp.2	Extinct	OU 22108
Squalodontidae, Unnamed sp.1	Extinct	OU 22457
Squalodontidae, Unnamed sp. 2	Extinct	OU 22257
Squalodelphinidae, Unnamed sp.	Extinct	OU 22306
Kekenodontidae, Unnamed sp.	Extinct	OU 22023

After surface-cleaning with ethanol, dental samples were mounted on metal holders using modeling clay with their apices facing upwards. Samples were scanned using a Skyscan 1172 Micro-CT desktop system (Skyscan, Kontich, Belgium). X-rays were generated at 100 kV, 100  $\mu$ A and 10W for extant samples, while fossils were scanned using 80 kV, 124  $\mu$ A and 10W. 0.5 mm thick aluminum and copper filters were placed in the beam path. The resolution was set at 8.6  $\mu$ m pixel size. The rotation was set to 0.5-degree

steps, creating 393 two-dimensional projections over a 180-degree rotation of the specimen. On average, 5 hours were required to complete each scan at this resolution.

Images were reconstructed using the Skyscan NRecon software (NRecon, version 1.4.4, Skyscan) in order to create cross-sectional slices of the specimens. Reconstruction settings that better resolved the images were previously tested and standardized for all specimens. Smoothing was set at 4, ring artifact correction at 68%, and beam hardening at 20. Reconstructed images were then qualitatively analyzed and re-sliced in orthogonal views using ImageJ (ImageJ 1.46, National Institutes of Health, Bethesda, USA).

For some specimens, a system of 2-phase resin-hydroxyapatite phantoms was used to calibrate the greyscales and mineral densities of the dental tissues (Schwass *et al.* 2009). Teeth were scanned with phantoms of known mineral density and then analyzed with Skyscan CTAn (CTAn, version 1.5.0, Skyscan) after being reconstructed. The calibration of grey scales and mineral densities followed Schwass *et al.* (2009).

