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Ultrastructural analysis of Odontocete cochlea

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Ultrastructural analysis of Odontocete cochlea

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Abstract

ABSTRACT

The morphological study of the Odontocete organ of Corti including possible pathological features resulting from sound over-exposure, represent a key conservation issue to assess the effects of acoustic pollution on marine ecosystems. Through the collaboration with stranding networks belonging to 26 countries, 150 ears from 13 species of Odontocetes were processed. In this dissertation, we present a standard protocol to 1) compare the ultrastructure of the cochlea in several Odontocete species and 2) investigate possible damage as a consequence of sound exposure, using scanning (SEM) and transmission (TEM) electron microscopy, and immunohistochemistry.

In a preliminary study, computerized tomography scans were performed before decalcification with ears of 15 odontocete species, proposing a set of standard measurements which classified very well the species. In addition, the constant ratio between measurements of inner and middle ear structures contributed to confirm the active role of the odontocete middle ear in sound reception mechanism.

We established a decalcification protocol using the fast commercial decalcifier RDO® and EDTA (Ethylendiaminetetraacetic acid). Although further experiments should be conducted to assess the suitability of using one or the other method (because the number of samples treated with EDTA was comparatively small), RDO® at specific dilutions decreased the decalcification time of cetacean ear bones with control of the decalcification endpoint, helping a faster access to inner structures.

The complementary use of electron microscopy and immunofluorescence allowed the description in odontocetes of new morphological features of tectorial membrane, spiral limbus, spiral ligament, stria vascularis, hair cells and their innervation. Furthermore, this study revealed qualitative and quantitative morphological characteristics of the organ of Corti in high-frequency hearing species, including 1) an outer hair cell (OHC) small length, 2) a thick cuticular plate in OHC, and a thick reticular lamina, 3) robust cup formation of the Deiters cell body, 4) the high development of cytoskeleton in Deiters and pillar cells and 5) the basilar membrane high stiffness. Interestingly, all these features, including a common molecular design of prestin, are also shared by echolocating bats, suggesting a convergent evolution in echolocating species.

The presence of scars among hair cell rows, the pattern of stereocilia imprints in the tectorial membrane and the condition of fibrocytes II and IV were criteria suitable to determine or discard possible acoustic trauma, despite the numerous artefacts that rapidly develop as a consequence of tissue autolysis.

Consequently, matching the preliminary approximation of the cochlear frequency map with the damaged region would bring information on the sound source that would have triggered a possible lesion.

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Framework of the dissertation and objectives

FRAMEWORK OF THE DISSERTATION AND OBJECTIVES

Noise pollution

The noise in the sea has always existed naturally or biologically. However, due to the recent, uncontrolled and massive introduction of sound sources produced by human activities, it has become a threat to the natural balance of the oceans.

The anthropogenic sources of marine noise pollution include, amongst others, maritime transport, offshore oil and gas exploration and exploitation, industrial and military sonar, experimental acoustic sources, undersea explosions, military and civilian engineering activities, supersonic aircraft noise, the construction and operation of sea-based wind farms, and acoustic deterrent and harassment devices.

These sound sources invade the acoustic and physical space of marine organisms (Figure 1) and there is no actual field of reference in which to foresee the negative consequences of these interactions on the ocean's natural equilibrium, and their short, medium and long term effects on marine biodiversity.
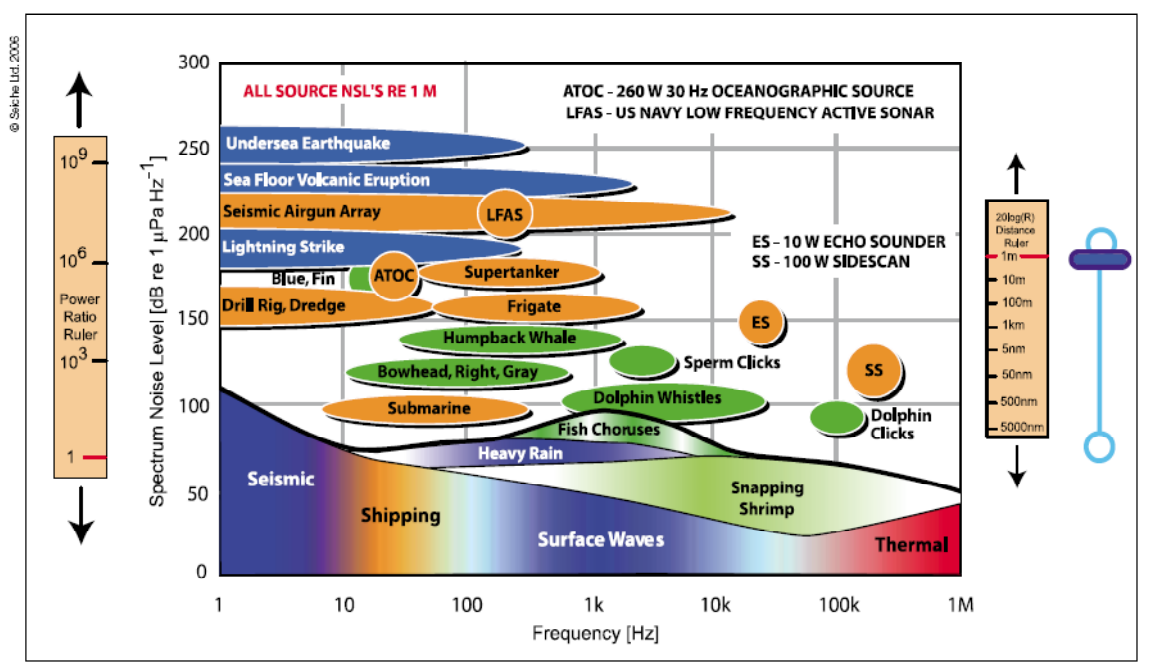
Figure 1. - Sound levels and frequencies from anthropogenic and natural sound sources in the marine environment (source: Boyd *et al.*, 2008)

Sound sources as a result of human activity/action have shown physical, physiological and behavioural effects on marine fauna (mammals, reptiles, fishes and invertebrates). The level of these impacts depends on the characteristics of the source and the proximity of an animal to the sound source (Bohne *et al.*, 1985; Skalski *et al.*, 1992; Engås *et al.*, 1993; Gordon *et al.*, 1998b; McCauley *et al.*, 2000; Guerra *et al.*, 2004; André *et al.*, 2011; see chapter 1.3.2 for further information on effects on cetaceans).

Assessing the acoustic impact of artificial sound sources in the marine environment is not a trivial task for several reasons: the first of them is the relative lack of information on the mechanism of processing and analysis of sounds by marine organisms. Furthermore, although we are able to record and catalogue the

majority of these signals, we do not know their role and importance in the balance and development of populations. Second, the potential noise impact concerns not only the auditory system but can intervene at other sensory or systemic levels and be lethal for the affected animal. If we add to these two reasons the fact that a punctual or prolonged exposure to a certain noise source can have negative consequences in the medium and long term and therefore could not be immediately seen, it makes difficult to obtain objective data that allows controlling the introduction of anthropogenic noise in the sea on a effective way.

Bioindicators

To answer some of these questions, the choice of cetaceans is not coincidental.

We choose the cetaceans as bioindicators in front of pollution source mainly for two reasons. The first is for their role as top predators in the food chain. A mismatch in any of its levels will unbalance the chain, in both directions. The second reason is the relationship and vital and almost exclusive dependence of cetaceans to acoustic information. Because they use the sounds for navigation, feeding and communication, they represent to date the best bioindicator of the effects of marine noise pollution.

Why are we conducting this research?

Based on previous studies on land mammals, structural alterations of the organ of Corti and its associated hair cells have been found as a consequence of sound exposure (see chapter 1.3.2). For this reason the study of the hearing system of cetaceans, and more specifically their cochlea, is a key issue to assess the impact of anthropogenic acoustic sources on these marine organisms. Currently little is known about cetacean hearing capacities, the functionality of sound reception pathway (see the chapter 1.2) and there are no morphological descriptions in the literature of the cochlear ultrastructure of any of these species under electron microscope. In addition, since the toothed whales (or odontocetes) have species-specific acoustic repertoire, it is expected to find differences in their cochlear morphology.

Stranding events represent a unique opportunity that allows increasing the knowledge of hearing system morphology and potential sensibility to cetaceans that have been exposed to noise. This study requires ears of very fresh animals (because of the organ of Corti cells begin the decomposition process very fast after the death of the animal) of many species and different seas because it is expected to have a set of representative data of the different acoustic pressures that toothed cetaceans suffer around the world. We have been building and managing a network to collect ear samples in collaboration with the stranding networks and rehabilitation centres from twenty-six countries.

Objectives

The main objectives of this thesis are:

1) to develop a standard protocol to analyze the ultrastructure of the cochlea using electron microscopy and immunohistochemistry:

1a) to describe the organ of Corti of several Odontocete species and further contrast ultrastructural similarities with bat echolocating species,

1b) to investigate possible structural alterations as a consequence of sound exposure.

To achieve this goal other intermediate objectives are considered:

2) to set up a network to collect odontocete ears from different countries,

3) to establish a decalcification protocol for several odontocete species using the fast commercial decalcifier RDO and EDTA (Ethylendiaminetetraacetic acid),

4) to describe the tympanic-periotic complex of 15 odontocete species before decalcifying the samples using computerized tomography scan 3D reconstructions and propose a set of standard measurements to classify the species.

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1. Introduction

1. INTRODUCTION

1.1. Acoustic signals produced by cetaceans

Cetaceans diverged from land mammals and became definitely marine 49 million of years ago. During their evolution, they developed many features to specialize in underwater life (see Pilleri, 1990; Fordyce and Barnes, 1994 among others). One of the most notorious is their unique capacity to produce, receive and process sounds. Cetaceans produce acoustic signals, either for communication between members of a social group, or for echolocation (biological sonar). These signals vary within the two sub-orders of cetaceans, the odontocetes or toothed whales and the mysticetes or baleen whales.

The toothed whales include about seventy species with representatives in every sea and some rivers. Their communication signals typically include mid frequency sounds (1-20 kHz). Most of these species have also developed a system of echolocation (Au, 1993; Thomson and Richardson, 1995; Ketten, 2000) that operates at high and very high frequencies (20-150 kHz), used to navigate, detect and locate obstacles, preys and congeners. The acoustic signals of odontocetes can be classified into three categories: tonal whistles, very short pulsed signals used in echolocation (clicks) and other less defined pulsed signals such as grunts, moans, croaks, growls and so forth. The majority of tonal whistles have a narrow bandwidth and its energy is found below 20kHz. They are used mainly for communication (Herman and Tavolga, 1980). Some authors defend that dolphins develop individually distinctive signature whistles that they use to transmit identity (Caldwell and Caldwell, 1965; Tyack, 1986; Caldwell *et al.*, 1990). However, other authors disagree with this hypothesis and state that dolphins produce a predominant shared whistle type that probably contains individual variability in the acoustic parameters of this shared whistle type, contributing to 'regional' dialects in dolphins (Dreher and Evans, 1964; Dreher, 1966; McCowan and Reiss, 1995a; McCowan and Reiss, 1995b; McCowan and Reiss, 1997; McCowan and Reiss, 2001). On the other hand, the clicks are short pulsed signals (between 50 and 200 µs in duration) of very high intensity (220-230 dB re 1 µPa a 1m) and frequency, very directional and projected forward. In some odontocete species, the pulses are separated enough to allow the echo returning sound before producing the next pulse, and other species, such as the belugas (*Delphinapterus leucas*), emit the following pulse without having time to receive the information from the first pulse. An exception to the correlation between high energy-high frequency clicks is found in non whisteling species like the harbour porpoise and the sperm whale. The harbor porpoise produces high frequency (110-120 kHz) low intensity (120 dB re 1 µPa) echolocation click trains while the sperm whale sonar clicks are centered around 15 kHz with up to 230 dB re 1 µPa source level.

On the other hand, the baleen whales include eleven species with representatives in all oceans of the world. Apparently, they are sensitive to low and medium frequencies (12Hz - 8 kHz) and until the date, it has not been possible to demonstrate that these signals can be used for echolocation. The acoustic repertoire of cetaceans is very varied and variations are found in both inter-and intra-specific.

Each of the species that makes up the order of cetaceans offers a unique acoustic repertoire in direct relation with the habitat where it has evolved over millions of years. However, the acoustic signals have only been studied in a few species (see Table 1.1.1). It is understood that, in order to detect prey, a coastal species will need to extract precise short distance details of the surrounding relief, while the absence of such relief will require pelagic cetaceans (those living in the open sea) to obtain information over medium and long distances. In spite of, all toothed whales share the same acoustic production mechanism (reviewed in Cranford, 2000; Cranford and Amundin, 2003). Generally, odontocetes produce echolocation signals using particular connective tissues called the phonic lips (Evans and Prescott, 1962; Cranford *et al.*, 1996;

Reidenberg and Laitman, 2008) that lie just superior to the nasal plug and project the signals out through the fatty forehead tissues (the melon) into the water.

Table 1.1.1. Characteristics of known echolocation signals produced by cetaceans, some of them published in Au, 1993. An asterisk indicates the species described in our study.

Species	Peak frequency (kHz)[1]	Bandwidth (kHz)	Signal duration (µs)	Source level (dB)	Conditions	References
Cephalorhynchus commersonii	120-130	17-22	180-600	160	tank	Kamminga and Wiersma, 1982 Evans *et al.*, 1988
Cephalorhynchus hectori	112-130	≈ 14	≈ 140	151	sea	Dawson, 1988
Delphinapterus leucas	100-115	30-60	50-80	225	bay	Au *et al.*, 1985; Au *et al.*, 1987
Delphinus delphis *	23-67	17-45	50-150	-	sea	Dziedzic, 1978; Madsen *et al.*, 2004b
Globicephala melas*	30-60	-	-	180	tank	Evans, 1973
Grampus griseus	65 50	72	40-100 50	~ 120 202-222	bay	Not published data Madsen *et al.*, 2004b
Inia geofrensis	95-115	-	200-250	-	river	Kamminga *et al.*, 1989
Lagenorhynchus obliquidens	30-60 59	-	34-52	180 170	tank tank	Evans, 1973 Fahner *et al.*, 2004
Lipotes vexilifer	100-120	37	-	156	tank	Youfu and Rongcai, 1989
Monodon monoceros	40	27	29-45	227	sea	Møhl *et al.*, 1990; Miller *et al.*, 1995
Neophocaena phocaenoides	128	11	127	-	tank	Kamminga, 1988
Orcaella brevirostris	50-60	≈ 22	150-170	-	tank	Kamminga *et al.*, 1983
Orcinus orca	14-20	≈ 4	210	178	tank	Evans, 1973
Phocoena phocoena*	120-140 128	10-15 16	130-260	162 average:157 max:172	tank tank	Møhl and Andersen, 1973 Kamminga and Wiersma, 1981 Hatakeyama *et al.*, 1988 Au *et al.*, 1999
Phocoenoides dalli	120-160 90-115 135-149	11-20	180-400	170 15-60 50-60	sea tank sea	Awbrey *et al.*, 1979 Hatakeyama and Soeda, 1990 Hatakeyama and Soeda, 1990
Platanista gangetica	15-60	-	-	-	tank	Herald *et al.*, 1969
Pseudorca crassidens	100-130 40	15-40	100-120 30	228 201-225	bay sea	Thomas and Turl, 1990 Madsen *et al.*, 2004b
Steno bredanensis*	5-32	-	-	-	tank	Norris and Evans, 1966
Sotalia fluviatilis	95-100	~ 40	120-200	-	tank river	Wiersma, 1982 Kamminga *et al.*, 1989
Tursiops truncatus*	110-130 52	30-60	50-80 50-250	228 170	bay tank	Au, 1980 Evans, 1973

[1] The peak frequency corresponds to the maximum amplitude of the energy contained in the signal

Ziphius cavirostris*	42		200	214	sea	Zimmer *et al.*, 2005
	13-17		70-160		sea	Frantzis *et al.*, 2002
Mesoplodon densirostris*	30-40		270	200-220	sea	Johnson *et al.*, 2004; Madsen *et al.*, 2005; Johnson *et al.*, 2006
Stenella frontalis*	40-50 and 110-130		Less than 70	200-210 (max 230)	sea	Au and Herzing, 2003
Stenella longirostris	70		31	222	sea	Schotten, 1997
Stenella attenuata	69		43	220	sea	Schotten, 1997
Lagenorhynchus albirostris	120		13-30	194-219	sea	Rasmussen *et al.*, 2002
Physeter macrocephalus*	20		200-300	232	sea	Møhl *et al.*, 2000
Feresa attenuata	45 and 117		25	197- 223 $_{pp}$	sea	Madsen *et al.*, 2004a

1.2. Cetacean hearing

Since cetaceans returned secondarily to the sea, their hearing system present some changes compared to that of terrestrial mammals, and it is characterized by unique morphological adaptations to underwater environment (Kellogg, 1928; Reysenbach de Haan, 1957; Dudok van Heel, 1962; McCormick *et al.*, 1970; Kasuya, 1973; Oelschläger, 1986; Ketten, 1992; Thewissen and Hussain, 1993; Ketten, 1994; Ito and Nakamura, 2003; Nummela *et al.*, 2004; Nummela *et al.*, 2007; Fahlke *et al.*, 2011).

In land mammals, the outer ear captures the sound, often with the help of a pinna, the acoustic waves are transmitted through the external auditory canal and make the tympanic membrane to vibrate. These vibrations are transmitted and amplified by the ossicles of the middle ear, malleus, incus and stapes. The stapes is connected to the oval window and this in turn to the inner ear. Sound travels through the air to the stapes, but the inner ear is bathed in perilymph and endolymph. Therefore the acoustic waves suffer a loss of intensity due to the change in the impedance when changing from aerial to aquatic environment, which is remedied by an amplification of 40 dB re 1 µPa in the ossicles of the middle ear. In the inner ear, the organ of Corti is the sensorineural end organ for hearing. It includes polarized epithelial cells (hair cells and supporting cells), a specialized basement membrane called basilar membrane, nerve endings and the tectorial membrane. The hair cells perform the transduction of the acoustic signal into electrochemical information but only the inner hair cells initiate the depolarization of the spiral ganglion neurons (see Moser and Beutner, 2000 among others), which send the impulses to the brain via the auditory nerve (see chapter 1.2.4 for further details). The outer hair cells have motile properties that amplify the acoustic vibration (Dallos, 1992).

Water is about thousand times denser than air and incompressible. Sound travels approximately five times faster than in air (1450m/s in water against 340m/s in air); the acoustic impedance of soft body tissues almost matches that of the surrounding medium, thus allowing sound energy to flow from water to body tissue, reaching the inner ear, with little energy loss.

This fact, together with the characteristics of the acoustic signals produced by cetaceans (Table 1.1.1), is reflected in the anatomical structures of the auditory system. Odontocetes possess a highly developed and intricate auditory system, perhaps the most developed of all auditory systems in the animal kingdom, considering their large frequency range of hearing (from below 100 Hz to greater than 150 kHz) and their ability to perceive very short signals on the order of tens of microseconds. Most of the knowledge of the odontocete auditory capabilities has been obtained from behavioural and electrophysiological studies (Table 1.2.1). The hearing capabilities of animals can be assessed by measuring the evoked potential response of the auditory nervous system along the auditory pathway. An auditory evoked potential (AEP) is an electrical response of the auditory nerve to an external acoustic stimulus. AEP can be measured as voltage by measuring electrodes. In all vertebrate species that have been studied, the animal's sensitivity to sound varies as a function of frequency. Most species show low sensitivity at very low frequencies and at very high frequencies in a kind of U-shaped pattern. An audiogram shows the minimum detectable sound intensity, i.e. the threshold, as a function of frequency. Out of the few species tested so far (see Table 1.2.1), most of them have the maximum sensitivity between 20 and 90 kHz, with the possible exception of the orca (Hall and Johnson, 1972), which is lower.

Table 1.2.1. Known audiograms of some species (modified from Morell *et al.*, 2007)

Species	Frequency range (kHz)	Maximum sensitivity (kHz)	Reference
Stenella coeruleoalba	0,5 - 160 (B)	64 (B)	Kastelein *et al.*, 2003
Delphinus delphis	11 – 152 (E)	60-70 (E)	Popov and Klishin, 1998
Tursiops truncatus	5 – 140 (E)	80 (E)	Popov and Supin, 1990b
	0,075 - 150 (B)	45 (B)	Johnson, 1967
	10-150 (B and E) at least	50 (B and E)	Schlundt *et al.*, 2007
	8-152 (E) at least	45 (E)	Popov *et al.*, 2007
Tursiops truncatus gilli	10 – 180 (E) at least	40 and 60-115 (E)	Houser *et al.*, 2008
Phocoena phocoena	10 - 160 (E)	30 and 125 (E)	Popov *et al.*, 1986
	0,25 - 180 (B)	100 - 140 (B)	Kastelein *et al.*, 2002
Orcinus orca	1,2 – 120 (E)	20 (E)	Szymanski *et al.*, 1999
	4 – 120 (B)	12 - 20 (B)	Hall and Johnson, 1972
Delphinapterus leucas	~16 - 110 (E)	60 – 80 (E)	Popov and Supin, 1987; Klishin *et al.*, 2000
	1 – 120 (B)	~30 (B)	White *et al.*, 1978; Awbrey *et al.*, 1988; Johnson, 1992
Inia geoffrensis	8 – 120 (E)	20-25 and 70-80 (E)	Popov and Supin, 1990a
	1 - 100 (B)	12 – 64 (B)	Jacobs and Hall, 1972
Pseudorca crassidens	2 – 115 (B)	20 (B)	Thomas *et al.*, 1988; Yuen *et al.*, 2005
	4-45 (E) at least	22,5 (E)	Yuen *et al.*, 2005
Grampus griseus	1,6 – 110 (B)	8 – 64 (B)	Nachtigall *et al.*, 1995
	4-150 (E)	32, 64 and 90 (E)	Nachtigall *et al.*, 2005
Lipotes vexillifer	1 – 200 (B)	16 – 64 (B)	Wang *et al.*, 1992
Lagenorhynchus obliquidens	0,10 – 140 (B)	64 (B)	Tremel *et al.*, 1998
Lagenorhynchus albirostris	min 16-181 (E)	50-64 (E)	Nachtigall *et al.*, 2008
Tursiops gilli	2 - 135(B)	30 – 80 (B)	Ljungblad *et al.*, 1982
Sotalia fluviatilis	4 – 135 (B)	85 (B)	Sauerland and Dehnhardt, 1998
Feresa attenuata	5-120 (E)	40 (E)	Montie *et al.*, 2011
Globicephala melas	4-100 (E) at least	40 (E)	Pacini *et al.*, 2010
Mesoplodon europaeus	5-160 (E) at least	40-50 (E)	Cook *et al.*, 2006; Finneran *et al.*, 2009; Pacini *et al.*, 2011

E, electrophysiological audiogram; B, behavioural/psychophysical methods

The outer, middle and inner ear from odontocetes will be presented in more details, as well as their differences compared to terrestrial mammals.

1.2.1. Outer ear

The morphological adaptations of cetaceans to the marine environment featured the pinna disappearing as well as other morphological structures that could generate friction with the water and interfere with the hydrodynamics of cetaceans (Figure 1.2.1a).

The external auditory meatus results to be very small, usually presenting less than 3mm in diameter even in the largest whales.
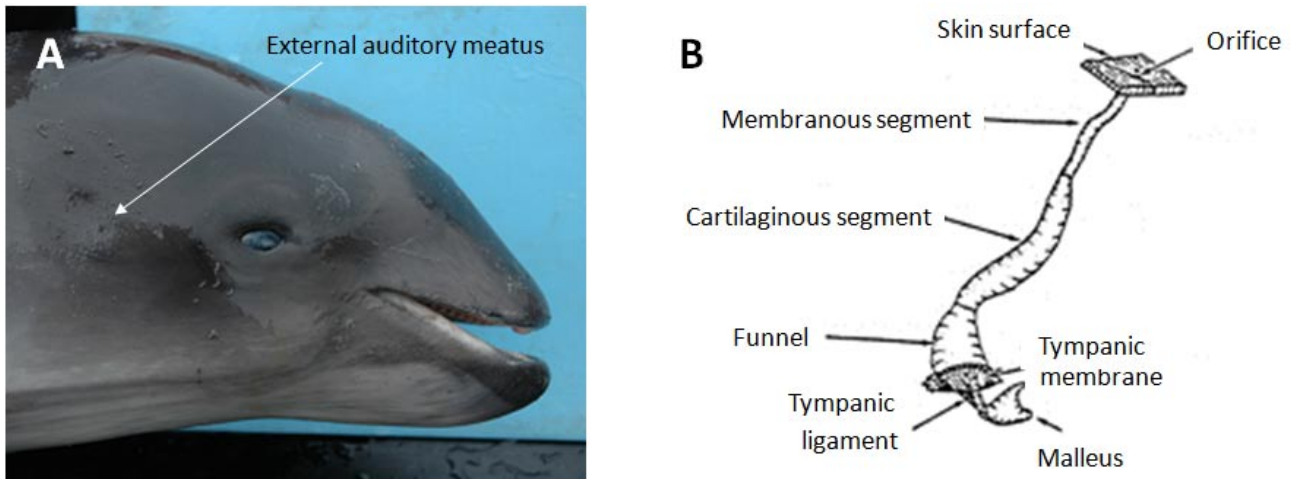
Figure 1.2.1- A) Localization of the external auditory meatus in the harbour porpoise *Phocoena phocoena* (photographer: Kees Camphuysen). B) Scheme of the outer ear and the connections with the middle ear in the bottlenose dolphin *Tursiops truncatus* (source: McCormick *et al.*, 1970).

In toothed whales the external auditory canal has a reduced diameter, S-shape and it is mainly occluded by cellular debris and wax (Reysenbach de Haan, 1957; Fraser and Purves, 1960; Dudok van Heel, 1962; McCormick *et al.*, 1970). While some authors argue that with regard to the sound reception is vestigial (McCormick *et al.*, 1970) others suggest that it could play an important role (Popov and Supin, 1990c). McCormick and colleagues (1970) studied the role of each structure of the auditory system by electrophysiological methods, measuring whether there was any change in cochlear potential when removing any of the elements. The external auditory canal was cut and no changes were observed, so it was concluded that the outer ear was not involved in sound reception. However, recent studies (Sassu and Cozzi, 2007) suggest that the auditory meatus is not a vestigial structure whose changes in shape and disposition may have been adapted to sudden and extensive variations of the environmental pressure.

Reysenbach de Haan (1957) and Dudok van Heel (1962) were among the first to propose that odontocetes did not receive the sound through the outer ear as in terrestrial mammals, but through fatty channels of the lower jaw (see Figure 1.2.2). Subsequently, it was observed that the posterior part of the lower jaw was very thin and was called the *pan bone*, which was surrounded by a fatty tissue (Norris, 1968). The composition of this fat, rich in isovaleric acid (Varanasi and Malins, 1970; Varanasi and Malins, 1971), was very similar to the melon, known for its role in the propagation of sound in the sound production process. It was suggested that the fat in the lower jaw acted as a low impedance path to the middle ear and the pan bone would provide an "acoustic window" for its thinness and position on the fatty channel and proximity to the tympanic bone (Norris, 1968; Brill *et al.*, 1988; Brill and Harder, 1991; Ridgway, 1999; Brill *et al.*, 2001). Other data

supported this hypothesis obtained using evoked potentials (Bullock *et al.*, 1968; Møhl *et al.*, 1999) and cochlear potentials (McCormick *et al.*, 1970) for stimuli above 20 kHz.

In addition to the lower jaw hypothesis a "lateral acoustic pathway", near the rudimentary external auditory meatus, was defined for lower frequencies reception (under 22,5 kHz; Popov and Supin, 1990c; Popov *et al.*, 2008). Thus, at least two sound-receiving areas (acoustic windows) with different frequency sensitivity were identified.

Recent modelling studies suggest a third pathway ("gular pathway") for sound reception in the beaked whale species *Ziphius cavirostris* (Cranford *et al.*, 2008). Propagated sound pressure waves would enter the head from below and between the lower jaws, passing through an opening created by the absence of the medial bony wall of the posterior mandibles, and proceeding toward the tympanic-periotic complex through the internal mandibular fat bodies.

Another modelling research proposed that the conical shape and very regular spacing of odontocete lower jaw teeth and the mandibular nerve would form part of high frequency echo pulse receptor, which could accurately match the transmitted signal parameters (Goodson and Klinowska, 1990). However, much research should be done in this direction to validate this model.

In addition, Ryabov (2003 and 2010) considered the mental foramens (asymmetric oblique orifices in the lower jaw where the branches of mental nerve and blood-vessels go out of the mandibular canal) like the traveling wave antenna and the lower jaw as a peripheral part of a dolphin's hearing system. The mental foramens would be acoustically narrow waveguides within the range of dolphin's hearing frequencies and would conduct the sound into the fat body of mandibular canal without distortion, defining its intensity. The mental foramens would function as elementary receivers of arrays and the structure of their location would define the beam pattern of each antenna array.

Regardless of the exact sound pathway to the middle ear, most studies concluded that sound is transmitted from the internal mandibular fat body to the tympanic bulla (Hemila *et al.*, 1999; Ridgway and Au, 1999; Ridgway, 1999; Cranford *et al.*, 2010; Hemilä *et al.*, 2010). A recent study showed that this fat was not homogeneous, containing an inner core, surrounded by a denser outer core. The inner core continued caudally and reached the tympanic-periotic complex (Montie *et al.*, 2011).
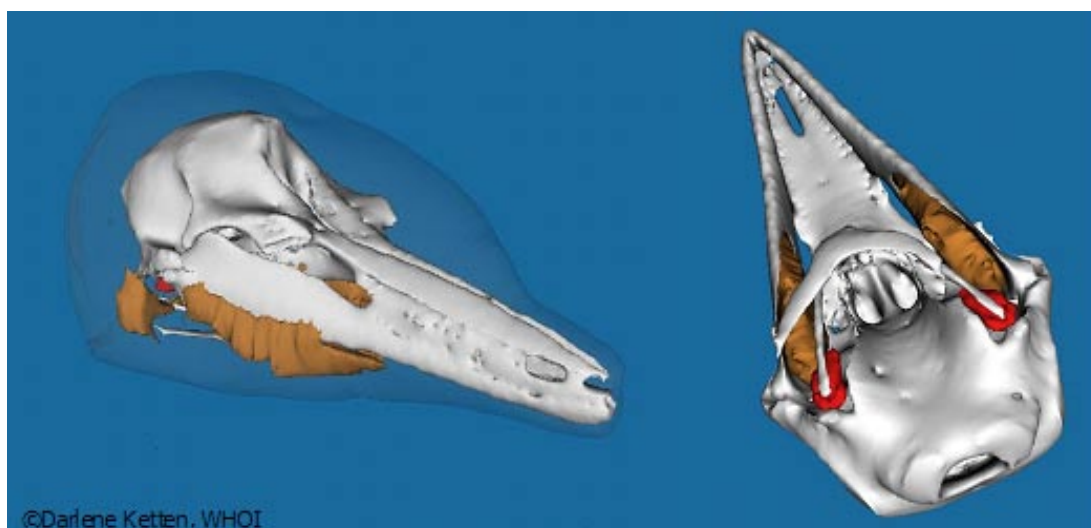
Figure 1.2.2- Computerized tomography 3D reconstruction of an odontocete head. It is represented in red the location of the tympanic-periotic complex and in brown the position of the fatty channel around the lower jaw.

1.2.2. Tympanic-Periotic Complex

The tympanic and periotic bones - the latter formed by the fusion of the petrosus and mastoid bones - house the middle and inner ear, respectively. These structures are partially fused by the *processus petrosus* forming the tympanic-periotic (T-P) complex (Figure 1.2.3). The T-P complex is surrounded by air sinuses called peribullar sinuses and suspended by ligaments in the peribullar cavity that keep each ear fixed and acoustically isolated from other skull bones (Reysenbach de Haan, 1957; Ketten and Wartzok, 1990; Nummela *et al.*, 1999; Houser *et al.*, 2004; Cranford *et al.*, 2008), except for sperm whales and some beaked whales in which the T-P complex is fused at some points to the temporal bone. The presence of air around the T-P complex forms a sound-reflective barrier that would protect the ear from self-made sounds originating at the nasal passages (Cranford *et al.*, 2008; Li *et al.*, 2012).

Peribullar sinuses are part of the paraotic sinuses, extending parallel to the bones of the skull. Paraotic sinuses receive different names depending of the related bone structure. Therefore, there are pterygoid, anterior, medial, peribullar and posterior sinuses. Cranford and colleagues (2008) were among the first researchers who described the paraotic sinuses in cetaceans and suggested that large peribullar spaces were an adaptation to mechanical stress due to high pressures on the environment and they were correlated with the ability to dive. In addition, the air spaces may aid in hearing directionality by contributing to the animal's ability in timing sound arrival differences between the ear, as the air sinuses would impede sound conduction through soft tissues that exist between the ears (Houser *et al.*, 2004).

Peribullar cavity in odontocetes contains a net of capillaries called the *corpus cavernosum*, which has the ability to be filled with blood and occupy some space in the cavity of the tympanic bone. It is joined by long bundles of nerve fibers belonging to the trigeminal nerve. It has been suggested that the *corpus cavernosum* may have a function related to adaptation to changes in pressure during dives (Sassu and Cozzi, 2007).
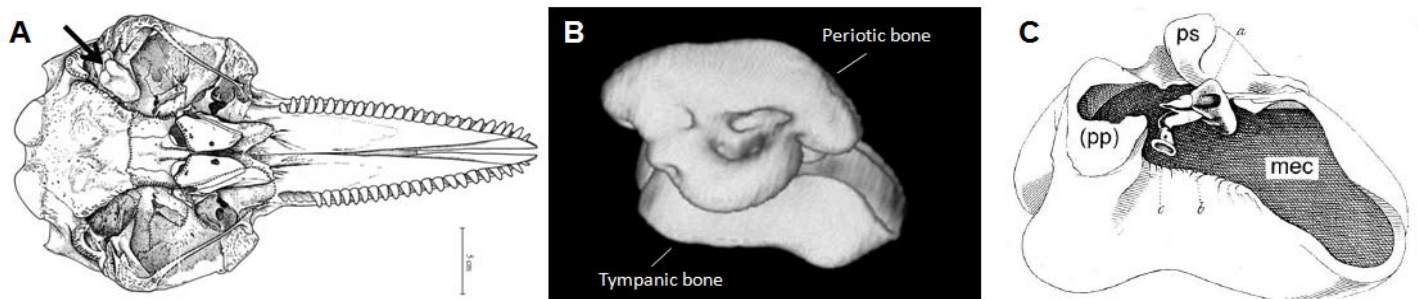
Figure 1.2.3- A) Drawing of the ventral view of an adult skull after removing the lower jaw, modified from Mead and Fordyce, 2009. The arrow indicates the location of the tympanic-periotic complex. B) 3D reconstruction of a tympanic-periotic complex of a stripped dolphin *Stenella frontalis.* C) Schematic representation of a tympanic bone of a short-finned pilot whale *Globicephala melas* (source: Hyrtl, 1845). mec: middle ear cavity, pp: *processus petrosus,* ps: *processus sigmoideus*

1.2.3. Middle ear

In all species of toothed whales the ossicles of the middle ear are well formed, denser and more rigid than in terrestrial mammals (McCormick *et al.*, 1970; Ketten, 1984; Ketten, 1992; Miller *et al.*, 2006), which could suggest an adaptation to the very high frequency hearing, allowing the tolerance of high pressures and energy transfer (Ketten, 2000). The eardrum has been transformed into a conoidal hyaline and fibrous

structure called tympanic plate, which is connected to the malleus by the tympanic ligament (see Figure 1.2.1b). The malleus is partially fused to the inner wall of the tympanic bone by a bone formation called the *processus gracilis*. The malleus is the stiffest bone of the middle ear. The incus and stapes are also attached by ligaments and a membranous sheath but have more flexibility. The stapedial ligament attaches the stapes to the oval window.

The mandibular fat bodies bifurcate posteriorly, attaching to the T-P complex in two branches (Cranford *et al.*, 2010; Montie *et al.*, 2011), one of them reaching a thin-walled bone anterior to the processus sigmoideus (Figure 1.2.3c), which could act as an acoustic window for the sound propagation to the middle ear (Cranford *et al.*, 2010).

The role of the middle ear is not yet known very well, and some authors have expressed doubts about its functionality (Fraser and Purves, 1954; Reysenbach de Haan, 1957; Fleischer, 1978; Ridgway *et al.*, 1997). However, some morphological studies (Nummela *et al.*, 1999; Nummela *et al.*, 1999; Ketten, 2000; Morell *et al.*, 2007) and modelling (Hemila *et al.*, 1999; Hemila *et al.*, 2001; Cranford *et al.*, 2010) supported the active role of the middle ear in sound transmission. Last models showed a different vibration pattern depending of the perceived frequency: low frequency (below 20 kHz) produce bulk motions of the entire T-P complex and little or no relative motion of the ossicles, but by contrast, high frequency vibrations produce intricate vibrational patterns in the wall of the bulla that result in complex and varied motions of the individual ossicles (Cranford *et al.*, 2010). With electrophysiological methods it could be demonstrated that neither the tympanic plate nor the tympanic ligament played an important role in sound reception (McCormick *et al.*, 1970). The stapes was essential to reception but the malleus could be removed without serious consequences, at least for high frequencies.

1.2.4. Inner ear

The inner ear is subdivided into the vestibular system and the cochlea.

Vestibular system

The vestibular system is formed by the semicircular canals, which function is to keep the equilibrium. In case of odontocete cetaceans these canals are extremely small with very reduced innervation. This characteristic could be an adaptation to the marine live. The first vertebras are fused limiting the head movement and the reduction of semicircular canals would permit only the lineal acceleration but not the tridimensional rotation, allowing the odontocetes to perform rotations and fast movements without losing the equilibrium sense.

Cochlea

The auditive system is formed by the cochlea, a spiral shape structure composed by three canals or *scalae* separated between them through membranes: the vestibular scala, the cochlear scala or scala media and the tympanic scala (Figure 1.2.4.1). The vestibular scala starts in the basal end of the cochlea with the vestibule and the oval window, place where it is attached to the stapes foodplate, and it communicates with the tympanic scala in the apex or helicotrema. From the helicotrema, the tympanic scala extends to the base

until it reaches the round window. Vestibular and tympanic scalae are filled with perilymph, while the cochlear scala is filled with endolymph. The endolymph is composed by a high potassium and a low sodium concentration. In contrast, the perilymph composition is closer to the extracellular medium, with a low potassium but a high sodium content. The differing ionic composition results in a roughly 80 mV difference in potential between the endolymph and the perilymph.
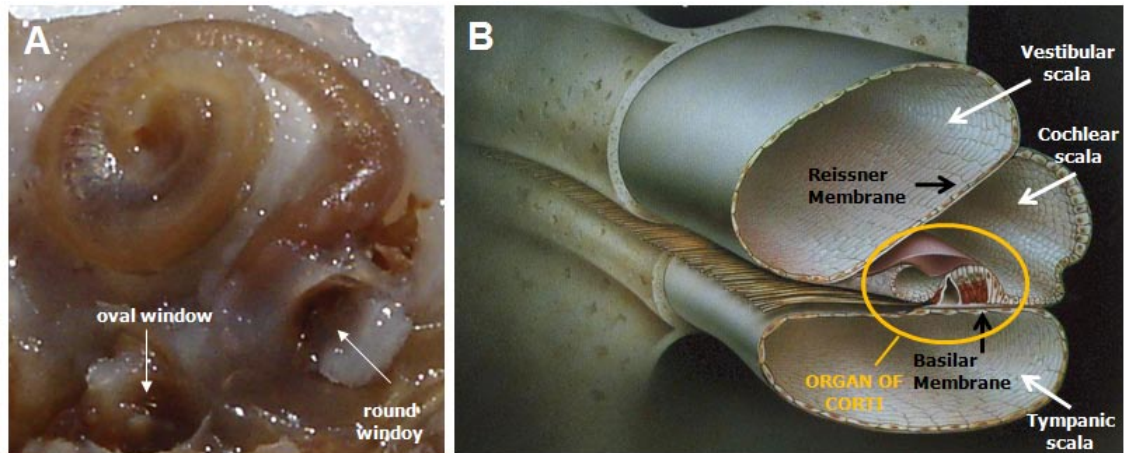
Figure 1.2.4.1. A) Harbour porpoise cochlea after decalcification of the periotic bone with RDO®. B) Scheme of a transversal cut of the cochlea (Source: Boys Town National Research Hospital).

The cochlea of odontocetes has the same fundamental organization as in the rest of land mammals but with some modifications that we will focus on below, presumably as an adaptation to the very high frequencies hearing.

1.2.4.1. Organ of Corti

The cochlear scala is separated from the vestibular scala through the Reissner membrane and from the tympanic scala by the basilar membrane (Figure 1.2.4.2). On the basilar membrane is placed the organ of Corti, the sensorineural end organ for hearing (for review: Lim, 1986a). It includes the inner and outer hair cells, their supporting cells (from internal to external side: first border cells, inner phalangeal cells, pillar cells, Deiters cells and Hensen cells) and annexe cells such as the border cells in the inner spiral sulcus, the Claudius cells and the Boettcher cells. The apical surface of hair cells and supporting cells are joined together by an elaborate set of junctional complexes to form the reticular lamina, which maintains the ion barrier between the endolymph of the cochlear scala and the perilymph –like fluid in the intracellular spaces bath is the basolateral domains of these cells (Gulley and Reese, 1976). The pattern of cell organization in the reticular lamina is known as "mosaic" epithelium, in which every hair cell is surrounded by four supporting cells (Leonova and Raphael, 1997). Tight junctions contribute to the polarized segregation of membrane proteins in hair cells and to sealing the reticular lamina against leaks, thus preventing mixing of endolymph and perilymph.

Reissner's membrane is made up of two cell layers, forming together an avascular membrane (Duvall III and Rhodes, 1967; Iurato and Taidelli, 1967). The cells of the membrane and the junctional complexes between them form an ionic barrier to the flow of ions. In addition, the membrane can most likely regulate ionic balance (and volume) of the fluids by selectively pumping ions

When sound is transmitted to the cochlea via the movement of the stapes footplate, it initiates a traveling wave that moves from the oval window to some point of maximum vibration along the basilar membrane (von Békésy, 1960). The actual vibration patterns on the basilar membrane are determined by the physical characteristics of stiffness and mass. The mechanical (impedance) characteristics of the organ of Corti change systematically along its length. This impedance gradient along the length of the basilar membrane leads to a tonotopic encoding of the frequency of the incoming sound. That is, the peak of the traveling wave is distributed such that high frequency stimuli cause maximal disturbance near the oval window, and as the frequency is lowered, the peak of basilar membrane motion systematically shifts apically.

Very little is known about cochlear morphology in odontocetes. Wever and colleagues (1971a, b and c; 1972) were amongst the first to study the cochlear morphology of the bottlenose dolphin (*Tursiops truncatus*) and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)*,* using histology, focussing their analysis on the description of the basilar membrane, hair cells and spiral ganglion cells.
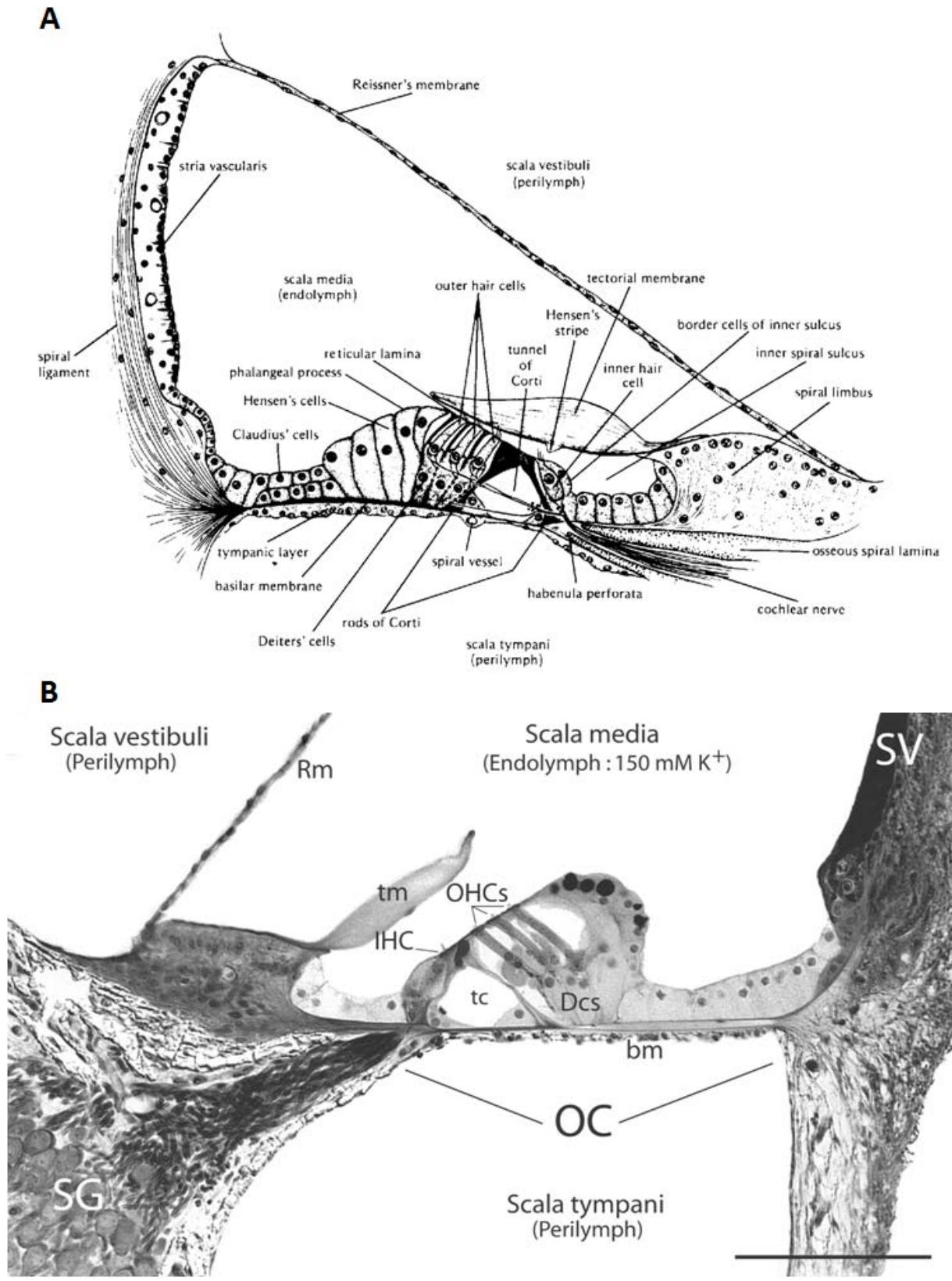
Figure 1.2.4.2. A) Schema of the organ of Corti drawn by Nancy Sally (source: Lim, 1986) B) Organ of Corti of the third turn of the cochlea of a guinea pig (Courtesy of Marc Lenoir). Scale bar: 100 µm OC: organ of Corti, bm: basilar membrane, tc: tunnel of Corti, Dcs: Deiters cells, OHCs: outer hair cells, IHC: inner hair cells, tm: tectorial membrane, Rm: Reissners membrane, SV: stria vascularis.

1.2.4.1.1. Sensory cells

There are two types of auditory sensory cells, the inner (IHCs) and the outer hair cells (OHCs), with the IHCs being the true sensory cell type, sending impulses via the auditory nerve. In contrast, OHCs are effector cells that enhance the performance of the cochlea, qualitatively (increased selectivity) and quantitatively (increased sensitivity) through electromotile properties. The name "hair" cell as chosen because of the bundle of stereocilia that protrude from the apical domain of every cell.

Hair cells are innervated by nerve fibers that send auditory signals into the brainstem (afferent neurons) and to other nerve cells that carry signals from the brain into the ear and influence cochlear function in a feedback loop (efferent neurons).

The hair cell number counted in bottlenose dolphins (17384, 3451 IHCs and 13933 OHCs, Wever *et al.*, 1971a) was found to be in same order of magnitude as in humans (14975 cells, Retzius, 1884).

Stereocilia are mechanosensitive organelles of the sensory hair cells of the inner ear of approximately 250 nm in diameter that can detect displacements on a nanometre scale and are supported by a rigid, dense core of actin filaments (Flock and Cheung, 1977; Flock *et al.*, 1977; Flock *et al.*, 1977; Itoh, 1982; Slepecky and Chamberlain, 1982). The actin filaments array in the stereocilia are organized in parallel paracrystalin array with all the filaments with the same polarity with the tip of the arrowheads pointing towards the cuticular plate (DeRosier *et al.*, 1980; Flock *et al.*, 1981; Slepecky and Chamberlain, 1982). Among the molecules that have been identified in stereocilia are: 1) Myosin VIIa (Hasson *et al.*, 1997; Hasson, 1999), 2) possibly fimbrin (Flock *et al.*, 1982), 3) 2E4, a novel actin-binding protein which is thought to play a unique role in the actin rearrangement during stereocilia formation (Bearer and Abraham, 1999), 4) stereocilin (Verpy *et al.*, 2001), 5) $\alpha 8\beta 1$ integrin (Littlewood Evans and Muller, 2000) and 6) espin (Zheng *et al.*, 2000b). The actin/crossbridge array imparts enormous rigidity to the stereocilia, and allows them to act as higly efficient accessory structures for transferring vibrational energy to the hair cell (Saunders and Dear, 1983; Saunders *et al.*, 1985b).

Stereocilia are disposed in 3-4 rows depending on the species with the tallest row being positioned towards the lateral wall (see review in Lim, 1986a). Stereocilia are linked to neighboring stereocilia of the same row with side links. The apical tips of stereocilia are also connected to their neighboring stereocilia (of the adjacent row) with a tip link (Pickles *et al.*, 1984). Sound-induced deflections of stereocilia induce tension on tip links, which play a critical role in the sensory function of hair cells in directing the opening of transduction channels that allows ion entrance (mostly K^+ and Ca^{++}) into the hair cells and their depolarization (Pickles *et al.*, 1984; Howard and Hudspeth, 1988; Assad *et al.*, 1991; Gillespie and Walker, 2001; Gillespie and Mueller, 2009).

