Final Report: Postmortem Research Feasibility Study on Cetacean Ears

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Executive Summary

The objective of this study is to assess the feasibility of investigating potential anthropogenic acoustic damage to cetaceans using histological examination of cetacean ears removed on post-mortem examination, predominantly from stranded or by-caught harbour porpoises (*Phocoena phocoena*) in UK waters. The research was conducted in collaboration with ongoing investigations at the Research and Technology Centre Westcoast, Büsum, Germany into traumatic damage, disease or other abnormalities potentially associated with exposure to loud noise or blast trauma. In this study, pairs of ears from 25 UK-stranded harbour porpoises were sent to Germany and 11 pairs were prioritised for histological analysis.

Of the 11 ears prioritised for processing, three were analysed in detail, four were not analysed further due to heavy over-decalcification artifacts during preparation (i.e. broken and torn bone tissue, collapsed inner ear), and four were celloidin-embedded pending detailed examination. Of the three examined in detail, none had evidence of acoustic trauma although one animal had some missing structures in the inner ear due to mechanical artifacts, and so it was not possible to completely rule out noise-induced auditory lesions in this case. It would also be desirable to conduct surface preparations and immunohistochemical staining of tissue sections to examine the stereocilia attached to the outer and inner hair cells and the tectorial membrane in more detail. One porpoise did have total degeneration of the organ of Corti, but this was considered more likely to be an abnormal development of the organ of Corti and not induced by noise exposure.

Important criteria for the examination of ear tissues in general, and especially for the assessment of potential noise-induced lesions, are the degree of fixation, the freshness of the tissue, and the state of decalcification. Soft ear tissue gradually decomposes within hours after death. In order to maximise tissue preservation, the auditory tissues should be fixed in formalin as soon after death as possible. It is also important to avoid introducing any additional changes (artifacts) due to mechanical and/or chemical steps while processing the ear in the laboratory. In this study, the degree of fixation or freshness of all three specimens examined in detail varied from very good to moderate, even though all three individuals were classed as “freshly dead” on initial post-mortem examination.

Because the ear structures are encased in bony tissue, the decalcification of these tissues was necessary in order to prepare sections of the auditory tissues for microscopic examination. Unfortunately, the decalcification period can also have a major impact on the quality of tissue sections. The decalcification procedure dissolves calcium complexes but can also impact on the composition of the remaining soft tissues. A decalcifying agent that is used for too long can soften tissue excessively and induce “compression artifacts”. Storing the ear bones in formalin for too long a period prior to decalcification can have similar effects. This additional softening may lead to unwanted ruptures of the thin sections. Ultimately, it is important to keep the period from post-mortem extraction and formalin fixation to decalcification as short as possible. Several months up to one year maximum would be optimal to achieve reliable and comparable results.
Another factor in this study was the effect of the varying storage time in formalin fixative and the potential impact of this on tissue quality. The combined results of this study and the studies conducted on German harbour porpoises have shown that a short storage period in formalin of up to a year will be workable. Longer periods such as 4 to 5 years will probably be more difficult but may be compensated in future via a shorter decalcification period. In order to avoid this uncertainty, it is recommended to decalcify and section cetacean ear samples as soon after extraction as possible. Studies (in human otolaryngology) have shown once celloidin sections have been produced, tissue preservation will be maintained so that staining and analysis of tissue sections can be conducted up to 30 years later.

In these UK cases, premortem changes (inflammation, nematodes and fungus) and artifacts due to autolysis and/or mechanical and chemical histological processes were identified that can complicate histological interpretations. The establishment of these methods and the experience gained will enable better identification and monitoring of diseases of the ears of harbour porpoises and other cetaceans, and will generate more reliable assessment of potential acoustic impacts on these species in British and other waters. Additional methods like immunohistochemical staining and surface preparation will be needed to identify and classify the type of hazardous noise impact (e.g. acute vs chronic acoustic trauma, trauma caused by blast or explosion) if noise-induced hearing loss occurs. These techniques should be conducted in conjunction with rigorous assessment of the whole animal to help identify lesions in other tissues that may be correlated with auditory disease or trauma.