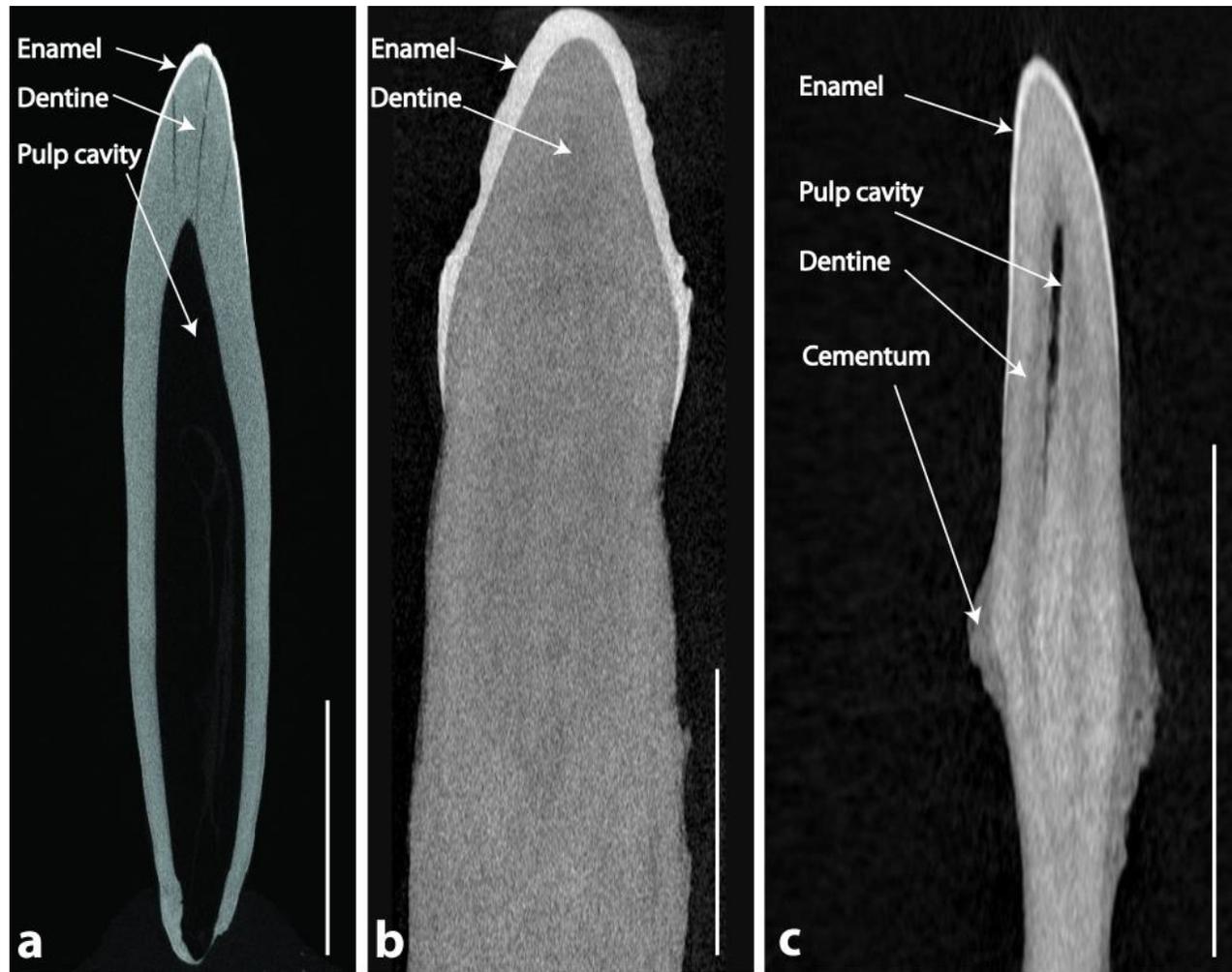
## RESULTS

### Qualitative assessment

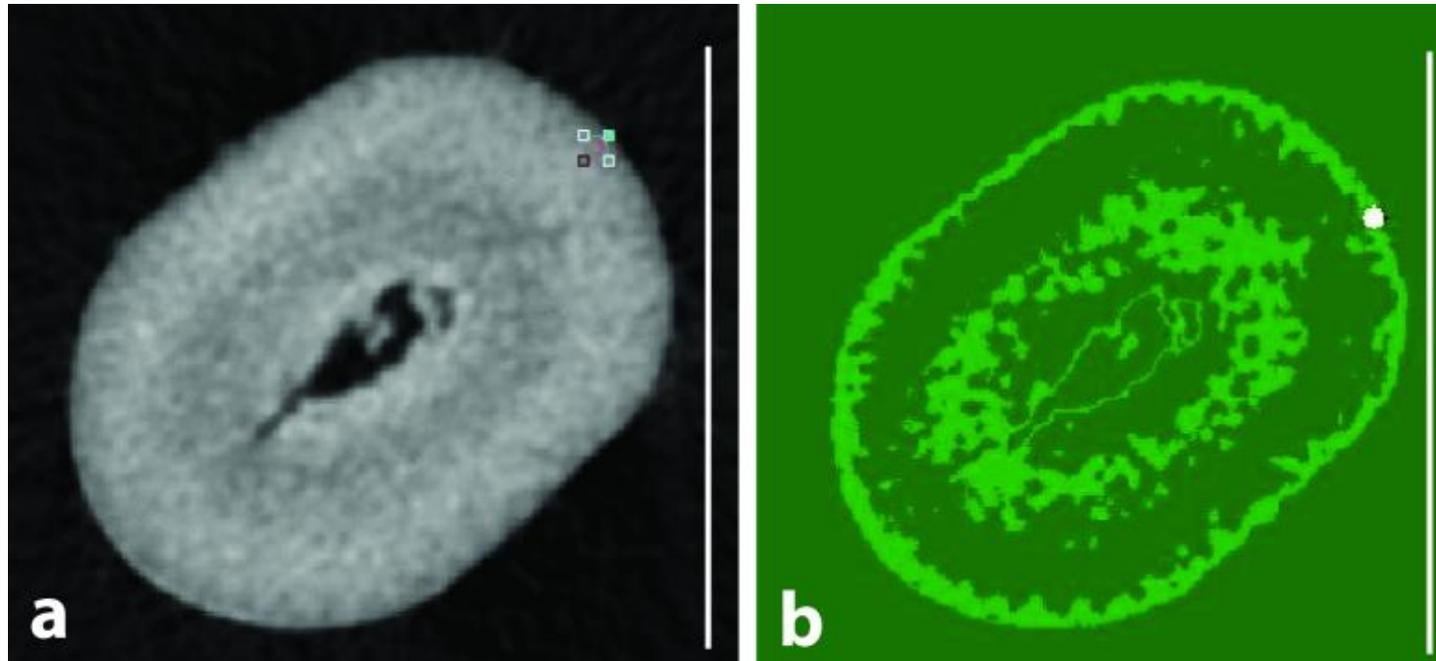
#### *General morphology*

Reconstructed images were well resolved for the major regions of the teeth for all extant species, allowing visualization and individualization of the enamel, dentine and pulp cavity regions ([Fig. 1](#)). Different stages of obliteration of the pulp cavity were observed among specimens, suggesting different stages of ontogenetic development. The dentinal region, although well-defined and differentiated from enamel and cementum, was relatively homogeneous and did not show any evidence of inner structure such as dentinal growth layers. Some of the specimens scanned showed a slight variation in greyscale in the dentinal area, enhanced by manipulation of thresholds using imaging software ([Fig. 2](#)). However the difference in greyscale could not be related to GLGs with confidence.

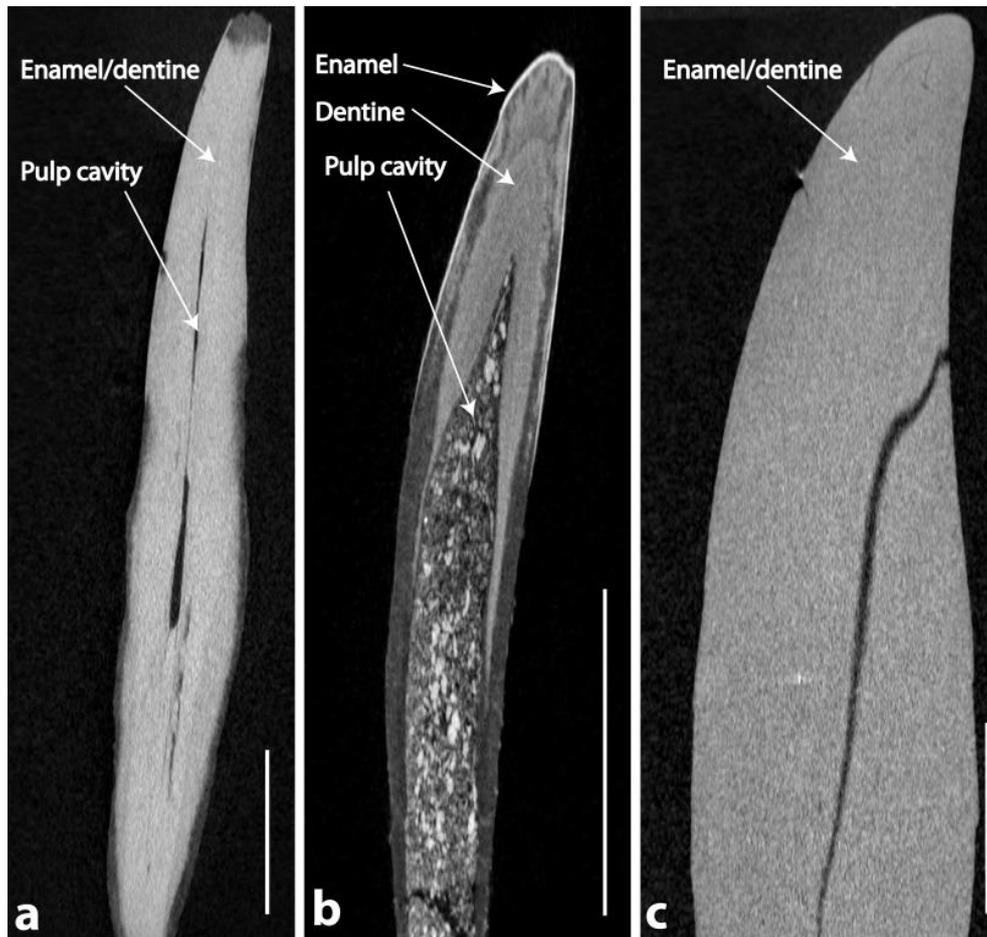
For the fossil cetaceans, mixed results were obtained. Some specimens showed fine resolution, resolving the enamel, internal layers of dentine and the pulp cavity ([Fig. 3b](#)), but other specimens revealed a poor resolution of dentine, and in some cases the enamel-dentine junction was not visible ([Fig. 3a and c](#)). For the latter specimens, enamel and dentine had the same x-ray contrast and were indistinguishable. Micro-CT also allowed the



**Fig. 1.** Internal morphology of extant odontocete teeth revealed by Micro-CT. Note the resolution of enamel, dentine, cementum and the different stages of obliteration of the pulp cavity, but no clear growth layer groups. Scale bar = 10 mm. a) Pilot whale *Globicephala* sp. (REF 6.5.76.1). b) Amazon river dolphin *Inia geoffrensis* (IEPA 1899). c) Franciscana *Pontoporia blainvillei* (UFSC 1310).