The length of stereocilia varies significantly between species, with ears specialized for high frequency hearing exhibiting the shortest stereocilia. Stereocilia are usually tallest in the apical end of the cochlea and decrease in a gradient towards the base.

The *cuticular plate* is an organelle located under the apical cell membrane of cochlear hair cells, which serves to anchor and support the actin rootlet of stereocilia. It may also be involved in regulating stereociliary stiffness by their contractile proteins (actin, α-actinin, myosin, tropomyosin, fimbrin, calbindin, etc; see Flock, 1983; Nielsen and Slepecky, 1986 for review).

1.2.4.1.1.A. Inner hair cells (IHCs)

Inner hair cells are pear shaped cells with a round centrally located nucleus (Figure 1.2.4.3). One row of IHC runs along the cochlear scala (Figure 1.2.4.4). IHC are positioned on the zone of the basilar membrane which is enclosed by bony shelves of the osseous spiral lamina. Thus, the basilar membrane is immobile in this region, and the IHC body probably does not vibrate in response to sound stimulation. Each IHC has between 20 and 50 (or more) stereocilia, depending on the species and the location along the cochlear scala, with more stereocilia closer to its basal end. In land mammals, the length of IHC does not varies very much from base to apex and is around 30-35 µm (see Nadol, 1988 for review, even 29-30 µm in horseshoe bats, Vater *et al.*, 1992). By contrast the typical length of their stereocilia (the tallest row) ranges between 2-8 µm long.

The junctional complexes of IHC include tight junction near the apical surface, followed by an adherens junction. Mature IHC do not have gap junctions or desmosomes.

Movement of the hair cell stereocilia in the direction of the taller row opens transduction ion channels, allowing entry of potassium and calcium ions and generating a depolarizing transduction current. The transduction current then activates voltage sensitive calcium channels along the IHC lateral wall and base as well as Ca^{2+} activated K^+ channels (Kros and Crawford, 1990; Roberts *et al.*, 1990; Issa and Hudspeth, 1994; Zhang *et al.*, 1999). There are both slow and fast activating K^+ currents (Jagger and Ashmore, 1999). The end result is release of neurotransmitter at the hair cell base. Movement of stereocilia in the opposite direction closes the stereocilia-related channels and stops the release of neurotransmitter.

The frequency of movement of stereocilia matches the frequency of the sound stimulus.

The inner hair cells perform the transduction of the acoustic signal and transmit the information to the auditory nerve and initiate the depolarization of the spiral ganglion neurons (see below in 1.2.4.1.1.C, Innervation)
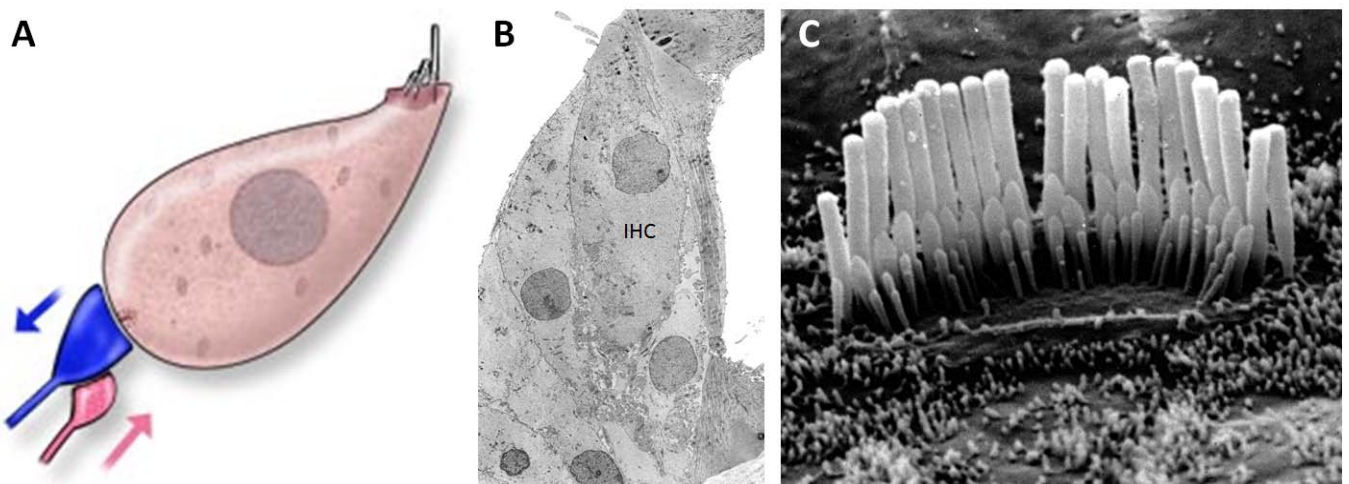
Figure 1.2.4.3. A) Schematic representation of an inner hair cell (IHC; drawing from S. Blatrix, extracted from "Promenade `round the cochlea"). B) Transmission electron microscopy image of an IHC. C) Scanning electron microscopy image of the surface of an IHC. Note the disposition of stereocilia. B and C images are from rat (courtesy of Marc Lenoir)
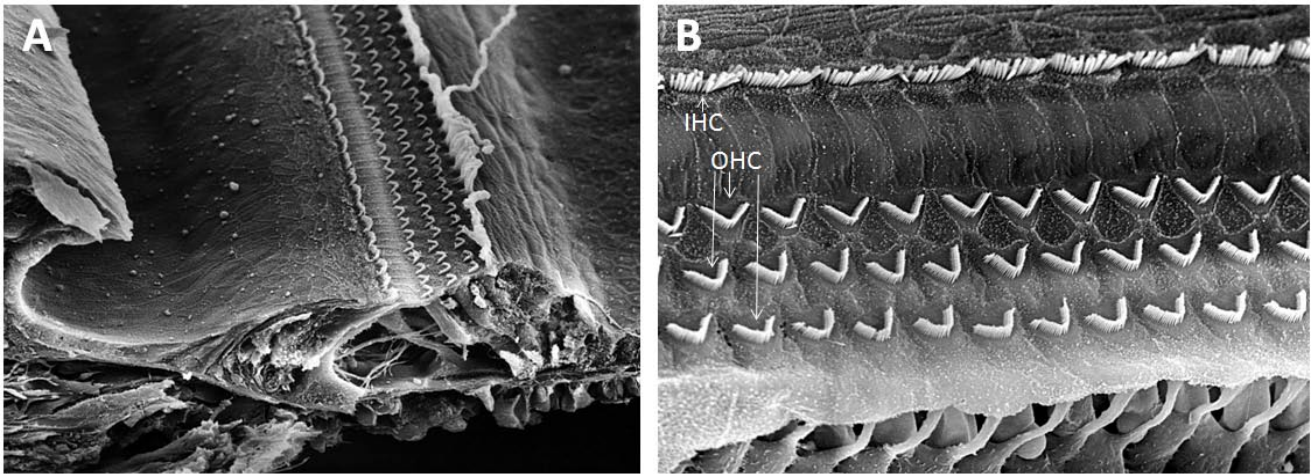
Figure 1.2.4.4. SEM image of the upper surface of a rat organ of Corti. Courtesy of Marc Lenoir (INM, Montpellier). IHC: inner hair cells, OHC: outer hair cell.

1.2.4.1.1.B. Outer hair cells (OHCs)

OHCs are known to enhance and modulate the function of the true auditory sensory cell, the IHC. OHCs have evolved an elaborate set of structural and functional features, some of them unique, which allow them to facilitate the exquisite sensitivity and selectivity of the cochlea (Davis, 1983). The general shape of OHCs is cylindrical, with a flat apical membrane. The nucleus is round and located in the basal portion of the cylinder (Figure 1.2.4.5). The basal end rests on a special "seat" provided by a Deiters cell. There are three rows of OHCs (Figure 1.2.4.4), but in some cases like in bottlenose dolphin some cells in irregular disposition were found in a fourth apical row (Wever *et al.*, 1971a). The fourth and even a fifth row are found in land mammals, especially in the apical turn or in those specialized in low frequency hearing (Raphael *et al.*, 1991). The lateral membrane of these cells is bathed in the fluid of the spaces of Nuel, which is biochemically continuous with perilymph. The apical domain of each OHC is in contact with four different supporting cells, i.e. one outer pillar cells and 3 Deiters cells for the first row of OHC and 4 Deiters for the two other rows (Leonova and Raphael, 1997).
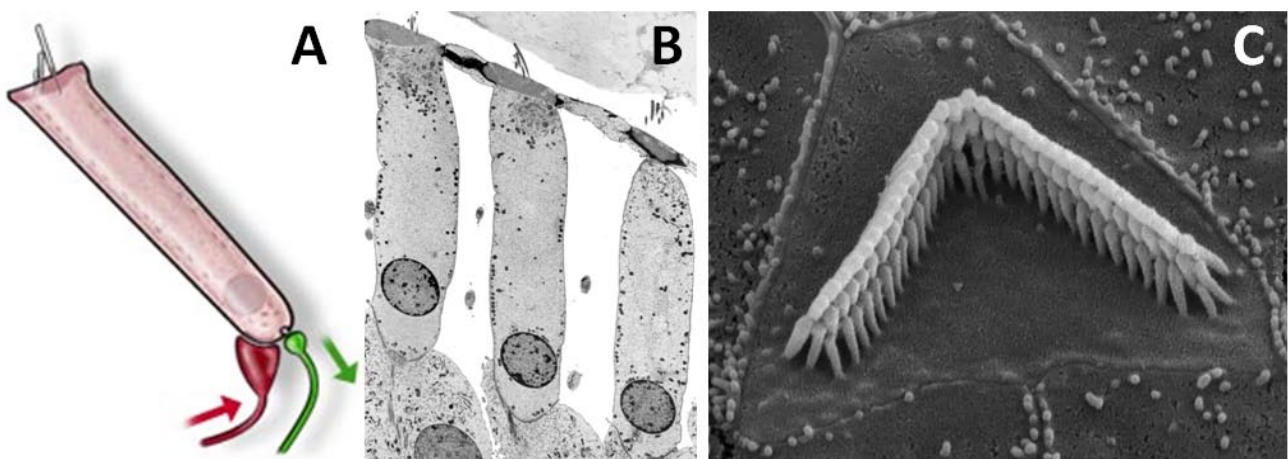
Figure 1.2.4.5. A) Schematic representation of an outer hair cell (OHC; drawing from S. Blatrix, extracted from "Promenade `round the cochlea"). B) Transmission electron microscopy image of the three rows of OHC in rat. C) Scanning electron microscopy image of the surface of an OHC in rat. Note the disposition of stereocilia. B and C images were courtesy of Marc Lenoir.

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OHCs vary in length among mammals and along the cochlea (Figure 1.2.4.6, Pujol *et al.*, 1991). OHCs of the basal turn are invariably shorter than those located in the apical turn. OHCs in low frequency adapted mammals (Raphael *et al.*, 1991 for mole rat) are longer than those specializing in high frequency (Vater *et al.*, 1992 for bat). Typically, in land mammals OHCs are not shorter than 20 μm and not longer than 70 μm, while in horseshoe bat are 12-15 μm to 28-30 μm (Vater *et al.*, 1992) and bottlenose dolphin are between 8 μm at the lower basal region and 17 μm in the apical region (Wever *et al.*, 1971a) .
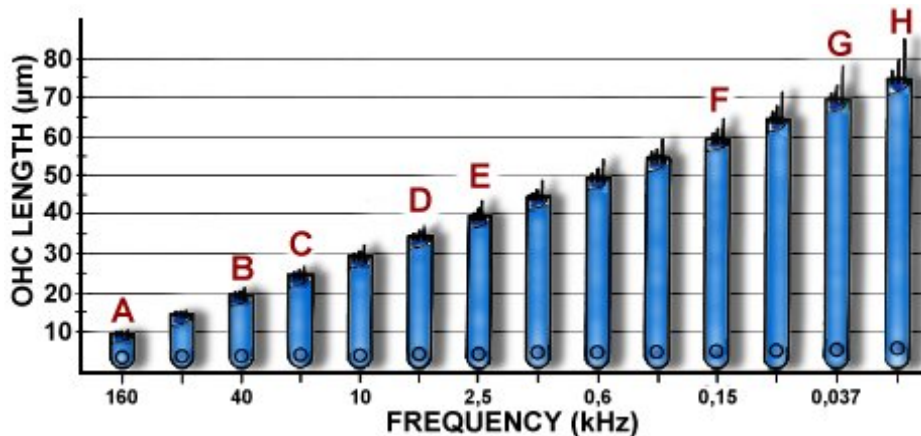
Figure 1.2.4.6. Schematic drawing representing OHCs from different mammalian species and different cochlear turns. While OHC diameter keeps a constant value (7 μm), their length regularly varies according to frequency. In the human cochlea, a 25 μm basal OHC (**C**) is found at a place which codes for 20 kHz; conversely a 70 μm OHC (**G**) is found apically at the site coding for a very low frequency (< 100 Hz). **A** = shortest OHC in basal turn of a bat cochlea (at a place coding for 160 kHz), **B** = basal OHC from a cat cochlea (at a place coding for 40 kHz), **D** = OHC from second turn of a guinea pig cochlea (at a place coding for 5 kHz), **E =** OHC from start of third turn of a guinea pig cochlea (at a place coding for 2.5 kHz), **F =** OHC from end of third turn of a guinea pig cochlea (at a place coding for 150 Hz), **H** = apical OHC from a mole rat cochlea (at a place coding for 15 Hz). Source: Adapted from Pujol *et al.*, 1991

The apical domain includes the stereocilia, which forms a W-shape pattern. The angle of the W varies in gradient along the cochlear duct, with the most acute angle at the apical turn. Every OHC have 3-4 rows of stereocilia (Lim, 1986a). The length of each stereocilium is slightly shorter than those of the IHCs (Wright, 1984). Stereocilia on OHCs are implanted within the undersurface of the tectorial membrane (see below) but not IHC stereocilia that are free into the perilymph.

The junctional complexes connecting OHCs to their neighboring supporting cells are similar, to those found in IHCs. The most apical complex, the tight junction, forms a mixed complex with the adherens junction, so that they alternate along an extensive length, sealing the reticular lamina and preventing passage of molecules between the lumen (scala media) and the perilymph in the space of Nuel. There are no desmosomes or gap junctions in OHCs.

The cytoskeleton of OHCs includes actin (and associated proteins), fodrin and microtubules (with their associated proteins). As in inner hair cells, intermediate filaments are absent.

The presence of a <u>cochlear amplifier</u> has been postulated by Davis (1983). Motility (length changes) in OHCs was discovered two years later (Brownell *et al.*, 1985; Zenner *et al.*, 1985; Ashmore *et al.*, 2000; Kakehata *et al.*, 2000). Because the reticular lamina is rather stiff, length changes in the OHC are likely to modulate the

distance between the reticular lamina and the basilar membrane, and the characteristics of the mechanical vibration presented to the IHC stereocilia. To generate the motility and deliver the length changes to the receiving parts of the organ of Corti, OHCs evolved to be stiff and motile. The lateral plasma membrane (*lateral wall*) participate in maintaining the shape and the stiffness of the OHC and generate their motility (Meech and Holley, 2001; Ashmore *et al.*, 2002; Dallos and Fakler, 2002).

OHCs have a dual response to the transduction current generated by the opening of transduction channels in the stereocilia. The major response is a rapid change in the length and stiffness of the OHC, closely coupled to the changing transduction current. The motile response of OHCs then provides a region specific amplification in the movement of the organ of Corti that enhances transduction at the inner hair cells in that specific region of the cochlear spiral (thus increasing both sensitivity and specificity). The length change is generated by conformational changes in motor protein or proteins located in the lateral wall of the OHC (Kalinec *et al.*, 1992; Frolenkov *et al.*, 1998; Kakehata *et al.*, 2000). The putative motor protein was identified as prestin, a protein related to pendrin and other suphate/anion transport proteins (Zheng *et al.*, 2000a; Zheng *et al.*, 2001; Dallos and Fakler, 2002).

1.2.4.1.1.C. Innervation

The nerve fibers within the organ of Corti are classically divided into two main classes: afferents and efferents. Afferents refer to the dendrites from spiral ganglion cells (SGCs) which carry messages from hair cells to the brain. There are two classes of afferent auditory neurons, called type I and type II spiral ganglion neurons, with the differentiation termed by morphological characteristics of their cell bodies. Efferents refer to the axonal endings of neurons located in the brain stem which carry messages from the brain to the cochlea. Here again, there are two types of efferent sytems, consisting of the lateral and the medial efferent neurons, each of them located in respectively the lateral superior olive nucleus and ventro-medial trapezoid nuclei.

All the information about innervation presented here was mainly extracted from reviews of Raphael and Altschuler (2003), Eybalin (1993) and Pujol and Lenoir (1986).

Inner hair cell innervation

1. IHC-afferent innervation

The Inner hair cells contact the peripheral dendritic processes of Type I spiral ganglion cells, SGCs (Morrison *et al.*, 1975; Kiang *et al.*, 1982), large bipolar neurons that comprise the major population (90–95%) of SGCs, which send auditory signals into the brainstem. These dendrites are called radial afferent dendrites. The axons of type I afferent neurons form the cochlear nerve which send auditory signals to the cochlear nucleus, into the central nervous system.

1.1. Structural organization of the synapse

The inner hair cell makes a ribbon synapse with the peripheral process endings of Type I SGCs (Figure 1.2.4.7). Presynaptically, multiple large round vesicles are lined up around a dense body, called a ribbon. The ribbons are believed to facilitate a continuous supply of synaptic vesicles containing glutamate allowing a continuous multivesicular release of transmitter. A Type I SGC has only one peripheral process, which

contacts a single inner hair cell, while each inner hair cell receives connections from multiple (10–30) SGCs (Spoendlin, 1969; Spoendlin, 1972; Liberman, 1980b; Liberman *et al.*, 1990) with the number varying, depending on the species. For example, the average SGC to IHC ratio is 27:1 for cetaceans, more than twice the average ratio in bats (Vater *et al.*, 1992) and gerbils (Wang J, personnal communication), and three times that of humans (Ketten, 2000).

Many differences appeared when comparing the innervation in cetacean odontocetes with land mammals. The number of SGC (Type I + II) in bottlenose dolphin was 95004, three times as much as in humans (Wever *et al.*, 1971a). In addition, SGCs are larger in odontocetes than in terrestrial mammals. For example, in porpoises (*Phocoena phocoena*) SGC have a mean of 35 by 25 µm and in the dolphins studied were 40 by 25 µm.

It is reasonable to assume that the high number of SGCs is related to the complexity of the information from echolocation signals. That is, by having a greater number of SGCs odontocetes can discriminate sounds better. The anatomy of the inner ear would be consistent with the extent of sensitivity to the very high frequencies.

Moreover, the diameter of the nerve fibers in odontocetes (with a mean of 12 µm) is much thicker than in other species of terrestrial mammals, with an average of 3 µm (Bullock and Gurevich, 1979; Morgane and Jacobs, 1972; Ketten, 1984; Nadol, 1988; Gao and Zhou, 1992; Ketten, 1992; Gao and Zhou, 1995). While the auditory nerve is clearly important to cetaceans, it is also remarkably vulnerable. The extracranial position of the periotic requires the eighth nerve to cross the retro-bullar space without the protection of bony canals before entering the brain case. This "externalization" of the auditory nerve may be unique in cetaceans. In odontocetes, the nerve has a dense fibrous sheath covering its exposed segments as well as thick, fibrous gaskets at is entry to the periotic, but, curiously, not at its entry point in the basi-cranium (Ketten, 1992).

1.2. Neurotransmitters

Evidence supports an excitatory amino acid, most likely glutamate (and/or aspartate), as the IHC neurotransmitter (review by Eybalin and Pujol, 1983; Eybalin, 1993; Ruel *et al.*, 2007). Glutamate is a fast neurotransmitter that allows the rapid transmission process requires for sound coding. However, when released in excess during acoustic overstimulation or when the IHCs are damaged, it induced the swelling or destruction of the afferent dendrites synapsing with the IHCs through an excitotoxic process (Robertson, 1983; Pujol *et al.*, 1985; Juiz *et al.*, 1989; Puel, 1995; Puel *et al.*, 2002). Generally, repair mechanisms occur at the dendritic extremity and new functional synapses are formed. However, in case of sustained excitotoxicity, their repair process is far to be not complete and since around 50 percent ganglion neurons may progressively die (Kujawa and Liberman, 2009).

There are several amino acid receptors associated to ionic channels and recognized by glutamate, which has a better affinity than aspartate. They are: NMDA (for N-methyl-D-aspartate), AMPA (for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors. It has been shown that strong or repetitive neuronal depolarizations are required to activate the NMDA receptor (Herron *et al.*, 1986; Collingridge *et al.*, 1988). In the cochlea, these conditions may correspond to sound stimuli of high intensities.

In normal hearing conditions, glutamate released from IHCs both AMPA and kainate receptor types when activating the dendrites of the type I primary auditory neurons (Bledsoe *et al.*, 1981; Bobbin *et al.*, 1981; Bobbin *et al.*, 1984; Jenison and Bobbin, 1985; Jenison *et al.*, 1986; Bobbin and Ceasar, 1987; Littman *et al.*, 1989; Puel *et al.*, 1989).

2. IHC-efferent innervation

2.1. Lateral olivocochlear efferent connections

Lateral olivocochlear (LOC) efferents arise in the lateral superior olive of the auditory brain stem, send terminals to the ipsilateral cochlea and terminate by forming axodendritic synapses with the radial afferent dendrites connected to the IHCs (Smith, 1961; Smith and Rasmussen, 1963; Spoendlin, 1966; Iurato, 1974; Warr, 1975; Warr and Guinan, 1979; Liberman, 1980a; Warr, 1980; Guinan *et al.*, 1983; White and Warr, 1983; Guinan *et al.*, 1984; Warr *et al.*, 1997; Nadol, 1983, Figure 1.2.4.7). However, in echolocating bats *Rhinolophus rouxi* (Aschoff and Ostwald, 1988) and *Pteronotus parnellii* (Bishop and Henson, 1987), these neurons most probably form another individualized nucleus in the superior olivary complex, apposed to the lateral superior olive, termed nucleus olivocohlearis or interstitial nucleus. This nucleus is located between the lateral and medial superior olives.

In the cat, Liberman (1980a) reported that each efferent fiber synapses with more than one radial afferent dendrite and that every dendrite has at least one efferent synapse.

2.2. Neurotransmitters

The LOC was shown to contain acetylcholine as a transmitter (Warr, 1975; Eybalin and Pujol, 1984; Altschuler *et al.*, 1985; Eybalin and Pujol, 1987; Vetter *et al.*, 1991). The LOC has also been shown to contain a large number of additional neurotransmitters (Eybalin, 1993) including enkephalin (Fex and Altschuler, 1981; Altschuler *et al.*, 1984; Eybalin and Pujol, 1984), dynorphin (Altschuler *et al.*, 1985; Hoffman *et al.*, 1985), dopamine (Jones *et al.*, 1987; Usami *et al.*, 1988), CGRP (Kitajiri *et al.*, 1985; Lu *et al.*, 1987; Takeda *et al.*, 1987; Silverman and Kruger, 1989; Sliwinska-Kowalska *et al.*, 1989; Vetter *et al.*, 1991; Simmons and Raji-Kubba, 1993) and GABA (Fex *et al.*, 1986; Eybalin *et al.*, 1988; Vetter *et al.*, 1991). Many of these neurotransmitters are co-localized within single LOC neurons and neuronal processes (Altschuler *et al.*, 1983; Altschuler *et al.*, 1984; Abou-Madi *et al.*, 1987; Altschuler *et al.*, 1988; Safieddine and Eybalin, 1992; Safieddine *et al.*, 1997). Because these unmyelinated fibers are difficult to record from or to stimulate, it has been difficult to determine the function of the LOC. Studies suggest that the lateral efferents can change the resting potential or "set-point" within the Type I SGC auditory nerve post-synaptic terminals, depending on which transmitter or transmitters are acting on the auditory nerve. Acetylcholine, dynorphin and CGRP are all capable of lowering the set point and potentiating the action of glutamate in achieving depolarization and auditory nerve activity. On the other hand dopamine, enkephalin and GABA are inhibitory and will hyperpolarize, raise the set-point and make the peripheral processes they influence less sensitive to glutamate activation by inner hair cells (Felix and Ehrenberger, 1992; Burki *et al.*, 1993; Oestreicher *et al.*, 1997; Arnold *et al.*, 1998; Ruel *et al.*, 2001).

2.3. Function

The function of the LOC may therefore be to produce a range of set-points, generating a continuum of spontaneous activities and sensitivities, which in turn provides a greater dynamic range for the driven activity of the auditory nerve. An additional function may be a lateral efferent loop or reflex that can change set-points and/or receptor trafficking and allow the dynamic range to be adapted to different levels of activity. In this way, it may provide protection (Pujol *et al.*, 1993). In fact, dopamine modulates tonically the activity of AMPA receptors and regulates spontaneous activity of auditory nerve neurons and their

responsiveness to sound stimulation. A removal of tonic inhibitory action of dopamine leads to the development of early signs of glutamate-induced exocitosis (Ruel *et al.*, 2001; Puel *et al.*, 2002).
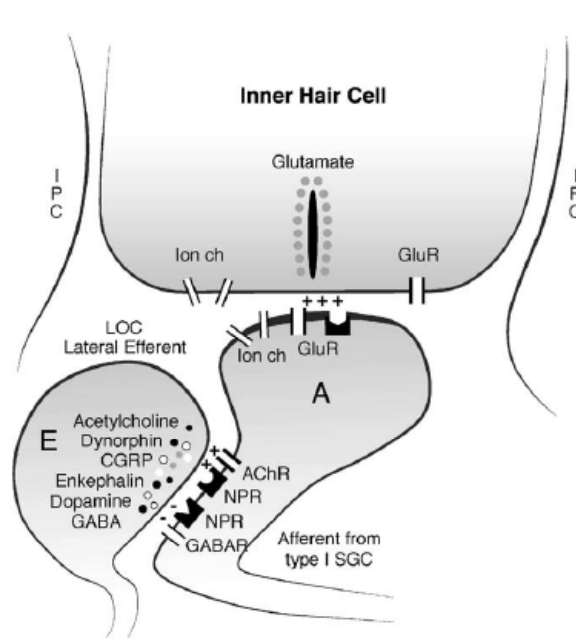
Figure 1.2.4.7. Schematic of the efferent (E) and afferent (A) connections to the inner hair cell. There are ion channels (Ion ch) including voltage sensitive calcium channels and Ca^{2+} activated K channels. The inner hair cell transmitter, an excitatory amino acid most likely to be glutamate, is sequestered in large round vesicles around a ribbon and released into the synaptic cleft. A connection is made to an afferent (A) terminal, the peripheral process of a Type I spiral ganglion cell (SGC). Glutamate receptors (GluR) are placed in the active zone of the post-synaptic membrane, with an AMPA type ionotropic membrane spanning receptor. There are also post-synaptic ion channels including K+ channels. The efferent (E) connection is made by the LOC system and is largely onto the afferents. There are multiple neurotransmitters (listed in diagram). These can have excitatory (+) or inhibitory (−) actions on post-synaptic receptors. There are ionotropic (membrane spanning) receptors, the acetylcholine receptors (AChr) and GABA-A receptors (GABAR) with an inhibitory action. There are also metabotropic (second messenger linked) receptors for the different neuropeptides (neuropeptide receptors—NPRs) with excitatory, inhibitory and other types of actions possible. IPC: inner phalangeal cell. Source: Raphael and Altschuler, 2003.

Outer hair cell innervation

By contrast with IHCs, OHCs are mainly innervated by efferent terminals whereas they show a discrete afferent innervation in most mammals

1. *Outer hair cell- afferent innervation*

1.1. *Structural organization of the synapse*

OHCs make synaptic connection with Type II SGCs (Brown, 1987; Figure 1.2.4.8). Type II SGCs are smaller, less numerous (5–15%; Spoendlin, 1969; Spoendlin, 1972; Morrison *et al.*, 1975; Kiang *et al.*, 1982; Berglund

and Ryugo, 1987; Brown, 1987) and less myelinated than the Type I SGCs. Each dendrite of a Type II neuron sends collaterals contacting from 6 to 100 OHCs, most often from the same row (Spoendlin, 1972; Perkins and Morest, 1975; Smith, 1975; Kiang *et al.*, 1982; Ginzberg and Morest, 1983; Berglund and Ryugo, 1987; Brown, 1987; Simmons and Liberman, 1988; Ryugo *et al.*, 1991). These afferent dendrites are called spiral afferents since, as they cross the tunnel of Corti, they run along the cochlear spiral towards the basal part before reaching the OHCs.

There are few presynaptic vesicles at the synapses between OHCs and Type II SGCs in basal and middle cochlear region and no pre-synaptic ribbons (Dunn and Morest, 1975; Liberman *et al.*, 1990). Multiple vesicles and ribbon synapses, as found in inner hair cells, are only seen for OHCs in the most apical parts of the cochlea (Pujol and Lenoir, 1986). At the present time, very few is known about its biochemical properties and physiology.

1.2. *Functionality*

It has been suggested by Robertson (1984) Type II neurons lack spontaneous activity and do not respond to stimulatory sounds. So, by contrast with IHCs, OHCs do not send auditory information to the brain. However, recent research (Weisz *et al.*, 2009) demonstrated that type II peripheral processes conduct action potentials, but the small and infrequent glutamatergic excitation indicates a requirement for strong acoustic stimulation. In addition, they showed that type II neurons are excited by ATP.

Functional hypothesis reports that this OHC afferent innervation may monitor the motile state (tension) of the OHC. This would imply that this innervation actually conveys "sensations of auditory pain" (Brown *et al.*, 1988) or messages of OHC damage (Simmons and Liberman, 1988) to the cochlear nucleus in response to high-intensity noises (>110 dB) that significantly depolarize the OHC (Cody and Russell, 1985), and probably alter greatly its motile state. A neurotransmitter release could occur at that moment which would excite the spiral dendrites of the Type II neurons.

One major function of their central connection may be to contribute to an efferent feed-back loop, the medial olivocochlear reflex. As part of this pathway, Type II SGCs make a central connection to the shell region of the cochlear nucleus which in turn projects to the superior olivary complex (Brown and Ledwith, 1990; Berglund and Brown, 1994; Morgan *et al.*, 1994; Berglund *et al.*, 1996; Warr *et al.*, 1997; Ye *et al.*, 2000).

1.3. Neurotransmitters

While there is evidence showing glutamate in OHCs (Altschuler *et al.*, 1989; Eybalin and Altschuler, 1990; Usami *et al.*, 1992) and glutamate receptors are expressed in Type II SGCs (Kuriyama *et al.*, 1993; Kuriyama *et al.*, 1994; Ottersen *et al.*, 1998), evidence for placement of the receptors into the postsynaptic complex (Matsubara *et al.*, 1996) and for an action of glutamate is still lacking. It is suggested that the OHCs only use glutamate as a metabolic intermediate and not as a neurotransmitter (Eybalin *et al.*, 1990; Eybalin *et al.*, 1991).

2. OHC - efferent innervation

2.1. *Structural organization of the synapse*

The basal pole of the OHCs is directly connected by terminals from efferent fibers forming large axosomatic synapses. Most of these fibers belong to the medial efferent system or medial olivocochlear efferent (MOC, defined by Warr, 1980). The MOC originates in various subnuclei of the superior olivary complex. They travel with the vestibular nerve until they join the auditory nerve close to the cochlea and enter the cochlea with the auditory nerve. They course within the inner spiral bundle below IHC, cross the tunnel of Corti, and reach the base of OHCs almost radially. These efferent fibers are myelinated up to the habenula perforata.

The MOC-OHC synapse is characterized by post-synaptic cisterna, along the length of the synapse. In many, but not all species, this medial olivocochlear efferent (MOC) innervation of OHCs has a basal bias, with more terminals on OHCs of the basal turn and the first row, with the number gradually decreasing more apically.

In the echolocating horseshoe bat, *Rhinolophus rouxi*, no medial efferent innervation was encountered (Bruns and Schmieszek, 1980; Aschoff and Ostwald, 1988; Vater *et al.*, 1992). The lack of medial efferent terminals on the OHCs was also found in the subterranean mole rat *Spalax erhenbergi* (Lenoir *et al.*, 1990; Raphael *et al.*, 1991) which, conversely to *Rhinolophus*, has low-frequency adapted hearing (Bruns *et al.*, 1988).

2.2. *Neurotransmitters*

Many studies support Acetylcholine (ACh) as a MOC transmitter (Schuknecht *et al.*, 1959; Bobbin and Konishi, 1971; Galley *et al.*, 1972; Bobbin and Konishi, 1974; Warr, 1975; Fex and Adams, 1978; Robertson and Johnstone, 1978; Eybalin and Pujol, 1984; Altschuler *et al.*, 1985; Klinke, 1986; Eybalin and Pujol, 1987; Vetter *et al.*, 1991). The effect of ACh release is a hyperpolarization of the OHC, which changes its set-point (resting potential; Bobbin and Konishi, 1971; Galley *et al.*, 1972; Bobbin and Konishi, 1974; Bobbin, 1979; Klinke, 1986; Dallos *et al.*, 1997; Evans *et al.*, 2000; Sziklai *et al.*, 2001), thus modulating outer hair motility and changing the gain of the cochlear amplifier. ACh also has a direct effect on OHC motility by influencing the OHC axial stiffness (Dallos *et al.*, 1997; Sziklai *et al.*, 2001). The MOC may act in "reflex" fashion by changing the cochlear amplifier as a consequence of the amount of auditory pathway activity and may also act to provide protection from overstimulation by noise (Maison and Liberman, 2000).

GABA has also been shown in medial efferents (Fex *et al.*, 1986; Eybalin *et al.*, 1988; Altschuler *et al.*, 1989; Vetter *et al.*, 1991; Matsubara *et al.*, 1996). There are evidences of the presence of GABA receptor (Drescher *et al.*, 1993) and CGRP receptor complex (Luebke and Dickerson, 2002) and GABA has been shown to hyperpolarize OHCs and thus may also function to modulate the set-point (Sziklai *et al.*, 1996; Oliver *et al.*, 2000), while the action of CGRP remains unknown.

For more details on the afferent and efferent pathways of the cochlea and cochlear neurotransmission see the reviews by Eybalin (1993), Fechner and colleagues (2001) and LePrell and colleagues (2001 and 2003).
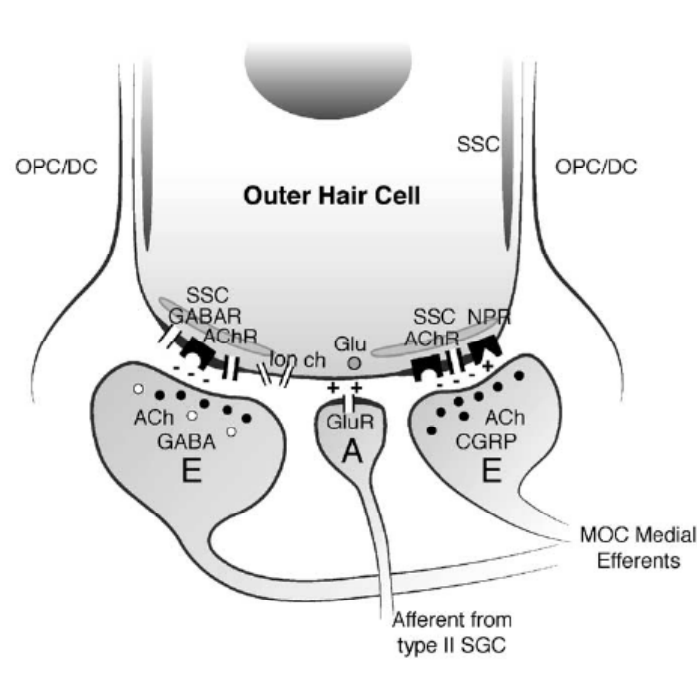
Figure 1.2.4.8. Schematic of the efferent (E) and afferent (A) connections to the outer hair cell (OHC), typical for the basal half of the cochlea. Efferents (E) from the medial olivocochlear (MOC) system make multiple connections to the OHC base. Efferent terminals contain acetylcholine (ACh) and perhaps also GABA and CGRP. In the active zone in the post-synaptic membrane of the OHC, the efferent terminals are apposed by acetylcholine receptors. GABA receptors (GABAR) and a neuropeptide receptor (NPR) for CGRP provide for their action. Specific types of ion channels (Ion ch), including K+ channels, help to generate the post-synaptic response to efferents. The OHC makes an afferent connection to the peripheral process of type II spiral ganglion cells (SGC). Presynaptic glutamate (Glu) is released from a small vesicular pool at the outer hair cell base and acts at glutamate receptors (GluR) in the postsynaptic afferent. OPC: outer phalangeal cell; DC: Deiters cell; SSC: subsurface cisternae. Source: Raphael and Altschuler, 2003

1.2.4.1.2. Supporting cells

The supporting cells of the organ of Corti are highly differentiated epithelial cells. IHC are surrounded by phalangeal cells and first border cells, the basal and the apical pole of OHCs are in contact with Deiters and outer pillar cells while Hensen cells are positioned further laterally in the organ of Corti.

In their basolateral aspect, supporting cells exhibit different sets of cell–cell junctions (Wersall *et al.*, 1965; Engstrom, 1967; Kimura, 1975; Spoendlin, 1979; Kikuchi *et al.*, 1995). In homologous junctions (supporting cell to supporting cell) there are desmosomes and gap junctions, in addition to the tight and adherens type junctions. In contrast, gap junctions and desmosomes are absent in heterologous junctions (supporting cell to hair cell, Kikuchi *et al.*, 1995; Forge *et al.*, 1999). The molecules that mediate cell-cell communication via the junctional complexes belong to different families of cell adhesion molecules, like E-Cadherin (Whitlon, 1993).

The supporting cells shape is most likely dependent on an elaborate network of cytoskeletal filaments. Indeed, microfilaments, intermediate filaments and microtubules are all present in supporting cells in conspicuous amounts and strict organization.

The microtubules provide stable, long-lived structural support (Slepecky *et al.*, 1995). The bundles of keratin intermediate filaments (Raphael *et al.*, 1987; Anniko *et al.*, 1989; Arnold and Anniko, 1989; Bauwens *et al.*, 1991; Kuijpers *et al.*, 1992; Mogensen *et al.*, 1998) arrays appear to anchor microtubule bundles to cell surfaces, especially in areas where supporting cells contact hair cells (Mogensen *et al.*, 1998).

Deiter cells

Deiters cells have three distinct compartments: cell body, stalk and apical head plate (phalangeal process). They provide support along the longitudinal direction because their phalangeal processes are positioned one or two cells apart longitudinally, depending the level along the cochlear spiral, filling the space between OHCs. They are also coupled to the basal portion of the OHC. Thus, each Deiters cell is in contact with 4-5 different OHCs, depending on the row: one that sits on it and four others that are connected to its apical head plate (except for the 3rd row of Deiters that are in contact with 4 OHCs). When the sensory cell is degenerated by acoustic trauma or ototoxicity the supporting cells swell to fill the opening in the reticular, sealing the potential perilymphatic leak and forming a "scar" (Lim and Dunn, 1979; Lim, 1986b).

Hensen cells

Hensen cells form the lateral border of the organ of Corti. These cells have less complex cytoskeletal organization than the Deiters and pillar cells. Some Hensen cells rest on the basement membrane while others appear to form a second layer, usually on top of Boettcher cells (on the lateral aspect of the Hensen cell area).

Pillar cells

Inner and outer pillar cells form the pillars of the organ of Corti and provide the most rigid support along the radial direction due to their unique architecture. The heads of the inner and outer pillar cells are interlocked, but their cell bases are widely separated.

Function of supporting cells

Supporting cells have functions beyond structural support, such as the regulation of the ionic environment within and around the organ of Corti (Kikuchi *et al.*, 2000). They are thought to recycle K$^+$ by removing it from the organ of Corti to fibrocytes that, in turn, transport it back to the stria vascularis supporting cells receive synaptic connections; In human and a few species, Hensen and Deiters cells receive a significant innervation that is predominantly derived from collaterals of Type II spiral ganglion cell afferents (Fechner *et al.*, 2001), with small additional contributions from the efferent system. The function of this innervation remains unknown. Deiters cells are also capable of a motile response (Dulon *et al.*, 1994), have P2X receptors (Chen and Bobbin, 1998) and ATP can induce their movement (Bobbin, 2001). Inner phalangeal

cells, Deiters cells and Hensen cells may be involved in the production of certain components of the tectorial membrane during development (Lim and Anniko, 1985).

In addition to their apical intercellular junction, gap junctions couple the cytoplasm of supporting cells and allow them to share a micromolecular cytoplasmic environment (Kikuchi *et al.*, 1995; Forge *et al.*, 1999). The role of gap junctions for hearing is crucial. Four genes, *GJB1*, *GJB2*, *GJB3* and *GJB6*, encode for connexin proteins (Connexin32, Connexin26, Connexin31 and Connexin30, respectively; Lefebvre and Van De Water, 2000).

1.2.4.1.3. Basilar membrane

The basilar membrane is a complex strand of connective tissue composed of cellular and extracellular components. The side of the BM facing the scala media features the basement membrane of the epithelium of the organ of Corti. On the opposite side, facing the perilymph of the scala tympani, several cellular and acellular components of connective tissue can be found. The cellular component is made up of a layer of mesothelial cells that line the scala tympani. The BM is composed of matrix and fibers. Specifically it has been identified Collagen Type II, IV and XI, (Thalmann, 1993; Cosgrove *et al.*, 1996; Dreiling *et al.*, 2002), fibronectin (Santi *et al.*, 1989; Cosgrove and Rodgers, 1997), proteoglycans (Tsuprun and Santi, 2001), tenascin (Swartz and Santi, 1999).

There are several structural domains within the BM. The main distinction is between the medial portion of the BM, called arcuate zone (pars tecta) and the lateral portion called pectinate zone (pars pectinata). The arcuate zone is partly enclosed in osseous spiral lamina, and therefore its ability to vibrate upon sound-induced displacement of the cochlear fluids is restricted. In contrast, the pectinate zone is free to vibrate in response to sound, within the physical constraints of its mass, stiffness and the effects of the active cochlear mechanisms. The border between the arcuate and pectinate zones is usually under the outer pillar cell. The arcuate zone of the BM has small perforations, collectively referred to as the habenula perforata, that accommodate the auditory nerve fibers as they extend from Rosenthal's canal to the organ of Corti.

The width of the BM (distance from the modiolar side to the lateral end) and its thickness (the distance from the tympanic border to the basal lamina) were measured in several mammal species (Nadol, 1988; Roth and Bruns, 1992; Sato *et al.*, 1999; Keiler and Richter, 2001). A clear gradient of size (thickness and width) is found in most mammals, proportional to the distance from the basal end of the cochlea, being narrower, thicker and relatively stiff in the base (place where the high frequencies are encoded) and broader and thinner in the apex (where the low frequencies are encoded). For example, in the bottlenose dolphin with a known hearing range of 75 Hz to 152 kHz (Johnson, 1967; Popov and Supin, 1990b; Popov *et al.*, 2007), the width of the basilar membrane increase about 14 times from base to apex (from 24 to 350 µm, Wever *et al.*, 1971b), while the thickness diminish 5 times (from 25 to 5 µm). However, in human that presents a hearing range from 20 Hz to 20 kHz, the basilar membrane with a length of 32 (Keen, 1940) to 33,5 mm (Held, 1926) increase the width only 5 times (from 125 to 500 µm) and threefold decrease in thickness (from 7 to 2 µm) from base to apex (Schuknecht, 1993; Ketten, 1998).

Interspecific differences in odontocetes in the hearing range and habitat have been reflected in differences in mass and stiffness of the basilar membrane (Ketten and Wartzok, 1990; Ketten, 1992; Ketten, 1994). That

is, coastal and river species little gregarious that produce very high frequency signals present the basilar membrane even narrower and stiffer than those pelagic species that live off-shore in large groups. In view of these results odontocetes were divided into two groups according acoustic sound production capabilities: Type I odontocetes included species with a peak frequency (ie, the frequency with highest energy) of the echolocation pulses above 100 kHz and Type II, less than 100kHz. Cochlear morphometry was significantly different between these two groups, especially the spiral geometry and stiffness of the basilar membrane (see Figure 1.2.4.9). It appears that an increase in the stiffness of the basilar membrane is related to adaptation to the hearing to the very high frequencies, as also seen in bats that use an echolocation system (Vater *et al.*, 1992).

In odontocete species, specially the type I odontocetes, the basilar membrane is very stiff in the base, fixed to both sides by a very well developed inner and outer osseus spiral laminae (Ketten and Wartzok, 1990; Ketten, 1992; Ketten, 1994, Figure 1.2.4.9). Poor developed inner osseus spiral laminae and reduced or absent outer osseus spiral laminae is found in low frequency hearing mammals. The spiral lamina is a structure containing fibroblasts that anchor and tension the basilar membrane. In odontocetes is observed that the spiral ligament cells are much more compact than in other mammals. In particular, the collagen fiber is 2 to 5 times more dense, especially at the base of the cochlea, reducing their density in the apical parts.
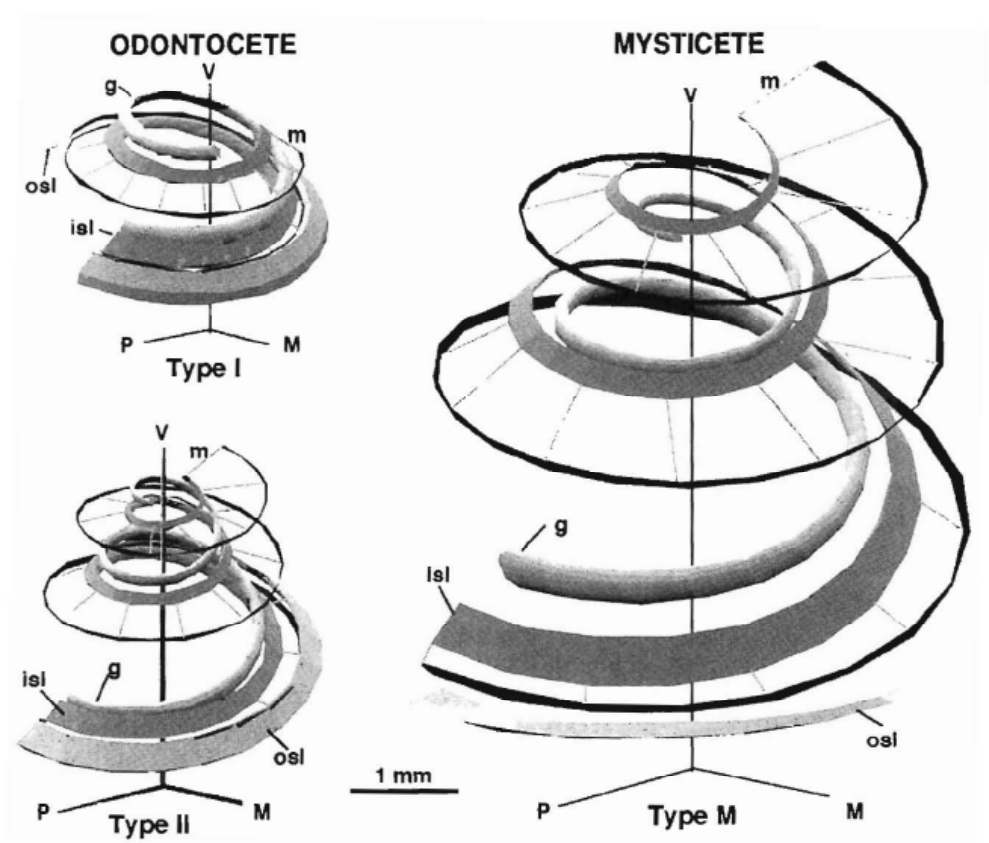
Figure 1.2.4.9. Basilar membrane and spiral laminae distribution in type I and II odontocete cetaceans and in mysticetes. *V*: ventral, *P*: posterior, *M*: medial, *g*: spiral ganglia, *isl*: inner osseus spiral lamina, *m*: mandible, *osl*: outer spiral lamina (Source: Ketten, 1992)

1.2.4.1.4. Tectorial membrane

The Tectorial membrane (TM) is an acellular connective tissue which lies over the sensory cells of the cochlea from base to apex (Lim, 1972; Steel, 1983). It is medially attached at the spiral limbus to *interdental cells* which secrete the TM matrix (Kimura, 1966; Lim, 1972, Figure 1.2.4.2). The lower (inferior) aspect of the TM is in contact with the stereocilia of OHCs. The dimensions and mass of the TM increase in reverse proportion to the frequency along the cochlear duct. Several distinctive regions have been defined in the TM, including the *cover net* (superior region), *marginal net* (lateral aspect, attached to the outermost row of Deiters cells and Hensen's cells during the period of development), *limbal zone* (medial, near the insertion of the TM to the interdental cell area) and the *middle zone* (above the inner hair cell area, see Figure 1.2.4.2). *Hensen's stripe* is an amorphous stripe in the inferior aspect of the TM, that is thought to be located apposing the IHC region (Lim, 1980; Lim, 1977). The area in the inferior surface of the TM where OHC stereocilia are embedded is known as *Hardesty's (or Kimura's) membrane*.

The TM is composed of distinct types of collagens (Types II, V, IX and XI; Thalmann *et al.*, 1987; Slepecky *et al.*, 1992b; Thalmann, 1993; McGuirt *et al.*, 1999; Goodyear and Richardson, 2002; Shpargel *et al.*, 2004) and non collagen-proteins (alpha and beta tectorin and otogelin; Richardson *et al.*, 1987; Cohen-Salmon *et al.*, 1997; Legan *et al.*, 1997), which appear as fibers and matrix. There are at least two types of fibers called fibrils (Type A and Type B; Kronester-Frei, 1978) and a non-fibrilar matrix.