Other considerations when planning future research on cetacean ears are the costs of the research, both financially and in terms of expertise and labour. In this study, only three UK-stranded animals were examined in detail, although this research involved months of tissue processing/decalcification and the production and reading of many thousands of tissue sections by experts in the field. Finally, assuming that there are only a small percentage (if any) of auditory defects in the population induced by exposure to high-intensity anthropogenic noise sources, and very fresh carcasses are needed to detect these abnormalities, these could take many years to begin to detect. Nonetheless, there are currently no other ways to examine auditory abnormalities in dead marine mammals.
Background

A range of anthropogenic activities, including commercial fisheries, shipping traffic, oil and seismic exploration, scientific and military activities, input acoustic noise into the marine environment around the UK. Harbour porpoises are highly dependent on their hearing for vital activities such as navigation, prey detection, avoidance of obstacles and predators (Au 1993), and for communication and mating behaviour with conspecifics. The acoustic specialisation of harbour porpoises did not evolve in a silent ocean, and these animals took advantage of a range of frequencies to which they have rarely been exposed as background noise over an evolutionary time-scale. Harbour porpoises have a hearing sensitivity ranging from 1 kHz to 150 kHz (Andersen 1970, Bibikov 1992). Moreover, harbour porpoises are known to echolocate (Busnel et al. 1965, Möhl and Andersen 1973, Kamminga and Wiersma 1981, Akamatsu et al. 1994) by creating an acoustic image from the received echoes of emitted ultrasound.

The degree of any auditory impairment of harbour porpoises due to anthropogenic noise may depend on the intensity, frequency and type (e.g. discrete impulse vs tone) of acoustic exposure. Acoustic emissions caused by shipping traffic are generally broad-band, continuous, and in the range of 1 Hz to 10 kHz (Richardson et al. 1995), i.e. they are mainly low to mid-frequency sounds. Oil exploration, seismic, scientific and military actions as well as the construction and operation of offshore windmills emit continuous or frequently repeated sounds of short duration. Their main frequency range lies below 20 kHz. Although it has been suggested that such anthropogenic noise sources may impair the hearing abilities of cetaceans in UK waters (such as the harbour porpoise) (Simmonds, Dolman, Weilgart 2004), systematic investigation of these potential impacts has not been undertaken.

The Institute of Zoology (Zoological Society of London)(IoZ), in collaboration with the Scottish Agricultural College (Inverness) and the Natural History Museum, have conducted research on dead by-caught or stranded marine mammals along the UK coastline under contract to the UK Department for Environment, Food and Rural Affairs (Defra) since 1990. One of the principal aims of the Defra-funded strandings project is to evaluate the health status of the marine mammal populations in UK waters and to investigate potential anthropogenic impacts that may affect their health. This feasibility study on auditory anatomy and pathology would integrate fully with the existing Defra-funded UK cetacean stranding research programme, and would develop a baseline/pilot study of auditory system health in UK-stranded harbour porpoises from which future investigations into UK-stranded cetaceans could be expanded.
Aims and Objectives

The objective of this study is to assess the feasibility of researching induced anthropogenic acoustic damage to cetaceans in British waters through the detailed examination of the auditory apparatus of stranded and by-caught animals.

This will involve the histological examination of cetacean ears removed on post-mortem examination, predominantly from stranded or by-caught harbour porpoises (*Phocoena phocoena*) in UK waters. The project will examine the feasibility of obtaining tissue of sufficiently good quality to identify signs of traumatic damage, disease or other abnormalities that may be associated with exposure to loud noise or blast trauma.

The research will be conducted in collaboration with ongoing investigations in Germany at the Research and Technology Centre Westcoast, Büsum (FTZ) under the co-ordination and management of Dr Ursula Siebert. Dr Darlene Ketten from Woods Hole Oceanographic Institution (WHOI) and Harvard Medical School (HMS) acted as a consultant on the project.

Project Start Date and Duration

The project ran from 1\textsuperscript{st} October 2004 to 31\textsuperscript{st} May 2006.

Materials and Methods

*Collection of ear tissues for analysis*

Cetacean post-mortem examinations have been conducted in the UK using internationally standardised methodology (Law 1994). Of these, the auditory apparatus (ears) have been collected in 10% neutral buffered formalin from a range of species including 187 harbour porpoises (*Phocoena phocoena*), 36 common dolphins (*Delphinus delphis*), 17 striped dolphins (*Stenella coeruleoalba*), three bottlenose dolphins (*Tursiops truncatus*), one pilot whale (*Globicephala melas*), one Risso’s dolphin (*Grampus griseus*), one Atlantic white-sided dolphin (*Lagenorhynchus acutus*), one white beaked dolphin (*Lagenorhynchus albirostris*) and one Sowerby’s beaked whale (*Mesoplodon bidens*). It is unlikely that any of these carcasses were examined within a 17 hours postmortem interval (*Table 1*).
### Table 1: Preserved (formalin-fixed) ears from UK-stranded cetaceans

