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Decalcifying odontocete ears following a routine protocol with RDO®

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ABSTRACT

The study of the organ of Corti is essential to assess the impact of underwater noise on cetaceans. While classical histology techniques (including EDTA decalcification) have been previously considered, the process is time consuming. Independently from the histological technique, one of the challenging steps after extraction and fixation of the samples is to decalcify the bone envelope to access the cochlea without damaging the soft tissues. Here, we propose to use a fast commercial decalcifier (RDO®). 93 ears from 11 different odontocetes species stranded in the Mediterranean, Spanish North Atlantic and North Sea were used to precisely determine the decalcification time. Depending on the tympanic–periotic volume of the species, the decalcification time ranged from several hours to a few days, allowing a subsequently faster observation of the cochlear structures through routine microscope techniques.

1. Introduction

While there is an increasing pressure of human impacts on the oceans, very little is known about the effect of underwater noise on marine organisms. Because of their vital dependence on acoustic information and their role in the food chain as top predators, the study of the effects of noise on cetaceans (Mammalia, Cetacea) has recently become ecologically essential (Richardson et al., 1995). Although some of these effects can be found in organs not directly related to the acoustic pathways (Jepson et al., 2003), other lesions are expected to affect the acoustic pathways, particularly the organ of Corti and its associated hair cells (Lurie et al., 1944). The examination of these structures implies accessing fresh samples and determining possible correlations between a pathological change of cochlea morphology and a sound exposure. Moreover, basic morphological and comparative descriptions of the cetacean ears are still lacking, probably because of the difficulty in obtaining a suitable material, and a reliable protocol for analysis.

A detailed description of the cochlea morphology was presented for *Tursiops truncatus* (Wever et al., 1971a,b,c) and studies of the basilar membrane and *osseus spiral laminae* in different odontocete species have been conducted to compare their hearing capabilities (Ketten and Wartzok, 1990; Ketten, 1992, 1994). Nevertheless, much work is still needed to describe the morphology of other species hearing structures.

The middle and inner ear are enclosed by the tympanic and periotic bones, respectively, forming the tympanic–periotic (T-P) complex. The T-P complex, which is suspended through ligaments outside the skull in a peribullar cavity, is surrounded by air filled sinuses, allowing the acoustic isolation from the skull (Reysenbach de Haan, 1957; Fleischer, 1976; Ketten and Wartzok, 1990; Nummela et al., 1999b). While some authors expressed doubts about the functionality of the middle ear (Fraser and Purves, 1954; Reysenbach de Haan, 1957; McCormick et al., 1970; Fleisher, 1978; Ridgway et al., 1997) others presented morphological evidences supporting the active role of the middle ear in sound transmission (Nummela et al., 1999a,b; Hemila et al., 1999, 2001; Ketten, 2000; Morell et al., 2007).

Despite of these previous findings in a limited number of cetacean species, little data are available to comparatively describe inner ear structures. Stranding events may represent a unique opportunity to help building knowledge on the morphology of cetacean hearing organs and their potential sensitivity when exposed to noise.

One of the challenging steps after extraction and fixation of the ear samples is to decalcify the very dense bone envelope (T-P complex) to access the cochlea without damaging the soft tissues. While classical histology techniques (including EDTA decalcification) have been previously considered, the process is time consuming (Ketten, 1984). However, a rapid assessment of possible lesions in the Organ of Corti after sound exposure, in addition to allow basic morphological and comparative studies at larger scales, is crucial for decision making when facing a conservation problem.

We chose to use a fast commercial decalcifier (RDO®), based on hydrochloric acid, to shorten the decalcification times observed with other methods. Previous studies compared the effects of HCl and EDTA on the mouse mandible tissue and concluded that, morphologically,

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Table 1
Total number of ears used for the study to establish the decalcification protocol and to determine the decalcification times for each species.

Species	Number of ears	Number of animals	Conservat1on state	Protocol
Phocoena phocoena	48	27	37 ears of state 2 7 ears of state 3 4 ears unknown state	Freezing + defreezing + fixation with 10% formalin
Tursiopstruncatus	8	8	1 ear of state 1 3 ears of state 2 2 ears of state 3 1 ear of state 5 1 ear unknown state	Fixation with 10% formalin
Stenella frontalis	13	7	4 ears of state 2 4 ears of state 3 4 ears of state 4 1 ear unknown state	Fixation with 10% formalin
Stenella coeruleoalba	13	8	5 ears of state 1 5 ears of state 2 3 ears of state 5	Fixation with 10% formalin
Delphinus delphis	2	2	1 ear of state 2 1 ear of state 5	Fixation with 10% formalin
Kogia simus	2	2	1 ear of state 2 1 ear of state 5	Fixation with 10% formalin
Kogia breviceps	2	2	1 ear of state 3 1 ear of state 5	Fixation with 10% formalin
Steno bredanensis	2	2	1 ear of state 2 1 ear of state 3	Fixation with 10% formalin
Lagenodelphis hosei	1	1	1 ear of state 2	Fixation with 10% formalin
Globicephala macrorhynchus	1	1	1 ear of state 4	Fixation with 10% formalin
Globicephala melas	1	1	1 ear of unknown state	Fixation with 10% formalin