The fluids above and below the TM are distinct, which presumably necessitates a seal at the margin of the TM (Ross, 1974; Anniko and Wroblewski, 1980; Burgio and Lawrence, 1980). It seems to be a close relationship between the TM and its surrounding fluids, because of the extreme sensitivity of the TM to changes in its ionic environment, such as swelling when the calcium ion concentration surrounding was reduced by the addition of EDTA of the same osmolarity (Kronester-Frei, 1978), sodium-induced shrinkage (Lim, 1977; Kronester-Frei, 1978; Kronester-Frei, 1979), or shrinkage and swelling under the influence of varying pH (Kronester-Frei, 1979). These observations implied than the matrix of the TM is in intimate contact with the endolymph (Steel, 1983).

It is unclear whether the stereocilia of inner hair cells are attached to the TM. In rat, Lenoir and colleagues (1987) showed IHC stereocilia hanging to the undersurface of the TM in the basal region of the cochlea. In bat (Vater and Lenoir, 1992) prints of IHC stereocilia were seen in the undersurface of the tectorial membrane in the basal cochlear region (Figure 1.2.4.10d). However, in most mammals studied, data were unable to demonstrate convincingly a direct physical attachment (Matsumura, 2001). In contrast, attachment links between the tips of the tallest row of stereocilia on each OHC and the TM were documented in several species (Kimura, 1966; Spoendlin, 1966; Iurato, 1967; Lim, 1972; Hoshino, 1974; Ross, 1974; Hoshino, 1977; Raphael *et al.*, 1991; Vater and Lenoir, 1992; Tsuprun and Santi, 2002, Figure 1.2.4.10). The attachment was to the Type B fibrils of the TM (Tsuprun and Santi, 2002). Imprints of these stereocilia tips are arranged in W patterns which precisely coincide with the arrangement of the cilia directly below (Figure 1.2.4.10b). Occasionally two or three rows of imprints may be seen over a single hair cell in certain regions (Kimura, 1966; Hoshino and Kodama, 1977; Hoshino, 1977; Kawabata and Nomura, 1981, Figure 1.2.4.10c), and specialized elevations bearing the imprints have been observed on the underside of the TM in bats, man (Bruns and Goldbach, 1980; Kawabata and Nomura, 1981, Figure 1.2.4.10c), and in the apical region of rats (Lenoir *et al.*, 1987).
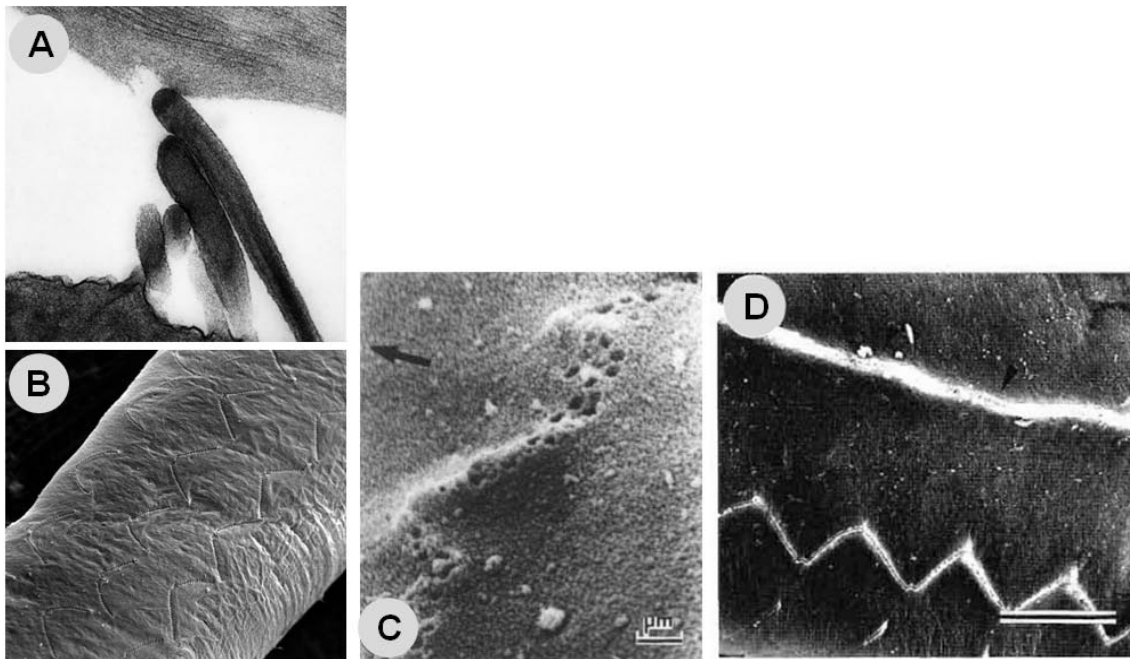
Figure 1.2.4.10. Tectorial membrane. A) Attachment of the tallest row of OHC of the apex to the undersurface of the tectorial membrane through TEM (courtesy of Remy Pujol); B) stereocilia imprints of the three rows of OHC through SEM in the base of a rat cochlea (reprinted from Lenoir *et al.*, 1987); C) imprints of man cochlea showing the coupling of more than the first row of OHC stereocilia in the W-shaped elevated zone (reprinted from Kawabata and Nomura, 1981); D) IHC stereocilia imprints in the upper basal turn of horseshoe bat marked with the arrow heat (bar: 5 µm, Vater and Lenoir, 1992)

Function of the tectorial membrane

The coupling of the TM with the OHC stereocilia might serve to hold the TM in a position suitable for normal stimulation of the IHC (Dallos, 1978). When the OHC stereocilia-TM coupling is adversely affected or completely eliminated by damaging the OHC, the features of the TM critical to IHC stimulation may be altered, leading to abnormal responses. Accordingly with Dallos and colleagues (1972) IHC are stimulated by the viscous drag of fluid flowing past their cilia. Ter Kuile (1900 a and b) suggested that a shearing action between the tectorial membrane and the reticular lamina was an essential step in the transduction process, implying a purely mechanical role for the TM. This view is widely accepted today. So, the TM provides mass loading on top of the organ of Corti, facilitating deflection of the stereocilia (Raphael and Altschuler, 2003).

The TM may also be involved in energy processing before transduction occurs. For example, it's generally believed that OHC influence the response of IHC (Sokolich *et al.*, 1976; Dallos *et al.*, 1977; Manley, 1978; Sellick and Russell, 1978; Dallos, 1981). Some hypotheses involve either electrical interaction (Manley, 1978; Brownell, 1982) or micro-mechanical interaction (Steele, 1973; Zwislocki and Kletsky, 1979; Duifhuis and van de Vorst, 1980), both of which may involve the TM.

The interaction between longitudinal and radial coupling within the TM could improve frequency analysis (Zwislocki, 1979; Zwislocki, 1980). TM may take part in the localization of sound energy to the appropriate point along the length of the cochlea and its presumed capacity for compressing the wavelength of the signal due to the reduced velocity of sound within the membrane (Naftalin and Jones, 1969; Naftalin, 1976;

Naftalin, 1977). The TM was also proposed to act as a second resonator and a structure within which there is significant longitudinal coupling (Richardson *et al.*, 2008). This resonator would be tuned to a frequency one-half an octave lower than the basilar membrane at any one point (Goodyear and Richardson, 2002), and would act as an inertial mass against which the OHC could exert force and thus amplify the motion of the basilar membrane at its characteristic resonance frequency (Legan *et al.*, 2000).

It was suggested that the TM and its connections to the underlying cells would have a protective function (Flock, 1971). The attachment of the marginal zone might act as a "strain belt" to protect the hair cells from excessive stimulation. In addition, the apparent decoupling of the IHC stereocilia from the TM at low frequencies may protect them from large amplitude displacements of the basilar membrane (Sellick and Russell, 1978).

The ionic content of the TM might be relatively independent of that of endolymph. The TM might presumably be able to impede the passive diffusion of ions between inner sulcus and endolymph. It might also be capable of separating a potential difference. The TM can interact mechanically by channeling any fluid flowing in the vicinity of the stereocilia (Steele, 1973), or by directly modifying the nature of any shearing force delivered to the hair cells.

In addition, experimental *in vitro* findings suggest the ability of the TM to propagate travelling waves along its length (Ghaffari *et al.*, 2007).

1.2.4.2. Lateral wall: stria vascularis and spiral ligament

The lateral wall, consisting in the stria vascularis and the spiral ligament defines the lateral aspect of the cochlear scala.

Stria vascularis

The stria vascularis generates the endocochlear potential and maintains the ionic composition of the endolymph (Wangemann, 1997), the fluid in which the apical surface of the hair cells, including the stereocilia, is bathed. The ultrastructure of the cells of the stria vascularis has been studied for many years in guinea pigs (Engstrom *et al.*, 1955; Smith, 1957; Rodriguez-Echandia and Burgos, 1965), cats (Hinojosa and Rodriguez-Echandia, 1966) mice (Spoendlin, 1967) and human (Kimura and Schuknecht, 1970). The cytoarchitectures differ somewhat in different species, but consistent agreement is noted in the presence of three types of cells, from medial to lateral: marginal cells, intermediate cells and basal cells.

Marginal cells

The marginal cells are a homogenous layer of polarized epithelial cells that derive from the membranous labyrinth. These cells are organized as one layer that lines the scala media fluid space.

One of the roles of the stria vascularis is to pump Na^+ away from the endolymph (Iwasa *et al.*, 1994) and provide it with high K^+ concentration. NaK-ATPase plays an important role in stria vascularis function for

generation of the endocochlear potential and maintenance of the ionic composition of endolymph (Offner *et al.*, 1987; Sakaguchi *et al.*, 1998).

In odontocetes, the stria vascularis is exceptionally dense; in observations in transmission electron microscopy were found up to five layers of marginal cells in the base (Ketten, 2000).

Intermediate cells

Lateral to the marginal cells are the intermediate cells. These interdigitate with the basal aspect of the marginal cells but do not reach the luminal surface. The intermediate cells contain melanin, and are often referred to as melanocytes (Hilding and Ginzberg, 1977). It was suggested by Steel and Barkway (1989) that melanocytes play an important role in the generation of endocochlear potential and that this function is independent of melanin.

Basal cells

Basal cells are located lateral to the intermediate cell layer, adjacent to the spiral ligament. This flat cells lack NaK-ATPase, suggesting that their main role may be related to establishing a barrier between the stria vascularis and the spiral ligament.

Some changes were observed at the level of stria vascularis after noise-induced hearing loss, such as an acute swelling, accompanied by an degeneration of strial intermediate (irreversible), and marginal cells, disruption of the basal layer, with gaps between basal cells, and a drastic reduction in membrane surface area of marginal and intermediate cells (Hirose and Liberman, 2003).

Spiral ligament

The spiral ligament is located between the stria vascularis (medially) and the otic capsule. It is composed mainly of connective tissue elements including extracellular material.

In addition to containing the capillary bed and providing mechanical support to the stria vascularis, the spiral ligament has other important functions:

1) It anchors the lateral aspect of the basilar membrane. Fibroblasts with stress fibers (tension fibroblasts) that contain contractile proteins (myosin and actin, Henson and Henson, 1988) are present in the tissue that anchors the spiral ligament to the basilar membrane, suggesting that the spiral ligament can generate and/or regulate basilar membrane tension (Henson *et al.*, 1984).

Hsp27 immunostaining has been demonstrated in tension fibroblasts of the spiral ligament, suggesting a potential role in the regulation and maintenance of the actin cytoskeleton in these cells (Leonova *et al.*, 2002). Hsp27 may also play a protective role in these cells, which may be required due to their tensile properties and potential for mechanical injury.

2) It maintains the ionic balance in the cochlea. Aided by gap junctions and NaK-ATPase pumps, the spiral ligament is thought to pump K^+ out of the perilymph and transport it for maintaining the high concentration of K^+ in the endolymph (Spicer and Schulte, 1991).

Type II, IV and V fibrocytes function to pump K^+ from the perilymph and produce a K^+ flow through gap junctions to Type I fibrocytes and strial basal cells (Spicer and Schulte, 1991; Spicer and Schulte, 1996).

Pathological changes in fibrocyte subtypes have been linked to noise-induced hearing loss as well as age related hearing loss, where fibrocyte pathology, particularly Type IV, was shown to precede hair cell loss (Hequembourg and Liberman, 2001). In addition, massive loss of type II fibrocytes was observed in chronic state of noise-damaged cochleas, which could regenerate over time, as well as irreversible degeneration of type IV fibrocytes (Hirose and Liberman, 2003).

The fibrocytes in odontocetes are heavily packed, specially the tension fibroblasts throughout the cochlea. The collagen fiber density is two- to fivefold that of most mammals with only moderate decreases in the cell packing density in the most apical regions (Ketten, 1995). In the echolocating horseshoe bat the tension fibroblasts are also very prominent and dense (Henson and Rubsamen, 1996). The most likely effect of increased tension would be greater stiffness of the basilar membrane and an increased speed of travelling waves.

1.3. Acoustic pollution effects on marine organisms

There is an increasing consensus about the potential impact of man-made sound on marine organisms. The conscious awareness of this issue has been reinforced by a series of strandings (especially in the case of cetaceans and cephalopods, see below) coinciding with the exposure to man-made sound sources.

1.3.1. Invertebrates and fishes

Very little is known about effects of anthropogenic sounds on fishes (see reviews in National Research Council, 1994; National Research Council, 2000; National Research Council, 2003; Popper, 2003; Popper *et al.*, 2004; Hastings, 2008; Popper and Hastings, 2009; Slabbekoorn *et al.*, 2010). Some studies report an effect of ship noise on fish flight behaviour (Vabø *et al.*, 2002; Handegard *et al.*, 2003; Sarà *et al.*, 2007), while others have shown an increase in secretion of the stress hormone cortisol during exposure to white noise or simulated boat noise (Smith *et al.*, 2004; Wysocki *et al.*, 2006). Impeding the ability of fish to hear biologically relevant sounds might interfere with critical functions such as acoustic communication, predator avoidance and prey detection, and use of the 'acoustic scene' or 'soundscape' (Fay and Popper, 2000; Slabbekoorn and Bouton, 2008) to learn about the overall environment. In more severe cases, fish died during exposure to underwater sound from pile driving operations (Caltrans, 2001). However, other studies on pile driving showed no significant differences in exposed animals (Nedwell *et al.*, 2003; Caltrans, 2004; Abbott *et al.*, 2005; Nedwell *et al.*, 2006; Ruggerone *et al.*, 2008). In addition, the study of hair cells after exposure of sounds of seismic air gun (McCauley *et al.*, 2003) or continuous exposure to a 300 Hz pure tone with a peak level of 180 dB *re* 1 µPa (Hastings *et al.*, 1996), showed severe damage of the sensory hair cells.

There is growing interest regarding the effect exposure to anthropogenic noise can also have on invertebrates, especially after the two incidents of multiple strandings of giant squids (*Architeuthis dux)* affecting nine specimens in 2001 and 2003. They appeared to be linked spatially and temporally to geophysical prospecting using air-gun arrays (Guerra *et al.*, 2004). The specimens presented evidence of acute tissue damage, especially in the microvascular branchial system and the hair cells of the angular acceleration receptors or statocysts. Recently, André and colleagues (2011) analysed under electron microscope the hair cells of the statocysts in four cephalopod species subjected to low-frequency controlled-exposure experiments. They found evidences of massive acoustic trauma, such as changes in the kinocilia, rupture of plasma membrane, ejection of hair cells, modification in the cytoplasmatic content (increasing of vacuole number and presence of electron dense inclusions) and degeneration of afferent nerve fibres.

More studies have showed low-frequency hearing in other invertebrate species and their reaction to sound, which increment the number of phylums susceptible to be affected by man-made noise. For example, the larvae of coral (Vermeij *et al.*, 2010) and of a number of crab species (Stanley *et al.*, 2010) were found to orientate and swim toward ambient underwater sound emanating from coastal settlement habitats, the latter changing their swimming behaviour and decreasing time to metamorphosis.

1.3.2. Marine mammals

Marine mammals, notably cetaceans, depend on acoustic exchange for a great number of activities and vital behaviors such as communication, geographical orientation, habitat relationships, feeding and a wide range endeavors within the broader social group (cohesive action, warnings and maternal rapports). On account of their fundamental role in the balance of the marine food chain, cetaceans were chosen as bioindicators of the interaction with noise of anthropogenic origin.

Anthropogenic originated sound can affect cetaceans in different ways (see Table 1.3.1 and Richardson *et al.*, 1995; Perry, 1998; Hildebrand, 2005; Southall *et al.*, 2007; Weilgart, 2007; Boyd *et al.*, 2008; Hastings, 2008 for review), and these effects can be at individual or group level. One of them is the acoustic **masking** of vital information, i.e., any sound at a determinate intensity level and frequency can be contaminant if it prevents or interfere the good reception of sonar echoes or acoustic communication signals. The direct consequences of this masking of communication and related signals can be diverse: group dispersal, reducing a fundamental part of their interaction with the natural environment (echolocation, André and Nachtigall, 2007), impaired feeding ability and the separation of mothers-calves. The responses of different cetacean species to the presence of ambient noise have different results, some of which have been documented (see Appendix 2.1). For example, while sperm and pilot whales have been observed to cease vocalizations during the exposition of intense noise sources (Bowles *et al.*, 1994; Andre *et al.*, 1997), beluga whales (Au *et al.*, 1985; Lesage *et al.*, 1999) and dolphins (Au, 1993) increased the intensity and frequency of their vocalizations to compensate for the presence of ambient noise. Despite these strategies, it is likely that the level of efficient communication has been reduced and that this reduction has limited their ability to react to stressful or dangerous situations.

The acoustic pollution can also affect their **behaviour**. The behavioural change responses to noise are complex and still not fully known (Richardson *et al.*, 1995). It may be that they are conditioned by certain factors such as hearing sensitivity, behavioural state, habituation or desensitization, age, sex, presence of offspring, proximity to exposure and distance from the coast (Richardson and Wursig, 1997; Ketten and Finneran, 2004; Richardson and Tyack, 2004).

Short term reactions to man-made sounds on cetaceans include sudden dives, orientation away from the sound source, changes in vocal behaviour, longer dive times, shorter surface intervals with increased blow rates, attempts to protect the young, increased swimming speed and departure from the ensonified area (see Appendix 2.2). In general, cetaceans are more susceptible to a specific noise when it is new or when or its intensity level is increasing (Edds and Macfarlane, 1987). Disturbance is the most commonly observed effect of noise on cetaceans, and probably the most difficult to assess in the long term. It is often assumed that the zone of disturbance, in the case of continuous sounds, corresponds to a broadband sound pressure level of 120 dB *re* 1 µPa at 1m (Erbe and Farmer, 2000).

Little is known with respect to the long term effects on behavioural changes in individuals or populations. Nevertheless, it is possible to confirm that the disruption of feeding activity, reproduction, migration or caring for the young induced by noise, has the potential to result in reduced food intake, breeding success or survival rate of offspring. These detrimental impacts will be more severe in cases where cetaceans have been displaced (permanently or temporarily) from important breeding and feeding zones.

Following exposure to sound can also induce **stress**, i.e., describe physiological changes that transpire in immune (and neuroendocrine) systems. Prolonged stress brought about by noise may weaken resistance to illnesses and endocrine imbalances that could affect an animal's ability to reproduce (Geraci and St. Aubin, 1980). Elevated levels of cortisol, for example, result in a reduction of the white blood cells essential to a functioning immune system and thus resistance to infections (Gwazdauskas *et al.*, 1980; Appendix 2.3).

In addition, **physical damage in non-auditory structures** can also be a consequence of an overexposure to anthropogenic sound (see Appendix 2.3). The post-mortem examination of animals stranded after exposure to low frequency sonar in the Canary Islands in 2002 (Martín, 2002; Martín *et al.*, 2004), in 2004 (Espinosa De Los Monteros *et al.*, 2005; Fernández, 2006b) and in Almeria in 2006 (Dalton, 2006; Fernández, 2006a; Fernández, 2006b) showed syndromes in line with a fat and gas embolism (Jepson *et al.*, 2003; Fernández, 2004; Fernández *et al.*, 2005b; Fernández *et al.*, 2005a; Fernández, 2006b) with symptoms that manifested a certain analogy with sicknesses associated with decompression in human beings (DSC Syndrome), although there is no scientific consensus on this subject (Piantadosi and Thalman, 2004; Jauniaux *et al.*, 2011).

Another way in which noise pollution may affect cetaceans is the repeated exposure to certain levels and perceptible frequencies (see below). As demonstrated in humans and other terrestrial mammals (see Saunders *et al.*, 1985a; Borg *et al.*, 1995; Salvi *et al.*, 1995 for review) these expositions can cause lesions leading to **hearing loss**.

Yet, the current scientific knowledge on the effect on noise on marine mammals (and marine fauna in general) and their habitat is still insufficient to understand the relationships between frequencies, intensities, and duration of exposures and the damage produced.

Table 1.3.1. Types of anthropogenic sound that can affect marine mammals (source: Boyd *et al.*, 2008)

Source	Effects of greatest concern
Ships	Masking Habitat displacement
Airguns (compressed air)	Masking Physical trauma Auditory loss Behavioural changes Habitat displacement Behaviour conditioning effects
Intense low or mid frequency sonar activity	Physical trauma Auditory loss Behavioural change Behaviour conditioning effects
Pile driving	Physical effects Auditory loss Behavioural change Behaviour conditioning effects
Other types of sonar (deepwater soundings,	Masking

trawlers, fishing boats)	Auditory loss
	Behavioural change
	Behaviour conditioning effects
Dredgers	Behavioural change
	Habitat displacement
	Behavioural conditioning effects
Drilling	Auditory loss
	Behavioural change
	Behaviour conditioning effects
Towed fishing materials	Behavioural change
	Behaviour conditioning effects
	Habitat displacement
Explosions	Physical trauma
	Auditory loss
	Behavioural change
	Behaviour conditioning effects
Recreational boats	Masking
	Behavioural change
	Behaviour conditioning effects
Acoustic hardware	Behaviour conditioning effects
Airplanes	Behaviour conditioning effects

Acoustic trauma

Treshold shift

The minimum level at which a sound can be perceived is called the auditory 'threshold'. If an individual needs a significantly greater sensitivity than is normal for its species to perceive a particular frequency, an auditory deficit marked by a change in the threshold level or *threshold shift* occurs.

This threshold change can be reversible or permanent. If the received emission causes a temporary loss, i.e. a *temporary threshold shift* (TTS), it will eventually return to normality sometime after exposure. If a received emission produces a permanent hearing loss (permanent change of auditory threshold) this is considered to be a *permanent threshold shift* (PTS). A PTS is an auditory injury.

Terrestrial mammals

By means of studies performed in land mammals, it is known that the influencing factors on the magnitude of auditory threshold change or threshold shift include intensity, duration, and frequency content, temporal pattern and energy distribution of the exposure to noise. Continuous noise causes greater damage to the cochlea than intermittent noise of the same intensity (Eldredge *et al.*, 1959; Kryter *et al.*, 1966; Sataloff *et al.*, 1969; Schmidek and Carpenter, 1974; Schmidek *et al.*, 1975; Sataloff *et al.*, 1983; Ward, 1970; Fredelius

and Wersall, 1992) and that, at the intensities tested, damage to the cochlea is not proportional to the total noise energy (Pourbakht and Yamasoba, 2003). In relation to the received frequency has been shown that high frequencies are much more damaging than low frequencies at the same level of intensity (Davis *et al.*, 1950).

At present, at least two mechanisms have been proposed for noise induced hearing loss (NIHL): a) mechanical injuries to the receptor cells induced by excessive movement of the cochlear partition (Saunders *et al.*, 1985a) and b) damage due to metabolic exhaustion resulting in distortion of the homeostasis of the organ of Corti (Slepecky, 1986).

The following ultrastructural changes in cochlear hair cells as a consequence of a TTS were described in land mammals: 1) damage to stereocilia on sensory cells, such as floppy, disarrayed, loss in stiffness (Saunders and Flock, 1986), 2) disruption of cuticular plates (Lim and Melnick, 1971; Lim, 1986b), 3) an increase in formation of blebs on the surface of the sensory hairs, 4) vesiculation proceeding to vacuolization of the smooth endoplasmic reticulum system and subsurface cisternae; 5) heavy accumulation of lysosomal granules in the subcuticular region; 6) changes in Golgi's apparatus (Lim and Melnick, 1971), 7) increase in lysosomes, multivesicular bodies, and 8) proliferation of Hensen bodies (Slepecky *et al.*, 1981). Intense sound can also induce excitotoxicity, which is the excessive release of neurotransmitter from the IHCs to the underlying post-synaptic element (see: Eybalin, 1993 for review). The dendritic swelling caused by excitotoxicity is reversible (Robertson, 1983; Puel *et al.*, 1998; Pujol and Puel, 1999), although recent data suggest that it can be followed by delayed ganglion cell death (Lin *et al.*, 2011). 8) It is also possible a reversible blockage of the transduction channels from the endolymph side of the hair cells (Lim, 1986b).

Some of the apparent causes of PTS in mammals are severe extensions of the underlying effects of TTS. PTS lesions as a consequence of noise exposure include: 1) damage in stereocilia, like buckling, fracturing, folding, fusion, formation of giant hairs, loosening of the stereocilia membranes, disintegration of the rootlets and or a complete disappearance of hairs (Spoendlin, 1971; Bredberg *et al.*, 1972; Slepecky *et al.*, 1981; Engstrom *et al.*, 1983; Engstrom *et al.*, 1984; Figure 1.3.1b-d), 2) degeneration of sensory cells (karyorrhexis, karyopyknosis; swelling of the nuclei and with vacuolization of the cytoplasm and mitochondria degeneration; Spoendlin and Brun, 1973; Hu *et al.*, 2000) that leads to a loss and scar formation (see below, Hawkins *et al.*, 1976) , 4) rupture of dendrites with incipient retrograde nerve degeneration (Spoendlin, 1971), 5) accumulation of synaptic vesicles in the medial efferent endings (M. Lenoir, personnal communication) or in the most severe cases 6) the lesions can be from a loss of adjacent supporting cells to a complete degeneration of the organ of Corti (Bredberg *et al.*, 1972; Figure 1.3.1i). When a hair cell dies in mammals do not regenerate, but the neighbouring supporting cells actively participate in the process of hair cell elimination and scar formation by rapidly expanding and sealing the reticular lamina (Figure 1.3.1e). This scarring process prevents the potassium-rich endolymph leakages into the fluid bathing the basal domain of hair cells where terminals of the auditory nerve reside, which would depolarize the neurons, abolish hearing and lead to further tissue damage (Raphael, 2002). Following noise exposure, it may be an activation of a Scr-protein tyrosine kinase signaling cascade that may be involved in mechanically and metabolically induce hair cell apoptosis (Harris *et al.*, 2005).

At the level of the tectorial membrane, morphological changes on the outer hair cell stereocilia imprints were also shown (Morisaki *et al.*, 1991) after acoustic overstimulation. They consisted in a transformation from circular to oval shape or irregular shape, fusion of adjacent concavities to form a larger concavity, and

occasionally the appearance of filamentous material (Figure 1.3.1g). This modification on the imprints remained for a considerably long period of time after sensory hairs had disappeared.

In addition, at the level of the lateral wall, chronic changes after acoustic overstimulation consisted in massive loss of type II and IV fibrocytes and degeneration of strial intermediate and marginal cells; type II fibrocytes may regenerate over time, but type IV do not (Hirose and Liberman, 2003).
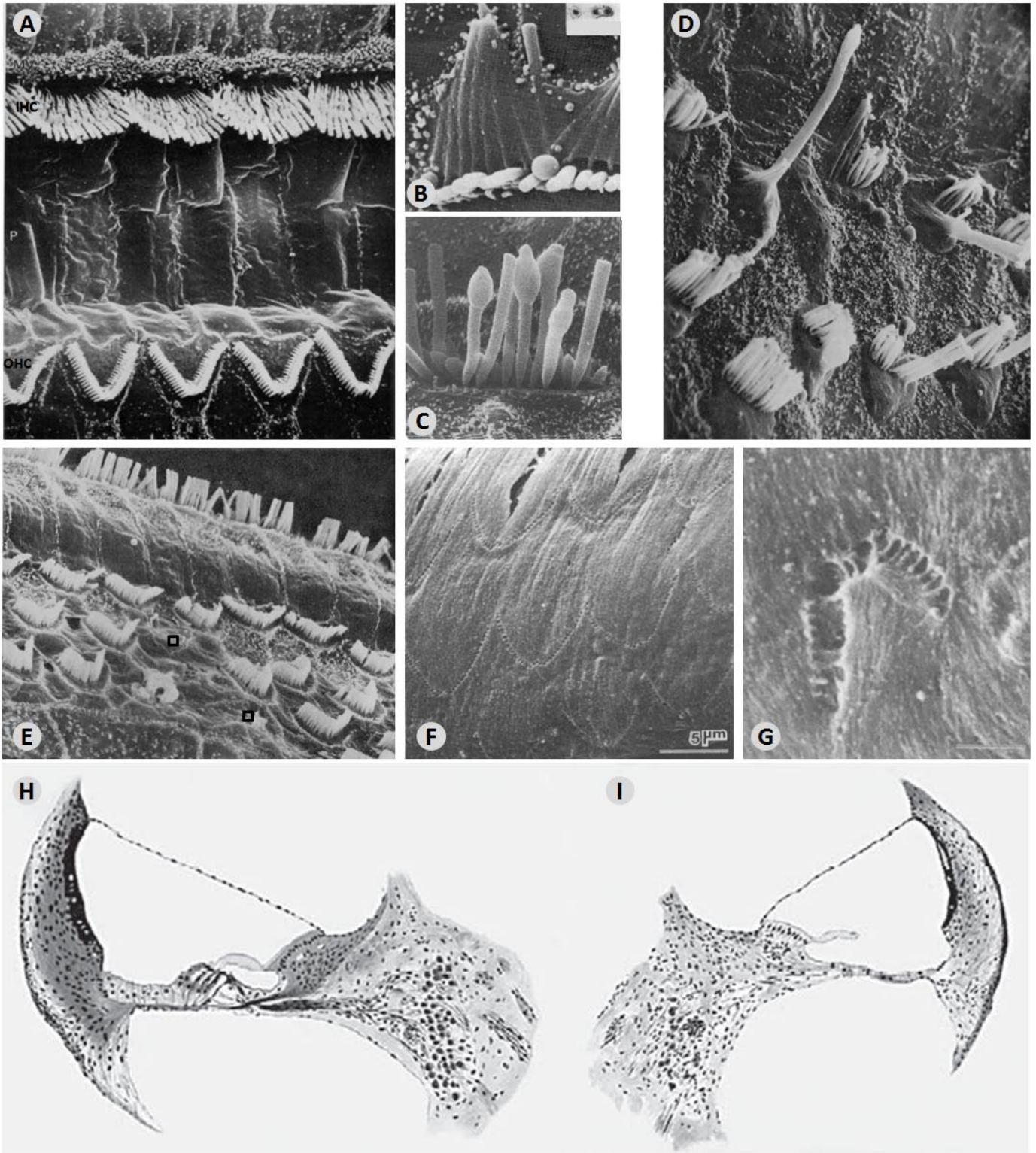
Figure 1.3.1. Changes in the organ of Corti after acoustic overstimulation. A-G) Scanning electron microscope (SEM) images of the cat (A-E) and the guinea-pig (F, G) cochlea. A) Image of the reticular lamina, where the normal aspect of the stereocilia of inner hair cells (IHCs) and the first row of outer hair cells (OHCs) can be seen. B-E) Damage to the stereocilia after noise exposure. B) fusion (insert : transmission electron microscopic image), C) formation of blebs and D) giant hairs. E) Squares mark missing OHCs and formation of scars after noise exposure. F) Undersurface of the tectorial membrane: the OHC stereocilia imprints are regular. G) Deformed and fused imprints after exposure to high intensity sound (blast of starting pistol). H, I) Light microscopy images of the chinchilla H) Normal aspect of the cochlear duct. I) Almost complete disappearance of the organ of Corti after a severe sound exposure (sources: A, D and E) Bredberg *et al.*, 1972; B and C) Engstrom *et al.*, 1984; F and G) Morisaki *et al.*, 1991; H and I) Hössli, 1912; Hawkins and Schacht, 2005).

Cetaceans

The relationship between TTS and PTS depends on a great number of variable complexities that concern the subject of study and the exposure to which it has been subjected (see Southall *et al.*, 2007 for review). A PTS may arise after a long period of exposure (Richardson *et al.*, 1995) or immediately following an exposure to highly elevated sound levels, such as those caused by explosions (Scheifele, 1997). Anatomical and behavioral studies suggest that cetaceans may be far more resistant to TTS than land mammals from having evolved in a relatively noisy environment (Perry, 1998). It is important to bear in mind that cetaceans also suffer from hearing loss (Mann *et al.*, 2010), some of them as a consequence of old age (Ridgway and Carder, 1997; Ketten, 1998; Kloepper *et al.*, 2010). Finally, a severe change in threshold shift has been linked with hydrocephalic sickness in one example of a stranded striped dolphin, indicating that lesions in the central nervous system could be at the origin of a PTS (André *et al.*, 2003; André and Nachtigall, 2007).

Auditory loss, whether temporary or permanent, can affect these animals in many ways. A temporal loss can impede the animal in detecting its prey or predators, or result in the animal entering an area that would be dangerous for its survival. In addition to these effects, permanent loss of hearing could result in loss of an animal's ability to communicate with conspecifics, find mates, care for young, or find food. Over the long term, loss of hearing capabilities by large numbers of a species could lessen reproductive potential and survival of the species. These damages have been deemed to be the result of receiving intense sound pressure (D'Amico, 1998; Gordon *et al.*, 1998b; Ketten, 1998; Finneran *et al.*, 2002; Degollada *et al.*, 2003; Ketten and Finneran, 2004; Ketten *et al.*, 2004; Ketten, 2004) and could in turn be the cause of successive strandings. In the long term, any loss in hearing capacity of large numbers of individuals of any species may diminish its reproductive potential and thus its survival as species.

Data from PTS and TTS of land mammals has been used in developing safe exposure guidelines in the workplace (example, National Institute for Occupational Safety and Health (NIOSH), 1998). Recently published data on sounds that cause light TTS (generally lower than 20 dB in auditory sensitivity) in toothed whales and pinnipeds has established a sound exposure level of 192-195 dB re 1 µPa2s as the threshold beyond which a TTS is created in dolphins and belugas exposed to mid frequency tones (Ridgway *et al.*, 1997; Schlundt *et al.*, 2000; Finneran *et al.*, 2005; Finneran and Schlundt, 2010). In addition, it was suggested that a received level of 80 to 140 dB over species-specific threshold for a narrow band source will induce temporary to permanent loss for hearing in and near that band in pinnipeds and delphinids (Ketten, 1998).

Shift in the auditory medium threshold of 4 dB at 8 kHz and a change of 8 dB at 16 kHz have been observed following exposure to noise in the octave band centred in 7.5 kHz (Nachtigall *et al.*, 2004). A similar change in threshold shift was observed with higher frequencies (Schlundt *et al.*, 2000; Finneran *et al.*, 2007) in the

case of cetaceans that have their maximum sensitivity in medium frequencies. It has also been noted that if octave band levels of a received signal noise are more than 96 dB above the central frequency of an audiogram, TTS could occur from 12 to 18 dB after an exposure of 30 minutes (Au *et al.*, 1999). In controlled experimental studies, mid-frequency sonar could induce temporary hearing loss in a bottlenose dolphin, following repeated exposure to intense sonar pings with total SEL of 214 dB re1 µPa²s (Mooney *et al.*, 2009). There is still no data on the characteristics of the exposures which may cause PTS in cetaceans.

PTS levels are very difficult to establish experimentally because, for ethical reason, cetacean can not be submitted to acoustic tolerance tests, which may cause extreme suffering or death of individuals. In addition, data are lacking for most species of cetaceans due to the difficulty of keeping them in captivity or to access them in their natural environment. The accessibility of stranded cetaceans, belonging to different species, is today the largest source of information on these mammals.

In necropsies performed on beaked whales that had atypically stranded in the Bahamas (NOAA and U.S. Navy, 2001; Ketten *et al.*, 2004) and the Canary Islands (Fernández, 2004; Fernández *et al.*, 2005a; Fernández *et al.*, 2005b; Fernández, 2006b), multiple haemorrhages were found to affect, particularly the kidneys, lungs, eyes, oral cavities, peribular tissues and the inner ear cranial cavities, tissue surrounding inter-cranial membranes and along the length of the acoustic fatty tissue (mandibles and peribular sinuses).

Nevertheless, some atypical cases of beaked whale strandings occurred due to exposure to sound levels inferior to those considered to cause TTS (Finneran *et al.*, 2002). Acoustic field models of beaked whale strandings (Bahamas Islands 2000) showed that the affected individuals were probably exposed to levels inferior to 150- 160 dB$_{RMS}$ for 50-150 s, however the received levels were certainly far less most of the time (Hildebrand *et al.*, 2004; Hildebrand, 2005; Balcomb, 2006). These levels are far lower to those that are suspected to be the cause of hearing loss in small toothed whales, or to those that are used by some regulatory authorities as acceptable or safe for use in management guidelines (E.g. California Coastal Commission, 2002).

In Appendix 2.4 details are shown of up-to-date published works on hearing loss on cetaceans.

Very little is known on cetacean auditive capacities and the functionallity of the acoustic reception pathway. There are only few anatomical studies of the organ of Corti in odontocetes (*Tursiops truncatus*: Wever *et al.*, 1971a; Wever *et al.*, 1971b; Wever *et al.*, 1971c; *Lagenorhynchus obliquidens*: Wever *et al.*, 1972, see Ketten, 1997; Ketten, 2000). Its ultrastructure has not been studied in detail due to the great difficulties in collecting specimens and typically rather long post-mortem times prior to tissue fixation. Since toothed whales (or odontocete) have species-specific acoustic repertoir, it is expected to find differences in their cochlear morphology.

This dissertation will focus on the examination of the organ of Corti cells under electron microscopy as a contribution to the knowledge of the odontocete ultrastructural cochlear features with a reference to possible changes in cochlear hair cells due to noise exposure.

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2. Materials and methods

2. MATERIALS AND METHODS

2.1. Species

We analyzed 150 ears from 13 odontocetes species that stranded in the Mediterranean Sea, Spanish, French and Portuguese North Atlantic, North Sea and Celtic Sea (Table 2.1.1)

Table 2.1.1. Total number of samples by species and location (*one of them comes from the Adriatic Sea)

species	Mediterranean Sea	Spanish North Atlantic	Portuguese North Atlantic	French North Atlantic	North Sea	Celtic Sea	n
Phocoena phocoena			1		67		68
Stenella coeruleoalba	14	16		1		1	32
Stenella frontalis		13					13
Tursiops truncatus	2*	7					9
Delphinus delphis		3	5	2		2	12
Steno bredanensis		2					2
Kogia breviceps		2		1			3
Kogia simus		2					2
Globicephala macrorhynchus		1					1
Globicephala melas		3					3
Lagenodelphis hosei		1					1
Hyperoodon ampullatus				2			2
Ziphius cavirostris	1			1			2
Total							**150**

2.2. Ear extraction and fixation

The ears were extracted according to the protocol presented at the European Cetacean Society Conference in Istanbul Morell and André, 2009, and adopted at the Necropsy Workshop, Liège 2009:

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UPC
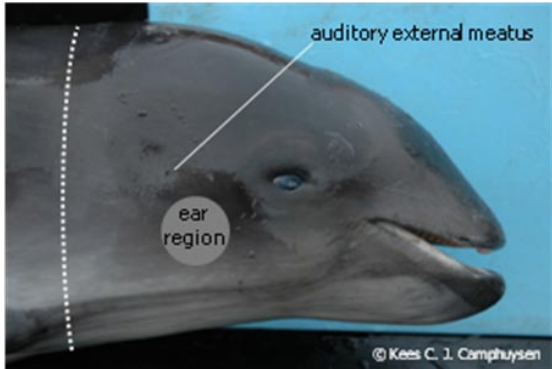
CETACEAN EAR EXTRACTION AND FIXATION PROTOCOL

Extraction

1. With small specimens, it is recommended to cut the head of the animal for an easier manipulation (Figure 1).

Figure 1. The position of the T-P complex and auditory external meatus is are indicated. The dotted line marks the incision path to separate the head from the rest of the body. Alternatively, the digestive system can be extracted from the head to facilitate the access to the ears.

2. Taking into account the localization of the tympanic-periotic complex (Figures 1 and 2), the easiest way to access the ears is to carefully remove the lower jaw.

Figure 2. Sagital cut of a bottlenose dolphin head where the location of the tympanic-periotic complex is indicated.

4. Situating the head in a ventral position and removing the soft tissues and ligaments (Figure 3) allows to proceed to the tympanic-periotic complex extraction.

Figura 3. Image taken during the necropsy of a *Phocoena phocoena*. This image reflects how the tympanic-periotic complex appears after removing the lower jaw (no effort has been made here to clean the area of extraction)
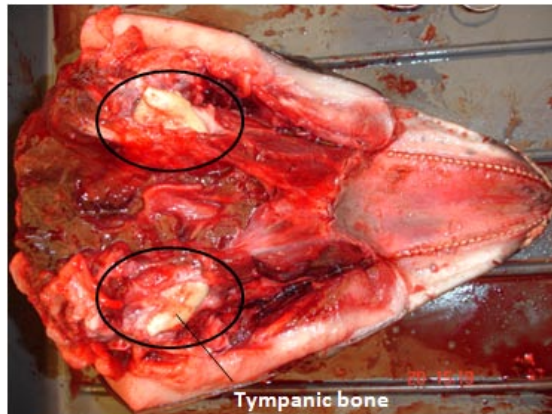
5. Incise **gently around** the tympanic-periotic complex with a small knife (a scalpel can be used for the final stage of the extraction) to cut the ligaments that mantain the ears in the paraotic sinus (see Figure 4).

Figura 4. Image taken during a *Phocoena phocoena* necropsy. The dotted line illustrates the location where the knife should be placed to extract the tympanic-periotic complex.
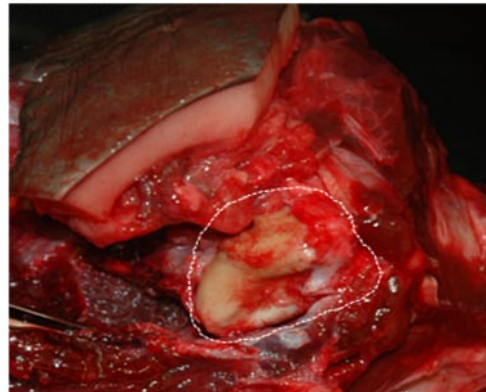
Fixation

6a. At that stage, the ear could be fixed simply placing it in a fixative solution: glutaraldehyde 2,5% with phosphate buffer 0,1M (these solutions will be provided). The ears can also be injected with a mixture of paraformaldehyde 0,5% with glutaraldehyde 1% with phosphate buffer 0,1M or alternatively be injected with formaldehyde 10%.

However, for a better result we recommend to follow the protocol described in point 6b.

6b. If already experienced with the injection protocol, you may want to:

>   separate the periotic from the tympanic bone (Figure 5);

>   cut the stapedial ligament and remove the stapes. If it does not come off easily, it helps passing a scalpel through the junction;

>   make a **little and very superficial** hole to the oval and round window membranes;

using a soft catheter from the same diameter as the windows size, **progressively and very slowly (with very little pressure)** introduce the fixative solution (glutaraldehyde 2,5% with phosphate buffer 0,1M; Figure 6) through the oval window and the round window until the solution gets out through the other one during some seconds.
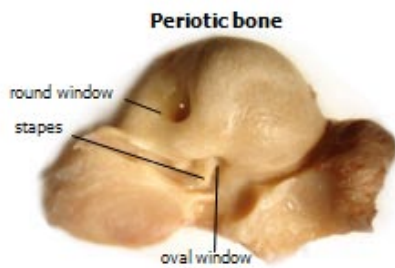
Figure 5. Localization of the oval and round windows in the periotic bone.

The ears can also be injected with a mixture of paraformaldehyde 0,5% with glutaraldehyde 1% with phosphate buffer 0,1M or alternatively be injected with formaldehyde 10%.

The injection is a very delicate process and if you do not feel comfortable with it, please do not perform it. It is important to mention if the ear has been injected or not when sending it. In any case, before performing the injection, you are welcome to contact us.
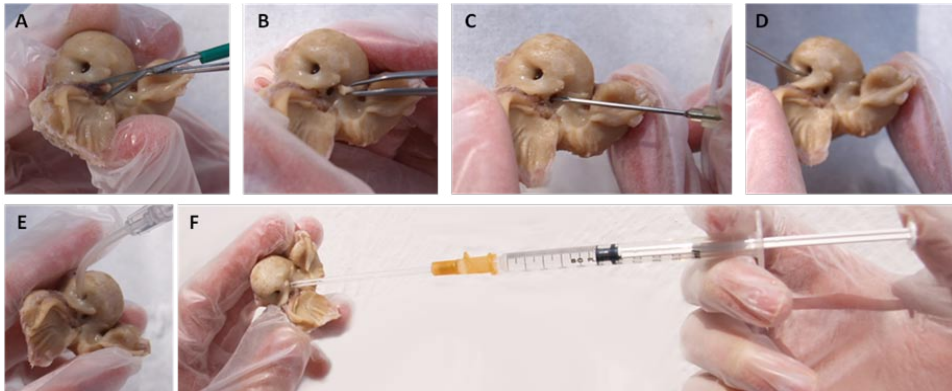
Figure 6. *Tursiops truncatus* periotic bone used to illustrate all the injection process: A) cut of the stapedial ligament, B) stapes extraction, C and D) realization of a little and very superficial hole to the oval and round window membranes respectively, E and F) very slow and progressive perfusion (with very little pressure) of the fixative through the oval window and the round window until the solution gets out through the other one during some seconds.

7. Place the ears in jars that contain the fixative liquids (see point 6).

After extraction, the samples were fixed with 10% buffered formalin or 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.3-7.4) and some of them perfused through the oval and round window (see Table 2.2.1 for more details).

Table 2.2.1. Total number of samples by species (n), decalcification agent and type of analysis performed. RDO®: commercial rapid decalcifier (Apex Engineering Products Corporation, Aurora, Illinois, USA), EDTA: Ethylenediaminetetraacetic Acid, Mw: microwave oven, SEM: scanning electron microscope, TEM: transmission electron microscope, Immuno: Immunohistochemistry.

species	n	Perfused	RDO®	EDTA	EDTA + Mw	Technovit	SEM	TEM	Immuno
Phocoena phocoena	68	18	56	9	3		17	2	1
Stenella coeruleoalba	32	18	23	6	1	2	25	1	
Stenella frontalis	12	4	13				4		
Tursiops truncatus	9	1	9				3		
Delphinus delphis	12	9	7	2	2	1	7		
Steno bredanensis	2	0	2						
Kogia breviceps	3	1	3				1		
Kogia simus	2	0	2					1	
Globicephala macrorhynchus	1	0	1						
Globicephala melas	3	2	2	1			3		
Lagenodelphis hosei	1	0	1				1		
Hyperoodon ampullatus	2	2	1	1			1		
Ziphius cavirostris	2	1	2				1		
Total	**150**	**55**	**122**	**19**	**6**	**3**	**63**	**4**	**1**

2.3. Decalcification

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. Decalcification procedure was used to subsequently observe the ears using several imaging techniques (see below) except for the computerized tomography. Two different solutions were tested: RDO® and Ethylenediaminetetraacetic acid (EDTA).

2.3.1. RDO®

RDO® is a rapid decalcifier based on hydrochloric acid (Apex Engineering Products Corporation, Aurora, Illinois, USA). A decalcification protocol to precisely determine the decalcification time with different concentrations of RDO® was developed Morell *et al.*, 2009. 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).

2.3.2. Ethylendiaminetetraacetic Acid (EDTA)

A solution of 14% Ethylenediaminetetraacetic acid (EDTA) tetrasodium salt at pH 7,4 and 7,6 was used either at room temperature (changing the media once a week; Callis and Sterchi, 1998 or in a microwave oven (Leica AM AMW, 45ºC, 30W in the Centre de Ressource en Imagerie Cellulaire of Montpellier, CRIC, France

and Milestone Ethos Plus, 45ºC, in the Research Technical Service of Girona University, Spain), changing the solution every 1-2 hours and overnight at room temperature Madden and Henson, 1997; see Table 2.2.1).

2.3.3. Experiments with Technovit 7200 VLC

To test the possible effects of the decalcification process on the results, some preliminary experiments were conducted without decalcifying the bone using Technovit 7200 VLC®. Technovit 7200 VLC® (Heraus Kulzer GMBH, Werheim, Germany) is an acrylic resin (light-curing methacrylate-based one-component resin) formulated for embedding undecalcified hard tissues. This resin has the advantage of completely penetrate the hard tissue with a minimal thermal stress (a temperature of 40 C is not exceeded).

The protocol used to embed the periotic bones was the following:
1 hour in 2.5% glutaraldehyde
2 hours in PBS 1X + 2 hours in PBS 1X
1 day in ethanol 70%
1 day in ethanol 96% + 1 day in ethanol 96%
1 day in ethanol 100% + 1 day in ethanol 100%
5 hours in xylol + 19 hours in xylol
1 day in Technovit 7200 VLC®
20 days minimum in Technovit 7200 VLC® with agitation and overnight in the fridge

Once the tissue was embedded, it was polymerized under ultraviolet light for 5 hours. The blocs were cut using an electric saw, polished to 60-80 micron, stained with hematoxylin and eosine and mounted in DPX. The observation was conducted under light microscope.

Three periotic bones were used for this preliminary test from: 1) a striped dolphin stranded in Catalonia that was very autolytic to set up the protocol, 30 days in the resin, 2) a common dolphin that stranded in France, that was fixed in formalin 10 hours post-mortem and injected with glutaraldehyde 20-22 hours pos-mortem, that stayed 35 days in the resin, and 3) a striped dolphin stranded in the Spanish Mediterranean coast, injected at least 22 hours post-mortem, immersed 2 months in the resin.