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of individuals with preserved ears</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harbour porpoise (<em>Phocoena phocoena</em>)</td>
<td>187</td>
</tr>
<tr>
<td>Common dolphin (<em>Delphinus delphis</em>)</td>
<td>36</td>
</tr>
<tr>
<td>Striped dolphin (<em>Stenella coeruleoalba</em>)</td>
<td>17</td>
</tr>
<tr>
<td>Bottlenose dolphin (<em>Tursiops truncatus</em>)</td>
<td>3</td>
</tr>
<tr>
<td>Pilot whale (<em>Globicephala melas</em>)</td>
<td>1</td>
</tr>
<tr>
<td>Risso’s dolphin (<em>Grampus griseus</em>)</td>
<td>1</td>
</tr>
<tr>
<td>Atlantic white-sided dolphin (<em>Lagenorhynchus acutus</em>)</td>
<td>1</td>
</tr>
<tr>
<td>White beaked dolphin (<em>Lagenorhynchus albirostris</em>)</td>
<td>1</td>
</tr>
<tr>
<td>Sowerby’s beaked whale (<em>Mesoplodon bidens</em>)</td>
<td>1</td>
</tr>
</tbody>
</table>

In mid-April 2005, the IoZ sent 25 pairs of the best preserved ears from UK-stranded harbour porpoises to FTZ. Details of these animals are included in Table 2.

#### Decalcification and analysis of ear tissues

From the 25 pairs of ears sent to FTZ, 11 of the freshest pairs were selected for further processing (Table 3). Ears chosen for processing included those from a live-stranded porpoise (ref. SW2004/76A) that was euthanased and examined post-mortem within 4 days of death and two porpoises with otitis media (refs. SW2001/36, SW1999/40).

All 11 pairs of ears prioritised for processing were decalcified and embedded in celloidin using the following standardised embedding technique.

#### Celloidin Embedding Method

The technique for histological processing of the auditory tissues (i.e. ears and associated structures) is based on the methods of Schuknecht (1993). Decalcification was conducted by immersion in 0.27 M EDTA (disodium ethylenediaminetetraacetate) at room temperature. The decalcification solution also contained 1% formalin and was changed at weekly intervals. Decalcification periods varied with each specimen (see results). At the end of the decalcification period the ears were checked by x-rays. After that all specimens were neutralised in tap water for 24 hours, then in distilled water for 4 to 6 hours followed by dehydration with 50% / 70 % / 80 % / 95 % / 100 % alcohol. The last two concentrations were changed twice before the samples were finally placed into ether-alcohol (1:1) twice.

The ear tissues were then embedded into 1.5 % celloidin for a week, into 3 % celloidin for another three weeks, into 6 % celloidin for three to four weeks, and finally into 12 % celloidin for another three to four weeks. While in the highest concentration of celloidin the jar had to be opened for 10 minutes three times a day to allow evaporation of alcohol and ether. At this stage, the celloidin solution became denser and harder. After about four weeks, the celloidin forms a block that was further hardened with chloroform in a desiccator at 4°C for two weeks followed by further hardening with fresh chloroform for another two weeks. At
this stage the block was trimmed. When the blocks took on a yellowish clear appearance and sank to the bottom of the jar, the hardening procedure was finished. The blocks were then set in cedarwood oil for at least a week which was refreshed for another week. The blocks were then sectioned using a microtome to produce 20µm thick tissue sections. Every 10th section was stained with haematoxylin/eosin and mounted while the rest of the tissue sections were stored in 80 % alcohol.

After decalcified and embedding in celloidin, up to one hundred haematoxylin and eosin stained slides were prepared from each ear from seven porpoises (SW2000/33, SW2001/36, SW2004/76A, SW2000/73, SW1999/26, SW1999/40 and SW2001/149) were produced in order to evaluate the state of preservation and suitability of the inner ear tissue for microscopic examination and interpretation. The other four priority ear samples (SW1999/17, SW2001/23, SW2001/127, SW2004/270 – see Table 3) are still being processed (October 2006). All ear tissues found to be in a suitable state of preservation will eventually be subjected to a complete systematic serial examination.