The preservation state is expressed as a scale from 1 (very fresh) to 5 (autolytic).

both decalcifiers preserved well the cellular structures (Shibata et al., 2000). However, for DNA or RNA studies the use of HCl for decalcification was not suited, while EDTA gave better results (Alers et al., 1999; Shibata et al., 2000; Moore et al., 2002). Callis and Sterchi (1998), when comparing 25%, 50% RDO® and EDTA pH 3.2, 7.0, 10.3 amongst other decalcifier agents, concluded that "all types of decalcification solutions and methods will produce excellent results if carefully watched".

Here, we propose to test the inclusion of RDO® in a standard protocol that will allow the rapid and direct observation of inner ear structures with microscopy techniques and determine the correlation between the periotic decalcification time and the tympanic–periotic volume.

2. Materials and methods

Ninety three (93) ears from 11 different odontocetes species stranded in the Mediterranean Sea, Spanish North Atlantic and North Sea have been extracted. Specifically, the species processed were: *Phocoena phocoena* ($n=48$), *Stenella coeruleoalba* ($n=13$), *Stenella frontalis* ($n=13$), *T. truncatus* ($n=8$), *Delphinus delphis* ($n=2$), *Kogia simus* ($n=2$), *Kogia breviceps* ($n=2$), *Globicephala macrorhynchus* ($n=1$), *Globicephala melas* ($n=1$), *Steno bredanensis* ($n=2$) and *Lagenodelphis hosei* ($n=1$, Table 2).

After extraction, the samples were fixed with 10% buffered formaline and used subsequently to precisely determine the decalcification time with different concentrations of RDO®.

All the ears were fixed using this protocol except in three cases:

- 48 *P. phocoena* ears were frozen and defrosted before being fixed in 10% buffered formalin
- One *S. coeruleoalba* ear was fixed in 2.5% glutaraldehyde with a phosphate buffer 0.1 M
- One *S. coeruleoalba* ear was fixed in 2.5% glutaraldehyde with 0.5% paraformaldehyde and a phosphate buffer 0.1 M (Table 1).

RDO® is a rapid decalcifier based on hydrochloric acid (Apex Engineering Products Corporation, Aurora, Illinois, USA). Specifically we tried with 100% RDO®, 80% RDO® (diluted with 80% ethanol), 75% RDO® (diluted with distilled water) and 50% RDO® (diluted with distilled water and changing the media after 24 h by or 50% RDO® or 25% RDO®, also diluted with distilled water).

Because the protocol assumes that there is no need to decalcify completely the periotic bone to access the Organ of Corti cells through scanning electron microscopy (SEM), the analysis was based on the time range necessary to uncover the *vestibular scalae* and the *stria vascularis* of the cochlea. This is what we have called the endpoint, which represents, in the frame of this study, the minimum necessary time of decalcification. Therefore, techniques like X-ray observation or chemical tests (e.g. using ammonium oxalate/ammonium hydroxide to precipitate calcium as calcium oxalate) were not used, and a mechanical dissection was necessary to establish the decalcification endpoint.

3. Results

Preliminary decalcification experiments were conducted with 100% RDO® on samples from *P. phocoena* (29% of $N=$ total number

Table 2
Decalcification times of the periotic bones analyzed during the study.

Species	Mean	Min–max	*n*	*N*
Phocoena phocoena	26h 39′	26h–28h 47′	11	48
Tursiops truncatus	66h 48′	65h 51′–67h 44′	2	8
Stenella frontalis	45h 54′	41h 40′–49h 27′	5	13
Stenella coeruleoalba	45h 39′	40h 07′–49h 23′	8	13
Delphinus delphis	52h 02′		1	2
Kogia simus	32h 16′		1	2
Kogià breviceps	32h 03′	31h 27′–32h 39′	2	2
Steno bredanensis	65h 50′	59h 13′–72h 27′	2	2
Lagenodelphis hosei	30h 10′		1	1
Globicephala macrorhynchus	87h 15′		1	1
Globicephala melas	88h 58′		1	1

Mean; *Min–max*, minimum and maximum decalcification time of periotic bones for each species; *n*, number of samples used to determine the final decalcification time, with a 50% RDO® and 25% RDO® after 24 h routine protocol; *N*, total number of samples used to establish the decalcification protocol.