This experiment was performed in collaboration with the Anatomy and Human Embryology Unit (University of Barcelona), under the supervision of Dr. Maria Cristina Manzanares and the technical assistance of Eva María Sánchez. The polishing was done at the facilities of the Department of Materials Science and Metallurgical Engineering (Technological Center of Vilanova i la Geltrú, UPC, Barcelona Tech).

2.4. Imaging techniques

2.4.1. Computerized tomography

In a preliminary study, a comparative analysis of the odontocete ear morphology using the diagnosis image method computerised tomography (CT) was conducted. CT is an x-ray exploration based on the protons differential attenuation of different atomic species that produces detailed images of axial cuts. The CT image is obtained through the combined movement of the x-ray tube and the detector that rotate around the

sample to be scanned. The attenuation coefficients are received and measured by the detectors that send the signal to a processor, which transforms the images following a grey scale, depending on the density. Amongst other advantages, CT is a non-invasive technique and allows the information, obtained in a series of slices, to be further rendered in 3D. Images were stored in Hounsfield Units, allowing a mapping of tissue densities via phantom calibration (see review of the technique in Alonso, 2005).

Before decalcifying the ear bones, 71 CT scans of the Tympanic-Periotic (T-P) complex of 15 odontocete species were performed using the Siemens Somatom Emotion Duo (Hospital Clinic, Barcelona). Specifically, we analysed the T-P complex of *Tursiops truncatus* (12), *Stenella coeruleoalba* (11), *Stenella frontalis* (12), *Steno bredanensis* (3), *Delphinus delphis* (8), *Globicephala melas* (6), *Globicephala macrorhynchus* (2), *Lagenodelphis hosei* (2), *Kogia breviceps* (1), *Kogia simus* (2 periotic bones), *Physeter macrocephalus* (1 T-P and 1 periotic bone), *Phocoena phocoena* (7), *Ziphius cavirostris* (1), *Mesoplodon europaeus* (1) and *Mesoplodon densirostris* (1).

For this study, some ears were preserved in formalin, but other kept dried after removal of soft tissues.

Following a standardized protocol, the samples were scanned in the same orientation in a helicoidal CT with spiral image acquisition, 130 kV voltage, 200 mA/s exposure, 760 projections every 360° for each slice, 1 mm section thickness with a reconstruction advance of 0.5 mm and resolution of 512 × 512 pixels (being the pixel size 0.1269 × 0.1269 mm^2).

To systematically orientate the T-P complex in the same position three reference points were dye-marked at the ear surface, two on the periotic and one on the tympanic, as can be seen in Figure 2.4.1. To hold up the ear bones within the transparent jars, a polyurethane foam was used. It has the peculiarity of being almost transparent to X-rays and at the same time it can take any shape and sustain the T-P complex. Once the ears were fixed a vertical plane was marked in the passing through two of the three points (highlighted in blue in Figure 2.4.1). This vertical plane coincided with a reference system located on the scanner table.
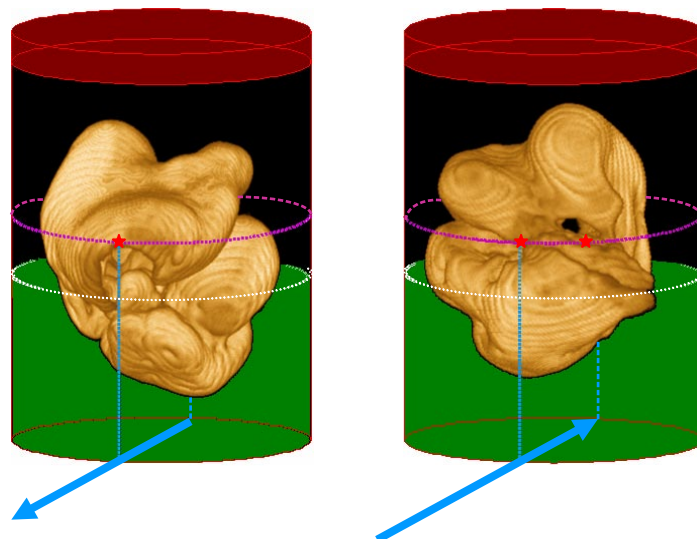
Figure 2.4.1. Standard positioning of the ears to be scanned by CT. The stars represent the 3 points that define the horizontal plane (pink), blue lines the vertical plane and the arrow the direction of the scan.

The images were stored in Digital Imaging and Communication in Medicine (DICOM) format, processed using the computer 3D rendering software Analyze® 5.0 and presented with the 3D image viewer MRIcro® and Adobe Photoshop®.

Analyze® is a multidimensional image processing, visualization and analysis programme that interprets and translates the differences in tissue densities in grey scale intensities. Bone threshold intensity value was set to be 645 to obtain a better image contrast. Following a determined intensity range the volume of a targeted organ could therefore be calculated. Using 3D tools in Analyze® 5.0, the following volumes and main lengths (see Figure 2.4.2) were measured:

T-P complex volume
Periotic (P) volume
Tympanic (T) volume
Cochlear volume
T-P complex total length (maximum length between the tympanic medial end and the periotic lateral end)
Tympanic total length (maximum length between the tympanic medial end and the tympanic latero-anterior end)
Periotic total length (maximum length between the periotic medial end and the periotic lateral end).

Working with volumes permitted rotating the reconstructed ears until reaching the best projection to measure any length. Analyze® allowed us to calculate the direct linear length between two points selected on the screen.

To calculate the volume, the program extrapolated it by counting the voxels of the selected density. To calculate the cochlear volume it was necessary to manually select the oval and round windows and then close the space occupied by the cochlea.

The total length of the animal (length from the anterior edge of the rostrum to the caudal fluke) was used as an external measurement.
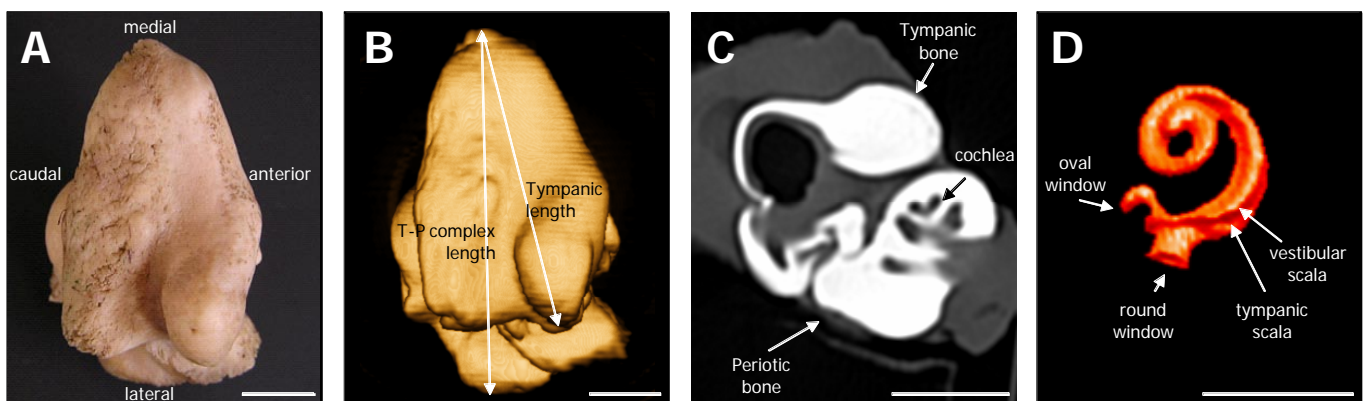
Figure 2.4.2. A) Photograph of the ventral view left ear of a *Tursiops truncatus*; B) T-P complex total length and tympanic length measurements from the rendered 3D volume of the same ear as in A). The periotic length was also measured using the same methodology; C) image obtained through CT scan of a *Tursiops truncatus* right ear; D) 3D rendered cochlea of a *Steno bredanensis*. Scale bars: 1 cm.

We chose two methods to statistically analyse the data:

— the linear correlation coefficients to compare the relationship between all double combinations of measurements;

— the Fisher's discriminant analysis, a multivariant test which allows a comparison of all measurements together and classification of the species by these measurements. The power of discrimination (i.e. the weight) of each variable was calculated with the Fisher discriminant ratio comparing the species which contained a larger number of replicates (*T. truncatus*, *S. coeruleoalba*, *D. delphis*, *P. phocoena* and *S. frontalis*).

All the statistical tests and mathematical analysis were performed with the SPSS® software package, Matlab® 7.0 and Microsoft Excel®.

To assess the effect of age and because the species had different sizes, the statistical analysis was performed for the situations detailed in Table 2.4.1.

Mean and standard deviation estimates were calculated for each variable, giving a basis to build species-specific standard morphological measurements.

Table 2.4.1. Specification of the four situations considered to compare the samples. All data were typified ([value - $\overline{x}$]]/ σ), being $\overline{x}$ the mean and σ the standard deviation.

Situation	Specification
1	only the adults typified data
2	only the adult data normalized by the animal length
3	adults and juveniles typified data
4	adult and juvenile data normalized by the animal length

2.4.2. Electron and light microscopy

2.4.2.a- Scanning Electron Microscopy (SEM)

Since there is no need to decalcify completely the periotic bone to access the Organ of Corti cells using scanning electron microscopy, the decalcification of the periotic bone was stopped when the *vestibular scalae* and the *stria vascularis* of the cochlea were uncovered. 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.

63 cochleas and their tectorial membranes were dissected, removing the remains of the decalcified bone, the tympanic scalae and part of the cochlear scala including the stria vascularis with microscisors and dissecting the tectorial membrane with fine forceps (allowing it to be observed separately) to expose the reticular lamina of the organ of Corti. Subsequently they were dehydrated in increasing series of ethanol (see Table 2.4.2), critical point dried with CO_2, and gold-palladium sputtered (Table 2.2.1). The samples were observed under SEM of the Institute of Marine Sciences - CSIC (Hitachi S-3500N), CRIC (Hitachi S-4000),

Centre Tecnològic de Vilanova i la Geltrú (Jeol JSM 5600) and Universitat Autònoma de Barcelona (Hitachi S-570) for morphological description and possible acoustic trauma assessment.

Table 2.4.2. Steps followed to progressively dehydrate the samples

step	reagent	minimum time
1	30% ethanol	10 min
2	50% ethanol	10 min
3	70% ethanol	15 min
4	80% ethanol	15 min
5	95% ethanol	2 baths = 2 x 1h
6	100% ethanol	One bath overnight and a final bath (1h)

2.4.2.b- Transmission Electron Microscopy (TEM) and Light Microscopy

Only the freshest samples that were perfused with 2,5% glutaraldehyde were used for morphological description through TEM. These were completely decalcified and the endpoint was determined using the weight loss/weight gain procedure Mawhinney *et al.*, 1984; Hornbeck *et al.*, 1986; Sanderson *et al.*, 1995 and X rays. Specifically, the images we present here belong to 1) an adult striped dolphin ear from the North of Spain fixed 5 hours 30 minutes post-mortem and perfused with the same fixative solution 9h post-mortem, 2) a juvenile harbour porpoise stranded on the Belgium coast, perfused 22 hours 10 minutes post-mortem and 3) an adult harbour porpoise stranded on the Netherlands coast, perfused at least 3 hours 15 minutes post-mortem (Table 2.2.1). A cochlea of a dwarf sperm whale was used to set up the protocol and study the feasibility of this technique.

After the first fixation with glutaraldehyde, the samples were double fixed for one hour with 2% osmium acid, dehydrated with ethanol increasing concentrations (first steps of Table 2.4.2, until 70% Ethanol). The cochleas were then micro dissected to isolate blocks containing the organ of Corti, the stria vascularis and the spiral ganglion from the basal, middle and apical regions of the cochlear spiral. The blocks were embedded in EPON resin using automatic microwave tissue processor for electron microscopy Leica AM AMW (see Figure 2.4.3 and Table 2.4.3 for further details). Semi-thin and ultra-thin transverse sections of the blocks were cut and examined under a light microscope and a Hitachi H-7100 TEM (CRIC) respectively.

Table 2.4.3. Steps followed by the automatic microwave tissue processor for electron microscopy Leica EM AMW at the facilities of CRIC

vial	reagent	time (sec)	max. temp. (ºC)	max. power (W)	mode
1	70% Ethanol (solvent)	40	35	20	slope
2	95% Ethanol (solvent)	59	35	20	slope
3	95% Ethanol (solvent)	59	35	20	slope
4	100% Ethanol (solvent)	300	35	20	slope
5	100% Ethanol (solvent)	300	35	20	slope
6	100% Ethanol (solvent)	300	35	20	slope
7	100% Acetone (solvent)	180	35	20	slope
8	100% Acetone (solvent)	180	35	20	slope

9	100% Acetone (solvent)	180	35	20	slope
10	Resin 3:1 (resin)	420	37	10	continuous
11	Resin 1:1 (resin)	420	40	10	continuous
12	Resin 1:3 (resin)	420	45	10	continuous
13	Epon (resin)	420	50	12	continuous
14	Epon (resin)	420	50	12	continuous
15	Epon (resin)	420	50	12	continuous
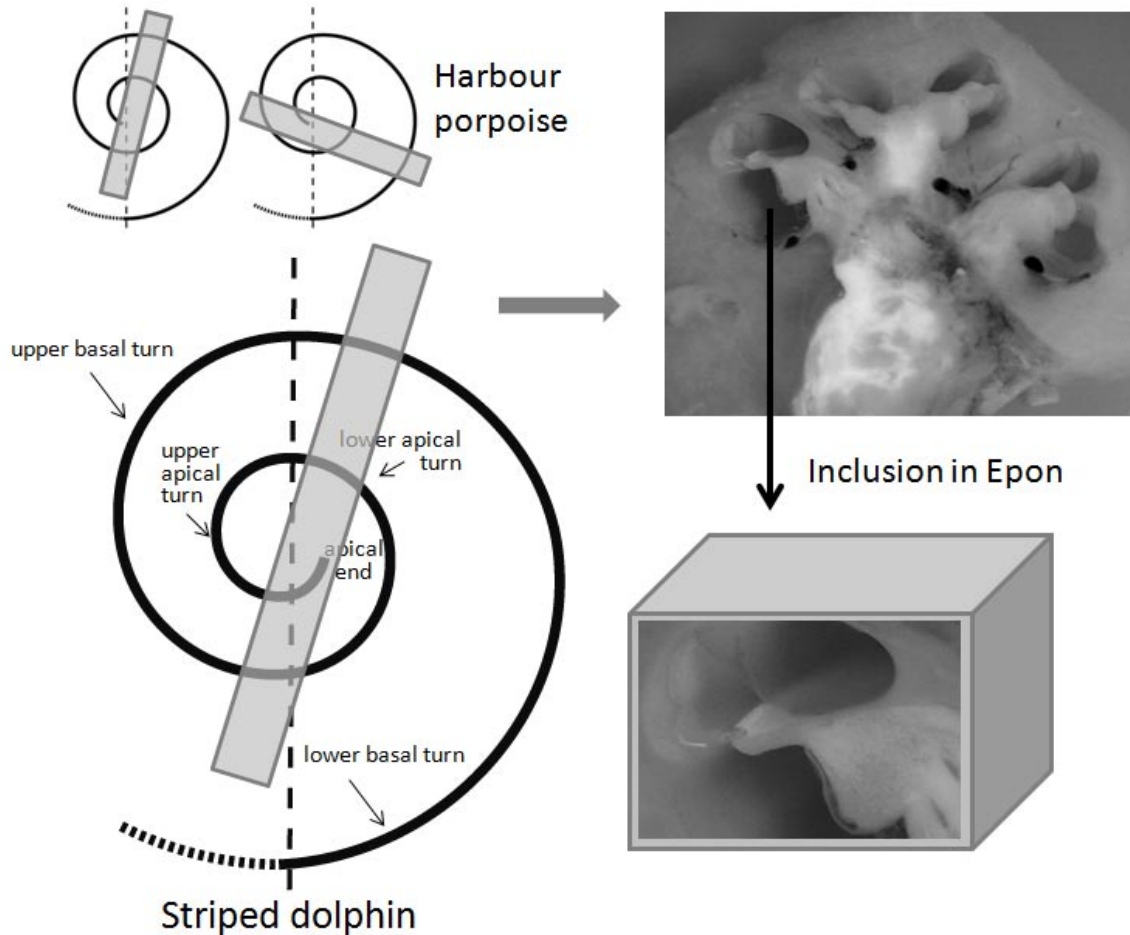
Figure 2.4.3. Schematic representation of the steps followed to process the sample using TEM. The cochleas of striped dolphin and harbour porpoise were cut with a blade in the directions marked with the grey rectangles, obtaining sections as shown in the right upper image. After, the sections were cut isolating each turn and embedded with Epon. The blocks were cut, first for the observation under light microscope, and when the orientation was well adjusted, ultra-thin sections were obtained for the observation with transmission electron microscopy.

2.4.2.c. Metric measurements

Several measurements of the cochlear structures were performed (see Figure 2.4.4 and the Results chapter for details) using either specific tools of the electron microscope software or the software ImageJ[2].

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[2] ImageJ is a public domain Java image processing and analysis program inspired by NIH Image for the Macintosh.

a) Spiral ligament length
b) Basilar membrane width
c) Basilar membrane thickness
d) OHC maximum length
e) OHC maximum diameter
f) OHC cuticular plate arms
g) OHC cuticular plate thickness
h) Stereocilia length

i) Stereocilia diameter
j) Stereocilia rootlet
k) Deiters cell length
l) Deiters cell cup length
m) OHC1 maximum length
n) OHC2 maximum length
o) OHC3 maximum length
p) Distance OHC1-OHC2
q) Distance OHC2-OHC3

r) Distance OHC1-OHC1
s) Distance OHC2-OHC2
t) Distance OHC3-OHC3
u) IHC maximum length
v) Distance between IHCs
w) IPC maximum length
x) IPC minimum length
y) OPC maximum length
z) PC maximum length

Figure 2.4.4. Scheme of the measurements of the cochlear structures that were performed using light microscope (A and B), transmission electron microscope (C) and scanning electron microscope (D). These measurements are reflected in Table 2 (a-l) and Table 4 (n-z). A and B represent a transversal cut of the upper basal turn of the cochlea of a harbour porpoise that was stained with osmium acid. C is a schematic representation of an OHC at the base of the cochlea and the apical part of the Deiters cell body. D represents the reticular lamina of the Organ of Corti epithelium. IHC: inner hair cell, OHC: outer hair cell, IPC: inner pillar cell, OPC: outer pillar cell, PC: phalangeal cell. Scale bars: A) 200 µm, B) 100 µm.

2.4.3- Immunohistochemistry

Immunohistochemistry technique is used to locate antigens in tissues, cells, organelles, etc. In this study immunofluorescense was applied. The primary antibody recognises the target molecule and binds to it, and the secondary antibody, which carries the fluorophore, recognises the primary antibody and binds to it. The results can be observed under a fluorescence microscope.

Since this method is very sensitive, only those samples from very fresh animals can be used. For this preliminary study, an ear sample from a harbour porpoise that stranded in the Netherlands was injected with formalin 5 hours post-mortem. The periotic bone was completely decalcified using the same methodology as in TEM (see chapter 2.4.2.b).

After decalcification, the periotic bone was immersed in a solution with PBS, 20% sucrose and 0.1% Na acid (to avoid bacteria proliferation) for 5 days, until the sample descended to the bottom of the jar. Subsequently, the solution was changed by half OCT® and half PBS with 20% sucrose and 0.1% Na acid for 7 hours. The sample was then submerged in OCT® and ultra-frost at -80ºC for 3 days. Finally, the periotic bone was cut using a cryomicrotom (Leica Jung Frigocut 2800E) in slices 20 µm thick. They were mounted in microscope slides and kept in the freezer at -20ºC.

Several primary antibodies at different dilutions were tested to study which one worked better for this specific tissue and species:

- Mouse anti-Neurofilament 200 (phoshorylated and non-phosphorylated) monoclonal antibody (Sigma-Aldrich ref. N0142) IgG1 isotope: it stains neurofilaments (one of the five major groups of intermediate filaments found predominantly in cells or tissues of neuronal origin) of 200 kDa, labelling fibrous profiles in neuronal perikarya, dendrites and axons. In our samples, it is expected to stain the myelinated fibres: type I neurons and medial efferent. We tried the dilutions 1:800, 1:400 and 1:200.

- Rabbit anti-Peripherin polyclonal antibody (Chemicon International ref. AB1530): it stains a 57 kDa band specifically from peripherin, allowing the visualization of type II neurons. We tried the dilution 1:400.

- Guinea pig anti-VGlut 3 (Vesicular glutamate transporter 3; courtesy of Salah El Mestikawy): it stains VGLUT3, a multipass membrane protein restricted to synaptic vesicles of glutamergic neurons. This staining highlights the radial afferent fibres that innervate the IHCs. We tried the dilution 1:100.

- Mouse anti – CtBP2 (c-terminal binding protein 2; BD Biosciences ref. 612044) IgG1 isotope: it marks the cell nuclei and the domain B of Ribeye[3] without the 20 first amino acids, labelling the synaptic ribbon. We tried the dilution 1:1000.

- Rabbit anti -Myosin VIIa polyclonal antibody (Proteus Biosciences ref. 25-6790): it labels Myosin VIIa, one of the unconventional members of the myosin molecular motor superfamily that move along filamentous actin, expressing mainly in receptor cells of the inner ear. We tried the dilutions 1:200 and 1:100.

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[3] Ribeye is a component of the synaptic ribbons that shares identity of sequences with CtBP2

- Goat anti-Prestin polyclonal IgG antibody (Santa Cruz ref. SC-22692): it stains specifically the prestin, a motor transmembrane protein specific of the outer hair cells. We tried the dilution 1:200.

- Sheep anti-VAcht (vesicular acetylcholine transporter) polyclonal antibody (Abcam ref. ab31544): it is specific to acetylcholine transport into synaptic vesicles, staining lateral and medial efferent. We tried the dilution 1:1000.

In addition to these primary antibodies, the fungal toxin Phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma-Aldrich ref. P1951) dilution 1:2000 and 1:5000 was used to observe the actin filaments of stereocilia of hair cell.

To recognize the primlary antibodies, we used the following secondary antibodies that were tagged with a fluorophore and diluted 1:2000:

- Goat anti-mouse Alexa 594 (red)
- Goat anti-rabbit Alexa 488 (green)
- Goat anti-guinea pig Alexa 647 (blue)
- Donkey anti-mouse Alexa 594 (red)
- Donkey anti-goat Alexa 488 (green)

Since the samples displayed autofluorescence (see below in the Results chapter), Sudan Black B, which reduces autofluorescence, was used to optimize contrast. The following staining protocol for immunofluorescense was applied:

- Rinse with PBS three times for 10 minutes
- Incubate in blocking serum (Aurion blocking solution code 905.002) for 1 hour to block unspecific binding of the antibodies. This solution can only be used with goat conjugates. PBS + 30% normal donkey serum is used as a blocking solution for goat and sheep primary antibodies.
- Rinse with incubation buffer[4] three times for 10 minutes
- Incubate in primary antibody overnight (it can stay also 2-3 nights) in the fridge and with low agitation
- Agitation at room temperature
- Rinse with incubation buffer three times for 10 minutes
- Incubate in secondary antibody (and Phalloidin-Rhodamine when convenient). From now on all the steps are in the dark.
- Rinse with PBS 5 minutes
- Rinse with etanol 70% 5 minutes
- Sudan Black B (Autofluorescent eliminator reagent, Chemicon International) for 5 minutes
- Rinse with ethanol 70% three times for 1 minute
- Mount with DAKO mounting media

The sections were observed with the fluorescent microscopes Zeiss Apotome, Leica DMRA and Zeiss LSM 5 Duo confocal at the cell imaging platform of Institute of Neurosciences of Montpellier.

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[4] To prepare 250 ml of incubation buffer: mix 250 µl of BSAc (Aurion) + 2 ml goat normal serum (or donkey normal serum for goat and sheep primary antibodies) + 2.5 ml of 10% CWFSG + 2.5 ml of 10% NaN_3 + PBS until 250 ml

3. Results

3. RESULTS

This section will present the results obtained from Computerized Tomography (comparative analysis of the odontocete ear morphology), decalcification techniques, SEM, TEM and immunohistochemistry.

3.1. Computerized Tomography

The results of this imaging technique presented here represent a follow-up from Morell and colleagues (2007).

3.1.1. Linear correlation coefficients

The correlation coefficients between measurements were calculated for all animals (situation 3, Table 2.4.1) as well as for the adults separately (situation 1, Table 2.4.1). The results are shown in Table 3.1.1. The results obtained with adults were very similar to those obtained when juveniles were also taken into account (Figure 3.1.1 and Table 3.1.1b).
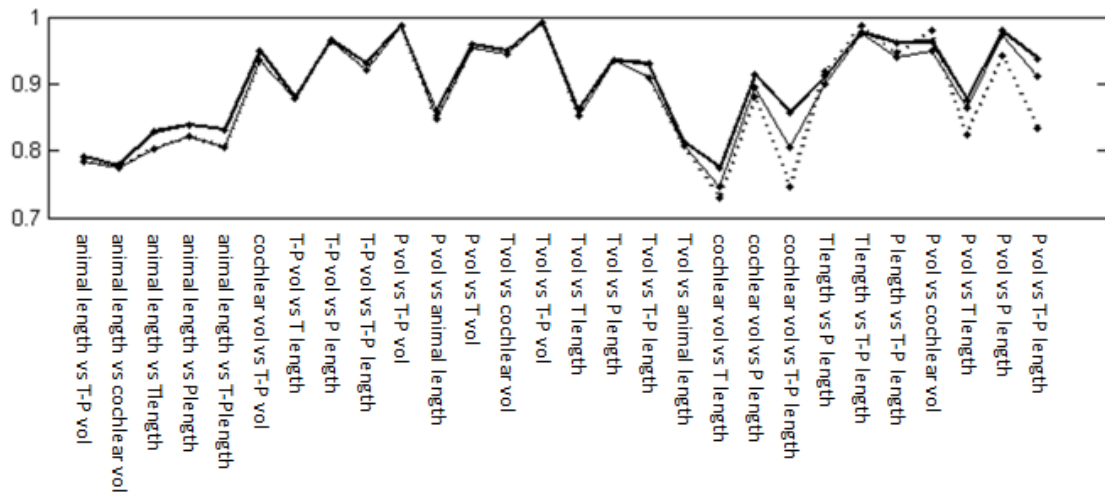
Figure 3.1.1. Representation of the correlation coefficients for the adults (thick line), all the data with the juveniles (dotted line) and all the data without the sperm whales, *Physeter macrocephalus* (thin line), between the two pairs of variables detailed on the x axis (see Table 3.1.1).

Table 3.1.1. Correlation coefficients between measurements done in: (A) situation 3 and (B) situation 1 (see Table 2.4.1). It is also specified the number of individuals in each case (N)

(A)	T-P vol	Cochlear vol	P vol	T vol	T length	P length	T-P length	Animal length
T-P vol	1							
Cochl vol	0.934 (N=62)	1						
P vol	0.986 (N=35)	0.979 (N=39)	1					
T vol	0.991 (N=34)	0.945 (N=34)	0.955 (N=35)	1				
T length	0.878 (N=64)	**0.730** (N=64)	0.825 (N=37)	0.853 (N=34)	1			
P length	0.964 (N=64)	0.881 (N=66)	0.941 (N=39)	0.935 (N=34)	0.918 (N=69)	1		
T-P length	0.922 (N=62)	**0.745** (N=62)	0.834 (N=35)	0.910 (N=33	0.986 (N=67)	0.946 (N=67)	1	
Animal length	**0.785** (N=62)	**0.775** (N=60)	**0.847** (N=34)	**0.807** (N=33)	**0.803** (N=63)	**0.821** (N=63)	**0.805** (N=61)	1

(B)	T-P vol	Cochlear vol	P vol	T vol	T length	P length	T-P length	Animal length
T-P vol	1							
Cochl vol	0.950 (N=47)	1						
P vol	0.987 (N= 27)	0.963 (N=27)	1					
T vol	0.992 (N=26)	0.950 (N=26)	0.959 (N=26)	1				
T length	0.879 (N=47)	**0.775** (N=47)	0.875 (N=27)	0.861 (N=26)	1			
P length	0.965 (N=47)	0.913 (N=47)	0.979 (N=27)	0.936 (N=26)	0.911 (N=47)	1		
T-P length	0.931 (N=45)	0.858 (N=45)	0.938 (N=25)	0.930 (N=25)	0.977 (N=45)	0.962 (N=45)	1	
Animal length	**0.792** (N=47)	**0.778** (N=47)	**0.859** (N=27)	**0.813** (N=26)	**0.828** (N=47)	**0.839** (N=47)	**0.832** (N=45)	1

When compared with other species, sperm whale (*Physeter macrocephalus*) measurements showed greatest separation from the regression line, for the correlation coefficients were again calculated without taking into account this species. As shown in Figure 3.1.1, juveniles and adults together without sperm whales (thin line) gave a closer approximation to the values obtained with adults only (thick line).

All measurements were highly correlated (r >0.9) except in two scenarios (highlighted in bold in Table 3.1.1):

— correlation was lower when comparing the animal length with the rest of the measurements (0.77< r <0.86);

— as for the cochlear volume, it proved to be highly related to all volumes and P lengths and to a lesser extent to all other lengths.

For all species, the proportion between the tympanic and periotic bones appeared to remain constant meaning that any change in either structure (tympanic or periotic) is reflected in the other in the same proportion both in juveniles and adults.

With this one dimensional statistical test we could not differentiate between all the species or calculate the weight of each variable to classify them. The results (see below) from a multi-discriminant analysis allowed us to find the most discriminant variables from our measurements.

3.1.2. Fisher discriminant analysis (FDA)

We first calculated the individual variables discriminant power with Fisher's discriminant ratios. To make the analysis more robust, ratios were only evaluated for the five species presenting more replicates: bottlenose dolphin *Tursiops truncatus* (Tt), striped dolphin, *Stenella coeruleoalba* (Sc), common dolphin *Delphinus delphis* (Dd), harbour porpoise *Phocoena phocoena* (Pp) and Atlantic spotted dolphin *Stenella frontalis* (Sf). These ratios, which give the weight of the variables — a higher ratio means a stronger discriminant power — are presented in columns 1 to 6, Table 3.1.2a.

All measurements were considered except the T and P volumes. In addition, it was eliminated from the analysis those individuals who did not contain data from all measurements, leading to 58 individuals from 13 species.

In all four situations (Table 2.4.1), the cochlear volume appeared to be the variable with the lowest weight. Additionally, in situation 3 (adults and juveniles) the animal length also seemed to have little importance despite being a strong discriminator in situation 1 (only adults). The variable that generally displayed the strongest discriminant power was the T-P complex length.

Next, we performed a FDA on the combination of all variables (Figure 3.1.2 and 3.1.3). The four plots show classification results in the four different situations (Table 2.4.1). The classification errors, i.e. those individuals that are from one species but, according to the measurements, are closer to other species individuals, are listed in column 7 of Table 3.1.2. When juveniles are taken into account the number of misclassifications increases.

In Table 3.1.2, function 1 and function 2 are the two most discriminant projected dimensions resulting from FDA. Table values reflect the percentage of the data variability. Since most of the variability is explained by the two first functions, we can assume that there is strong dependency between the variables.

The last column in the table shows the Wilks' λ: a very low value would indicate that the means of the classes are well separated, which is the case here (Table 3.1.2a and 3.1.2b).

These results confirm that all combined variables classify the species very well.

Because the results for adults and juveniles did not lead to the same relationship between variables, we used adults for standard ear mean and standard deviation calculations. In cases when we had no adults, juveniles were used. Figure 3.1.4 shows these values as species-specific standard measurements, as well as the scale reconstructions of all the T-P complex and cochlea volumes.

Ketten and Wartzok (1990) and Ketten (1992, 1994) divided the odontocete ears into two Types: I and II, depending on click peak frequency production. In our study there is just one type I species, harbour porpoise *Phocoena phocoena* (which presents a peak frequency >100 kHz), the rest of the species being all of type II. When conducting the FDA considering these two types, some Type II individuals were misclassified as Type I (see column 7, Table 3.1.2c and 3.1.2d). The high Wilks' λ values presented in Table 3.1.2c and 3.1.2d support the fact that the variables do not classify well between Type I and II species.

 Table 3.1.3 shows a summary of the results obtained with the two statistical tests.

Table 3.1.2. Comparative results of the Fisher discriminant analysis for the 4 situations (see Table 2.4.1). The Fisher discriminant ratios for every variable are shown in columns 1 to 6. The maximum values for every situation are indicated in bold letters. Column 7 specifies the number of bad classified (Bc) samples. Columns 8 and 9 reflect the percentage of variance for the two first functions while column 10 shows the Wilks' λ values. In cases A and B the populations were classified by species and in cases C and D by their acoustic characteristics in sound production (Type I and II, Ketten and Wartzok, 1990)

A) Sf Sc Dd Pp Tt	1 T-P vol	2 Cochlear vol	3 T length	4 P length	5 T-P length	6 Animal length	7 Bc	8 Function 1	9 Function 2	10 Wilks' λ
Situation 1 (n= 38)	45.66	27.01	74.09	79.10	87.48	**203.22**	0	94.4	4.1	0.002
Situation 2 (n= 38)	15.94	15.43	45.21	46.30	**78.67**		1	92.2	5.7	0.003
Situation 3 (n= 46)	47.97	29.20	72.31	**77.24**	75.08	27.17	4	83.7	11.5	0.009
Situation 4 (n= 46)	6.91	4.90	9.20	9.42	**11.20**		6	61.0	29.6	0.032
B) All data										
Situation 1 (n=46)							0	94.6	4.2	0.000
Situation 2 (n=46)							1	94.9	3.1	0.000
Situation 3 (n=58)							3	79.1	14.2	0.000
Situation 4 (n=58)							14	74.0	13.4	0.003
C) All data (classified byType I and II)										
Situation 1 (n=46)							7	100	-	0.785
Situation 2 (n=46)							2	100	-	0.597
Situation 3 (n=58)							11	100	-	0.734
Situation 4 (n=58)							2	100	-	0.546
D) Sf Sc Dd Pp Tt (classified byType I and II)										
Situation 1 (n= 38)							0	100	-	0.550
Situation 2 (n= 38)							0	100	-	0.456
Situation 3 (n= 46)							3	100	-	0.549
Situation 4 (n= 46)							2	100	-	0.480
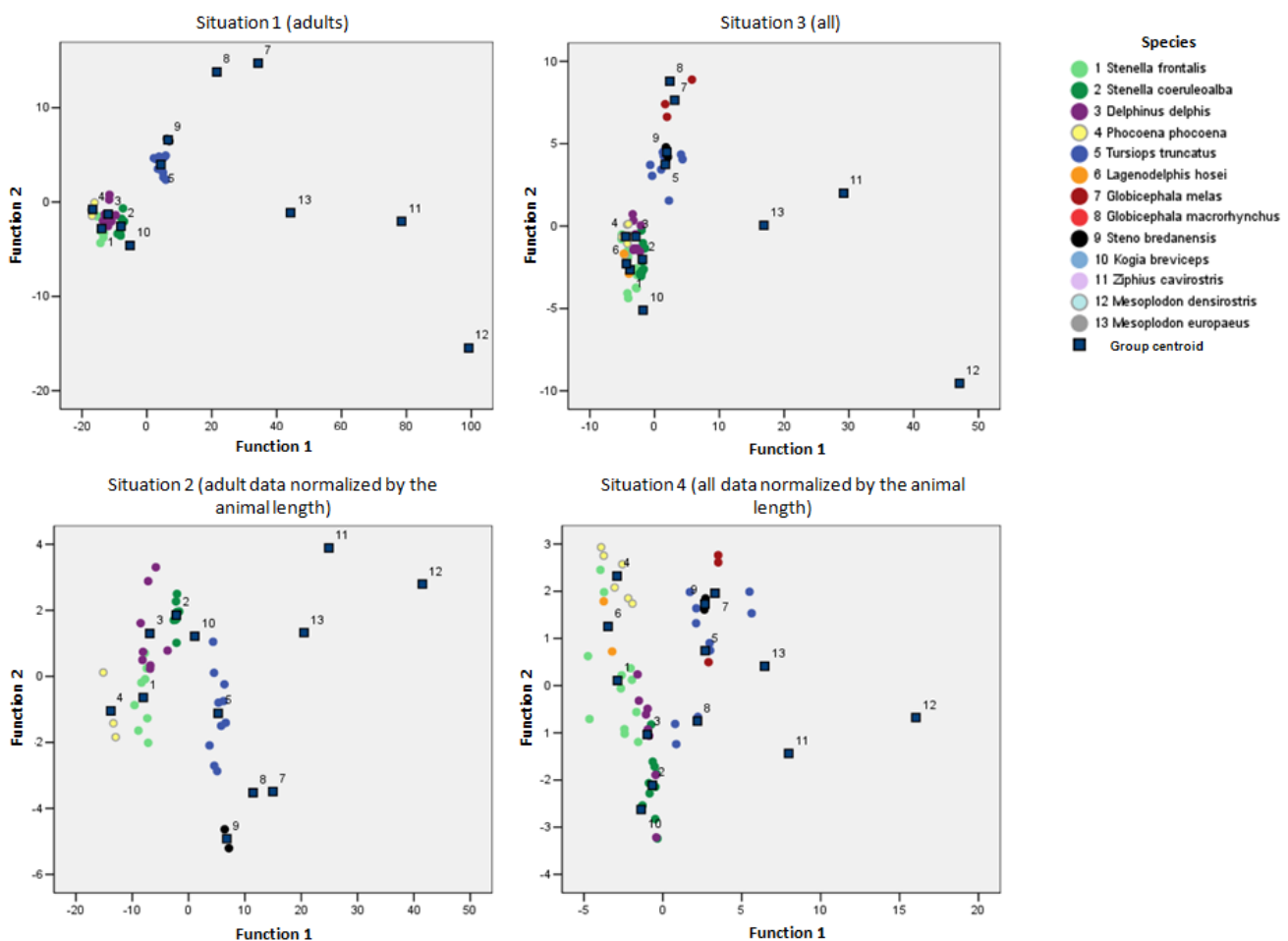
Figure 3.1.2. Plot of the 13 species in the two most discriminating projected dimensions (functions 1 and 2) resulting from the Fisher discriminant analysis for the four situations (Table 2.4.1).
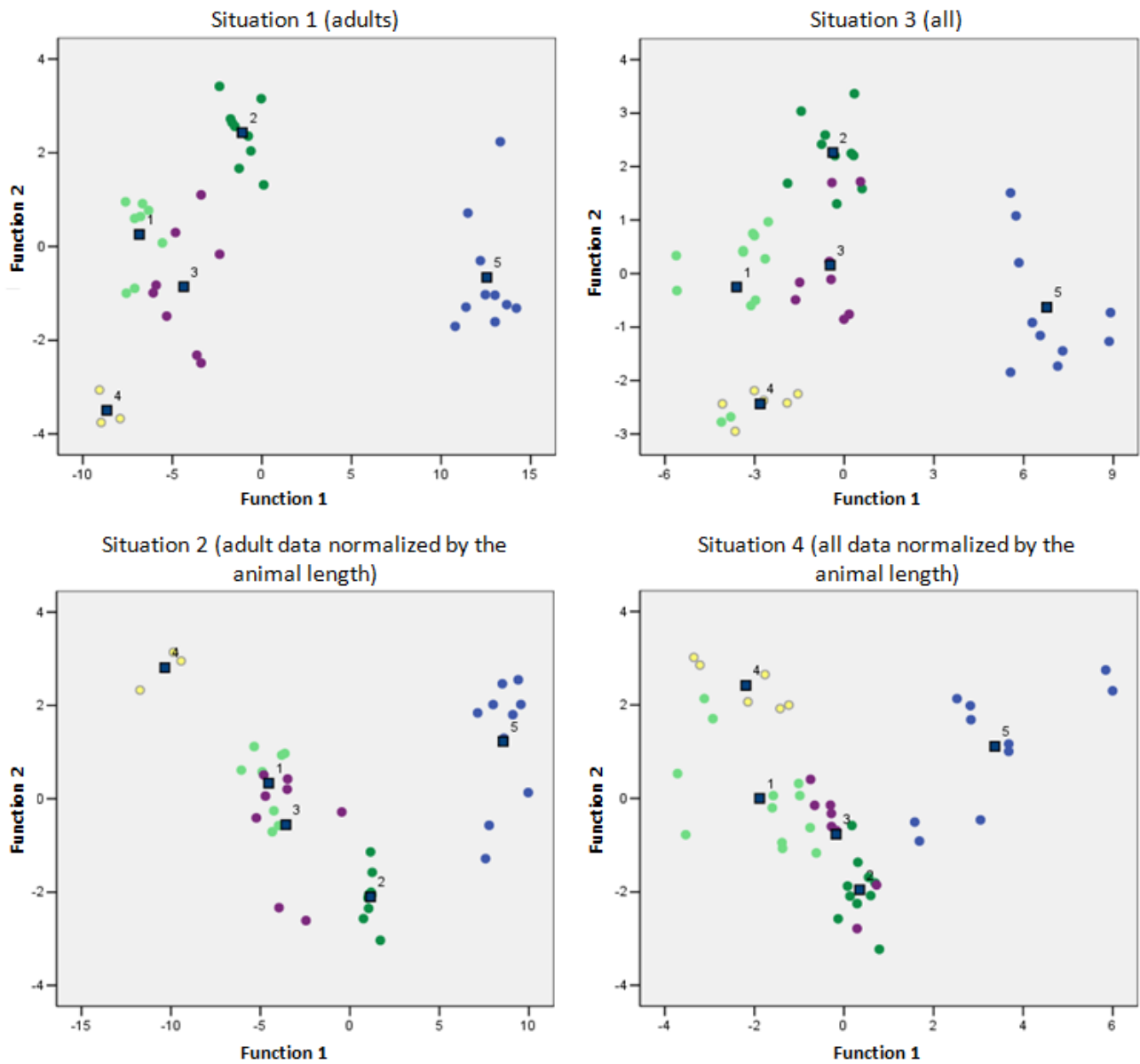
Figure 3.1.3. Plot of the 5 species with more replicas in the two most discriminating projected dimensions (functions 1 and 2) resulting from the Fisher discriminant analysis for the four situations (see legend in Figure 3.1.2).

	T-P vol	Cochlear vol	P vol	T vol	T length	P length	T-P length	Animal length
Physeter macrocephalus	-	858.570* (σ= -)	26975.60* (σ= -)	-	56.143* (σ=0.650)	62.163* (σ=1.121)	67.785* (σ=5.593)	-
Mesoplodon densirostris	37013.470 (σ= -)	496.660 (σ= -)	16029.270 (σ= -)	20984.170 (σ= -)	53.096 (σ= -)	55.161 (σ= -)	65.016 (σ= -)	4350.0 (σ= -)
Ziphius cavirostris	27268.120 (σ= -)	410.410 (σ= -)	14553.250 (σ= -)	12714.820 (σ= -)	50.258 (σ= -)	56.706 (σ= -)	63.522 (σ= -)	5640.0 (σ= -)
Mesoplodon europaeus	18917.780 (σ= -)	281.610 (σ= -)	8232.270 (σ= -)	10685.510 (σ= -)	41.847 (σ= -)	43.910 (σ= -)	53.909 (σ= -)	4090.0 (σ= -)
Globicephala melas	13668.135 (σ=1326.624)	184.035 (σ= 9.256)	6526.550 (σ= -)	6550.990* (σ=50.883)	45.651 (σ= 3.164)	39.138 (σ= 0.929)	55.651 (σ= -)	4550.0 (σ= 353.553)
Globicephala macrorhynchus	12956.460 (σ= 159.651)	141.445 (σ= 0.262)	6280.615 (σ= 18.576)	6675.845 (σ=141.075)	48.187 (σ= 0.035)	38.581 (σ= 0.264)	52.726 (σ= -)	3650.0 (σ= -)
Steno bredanensis	12032.470 (σ= 21.510)	100.780 (σ= 0.636)	5704.085 (σ= 22.253)	6328.385 (σ= 0.742)	41.846 (σ= 0.212)	36.562 (σ= 0.559)	48.842 (σ= 0.260)	2470.0 (σ= -)
Tursiops truncatus	11019.790 (σ=1802.143)	141.070 (σ= 21.243)	5161.973 (σ= 82.305)	6198.823 (σ=494.443)	39.831 (σ= 1.474)	37.203 (σ= 1.532)	47.703 (σ= 2.127)	2457.5 (σ= 355.075)
Stenella coeruleoalba	6211.381 (σ= 641.363)	88.561 (σ= 14.437)	3830.160 (σ= 66.059)	3049.740 (σ= 27.279)	32.998 (σ= 1.358)	31.136 (σ= 1.264)	37.801 (σ= 1.166)	2207.8 (σ= 27.739)
Delphinus delphis	5910.505 (σ= 565.997)	106.193 (σ= 16.841)	3350.373 (σ=442.168)	2973.750 (σ=339.864)	33.353 (σ= 1.153)	30.383 (σ= 1.709)	38.779 (σ= 0.962)	1827.5 (σ= 138.538)
Stenella frontalis	5156.086 (σ= 367.356)	65.693 (σ= 6.142)	2653.413 (σ=109.811)	2346.935 (σ=158.500)	31.277 (σ= 0.905)	28.402 (σ= 0.392)	36.301 (σ= 0.595)	1792.5 (σ= 73.046)
Lagenodelphis hosei	5053.050* (σ=21.185)	59.250* (σ=3.224)	3079.800* (σ=14.835)	1973.250* (σ=6.350)	29.335* (σ=0.505)	30.387* (σ=0.033)	36.538* (σ=0.676)	1300.0* (σ= -)
Kogia breviceps	4748.620 (σ= -)	72.770 (σ= -)	2470.950 (σ= -)	2277.670 (σ= -)	30.539 (σ= -)	27.929 (σ= -)	33.922 (σ= -)	2580.0 (σ= -)
Kogia simus	-	57.620* (σ=1.527)	2311.965* (σ=1.011)	-	-	25.444* (σ=0.016)	-	-
Phocoena phocoena	5486.483 (σ= 235.367)	83.565 (σ= 11.348)	2050.025 (σ=88.028)	3475.590 (σ=124.875)	31.883 (σ= 0.848)	29.655 (σ= 0.565)	38.801 (σ= 0.646)	1477.5 (σ= 95.000)

Figure 3.1.4. Macroscopical 3D reconstructions of all the species rendered bullae and cochlear volumes. The rendered T-P volume was not shown when the complete T-P complex was not available (either T or P were missing). The above table shows the means and standard deviations of the adult variables, giving a basis to build species-specific standard morphological measurements. All the volumes were presented in mm3 and all the lengths in mm. The values marked with * were calculated from juveniles.

Table 3.1.3. Results summary.

Test	Objectives	Results
Linear correlation coefficient	Compare one to one all double combinations of measurements	Very high correlations 0.9 < r < 0.99 (except animal length with the rest of measurements and cochlear volume with some lengths) Adults and juveniles lead to similar results Without *Physeter macrocephalus* the coefficients are higher
Fisher discriminant analysis	Compare all the variables together to use all the available information from the measurements. Determine the weight of each variable	The measurements classify well the species. There is a strong dependency between the variables When juveniles are taken into account the number of errors in the classification increases

3.2. Decalcification

3.2.1. RDO®

The first results that derive from a decalcification protocol using RDO® are published in Morell *et al.*, 2009.

Preliminary decalcification experiments were conducted with 100% RDO® on samples from harbour porpoise (27% of N=total number of samples per species, see Table 3.2.1), bottlenose dolphin (56% of N), spotted dolphin (23% of N) and striped dolphin (9% of N). The decalcification time with this RDO® concentration was considerably reduced (from 54% to 66% depending on the species, except in the case of harbour porpoise, that was increased by 9%) compared to the results shown below with 50%, 75%, and 80% RDO® concentration. Despite the reduction in decalcification times with 100% RDO®, the determination of the endpoint times was 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 Figure 3.2.1.
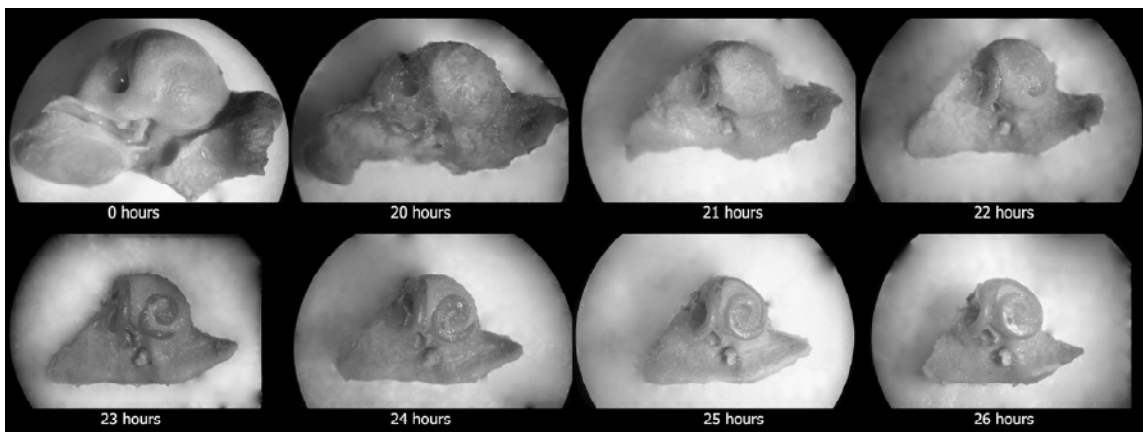
Figure 3.2.1. Decalcification process of a harbour porpoise periotic bone using 50% RDO® and 25% RDO® after 24 h. The decalcification time is shown below each picture. In this example, the vestibular scalae and the stria vascularis of the cochlea were uncovered after 26 h.

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 3.2.1.

Table 3.2.1. Decalcification times of the periotic bones using RDO® analyzed during the study.