**Fig. 2.** a) Reconstructed Micro-CT cross section of the Franciscana *Pontoporia blainvillei* (UFSC 1310). b) Same specimen after threshold manipulation. Scale bar = 2 mm.



**Fig. 3.** Internal morphology of fossil odontocete teeth revealed by Micro-CT. Note the poor resolution of dentine and enamel-dentine junction in two of the specimens (a, c) and the different stages of obliteration of the pulp cavity. Scale bar = 5 mm. a) Squalodelphinidae, unnamed sp. (OU 22306). b) Delphinoidea, unnamed sp. 2 (OU 22108). c) Delphinoidea, unnamed sp. 1 (SGO-PV-754).

visualization of the pulp cavity, which could furnish an indirect proxy for ontogenetic development. Sometimes the pulp cavity was also infilled with sedimentary matrix (Fig. 3b). Cracks provoked by desiccation or even due to burial-related compaction and tectonic distortion were also apparent (Fig. 3c).

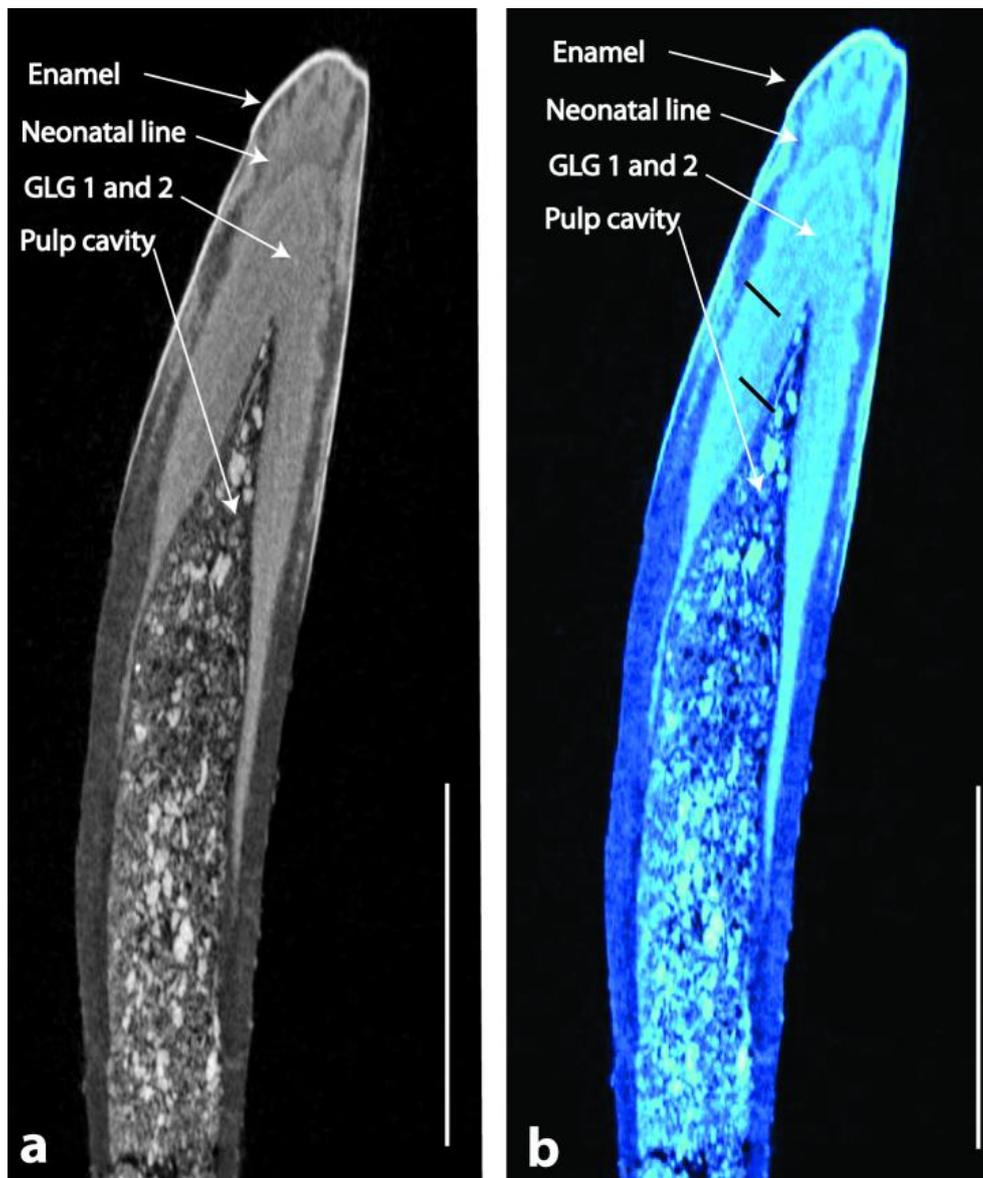
Reconstructed orthogonal Micro-CT images of the unnamed Delphinoidea (OU 22108) showed a fine resolution of the enamel-dentine junction, together with definition of internal layers in dentine (Fig. 4a). By changing the appearance of the image using Lookup tables from ImageJ, it was possible to resolve some details of these features (Fig. 4b). Lookup tables attribute colors to the different greyscale values obtained, thus changing the appearance but not the pixel values of the image. The neonatal line was visible below (internal to) the enamel, followed by two growth layers of dentine in the crown region. The pulp cavity was open and infilled with sediment, which suggests that the dolphin was a young animal.

