Investigation into hearing damage and life history of Dutch stranded harbour porpoises

Lonneke L. IJsseldijk and Andrea Gröne

Faculty of Veterinary Medicine
Department of Pathobiology
Utrecht University

Utrecht University

Titlepage

Lonneke IJsseldijk, BSc
Yalelaan 1, 3584 CL Utrecht
The Netherlands
T: 030-2535312
M: 06-24455698
W: www.uu.nl/strandingsonderzoek
E: L.L.IJsseldijk@uu.nl

Prof. Dr. Andrea Gröne
Yalelaan 1, 3584 CL Utrecht
The Netherlands
T: 030-2534364
E: a.grone@uu.nl

In collaboration with:

Dr. F. Read	University of St. Andrews	Scotland
Dr. M. Morell	University of British Colombia	Canada
Dr. S. Murphy	Galway Mayo Institute of Technology	Ireland

Table of contents

1. Introduction

In the southern North Sea, the harbour porpoise (*Phocoena phocoena*) is currently a common resident (Camphuysen & Peet 2007). Although historically not always a common species, harbour porpoise sightings nowadays in near shore waters are frequent and year-round, with peak abundance in late winter and early spring (Camphuysen 2004; Camphuysen 2011). Abundance estimates in the Dutch sector of the North Sea range from 26,000 (mid-summer) to 85,000 (spring) individuals (Geelhoed et al. 2013; Scheidat et al. 2013). An exponential increasing in stranding frequency is recorded since 2000, which is more or less in parallel with the increase in sightings (Camphuysen & Siemensma 2011). Over the past 15 years, almost 6,000 harbour porpoises were found dead along the Dutch shores, with peak numbers in 2011 (n=882) and 2013 (n=881) (www.walvisstrandingen.nl).

Harbour porpoises are protected under several international agreements with conservation objectives (e.g. ASCOBANS; European Union Habitat Directives; Marine Strategy Framework Directives and Common Fisheries Policy; the OSPAR convention) (Siebert et al. 2006; Peltier et al. 2013; Scheidat et al. 2013). To identify appropriate management measures, it is important to understand the factors underlying the observed population trends and shifts in distribution. Also, identifying time periods during which animals are more sensitive to impacts caused by human activities, such as wind farm construction, is vital to the effective conservation and management of harbour porpoises.

Since 2008, post mortem investigation on stranded harbour porpoises in The Netherlands has been conducted at the faculty of Veterinary Medicine at Utrecht University, commissioned by the Ministry of Economic Affairs. Since 2016, this research is embedded in the law as a so-called WOT ('Wettelijke Overheidstaak'). Utrecht University is particularly suitable to conduct this research, due to its experience with veterinary medicine and pathology, the existing facilities and its central location in The Netherlands.

Rijkswaterstaat established the 'Wind op Zee Ecologisch Programma' (WoZEP) commissioned by the Ministry of Economic Affairs, in order to investigate the knowledge gaps concerning the effects of wind farms on the ecology in the North Sea in 2016 (Rijkswaterstaat 2016). One of the key species within this program is the harbour porpoise. As being one of the top predators (Peltier et al. 2013), these animals may reflect environmental changes, also as an effect of anthropogenic activities in their habitat. Within the frameworks of WoZEP, a set of 'no-regret' studies was requested in 2016 by Rijkswaterstaat. This included research on direct impact of windfarm construction and cumulative threats on the harbour porpoise population. Utrecht University was commissioned by Rijkswaterstaat to facilitate this research, and this report was therefore made on request of, and funded by Rijkswaterstaat.

This report contains the results on the analysis of acoustic trauma and life history (age composition and reproduction) of subsets of harbour porpoises stranded on the Dutch coast. The data on the contaminants is presented elsewhere (van den Heuvel-Greve et al. in prep).

2. Hearing damage

Dr. Maria Morell from the University of British Colombia was contracted by Utrecht University to perform the hearing damage analysis. The ears were shipped from The Netherlands to Canada with appropriate CITES permits (16NL234380/12).

2.1 Background

Cetaceans are the only mammals fully modified to life in water and the numerous adaptions to this environment represent a large evolutionary level. Among these adaptions is the well-developed auditory organ, which is the basis of the echolocation system (Cozzi et al. 2016). Echolocation in odontocetes is used for the detection of prey and predators, for orientation and communication and therefore their primary sensory indicator (Mooney et al. 2012, Morell et al. 2015). Therefore, cetaceans have a sensitive and sophisticated hearing which is key to their survival underwater. Their echolocation allows them to function well, also on great depths and at night, when light is limited, and in murky waters like the North Sea (Mooney et al. 2012). All mysticete vocalizations are significantly lower in frequency than those of odontocetes and differences in produced sounds imply different perceptual abilities (Ketten 1993). Odontocetes have evolved a mechanisms that protects them from their own high frequency sounds, but this mechanisms is not sufficient to overcome the exposure to certain anthropogenic sounds (human-made sounds) (Mooney et al. 2012).

Physiological damage of sound can be observed in organs (Jepson et al. 2003), but the lesions affecting cetaceans hearing can be expected mainly in their inner ears (Morell et al. 2015). Cetaceans have very characteristic ear bones, which differ in appearance from other mammals. There are two distinct components: the periotic and tympanic complexes (Ketten 1993; Morell et al. 2015) (Figure 1). The inner ears of odontocetes contain the vestibular system and the cochlea. The latter compromised the auditory system and features the parts that are influenced by frequency perception (Ketten 1993). Noise induced hearing loss is due to overstimulation of the inner ear sensory cells. This can result in damage of the hair cells and organ of Corti, which can result in prevention of production of neurochemical releasers that initiate auditory fiber impulses. Additional conditions, like exposure to contaminants and physiological stress, may accelerate such hearing loss. Damage to the inner hair cells (IHCs) results in a total lack of response, whereas the loss of outer hair cells (OHCs) produces elevated thresholds. If hair cells recover from noise insults, the hearing loss is a temporary threshold shift (TTS) (Ketten 1993; Ketten 2012).

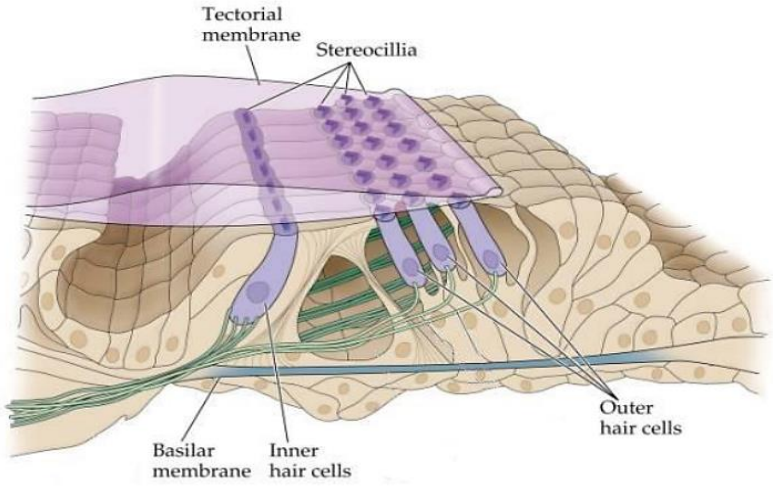

Figure 1: Schematic figure of the organ of Corti, characterized by one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). Source: M. Morell via flipper.diff.org.

A description of the morphology of odontocetes inner ears was given by Morell et al. (2015), who investigated inner ears of 150 cetaceans. Among this sample size, inner ears of 71 harbour porpoises were included for analysis and the general ultrastructure of the harbour porpoise' cochlea was described. This allows us to detect structural alterations by investigating the morphological features of the inner ear that might result from sound overexposure. In The Netherlands, yearly hundreds of harbour porpoises are found dead, and a selection of these stranded animals are submitted for necropsy. Of the freshest cases, the inner ears are collected for analysis of hair cells and organ of Corti to assess hearing damage. Here, we report the findings of the analysis of the hearing organs of ten harbour porpoises, stranded between February and July 2016. We also provide a general perspective on the current technique and future opportunities of inner ear analysis to extend the research on hearing damage in harbour porpoises and other odontocetes.