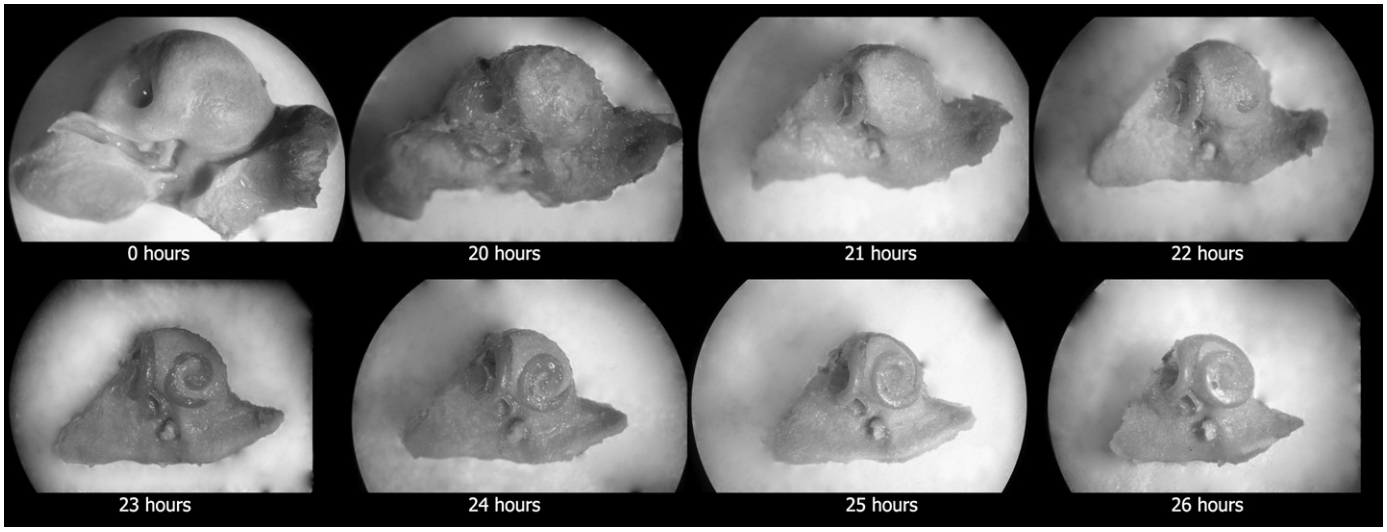

Fig. 1. Decalcification process of a harbor porpoise periotic bone using 50% RDO® and 25% RDO® after 24 h. The decalcification time is shown below each picture. In this example, the *vestibular scalae* and the *stria vascularis* of the cochlea were uncovered after 26 h.

of samples per species, see Table 2), *T. truncatus* (62% of *N*) and *S. coeruleoalba* (8% of *N*). Despite the fact that decalcification time with this RDO® concentration was considerably reduced (from 68% to 84% depending on the species) compared to the results shown below with 50%, 75%, and 80% RDO® concentration, the determination of the endpoint times resulted less accurate thus leading to the possibility of tissue overdecalcification and the introduction of consecutive artefacts.

More accurate decalcifying endpoint times were obtained using 50% RDO® (diluted with distilled water) and by changing the medium and the concentration (25% RDO® diluted with distilled water) after 24 h than with the other dilutions:

- 100% RDO®
- 80% RDO® diluted with 80% ethanol
- 75% RDO® diluted with distilled water
- 50% RDO® diluted with distilled water and changing the media after 24 h by 50% RDO®.

The dilution of 50% RDO® and 25% RDO® after 24 h, allowed slowing down the decalcification at the end of the process and stopping it accordingly. The decalcification times are shown in Fig. 1.

From the total samples that were analyzed, the mean and the minimum and maximum values of the decalcification time of all species studied following the best decalcification protocol (that was 50% RDO® for the first 24 h and 25% RDO® for the rest of the time) is shown in Table 2.

A highly linear correlation is observed comparing the periotic decalcification times with:

- the tympanic–periotic complex volumes extracted from previous CT scans (Morell et al., 2007) ($r = 0.935$, $n = 12$, Fig. 2A) or,