#### ***Pathology and wear***

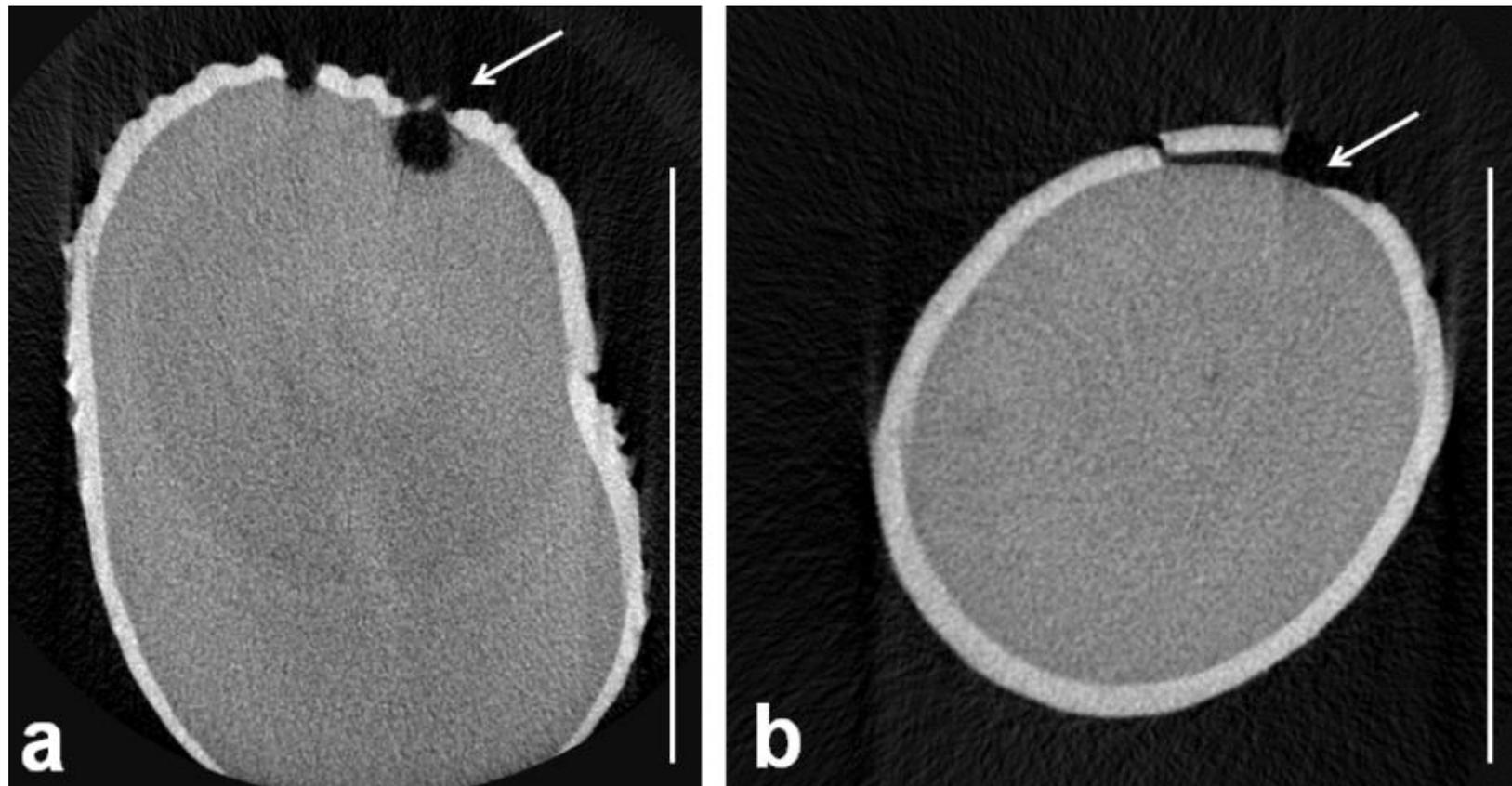
Apart from revealing the morphology and inner structure of dental tissues, pathological processes and tooth wear were also evidenced in Micro-CT slices. Cross-sections and orthogonal slices of reconstructed Micro-CT images provided internal details that allowed the diagnosis of alterations observed at the enamel surface. These include a potential case of a caries-like lesion (*sensu* Loch et al. 2011) in the Amazon river dolphin (*Inia geoffrensis*) (Fig. 5a) and wear and loss of enamel due to desiccation in the bottlenose dolphin (Fig. 5b). Potentially, Micro-CT could also help reveal and characterize internal anomalies that could interfere in age estimation techniques, such as resorption of dentine and cement, and pulp stones.

#### ***Variation in greyscales***

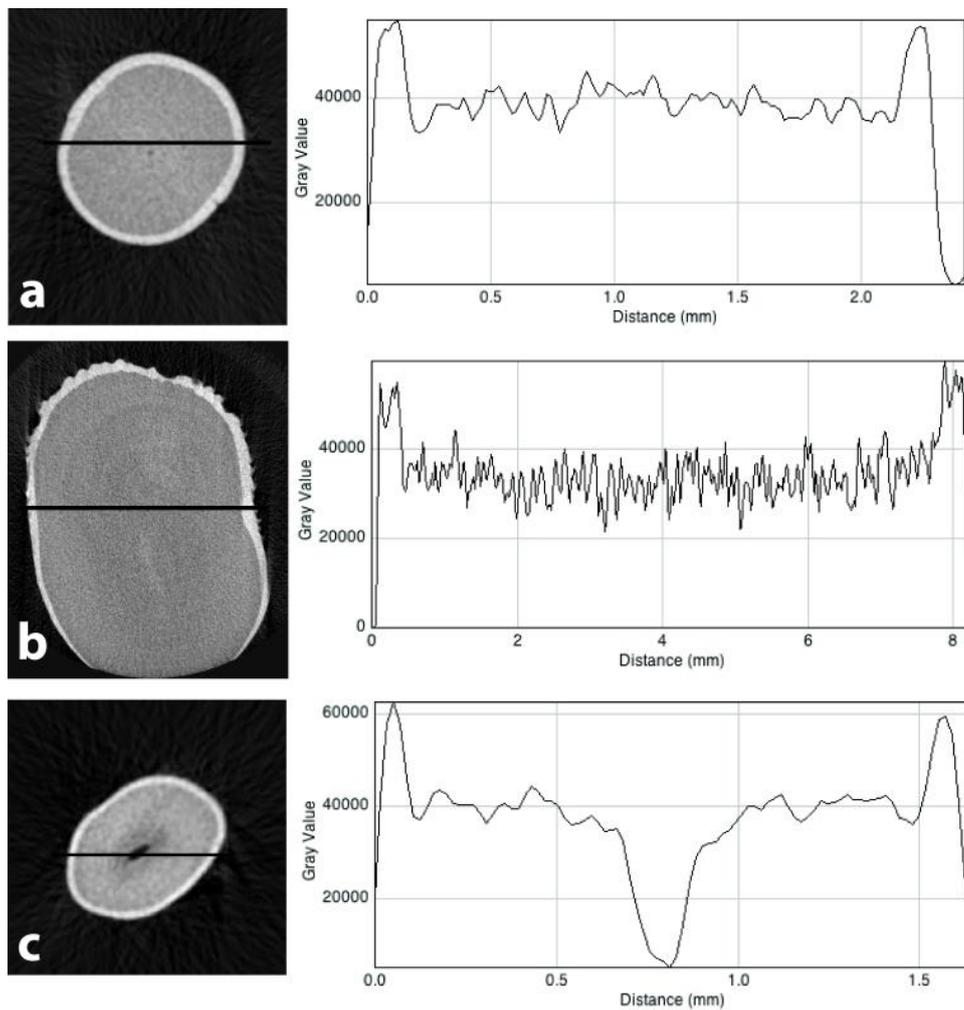
The assessment of the variation in greyscale values in Micro-CT cross-sections permitted a qualitative approach to the differences in mineral density among dental tissues and across tooth regions (Fig. 6). Greyscale values were measured across distance profiles using 16 bit images, in which the maximum grey value is 65,535 in contrast with the 255 grey values possible with 8 bit images. Variations in greyscale were more evident in the transition from enamel to dentine and in the transition from dentine to the pulp cavity. In these two interfaces there was an abrupt change in grey values. In cases where the pulp cavity was obliterated, no change was evident in the grey values. Qualitative analysis of dentine and enamel through Micro-CT showed that enamel has higher greyscale values than dentine, reaching about 60,000 grey values, and that dentine averages about 40,000 or slightly less. For enamel, greyscale values seemed to be lower at the outer and inner enamel reaching its maximum at mid enamel (Fig. 6a)