Species	Mean	Min–max	n	N
Stenella coeruleoalba	46h 47′	34h–53h 31′	19	23
Phocoena phocoena	29h 52′	26h–38h 27′	17	56
Stenella frontalis	45h 52′	41h 40′–49h 27′	4	13
Delphinus delphis	49h 03′	46h 43′- 54h 04′	6	7
Tursiops truncatus	65h 16′	62h 13′–67h 44′	3	9
Kogia breviceps	37h 22′	31h 27′–48′	3	3
Steno bredanensis	65h 50′	59h 13′–72h 27′	2	2
Kogia simus	32h 16′		1	2
Lagenodelphis hosei	30h 10′		1	1
Globicephala macrorhynchus	63h 15′		1	1
Globicephala melas	66h 58′		1	2
Ziphius cavirostris	4d 10h 10′		1	1
Hyperoodon ampullatus	7d 18h 53′		1	2

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.

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

– the tympanic–periotic complex volumes extracted from CT scans (r=0.935, n=12, Figure 3.2.2a) or,

 – the average of the tympanic–periotic complex volumes for each species (see Figure 3.1.4) when data were not available (r=0.891, n=34, Figure 3.2.2b).

The remaining specimens ("N–n" samples in Table 3.2.1) 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).
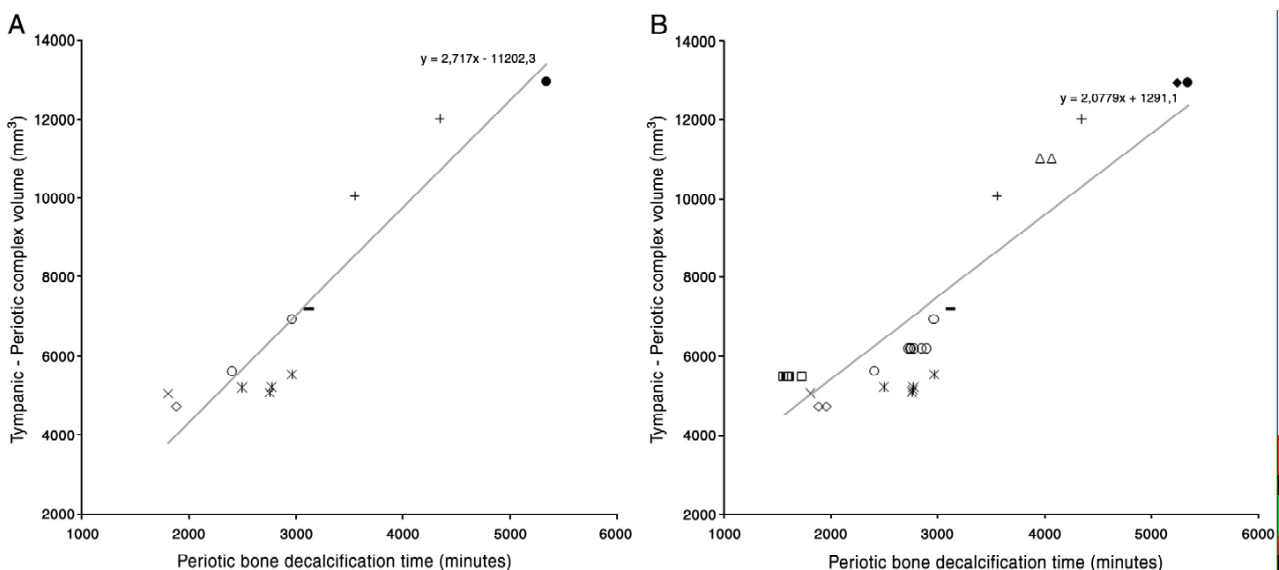
Figure 3.2.2. Correlation between the periotic decalcification time with: A) the tympanic–periotic volume (r=0.935, n=12) and B) the mean of T-P volume for the species (see Figure 3.1.4; Morell *et al.*, 2007; r=0.891, n=34) when data were not available. □ *Phocoena phocoena*, Δ *Tursiops truncatus*, Җ *Stenella frontalis*, ○ *Stenella coeruleoalba*, – *Delphinus delphis*, ◊ *Kogia breviceps*, + *Steno bredanensis*, × *Lagenodelphis hosei*, ● *Globicephala melas*, ♦ *Globicephala macrorhynchus*

3.2.2. EDTA

When samples were decalcified with EDTA, the decalcification times increased, as is shown in Table 3.2.2. However, the periotic bones decalcified with EDTA in a microwave oven reduced substantially the time of decalcification compared to those decalcified with EDTA at room temperature. As an example, an averaged harbour porpoise ear will take around 30 hours with RDO® to be partially decalcified for the subsequent analysis using SEM (Table 3.2.1), 42 hours with EDTA in a microwave oven (and at room temperature overnight) and 17 days with EDTA at room temperature (Table 3.2.2).

Table 3.2.2. Average, minimum and maximum values of the decalcification time using EDTA at room temperature and in a microwave oven. The samples were decalcifying partially or completely in the first case, depending if the subsequent analysis was using SEM or TEM/immunohistochemistry respectively.

| | EDTA at room temperature | | | | | | EDTA + MW | | |
| | Samples for SEM | | | Samples for TEM or immunohistochemistry | | | Samples for SEM | | |
Species	average	min-max	n	average	min-max	n	average	min-max	n
Phocoena phocoena	17d 8h	15d 16h-19d 4h	4	38d 1h	19d 17h-54d 19h	4	41h 40'	24h-60	3
Stenella coeruleoalba	24d 12h	18d 18h-28d 23h	5	67d 1h		1	64h		1
Delphinus delphis	29d 2h	17d 6h-40d 21h	2				52h		1
Globicephala melas	40d 2h		1						
Hyperoodon ampullatus	122d 23h		1						

Min–max, minimum and maximum decalcification time of periotic bones for each species; n, number of samples used to determine the final decalcification time; d:days; h:hours; MW: microwave

3.3. Technovit

Preliminary experiments embedding the periotic bones in Technovit 7200 VLC® resin did not lead to very good and promising results. This resin has the advantage to penetrate the bone at low temperature. However, we found that, while we obtained very good bone preparations, it was not optimal to fill internal cavities, even when the embedding time was duplicated (from 20 days to two months). In addition, no differences were obtained when observing under the light microscope an autolytic sample (Figure 3.3.1a and b) and the other two samples fixed between 20 and 22 hours or over 22 hours (Figure 3.3.1c).

For each cochlea, we obtained only 4-5 slices with the organ of Corti. The majority of the slices presented artefacts, such as bubbles, cracks, black spots, or places without resin.
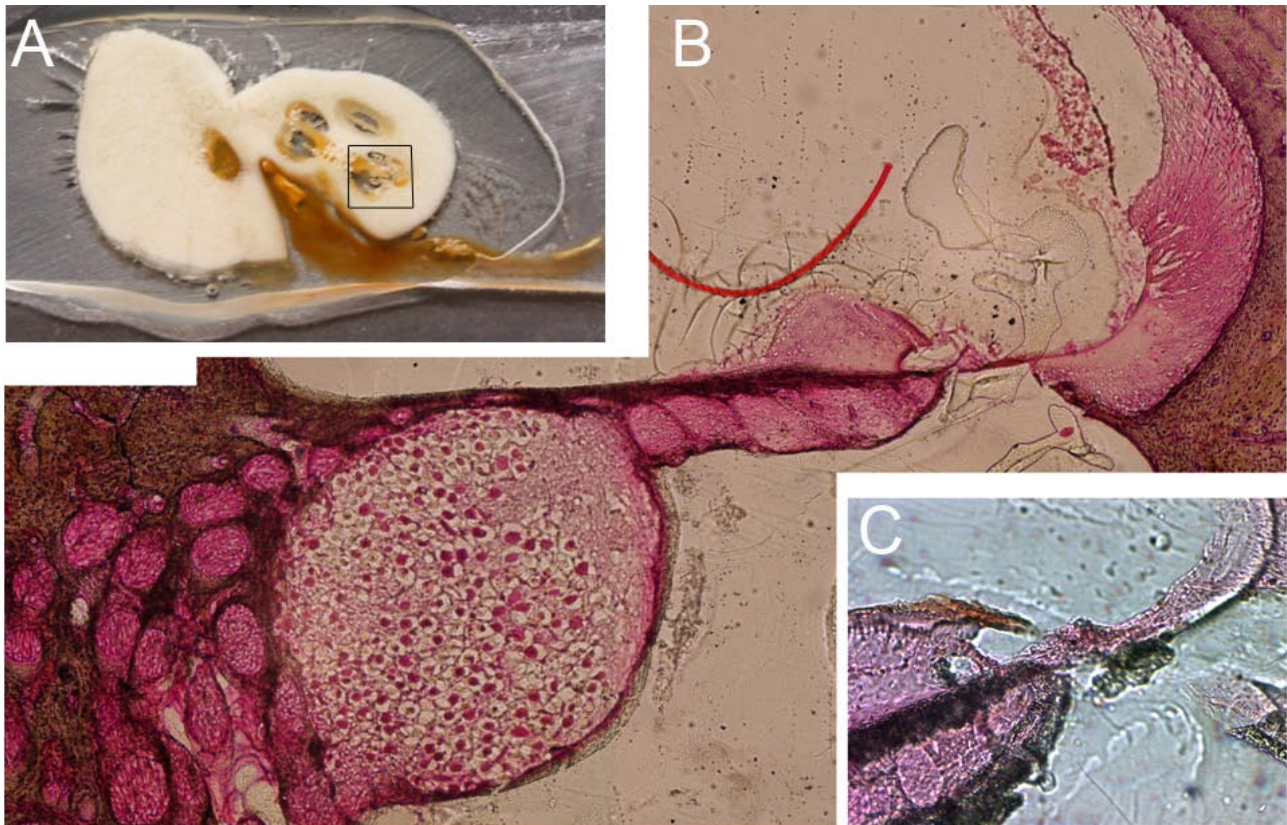
Figure 3.3.1. A) Section of striped dolphin periotic bone in autolytic condition before the polishing process. B) High magnification of the insert in A) under light microscope and hematoxylin-eosine staining. C) Remains of the organ of Corti of a striped dolphin injected at least 22 hours post-mortem. Both samples were provided by the Generalitat de Catalunya – Fundació CRAM.

3.4. TEM

Common findings between SEM and TEM concern the general morphology of the organ of Corti, which was typically formed by one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). In a harbour porpoise, some additional OHCs were observed forming a fourth row in the lower apical turn.

Table 3.4.1 and 3.4.2 show the averaged measurements performed by TEM and light microscope of the organ of Corti, spiral ganglion cells and spiral ligament at different baso-apical locations (Figure 3.4.1). We present below the most interesting features of each structure.

Table 3.4.1 - Mean measurements using transmission electron microscopy or light microscopy (marked with an asterisk) of the outer hair cells (OHCs), Deiters cells, basilar membrane and spiral ligament of different baso-apical locations. All measurements are expressed in µm. See Figure 2.4.4a-c for more details on the measurements.

	OHCs max. length	OHCs max. diameter	OHC cuticular plate		First row of the OHC stereocilia			Basilar membrane		Spiral ligament length	Deiters cells	
			arms	thickness	length	diameter	rootlet	width	thickness		length	cup length
Striped dolphin												
Lower basal turn	9,965	5,165	2,472	0,929				117,856* / 112,056	13,947*/ 12,981	336,843*	25,35	2,205
Upper basal turn		5,420						199,483* / 148,161	11,906*	285,710*		3,13
Lower apical turn	14,904	6,250	2,647	1,476	0,988	0,169	0,659	222,051*	7,871*	205,804*	35,667	
Apical end	22,099	13,064	1,767	2,705	1,759	0,289	0,660	377,025*	5,382*	86,349*		
Harbour porpoise												
Lower basal turn	8,898	4,323	3,148	1,271				91,120*/ 76,152	11,057*/ 9,800	335,180*	27,565	3,736
Upper basal turn	9,071	4,580	2,642	1,262				136,535* / 114,017	9,919*/ 8,892	214,477*	32,35	2,469
Lower apical turn	14,967	6,509	1,990	1,352	0,982	0,206	0,741	225,524* / 202,032	8,071*	141,899*	37,709	
Upper apical turn								257,392				

Table 3.4.2. Mean measurements of the innervation using transmission electron microscopy or light microscopy (marked with an asterisk) in different baso-apical locations.

	Spiral ganglion cells			Nerve fibers						
	Density (cells/ 10000µm²)	max length (µm)	min length (µm)	Myelin sheath length (nm)	Number of myelin layers	Thickness of myelin layers (nm)	Diameter of afferent fibers before habenula (nm)	Diameter of nerve fibers of the spiral lamina (µm)	Density (fibers/ 1000 µm²)	Number of rows of nerve fibers in the spiral lamina and habenula perforata
Striped dolphin										
Lower basal turn	4,558*	45,972	23,214	670	42,5	12	329	3,073	38,798	14,8
Upper basal turn	4,431*	37,090	22,483	541	38	14	296	3,126		
Lower apical turn	4,595*	46,179	29,748	680	39	16	338	4,182		
Apical end	5,773*			403	29			2,252	58,624	5
Harbour porpoise										
Lower basal turn	6,804*	44,985	25,142	439	34	11	676	2,900	42,148	13,231
Upper basal turn	7,590*	37,627	22,227	380				2,402	47,747	14,917
Lower apical turn	7,126*	34,814	23,688	443	26,25	16		1,851	90,841	9,818
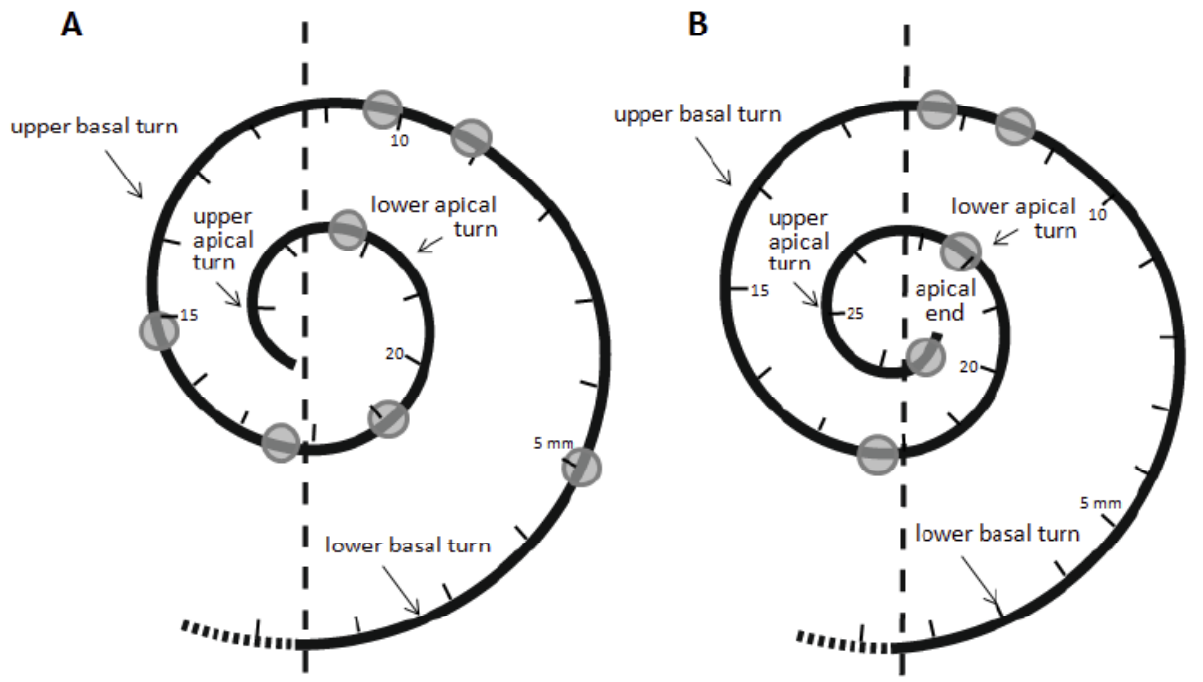
Figure 3.4.1. Schematic plot of the cochlear shape of A) harbour porpoise and B) striped dolphin with the different locations. Note the millimetre scale on each cochlea. Shadow regions indicate where the analysis was performed.

3.4.1. Basilar membrane

The basilar membrane (*pars tecta* and *pars pectinata*, see Figure 2.4.4b, section 2.4.2 in Material and Methods) increased in width (length of the segment comprised between the foramen of the *habenula perforata* and the spiral ligament) and decreased in thickness towards the apex, being especially short and thick in the lower basal turn. From the base to the apical region, in striped dolphin the average values ranged from around 112 to 377 μm in length and 13 to 5 μm in thickness, while in harbour porpoise the average values ranged from around 76 to 257 μm in length and 10 to 8 μm in thickness (Table 3.4.1).

3.4.2. Lateral wall: spiral ligament and stria vascularis

The spiral ligament (see Figure 2.4.4a) was also interestingly large in the lower basal turn (maximum values of 336,84 μm in striped dolphin and 335,18 μm in harbour porpoise) where the five types of fibrocytes were found (see Figure 3.4.2).

The stria vascularis (Figure 3.4.3) was badly preserved in all the sections. However, it was possible to identify the 3 layers of cells and to appreciate its exceptional density. The basal layer was formed by a line of cells, separating the stria vascularis from the spiral ligament. We could not identify the cell limits that form the marginal layer cells (Figure 3.4.3b). The marginal and intermediate layers in the harbour porpoise presented an average thickness of 22,8 μm and 44,4 μm, respectively.
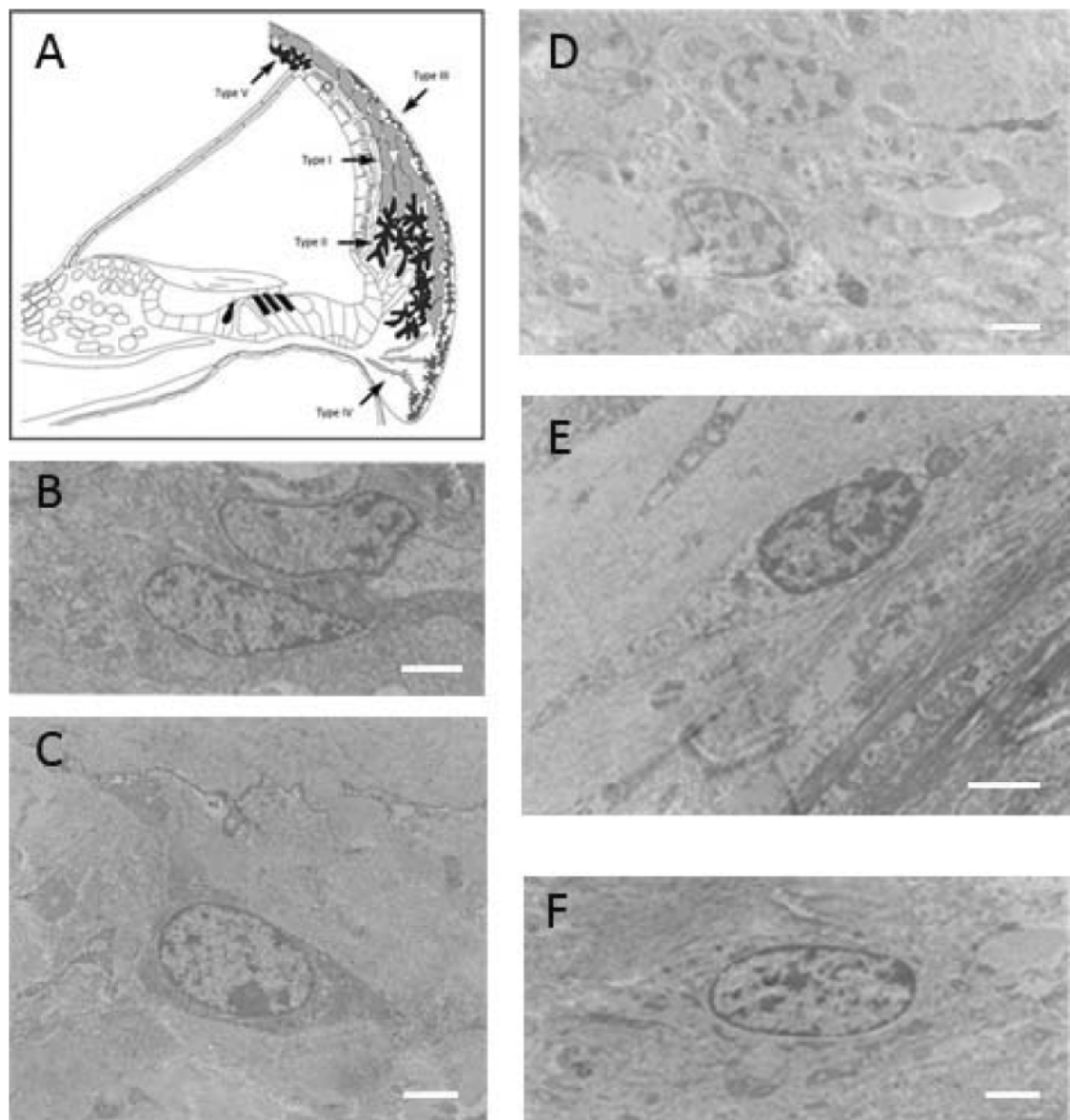
Figure 3.4.2. A) Schematic representation of the spiral ligament (modified from Hirose and Liberman, 2003). Transmission electron microscopy images of the five types of fibrocytes from the basal turn of harbour porpoise: type I (B), type II (C), type III (D), type IV (E) and type V (F). The sample was provided by Utrecht University and fixed at least 3 hours post-mortem. Scale bars: 2 µm.
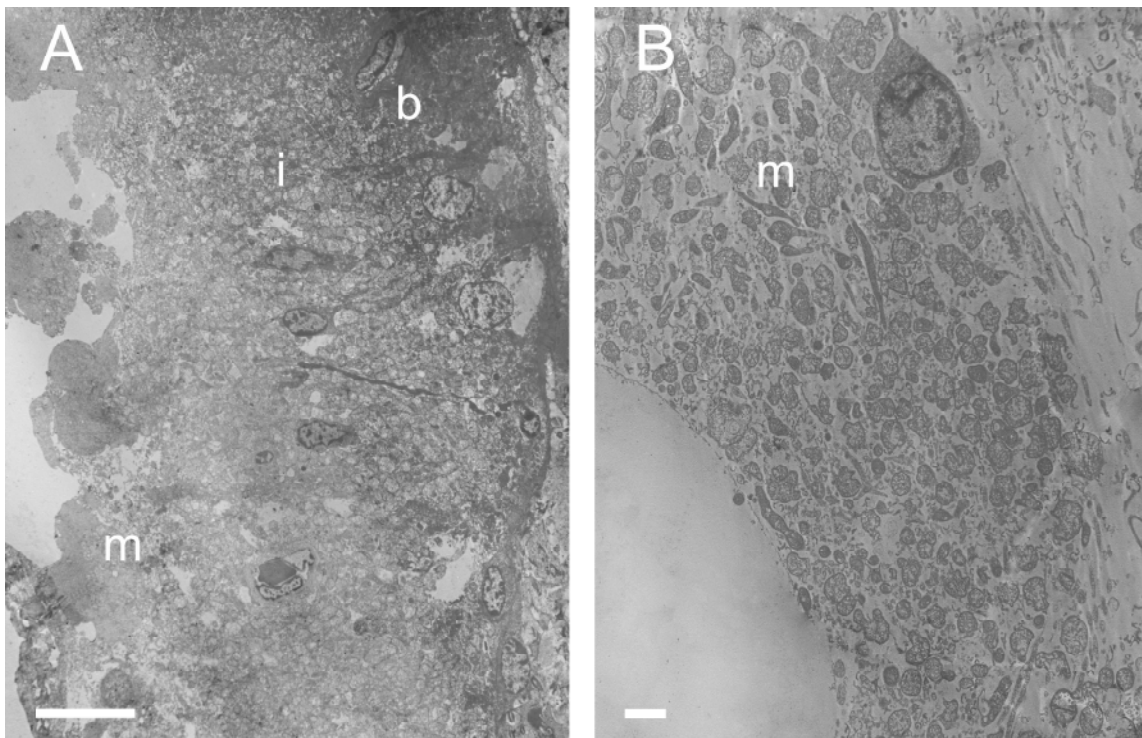
Figure 3.4.3. Transmission electron images of the stria vascularis of the upper (A) and lower (B) basal turn of a harbour porpoise. B shows a higher magnification of the marginal layer. The sample was provided by Utrecht University and fixed at least 3 hours post-mortem. Scale bars: 10 μm (A) and 2 μm (B). b: basal layer, i: intermediate layer, m: marginal layer.

3.4.3. Sensory cells

3.4.3.a. Outer hair cells

The ultrastructure of the OHCs is illustrated in Figure 3.4.4. The length of the cell body measured in OHCs of the lower basal turn (Figure 3.4.4a) is very small, with an average of 9,96 μm reaching 22,1 μm in the apical end in striped dolphin (Figure 3.4.4b) and from 8,9 in the base to 14,97 μm in the lower apical turn in harbour porpoise. The OHCs are also very narrow and increasing in diameter while approaching to the apex (from 5,16 to 13,06 μm in striped dolphin and 4,32 to 6,51 μm in the lower apical turn in harbour porpoise).

Stereocilia and cuticular plates. Although stereocilia were not very well preserved and only partial data could be extracted, stereocilia were also very short, being larger in the apical end (1,8 μm length and 289nm diameter in striped dolphin) than in the lower apical turn (0,99 and 0,98 μm large and 169 and 206nm diameter in striped dolphin and harbour porpoise, respectively). The OHCs usually present 3 rows of stereocilia. However, in one case, four rows were observed (insertion of Figure 3.4.4d).

The cuticular plates of OHCs established tight junctions with the outer pillar cells and phalangeal processes of the 3 rows of Deiters cells. These attachment zones were extremely long in the OHCs of the basal turn forming two "arms" (Figure 3.4.4c and 3.4.4d).

Subsurface cisternae. OHCs posessed a single layer of subsurface cisternae, which started just below the cuticular plate and continued down to the beginning of the synaptic region, below the nucleus (Figure 3.4.4e).

3.4.3.b. Inner hair cells

Inner hair cells (IHCs) were very poorly preserved, and were only observed in the lower apical turn of the striped dolphin and in the lower basal turn of the harbour porpoise.

They presented the typical pear shape (Figure 3.4.4f) with a cuticular plate thickness of 1,57 µm, longest stereocilia length of 2,97 µm and 308 nm in diameter.
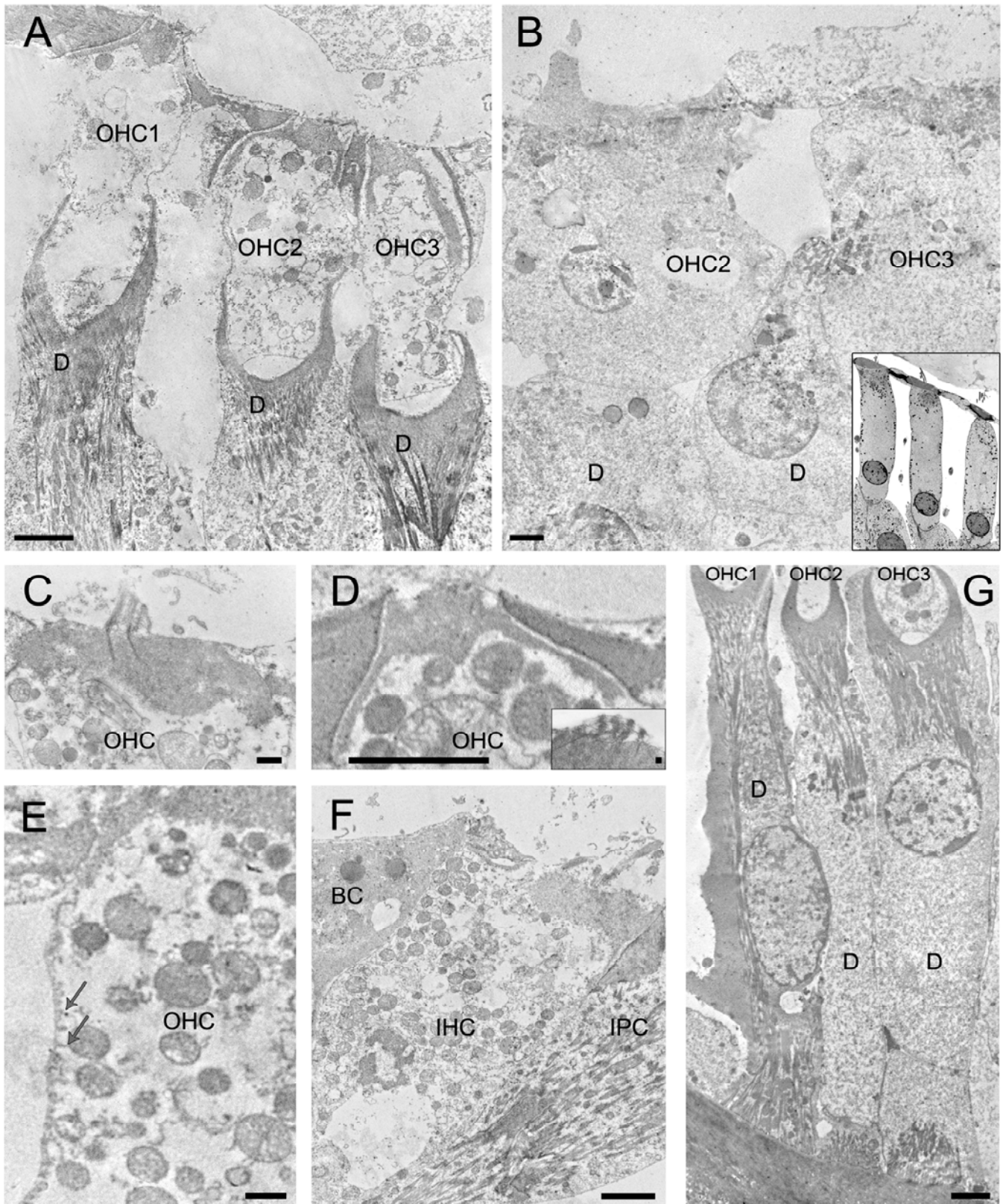
Figure 3.4.4. Ultrastructure of outer hair cells (OHC) and Deiters cells (D) of upper (insertion in D) lower basal turn of harbour porpoise (A, D and G) and apical end (B) and lower apical turn (C) of striped dolphin. Insertion in B): outer hair cells of rats in the apical turn (courtesy of Marc Lenoir). Outer hair cell subsurface cisternae (arrows in E) and inner hair cell (F) of the lower apical turn of a striped dolphin. Scale bars: A, B, F and G) 2 µm, C, D and E) 500 nm, insertion in D) 100 nm. BC: border cell, IPC: inner pillar cell

3.4.4. Supporting cells

Pillar and Deiters cells, especially at the base of the cochlea, presented an extremely well developed microtubular cytoskeleton (Figure 3.4.4a and 3.4.4g). In addition, in Figure 3.4.5d-f the differences in the microtubular disposition along the cochlear spiral can be observed, as well as the high packing of inner and outer pillar cells in the basal turn (Figure 3.4.5e) in comparison with the large extension on both cells in the apical turn (Figure 3.4.5d and 3.4.5f), where the space in the tunnel of Corti is increased.

The phalangeal process of Deiters cells and outer pillar cells had exaggerated long contact zone with the outer hair cells. This feature was especially evident on the basal turn of odontocete cochlea (Figure 3.4.4a and 3.4.4d).

The lower parts of the OHC bodies were completely surrounded by a large cup formed by the Deiters cells. The ultrastructure of the Deiters cup attachment is shown for basal (Figure 3.4.4a) and apical cochlear locations (Figure 3.4.4b). In the lower basal turn, this cup could reach up to 45% of the total length of the OHCs.

The apical pole of Hensen cells presented microvilli and large invaginations in the lateral cellular wall (Figure 3.4.5a). Hensen and Claudius cells were not easy to distinguish. In the basal turn, Hensen cells presented the nucleus in a lower position than Claudius cells (Figure 3.4.5c and 3.4.5g). However, in the apical turn we could not use the same criteria, but the position (Figure 3.4.5b and 3.4.5h). The length and inclination of Claudius cells were notably higher in basal positions than in apical ones (see Figure 3.4.5g and 3.4.5h that are at the same scale for comparison), ranging from up to 112 µm in the base of harbour porpoise to 12 µm in the apical coil of striped dolphin. On the other hand, Hensen cells length varies from 35 to 63 µm in both species and in all cochlear locations.

The inner sulcus cells usually fill most of the space between the basilar membrane and the limbus, extending from the border cells to the lower edge of the limbus (Figure 3.4.6f). The border cells (Figure 3.4.6g) were poorly preserved in all the sections.
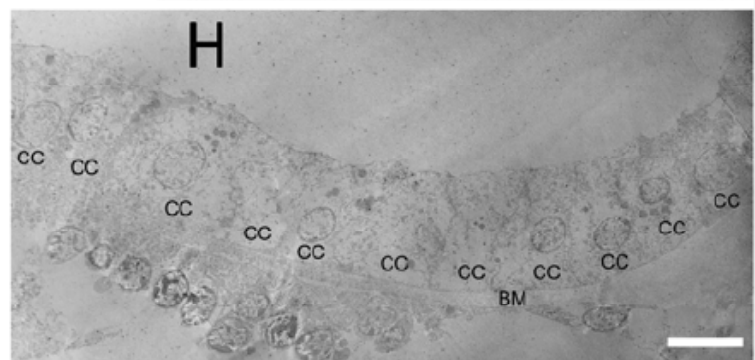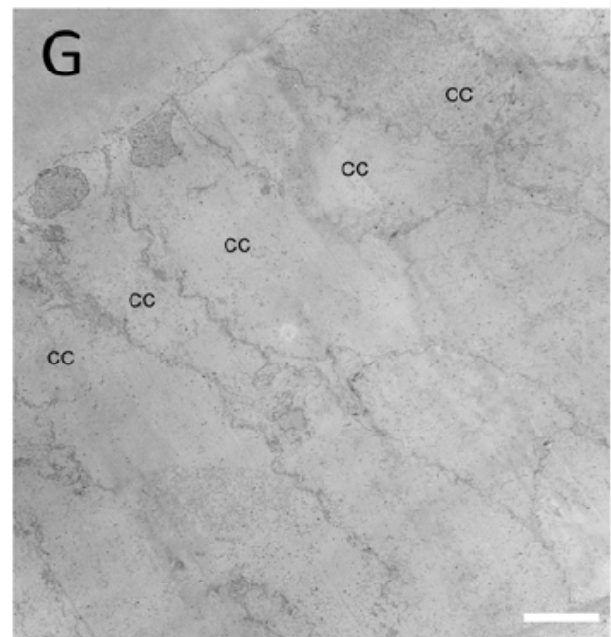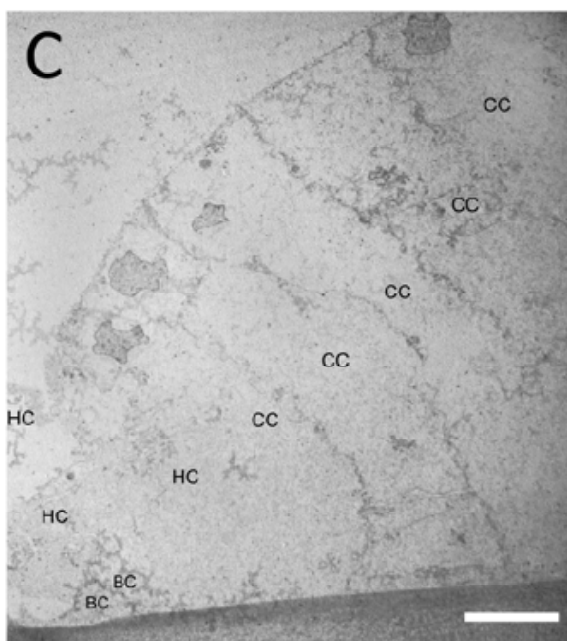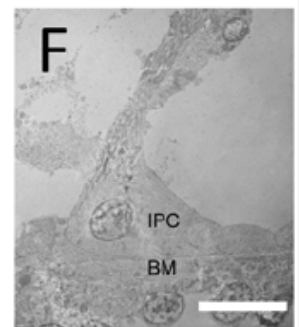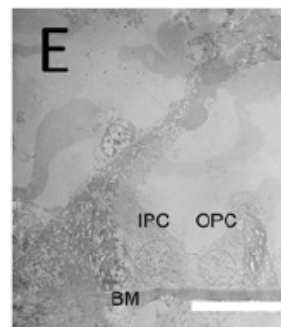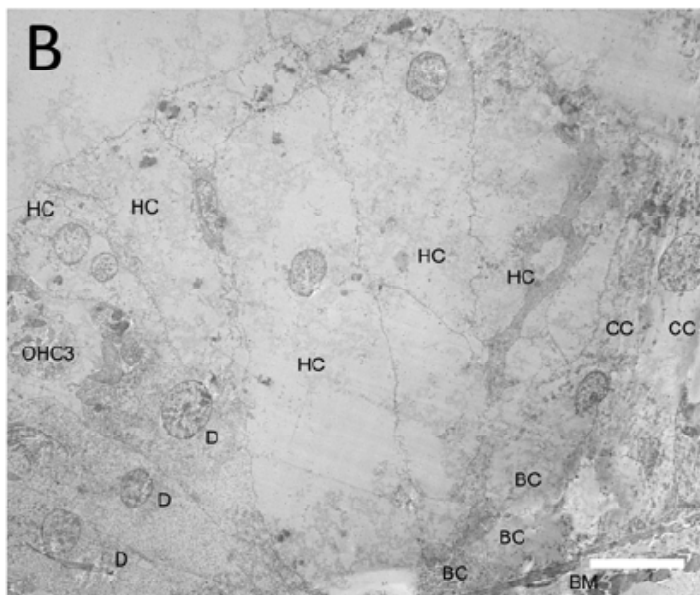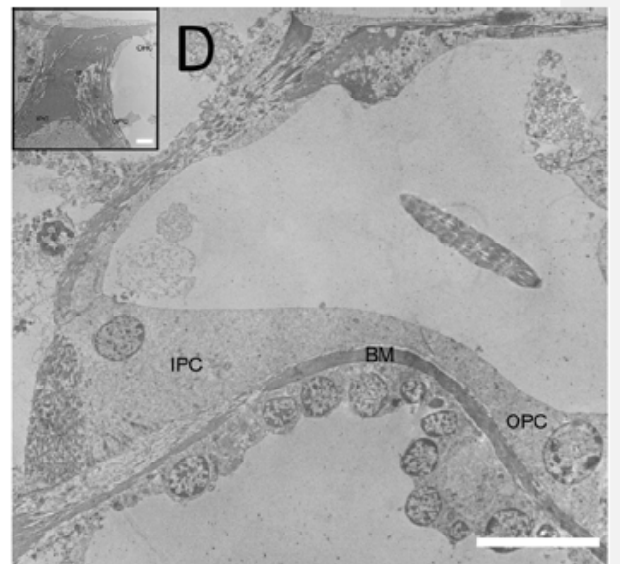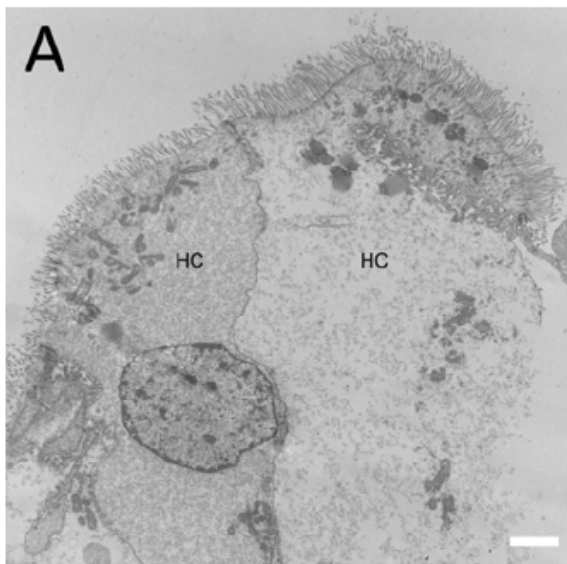
Figure 3.4.5. Transmission electron images of several types of supporting cells from a harbour porpoise (A, C-E, G) and a striped dolphin (B, insert in D, F and H). A) Detail of the Hensen cells of the lower basal turn. Hensen cells of lower apical turn (B) and lower basal turn (C). Pillar cells from the lower apical turn (D), lower basal turn (E) and apex (F). The insertion in D shows the apical pole of inner and outer pillar cells of the lower basal turn. Claudius cells of the lower basal turn (G) and apex (H). Note that the pairs of images B - C and G - H are in the same proportion, respectively. The samples were provided by: Utrecht University, fixed at least 3 hours post-mortem (A); AMBAR, fixed over 5 hours post-mortem (B, insert in D, F and H); and University of Liège, fixed 22 hours post-mortem (C-E, G). Scale bars: 10 μm (B-D, G, H), 2 μm (A, insert D, E, F). BM: Basilar membrane, CC: Claudius cell, D: Deiters cell, HC: Hensen cell, IPC: inner pillar cell, OHC: outer hair cell, OPC: outer pillar cell.

3.4.5. Spiral limbus

The cells that form the spiral limbus (see a schematic representation in Figure 3.4.6a) were generally well preserved. With the exception of the light and supralimbal fibrocytes, which used to remain out of the section, we could identify the rest of the cells contained in the spiral limbus. The epithelium lining cochlear scala and covering the limbus from the last inner sulcus cell to Reissner's membrane consisted of interdental cells. Morphologic differences permitted differentiation of three types of interdental cells located in the upper lateral (Figure 3.4.6c), central (Figure 3.4.6b), and medial (Figure 3.4.6c) parts of the limbus, respectively. The lateral interdental cells were only found in the superior lateral edge, and, instead of having a tubular shape, in our case they presented a shape more ocellated.

The stellate fibrocytes (Figure 3.4.6e) were located in the medial area of the spiral limbus and were in contact with the central interdental cells.

Interestingly, we could only observe a well formed apposite in the most basal section (around 5 mm of the basal end), in contact with the lateral edge of the spiral limbus. This structure is highlighted with an asterisk in Figure 3.4.8b-d.
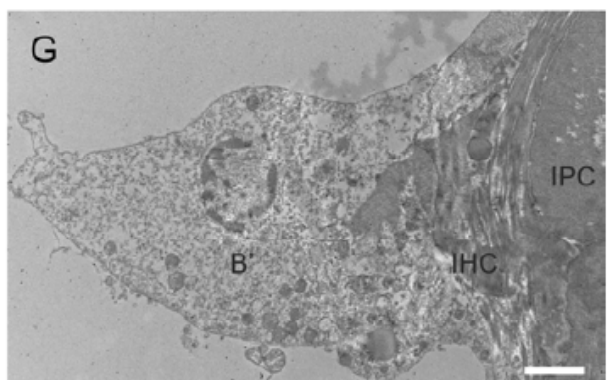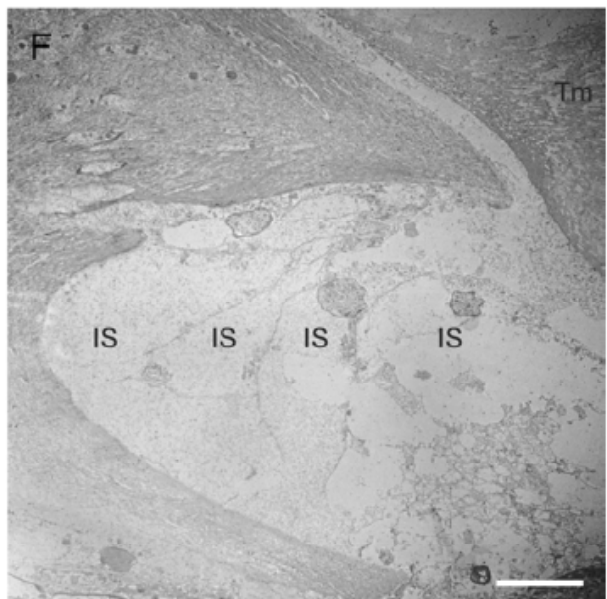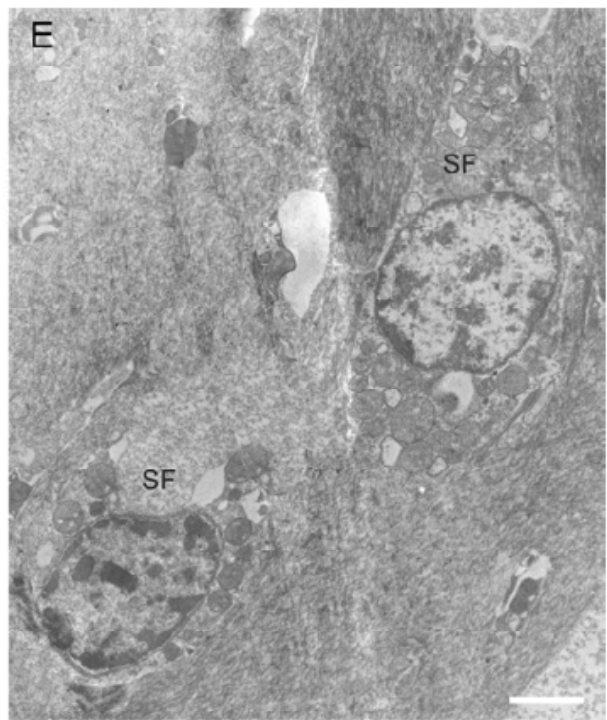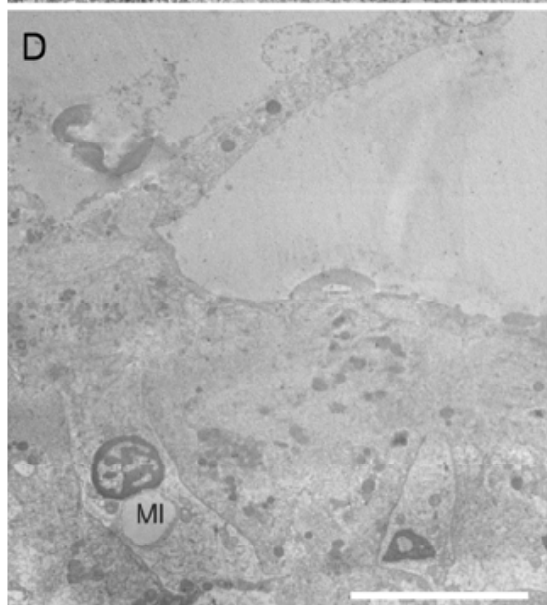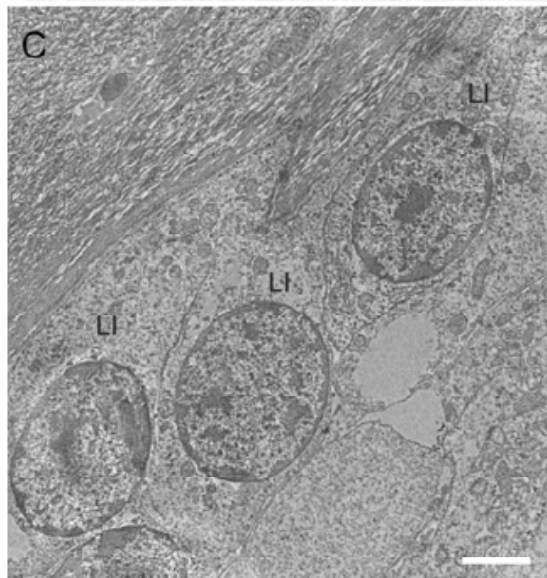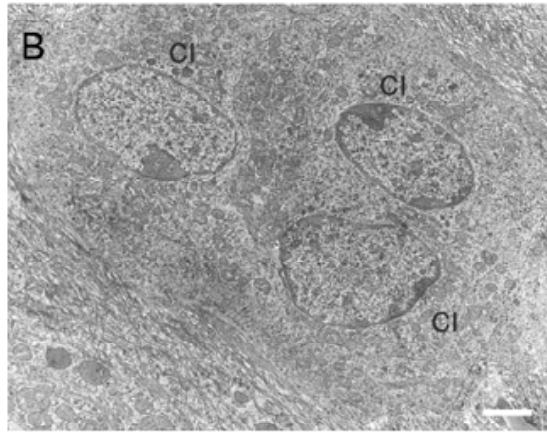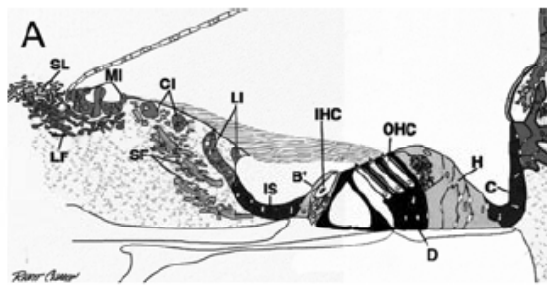
Figure 3.4.6. A) Schematic representation of the spiral limbus and organ of Corti cells (modified from Spicer and Schulte, 1998). Transmission electron images of the spiral limbus (B-E) and supporting cells (F, G) in the lower (B-E) and upper (G, F) basal turn of the cochlea of a harbour porpoise (B-E, F) and a striped dolphin (G). Central (B), lateral (C) and medial (D) interdental cells of the spiral limbus. E) Stellate fibrocytes. F) Inner sulcus cells. G) Apical pole of a border cell. The samples were provided by: Utrecht University, fixed at least 3 hours post-mortem (B, C, E); University of Liège, fixed 22 hours post-mortem (D and F); and AMBAR, fixed over 5 hours post-mortem (G). Scale bars: 10 µm (C, D, F) and 2 µm (B, E, G). B': border cells, C: Claudius cell, CI: central interdental cell, D: Deiters cell, IHC: inner hair cell, IS: inner sulcus cell, H: Hensen cell, LF: light fibrocyte, LI: lateral interdental cell, MI: medial interdental cell, OHC: outer hair cell, SF: stellate fibrocytes, SL: supralimbal fibrocyte.

3.4.6. Innervation

Throughout the odontocete cochlea analyzed, the OHCs were contacted by nerve fibers, which passed between the Deiters cells. These fibers were clearly identified as afferent fibers by the abundant microtubules content of their cytoplasm (Figure 3.4.7b). In contrast, there were no signs of either vesiculated terminals below OHC (Figure 3.4.7a) or upper-crossing fibers in the tunnel of Corti, belonging to the medial efferent system. Moreover, there were no remains of postsynaptic cisterns at the basal pole of the OHCs (Figure 3.4.7a).