2.2 Materials and methods

Between 17th February and 28th July 2016, ten very fresh harbour porpoises were found dead, or died on the Dutch coastline, and were submitted for immediate necropsy at the Faculty of Veterinary Medicine (Table 1). During the necropsies the head was disarticulated at the atlanto-occipital joint, the ears were exposed and extracted according to conventional techniques (Figure 2). The cochlea was infused via the round and oval windows following the protocol optimized by Morell and André (2009). Next, the ears were fixed in 10% neutral buffered formalin. At the time of necropsy, both ears were collected and temporarily stored, prior to the shipment to Canada. Of each individual, one ear was selected for ultrastructural analysis, including 7 right and 3 left ears.

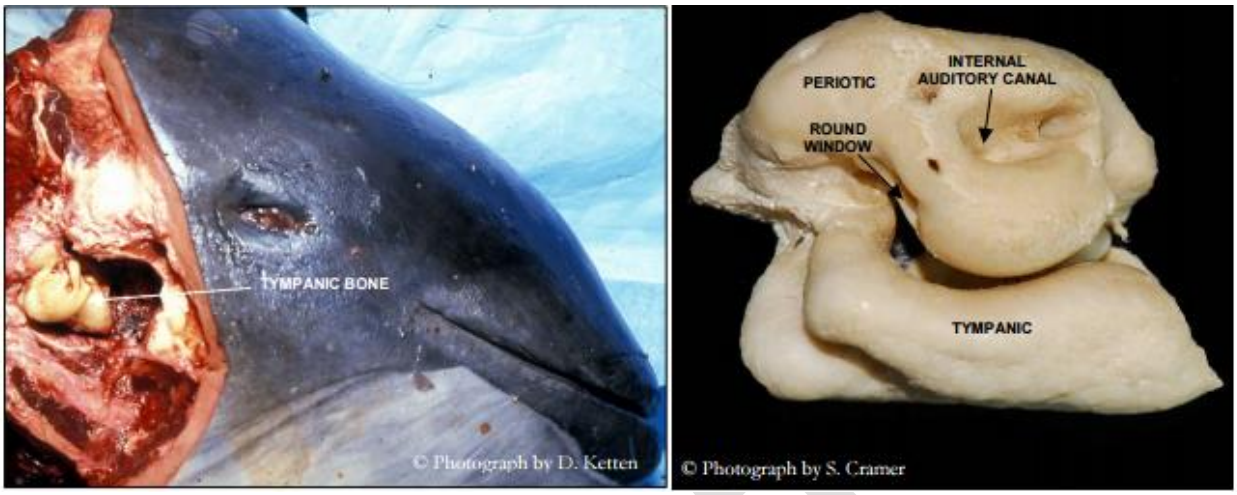

Figure 2: Lateral view of harbour porpoises head to indicate the location of the ear bone (left picture) and a dissected and cleaned delphinid left ear bone (right picture) (Ketten et al. 2007).

In short: the cochlea was decalcified using 14% ethylenediaminetetraacetic acid (EDTA) tetrasodium salt at pH 7.4 at room temperature (changing the solution once every 7-9 days; Callis and Sterchi 1998), with decalcification times between 30-37 days. To investigate the organ of Corti throughout the cochlear spiral, the ten cochleas were dissected. Subsequently, the ears were dehydrated through ethanol, dried with CO_2, and coated with platinum-palladium for analysis. The samples were evaluated by Scanning Electron Microscopy (SEM) by a Hitachi S-4700 electron microscope (University of British Columbia Bioimaging Facility, Canada).

Table 1: Basic data of stranded harbour porpoises selected for hearing damage analysis, including their ID codes; stranding date(Dd/Mm/Yy); stranding location; Gender (Male/Female); Total length (cm); and time between death and fixation, with all confirmed lived stranded animal which died as ' real' , while other were estimated times between death and fixation.

Idcode	Dd	Mm	Yy	Stranding location	Gender	Length (cm)	Time death to fixation
UT1495	17	2	2016	Scheveningen	M	105	4 h (real)
UT1509	11	3	2016	Ouddorp	F	113	6 h (estimate)
UT1513	22	3	2016	Oostdijk	F	106	26-48 h (estimate)
UT1517	5	4	2016	Terschelling	M	109	7 h (real)
UT1518	6	4	2016	Katwijk	F	107	6-18 h (estimate)
UT1527	29	6	2016	Texel	F	157	12.5 h (real)
UT1528	30	6	2016	Noordwijk	M	75	<12 h (estimate)
UT1531	7	7	2016	Bergen aan Zee	M	139	10-12 h (estimate)
UT1532	18	7	2016	Vlissingen	M	82	12 h (estimate)
UT1535	28	7	2016	Domburg	F	146	4 h (real)

2.3 Results

On gross dissection there were four cases with focal to segmental vascular congestion of the vein at the cochlear aqueduct (UT1527, UT1528, UT1531, and UT1535) and two cases with mild focal hemorrhage in the vestibular scala (UT1531 and UT1532). Macroscopically, no abnormalities were found in the other cases (UT1495, UT1509, UT1513, UT1517 and UT1518).

SEM revealed that the organ of Corti had varying degrees of decomposition, and although sensory cells were not intact, their morphology was clearly visible for all cases. No conclusive evidence of acoustic trauma was found in seven cases: UT1495, UT1509, UT1513, UT1517, UT1528, UT1531 and UT1532. Ultrastructural alterations that could be related to hearing damage was however found in the ears of three porpoises: UT1518, UT1527 and UT1535. These three animals presented with absence of OHCs between the first 200 to 815 µm from the apex in the upper apical turn, which can be compatible with noise overexposure, and are further discussed below.

2.3.1 UT1518

SEM examination at the apex of the spiral revealed two rows of OHCs, instead of the expected three. This extended through the first 400 µm of the apex and could be the results of hearing damage. In general, the cochlea of this case was reasonably well preserved, although the lower basal turn was partially obscured by a mild hemorrhage and cellular debris overlying the apical aspect of the organ of Corti. In the apex, there were no stereocilia imprints in the undersurface of tectorial membrane. However, regular imprints were identified throughout the spiral and in the most basal portion of the spiral, indicating intact stereocilia in these regions. Irregular imprints in the base is a good indicator of cases of age related hearing loss or ototoxic drugs overexposure, however neither are the case here. In this case, some loss of ability to detect high frequencies is most likely, but since this individual presented good body condition with signs of recent predation at necropsy, the potential hearing loss had little consequence to this individual.

2.3.2 UT1527

In this case, the apex of the cochlea was not well preserved. Although potential missing OHCs were detected in the first 200-250 µm from the apex, the poor preservation hindered assessment of the ultrastructure and assessment of a possible acoustic trauma. The remaining portions of the cochlea were better preserved and one row of IHCs and three rows of OHCs were apparent through the spiral. There was scattered cellular debris that partially overlaid and obscured the organ of Corti. Here, also the tectorial membrane was processed for SEM analysis. There were no OHC stereocilia imprints apparent at the undersurface of the tectorial membrane of the apex, but regular imprints at the base, indicating that this individual had healthy OHCs that encoded for the high frequencies. These observations indicate that age related hearing loss is unlikely, besides this animal was 8 years of age and therefore a relative young adult. The ultrastructural observations and potential lesions of the apex must be placed in context of clinical history, systemic pathology and ancillary diagnostic findings to better understand if there was any contribution to the stranding of this individual. The lack of the third row of hair cells may also represent a normal apical anatomic variation in this case. To understand this particular case better, currently the other inner ear is processed for SEM analysis, with pending results.

2.3.3 UT1535

With SEM, the organ of Corti was absent in the first 525 µm from the apex and moderately well preserved through the rest of the spiral turn, three rows of OHCs and one row of IHCs could be identified. Focal mild hemorrhage was observed in the vestibular scala of the lower basal turn. It is possible that artefactual transfer of erythrocytes from around the vein at the cochlear aqueduct during the dissection or critical point drying process may have occurred. In the first region where the organ of Corti cells were present, there were scars as a result of missing OHCs in a 290 µm area and in a focal area around 1.5 mm from the apex. The missing OHCs of this case are consistent with noise overexposure. In the undersurface of tectorial membrane in the apex, there were no stereocilia imprints, but regular imprints were identified at the base indicating intact OHC stereocilia in this region. It is unlikely that this observation was related to an older age, as age related hearing loss is observed primarily at high frequencies (Johnsson and Hawkins 1972) encoded at the cochlear base and this animals was a relative young adult of 6 years of age.