**Fig. 4.** Internal structure of fossil unnamed Delphinoidea tooth (OU 22108) finely resolved by Micro-CT. Scale bar = 5 mm. a) Orthogonal view of the specimen. b) Same specimen after colorizing treatment to enhance features.



**Fig. 5.** Micro-CT cross-sections of odontocete teeth evidencing pathological conditions and abnormalities. Scale bar = 10 mm. a) Caries-like lesion in the Amazon river dolphin *Inia geoffrensis* (IEPA 1899). b) Worn desiccated enamel of the bottlenose dolphin *Tursiops truncatus* (UFSC 1349).



**Fig. 6.** Reconstructed Micro-CT cross-sections of extant odontocete teeth and greyscale value/distance profile. a) Striped dolphin *Stenella coeruleoalba* (UFSC 1344). b) Amazon river dolphin *Inia geoffrensis* (IEPA 1899). c) Franciscana *Pontoporia blainvillei* (UFSC 1310).

and c). However, the Amazon river dolphin ([Fig. 6b](#)) showed a different trend, with greyscale values peaking at the extremities and decreasing slightly at mid-enamel. Variation in greyscale values in dentine were relatively homogeneous, with small peaks followed by small drops in greyscale values.

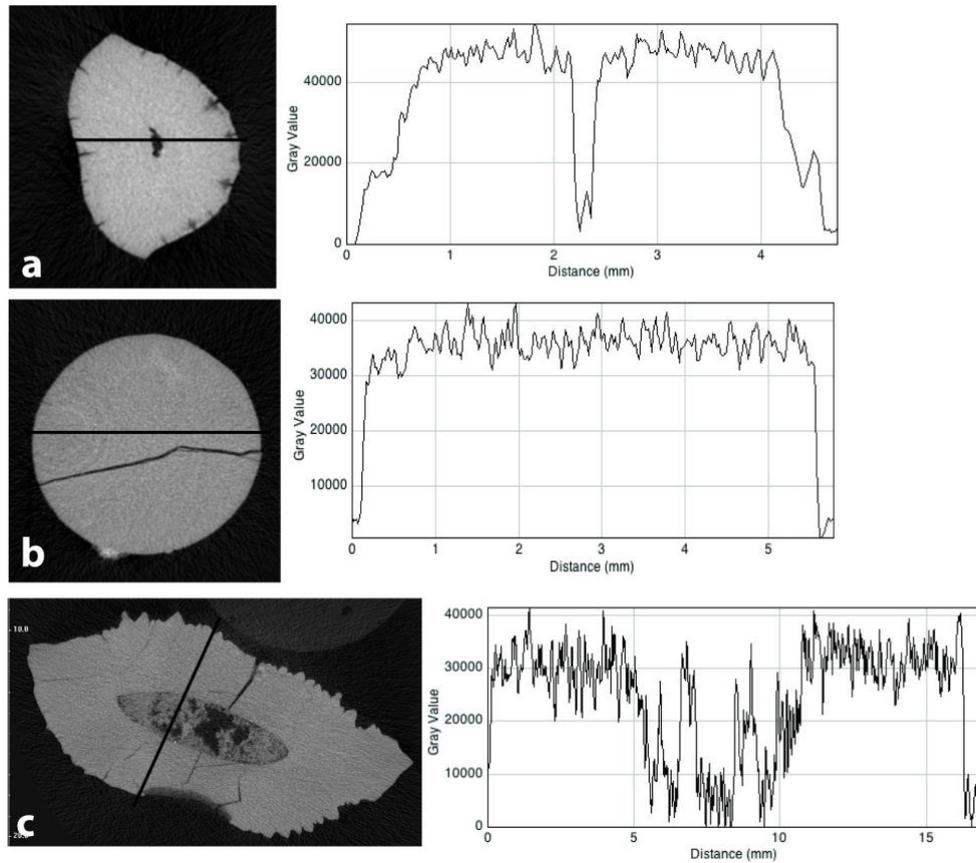
For most of the fossil cetaceans ([Fig. 7](#)), the variation in greyscale values was not consistent with the trends observed for extant odontocetes. Apart from the abrupt change in greyscale observed in the pulp cavity area, no other consistent or reliable trend could be identified. Greyscale values for enamel were lower or similar to dentine values, not allowing the characterization of the enamel-dentine junction. Grey values on dentine were also relatively homogenous and followed the trend of small peaks followed by abrupt drops as seen in extant odontocetes.