Table 2: Harbour porpoises from England and Wales from which ears were sent to FTZ (those prioritised for histological processing in bold)

<table>
<thead>
<tr>
<th>Ref_number</th>
<th>Species</th>
<th>Date found</th>
<th>Condition</th>
<th>County</th>
<th>Sex</th>
<th>Age cat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW1999/17</td>
<td>P. phocoena</td>
<td>28/01/99</td>
<td>Fresh</td>
<td>Humberside</td>
<td>F</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW1999/26</td>
<td>P. phocoena</td>
<td>13/02/99</td>
<td>Fresh</td>
<td>Cornwall</td>
<td>M</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW1999/33</td>
<td>P. phocoena</td>
<td>22/02/99</td>
<td>Fresh</td>
<td>Lincolnshire</td>
<td>F</td>
<td>Adult</td>
</tr>
<tr>
<td>SW1999/40</td>
<td>P. phocoena</td>
<td>04/03/99</td>
<td>Fresh</td>
<td>Greater London</td>
<td>F</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2000/27</td>
<td>P. phocoena</td>
<td>20/02/00</td>
<td>Fresh</td>
<td>Ceredigion</td>
<td>M</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW2000/33</td>
<td>P. phocoena</td>
<td>29/02/00</td>
<td>Fresh</td>
<td>Northumberland</td>
<td>F</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW2000/50</td>
<td>P. phocoena</td>
<td>14/03/00</td>
<td>Fresh</td>
<td>Greater London</td>
<td>F</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2000/73</td>
<td>P. phocoena</td>
<td>04/04/00</td>
<td>Fresh</td>
<td>Suffolk</td>
<td>F</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2000/146(2)</td>
<td>P. phocoena</td>
<td>29/08/00</td>
<td>Fresh</td>
<td>Humberside</td>
<td>M</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2001/23</td>
<td>P. phocoena</td>
<td>26/01/01</td>
<td>Fresh</td>
<td>Essex</td>
<td>M</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2001/36</td>
<td>P. phocoena</td>
<td>17/02/01</td>
<td>Fresh</td>
<td>Kent</td>
<td>F</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW2001/127</td>
<td>P. phocoena</td>
<td>21/06/01</td>
<td>Fresh</td>
<td>Humberside</td>
<td>M</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2001/139</td>
<td>P. phocoena</td>
<td>04/07/01</td>
<td>Fresh</td>
<td>Humberside</td>
<td>M</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2001/149</td>
<td>P. phocoena</td>
<td>12/07/01</td>
<td>Fresh</td>
<td>Humberside</td>
<td>F</td>
<td>Adult</td>
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<tr>
<td>SW2002/5</td>
<td>P. phocoena</td>
<td>04/01/02</td>
<td>Fresh</td>
<td>Suffolk</td>
<td>F</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2002/19</td>
<td>P. phocoena</td>
<td>16/01/02</td>
<td>Fresh</td>
<td>Suffolk</td>
<td>M</td>
<td>Adult</td>
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<td>SW2002/103</td>
<td>P. phocoena</td>
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<td>Kent</td>
<td>M</td>
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<td>SW2002/250</td>
<td>P. phocoena</td>
<td>09/07/02</td>
<td>Fresh</td>
<td>Humberside</td>
<td>F</td>
<td>Adult</td>
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<tr>
<td>SW2002/262A</td>
<td>P. phocoena</td>
<td>15/07/02</td>
<td>Fresh</td>
<td>Humberside</td>
<td>F</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW2003/257C</td>
<td>P. phocoena</td>
<td>08/06/03</td>
<td>Fresh</td>
<td>Humberside</td>
<td>M</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW2003/271</td>
<td>P. phocoena</td>
<td>25/06/03</td>
<td>Fresh</td>
<td>Humberside</td>
<td>F</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2004/76A</td>
<td>P. phocoena</td>
<td>08/02/04</td>
<td>Fresh</td>
<td>Kent</td>
<td>F</td>
<td>Juvenile</td>
</tr>
<tr>
<td>SW2004/267</td>
<td>P. phocoena</td>
<td>08/09/04</td>
<td>Fresh</td>
<td>Humberside</td>
<td>F</td>
<td>Subadult</td>
</tr>
<tr>
<td>SW2004/270</td>
<td>P. phocoena</td>
<td>13/09/04</td>
<td>Fresh</td>
<td>Humberside</td>
<td>M</td>
<td>Adult</td>
</tr>
<tr>
<td>SW2005/15</td>
<td>P. phocoena</td>
<td>20/01/05</td>
<td>Fresh</td>
<td>Norfolk</td>
<td>M</td>
<td>Adult</td>
</tr>
</tbody>
</table>