The nerve fibers in the inner spiral bundle (below IHCs) were in a very bad conservation status (Figure 3.4.4f), without remains of nerve terminals.

Some morphometric information was extracted on the nerve fibers passing in the habenula and spiral lamina, including the diameter of the fibers, the thickness of the myelin sheath, the number of myelin layers and the number of rows of nerve fibers (Table 3.4.2).

The density of spiral ganglion cells (SGCs) expressed as the number of cells/ 10000 μm^2, was calculated. Although, by looking at the average data in Table 3.4.2, it seems there is a tendency of increasing density when getting closer to the apex, in a visual representation of all data (not shown here) a high variability on the measurements could be observed, without any clear tendency. However, the density of SGC in harbour porpoises (from 6,8 to 8,1 SGC/10000 μm^2) was systematically higher than in striped dolphin (from 4,4 to 5,8 SGC/10000 μm^2).

The SGCs were well preserved (Figure 3.4.7d), were very large in size, around 37-46 µm in the longest axis, and 22,4-29,7 µm in the shortest, in striped dolphin and from 34,8 - 45 µm to 22,2-25 µm in harbour porpoise.
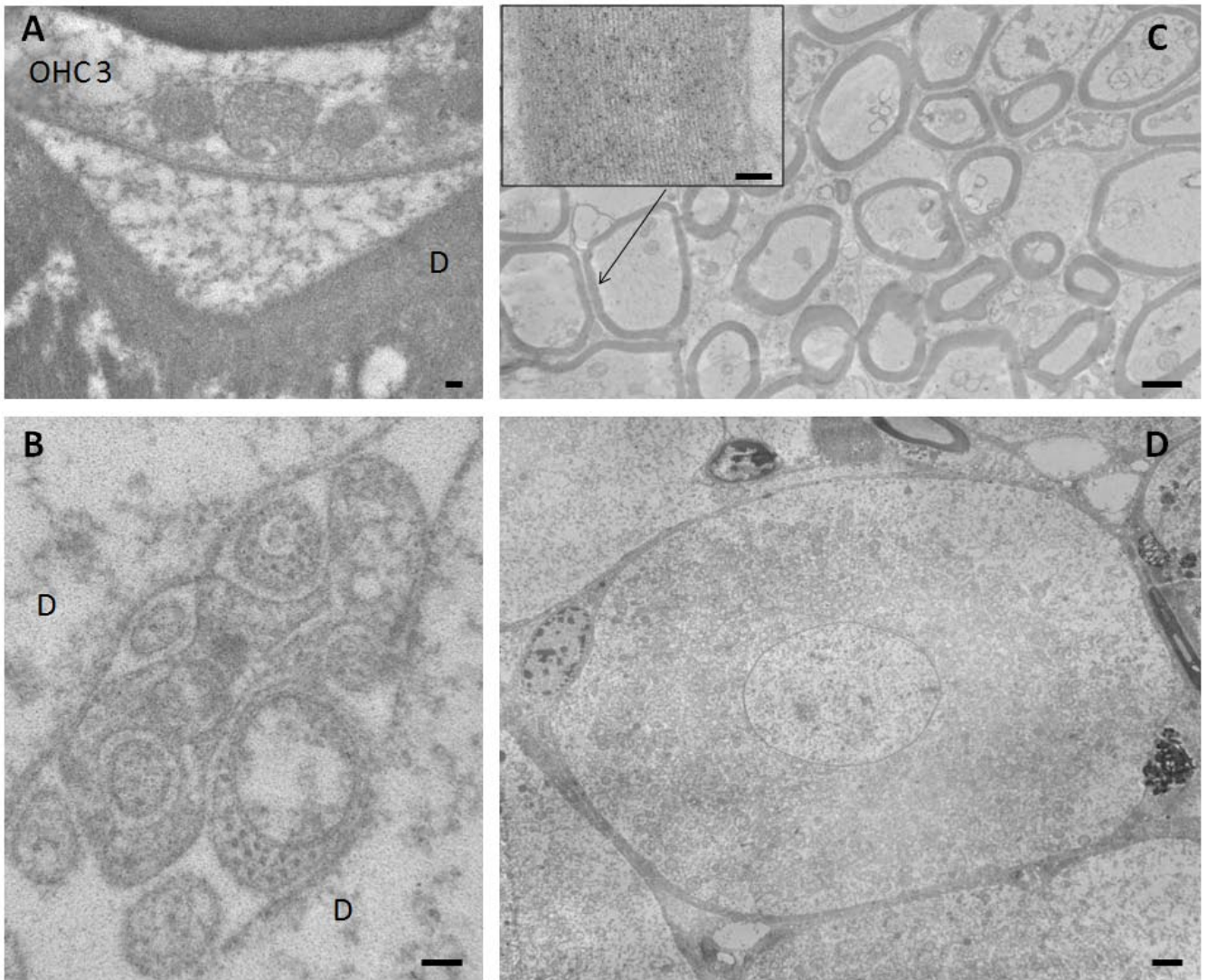
Figure 3.4.7. A) innervation of the third row of outer hair cells (OHC3) and cup of Deiters cells, B)Transversal cut of several afferent fibers containing microtubules among two Deiters cells (D), C) nerve fibers of the spiral lamina, including a high magnification of the myelin sheath. D) Spiral ganglion cell and satellite cell of the lower basal turn of harbour porpoise fixed at least 3 hours 15 minutes post-mortem. B) is from the lower apical turn, while A) and C) are from the basal turn of a striped dolphin, fixed 7 hours *post-mortem*. Scale bars: 100 nm (A, B and zoomed area in C) and 2 μm (C and D)

3.4.7. Tectorial membrane

The tectorial membrane was clearly larger while approaching to the apical region. Figure 3.4.8a and b illustrate this feature; Figure 3.4.8a was taken from around 18 mm from the basal end and Figure 3.4.8b from around 5 mm of the basal end of harbour porpoise cochlea. They were taken at the same magnification for comparison.

Only the Hensen stripe of the lower basal turn presented a notch where the stereocilia of inner hair cell could fit in (see arrow in Figure 3.4.8e). We could also observe the notches in the undersurface of the tectorial membrane where the tallest outer hair cell stereocilia fit (arrow in Figure 3.4.8f).
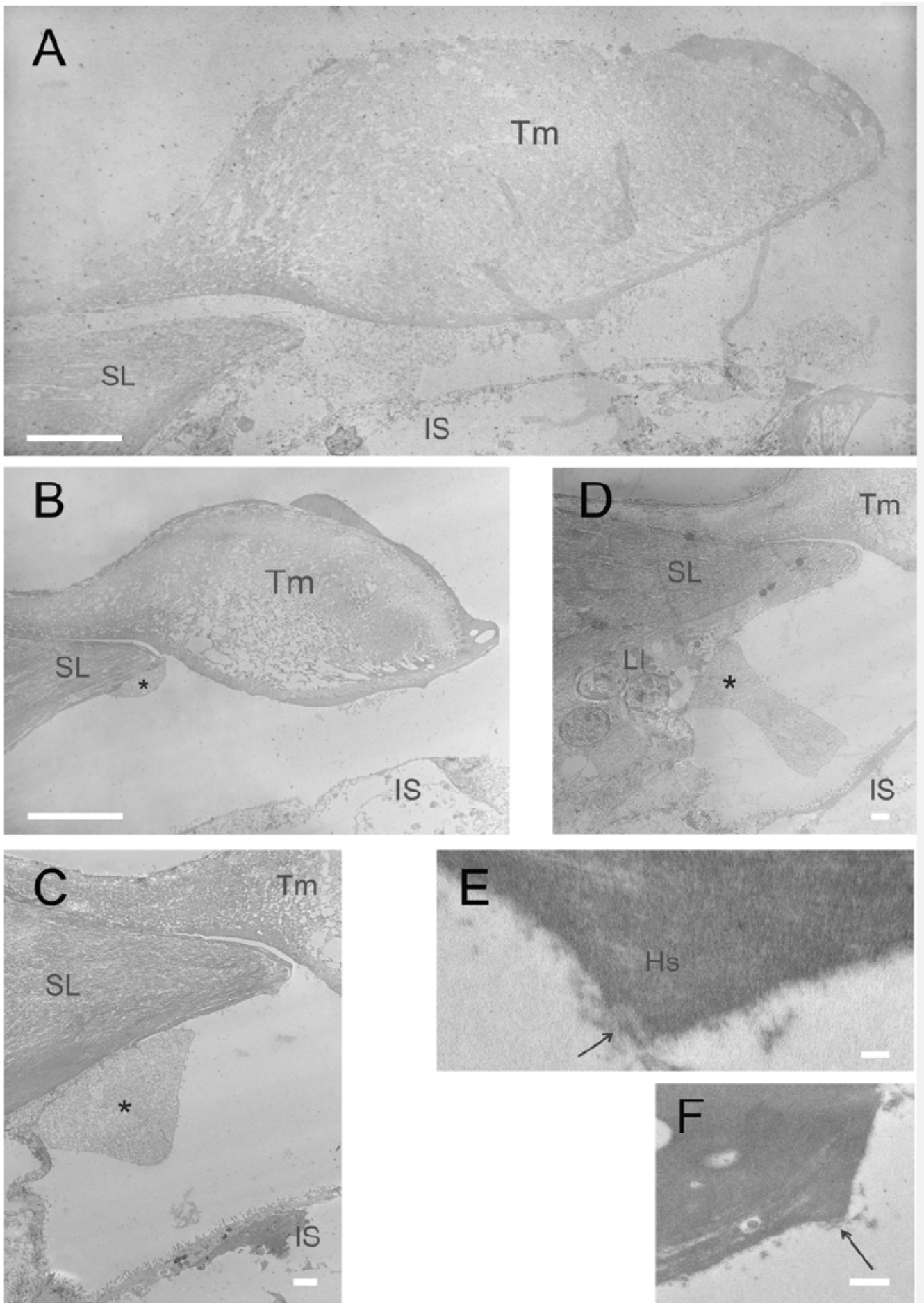
Figure 3.4.8. Transmission electron images of the tectorial membrane of the upper (A) and lower (B-F) basal turn of a harbour porpoise. Note that A and B are in the same proportion. The asterisk (B, C, D) highlights an apposite in different sections. E is a high magnification of the Hensen stripe, where the arrow marks a possible imprint of inner hair cell stereocilia. F is a high magnification of the marginal net with the imprint of outer hair cell stereocilia (arrow). The samples were provided by University of Liège, fixed 22 hours post-mortem (A) and Utrecht University, fixed at least 3 hours post-mortem (B-F). Scale bars: 10 µm (A and B), 2 µm (C and D), 500 nm (F) and 100 nm (E). Hs: Hensen stripe, IS: inner sulcus cell, LI: lateral interdental cells, SL: spiral limbus, Tm: tectorial membrane.

3.5. SEM

For some yet unexplained reason, the best preserved region in all cochleae that were observed in SEM was the apical end, apical turn and in some cases, part of the upper basal turn. All the results presented here come therefore from these locations. There were no significant differences among the measurements on the apical pole of the sensory and supporting cells presented below in the different measured positions. For this reason, all the values in Table 3.5.1 are averaged values, giving information of the general features of this area.

Table 3.5.1. Mean morphometric measurements of the reticular lamina performed with the images obtained using scanning electron microscopy from the lower and upper apical turn, apical end and in some cases, part of the upper basal turn. See Figure 2.4.4d for more details on the measurements. OHC: outer hair cell, IHC: inner hair cell, IPC: inner pillar cell, OPC: outer pillar cell, PC: phalangeal cell.

	OHC1/ mm	Total number OHC	IHC/mm	Total number IHC	IPC/mm	Maximum length			Distance between OHC of different rows		Distance between OHC of the same row		
						OHC1	OHC2	OHC3	OHC1-OHC2	OHC2-OHC3	OHC1-OHC1	OHC2-OHC2	OHC3-OHC3
Striped dolphin	175,370	15012,023			232,681	5,251	5,038	5,603	12,750	9,549	0,582	0,612	0,649
Harbour porpoise	147,672	11405,447	121,459	3126,962	217,858	6,196	6,102	5,468	11,199	8,203	0,512	0,552	0,886

	IHC maximum length	Distance between IHC	IPC maximum length	IPC minimum length	OPC maximum length	PC maximum length
Striped dolphin	8,430		16,941	4,406	10,715	
Harbour porpoise	8,244	1,154	14,997	4,714	8,944	7,373

3.5.1. Cochlear length- preliminary frequency map

In our study, the cochlear length was measured in some species (Table 3.5.2). In cases when the audiogram is known it is possible to build a preliminary approximation of their frequency map (Figure 3.5.1).

Table 3.5.2. Measurements of cochlear length of several species from SEM images, performed along the limit of the first row of outer hair cells with the inner pillar cells. The cochlear length measurements of juvenile individuals were not taken into account for this study except in the cases marked with an asterisk.

Species	Cochlear length (mm)	n
Striped dolphin (*Stenella coeruleoalba*)	26,878	2
Harbour porpoise (*Phocoena phocoena*)	24,813	3
Bottlenose dolphin (*Tursiops truncatus*)	34,418	1
Common dolphin (*Delphinus delphis*)	27,123	1
Northern bottlenose whale (*Hyperoodon ampullatus*)	32,818	2
Pilot whale (*Globicephala melas*)	42,107*	1
Fraser's dolphin (*Lagenodelphis hosei*)	25,736*	1

Atlantic spotted dolphin (*Stenella frontalis*)	22,38*	1
Pygmy sperm whale (*Kogia breviceps*)	22,675*	1
Cuvier's beaked whale (*Ziphius cavirostris*)	30,076	1

The following empirical function that relates the characteristic frequency in kHz, F, to cochlear points, χ, fits several species and was originally suggested by Greenwood (1961) and also applied in the cat by Liberman (1982) and in the Mongolian gerbil by Müller (1996) was used:

$$F = A (10^{\alpha x} - k).$$

The value 0,021 was used for the slope of the straight-line portion of the frequency-position curve, α, in normalized form (i.e., when χ is expressed as a percentage of basilar length, from 0 at the apex to 100 at the base). α has shown to be conserved throughout several land mammal investigated species (Greenwood, 1961; Greenwood, 1990; Greenwood, 1996). *A* is a scaling constant between the characteristic frequency and the upper frequency limit of the species and *k* is a constant of integration that represents the divergence from the log nature of the curve and is determined by the lower frequency audible limit in the species. *A* and *k* were estimated for striped dolphin, harbour porpoise and bottlenose dolphin according to their audiogram (Table 3.5.3). However, the values proposed here should be considered as a first approximation.

Table 3.5.3. Values used for the empirical function $F = A (10^{\alpha x} - k)$ that relates the characteristic frequency in kHz, F, to cochlear points, χ (Greenwood, 1961) with the hearing frequency range considered to calculate them according to published audiograms (see text for details).

	α	A	k	Hearing frequency range considered
Striped dolphin	0,021	1,2771	0,6085	0,5-160 kHz (Kastelein *et al.*, 2003)
Harbour porpoise	0,021	1,439	0,8263	0,25-180 kHz (Kastelein *et al.*, 2002)
Bottlenose dolphin	0,021	1,217	0,9384	0,075-152 kHz (Johnson, 1967; Popov and Supin, 1990b; Popov *et al.*, 2007)
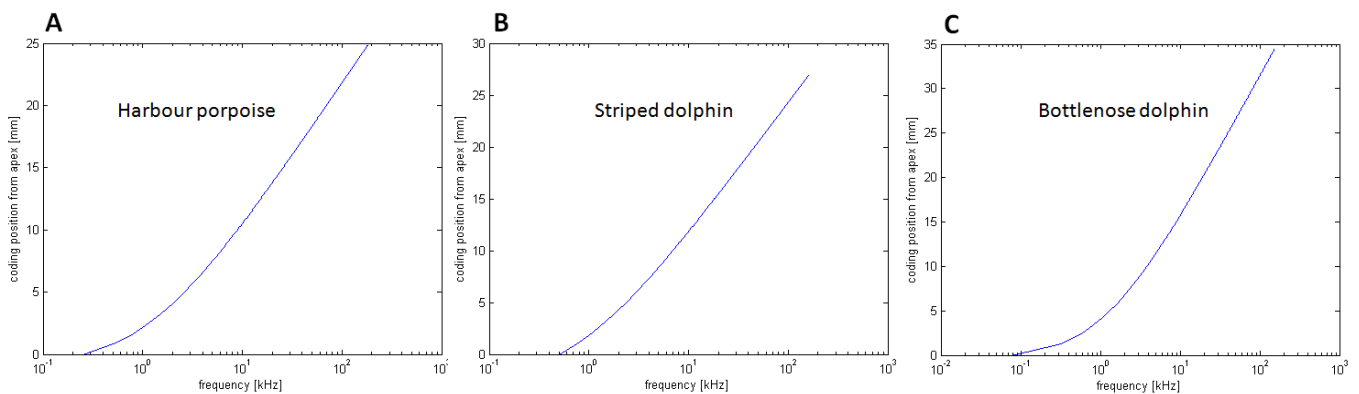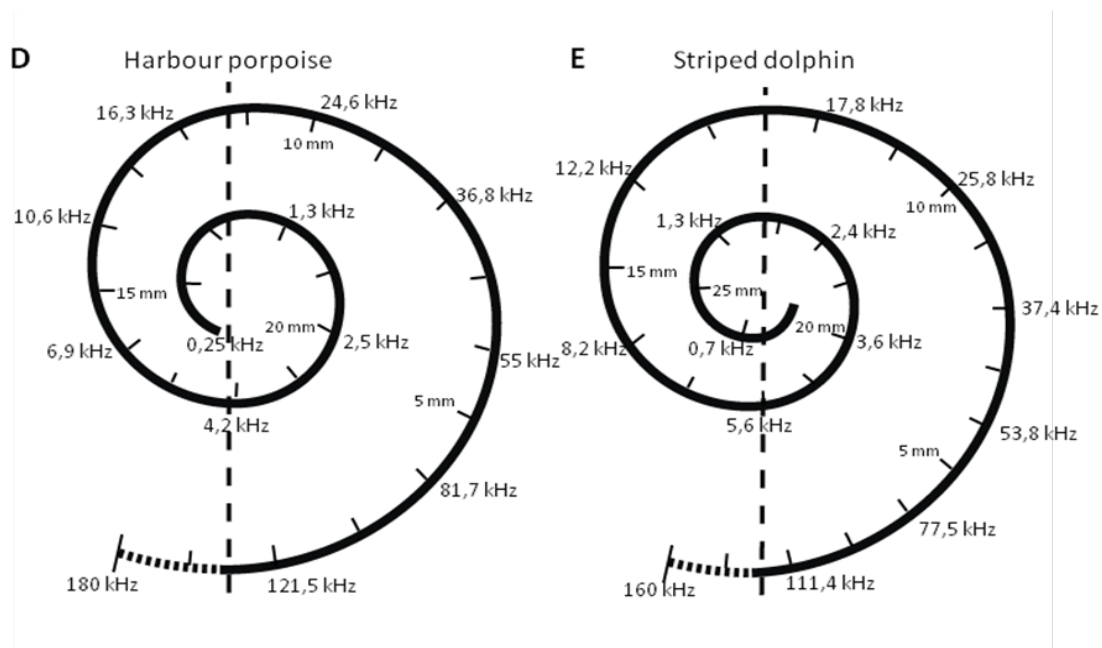
Figure 3.5.1. Preliminary cochlear frequency maps for harbour porpoise (A), striped dolphin (B) and bottlenose dolphin (C) extrapolated from the values of Table 3.5.3. The frequency (in kHz) is represented versus the distance from the apex (in mm). D and E are squematic drawings that display the same information as in the graphs A and B, respectively.

3.5.2. Sensory cells

3.5.2.a. Outer hair cells

The OHCs had narrow, wingshaped cuticular plates, between 5 and 6,2 µm long and separated from each other by 500-880 nm when they belonged to the same row (Table 3.5.1). The innermost row (OHC1) and the middle row (OHC2) were distinctly separated by the large hexagonal heads of the outer pillar cells. In contrast, there was very little space between the middle (OHC2) and the outermost (OHC3) rows of OHCs, significantly different in both species (p-value= 2,5 e^{-10} for harbour porpoise and 2,86 e^{-10} for striped dolphin).

From all the samples analyzed, we only found, in one harbour porpoise, few cells forming a fourth row of OHCs in the apical region (Figure 3.5.3b).

The OHCs had presumably three rows of stereocilia (Figure 3.5.2b) but more studies with better preserved samples should be conducted to confirm this result.

Total number of OHC was estimated taking into account the average density (175,37 OHCs mm^{-1} of cochlea for striped dolphin and 147,67 for harbour porpoise) and extrapolating it to all averaged cochlear lengths (28,534 mm for striped dolphin and 25,745 mm for harbour porpoise), resulting in 15012 and 11405 OHCs, respectively.

Even if considering that the samples were in autolytic conditions, it was possible to determine the shape of OHC and determine if there was presence of a scar resulting from the growth of supporting cells after the death of an OHC (Figure 3.5.2c, see a scheme of the scar formation process in Figure 3.5.2e). Moreover, in the case of one harbour porpoise, a high proportion of the apex, around 420 µm, was full of scars (Figure 3.5.2d).
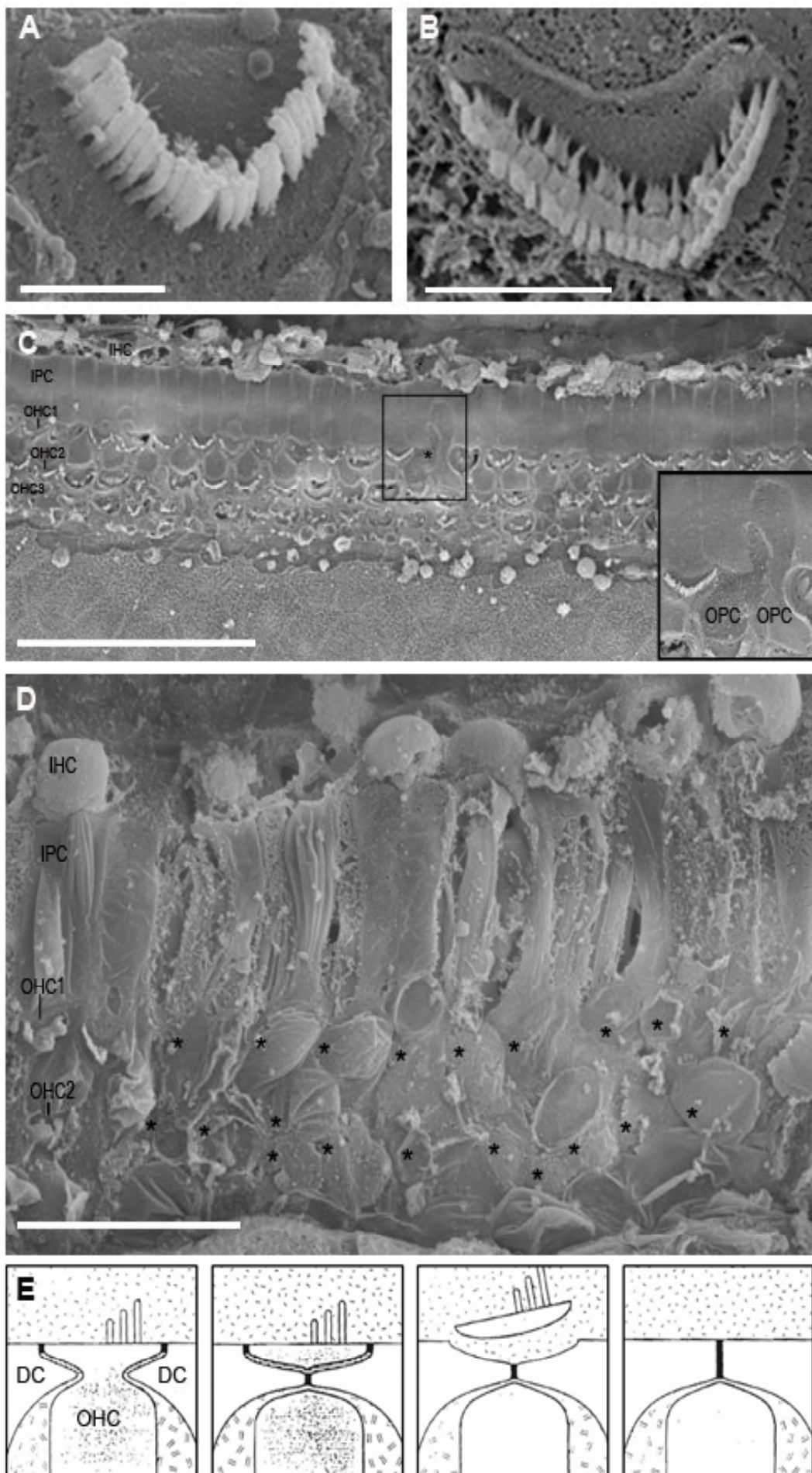
Figure 3.5.2-Scanning electron microscope of outer hair cells (OHCs) apical pole from the apical turn of A, C, D) harbour porpoise and B) common dolphin, where it is possible to observe three rows of stereocilia. In C, the rectangle highlights one scar (asterisk) formed with two expansions of outer pillar cells after the disappearance of one OHC in the first row. In D, all OHCs have disappeared and a large scar is formed by many supporting cells including outer pillar cells and Deiters cells. E) Squematic diagram showing the process of scar formation (source: Leonova and Raphael, 1997). Sample A) was fixed by SOCPVS before 18 hours post-mortem and B, C and D) by the University of Utrecht, 16 hours post-mortem (B and D) and at least 3 hours 15 minutes (C). Scale bars: 3 µm (A), 2 µm (B), 50 µm (A) and 20 µm (B). IHC: inner hair cell, OHC: outer hair cell, IPC: inner pillar cell, OPC: outer pillar cells, D: Deiters cells.

3.5.2.b. Inner hair cells

The IHCs were rarely well preserved in all the samples analyzed. They are the first structures that degenerate after anoxia, possibly because they are not strongly anchored in their base to phalangeal cells, as it occurs in OHCs with Deiters cells (Figure 3.4.4).

The IHC cuticular plates were ovoid (Figure 3.5.3a), with the maximum length on the longitudinal axis. In the striped dolphin, their average maximum length was 8,4 µm and 4,5 µm the minimum. In the harbour porpoise, the lengths were 8,2 and 5,2 µm respectively, the IHCs were 1,54 µm, this latter distance being larger than the separation found between OHCs of the same row.

The total number of IHCs was estimated following the same procedure as above, ending in 3127 IHCs in harbour porpoise.
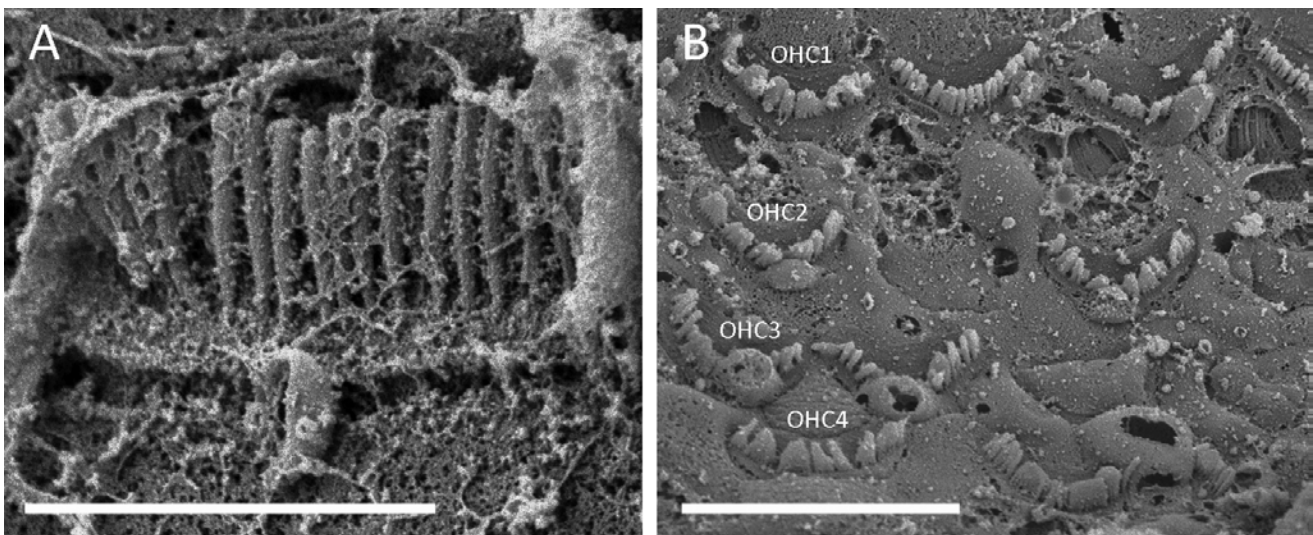
Figure 3.5.3. Scanning electron microscopy image of: A) an inner hair cell stereociliary bundle in the upper basal turn of a striped dolphin cochlea perfused 7 hours *post-mortem;* and B) a few cells forming a fourth row of outer hair cells in the apical turn of a harbour porpoise cochlea fixed 9 hours *post-mortem.* Scale bars: 5 µm (A) and 10 µm (B). The samples were provided by AMBAR (A) and University of Liège (B).

3.5.3. Tectorial membrane

Despite the relatively bad preservation status of the organ of Corti, the tectorial membrane was comparatively in very good condition. Even in a sample of a bottlenose whale *Hyperoodon ampullatus* where there were no remains of the organ of Corti, the imprints of the OHC stereocilia were present in the tectorial membrane (Figure 3.5.4c). Figure 3.5.4 shows these imprints that were found in the tectorial membrane of different species. In some cases, a double imprint pattern was also detected.

By counting the number of imprints, it is possible to know the number of the longer stereocilia per OHC, and assuming that each OHC presents three rows of stereocilia (see Figure 3.5.2b), the total number of stereocilia can be extrapolated. This number ranged from 130 to 168, depending on the species (see Table 3.5.4).

In a case of a common dolphin impressions of the inner hair cell stereocilia were found for the first time in an odontocete species in the very basal partition of the tectorial membrane (Figure 3.5.4h and i).

Table 3.5.4. Analysis of the outer hair cell (OHC) stereocilia imprints on the undersurface of the tectorial membrane.

Tectorial membrane	number of imprints	Number of stereocilia /OHC	Number of animals	Number of cells
Striped dolphin	56	168	2	6
Harbour porpoise	43,4	130,2	3	22
Northern bottlenose whale	49,6	148,8	1	15
Common dolphin	52,75	158,25	2	8
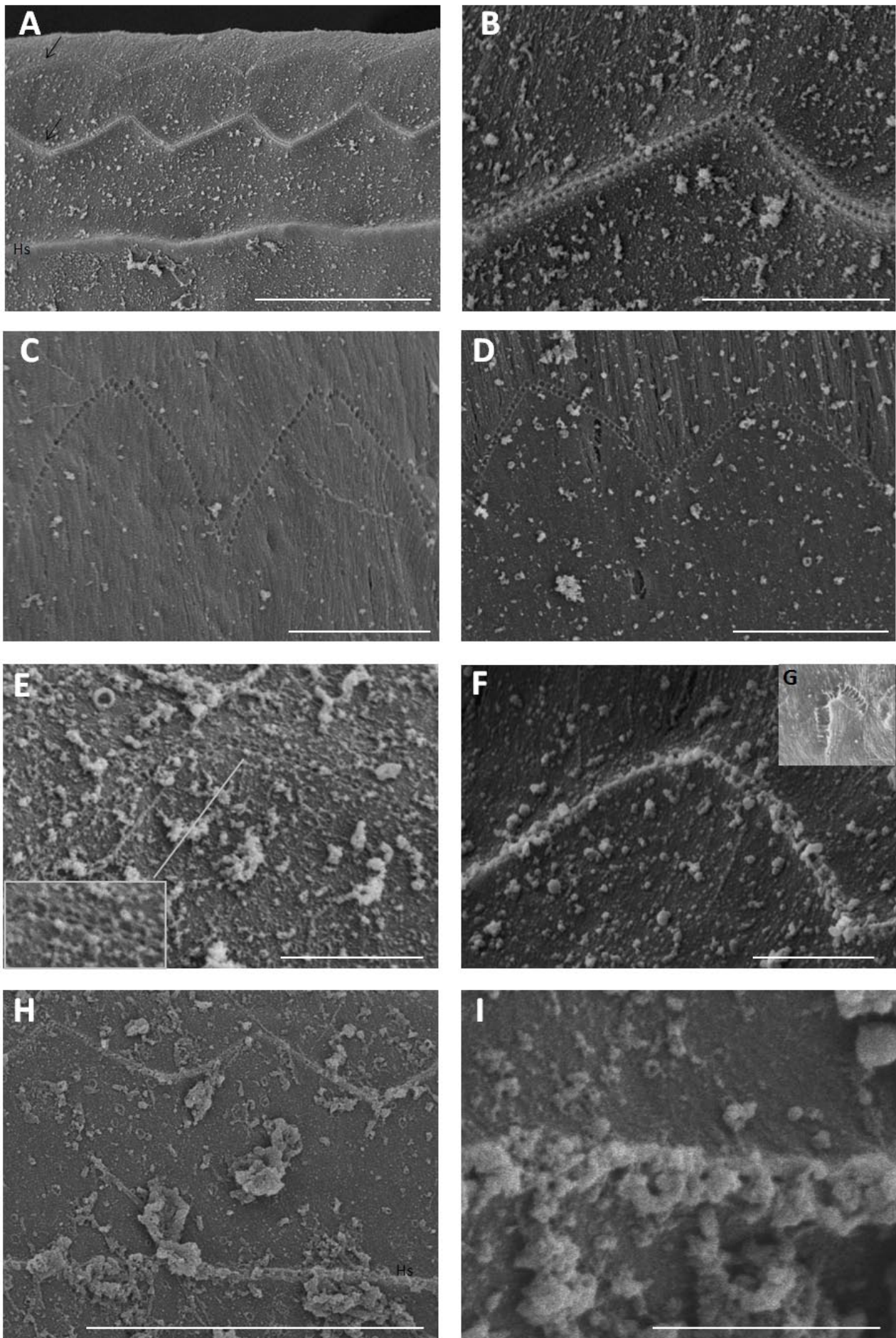
Figure 3.5.4. Scanning electron microscopy images of the outer hair cell stereocilia imprints on the undersurface of the tectorial membrane of a striped dolphin (A, highlighted with arrows, and B), northern bottlenose whale (C), harbour porpoise (D), common dolphin (E, F). Note the double imprint in E. G shows enlarged imprints in the tectorial membrane of a guinea pig cochlea after a loud sound exposure (reproduced from Morisaki *et al.*, 1991). H and I show inner hair cell stereocilia imprints on the Hensen stripe of the most basal portion of the tectorial membrane of a common dolphin. The samples were provided by Generalitat de Catalunya-Fundació CRAM (A and B), University of La Rochelle (C and E), University of Liège (D) and SOCPVS (F, H, I). Hs: Hensen stripe. Scale bar: 10 µm (A and H), 3 µm (B and C), 5 µm (D), 2 µm (E and I) and 1,5 µm (F).

3.6- Immunohistochemistry

One of the main problems we faced when analyzing the harbour porpoise cochlea was that it displayed autofluorescence (see Figure 3.6.1 from a section without being treated with antibodies).
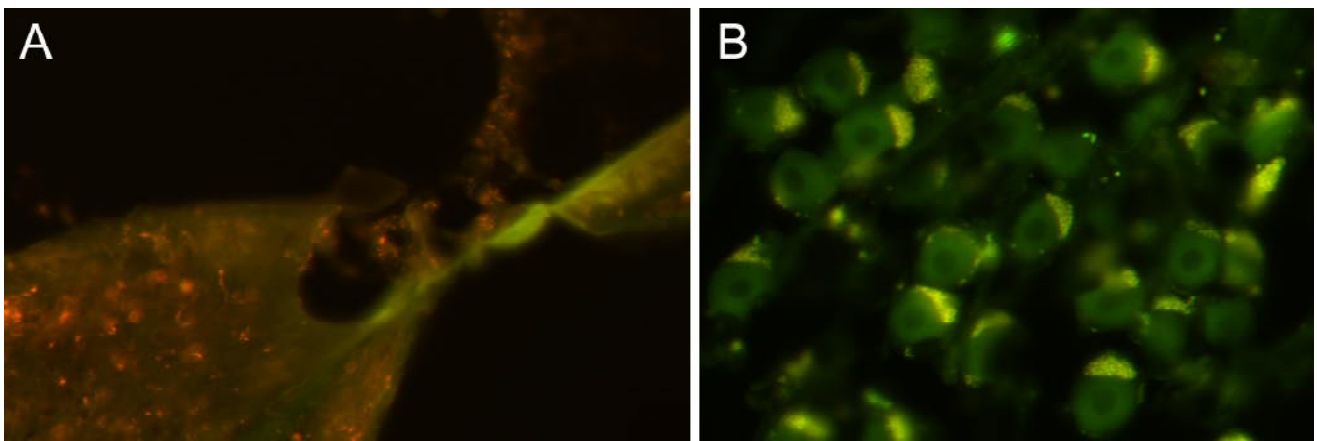
Figure 3.6.1. Result from the observation of a sample only treated with incubation buffer, but no antibodies. It is shown that the sample displays autofluorescence. A shows the organ of Corti, spiral limbus and part of the spiral lamina and B the spiral ganglion neurons of the upper basal turn of a harbour porpoise.

In this preliminary study, several agents at different exposition time were used in different slides to decrease autofluorescence or optimize contrast, such as: 1) Histo/Zyme proteolytic enzyme (5 minutes), 2) ammonium chloride (30 minutes), 3) sodium borohydride (20 and 40 minutes), 4) ultraviolet rays (10 minutes) and 5) Sudan Black B (3, 4, 5, 10, 20 and 30 minutes). From all of them, the one that worked better was Sudan Black B, specially the exposition of 5 minutes after the secondary antibody (see Methodology section 2.4.3 for the best optimized protocol).

Not all the tested antibodies stained specifically, or simply worked at the dilutions we used. In our study, the antibodies that did not work were:

- Mouse anti-myosin VIIa at 1:100 and 1:200

- Guinea pig anti-VGlut3 at 1:1000

- The fungal toxin Rhodamin-Phalloidine at 1:2000 and 1:5000

However, and despite the conservation status of the sample, we obtained positive staining for:

- Goat anti-prestin polyclonal antibody at 1:200. The transmembrane motor protein prestin labelled the OHC walls (Figure 3.6.2c and 3.6.2e-h in green). We found sometimes some nonspecific labeling at the spiral limbus and even IHC position. With this technique we could describe the same phenomenon exposed in section 3.4.3a, consistent in that the OHCs increased their length while approaching the apical region of the cochlear spiral.

- Mouse anti – ctBP2 at 1:1000. It recognized specifically the cell nucleus of the organ of Corti cells and spiral ligament fibrocytes (in red in Figure 3.6.2c-f and 3.6.2i-k), but not the glutamate vesicles.

- Sheep anti-VAcht (vesicular acetylcholine transporter) at 1:1000. It stained the efferent fibers (Figure 3.6.3b-d) instead of the synaptic vesicles of lateral and medial efferent system. We do not know if they are lateral (onto the inner hair cells) or medial (onto the outer hair cells). However, since we found not stained fibers in the tunnel of Corti, we believe that the ones recognized by this antibody are the lateral efferents.

- Mouse anti-neurofilament 200 monoclonal antibody at 1:200 and 1:400 (dilution 1:800 was used to adjust the protocol). We obtained specific marking in type I spiral ganglion cells and their axons that pass through the modiolus (Figure 3.6.4d). However, the dendrites of radial efferent fibers were not stained.

- Rabbit anti-peripherin polyclonal antibody at 1:400. It stained the type II spiral ganglion cells and their axons (Figure 3.6.4e and f).

With the combination of anti-neurofilament and anti-peripherin antibodies (Figure 3.6.4g and h) it was possible to determine the percentage of type I and II neurons for the first time in this species. In average, we find around 95% type I and 5% type II neurons (the values fluctuate between 90-99% type I and 1-10% type II SGCs). We did not find differences or trends in these proportions along the cochlear spiral. Type II spiral ganglion cells were extremely small, representing 10-24% (16% in average) of the area of type I spiral ganglion cells.
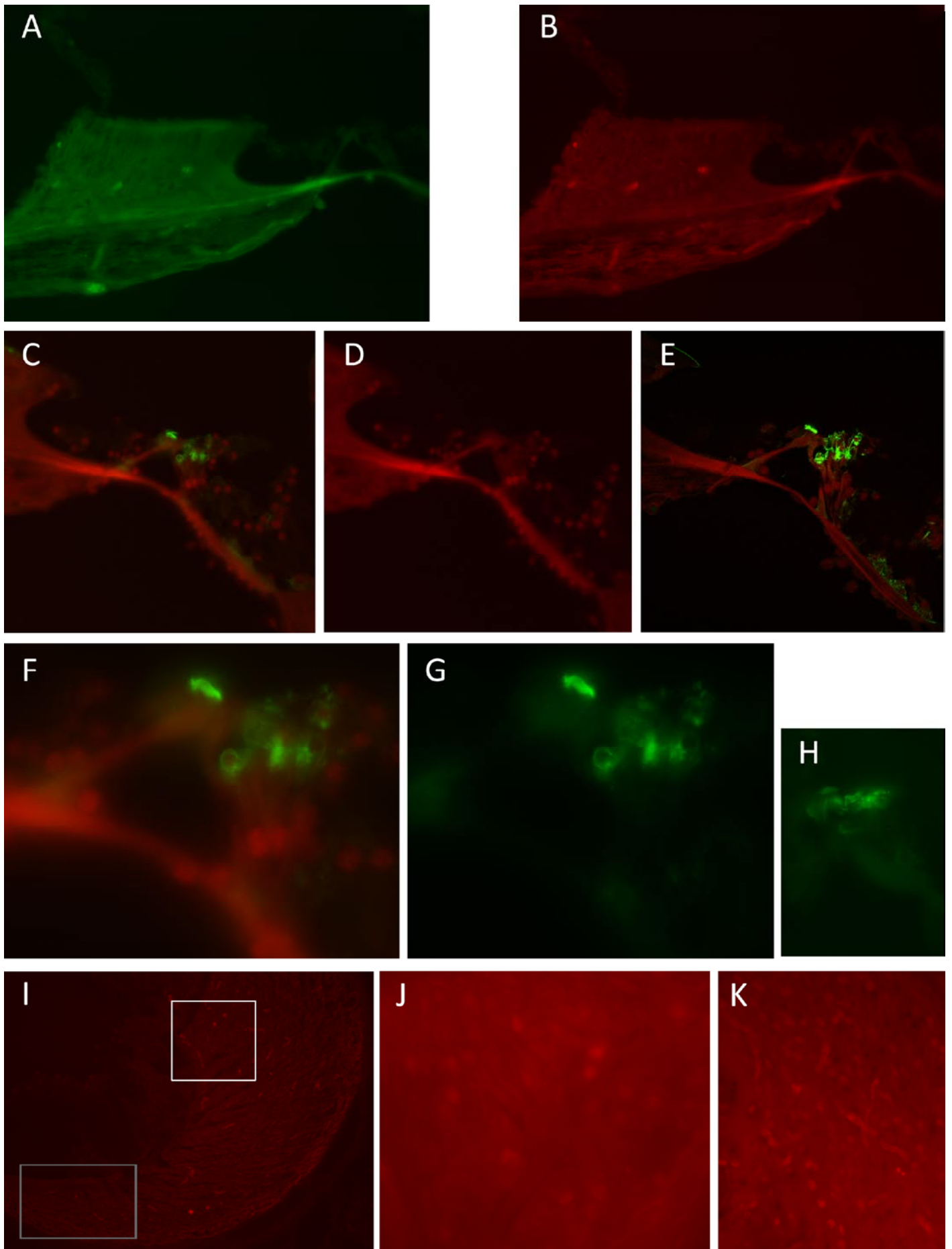
Figure 3.6.2. Immunofluorescense results of the cochlea of a harbour porpoise stained with goat anti-prestin (1:200, in green) and mouse anti – ctBP2 (1:1000, in red). A and B) Negative controls treated without the primary antibody, but only with the secondary antibodies donkey anti-goat Alexa 488 (A) and donkey anti-mouse Alexa 594 (B). C-G) Organ of Corti of the upper basal turn. E is a reconstruction obtained with the software ImageJ from 10 consecutive sections performed with a confocal microscope (each section was 0,28 μm thick). H) Organ of Corti of the lower basal turn. Note that the length of the outer hair cells in H, marked with the prestin staining, is much shorter than in F and G, which are at the same magnification. I-J) Spiral ligament. I was taken in the lower basal turn. The gray square marks the position of J in the upper apical turn and the white square the location of K in the upper basal turn. The high magnification of J and K allows observing the nucleus of fibroblasts.
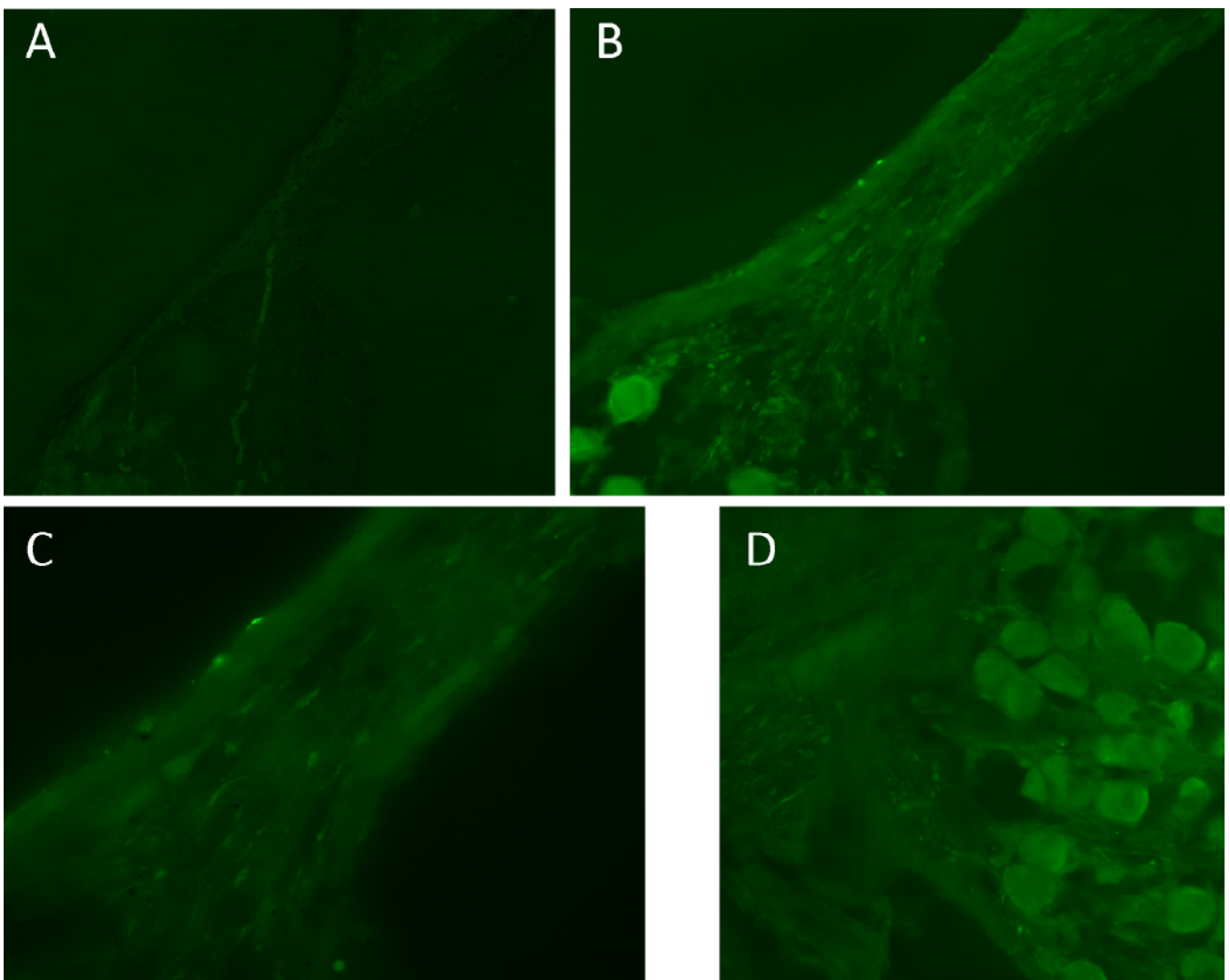
Figure 3.6.3. Immunofluorescense results of the cochlea of a harbour porpoise stained with sheep anti-VAcht (vesicular acetylcholine transporter) antibody at 1:1000. A) Negative control treated without the primary antibody, but only with the secondary antibody donkey anti-goat Alexa 488. B, C and D show the efferent fibers of the spiral lamina (B and C) and modiolus (D) recognized by anti-VAcht antibody. A, B and C are from the upper apical turn while D is from the upper basal turn. The black points observed in A and C are remains of the Sudan Black B.
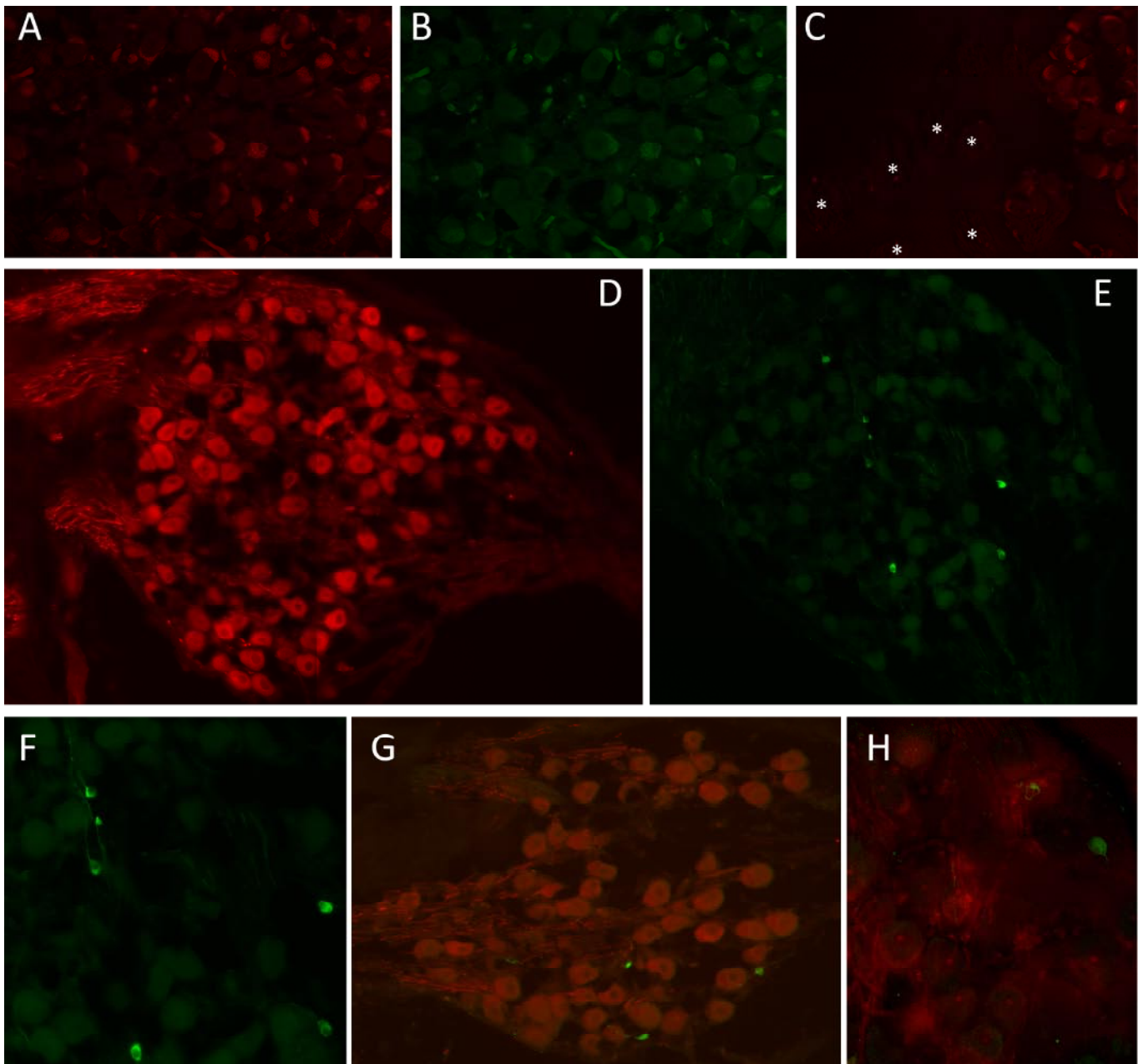
Figure 3.6.4. Immunofluorescense results of the cochlea of a harbour porpoise stained with mouse anti-neurofilament 200 (in red here) and rabbit anti – peripherin (in green in this figure). A-C) Negative controls treated without the primary antibody, but only with the secondary antibodies goat anti-rabbit Alexa 488 (B) and goat anti-mouse Alexa 594 (A and C). A and B pictures are from the spiral ganglion cells region and the asterisks in C mark the location of the nerve fibers of the modiolus. D) Anti-neurofilament antibody (1:400), which recognized the type I spiral ganglion cells and their axons in the modiolus. E and F) Anti-peripherin antibody (1:400) that stained the type II spiral ganglion cells and their axons. G and H) Combination of the two antibodies, anti-peripherin (1:400) and anti-neurofilament (1:200). H is a reconstruction obtained with the software ImageJ from 13 consecutive sections performed with a confocal microscope (each section was 1,28 µm thick). The pictures were taken from the lower (A, B, C, H) and upper (D, E, F, G) basal turns.