This individual was a lactating female in very poor nutritive condition, with no evidence of recent feeding. She was observed swimming in shallow water, against poles in the water and live stranded. Post-mortem examination revealed severe protozoal encephalitis, due to *Toxoplasma gondii* infection. Depending on the parasite genotype and stage of gestation at the time of in utero exposure, sensorineural hearing loss due to congenital infection by *Toxoplasma gondii* has been described in children (Noorbakhsh et al. 2008). However, the cochlear ultrastructural changes in cases of toxoplasmosis are not commonly recognized. Further studies are underway with the right ear of this individual by an alternative technique that will allow us to distinguish between acute to subacute (i.e. that occurred in the 7-9 days previous to the stranding) from more chronic injuries. If these lesions are visible in both ears, further analysis from the right ear may provide valuable insights into the possible contribution of hearing loss to this stranding.

2.4 Discussion

Anomalies in the inner ears were found in three out of ten cases included in this study; among one case (UT1535) the results were most conclusive. Missing OHCs, as observed in these three cases, can be consistent with noise overexposure. However, loss of hair cells can also be due to other factors, such as age, polychlorinated biphenyls (PCBs), congenital or immunological disorders, or species and individual variability in apex morphology. SEM analysis of the undersurface of the tectorial membrane of these three individuals showed a regular pattern of imprints of OHC stereocilia. This regularity indicates that stereocilia are intact in this region and it does not support age related hearing loss as potential factor. Besides, these three cases all were relatively young animals (appendix 1). The observation of missing OHCs of individuals UT1518 and UT1527 in the first 200-400 µm from the apex primarily involving the third row of OHCs, and coinciding with the lack of the third row of Deiters' cell phalangeal processes, suggests that these two cases represent individual variability within harbour porpoise, explaining the anomaly seen in these cases. In contrast, UT1535, absence of OHCs were localized between 525 – 815 µm and focally at 1.5 mm from the apex. The missing OHCs were from all 3 rows although more commonly from the third and Deiters' cell phalangeal processes from the third row were present. The

missing OHCs of UT1535 are suggestive of a noise overexposure or may be attributed to the *Toxoplasma gondii* infection and needs more research.

The anomalies in the first 200-400 µm of the apex as seen in cases UT1518 and UT1527, could suggest a congenital malformation which is very rare in humans (prevalence of around 0.1 %) or possible species apex variability, rather than a pathologic process. The first µm of the apex use to have the highest hair cell variability within mammals, where missing hair cells can be common depending on the species. The knowledge on normal species/individual apical morphological variability in cetaceans is extremely limited, in part because the first cells of the cochlea are very difficult to preserve during sample processing for SEM. A larger survey of harbour porpoise apex morphology is needed to better address ultrastructural finding, such as in this case, so differences can be related to biological characteristics, as age and gender.

Impacts from noise on cetaceans can also induce other anomalies which may or may not be visible in the inner ear. Potential affects can be physiological, pathological, acute or chronic, but also mainly induce a behavioral response (Ketten 2012), making it highly challenging to detect this in free-ranging cetacean. Therefore, there is a need for a better understanding of the many consequences of sound use and noise effects on marine mammals. Continuing a full-scale of research on fresh, stranded animals is highly recommended, as natural hearing loss from aging, trauma and disease, other than anthropogenic sounds may also occur.

In the case of specific acoustic trauma, further studies on cochlear frequencies distribution in harbour porpoise will help determine the possible sound sources that might have resulted in these lesions. Current work is conducted on creating cochlear frequency maps based on morphological features. The cochlea can be seen as a 'map', in which different frequencies are codified in specific regions: the high frequencies are encoded in the base and the low frequencies in the apex of the spiral. How these frequencies are distributed through the spiral is species specific. Besides, there is a high individual variation in it as well. Figure 3 shows that when an acoustic trauma is visible in a specific region of the cochlea, we can get information on the frequency characteristics of the source that might cause this lesion, like for example when finding a lesion in the orange region it will likely be caused by mid-frequency active sonar.

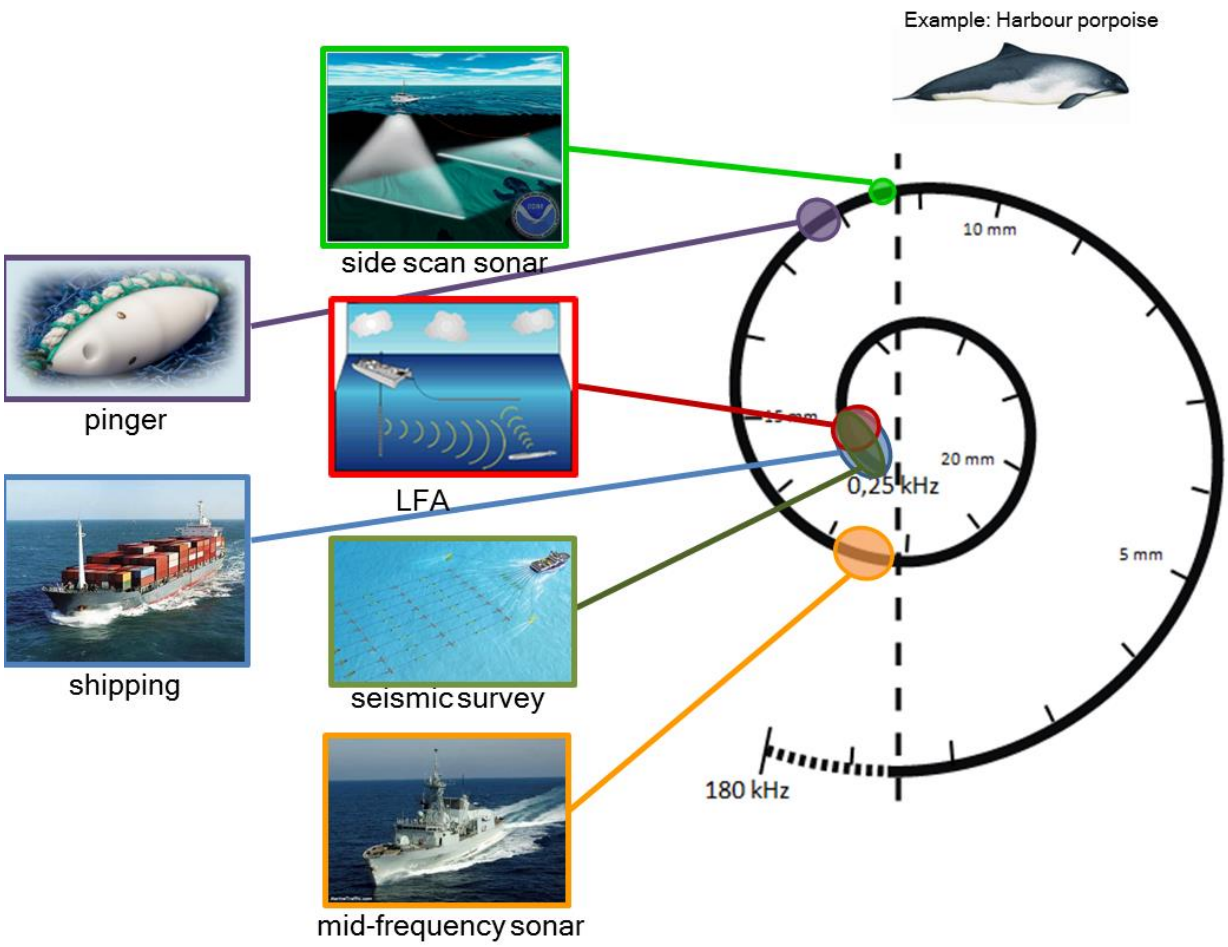

Figure 3: Schematic cochlear frequency map of harbour porpoise. This figure gives the first indication of the frequency distribution through the spiral and is currently finalized. Image provided by Maria Morell.