## **Quantitative assessment**

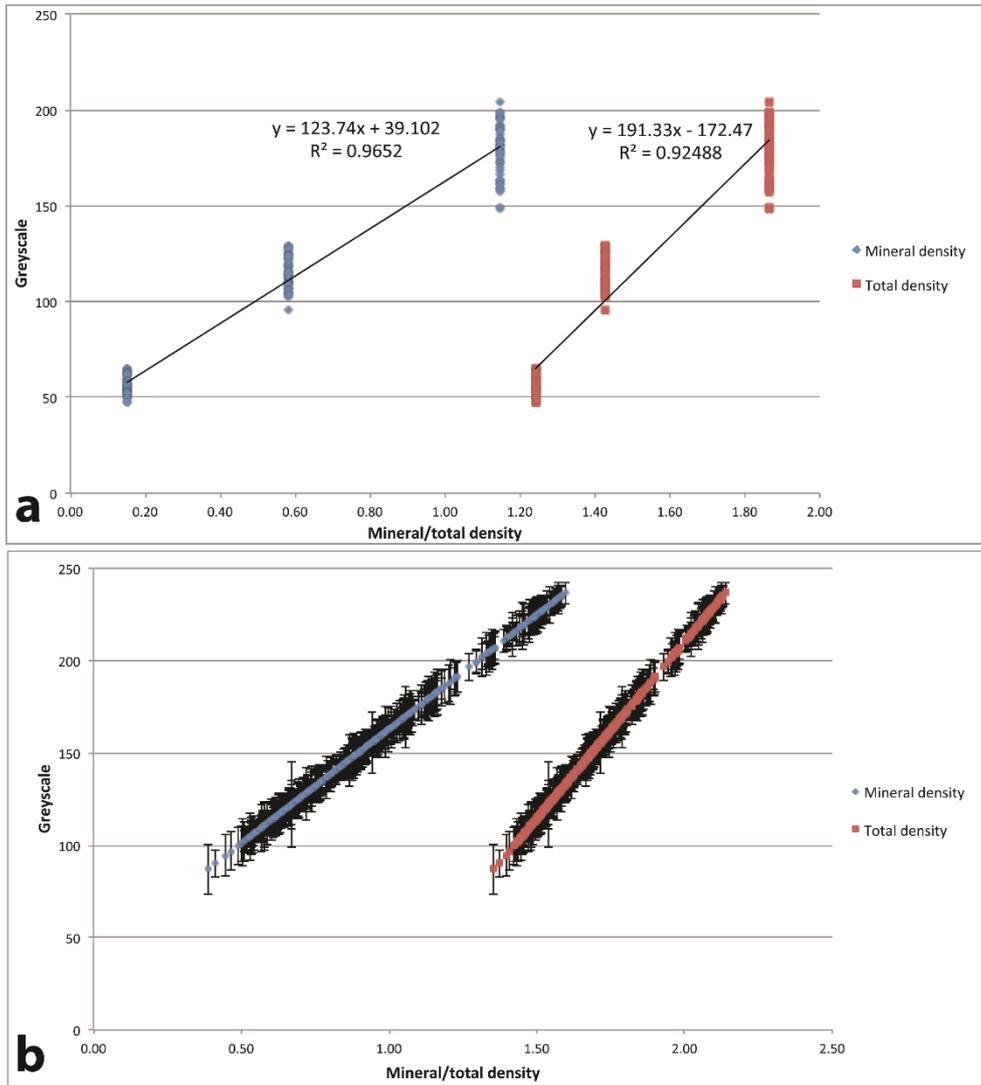
### ***Mineral concentration and density***

To provide a comparative analysis of mineral concentrations and densities in different regions of the tooth, greyscale measurements were taken in enamel (outer, mid and inner region), dentine (outer — near the enamel-dentine junction and inner — near the pulp) and cementum. Two extant cetaceans were analyzed, the delphinoid Guiana dolphin (*Sotalia guianensis*) and the inioid franciscana. Data similarly obtained from human dental tissues were also included in the comparison. These data were included because they were readily available and they could give breadth to our interpretation, as dental studies in humans are much more common than studies in other mammals. Greyscales were measured with a region of interest (ROI) of 20X20 pixels for the human specimen and for the Guiana dolphin. Due to the small tooth size of the franciscana, a ROI of 10X10 pixels was used. Greyscale measurements done with 20X20 and 10X10 ROIs for the Guiana dolphin showed that values obtained from different-sized regions were consistent and statistically similar (Mann-Whitney test for two independent samples,  $p > 0.05$ ). Thus greyscale values measured for the franciscana were comparable with datasets produced for the Guiana dolphin and for human dental samples.

Mean greyscale values obtained from three hydroxyapatite calibration standards showed a linear relationship with both mineral concentration and total density values ([Fig. 8a](#)). By using the linear regression equations obtained from the standards, it was possible to estimate the mineral concentration and total density of dolphin and human enamel and dentine based on their greyscale readings ([Fig. 8b](#)). Values for mineral concentration and total density in different regions of the tooth in dolphins and humans are summarized in Table 2.



**Fig. 7.** Reconstructed Micro-CT cross-sections of fossil odontocete teeth and greyscale value/distance profile. Note the preservation artifacts (irregular dark lines) in b and c. a) Unnamed Squalodontidae sp.1 (OU 22457). b) Unnamed Delphinoidea sp.1 (SGO-PV-754). c) Unnamed Squalodontidae sp.2 (OU22257).



**Fig. 8.** a) Scatter plot and linear regression of greyscale values versus mineral concentration and total density for hydroxyapatite calibration standards. b) Scatter plot of greyscale values versus mineral concentration and total density for the franciscana *Pontoporia blainvillei* (UFSC 1310).

**Table 2.** Summary of average mineral concentration and total effective density (in  $\text{g/cm}^3$ )  $\pm$  SD in human and odontocete dental samples. Maximum and minimum values observed are in parenthesis.