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4. Discussion

4. DISCUSSION

This section will discuss the findings at each structural level of the odontocete ears. The focus will be first on the tympanic-periotic complex, from the analysis of computerized tomography and decalcification techniques. Then, the results of the cochlear ultrastructure will be discussed from the analysis of both SEM and TEM, as well as from immunohistochemistry techniques.

4.1. Tympanic-periotic complex

4.1.1. Computerized Tomography

Being a non-invasive technique and supporting a very high resolution in 3D reconstructions, computerized tomography is confirmed to be a powerful tool for the study of the tympanic-periotic complex morphology and morphometry, leading to close-to-reality results (Figure 2.4.2a and b and Figure 3.1.4).

Linear correlation coefficients

Our results are generally consistent with previous studies (Ketten, 1992), although here the correlation coefficients between animal lengths and the total volume and lengths of the bullae (Table 3.1.1), are much lower (Ketten and Wartzok, 1990). This indicates that the length of the animals may not be a primary parameter to take into account when defining ear measurements.

Interestingly, without sperm whales, juveniles and adults together gave a closer approximation to the values obtained with adults only (Figure 3.1.1). Thus, the apparent differences in the coefficients between adults and juveniles could be due to the presence of sperm whales in juvenile data.

The high correlation coefficients between the measurements lead us to conclude that the ratio between the tympanic and periotic bones remains constant. This would mean that, if one of these structures changes, the other will also change in the same proportion, both in juveniles and adults, and in all odontocete species that were compared here.

A feature that has remained constant throughout the evolution and in all species is a good indicator that could confer functionality to the structure. The apparent functional relationship between the tympanic and periotic bones suggests a possible active role of the middle ear in the odontocete sound reception mechanism. This role has been controversial; while some authors have expressed doubts about its functionality (Fraser and Purves, 1954; Reysenbach de Haan, 1957; Fleischer, 1978; Ridgway *et al.*, 1997), our results support the conclusions of previous morphological and modelling analysis (Hemila *et al.*, 1999; Nummela *et al.*, 1999; Nummela *et al.*, 1999; Ketten, 2000; Hemila *et al.*, 2001; Cranford *et al.*, 2010).

Fisher discriminant analysis (FDA)

The results of this statistical analysis confirm that all combined variables classify very well the species, with low Wilks' λ values (see Table 3.1.2a and b, column 10). The classification power of each variable by the

Fisher discriminant ratio could also be determined. Different results were obtained depending on the situation. As shown in Table 3.1.2a (column 6), in situation 3 (adults and juveniles) the animal length also seemed to have little importance despite being a strong discriminator in situation 1 (only adults). This difference can be explained by the fact that these species present different lengths and that juveniles from a species could easily be misclassified as an adult from a smaller species. When the data was normalized by the animal length (situation 2 and 4), the variable with higher ratio was the T-P complex length.

When juveniles are taken into account the number of misclassifications increases (column 7 of Table 3.1.2). This would indicate that morphological changes of the ear are not linearly related to animal growth.

When conducting the FDA considering the differences in echolocation signals production characteristics (Type I and II odontocetes, Ketten and Wartzok, 1990; Ketten, 1992; Ketten, 1994), we observed that some Type II individuals were misclassified as Type I (see column 7, Table 3.1.2c and d), which is not consistent with the Ketten and Wartzok results (1990). The high Wilks' λ values presented in Table 4.1.2c and d support the fact that the variables do not classify well between Type I and II species. In addition, their analysis compared ear anatomy with sound production while conversely, our study aimed at focusing on the relationship between sound reception sensitivity and hearing structures.

Unfortunately, there is still a great need for data on species-specific audiograms (Table 1.2.1) as well as individual hearing measurements within the same species, before we can statistically explore the above dependence under a multidimensional analysis. Electrophysiological measures of hearing appear the most promising source of data.

The multivariant analysis showed that the odontocete ear morphometrics is a good species indicator and could therefore be used to classify them. It also suggests that the reported measurements can characterize standard ears and may therefore constitute a morphological basis for further species-specific acoustic comparison.

4.1.2. Decalcification

Following a routine protocol with a specific dilution of RDO®, the odontocete ear decalcification time ranged from several hours to a few days (see Table 3.2.1), depending on the volume of the periotic bone (Figure 3.1.4). This reduced the decalcification time from a few months using EDTA (Table 3.2.2) to a maximum of a week for the largest T-P complexes. The high correlation between the T-P complex volume and the periotic decalcification time (Figure 3.2.2) should allow a better approximation to the accurate decalcification time to analyze odontocete ears in the future.

The decalcification protocol developed for this study was adjusted to perform the examination of the Organ of Corti's cells through SEM and establish a fast diagnosis of possible lesions in freshly extracted ears. The respective decalcification time values may need to be increased if a complete decalcification of the periotic bone is needed for routine histology, immunohistochemistry or TEM techniques.

A priori, we did not observe morphological differences using SEM (see section 3.5) between the ears decalcified following any of the approaches. However, since the number of samples that were decalcified in

EDTA was small when compared to those that were treated with RDO® (see Table 2.2.1), more experiments are needed to assess the suitability of using one or the other method.

The resin Technovit 7200 VLC® did not properly embed the internal cavities of the cochlea. The fact that no differences were obtained between an autolytic sample (Figure 3.3.1b) and the other samples fixed between 20 and 22 hours or over 22 hours (Figure 3.3.1c) could have several explanations. One of them is that the best samples were not well fixed. Alternatively, the methodology could not work very well in this type of structures. Since all the process took so long, it was subjected to many inaccuracies when cutting, polishing, adjusting the final slight thickness, and we could only obtain 4-5 good slices per cochlea - most of them with artefacts (bubbles, cracks) - we decided to abandon this protocol.

4.2. Cochlea

Over the last 5 years, a lot of attention has been given in setting up and managing a network to collect ear samples under a standardized procedure that includes an ear extraction and fixation protocol (see section 2.2 in Materials and Methods). As a result of this constant effort, we nowadays receive contributions from stranding networks and rehabilitation centres of the following countries: Albania, Belgium, Canada, Croatia, Denmark, France, Greece, Green Cap, Greenland, Iceland, Ireland, Israel, Italy, Malta, Poland, Portugal, Romania, Scotland, Slovenia, Spain, Sweden, Russia, the Netherlands, Tunisia, Turkey and Ukraine. It is of most importance to notice that the quality of the samples has dramatically improved since the early days, due to faster extractions and fixations of the ears that we receive.

However, the rapid decomposition process of the organ of Corti after the death of the animal, together with the early difficulty of obtaining good material, did not allow us to analyse very fresh cochleas in the framework of this dissertation. We believed for instance that this is one of the reasons why some antibodies did not work, although sometimes they behave better in some species than others and at different concentrations. In addition, due to economical constraints, the number of slides processed in TEM could not be larger.

Despite these difficulties, it was possible to describe for the first time the ultrastructure of odontocete cochlea using electron microscopy and immunofluorescence (see the main achievements of the dissertation in the text box below). In this section, we compare our findings with previous cochlear morphological descriptions published on some odontocete species using histology (Wever et al., 1971a, b and c; Wever et al., 1972; review: Ketten, 2000).

4.2.1. Sensory cells

As a general feature, the pear-shaped form of IHCs and cylindrical shape of OHCs in odontocete cochlea resembles the typical form of these receptor cells in other mammals (see Lim, 1986c for review). Whereas the size of IHCs (around 30-35 µm; Nadol, 1988; Pujol *et al.*, 1997) appears to be quite constant amongst species, the data on the dimensions of the OHCs, however, deserve further comments.

Typically, OHCs in land mammals are not shorter than 20 µm and not longer than 70 µm (Pujol *et al.*, 1997). However, in horseshoe bat, a high frequency hearing specialist, they are 12-15 µm in the base to 28-30 µm in the apex (Vater *et al.*, 1992); optical microscope measurements in bottlenose dolphin revealed that OHCs range between 8 and 17 µm (Wever *et al.*, 1971a); in striped dolphin from 10 to 22 µm and harbour porpoise from 9 to 15 µm (present study), this last measurement being found in the lower apical turn.

There is a linear relationship between frequency and OHC length in land mammals (Pujol *et al.*, 1991; Figure 1.2.4.6). Indeed, the length of the OHCs increases while approaching the apex. Since the absolute length of OHC may play a role in an intrinsic tuning mechanism (Pujol *et al.*, 1991) the small size of odontocete OHCs is consistent with a morphological adaptation to the high frequency hearing. These results confirm that odontocetes present the shortest OHCs ever described in mammals, which is not unexpected taking into account their higher frequency hearing capabilities; harbour porpoises, for example, can hear up to 180 kHz (Kastelein *et al.*, 2002) while echolocating bats hearing goes up to 150 kHz (Neuweiler *et al.*, 1984; Grinnell, 1995). However, when comparing the OHC length and the codified frequency of these particular cells in other species, we found that odontocetes do not follow the same above relationship (Pujol *et al.*, 1991), being much shorter than expected all along the cochlear spiral. The reason of this discrepancy is not clear.

Besides their small length, the OHCs are also small in diameter. In land mammals, OHC diameter keeps a constant value (7 µm), but in the species of odontocetes that were studied here, the diameter ranges from 5 to 6,2 µm. These lengths and diameters might represent a decrease in the OHC membrane surface, which might also decrease the membrane cell capacitance, increase its conductance thus increasing the sensitivity to high frequency (Housley and Ashmore, 1992).

The total number of IHCs (2842) and OHCs (10367), estimated here for harbour porpoise and OHCs (13405) for striped dolphin, are consistent with previous results on other odontocete species. For example, 3272 IHCs and 12899 OHCs were calculated for the Pacific white-sided dolphin (Wever *et al.*, 1972), and 3451 IHCs and 13933 OHCs for the bottlenose dolphin (Wever *et al.*, 1971a). The magnitude of these hair cell estimations coincide with what was reported by Retzius (1884) for the human ear: 3475 IHCs and 11500 OHCs, which is about the same cochlear length.

The finding of some cells forming a fourth row of OHCs in the apical region of a harbour porpoise with SEM (Figure 3.5.3b) would support the description of the same phenomenon published by Wever and colleagues (1971c) in bottlenose dolphin using routine histology. Since both in Wever's and this present study, this characteristic was only found in one case and because other authors did not find it in their analysis, we suggest that this feature is subjected to individual variability. This indeed occurs with other mammalian species, such as mole rats that can present even a fifth row (Raphael et al., 1991).

Stereocilia

The number of stereocilia per OHC seemed to be constant along the cochlear length, thus ranging from 130 in harbour porpoises to 168 in striped dolphin (Table 3.5.4) if considering that each OHC has three rows of stereocilia. In one case, we found four rows of OHC stereocilia using TEM (see insert in Figure 3.4.4d). Since we have observed this feature only once, and SEM images show three rows of stereocilia, we assume that the fourth row was an oblique sectioning artefact. However, further research should be conducted to confirm these results.

On the contrary, in some land mammals, the number of stereocilia changes along the baso-apical locations and between the OHC rows. For example, in man, the number of OHC stereocilia varies from 50 to 120 from the apical to the basal coil (Kimura *et al.*, 1964), or in chinchilla, the number of OHC1 and OHC2 stereocilia remains quite constant (100-110 stereocilia in the basal turn and 90-100 in the apex) while in the OHC3 the number of stereocilia varies from 80 in the basal turn and 18-40 stereocilia in the apical turn (Lim, 1986c). The large number of stereocilia in cetaceans may have at least two consequences: to improve the functional relationship between the tectorial membrane and the organ of Corti; to increase the number of ion channels that would facilitate depolarization of the cell and promote its sensitivity (Assad *et al.*, 1991).

OHC longest stereocilia increased in length while approaching to the apex, as in all mammalian species. Nevertheless, in the harbour porpoise and the striped dolphin they were very short, ranging from about 1 to 1,76 μm in the apical turn (Table 3.4.1), compared to other species (as for example from base to apex: 1,3 to 5,4 μm in guinea pig; from 2,5 to 7,5 μm in man; or 0,7 to 5,5 μm in chinchilla; Lim, 1980; Wright, 1984).

Subsurface cisternae

In the horseshoe bat (Vater *et al.*, 1992), rats, mice or humans (Arnold and Anniko, 1990) there is only one layer of subsurface cisternae. In contrast, guinea pig OHCs display multiple layers of subsurface cisternae, especially in the apical region (Saito, 1983). Subsurface cisternae are believed to play an important role in OHC motilities by releasing in Ca^{++} (Tolomeo *et al.*, 1996; Oghalai *et al.*, 1998). The harbour porpoise and the striped dolphin showed the same pattern as the horseshoe bat.

Prestin

Prestin is a motor protein located in the lateral wall of mammalian OHCs of the organ of Corti (Zheng *et al.*, 2000a), responsible of rapid changes in the length and stiffness of the OHC. Although it is known that cetaceans do present the gene coding prestin, which shows a sequence convergence in the prestin gene with echolocating bats (Li *et al.*, 2010; Liu *et al.*, 2010b), there was no information of the cochlear expression of this protein in these animals. Our results show, for the first time, that harbour porpoise OHCs contain prestin all along the cochlear spiral, including in the basal turn (see Figure 3.6.2c and 3.6.2e-h). This strongly suggests that, like in land mammals, OHC electromotility is part of the process by which cetacean OHCs amplify and tune the acoustic stimulus. Nevertheless, the presence of prestin alone may not be sufficient to help understanding the apparent selective acoustic sensitivity of harbour porpoise in those frequencies. Morphological features encountered in the basal portion of the cochlea (see below, especially in supporting cells section), conferring a dense packing and high stiffening of all the organ of Corti structures, might bring pieces to the "puzzle" of this complex processing.

Scaring process

Considering the case where a large portion of the upper apical turn was formed by scars on the OHC position, and taking into account the preliminary frequency map built here, the area that codifies approximately the area of 470-630 Hz was damaged. If this damage was caused by acoustic overstimulation, the acoustic characteristics of the source could be extrapolated (310-420 Hz) because "the greatest hearing-

loss occurs at a frequency about half an octave above the exposure tone" (Davis *et al.*, 1950). This frequency range corresponds to several anthropogenic marine sources, as for example shipping, pile driving or seismic operations. However, other causes should be taken into account such as age-related hearing loss. This animal was old, and we know from studies of age-related hearing loss in land mammals that the base of the cochlea is always affected, as well as, in some cases, a small region of the apex (Johnsson and Hawkins, 1972; Spongr *et al.*, 1997).

Nevertheless, despite the basal turn of the cochlea was not well preserved, thus preventing us to determine the possible influence of aging in the analysis, we could clearly distinguish between disappearance of hair cell occurring prior to death and damage to hair cell due to autolysis effects. In the first case, the stereociliary bundle and cuticular were missing and a scar was made by neighboring epithelial cells. In the second case, the stereocilia were generally missing but the cuticular plate of the hair cells remained and the epithelial mosaic was retained.

4.2.2. Supporting cells

Deiters and Pillar cells

In the basal turn of odontocete and echolocating bat cochlea, the fact that the thickness of the reticular lamina, made of both Deiters cell phalanges and heads of outer pillar cells, is exaggerated may provide an advantage for motion at high frequencies (Vater *et al.*, 1992).

The arrangement of Deiters cells, pillar cells and Deiters cups in the basal cochlear turn showed robust cytoskeletal structures, suggesting that the OHCs mechanical anchorage is reinforced and extremely stiff compared to the arrangement in the apical turn or throughout most of the cochlea of other mammals. Again, in echolocating bats, the same disposition of Deiters cups was found in the basal turn (Henson and Henson, 1979; Dannhof and Bruns, 1991; Vater *et al.*, 1992). This feature may therefore be relevant for high-frequency processing (Reysenbach de Haan, 1957; Wever *et al.*, 1971c; Wever *et al.*, 1972; Vater *et al.*, 1992).

Wever and colleagues (1971c) found different pattern of inclination of Deiters cells in bottlenose dolphin, being inclined (around 60º) in the base and more oblique (more than 45º) and not aligned with the OHCs in the apical turn. We did not see a change in inclination in transversal cuts of the organ of Corti from harbour porpoise and striped dolphin: the OHCs and Deiters were aligned.

Hensen and Claudius cells

In the harbour porpoise and the striped dolphin, Hensen cells were very difficult to distinguish from Claudius cells in apical positions. Instead of using the criteria of the nucleus of Hensen cells, which is usually lower than the Claudius cells (Kolmer, 1908), we considered the criteria of the position, following Wever and colleagues (1971c), when describing these cell types on bottlenose dolphin. Hensen cells were closer to the third (the more external) row of OHCs.

Claudius cells were very large in the basal cochlear turn, as in the other species that are known to echolocate (Wever *et al.*, 1971c; Vater and Kössl, 2004), but the functional significance of this structural characteristic is unknown. However, in our case, the values of Claudius cell length (from 112 µm in the base to 12 µm in the apex) are not that extreme than those measured for the bottlenose dolphin (ranging from 145 µm to about 7 µm; Wever *et al.*, 1971c), but closer to the Pacific white-sided dolphin (from 110 µm to about 7 µm, Wever *et al.*, 1972). The discrepancy in the measurements could be due to species specificity or a lack of some information of the extreme locations of the cochlea in our study (first and last millimetres).

Inner sulcus cells

As also stated by Wever and colleagues (1971c), the inner sulcus cells almost fill the space between the basilar membrane and the limbus, extending from the border cells to the lower edge of the limbus (Figure 3.4.6f) in the basal turn. In the apical coil, the inner sulcus cells seemed not to cover all the space around the spiral limbus edge, but to end lower or at the level of the lateral interdental cells. This could be likely due to the smaller size of the inner spiral sulcus in the basal cochlear region compared to more apical ones.

4.2.3. Spiral limbus

We could identify for the first time the three types of interdental cells and the stellate fibrocytes on odontocete species (Figure 3.4.6). However, the light and supralimbal fibrocytes appeared to remain out of the observed section, further analysis is therefore needed to describe these cell types.

The lateral interdental cells, instead of forming two rows of tubular shape as in Mongolian gerbils (Spicer and Schulte, 1998), were found only in the upper lateral edge with an ocellated shape.

The stellate fibrocytes, which were in contact with the central interdental cells, could have a role in the K^+ effluxed from IHCs transport during auditory transduction, proposed by Spicer and Schulte (1998). Stellate fibrocytes express Na,K-ATPase, which would participate in the returning path of K^+ into the cochlear scala via the central interdental cells.

The fact that these odontocete species present the same type of interdental cells as in land mammals, could mean that they would accomplish the same biologic role in 1) anchoring (Hilding, 1952) and secretion (Belanger, 1953; Iurato, 1962; Santi, 1988) of the tectorial membrane, 2) pinocytotic uptaking of substances from the limbus and their transport to endolymph (von Ilberg, 1968) and secretion into tectorial membrane (Voldrich, 1967; Lim, 1970) and 3) moving electrolytes toward endolymph, specially K^+ (Spicer and Schulte, 1998).

Interestingly, we could describe for the first time for all mammalian species, the formation of an apposite of amorphous nature in the most basal section (around 5 mm of the basal end), in contact with the lateral edge of the spiral limbus (asterisk in Figure 3.4.8b-d). We do not know the functionality that this structure might contribute. It is possible that the apposite remained on the top of the organ of Corti after the dissection if the samples were not very fresh, and it could be one of the reasons why it is so difficult to be observed in the basal region under SEM.

4.2.4. Lateral wall: spiral ligament and stria vascularis

The spiral ligament had the conventional five divisions of fibrocyte types, but again, like other cochlear elements, cells were heavily packed (Ketten, 2000). The packing of fibroblasts could be also verified by CtBP2 positive labelling on their nucleus.

Although fibrocytes were not in perfect conservation status, it is expected than in fresher samples, type II and IV fibrocytes might be used as an indicator of possible acoustic trauma if degeneration or loss is observed (Hirose and Liberman, 2003). We could not differentiate the fibrocyte types by cytoplasmatic content or shape (Takahashi and Kimura, 1970; Santi, 1988; Spicer and Schulte, 1996), but by position (see **Figure 3.4.2a**). Further experiments with immunostaining for transport mediating enzymes, including Na,K-ATPase, carbonic anhydrase and creatine kinase (Spicer and Schulte, 1991) should be done to better differentiate the fibrocyte types, validate our study, compare the distribution all along the cochlear duct and help assessing the role of each type in ion transport (K^+, Na^+ and Cl^-) on odontocete species.

Previous histology studies demonstrated that the spiral ligament marginal region of odontocetes contains a high cellular density of tension fibroblasts throughout the cochlea (Ketten, 2000) that anchor and add tension to the basilar membrane (Henson *et al.*, 1984; Henson *et al.*, 1985; Henson and Henson, 1988). The same feature was described for horseshoe bats (Henson and Henson, 1988). Unfortunately, the marginal region of the spiral ligament appeared to be out of sight in our slides, so we could not describe the tension fibroblasts ultrastructure with TEM. Due to the large size of the spiral ligament in odontocete species, especially in the lower basal turn, we should consider to make preparations of only this structure in the future, to ensure that it will fit in the grid space for the subsequent observation. In addition, Hsp27 immunostaining can be used to describe tension fibroblasts in odontocetes (Leonova *et al.*, 2002).

Since the stria vascularis is one of the structures that starts the decomposition process faster, we recommend not to use it as an indicator of noise induced hearing loss, because the consecutive lesions, including acute swelling and chronic degeneration of strial intermediate and marginal cells (Hirose and Liberman, 2003), can be easily confused with post-mortem decomposition. Despite the stria vascularis was not very well preserved in our samples, we could confirm that it was very dense, and that the layer of marginal cells was particularly thick (average of 22,8 µm in our study, compared with approximately 5 µm in mouse; extracted from figure 6 in Hirose and Liberman, 2003).

4.2.5. Innervation

Medial efferent innervation

By focusing on the position of the nerve endings at the basal region of the OHC, it is not possible to differenciate the dendrites of afferent innervation and axons of efferent neurons (Figure 4). However, from studies performed in humans it was shown that the efferent nerve endings are very resistant to anoxia, and usually remain present, even when the OHCs are in a very advanced decomposition status (Bruns and Schmieszek, 1980; Lavigne-Rebillard and Pujol, 1988; Lavigne-Rebillard and Pujol, 1990). In our specimens, the absence of recognizable efferent vesiculated endings, (Figure 4), the lack of postsynaptic cistern in the OHCs and the absence of thick upper crossing fibers in tunnel of Corti, confirms the hypothesis that these

species do not receive the medial efferent innervation onto the OHCs. In addition, the lack of labelling with VAcht antibody in the upper part of tunnel of Corti, the usual way by which medial efferents reach the OHC basal pole, could help confirming this hypothesis. Nevertheless, more experiments with VAcht are recommended to verify that only the lateral efferent nerves were stained.

The lack of medial efferent innervation was previously found in two cases, in the echolocating horseshoe bats (Bruns and Schmieszek, 1980; Aschoff and Ostwald, 1988; Bishop and Henson, 1988; Vater *et al.*, 1992) and in the mole rat *Spalax ehrenbergi* (Raphael *et al.*, 1991), this latter being specialized for low-frequency hearing (Bruns *et al.*, 1988). This supports the notion that the medial efferent innervation of the OHCs is a regulating system for the mid frequencies, but that is not needed at extremely high and low frequencies (Vater *et al.*, 1992).

Spiral ganglion cells

The values of density of SGCs (4,4-8 SGCs/10000 μm^2) in the odontocetes are of the order of the values in guinea pigs (8 SGC/10000 μm^2; Jin *et al.*, 2006), but much lower than Mongolian gerbils (28,1 SGC/10000 μm^2, Fujita *et al.*, 2007) or mice (between 36,3 in the apex and 39,6 1 SGC/10000 μm^2 in the base, Sato *et al.*, 2006).

Since bottlenose dolphin and Pacific white-sided dolphin have three times, and almost twice as many folds SGC as humans, respectively (Wever *et al.*, 1971a; Wever *et al.*, 1972) we could expect a higher density of SGCs in the odontocetes that were studied here. However, the SGCs of the harbour porpoise and striped dolphin were much larger in size (maximum 45 µm x 25 µm and 46 x 30 µm, respectively, Table 3) than those of rodents (for exemple, 13-18 µm in rats; Bichler, 1984). They were even larger than in bottlenose dolphins, which in the basal turn were 38,4 x 25,2 µm and in the middle turn 34,4 x 21,1 µm in the longer and shorter dimension, respectively (Wever *et al.*, 1971a). In fact, odontocetes increase the number of SGC by increasing the space occupied in the Rosenthal's canal. In the striped dolphin the number of SGC in a transversal cut of the Rosenthal's canal ranged from 128 to 152 and in the harbour porpoises from 122 to 182 (not illustrated here), while in rats they were 81 and 88 SGC and in gerbils 57 and 67 (Ruttiger *et al.*, 2007). Our results are consistent with the large ratio of SGCs to hair cells found by Wever and colleagues (1971a), suggesting "unusual capabilities in the utilization of auditory information".

In our preliminary immunohistological study in harbour porpoise, we could confirm that peripherin constitutes a reliable marker for type II SGCs, neurofilament for type I SGCs and VAcht for the efferent system fibers, which presumably would be the lateral efferents (see above).

The same results were previously observed for VAcht immunostaining in lateral and medial efferent terminals and medial efferent crossing fibers (Altschuler *et al.*, 1985 in guinea pig, or Eybalin and Pujol, 1987 and Vetter *et al.*, 1991 in rat). However, no VAcht staining of the efferent fibers passing the spiral lamina was described in the literature. The labelling on the fibers instead of the terminals could be due to the delay in fixation post-mortem, with a lysis of the terminals enabling the diffusion of the vesicles. Antibody against peripherin was also used in land mammals to label type II central and peripheral processes (for example, Hafidi, 1998 in rat, or Liu *et al.*, 2010a in human). Antibody against neurofilament 200 kD is normally used to label Type I and II neurons and their processes as well as medial efferent axons. However, type II neuronal cell bodies are always more strongly labeled than type I cell bodies (Hafidi and Romand, 1989). In our case, neurofilament immunoreactivity was localized in type I neurons (central and peripheral processes,

particularly in fibers reaching the modiolus), but not in type II, characteristic that could be proved with the double labeling with anti-peripherin and anti-neurofilament antibodies. Our results suggest that in the harbour porpoise, type I neurons can be distinguished by their abundant neurofilament content.

Double immunostaining using peripherin and neurofilament subunit antibodies allowed us also to compare between type I and II SGCs subpopulations, establishing a proportionality of 95% type I and 5% type II neurons. This proportion is also very common in land mammals (Spoendlin, 1978). It would therefore be very useful to validate SGCs counts in odontocetes with serial sections. In addition, when comparing the area taken up for both types, we could describe, a species where type II SGCs surface only represented a 10-24% (16% in average) of the area of a type I SGC. This percentage is very low compared with other mammalian species, which consists in 43-44% in cats, 33-44% in monkeys and 30-33% in humans (Kiang *et al.*, 1984; Nadol, 1988). Since odontocetes have evolved in a natural noisy environment, we could expect a higher size of type II SGCs, increasing at the same proportion as type I. The fact that they are so small in comparison suggests that there might be another mechanism besides the type II afferent neurons to protect the hair cells from an acoustic overstimulation in these species, which would convey "sensations of auditory pain" (Brown *et al.*, 1988) or messages of OHC damage (Simmons and Liberman, 1988). In addition, our results support the conclusions of previous studies that state that type II neurons react differently to sound stimulation than type I afferent (Robertson, 1984; Weisz *et al.*, 2009)

4.2.6. Basilar membrane

The width of the basilar membrane and its thickness were measured in several mammal species (Nadol, 1988; Roth and Bruns, 1992; Sato *et al.*, 1999; Keiler and Richter, 2001). A clear gradient of size (thickness and width) was found in most species as well as in the species that were analyzed here, being narrower, thicker and relatively stiff in the base and broader and thinner in the apex. In the bottlenose dolphin, the width of the basilar membrane increased about 14 times from base to apex (from 25 to 350 μm, Wever *et al.*, 1971b), while the thickness diminished 5 times (from 25 to 5 μm). However, in humans, the basilar membrane showed an increase of the width of only 5 times (from 125 to 500 μm) and a threefold decrease in thickness (from 7 to 2 μm) from base to apex (Schuknecht, 1993; Ketten, 1998).

The values that were calculated for the harbour porpoise and the striped dolphin (Table 3.4.1) did not show the similar increase as in the bottlenose dolphin, even if the striped dolphin and the bottlenose dolphin present similar hearing sensitivities (Table 3.5.3). The reason of this difference in the measurements could be due to the lack of some information of the extreme locations of the cochlea (first and last millimetres, see Figure 3.4.1) or because the criterion to establish the limits of the basilar membrane was different. Further comparative research should be conducted to assess the measurement differences found here between these odontocete species. In odontocete species, the basilar membrane is very stiff at the base of the cochlea, fixed to both sides by a very well developed inner and outer osseus spiral laminae, conferring an adaptation for very high frequency hearing (Wever *et al.*, 1971b; Ketten and Wartzok, 1990; Ketten, 1992; Ketten, 1994).

4.2.7. Tectorial membrane

Despite the very fast autolysis process after death, the tectorial membrane structure remained longer in acceptable condition, which could be expected due to its nature, being an acellular connective tissue composed mainly of collagens (Slepecky *et al.*, 1992a). The high resistance of this structure to anoxia would allow the diagnosis of possible alterations on the OHC stereocilia due to an acoustic trauma (Morisaki *et al.*, 1991) even if the cochlea was fixed over 20 hours post-mortem. In all samples analyzed, the imprints were regular (Figure 3.5.4a-f and h). Since the shape of these imprints mirror the organization of the OHC stereocilia, there was here no evidence of acoustic trauma. This does not exclude that acoustic trauma did not occur in some other specimens as suggested by the presence of epithelial scars seen in SEM.

We discovered IHC stereocilia impressions in the very basal region of the striped dolphin tectorial membrane (Figure 3.5.4h and i). This feature was already described in rats (Lenoir *et al.*, 1987) and bats (Vater and Lenoir, 1992), but not in odontocete species. However, data were unable to demonstrate in most mammalian species a direct physical attachment between IHCs and the tectorial membrane (Matsumura, 2001). We believe that this characteristic was not easy to observe in all our samples because of the decomposed status of the organ of Corti, and possibly also because the remains of the apposite attached to the spiral limbus (asterisk in Figure 3.4.8b-d) could interfere in the observation of IHC stereocilia imprints.

Because of the difficulties of obtaining and analysing fresh samples of cetacean ears immediately after stranding, these results recommend to consider the analysis through imaging techniques of tectorial membrane, a procedure that is not common to date, as a diagnosis to detect possible lesions due to noise overexposure.

4.2.8. Cochlear map

LePage (2003) and Vater and Kossl (2011) stated that the function defined by Greenwood (1961; see section 3.5.1) cannot be used in hearing specialists, such as the echolocating bats (i.e. bats with Dopplersensitive sonar), mole rats and dolphins. Cochlear frequency maps of horseshoe bats (Vater *et al.*, 1985), mustached bats (Kössl and Vater, 1985) and African mole rats (Muller *et al.*, 1992) support this notion. Instead of following a smooth curve, in these specialist species there are some discontinuities in the areas of best sensitivity; i.e. they have very high resolution but only at a cost when the map (or its gradient) becomes discontinuous (LePage, 2003). Despite some ultrastructural morphological similarities in the cochlea of echolocating bats and the odontocetes described in our study, we decided to use this function for the following reasons:

- The number of octaves in the audiogram of odontocetes is very heterogeneous. "As observed by Ketten, odontocete's hearing may cover 12 octaves but this comes at a price of introducing a discontinuity into the map, or into the map gradient, resulting in specialized anisomorphic cochleae" (LePage, 2003). In fact, the number of octaves in striped dolphin, harbour porpoise and bottlenose dolphin are around 8.5, 9.5 and almost 11, respectively. This number of octaves are closer to guinea pig, human, cat and chinchilla (Fay, 1988; Vater and Kössl, 2011), which are around 9-10, than in horseshoe bat (over 5 octaves; Long and Schnitzler, 1975) or mustached bat (3,5 octaves; Marsh *et al.*, 2006). Therefore, we believe that the number of octaves in the audiograms of the odontocetes described in this study reinforced our hypothesis that in these species there might not be discontinuity in their frequency map.

- The ratio of afferent nerve terminals per IHC is a very important characteristic to determine the species sensitivity at different frequencies. A Type I SGC has only one peripheral process, which contacts with a single IHC, but each IHC receives connections from multiple SGCs. The number of afferent nerve terminals per IHC varies depending of the portion of the cochlea, being usually larger in those IHCs that codifies for the frequency in which the individual present better sensitivity; i.e. the auditory sensitivity is positively related to innervation density (Schuknecht, 1960). In cat, from 20-26 afferent nerve terminals per IHC have been observed (Spoendlin, 1969; Liberman, 1980b), while in humans, 9 are found in the base and 11 in the middle turn and the apex (Nadol, 1988). In horseshoe bats, the number of type I neurons per IHC ranged from 8,3 to 23,5 depending of the region of the spiral cochlea (Bruns and Schmieszek, 1980). Interestingly, the maximum in afferent innervation was observed at 5,5 mm of the base, which corresponded to the coding portion for 15-30 kHz (Vater *et al.*, 1985), coinciding with communication sounds, while the maximum sensitivity in those species are between 80-86 kHz (with the central frequency 83 kHz). The zone of 80-86 kHz did not present a higher density of SGCs, but took a larger portion of the cochlea (26% of the total BM length), thus increasing the number of SGCs that innervate the IHCs which respond to such frequencies. That is, the pitch discrimination can be improved by a large number of IHCs, or by a larger number of afferent nerve terminals per IHC. We believe that odontocetes could follow the latter because the average SGC to IHC ratio is 27:1 for cetaceans (Ketten, 2000), more than twice the average ratio in bats (Vater *et al.*, 1992). According to the proposed preliminary frequency maps, the area of maximum sensitivity for striped dolphin (64 kHz; Kastelein *et al.*, 2003) would be at 5,05 mm from the base, and at 1,28 to 3mm in harbour porpoise (maximum sensitivity between 100 - 140 kHz; Kastelein *et al.*, 2002). Unfortunately, we do not have information of these particular regions of the cochlea (see Figure 3.4.1). Regarding bottlenose dolphin, a SGC distribution along the cochlear spiral was published by Wever and colleagues (1971b). They calculated a cochlear length of 38,5 mm, while we obtained a length of 34,418 mm. Since it is not explained how it was calculated, it is difficult to perform right extrapolations from both measurements. The place that would correspond to the best frequency hearing (45-50 kHz; Johnson, 1967; Schlundt *et al.*, 2007) in our cochlea would be at 9,62 – 8,78 mm from the base. If we assume a proportional difference in cochlear size between Wever's cochlea and ours, the density of SGCs in this portion regarding their calculations would be 2500 SGCs/mm cochlea, or 33,16 SGC/IHC, which is not the higher. The higher SGC density would be in the area that would codify for 19 - 25,32 kHz if we make a proportional extrapolation. Further experiments should be conducted, focusing in 5,05 mm, 1,28 - 3mm and 9,62 – 8,78 mm from the basal end in striped dolphin, harbour porpoise and bottlenose dolphin, respectively. These would be a key study to accept or refuse our hypothesis that certain odontocete species could follow a smooth curve in their cochlear frequency map without discontinuities.

- Basoapical gradients of change in basilar membrane width and thickness are not uniform across species (Figure 4.2.1). While both parameters vary gradually from base to apex in some species such as cat (illustrated in Figure 4.2.1a; Cabezudo, 1978), rat (Burda *et al.*, 1988) or guinea pig (Fernandez, 1952), the basilar membrane of echolocating bats presents a nearly constant width and thickness separated by abrupt transitions (Figure 4.2.1.b) which sets them apart from all other mammals (Bruns, 1976a; Bruns, 1980; reviews: Kössl and Vater, 1995 and Vater and Kössl, 2011). When representing our fragmented data of basilar membrane width and thickness along the cochlear spiral from harbour porpoise and striped dolphin (see Figure 4.2.1c and d), we observed than the averaged values for each position follows a curve more similar than the one obtained for cats. The most

abrupt transitions are not that high as in horseshoe bats, and when increasing the number of measurements possibly the curve would be smoother. Nevertheless, the most basal part is still missing in our data, so we recommend again to focus the study on the first millimetres of the basilar membrane in the future to complete the picture for a better understanding of the hearing mechanism at cochlear level.
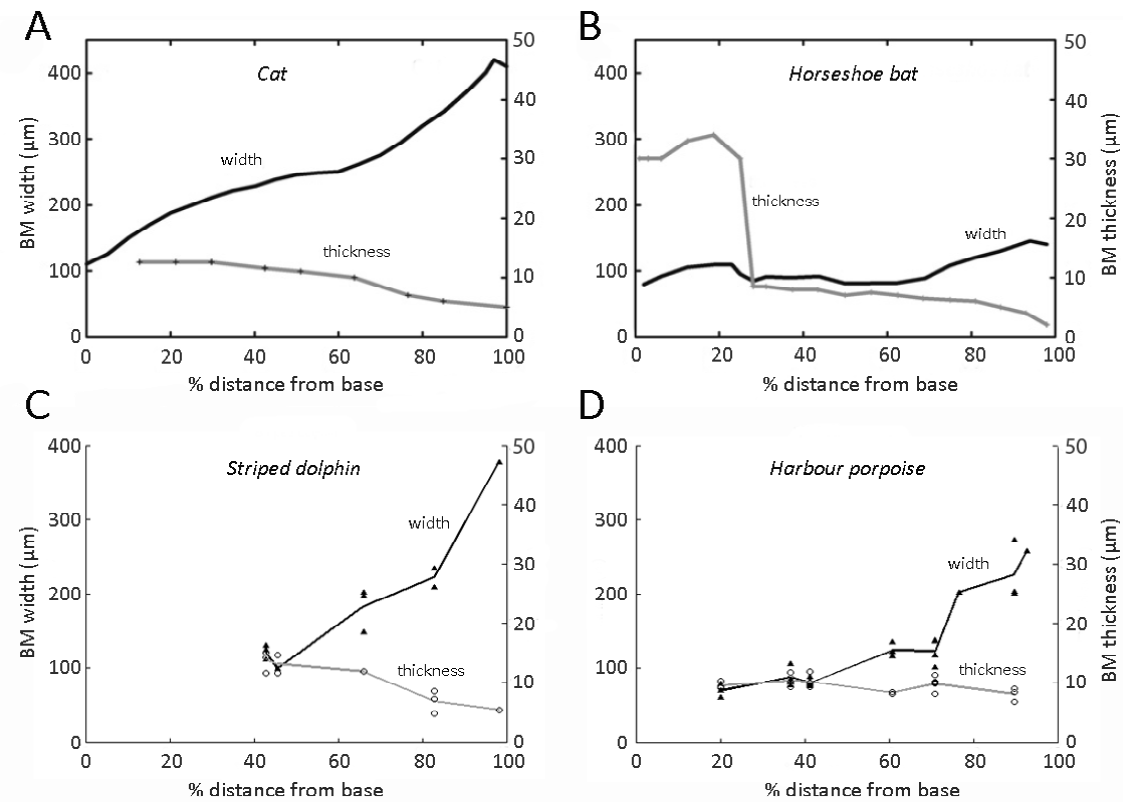
Figure 4.2.1. Baso-apical gradients in basilar membrane (BM) width and thickness in several species along the cochlear spiral: A) cat (after Cabezudo, 1978); B) horseshoe bat (after Bruns, 1976b); C) striped dolphin; and D) harbour porpoise. A and B are extracted from Vater and Kössl, 2011 and C and D are the representation of data from our study, taking in each position the averaged value. Note than triangles correspond to BM width data and circles to the BM thickness data.

In what concerns the striped and bottlenose dolphins, we believe that the proposed cochlear frequency map could be well adjusted, especially in the low and mid-frequencies region (under 20 kHz) used for communication. The area where we expect to find lesions in case of acoustic trauma originated by anthropogenic sources is in the low frequencies, an area where the cochlear frequency map could follow the function defined by Greenwood (1961).

Harbour porpoise is a special case in which the obtained curve (Figure 3.5.1a) is more questionable because, even if they are able to hear at low frequencies (down to 250 Hz, Kastelein *et al.*, 2002) the acoustic repertoire is very narrow banded, with a frequency peak at 120-140 kHz (Møhl and Andersen, 1973).

However, the values proposed here should be considered as a first approximation. Since odontocetes are protected species, no controlled exposure experiments that could lead to permanent hearing loss prior to sacrificing the specimen can be performed to adjust the previous function. This cochlear frequency map could be contrasted for consistency if a damaged portion of the cochlea could be described from an animal of known audiogram.

Dissertation major contributions to the field of odontocete hearing research

In summary, this dissertation appeared to be pioneer in describing the ultrastructure of the organ of Corti of different odontocete species using electron microscopy and immunofluorescence. The two species of odontocetes analyzed by TEM presented morphological characteristics of high-frequency hearing species, including 1) a OHC small length (stereocilia and cell body), 2) a thick cuticular plate in OHC, and reticular lamina Deiters cells and outer pillar cells, 3) robust cup formation of the Deiters cell body, 4) the high development of cytoskeleton in Deiters and pillar cells and 5) the basilar membrane high stiffness. All these features, in addition to a common molecular design of prestin (Li *et al.*, 2010; Liu *et al.*, 2010b) are also shared by echolocating bats (review: Vater and Kossl, 2011), suggesting a convergent evolution in echolocating species.

In addition, other features were described or observed for the first time in odontocete species, such as: the IHC and OHC stereocilia imprints in the undersurface of the tectorial membrane, the apposite on the edge of spiral limbus, the interdental cells, the stellate fibrocytes, the five types of fibrocytes in the spiral ligament, the stria vascularis, the relevant difference in size between type I and II neurons, the stereocilia of sensory cells, the presence of prestin in OHCs, the pattern of subsurface cisternae.

The morphological analysis of the inner ear was also conducted to detect possible structural alterations as a consequence of sound exposure. Due to delayed tissue fixation, the cochleas that were analysed in this study presented decomposition features and it was therefore not possible to assess a diagnosis of acoustic trauma by directly analyzing the hair cell stereocilia changes (Bredberg *et al.*, 1972; Engstrom *et al.*, 1984), degeneration of the stria vascularis (Hirose and Liberman, 2003), or other alterations at the sensory cell (Hu *et al.*, 2000) or innervation levels (Spoendlin, 1971).

However, the tectorial membrane, the spiral ligament fibrocytes and the presence/absence of scars appeared to be more resistant to *post-mortem* autolysis. Indeed, the presence of scars (Figure 3.5.2c and d), which indicates *pre-mortem* hair cell disappearance, could be clearly distinguished from autolysis artefacts. In one case (harbour porpoise, Figure 3.5.2d), the lesions would be compatible with an acoustic trauma. We therefore recommend here considering the analysis through electronic imaging techniques of stereocilia imprints on the tectorial membrane, apical poles of hair cells and fibrocytes II and IV as a diagnosis to detect possible lesions due to noise overexposure.

Consequently, matching the preliminary approximation of the cochlear frequency map with the damaged region would bring information on the sound source that would have triggered a possible lesion.

Finally, we believe the described protocol has brought evidence that its broad use would help diagnosing the effect of man-made noise shortly after the animal death, allowing decision-taking as well as future environmental policies on the development of human activities at sea.

Future research:

- We would like to increase the number of ear samples by training the responsible of new stranding networks, rehabilitation centres and oceanaria, on cetacean ear extraction and fixation using the perfusion technique.

- More experiments with EDTA are needed to assess the suitability of using it or RDO as a routine decalcification method.

- We will continue with the combined analysis of the inner ear using scanning (SEM) and transmission (TEM) electron microscopy. Specially, further research should be done on:

   o   hair cells and their stereocilia (morphological description and quantification and density of hair cells, together with the respective number of afferent synapses),
   o   density of spiral ganglion neurons in the very basal portion of the cochlea
   o   morphology on the spiral limbus, especially light and supralimbal fibrocytes
   o   good description of Type II and IV fibrocytes, tension fibroblasts, stria vascularis in fresher samples,
   o   basilar membrane extreme measurements (first and last millimeters of the cochlea)

- More experiments with new antibodies should be done to label hair cell stereocilia and their innervation, fibrocytes and fibroblasts. In addition, we will continue with the use of immunostaining that already worked, such as VAcht, peripherin, neurofilament 200kD, prestin and CtBP2.

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5. Conclusion

5. CONCLUSION

I - The analysis of the ear samples described here was only possible because a consistent relationship was built and has being consolidated across marine mammal stranding networks and rehabilitation centres belonging to 26 countries. A standard ear extraction and fixation protocol was adopted and is currently comparing tissues from a wide variety of species and geographies around the world.

II - Innovative complementary techniques to analyse the morphology of odontocete cochlea appeared to have been successful in determining that:

      1 - **Computerized tomography** was suitable to reconstruct very high resolution images, stating that odontocete ear morphometrics is a good species indicator and could therefore be used to classify them.

      2 - The constant ratio between measurements of inner and middle structures contributed to confirm the active role of the odontocete middle ear in sound reception mechanism.

      3 - The use of **RDO®** at specific dilutions decreased the **decalcification** time of cetacean ear bones with control of the decalcification endpoint, helping a faster access of inner structures.

      4 - A combined use of **electron microscopy (SEM & TEM) and immunohistochemistry** revealed specific morphologic characteristics of the organ of Corti of high-frequency hearing species, including echolocating bats, suggesting a convergent evolution in echolocating species;

      5 – The presence of scars in hair cells, of regular stereocilia imprints in the tectorial membrane and the condition of fibrocytes II and IV were found suitable to determine or discard possible alterations after sound exposure, despite the numerous artefacts that rapidly develop as a consequence of tissue autolysis.