Over the last decade, an increase in research has taken place investigating normal and impaired hearing in some marine mammals, like for harbour porpoises as presented here. There is a general concern about noise in the marine environment and the impact on species living here. This concern seems rather acute for marine mammals, with many populations declining and an increase in human activities or anthropogenic sound in the oceans. To understand the impacts of noise on cetacean and to put things in perspective, we need to know which species are exposed to what noise, for which period of time and how this affects their hearing abilities (Ketten 2012). Aiming to put the presented research in perspective, we present a SWOT analysis on the inner ear research on hair cells and the organ of Corti, helping to facilitate future research topics and providing recommendation aiming to increase knowledge on hearing damage analysis in harbour porpoises and other cetacean species (Figure 4).

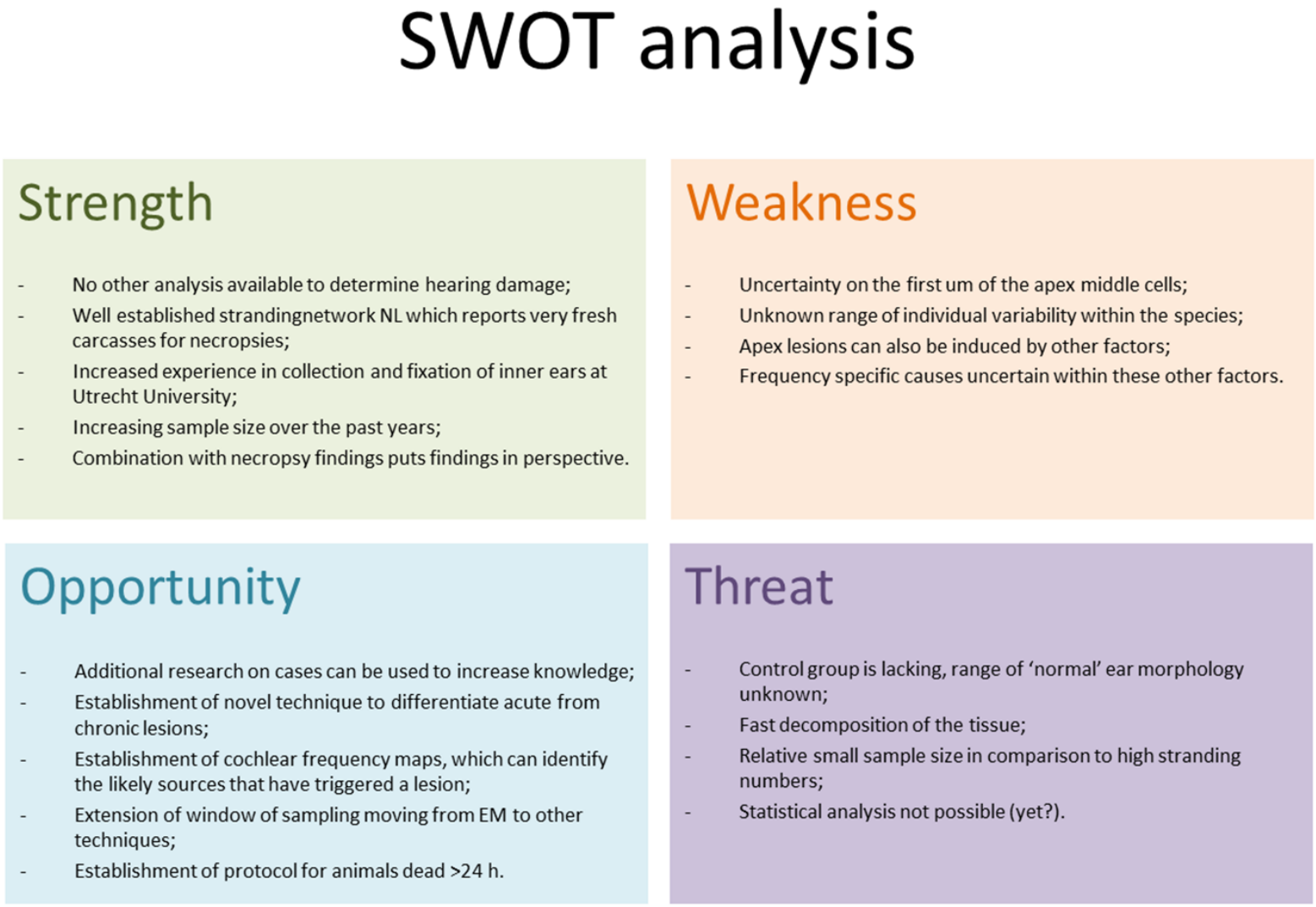

Figure 4: Strength, weakness, opportunity and threats (SWOT) of current inner ear research. Figure further explained in the text below.

Strength: Currently, no other existing analysis that link acoustic trauma to cetaceans and their causes of death is available. Besides, the lesions induced by acoustic trauma are not reflected elsewhere in the body, making the specialized method a highly important tool. If a lesion is found and once the cochlear frequency map is built, it will be possible to identify the more likely sources that have trigged this lesion. Besides, the available histopathology/necropsy findings in combination with the inner ear findings are very valuable to put things in perspective. The number of samples gained from porpoises stranded on the Dutch coast is increasing significantly, due to the well-established strandingnetwork that report and transport fresh cases to Utrecht, and due to the current research in place. Experience with collection and fixation of the inner ears has resulted in an extension of the window of sampling already, as proven by case UT1513 described here.

Weakness: There is an uncertainty among the morphology of the first um of the apex cells, and there seems to be variability in the samples processed so far. There is an unknown range of individual variability here, making it difficult to differentiate between hearing damage or this individual variation at

this location of the spiral. Besides, apex lesions can also be induced by PCBs, congenital malformations, a few diseases like osteoporosis or in some cases age, but it is unknown at which locations in the spiral these changes may become visible.

Opportunities: As the fresh cases go through a very extensive investigation at Utrecht University, including a range of tissue sampling and storage, these animals can be full screened for any other characteristic and factors, e.g. their exact age and contaminant load can be determined, but also all underlying diseases that might have caused anomalies elsewhere in the body. This can help interpreting the inner ear results and put things in perspective. Some novel techniques are currently finalized, like the use of immunofluorescence instead of Electron Microscopy (EM). EM is very sensitive, and if immunofluorescence proves to be reliable, using this technique will allow extension of the window of sampling and eventually increase the sample size. Also, immunofluorescence techniques will allow us to distinguish between new cochlear lesions (i.e. that occurred in the 7-9 days previous to the stranding) from older, more chronic injuries. Currently, optimizations of antibodies that detect changes in lesions within the first 9h post-exposure are conducted. Also, cochlear frequency maps are finalized, of which Figure 3 is an example. This will allows us in the future to identify the likely sources that have trigged a lesion based on its frequency. Other opportunities which can be investigated are the analysis of ears gained from porpoises >24 hours post mortem. These ears will have many artifacts in the organ of Corti making (S)EM unsuccessful. Currently, no other assessment than macroscopic analysis of these ears (e.g. assessment of parasites and abscesses) is conducted during necropsy. However, these ears can still be valuable to process for histopathology or scanned by CT-scans and reveal any pathologies or hemorrhages present.

Threats: As account for most studies on wildlife/cetaceans, no control group is available which allows the assessment of 'normal' ear morphology and the range of variation among these. The fast decomposition of the tissue only allows the freshest cases to be investigated, which poses a bias in the samples size to those animals which died in coastal waters. It will be highly challenging to link findings from the small sample size to the population, as the numbers do not allow any statistical analysis at this point.

Utrecht University has provided by far the most samples to investigate hearing damage in harbour porpoises, due to the access to fresh carcasses UU has. Continuing the research on hearing damage in harbour porpoises will increase the sample size and therefore increase our knowledge on inner ear morphology and anomalies in the future. Findings as presented here for case UT1535 are the first proves ever reported for this species that the method is valid and hearing damage is present and can be found, therefore highlighting the importance of this work. To put things in perspective, the extensive research which can be done on such fresh cases, including full diagnosis of diseases and other abnormalities together with assessment of biological data (reproduction and age) is therefore the way to move forward. The second ear of all animals submitted for this research will be used to finalize the novel techniques mentioned (immunofluorescence) and establish the cochlear frequency maps, which will shed more light in the (near) future on chronic and acute nature of lesions and the sources that triggered lesions. Besides, extending the research by using other available methods, as histopathology of ears or CT-scans to detect hemorrhages and infections should be further exploited.