Entries in **BOLD** were prioritised for processing by FTZ.
Once decalcified, the ear apparatus is serially sectioned and examined for:

a) suitability of tissue quality for histological analysis
b) presence or absence of auditory system lesions (i.e. abnormalities of the ears)

**Results**

Of the 11 ears prioritised for processing (Table 2), three were analysed in detail (SW2000/33; SW2001/36 and SW2004/76A), four (SW2000/73; SW1999/26; SW1999/40 and SW2001/149) were not analysed in detail due to heavy over-decalcification (see discussion) artifacts (i.e. broken and torn bone tissue, collapsed inner ear), and four (SW1999/17, SW2001/23, SW2001/127, SW2004/270) were celloidin-embedded but have not yet been examined.

**SW2000/33:**

56 H/E stained sections, total 560 sections.

**Fixation:**

Good to very good. All epithelia were in a good to very good state of preservation and were mostly non-autolytic. Even the sensory epithelium and the organ of Corti structures looked well preserved. Most of the stria vascularis was still attached and showed signs of early autolysis (i.e. the innermost layer was disintegrating). This structure has relatively high oxygen sensitivity and typically decomposes first. Inner and outer hair cells as well as supporting cells (Deiter’s, Hensen’s) could be clearly identified.

**Decalcification:**

Poor. The ear bone showed many “compression artifacts”, i.e. the periotic bone and the labyrinth appeared compressed. The effects of this are distortions and ruptures of bone tissue, sometimes involving the labyrinth. In some areas the basilar membrane was bent with the apex collapsing inwards forcing the stria vascularis down onto the spiral limbus with resultant loss of inner and outer hair cells. The Reissner’s and tectorial membranes were often torn. Although the decalcification period only took 8 weeks (the normal period of decalcification for German harbour porpoises was 7 to 25 weeks), the tissue looked heavily impacted by the decalcification process. It is possible that the relatively longer storage in formalin had a decalcifying impact on the tissues making it softer and therefore more vulnerable for chemical and mechanical processes (embedding, sectioning, staining and mounting).

Decalcification period: 8 weeks.

**Findings:**

Middle ear:

There was a moderate infestation of spiral first-stage parasitic larvae (probably *Stenurus minor*) surrounded by massive aggregations of lymphocytes. The ossicles appeared normal. The tympanic cavity was mostly lined with a pseudostratified columnar epithelium. Few areas had a stratified epithelium. A special feature in this ear was the prominence of intraepithelial multicellular...
glands (Fig.1A) in the pseudostratified columnar epithelium representing aggregates of secretory cells within an epithelial lining. The function of these secretory products in the middle ear is not yet known. In addition, there were aggregations of inflammatory cells in several submucosal locations (Fig.1B). The whole inner tympanic periosteum had a subepithelial layer of inflammatory cells, mainly lymphocytes and neutrophil granulocytes. The oval and round windows could not be evaluated due to compression artifacts.

Inner ear:
The eighth nerve had a normal appearance. Schwann cells, nerve cells and nucleoli were clearly visible and ganglion cells appeared normal. Nuclei, nucleoli were clearly visible and the cytoplasm had a normal dense staining. Outer and inner sulcus cells also appeared normal apart from those areas mechanically torn off. Inner/outer hair and phalangeal cells looked normal where the material has not been damaged due to over-decalcification. The scala tympani showed haemorrhage in all turns (Fig.1C).

Comments: Although the tissue showed numerous artifacts that were probably due to a long period of storage in the fixative and that presumably initiated over-decalcification, several pre-mortem changes were found. In areas not impacted by the artifacts the organ of Corti looked normal and healthy. There was no evidence of any noise-induced hearing loss (i.e. no degenerated hair cells and/or ganglion cells). The areas impacted by the artifacts could not be assessed. The bulla showed a subepithelial sheet-like distribution of chronic inflammatory cells throughout the whole tympanic cavity. Although these lesions are abnormal, they were considered unlikely to impair normal hearing capabilities.

SW2001/36:

83 H/E stained sections, total 830 sections.

Fixation:
Good to moderate. The epithelia in general showed signs of early autolysis. The stria vascularis is still attached to the spiral ligament, but the innermost layer was already disintegrated. Throughout the whole cochlea, sensory cells (inner and outer hair cells) were not identifiable, but this may not necessarily be a fixation phenomenon (see Findings). The outer and inner sulcus cells were visible and intact. The ganglion cells were shrunken but nuclei and nucleoli were otherwise normal in appearance.