	<b>Average Mineral Concentration</b>	<b>Average Total Effective Density</b>
<b>Human</b>		
Enamel Outer	2.50 +/- 0.07 (2.27-2.72)	2.54 +/- 0.04 (2.42-2.66)
Enamel Mid	2.59 +/- 0.06 (2.39-2.70)	2.60 +/- 0.04 (2.48-2.66)
Enamel Inner	2.59 +/- 0.09 (2.39-2.91)	2.59 +/- 0.09 (2.48-2.78)
Dentine Outer	1.51 +/- 0.09 (1.04-1.76)	1.96 +/- 0.08 (1.09-2.10)
Dentine Inner	1.53 +/- 0.08 (1.30-1.74)	1.97 +/- 0.05 (1.84-2.10)
Cementum	1.14 +/- 0.10 (0.86-1.44)	1.85 +/- 0.06 (1.68-2.02)
<b>Guiana dolphin</b>		
Enamel Outer	2.62 +/- 0.12 (2.33-2.80)	2.81 +/- 0.08 (2.62-2.93)
Enamel Mid	2.59 +/- 0.13 (2.13-2.80)	2.79 +/- 0.09 (2.49-2.83)
Enamel Inner	2.56 +/- 0.16 (1.44-2.79)	2.77 +/- 0.10 (2.04-2.92)
Dentine Outer	1.42 +/- 0.08 (1.24-1.84)	2.03 +/- 0.05 (1.91-2.30)
Dentine Inner	1.42 +/- 0.06 (1.28-1.63)	2.03 +/- 0.04 (1.94-2.17)
Cementum	1.25 +/- 0.04 (1.14-1.36)	1.92 +/- 0.03 (1.85-1.99)
<b>Franciscana</b>		
Enamel Outer	1.37 +/- 0.19 (0.88-1.58)	1.99 +/- 0.12 (1.67-2.13)
Enamel Mid	1.37 +/- 0.19 (0.88-1.60)	1.99 +/- 0.12 (1.67-2.14)
Enamel Inner	1.37 +/- 0.19 (0.86-1.58)	1.99 +/- 0.12 (1.66-2.13)
Dentine Outer	0.92 +/- 0.08 (0.73-1.12)	1.70 +/- 0.05 (1.57-1.83)
Dentine Inner	0.93 +/- 0.13 (0.54-1.16)	1.71 +/- 0.09 (1.46-1.86)
Cementum	0.64 +/- 0.09 (0.39-0.92)	1.52 +/- 0.06 (1.36-1.70)

Using the non-parametric Kruskal-Wallis test for multiple independent samples, it was shown that differences in mean values of mineral concentration and total effective density were statistically significant among the three species ( $p < 0.05$ ). The franciscana had the lowest mineral concentration and total effective density for all locations sampled, being considerably lower than the Guiana dolphin. For enamel, the highest mean values of both mineral concentration and total effective density were observed in the outer enamel layer of the Guiana dolphin, which were statistically different from human values ( $p=0.000$ ). The mineral concentration in the mid and inner enamel regions were similar between human and Guiana dolphin samples (non-parametric Mann-Whitney test for two independent samples;  $p=0.24$  and  $p=0.34$ , respectively). Dentine mineral concentration values were higher for human samples, but total densities were higher in the Guiana dolphin. For cementum, the Guiana dolphin had the highest mineral concentration and total density of all samples analyzed.

## DISCUSSION

Early work involving Micro-CT and dental morphology of fossil and living vertebrates has demonstrated this is a useful and versatile technique both in terms of range of taxa studied and diversity of applications (e.g. McErlain *et al.* 2004, Rossi *et al.* 2004, Plotino *et al.* 2006, Kim *et al.* 2007, Swain and Xue 2009, Abel *et al.* 2012, Davis 2012). The specimen preparation and handling for Micro-CT are quite easy compared to most other study methods, and most importantly, Micro-CT allows the recording and study of specimens by means of a non-destructive advanced analytical technique (Rossi *et al.* 2004, Schwass *et al.* 2009). In addition, internal features can be re-examined many times, as samples remain available after scanning (Swain and Xue 2009).

Micro-CT analysis has been shown to accurately reproduce details of tooth anatomy (Kim *et al.* 2007), having high correlation with histological data (Plotino *et al.* 2006). Micro-CT can also allow basic linear and volumetric measurements, being applicable to measurements of enamel thickness, in turn a basic procedure in the understanding of tooth biomechanics and function (Kim *et al.* 2007, Swain and Xue 2009). For the extant cetaceans analyzed here, Micro-CT sections resolved internal details of tooth morphology, including definition of enamel, dentine, cementum and the pulp cavity. These regions were resolved both through reconstructed sections and also by the assessment of the variation in greyscale levels.

The dentinal region is known to be formed by layers of dentine deposited with controlled periodicity, which are visible in histological sections after

decalcification (Lockyer 1995). These layers (growth layer groups, GLGs), however, were not clearly visible in reconstructed Micro-CT sections in extant odontocetes. The reconstruction settings used allowed images with a pixel size resolution of 8.6  $\mu\text{m}$ , which is considerably smaller than the spatial resolution of GLGs reported for some dolphin species (Myrick *et al.* 1984, Hohn *et al.* 1989, Pinedo and Hohn 2000). Thus, it is believed that the water and organic content of dentine, and the fact that we analyzed intact and whole specimens, may have obscured the definition of the growth layers, which are commonly evident in thin histological sections. Micro-CT cross sections and projected greyscale values/distance profiles showed a relatively uniform dentinal region with no clearly-defined dentinal layers. Manipulation of greyscale thresholds using imaging software revealed different zones identified by their greyscale/mineral densities. Nonetheless, further developments are necessary to show if and how this zonation is related to GLGs and if Micro-CT can be applied as a complementary method for age estimation.

For most paleontological and archaeological dental specimens, Micro-CT is the only non-destructive and non-invasive investigative method currently available (Rossi *et al.* 2004). It allows ‘digital preparation’ of fossil specimens, revealing material otherwise covered by layers of coating material or matrix, and facilitates visualization of hidden structures and observation of fine morphological variation (Rossi *et al.* 2004, Abel *et al.* 2012, Davis 2012). However, in heavily mineralized specimens (*e.g.* specimens secondarily mineralized or with natural high mineral density), Micro-CT may have clear limitations for reliably distinguishing dental tissues (Swain and Xue 2009). This was the case in some of the fossil specimens analyzed in this study, in which enamel and dentine were not distinguishable through Micro-CT images. When two materials have similar densities, the voxels that represent both tissues will have comparable grey values due to insufficient density contrast between them (Abel *et al.* 2012). Because of these issues, we had to adopt different equipment settings for fossils in comparison to extant specimens (80 kV vs. 100 kV). A slight decrease in equipment voltage was used to compensate the higher mineral content of fossil specimens, while still producing images with good resolution.

These fossil specimens could have been affected by the dissolution processes that would be expected in geological settings, with significant mobility of anions such as carbonate and phosphate, resulting in increased secondary mineral content in the dental tissues. Such changes are part of what is known as diagenesis, a postmortem early-burial process which results in a range of chemical and mineral changes to the organic and inorganic constituents of fossil bone and teeth. Diagenetic processes can include trace element enrichment in the sample, dissolution or precipitation of minerals, and

recrystallization of the original biological apatite, resulting in modifications to fossil specimens from different sites and ages (Schweitzer *et al.* 2008, Thomas *et al.* 2011). Diagenesis may also cause increase in crystallite size and crystallinity in the samples, a possible explanation for similar grayscale values for fossil enamel and dentine. However, even for the specimens in which dental tissues could not be reliably distinguished, Micro-CT revealed the internal morphology of the pulp cavity and the presence of desiccation cracks and other post-mortem alterations. Micro-CT images provide an important record of the internal structure of fossil specimens before other destructive analyses are performed, and may reveal diagenetic alteration that could interfere with results from further geochemical analysis and detailed ultrastructural studies (*e.g.* Schweitzer *et al.* 2008, Thomas *et al.* 2011). Chemical and mineral changes caused by diagenesis may compromise the use of Micro-CT to reveal the mineral density of dental tissues in fossils. For these specimens, qualitative analyses offer more scope than quantitative approaches.