3. Life history

Dr. Sinéad Murphy from Galway Mayo Institute of Technology (Ireland) was contracted by Utrecht University to perform the ovary analysis. The ovaries were shipped from The Netherlands to Ireland with appropriate CITES permit (16NL235413/20).

Dr. Fiona Read from Life History Studies (UK) was contracted by Utrecht University to perform the age analysis. The teeth were shipped from The Netherlands to the UK with appropriate CITES permit (16NL235412/20).

3.1 Background

Like in all mammalian species, reproduction and the care of a calf in cetaceans is fundamental for maintaining their populations (Whitehead and Mann 1999). In the case of the harbour porpoise in the (southern) North Sea, not much literature is available on their maximal population growth (Lockyer 2003). Estimates of mean age at sexual maturity reported for animals in the North Sea are three for males and four-five for females, with length of 135 cm in males and 143 cm in females. Gestation is estimated at ten to eleven months, with mating season in August and birth season in June (Lockyer 2003). Lockyer (2003) reviewed the literature available on the biological parameters of North Atlantic harbour porpoises, but for Dutch waters only limited information was available. Lockyer reported that neonates were born at approximately 74.3 cm and that the mating period occurred in August here. In Danish and adjacent North Sea waters, neonatal length ranged from 60-75 cm, and the lactation period was believed to last for more than eight months.

Following Lockyer (2003), additional information on biological parameters in Dutch porpoises can be gained from the EU BIOCET project that included samples from female porpoises between 2001 and 2003 (Learmonth et al. 2004). A sample size was available for the study which was composed of eight sexually immature and 11 sexually mature females and therefore relatively small. Results from the study suggested that females sampled in Dutch waters attain sexual maturity at body lengths greater than 130 cm and about 5 years of age, corresponding to Lockyer (2003). A low pregnant rate was observed in Dutch porpoises of 0.11 (1 of 9 mature females was pregnant), which was determined based on both the presence of a corpus luteum and an embryo or fetus. The low pregnancy rate may have been due to exposure to pollutants, as within the BIOCET study, highest PCB levels were reported in porpoises sampled along southern North Sea coasts (Pierce et al. 2008), which will be further discussed in (van den Heuvel-Greve et al. in prep). Further information on reproductive parameters in Dutch waters is available from sightings data. Recent surveys reported high sighting rates of calves in July, but some calves were sighted as early as March (Geelhoed et al. 2013). A similar peak in calving was observed in stranding data in the 1990s, as Addink et al. (1995) reported a pronounced peak in births in July (June- August), with some neonates reported in May and September also.

Knowledge on reproduction parameters, including age at sexual maturity, is important information when assessing population survival. Here, we report upon the age determination of 98 harbour porpoises

stranded along the Dutch coast. Also, we report upon the analysis of reproduction organs to assess reproduction status of ten adult female harbour porpoises. This information is building upon the EU BIOCET work and earlier literature on pregnancy and calving in the (southern) North Sea, aiming to increase knowledge on the reproduction parameters of harbour porpoises here.

3.2 Materials and methods

3.2.1 Age determination

Teeth of the harbour porpoises that were necropsied were collected and stored at UU. For this study, the teeth of 98 harbour porpoises were selected to determine their age in years. The sample size was carefully selected and compromised of both males (n=43) and females (n=55), with a minimum body size of 115 cm. The spatial distribution of this selection reflected the spatial distribution of all necropsied porpoises and so did their causes of death. During necropsy, age classes of all cases were assigned based on the assessment of their reproduction organs and categorized as 'neonate', 'juvenile' or 'adult'.

Teeth were prepared following the protocol adapted from Hohn and Lockyer (1995). In short: teeth were formalin-fixed for 24 hours, rinsed thoroughly in water and decalcified in commercial decalcifying agent Rapid Decalcifier (RDO©). Once decalcified, the teeth were rinsed thoroughly in water for at least 8 hours. One tooth from each individual was sectioned parallel to the mandible and a second sectioned perpendicular to the mandible. Sections of 25 µm thickness were cut using a cryostat set at -12°C, stained with Mayer's hematoxylin (modified by Grue) and 'blued' in a weak ammonia solution. The best sections were selected and mounted on glass slides using DPX©. Age was estimated by counting growth layer groups (GLGs) in the dentine of the tooth sections, using a binocular microscope (10x50). Duplicate age estimates were obtained by two independent readers, without reference to biological data of the cases (Dr. Fiona Read and Dr. Christina Lockyer).

3.2.2 Reproductive status

Ovaries of ten adult harbour porpoises were formalin fixed and stored after necropsy at UU. Assessment of reproductive status was based on procedures and terminology recommended by the International Whaling Commission (Perrin and Reilly 1984) and used in earlier studies (Read and Gaskin 1990, Murphy et al. 2009). The ten animals were classified into four categories: pregnant, lactating, pregnant and lactating, or resting. Pregnancy was established by the presence of an embryo/fetus during the necropsy. Additional pathological findings of the reproduction organs of these ten animals are also reported here.

The ovaries were measured and weighed and a macroscopic examination on the ovaries was conducted, including identification of corpora scars (corpora lutea (CL) and corpora albicantia (CA)). All corpora scars and the largest follicle were measured. The CL is an endocrine gland and recognizable on the ovary as a pronounced distension, usually yellow in colour as a result of the yellow pigments of the carotenoid luteins. CA appears as raised, wrinkled scars, which are recognizable on the cut surface as a pale fibrotic area. CA's are composed of white connective which that becomes fragmented with age, and reflect the amount of ovulations of an individual (Murphy et al. 2010). Activity of CL was assessed microscopically using histological techniques and a preliminary assessment of abnormalities in the ovaries was also undertaken.

3.3 Results

3.3.1 Age determination

A maximum total length of the male porpoises was 148 cm and a maximum age of 13 was established, with a mean length of 131.3 cm and a mean age of 4.6 years. For the females, a maximum total length of 168.5 and a maximum age of 24 was established, with a mean length of 143 cm and a mean age of 5.7 years. The full list with the case ID numbers, stranding dates and -locations, and basic biological data can be found in Appendix 1.

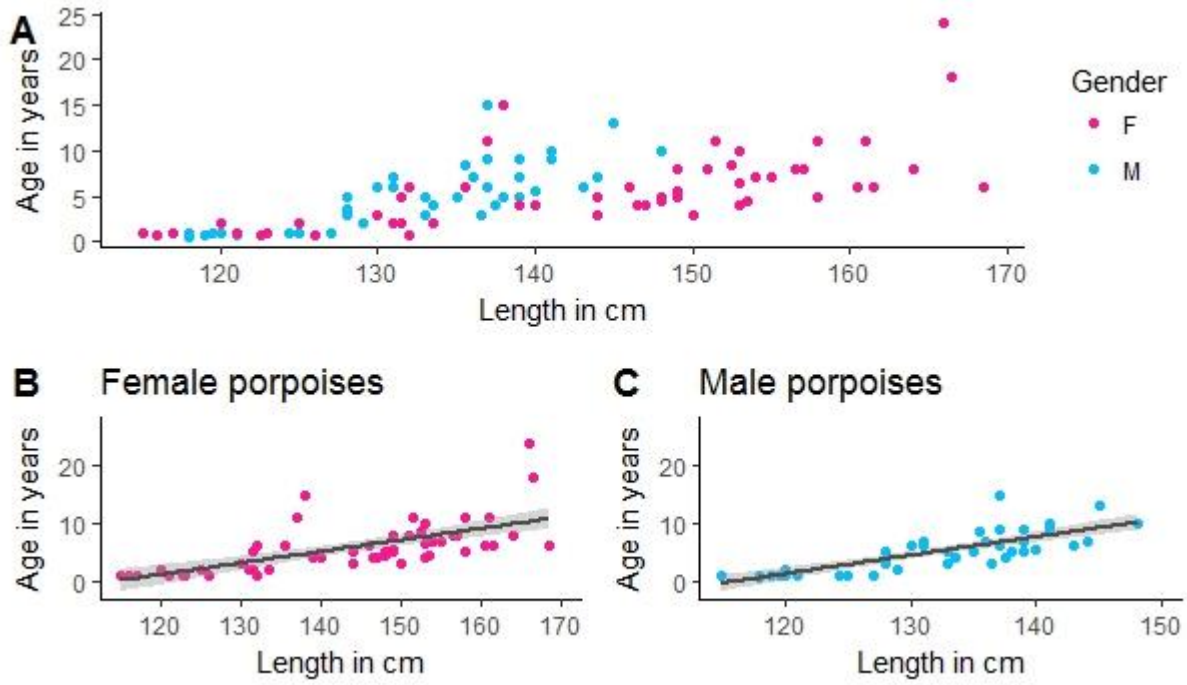

Figure 5: Ages at total length of harbour porpoises. **A**: scatterplot of all harbour porpoises included in this analysis with pink dots 'females' and blue dots 'males'. **B**: Female harbour porpoises with linear regression line (dark grey) and 95% confident interval (light grey area). **C**: Male harbour porpoises with linear regression line (dark grey) and 95% confident interval (light grey area).