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Appendix

APPENDIX 1

Abreviations

ADD: acoustic deterrent device

AHD: acoustic harassment device

ATOC: acoustic thermometry of ocean climate

BM: basilar membrane

EDTA: ethylenediaminetetraacetic acid

IHC: inner hair cell

LFA: low frequency sonar

LOC: lateral efferent olivocochlear

MOC: medial efferent olivocochlear

OHC: outer hair cell

p-p: peak to peak

PTS: permanent threshold shift

RMS: root mean square

SEM: scanning electron microscope

SGC: spiral ganglion cell

SPL: source pressure level

TEM: transmission electron microscope

TM: tectorial membrane

T-P complex: tympanic-periotic complex

TS: threshold shift

TTS: temporary threshold shift

VAcht: vesicular acetylcholine transporter

APPENDIX 2

Summary of relevant articles on masking, behavioural change and physiological effects due to man-made noise carried out on cetaceans

Table II.1. Summary of relevant articles on the masking of acoustic signals of cetaceans. Unless specified dB as it appears will refer to dB re 1µPa at 1 m.

Species	Experiment objectives	Results and conclusions	Source
Beluga (captivity)	Analyze the noise effects of icebreakers and the elaboration of *maskograms* to illustrate masking zones around various noises.	Masking radius: - 15 km for bubbler system noise of icebreakers (SPL 194 dB re 1µPa at1m) - 22 km from propeller cavitation noise (SPL 203 dB re 1 µPa at 1m) Melting ice does not seem to contribute to the masking of beluga signals.	Johnson *et al.*, 1989 ; Erbe, 1997
	Analyze the effects of icebreakers in masking noise and the construction of a model to process the effect.	Icebreaker noise from ramming, ice cracking, and bubbler systems produced masking at noise-to-signal ratios of 15–29 dB. The predicted zone of masking for beluga calls from ramming noise was 40 km.	Erbe and Farmer, 1998; Erbe *et al.*, 1999; Erbe and Farmer, 2000; Erbe, 2000
	Study the vocalizations of belugas when there is an increase in ambient noise.	Beluga whales' vocal output changes when they are moved to locations with higher background noise. With noise at low frequencies, an animal increases both the sound pressure level and the frequency of its vocalizations, perhaps in an attempt to avoid or overcome masking.	Au *et al.*, 1985
Beluga	Study the vocalizations of belugas as a response to boat noise.	The belugas increased call rates and shift to higher call frequencies in response to boat noise.	Lesage *et al.*, 1999
Sperm whale	Study the behavioral responses in sperm whales after the emission of different acoustic sources with the objective of diverting them from shipping lanes and avoiding collision	The sperm whales that were studied did not react to the majority of the emitted signals despite the very high level of the first exposure. They did momentarily cease making their 'clicking' echolocation signals after having been exposed to a series of artificial codas.	Andre *et al.*, 1997
Long fin pilot whale	Study pilot whales vocalizations as a response to the "Head Island Feasibility Test/HIFT" 1991.	Pilot whales ceased all vocalizations when exposed to HIFT.	Bowles *et al.*, 1994

Dolphins	Study the effect of masking noises in dolphins while using echolocation.	The capacity of distinguishing and detecting targets can be seen to be severely reduced by the introduction of masking noise.	Au and Nachtigall, 1993
	Study the effects of ambient and anthropogenic noise in dolphins.	The capacity to distinguish and detect objects diminished severely upon the introduction of making noise. On many occasions dolphins compensated for the presence of masking noise by emitting more "clicks" by sweep.	Au, 1993
Bottlenose dolphins	Demonstrate how natural sounds (snapping shrimp) can degrade the dolphin's echolocation detection range of fishes.	In an ambient noise of 55 dB re 1 μPa^2/Hz there is a reduction of 46% in the detection range (going from detecting a 28 cm cod from a distance of 173 m to detecting it from 93m away).	Au *et al.*, 2007
	Model the noise masking zone from pile driving and wind farms.	The masking zone for strong vocalizations is from 10-15 km, and up to 40 km for those weaker vocalizations.	David, 2006
Harbour porpoise	Study the 3 types of wind power generators in Denmark and Sweden (Middelgrunden, Vindeby, and Bockstigen-Valar). The turbine noise was only measured above the ambient noise in frequencies below 500 Hz.	It's unlikely that this noise reaches dangerous levels at any distance from the turbines, and this noise is not considered capable of masking porpoise communication.	Tougaard *et al.*, 2009
	50% of the detection of a porpoise's auditory threshold for a narrow band modulated frequency signal of 4.0 kHz where studied using behavioral methods, in the bottom noise level of a swimming pool and with two levels of masking noise.	The masking consisted in a noise in a 1/6 octave band with a frequency of 4.25 kHz. Its amplitude was reduced to 24 dB/octave on both sides of the respective spectrum plane. The auditory system of the animal responded in a linear form with the increase of the masking noise. Given that the narrow band noise was centered outside of the test frequency, the critical ratio of the porpoise for tonal signals of 4 kHz in target noise, can only be estimated to be between 18 and 21 dB re 1µPa.	Kastelein and Wensveen, 2008
	Study the reaction of harbour porpoises to pingers that emitted pulses of 10 kHz every 4 s at 132 dB.	Echolocation rate and occurrence were significantly reduced in the vicinity of the pinger	Cox *et al.*, 2001
Narwhal	Study the reaction of the narwhal to	The narwhal exhibited a totally silent behavior in contrast to the known state of alarm	JCNB/NAMMCO, 2005

	icebreaker noise.	behavior of belugas when they were exposed to icebreaker noise.	
Orcas	Study the vocalizations of orcas as a response to its interaction with whale watching boats.	It was suggested that orcas shift their call frequencies in response to the presence of whale-watching boats.	Foote *et al.*, 2004
Humpback whale	Study humpback vocalizations as a response to low frequency active sonar transmissions.	Some humpbacks were observed to cease vocalizations, while the songs of others were 29% longer at a maximum received level of 150 dB. Miller *et al*. 2000 signaled that perhaps this was to compensate for interference. Fristrup *et al* (2003) showed that humpback's songs were up to 10% longer, two hours after the exposure to sonar.	Miller *et al.*, 2000; Fristrup *et al.*, 2003
Grey whale	Responses of gray whales to increased levels of noise were documented during playback experiments.	They modified the calling to optimize signal transmission and reception.	Dahlheim, 1993

Table II.2. Summary of relevant articles on behavioral change due to man-made noise carried out on cetaceans. Unless specified dB as it appears will refer to dB re 1µPa at 1 m. SURTASS, sonar sweeping surveillance sensors of the US Navy; LFA, low frequency sonar; SPL, Source Pressure Level; ATOC, Acoustic Thermometry of Ocean Climate; ADD, Acoustic Deterrent Device; AHD, Acoustic Harassment Device; p-p, peak to peak; RMS, root mean square. Some information has been extracted from Perry, 1998 and Nowacek *et al.*, 2007.

Species	Experiment objectives	Results and conclusions	Source
Various cetacean species	Evidence of disturbance due to ships.	Moved among cetaceans in a specially silenced research boat without disturbing them, concluding that most reactions to vessels are a result of the noise emitted, rather than the physical presence of the boat.	Schevill, 1968
Blue whale	Evidence of disturbance due to ships.	Fast erratic approaches of boats close to blue whales caused flight reactions, separation of pairs of animals, shorter respiration rates, and displacement from the area.	MacFarlane, 1981
	Evidence of disturbance from seismic surveys. The acoustic tracking of a blue whale while carrying out an air-gun operation, producing a pulse at 215 dB re1µPa (10-60Hz band).	The blue whale started its call sequence when the air-gun ship was 15km away, and approached the ship to a range of 10km (where it was subject to an estimated received level of 143dB re1µPa). After a gap in calling, the whale started a new call series and moved diagonally away from the ship.	McDonald *et al.*, 1995
Blue whale	Evidence of disturbance due to ships.	Short term flight reactions in blue whales and fin whales in response to vessels in the	Edds and Macfarlane,

and fin whale		Saint Lawrence Estuary particularly if boats moved at high speed or erratically.	1987
Blue whale, fin whale and grey whale	Evidence of disturbance by sonar. A series of playback experiments were carried out to evaluate the impact of SURTASS LFA at received SPL levels not greater than 160 dB.	No overt responses have been observed in feeding blue and fin whales off southern California, however a consistent decrease in the number of whales producing long patterned sound sequences has been found Deflections in the migratory path of grey whales have been observed during playback.	Clark *et al.*, 1998
Minke whale, fin whale, humpback and right whale	Evidence of disturbance by whale watching ships.	Responses of baleen whales to boats in Cape Cod waters were variable with species and changed over time. In general, minke whales, humpback whales and fin whales appeared to habituate to boats, while right whale behaviour remained unchanged.	Watkins, 1986
Fin whale	Evidence of disturbance by whale watching ships.	Fin whales in the Gulf of Maine showed significantly reduced dive times, and reduced number of blows per surfacing sequence when whale watching boats were present.	Stone *et al.*, 1992
Humpback whale	Evidence of disturbance by ships.	Swimming speed, respiration and social behavior of wintering humpbacks were affected by maritime traffic, in particular to the speed, proximity and numbers of boats. A case study indicated that a calf was sensitised by a large vessel, the calf subsequently breaching in response to noise from a small boat engine, which had not previously elicited a response.	Bauer *et al.*, 1993
	Evidence of disturbance by ships. The response of feeding humpbacks to vessels was studied.	At 2-4km from the vessels the responses included shorter dive times, longer blow intervals and faster swimming speeds. At less than 2km, the responses were longer dive times, shorter blow intervals and slower swimming speeds, i.e. the whales avoided vessels by remaining submerged.	Baker *et al.*, 1982; Baker *et al.*, 1983
	Evidence of disturbance by ships. The same group of humpbacks was studied in their breeding grounds off Hawaii.	Attributed a consistent decrease in the percentage of mothers and calves in inshore waters to high levels of boating activity and aircraft.	Glockner-Ferrari and Ferrari, 1985
	Evidence of disturbance by ships.	Parasail boats displaced Hawaiian humpback whales, including cow/calf pods, from near shore areas.	Green, 1991
	Evidence of disturbance by commercial (C) and experimental (E) seismic surveys.	The received levels were 258 (C) and 227 (E) dBp-p re1 µPa. Avoidance responses were observed at 160-170 dBp-p re1 µPa for both arrays C and E.	McCauley *et al.*, 2000
	Evidence of disturbance by sonar.	Humpbacks in Hawaii showed avoidance behaviour in response to playbacks of sonar	Maybaum, 1993

		pulses of 3.3 kHz, and sonar sweeps of 3.1-3.6 kHz. It was postulated that the reactions stemmed from the resemblance between sonar signals and sounds that whales associate with threats or warnings.	
	Evidence of disturbance by sonar. A series of playback experiences were carried out to simulate and evaluate the impact of SURTASS LFA with 18 transducer array towed at 60–180 m depth, emitting at 130-160 Hz ("low" frequency component) and at 260-320 Hz ("high" frequency component).	The received levels were 130-150 dB $_{RMS}$. A significantly longer whale song was heard during playbacks than either before or after their emission.	Miller *et al.*, 2000
	Evidence of disturbance by sonar. A series of playback experiences were carried out to simulate and evaluate the impact of SURTASS LFA with 18 transducer array towed at 60–180 m depth, emitting at 130-160 Hz ("low" frequency component) and at 260-320 Hz ("high" frequency component).	The received levels were 130-150 dB $_{RMS}$. Songs were longer if overlapped by pings and these effects lasted up to 2 hours after the pings.	Fristrup *et al.*, 2003
	Evidence of disturbance by detonations at 1.8 km distance, 400 Hz.	The received levels were 140-153 dB $_{RMS}$. No detectable changes were found in in respiration rates, surface reactions or differences in re-sighting rates.	Todd *et al.*, 1996
	Evidence of disturbance by ATOC, that emitted a central frequency of 75 Hz.	The humpbacks found at a depth of 10-80 m and at 100-2000 m from the source dived longer and travelled farther between dives. The humpbacks which were at 8-12 km from the source showed an increase in dive time and in distances between dives with the estimated received level. Both situations were estimated to have received levels of ≤130 dB $_{RMS.}$	Frankel and Clark, 1998
Humpbacks and sperm whales	Evidence of disturbance by ATOC.	Aerial surveys off central California showed that humpback and sperm whales were distributed significantly further away from the ATOC source during sound broadcast.	Calambokidis *et al.*, 1998
	Evidence of disturbance by ATOC	Studies using playback of low intensity ATOC sounds have elicited few responses from sperm whales and humpback whales.	Gordon *et al.*, 1998a; Frankel and Clark, 1998
Grey whale	Evidence of disturbance by ships	Grey whales in San Diego bay responded to vessel noise by abandoning calving lagoons,	Reeves, 1977

		returning only after vessel traffic decreased	
	Evidence of disturbance by ships	Grey whales abandoned Guerrero Negro Lagoon over a period of various years whilst the bay was subjected to human activities (intense shipping traffic and intensive dredging). After a decrease in shipping activities, grey whales reoccupied the lagoon.	Bryant *et al.*, 1984
	Evidence of disturbance by industrial activities	Oil exploration playback noises were broadcasted underwater as 3500 migrating grey whales were passing. Avoidance responses began at broad-band received levels of around 110 dB re1μPa, and increased as noise levels were elevated. More than 80% of whales showed avoidance at received levels over 130 dB.	Malme *et al.*, 1984 ; Malme *et al.*, 1983
	Evidence of disturbance by seismic survey using a 4000 cubic inch (65.54 l) air-gun array.	10% of grey whales showed avoidance to received broad-band levels of 164dB re1μPa, 50% showed an avoidance reaction at 170dB, and 90% at 180dB. Whales were seen to move into the shallow surf zone and into sound shadows of rocks.	Malme *et al.*, 1983; Malme *et al.*, 1984
	Evidence of disturbance by aircraft. Grey whales reaction to helicopter playback noises was observed (excluding low frequency components).	Three simulated passes per minute elicited minor avoidance reactions in 50% of the whales, at received broad-band pressure levels of 120 dB re1μPa.	Malme *et al.*, 1984
Noth Atlantic Right whale	Evidence of disturbance from acoustic synthetic alert signal. The alerting signal device used emitted pure tones at 1000 Hz, a downsweep and amplitude modulated tones.	Estimated received levels were from 148 dB re 1 μPa/sgrt (Hz). 5 or 6 individuals swam upwards and maintained a depth of some 5 m below the surface.	Nowacek *et al.*, 2001
Bowhead whale	Evidence of disturbance by ships	The whales swam rapidly away from vessels at ranges of 0.8-3.4km, with shorter surface and dive times. The whales were effectively scattered, with mean inter-animal distance increasing from 7.5 to 37 whale lengths, this effect persisting for at least one hour.	Richardson *et al.*, 1985
	Evidence of disturbance from industrial activities. Comparison of Bowhead whale distribution and industrial activities in the Canadian Beaufort Sea.	It was speculated that a decrease in bowhead use of the main industrial area since 1980 was a result of cumulative effects of industrial activity which started in 1976. The effects of changing distributions of zooplankton and other environmental factors are not known.	Richardson *et al.*, 1987
	Evidence of disturbance from industrial activities.	Playback studies have found that most bowhead whales avoid drillship or dredging noise with broad-band (20-1000Hz) received levels around 115dB , levels that could occur 3-11km from typical drilling and dredging vessels. This equates to a response	Richardson *et al.*, 1990; Richardson and Greene, 1993

		threshold of about 110dB in the 1/3-octave band where industrial noise is most prominent. Higher intensity noise is endured by bowhead whales if the only migration route requires close approach to the sound projector.	
	Evidence of disturbance from seismic surveys.	The whales swam rapidly away from a seismic vessel at a distance of 24km.	Koski and Johnson, 1987
	Evidence of disturbance from seismic surveys.	A change in behavior began at more than 8 km from the source, with received levels of 142-157 dB.	Ljungblad *et al.*, 1988
	Evidence of disturbance from seismic surveys.	Subtle alterations in surfacing, respiration and dive cycles in response to seismic vessels, indicating that the absence of a conspicuous response does not necessarily prove that an animal is unaffected.	Richardson *et al.*, 1985
	Evidence of disturbance by seismic surveys.	Whales engaging in normal activities as close as 6km to the vessels, where estimated received levels were 158 dB.	Richardson *et al.*, 1986
	Evidence of disturbance by airplanes.	Avoidance reactions of bowheads when aircraft approached or circled at or below 305m above sea level.	Richardson *et al.*, 1985
	Evidence of disturbance by airplanes.	Bowhead whales were less responsive to passing aircraft when actively engaged in feeding, social activities or mating, than when resting	Richardson *et al.*, 1995
	Evidence of disturbance by aircrafts.	Received levels were 114 dB $_{RMS}$ at 3 m depth and of 120 dB $_{RMS}$ at 18 m depth. Short and abrupt dives, moving away from the sound source were observed.	Patenaude *et al.*, 2002
Belugas	Evidence of disturbance by ships. Belugas were monitored before, during and after exposure to noise from a small motor boat and a ferry.	Reactions to the approach of the vessels included reduced diversity of call types and call rates, and repetition of specific calls within 1 km of the vessels. Within 300m, the beluga whales shifted the peak frequency of their signals from 3.5 kHz to 5.2-8.8 kHz	Lesage *et al.*, 1993
	Evidence of disturbance by ships.	Avoidance reactions to the playback of boat noise at levels which were believed to be barely perceptible. It was concluded that the belugas seemed to be more influenced by the habitat and by activity at the moment of disturbance than by the intensity of the noise.	Stewart *et al.*, 1982
	Evidence of disturbance by ships.	Altered behaviors were shown in swim speed and direction, changes in immersion patterns, breathing and surfacing time and/or changes in vocalization patterns.	Lawson, 2005
	Evidence of disturbance by simulations of distant underwater explosions.	A perturbation threshold was established at 220 dBp-p. No TTS was predicted >6 dB above 221 dBp-p.	Finneran *et al.*, 2000

Belugas and narwhal	Evidence of disturbance by ships. Reactions of belugas and narwhals to ice-breaking ships in the Canadian High Arctic.	The belugas reacted with a flee response and the narwhals with a freeze response, the characteristics of which were typical of their responses to predation by killer whales. The belugas avoided approaching ships at ranges of 45-60km, and seemed aware of an approaching ship at a distance of 85km. The reactions began when broad-band (20-1000Hz) received levels of ship noise were 94-105dB. The belugas were aware of the ships at far greater ranges than would be predicted from calculations based on captive auditory thresholds 2, placing some doubt on the applicability of laboratory audiograms to natural situations. The belugas moved up to 80km from their original location in response to the ship, and remained absent for 1-2 days. Effects on narwhals appeared to be more transient, normal activities being resumed when received broad-band levels were as high as 120dB.	Finley *et al.*, 1990
Sperm whales	Evidence of disturbance by ships.	The responses observed in sperm whales faced to boats included reduced surface times, with fewer blows per surfacing, shorter intervals between blows and reduced frequency of dives with raised flukes	Gordon *et al.*, 1992
	Evidence of disturbance by whale watching ships.	Sperm whales in New Zealand avoided commercial whale watching boats at a distance of 2 km.	Cawthorn, 1992
	Evidence of disturbance by seismic surveys.	Sightings surveys show that sperm whales were displaced to a distance of 60km from an area in the Gulf of Mexico, where seismic surveys were taking place.	Mate *et al.*, 1994
	Evidence of disturbance by seismic surveys.	Sperm whales ceased vocalizations in response to relatively weak seismic pulses coming from a ship at hundreds of km's distance.	Bowles *et al.*, 1994
	Evidence of disturbance by seismic surveys.	Studies in the Northern Gulf of Mexico indicate that seismic exploration has a negative impact on aspects of communication and orientation behaviour of sperm whales, but no effects on the distribution of other odontocetes.	Rankin and Evans, 1998
	Evidence of disturbance by seismic surveys emitting at 210-260 Hz.	At estimated received levels of 146 dBp-p it was not observed avoidance. The whales stayed in the area for at least 13 days of exposure.	Madsen *et al.*, 2002
	Evidence of disturbance by seismic survey in the Gulf of Mexico.	Sperm whales in this area have been exposed to seismic survey sounds for many years (Wilson *et al.* 2006). By means of visual surveillance and satellite tracking, the animals showed no or only few detectable changes in behaviour.	Gordon *et al.*, 2006; Winsor and Mate, 2006

	Evidence of disturbance by seismic survey in controlled exposure experiments with specialized recorders or DTAG – digital acoustic recording tag's (Johnson and Tyack, 2003) attached to the animals.	The animals didn't demonstrate any avoidance behavior in a 1-13 km range from the source at received levels of 152-162 dBp-p (135-147 dB $_{RMS}$, 115-135 dB re 1 µPa²s). While most of them continued with their dive patterns, the behavioural change was indicated by reduced fluke pitch and vocalisation "buzz" rates during the dive.	Miller *et al.*, 2006
	Evidence of disturbance by sonar.	The sperm whales reacted to military sonar at a distance of 20 km or more from the source. Sonar at frequencies of 6-28 kHz caused cessation of calling and sometimes avoidance.	Watkins *et al.*, 1985; Watkins *et al.*, 1993
	Evidence of disturbance by acoustic devices.	A study to repel whales from ferry routes in the Canary Islands using playback of a variety of sounds found that sperm whales reacted strongly to 10kHz pulses, particularly when breathing at the surface after a long dive.	Andre *et al.*, 1997
	Evidence of disturbance by explosions.	The received levels were from < 179 dB $_{RMS}$. No behavioral or acoustic effects were noticed.	Madsen and Møhl, 2000
	Acoustic exposure and behavior of 8 tagged sperm whales, before, during and after 5 controlled sound exposures of air guns, separated by 1-2 hours.	None of the 8 sperm whales changed behavior (7 feeding, 1 resting) following the initial ramp-up at a distance of 7-13 km, or during the exposures (1-13 km). The animal closest to the source was resting during the experiment, but began to feed soon after the sound tests stopped, possible indicating a delay in foraging occurred during the exposure. The sperm whales did not exhibit any horizontal avoidance of the airguns. There was a 6% reduction in oscillations in pitch generated by swimming movements and a 19% decrease in buzz rates during feeding, but the latter didn't turn out to be significant.	Miller *et al.*, 2009
Sperm and pilot whales	Evidence of disturbance from acoustic thermometry. The Heard Island Feasibility Test transmitted sound for one hour of every three, with source levels of 209-220 dB at a depth of 175 m. The centre frequency was 57 Hz, with a maximum bandwidth of 30 Hz.	Sperm whale and pilot whale signals were heard in 23% of 1181 minutes of baseline acoustic monitoring before transmission, but were absent in 1939 minutes of monitoring during transmission. Sperm whale clicks were eventually heard 36 hours after the end of the transmission. Sighting samples were too small to estimate changes in densities of cetaceans.	Bowles *et al.*, 1994
Short-fined pilot whale	Evidence of disturbance by whale watching ships.	Significantly longer dive times and closer grouping of short-finned pilot whales in response to a large number of whale watching boats in the Canary Islands. Respiration patterns were found to normalize eventually, however examples of unusually aggressive behaviour were documented during the observations	Heimlich-Boran *et al.*, 1994

	Evidence of disturbance by ramp up used as a mitigation strategy in possible air gun sound impact. A group of 15 pilot whales were monitored before, during and after a seismic ramp up procedure of 30 minutes in a 2-D seismic survey in Gabon.	No behavioral changes were observed during the initial ramp up period. Nevertheless, 10 minutes after the start (air gun pistol volume of 940cu³) the nearest subgroup fled suddenly from the source. Subsequent behavior included milling, tail slapping and a 180º change of direction away from the seismic ship.	Weir, 2008
Common dolphin	Evidence of disturbance by seismic surveys with air gun at 80-100 m depth which emitted at ; a) 250 Hz, b) 2 kHz, c) 10 kHz, d) 20 kHz. The dolphins were monitored before, during and after seismic surveys in the southern Irish Sea.	Avoidance reaction of the dolphins in monitored area (1-2km from the survey vessel). The received levels were 170 (a), 140 (b), 115 (c) and 227 (d) dB re 1µPa/sgrt (Hz). The animals at 5 km from the source exhibited a greater number of vocalizations by hour before than during seismic surveys. The survey employed a 2120 cubic inch air-gun, which is smaller than the arrays typically used by prospecting companies	Goold, 1996
	Evidence of disturbance by seismic exploration with air gun emitted at: a) 200 Hz, b) 20 kHz.	Received levels were from 140 (a) and 90 (b) dB re 1 µPa/sgrt (Hz). The signal was estimated to be clearly audible for dolphins at a range of 8 km. The animals 750 m from the source showed a smaller proportion of acoustic contact during emissions (4%) then when air guns were not in use.	Goold and Fish, 1998
Bottlenose dolphin	Evidence of disturbance by ships. The sound effects of speedboats and the playback of their sound were examined in dolphins in Cardigan Bay.	Responses of shorter surface periods, longer dives and movement away from vessels at ranges of 150- 300 m. It has been suggested that quieter boats, travelling at high speed, disturb dolphins more than slower, larger boats that emit higher intensity noise, as the noise produced by a high speed boat rises above ambient levels for only a short time before its closest point of approach, thereby provoking a startle response.	Evans *et al.*, 1992
	Evidence of disturbance by recreational alboats which emitted levels of noise of 115-138 dB $_{RMS}$, (planing boats), 114–121 dB $_{RMS}$ (plowing boats) and 113–116 dB $_{RMS}$ (idling boats).	The boats maintained 20 m from focal dolphin. There were a higher whistle rate at onset of noise than during or after exposure	Buckstaff, 2004
	Evidence of disturbance by whale watching boats.	Significant decreases in surfacing frequencies of bottlenose dolphins in response to a dolphin watching boat which attempted to remain near the group. The dolphins showed little response to other boats in the area.	Janik and Thompson, 1996
	Evidence of disturbance by seismic survey. Small groups of cetaceans in the Irish Sea were monitored before, during and after seismic survey.	Although most sample sizes were too small for statistical analysis, a significant decline in the number of individual bottlenose dolphins was found, suggesting that a proportion of the population had moved out of the area during the period. It is not known if this movement reflected a response to seismic activity, or seasonal	Evans *et al.*, 1993

		movements.	
	Evidence of disturbance by acoustic devices. The ADD used emitted pulses of 10 kHz every 4 s at 132 dB.	The received levels were 120 dB $_{RMS}$ at approximately 100 m. No differences were seen between the maximum approach distance to the source when the device was activated or not.	Cox *et al.*, 2004
	Evidence of disturbance by simulations of distant underwater explosions.	An alteration threshold was established between 196 and 209 dBp-p. No TTS >6 dB was forecast above 221 dBp-p.	Finneran *et al.*, 2000
Hectors dolphin	Evidence of disturbance by acoustic devices. The ADD used emitted pulses of 10 kHz every 4 s at 132dB	The maximum estimated level in the closest approach to the source (552 m) was 86 dB $_{RMS}$. An avoidance response to the sound source was witnessed.	Stone *et al.*, 1997
	Evidence of disturbance by aircraft. Helicopter flew overhead and produced mainly tones between 10 and 500 Hz and was at 150m and 450 m altitude.	The received levels were of 120 dB $_{RMS}$ at 3 m depth and 112 dB $_{RMS}$ at 18 m depth. Short dive durations, abrupt dives, orienting away from noise was observed.	Stone *et al.*, 1997
Indo-Pacific dolphin	Evidence of disturbance by tourist boats in Zanzibar.	The 5 mother-calf pairs studied did not exhibit swim pattern changes where they were few boats in the area but showed a very significant number of erratic movements when they were scuba divers in the water. The proportion of "tail out" dives increased with the escalation of human (tourist) activity.	Stensland and Berggren, 2007
Franciscana	Evidence of disturbance by acoustic devices. The ADD used emitted 10 kHz pulses every 4 seconds at 132 dB.	The estimated level at the net was ≥104 dB $_{RMS}$. It was observed a reduction in the by-catch and increasing in depredation.	Bordino *et al.*, 2002
False killer whale and Risso's dolphin	Evidence of disturbance by ATOC. Hearing thresholds of a captive Risso's dolphin and a false killer whale were measured to a one-second pulsed ATOC signal.	Both species had relatively high thresholds to the sound (139-142dB), indicating that the dolphins would have to dive to a depth of around 400m, directly above the source, in order to detect the sound.	Au *et al.*, 1997
Harbour porpoise	Evidence of disturbance by ships.	The harbour porpoises exhibited an avoidance reaction to survey vessels.	Polacheck and Thorpe, 1990
	Evidence of disturbance by ships.	The porpoises of the South-East Shetland Islands evaded ships of all sizes, sometimes moving right out of the area. It was discovered that the porpoises were more likely to avoid infrequent vessels than routine vessels, such as the daily ferry.	Evans *et al.*, 1994
	Evidence of disturbance by industrial activity, specifically pile-driving during the construction of a	The harbour porpoises exhibited a flee response of up to 10-20 km from the source, decrease of density(visually recorded) and cease of acoustic activity.	Tougaard *et al.*, 2003; Tougaard *et*

	Danish offshore wind farm.		*al.*, 2005
	Evidence of disturbance by 3 types of wind turbines in Denmark and Sweden (Middlegrunden, Vindeby and Bockstigen-Valar). Wind turbine noise was measured only above the ambient noise in frequencies below 500 Hz.	The total SPL was in the 109-127 dB $_{RMS}$ range, at a distance between 14 to 20 m from the cement foundations. The maximum 1/3 octave levels were in the range of 106-126 dB $_{RMS}$. The audibility was low for the porpoises reaching 20-70 m away from the base. It appears improbable that the porpoises would react to the noise in behavior unless they were very close to the cement foundations.	Tougaard *et al.*, 2009
	Evidence of disturbance by seismic survey.	The porpoises showed avoidance behavior towards the source above received levels of 145 and 155 dB $_{RMS}$ up to more than 70 km distance.	Bain and Williams, 2006
	Evidence of disturbance by a single airgun stimulus at increasing received levels.	The animal exhibited constant reactions of aversive behavior in received SPL above 174 dBp-p or SEL of 145 dB re 1 µPa²s.	Lucke *et al.*, 2009
	Evidence of disturbance by acoustic devices. The ADD used emitted 8–16 kHz chirps, spreadspectrum blocks, frequency sweeps and modulated frequency shifts at 116-130 dB.	It was observed discomfort at received levels ≤116 dB $_{RMS}$ and avoidance of sound source as source levels increased.	Kastelein *et al.*, 2005
	Evidence of disturbance by acoustic devices. 4 experiments were carried out with different ADD's: 1) clicks, tones and sweeps of 17.5 to 140 kHz; 2) tones of 2.5 kHz and 110-131 dB; 3) 110 kHz, 158 dB; 4) 325 kHz, 179 dB.	Received levels were ≤107 dB $_{RMS}$. An avoidance reaction to the sound source was observed.	Kastelein *et al.*, 1997
	Evidence of disturbance by acoustic devices. 3 experiments with different ADD's were carried out: 1) pulses of 10 kHz every 4s at 132 dB; 2) pulses of 10 kHz, randomized production, 132 dB; 3) sweeps between 2 and 3.5 kHz, 100 dB.	Received levels ≤124 dB $_{RMS}$ in cases 1 and 2. In case 3 was estimated received levels of 90 dB $_{RMS}$ re 1 µPa at 1 m at 3.5 kHz. In all cases an avoidance behavioral response to the sound source was observed.	Kastelein *et al.*, 2000
	Evidence of disturbance by acoustic devices. 3 experiments were carried out with different ADD's: 1) 16 tones (constant pulse width and interval) between 9 and 15 kHz, 145 dB; 2) as above 1), but with randomized pulse width and interval; 3) 0.1s upsweep	Received levels were ≤138 dB $_{RMS}$ at 33 kHz in the first experiment, ≤140 dB $_{RMS}$ at 12 kHz in the second and of ≤90 dB $_{RMS}$ at 6 kHz in the third. In all cases an avoidance behavioral response to the source was observed with an increase in respiration rates.	Kastelein *et al.*, 2001

	at 0.2s downsweep between 20-80 kHz and 96-118 dB.	
	Evidence of disturbance by acoustic devices. The ADD used emitted sweeps between 20 and 169 kHz and at 145 dB.	The maximum estimated level in the closest approach to the source (130 m) was 102 dB $_{RMS}$. Avoidance behavior to the sound source was observed.	Culik *et al.*, 2001
	Evidence of disturbance by acoustic devices. The ADD used emitted tones of 115 dB at 2.5 kHz.	The maximum estimated level in the closest approach to the source (130 m) was 72 dB $_{RMS}$. Avoidance behavior to the sound source was observed.	Koschinski and Culik, 1997
	Evidence of disturbance by acoustic devices. The ADD used emitted pulses of 10 kHz every 4 s at 132 dB.	The porpoises were initially displaced 208m from the pinger, but this displacement diminished by 50% within four days. The probability of porpoises within 125m of the pinger (received levels of 118-122 dB $_{RMS}$) initially decreased when the pinger was turned on, but then increased to equal the control in 10-11 days.	Cox *et al.*, 2001
	Evidence of disturbance by acoustic devices.	Initially the porpoises reacted most strongly to the sonar pinger-like sounds by diminishing vocalizations, surface times and heart rates, entering below normal bradycardia. In the following test sessions the animals appeared to get used to the noise.	Teilmann *et al.*, 2006
	Evidence of disturbance by acoustic devices. The ADH used emitted at levels of 180-200 dB.	It was estimated that the animals received levels of 122 dB $_{RMS}$ at the maximum range of influence. Due to the large percentage of locations where ADH's were used, it could be possible habitat exclusion.	Johnston and Woodley, 1998
	Evidence of disturbance by acoustic devices. The ADH used emitted at levels of 180 dB.	The porpoises avoided the sound source. No animals were seen in the first 200 m. Received levels were estimated to be ≤134 dB $_{RMS}$ at 200 m (exclusion zone).	Olesiuk *et al.*, 2002
	Evidence of disturbance by acoustic devices. The ADH used emitted at levels of 180 dB.	The porpoises avoided the sound source; approaching a maximum distance of 645 m. Received levels were estimated to be of 125 dB $_{RMS}$ (distance of closest approach: 991m).	Johnston, 2002
	Evidence of disturbance by acoustic devices. The ADH used emitted levels of 180-200 dB.	Authors concluded that the ADH could exclude non- target species from important habitats. They estimated received levels greater than 130 dB $_{RMS}$ at 1 km from the source of 200 dB re1 µPa at 1m.	Taylor *et al.*, 1997
Harbour porpoise and striped dolphin	Evidence of disturbance by acoustic devices. The ADD used emitted 16 tones (constant pulse width and interval) between 9 and 15 kHz and 145 dB.	Received levels were ≤138 dB $_{RMS}$ at 33 kHz. The porpoises showed avoidance behavior towards the source; however the dolphins showed no reaction.	Kastelein *et al.*, 2006

| Orca | Evidence of disturbance by "leapfrogging" whale watching ships, which were at more than 100 m from the orcas and produced sounds at 100 Hz. | Received levels were 115 dB $_{RMS}$ measured at 100m. The orcas were noted to have movement paths less direct and less predictable | Williams *et al.*, 2002 |

Table II.3. Documented evidence of stress and other physiological effects induced by human activities on cetaceans

Species	Experiment objectives	Results and conclusions	Source
Belugas (captive)	Study stress produced in cetaceans by anthropogenic activities. 4 captive belugas were subjected to playback of noise from a drilling platform (source levels of 153 dB re 1 µPa ref 1m).	Blood levels of catecholamine (adrenaline and noradrenaline) were not higher after the experiment and no significant changes in behavior were noticed. The authors noted the possibility that captive whales are habituated to low frequency noise from water pumps, and advised caution in applying the results to free-ranging belugas in the absence of long term monitoring.	Thomas *et al.*, 1990
Irrawaddy River dolphin	Find evidence of non-auditory physical effects on cetaceans by man-made activities.	A decline in the number of Irrawaddy dolphins in Lao PDR and north-eastern Cambodia was linked to incidental mortalities from explosives used by fishermen	Baird *et al.*, 1994
Bottlenose dolphin	Study stress in cetaceans provoked by man-made activities.	When the dolphins were chased and captured they showed an increased level of cortisol and associated decreased levels of leucocytes. The animals which already showed high levels of cortisol due to their handling did not exhibit further cortisol increases in response to injections of ACTH, suggesting that the adrenal cortex was already maximally stimulated. Two of the dolphins administered with ACTH died.	Thomson and Geraci, 1986
	Find evidence of non-auditory physical effects produced on cetaceans by man-made activities	Close proximity of marine mammals or humans to low frequency noise at SPLs in excess of 210dB re1µPa at 500Hz could result in significant growth of existing bubbles in capillaries and other small blood vessels. Although noise of this intensity is rare, they suggested that considerably lower intensity noises could induce bubble growth if the body fluid was already super-saturated with gas. This occurs when human divers using breathing apparatus are near decompression limits	Crum and Mao, 1996
	Study evidence of non-auditory physical effects produced on cetaceans by man-made activities	Some cetaceans make repeated dives to great depths which could produce over-pressure of nitrogen in muscle tissues. It is theoretically possible for intense sounds to induce the pathological conditions associated with bubble growth ("the bends") in cetaceans	Ridgway and Howard, 1982; Ridgway, 1997
Harbour porpoise	Study evidence of non-auditory physical effects produced in cetaceans	The harbour porpoise may suffer tissue damage in the first 7 m from an acoustic harassment device.	Taylor *et al.*, 1997

| Beaked whales | Necropsies carried out on stranded beaked whales in Canary Islands in 2002 and Almeria in 2006 after Naval maneuvers where active mid frequency sonar had been in operation. | The stranded animals showed a syndrome of fat and gas embolic syndrome that manifested a certain analogy with sicknesses related to decompression in humans. | Jepson *et al.*, 2003; Fernández *et al.*, 2004; Fernández, 2004; Fernández *et al.*, 2005a; Fernández *et al.*, 2005b; Fernández, 2006b |

Table II.4. Summary of the relevant articles on hearing loss on cetaceans. Unless specified dB as it appears will refer to dB re 1µPa at 1 m. Abbreviations used: SEL, Sound exposure level; SPL, Source pressure level; PTS, Permanent threshold shift; TTS, Temporal threshold shift; p-p, peak-to-peak; OBN, octave-band noise.

Species	Experiment objectives	Results and conclusions	Source
Belugas (in the wild)	To convert human Occupational Safety and Health Administration (OSHA) standards to sub-aquatic standards for cetaceans.	Levels of noise that could cause PTS in belugas (at frequencies of 500 Hz, 1 kHz and 10 kHz) occurred in 2 of the 3 areas researched in the Saint Lawrence River Estuary. As noise levels varied over the day it is unlikely that beluga population was subjected to OSHA criteria (for PTS in humans, which is exposure for about eight hours a day, for ten years). Scheifele noted however that a number of the assumptions made in the conversion were sufficiently conservative to reasonably expect PTS to occur at lower noise levels than predicted.	Scheifele, 1997
Beluga	Software model estimating zones of impact on marine mammals around man-made noise was applied to the case of icebreakers affecting beluga whales in the Beaufort Sea. Two types of noise emitted by the icebreaker (with 1/3 octave band level centered at 5 kHz) were analyzed: bubbler system noise and propeller cavitation noise. The received levels would be from 81 dBRMS re 1 µPa a 1 m (corresponding to the disturbance threshold).	The audible zones would include from 35 to 78 km. Masking of beluga communication signals was predicted within 14-71-km range. TTS could occur if a beluga stayed within 1-4 km of the icebreaker for at least 20 min. (Specifically TTS of 12-8 dB in a 30 min exposure would be produced in the first 40 m for bubble noises and 120 m for ramming, or of 4.8 dB for a 20 min exposure). Bubbler noise impacts over the short ranged quoted; propeller cavitation noise accounted for all the long-range effects.	Erbe and Farmer, 2000
Sperm whale	Preliminary study of inner ear structures in 2 sperm whales died in a Canary Islands ferry collision.	The results are consistent with auditory nerve degeneration and increased fibrousness in response to inner ear injury. Combined with the experimental	André *et al.*, 1997

		playback results, these results suggest that low frequency sounds from ships may be affecting hearing and increasing the incidence of collisions around the Canary Islands.	
Harbour porpoise	To measure the TTS in a porpoise after exposure to single airgun stimuli at increasing received levels. Immediately after each exposure the animal's hearing threshold was tested for significant changes.	At 4 kHz the predefined TTS criterion was exceeded at a received SPL of 199.7 dBp-p re 1 µPa and a SEL of 164.3 dB re 1 µPa^{2}s. The animal consistently showed aversive behavioral reactions at received sound pressure levels above 174 dBp-p re1µPa or a SEL of 145 dB re 1µPa^{2}s. Elevated levels of baseline hearing sensitivity indicate potentially masked acoustic thresholds. Therefore, the resulting TTS levels should be considered masked temporary threshold shift (MTTS) levels.	Lucke *et al.*, 2009
Striped dolphin	Electrophysiological measurements of the hearing of a stranded striped dolphin.	The PTS was estimated to be over 60 dB re 1µPa at 1m along the measured audiogram, in comparison with the species reference. The PTS was attributed to severe hydrocephaly which was revealed *post-mortem*.	André *et al.*, 2007
Bottlenose dolphin and Beluga	Two bottlenose dolphins and one beluga were exposed to single pulses from an "explosion simulator" (ES). The ES consisted of an array of piezoelectric sound projectors that generated a pressure waveform resembling that from a distant underwater explosion	The pressure waveform was generally similar to waveforms predicted by the Navy REFMS model (Britt et al., 1991). The ES failed to produce realistic energy at frequencies below 1 kHz, however. No substantial (i.e., ≥ 6 dB) threshold shifts were observed in any of the subjects exposed to a single pulse at the highest received exposure levels (peak: 70 kPa [10 psi]; 221 dB dBp-p re1 µPa; SEL: 179 dB re1 µPa^{2}s).	Finneran *et al.*, 2000
	To repeat previous experiment with using a seismic watergun that produced a single acoustic pulse. Hearing thresholds were measured at 0.4, 4, and 30 kHz. The subjects were a bottlenose dolphin and a beluga.	The TTS measurements in the beluga were 7 and 6 dB at 0.4 and 30 kHz respectively, following an exposure length of 2 minutes of single intense pulses with peak pressures of 160 kPa, 226 dBp-p re1 µPa, and total energy fluxes of 186 dB re1 µPa^{2}s. Thresholds returned to within ± 2 dB of the pre-exposure value within 4 min of exposure. No TTS was observed in the bottlenose dolphin at the highest exposure conditions: peak pressures of 207 kPa [30 psi]; 228 226 dBp-p re1 µPa; SEL: 188 dB re1 µPa^{2}s. These studies demonstrated that, for very brief pulses, higher sound pressures were required to induce TTS than had been found for longer tones	Finneran *et al.*, 2002
	To study TTS in 4 dolphins and 2 belugas in masked hearing thresholds, using 1 s tones at 3, 10, 20 and 75 kHz.	The dolphins began to demonstrate measurable TTS at received levels from 192-201 dB re 1µPa, depending on frequencies and individuals. One beluga did not	Ridgway *et al.*, 1997

		show TTS at the highest intensity studied (201 dB re1 µPa), while the other showed a TTS at a level of 198 dB re1 µPa.	
	To measure TTS in 5 bottlenose dolphins and 2 belugas exposed to pure tones of 1 second (non- pulsed). Also include the data analysis of TTS from a technical report by Ridgway *et al.*, 1997.	At frequencies of 3 kHz, 10 kHz and 20 kHz, the SPLs necessary to induce TTS were 192 to 201 dB re at 1µPa, (SEL: 192 to 201 dB re 1 µPa^{2}s), The mean exposure SPL for TTS-onset was 195 dB re at 1 µPa (195 dB re 1 µPa^{2}s). At 0.4 kHz, no subjects exhibited shifts after exposures up to SPL exposures of 193 dB re at 1 µPa (193 dB re 1 µPa^{2}s). Data at 75 kHz were inconclusive: one dolphin exhibited a TTS after exposure at 182 dB SPL re at 1 µPa (182 dB re 1 µPa^{2}s) but not at higher exposure levels. The other dolphin experienced no threshold shift after exposure to maximum SPL levels of 193 dB re at 1 µPa (193 dB re 1 µPa^{2}s). The shifts occurred most often at frequencies above the fatiguing stimulus.	Schlundt *et al.*, 2000
Bottlenose dolphin	To measure TTS in bottlenose dolphins exposed to 3 kHz tones with 1, 2, 4, 8 second durations and various SPL value levels using behavioral methods. Experiments were conducted in a relatively quiet pool with ambient noise levels below 55 dB re 1 µPa/Hz at frequencies above 1 kHz.	Small amounts of TTS (3 to 6 dB) occurred in one dolphin following exposures with SELs of 190 to 204 dB re 1 µPa^{2}s. In general, the SEL necessary for TTS-onset was relatively consistent across the range of exposure durations, whereas exposure SPL values causing TTS-onset tended to decrease with increasing exposure duration. TTS magnitude was best correlated with exposure SEL rather than SPL.	Finneran *et al.*, 2005
	To report on the growth and recovery of TTS in a bottlenose dolphin exposed to 3 kHz tones with SPLs up to 200 dB re 1 µPa and durations up to 128 s.	The maximum exposure SEL was 217 dB re 1 µPa^{2}s, which produced a TTS4 of ~23 dB. All thresholds recovered to baseline values in the first 24 hours, most in the first 30 minutes. The growth of TTS4 with increasing exposure SEL was ~1 dB TTS per dB SEL for TTS4 of ~15 to 18 dB.	Schlundt *et al.*, 2006
	To measure TTS in a bottlenose dolphin after single and multiple exposures to 20 kHz tones. Hearing threshold were estimated in multiple frequencies (from 10 to 70 kHz) using behavioral or electrophysiological methods. 3 experiments were carried out. The first two with single exposures (20 kHz, 64-s tones at 185 and 186 dB re µPa) and the third with exposures of 20 kHz, 16 s separated by 11 and 12 min, with a mean SPL of 193 dB re µPa (SD = 0.8 dB).	Hearing loss was frequency dependent with the larger TTS at 30 kHz, lesser at 40 kHz and then at 20 kHz, and little or no TTS in the other frequencies measured. AEP threshold shifts reached 40 to 45 dB and were always larger than behavioral shifts, which were 19 to 33 dB. Complete recovery required up to 5 d, with the recovery rate at 20 kHz being ~2 dB/doubling of time and the rate at 30 and 40 kHz ~5 to 6 dB/ doubling of time.	Finneran *et al.*, 2007
	To measure TTS (*ca.* 20 min after noise cessation) in a bottlenose dolphin.	It was found an average 11 dB shift following a 30- min net exposure to OBN with a 7.5 kHz cent. No TTS was observed after exposure to the same OBN at maximum	Nachtigall *et al.*, 2003

		SPL values of 165 and 171 dB re 1 µPa (SEL: ~198 to 200 dB re 1 µPa^{2}s and 204 to 206 dB re 1 µPa^{2}s, respectively).	
	To measure TTS in a bottlenose dolphin using auditory evoked potentials (AEP). During each session, following an initial measure of threshold, noise of 160 dB re µPa at 4- 11 kHz bandwidth was presented for 30 min. After the noise exposure, thresholds were measured again at delays of 5, 10, 15, 25, 45, and 105 min. Measurements were made at test frequencies of 8, 11.2, 16, 22.5, and 32 kHz.	The maximum TTS occurred 5 min after exposure and rapidly recovered with a rate of around 1.5 dB per doubling of time. TTS Occurred at test frequencies from 8 to 16 kHz, with the maximum at 16 kHz. TTS was negligible at 22.5 kHz and absent at 32 kHz.	Nachtigall *et al.*, 2004
	To carry out controlled experimental studies to witness the effects of active mid-frequency sonar on a bottlenose dolphin.	Mid-frequency sonar can induce temporary hearing loss in a bottlenose dolphin, following repeated exposure to intense sonar pings with total SEL of 214 dB re1 µPa²s. Mild-behavioural alterations were also associated with the exposures.	Mooney *et al.*, 2009
	Temporary threshold shift in hearing at 7.5 kHz was studied with a bottlenose dolphin	The animal's hearing was not affected when the noise was 171 dB at 1 µPa with a total energy flux density of 205 dB at 1 µPa^{2}s. After 30 minutes of exposure, TTS of 12–18 dB were obtained when the noise increased to 179 dB with an energy flux density of 213 dB. The fatiguing stimulus was about 96 dB above the animal's pure tone threshold of 84 dB.	Au *et al.*, 1999
	To measure TTS after exposure to 16s tones at 3 and 20 kHz to examine the effects of exposure frequency on the onset and growth of TTS.	There were frequency-specific differences in TTS onset and growth, and increased susceptibility to auditory fatigue after exposure to 3-kHz tones	Finneran and Schlundt, 2010
Harbour porpoise	To calculate the TTS in harbour porpoise.	It was calculated that the harbour porpoise could suffer severe disturbance and temporary loss of hearing within 1km of an AHD used on fish-pens. Immediate auditory damage and injury could occur within 7m of the device. This is of particular concern as some devices can be triggered at full power, either manually or by net sensor.	Taylor *et al.*, 1997
	To calculate theoretical TTS zones depending on frequency noises during 1.5 MW wind farms construction/operation (wideband SL peak=228 dB$_{0-P}$ re µPa ref 1m/206 dB re µPa²s ref 1m).	The zone of audibility for pile-driving will most certainly extend well beyond 80 km, perhaps hundreds of kilometres from the source. Behavioural responses are possible over many kilometres, perhaps up to ranges of 20 km. Hearing loss might be a concern – on the basis of a regulatory approach - at 1.8 km	Thomsen *et al.*, 2006

		in porpoises	
Bottlenose dolphin, harbour porpoise and harbour seal	Review of studies on responses of marine mammals to sounds of windmill construction and operation	They recommended that operational guidelines for wind farms should include maximum noise levels that lower than 110 dB re 1 µPa RMS at 100 m.	Madsen *et al.*, 2006
Killer whale	To model the broadband sounds produced by a whale watching zodiac with twin engines of 150 hp with estimated received levels of 120 dBRMS re 1 µPa at 1 m in order to calculate potential auditory damage zones.	The zones with audible levels, of masking, with behavioral changes, TTS (5 dB after 30 to 50 min exposure) were 1600, 1400, 200 and 450 respectively. If an animal was exposed to this boat noise within 1 km range continuously for 8 h per day, 5 d a week, for 50 yr, a PTS of 2-5 dB could be expected based on Kryter's (1985) data for humans.	Erbe, 2002

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