The unnamed delphinoid (OU 22108) was the only fossil analyzed here that showed a clear definition of tooth regions, together with a good resolution of the dentinal region. In this specimen the neonatal line and two additional growth layers were observed. Of note, the neonatal line is the baseline for age determination and the line that represents the beginning of life as an individual (Lockyer 1995). In fossil tooth OU 22108, fossilization seemed to have enhanced the layers to allow finer resolution by Micro-CT. Fossilization commonly involves the dissolution and mobilization of minerals in surrounding sediments, leading to re-deposition of minerals in pore spaces within the fossil, as the saturated water and endogenous organic constituents are concomitantly removed. Fossilization can also involve actual replacement of original molecules of tooth minerals (Schweitzer *et al.* 2008). For the delphinoid OU 22108, the removal of the organic and water fraction and substitution by other minerals seemed to have improved the resolution of dentinal layers. Conversely, it is not apparent why only OU 22108 had a clear resolution of its dentinal layers, while 5 other fossils were unrevealing, presumably because of diagenetic alteration. One of the latter specimens, OU 22257, is from the same locality and sequence as OU 22108, and would have had a similar burial history. It is known, however, that fossils from the same strata may differ significantly in their degree of secondary mineralization; for example, some teeth from the locality for OU 22108 are cemented with secondary calcium carbonate, while others are free of adherent secondary minerals.

Micro-CT slices also allowed the observation and internal characterization of pathological lesions and processes of dental wear and post-mortem enamel desiccation. Similar alterations were also observed and characterized by McErlain *et al.* 2004, Rossi *et al.* 2004 and Schwass *et al.* 2009, involving

fossils, archaeological and extant specimens. In these studies, Micro-CT allowed a complementary approach to the characterization of dental caries and demineralization of dental tissues in contemporary humans (*e.g.* Schwass *et al.* 2009), and also allowed the identification of similar lesions in fossil and archaeological specimens (McErlain *et al.* 2004 and Rossi *et al.* 2004), in which the study by destructive methods would be undesirable due to the rarity of the samples.

Traditional methods for estimating the mineral concentration of dental tissues involve destructive and time-consuming chemical analysis (Swain and Xue 2009). Micro-CT analyses of teeth scanned together with resin-hydroxyapatite calibration standards of known densities allowed the quantification of mineral density of dental tissues in odontocetes without having to section and destroy specimens. Average mineral concentrations and effective densities were estimated for the Guiana dolphin and franciscana, providing the first report of these values for cetaceans. The overall low standard deviation observed in our results both for mineral concentrations and effective densities suggest that although the regions of interest were relatively small by necessity due to the small size of teeth (for the franciscana in particular), the results reliably indicate mean values for dental tissues in these two species of dolphins.

The franciscana, with its elongated rostrum and slender, needle-like and increased number of teeth (Flower 1867), is most likely capable of performing a fast snap action but possibly not very powerful bite. This species had the lowest values for mineral concentration and effective density of all specimens sampled. Loch *et al.* (2013a) reported that the franciscana also had the lowest values for mechanical properties among several species of extant delphinoids and inoids, suggesting teeth have an undemanding role in food processing. Although not phylogenetically related, the dental samples of the Guiana dolphin and human had generally similar values for mineral concentration. In the outer enamel layer, however, the mean values for mineral density and total density were higher in the Guiana dolphin than in the human sample. A prominent layer of prismless enamel in the outer layer of dolphin enamel in contrast with humans (Loch *et al.* 2013b) could explain why the Guiana dolphin had higher values of mineral and total density. Higher values of total effective density in the enamel of the Guiana dolphin suggest higher protein content in this species. The mineral densities of dentine were higher in the human sample than in the Guiana dolphin. Dentine, however, is deposited throughout the life of the tooth and can be deposited as secondary or tertiary dentine. Ontogenetic development can certainly interfere with the mineral concentration and density of dentine, making comparisons among species and sampling locations not as reliable as in enamel. Future studies should

determine if there are prominent density changes across dentine, and if these changes somehow are related to the GLG.

Apart from the obvious benefits of using Micro-CT for dental studies in odontocetes and other mammals, this technique has some disadvantages. The scanning time can be relatively long for bigger specimens scanned in high resolution (about 5 hours for the specimens in this study), followed by a similar amount of time for reconstruction. The time and high computer power and expertise required are one of the main constraints in using Micro-CT for routine analysis (Plotino *et al.* 2006). Equipment cost is reasonably high (around US\$225,000 to \$375,000 for a desktop model), which favors multidisciplinary research centers that use Micro-CT for both applied studies in humans and basic research in other mammals. The main factors that still keep Micro-CT away from mainstream research are affordability, productivity and computing power. However, once the equipment is purchased, the running and maintenance costs are quite low, allowing research centers to charge a reasonably low hourly rate for its users (Abel *et al.* 2012). Other limitations include physical dimensions of the equipment. For the system used in this study (Skyscan 1172), the chamber allowed objects with a maximum height of 70 mm and width of 55 mm, which could be a limitation in studying cetaceans with bigger teeth such as sperm whales, killer whales and some ziphiids.

Qualitative and quantitative exploratory analyses employed here showed that Micro-CT is a feasible and useful complementary technique in dental research in cetaceans. The report of strengths and weaknesses, above, may help others who might consider using such methods. However, the use of Micro-CT still needs refinement to allow resolution of internal structure and potential application in non-destructive aging techniques. Factors such as use of filters, voxel resolution and algorithm choice by the reconstruction software, need to be further explored to improve resolution of the images (Park *et al.* 2010). Similarly, advanced features of imaging software need to be extensively tested to elucidate greyscale values and their relation to GLGs. Paired studies should be run, to compare Micro-CT scans with the physical and chemical structure revealed by study of destructive sections of the previously-scanned teeth. Meanwhile, Micro-CT analyses has been proven to be a potentially useful tool to document internal morphology of unique fossil teeth and/or unique specimens that are either difficult to section or must remain intact, before they are subject to other biological and mechanical analyses which would affect the specimen integrity.

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