In our dataset, males were smaller at an age of 3(+) than earlier reported for the North Sea population: an average of 136.8cm for all adults in the data set, which had an average of 6.6 years of age. The same accounted for the females, with seven adults smaller than 143 cm; the reported total length at sexual maturity. An average age of 6.7 years was found for these cases, with an average total length of 136.2 cm. Average total length of all adult females (n=39) was 150.7 cm, with an average age of 6.6 years.

3.3.2 Reproductive status

The females in this sample size ranged from 132-164 cm in body length. There were three pregnant, one pregnant and lactating, four lactating (and recently pregnant), one mature and ovulating and one resting mature female. For the most part, females were reproductively active with only one female reported as resting (not pregnant, lactating or ovulating, UT1470) (Table 2). The measurements of the ovaries and their CL and CA can be found in Appendix 2.

Table 2: Reproduction data of ten adult harbour porpoises

Idcode	Dd	Mm	Yy	Stranding location	Length (cm)	Age	Reproductive status	Total number of ovary scars
UT1266	8	5	2013	Termunten	148	5	Pregnant	6
UT1271	13	9	2012	Texel	132	6	Pregnant	2
UT1316	25	6	2014	Texel	153	6.5	Lactating	6
UT1470	24	6	2015	Egmond a/z	152.5	8.5	Resting Mature	15
UT1482	1	8	2015	Wassenaar	155	7	Lactating	11
UT1484	18	8	2015	Terschelling	164	8	Pregnant	5
UT1496	29	2	2016	Sexbierum	150	4	Pregnant Lactating	8
UT1508	10	3	2016	Texel	158	11	Mature Ovulating	15
UT1527	29	6	2016	Texel	157	8	Lactating	11
UT1535	28	7	2016	Domburg	146	6	Lactating	12

Eight of the ten females died from infectious disease. Signs of infection (based on macroscopic analysis and histopathological examination) of the reproductive organs and/or mammary gland were seen in case UT1266; which died of dystocia, and case UT1482; which had a severe purulent mastitis (infection on the mammary gland). *Brucella spp* infection was detected in the lung and uterus lymph nodes of another case (UT1527). Poor body condition was confirmed during the necropsy for 6/10 cases (UT1316, UT1470, UT1482, UT1484, UT1527, UT1535), which all died of a presumably chronic infection of several organs. A good body condition, suggesting a more acute cause of death instead of chronic illness was determined in the additional four cases (UT1266, UT1271, UT1496, UT1508), among which one animal died of possible ship-strike (UT1271).

3.4 Discussion

Age at sexual maturity in harbour porpoises from the Atlantic have been reported at three years and 135 cm for males and four-five years and 143 cm for females (Lockyer 2003). The findings presented here however suggest a smaller length at age of maturity than earlier reported for both males and females from the (southern) North Sea. Ages of the cases corresponded to their age class assessed during necropsy almost fully (confirmed for n=96).

Total length for males had a maximum of 148 cm, while females reached sizes of almost 170 cm, with both genders generally reaching maximum ages of 10-15, with two outliers in the females (one animal of 20 years and one of 24 years of age). Earlier reports on sizes of harbour porpoises from the North Sea presented maximum lengths for females of 189 cm and for males 163-167 cm. Ages reported by Lockyer (2003) for the North Sea (British Isles and southern North Sea) harbour porpoise population have maxima of 22 for females and 24 for males. Male harbour porpoises, with their smaller lengths and lower ages than reported previously, therefore do not seem to reach full grown maturity in Dutch coastal waters. To justify this, the sample size needs to be enlarged, including animals >120 cm in size, which can

help generating a better age at total length and age at sexual maturity for both males and females from Dutch coastal waters.

From the animals analyzed for reproduction status, four of the porpoises that died between June and August, and which were not pregnant, showed no signs of ovulation. Three of these females were reported as recently pregnant and lactating and it appears that they had not re-commenced ovulating following parturition. All three females died due to infectious disease. Only one mature female had a largish fluid-filled follicle (approx. 10 mm in diameter) and that individual died in March, which suggests that the mating period could be extended to early summer.

All mature females showed ovarian asymmetry with corpora scars being reported on the left ovary only. This is common in harbour porpoises where the left ovary is larger and active, while the right is undeveloped in size and non-functional (Gaskin et al. 1984; Addink et al. 1995). Thus the left ovary weighs more than the right (see Appendix 2). Corpora scar number ranged from 2-15, with the resting mature female and the ovulating female exhibiting the highest number of corpora scars, while pregnant females had on average the lowest number of corpora scars. Relations with PCBs are further discussed elsewhere (van den Heuvel-Greve et al. in prep). Within the BIOCET study corpora scar number ranged from 1-12 in mature Dutch female porpoises (Learmonth et al. 2004). Within the current study, all CL of pregnancy were < 20.8 mm - the mean size of CL of pregnancy (with a fetus present) found in harbour porpoises in the Northwest Atlantic (Read 1990). A pregnancy rate could not be estimated for this sample as all females died between February and September, and therefore was not reflecting the entire reproductive cycle of porpoises. An increase of the sample size, including stranded adult female of all months in a year, is highly recommended in order to understand the phases of the productive cycle of harbour porpoises, and all additional reproduction parameters including e.g. pregnancy rate and fetus growth curves.

Porpoises mature at a relative young age, and therefore changes in age of sexual maturity will induce large changes in population growth rate (Hohn 1989; Learmonth et al. 2014). This makes the assessment of exact age of sexual maturity an important criterion when evaluating population status, and can be seen as an index of the population condition or the relative carrying capacity of an area (e.g. DeMaster 1984, Learmonth et al. 2014). Age of sexual maturity have been reported for porpoises of other locations, but is currently unknown for the southern North Sea. Spatial differences in age of sexual maturity can be affected by several causes, including differences in porpoise population density, prey availability, and other habitat characteristics including disturbance and anthropogenic activities (Learmonth et al. 2014). Therefore, a broad scale assessment of age at sexual maturity and other reproduction parameters should be gained in order to estimate the current status of the population, but also in order to detect any changes in the future that might affect the population growth and survival.