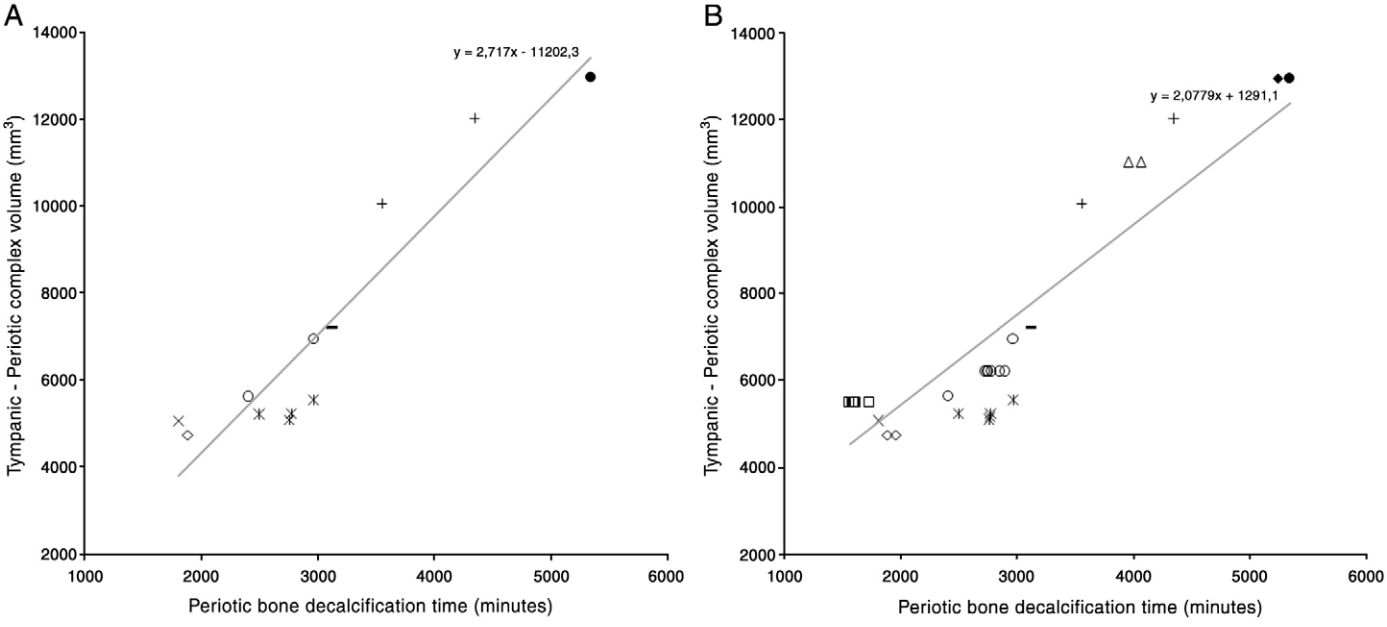

Fig. 2. Correlation between the periotic decalcification time with: A) the tympanic–periotic volume ($r = 0.935$, $n = 12$) and B) the mean of T-P volume for the species (Morell et al., 2007; $r = 0.891$, $n = 34$) when data were not available. □ *Phocoena phocoena*, Δ *Tursiops truncatus*, Ж *Stenella frontalis*, ○ *Stenella coeruleoalba*, – *Delphinus delphis*, ◊ *Kogia breviceps*, + *Steno bredanensis*, × *Lagenodelphis hosei*, ● *Globicephala melas*, ◆ *Globicephala macrorhynchus*.

– the average of the tympanic–periotic complex volumes for each species (Morell et al., 2007) when data were not available ($r = 0.891$, $n = 34$, Fig. 2B).

The remaining specimens ("*N–n*" samples in Table 2) were used to adjust the protocol (tests with different volumes, changes of media following different periods of time or overdecalcification, all conducting to unusable results) with different RDO® dilutions (100% RDO®, 50% RDO® diluted with distilled water and changing the media after 24 h by 50% RDO®, 75% RDO® diluted with distilled water and 80% RDO® diluted with 80% ethanol).

4. Discussion and conclusion

Following a routine protocol with a specific dilution of RDO®, the odontocete ear decalcification time ranged from several hours to a few days (Table 2), depending on the volume of the periotic bone, that, in turn, is highly correlated with the T-P complex volume (Morell et al., 2007). This reduced the decalcification time from a few months using EDTA (Ketten, 1984) to a maximum of a week for the largest T-P complexes. The high correlation between the T-P complex bone volume and the periotic decalcification time should allow a better approximation to the accurate decalcification time to analyze odontocete ears in the future (Fig. 2).

The decalcification protocol developed for this study was adjusted to perform an examination of the Organ of Corti's cells through scanning electron microscopy (SEM) and establish a fast diagnosis of possible lesions in fresh material. The respective decalcification time values may need to be increased if a complete decalcification of the periotic bone is needed for routine histology or transmission electron microscopy techniques.

In conclusion, the use of RDO® decreased the decalcification time of cetacean ear bones, thus allowing comparative morphological studies with a greater number of samples. Accordingly this method could represent a fast diagnostic tool to analyse possible alterations of the Organ of Corti, for example induced by sound exposure.

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