Decalcification:
Moderate over-decalcification. There were relatively few ‘compression’ artifacts but some sections showed ruptures in the bone tissue, probably due to the length of storage in formalin which can soften bone tissue over time. In some sections the basilar membrane was bending slightly. Decalcification period: 8 weeks.

Findings:
The Os petrosum and Os tympanicum were received as separate parts. The stapes was still attached while incus and malleus were absent. Middle ear:
The periotic and tympanic bones were received in separate pieces thus limiting the interpretation of the middle ear tissues. The ossicles are described below in conjunction with the inner ear. The periosteum of the tympanic bone showed both a pseudostratified and stratified epithelial lining. At several subepidermal spots lymphocytes accumulated within the tympanic cavity. A severe infestation of a fungus almost filled the entire tympanic cavity (Fig.1D). The fungus was isolated on culture during the necropsy and identified as *Aspergillus terreus*.

Inner ear:
The eighth nerve was abnormal. Only few areas had healthy Schwann cells, nerve cells and nucleoli but, due to autolysis, many lesions could not be identified. In and around the oval and round windows there was a massive infiltration of protein and inflammatory cells surrounding a large mycelium reaching far into the tympanic cavity. The lateral side of the petrous bone showed osteolysis near the oval window opening and adjacent bony structures like the innermost ossicle (stapes) were also affected (Fig.1E). Cyst-like structures were seen within the new epithelium, and severe accumulation of lymphocytes and newly formed vessels filled the usually empty space beyond the round window membrane. Beyond the oval window a massive net of fibrin fibres replaced three quarters of the perilymphatic fluid space extended into the cochlea (Fig.1F). Half to three quarters of the scala vestibuli and one third of scala tympani were filled with a net of fibrin fibers and both were infiltrated with inflammatory cells. Neutrophil granulocytes, lymphocytes, and plasma cells were attached to the intact Reissner’s membrane, spiral ligament and within the net of fibrin fibers. The scala media did not appear to be involved in the inflammatory process.

The whole organ of Corti appeared malformed. In most regions of the cochlea the inner and outer hair cells, the inner and outer phalangyeal and pilar cells, and the inner and outer tunnel and Nuel’s room were missing. Only the right upper turn had rudiments of inner and outer pilar cells on a 0.8mm length and again 1mm closer to the helicotrema for a length of 0.6 mm left and right. The outer and inner sulcus cells were intact and continuously lined the lower half of scala media from the stria vascularis, basilar membrane, spiral limbus to the tectorial membrane. The stria vascularis was moderately autolysed. The Rosenthal-Canal showed dilated vessels, representing hyperaemia. The ganglion cells appeared shrunken but intact with normal nuclei.

Comments:
The severe fungal infestation had caused an inflammatory reaction in the middle ear that extended into the oval and round window areas. There was massive infiltration with proteins, inflammatory cells and newly formed cysts, probably induced by the flexibility of the oval and round window membranes. The severe fibrin net and inflammatory cells within the perilymphatic spaces of scala tympani and scala vestibuli were symptoms of an acute fibrinous otitis. Both bullae were infected by *Aspergillus terreus*. The scala media did not show any signs of inflammation.

The additional finding of a malformed organ of Corti throughout the whole cochlea leads to the conclusion that hearing capabilities were severely impaired resulting in complete hearing loss. However, there is no clear evidence that the fungal infection had a direct impact on hearing because the changes in the
cochlea that were probably causing deafness appeared to have been generated prior to the fungal lesions. Ultimately, it is possible that the hearing impairment/deafness in at least one ear hampered prey detection and led to the poor nutritional status and eventual stranding of this animal.

**SW2004/76A:**

111 H/E stained sections, total 1101 sections.

**Fixation:**
Moderate to good. The epithelium around the ossicles and the mucosa in the tympanic cavity appeared fresh and normal. Parts of the corpus cavernosum appeared slightly decomposed. The stria vascularis was barely attached to the spiral ligament and in a progressing state of autolysis. Sensory and supporting cells of the organ of Corti had a normal appearance where not mechanically damaged.

**Decalcification:**
Moderate to severe over-decalcification. “Compression artifacts” in the bone tissue (i.e. all scalae looked slightly compressed). The softening had again caused ruptures due to mechanical handling. The basilar membrane was bending in some places. In some sections the organ of Corti was not intact and supporting cells look mechanically damaged. Decalcification period: 8 weeks.