4. References

Addink MJ, Sørensen TB, García Hartmann M (1995). Aspects of reproduction and seasonality in the harbour porpoise from dutch waters. Developments in Marine Biology, 4: 459–464

Callis G, Sterchi D (1998). Decalcification of bone: literature review and practical study of various decalcifying agents, methods, and their effects on bone histology. Journal of Histotechnology, 21, 49–58

Camphuysen CJ (2004). The return of the harbour porpoise (*Phocoena phocoena*) in Dutch coastal waters. Lutra, 47(2), 113-122

Camphuysen CJ (2011). Recent trends and spatial patterns in nearshore sightings of harbour porpoises (*Phocoena phocoena*) in the Netherlands (Southern Bight, North Sea), 1990-2010. Lutra, 54: 39-47

Camphuysen CJ, Peet GH (2007). Whales and dolphins of the North Sea. Fontaine Uitgevers BV, 's Graveland, The Netherlands, pp 159

Camphuysen CJ, Siemensma ML (2011). Conservation plan for the Harbour Porpoise *Phocoena phocoena* in The Netherlands: towards a favourable conservation status. NIOZ Report 2011-07, Royal Netherlands Institute for Sea Research, Texel, pp 183

Cozzi B, Huggenberger S, Oelschlager H (2016). Anatomy of dolphins: Insight into body structures and function. Academic Press Elsevier, London, pp 438

DeMaster DP (1984). Review of techniques used to estimate the average age at attainment of sexual maturity in marine mammals. Reports of the International Whaling Commission Special Issue 6: 175-179

Gaskin DE, Smith GJ, Watson AP, Yasui WY, Yurick DB (1984). Reproduction in the porpoises (Phocoenidae): implications for management. Reports of the International Whaling Commission Special Issue 6: 135-48

Geelhoed S, Scheidat M, van Bemmelen RSA, Aarts G (2013). Abundance of harbour porpoises (*Phocoena phocoena*) on the Dutch Continental Shelf, aerial surveys in July 2010-March 2011. Lutra, 56 (1): 45-57

Hohn AA (1989). Variation in life-history traits: The influence of introduced variation. PhD thesis, University of California, Los Angeles, California, pp 123

Hohn AA, Lockyer C (1995). Protocol for obtaining age estimates from harbour porpoise teeth. Reports of the International Whaling Commission Special Issue 16: 494-496

Jepson PD, Arbelo M, Deaville R, Patterson IAP, Castro P, Baker JR, Degollada E, Ross HM, Herráez P, Pocknell AM, Rodríguez F, Howie FE, Espinosa A, Reid RJ, Jaber JR, Martin V, Cunningham AA, Fernández A (2003). Gas-bubble lesions in stranded cetaceans. Nature, 425: 575-576

Johnsson LG, Hawkins JE (1972). Sensory and neural degeneration with aging, as seen in microdissections of human inner ear. Annals of Otology Rhinology and Laryngology, 81: 179-93

Ketten DR (1993). 'The cetacean ear: form, frequency and evolution'. In: Marine Mammal Sensory Systems. Plenum Press New York, 53-75

Ketten DR (2012). Marine Mammal Auditory System Noise Impacts: Evidence and Incidence. The Effects of Noise on Aquatic Life, 207-212

Ketten DR, Cramer S, Arruda J (2007). A manual for the removal, fixation and preservation of cetacean ears. Available from: http://csi.whoi.edu/biblio/manual-removal-fixation-and-preservation-cetacean-ears

Learmonth JA, et al. (2004). Measurement of reproductive output in small cetaceans from the Northeast Atlantic. BIOCET workpackage 5 - final report. Project Reference: EVK3-2000-00027, pp 53

Learmonth JA, Murphy S, Luque PL, Reid RJ, Patterson IAP, Brownlow A, Ross HM, Barley JP, Santos MB, Pierce GJ (2014). Life history of harbor porpoises (*Phocoena phocoena*) in Scottish (UK) waters. Marine Mammal Science, 30(4): 1427–1455

Lockyer C (2003). Harbour porpoises (*Phocoena phocoena*) in the North Atlantic: Biological parameters. NAMMCO Scientific Publications, 5: 71-90

Mooney TA, Yamato M, Branstetter BK (2012). 'Hearing in cetaceans: From natural history to experimental biology'. In: Advances in Marine Biology, 63: 198-234

Morell M, André M (2009). Ear extraction and fixation protocol. Available from: http://www.zoology.ubc.ca/files/Ear_extraction_and_fixation_protocol_UBC.pdf

Morell M, Lenoir M, Shedwick RE, Jauniaux T, Dabin W, Begeman L, Ferreira M, Maestre I, Degollada E, Hernandez-Milian G, Cazevieille C, Fortuño JM, Vogl W, Puel JL, André M (2015). Ultrastructure of the Odontocete Organ of Corti: Scanning and transmission electron microscopy. Journal of Comparative Neurology, 523 (3): 431–448

Murphy S, González AF, Read FL (2009). Importance of biological parameters in assessing the status of *Delphinus delphis*. Marine Ecology Progress Series, 388: 273-291

Murphy S, Pierce GJ, Law RJ, Bersuder P, Jepson PD, Learmonth JA, Addink M, Dabin W, Santon MB, Deaville R, Zegers BN, Mets A, Rogan E, Ridoux V, Reid RJ, Smeenk C, Jauniaux T, López A, Alonso Farré JM, González AF, Guerra A, García-Hartmann M, Lockyer C, Boon JP (2010). Assessing the effect of persistent organic pollutants on reproductive activity in common dolphins and harbour porpoises. Journal of Northwestern Atlantic Fisheries Science, 42: 153–173

Noorbakhsh S, Memari F, Farhadi M, Tabatabaei A (2008). Sensorineural hearing loss due to *Toxoplasma gondii* in children: a case–control study. Clinical Otolaryngology, 33(3): 269–273

Peltier H, Baagøe HJ, Camphuysen CJ, Czeck R, Dabin W, Daniel P, Deaville R, Haelters J, Jauniaux T, Jensen LF, Jepson PD, Keijl GO, Siebert U, Van Canneyt O, Ridoux V (2013). The stranding anomaly as population indicator: the case of harbour porpoise *Phocoena phocoena* in North-Western Europe. PLoS ONE, 8(4): e62180

Perrin WF, Reilly SB (1984). Reproductive parameters of dolphins and small whales of the family Deiphinidae. Reports of the International Whaling Commission Special Issue 6, 97-133

Pierce GJ, Santos MB, Murphy S, Learmonth JA, Zuure AF, Rogan E, Bustamante P, Caurant F, Lahayea V, Ridouxf V, Zegers BN, Mets A, Addink M, Smeenk C, Jauniaux T, Law RJ, Dabin W, López A, Alonso Farré JM, González AF, Guerra A, García-Hartmann M, Reid RJ, Moffat CF, Lockyer C, Boon JP (2008). Bioaccumulation of persistent organic pollutants in female common dolphins (*Delphinus delphis*) and harbour porpoises (*Phocoena phocoena*) from western European seas: Geographical trends, causal factors and effects on reproduction and mortality. Environmental Pollution, 153 (2): 401–415

Read AJ, Gaskin DE (1990). Changes in growth and reproduction of harbour porpoises, *Phocoena phocoena*, from the Bay of Fundy. Canadian Journal of Fisheries and Aquatic Sciences, 47(11): 2158-2163

Rijkswaterstaat (2016). Jaarplan 2016 Windenergie op zee ecologisch programma (version 2.2). March 2016, The Hague, The Netherlands

Scheidat M, Leaper R, van den Heuvel-Greve M, Winship A (2013). Setting maximum mortality limits for harbour porpoises in Dutch waters to achieve conservation objectives. Open Journal of Marine Science, 3: 133-139

Siebert U, Gilles A, Lucke K, Ludwig M, Benke H, Kock KH, Scheidat M (2006). A decade of harbour porpoise occurrence in German waters – Analyses of aerial surveys, incidental sightings and strandings. Journal of Sea Research, 56: 65-80

Whitehead H, Mann J (1999). 'Female reproduction strategies of cetaceans'. In: Cetacean Societies: Field Studies of Dolphins and Whales. University of Chicago Press, pp 219-246