**Findings:**
**Middle ear:**
There were no abnormalities of the ossicles detected. The tympanic cavity was mostly lined with a pseudostratified columnar epithelium. The corpus cavernosum was partially maintained. Over a length of 2.4mm (section 511 – 632) a submucosal rounded aggregation of lymphocytes filled part of the corpus cavernosum. This phenomenon has also been observed in some harbour porpoises from German and Danish waters and probably indicates an inflammatory reaction. This mucosa-associated lymphoid tissue (MALT) in humans is often found in the eustachian tube and middle ear, and is often correlated with otitis media. Studies suggest that the presence of MALT is closely correlated to repeated infection (Matsune et al. 1996, Kamimura et al. 2001).

**Inner ear:**
The Reissner’s membrane and basilar membrane were intact. In some places, the basilar membrane was bending. The spiral ligament, tectorial membrane and spiral limbus had a normal appearance. The outer sulcus cells were mostly intact, but there were some damages to the outer sulcus cells, inner sulcus cells and the inner hair cells due to mechanical processing. The outer phalangeal cells and outer hair cells were mostly intact but a few outer hair cells were missing. Due to autolysis the ganglion cells had a shrunken cytoplasm but were intact with normal nuclei.

**Comments:**
The aggregation of lymphocytes in the corpus cavernosum in the tympanic cavity was indicative of some inflammatory reaction. Due to mechanical damage, some structures were missing in the inner ear. The preserved structures had a normal appearance with no evidence of any general pathologic change in the inner ear. Due to the incomplete nature of the relevant structures, however, this ear was not suitable for a complete analysis in relation to potential noise-induced auditory lesions consistent with hearing loss or acoustic trauma.

The following histological series were not analysed in detail, due to heavy over-decalcification artifacts (i.e. broken and torn bone tissue, collapsed inner ear):

**SW2000/73** (501 sections)
*Fixation*: Good.

*Decalcification*: Heavy over-decalcification artifacts (7 weeks decalcification).

*Results*: Heavy parasitism in the tympanic cavity.

**SW1999/26** (771 sections)
*Fixation*: Good.

*Decalcification*: Heavy over-decalcification artifacts (8 weeks decalcification).

*Results*: None recorded.

**SW1999/40** (681 sections)
*Fixation*: Moderate to poor.

*Decalcification*: Heavy over-decalcification artifacts (7 weeks decalcification).

*Results*: Sensory cells changed due to post mortem artifact.

**SW2001/149**: (831 sections)
*Fixation*: Good.

*Decalcification*: Heavy over-decalcification artifacts (8 weeks decalcification).

*Results*: None recorded.

Finally, animals SW1999/17, SW2001/23, SW2001/127 and SW2004/270 were celloidin-embedded but are pending sectioning, staining and mounting.

**Discussion**

The aim of this study was to conduct detailed examination of UK-stranded cetacean inner and middle ear structures taken at post-mortem examination and
preserved in buffered formalin to assess the feasibility of researching damage to cetacean ears potentially induced by anthropogenic acoustic injury. This approach included histological investigation of ear structures to assess normal anatomy and to characterise any traumatic damage or other abnormalities. The tissues were particularly scrutinised for any signs of traumatic or degenerative changes that could be associated with exposure to loud noise and/or blast trauma (e.g. from the use of explosives).

In human otolaryngology, the effects of noise are mainly differentiated into acoustic trauma and noise induced hearing loss (Strutz & Mann 2001). For most cetacean species, combinations of received sound pressure levels and sound durations capable of inducing these acoustic injuries are not known. For the histopathological investigations of the cetacean inner and middle ear in this study, a method was adapted from one previously established in human otolaryngology (Schuknecht 1993) enabling mechanical (acoustic) damage to the organ of Corti and surrounding structures to be detected. This method also permits a more general pathological assessment of the ear structures so that inflammatory, parasitic and haemorrhagic abnormalities could be clearly identified in the tympanic cavity and inner ear.

In this study, we investigated three UK-stranded harbour porpoises in detail. None of them had evidence of acoustic trauma. However, one animal (SW2004/76A) had some missing structures in the inner ear due to mechanical artifacts, and so it was not possible to completely rule out noise-induced auditory lesions. In relation to noise induced hearing loss, we could not identify any degenerated hair cells or nerve fibres. However, it would be desirable to conduct surface preparations and immunohistochemical staining of tissue sections to examine the stereocilia attached to the outer hair cells and the tectorial membrane in more detail. One porpoise did have total degeneration of the organ of Corti, but this was considered more likely to be an abnormal development of the organ of Corti and not induced by noise exposure.