5. Appendix

Appendix 1: Harbour porpoises selected for age determination

Idcode	Dd	Mm	Yy	Stranding location	Age class	Gender	TL (cm)	Final age	Age range
UT221	5	3	2009	Den Helder	J	M	127	1	
UT231	10	4	2009	Groote Keeten	J	F	121	1	
UT239	27	4	2009	Westkapelle	A	M	128	3	
UT261	9	6	2009	Texel	A	F	151	7	7-9
UT275	17	10	2009	Terschelling	A	F	144	3	
UT277	4	11	2009	Wijk aan Zee	A	M	144	7	7-9
UT278	11	11	2009	Bloemendaal	J	F	130	3	
UT289	7	1	2010	Texel	A	F	147	4	
UT291	11	1	2010	Texel	J	F	125	2	
UT298	16	3	2010	Kerkwerve	A	M	137.5	4	
UT303	13	10	2009	Ameland	A	M	135	5	
UT305	6	4	2010	Bloemendaal	J	M	133	3	
UT312	13	10	2009	Texel	A	M	131	6	
UT318	3	6	2009	Renesse	A	F	146.5	4	
UT332	5	12	2009	Texel	A	F	156.5	min. 8	7+
UT340	28	7	2010	Hondsbossche zeewering	A	F	151.5	11	
UT344	2	12	2009	Texel	A	M	141	9	
UT347	8	8	2010	Julianadorp	J	F	131	2	
UT357	29	6	2010	Noordwijk	A	F	161	min. 10	9-13
UT373	13	11	2010	St.Maartenzee	A	F	140	4	
UT379	4	12	2010	Vlieland	A	F	160.5	6	
UT388	2	4	2010	Vlissingen	J	M	128	3	3-4
UT393	26	1	2011	Katwijk	J	M	120	2	
UT400	20	5	2010	Ameland	A	M	140	5	5-6
UT404	8	2	2011	Ter Heijde	A	M	139	7	
UT410	30	1	2011	Langevelderslag	A	F	166.5	20	17-20
UT411	19	12	2010	Dombrug	J	M	129	2	
UT421	28	3	2011	Noordzee	J	F	117	1	
UT423	28	3	2011	Wijk aan Zee	A	M	136	7	
UT428	8	5	2011	Cadzand- bad	J	F	115	1	
UT433	31	5	2011	Nieuw Haamstede	A	F	139	4	
UT469	6	8	2011	Monster	J	M	120	<1	

UT522	14	9	2011	Terheijde, zandmotor	A	F	153.5	4	4-5
UT528	18	10	2011	Hoek van Holland	A	F	166	ca. 24	
UT529	11	9	2011	Bath	A	F	135.5	6	
UT532	14	9	2010	Callantsoog	A	M	131	8	6-8
UT549	8	1	2012	Colijnsplaat	J	M	118	ca. 1	<1 or just 1
UT564	10	1	2012	Maasvlakte 1	A	F	153	10	
UT623	17	10	2011	Texel	A	F	168.5	6	
UT624	29	8	2011	Texel	J	M	125	1	
UT703	4	3	2012	Kwadenhoek	A	F	132	<1	
UT760	24	8	2011	Westenschouwen	J	F	123	1	
UT775	31	8	2012	Texel	A	F	137	7	approx.
UT855	10	3	2012	Borselle, Kaloot	J	F	116	<1	
UT867	21	3	2013	Zoutelande	J	M	121	<1	
UT917	24	3	2013	Eemshaven	J	F	122.6	<1	
UT946	17	4	2013	Westkapelle	J	M	119	<1	
UT955	27	4	2013	Renesse	A	F	148	5	
UT956	27	4	2013	Breskens	J	F	126	<1	
UT977	9	5	2013	Ter Heijde	A	M	135.5	8	8-9
UT1003	6	7	2013	Ameland	A	M	137	9	
UT1007	1	10	2013	Zandvoort	A	F	144	5	
UT1008	9	8	2013	Scheveningen	J	F	120	2	
UT1017	15	9	2013	Ouwerkerk	A	M	130	6	
UT1019	1	12	2012	Noordwijk	A	F	149	6	5-6
UT1020	19	11	2013	Hondsbossche zeewering	J	F	130	3	
UT1024	24	4	2012	Noordwijk	A	M	133.5	4	
UT1266	8	5	2013	Termunten	A	F	148	5	4-5
UT1267	29	2	2012	Texel	A	F	149	5	
UT1268	16	2	2012	Callantsoog	A	F	138	15	
UT1270	6	2	2012	Zandvoort	A	M	137	6	
UT1271	13	9	2012	Texel	A	F	132	6	
UT1272	28	12	2012	Groote keeten	A	M	133	5	
UT1274	2	3	2013	Noordwijk	A	M	136.5	3	
UT1276	27	6	2012	Bloemendaal	A	M	148	10	
UT1293	7	4	2013	Noordwijk	J	M	118	0.5	
UT1294	27	3	2013	Texel	J	M	118	1	
UT1300	11	1	2014	Scheveningen	A	M	145	13	
UT1301	11	1	2014	Westenschouwen	A	M	139	10	8-10
UT1302	13	1	2014	Noordwijk	J	F	133.5	2	
UT1306	24	1	2014	Vlissingen	J	M	119.5	1	

UT1311	3	3	2014	Oostkapelle	A	F	158	5	
UT1312	5	3	2014	Westkapelle	J	F	131.5	2	
UT1316	25	6	2014	Texel	A	F	153	6	6-7
UT1325	22	1	2013	Hoek van Holland	A	F	161.5	6	
UT1332	8	11	2011	Scheveningen	A	F	154	7	
UT1418	21	12	2014	Den Haag	A	F	151	8	
UT1448	4	3	2015	Zwanenwater	A	F	153	4	
UT1458	23	3	2015	Noordwijk	J	M	124.3	1	
UT1464	25	5	2015	Kamperland	J	M	118	1	
UT1467	31	5	2015	Egmond aan zee	A	M	141	10	
UT1470	24	6	2015	Egmond aan zee	A	F	152.5	ca. 7	7-10
UT1471	27	6	2015	Noordzee	J	M	115	1	
UT1472	3	7	2015	Domburg	A	M	138	5	
UT1480	29	7	2015	Egmond aan zee	J	F	131.5	5	
UT1482	1	8	2015	Wassenaar	A	F	155	7	
UT1484	18	8	2015	Terschelling	A	F	164	8	
UT1485	20	8	2015	Noordzee	J	F	115	1	
UT1492	3	1	2016	Scheveningen	A	M	137	5	
UT1494	2	2	2016	Schiermonnikoog	A	M	128	5	
UT1496	29	2	2016	Sexbierum	A	F	150	4	
UT1508	10	3	2016	Texel	A	F	158	11	
UT1512	22	3	2016	Texel	A	M	130	6	
UT1522	18	5	2016	Maasvlakte 2	A	M	143	ca. 6	
UT1527	29	6	2016	Texel	A	F	157	8	
UT1530	26	6	2016	Westenschouwen	A	F	149	8	
UT1531	7	7	2016	Bergen aan Zee	A	M	139	ca. 5	
UT1535	28	7	2016	Domburg	A	F	146	6	

Appendix 2: Ovary and - scar measurements

Ovaries				Left ovary (mm)			Right ovary (mm)		
Idcode	**Reproductive status**	**Weight left ovary (g)**	**Weight right ovary (g)**	**Length**	**Width**	**Depth**	**Length**	**Width**	**Depth**
UT1266	Pregnant	6.254	1.214	44	19.2	13.8	18	11	6
UT1271	Pregnant	8.974	1.684	38.2	25.1	16	26.9	9.4	7
UT1316	Lactating	5.884	2.284	40.2	18	12.1	27.5	14.1	7.1
UT1470	Resting Mature	2.124	0.894	21.9	14.4	10.8	20.1	10.2	5.2
UT1482	Lactating	3.014	0.994	25.2	15	14	23.9	18.2	7
UT1484	Pregnant	3.654	0.674	28.3	17	11.7	23.7	9.7	3.9

UT1496	Pregnant Lactating	6.504	1.994	40.4	20.7	13.7	27.4	17	6
UT1508	Mature Ovulating	4.574	1.544	32	15.5	11	21.7	7.8	7
UT1527	Lactating	4.104	1.484	32.1	16.9	10.5	29.5	14.7	4.9
UT1535	Lactating	7.774	1.844	29	28.9	17	23.8	12.2	8.8

Scars	Left		Right			
Idcode	**CL**	**CA**	**CL**	**CA**	**Total scars**	**Size of CL (mm)**
UT1266	1	5	0	0	6	19.1x18.3x13.6
UT1271	1	1	0	0	2	19.7x18.1x16.1
UT1316	0	6	0	0	6	
UT1470	0	15	0	0	15	
UT1482	0	11	0	0	11 approx.	
UT1484	1	4	0	0	5	14.2x12.5x14.4
UT1496	1	7	0	0	8	17.0x16.4x13.7
UT1508	0	15	0	0	15 approx.	
UT1527	0	11	0	0	11	
UT1535	1	11	0	0	12 approx.	18.6x15.0x17.9