Important criteria for the examination of ear tissues in general, and especially for the assessment of potential noise-induced lesions, are the degree of fixation, the freshness of the tissue, and the state of decalcification. Soft ear tissue gradually decomposes within hours after death. In order to preserve the in vivo state, the auditory tissues would have to be perfused with formalin fixative while the animal was still alive. Obviously such a method is neither possible nor desirable in cetaceans, and so it is necessary to achieve formalin fixation as soon after death as possible. It is also important to avoid introducing any additional changes (artifacts) due to mechanical and/or chemical steps while processing the ear in the laboratory. In this study, the degree of fixation or freshness of all three specimens examined in detail varied from very good to moderate, even though all three individuals were classed as “freshly dead” on initial post-mortem examination.

Because the ear structures are encased in bony tissue, the decalcification of these tissues was necessary in order to prepare sections of the auditory tissues for microscopic examination. Unfortunately, the decalcification period can also have a major impact on the quality of tissue sections. The decalcification
procedure dissolves calcium complexes but can also impact on the composition of the remaining soft tissues. A decalcifying agent that is used for too long can soften tissue too much and induce “compression artifacts”. This causes destruction of the bone tissue first, followed by the soft tissue of the labyrinth. Storing the ear bones in formalin for too long a period prior to decalcification can have similar effects. This additional softening may lead to unwanted ruptures of the thin sections. Ultimately, it is important to keep the period from post-mortem extraction and formalin fixation to decalcification as short as possible. Several months up to one year maximum would be optimal to achieve reliable and comparable results.

Another factor in this study was the effect of the varying storage time in formalin fixative and the potential impact of this on tissue quality. The combined results of this study and the studies conducted by FTZ on German harbour porpoises have shown that a short storage period in formalin of up to a year will be workable. Longer periods like 4 to 5 years will probably be more difficult but may be compensated in future via a shorter decalcification period. In order to avoid this uncertainty, it is recommended to decalcify and section cetacean ear samples as soon after extraction as possible. Studies have shown once celloidin sections have been produced, tissue preservation will be maintained so that staining and analysis of tissue sections can be conducted up to 30 years later (Shi et al. 1992).

In these UK cases, premortem changes (inflammation, nematodes and fungus) and artifacts due to autolysis and/or mechanical and chemical histological processes were identified that can complicate histological interpretations. It is therefore necessary not only to have access to adequately fresh material in future studies, but also to compare specimens among different individuals. The establishment of these methods and the experience gained will enable better identification and monitoring of diseases of the ears of harbour porpoises and other cetaceans, and will generate more reliable assessment of potential acoustic impacts on these species in British and other waters. Additional methods like immunohistochemical staining and surface preparation will also be needed to determine accurately potential noise induced hearing loss. These techniques should be conducted in conjunction with rigorous assessment of the whole animal to help identify lesions in other tissues that may be correlated with auditory disease or trauma.

Other considerations when planning future research on cetacean ears are the costs of the research, both financially and in terms of expertise and labour. In this study (costing £30,000), only three UK-stranded animals were examined in detail, although this involved months of tissue processing/decalcification and the production and reading of many thousands of tissue sections by experts in the field. Finally, if there are only a small percentage of auditory defects in the population induced by exposure to high-intensity anthropogenic noise sources, and very fresh carcasses are needed to detect these abnormalities, these could take many years or even decades to detect. Nonetheless, there are currently no other ways to examine auditory abnormalities in dead marine mammals, and so these techniques are the only option currently available to assess the potential auditory effects of noise pollution.
References


FIGURE 1. (A) SW2000/33; tympanic cavity. Intraepithelial multicellular glands in the pseudostratified columnar epithelium. Bar = 20µm. (B) SW2000/33; tympanic cavity. Inflammatory cells in submucosa. Bar = 50µm. (C) SW2000/33; Cochlea. Haemorrhage in scala tympani. Bar = 200µm. (D) SW2001/36; tympanic cavity. Fungal hyphae (Aspergillus species) causing mycotic otitis. Bar = 20µm. (E) SW2001/36; osteolysis of petrous bone and nearby ossicle (stapes). (F) SW2001/36; Cochlea. Net of fibrin fibres within the perilymphatic fluid space within the cochlea.